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**GELATIN FUNCTIONALIZATIONS FOR CELL
EMBEDDING IN 3D GEOMETRIES USING RAPID
PROTOTYPING TECHNOLOGY**

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I always found the acknowledgement part of a PhD dissertation rather intriguing. The finalization of a PhD encompasses several years of research, thus PhD students are bound to come across a variety of “helping hands”. Peronally, I prefer visual representations to grasp a phenomenon rather than spitting through text to comprehend the meaning behind. Therefore, I opted to graphically represent my “helping hands”.

Nevertheless, I would like to take the opportunity to give my special appreciation to Elien Gevaert from the lab of Prof. M. Cornelissen, who supplied the necessary biological input for this work. Also to my parents and Hanne. My parents were of utmost importance paving the path towards an enjoyable and interesting training. Hanne, thanks for letting me cherish the person you are!

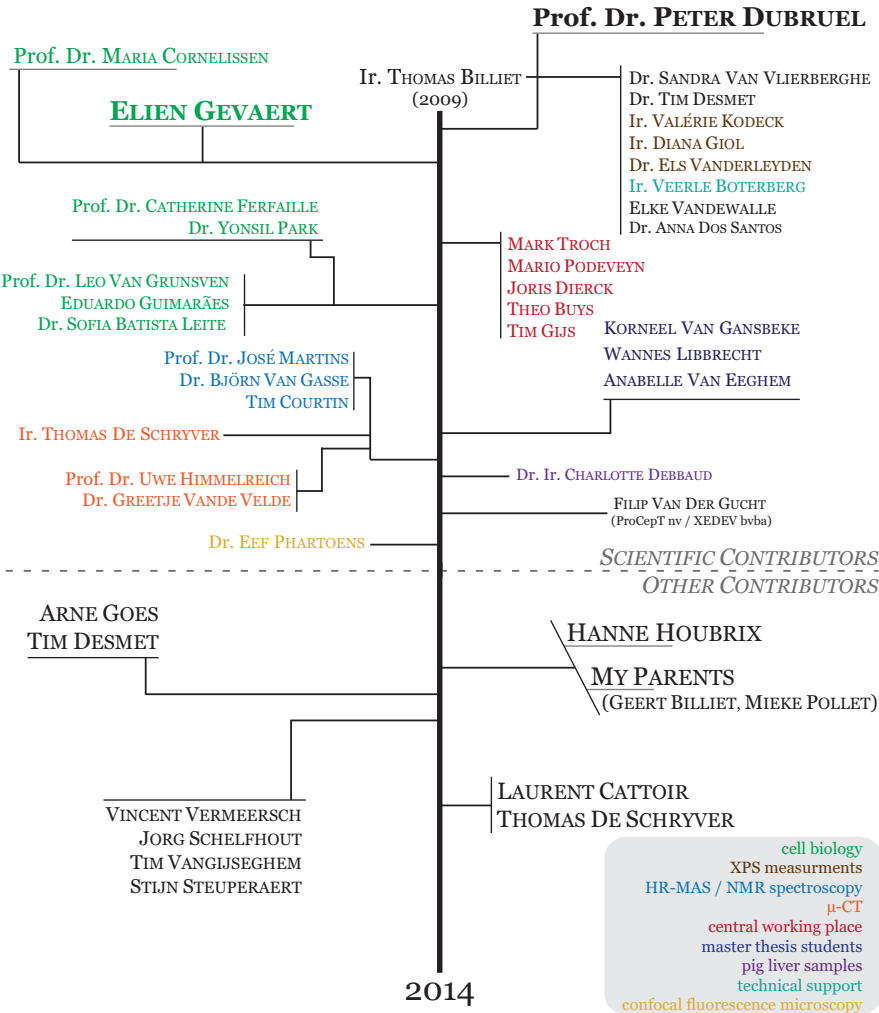


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1 | A REVIEW OF TRENDS AND LIMITATIONS IN HYDROGEL-RAPID PROTOTYPING FOR TISSUE ENGINEERING

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*This chapter serves as an introduction describing concepts such as hydrogels, rapid prototyping and tissue engineering together with the current trends and limitations. This work was partly published as review article in Biomaterials¹ and as a book chapter in “Biologically Responsive Biomaterials for Tissue Engineering (Springer)”.*²

1.1 | GENERAL FRAME OF REFERENCE

To date, organ and tissue transplantation remains one of the most important while complex options in order to restore or enhance life expectancy. A recent annual report prepared by the Scientific Registry of Transplant Recipients (SRTR) in collaboration with the Organ Procurement and Transplantation Network (OPTN) registered 112,905 patients in the USA awaiting transplantation at the end of 2011, while only 26,246 transplantations were performed.³ If we keep the steady increase in life expectancy in mind, these numbers emphasize the shortage of organ donors.⁴ In addition, diseases, infections and rejection of the tissue by the host often complicate transplantation.⁵

As such, there is a need for technological development in the field of regenerative medicine, capable of restoring and/or replacing malfunctioning tissues. The origin of regenerative medicine stems from a variety of medical activity, including surgery, medical prosthetics, transplantation, and biomaterial scaffolds.⁶ Therefore, the term ‘regenerative medicine’ refers to a group of biomedical approaches centrally focussed on human cells with the aim to provide a platform for the restoration, enhancement and/or establishment of normal, healthy tissue function. For instance:

- Restoring tissues using therapies based on the administration of biologically active molecules such as genes, proteins, and antibodies.;
- The injection of stem cells;
- The use of medical prosthetics to substitute for organs or tissue functions;
- Transplantation of *in vitro* grown organs or tissues;
- ...

Regenerative medicine not only aims at compensating the donor shortage, but additionally targets on improving the standard of care.⁷ Ideally, in structure as well as in function, the replaced or repaired tissue should be reciprocal to normal tissue. Additionally, regenerative medicine is not only restricted to therapeutic applications, but also encompasses diagnostic and research developments (i.e. drug screening, phatogenicity testing, toxicity testing, ...). One of those branches in regenerative medicine, called ‘Tissue Engineering’, is applied in the present work.

This dissertation is situated within the framework of the IWT-SBO called Hep-Stem (Functional Human Hepatocytes, Stellate Cells and Sinusoidal Endothelial Cells from Stem Cells). As a subpart of this national project, this work aimed at

the exploration and development of 3D biodegradable hydrogel cell carriers, possessing extended control over the matrix architecture. The developed strategies are targeted to hepatic tissue engineering applications.

1.2 | THE CONCEPT OF TISSUE ENGINEERING (TE): AN INTRODUCTION

1.2.1 Definition, scope and classification

To overcome these problems associated with transplantation, the last few decades, tissue engineering (TE) has grown as a new inter- and multidisciplinary scientific field.⁸ This discipline has rapidly emerged and combines the principles of engineering and life sciences. It holds as main objective the recovery, maintenance and improvement of tissue performance.^{8–10} The European Commission on Health and Consumer Protection defines TE as the persuasion of the body to heal itself through the delivery, to the appropriate site, independently or in synergy, of cells, biomolecules and supporting structures.¹¹

This definition nicely illustrates the core hypothesis, which serves as the basic concept of tissue engineering.⁹

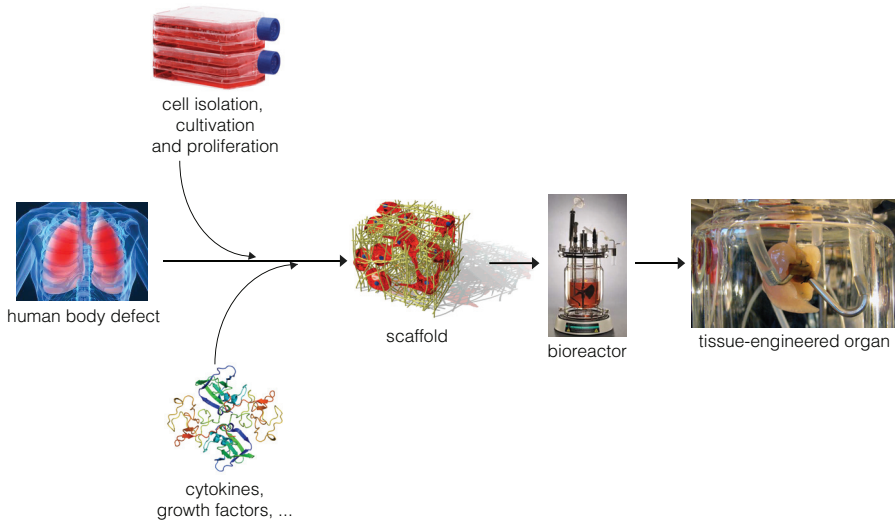


Figure 1.1: The workflow of the conventional tissue engineering approach.

By and large, two approaches can be distinguished:¹² (i) *in vitro* construction of bioartificial tissue employing cells isolated from donor tissue, and; (ii) *in vivo* manipulation of the cell growth and function. The former focusses on the development

of alternatives for tissue transplantation, the study of complex tissue functions and on tissue morphogenesis *ex vivo*. The latter aims at the *in situ* modification of the growth/function of cells. Independent from the methodology, the production of extracellular matrix (ECM) is the premise for tissue regeneration and repair. Hence, having cells with the correct/desired phenotype at one's disposal is crucial. This aspect is partly translated by material interactions.

Researchers will strive to fulfill those afore mentioned objectives through the utilization of:

1. Isolated cells or cell substitutes;^{13–16}
2. Tissue inducing substances,^{17–19} and/or;
3. Three-dimensional scaffolds, either *in vivo* or *in vitro*.^{5,8,10,20}

Although, conventionally, the application of a supporting scaffold is preferred in circumstances where the defect acquires certain dimensions. Post-processing cell seeding and maturation to tissue has therefore been implemented as a commonly applied TE strategy.^{20–24} Expanding the cell population and maturation to tissue is performed in bioreactors, which can be described as devices, in which biological and/or biochemical processes are manipulated through close control of environmental and process-bound factors such as pH, temperature, pressure, and nutrient and waste flow.²⁵ The general principle behind the concept of the conventional TE approach is illustrated in Figure 1.1.

A primary, rough classification can be performed based on the mechanical properties of the target tissue. As such the so-called ‘hard’ and ‘soft’ tissue engineering relate to this distinction. In order to give an impression on this difference, Table 1.1 gives some examples of tissues and their elastic moduli. The presented values were collected from different sources.^{26–28}

Table 1.1: Mechanical properties of some hard and soft tissues.

HARD TISSUES		SOFT TISSUES	
Tissue sample	Elastic modulus [GPa]	Tissue sample	Elastic modulus [kPa]
Vertebral trabecular bone	3.8	Liver	0.4 – 6
Tibia trabecular bone	1 – 15	Breast	18 – 24
Femur trabecular bone	5 – 12	Prostate	55 – 63
Cortical bone	18 – 22	Tendon	0.4 GPa
Dentine	11	Skin	10 – 20

1.2.2 Biomaterials for tissue engineering

Throughout history, examples of glass eyes and wooden and gold-filled teeth can be found.²⁹ Table 1.2 relates to some important milestones achieved in the history of implants and their correlated materials.³⁰ From this, we can deduct that tissue engineering and modern biomaterial development is a rather young science. Evidently, the mechanical properties of the target tissue will translate into the screening and/or the design of suitable biomaterials. The definition of what exactly a biomaterial encompasses has been subtly altered over the last few years. A biomaterial can be described as every material (natural, synthetic or a combination) applied for the fabrication of apparatuses, bound for the replacement of biological systems or to operate in close contact with biological tissues.

Table 1.2: Some milestones in the history of implants.³⁰

YEAR	RESEARCHER	DEVELOPMENT
Late 18 th - 19 th century		Metal devices for fracture fixations; wires and stems from Fe, Au, Ag, and Pt
1860-1870	J. Lister	Aseptic surgical techniques
1886	H. Hansmann	Ni-plated steel fracture-plate
1893-1912	W.A. Lane	Steel plates and screws for fracture fixation
1909	A. Lambotte	Al, Ag, and Cu plate
1912	Sherman	Vanadium plate, first alloy exclusive for medical applications
1924	A.A. Zierold	Stellite (CoCrMo alloy)
1926	M.Z. Lange	18-8sMo (2-4 %) stainless steel for enhanced corrosion resistance
1926	E.W. Hey-Groves	Carpenter's screw for femur neck fracture
1931	M.N. Smith-Petersen	Development of first femur neck fracture fixation screw, later on replaced by Vitallium
1936	C.S. Venable, W.G. Stuck	Vitallium; 19 w/o Ni stainless steel
1938	P. Wiles	First total hip replacement
1946	J. and R. Judet	First biomechanically designed hip prosthesis; first application of plastics for joint replacement
1940s	M.J. Dorzee, A. Franceschetti	Acrylates for cornea replacement
1947	J.Cotton	Ti and Ti alloys
1952	A.B. Voorhees, A. Jaretzta, A.H. Blackmore	First cotton fabricated artery replacement
1958	S. Furman, G. Robinson	First successful direct heart stimulation
1958	J. Charnley	First application of acrylic bone cement for total hip replacements
1960	A. Starr, M.L. Edwards	Heartvalve
1970s	W.J. Kolff	Experimental total heart replacement
1990s		Implants which enable bone ingrowth
2000s		Introduction of the concept Tissue Engineering
2000s		Nanoscale materials

This description focuses on structural biomaterials, however, a recent and widely adopted definition broadens the definition:³¹ a biomaterial is a substance engineered to take a form, which alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine.

This definition even incorporates engineered tissues and cells. For simplicity, in the present work, we refer to biomaterials as the structural materials used in the field of regenerative medicine.

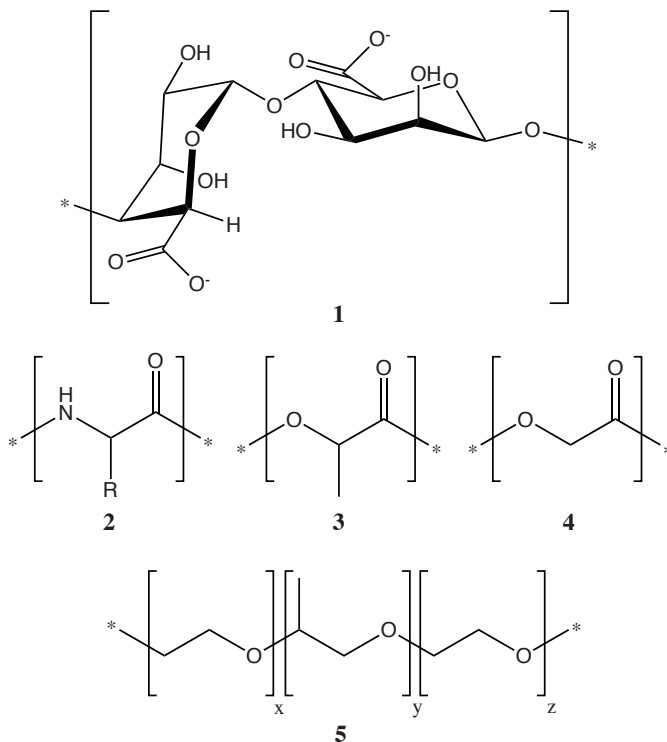


Figure 1.2: The chemical structure of some natural (1,2) and synthetic (3-5) polymers applied in the biomedical field: 1) alginate, 2) poly(α -amino acids), 3) poly(glycolic acid) (PGA), 4) poly(lactic acid) (PLA), 6) ethylene oxide (EO) and propylene oxide (PO) block copolymers.

Biomaterials can be differentiated based on function or based on the anatomical purpose. However, they can also be classified based on the material on its own. Metals, ceramics, polymers, and composites are the four major biomaterial classes.³²⁻³⁵ The utilization of metals and ceramics is mostly aimed at the disciplines of hard tissue engineering.^{32,33} Polymers however form a versatile group with a wide variety of tissue engineering applications in both the hard and soft disciplines. In recent past, a shift from biostable towards biodegradable and/or bioresorbable biomaterials as a model for TE has occurred. Independent from their purpose, biomaterials design should exhibit as little as possible adverse body reactions, a requirement called ‘biocompatibility’.^{29,36} Because of this, polymer development for biomedical purposes has boosted over the years. In Figure 1.2,

the chemical structures of some widely applied natural and synthetic polymers are provided.

In general, natural polymers will exhibit intrinsic advantages such as biocompatibility, possess the ability to present receptor-binding ligands to cells, are biodegradable, and are susceptible to natural remodeling. Nevertheless, antigenicity, instability/deterioration, complexity of purification and disease transmission should be considered next to the reduced mechanical properties. The properties (mechanical, degradation, processability) of synthetic polymers on the other hand can more readily be adjusted but present more difficulty with biocompatibility and cell adhesion issues.³⁷

1.2.3 Hepatic tissue engineering

The successful progresses obtained in the tissue engineering of bone, cartilage, and skin were reached because they do not require an extensive vasculature and due to their limited metabolic needs. Contrarily, as a highly metabolic tissue, the liver is, among other, involved in detoxification, glycogen storage, protein synthesis, metabolic homostasis, release of proteins, carbohydrates, lipids, and metabolic wastes.^{38,39} The heterogeneous structure and functionality of the liver has been considered second only to the brain in its complexity.⁴⁰ Figure 1.3 illustrates a hepatic lobule, the functional unit of the liver and highlights the intricate organization. For a better understanding on the anatomy and functional aspects of the liver, readers are referred to more detailed literature.^{29,40,41}

Remarkably, the liver has the capacity to fully restore itself after a significant hepatic tissue loss either from partial hepatectomy or acute liver injury. Therefore, in classical liver regeneration, a partial hepatectomy resecting $\sim 70\%$ of the liver is performed. Next, the remaining lobes enlarge and reconstitute the original size, orchestrated by a myriad of signals exerting modulatory effects (positive or negative). Typically, this process takes 5-7 days for rats, but has also been demonstrated in larger animals and humans.⁴³

Nevertheless, full hepatic failure caused by disease, genetic complications, or adverse drug reactions still make hepatic transplantations necessary. On the other hand, the *in vivo* regenerative capacity of mammalian liver supports the hypothesis that within the correct stimulatory environment *in vitro* culture of large quantities of liver tissue should be possible.⁴⁴ Liver tissue engineering addresses three major applications:^{38,41,44}

1. Model tissues for drug testing/toxicology;
2. Bioartificial Liver (BALs)/Liver Assist Devices (LADs), which use functional hepatocytes to temporarily sustain a patient with a recent dysfunctional

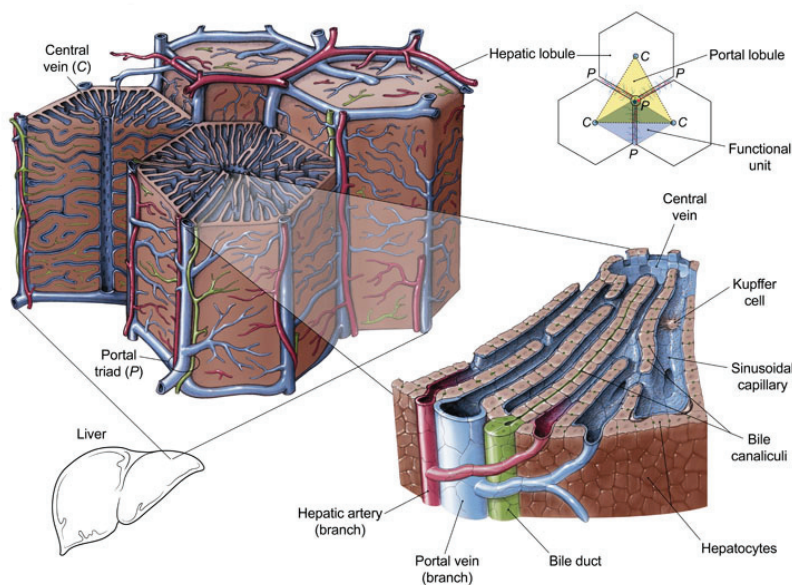


Figure 1.3: Structure of a hepatic lobule, the functional unit of the liver.⁴² The complexity of the structure of this organ is immediately apparent.

transplant, hepatic failure, or the progress towards failure due to a chronic liver disease. As such, they serve as temporary support;

3. Engineered organs for implantation into patients.

A desired solution is to replace endogenous hepatic function using cells, which are subsequently seeded on a polymer, cultured in a bioreactor and transplanted later *in vivo*. This methodology has demonstrated to maintain functionality for many weeks.^{45–47}

The major challenge in hepatic tissue engineering is to determine the minimal environmental signals that are required to grow long-term functional liver tissue.⁴¹ Hepatocytes, hepatocyte precursor cells (oval cells or Ito cells), sinusoid endothelial, Kupffer, stellate, biliary epithelial, fibroblasts and pit cells make up the bulk of the liver.^{38,39} Hepatocytes, anchorage-dependent cells composing 60 % of the liver cells and 80 % of the volume, primarily exhibit the characteristic hepatic functions and require a substratum to survive and function properly.^{38,44} Tight junctions and connective tissue such as type I and IV collagen and laminins, connect the cells.

When working with low-water content polymers, post-processing cell seeding is the only available seeding mechanism. However, insufficient cell seeding and/or non-

uniform cell distribution have been reported using this methodology.^{25,48} There is thus a need for better and more uniform seeding principles. Enhancing the seeding efficiency can, among other, be accomplished by cell encapsulation strategies. This method requires a high-water content environment.

1.3 | HYDROGELS: A ROUTE TO “SOFT” TE

1.3.1 The extracellular matrix

The extracellular matrix (ECM) is the non-cellular, complex meshwork of fibrous proteins and proteoglycans that is present within all tissues and organs. It provides the essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis.^{49,50}

Fundamentally, water, proteins and carbohydrates make up the ECM, although, each tissue has its own unique composition and topology. Additionally, the ECM is not only tissue-specific but also markedly heterogeneous. A simplified diagram is presented in Figure 1.4. Two main classes of macromolecules comprise the ECM:⁵¹

1. Proteoglycans - which are composed of glycosaminoglycan (GAG) chains covalently linked to a specific protein core (with the exception of hyaluronic acid). The GAG chains are unbranched polysaccharide chains comprised of disaccharide repeating blocks (either sulfated or non-sulfated) that adopt highly extended conformations.⁵²
2. Fibrous proteins - which can be either specialized (i.e. laminins, fibronectins) or structural (i.e. collagens, elastins) in function.

Generally, proteoglycans form hydrated gels and as such fill the majority of the extracellular interstitial space within the tissue.⁵³ Small leucine-rich proteoglycans, modular proteoglycans, and cell-surface proteoglycans form the three main families.⁵² Their functionality stems from the unique buffering, hydration, binding and force-resistance properties, and is quite diverse.^{54–56} Some of these specialized relevances are:⁴⁹ regulation of the inflammatory response, binding and activation of transforming growth factor beta ($TGF\beta$), modulation of cell adhesion/migration/proliferation, serve as pro- and anti-angiogenic factor, . . .

Being the most abundant fibrous protein, collagen makes up for up to 30 % of the total protein mass and is the main structural component of the ECM. Collagen has been reported to regulate cell adhesion, support chemotaxis and migration, provide tensile strength, and plays a role in direct tissue development.⁵⁷ Collagen fibers, predominantly secreted and organized by fibroblasts, are generally a heterogeneous

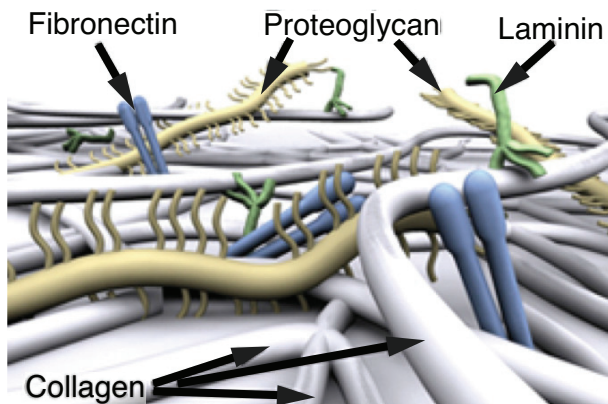


Figure 1.4: A simplified diagram illustrating the composition of the ECM.

mix of different types.⁴⁹ However, within a given tissue, one type usually outweighs the others.

Altogether, the unique characteristics of the ECM provide the biochemical, biomechanical, protective and organizational properties of each organ.

1.3.2 Hydrogels: definition, classification, and general properties

The term hydrogel stems from “hydro” (water) and “gel” and can be defined as polymer networks that are insoluble in water, but will swell till an equilibrium volume while retaining their shape.¹⁷ Insolubility is obtained through the formation of three-dimensional networks (due to so-called cross-links), whereby an equilibrium exists between dispersing (acting on hydrated chains) and cohesive (limiting water molecule penetration) forces.⁵⁸ On the other hand, the presence of hydrophilic chemical residues such as hydroxyl (-OH), carboxyl (-COOH), amide (-CONH-), primary amide (-CONH₂), sulfonic (-SO₃H), and others that can be found within the polymer backbone or as lateral chains result in the swelling behavior. Next to contact with aqueous solvents, swelling/shrinking behavior can be designed and triggered by other stimuli such as temperature, pH, ionic strength, electrical field, or light.^{17,59,60}

By virtue of their interesting properties, hydrogels found a variety of applications, of which some examples are provided in Figure 1.5.

Hydrogels based on natural or synthetic polymers have been of great interest regarding cell encapsulation.^{58,61–75} For the past decade, such hydrogels have become especially attractive as matrices for regenerating and repairing a wide variety of tissues and organs because of their innate structural and compositional similarities



Figure 1.5: Some applications of hydrogels in the biomedical field.

to the extracellular matrix.^{11,17,58,72–130} Depending on the hydrophilicity, they can absorb up to thousands of times of their dry weight and form chemically stable or (bio)degradable gels. Thanks to their thermodynamic compatibility with water these hydrophilic homopolymers/copolymers/macromers are typically soft and elastic.⁷ Depending on the nature of the hydrogel network (i.e. hydrogel network formation), ‘physical’ and ‘chemical’ gels can be distinguished. The cross-linked network structure is characterized by their junctions or tie knots. Hydrogels are called ‘physical’ when the network formation is reversible. In contrast to ‘chemical’ hydrogels, which are established by irreversible, covalent cross-links. Combinations of both physical and chemical networks can also be achieved, e.g. gelatine modified with methacrylamide groups.¹³¹ Table 1.3 presents some examples of hydrogels classified by their preparation method.^{17,132–134} As a mechanism for the formation of chemical gels, polymerization remains the most common case and is routinely induced by irradiation (gamma-rays, electron beams, UV or visible light, X-rays) of the monomers and/or macromonomers.¹³⁵

The characteristic properties of hydrogels make them especially appealing for repairing and regenerating soft tissue.^{71,75–77,123–130,136–139} Nevertheless, one of the main disadvantages of processing hydrogels is the difficulty to shape them in pre-

Table 1.3: Some examples of hydrogel network formation mechanisms.

CLASSIFICATION	PREPARATION METHOD	EXAMPLES
Physical hydrogels	Ionic interaction	chitosan, alginate, hyaluronan
	Hydrogen bonding	PVA, gelatin
	Crystallization	PVA after freeze-thaw cycles
	Self-assembly	Plu F127, PLGA-PEG-PLGA, PEG-PLGA-PEG
	Hydrophobic interactions	PNIPAAm, hydroxypropylmethyl cellulose
Chemical hydrogels	Polymerization	HEMA, NVP, glycidyl acrylate
	Reaction of complementary groups	glutaraldehyde + chitosan
	Enzymes	transglutaminase (TGase) + gelatin

designed geometries. The following sections of this chapter will provide a detailed overview of the different rapid prototyping techniques compatible with hydrogel manufacturing and that allow to accurately shape external and internal geometries. It is the purpose to highlight the advantages, but more importantly also the current limitations of the distinctive techniques, together with the respective hydrogels used so far.

1.3.3 Gelatin as hydrogel building block

Proteins serve as structural materials and catalysts. Potentially directing cell migration, growth and organization during tissue regeneration, as well as stabilization of encapsulated cells have been important advantages correlated to protein-based matrices.¹⁴⁰ Collagen is the most widely applied tissue-derived natural polymer, and, as described previously, is a main ECM constituent of mamalian tissues.¹⁰⁴ Being a derivative of collagen, gelatin has been extensively explored in a variety of tissue engineering approaches: it has been used as the main component in the design of tissue engineering matrices,^{138,141–145} but also applied as a coating layer in order to enhance cell-material interactions of less bioactive surfaces.^{146–152}

As a water-soluble protein, gelatin is composed of amide linked amino acids. It is a linear polymer formed by breaking the natural triple-helix structure of collagen into single strand molecules, having a molecular weight between 15,000 and 250,000 Da.¹⁵³ The amino acid composition is mainly dominated by glycine, proline and hydroxyproline, which make up for more than 50 % of the average amino acid composition.

Manufacturers offer gelatins in a variety of isoelectric points, of which basic gelatin (type A, isoelectric point of 7-9) and acidic gelatin (type B, isoelectric point of 4.6-5.4) are the most commonly used.^{104,140} Differences are related to the extraction process:

- Type A gelatin is obtained by acidic hydrolysis of collagen (pH 3.8-6);
- Type B gelatin is obtained by alkaline treatment (pH 5-7.4).

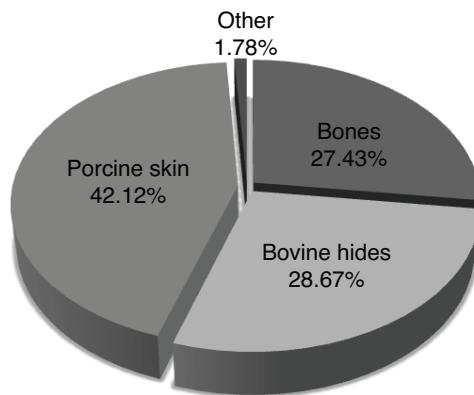


Figure 1.6: Gelatin volume by raw materials in 2011.

The reduction of the isoelectric point of type B gelatins relates to the amidolysis whereby asparagine and glutamine amino acids are converted to their respective acids (aspartic and glutamic acid). These conversions do not or only slightly occur in acidic medium. Furthermore, ornithine can be derived from arginine under alkaline conditions.

The principle raw materials are cattle bones and hides, and porcine skins and bones (Figure 1.6). However, recently, other sources such as fish derived gelatin gains interest.^{154,155} The raw materials are subjected to a series of treatment steps in order to derive a purified dry end-product.¹⁵⁶ A simplified workflow is provided in Figure 1.7. Prior to alkali or acid hydrolysis, substances such as minerals, fats, and albuminoids are removed by chemical and/or physical treatments in order to derive a purified collagen. The selected pretreatment will depend on the raw material source.

Recently, biotechnologically produced recombinant gelatins are receiving attention.¹⁵⁷ As described above, gelatins are harvested by proteolysis under acidic or alkaline conditions. As a result, heterogeneous mixtures of molecules with different molecular weights and isoelectric points are obtained. Recombinant gelatins, however, have well-defined molecular weights, amino acid sequences, and isoelectric points, determined by the gelatin-encoding gene that is incorporated in the expression system (i.e. yeast cells).¹⁵⁷ However, these remain much more expensive compared to natural gelatin.

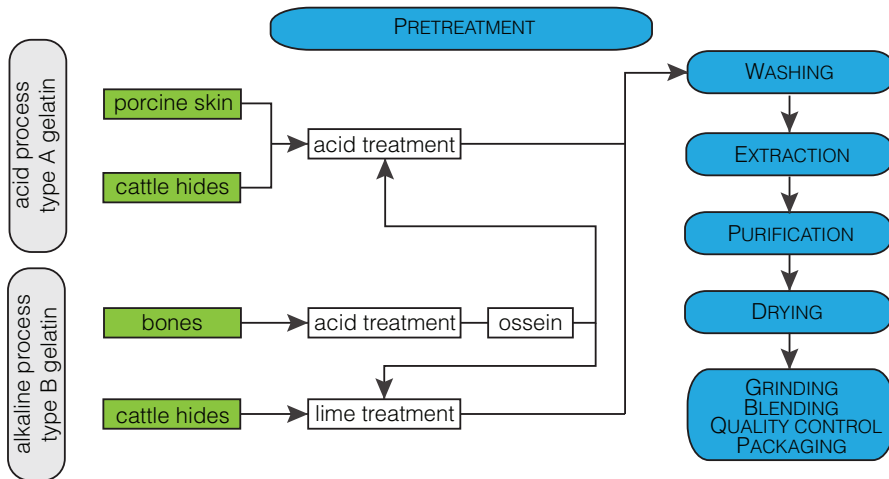


Figure 1.7: Simplified workflow for the extraction of type A and B gelatins.

In the present work, mainly gelatin-based hydrogel building blocks will be researched and designed to explore their potential as hepatic tissue engineering bio-material. Recombinant gelatins will, however, be excluded from this work.

1.4 | ECM MIMETICS: CURRENT CONCEPTS

1.4.1 Scaffold-free versus scaffold-based tissue engineering

From a cell biology perspective, 2D cell culture models only provide physiologically compromised cells induced by an unnatural environment,¹⁵⁸ and the lack of a 3D structure will cause cells to form a random 2D mono-layer.²²⁻²⁴ *In vivo*, cells are subjected to growth in three dimensions and complex cell-cell interactions. This observation encouraged a paradigm shift from conventional 2D cell culture models towards 3D microenvironments.¹⁵⁹ To obtain a more realistic understanding of cell-cell and cell-biomaterial interactions, Kirkpatrick et al.¹⁶⁰ proposed the use of co-culture models *in vitro*. Independent of the applied strategy, the ultimate goal of TE remains the same. Nevertheless, regarding the aspect of 3-dimensional cell migration, proliferation and differentiation behavior and requisites, one can distinguish two major premises. Currently both of them are being heavily explored. The first one is based on the presumption that cells require a 3D biomaterial scaffold that closely mimics the corresponding extracellular matrix (ECM).^{159,161} In this approach, the biomaterial construct acts as a necessary cell guide and supporting template. The second one finds its roots in the hypothesis that cells have a considerable potency to self-organize through cell-cell interactions and is

referred to as ‘scaffold-free TE’.¹⁶² While the former theory maximizes the role of a supporting structure as a cell guide and minimizes the potency to self-assembly, the latter reverses the importance of both contributions.

1.4.2 Scaffolds

Ideally, scaffolds can be seen as ECM biomimetic structures with three main objectives:^{22,23}

1. Defining a space that moulds the regenerating tissue;
2. Temporary substitution of tissue functions, and;
3. Guide for tissue ingrowth.

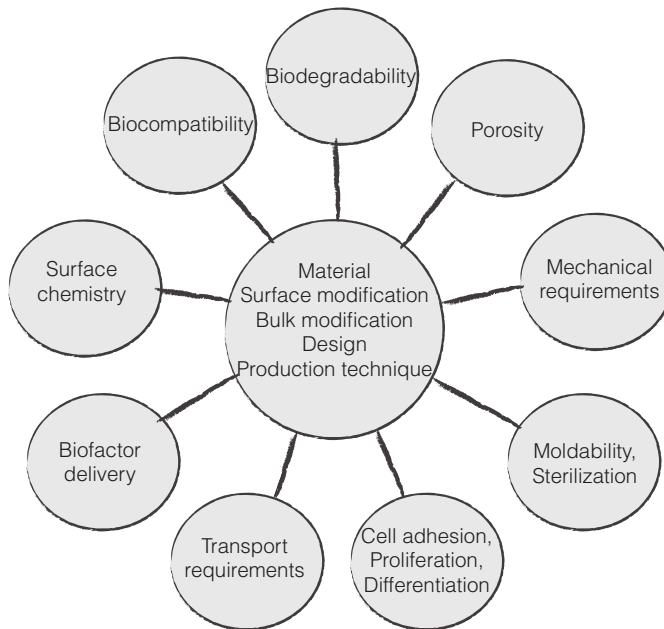


Figure 1.8: Schematic illustration integrating the complex multi-disciplinary needs, which determine the constraints for the ideal scaffold fabrication design.

It is clear that scaffold design should meet the needs of some basic requirements to be able to meet those objectives, including:^{5,20,22–24}

- High porosity (preferably 100% interconnectivity for optimal nutrient/waste flow and tissue ingrowth);

- Relevant geometry and pore dimensions (5-10 times the cell diameter);
- Biodegradable with adjusted degradation time;
- Maintaining the mechanical integrity during a prefixed time frame;
- It should have suitable cell-biomaterial interactions, and;
- Be easy to manufacture.

Adjusting the mechanical and degradation properties to the desired tissue is essential. Either enzymatic or non-enzymatic hydrolytic processes control the degradation profile. Specifically, TE requires biomaterials that provoke cell interactions ($\sim$ bioactivity)¹⁶³ and as little as possible adverse body reactions ($\sim$ biocompatibility).³⁶ Control over the material bioactivity can be achieved by incorporating growth factors,¹⁶⁴ enzymatic recognition sites,¹⁶⁵ adhesion factors,^{136,166} or material modifications.¹⁶⁵ Material modification is a general term indicating either bulk modification^{163,167} or surface modification.^{151,152,163} Modifying the bulk properties is closely related to material biocompatibility, the physical and chemical properties covering the life-span of the implant,¹⁶⁸ while varying the surface chemistry reflects on the initial cell/tissue-material interactions.^{168,169} Figure 1.8 illustrates schematically the complex multi-disciplinary interactions inherent towards scaffold fabrication.

In the sub-science of scaffolding, both conventional and rapid prototyping (RP) techniques have been explored (Figure 1.9). Conventional scaffold fabrication setups include techniques such as particulate leaching,^{123,171–173} gas foaming,^{172–175} fiber networking,^{176,177} phase separation,^{178,179} melt moulding,^{180,181} emulsion freeze drying,^{182,183} solution casting,^{184,185} freeze drying^{119,125,186} and combinations of those. Conventional/classical approaches are defined as processes that create scaffolds with a continuous, uninterrupted pore network. Nonetheless, they completely lack long-range micro-architectural channels.²⁴ Other reported disadvantages involve low and inhomogeneous mechanical strength, limited porosity and insufficient interconnectivity, inability to spatially design the pore distribution (internal channels) and pore dimensions, and difficulty in manufacturing patient specific implants (control over external geometry is limited).^{24,187} Furthermore, the use of organic solvents during processing is seen as a second major drawback in addition to the above-mentioned architectural drawbacks. The presence of organic solvent residues can pose significant constraints related to toxicity risks and carcinogenetic effects.²⁴ Despite some adaptations, over the years the scaffold design remains process-dependent by means of classical approaches. A more design-dependent method would be attractive, and this can be attained by RP techniques.

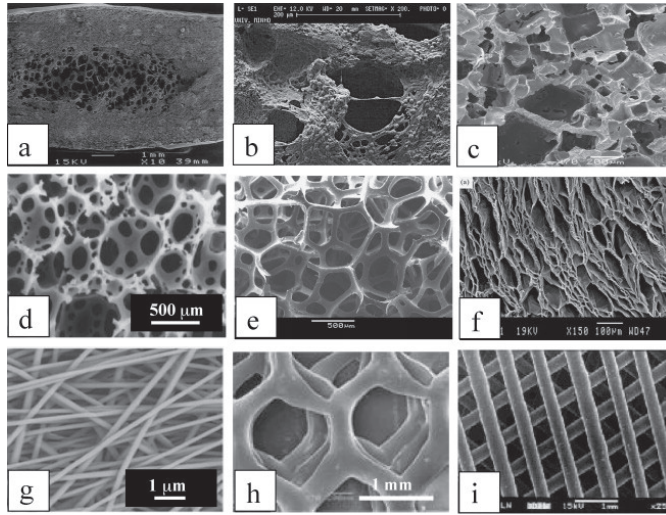


Figure 1.9: Some typical porous scaffold examples fabricated by: (a) melt moulding, (b) extrusion with gas blowing, (c) particulate leaching, (d) paraffin microsphere leaching, (e) gas foaming, (f) phase separation/freeze drying, (g) electrospinning, (h) 3D printing, and (i) fused deposition modeling. Reprinted from¹⁷⁰ with permission from Elsevier.

1.4.3 Controlling the external and internal geometry

Solid freeform fabrication (SFF) is the general term covering all techniques that produce objects through sequential delivery of energy and/or material. When rapid fabrication of a prototype, a finished object or a tool is pursued, they are respectively called rapid prototyping (RP), rapid manufacturing (RM) and rapid tooling (RT).¹⁸⁸

By means of RP, an additive computer-controlled layer-by-layer process generates a scaffold. 3D computer models shape the external design, and such models can either be designed by computer aided design (CAD) software or by modeling imaging data (computer tomography (CT), magnetic resonance imaging (MRI)). On the other hand, the internal architecture is determined by the processing of the CAD data into a STereoLithography (STL) file and subsequent slicing of the STL data (generation of the machine parameters). This directly indicates one of the greatest assets of RP: direct fabrication of scaffolds with a complex, patient specific external geometry in combination with a precise control over the internal architecture (limited by the resolution of the system).^{22,24,103} Other advantages comprise: high degree of interconnectivity, possibility to use heterogeneous materials, high speed due to a high degree of automatization and the limited number of process steps, and a superior cost-efficiency.^{5,103}

Both direct and indirect RP methods exist. In the former case, the scaffold is directly processed from a biomaterial, in the latter case the scaffold is processed out of a RP mould. Worldwide more than 30 different RP techniques are being applied in the most diverse industries, and around 20 of them found applications in the biomedical field.¹⁸⁸ Several authors have reviewed this particular subject.^{5,188,189} Despite the wide diversity of RP technologies, only some of them seem to be compatible for the processing of hydrogels. The following section describes the different RP scaffolding techniques compatible with hydrogels.

1.5 | RAPID PROTOTYPING HYDROGELS: POWERFUL AID IN MAKING SCAFFOLD-BASED TISSUE ENGINEERING WORK

A primary classification of the SFF techniques supporting biomedical applications can be made hinged on the working principle:

- (i) Laser-based systems;
- (ii) Nozzle-based systems, and;
- (iii) Printer-based systems.

Laser-based systems benefit from the photopolymerization pathway as a basis to fabricate cross-linked polymeric TE scaffolds. The well-known processing of (pre)polymers by dint of extrusion/dispension supports the second category of RP systems. The last subclass works with powder beds and deposition of a binder that fuses the particles, or directly depositing material using inkjet technology.

An important characteristic feature of every technique will be its resolution. Every technique is subdued to a lower technical limit size of the smallest details producible. This so-called lower limit shows a clear relationship with the feasible scale of the object: the higher the resolution of the smallest details, the smaller will be its maximum object size.¹⁹⁰ However, since not all RP techniques are applicable for the processing of hydrogel materials, some more than others, the amount of RP technologies is further diluted. Figure 1.10 classifies the different RP techniques with biomedical applications. The fabrication of hydrogel scaffolds requires mild processing conditions. Some of the techniques mentioned in Figure 1.10 are not able to meet those constraints due to the rather harsh processing conditions. Exploring on all of those techniques is not the purpose of this introductory chapter in which the hydrogel compatible systems only will be explained in detail.

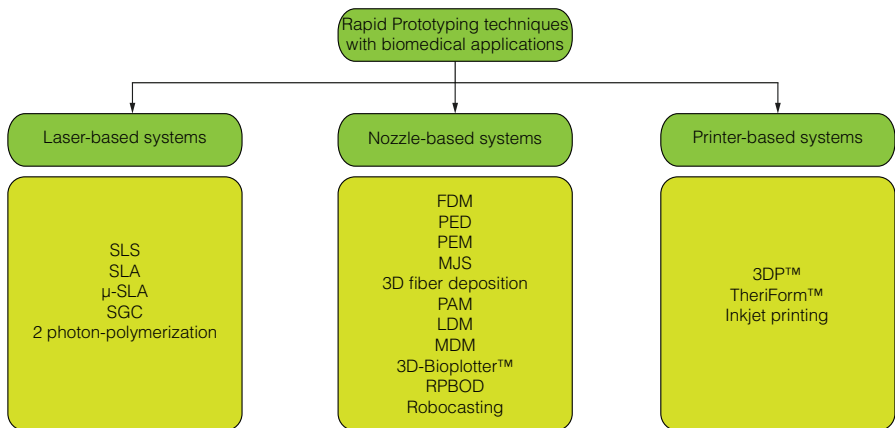


Figure 1.10: Classification of RP techniques with biomedical applications into laser-, nozzle-, and printer-based systems.

1.5.1 Laser-based systems

1.5.1.1 Working principles and recent trends of laser-based systems

With the exception of selective laser sintering (SLS), all of the laser-based systems are suitable for hydrogel processing. Unlike the nozzle- and printer-based systems that sequentially deposit material, this subclass sequentially deposits light energy in specific predefined patterns. This directly implies that only photocross-linkable prepolymers can be employed to finally obtain a cross-linked hydrogel network.

SLA: Stereolithography (SLA) is considered to be the first commercially available SFF technique, developed by 3D systems in 1986.¹⁹⁰ A SLA apparatus (Figure 1.11) consists of: a reservoir to be filled with a liquid photocurable resin, a laser source (commonly UV light), a system that controls the XY-movement of the light beam, and a fabrication platform that permits movement in the vertical plane. Scanning the surface of the photosensitive material produces 2D patterns of polymerized material through single photon absorption at the surface of the liquid material. The build-up of a 3D construct is made possible using a layer-by-layer approach, whereby the fabrication platform moves stepwise in the Z-direction after a 2D layer is finished. The step height of the fabrication platform is typically smaller than the curing depth, ensuring good adherence of subsequent layers. Post-treatment steps involving washing-off excess resin, and further curing with UV light are in most cases necessary. Arcaute et al.¹⁹¹ demonstrated the possibility to alter the resolution of the cure depth by varying the laser energy, the concentration of poly(ethylene glycol) dimethacrylate (PEG-DMA) as photocross-linkable material, and the type and concentration of the photo-initiator (PI). In addition,

adjusting the scanning speed influences the cure depth.

μ -SLA: The working mechanism of micro-stereolithography (μ -SLA) can be considered the same as that of a normal SLA. The difference between both involves the resolution of the system. μ -SLA systems are typically able to build very accurately (a few microns) objects of several cubic centimeters [134-137].¹⁹²⁻¹⁹⁵

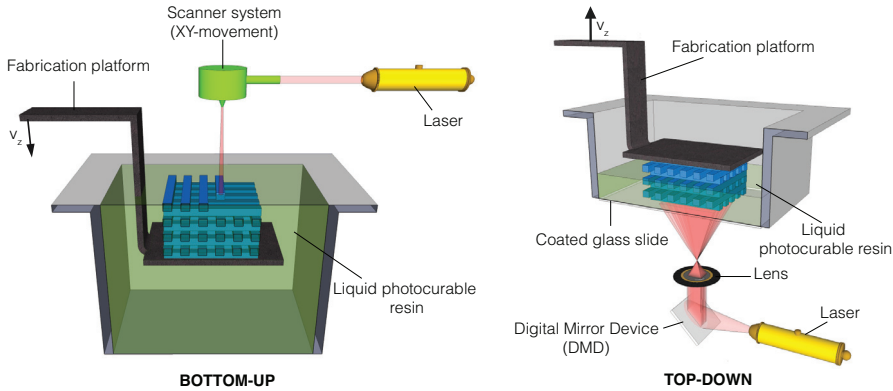


Figure 1.11: Scheme of bottom-up and top-down stereolithography setups. The bottom-up setup shown is an example of a system whereby the laser scans the surface for the curing of the photosensitive material. In the example of the top-down setup, dynamic light projection technology is used to cure a complete 2D layer at once.

Figure 1.11 (left) shows a scheme of a so-called bottom-up setup, in which an object is built from a fabrication support just below the resin surface. Subsequent layers are being cured on top of the previous layers by irradiation from above. Although, to date this is the most applied setup,^{191,193-198} a top-down approach (Figure 1.11 right) is gaining interest.^{109,190,192} Top-down setups have a non-adhering, transparent plate acting as the bottom of the liquid reservoir. Polymerization of the photosensitive material occurs through irradiation from underneath, and the fabrication platform moves in the opposite direction as in the bottom-up approach. In this way, every newly formed layer is located beneath the previous one. Separating every newly formed layer from the bottom plate will subject the structure to larger mechanical forces but on the other hand the reservoir content can be minimized, the irradiated surface will not be exposed to the atmosphere (cross-linking efficiency limitation due to oxygen will be minimized), recoating the structure with a new resin layer is not required, and the illuminated area is always smooth.¹⁹⁰ Another recent and more fundamental trend in the field of SLA is the emerging use of digital light projection (DLP) technology.^{192,198-200} The working principle

is illustrated in Figure 1.11 (right) in the top-down scheme, but is also applicable for bottom-up setups. Projection technology enables the curing of a complete layer of resin in one go, which obviously reflects on the building time. A Digital Micro-mirror Device[™] (DMD) consists of an array of mirrors, which can independently be tilted in an on/off state. In this way the DMD serves as a dynamic mask that projects a 2D pattern (often designed in PowerPoint slides) on the surface. Instead of DMD, LCD displays have also been employed as a dynamic mask projector,^{201,202} however, DMD offers better performance in terms of optical fill factor and light transmission.¹⁹⁸

SGC: A projection technology somewhat similar to DMD technology is solid ground curing (SGC) developed by Cubital Inc.^{203,204} Coating the fabrication platform by spraying a photosensitive resin is the first step in the SGC workflow. Meanwhile, the machine prints a photomask of the layer to be built on a glass plate above the fabrication platform. The printing process resembles the one applied in commercial laser printers. Solidification of the sprayed layer occurs when the mask is exposed to UV light, only permitting irradiation of the transparent regions. After the layer is completed, excess liquid resin is removed by vacuum and replaced by a wax to support the next layer. Before repeating the cycle, the layer is milled flat to an accurate, reliable finish for the next layer.

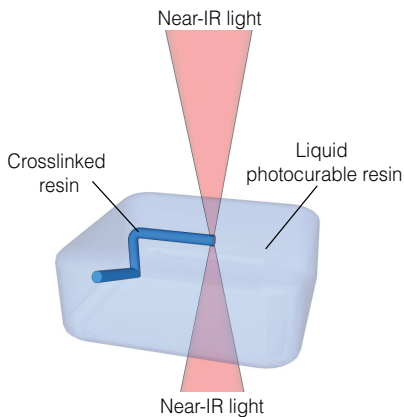


Figure 1.12: Working principle of two-photon photopolymerization. In the focal point of the near-IR laser beams, the photo-sensitive polymer is cross-linked. A ‘true’ 3D object is obtained.

2PP: Two photon-polymerization (2PP) is an emerging state-of-the-art laser-based technique. In this process, light is used to trigger a chemical reaction leading to polymerization of a photo-sensitive material. Unlike other light curing systems (single photon polymerization), 2PP initiates the polymerization through radia-

tion with near-infrared femtosecond laser pulses of 800 nm (Figure 1.12). In the focal point, a suitable PI absorbs two photons, with a wavelength of 800 nm, simultaneously, causing them to act as one photon of 400 nm, and thus starting the polymerization reaction.¹⁹² The nonlinear excitation nature triggers polymerization only in the focal point, while other regions remain unaffected. This approach has potential solidification resolutions below the diffraction limit of the applied light. Moving the laser focus enables the fabrication of a direct ‘true’ 3D object into the volume of the photosensitive material. Creating reproducible micron-sized objects with feature sizes of less than 100 nm is attainable, thus being superior to all other SFF techniques regarding accuracy and resolution.²⁰⁵

1.5.1.2 Current limitations and hydrogel feasibility for laser-based systems

SLA has already been frequently applied to develop porous hydrogel-based scaffolds (Table 1.4). Yu et al.²⁰⁶ have described the patterning of 2-hydroxyethyl methacrylate (HEMA) followed by drying and subsequent rehydration to enable cell adhesion. Initially, the procedure was applied to create single-layer structures, however, at present, multiple layers can be superimposed to generate porous 3D scaffolds. Liu et al.⁷⁰ selected poly(ethylene glycol) diacrylate (PEG-DA) hydrogels to construct 3D scaffolds layer by layer using emulsion masks. In that study, three hydrogel layers were fused at a resolution of several hundreds of microns. Interestingly, the pores were interconnected enabling cell survival through convective flow of culture medium. However, this procedure is time consuming, requires a great number of prefabricated masks depending on the required shape and is not completely automated. Therefore, one can discuss whether this can be considered as a genuine rapid prototyping technique. Lu et al.^{198,207} have adopted the above mentioned procedure to develop scaffolds possessing complex internal architectures and spatial patterns within a Z-range of several hundreds of microns. Another research group even produced PEG-based scaffolds in the millimeter scale.^{191,208–210} Several other research groups also selected PEG-DA as starting material.^{71,211} Tsang et al.⁷¹ successfully plotted 100 μm -sized complex scaffold architectures. The swelling effects of the PEG-DA, however, prevented the fabrication of highly reproducible samples below 100 μm . Higher resolutions could be obtained in the presence of UV absorbers since they prevent the internal reflection of the UV light within the polymer solution. More recently, the feasibility of plotting cell-encapsulated hydrogels has been evaluated. Several research groups have already reported on the cell encapsulation in photocross-linkable poly(ethylene glycol) (PEG) microgels.^{70,212} In addition to synthetic polymers, photocross-linkable biopolymers including hyaluronic acid (HA)²¹³ and gelatin¹⁰⁹ derivatives have already been printed with or without cells using SLA.

Table 1.4: Hydrogel materials explored in laser-based systems.

LASER-BASED SYSTEMS	HYDROGEL MATERIALS	CELL ENCAPSULATION	REFERENCE
SLA	Gelatin-methacrylate	✗	109,192,214
	Gelatin-methacrylamide		
	Hyaluronic acid-methacrylate	Murine fibroblasts (NIH 3T3) Murine embryonic stem (ES) cells	213,215
	Cystein-modified agarose	✗	216
	HEMA	✗	206
		Human hepatoma cells (HepG2) Hepatocytes NIH 3T3	
	PEG-D(MA)	Human dermal fibroblasts (HDFs) Chinese hamster ovary (CHO) cells Murine OP-9 marrow stromal cells Mammalian cells	70,71,191,196,198,212
	PEG-D(MA)	✗	200,208–211
	Alginate + Acrylated TMC/TMP	Chondrocytes	193–195
	Gelatin-methacrylamide	✗	192
2PP	PEG-DA	✗	214,217
	Gelatin-methacrylamide	✗	192,218–220
	Fibronectin	✗	220
	Bovine serum albumin (BSA)		
	PEG-DA	✗	221,222

In order to enhance the cell-interactive properties of a material, different surface functionalization strategies can be elaborated.⁷¹ Luo et al.²¹⁶ grafted Arginine-glycine-aspartic acid (RGD)-containing peptide sequences on the surface of scaffolds composed of cystein-modified agarose. Han et al.²⁰⁰ applied a fibronectin coating on the surface of a PEG-DA scaffold to improve the attachment of murine marrow-derived progenitor cells. However, important limitations of laser-based systems include both the need for photocross-linkable materials as well as the effect of the applied UV light on the encapsulated cells.²¹⁵ Since shrinkage occurs after post-processing of scaffolds developed using SLA, a major drawback is its limited resolution.¹⁹⁷ In addition, due to scattering phenomena of the applied laser beam, a significant deformation occurs when relatively small objects are developed. The produced hydrogel is often weak upon removal and post-curing is often essential.

Therefore, μ -SLA was introduced to counter the limitations of SLA from a resolution point-of-view. For example, Lee et al.^{193–195} developed a hybrid scaffold con-

sisting of an acrylated trimethylene carbonate/trimethylolpropane (TMC/TMP) framework and an alginate hydrogel for chondrocyte encapsulation. The encapsulated cells retained their phenotypic expression within the structure and the scaffold remained mechanically stable up to 4 weeks after implantation in mice. Barry et al.²¹⁷ have combined direct ink writing (DIW) with in situ photopolymerization to create hydrogel scaffolds possessing μm -sized features. Using this approach, another research group even realized submicron range structures based on PEG-DA.²¹⁴

In addition to SLA techniques, SGC also shows potential to be applied in the development of porous hydrogel-based scaffolds for tissue engineering, as already indicated before.¹⁹⁷ However, up to now, no literature data regarding this application and hydrogel processing can be found.

In order to achieve 3D subcellular resolution during scaffold development, 2PP can offer a suitable alternative for SLA. Since this technique was only introduced recently, only few reports can be found in literature regarding the application of 2PP to produce porous hydrogels. For example, Schade et al.²²¹ developed hydrogel-like scaffolds possessing well-defined 3D structures using a methacrylated polyurethane and PEG-DA as starting materials. Ovsianikov et al.²²² also selected PEG-DA as starting material for 2PP scaffolds. More recently, they evaluated the feasibility to produce porous scaffolds using methacrylamide-modified gelatin developed in our research group.²²³ The results were very recently published and demonstrated the techniques potential in the processing of biopolymers.^{218,219} Table 1.8 summarizes more technical details on the implementation of hydrogels in the different RP technologies discussed.

1.5.2 Nozzle-based systems

1.5.2.1 Working principles and recent trends of nozzle-based systems

The class of nozzle-based systems is characterized by a wide diversification (Figure 1.10). Fused deposition modeling (FDM), 3D fiber-deposition, precision extrusion deposition (PED), precise extrusion manufacturing (PEM), and multiphase jet solidification (MJS) are techniques based on a melting process. Generally, the melt process involves elevated temperatures, which are undesirable from the perspective of scaffold bioactivity.²³ Researchers have therefore tried to bring forth several other techniques that overcome this limitation by applying a dissolution process, which is attractive for the processing of hydrogels. Four major nozzle designs have been described in literature: pressure-actuated, solenoid-actuated, piezoelectric, and volume-actuated nozzles.^{224,225} These nozzle types can be found in the following systems.

PAM: A technique that resembles FDM without the need for heat is the pressure-assisted microsyringe (PAM) technique, developed by Vozzi et al.²²⁶ The setup consists of a 5-20 (μm) pneumatic-driven glass capillary syringe that can move in the vertical plane and deposits material on a substrate. The substrate proceeds in the planar field relative to the syringe. Transforming jpeg or bitmap images into a sequential list of linear coordinates easily allows depositing practically any type of structure in subsequent layers.²²⁷ Material viscosities, deposition speed, tip diameter and the applied pressure correlate with the final deposited strand dimensions. The PAM system has been described in several publications.^{228,229} Recently, the fabrication of hydrogel scaffolds was successful with the PAM method.^{230,231}

LDM: Proposed by Xiong et al.²³² in 2002, low-temperature deposition modeling (LDM) found his way as a RP system with biomedical applications. The key feature of this technique is a non-heating liquefying processing of materials.²³² Using temperatures below 0 °C, the material solution is solidified when deposited on the fabrication platform.²³³ The material gets extruded out of a nozzle capable of moving in the XY-plane onto a build platform movable in the Z-direction. Incorporating multiple nozzles with different designs into the LDM technique gave existence to multi-nozzle (low-temperature) deposition modeling (MDM, M-LDM).^{224,225,234,235} A multi-nozzle low-temperature deposition and manufacture (M-LDM) system is proposed to fabricate scaffolds with heterogeneous materials and gradient hierarchical porous structures by the incorporation of more jetting nozzles into the system.²³² Biomolecules can be applied in the LDM process to fabricate a bioactive scaffold directly.

3D-Bioplotter™: This 3D dispensing process, displayed in Figure 1.13, has been introduced by Landers and Mülhaupt in 2000 at the Freiburg Research Centre.²³⁶ The technique was specifically developed to produce scaffolds for soft-tissue engineering purposes, and simplifying hydrogel manufacturing. The three-dimensional construction of objects occurs in a laminar fashion by the computer-controlled deposition of material on a surface. The dispensing head moves in three dimensions, while the fabrication platform is stationary. It is possible to perform either a continuous dispensing of microstrands or a discontinuous dispensing of microdots. Liquid flow is generated by applying filtered air pressure (pneumatic nozzle), or using a stepper-motor (volume-driven injection (VDI) nozzle). The ability to plot a viscous material into a liquid (aqueous) medium with a matching density is the key feature of this process. Low viscous materials in particular benefit from this buoyancy compensation principle. Since heating is not required, the system can process thermally sensitive biocomponents, and even cells. Curing reactions can be performed by plotting in a co-reactive medium or by two-component dispensing using mixing nozzles. The strand thickness can be modulated by varying

material viscosity, deposition speed (speed in the planar field), tip diameter, or the applied pressure. Constructs build by this plotting technique mostly have smooth strand surfaces, which are not desired for appropriate cell attachment. Therefore, further surface treatment is required to render the surface favourable for cell-adhesion. Recently Kim et al.²³⁷ adapted the Bioplotting device with a piezoelectric transducer (PZT) generating vibrations while plotting PCL. Scaffolds build had a rougher surface and showed better cell adhesion than the ones build with the conventional setup.

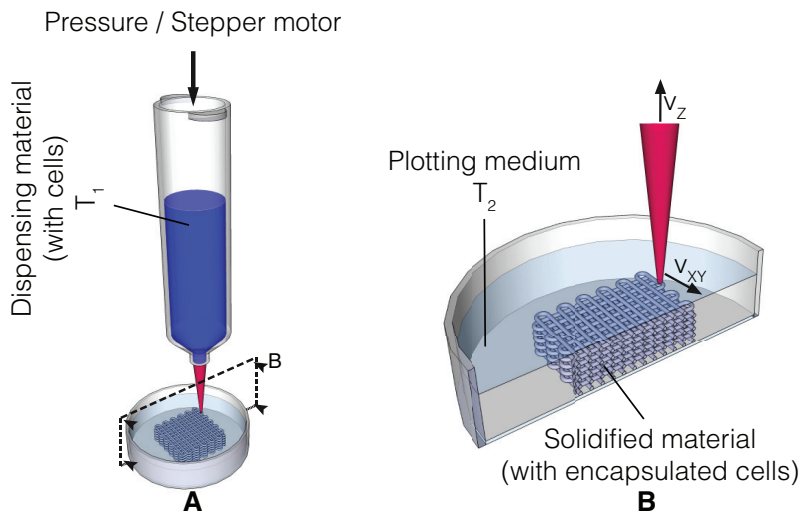


Figure 1.13: Scheme of 3D-Bioplotter™ dispensing principle. In the 3D-Bioplotter™ system, the nozzle works pneumatically or via volume-driven injection. This also illustrates the principle of nozzle-based systems in general, where a nozzle is used for the deposition of material. Key difference with other nozzle-based systems is the ability to plot into a liquid medium with matching density, thus introducing buoyancy compensation.

RPBOD: Ang et al.²³⁸ adopted an analogous concept of the 3D-Bioplotter™ technology to develop a robotic dispensing system: the rapid prototyping robotic dispensing (RPBOD) system. The setup consists of a one-component pneumatic dispenser.

Robocasting: The laminar deposition of highly loaded ceramic slurries (typically 50 – 60 vol% ceramic powder) to build a 3D construct using robotics is called robocasting.²³⁹ Unlike the bioplotting process, in most cases the robocasting setup has a stationary dispensing head, while the fabrication platform moves in the planar and vertical field. Inks used for robocasting have to flow under stress and

recover enough stiffness such that, when the stress is released, they can bear both the extruded filament weight and the weight of successive layers.^{240,241} Thus, in contrary to other SFF techniques, the concept of robocasting relies essentially on the rheology of the slurry and also on the partial drying of the deposited layers. Building-up constructs from hydrogel material alone is therefore not possible using this technique.

Direct ink writing (DIW): Direct ink writing (DIW) or direct-write assembly enables a wide variety of materials to be patterned in nearly arbitrary shapes and dimensions.^{242–244} Colloidal-based inks are patterned in both planar and 3D forms with lateral feature sizes that are at least an order of magnitude smaller than those achieved by ink-jet printing and other rapid prototyping approaches.²⁴⁵ The colloidal gels are housed in individual syringes mounted on the z-axis motion stage and deposited through a cylindrical nozzle (diameter ranging from 100 μm to 1 mm) onto a moving XY stage. The inks used in this technique must meet two important criteria. First, they must exhibit a well-controlled viscoelastic response, i.e., they flow through the deposition nozzle and then ‘set’ immediately to facilitate shape retention of the deposited features even as it spans gaps in the underlying layer(s). Second, they must contain a high colloid volume fraction to minimize drying-induced shrinkage after assembly is complete, i.e., the particle network is able to resist compressive stresses arising from capillary tension.²⁴⁶

Extruding/aspiration patterning system: The extruding/aspiration patterning system offers both extrusion, and aspiration on the basis of Bernoulli suction. The extrusion and aspiration modes are easily switchable, leading to a variety of applications in cell patterning by combining extrusion/aspiration and using a thermo-reversible hydrogel.²⁴⁷ This fabrication method makes it possible to produce cell-loaded scaffolds. One of the advantages is the possibility that cell patterns can be filled into another cell matrix. A schematic illustration of an extruding/aspiration patterning system is given in Figure 1.14. Nozzle and substrate are placed in a space that controls temperature and humidity. The nozzle is connected to the dispenser, which regulates the pressure of the compressed air, and the valve. The system operates in three modes: extrusion, aspiration, and refilling mode. Closing or opening the valve enables switching between the modes. In a closed configuration, compressed air is supplied to the nozzle and the hydrogel is extruded onto the substrate, or refilled into the aspirated groove. By opening the valve, the compressed air is directed through the valve, and the hydrogel solution is aspirated by Bernoulli suction. To prevent desiccating (to maintain the viability of the cells), a mist spray is used to create a supersaturated atmosphere inside the box.

The most recent trend is the use of hydrogel systems and cell encapsulation strate-

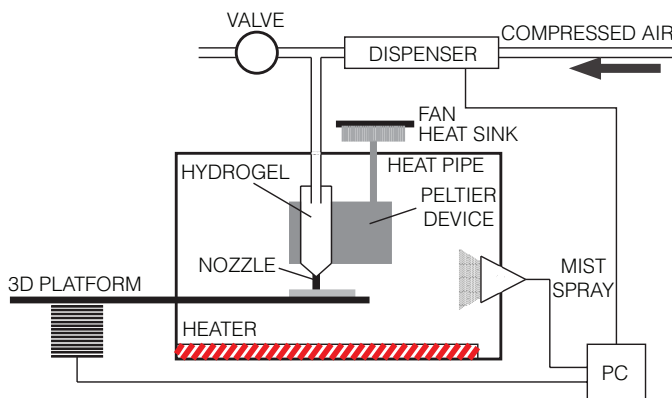


Figure 1.14: Schematic illustration of an extruding/aspiration patterning setup. The main difference with other nozzle-based systems is established through the incorporation of a mist spray, thus preventing desiccation of the fabricated constructs.

gies to fabricate gel/cell hybrid constructs.^{142,248–253} The 3D-Bioplotter and other similar techniques can be seen as the most straightforward hydrogel/cell manufacturing method for designing complex inner and outer architectures, without the need for an additional support structure. In some cases, the developed machine contains multiple nozzles with different designs (sometimes even printer-based). In literature, ‘Cell Assembler’ and ‘Bioassembly Tool’ are common machine names that have a similar working principle. Work of Chang et al.²⁵⁴ examined the effect of nozzle pressure and size on cell survival and functionality. A quantifiable loss in cell viability was seen, which was caused by a process-induced mechanical damage to cell membrane integrity. It was suggested by the authors that cells might require a recovery period after manufacturing.

1.5.2.2 Current limitations and hydrogel feasibility for nozzle-based systems

An overview of the combination nozzle-based systems/hydrogel materials is summarized in Table 1.5. Lately, cell encapsulation strategies have been frequently applied to fabricate gel/cell hybrid constructs.^{248–252} For each hydrogel material, it is indicated whether or not cell encapsulation experiments were performed. In addition, Table 1.8 comprises the more technical details, focused on the disadvantages specific for hydrogels. As shown, scaffolds produced by a 3D-Bioplotter have limited resolution and mechanical strength. Material rigidity was shown to influence cell spreading and migration speed, as demonstrated by Wong et al.²⁷⁰ Vascular smooth muscle cells displayed a preference for stiffer regions, and tended

Table 1.5: Hydrogel materials explored in nozzle-based systems.

NOZZLE-BASED SYSTEMS	HYDROGEL MATERIALS	CELL ENCAPSULATION	REFERENCE
3D BIOPLOTTER	Gelatin/Hyaluronan	X	255
	Gelatin/Alginate/Fibrinogen		
	Gelatin/Fibrinogen	Adipose-derived stromal cells (ADSC),	142,256–258
	Gelatin/Alginate	Pancreatic islets, Hepatocytes	
	Gelatin/Chitosan		
	Collagen-chitosan-hydroxyapatite	X	259
	Gelatin	Hepatocytes	260
	Gelatin-ethanolamide methacrylate,	HepG2, NIH 3T3	
	Hyaluronan methacrylate	Human intestinal epithelial cells (Int-407)	253
	PCL hybrid with alginate	chondrocyte cell line (C20A4)	261
	Collagen	X	250,251
3D BIOASSEMBLY TOOL	PCL/PLGA hybrid with acetocollagen,	Hepatocytes	141
3D CELL ASSEMBLER	Gelatin or hyaluronic acid	MC3T3-E1 preosteoblast	
	Gelatin, agar	X	262
	PEG-DA/alginate	X	263
	PEG-DA	X	264
	Plu [®] F127 AlaL	Multipotent stromal cells (MSCs)	265
		Bone marrow stromal cells (BMSCs)	
	Plu [®] F127	Human fibroblasts	82,250
		Bovine aortic endothelial cells (BAECs)	
	Alginate		
	Matrigel	BMSCs	
	Methylcellulose	Hepatocytes	82,266,267
	PEO-PPO-PEO block copolymer	Endothelial cells	
	Agarose		
	Silicone sealant Silicon SE [®]	X	236
	Polyurethanes	X	233
RPBOD	Chitosan/HA	X	238
DIW	Acrylamide/Glycerol/Water	X	217
	Polyacrylate latex particles in Plu [®] F127	X	268
	PEI-coated silica microspheres	X	242
	Polyelectrolyte complexes of polyanions (PAA) and polycations (PEI, PAH)	X	243
	Titanium diisopropoxide		
	bisacetylacetonate (TIA) and PVP	X	244
(M) - LDM	Gelatin/Alginate/Fibrinogen	hepatocytes	248
	Gelatin/Alginate/Chitosan	Primary rad ADSC	
	Gelatin, Sodium alginate	Schwann cells	249
		Primary neuron cells	
	Gelatin/Chitosan and type I Collagen	X	225
PAM	Polyurethane elastomer (Polytek 74-20)	X	269
ROBOCASTING	Plu [®] F127	X	240
EXTRUDING/ASPIRATION PATTERNING SYSTEM	Mebiol gel(N-isopropylacrylamide and polyoxyethyleneoxide)	Sf-9 insect cells	247

to migrate faster on surfaces with lower compliance. In addition, the 3D-Bioplotter technology is a time consuming technique due to the optimization of the plotting conditions for each different material. Ang et al.²³⁸ reported 3D chitosan and chitosan-HA scaffolds using the RPBOD. Solutions of chitosan or chitosan-HA were extruded into a sodium hydroxide and ethanol medium to induce precipitation of the chitosan component. The concentration of sodium hydroxide was identified as important in controlling the adhesion between the layers. The scaffolds were then hydrated, frozen and freeze-dried. Prior to cell culturing with osteogenic cells, the scaffolds were seeded with fibrin glue. Drawbacks of this technique largely follow those of the 3D-Bioplotter and analogues. The inks used in the direct ink writing (DIW) are limited by their flow criteria itself.

Khalil et al.²²⁴ developed a multi-nozzle low-temperature deposition system with four different micro-nozzles: pneumatic microvalve, piezoelectric nozzle, solenoid valve and precision extrusion deposition (PED) nozzle. The system consisted of an air pressure supply. Multiple pneumatic valves were simultaneously operated for performing heterogeneous deposition in the development of the 3D scaffold. With this technique, multi-layered cell-hydrogel composites can be fabricated.²⁵¹ Hydrogels have also been processed with the PAM technique.^{230,231} Of the 3D rapid prototyping micro-fabrication methods available for tissue engineering, PAM has the highest lateral resolution. Recently, it has been demonstrated that the performance of this method is comparable to that of soft lithography.²⁷¹ However, capillaries with a very small diameter require careful handling to avoid any tip breakage. In addition, pressures are needed to expel the material from a small orifice. Robocasting relies on the rheology of the slurry and partial drying of the deposited layers. This implies that a pure hydrogel composition cannot be processed via this particular technique, being the most fundamental drawback of the technique. A last nozzle-based system is the extruding/aspiration patterning system. One of the advantages is that its setup is favourable for cell encapsulation purposes and the fact that cell patterns can be filled into another cell matrix. However, the hydrogel materials require a small temperature hysteresis, so it has limited applicability.

Concerning the nozzle-based systems in rapid prototyping of hydrogels, several challenges need to be addressed. Looking at the limited range of materials, the following topics should be addressed: optimal scaffold design, bioactivity of the scaffold as well as the issues of cell seeding and encapsulation possibilities. So, future development will need to focus on the engineering of new materials, the scaffold design and the input of cell biologists. Keeping this in mind, rapid prototyping still remains a promising technique as a methodical interface between tissue and engineering.

Concerning (non home-made) RP devices in general, high equipment purchase costs can be considered a substantial disadvantage. Therefore, it is also noteworthy to mention that very recently open-source low-budget nozzle-based systems have found their way to this research domain. A hydrogel compatible example is the Fab@Home (\$ 1,000–3,000) system that was developed at Cornell University. Cohen et al.²⁶⁶ tested a proof-of-concept for in situ repair of osteochondral defects using alginate as scaffolding material. Lixandrao et al.²⁶³ demonstrated the feasibility of complex architecture scaffolding with the Fab@Home. They constructed aortic valves based on a PEG-DA/alginate blend.

1.5.3 Printer-based systems

1.5.3.1 Working principles and recent trends of printer-based systems

In literature, ‘printing’ is often used as a general term for both the construction of a scaffold or to indicate printer-based systems. To differentiate between both, we define the latter as manufacturing techniques that implement inkjet technology. Inkjet printers can be divided in drop-on-demand or continuous ejection types. In drop-on-demand systems, electrical signals are used to control the ejection of an individual droplet. In continuous-drop systems, ink emerges continuously from a nozzle under pressure. The jet then breaks up into a train of droplets whose direction is controlled by electrical signals.²⁷² Both drop-on-demand and continuous-jet systems can be operated with droplets ranging in size from 15 to several hundred microns.²⁷² Many commercial (adapted) printers fall in the former category, and will only eject ink when receiving a demand signal from the computer. Table 1.6 classifies the existing inkjet printers (modified from Nakamura et al.²⁷³). Like the nozzle-based systems, building a construct occurs in an additional computer-controlled layer-by-layer sequence with deposition of material.

Table 1.6: Classification of inkjet printers.

Inkjet printers	Drop-on-demand	Electromechanical (piezo-actuated and electrostatic actuated)
		Electrothermal (thermal actuated)
		Electrostatic vacuum
	Continuous ejection	Electro-field controlled inkjet
		Hertz continuous inkjet (mutual charged droplet repulsion type)

3DP™: Prof. Sachs from the Massachusetts Institute of Technology (MIT) introduced the 3D Printing™ technology.²⁷⁴ It is an example of a solid-phase RP

technology. 3D Printing can be used to fabricate parts in a wide variety of materials, including ceramic, metal, metal-ceramic composite and polymeric materials. 3D printing is the only of the solid-phase RP techniques compatible with hydrogel manufacturing. A scheme of a typical 3DP™ setup is given in Figure 1.15. The technique employs conventional inkjet technology. The workflow can be described in 3 sequential steps: (1) the powder supply system platform is lifted and the fabrication platform is lowered one layer; (2) the roller spreads the polymer powder into a thin layer (excess powder falls in an overflow reservoir), and; (3) an inkjet print head prints a liquid binder that bonds the adjacent powder particles together. The binder can dissolve or swell the powder particles, causing bonding via the inter-diffusion of polymer chains or via infiltration of the binder into the powder.²⁷⁵ Cycling steps 1–3 fabricate a 3D object. A key requirement for 3D printing is the availability of biocompatible powder-binder systems.²² The powder utilized can be a pure powder or surface-coated powder, depending on the application of the scaffold. It is possible to use a single, one-component powder, or a mixture of different powders blended together.²³ After the finished construct is retrieved, any unbound powder is removed, resulting in a complex three-dimensional part. Basic requirements that the binder system must satisfy: (i) the binder solution must have a high binder content while still having a low viscosity so that it is capable of being deposited by the print head; (ii) minimal conductivity may be required for continuous jet printing heads; (iii) the binder must dry or cure rapidly so that the next layer of particles can be spread.²⁷²

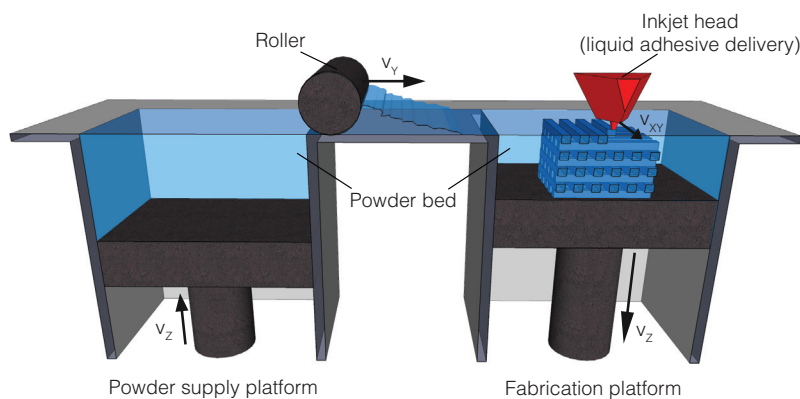


Figure 1.15: Schematic representation of a typical 3DP™ setup. A roller spreads a thin layer of polymer powder over the previously formed layer, and is subsequently solidified by the spatially controlled delivery of a liquid binder.

3D Printing has four steps that can limit the rate of the process: the application of

the powder layers, the printing of the binder, the infiltration of the binder into the powder and the drying of the binder.²⁷² An important advantage of powder-based systems is the production of rougher surfaces, which may enhance cell adhesion.²²

Zcorp developed a 3D printer (Z402) that uses natural polymers as well as plaster of Paris in combination with a water-based ink.^{275,276} This opens perspectives towards hydrogel manufacturing. A recent detailed review on 3DPTM concerning all process development steps, such as powder-binder selection and interaction, is given by Utela et al.²⁷⁷

In 3DP, control over the geometry is realized by two distinct issues: the minimum attainable feature size, and the variability of part dimensions.²⁷² Both depend strongly on the binder droplet-powder particle interactions. Factors controlling the interaction of powder and binder include: powder material, powder surface treatment, powder size and size distribution, powder shape, powder packing density, binder material, binder viscosity, binder surface tension, droplet size, droplet velocity, temperature of the powder and binder, and ambient temperature.²⁷² Factors that determine the final object dimensions are: local and accumulative accuracy of deposited layer thickness, accuracy of drop placement, reproducibility of the spread of the printed droplets, and reproducibility of the dimensional changes that accompany binder cure. Sometimes, resolution of the machine is mentioned. Resolution in this context refers to the smallest pores and the thinnest material structures that are obtainable with the equipment.²⁷²

Inkjet printing: This printer-based subclass comprises all liquid-phase inkjet technologies. It can vary from setups similar to the 3DPTM system in which the powder bed is replaced by a liquid hydrogel precursor,²⁷⁸ or systems that use direct inkjet writing.^{18,279} In the case of direct inkjet writing, the construct is build up by the deposited liquid itself.

1.5.3.2 Current limitations and hydrogel feasibility for printer-based systems

Printer-based systems can perhaps be regarded as the least hydrogel/cell suitable of the systems that allow hydrogel processing. Tables 1.7 and 1.8, summarize the hydrogel feasibility respectively limitations towards hydrogel manufacturing. Wu et al.²⁸⁰ described the use of polyethylene oxide (PEO) and poly- ϵ -caprolactone (PCL) as matrix materials and a 20 % PCL-lipopolysaccharide(LPS)/chloroform binder solution to create a 3D device for controlled drug release. Top and bottom layer of the tabular device was made out of slowly degrading PCL, while the interior layers were composed of PEO bound by printing binder solutions. The local microstructure of the device could be controlled by either changing the binder

or by changing the printing parameters (velocity). Typical powder particle size ranged from 45 to 75 μm for PCL and 75-150 μm for PEO. The binder droplets had a diameter in the order of 60-80 μm . After 20 hours, significant swelling of the PEO was experimentally observed.

Table 1.7: Hydrogel materials explored in printer-based systems.

PRINTER-BASED SYSTEMS	HYDROGEL MATERIALS	CELL ENCAPSULATION	REFERENCE
3DP™	Starch/Cellulose/Dextrose	✗	275,276
	Starch/Cellulose fiber/ Sucrose/Maltodextrin	✗	281
	Corn starch/Gelatin/Dextran	✗	276
	Starch/Polyurethanes/PEG	✗	275
	PEO/PCL	✗	280
	PLLGA/Pluronic® F127	✗	282
	HA/Cellulose/Starch	✗	283
	PEG/Collagen/PDL	✗	284
INKJET PRINTING	PNIPAM, Collagen	bovine aortal endothelial cells	285
	Alginate/Gelatin	mouse endothelial cells (ATCC CRL-2581)	278
		rat primary hippocampal and cortical cells	
	Fibrin	human microvasculature endothelial cells (HMVEC)	286,287

Landers et al.²³⁶ studied the use of water-soluble polymers, which are bonded together by means of water-based saccharide glues. Although the choice of powders and the corresponding adhesives appears to be unlimited, this technology requires post-sintering or post-curing to improve mechanical as well as environmental stability.

Lam et al.²⁷⁶ developed a blend of starch-based powder containing cornstarch (50 wt %), dextran (30 wt %) and gelatin (20 wt %). Distilled water was used as a suitable binder material. Cylindrical scaffolds ($\emptyset$ 12.5 $\times$ 12.5 mm) were produced having either cylindrical ($\emptyset$ 2.5 mm) or rectangular (2.5 $\times$ 2.5 mm) pores. Using water as the binder means that the problem of a toxic fabrication environment was eliminated and the problem of residual solvent in the construct was solved. Other advantages of using a water-based binder include the possibility to incorporate biological agents (e.g. growth factors) or living cells. Post-processing of the scaffolds was necessary to enhance the strength of the scaffolds and increase the resistance against water uptake. The scaffolds were dried at 100 °C for 1 hour after printing and infiltrated with different amounts of a copolymer solution consisting of 75 % poly(L-lactide) and 25 % PCL in dichloromethane.

Sanjana et al.²⁸⁴ report on the use of ink-jet printing to fabricate neuron-adhesive

patterns such as islands and other shapes using poly(ethylene) glycol (PEG) as cell-repulsive material and a collagen/poly-D-lysine (PDL) mixture as cell-adhesive material. They worked with a positive relief: PEG used as background and anti-fouling material was bonded covalently to the glass surface while the collagen/PDL mixture was used as the printed foreground and cell-adhesive material. They also suggest that the ink-jet printing technique could be extrapolated to building 3D structures in a layer-by-layer fashion.

Xu et al.²⁸⁶ use the inkjet printing technology for the construction of three-dimensional constructs, based on fibrin gel. Fibrin was used as a printable hydrogel to build 3D neural constructs. The fibrin is formed by the enzymatic polymerization of fibrinogen by addition of thrombin and calcium chloride CaCl_2 . First, a thin sheet of fibrinogen was plated and subsequently, thrombin droplets were ejected from the print cartridge onto the pre-plated fibrinogen layer. Fibrin gel formation was observed immediately after thrombin ejection. Subsequently, NT2 neurons were printed on the gelled fibrin. The whole procedure was repeated 5 times, resulting in a 3D neural sheet.

Koegler et al.²⁸² described the fabrication of 3DP scaffolds based on poly(L-lactide-co-glycolide). Surface chemistry of these scaffolds was modified by reprinting the top surface with a solution of Pluronic F127 in CHCl_3 .

Cui et al.²⁸⁷ reported on the fabrication of micron-sized fibrin channels using a drop-on-demand polymerization. A thrombin/ Ca_2^+ solution together with human micro-vascular endothelial cells (HMVEC) cells was used as ‘bio-ink’ and sprayed by the inkjet technology onto a fibrinogen substrate. They suggest that these constructs show potential in building complex 3D structures. Examples of the direct use of printer-based systems together with hydrogels are rather limited. In some cases, the use of an indirect system is mentioned.

Sachlos et al.²⁸⁸ use an indirect approach to produce collagen scaffolds with pre-defined and reproducible complex internal morphology and macroscopic shape by developing a sacrificial mould, using 3D printing technology. This mould is then filled with a collagen dispersion and frozen. The mould is subsequently removed by dissolution in ethanol and a solid collagen scaffold was produced using critical point drying.

Yeong et al.²⁸⁹ also utilized a similar indirect approach to fabricate collagen scaffolds. In addition, they investigated different drying routes after removal of the sacrificial mould with ethanol. The effects of a freeze-drying process after immersion of the scaffold in distilled water and critical point drying with CO_2 reflected onto dimensional shrinkage, pore size distribution and morphology in general.

Boland et al.²⁷⁸ described the use of the inkjet printing technique for the construction of synthetic biodegradable scaffolds. They used a 2% alginic acid solution, a liquid that is known to cross-link under mild conditions to form a biodegradable hydrogel scaffold. The ink cartridge was filled with 0.25 M (CaCl_2), which is known to promote the cross-linking of the individual negatively charged alginic acid chains resulting in a 3D network structure. This cross-linker was printed onto liquid alginate/gelatin solutions.

The biggest obstacles for RP technologies, thus also printer-based systems, are the restrictions set by material selection and aspects concerning the design of the scaffold's inner architecture. Thus, any future development in the RP field should be based on these biomaterial requirements, and it should concentrate on the design of new materials and optimal scaffold design.²³ The selected scaffold material must be biocompatible, compatible with the printing process, and it must be easily manufactured in the form required (powder or liquid).²⁹⁰ In the case of powder material, the particle size must be controllable. Another issue is the sterility of the manufacturing process and products and their ability to withstand sterilization processes.²² Off course, this plays a pivotal role for all systems when embedding cells during the process.

Some limitations are caused by material trapped in small internal holes. These trapped liquid or loose powder materials may be difficult or even impossible to remove afterwards, and in some cases, these residues may even be harmful to cells and tissues. Experimental results show that the smoother the surface generated, the easier the removal of trapped material.^{22,23} Smoother surfaces are on the other hand less desirable for cell adhesion purposes.

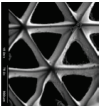
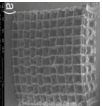
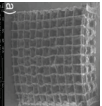
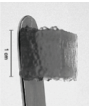
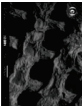
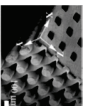
Limitations of 3DP include the fact that the pore sizes of fabricated scaffolds are dependent on the powder size of the stock material. As such, the pore sizes available are limited to smaller pore values ($< 50\ \mu\text{m}$) widely distributed throughout the scaffold. More consistent pore sizes, including larger pores, can be generated by mixing porogens (of pre-determined sizes) into the powder prior to scaffold fabrication. However, incomplete removal of the porogens is sometimes observed due to incomplete leaching. The mechanical properties and accuracy of 3DP fabricated scaffolds are other considerations that need to be addressed.²⁸³

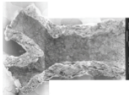
Despite the idea of using a water-based ink in order to eliminate a toxic fabrication environment, and thus creating an opportunity to incorporate biological agents or even living cells, toxic post-processing of the constructs is often needed to improve the mechanical properties. Suwanprateeb²⁸¹ described a double infiltration technique to increase the mechanical properties of natural polymers fabricated by three-dimensional printing using a water-based binder. The 3DP parts were

porous in nature since the powder bed was only lightly packed during the process and only the surface of the powder granules was connected by a binder. Porosity typically ranged between 50 and 60 %. To enhance the performance of the 3DP parts based on a mixture of 40 wt % starch, 15 wt % cellulose fiber, 25 wt % sucrose sugar and 20 wt % maltodextrin, infiltration by some other material was performed. The infiltration material used in this experiment was a heat-cured dental acrylate prepared by mixing triethylene glycol dimethacrylate, 2,2-bis[4(2-hydroxy-3-methacryloyloxypropyloxy)-phenyl]propane and a polyurethane dimethacrylate in a 40 : 40 : 20 wt % proportion. Benzoylperoxide was used as initiator. After infiltration, the specimen was cured at 105 °C for 30 min. From the results, it was found that double infiltration and curing of 3DP samples increased the performance of specimens in wet conditions.

Pfister et al.²⁷⁵ described the fabrication of biodegradable polyurethane scaffolds by 3D Printing. In this case, the polyurethane formation is the post-treatment step. Commercially available powder ZP11 (a powder blend of starch, short cellulose fibers and dextrose as a binder) was processed by the printing of an aqueous ink, which activates the dextrose binder. The resulting objects were very fragile and highly water-soluble. Therefore, the authors selected an additional post-treatment step involving infiltration and partial cross-linking with lysine ethylester diisocyanate . The starch polyols react with the isocyanate to form network structures. The obtained structures exhibited much improved mechanical stability. Because of the presence of starch incorporated in the network, the structures could swell and the resulting lysine-based polyurethane networks were biodegradable upon exposure to water and body fluids.

Table 1.8: General properties of hydrogel compatible RP techniques.

TECHNIQUE	DRAWBACKS	RESOLUTION	POROSITY	EXAMPLE ^a	REFERENCE
SLA	Scaffold shrinkage due to water evaporation Incomplete conversion thus post-curing essential Limited resolution Limited availability of cells, non-homogeneous cell distributions, cytotoxic photo-initiator Complex architectures with tunable micro- and macroscale features are difficult to achieve	30 μ m	< 90%	 Source: 109	109, 192 190 197 196 222
	Lower resolution than 2PP Complex architectures with tunable micro- and macroscale features are difficult to achieve Post-curing can be necessary	1 μ m	< 90%	 Source: 198	199 222
	Not feasible to produce large scaffolds Time consuming adjustment to new materials Scaffold shrinkage due to water evaporation Diffusion driven polymerization is possible resulting in cross-linking of non-irradiated material	< Diffraction limit of applied light	Not specified	 Source: 219	192 137, 275 219 219
LAZER-BASED SYSTEMS					
2PP	Not feasible to produce large scaffolds Time consuming adjustment to new materials Scaffold shrinkage due to water evaporation Diffusion driven polymerization is possible resulting in cross-linking of non-irradiated material	< Diffraction limit of applied light	Not specified	 Source: 219	192 137, 275 219 219
3D BIOPLOTTER 3D BIOASSEMBLY TOOL, 3D CELL ASSEMBLER	Low mechanical strength Smooth surface Low accuracy Slow processing Precise control of properties of materials and medium (i.e., mechanical properties of the solution must be high enough to form the 3D structure) Calibration for new material Fused horizontal pores mentioned in some cases	45-1600 μ m	< 45-60%	 Source: 262	82, 202, 205
RPBOD	Item 3D Bioplotter Precise control properties of material and medium Requires freeze drying	Not specified	Not specified	 Source: 238	238
NOZZLE-BASED SYSTEMS					
DIW	Low mechanical strength smooth surface Low accuracy Slow processing Precise control of properties of materials and medium	5-100 μ m	< 90%	 Source: 291	292, 293

TECHNIQUE		DRAWBACKS	RESOLUTION	POROSITY	EXAMPLE ^a	REFERENCE
NOZZLE-BASED SYSTEMS	(M)-LDM	Solvent is used Requires freeze drying	300-500 μm	75-90%	 Source: ²²⁵	232, 294
	PAM	Small nozzle inhibits incorporation of particle Narrow range of printable viscosities Solvent is used Highly water soluble materials cannot be used	7-500 mm	71-94%	 Source: ²²⁹	228
	ROBOCASTING	Precise control of ink properties is crucial	100-150 mm	< 45%	 Source: ²⁴⁰	295
	EXTRUSION/ASPIRATION PATTERNING SYSTEM	A small thermal hysteresis of the products is required Limited applicability	141-300 mm	Not specified	 Source: ²⁴⁷	295
PRINTER-BASED SYSTEMS	3DP INKJET PRINTING	Mechanical strength: post-processing often necessary Powder entrapment Availability in powder form	100 μm	33-60%	 Source: ²⁷⁵	236, 275, 276, 281 23 275, 276, 280-282
		Pore size: dependent on powder particle size	50-250 μm		 Source: ²⁸³	22, 283, 390
		Binder droplet size and accuracy of drop placement (resolution of the machine) Clogging of small binder jets	Droplet Volume: 80-130 μL	50%	 Source: ²⁸⁸	272, 276, 280, 284, 286, 287 272
		Indirect methods needed (sacrificial molds)	200-500 μm			288, 289

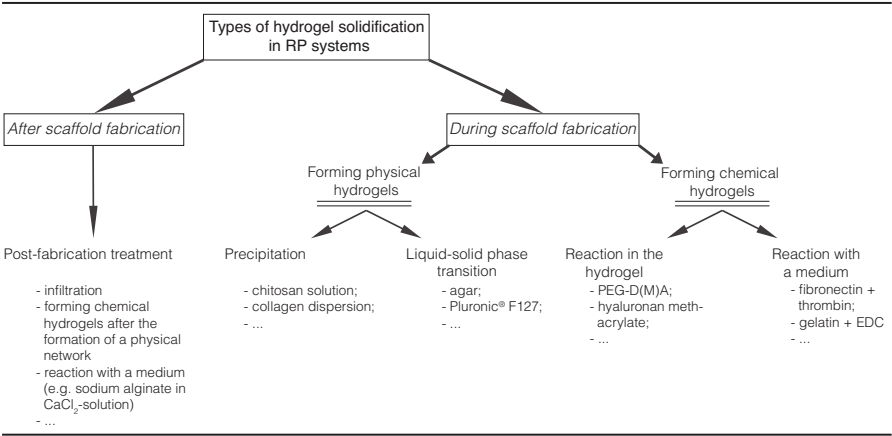
(a) All examples figures are reprinted with permission from the respective publishers as indicated in the source.

1.5.4 Preserving the mechanical integrity of RP processed hydrogels

As previously mentioned, the mechanical properties of a certain biomaterial play a partial, although crucial role in its potential success as a scaffolding material. More specific, it has been generally accepted that in designing a proper scaffold biomaterial, one must strive to translate the mechanical characteristic features of the target tissue into the mechanical features of the fabricated construct. For instance, hard tissue regeneration requires strengths of 10-1500 MPa, while soft tissue strengths are typically located between 0.4 and 350 MPa.²⁹⁶ As a part of it, preserving the mechanical integrity of the scaffold contributes substantially to the completion of this demand. The latter appears to be of utmost importance with respect to hydrogels and will for that reason be discussed in this subsection. Moreover, it is possible to enhance the control over not only the mechanical properties, but even the biological effects and degradation kinetics by hierarchical design of scaffolds with micron to millimeter features.²⁸³ In the case of hydrogel performance, degradation rates are controlled by hydrolysis, enzymatic reactions or simply by dissolution of the matrix (e.g. ion exchange in Ca_2^+ cross-linked alginate systems).

The process of obtaining a construct with suitable strength starts with the solidification and simultaneous shaping of the material in a certain pattern. An overview summarizing the most frequently applied and different solidification mechanisms is given in Table 1.9. Although our discussion focuses on hydrogel materials, other materials could easily be incorporated into this scheme.

Table 1.9: Classification of the most frequently applied hydrogel solidification mechanisms in RP processing.



The application of natural as well as synthetic hydrogels in TE and as cell embedding materials has been a review topic of several authors.^{17,58,65,104} The solidification or gelling mechanisms of hydrogels include inherent phase transition behaviour and cross-linking (ionic or covalent) approaches. Regarding the former mechanism, careful control over the printing temperatures can provide for some hydrogels a phase transition from solution to gel state, in particular for polymers with a lower critical solution temperature (LCST) behaviour (e.g. Plu[®] F127). However, this behaviour is reversible. The formation of an ionically cross-linked network through the use of multivalent counterions, e.g. sodium alginate and Ca₂⁺ ions, provides more control over the mechanical integrity. Nevertheless, these ions could be leached out in long-term culture, or even be exchanged by other ionic molecules, compromising the control over the construct properties. Therefore, in most cases, covalent network formation is required in order to precisely enhance the mechanical stability and reproducibility of the constructs. The light-induced radical cross-linking of monomers and/or prepolymer solutions have established a quasi monopoly as a hydrogel solidification strategy in combination with RP fabrication schemes. In the case of the laser-based systems, this is even the fundament on which the respective techniques are based and those are striking examples of one-step cross-linking approaches. The chemical structures of some well-known photo-initiators (PIs) are shown in Figure 1.16. The first structure (Figure 1.16(A)) is an example of a D- π -D chromophore, known for its high sensitivity in 2PP processes.^{297,298} The π -part is a conjugated backbone symmetrically substituted at the ends by electron-donating D-parts. Figure 1.16(B) represents the chemical formula of Irgacure[®] 2959 (I2959), a commonly applied photo-initiator thanks to its high biocompatibility. It is known to absorb light in the UV range. The last structure represents camphorquinone (CQ; Figure 1.16(C)), an initiator with many dental applications and visible light working range.

Different reactive thermo-sensitive thiolated systems were developed in the past.^{299–306} Thiol, vinyl or allyl ether polymerization display some advantages: mild reaction conditions, photo-initiator not necessary, low or even no oxygen inhibition effects, fast process, forming of cross-linked networks with good physical and mechanical properties³⁰⁷ and degradable in (mimicked) physiological conditions.³⁰⁸ The so-called ‘tandem reaction’ consists of two steps. The first step is the occurrence of rapid gelation kinetics. The second step involves a covalent curing based on the Michael-type addition. This technique allows to create covalently bonded hydrogels in combination with functional cells under physiological conditions, with no side reactions with bioactive molecules.³⁰⁹

When performing cross-linking in general, be it radical (light, redox, temperature induced) or non-radical (enzymatic, ionic, carbodiimide, glutaraldehyde,

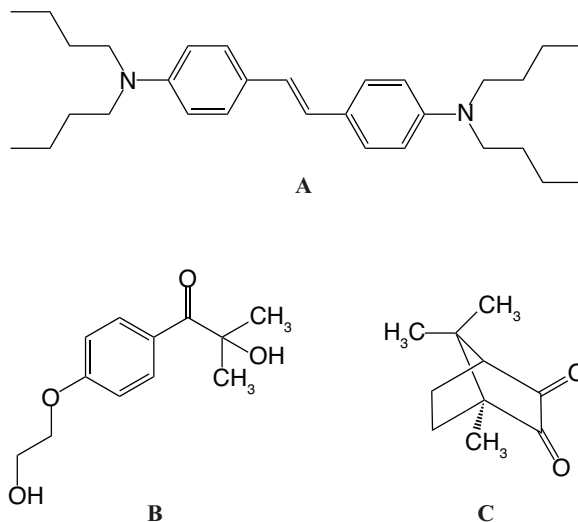


Figure 1.16: Some typical photo-initiators used for (A) 2PP – an example of a strongly absorbing two photon absorbing D- π -D chromophore –, (B) general UV curing – Irgacure® 2959–, and (C) visible light curing – camphorquinone –.

genipin,...), the toxicity of the cross-linkers should be carefully considered. This aspect is of crucial importance towards the success of a construct. For instance, comparing the biocompatibility of genipin and glutaraldehyde as a way to non-radically cross-link gelatin demonstrated the cytotoxic effect of glutaraldehyde and to a lower extent that of genipin.^{72,249} A possible aldol condensation cross-linking mechanism for genipin was proposed by Liang et al.³¹⁰ and the authors also provided evidence for its better biocompatibility compared to carbodiimide cross-linking with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). However, genipin has only few applications because of its high cost, and dark blue staining, which could interfere with cell characterization techniques.²⁴⁹ Thrombin, on the other hand, did not show any substantial adverse effects.^{249,256} Next to those non-radical cross-linking mechanisms, radical cross-linking induced through light irradiation is accepted as a common strategy. Cytotoxicity studies of several frequently used UV and visible light initiating systems have been performed.^{311,312} CQ at concentrations $\leq 0.01\%$ (w/w) and low intensity irradiation ($\sim 60\text{ mW cm}^{-2}$; 470–490 nm) appeared to have less toxic effects than isopropyl thioxanthone (ITX) as visible light initiator. Another promising visible light initiator that absorbs at 512 nm is eosin Y,^{313,314} although up to date no relevant cytotoxicity studies have been performed. Out of three different Irgacure® initiators, it was the I2959 ini-

tiating system ($\sim 6 \text{ mWcm}^{-2}$; 365 nm) that held the greatest potential at concentrations below 0.05 % (w/w). Additionally, it was also found that those cytotoxic effects varied depending on the cell types.^{312,315} Performing radical cross-linking reactions without the use of either visible or UV light can also be mediated through the use of redox systems. Duan et al.³¹⁶ reported on the negative cooperative effect of a water-soluble redox initiating system, consisting out of ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediamine (TEMED), on NIH/3T3 fibroblasts. The system was used to trigger PEG-DA polymerization. Redox initiating systems have been extensively reviewed before.³¹⁷

In other cases, the formation of chemical hydrogels can only be attained after scaffold fabrication. Post-fabrication UV curing of a physically cross-linked photosensitive Plu F127 AlaL construct with Irgacure[®] 2959 as initiator was for instance performed by Fedorovitch et al.²⁶⁵ in order to acquire a chemical network. This example directly serves as an easy template into attaining multiple-step cross-linking. When working with blends of different hydrogels and combinations of physical as well as chemical network formations, multiple-step cross-linking systems of higher complexity are needed. Very recently, Xu et al.¹⁴² demonstrated a fascinating state-of-the-art example of such an approach by blending gelatin, sodium alginate and fibrinogen with cells and stepwise cross-linking of the sodium alginate with a CaCl_2 solution and the fibrinogen with a thrombin solution. The gelatin component served as a necessary co-material blend in to provide sol to gel phase transition during the fabrication process.²⁴⁹ Other examples can be found in the work of Xu et al.,²⁵⁶ Zhang et al.,²⁵⁵ Skardal et al.²⁵³ and, Yan et al.²⁵⁷

1.6 | FUNDAMENTALS ON RAPID PROTOTYPING IN SCAFFOLD-FREE TISSUE ENGINEERING

In general, the application of scaffolds in a TE approach is straightforward, but still subject to some challenges.^{318,319} These can be divided in two distinct categories: (i) complications posed by host acceptance (immunogenicity, inflammatory response, mechanical mismatch), and; (ii) problems related to cell cultures (cell density, multiple cell types, specific localization). It is surprisingly interesting that, during embryonic maturation, tissues and organs are formed without the need for any solid scaffolds.³²⁰ The formation of a final pattern or structure without externally applied interventions, in other words the autonomous organization of components, is called self-assembly.^{321,322} A premise concerning the self-assembly and self-organizing capabilities of cells and tissues is worked out in the field of scaffold-free TE. This idea poses an answer to the immunogenic reactions and other unforeseen complications elicited through the use of scaffolds. One

way of implementing this self-assembly concept is the use of cell sheet technology, which was demonstrated by L'Heureux and colleagues for the fabrication of vascular grafts.^{323,324} In a similar way, the group of Okano engineered long-lasting cardiac tissue based on cell sheet strategies.^{325–327} Remarkably, some have already reached clinical trials.^{327,328} An alternate approach was selected by McGuigan and Sefton, who encapsulated HepG2 cells in cylindrical sub-millimetre gelatin modules, followed by endothelializing the surfaces.⁷² A construct with interconnected channels that enabled perfusion was generated through random self-assembly of the cell/hydrogel modules. However, the implementation of RP technologies offers another even more fascinating perspective on scaffold-free TE, and is commonly termed ‘bioprinting’ or ‘organ printing’.

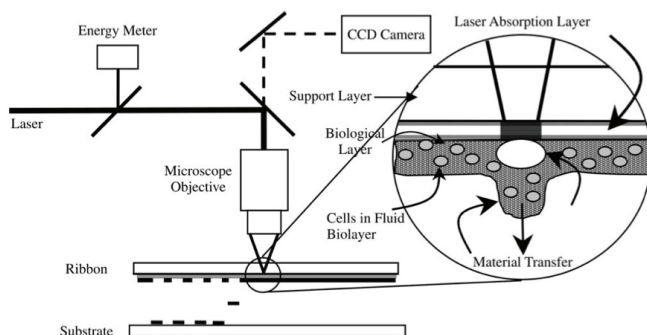


Figure 1.17: Schematic illustration explaining the working principle of BioLP. A focussed laser beam initiates material transfer towards the substrate. Interestingly, a laser absorption layer prevents direct interaction between the laser and the biological materials. The scheme was reused from³²⁹ with the permission from Springer.

We define organ printing as the engineering of three-dimensional living structures supported by the self-assembly and self-organizing capabilities of cells delivered through the application of RP techniques based on either laser,^{329–332} inkjet,^{273,285–287,333–338} or extrusion/deposition^{250,253,339–344} technology. An emerging laser based RP technique called biological laser printing (BioLP) stems from an improved matrix assisted pulsed laser evaporation direct write (MAPLE DW) system. The improvement is realized by incorporation of a laser absorption layer and thus eliminating the direct interaction with the biological materials. The principle is illustrated in Figure 1.17. Prior to laser exposure, a cell suspension layer is formed on top of the absorption layer. Then, a laser beam is focused on the interface of the target, which causes a thermal and/or photomechanical ejection of the cell suspension towards the substrate.³³¹ Target and substrate are both able to move in the planar field.

The workflow of inkjet or extrusion based bioprinting can be represented by Figure 1.18. In short, balls of bio-ink are deposited in well-defined topological patterns into bio-paper sheets. The bio-ink building blocks typically have a spherical or cylindrical shape, and consist of single or multiple cell types. Several bio-ink preparation methods have been described.^{318,320,339,343} In a post-processing step, the construct is transferred to a bioreactor and the bio-ink spheres are fused. The bio-paper, a hydrogel, can be removed after construction if required. Bio-printers can either have a jet design or work like a mechanical extruder.^{162,345} This implies that several RP apparatuses described in the previous part can serve as a bio-printer (e.g. the Bioassembly Tool, 3D-Bioplotter™, ...), if sterile conditions can be acquired. In the case of inkjet technology, individual or small cell clusters are printed. Despite the advantageous speed, versatility and cost, high cell densities are difficult to obtain and considerable cell damage is induced.^{318,345} On the other hand, extrusion-based bio-printers are more expensive but offer a more gentle approach towards cells.

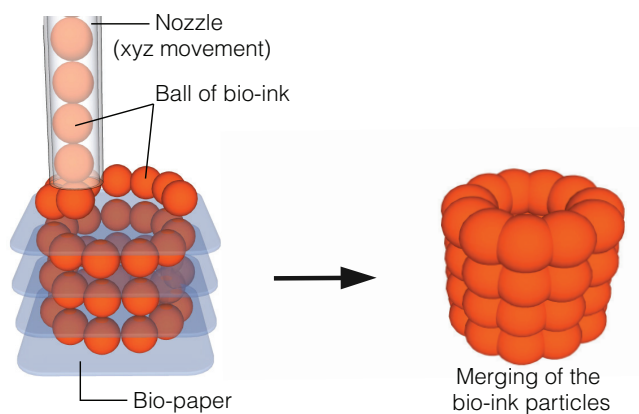


Figure 1.18: Basic concept of bioprinting bio-ink particles into bio-paper (hydrogel) sheets. The bio-ink particles are deposited in a tubular geometry (left). After the deposition is finished, the construct is transferred to a bioreactor to fuse the bio-particles and further maturation made possible (right).

In this context, hydrogels are employed as bio-paper and only provide a temporary support for the deposited bio-ink particles. In other words, the bio-paper is clearly different from scaffolds used in classical scaffold-based TE. Arai et al.³⁴⁶ made use of a hydrogel consisting of 2.0 % CaCl_2 , 20 wt % PVA and 3 wt % hyaluronan for the deposition of alginate based bio-ink particles. In most cases, this bio-paper hydrogel will have a sheet-like design (e.g. Figure 1.18). For instance, Boland et al.²⁸⁵ made use of thermo-sensitive gels to generate sequential layers for cell printing. The group developed a cell printer, derived from commercially

available ink-jet printers that enable to place cells in positions mimicking their spatial location in an organ.³³⁴ The printer can put up to nine solutions of cells or polymers into a specific place by the use of specially designed software, and print two-dimensional tissue constructs. Extending this technology to three dimensions is performed by the use of thermo-reversible gels. These gels were a fluid at 20 °C and a gel above 32 °C and serve as bio-paper on which tissue structures can be printed. Dropping another layer of gel onto the already printed surface could generate successive layers. The thermo-sensitive gel used for the experiments was a poly[N-isopropylacryamide-co-2-(N,N-dimethylamino)-ethyl acrylate] copolymer in a concentration of 10 wt % polymer in cold, deionized water.

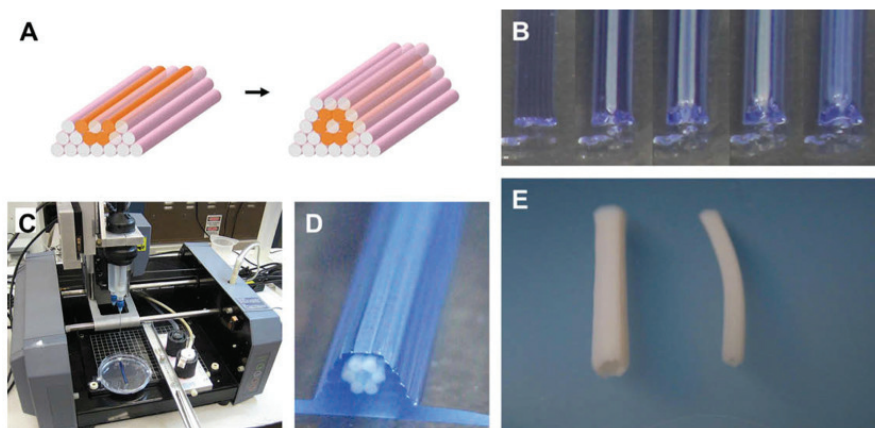


Figure 1.19: Bioprinting tubular structures with cellular cylinders. (A) Designed print template. (B) Layer-by-layer deposition of agarose (blue) cylinders and multicellular pig SMC cylinders (white). (C) The bio-printer outfitted with two vertically moving printing heads. (D) The printed construct. (E) Engineered pig SMC tubes of distinct diameters resulted after 3 days of post-printed fusion (left: 2.5 mm OD; right: 1.5 mm OD). Pictures were reprinted from³³⁹ with permission from Elsevier.

However, collagen used in a sheet-like design appeared to have integrated into the final structure, posing difficulties in its removal.³⁴³ Depending on the target tissue design, the bio-paper can also have other geometries. For instance, agarose rods were plotted and easily removed after post-printing fusion of a multicellular construct in order to fabricate tubular constructs (Figure 1.19).³³⁹ Agarose, being an inert and biocompatible hydrogel, is not remodelled by the cells and can easily be removed after fusion of the bio-ink. In situ cross-linkable synthetic ECM (sECM) mimetic hydrogels formed by co-cross-linking PEG-DA and modified hyaluronic acid (HA)/gelatin to the corresponding thiolated dithiopropionyl-

hydrazide (DTPH) derivatives were developed as bio-paper by Mironov et al.³⁴⁵ Other examples of hydrogels that served as bio-paper include fibrin, Matrigel™, fibrinogen, poly(N-isopropylacrylamide) (PNIPAM) and polyethylene glycol tetraacrylates.^{285,287,329,340}

It is somewhat unclear as to whether the fabrication of cell/gel hybrid constructs, which has already been brought up in the previous part, falls under the category of bioprinting or if it can be regarded, as it is our understanding, as a bridge between pure scaffold-based and pure scaffold-free TE strategies. For instance, Skardal et al.²⁵³ methacrylated HA and gelatin, in order to fabricate tubular photocross-linkable constructs from partially pre-cross-linked hydrogels and cells suspended within. The importance that is correlated to the biomaterial(s) used, as is clarified in Figure 1.20, notes the primary distinction with pure scaffold-free TE. Xu and colleagues made 3D cell/gel hybrid scaffolds from adipose derived stem (ADS) cells and gelatin/alginate/fibrin hydrogels to model the metabolic syndrome (MS) in 3D.¹⁴² Their work revealed the potential use of this 3D physiological model for drug discovery and the use of fibrin as an effective material to regulate ADS cell differentiation and self-organization into adipocytes and endothelial cells. Nevertheless the importance appointed to the biomaterial, cells are being encapsulated within the structure, and therefore this approach deflects somewhat from pure scaffold-based engineering. Maybe the future shows that this state-of-the-art TE concept and crossover approach of the two main distinct TE premises leads to some fascinating results and research.

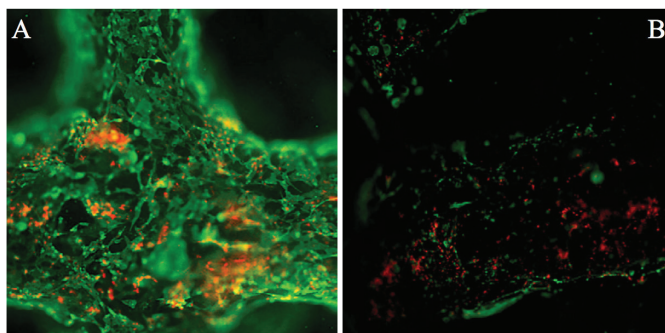


Figure 1.20: ADC cells, cultured with EGF, in a 3D gelatin/alginate/fibrin scaffold to differentiate into endothelial cells and adipocytes. (A, B) Immunostaining with mAbs for mature endothelial cells in green and PI for nuclear in red. (A) ADC cells within a gelatin/alginate/fibrin construct, and (B) without fibrin. The pictures were reprinted from¹⁴² with permission from Elsevier.

1.7 | CONCLUSIONS

In the course of this introductory chapter, the emerging field of Tissue Engineering has been described. The role of biomaterials within this scientific field has also been situated. As a small subdivision of this broad field, some more background information on hepatic tissue engineering was given and within this so-called soft Tissue Engineering segment, especially the potential advantages of hydrogel biomaterials, and more specific gelatin hydrogels, was explained.

It has been made clear that a shift from 2D geometries towards 3D scaffolds offers significant benefits. As a method of producing 3D geometries, rapid prototyping was introduced. This is a known technology domain which first arose in the automotive industry, but lately, the applications have been expanding. Several of these techniques can be used for the construction of 3D scaffolds related to tissue engineering. Limitations and future trends correlated to the manufacturing of hydrogel scaffolds by rapid prototyping techniques have been described.

As a relatively new approach, the processing of hydrogels using rapid prototyping technologies faces still some major disadvantages and/or technological drawbacks. On the other hand, it is believed that the importance of hydrogels, which enable cell encapsulation and in some instances the fabrication of cell-gel constructs, will increase.

2 | PROBLEM STATEMENT AND PhD BLUEPRINT

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2.1 | PROBLEM STATEMENT

Tissue regeneration and engineering are relatively new developments in the biomedical field, which try to give an answer to the chronic organ donor shortage. Mechanistic insight in cell-material interactions have led to the expansion of biomaterial research for biomedical applications. However, close interactions between fundamentally different scientific domains resulting in interdisciplinary feedback loops are still prone to amelioration. This is especially emphasized in the engineering of metabolic tissues, in contrast to (mostly hard) tissues such as cartilage and bone where primarily structural functions should be tackled. Being a highly metabolic tissue, liver failure (acute and chronic) has a drastic impact on the life standard. Among other, the liver is involved in detoxification, glycogen storage, protein synthesis, metabolic homostasis, release of proteins, carbohydrates, lipids, and metabolic wastes. Sometimes, the heterogeneous structure and functionality of the liver has been considered second only to the brain in its complexity.

Since its structure closely mimicks the extracellular matrix, hydrogel materials have been considered as suitable candidate materials. As a common guideline, highest cytocompatibility is mostly achieved by applying naturally derived polymers. Moreover, encapsulation of cells to provide three-dimensional environments have been reported to benefit cell culture. These findings are causing a paradigm shift from seeding toward encapsulation of cells. Gelatins, as a natural protein

extracted from collagen, have excellent biocompatibility. It is a bioactive material of which the production cost is low and readily available in large quantities.

Conventional fabrication techniques for the introduction of porosities have some drawbacks that can be overcome by using RP techniques and/or electrospraying/electrospinning. On the downside, hydrogels in general, but specifically natural hydrogels (e.g. gelatins), tend to exhibit difficulty in shaping ability and in the maintenance of the mechanical integrity. For instance, internal collapse of the designed pore network during RP processing is a common problem. As a result, highly controlled 3D shaping of gelatin matrices is mainly limited to expensive specialized machinery.

2.2 | PHD BLUEPRINT

In this work, gelatin was selected as a starting material. The aim was to develop three-dimensional shapes having high dimensional reliability for cell encapsulation and seeding purposes. Gelatins have a temperature dependent sol-to-gel transition upon cooling, limiting the biomedical applications at physiological conditions. In addition, gelatin is a protein composed of a variety of amino acids, containing various functional side groups.

The layout of the present work can be divided in subparts composed of multiple chapters that each emphasize specific questions related to the overall study.

2.2.1 Gelatin hydrogel network formation

In **chapter 3**, the origin of the applied gelatins is evaluated for their influence on the structural, physical and biocompatibility properties. The data from this chapter serves as background information that supports further experiments. The formation of chemical networks using photo-initiated polymerization reactions are described in **chapter 4**. Two vinyl-based gelatin derivatives are compared for their impact on the network structure, curing kinetics and potential as a cell encapsulation material.

2.2.2 Rapid prototyping as a tool to generate 3D constructs

Chapter 5 provides a study on the 3D printing of cell-laden structures. A post-fabrication curing strategy (discussed in chapter 4) is evaluated for two photo-initiators. In this part, special attention is given to the interdisciplinary nature to relate material, processing and biology parameters together. For this work, a close collaboration was performed with the lab of Prof. M. Cornelissen. The introduction of a porous network will lead to deflections in the degradation, metabolite flow

and mechanical strength of the fabricated constructs. These factors will be discussed in **chapter 6**, together with pore network design, cell seeding applications and the introduction of co-culture systems.

2.2.3 Galactose as biological ligand for hepatic tissue engineering

Chapters 7 and 8 handle gelatin bulk and surface functionalization with a biological ligand targeted to hepatocyte specific tissue engineering. Galactosylation of gelatin free amines is discussed in chapter 7, with respect to the biological response of HepG2 cells. During chapter 8, a glycomonomer is synthesized which can be used for the *in situ* functionalization of either the bulk or the surface. Parameters affecting the functionalization are investigated.

2.2.4 Reversible networks for the production of microspheres

As an alternative to large geometry printed constructs, the generation of microspheres using all-aqueous solutions by means of the electrospraying technique are presented in **chapter 9**. Some preliminary results discuss the potential of the technique for the spraying of cell suspensions.

2.2.5 Conclusive summary and future directions

An outline summary of the general conclusions of this work are presented in **chapter 10**. Finally, some interesting future perspectives and opportunities are tackled as well.

3 | INFLUENCE OF GELATIN ORIGIN ON THE STRUCTURAL, PHYSICAL, AND BIOCOMPATIBILITY PROPERTIES

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This chapter stems from an evaluation on potential gelatin batch-to-batch differences originating due to the selected raw materials and their corresponding extraction treatment. As such, the goal of this chapter is to provide more scientific background information, which will be referred to in subsequent chapters.

3.1 | INTRODUCTION

In the field of tissue engineering, the selection of an appropriate starting material is one of the most crucial elements in biomaterials development. Several approaches can be followed, of which “biomimetism” is based on the hypothesis that organ functional restoration can be reached if the target tissues are imitated.³⁴⁷

As a key structural fibrous protein, collagen as well as its degradation product, gelatin, have therefore widely been applied as starting materials.^{138,143,148,288,348,349} In spite of the identification of 29 collagen types to date, Type I contributes for over 90 % of the collagen in the body.³⁴⁷ Type I collagen is found in bones, skins, tendons, vascular, ligatures, and organs.

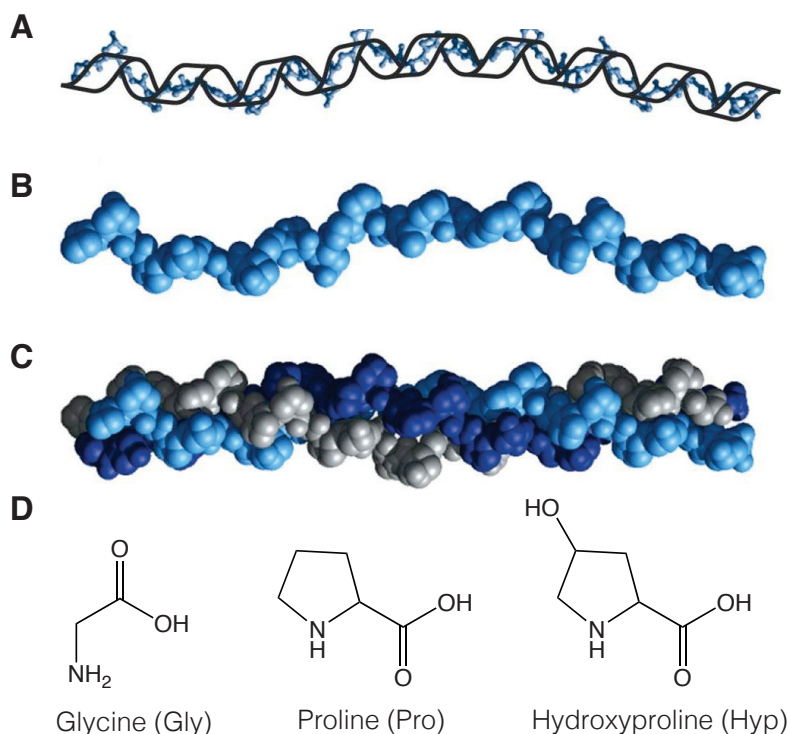


Figure 3.1: The molecular structure of collagen. (A) The polypeptide α -chain adopts a left-handed helical conformation due to the repeating tripeptide sequences Gly-Pro-Y or Gly-X-Hyp. This model was generated using a Gly-Pro-Hyp sequence. (B) Space-filling model of the α -chain. (C) Representation of the collagen quaternary structure (tropocollagen) whereby three left-handed α -chains are twisted into one right-handed triple-helix. (D) The chemical structure of the main amino acids glycine, proline and hydroxyproline. This figure was adopted from.³⁵⁰

Independent from the type, the primary structure as a regular arrangement of repetitive tripeptide units of glycine (Gly)-X-Y is a distinctive feature of all collagens (Figure 3.1).^{347,351,352} Hereby X and Y represents any other amino acid residue. Nevertheless, the X- and Y-positions are mostly occupied by proline (Pro) respectively hydroxyproline (Hyp). A left-handed helical conformation is formed: the α -chain. Collagen α -chains have a MW $\sim$ 100 kDa. Hydrogen bonds stabilize the super-coil, while hydrophobic and/or electrostatic interactions between amino acids at the X- and Y-positions can participate in intermolecular stabilization. On the other hand, the non-helical domains present at the sides (telopeptides) are pivotal for the natural cross-linking between chains.

Gelatin is a denatured collagen of which the MW, the isoelectric point (IEP), and the amino acid composition depends on the source of collagen and the manufacturing process. They are derived primarily from Type I collagen that generally does not contain the amino acid cysteine (Cys).³⁴⁷ The amino acid tryptophan (Trp) is also absent in gelatins.³⁵¹ Gelatins exhibit an Upper Critical Solution Temperature (UCST) and thus undergo a first order thermo-reversible sol-to-gel transition at temperatures lower than the critical temperature ($\sim$ 30°C) for solutions in hydrogen-bond friendly solvents and concentrations larger than the chain overlap concentration ($\sim$ 2 w/v %). During this, a conformational transition from random coil to triple helix occurs.^{353–355} Upon cooling, small triple-helical segments may further aggregate, however, the lateral aggregation typical for collagen (arrangement of fibrils) does not manifest in gelatin gels.

Despite an extensive exploration of gelatin as a polymer, the exact structure and functionality are still being discussed today. Notwithstanding the recognition of batch-to-batch heterogeneity, research comparing gelatin source for cell culture applications is limited, and gelatins originating from a variety of collagen sources are employed. For example, Hwang et al.³⁵⁶ applied type A gelatin, while Hong et al.³⁵⁷ selected type B gelatin for their respective hepatocyte cultures. In addition, the UCST thermo-reversible material property generates the need for covalent cross-linking strategies. Therefore, a manifold of cross-linking approaches has been explored, commonly but not exclusively accompanied by a chemical modification step.^{72,109,223,310,358–360} Logically, in the condition of a modification approach, knowledge on the chemical composition of the practiced gelatin will be essential.

In the following chapter, we provide background information on the physico-chemical properties of gelatins having distinctive origins. Next, the biological response of HepG2 cells as a function of gelatin origin and gelatin concentration is assessed as an indication for their cytocompatibility. Finally, two natural cross-linkers are evaluated in the perspective of future cell encapsulation and bioplotting

applications.

3.2 | EXPERIMENTAL PART

3.2.1 Gelatin materials

Gelatins hailed from different species and extracted using acid or alkali pre-treatment were evaluated. One type A gelatin, derived from porcine skin (Bloom value of 202 and approximate IEP of 8.8), and three bovine type B gelatins, derived from different raw material sources (Bloom values of 257, 226 and 254 and approximate IEP of 5), were kindly supplied by Rousselot (Ghent, Belgium). The manufacturers specifications are provided in Table 3.1.

3.2.2 Amino acid analysis of gelatin

Amino acid analysis was performed using the protocol Kerkaert et al.³⁶¹ Briefly, the gelatins were hydrolysed to the constituent amino acids using HCl solution (8 M), and hydrolysate was subsequently neutralized using NaOH solution (8 M) prior to high-performance liquid chromatography (HPLC) separation. All of the amino acids were automatically derivatized with 2-orthophthalic dialdehyde (OPA, Sigma-Aldrich), except proline, which was derivatized with 9-fluorenylmethylchloroformate (FMOC, Sigma-Aldrich), in the injector of an Agilent 1100 system (Agilent Technologies, Switzerland). Separation of the derivatized amino acids occurred on a Zorbax Eclipse AAA RapidResolution column (4.6×150 mm, Agilent Technologies) and was fluorometrically detected. Norvaline and sacrosine were both used as internal standards for the measurements. A flow rate of 2 mL min^{-1} was applied with a gradient solvent A (45 % methanol, 45 % acetonitrile, and 10 % water) and solvent B (45 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.02 % NaN_3 , pH 7.8). Samples values are expressed as averages $\pm$ S.D. of three repeated measurements.

3.2.3 Size exclusion chromatography (SEC)

Molecular weights were determined by means of size-exclusion chromatography (SEC) using a Millipore-Waters 510 pump and a Waters 410 Differential Refractometer for detection. Waters Ultrahydrogel columns 250–500 (300×7.8 mm) at 80°C (internal temperature 50°C) were used. Phosphate buffer (pH 7.4; 0.2 M) was applied as eluent with a flow rate of 1 mL min^{-1} . Calibration curves were recorded by means of pullulan standards (10 mg mL^{-1} , SDK Showa Denko). Gelatin samples (10 mg mL^{-1}) were injected and their molecular weight and polydispersity indices were compared.

3.2.4 Sol-to-gel transition

The UCST sol-to-gel transition was monitored applying rheological experiments using a Physica MCR 350 (Anton Paar) with plate-plate geometry. In order to investigate the influence of mixture concentration on the physical cross-linking of gelatin solutions, G' and G'' were recorded by small strain oscillatory measurements as a function of the temperature at a constant frequency (1 Hz) and strain (0.1 %) for type A and type B gelatins. Mechanical spectra of physically gelled gelatin samples (1 mm thick) were recorded at a constant deformation of 0.1 % strain in the frequency range of 0.1 – 100 rad s⁻¹ at 20 °C. Prior to the measurements, the linear visco-elastic range was determined by isothermal measurements of the storage and loss moduli G' and G'' as a function of the deformation ($\gamma = 0.01\% \rightarrow 1\%$) at a constant frequency (1 Hz).

3.2.5 Surface topography

Thin gelatin films were prepared using a table-top spincoater system (SPIN15, SPS-Europe B.V.). The protocol consisted of spincoating 50 μ L from an aqueous gelatin solution at a speed of 8000 rpm for 60 s on round glass slides ($\emptyset = 15$ mm). Thin films were prepared from 10 – 15 – 20 w/v % gelatin precursor solutions.

Atomic force microscopy (AFM) was used to analyze the surface topography (see section 11.2.4). AFM images were obtained in ambient conditions with a multimode scanning probe microscope (Digital Instruments-USA) equipped with a Nanoscope IIIa controller. Scans (1 μ m²) were recorded in tapping mode with a silicon cantilever (OTESPA-Veeco). The recorded images were modified with an X and Y Plane Fit Auto procedure.

3.2.6 Influence of the gelatin type and concentration on the *in vitro* cytocompatibility

The effect of gelatin origin and electrical nature on HepG2 cytocompatibility was investigated by performing titration experiments. HepG2 cells were maintained in a humidified 5 % CO₂-containing atmosphere (37 °C) with cultivation medium consisting of dulbecco's modified eagle medium (DMEM), supplemented with 10 v % fetal bovine serum (FBS), 50 U mL⁻¹ penicillin and 50 μ g mL⁻¹ streptomycin, all provided by Life technologies. Native gelatins were dissolved in culture medium at various concentrations in the range of 5 – 30 w/v %. Prior to cell mixing, the hydrogel precursor mixtures were pasteurized three times at 70 °C. Subsequently, equal cell numbers (1.5 $\times 10^6$ cells mL⁻¹) were mixed with these gelatin solutions and cast in 48-well cultivation plates (Greiner-Bio one). After 24 hours, cell survival was determined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay. Cell survival was expressed as percentage survival, compared to cells cultivated in absence of gelatin. The MTT assay was conducted as described by the manufacturer. Briefly, cells were exposed for 4 h to 0.5 mg ml⁻¹ MTT (Calbiochem) dissolved in culture medium. After discarding the solution, the formed formazan crystals were released by treatment (30 min) with isopropanol-0.04 N HCL, supplemented with 1 v % Triton X100 (Sigma). The absorbance was measured at 580 nm with an EL800 Universal microplate reader (BioTek instruments Inc.).

3.2.7 Cross-linking of gelatin with natural cross-linkers

Two natural cross-linkers were evaluated for the cross-linking of gelatins with the aim at cell encapsulation and bioplotting. Ten w/v % gelatin type B was prepared by dissolving 1 g gelatin in 10 mL PBS at 40 °C. Genipin covalent cross-linking was carried out at 37 °C by blending methyl-(1R,2R,6S)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate (Genipin, Sigma-Aldrich) to obtain a final concentration of 4 mM. Enzymatic covalent cross-linking was evaluated using the food enzyme ACTIVA WM[®], kindly gifted by Ajinomoto. It consists out of 99 % maltodextrin and 1 % microbial transglutaminase (mTGase) with an enzyme activity of 100 U g⁻¹. An enzyme stock solution of 10 U mL⁻¹ was prepared, and subsequently added to the gelatin precursor solution to obtain final concentrations of 0.1 – 1 U mL⁻¹.

The hydrogel precursor solutions were poured in 24 well-plates (Greiner Bio One) and allowed to cross-link for certain time intervals after which the formed gels were frozen in liquid nitrogen and freeze-dried. The dry gels were weighed (m_{di}) and subsequently incubated at 37 °C, immersed in double distilled water. After equilibrium swelling was reached (e.g. 24 h), the gels were removed, gently dabbed with paper and weighed again (m_{he}). Subsequent freeze-drying gave the final dry weight (m_{de}). The loss of dry mass of the hydrogel during incubation at 37 °C enabled us to calculate the gel fraction (see section 11.2.1). For mTGase gels, the influence of temperature on the cross-linking was followed using small strain dynamic oscillatory rheology time sweeps with $\gamma = 0.1\%$ at a constant frequency of 1 Hz. Rheology is described in section 11.2.2.

The cross-linking extend was determined by monitoring the free amine-group content with the ninhydrin assay. Lyophilized samples were subjected to a 2 wt % ninhydrin (Sigma-Aldrich) solution for 30 min at 100 °C, after which the optical absorbance of the solution was recorded spectrophotometrically at a wavelength of 560 nm (Biotek Uvikon XL). A calibration curve with pure, distilled aminoethanol was established. Finally, cell encapsulation was investigated by mixing HepG2 cells or primary mouse hepatocytes at a final concentration of 1.5×10^6 cells mL⁻¹ and the cell viability was assessed by MTT assay. Primary mouse hepatocytes were iso-

lated from adult mouse livers (8-14 weeks of age) using a two-step collagenase perfusion method followed by percoll gradient purification as described by Goncalves et al.³⁶² Primary hepatocytes were cultured in William's E medium (Life Technologies), supplemented with L-glutamine (292 mg mL^{-1}) (Invitrogen), glucagon (7 ng mL^{-1}) (sigma), insulin ($0.5 \text{ } \mu\text{g mL}^{-1}$) (sigma), hydrocortisone ($25 \text{ } \mu\text{g mL}^{-1}$), epidermal growth factor (EGF) (10 ng mL^{-1}), 10 v/v % FBS (Life Technologies), 50 U mL^{-1} penicillin (Life Technologies) and $50 \text{ } \mu\text{g mL}^{-1}$ streptomycin (Life Technologies).

3.2.8 Statistical analysis

The experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical analysis was performed with the Student's *t*-test and significance was determined at $p < 0.05$.

3.3 | RESULTS AND DISCUSSION

3.3.1 Collagen vs. gelatin: the amino acid composition of type A and type B gelatins

Amino acid analysis of gelatins are variable depending on the raw material and extraction process, and generally the largest variations can be found in the minor constituents.^{347,352} Table 3.1 provides the amino acid composition of one type A gelatin (porcine origin) and three type B gelatins (bovine origin). As a reference, the amino acid profiles of bovine bone and human bone collagen found in literature is included. During treatment of collagen to extract gelatin, the amino acid content is largely undisturbed, therefore the amino acid content of gelatin is quite similar to that of collagen. Nevertheless, the amino acid composition affects the gelatin's chemical and physical properties.

In correspondence to collagen, glycine (Gly), proline (Pro) and hydroxyproline (Hyp) are the three major contributors to the amino acid composition. The high content of (hydroxy)proline contributes substantially to the well-known gelling properties of gelatin.^{347,352,353} Other amino acids can be regarded as minor constituents. The mammalian gelatins, as they are extracted from Type I collagen rich sources, were deficit in the amino acids tryptophan (Try) and cysteine (Cys). Ornithine (Orn), a non-essential α -amino acid that is naturally synthesized from arginine in the liver, could only be found in type B gelatins owing to the alkaline hydrolysis of arginine during the extraction process. This amino acid has been reported to be able to mediate hepatocytes albumin and transferrin secretion.³⁶³

Table 3.1: Comparison of the amino acid composition of gelatins and collagen. Values are given as g of amino acid per 100 g of protein.

AMINO ACID	TYPE A GELATIN	TYPE B GELATIN			REFERENCE DATA [†]	
	Porcine skin	Bovine	Bovine hides	Bovine bone	Ox bone collagen	Human bone collagen
Aspartate (Asp)	4.40 ± 0.12	4.95 ± 0.18	5.01 ± 0.14	4.20 ± 0.17	7.10	6.70
Glutamate (Gln)	8.14 ± 0.34	9.31 ± 0.35	9.20 ± 0.20	7.99 ± 0.37	11.90	11.40
Serine (Ser)	3.12 ± 0.09	2.66 ± 0.07	2.76 ± 0.05	2.84 ± 0.06	4.24	4.06
Histidine (His)	0.69 ± 0.02	0.56 ± 0.03	0.61 ± 0.01	0.53 ± 0.01	0.96	0.96
Glycine (Gly)	21.63 ± 0.71	21.99 ± 0.89	22.12 ± 0.59	21.88 ± 0.61	25.30	25.80
Threonine (Thr)	1.77 ± 0.03	2.24 ± 0.07	2.18 ± 0.05	1.77 ± 0.08	2.52	2.35
Arginine (Arg)	7.32 ± 0.22	7.37 ± 0.25	6.74 ± 0.14	6.95 ± 0.24	9.20	8.80
Alanine (Ala)	8.18 ± 0.24	9.06 ± 0.32	8.76 ± 0.18	8.69 ± 0.31	10.50	10.90
Tyrosine (Tyr)	0.64 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.17 ± 0.02	0.56	0.86
Valine (Val)	2.49 ± 0.13	2.69 ± 0.11	2.63 ± 0.08	2.59 ± 0.10	2.65	2.97
Methionine (Met)	0.95 ± 0.03	0.76 ± 0.02	0.86 ± 0.02	0.68 ± 0.02	0.80	0.84
Hydroxylysine (Hly)	1.24 ± 0.03	1.17 ± 0.05	1.26 ± 0.03	1.21 ± 0.04	1.12	0.62
Phenylalanine (Phe)	1.92 ± 0.07	1.81 ± 0.06	1.76 ± 0.04	1.75 ± 0.04	2.88	2.49
Isoleucine (Ile)	1.42 ± 0.05	1.67 ± 0.06	1.68 ± 0.04	1.62 ± 0.04	1.73	1.88
Ornithine (Orn)	-	0.55 ± 0.05	0.97 ± 0.04	0.43 ± 0.07	‡	‡
Leucine (Leu)	3.42 ± 0.12	3.41 ± 0.09	3.24 ± 0.07	3.47 ± 0.06	3.93	3.60
Lysine (Lys)	3.85 ± 0.11	3.75 ± 0.09	3.49 ± 0.08	3.99 ± 0.09	4.11	4.90
Proline (Pro)	13.57 ± 0.23	13.49 ± 0.43	14.35 ± 0.40	12.54 ± 0.39	14.70	15.30
Hydroxyproline (Hyp)	10.25 ± 0.33	10.91 ± 0.39	11.17 ± 0.28	10.71 ± 0.51	14.10	14.10
Bloom strength [§]	202	257	226	254		
Viscosity (6.67 %, 60 °C) [§]	2.97	4.88	4.79	4.62		
pH (6.67 %, 45 °C) [§]	5.14	5.69	5.63	5.73		
IEP [§]	~ 8.8	~ 5	~ 5	~ 5		

[†] Eastoe³⁵²

[‡] Included in the lysine value

[§] Obtained from data sheets

Gelatin derivatization mostly occurs via reactions with the amine groups available on some amino acid side chains (Figure 3.2). Under mild basic conditions however, the guanidinium group of arginine will be protonated, making nucleophilic attack impossible, while the imidazole group of histidine reacts, but leads to the formation of unstable products.³⁶⁴ As a result, type B gelatins have a slightly higher amount of reactive free amines stemming from Lys, Hly and Orn compared to type A gelatin: $38 \pm 1 \text{ mmol g}^{-1}$ for type B vs. 34 mmol g^{-1} for type A gelatins.

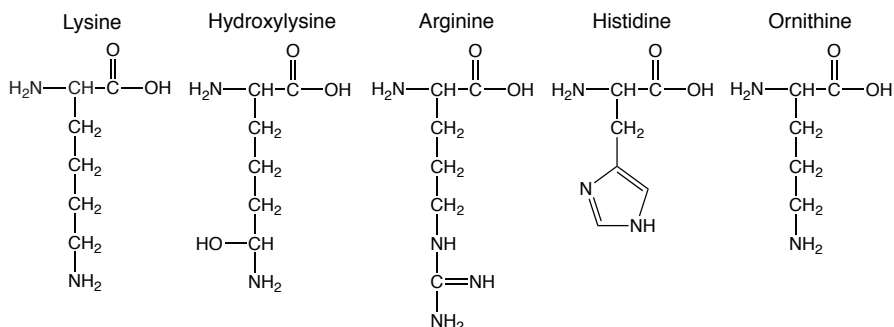


Figure 3.2: Amino acids of gelatin having amine groups in the side chain.

Only small amounts of the aromatic amino acids tyrosine (Tyr) and phenylalanine (Phe) were found, with higher levels for porcine-derived type A gelatin. Even up to three-fold in the case of tyrosine ($p < 0.02$). Mostly non-significant differences were detected between the profiles of the three bovine-derived type B gelatins. However, the levels of aspartate (Asp) and glutamate (Glu) were significantly lower for the bone derived gelatin ($p < 0.05$). Comparing both skin derived gelatins from either porcine or bovine source, and leaving out amino acid differences originating from the extraction process (i.e. higher levels of Asp and Glu, and the presence of Orn in type B gelatins), only the minor constituents Arg and Tyr were higher for porcine skin gelatin while the amount of Thr was slightly lower.

In contradiction to our amino acid profiles, distinct differences for Arg, Gly, and Pro were reported by Hafidz et al.³⁵¹ for porcine and bovine skin gelatins. The authors could not detect His, and both gelatins had low levels of Tyr. It was not clear whether both gelatins were extracted by the same manufacturer and/or in the same country. Our mammalian gelatins all had approximately similar levels of Gly and the imino acids Pro and Hyp, however, fish derived gelatins are reported to have lower imino acid (Pro, Hyp) content resulting in decreased gelling potential.^{347,353}

3.3.2 The influence of the extraction process on the molecular weight and molecular weight distribution

During gelatin extraction, cleaving of the triple helical structure occurs on a different way depending on the type of pre-treatment (alkaline vs. acidic) and length of the thermal denaturation-hydrolysis process, resulting in distinct molecular weight (MW) and molecular weight distribution (MWD) profiles. The MWD will depend on the intra- and inter-molecular covalent cross-linkages present in the starting raw material, and the balance between the relative amount of unhydrolyzed cross-linkages and the resulting length of the polypeptide chains. These cross-linkages stem from a condensation reaction between the amino groups of Lys and an aldehyde group formed by an enzymatic oxidation of a Lys or Hly residue.³⁵³ Figure 3.3 represents size exclusion chromatography (SEC) elution profiles of the four examined gelatin types.

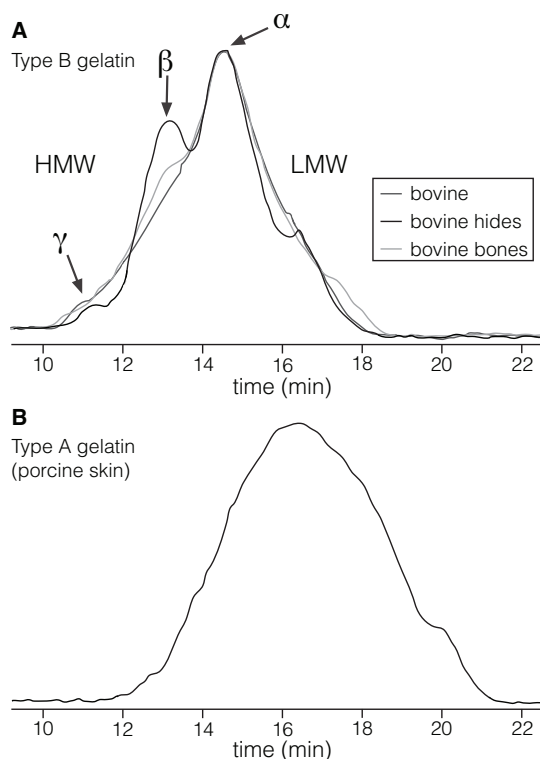


Figure 3.3: SEC chromatograms showing the elution profiles of three bovine-derived type B gelatins (A) and one porcine-derived type A gelatin (B). Arrows indicate peaks/shoulders of α -, β -, γ -chains.

As can be derived from the type B SEC chromatograms, covalent cross-linkages

between α -chains can survive the manufacturing treatments, leading to fractions of β -chains (two covalently linked α -chains), γ -chains (three covalently linked α -chains) and even higher molecular weight (HMW) components. The main peak of the type B gelatins corresponded to a MW 88 ± 2 kDa ($\sim \alpha$ -chain). In the profile of the bovine hides gelatin, a clear peak attributed to β - and to a lesser extend also γ -chains could be observed. These were less pronounced for both other type B gelatins. Nevertheless, rupture of the peptide bonds in the primary structure can also occur during pre-treatment and extraction, resulting in subunits of the original α -chains: the low molecular weight (LWM) compounds. The latter was more pronounced in the SEC chromatogram of type A gelatin, of which its main peak corresponded to a MW 45 ± 1 kDa, indicating a more intensive hydrolysis. As a small remark, not all type A gelatins have a main contribution of LMW molecules as in our case, as evidenced from the MWD profiles of other gelatin extraction intensiveness.³⁵³ The examined type B gelatins had a more narrow MWD compared to the one of type A gelatin, nevertheless, the polydispersity indices (PDI) were for all gelatins > 2 . The highest fractions of β - and γ -chains was found for bovine hides gelatin, while the highest amount of LMW molecules was observed for the porcine skin gelatin.

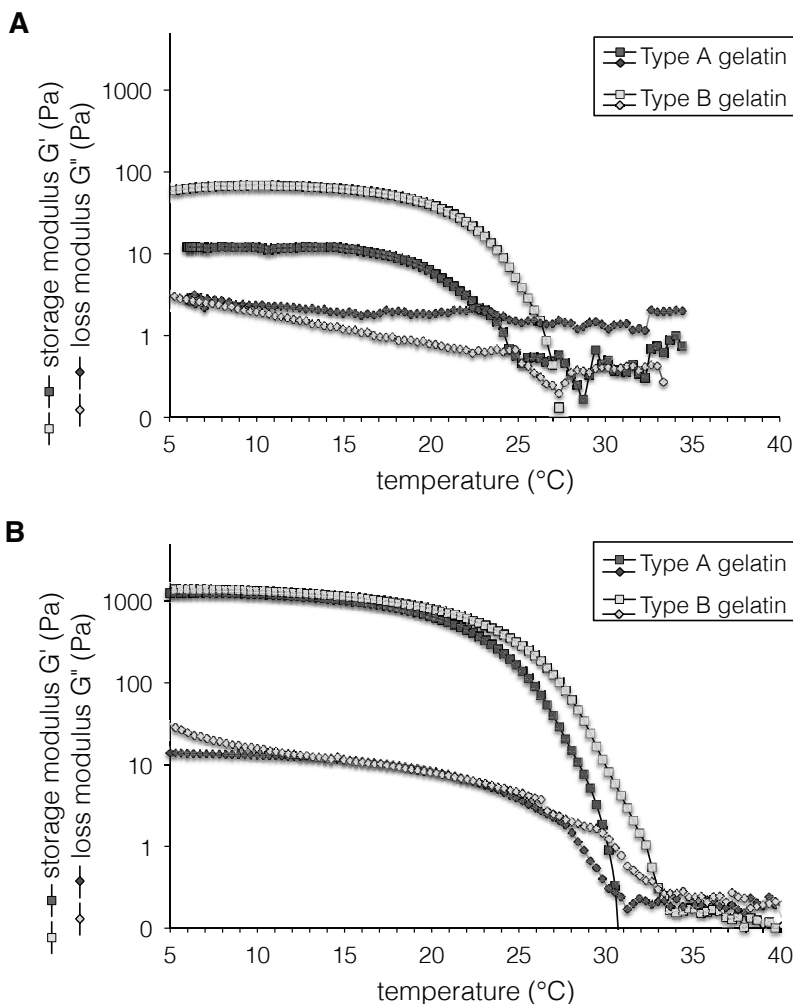


Figure 3.4: Dynamic oscillatory temperature sweep recordings of the storage G' and loss G'' moduli for 2 w/v % (A) and 5 w/v % (B) gelatin solutions illustrating the transition from ‘gel’ to ‘sol’. Measurements were performed with $\gamma = 0.1\%$ at a frequency of 1 Hz ($F_N = 0.1$ N).

3.3.3 The effect of α -chains, HMW and LMW on the gelling and the G' value

The gelation property inherent to gelatins stems from the partial recovery of the triple helical structure of collagen. Measurement of the storage (G') and loss (G'') moduli allows to follow the gelation kinetics *in situ*. The $\tan(\delta)$ (ratio of G'' to G') value gives an indication on the resulting gel strength. As illustrated in Figure 3.4, the gelation is concentration, temperature, as well as gelatin type dependent.

At a concentration of 2 w/v %, the temperature influences only to a very limited amount the G'' modulus evolution, while at higher concentrations (i.e. 5 w/v %) the temperature clearly affects the G'' as well as the G' moduli, typically indicating the formation of a gel network at lower temperatures. For 2 w/v % gelatin solutions, the $\tan(\delta)$ value is too high even at a temperature of 5 °C to consider this as a hydrogel network. At a concentration of 5 w/v %, we observe a transition zone between 25 – 35 °C whereby G' as well as G'' significantly drop/increase with increasing/decreasing temperature: the sol-to-gel transition. The higher the difference between the G' and G'' values, the stronger the formed physical hydrogel network.

Figure 3.5 demonstrates the evolution of the storage modulus G' as a function of concentration and gelatin type. Logically, higher concentrations lead to stronger physical gelation (thus higher G' values) due to both an increase in solid content as well as an increase in the formation of hydrogen bonding. Regarding gelatin type, we observe that physical hydrogels of type A gelatin form softer hydrogel networks when compared to type B gelatin.

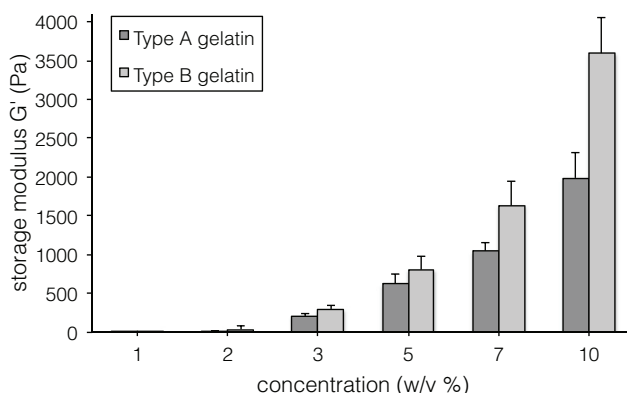


Figure 3.5: Evolution of the storage moduli (G') of gelatin hydrogels at 20 °C as a function of the concentration and gelatin type (acid porcine skin vs. alkaline bovine gelatin).

It should be commented that the G' of the bovine hides derived type B gelatin was lower (~ 2.7 kPa) than both other type B gelatins, however still substantially higher compared to the porcine skin type A gelatin. As an alternative and industrially accepted measure for the gel strength, the Bloom numbers followed these findings.

In general, a lower mean molecular weight lowers the gel strength and viscosity of the solution. This is clearly illustrated comparing the results of the selected type A

and B gelatins. However, interestingly, the bovine hides derived gelatin had a similar viscosity as the other bovine gelatins (Table 3.1) but lower gel strength despite having more pronounced β -, and γ -chain peaks in its MWD profile (Figure 3.3). Some contradiction is found in the literature: Normand et al.³⁶⁵ suggested that the α -, β - and γ -chains contributed equally to the elasticity. Johnston-Banks³⁶⁶ correlated gel strength to the sum of the α - and β -chains. Others reported the α -chains as the primordial contributors of the resulting gel strength, and perturbations of the helix reformation could occur due to the presence of subunits, β -, γ - and highly branched chains (non-linear chains).^{367,368} The work of Eysturskarð on the mechanical properties of mammalian and fish gelatins suggested a positive correlation with the fraction of β -chains and HMW molecules and a negative correlation with the LWM molecules.

Our findings suggest that the acid porcine skin gelatin had a lower G' due to a higher fraction of LWM molecules, as well as a higher PDI. For the type B gelatins, which had similar MW and PDI, the higher fraction of β - and γ -chains present in the bovine hides gelatin reflected in a lower Bloom and G' value, but similar viscosity. This correlates to α -chains as the main contributor of gel strength, while β -, γ - and HMW molecules perturb the helix reformation but contribute to viscosity.

3.3.4 The effect of MWD on the surface topography

As described in the previous paragraph, the presence of β -, γ -chains and HMW molecules are proposed to impact the gelification potential of the hydrogel building blocks. If this statement would be correct, it can be anticipated that the surface topography could alter as a result of increased/decreased globular domain formation during gelification. As demonstrated in Figure 3.6, AFM images of the three bovine gelatins reveal less large globular domains for bovine hides gelatin, further confirming this hypothesis.

Similar results were obtained for other concentrations (10 – 15 – 20 w/v%). Nonetheless, the surface wettability, partially influenced by the surface topography, revealed no statistically significant differences between the four gelatins (data not shown).

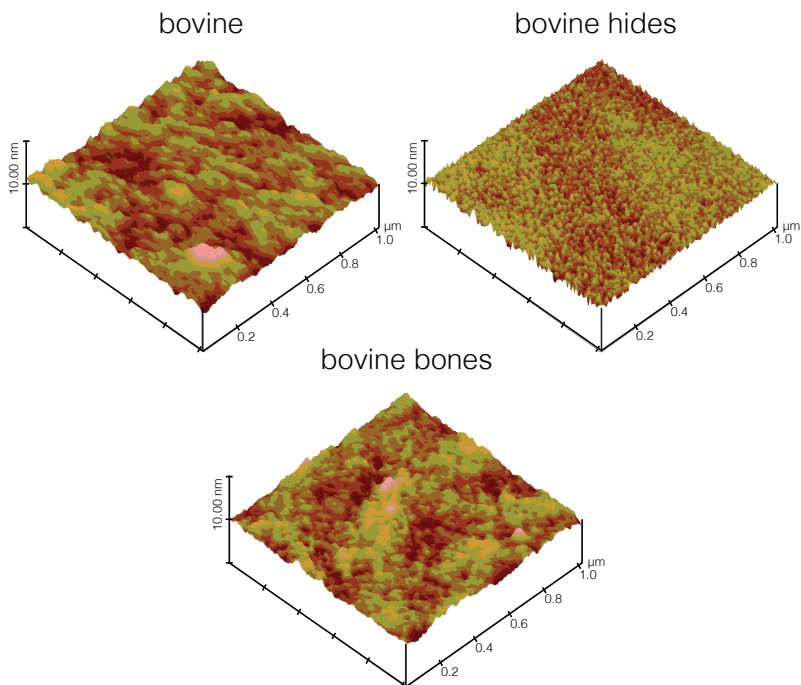


Figure 3.6: AFM images of physical type B gelatin hydrogels representative for a concentration of 10 w/v %.

3.3.5 Type A vs. Type B gelatins: *in vitro* cytocompatibility

Gelatins have since long been applied as cell-interactive matrices for TE purposes. Generally, gelatin is regarded as a biopolymer with high biocompatibility. The view most commonly held on gelatin's non-immunogenic and non-antigenic property is suggested to be due to the very small amount of aromatic rings from Tyr, Trp, and Phe amino acids, and the absence of aromatic radicals.^{347,369} Nevertheless, no research could be found regarding biocompatibility issues of gelatins stemming from different origin and/or extraction processes.

Figure 3.7 depicts the *in vitro* cytocompatibility results as a function of the added amount of gelatin ranging between 5 and 30 w/v %. Concentrations < 5 w/v % were excluded since these were not considered relevant for future cell seeding and encapsulation whereby gelatin-only matrices will be evaluated. A decrease in cell viability as a function of increasing concentrations could be observed for all gelatins. Type B gelatins derived from bovine hides and a “combination” of bovine materials demonstrated viability levels of $\geq 70\%$ up to 30 w/v %, whereas

the one derived from bone only remained $\geq 70\%$ up to 10 w/v %. Viability levels using porcine skin derived gelatin type A were similar to bovine bone derived type B gelatin and rapidly decreased with increasing hydrogel precursor concentration. It should be remarked that the described differences were only investigated for HepG2 cells.

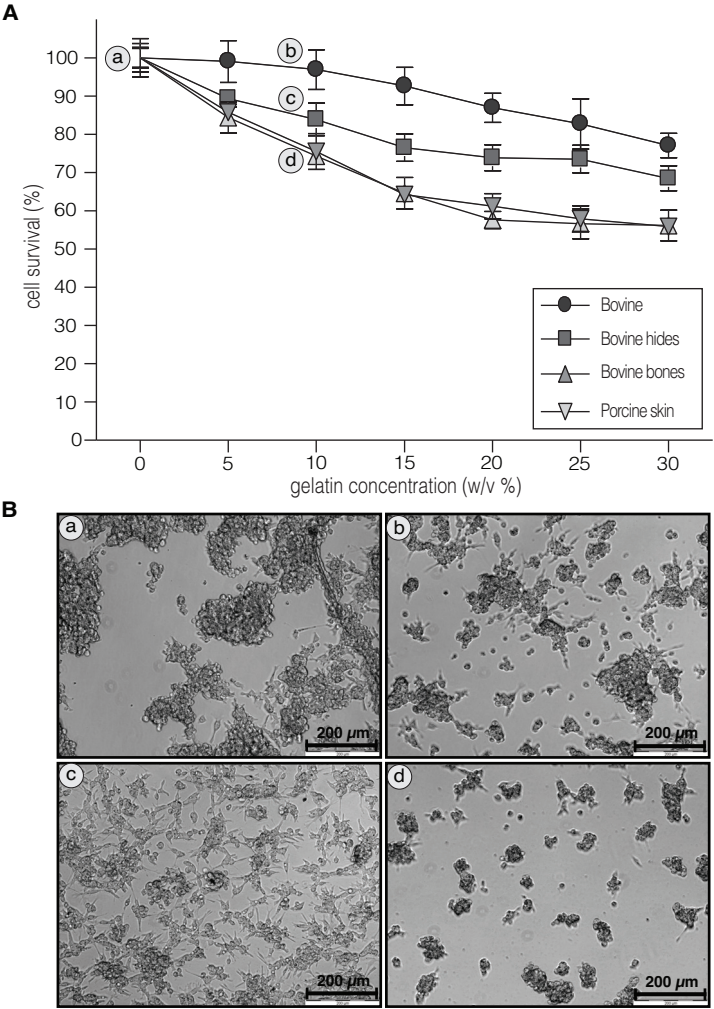


Figure 3.7: (A) The effect of gelatin type and concentration on the cell viability of HepG2 cells. (B) Brightfield microscopy images corresponding to the points indicated on part A. Scale bar represents 200 μm .

We observed a difference between gelatin type A and gelatin type B, with the former exhibiting clearly less cytocompatibility. Therefore, further testings on cell encapsulation of unmodified gelatins in this chapter will focus on type B gelatin.

Looking at the amino acid profile, as described before, the fraction of the aromatic amino acid Tyr, and to lesser extend also Phe, is significantly higher for the examined type A gelatin. On the other hand, all type B gelatins had similar fractions of aromatic amino acids. The bovine bone gelatin, which showed lowest cytocompatibility, had the lowest amount of Asp, Glu and Thr. These values were close to the values for the porcine type A gelatin, suggesting that not only the aromatic rings as a general marker but perhaps also these amino acids impose an influence on the examined cell type.

Additionally, the MWD of the four gelatins showed clear differences. Especially in the LMW domain. Type A gelatin mainly consisted out of LMW molecules (MW $\sim$ 45 kDa (peak) and $\sim$ 15 kDa (shoulder)), while the bovine gelatin had the lowest contribution of LMW molecules but the highest biocompatibility. Furthermore, the bovine hides and bovine bone gelatin had respectively a clear LMW peak (MW $\sim$ 48 kDa) and shoulder (MW $\sim$ 24 kDa). Most likely, the extraction process and its effect on the MW and MWD also impacts the resulting cytocompatibility.

3.3.6 Natural cross-linkers for gelatin: genipin vs. transglutaminase

The reversible UCST temperature-dependent sol-to-gel transition of gelatins limits its applicability in TE applications. In order to guarantee a sustainable TE matrix at physiological temperatures, researchers developed several cross-linking strategies to form covalent gelatin networks. These can be roughly divided into two groups, namely strategies employing native gelatin and a cross-linker or strategies that are based on gelatin modifications.

Two natural cross-linkers that can form covalent networks are the compound Genipin and the enzyme transglutaminase. These are considered highly biocompatible,^{310,347,348} and therefore we evaluated their potential use for the cross-linking of cell-laden gelatin hydrogel precursors.

3.3.6.1 Genipin

Genipin is a natural product obtained from its parent compound geniposide (an iridoid glucoside), abundantly present in *Genipa Americana* and *Gardenia jasminoides Ellis*.^{310,347} The spontaneous cross-linking mechanism of Genipin with free amino groups of proteins such as Lys, Hyl, Arg and Orn to form dark blue pigments is still under investigation (Figure 3.8). A mechanism through a nucleophilic attack of the amines of gelatin, inducing an opening of the dihydropyran ring of Genipin followed by the attack of a second amine on the resulting aldehyde was proposed.³⁴⁷ Genipin has been used to fix biological tissues or amino-group

containing biomaterials for biomedical applications, and has been reported to have enhanced biocompatibility over carbodiimide cross-linking.³¹⁰

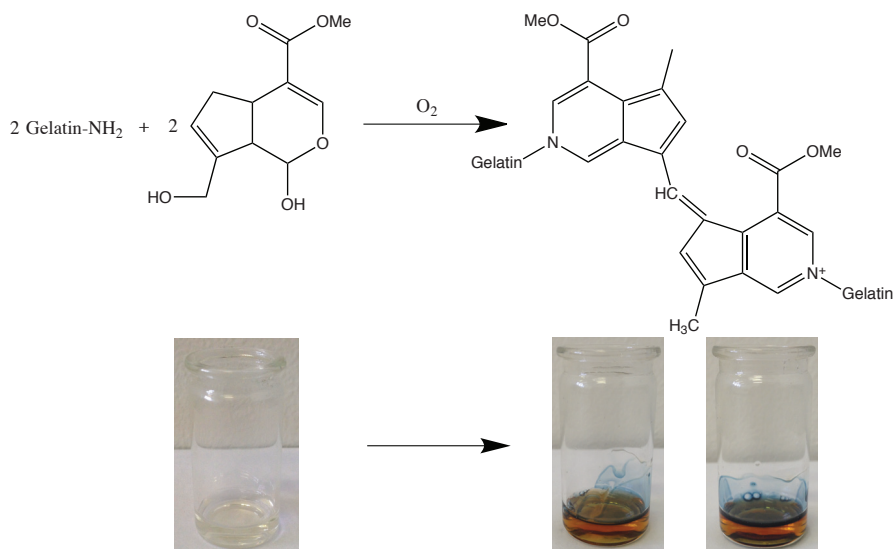


Figure 3.8: Reaction mechanism of Genipin proposed by Mi et al.

During Genipin cross-linking, the gelatin hydrogels are pigmented brown to dark blue as seen in Figure 3.8. The cross-linking occurs on a steady, however slow pace. Table 3.2 presents some values on the conversion and swelling properties. After a cross-linking time of 60 min (t_{60}), only $\sim 9 - 14\%$ of the initial free amines are consumed in the reaction. A plateau value whereby $< 35\%$ of the initial NH_2 groups are consumed and a maximal gel yield of $\sim 80\%$ is reached (type B gelatin). For type A the gel yield was lower as this gelatin type has a lower NH_2 content and a lower MW. These results were in close correlation to the experiments of Liang et al.³¹⁰ Moreover, it should be put under attention that the standard deviations (S.D.) are rather big, suggesting a relatively difficult control over the cross-linking process.

Table 3.2: Evolution of Genipin cross-linking as determined by the ninhydrin assay and equilibrium swelling experiments for 10 w/v % gelatin solutions.

SAMPLE	NH_2 100 g ⁻¹ gelatin			CONVERSION [%] [†]	GEL FRACTION [%] [†]
	t_0	t_{60}	t_{300}		
Type A	33.98	31 ± 2.6	27 ± 2.1	~ 21	56 ± 6
Type B	37.04	32 ± 1.9	26 ± 2.0	~ 30	80 ± 5

[†] at t_{300}

With respect to adopting this strategy, some major drawbacks arise: i) the pigmentation could interfere with biological characterizations, especially in the case of encapsulation purposes (which is one of the present thesis’s aims), mainly limiting the applicability to cell seeding strategies, and; ii) the poor cross-linking speed and rather large uncross-linked fraction will impose substantial problems when aiming at cross-linking rapid prototyped architectures. Therefore, this system was not further evaluated.

3.3.6.2 Microbial transglutaminase (mTGase)

A second natural cross-linker, which excludes the necessity for gelatin modification, is the enzyme transglutaminase (TGase). We applied a calcium-independent microbial transglutaminase (mTGase) frequently applied in food chemistry as a ‘meat glue’. From the FDA, it received the ‘Generally Recognized As Safe’ (GRAS) notification. Little is known of its potential use as a cross-linker of proteins for biomedical applications. One application is the use of transglutaminase-gelatin injectable system, which enables the cross-linking with endogeneous collagen to create a strong bond between the gel and the tissue ECM.³⁶⁰ As every enzyme, mTGase has a temperature and pH range and optimum (data sheet Ajinomoto):

- Temperature: 0 °C – 65 °C (optimum: 50 – 55 °C);
- pH: 4–9 (optimum: 6–7)

Transglutaminase catalyses the cross-linking of gelatin by reaction between the γ -carbonyl group of a glutamine residue and the ε -amino group of Lys/Hyl releasing one molecule of ammonia per cross-linking as by-product (Figure 3.9 (A)). Cysteine is the enzyme’s active center and the formed isopeptide bonds exhibit high resistance against proteolytic degradation (proteolysis).^{347,348,360,370}

Small strain oscillation time sweeps were recorded at three distinct temperatures (4 – 20 – 37 °C) for 10 w/v % gelatin hydrogel building blocks with a fixed enzyme activity of 0.1 U mL⁻¹ to evaluate the cross-linking potential at temperatures away from the optimum. The evolution of the storage modulus G' is presented in Figure 3.9 (B). As anticipated, a cross-linking temperature of 37 °C rapidly promotes the formation of ε -(γ -L-glutamyl)-L-lysine isopeptide bonds. At lower temperatures, the hydrogel building blocks have already formed a physical network, explaining the initially higher G' value, interfering with the enzyme mobility and thus slower/less complete cross-linking. This is also illustrated by equilibrium swelling experiments (Figure 3.9 (C)). Temperatures below the gelification point are relevant in the framework of three dimensional prototyping whereby an initial physical gelation would be necessary prior to chemical cross-linking.

At elevated temperatures, no significant effect of the enzyme concentration on the gel yield could be observed for the tested concentration range, and gel fractions of > 85 % were measured. Lowering the temperature, for all enzyme concentrations,

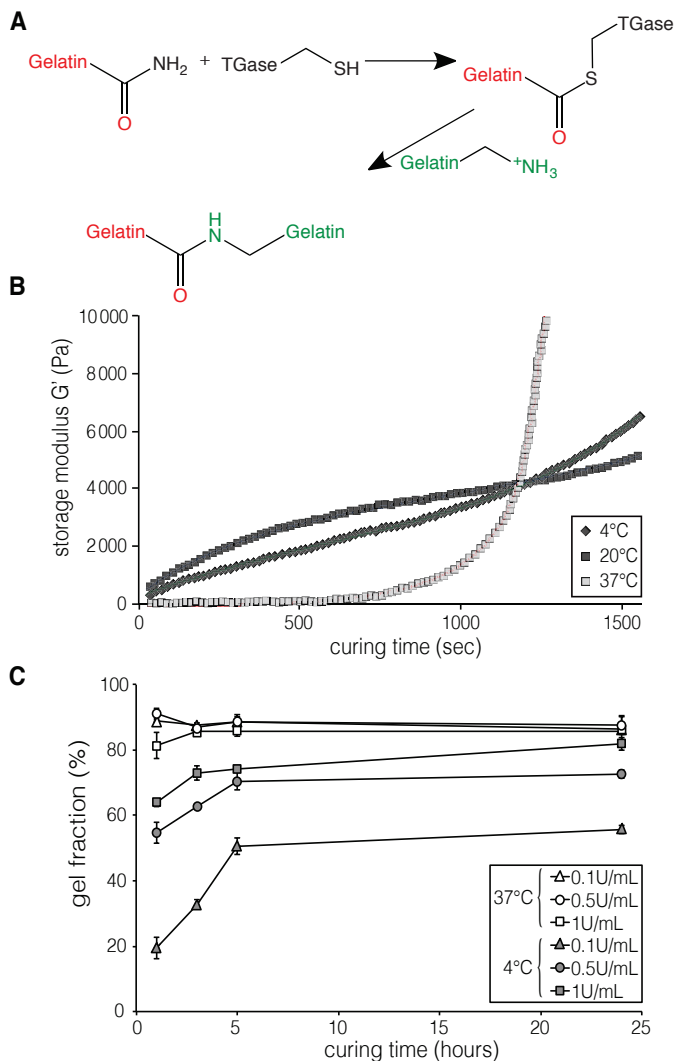


Figure 3.9: The use of mTGase as a natural cross-linker for gelatin hydrogel building blocks: (A) the formation of isopeptide bonds promoted by transglutaminase, (B) rheological time sweep recordings of G' as a function of the cross-linking temperature, and (C) the gel fraction as a function of the cross-linking temperature and enzyme activity. Ten w/v % gelatin type B solutions were used for all experiments.

the gel yield plateau value dropped and was only reached for cross-linking periods exceeding 5 h. In contrast to Genipin, mTGase cross-linking appeared sufficiently controllable to be considered as a potential strategy for rapid prototyping applications. Therefore, some initial cell culture experiments were performed to assess the applicability for cell encapsulation of HepG2 cells. In accordance to the cytocompatibility results presented earlier in section 3.3.5, bovine type B gelatin at a concentration of 10 w/v % was selected for these initial experiments. The results are presented in Figure 3.10.

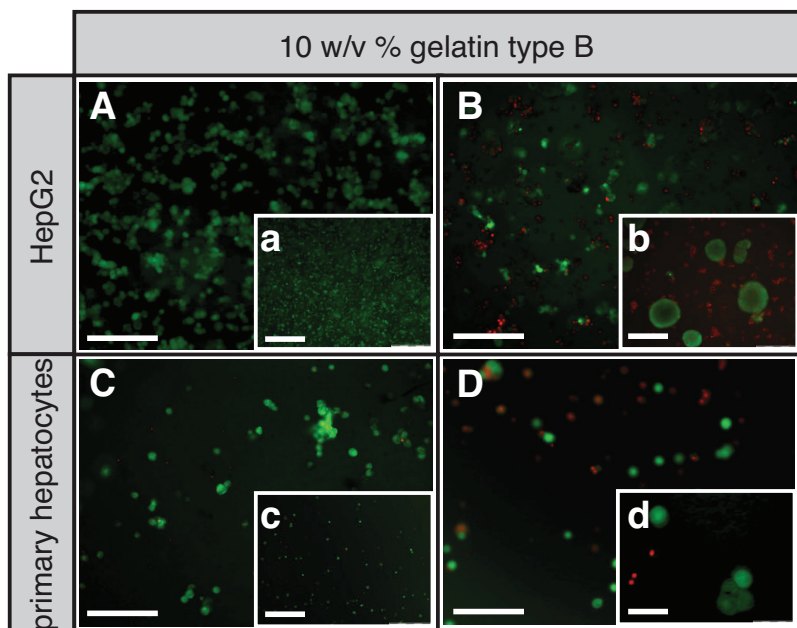


Figure 3.10: The use of mTGase as a natural cross-linker for the production of cell-laden sheets using 10 w/v % gelatin type B as cell embedding matrix. Live and dead stain of encapsulated HepG2 cells at day 1 (A,a) and day 7 (B,b). Live and dead stain of encapsulated mouse primary hepatocytes at day 1 (C,c) and day 3 (D,d). Scale bar represents 200 μm (A-D), 500 μm (a,c), 50 μm (b,d).

Initially, HepG2 cells showed promising viability levels ($> 95\%$) after encapsulation in 10 w/v% type B gelatin. Unfortunately, the cell survival rate dropped significantly with cultivation time. It is highly likely that the resulting hydrogel network has a dense skeletal framework and thus interferes on the long-term viability due to the generation of too highly constricting gels. To solve this, two options are available: either lowering the gelatin concentration or deactivating the enzyme after a certain reaction time. Traditionally, mTGase enzyme is inacti-

vated by cooking, which cannot be done with cell-laden matrices. While lowering the gelatin concentration seems appealing and could help in the generation of 2D cell-laden sheets, this strategy cannot be followed for the production of 3D open porous Bioplotting cell-laden scaffolds. Up to date, we could not find a relevant alternative solution meeting the needs for this work.

Kuwahara et al.³⁶⁰ encapsulated MSCs with high viability in a 10 w/v% gelatin using transglutaminase as a cross-linker, and observed a cell morphology change from round to network-like structures having high proliferation. In this case, the authors cited the advantage of using mTGase as this enabled co-crosslinking of the native collagen with the injected cell-laden gelatin matrix. However, they stated a potential drawback of applying high gelatin concentration by constricting the cells as has been observed by Hoshikawa et al.³⁷¹ in the encapsulation of chondrocytes in gelatins. However, for their MSCs (smaller than hepatocytes) they did not observe adverse effects. Recently, Ijima et al.³⁷⁰ reported the successful use of gelatin-transglutaminase for the cultivation of encapsulated primary hepatocytes with promising results. Unfortunately, the gelatin working concentration was not reported but since the hot gelatin solution was filter sterilized, it is safe to assume that concentrations far below 10 w/v% were applied. These findings suggest that mTGase cross-linking could be a promising alternative, however since we are limited to higher concentrations in order to enable Bioplotting, and these high concentrations result in too confined networks for hepatocyte-like cells (which are rather big), this strategy was abandoned.

3.4 | CONCLUSIONS

Four gelatins having different origin and extraction processes were investigated for their difference on the primary structure, molecular weight (MW), molecular weight distribution (MWD), gelification, and *in vitro* cytocompatibility for HepG2 cells and primary hepatocytes.

The amino acid composition of porcine and bovine derived mammalian raw materials differed mainly in the amount of aromatic amino acids, however, the influence of the acid or alkaline pre-treatment proved to be more obvious. Three bovine type B gelatins had similar amino acid compositions. As a result of the extraction process, the MW and MWD of the gelatins showed clear differences. Type A gelatin mainly consisted of low molecular weight (LMW) molecules, while type B gelatins all had a clear α -chain peak. The bovine hides type B gelatin additionally had a clear β - and γ -chain contribution. The observed differences resulted in variations of the gelification potential. It was observed that mainly the α -chains contributed to the final gel strength, while the β -, γ -chains and HMW molecules are able to perturb the physical helix reformation but contribute highly to the solution viscosity.

Evaluating the cytocompatibility of HepG2 cells in physically cross-linked gelatin hydrogels, a clear relation between cell death and gelatin concentration could be observed. An optimal balance between cell viability and gelatin physical properties is situated at around 10 w/v%. Overall, the tested bovine type B gelatin demonstrated lowest toxicity levels even up to a concentration of 30 w/v%.

Finally, the unmodified gelatins were subjected to two types of natural cross-linkers to examine the chemical cross-linking needed for cell biology applications. Genipin cross-linking proved to be difficult to control, thus excluding this approach for rapid prototyping applications where an extended control over the curing should be feasible. On the other hand, enzymatic cross-linking using microbial transglutaminase showed promising results. However, the inability to halt the curing under cell friendly conditions hampers strategies employing cell-laden hydrogels and enzymatic cross-linking. Nevertheless, for lower gelatin concentrations and sheet-like structures, enzymatic cross-linking would certainly deserve a thorough investigation.

4 | QUANTITATIVE CONTRASTS IN THE PHOTOPOLYMERIZATION OF ACRYLAMIDE AND METHACRYLAMIDE-FUNCTIONALIZED GELATIN HYDROGEL BUILDING BLOCKS

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This chapter tackles the synthesis and free-radical initiated photo-polymerization process of cross-linkable, photo-sensitive, gelatin derivatives. The cross-linking strategy is evaluated for its effects on the important hydrogel network properties. This work was partly, the data on type B gelatin, published in Macromolecular Bio-science.³⁷²

4.1 | INTRODUCTION

In the field of tissue engineering, material engineers together with various researchers from other disciplines aim to closely mimic the extracellular matrix (ECM) composition of a certain target tissue. As such, it is understandable that naturally derived macromolecules are being employed in this rapidly evolving scientific field. In particular hydrogels, of which the hydrated states resemble that of the ECM, allow for an easy exchange of gasses, nutrients, and metabolites.⁷ Generally, the liquid precursory state can be solidified by means of physical and/or chemical cross-linking in the presence of water.

The use of gelatin-hydrogel systems is limited by a reversible temperature dependent sol-to-gel transition (upper critical solution temperature (UCST) thermo-reversible type of material). As described in the previous chapter, a variety of chemical cross-linking approaches have been developed over the years to tackle this limitation. For example, Genipin cross-linking,^{310,373} carbodiimide chemistry,^{148,310,374} the use of aldehyde cross-linkers,^{152,375,376} and enzyme-triggered gelation^{133,155,377,378} strategies have been explored. General agreement regarding one standard cross-linking protocol that satisfies the needs of material scientists and cell biology experts alike, has yet to be achieved. In most approaches, native gelatin is used for the formation of a chemically cross-linked hydrogel. One of the major drawbacks is, however, that cross-linking kinetics are less controllable and possible cytotoxic side effects of some cross-linkers cannot be ignored (e.g., glutaraldehyde³⁷⁹).

Interestingly, the chemical composition of gelatin allows for straightforward modifications of the inherent functional groups towards desired (reactive) side groups or coupling with other bioactive ligands.^{109,223,357–359} This has aided researchers to modify gelatin for light-activated free-radical cross-linking purposes, which can be regarded as more controllable and reproducible processes. Hereby, a biocompatible photo-initiator (PI) is needed. Several studies have claimed the merits associated with 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, I2959) compared to similar PIs.^{311,312,380} These merits comprise, among other, a favorable use in biocompatibility studies due to the absence of toxicity in its native form, while only slight viability decreases are observed in the activated form. In the case of gelatin, the choice of modification is mostly a derivatization with methacryl-based side groups. Nonetheless, it is known that acryl-based curing kinetics are considerably faster compared to methacryl-based ones.^{381,382} For example, Anseth et al.³⁸² observed up to three orders of magnitude larger kinetic constants comparing the photopolymerization of acrylates versus methacrylates. However, gelatin modification with acryl-based side groups

has not yet been reported, while it could potentially offer advantages when rapid curing reactions are desired (e.g., injectable scaffolds, cell encapsulation).

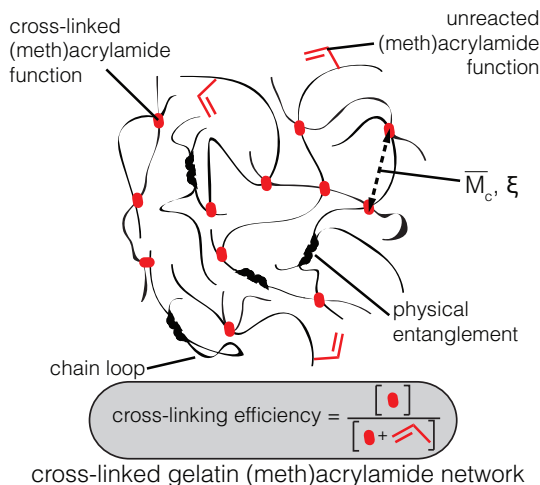


Figure 4.1: Schematic representation of chemical and physical junctions and their characteristic units.

The current chapter evaluates the synthesis and characterization of methacrylamide and acrylamide-modified gelatin. The focus of this study was aimed at a qualitative and quantitative comparison between both cross-linkable functionalities in terms of cross-linking kinetics and overall structural properties of the resultant gels (Figure 4.1). Also, the use of NMR techniques for the determination of the effective cross-linking efficiency was explored and compared with more conventional swelling experiments.

4.2 | EXPERIMENTAL SECTION

4.2.1 Materials

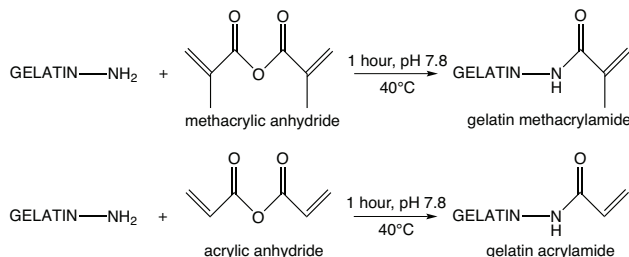
Bovine type B gelatin (approximate isoelectric point of 5 and Bloom strength of 257), isolated by alkaline treatment, and porcine skin type A gelatin, isolated by acidic treatment, were kindly supplied by Rousselot (Ghent, Belgium). Methacrylic anhydride (Sigma-Aldrich, Belgium), acrylic anhydride (ABCR, Germany), (meth)acryloyl chloride (Fluka, Belgium) were used without further purification. Dialysis membranes Spectra/Por (molecular weight cut-off (MWCO) 12–14 kDa) were obtained from Polylab (Antwerp, Belgium). Sodium phosphate (dibasic), potassium phosphate (monobasic), and tertiar-butylcatechol were purchased from Acros (Geel, Belgium). 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-

2-methyl-1-propane-1-one (I2959) was obtained from Ciba Specialty Chemicals (Groot-Bijgaarden, Belgium). A long-wave ultraviolet (LWUV) lamp model VL-400L (Vilber Lourmat, Marne La Vallée, France) was used for sample curing (UV-A light, 365 nm, 4 mW cm⁻¹). Pullulan standards (P-50, P-100, P-200, and P-400) were purchased from SDK Showa Denko. N-hydroxysuccinimide and deuterated solvents were purchased from Sigma-Aldrich (Belgium). HepG2 cells were maintained in a humidified 5% CO₂-containing atmosphere (37 °C) with cultivation medium consisting of DMEM, supplemented with 10 v % FBS, 50 U mL⁻¹ penicillin and 50 µg mL⁻¹ streptomycin, all provided by Life technologies.

4.2.2 Synthesis of cross-linkable gelatins

4.2.2.1 Synthesis of (Meth)acrylamide-Modified Gelatin

Gelatin was chemically modified with methacrylamide side groups, as described by Van Den Bulcke et al.²²³ The modification strategy with acrylic anhydride was performed in an analogous fashion in order to investigate the difference between methacrylamide and acrylamide side groups as photocross-linkable sites. The two modification strategies are schematically represented in Scheme 4.1.



Scheme 4.1: Schematic representation of the methacrylamide and acrylamide modification reactions yielding photo-sensitive gelatin derivatives.

Typically, 20 g of type B gelatin (7.6 mmol free amines) was dissolved in 200 mL phosphate buffer (pH 7.8) at 40 °C until complete dissolution was achieved. Next, 0.88 mL (7.6 mmol, 1 eq, 29 ppm MeHQ radical inhibitor) of acrylic anhydride or 1.13 mL (7.6 mmol, 1 eq, 23 ppm topanol A radical inhibitor) of methacrylic anhydride was added drop-wise to the gelatin mixture during vigorous stirring. A series of (meth)acrylic anhydride additions (1.9 – 3.8 – 7.6 – 11.4 – 15.2 mmol) were performed to investigate the reaction proceedings. The pH of the mixture was adjusted with NaOH solution (1 M) to be kept constant at pH 7.8. After a reaction period of 1 h under vigorous stirring at 40 °C, the mixture was diluted with 200 mL double distilled water until a homogeneous mixture was obtained. The solution was transferred to dialysis membranes and dialyzed against distilled water for 24 h

at 40 °C. Lyophilization (Christ alpha I-5) yielded the purified gelatin derivate. An identical setup was applied for the modification of type A gelatin. The methacrylamide and acrylamide-modified gelatins were respectively stored at room temperature and -20 °C until further use. The amount of reagent is expressed as equivalents to the amount of free amines on the gelatin backbone. The modification step with acrylic anhydride was analyzed for five different reagent additions (between 0.25 and 2 equivalents), and compared to modification with methacrylic anhydride.

4.2.2.2 Preparation of (thin) gelatin films

Typically, 0.5 – 2 g of gelatin was dissolved in 10 mL phosphate buffered saline (PBS, pH 7.4) at 45 °C. After complete dissolution, 2 mol% (relative to the amount of double bounds) of an 8 mg mL⁻¹ stock solution I2959 in distilled water was added to the mixture, followed by degassing for at least 5 min. The heated gelatin solution was injected between two parallel glass plates, separated by a 1 mm thick silicon spacer. In order to prevent the sticking of the gels to the glass plates, they were covered with a thin Teflon sheet. Finally, the hydrogel was irradiated on both sides with UV-A light. The chemically cross-linked gelatin films were either directly used for further characterization, or stored at 5 °C.

For a number of characterization techniques, thin gelatin films were prepared using a table-top spincoater system (SPIN15, SPS-Europe B.V.). The spincoating protocol was described in section 3.2.5. Thin films were prepared starting from different concentrations (5, 10, 15 and 20 w/v %). Spincoated samples containing 2 mol% initiator were UV-A cured unless indicated otherwise. Samples were stored in a desiccator at room temperature until further analysis.

4.2.3 Characterization of the physico-chemical properties

4.2.3.1 Effect of Modification Reagent on the Properties of Cross-Linkable Gelatins

The amount of free amines, converted into cross-linkable sites is expressed as the degree of substitution (DS). This value can be calculated from ¹H NMR spectra after gelatin modification with either methacrylamide or acrylamide side groups. The ¹H NMR spectra were recorded at 45 °C in deuterated water using a Bruker AVANCE II 500 MHz. The degree of substitution was obtained after comparison of the integrations of the characteristic peaks of the methacrylamide (5.7 ppm and 5.95 ppm) or the acrylamide substituent (5.8 ppm and 6.25 ppm) and the integration of the area corresponding to the combined peaks of valine, leucine and isoleucine (at 1.2 ppm). The hydrophobic alkyl side chains of the latter amino acids can be considered chemically inert and their signal serves as an internal standard.

These amino acids have a known fixed concentration and can be found in section 3.3.1. The results are expressed as the calculated DS (%) as a function of the added amount of reagent (in equivalents).

Changes in the molecular weights after the modification step were analyzed by means of size-exclusion chromatography (SEC). The setup and protocol was described in section 3.2.3. The results are presented as relative changes to the values for the respective non-modified gelatins.

Bulk changes after the introduction of cross-linkable groups were analyzed by means of FT-IR spectroscopy in attenuated total reflection (ATR) mode (Spotlight 400 Imaging System). FT-IR spectra of the gelatins were measured over a frequency range of $750 - 4000\text{ cm}^{-1}$. All spectra were recorded in the spectral range by accumulation of 64 scans with a spectral resolution of 8 cm^{-1} . Non-modified gelatin was chosen as reference sample.

Alterations in the elemental chemical composition due to the modification step were investigated by means of X-ray photoelectron spectrometry (XPS, see section 11.2.5). Spincoated samples, before and after modification, were analyzed. Measurements were performed on an ESCA S-probe VG monochromatized spectrometer with an Al $K\alpha$ X-ray source (1486 eV). A survey scan spectrum was collected and from the peak-area ratios, the relative elemental composition of the material top layer was determined. The generated data are displayed as a plot of the electron counts versus the electron binding energy. *CasaXPS* software was used for data analysis.

4.2.3.2 Equilibrium swelling and cross-linking efficiency

Cross-linked hydrogel films (1 mm thick, $\emptyset = 10\text{ mm}$) were freeze-dried and weighed (m_{di}). The dry disks were incubated at 37°C , and immersed in double distilled water. After equilibrium swelling was reached (e.g. 24 h), the disks were removed, gently dabbed with paper and weighed again (m_{he}). Subsequent freeze-drying gave the final dry weight (m_{de}). The loss of dry mass of the hydrogel as well as the water uptake capacity during incubation at 37°C enabled us to calculate the gel fractions and the swelling rates. During the swelling experiments, changes in macroscopic geometry were determined with an electronic calliper. He-pycnometry (Accupyc 1330, Norcross, GA) was used for the determination of the volumetric changes. One cycle consisted out of 10 measurements to obtain an average volume.

The effective cross-linking efficiency was studied using high-resolution magic angle spinning (HR-MAS) NMR (see section 11.2.6). Previous work from our group demonstrated the use of HR-MAS spectroscopy as a powerful tool to characterize

hydrogel cross-linking.³⁸³ Freeze-dried gelatin films were cut up into small pieces and inserted into a 4 mm 50 μ L MAS rotor. Solvent (D_2O) was added, causing the gelatin film to swell. The sample was homogenized by manually stirring the solvent and the gelatin inside the rotor. All 1H NMR spectra were recorded on a Bruker Avance II spectrometer operating at a 1H frequency of 700.13 MHz and equipped with a 1H , ^{13}C , ^{119}Sn triple resonance 4 mm HR-MAS probe. A spinning rate of 6 kHz was used throughout. The samples were characterized using one-dimensional (1D) 1H spectra and diffusion filtered 1D 1H spectra. The 1D 1H spectra were accumulated from 32 scans of 32768 fid points each with an acquisition time of 1.136 s and a spectral width of 20.6 ppm. A relaxation delay of 30 s was inserted between each of the 32 scans to fulfill quantitative conditions for integration. The water signal was suppressed using presaturation during relaxation delay. The diffusion filtered spectra were accumulated from 64 scans of 8192 fid points each with an acquisition time of 0.284 s with a spectral width of 20.6 ppm. A relaxation delay between the individual scans was used. A double stimulated echo sequence with adapted phase cycle was used.^{384,385} The diffusion filter was optimized to show only signals of protons bound to an immobile entity (no leftover signals originating from small compounds, such as solvent, non-reacted molecules, or impurities). For this purpose, the gradient pulse was applied using a sine bell shape with a length δ of 2 ms and a maximum gradient strength of 0.33 T m $^{-1}$. Fourier transformation, phasing, baseline correction and integration were done using *TopSpin 2.1* software. Gelatin films (1 mm thick) modified with either methacrylamide or acrylamide side groups, with concentrations between 5 – 20 w/v % were used for this study. The generated data are expressed as the conversion in function of the UV-A irradiation dose and the initial gelatin concentration. Each condition was measured in duplicate.

4.2.3.3 Static contact angle measurements

Static water contact angle measurements were performed on the non-modified and modified spincoated thin films in air using the sessile drop method (1 μ L) on a contact angle instrument, OCA 20 (Dataphysics, Benelux Scientific). A video was collected and Laplace-Young fitting was used in order to determine the contact angles 30 s after positioning the drop. This method was used to determine the wettability of gelatin substrates (see section 11.2.3).

4.2.3.4 Atomic force microscopy analysis

Topographical changes induced by the gelatin concentration or the curing conditions were studied by atomic force microscopy (AFM) on spincoated samples. AFM images were obtained according to the conditions as described in section

3.2.5. *Nanoscope* software version 4.43r8 was used for surface roughness analysis after the recorded images were modified with an X and Y Plane Fit Auto procedure.

4.2.3.5 Rheological properties of hydrogels

Rheological characterization of cross-linked gelatin films (1 mm thick) was performed using a Physica MCR 350 (Anton Paar) with plate-plate geometry. The linear visco-elastic range was determined by isothermal measurements (37°C) of the storage and loss moduli G' and G'' as a function of the deformation ($\gamma = 0.01\% \rightarrow 1\%$) at a constant frequency (1 Hz). Mechanical spectra were recorded at a constant deformation of 0.1% strain in the frequency range of 0.1 – 100 rad s⁻¹ at 37°C. Prior to loading, cross-linked samples were incubated in double distilled water at 37°C for at least 24 h to ensure complete leaching of the non cross-linked hydrogel fraction.

Additionally, *in situ* curing of (meth)acrylamide-modified gelatin precursor solutions (10 w/v %) were carried out at 20°C. For this, non-irradiated 2D slabs were placed between the two plates. Irradiation was realized from the bottom by means of an optical fiber (365 nm).

4.2.4 *In vitro* cytocompatibility

(Meth)acrylamide gelatin precursor solutions were prepared as previously described and mixed with HepG2 cells at a final concentration of 1.5×10^6 cells mL⁻¹. Prior to cell mixing, the hydrogel precursor mixture was pasteurized three times at 70°C, while PBS and I2959 stock solutions were filter sterilized. The cell-gel mixtures were cast in 48-well cultivation plates (Greiner-Bio one), cured as previously described and maintained in 500 µL cultivation medium. Medium was refreshed every 24 h. Prior to the live/dead assay, the hydrogels were washed twice with PBS and subsequently incubated for 10 min with 1 mg mL⁻¹ calcein-acetoxymethyl (calcein-AM) (AnaSpec) and 1 mg mL⁻¹ propidium iodide (Sigma). After washing with PBS, fluorescence was evaluated and recorded using an inverted fluorescence microscope (Olympus IX81) equipped with *Xcellence* software (Olympus).

4.2.5 Statistical analysis

All measurements were performed with $n \geq 3$, unless otherwise stated, and the experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical analysis was performed with the Student's *t*-test and significance was determined at $p < 0.05$. Statistical analysis was used in order to evaluate both the reproducibility of the respective techniques and the significance of differences between

samples.

4.3 | RESULTS AND DISCUSSION

4.3.1 Effect of the reagent on the modification of gelatins

Initially, we studied the influence of the applied reagent, methacrylic versus acrylic anhydride, on the finally obtained gelatins. The modifications as the reaction proceeds can be followed by expressing the DS as a function of the added amount of reagent, and the respective effects on the molecular weight and polydispersity index. Gelatin is a protein composed out of, among other, lysine (Lys), hydroxylysine (Hly), ornithine (Orn), valine (Val), leucine (Leu), and isoleucine (Ile). Of those, Lys, Hly and Orn contain free amines, which are the reactive sites consumed during the targeted modification step. The ^1H NMR spectra given in Figure 4.2 represents the spectra of gelatin (part A), gelatin methacrylamide (part B), and gelatin acrylamide (part C).

The peaks can be assigned as follows: peak a (1.15 ppm) to the methyl residues of Val, Leu and Ile; peak b (5.70 ppm) to the methylene protons $\text{H}_2\text{C}=\text{C}(\text{CH}_3)-$ of methacrylamide grafts confirming successful modification with methacrylic anhydride; peak c (5.83 ppm) to the methylene protons $\text{H}_2\text{C}=\text{CH}-$ of acrylamide grafts confirming successful modification with acrylic anhydride; peaks (1.45, 1.62, 1.91 and 2.01 ppm) to the methylene residues of threonine (peak 1), alanine (peak 2), lysine (peak 3), and arginine (peak 4); peaks (2.90, 3.21, 3.44 and 3.85 ppm) to methylene resonances of aspartate (peak 5), lysine (peak 6), arginine (peak 7), and proline (peak 8).¹⁴⁴ The total amount of free amines present in gelatin B is 0.38 mmol g^{-1} , which is in excellent correlation with earlier published data.²²³ For type A gelatin, a free amine content of 0.34 mmol g^{-1} was determined (chapter 3, section 3.3.1) On the other hand, the hydrophobic alkyl side chains of Val, Ile and Leu can be considered chemically inert, and their methylene signals (at 1.12 ppm) can be used as an internal standard for the calculation of the DS by means of ^1H NMR spectroscopy. The integration of this combined peak (18 protons) corresponds to 3.69 mmol g^{-1} for type B and 3.49 mmol g^{-1} for type A gelatin, allowing the following formulae to be used to calculate the DS (Equations 4.1 and 4.2):

$$DS_{\text{gelA}}(\%) = 0.3492 \text{ mol/100g} \times \frac{\text{integration at 5.7 or 5.8 ppm}}{\text{integration at 1.2 ppm}} \times \frac{100}{0.03398 \text{ mol/100g}} \quad (4.1)$$

$$DS_{\text{gelB}}(\%) = 0.3699 \text{ mol/100g} \times \frac{\text{integration at 5.7 or 5.8 ppm}}{\text{integration at 1.2 ppm}} \times \frac{100}{0.03798 \text{ mol/100g}} \quad (4.2)$$

The formula for the calculation of type B gelatin modifications is slightly altered in the case of bovine hide and bovine bone derived gelatins, according to their respective amino acid composition.

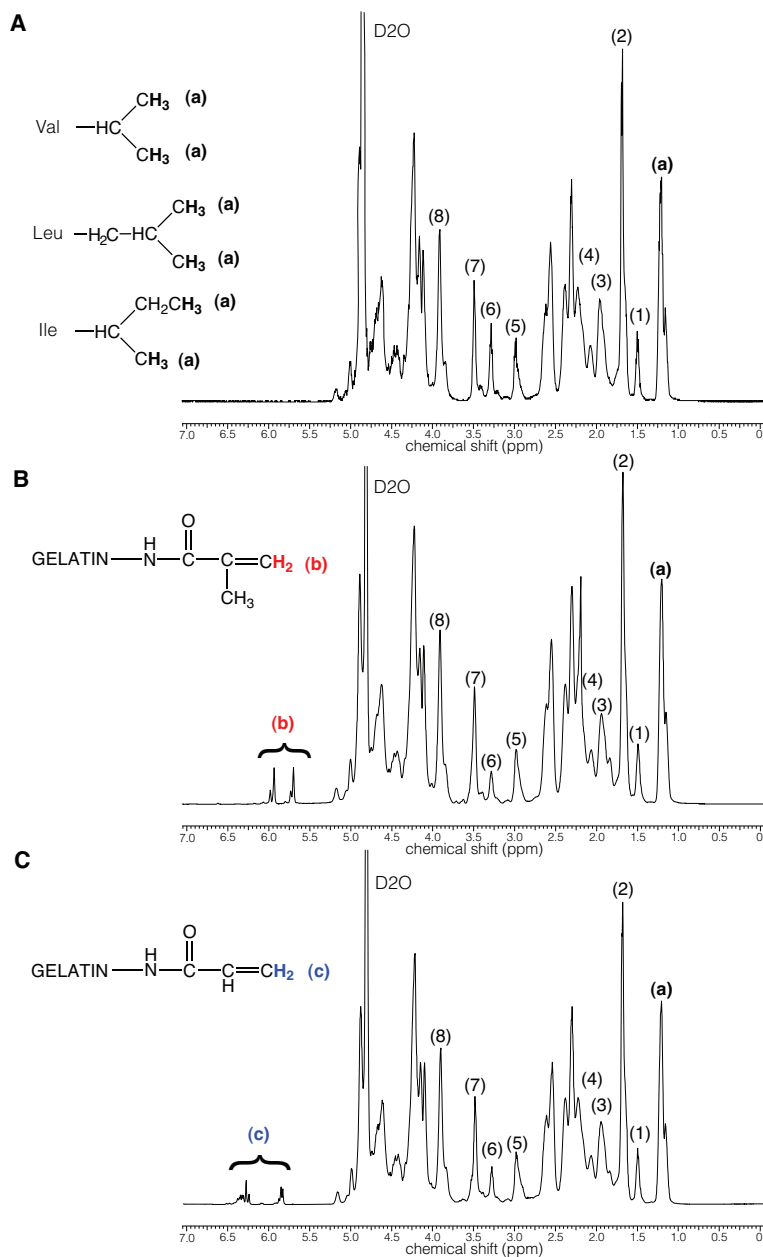


Figure 4.2: ^1H NMR spectra representative for gelatin (A), methacrylamide modified gelatin (B), and acrylamide modified gelatin (C).

Figure 4.3 illustrates both the changes in DS (e.g. modification curves) and alterations in the molecular weight and PDI. For simplicity, the modification curves for the bovine hides and bone type B gelatins were left out since the results were

analogue to the bovine type B mastercurve.

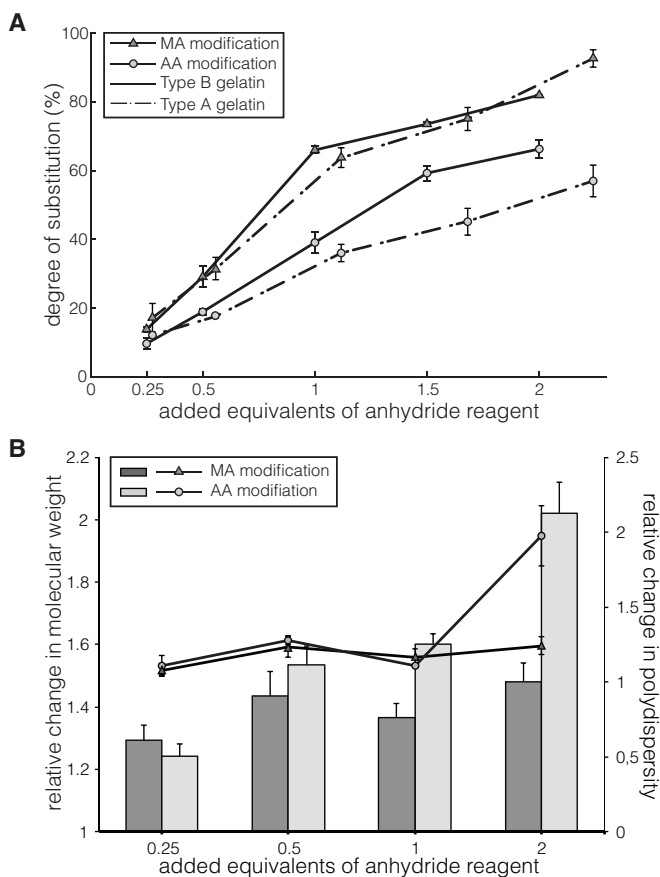


Figure 4.3: Influence of the applied reagent on the modification proceedings (top) and on differences in the molecular weights of the purified end products (bottom).

Statistically significant differences were observed between both modification curves, and the amount of introduced methacrylamide functionalities was higher for the whole range investigated ($p < 0.02$). This can most likely be attributed to the increased reactivity of acrylic anhydride compared to methacrylic anhydride. Since the reaction takes place in an aqueous environment, the anhydrides will decompose. However, the acrylic counterpart will do so more rapidly, leading to a higher consumption yet lower modification degree. Ultimately, these findings lead us to state that the modification efficiency with methacrylic anhydride is significantly higher than that with acrylic anhydride when the same numbers of equivalents are compared.

Furthermore, a significant increase in the molecular weight (a factor of 1.3–1.5), as well as the PDI (around 1.3) was observed after the introduction of both functional groups, compared to the native gelatin ($p < 0.03$). In contrast, when comparing methacrylamide modified gelatins with increasing modification degree, no significant differences could be found between the modified products (e.g. the DS did not impact the molecular weight). However, for the acrylamide modified products, an increasing trend could be observed with increasing cross-linkable groups (up till two-fold that of native gelatin). Differences between both functional groups became only significant for reactions with more than one equivalent of reagent. From this point onwards, molecular weight as well as the PDI rapidly increased. The above-mentioned observations could also be explained by the auto-initiated reactivity of the introduced functionalities. Since after the modification, a dialysis step of at least 24 h at elevated temperatures (40 °C) is performed, it would appear logical that some of the introduced groups react. This would be more pronounced with increasing functionalities, and/or with increasing reactivity. The described trends on the molecular weight and PDI were also valid in the case of type A gelatin derivatives (data not shown).

The degrees of substitution for gelatin methacrylamides correlate well with previous work from our lab, Van den Bulcke et al.,²²³ who originally established the synthesis of methacrylamide modified type B gelatin. Nichol et al.¹⁴³ fabricated gelatin type A methacrylamides containing degrees of substitution of 20, 54, and 80 % for the generation of cell-laden hydrogels. Hu and co-workers¹⁴⁴ coupled methacrylic acid via EDC-activated amidation and reported a DS of 49 % of the free amino groups. Recently, Hoch et al.³⁸⁶ synthesized a series of highly substituted type B gelatin methacrylamides by adding methacrylic anhydride in molar excess up to 20-fold with respect to the gelatin free amino groups. To the best of our knowledge, the modification with acrylamide functionalities has not been reported yet.

In order to enable further comparison between the two cross-linkable groups, two respective derivatives, of each gelatin type, with similar degree of modification were selected, whereby gelatin modified with one equivalent methacrylic anhydride was compared to gelatin modified with two equivalents of acrylic anhydride. Both products have a DS of ± 66 % in the case of type B gelatin, which means each of those derivatives contains ± 251 μmol photo-sensitive groups per gram of gelatin. For type A gelatin, the DS was ~ 63 % corresponding to ~ 214 μmol photo-sensitive groups per gram of gelatin. An indirect determination of the modification degree based on the spectrophotometric quantification of the amount of free amines was performed shortly after the modification step. These tests were especially performed since Michael Addition reactions could occur between the acrylic anhydride

reagent and the amines on the gelatin backbone. However, these results (data not shown) correlated well with the NMR method above. This means that the amount of consumed NH_2 - groups correlated well with the amount of calculated acrylamide groups via ^1H NMR. Therefore, Michael Addition side reactions were very limited ($< 1 - 2\%$).

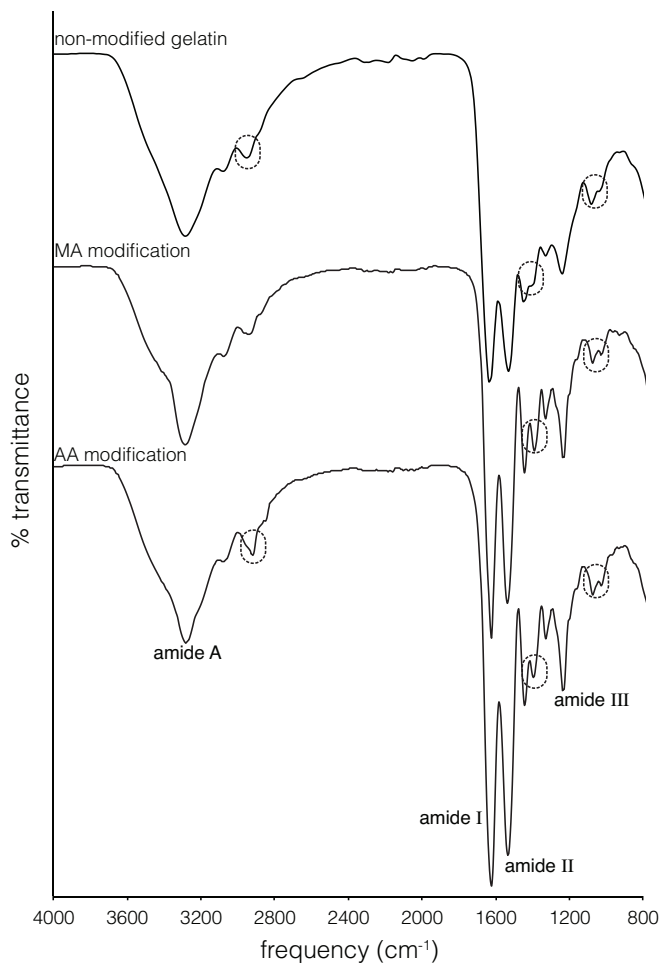


Figure 4.4: Changes in the FT-IR spectra of gelatin type B before and after modification with either methacrylic (MA) or acrylic (AA) anhydride.

Following material selection, FT-IR spectroscopy and XPS analysis were performed to gain a better understanding of possible compositional changes. The results are displayed in Figure 4.4 and Table 4.1. FT-IR analysis clearly showed the key absorbance peaks: native gelatin was characterized by the amide A peak (N-H stretching vibration) at 3280 cm^{-1} , the amide I peak (C=O stretch) at 1630 cm^{-1} ,

the amide II peak (N-H bend and C-H stretch) at 1547 cm^{-1} , and the amide III peak (C-N stretch plus N-H in phase bending) at 1240 cm^{-1} , which are characteristic peaks of gelatin.³⁸⁷ The incorporation of (meth)acrylamide moieties should be noticeable by an absorption band typical for vinyl groups (C=C stretching vibration at $1680 - 1620\text{ cm}^{-1}$), however, in our case this peak was concealed by the signal of the amide I peak. Nevertheless, the intensities of the amide I, II and III peak increased, which can be attributed to the incorporated amide-bonds coupled with a C=C stretching vibration. The peak at 3280 cm^{-1} served as internal reference. Only some minor differences could be observed in the rest of the spectra (indicated by dashed circles).

The relative elemental composition before and after modification, as measured by XPS, is depicted in Table 4.1. For example, for type B gelatins, the relative amount of nitrogen decreased significantly after modification from 16.3 to 14.9% (methacrylamide modified, $p < 0.001$) respectively 14.0% (acrylamide modified, $p < 0.001$). The relative amount of carbon slightly increased, while no significant changes in the oxygen signal could be observed. The atomic ratios N/C and O/C give more intuitive information. Especially for the N /C ratio, a decrease was expected after modification, since the amount of nitrogen remained the same, while more carbon units were incorporated. Between both modifications, this ratio was not significantly different however it was different when compared to native gelatin (control condition). The O/C ratio is more difficult to interpret since the amount of both atoms is increasing by the applied modification. Therefore, a less pronounced decrease of the ratio was expected, which was also evidenced by the data. Derivates of type A gelatin illustrated similar trends.

Table 4.1: Influence of the applied reagent, acrylic anhydride (AA) versus methacrylic anhydride (MA), on the elementary composition of the derivatives as determined by XPS.

SAMPLE	SURFACE COMPOSITION [%]			ATOMIC RATIO	
	O 1s	C 1s	N 1s	N/C	O/C
Type B Gelatin	18.4 ± 1.2	65.3 ± 1.3	16.3 ± 0.1	0.25	0.28
MA modification	18.5 ± 0.6	66.6 ± 0.4	14.9 ± 0.2	0.22	0.27
AA modification	18.5 ± 0.3	67.5 ± 0.2	14.0 ± 0.2	0.21	0.27
Type A Gelatin	20.7 ± 0.4	64.2 ± 1.1	15.1 ± 0.9	0.23	0.32
MA modification	20.5 ± 0.7	65.3 ± 0.7	14.1 ± 0.1	0.20	0.31
AA modification	20.0 ± 0.3	67.1 ± 0.3	13.0 ± 0.4	0.19	0.30

4.3.2 Effect of cross-linkable group on the reaction behavior of gelatin: acrylamide versus methacrylamide

The scope of our studies then further focused on the evaluation of two cross-linkable groups with different reactivities. Acrylate functionalities are regarded as the more reactive group compared to methacrylates.³⁸² To the best of our knowledge, no study has been performed comparing both methacrylamide and acrylamide reactive groups with the aim to develop gelatin derivatives for biomedical applications. Comparing acrylamide with methacrylamide modified gelatin, we anticipated differences in cross-linking degree (DC) and the curing kinetics.

4.3.2.1 Hydrogel Swelling studies

A conventional method (swelling studies) was used to determine the sol-gel fraction of the different derivatives developed for a dual reason: (i) to determine the degree of cross-linking as an indicator of (meth)acrylamide functionality after coupling, and (ii) to obtain a set of data that can be referenced against a HR-MAS method which is more powerful to study hydrogels (see below). These values can be used as a qualitative indication for the cross-linking efficiency. Furthermore, the water uptake capacity or swelling ratio of the hydrogels can be derived. The results are expressed as the gel fraction and the equilibrium mass swelling ratio (q) according to following formulas:³⁸⁸

$$gel\ fraction(\%) = \frac{m_{de}}{m_{di}} \times 100\% \quad (4.3)$$

$$q = \frac{m_{he}}{m_{de}} \quad (4.4)$$

here, m_{di} , m_{de} , and m_{he} represent the weights of respectively the dry gelatin after cross-linking, the dry gelatin after equilibrium swelling, and the swollen hydrogel after equilibrium swelling. In Figure 4.5, the gel fractions (part A and part B) and the swelling ratios (part C and D) of four different type B polymer concentrations selected to produce hydrogels (5, 10, 15 and 20 w/v %) are presented as a function of the applied UV-A irradiation dose. A relationship can be established between the hydrogel swelling, the hydrogel network and mass transport properties.¹⁴⁴ As anticipated, an increasing gelatin concentration and/or irradiation dose increases the final gel fraction and decreases the swelling ratio. For both modification reagents and each concentration applied, a plateau value for the gel fractions is obtained. Typical gel fractions of maximum 97% and final swelling ratios situated between 4 and 25 were obtained for all conditions. In the case of methacrylamide-modified gelatin, only the highest concentrations (15 and

20 w/v %) of precursor solution resulted in a measurable gel fraction for an irradiation dose as low as 240 mJ cm^{-2} . In contrast, all acrylamide modified precursor solutions yielded hydrogel networks. Furthermore, the minimal irradiation dose required to obtain the plateau gel fraction value was substantially lower in the case of acrylamide containing gelatins. These observations further prove the premise of a more reactive cross-linkable group, confirming the successful gelatin modification in both cases. The high swelling ratios up to 16 for the acrylamide- and 25 for the methacrylamide-functionalized fully cured gelatins illustrate the high water absorption capacity of these hydrogels. Furthermore, these data demonstrate the possibility to adjust the swelling behavior of cross-linked gelatins over a broad range by controlling the irradiation dose and mass fraction.

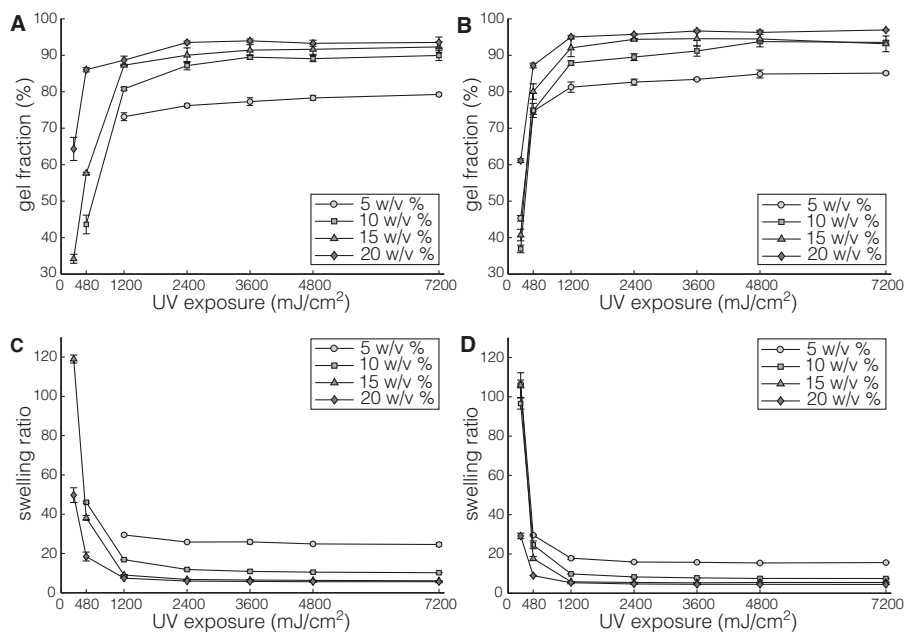


Figure 4.5: Cross-linking behavior of (A, C) methacrylamide versus (B, D) acrylamide modified type B gelatin based on swelling studies. (A, B) Gel fraction (%) as a function of UV-A exposure and (C, D) swelling ratio as a function of UV-A exposure.

As for the type A gelatin derivatives, similar trends are observed, whereby reactivity and final gel yields are enhanced for the acrylamide variant. Figure 4.6 presents the equilibrium swelling experimental data for type A gelatin (meth)acrylamides. The discrepancy between both photocross-linkable moieties is predominantly expressed at a hydrogel precursor concentration of 10 w/v % whereby for the gelatin methacrylamide even at an irradiation dose of 7200 mJ cm^{-2} the gel yield plateau

value is not yet reached in contrast to the acrylamide variant. With regard to the difference between type A and type B gelatin building blocks, generally, the type B derivatives displayed improved cross-linking kinetics leading to higher gel yields. As expected, the swelling ratios are slightly higher for type A gelatins although still in the same order of magnitude.

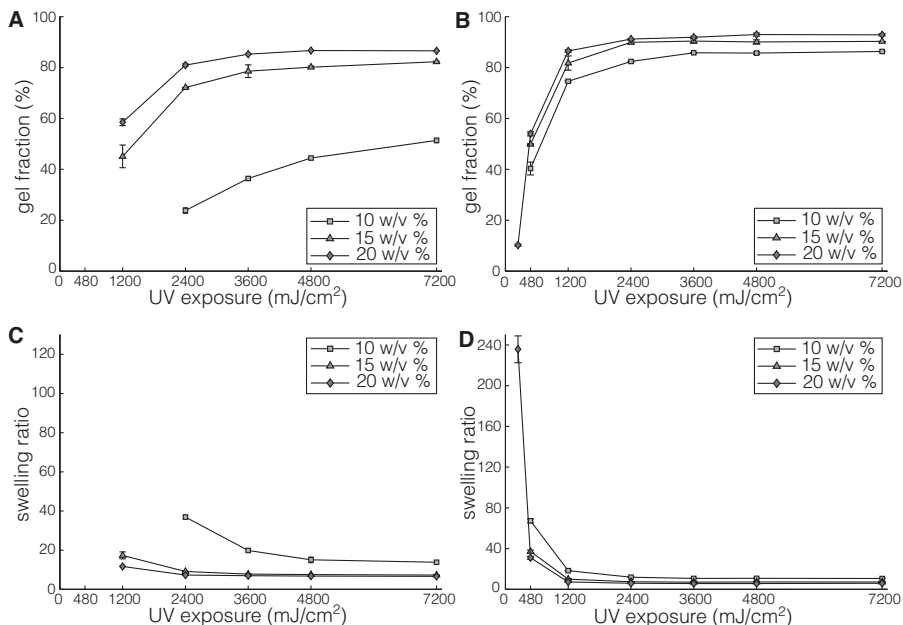


Figure 4.6: Cross-linking behavior of (A, C) methacrylamide versus (B, D) acrylamide modified type A gelatin based on swelling studies. (A, B) Gel fraction (%) as a function of UV-A exposure and (C, D) swelling ratio as a function of UV-A exposure.

The work of Hoch et al.³⁸⁶ reported gel fractions between 70 – 85 % for bovine type B methacrylamide gelatins having a DS of around 68 %, concentrations between 10 and 20 wt% and an irradiation dose of 9000 mJ cm⁻². Given the lower concentration of PI used, these results are comparable with the present study whereby higher gel fractions are obtained at lower irradiation dose. In accordance, hydrogel water uptake capacities were lower compared to the values obtained in the present study, which can be correlated to the lower gel fractions. Swelling studies performed on gelatin methacrylamide having a lower DS value (49 %) resulted in higher swelling ratios, as has been reported by Hu et al.¹⁴⁴ The mass swelling ratios in the work of Nichol et al.¹⁴³ on porcine skin type A gelatin methacrylamide were similar to our findings. From this, the advantage of a more rapidly curing gelatin derivative having good swelling properties could be useful, for instance in the field of rapid prototyping. Additionally, UV exposure times can be decreased

which could potentially benefit cytocompatibility.

4.3.2.2 Rheological analysis of cross-linked hydrogels

Despite the semiquantitative information that can be obtained from swelling tests, a quantitative description of the effective (chemical) cross-linking degree cannot be derived from these studies. These data are, however, very relevant when comparing cross-linkable groups of different reactivity or various amounts of one single type of cross-linkable entity. Rheological time-sweep tests coupled with in situ photocross-linking were performed to observe the increase in storage moduli (G') as the gel network is formed. Figure 4.7 depicts the observed effects for both types of cross-linkable groups using 10 w/v % type B hydrogels while keeping all other conditions fixed.

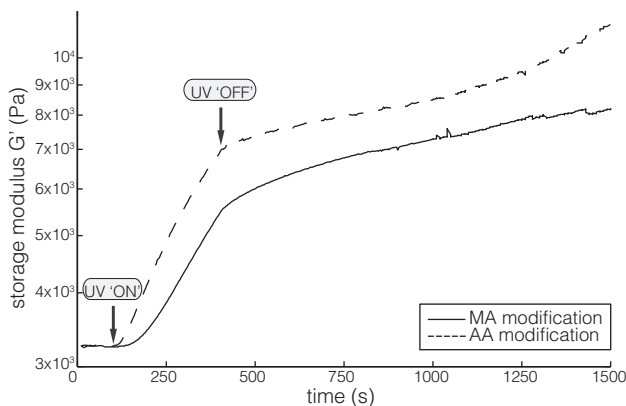


Figure 4.7: The cross-linking kinetics of type B gelatin (meth)acrylamides monitored by rheological time-sweep recordings for the storage moduli (G') of 10 w/v % hydrogel precursors. Dashed line represents acrylamide-modified gelatin (i.e., AA modification), full line represents methacrylamide-modified gelatin (MA modification). The zone in between arrows indicates UV irradiation.

From the obtained data, some interesting findings were deduced: (i) Acrylamide-modified gelatin directly starts to cross-link upon irradiation with UV-A. This is in contrast to the methacrylamide-modified gelatins, which showed a small lag phase before an increase in storage modulus could be observed. (ii) The slope of the curve is steeper for the acrylamide variant indicating faster cross-linking kinetics. (iii) The strength of the acrylamide based hydrogels was higher at any given time point. (iv) In both cases, post-curing occurs after halting UV-A irradiation. All of the above mentioned observations suggest a faster and more complete (i.e. more groups consumed) network formation in the case of the acrylamide-modified gelatins. It should be noted that the UV-A light source used in this rheological setup had a

different intensity and wavelength as the one used to produce the 2D films. Hence, despite the correctness of the sigmoid trends observed in the curing rates, kinetics as determined with this setup cannot be translated directly to predict curing under the other setup. It is however clear that the observed trends remain valid.

4.3.2.3 Analysis of cross-linked hydrogels by HR-MAS NMR spectroscopy

In addition to the rheological evaluation, quantitative HR-MAS NMR spectroscopy was performed in order to derive the absolute cross-linking efficiency. Changes in the intensities of the geminal ^1H (meth)acrylide resonances of $\text{H}_2\text{C}=\text{C}(\text{CH}_3)-$ (5.7 and 5.95 ppm) and $\text{H}_2\text{C}=\text{CH}-$ (5.8 and 6.25 ppm) were followed as a function of the irradiation dose, in this way quantitatively determining the conversion of respectively the methacrylamide and the acrylamide groups (Figure 4.8). The samples

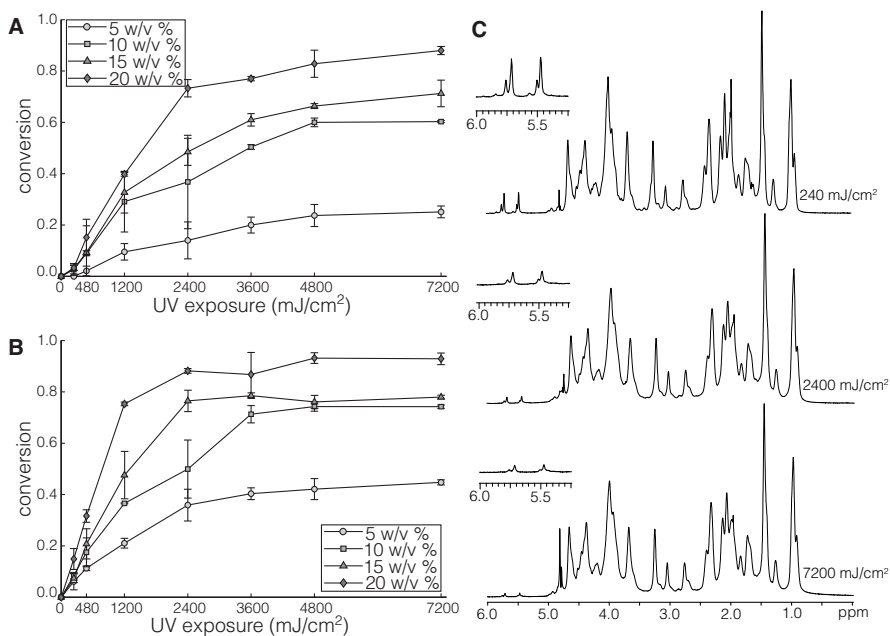


Figure 4.8: Effective cross-linking efficiency expressed as double bond conversion for (A) methacrylamide and (B) acrylamide-modified gelatin (type B). Values are based on HR-MAS ^1H NMR studies. (C) Examples of such data obtained with the resonances typical for the (meth)acrylamide shown as inset.

still contained the remaining sol fraction, and the corresponding resonance integrals were used to quantitatively calculate the total amount of reacted groups versus the total amount of available groups (i.e., the conversion). The combined

data from the swelling studies and HR-MAS ^1H NMR measurements demonstrated that a measurable gel fraction could be derived once $> 5\%$ of the double bonds were converted, which corresponded to a gel fraction of around 40% for type B gelatins. This stipulates that very low double bond conversions are sufficient for forming hydrogel networks. In correlation to the rheological findings, a delay in conversion for the methacrylamide-modified gelatins was observed, while a direct steep increase characterized the acrylamide conversion. In addition, it could be proven that even after obtaining the final gel fraction value (i.e., the plateau value during swelling studies), the absolute conversion further increased significantly ($p < 0.02$). These results strongly implicate the existence of a non-linear correlation between the qualitative and quantitative methods to monitor cross-linking efficiencies. When defining the cross-linking efficiency as a measure for the amount of reacted groups, one clearly overestimates this value when solely relying on results of swelling studies. It should be commented that the conversion values are depicted in reference to the initially available cross-linkable groups. When measuring hydrogels in fully swollen state (i.e., when the sol fraction will have leached out), these values will increase significantly. For example, a $10\text{ w/v}\%$ hydrogel will result in a conversion degree of $> 90\%$ only taking the gel fraction into account (compared to a value around 60% when the feed components are considered). Nevertheless, both sets of data yield very useful information about the network formation as a function of the applied irradiation dose. As previously mentioned, the acrylamide-modified gelatins cross-link faster, leading to gels with a higher conversion degree and better mechanical properties.

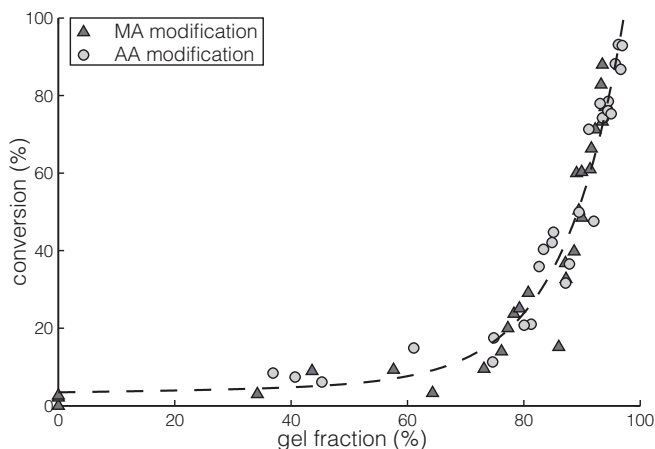


Figure 4.9: Correlation between the conversion degree and gel fraction of gelatin type B acrylamide (AA) and gelatin type B methacrylamide (MA). Exponential regression was performed on the total population (i.e., for four concentrations and for both modifications.)

In a last part of this comparative study into the reactivity differences between acrylamide and methacrylamide-modified gelatin we were interested to establish a possible correlation between the degrees of reacted groups, the gelatin modification type, the gel fractions, and the precursor concentration. Figure 4.9 displays a scatter diagram in which every (gel fraction, conversion) combination is plotted for type B gelatins, thus excluding the irradiation dose factor. For both modifications, the data sets of the four concentrations were grouped. Regression analysis revealed an exponential correlation between the gel fractions and the conversion degrees ($R^2 > 0.95$). Both data sets followed the same trend. In other words, knowing the correlation, one can predict the effective amount of reacted groups thus providing information about the gel fraction, or vice versa, independent of the precursor concentration or the modification type. Of course, this correlation can only be derived in cases where the same DS is used.

In accordance to the swelling studies, the conversion factors for the type A gelatins were lower than for type B derivatives, as depicted in Figure 4.10. Despite having slightly lower DS values, the (gel fraction, conversion) pairs for (meth)acrylamide-modified type A gelatins by and large follow the exponential correlation further proving the above-mentioned hypothesis.

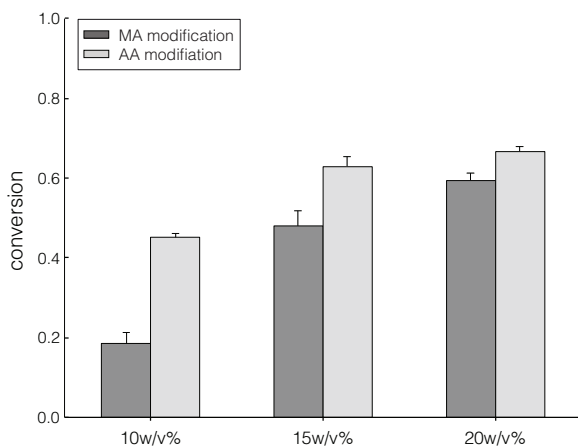


Figure 4.10: Effective cross-linking efficiency expressed as double bond conversion for type A (meth)acrylamide-modified gelatins. Values are based on HR-MAS ^1H NMR studies at a curing dose of 7200 mJ cm^{-2} .

4.3.3 Effect of cross-linkable group on the physico-chemical hydrogel behavior: acrylamide versus methacrylamide

Most often, dynamic force measurements are used to determine the visco-elastic behavior of hydrogels. The storage (G') and loss (G'') moduli were recorded over a frequency range of $0.1 - 100 \text{ rad s}^{-1}$ at 37°C after equilibrium swelling was reached. For all measured conditions, the storage moduli were 5-10 times higher than the loss moduli, indicating an elastomeric hydrogel network. No significant influence of frequency changes could be observed for both kinds of moduli, indicative of cross-linked systems. As shown in Table 4.2, the storage moduli are closely correlated to the concentration of the precursor mixture, and to the combined actions of the swelling capabilities and the amount of reacted groups in the final hydrogels.

Both moduli increased with increasing concentration, where higher cross-linking densities per volume could be obtained, resulting in a stronger ability to resist deformation. Comparing the data from both modification types, it could not only be confirmed that acrylamide-modified gelatins resulted in a faster cross-linking, but also that the resulting hydrogels were significantly stronger compared to the methacrylamide-modified counterparts at equal curing conditions. A broad range of storage moduli was obtained ranging between 7 and 152 kPa for the acrylamide- and between 1.5 and 107 kPa for the methacrylamide-functionalized type B gelatins. Type A gelatins had substantially lower mechanical strength with G' values ranging between 0.9 and 20 kPa.

It should be noted that the stiffness of gelatin hydrogels can be adjusted by varying the amount of photo-sensitive groups as has been demonstrated in the work of Hoch et al.,³⁸⁶ Van den Bulcke et al.,²²³ and Nichol et al.¹⁴³ Especially the work of the former illustrates the possibility to fabricate high stiffness gelatins (up to 368 kPa) by increasing the DS up to 98 %, which could become promising for engineering of “stiff” soft tissues. As indicated by the present results, stiffer gelatin hydrogels can also be obtained by varying the cross-linkable group, broadening possible applications. On the other hand, cross-linked methacrylamide gelatins were observed to be stable in the work of Van den Bulcke et al.,²²³ indicating potential as drug delivery matrix for wound healing.

When describing the nanostructure of cross-linked hydrogel materials, generally, the polymer volume fraction in the swollen state ($v_{2,s}$), the average number molecular weight between cross-linking points ($\overline{M}_c$), and the network mesh size (ξ) represent three important parameters. The polymer volume fraction in the swollen state ($v_{2,s}$) and the volumetric swelling ratio (Q) both describe the amount of liquid that can be imbibed in a hydrogel:^{132,389}

Table 4.2: Summary of experimentally measured variables of gelatin methacrylamide (MA) and gelatin acrylamide (AA) hydrogels. The gel fractions and mass swelling ratios were determined by swelling experiments, the percentage reacted groups was measured by HR-MAS spectroscopy, and storage moduli were derived from rheology on fully swollen samples. All values represent samples with 7200 mJ cm^{-2} UV-A irradiation.

SAMPLE TYPE	DS [%]	INITIAL CONC. [% , w/v]	GEL FRACTION [%]	REACTED GROUPS [%]	q	G' at 37°C [kPa]
TYPE B GELATIN	MA	5	79.25 ± 0.43	25.11 ± 2.30	24.55 ± 0.90	1.5 ± 0.4
		10	89.94 ± 1.40	60.30 ± 0.42	10.19 ± 0.19	14 ± 0.6
		15	92.28 ± 0.89	71.27 ± 5.17	6.09 ± 0.05	35 ± 1.5
		20	93.51 ± 1.48	87.99 ± 1.58	5.59 ± 0.06	107 ± 6.1
	AA	5	85.11 ± 0.31	44.73 ± 0.99	15.61 ± 0.10	7 ± 1.3
		10	93.52 ± 0.41	74.23 ± 0.63	7.45 ± 0.16	34 ± 1.8
		15	93.12 ± 1.85	77.95 ± 0.72	5.47 ± 0.18	64 ± 2.4
		20	96.92 ± 0.19	92.88 ± 2.26	4.44 ± 0.03	152 ± 3.1
TYPE A GELATIN	MA	10	51.36 ± 0.89	26.54 ± 2.92	13.84 ± 0.72	0.95 ± 0.2
		15	82.29 ± 0.25	47.96 ± 3.71	7.50 ± 0.14	8 ± 1.2
		20	86.69 ± 0.26	59.52 ± 1.76	6.63 ± 0.07	13 ± 1.5
	AA	10	86.33 ± 0.12	35.11 ± 1.15	10.65 ± 0.27	4 ± 0.3
		15	90.41 ± 0.23	63.01 ± 2.24	7.23 ± 0.12	11 ± 2.1
		20	92.95 ± 0.42	68.76 ± 1.15	5.88 ± 0.30	21 ± 1.7

$$v_{2,s} = \frac{V_p}{V_g} = \frac{1}{Q} = \frac{\frac{1}{\rho_{gelatin}}}{\frac{q}{\rho_{H_2O}} + \frac{1}{\rho_{gelatin}}} \quad (4.5)$$

where, V_p is the polymer volume, V_g the hydrogel volume at equilibrium swelling, q the mass swelling ratio, and $\rho_{gelatin}$ and ρ_{H_2O} the densities of gelatin and water. $\rho_{gelatin}$ was estimated to be 1.36 g cm^{-3} based on reports from literature.^{144,157,390} The volumetric swelling ratio Q was used to determine the average molecular weight between cross-links ($\overline{M}_c$) as network chains in all the hydrogels used in this experiment follow Gaussian statistics model (Figure 4.11):³⁹¹

$$G = \frac{cRT}{\overline{M}_c} \times \left(1 - \frac{2\overline{M}_c}{\overline{M}_n}\right) \times Q^{-\frac{1}{3}} \quad (4.6)$$

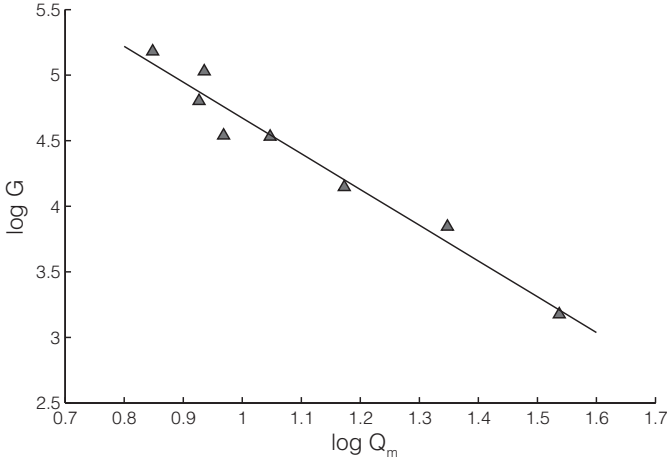


Figure 4.11: Log G versus log Q of all modified type B gelatin hydrogels used for the calculation of the molecular weight between cross-linking points.

where R is the universal gas constant, T is the absolute temperature, G is the shear modulus, c is the concentration of gelatin in the solution, and $\overline{M}_n$ the average molecular weight of gelatin. The hydrogel shear modulus may be derived from the mean peak value of the storage modulus G' .³⁷⁴ An estimation of the average mesh size (ξ), expressed in $\text{\AA}$, of the gelatin hydrogels at the equilibrium swollen state was based on the knowledge of $\overline{M}_c$, using the following formula:^{144,392}

$$\xi = 2\alpha' \times \left(\frac{\overline{M}_c}{M_r}\right)^{\frac{1}{3}} \times (2.21 \text{\AA}) \times Q^{\frac{1}{3}} \quad (4.7)$$

here, M_r is the average molecular weight of one amino acid (estimated to be 100 g mol^{-1}). α' is an expansion factor assumed to be 2.0 based on reports of gelatin conformation in solution similar to our conditions.^{157,392} Multiplication of

the freely rotating root-mean-square end-to-end distance, $(\frac{\bar{M}_c}{M_r})^{\frac{1}{3}} \times (2.21\text{\AA})$, with $2\alpha'$ gives the experimental root-mean-square end-to-end chain distance.³⁹² Finally, the cross-linking density (ρ_χ) was calculated according to:^{388,391}

$$\rho_\chi = \frac{1}{\bar{v}\bar{M}_c} \quad (4.8)$$

in which $\bar{v}$ denotes the specific volume of gelatin ($0.735\text{ cm}^3\text{ g}^{-1}$).

Corresponding to the experimentally determined data from the swelling studies and shear storage moduli, a summary of the calculated physico-chemical parameters is presented in Table 4.3.

Table 4.3: Influence of modification type, gelatin methacrylamide (MA) and gelatin acrylamide (AA), and gelatin concentration on $v_{2,s}$, Q , $\bar{M}_c$, ξ , and ρ_χ .

	SAMPLE TYPE	DS [%]	INITIAL	$v_{2,s}$	Q	$\bar{M}_c$ [g mol ⁻¹]	ξ [Å]	ρ_χ ($\times 10^{-4}$) [mol cm ⁻³]
			CONC. [% w/v]					
TYPE B GELATIN	MA	66 ± 1.0	5	0.029	34.45	15 429	357	0.88
			10	0.067	14.89	7 411	187	1.83
			15	0.108	9.30	5 562	139	2.44
			20	0.116	8.62	2 707	94	5.02
	AA	66 ± 2.8	5	0.045	22.27	10087	250	1.35
			10	0.090	11.15	4 969	139	2.73
			15	0.118	8.45	4 016	114	3.39
			20	0.142	7.05	2 609	87	5.22
TYPE A GELATIN	MA	64 ± 2.9	10	0.050	19.86	19 454	334	0.70
			15	0.089	11.22	13 611	231	1.00
			20	0.100	10.03	11 172	202	1.22
	AA	61 ± 3.2	10	0.064	15.51	15 067	271	0.90
			15	0.092	10.85	12 026	215	1.13
			20	0.111	9.01	9 977	184	1.37

As anticipated, increasing concentrations affect the hydrogel structure leading to decreasing mesh sizes. As an example, the average mesh size of a methacrylamide modified type B gelatin hydrogel decreases from 357 to 94 Å when the gelatin concentration is increased from 5 to 20 w/v %. In general, the acrylamide-modified gelatins results in hydrogels with a tighter network structure as evidenced by: (i) smaller (volumetric) swelling ratios, (ii) lower average molecular weights between adjacent cross-links, (iii) lower average mesh sizes, and (iv) higher cross-linking densities. It is important to stress that the determined cross-linking densities account for the physical as well as the chemical junction knots. According to the data

derived from HR-MAS experiments, the cross-linking density of chemical junction knots can also be derived and compared. It was noticed that the contribution to the cross-linking density due to reaction of the (meth)acrylamide functionalities decreases with increasing concentration. For example, approximately half of the total cross-links in a 20 w/v % gelatin type B hydrogel can be attributed to chemical junctions, while for a 5 w/v % hydrogel the contribution gets as high as almost 90 %. In summary, the contribution of chemical structuring due to double bond conversion, to the hydrogel network properties will decrease with an increasing concentration.

As for the topography of the hydrogels, AFM images were recorded for all hydrogel samples developed. Typical images for 10 w/v % hydrogels are represented in Figure 4.12. As can be seen, no large differences could be distinguished between non-modified gelatin with either of the modifications. Concerning the roughnesses of the samples, methacrylamide modification slightly decreased the roughness, while for the acrylamide modification roughness values slightly increased. The observed trends were, however, not statistically significant.

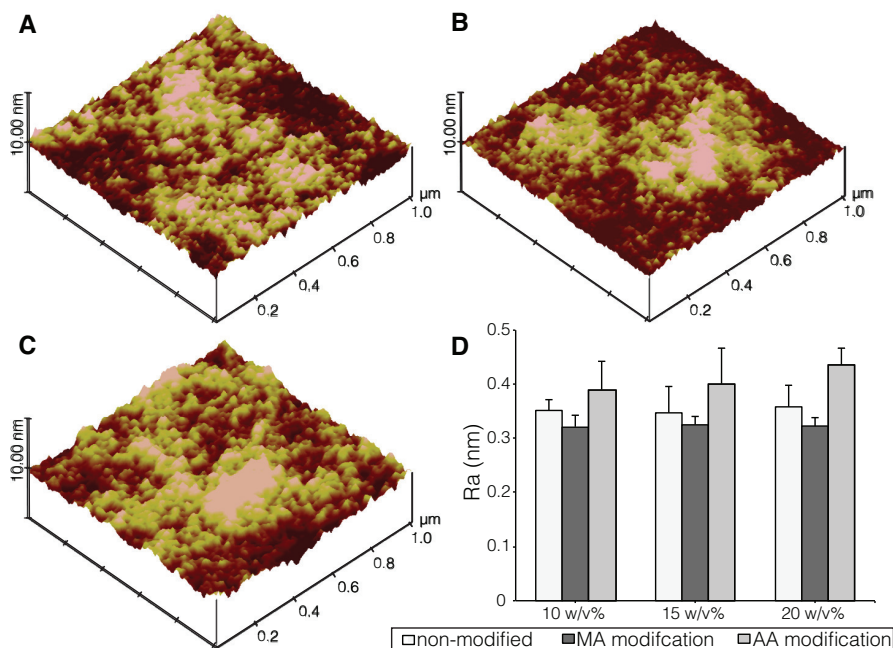


Figure 4.12: AFM images of (A) non-modified gelatin, (B) gelatin methacrylamide, and (C) gelatin acrylamide. Images all represent 10 w/v % type B hydrogels. The roughness is expressed by the R_a values as determined from $1 \mu\text{m}^2$ scan areas.

4.3.4 *In vitro* cytocompatibility study: acrylamide versus methacrylamide-functionalized gelatin

For a number of applications, fast curing materials could be beneficial or even required (i.e., rapid prototyping technologies). In the present study, we evaluated acrylamide side groups as a fast curing alternative for the more conventional methacrylamide gelatins. With regard to biological applications, preliminary cell encapsulation cytocompatibility testing was performed using HepG2 cells. Additionally, the influence of gelatin precursor concentration was taken into account since we anticipated the impact on the network structure and material stiffness. Type B gelatins were selected for the evaluation of both cross-linkable functionalities, since these revealed lower cytotoxicity compared to type A gelatin (chapter 3, section 3.3.5). Figure 4.14 represents live/dead staining fluorescence images for 10 and 20 w/v % (meth)acrylamide hydrogels. The results for viable cell counts are represented by Figure 4.13.

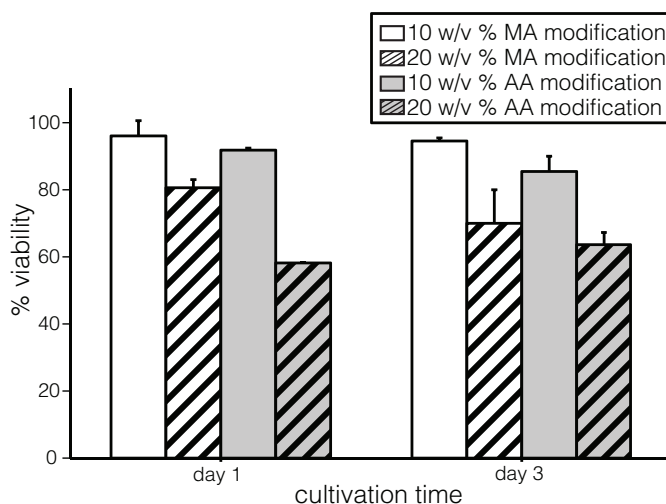


Figure 4.13: Viability values (%) of encapsulated HepG2 cells at day 1 and 3 of the respective hydrogels (type B gelatins).

As a first observation, it is clear that for both derivatives, a concentration of up to 10 w/v % poses no adverse effects on the encapsulated cells, and cell viabilities of $> 90\%$ were observed. However, increasing the gelatin concentration resulted in a small drop of the cell viability, which was more pronounced for the acrylamide-modified gelatins. This experimental setup was mainly used to pinpoint qualitative trends to differentiate gelatin concentration and modification type. These observations can be explained as follows: both material derivatives and concentrations used were cross-linked using the same irradiation dose (1800 mJ cm^{-2}) to enable

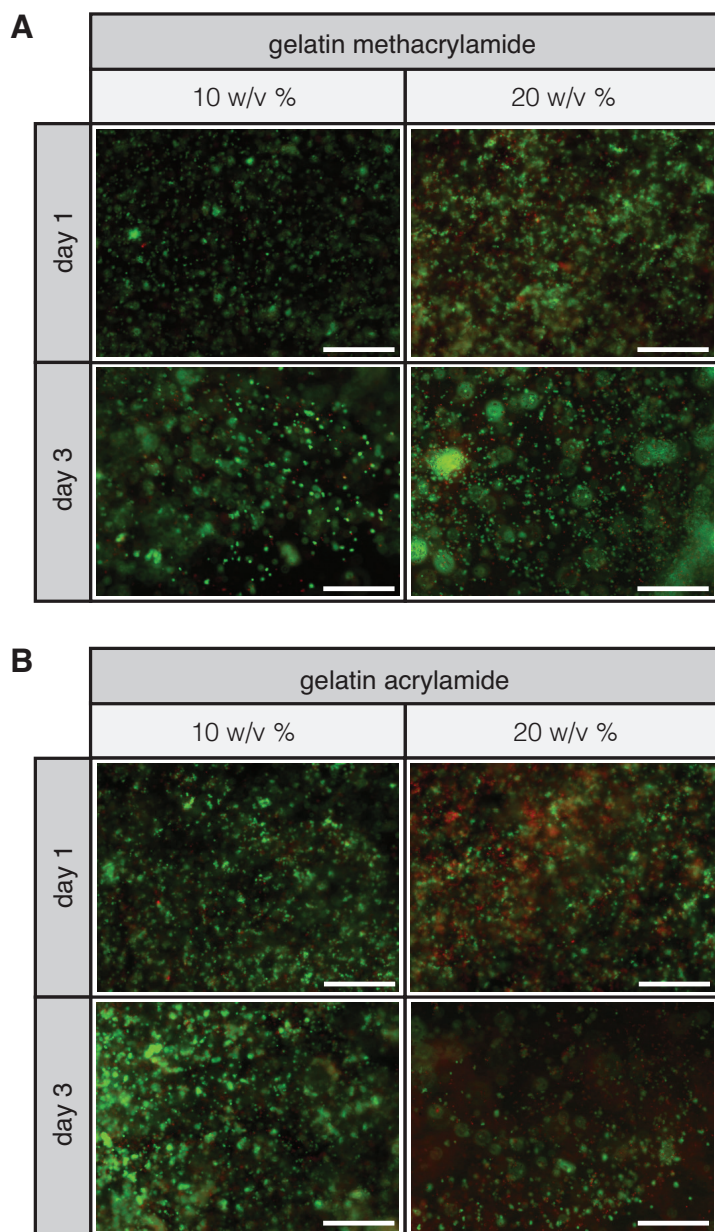


Figure 4.14: HepG2 cells with live (green) and dead (red) cell stain encapsulated in gelatin methacrylamide (A) and gelatin acrylamide (B) of different type B gelatin concentration after 1 and 3 days of cultivation. Scale bar 500 μm .

material comparison. As such, it would be expected that a drop in cell viability caused by stiffening the construct would be more pronounced for the acrylamide derivatives since these will possess the highest G' values. Additionally, Michael Addition reaction of the residual acrylamide groups could increase toxicity due to reaction between the acrylamide and $-NH_2$ function of proteins. We anticipate this effect to be more pronounced at higher concentrations. However, one should keep in mind that the irradiation dose for gelatin acrylamide can be reduced without a significant loss in gel fraction.

McGuigan and Sefton⁷² encapsulated HepG2 cells in non-modified gelatin constructs which were subsequently cross-linked using glutaraldehyde (0.025 %). They observed a significant loss in cell viability due to the applied glutaraldehyde. Material-cell cytocompatibility studies on gelatin methacrylamides for different gelatin mass fractions demonstrated favorable cytocompatibility using porcine chondrocytes.³⁸⁶ Promising results were also obtained in the micropatterning of NIH 3T3 fibroblasts using gelatin methacrylamides with cell viabilities of 92 % in 5 % gelatin methacrylamide.¹⁴³ The authors furthermore reported on the loss in cell viability as a function of increasing gelatin mass concentration, being a similar effect compared to our tests. In the present study, the use of gelatin methacrylamide as well as gelatin acrylamide as a promising matrix material for biological applications was demonstrated.

4.4 | CONCLUSIONS

The present investigation focused on unravelling possible differences in (i) the curing kinetics of various vinyl-based gelatin precursors undergoing photopolymerization and (ii) the physico-chemical properties of the obtained networks. Two photocross-linkable side-groups, methacrylamide and acrylamide, were successfully introduced using the gelatin primary amine functions. The acrylamide derivate was identified as the more reactive analogue. As a result, curing times could be decreased and stronger hydrogels could be produced as compared to the methacrylamide counterpart. In addition to the classical swelling studies, HR-MAS ^1H NMR spectroscopy was used to unravel the absolute conversion degree. Comparison between both of the aforementioned techniques indicated a non-linear correlation between the results of both techniques. Interestingly, when excluding the irradiation dose factor, the (conversion, gel fraction) pairs for both modifications follow the same exponential trend. It was shown that a conversion of $> 5\%$ of the introduced groups was necessary to obtain a stable hydrogel (type B derivatives). At this stage, the amount of gelatin that remained in the matrix was already 40% . Rheological time-sweeps revealed a direct conversion of the acrylamide moieties following irradiation, however, gelatin methacrylamide curing showed a lag-phase. More fundamentally, it could be concluded that a tighter network can be generated when using gelatin acrylamide. As a result, a decrease in average mesh size could be calculated at the nanomolecular level, while this translated towards a decreased (volumetric) swelling capacity at the macroscopic scale. In this way, hydrogels can be engineered with a predesigned network density which is important, for instance, in the fields of drug delivery and cell biology.

It has been shown that highly viable ($> 90\%$) cell-laden constructs could be obtained using $10\text{ w/v } \%$ (meth)acrylamide gelatins. Therefore, the acrylamide derivative could be a promising alternative to the more conventional methacrylamide derivative in terms of curing kinetics.

5 | AN INTERDISCIPLINARY STUDY ON THE 3D PRINTING OF GELATIN METHACRYLAMIDE CELL-LADEN TISSUE-ENGINEERED CONSTRUCTS WITH HIGH CELL VIABILITY

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*The current chapter describes the general workflow regarding the generation of 3D open porous (cell-laden) gelatin scaffolds on a parametric base. This work was previously published in Biomaterials.*³⁹³

5.1 | INTRODUCTION

In recent years, biocompatible cell culture substrates have been extensively revised. A steady paradigm shift from conventional 2D cell culture models toward 3D microenvironments has been observed.¹⁵⁹ Cell responses due to the microenvironment (mechanical and chemical) tend to differ between both models.³⁹⁴ Developments in (rapid) prototyping techniques, which were already well established in other industries (e.g. automotive industry), have enabled researchers to expand their *in vitro* tissue models towards highly controlled three-dimensional (porous) scaffold architectures.^{1,5,142,190,329,395} Three-dimensional porous scaffold designs allow for improved cell-cell contact, cell-matrix interactions, and increased cell densities.^{1,396} Furthermore, more efficient blood vessel ingrowth and enhanced oxygen, nutrient and waste diffusion are plausible.

Post-fabrication cell seeding benefits from these advantages but is often correlated with insufficient seeding efficiency and/or a non-uniform cell distribution.^{25,48} To tackle these problems, combining prototyping techniques with high-water content polymers and cell encapsulation strategies can serve as an alternative. Nevertheless, the generation of cell-laden prototyped scaffolds remains challenging and is mainly limited to hydrogel processing.¹ During processing of cell-laden hydrogel mixtures two major disadvantages can be described: first, the loss of cell viability due to dispensing pressure and nozzle diameter, which has been described by Chang and Sun³⁹⁷ for the encapsulation of HepG2 during alginate plotting, and second, hydrogel mechanically stable construct built-up without internal pore collapse remains the main challenge, even in the absence of cells.^{82,224,260} Generally, the latter is tackled by blending gelatins with other hydrogel materials, and/or co-deposition of thermoplastic materials.^{76,141,142,248,249,253,257} For instance, Schuurman et al.³⁹⁸ recently opted for blending of hyaluronic acid and/or co-deposition of poly- ϵ -caprolactone in order to be able to process 20 w/v % gelatin hydrogels without internal collapse. Another approach consist of printing sacrificial material at the future pore locations.³⁹⁵

Additionally, enhanced control over matrix stiffness and liquid flow (e.g. shear stress responses) is reported applying 3D culture models.⁴⁶ In this manner, close interactions between biological, chemical and engineering cues are important in the feedback process for the generation of, for example, drug screening tools or implantable devices (Figure 5.1). For example, Chang et al.³⁹⁷ developed an *in vitro* drug screening system via integration of a 3D cell-laden alginate hydrogel environment integrated within a microbioreactor.

Besides the previously mentioned advantages of 3D porous scaffolds, the introduc-

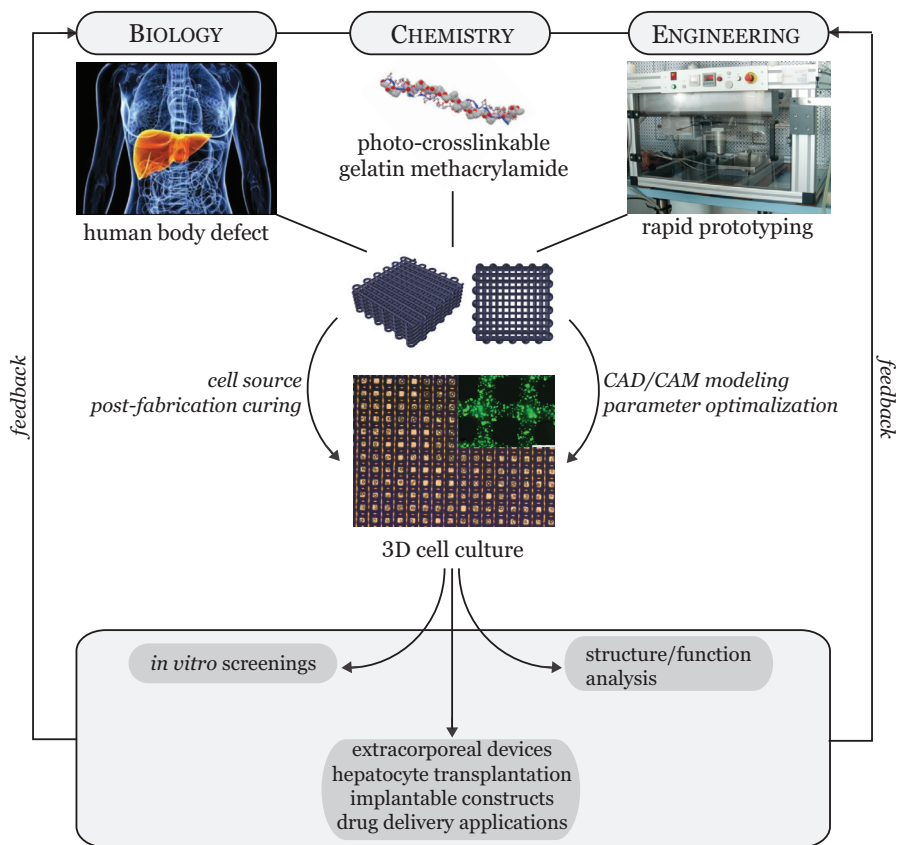


Figure 5.1: Schematic illustration of multi- and interdisciplinary workflow related to the use of rapid prototyping in the field of tissue engineering.

tion of pores has been reported to enhance both cell proliferation and albumin production of HepG2 cells in porous alginate hydrogels.³⁵⁶ Earlier work demonstrated good *in vitro* cytocompatibility of photosensitive gelatin methacrylamides for the encapsulation of fibroblasts, myoblasts, chondrocytes endothelial cells, and cardiac cells.^{143,398–400} Taking all this into account, the generation of a highly viable cell-laden gelatin scaffold with sufficient mechanical stability would be desirable.

The Biplotter technology enabled researchers to generate hydrogel-based constructs. The present study aims to develop a 3D microenvironment applying post-processing photo-induced free radical cross-linking of cell-laden gelatins. An evaluation of two photo-initiating systems is performed, introducing a novel photo-initiator, and a parametric study on the printing of (cell-laden) gelatin hydrogels is presented.

5.2 | EXPERIMENTAL PART

5.2.1 Materials

5.2.1.1 Cell culture

HepG2 cells were maintained in a humidified 5% CO₂-containing atmosphere (37 °C) with cultivation medium consisting of DMEM, supplemented with 10 v % FBS, 50 U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin, all provided by Life technologies.

5.2.1.2 Hydrogels

Bovine type B gelatin (approximate iso-electric point of 5 and Bloom strength of 257), isolated by alkaline treatment, was supplied by Rousselot (Ghent, Belgium). Photosensitive gelatin, gelatin methacrylamide, was synthesized (Scheme 4.1) as described in detail (chapter 4). The purified gelatin methacrylamide had a degree of substitution of 62 %, as determined by ¹H NMR (Bruker AVANCE II 500 MHz) in deuterated water (Sigma- Aldrich) at 45 °C. Prior to use, the hydrogel building blocks were sterilized by ethylene oxide (EO) treatment (cold cycle, Maria Middelaers hospital, Ghent, Belgium).

5.2.1.3 Photocross-linking presets

Two types of photo-initiators (PIs) were used in this study: (i) 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure® 2959, I2959) was obtained from Ciba Specialty Chemicals (Groot-Bijgaarden, Belgium); (ii) 2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) photo-initiator was purchased from Wako Specialty Chemicals. I2959 or VA-086 stock solutions in PBS were filter sterilized and added to the culture medium or hydrogel building blocks to create respectively final concentrations of 2 mol % or 20 mol % (with respect to the amount of double bonds). These precursor solutions were thoroughly degassed for at least 10 min prior to loading with HepG2 cells. A LWUV lamp model VL-400L (Vilber Lourmat, Marne La Vallée, France) was used for (cell-laden) hydrogel sample curing (UV-A light, 365 nm, 4 mW cm⁻²).

5.2.1.4 Rheological evaluation of gelatin methacrylamides

The effects of the applied PI on the *in situ* curing of gelatin methacrylamide precursor solutions (10 w/v %) as well as recordings of the mechanical spectra of cross-linked films (1 mm thick) were investigated following the protocol described in section 4.2.3.5. In order to investigate the influence of mixture concentration

and cell densities on the physical cross-linking of (cell-laden) gelatin methacrylamide solutions, G' and G'' were recorded as a function of the temperature at a constant frequency (1 Hz) and strain (0.1 %) for different gelatin methacrylamide concentrations (5 – 20 w/v %) and cell densities ($0 - 2.5 \times 10^6$ cells mL⁻¹). Additionally, shear stress and viscosity measurements as a function of shear rate ($0 \rightarrow 2000$ s⁻¹) and temperature ($15 \rightarrow 37$ °C) of (cell-laden) gelatin methacrylamide solutions were obtained by rotational viscosimetry at a gap size of 400 µm.

5.2.1.5 Equilibrium swelling experiments

Information on the PIs effects on cross-linking and swelling behavior was evaluated by equilibrium swelling experiments, as described in section 4.2.3.2.

5.2.2 3D strand deposition of gelatin methacrylamide hydrogels

Three-dimensional porous scaffolds were produced by sequential strand (also called strut or fiber) deposition using the Bioplotter pneumatic dispensing system (Envisiontec, GmbH, Gladbeck, Germany). A detailed description can be found elsewhere^{236,262} and in the techniques chapter section 11.1.2. ‘Strut’ and ‘strand’ are alternative terms commonly used to indicate the deposited fiber. Briefly, the Bioplotter operates as a three-axis dispensing machine that deposits (cell-laden) hydrogel precursors pneumatically on a stationary platform. Designed computer aided design/computer aided modeling (CAD/CAM) models are translated into a numerical code (NC code) by the Bioplotter software and contain the information for the layer-by-layer scaffold build-up. Since gelatins have a temperature dependent sol-gel transition, temperature control was of utmost importance. Therefore, two adaptations on the device were performed to ameliorate the processing of gelatin hydrogels:

- (i) The syringe heating mantle was adjusted to ensure a homogeneous plotting temperature till the tip of the dispensing needle;
- (ii) The stationary platform was equipped with Peltier elements which enabled cooling of the platform to temperatures far below the gelling point.

In the present research, a parametric study on the obtained strand diameters as a function of the gelatin methacrylamide initial concentration (5 – 20 w/v %), the applied pressure (0 – 5 bar), temperature (24 – 30 °C), nozzle internal diameter (150 – 200 µm), nozzle type (cylindrical vs. conical), and plotting speed ($0 - 1000$ mm min⁻¹) was performed. Constructs were configured in a 0°/90° fiber lay-down pattern. The fabricated constructs were examined using light microscopy,

to extract information regarding the pore networks. A Zeiss (KS 100, Axiotech) light microscope in combination with an Axiovision digital camera was used for top view, cross-section, and side-view imaging. Image processing was performed using *Carl Zeiss AxioCam* software.

5.2.3 Printing of cell-laden gelatin methacrylamide constructs

Hydrogel precursor solutions were prepared as previously described, and mixed with HepG2 cells to create a cell density of 1.5×10^6 cells mL⁻¹. The cell-gel mixtures were transferred into a syringe and loaded into the Bioplotter. In order to perform a parametric study on the plotting process, the mixtures were dispensed at various plotting speeds, air pressures, and temperatures. Finally, after optimization, 1 – 3 mm thick constructs of 13×13 mm were created with a layer thickness of 150 – 200 μ m and fiber spacing of 350 μ m and 550 μ m. The Bioplotter was placed in a flow cabinet, however, since other materials are being processed by the device (e.g. poly- ϵ -caprolactone), the risk of scaffold contamination was limited by autoclaving all materials in direct contact with the cells prior to processing. Furthermore, specific measures against bacterial contamination were taken by equipping the syringe inlet with a 0.2 μ m sterile air filter (TETPOR AIR 50, Parker). Constructs were printed on sterile glass cover slips, transferred to 6-well culture plates (Greiner-Bio One), cured as previously described, and maintained in 4 mL cultivation medium. Medium was refreshed every 24 hours.

5.2.4 Finite element simulation of syringe needle flow of gelatins

Finite element fluid flow simulations were conducted in COMSOL 4.0a (Comsol, Palo Alto, CA) using a mesh of free tetrahedral elements and boundary conditions of 1 bar inlet pressure and atmospheric pressure at the outlet. An incompressible, non-Newtonian fluid model was used to simulate the flow of noncross-linked gelatin using a power-law, shear-rate dependent relationship. The density, constants n (the flow behavior index) and K (the consistency coefficient) were empirically obtained. Gelatin solutions were assumed to obey a power-law following:

$$\tau = K\dot{\gamma}^n \quad (5.1)$$

with, τ the shear stress, $\dot{\gamma}$ the shear rate, K the consistency coefficient, and n the flow behavior index. For Newtonian fluids, $n = 1$, while for shear thinning fluids, $0 < n < 1$. Smaller n values represent more shear thinning behavior. Dynamic oscillatory rheometry data was curve fitted and the obtained coefficients

are used as the n and K values. Zero-shear viscosity is also reported as product of n and K . All simulations were found to be grid-independent and convergence criterion independent after mesh settings were studied, and all discretization errors converged to zero.

5.2.5 Scaffold characterization

5.2.5.1 Micro-computed tomography (μ -CT)

The printed scaffolds were also scanned in a micro-CT setup at the UGhent Centre for X-ray Tomography (UGCT) using a VARIAN Paxscan 2520 V Flatpanel detector and a Feinfocus X-ray tube. The resulting tomography data has been reconstructed at a voxel size of 8 μm . A detailed description on the system can be found elsewhere.⁴⁰¹ Extra information is included section 11.3.2.

5.2.5.2 Scanning Electron Microscopy (SEM)

Gelatin methacrylamide scaffolds were freeze-dried to remove the aqueous phase. Scaffolds containing HepG2 cells were fixed with 2 % glutaraldehyde in PBS at 4 °C, thoroughly rinsed with double distilled water, dehydrated with ethanol dilution series, and freeze-dried overnight. The dry scaffolds were subsequently coated with a (approximately) 20 nm thin gold layer. SEM images were recorded using a tabletop SEM (PHENOM™, FEI Company). The technique is described in section 11.3.1.

5.2.5.3 Live/Dead fluorescence staining

Cell-laden scaffolds were washed twice with PBS and stained following the protocol in section 4.2.4. Then the probes were discharged and the scaffolds were thoroughly rinsed (2×5 minutes) with PBS. To visualize and determine the ratio of living and dead cells, fluorescence was evaluated and recorded using an inverted fluorescence microscope (Olympus IX81) equipped with *Xcellence* software (Olympus).

5.2.5.4 Immunohistochemistry staining

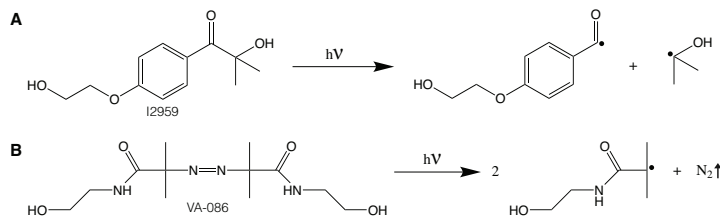
The cell-laden scaffolds were embedded in paraffin after fixation in 4 % paraformaldehyde at room temperature. Sections of 5 μm thickness were used for the different stainings.

PAS stain - After hydration, the sections were exposed to 1 % periodic acid for 15 min, washed (3×5 min) in PBS and exposed to Schiff reagent (Sigma) for 30 min in dark. After rinsing in water, the sections were stained using hematoxylin.

IHC staining - Endogenous peroxidase of the hydrated sections was quenched using 3 % H_{202} for 1 h. Subsequently, the samples were treated with blocking reagent (1 % BSA, 0.2 v % Tween 20 in PBS) for 30 min and then incubated for 2 h with the primary antibody (dilution 1:100). Next, the samples were incubated with the secondary antibody (dilution 1:200) for 30 min and visualized by the activity of conjugated horseradish peroxidase using a 3,3-diaminobenzidine tetrahydrochloride substrate. Finally, the sections were counterstained with hematoxylin. For hepatocyte nuclear growth factor 4 alpha (HNF4 α), Ki67 and proliferating cell nuclear antigen (PCNA), the entire procedure was preceded by antigen retrieval using citrate buffer (pH 6.00). Goat anti-human albumin antibody (p20, Santa Cruz), rabbit anti-human HNF4 α (H-171, Santa Cruz), mouse anti-human Ki67 (M7240, Dako) and mouse anti-human PCNA (PC10, Novo Castra) were used as primary antibodies.

5.2.6 Statistical analysis

Unless stated otherwise, all characterizations were performed at least in triplicate. Data analyses were performed applying the commercially available statistical package GraphPad Prism 4. Differences between groups were explored by one-way ANOVA, followed by a Student *t*-test.



Scheme 5.1: Radicals generated from I2959 photo-dissociation (A). Radicals generated from VA-086 photo-dissociation (B).

5.3 | RESULTS

5.3.1 Effect of photo-initiator type on the network properties and cell viability

To overcome the UCST material behavior of gelatin, gelatin was modified with methacrylamide side groups to yield a photosensitive gelatin derivate, as described previously. In the present part of our study, we opted not to apply acrylamide modified gelatins in order to exclude potential premature cross-linking as well as to avoid a mismatch in mechanical properties with encapsulated cells. In a first

series of experiments, the applicability of a less known PI (VA-086) was compared to the use of the widely well-known PI I2959. The radicals generated due to PI photo-dissociation are schematically shown in Scheme 5.1.

The applied concentration of a biocompatible PI should be the lowest concentration that still ensures desired cross-linking kinetics. Optimization of the VA-086 PI with respect to the finally obtained gel strengths, gel fractions, and curing kinetics led to a concentration of 20 mol % (in respect to the amount of double bonds on gelatin methacrylamide). In Figure 5.2 and 5.3, a comparison between the *in situ* curing kinetics and final gel strength of the two applied PIs is illustrated.

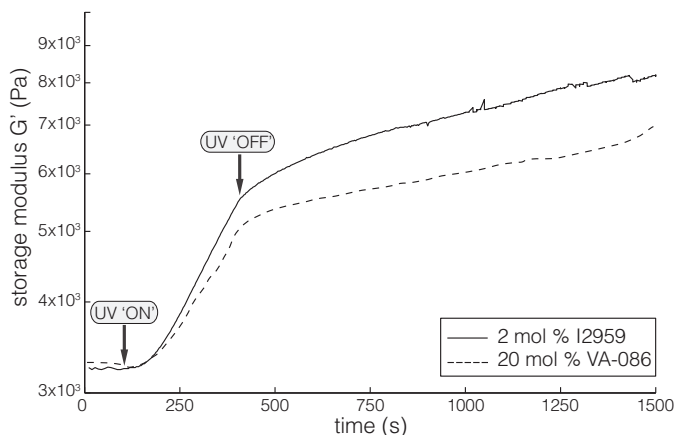


Figure 5.2: The cross-linking kinetics of gelatin methacrylamides monitored by rheological time-sweep recordings for the storage moduli (G') of 10 w/v % hydrogel precursors. Full line represents cross-linking using 2 mol % I2959 as photo-initiator, dashed line represents cross-linking using 20 mol % VA-086 as photo-initiator. The zone in between the arrows indicates UV irradiation.

First of all, in all cases, a curing lag time was observed. This meant that the substantial increase in storage moduli G' , indicating chemical cross-linking, was not observed simultaneously with the start of UV-A irradiation. Furthermore, an after-curing effect could be observed indicating continued cross-linking after UV-A irradiation was stopped. Secondly, the use of VA-086 demonstrated less efficient curing rates and lower curing end-point at “UV-A off” despite the 10-fold higher concentration. It is highly probable that this can be attributed to the generation of distinctive radicals, of which the tertiary alkyl radical intermediate next to a stabilizing carbonyl functionality has high stability thus exhibiting lower reactivity (Scheme 5.1). On the other hand, comparing the storage moduli of the equilibrium-swollen hydrogels, only a slightly lower mechanical strength was observed for the latter (10.5 ± 1.7 kPa versus 14 ± 0.6 kPa). It should be remarked that the use of

VA-086 PI results in increased porosity by means of N_2 gas evolution as a result of the UV induced radical formation, as can be seen in Figure 5.3.

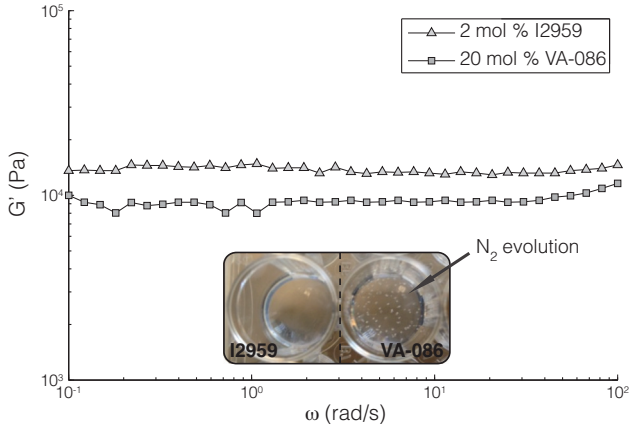


Figure 5.3: Mechanical spectra of equilibrium swollen 10 w/v % gelatin methacrylamide hydrogels prepared with two photo-initiators. Samples were measured at 37°C with $F_N = 0.1\text{ N}$ and $\gamma = 0.1\%$. Inset images represent cell-laden 2D sheet samples.

Figure 5.4 demonstrates the evolution of the gel yield and water uptake capacities as a function of the UV-A irradiation dose for both PIs on 1 mm thick 2D films. For both PIs, a steep increase up to an irradiation dose of 2400 mJ cm^{-2} occurred, followed by a non-significant gel yield increase up to $\geq 90\%$. Additionally, equilibrium mass swelling ratios (q) decreased with increasing irradiation dose.

The influence of the applied photo-initiator on the cell viability was initially evaluated by encapsulation in 2D sheets (1 mm thick). The results are represented by the live/dead images from Figure 5.5. No significant differences were observed at day 1 after encapsulation, and for both photo-initiators HepG2 cells maintained their viability. At day 3 respectively day 7 after encapsulation, cell viability demonstrated a modest significant improvement for the VA-086 cross-linked 2D sheets ($p < 0.01$ respectively $p < 0.001$) likely due to a lower cross-linking density as radical generation is less efficient.

5.3.2 Parameters affecting scaffold architecture

Scaffold architecture, and specifically pore architecture will greatly influence overall construct strength and nutrient/waste flows. However, it is the deposited strut (diameter), which will impact the porosity, strength and layer height. On the other hand, the solution viscosity, applied pressure, XY plotting speed, needle

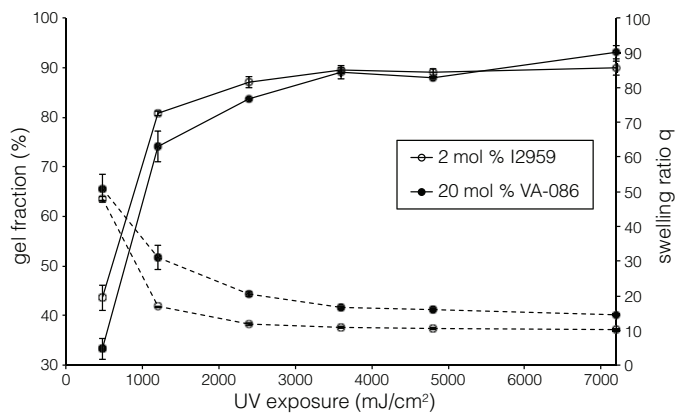


Figure 5.4: The effect of the applied photo-initiator (I2959 versus VA-086) on the cross-linking behavior of 10 w/v % gelatin methacrylamide films based on swelling studies as a function of UV-A exposure. 2D films were 1 mm thick. Full lines represent the gel fraction values, dashed lines represent the equilibrium mass swelling ratio q .

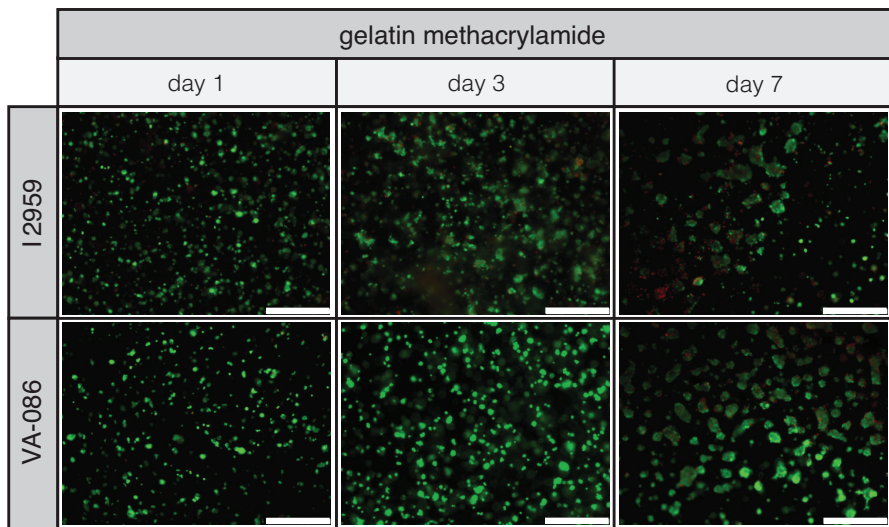


Figure 5.5: Live (green) and dead (red) stain illustrating the effect of the applied photo-initiator (I2959 versus VA-086) on the viability of encapsulated HepG2 in 10 w/v % gelatin methacrylamide films. The irradiation dose was 1800 mJ cm^{-2} . Scale bar represents $500 \mu\text{m}$.

type and diameter, cell density and temperature drop, mainly control the strut diameter itself. All those parameters are closely interconnected and need parametric optimization to ensure a reproducible plotting process.

5.3.2.1 Rheological evaluation

As mentioned, solution viscosity will greatly impact the overall performance of the fiber deposition process. Therefore, a rheological study for the evaluation of concentration, temperature and cell density effects was performed to gain an in depth insight on the plotting. Figure 5.6 depicts rheological data on (cell-laden) gelatin mixtures. The shear rate dependent behavior of liquid gelatin solutions is provided for increasing gelatin concentrations (Figure 5.6 A) and increasing cell densities (Figure 5.6 B), illustrating the non-Newtonian liquid behavior. Additionally, the temperature dependence of the solution viscosity is demonstrated in Figure 5.6 C and D for respectively increasing gelatin concentrations and increasing cell densities. Finally, cross-over points, i.e. the temperature at which the sol-to-gel transition occurs, was investigated and presented in Figure 5.6 E and F.

The solutions all showed shear thinning behavior, and as expected, solution viscosity dropped with decreasing gelatin concentration and increasing temperature (Figure 5.6 A and C). During cross-over point determination, the contributions of “liquid” and “gel” on the mixture rheology can be followed. The gel point decreased from 35 °C to 32 °C after decreasing the gelatin concentration from 20 w/v % to 5 w/v %. Furthermore, below the gel point, the ratios of the loss moduli to the storage moduli (i.e. the loss factor $\tan(\delta)$) increased for decreasing gelatin concentration, indicating more “liquid-like” behavior. Especially 5 w/v % storage moduli decreased significantly, limiting the overall plotting potential. However, since the final aim is the construction of hybrid constructs, it was interesting to investigate the influence of increasing cell densities on the rheological behavior of gelatin solutions. Ten w/v % was selected since highly viable cell encapsulation of HepG2s could be performed and plotting with high shape fidelity remained possible. The blending of cells proved to impact solution viscosity as a result of a particle-like component. For temperatures above the gelification point, the viscosity was decreased by a factor of 2 up till a cell density of 1.5×10^6 cells mL⁻¹. Increasing the cell density further to 2.5×10^6 cells mL⁻¹ increased this factor to 4. Below the gelification point, in order to obtain similar viscosities (i.e. physical gelation), cell-gel mixture temperatures had to be decreased by > 4 °C when increasing the cell density to 2.5×10^6 cells mL⁻¹. From the evolution of the elastic (i.e. storage moduli) and viscous (i.e. loss moduli) contributions of the cell-laden hydrogels (Figure 5.6 F), it could be derived that the differentiation due to alteration of the storage moduli was more pronounced, ultimately rendering higher physical

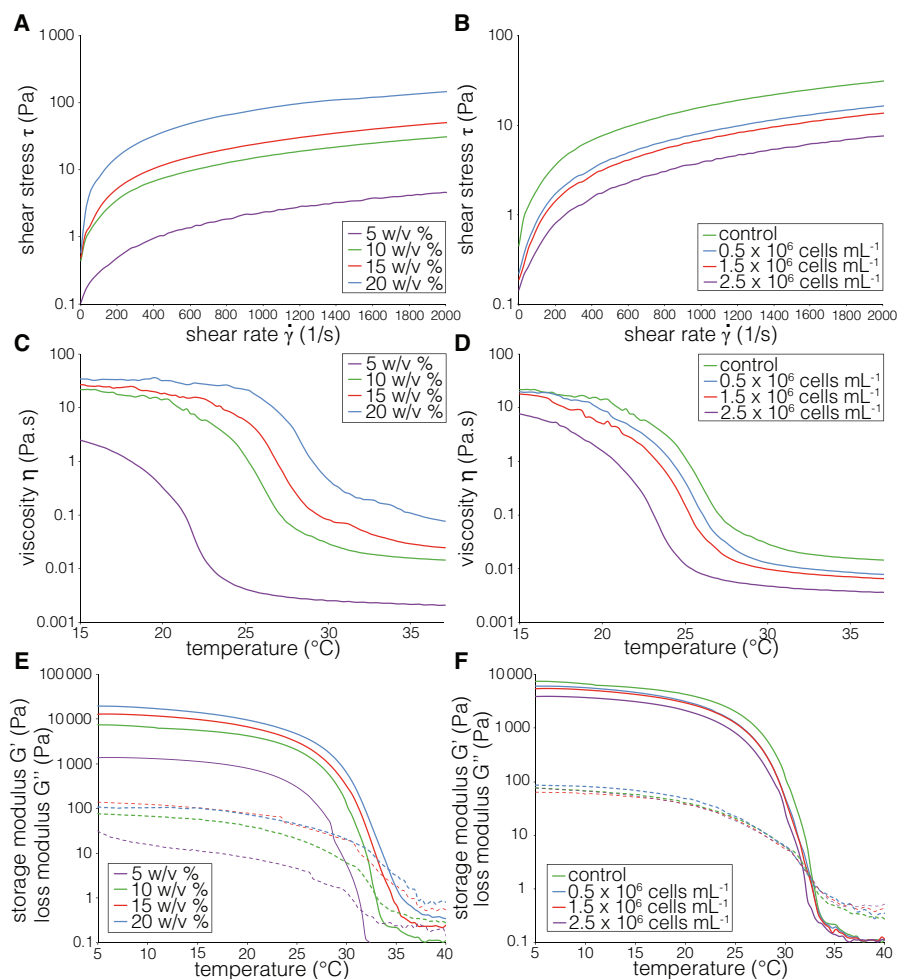


Figure 5.6: Rheological data representing the influences of gelatin methacrylamide concentration and cell density on the physical properties of the gelatin(-cell) precursor solutions. Rotational viscosimetry measurements (A-D) illustrating i) the influence of gelatin methacrylamide concentration on the shear stress as a function of shear rate (A) and viscosity as a function of temperature (C); ii) the influence of the cell density on the shear stress as a function of shear rate (B) and viscosity as a function of temperature (D). Oscillatory measurements representing the cross-over points between the gel and sol state ($G' = G''$, T_{gel}) for different concentrations (E) and different cell densities (F). For the graphs on cell density influences (B, D, F), a 10 w/v % gelatin methacrylamide solution without cells was selected as control condition.

gelation without cells while only a subtle shift in the gel point was noticed.

5.3.2.2 Pressure, needle type, temperature and cell density affect deposited strand diameter

Figure 5.7 explores the potential impact of inlet pressure, needle internal diameter, plotting temperature and cell density on the diameter of dispensed 10 w/v % (cell-)gel strands. As can be derived from these graphs, combining these data allows targeting of a desired strut diameter. The data additionally clearly illustrates the viscosity drop resulting in increasing strut diameters in the case of cell-laden hydrogel dispensing. Therefore, these data correlate well with the rheological analysis. Inlet pressure and plotting temperature (affecting viscosity) proved to be the utmost important parameters. Additionally, the needle type (conical versus cylindrical) and gelatin building block concentration affects the diameter of the dispensed strands (data can be found back in the *Techniques* chapter, section 11.1.2.1).

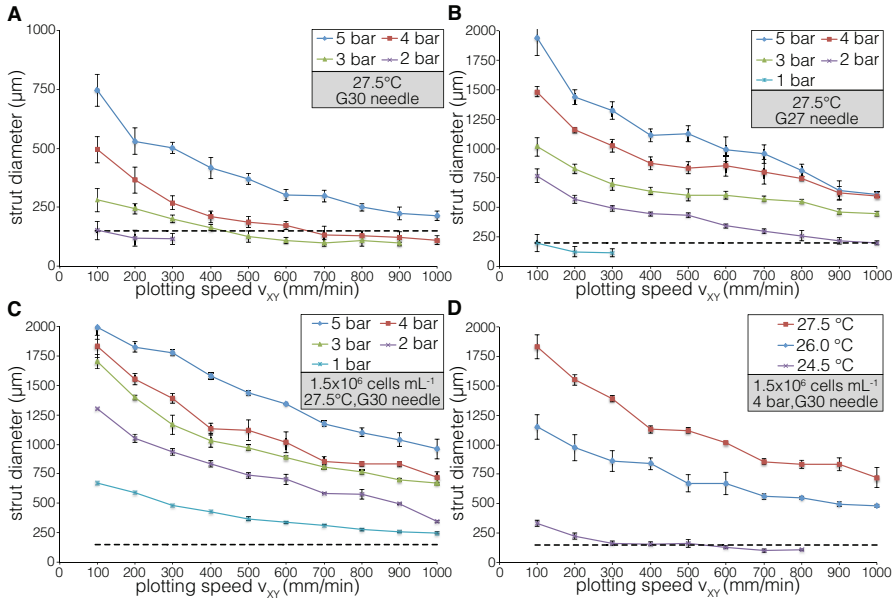


Figure 5.7: Evolution of the deposited strut diameters as a function of the applied plotting speed and dispensing inlet pressure for cylindrically shaped needles. A gelatin methacrylamide concentration of 10 w/v % was used for all experiments. Plotting at 27.5 °C with a needle internal diameter of 200 μm (A) and 150 μm (B). The influence of the plotting temperature of cell-gel hybrid mixtures is illustrated for a needle internal diameter of 150 μm (C, D). Dashed lines represent the needle internal diameter.

The temperature/viscosity influence was anticipated from the rheological data and therefore the Bioplotter device was altered. Two major changes made plotting more reproducible:

1. First of all, as depicted schematically in Figure 5.8, the original cooling/heating mantle of the dispensing head was extended till ~ 1 mm from the needle tip outflow, and;
2. The stainless steel build platform was coupled to Peltier elements and ventilators to enable cooling.

The first adaptation rendered a homogeneous solution viscosity till outflow, allowing plotting at lower solution temperature. The second adaptation proved to be necessary when trying to plot hydrogels with a concentration < 20 w/v %. The cooling (down to ~ 5 °C) ensured enhanced physical cross-linking and thus layer-build up. Without cooling, 10 and 15 w/v % constructs collapsed completely due to insufficient mechanical integrity.

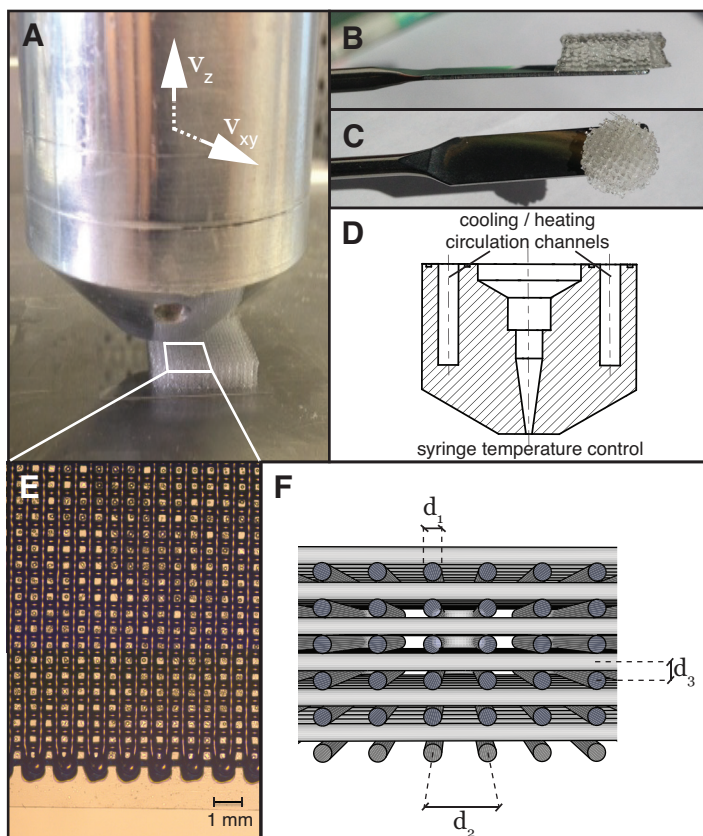


Figure 5.8: Images representing the plotting of 10 w/v % constructs (A-C). To ensure a stable plotting process, the heating mantle was adjusted till the tip of the dispensing syringe, as schematically illustrated in the cross-section of the mantle in (D). Light microscopy image demonstrating the porous nature of the finalized scaffolds in top view (E). Theoretical construct dimensions and scaffold build-up in cross-section (F).

5.3.2.3 Pore architecture and curing kinetics of 3D porous gelatin methacrylamide scaffolds

Combining the above-mentioned results, a parameter set of processing conditions was extracted for the plotting of 3 mm thick scaffolds as can be found in Table 5.1.

Table 5.1: Processing conditions for the 3D fiber deposition of (cell-laden) gelatin hydrogel precursors.

PARAMETER	GELATIN CONCENTRATION				
	10 w/v %		15 w/v %	20 w/v %	
Cells [$\times 10^6 \text{ mL}^{-1}$]	-	1.5	1.5	-	-
Needle gauge	G30	G30	G27	G27	G27
$\emptyset$ [μm]	150	150	200	200	200
Material temperature [$^{\circ}\text{C}$]	27.5	24.5	24.5	28.5	30
Layer height [μm]	110	110	160	170	180
Pressure [bar]	3	3	0.5*/1	4	4
XY-speed [mm min^{-1}]	450	400	600	550	550
Z-speed [mm min^{-1}]	500	700	700	300	300
Preflow [sec]	0.2	0.1	0.1	0.2	0.2
Postflow [sec]	0.2	0.2	0.05	0.2	0.1
Corner delay [sec]	0.2	0.1	0.05	0.2	0.1
Pullback time [sec]	0.5	0.5	0.5	0.5	0.5
Interstrut distance [mm]	0.60	0.80	0.80	0.60	0.60

* For conical needle scape

For the 10 w/v % gelatin constructs, a needle with 150 μm internal diameter was selected, while for higher concentrations a needle of 200 μm was preferred. As described during the rheology results, temperature had to be adjusted from 27.5 $^{\circ}\text{C}$ for 10 w/v % hydrogels to 30 $^{\circ}\text{C}$ for the plotting of 20 w/v % hydrogels in order to ensure optimal sol-to-gel ratio. These cell-free constructs were used for analysis of the pore architecture and chemical cross-linking kinetics. Figure 5.8 represents the dispensing process of 10 w/v % gelatin hydrogels and schematically describes the theoretical architecture of a scaffold. In analogy to the 2D curing of gelatin films, curing kinetics of 3D plotted structures was evaluated as a function of the UV-A irradiation dose, as well as the initial gelatin concentration. Figure 5.9 summarizes the gel yields and equilibrium mass swelling ratios for the swelling studies on 3D porous scaffolds.

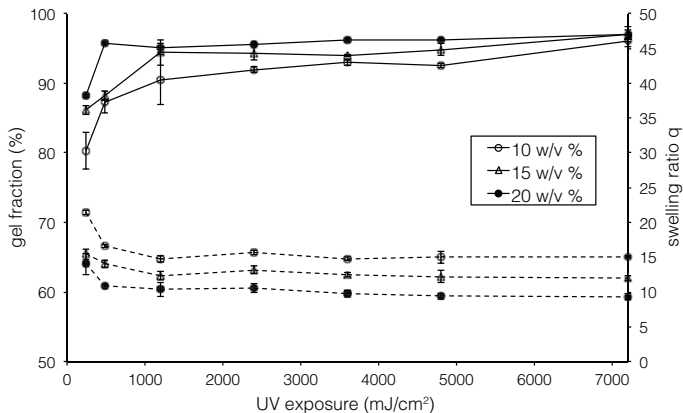


Figure 5.9: Influence of the initial gelatin methacrylamide concentration on the cross-linking kinetics of 3 mm thick printed scaffolds. Full lines represent the gel fraction values, dashed lines represent the equilibrium mass swelling ratio q .

Generally, the observed trends from 2D swelling studies were translated to the 3D constructs. However, some small differentiations could be noticed:

1. The finally obtained (i.e. full curing) gel fraction values were slightly higher for the 3D constructs compared to 2D sheets;
2. Remarkably, at any evaluated irradiation dose the swelling ratios were higher than their 2D counterparts, despite the higher gel yields of the latter, and;
3. Curing of 3D constructs occurred at a faster rate compared to 2D films.

These observations were valid for both PI types.

Finally, pore geometry and overall strand deposition architecture was evaluated for varying gelatin concentrations (Figure 5.10). Light microscopy images reveal the highly regular deposition of strands regarding top view (Figure 5.8 E and 5.10 A).

It should be remarked that final strand diameter dimensions will slightly increase compared to the ones derived in Figure 5.7, due to the immersion-based cross-linking step. Additionally, equilibrium swelling will further increase these dimensions corresponding to their swelling ratio. Cross-sections of constructs with increasing gelatin concentration (Figure 5.10 B, E, and F) reveal partial collapse of subsequent strands due to the combined effects of flow behavior/gelification of the deposited strands and gravitational forces. The lower the concentration, the

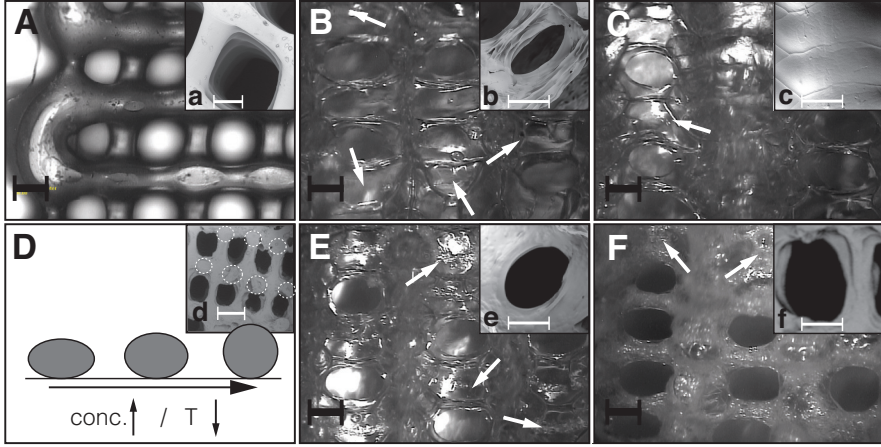


Figure 5.10: Scaffold pore architecture is influenced by the hydrogel concentration. Light microscopy and SEM images representing 10 w/v % scaffold pore geometry in top view (A, a), cross-section (B, b), and side view (C, c). Cross-section images of 15 w/v % (E, e) and 20 w/v % (F, f) scaffolds. White arrows indicate partial collapse of the subsequent layers. Scale bars indicate 200 μm . Schematic representation of the deposited strand geometry as a function of initial concentration and plotting temperature (D), accompanied by a cross-section SEM image indicating the strut geometry of 20 w/v % scaffolds (d). Scale bar of (d) indicates 400 μm .

more this phenomenon occurred. At 5 w/v %, complete collapse of the subsequent layers was observed, and are therefore left out of consideration. Figure 5.10 C illustrates a side view for 10 w/v % scaffolds indicating the complete collapse of the meander feature (also indicated by the SEM image Figure 5.10 c) at the sides of the scaffold. SEM images corresponding to the respective microscopy images (Figure 5.10 a-f) clearly demonstrate the impact of plotting concentration on the pore architecture. Increasing the gelatin concentration will result in faster physical gelation and therefore, more spherically deposited strands and pores. This is schematically illustrated in Figure 5.10 D. Three-dimensional reconstruction of micro-computed tomography data (Figure 5.11) revealed a regular material deposition matrix (green) with an interconnected pore network (yellow) for a 10 w/v % construct. It is clear that the pore volume is still interconnected, which confirms the successful printing of gelatin methacrylamide hydrogels. The pore volume has the largest dimensions along the Z-axis closely resembling the designed dimensions, while along the XY-axis (volumes in between every $0^\circ/90^\circ/0^\circ$ fibers) the pore volume is somewhat less than the theoretically designed d_3 (Figure 5.8 F and Figure 5.11).

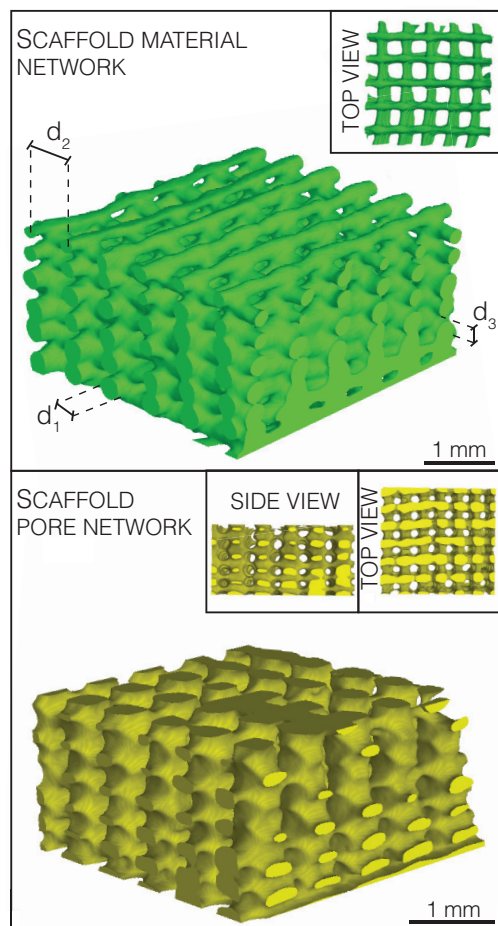


Figure 5.11: Micro-computed tomography reconstruction of hydrated 10 w/v % scaffolds. Gelatin methacrylamide material architecture is represented in the top part (green), while the scaffold pore network is visualized in the bottom part (yellow).

5.3.3 Plotting of cell-laden 3D constructs

5.3.3.1 Pressure, needle type, PI and UV-A irradiation dose affect cell viability

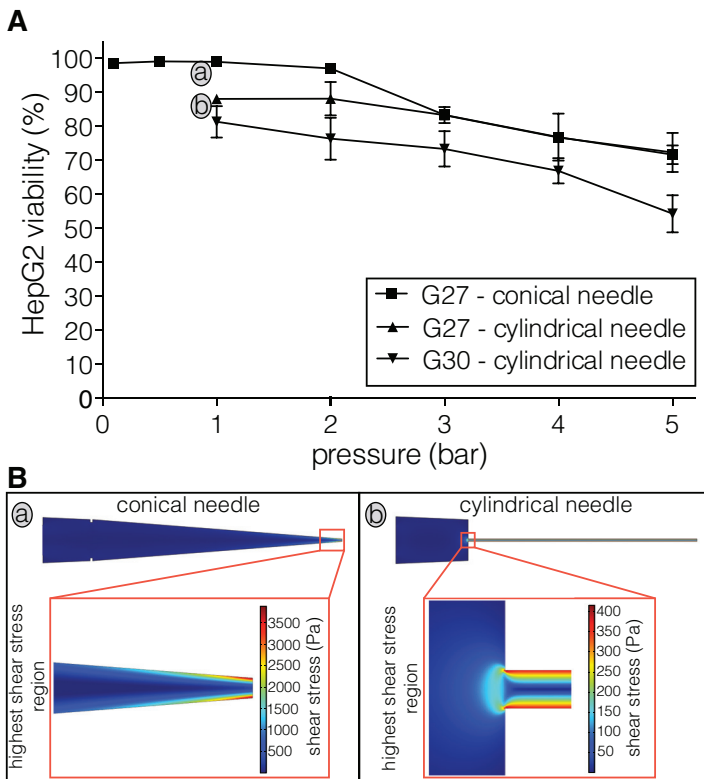


Figure 5.12: Cell-gel flow during syringe needle deposition. (A) Evolution of HepG2 viability during the printing process as a function of the applied inlet pressure and needle type. (B) Heat map of the shear stress at 1 bar inlet pressure for a conical needle (a) and cylindrical (b) needle, obtained by finite element modeling of noncross-linked cell-gel (10 w/v %) mixture. Figures are to scale for needle internal diameter of 200 μm .

Based on earlier performed cell tests, 10 w/v % gelatin methacrylamide was selected for the printing of cell-laden constructs. Prior to the printing of full sized constructs, parameters influencing cell viability were investigated in order to select the most appropriated conditions to construct a highly viable cell-laden scaffold. First, the influence of needle type (conical vs. cylindrical), needle internal diameter, and dispensing pressure on the viability of HepG2 cells was examined (Figure 5.12 A). It appeared that cell viability was dependent on the applied needle type,

with enhanced viability levels for conically shaped needles. For all tested needles, higher inlet pressures resulted in decreased cell survival. In the case of cylindrically shaped needles, cells printed with bigger needle internal diameter maintained higher cell survival than those printed with a smaller diameter. The highest viabilities, $> 97\%$, were observed at low dispensing pressures (≤ 1 bar) using a conical needle type ($\emptyset = 200\ \mu\text{m}$). Nevertheless, at higher inlet pressures, viability dropped faster compared to the cylindrical needle type.

To understand the discrepancy between the two needle types, finite element analysis (FEA) simulations were performed. Figure 5.12 B (a and b) shows heat maps of the simulated fluid flow induced shear stresses for both needle types using the same inlet pressure (1bar). Empirical data used in the FEA simulations were determined by rheology (example presented in Figure 5.13) and the derived parameters are presented in Table 5.2. Additional FEA results can be found in the *Techniques* chapter, section 11.1.2.1.

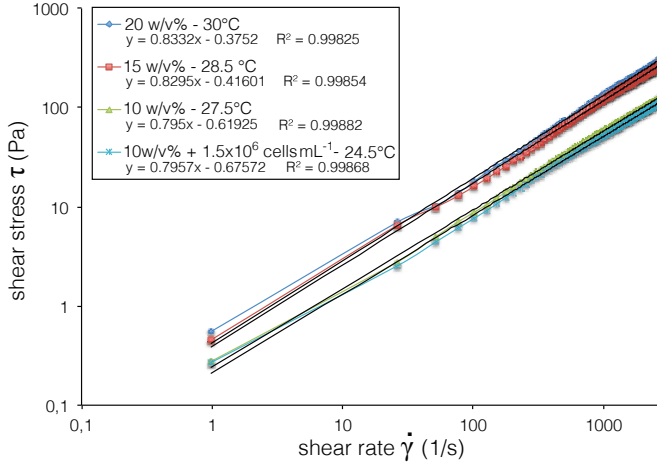


Figure 5.13: Empirical determination of flow behavior index, n , and consistency coefficient, K , from rheological data.

With increasing temperature, a decrease in the consistency coefficient K was observed, indicative for the decreasing viscosity as temperature increases. On the other hand, K increased with increasing gelatin concentration but decreased with increasing cell density. As such, consistency coefficient is a strong function of concentration (hydrogel building block and cell density) and temperature.

The flow behavior index n was for all conditions < 1 confirming non-Newtonian shear thinning behavior whereby the apparent viscosity (η_{app}) is a function of the shear rate:

$$\eta_{app} = K \dot{\gamma}^{n-1} \quad (5.2)$$

Table 5.2: Flow behavior index n , consistency coefficient K , and zero-shear viscosity η describing gelatin solutions of various concentration and temperature.

GELATIN SAMPLE		T [°C]	n	K [Pa.s ^{n}]	η [Pa.s]
5 w/v %			0.9719	0.0029	0.0028
10 w/v %		35.0	0.9565	0.0219	0.0209
15 w/v %			0.9558	0.0352	0.0336
20 w/v %			0.9593	0.1025	0.0983
10 w/v %	no cells		0.9565	0.0219	0.0209
	0.5×10^6 cells mL ⁻¹	35.0	0.9784	0.0097	0.0094
	1.5×10^6 cells mL ⁻¹		0.9804	0.0079	0.0077
	2.5×10^6 cells mL ⁻¹		0.9789	0.0049	0.0047
10 w/v %	no cells	27.5	0.7950	0.2403	0.1910
	1.5×10^6 cells mL ⁻¹	24.5	0.7957	0.2110	0.1678
15 w/v %		28.5	0.8295	0.3837	0.3182
20 w/v %		30.0	0.8332	0.4215	0.3512

The flow behavior index did not display a similar strong dependency with the concentrations. Only upon decreasing the temperature, a notable decrease in n was observed. Furthermore, these data nicely illustrate the necessary processing temperature drop for the printing of cell-laden mixtures in order to obtain similar flow behavior as without cells.

Highest shear stresses were obtained for the conical needle type. However, shear stress built-up was only observed close to the fluid outlet (≤ 1 mm), limiting the passage time for this region. On the other hand, flow through a cylindrical needle type resulted in lower peak shear stresses but for an increased passage length (> 16 mm). These data stress that, besides feedback based on rheology and gelification behavior, the selected conditions had to incorporate feedback based on cell viability as well (Figure 5.1).

Since curing of 3D constructs occurred at a faster rate compared to 2D films, the influence of UV-A irradiation dose on cell survival was evaluated as well. When using the conventional PI I2959, a gradual improvement of cell viability with decreasing UV-A irradiation dose (without impairing scaffold stability) was observed in the cell-laden scaffolds.

As illustrated in Figure 5.14, viable cell fractions of $55.7 \pm 7.3\%$, $70.6 \pm 12.6\%$ and $89.1 \pm 2.1\%$ were determined for UV-A irradiation doses of 5400, 2700 and 1350 mJ cm^{-2} respectively. An irradiation dose of 1800 mJ cm^{-2} , yielding long-term stable cell-laden scaffolds, was selected for further evaluation. A comparison between I2959 and VA-086 PIs was made and cell survival values for cells encapsulated in 2D disks and printed 3D constructs are provided in Figure 5.15. For the VA-086 PI, a significant increase ($p < 0.01$) in viability is observed for 3D fabricated constructs, as well as increasing viability when compared to 2D sheet encapsulation ($p < 0.01$).

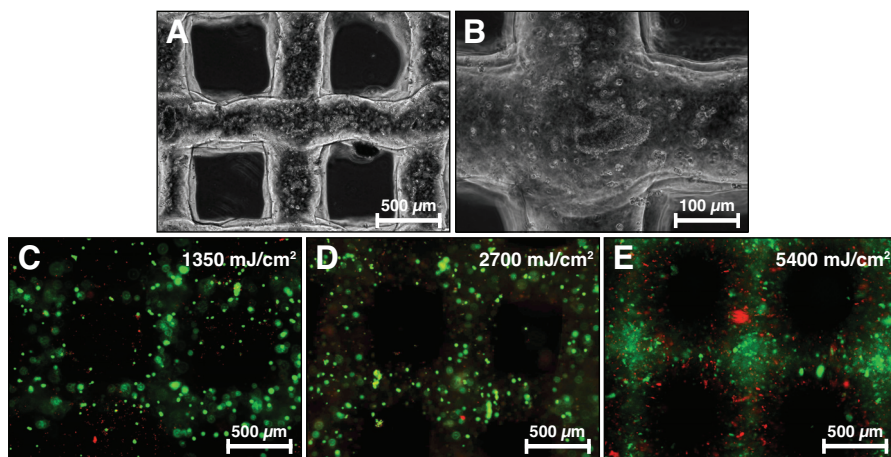


Figure 5.14: Optimal scaffold parameters and the effect of UV-A irradiation dose on the cell viability. The optimized parameters for the generation of gelatin scaffold yield optimal scaffold characteristics (A, B). HepG2-gelatin constructs were cured using I2959 and an irradiation dose of 1350 (C), 2700 (D) and 5400 (E) mJ cm^{-2} . After 24 hours, cell survival was evaluated using a live/dead assay. Scale bar indicates 500 μm (A, C-E) and 100 μm (B).

5.3.3.2 The production of highly viable cell-laden scaffolds: the result of balancing parameters

As the rheological and gelification behavior altered, processing conditions were adjusted based on the data derived from the above-mentioned studies. The selected conditions are summarized in Table 5.1. The final decision to use VA-086 as photoinitiator with a UV-A irradiation dose of 1800 mJ cm^{-2} led to an excellent cell viability of $98.6 \pm 1.3\%$ at day 1, $98.8 \pm 1.5\%$ at day 7 and $98.9 \pm 0.8\%$ at day 14 within the gelatin scaffold (Figure 5.16).

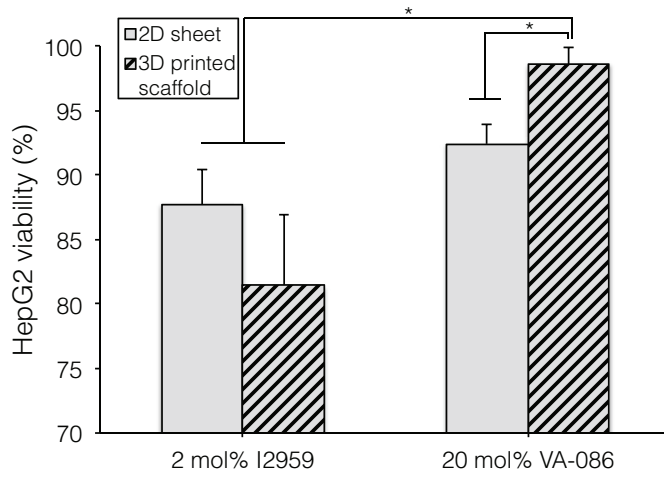


Figure 5.15: Viability of encapsulated HepG2 for 2D sheets versus 3D printed scaffolds using two different photo-initiators. (*) $p < 0.01$

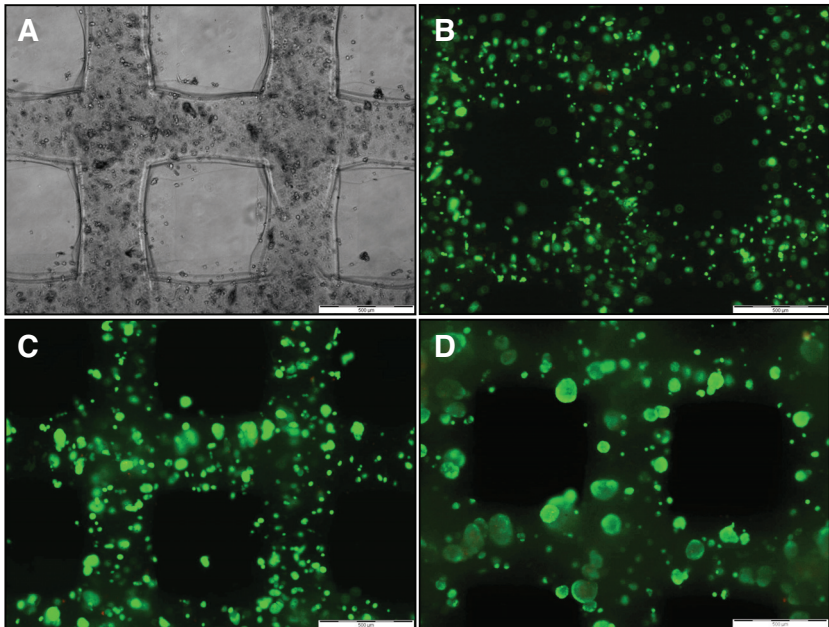


Figure 5.16: High viability cell-laden gelatin scaffolds. HepG2-gelatin constructs, cured using the VA-086 PI, with well defined dimensions were obtained as shown by the BF image (A). Cell viability within the scaffold was evaluated at day 1 (B), day 7 (C) and day 14 (D) using a live/dead stain. Scale bar = 500 μm .

The cell viability was constant in time and scaffold stability was maintained for at least 21 days as demonstrated by the BF image and live/dead stains. SEM images of cell-laden constructs are represented by Figure 5.17. Cells on the outer part of the strands were covered by a hydrogel layer and had a round morphology. As described by several authors, round morphology of HepG2 cells is taught to enhance and prolong hepatocyte function.^{402–404}

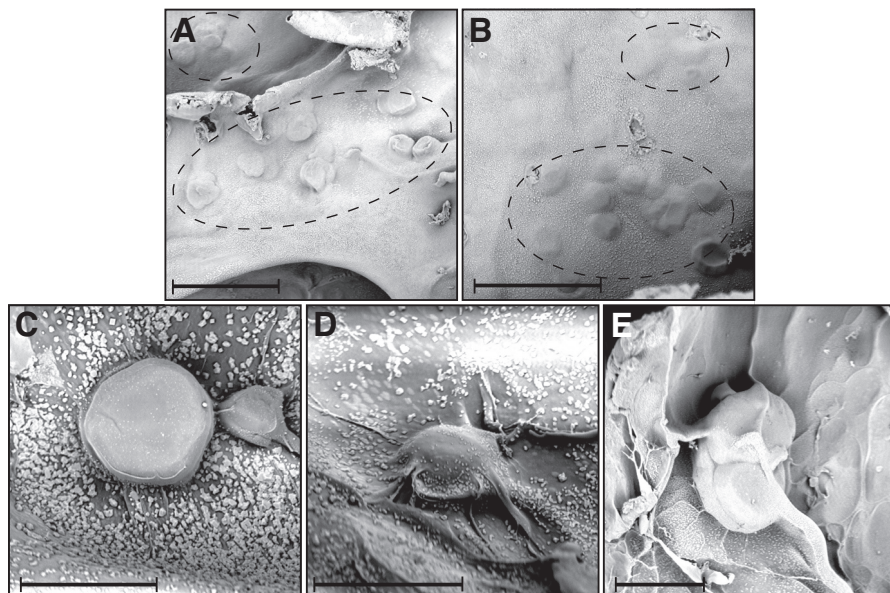


Figure 5.17: SEM images of encapsulated HepG2 cells in 10 w/v % gelatin methacrylamide plotted constructs. The scaffolds were fixed with glutaraldehyde and lyophilized to remove the liquid fraction prior to sample loading. Multiple cells visualized on the surface of a strut (A, B). Scale bars represent 50 μm . Examples of single cells covered by a gelatin layer (C, D) and an example of a cell cluster (E). Scale bars represent 20 μm .

At all investigated time points, the cells displayed viable and euchromatic nuclei as indicated by HE staining (Figure 5.18 A, a). Moreover, the cells maintained their potential to express liver specific functions such as the production of albumin (Figure 5.18 B, b) and HNF4 α (Figure 5.18 C, c) and the storage of glycogen (Figure 5.18 D, d). Also the maintenance of proliferative capacity was confirmed both by positive Ki67 and PCNA staining (Figure 5.18 E, F).

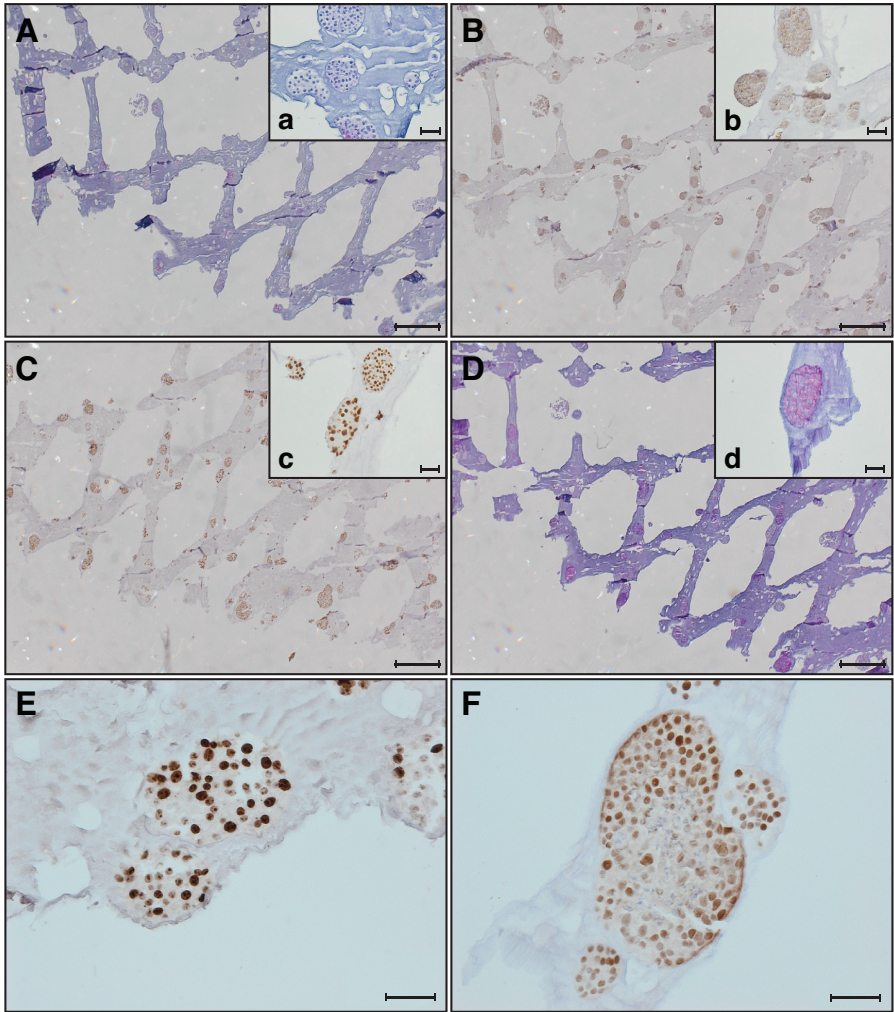


Figure 5.18: Representation of the cell morphology and phenotype maintenance in cell-gelatin constructs 14 days post-fabrication. Cell morphology was examined using hematoxylin/eosin stainings (A, a). IHC staining for albumin (B, b) and HNF4 α (C, c) and PAS (D, d) were performed to assess phenotypical behavior. IHC staining for Ki67 (E) and PCNA (F) were assessed to confirm proliferation. Scale bars indicate 50 μ m (a-d, E, F), and 500 μ m (A-D).

5.4 | DISCUSSION

In the previous chapter, the use of I2959 PI initiated gelatin (meth)acrylamide hydrogels, as HepG2 encapsulation matrix material was assessed as sheets. The materials demonstrated high cell survival levels.³⁷² From all PIs used for the photo-initiated free-radical polymerization of cell-laden hydrogels, I2959 is most commonly applied for several reasons:^{311,398,405}

1. It is slightly water soluble, enabling applications in aqueous environments;
2. In the absence of UV-A irradiation, no significant cytotoxic effects are correlated to the use of this PI, and;
3. The generation of radicals is highly efficient thus reducing the required concentration for cross-linking down to a level with lead to acceptable cell survival levels.

However, the search for even better PIs remains. For instance, Rouillard et al.⁴⁰⁵ evaluated a variety of water soluble PIs in order to increase the viability of bovine chondrocytes during 2D encapsulation in methacrylated alginate. From their research, the potentially superior cell viability levels using a newly introduced bio-compatible PI, VA-086, was postulated. Unfortunately, a quantitative comparison on the cross-linking lacked. Furthermore, to the best of our knowledge, studies broadening the applicability of this PI toward other cell types are limited to human microvasculature endothelial cells (HUVEC) and bone marrow stromal cells (BMSCs) using methacrylated alginate.⁴⁰⁶ The current study presents quantitative data comparing this novel photo-initiator with the conventional I2959 PI for the encapsulation of HepG2 cells in gelatin methacrylamides. The gel yield and water uptake capacity were examined as two important factors affecting network properties and overall cross-linking. Fine-tuning resulted in a 10-times higher PI concentration in order to reach comparable hydrogel network properties, illustrating the lower efficiency of VA-086. This was further confirmed by the decreased *in situ* cross-linking kinetics assessed by recordings of the increasing storage moduli during UV-A irradiation. Final gel strength, however, only slightly decreased. Cell-laden two-dimensional films demonstrated a modest, significant ($p < 0.04$) improvement in cell viability. These data further confirm the applicability of VA-086 as an alternative, highly biocompatible PI for cell encapsulation purposes. For both PIs, increasing the irradiation dose decreases the equilibrium mass swelling ratios (q) and increases the gel yield since more chemical network junctions are built in, resulting in tighter network structures (as discussed in the chapter 4).

Due to the low production costs, its natural origin, and excellent biocompatibility, gelatin is a widely explored biomaterial.^{141,142} Nevertheless, application-wise, the

limited manufacturability causes difficulty for the fabrication of complex porous 3D architectures using low-cost dispensing approaches. In order to enable 3D fiber deposition of (cell-laden) gelatin methacrylamide, an in depth evaluation of the rheological properties was performed. From these data, it was demonstrated that next to the hydrogel building block concentration, the incorporation of cells altered the rheological properties. As such, these parameters will affect the printing process since the viscosity as well as the gelification potential is altered. These findings emphasize the importance of precise temperature control of the printing solution as well as the printing substrate. Small adaptations to the Bioplotter device enabling improved temperature control on the build platform (i.e. enhanced physical gelation due to cooling) and improved control over the printing solution till needle tip were performed. Finally, 3D porous (cell-laden) gelatin methacrylamide constructs could be generated in the range of 10 – 20 w/v % gelatin. Lower concentrations lead to severe internal pore collapse, mainly caused by insufficiently fast physical gelation. The printing speed, the needle internal diameter, the plotting temperature and the applied pressure were optimized to obtain a set of data on the deposited strut diameters. Keeping oxygen diffusion and metabolite transport in mind, especially the processing window resulting in strand diameters of $\leq 200\text{ }\mu\text{m}$ was of particular interest. Hereby, the successful photo-initiated cross-linking of 3D porous constructs was confirmed. Compared to sheets, cross-linking was enhanced, but remarkably higher swelling degrees were obtained. Most likely, this can be attributed to the smaller dimensions of the deposited strands on one hand, and the corresponding increased surface area on the other, compared to the rather bulky (1 mm thick) sheets.

Fedorovitch et al.⁸² plotted alginate hydrogels describing the collapse of internal pore networks during layer-by-layer deposition. Very recently, gelatin methacrylamides were plotted using the Bioplotter device.³⁹⁸ However, construct built-up using only-gelatin containing mixtures proved impossible up to 20 w/v % gelatin due to severe viscosity issues stemming from difficult temperature control. As a result, the authors had to blend viscosity-enhancing hyaluronic acid in order to build mechanically stable 3D porous scaffolds. Other researchers plotted gelatin applying viscosity-enhancing additives such as alginate, chitosan, fibrinogen, or co-deposition of polyester biomaterials.^{141,248,249,257,258,261,398} Wang et al.²⁶⁰ plotted 20 w/v % glutaraldehyde cross-linked gelatin but had to decrease the temperature of the printing solution below 20 °C resulting in a highly physically gelated printing solution, thus high pressure and high internal diameter dimensions were necessary. In the present paper, gelatin methacrylamides were successfully printed in a range of concentrations, without severe internal collapse or the need for stabilizing co-deposition of other materials. As a result of our adaptations and parametric approach, gelatin-only 3D scaffolds having a 100 % interconnected pore network could

be fabricated. Our results contrast with other studies whereby viscosity-enhancing additives were obligatory to obtain porous constructs, without compromising scaffold architecture.

For the printing of cell-gel hybrid constructs, a 10 w/v % concentration was selected based on earlier performed experiments. Only partial pore collapse occurred for this concentration, still revealing an 100 % interconnected pore network. Therefore, easy diffusion of culture medium through the photo-initiated cross-linked gelatin methacrylamide construct can be achieved. Furthermore, the obtained constructs can undergo biodegradation stemming from enzyme secretion of living cells. To enable the creation of cell-laden constructs, it was clear that a careful adjustment of the plotting parameters was needed in order to meet two goals:

1. The creation of well-defined scaffolds with an interconnected pore network, and;
2. Optimal cell viability within the construct.

To meet both goals, the influence of different parameters on cell survival was investigated by a step-by-step approach enabling us to understand the individual parameters influencing both the construct and the cell behavior. The first investigated parameters affecting cell viability were the needle type, the needle size and the inlet pressure. A significant pressure and needle type dependence of the cell viability was observed. At low inlet pressure, conically shaped needles are preferred over cylindrically shaped ones. However, this advantage disappears at higher inlet pressures. Finite element fluid flow simulations can help to find an explanation. A higher peak shear stress was obtained by flow through the conical needle. However, keeping in mind the substantially lower passage distance and higher overall flow velocity, the passage time of cells in this high shear regime is substantially reduced compared to flow through a cylindrical needle. It is our belief that at lower pressures (i.e. higher passage time) cells will be more affected by the increased passage of high shear for the cylindrical needle, while at higher pressures, the significantly lower passage time for the conical needle cannot longer make up for the substantially higher shear stresses induced. As a result, initially higher viability levels are obtained for the conical type, however, pressure increase will result in a more pronounced viability drop compared to the cylindrical type. Aguado et al.⁴⁰⁷ evaluated cell viability drops caused by mechanical membrane disruption due to injection. Their data suggested that extensional flow at the entrance of the syringe needle (i.e. higher shear stress) was the main cause for acute cell death. Our findings on needle type as a function of dispensing pressure could provide mechanistic insight into the role of mechanical forces during construct printing.

The second investigated parameter was the influence of the UV-A irradiation dose on cell survival for I2959 PI. In the absence of a radical initiator, short exposure to long-wave UV irradiation did not prove to be cytotoxic.³⁸⁰ As expected, the presence of a radical initiator can compromise cell survival. Lower irradiation doses resulted in improved cell survival. Subsequently, we aimed to compare I2959 with VA-086 PI for an irradiation dose of 1800 mJ cm^{-2} . The results indicated a significantly higher fraction of viable cells when using the VA-086 PI, and this fraction was even higher than the viable fraction observed with I2959 PI for lower irradiation doses of 1350 mJ cm^{-2} . These conclusions are in close correlation to the results of Rouillard et al.⁴⁰⁵ on the encapsulation of chondrocytes in methacrylated alginate. As such, the lower cytotoxicity of the generated radicals due to photodissociation of VA-086 make it a good candidate for photo-initiated cross-linking of other cell-laden biomaterials.

As illustrated in Figure 5.1, our findings stress the importance of feedback loops between different scientific domains in order to fabricate cell-laden constructs having the appropriate properties combined with high cell viability. Although the individual improvements in cell survival sometimes seem quite modest, the synergistic effect of the combined adjustments eventually lead to the fabrication of highly viable cell-laden gelatin methacrylamide scaffolds having a 100 % interconnected pore network. Previously, the generation of 3D hepatocyte-gelatin constructs cross-linked with glutaraldehyde were reported to have a viability of 93 %.²⁶⁰ However, increasing the gelatin concentration up to 20 w/v % and needle internal diameter to $300 \mu\text{m}$ was necessary, resulting in strands of $> 350 \mu\text{m}$ with lower dimension fidelity. In our approach, we avoided exposure to toxic glutaraldehyde by the use of photo-curable gelatins, resulting in somewhat higher cell survival rates (98 %). Additionally, softer matrices could be generated (10 w/v % constructs), still possessing an interconnected pore network and smaller strand dimensions. Furthermore, the SEM images of cell-laden constructs revealed a round cell morphology, preferential and typical for encapsulated HepG2 cells.

Although cell survival is an important criterion, in our opinion this methodology does not cover full evaluation for the overall quality of a cell-laden construct. To validate the construct, it was necessary to demonstrate the cells' capability of displaying their normal cell behavior post-construction of the scaffold. For HepG2 cells, a hepatocellular carcinoma cell line, this includes maintained expression of hepatocyte markers and proliferative capacity. The cell-laden scaffold was therefore analyzed as such. The expression of albumin, HNF4 α , Ki67 and PCNA was confirmed, indicating that cell behavior was not impaired due to the printing process and exposure to increased fluid shear stresses for the duration of the study.

5.5 | CONCLUSION

The RP Bioplotter technique provides a useful tool for the printing of cell-laden constructs. Precise temperature control during the printing process results in the possibility to fabricate constructs displaying an interconnected pore network in the range of 10 – 20 w/v % gelatin methacrylamide. Control over the deposited strand dimensions can be guaranteed due to the physical properties of gelatin methacrylamide hydrogels and machine operating parameters. As a result, constructs having the desired stiffness and high shape reliability can be designed. Further applying a feedback strategy combining engineering, chemistry and biology, enabled the printing of cell-laden gelatin methacrylamide hydrogels without the need for viscosity-enhancing additives. The induced shear stress, curing irradiation dose and the applied photo-initiator are influential for the resulting cell viability. High viability, > 97 %, constructs displaying a maintained expression of liver specific functions were obtained using the novel VA-086 photo-initiator.

6 | FROM FILMS TO 3D DESIGNED (METH)- ACRYLAMIDE CONSTRUCTS: A ROUTE TO CO-CULTURE SYSTEMS

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Current chapter serves as an extension on the previous chapter. The network properties of 3D printed constructs are evaluated. The plotting of cell aggregates is presented with the aim for subsequent seeding to obtain co-culture systems. The formation of hepG2 and primary hepatocyte cell aggregates and their subsequent embedding in gel-MOD hydrogel films for modular tissue engineering purposes was published in PLOS one.⁴⁰⁸ However, since this mainly governs cell biology, the data produced for this article is only given where relevant. For the complete study, we refer to the PhD thesis of Elien Gevaert.

6.1 | INTRODUCTION

Hepatocytes are characterized by a rapid decrease in viability and metabolic functions once isolated from their native micro-environment. This presents limitations for clinical, engineering and research purposes. Achieving a prolonged maintenance of hepatocyte phenotype could e.g. help in the development of a tissue engineered construct, BAL, more efficient transplantation or more reliable *in vitro* models thereby improving drug toxicity screening. Cultivation of hepatocytes in the form of aggregates, thus enhancing cell-cell contact in a 3D environment, is a proposed approach to improve hepatocyte performance.⁴⁰⁹ Different studies have reported an improvement of many metabolic functions, including cytochrome P450, albumin secretion, urea production and glutathione S-transferase activity.^{409,410} These cell-aggregates could potentially serve as building blocks in liver repair and be assembled using embedding in appropriate biomaterials, while preserving mass transport of nutrients and metabolites.^{320,411}

The 3D Bioplotter device, first introduced by Landers and Mülhaupt in 2000, has since frequently been applied to fabricate 3D cell substrates for biomedical applications. Using the Bioplotter, the scaffold internal pore architecture can be designed. In addition, the nutrient waste and metabolite flow, as well as degradation rates are affected by the hydrogel network microscopic and macroscopic properties.^{364,388,396,412,413} These are important parameters when aiming at, for instance, drug release applications.

Nonetheless, in the development of hydrogel constructs, 0°/90° patterns are typically selected for the layer-by-layer material deposition pattern.^{82,262,265,398} Very recently, Almeida et al.⁴¹⁴ demonstrated that rapid prototyped platforms having distinct scaffolding features and/or material properties (PLA, PLA/calcium phosphate glass, chitosan) led to distinct macrophage morphology and cytokine profile. For both sets of parameters, macrophages were mostly affected by the material properties and only slightly by the scaffolding features (e.g. pore geometry).

Liver pathology shows a correlation to the mechanical properties of the liver tissue. For instance, Yeh et al.⁴¹⁵ graded fibrosis of human livers and found a correlation between the fibrosis severity and elastic modulus of the tissue. Other research demonstrated myofibroblastic differentiation of hepatic stellate cells with increasing substrate stiffness.⁴¹⁶ Schuurman et al.³⁹⁸ tailored the mechanical properties of gelatin-HA constructs by selection of the precursor concentration. However, literature on scaffold lay-down pattern related to construct mechanical properties is limited for hydrogels. Hutmacher et al.⁴¹⁷ reported a clear influence of architecture on the compressive moduli of PCL scaffolds processed by FDM technology.

The following chapter compares micro- and macroscopic network properties of 2D films with 3D bioplotted constructs. Variations in the pore network architecture are evaluated in the framework of compressive strength, while hydrogel precursor parameters are related to degradation and drug release properties. Next, the plotting of cell aggregates is tackled and optimized to obtain high viability aggregate assembly into 3D shapes. As a final part, co-culturing of 3D embedded HepG2 aggregates and seeded HUVECs is illustrated. This is schematically presented in Figure 6.1.

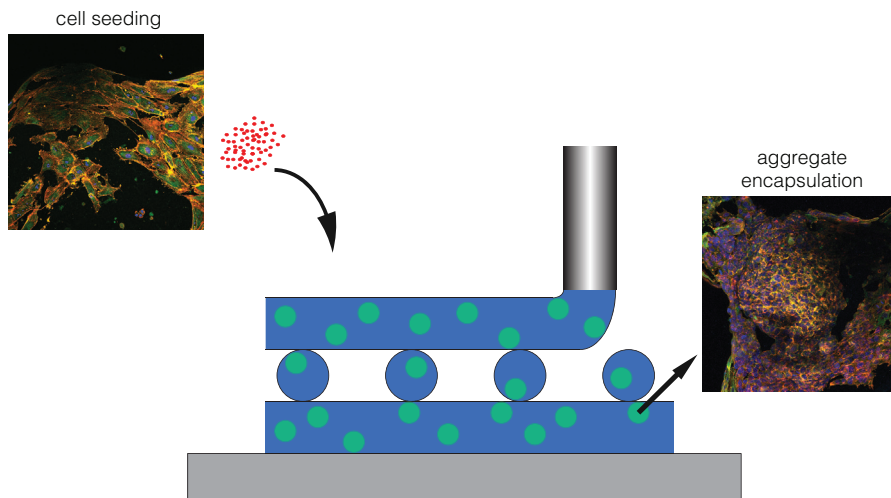


Figure 6.1: Schematic illustration of the co-culture of embedded aggregates with seeded cells. In the confocal images, cells were stained for the cell nuclei (DAPI, blue), the actin skeleton (phalloidin, red), and the focal adhesion points (vinculin, green).

6.2 | MATERIALS AND METHODS

6.2.1 Preparation of hydrogel sheets and fabrication of 3D printed constructs

GelA-MA, gelA-AA, gelB-MA and gelB-AA were synthesized according to the protocol described in section 4.2.2. Hydrogel sheets having a thickness of 1 mm were generated by injection of gelatin derivate solution containing photo-initiator as described in section 4.2.2.2. 3D fiber deposition of gelB-MA and gelB-AA precursor solutions to obtain 3D open porous scaffolds was performed employing the optimized parameters from chapter 5. SEM images were recorded according to section 5.2.5.2.

6.2.2 Properties of modified gelatin films and 3D constructs

6.2.2.1 Uniaxial unconfined compression

The mechanical properties of porcine liver and 3D plotted constructs were studied using a TA500 Texture Analyser (Lloyd). Freshly isolated pig livers were cut into small pieces ($\sim 1 \times 1 \times 0.5 \text{ cm}^3$) for uniaxial unconfined compression testing. Specimen samples were collected with and without capsule, i.e. from the outer and inner parts. GelB-MA and gelB-AA scaffolds ($1 \times 1 \times 0.5 \text{ cm}^3$) were swollen to equilibrium before measurements. Samples were compressed (1 mm min^{-1}) until a strain of 25 % was reached. A load cell of 100 N was applied and a preload of 0.01 N prior to data collection was selected. Next, the stress (MPa) corresponding to each strain (%) was collected and the compression modulus was calculated from the slope of the initial linear part of the compression curve.

6.2.2.2 *In vitro* degradation

The *in vitro* degradation behavior of hydrogel films and scaffolds was studied by enzymatic degradation. Freeze-dried samples ($\sim 5 - 10 \text{ mg}$) were reswollen in 0.5 mL Tris-HCl buffer (0.1 M, pH 7.4) in the presence of 0.005 w/v % NaN_3 and 5 mM CaCl_2 at 37°C . Collagenase (enzym activity of 388 U mg^{-1} , Sigma-Aldrich) stock solution of 200 U mL^{-1} in Tris-HCl was prepared and added to the samples to obtain final concentration of 2 U mg^{-1} gelatin. At different time intervals, the degradation was stopped by the addition of ethylenediamine tetraacetic acid (EDTA) solution (0.2 mM, Sigma Aldrich) and subsequent cooling of the sample on ice. The partially degraded scaffolds were washed with ice-cooled Tris-HCl buffer and double distilled water, and freeze-dried for the determination of the gel fraction.

6.2.2.3 Release of polysaccharides and proteins

The release properties of gelatin films and scaffolds were investigated by monitoring the release of two model fluorescein isothiocyanate (FITC) labeled molecules: (i) dextrans as a model for polysaccharide release (FITC-DEX), and; (ii) bovine serum albumin as a model for polypeptide release (FITC-BSA). Samples were prepared as described above, with the addition of 10 mg mL^{-1} of the FITC labeled molecules. Samples were incubated in distilled water at 37°C . At different time intervals, the cumulative release of DEX and BSA molecules was monitored by means of spectrophotometric absorbance measurements using an UVIKON XL UV-VIS spectrophotometer (BioTek).

Dextran release - Three FITC labeled dextrans were studied to investigate influences of the molecular weight: 4 000 Da (FITC-DEX₄₀₀₀), 40 000 Da (FITC-

DEX₄₀₀₀₀), and 250 000 Da (FITC-DEX₂₅₀₀₀₀).

BSA release - FITC-BSA with a MW of 66 kDa (FITC-BSA₇₀₀₀₀) was evaluated.

6.2.2.4 Dynamic Vapor Sorption (DVS)

A Dynamic Vapor Sorption apparatus (DVS-1, Surface Measurement Systems) was used for examination of the water vapor sorption and desorption behavior of hydrogels. The apparatus is placed in a temperature-controlled housing. Samples were exposed to stepwise changes (10 %) of the relative humidity (RH) (RH 0 – 100 %) at 37 °C, and the water sorption/desorption profile was determined gravimetrically (Cahn microbalance)

6.2.3 Cell culture and characterization

6.2.3.1 Micro-aggregate formation

Primary mouse hepatocytes were isolated from adult mouse livers (8-14 weeks of age) using a two-step collagenase perfusion method followed by percoll gradient purification as described by Goncalves et al.³⁶² Primary hepatocytes were cultured in William's E medium (Life Technologies), supplemented with L-glutamine (292 mg mL⁻¹) (Invitrogen), glucagon (7 ng mL⁻¹) (sigma), insulin (0.5 µg mL⁻¹) (sigma), hydrocortisone (25 µg mL⁻¹), EGF (10 ng mL⁻¹), 10 v/v % FBS (Life Technologies), 50 U mL⁻¹ penicillin (Life Technologies) and 50 µg mL⁻¹ streptomycin (Life Technologies). HepG2 cells were maintained in DMEM Glutamax supplemented with 10 v/v % FBS, 50 U mL⁻² penicillin and 50 mg mL⁻² streptomycin, all provided by Life Technologies. Both the HepG2 cells and the primary hepatocytes were maintained in a humidified 5 % CO₂-containing atmosphere at 37 °C.

To produce the agarose micro-wells for the formation of micro-aggregates, sterilized powder Ultrapure Agarose (Life Technologies) was dissolved (3 w/v %) and heated in PBS. The liquid agarose solution was added to the mold and left to solidify at room temperature. After cooling, the gels were separated from the moulds and subsequently transferred into 12 well culture plates. Single cell suspensions of various densities were seeded into the micro-wells. After seeding, formation of micro-aggregates was supported by centrifugation for 1 min at 1500 rpm to let the cells settle into the bottom of the micro-wells. Culture medium was replenished 24 h after seeding. Cells were left in culture for 3 days to allow formation of stable micro-aggregates.

For the embedding in gelatin hydrogels, aggregates were mixed with predissolved gel-MOD precursor solutions to obtain a density of 2300 aggregates mL⁻¹ Aggregate-

gel mixtures were further processed as previously described.

6.2.3.2 3D printing of aggregate-laden gelatin methacrylamides and HUVEC seeding

For the fiber deposition of aggregate-laden gelB-MA solutions, a hydrogel building block precursor concentration of 15 w/v % containing 2300 aggregates mL^{-1} was selected using the plotting setup as described in section 5.2.3. VA-086 was used as photo-initiator, as described in section 5.2.1.3.

HUVEC-Umbil Vein, pooled amp-P1 (CC-2519, Lonza) cells were cultivated in endothelial growth medium, ordered as EBM Bulletkit (CC-3124, Lonza). At day 20, P8 HUVECs were seeded on the aggregate-laden (HepG2) scaffolds: 250 000 HUVECs per 1/4 scaffold in 48 well plates in a total volume of 300 μL . After 24 h, the scaffolds were transferred to new wells (total volume 500 μL). Seeding and further cultivation of the scaffolds occurred in endothelial growth medium.

6.2.3.3 Characterization of the aggregates

Assesment of the viability was performed according to the protocol described in section 4.2.4. PAS and IHC stainings were performed following the description from section 5.2.5.4.

To assess albumin secretion, media samples were collected at different time points. After omitting cross-reactivity for bovine albumin, the amount of secreted albumin was determined using a mouse albumin enzym-linked immunosorbent assay (ELISA) quantitation kit or a human albumin ELISA quantitation kit (Bethyl laboratories, Inc., UK) and normalized for the amount of cells using an MTT assay.

Cytochrom P450 3A4 (Cyp3A4) activity of the primary hepatocyte aggregates was analyzed using a P450-GloTM-CYP3A assay (Promega) according to the manufacturer's protocol for cell-based assays. After 72 h exposure to hydrocortisone, an inducer of Cyp3A4, the aggregates were cultured for 1 h in media containing luciferin-isopropyl alcohol (1:1000). After 1 h, an equal volume of the liquid was transferred to a 96 well plate and incubated with an equal volume of detection reagent. After 20 min, luciferase activity was detected using a Wallac 1420 Victor multilabel counter (PerkinElmer). The detected luminescence was normalized for the amount of cells (MTT assay). Background subtraction was performed with culture medium considered as negative control.

6.2.3.4 Real time-PCR

Total RNA was extracted from the aggregates using TRI Reagent (Sigma-aldrich) and treated with DNase digestion kit (Invitrogen). RT Core Kit (Eurogentec)

was used to synthesize cDNA, according to the manufacturer's protocol. A 7500 Fast Real-Time polymerase chain reaction (RT-PCR) system (Applied Biosystems) and a SYBR Green PCR kit (Eurogentec) was used to perform the RT-PCR, all according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as stable housekeeping marker. The $2^{-\Delta\Delta C_t}$ method was used to determine the relative gene fold changes, compared to gene expression in two dimensional cultures. The primer sequences were previously described and taken from Roelandt et al.⁴¹⁸

6.2.3.5 Confocal Fluorescence Microscopy (CFM)

Aggregate-loaded (HepG2) scaffolds having HUVECs seeded on top were analyzed by CFM measurements in wet conditions. The images were collected on a Leica SP5 laser scanning confocal microscope with a 40X 1.25 oil immersion objective. Digital Z slices were reconstructed using Velocity image analysis software.

Prior to the measurements, the actin skeleton, focal adhesion points and cell nuclei were stained using the actin cytoskeleton and focal adhesion staining kit from Millipore (FAK100). Samples were stained at 28 days of cultivation. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed (2×5 min with PBS containing 0.05 % tween20) and permeabilized with 0.1 % Triton X-100 in PBS for 5 min. After washing (2×5 min with PBS containing 0.05 % tween20), the samples incubated for 30 min in blocking buffer (PBS + 1 % BSA), and subsequently incubated with the anti-vinculin antibody (1:100 in blocking buffer) for 1 h, the secondary antibody FITC-goat anti-mouse IgG antibody (GAM) (1:500 in blocking buffer) and tetramethyl rhodamine-conjugated phalloidin (1:500 in blocking buffer) for 1 h, and 4',6-diamidino-2-phenylindole (DAPI) (1:1000 in PBS) for 5 min. Extensive washing with PBS containing 0.05 % tween20 (3×10 min) was performed after each incubation step.

6.2.4 Statistical analysis

All measurements were performed with $n \geq 3$, unless otherwise stated, and the experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical analysis was performed with the Student's *t*-test and significance was determined at $p < 0.05$. Statistical analysis was used in order to evaluate both the reproducibility of the respective techniques and the significance of differences between samples.

6.3 | RESULTS AND DISCUSSION

6.3.1 Scaffold internal pore architecture: theoretic design vs. practical dimensions

Three scaffold designs were fabricated, to study the deflections from the theoretically designed lay-down pattern. Units representing the theoretical design are displayed in Figure 6.2: (i) two $0^\circ/90^\circ$ patterns whereby one has a shifted pattern, i.e. each 0° and 90° layer are shifted so that in top view these are located precisely in the middle of the interstrut voids, and; (ii) a pattern whereby each subsequent layer is rotated by 45° . The theoretical models were drawn using COMSOL software.

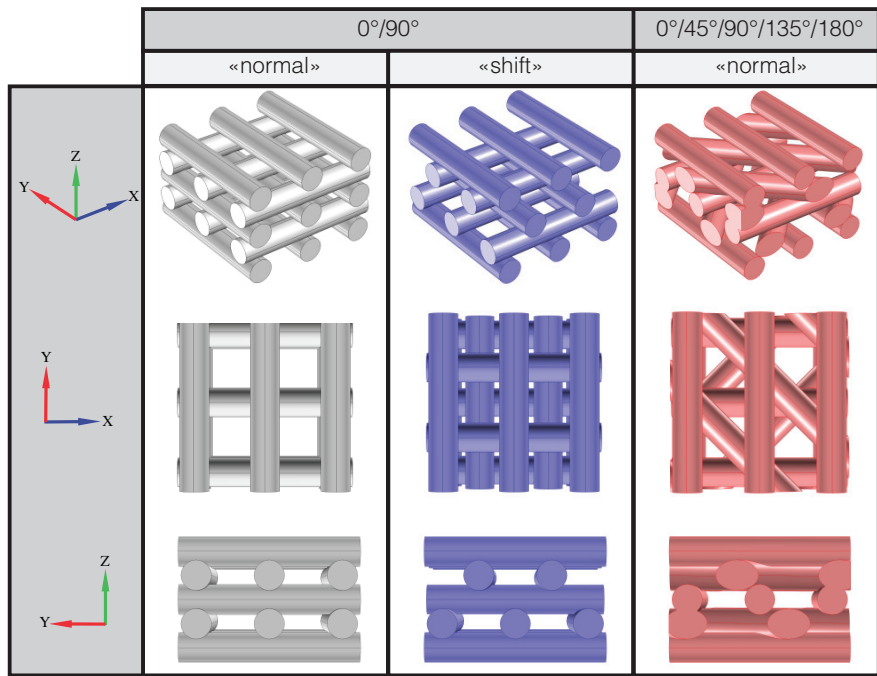


Figure 6.2: Theoretical scaffold designs viewed from 3 different angles.

The idealized models clearly demonstrate the influence of only adapting the layer deposition pattern. While the material strut volumes are kept identical, the void architecture is altered substantially. In Figure 6.3, optical microscopy (A-C) and SEM images (D-I) of the 3D printing outcome of these designs are displayed. The scaffolds were plotted using a 20 w/v % precursor solution. The $0^\circ/90^\circ$ “normal” pattern has been described in chapter 5, and a good agreement with the designed

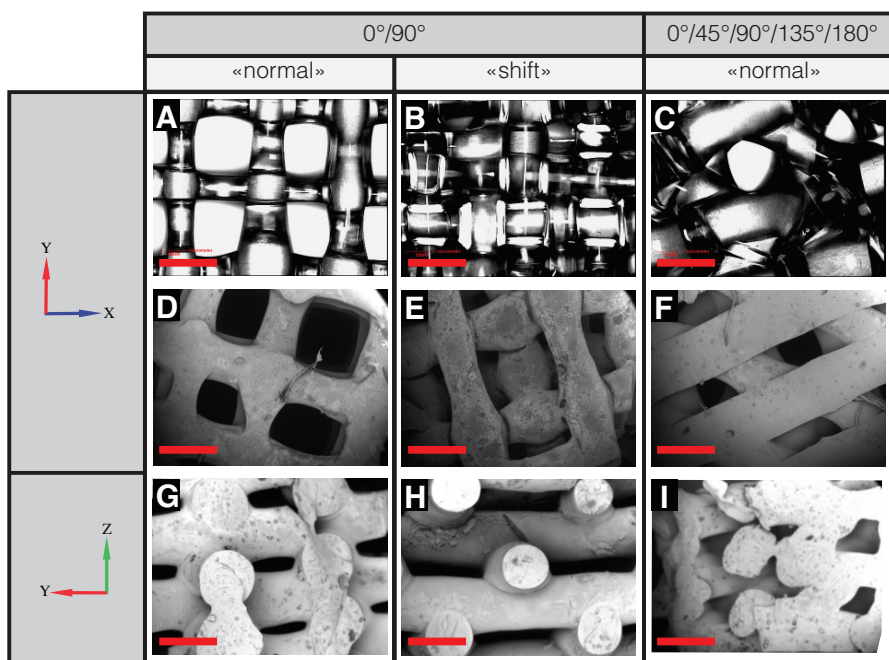


Figure 6.3: Scaffold desing for the 3D printing of 20 w/v % precursor solutions using G27 needle: optical microscopy (A-C) and SEM images (D-I). Scale bars represent 250 μm (A-C), 350 μm (D-F) and 260 μm (G-I).

geometry can be expected. For the 0°/90° “shift” pattern, porosity in top view is difficult to visualize with optical microscopy, but looking at the SEM pictures, it can be deduced that the final geometry closely resembles the objective. On the other hand, the 0°/45° pattern is best visualized in top view. Especially in cross-section (I), the cutting position greatly affects the obtained image (cutting deformation). These cutting defects are also seen in the cross-sectional image of the 0°/90° “normal” scaffold (G). Nonetheless, this particular pattern resulted in a slightly lower shape fidelity. Overall, an acceptable shape fidelity is observed.

The water vapor uptake was studied at 37 °C to investigate the difference between 10 w/v % 2D sheets and 3D scaffolds (0°/90° “normal” pattern). The increase in hydrogel masses (%) as a function of the relative humidity (RH, %) results in a typical DVS plot as presented in Figure 6.4. Especially for $\text{RH} \geq 80\%$, the hydrogel mass rapidly increases. The results are summarized in Table 6.1. Since the bulk material composition remained the same for both the 2D sheet and the 3D scaffold, it was expected to obtain by and large similar vapor uptake values at high RH. As can be deduced from the table, at higher target RH values ($\geq 50\%$) the mass change converges for both samples. However, the macropores present in

Table 6.1: Comparing dynamic vapor uptake of 10 w/v % 2D sheets and 3D 0°/90° “normal” scaffolds. Mass change (%) during sorption as a function of the relative humidity (RH, %).

TARGET RH [%]	Δm - dry [%]	
	2D SHEET	3D SCAFFOLD
0	0.00	0.00
10	2.44	6.40
20	4.88	8.74
30	6.91	10.18
40	8.80	11.48
50	10.34	12.95
60	12.76	15.52
70	15.99	17.81
80	23.92	27.56
90	35.60	37.68
100	63.19	67.52

3D scaffolds result in a slightly higher water vapor uptake at low RH values. This can be attributed to the increased surface area.

Our findings are in close correlation to previous experiments performed in our group on cryogenic scaffolds exploring the effect of scaffold hydrogel concentration and porosity (i.e. the work of Vanvlierberghe et al.⁴¹²).

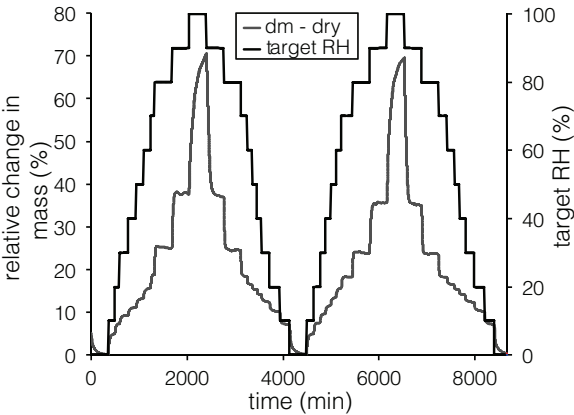


Figure 6.4: Typical DVS diagram representing the change in mass (%) of 10 w/v % 3D constructs in function of the relative humidity (RH, %).

6.3.2 Hydrogel material properties and the designed internal pore network affect scaffold mechanical properties

In chapter 4, two photocross-linkable gelatin hydrogel building blocks were compared for their shear moduli of 2D sheets (1 mm thickness). For scaffolds, compression measurements are more meaningful. Some typical stress-strain curves for liver samples and 20 w/v % gelB-MA constructs are depicted in Figure 6.5 A-B. The compression moduli of 3D constructs fabricated from gelB methacrylamide (gelB-MA) and gelB acrylamide (gelB-AA) as a function of the hydrogel building block concentration are presented in Figure 6.5 C. As discussed, the scaffold build-up pattern exerts an influence over the void/material geometry. Therefore, we anticipate an effect on the mechanical properties of the constructs. Figure 6.5 D demonstrates the impact of the layer deposition pattern on the compression moduli of 20 w/v % 3D printed constructs.

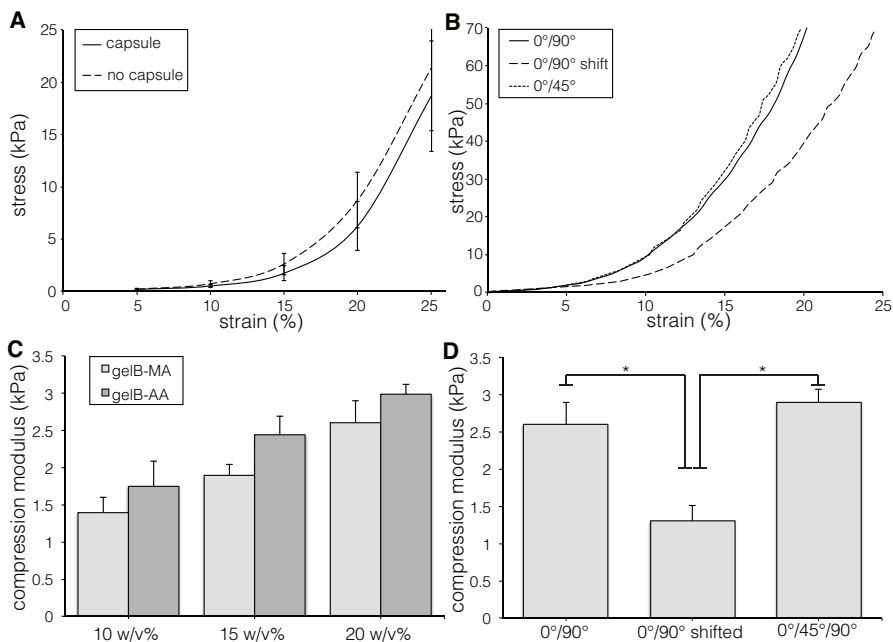


Figure 6.5: Stress-strain curves of liver samples (A) and 20 w/v % 3D constructs. Compression moduli gelatin scaffolds in function of the precursor concentration and photocross-linkable function for 0°/90° patterns (C) and in function of the fiber lay-down pattern for 20 w/v % constructs (D). All scaffolds were cross-linked with an UV irradiation dose of 7200 mJ cm². All compression tests were performed using a speed of 1 mm min⁻¹ at 37 °C.

The scaffolds were constructed using G30 (10 w/v %) or G27 (15 and 20 w/v %) needles following the parameters from chapter 5. The cross-linking kinetics, as

compared between methacrylamide and acrylamide gelatins, largely followed the findings of 2D films. However, faster curing was observed, as discussed in chapter 5 for methacrylamide constructs, with even faster curing rates for the acrylamide variant (i.e. a gel fraction of $92.3 \pm 2.6\%$ was reached at 240 mJ cm^{-2}). In line with the expectations, increasing concentrations lead to constructs with increased compression moduli. In contrast to the 2D films on the other hand, the discrepancy between constructs of different hydrogel concentration are less pronounced. Moreover, despite the slightly increased compression moduli of gelB-AA constructs, the difference was not significant. It is presumed that the relatively high curing dose for 3D printed constructs, levels off the differences. It can be expected that smaller exposure windows to UV-A light would enable tuning of the construct mechanical properties. Contrarily, when evaluating the layer pattern with respect to the resulting compressive strength, the “shift” pattern demonstrated a significant reduction in compressive strength.

Freshly isolated pig liver tissue samples were subjected to an identical measurement protocol, since the recorded mechanical properties of poroviscoelastic materials will greatly be affected by the measurement protocols. The livers were collected at the KU Leuven, transported on ice, cut in pieces and measured in the hours upon collection to limit changes in the mechanical properties. Two types of samples were analyzed: samples containing the capsule (i.e. the outer parts), and samples without capsule (i.e. the inner tissue). As can be seen in the averaged stress-strain curves, samples without capsule have a slightly higher compression strength. This can be attributed to the high density of arteries and veins present in the inner liver tissue. When taken the average for both types of samples, a compression modulus of $0.76 \pm 0.3 \text{ kPa}$ was calculated. This value is lower than the value for 10 w/v % gelB-MA constructs ($1.4 \pm 0.2 \text{ kPa}$).

Research studies towards the mechanical properties of animal and human livers mostly target static material properties. Reports on dynamical approaches are much less. For instance, the linear viscoelastic properties by means of oscillatory shear experiments demonstrated a storage modulus increase from 1 kPa to 6 kPa with increasing frequency, and in addition, an increase in storage and loss modulus is observed with increasing preservation times.⁴¹⁹ This value corresponds to a 5 w/v % fully cured gelB-MA hydrogel (chapter 4). Unfortunately, adapting the bioplotter device enabled us to process modified gelB hydrogel building blocks with a lower limit at around 10 w/v %.

In our experiments we conducted uniaxial compression experiments in order to compare (pig) liver tissue to gelB-MOD hydrogel scaffolds. Yeh et al.⁴¹⁵ reported a compression modulus of $0.64 \pm 0.08 \text{ kPa}$ of healthy liver tissue and $1.65 \pm 0.11 \text{ kPa}$ for fibrotic tissue (5 % preload strain) using ultrasonic imaging. Our results sug-

gest that 10 w/v % gelB-MA hydrogels were closest to the value of pig liver, and that this value can be lowered even further when taking the scaffold pattern into consideration.

Finite element simulations were performed on the theoretical designs in order to obtain a qualitative understanding on the differences between scaffold desing under uniaxial compression. Qualitative heat mappings of the resulting Von Mises stresses at a deformation of 10 % are given in Figure 6.6. The images give a qualitative impression of the localized surface stress magnitudes and field displacements upon compression. As can be seen, highest local stress distributions are observed for the highly regular $0^\circ/90^\circ$ “normal” and the $0^\circ/45^\circ$ “normal” patterns. For the shifted pattern, the surface stresses are more homogeneously distributed. Despite the qualitative nature, the images correspond with the compression data.

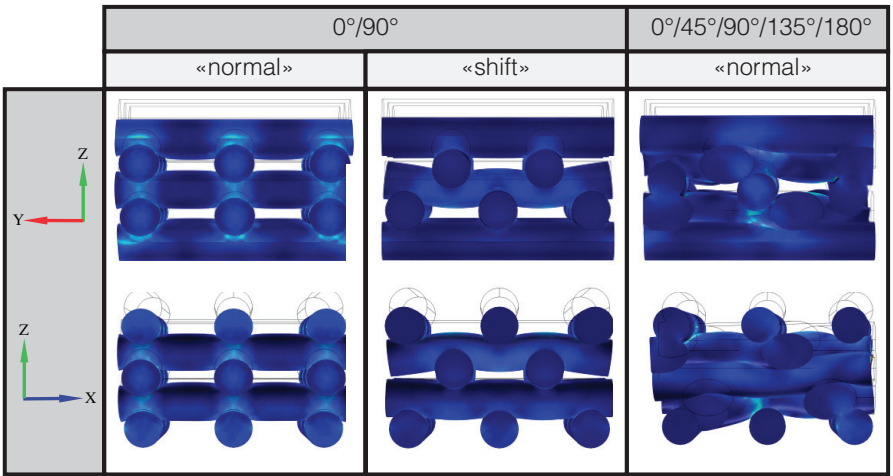


Figure 6.6: Qualitative heat mapping of the relative Von Mises stress profile comparing scaffold design upon a deformation of 10%. Black lines show the initial design.

6.3.3 Enzymatic degradation behavior of gelatins: 2D films versus 3D constructs

The enzymatic degradation behavior of gelatin (meth)acrylamide hydrogels was studied to investigate the effect of the gelatin precursor, hydrogel building block concentration, and to compare 2D films and 3D scaffolds. The degradation was evaluated by determination of the gel yield after incubation in collagenase solution (Figure 6.7).

The results can be summarized as follows: (i) gelA derivatives degrade faster than gelB derivatives; (ii) gel-MA hydrogels degrade faster than gel-AA hydrogels; (iii) 3D scaffolds degrade faster than 2D films; (iv) increasing the hydrogel precursor concentration leads to slower degradation rates, and; (v) 0°/45° scaffolds degrade slightly slower. These results suggest that the degradation rates can be tailored not only by varying the material properties, but also by changing the internal macroscopic pore network of 3D scaffolds.

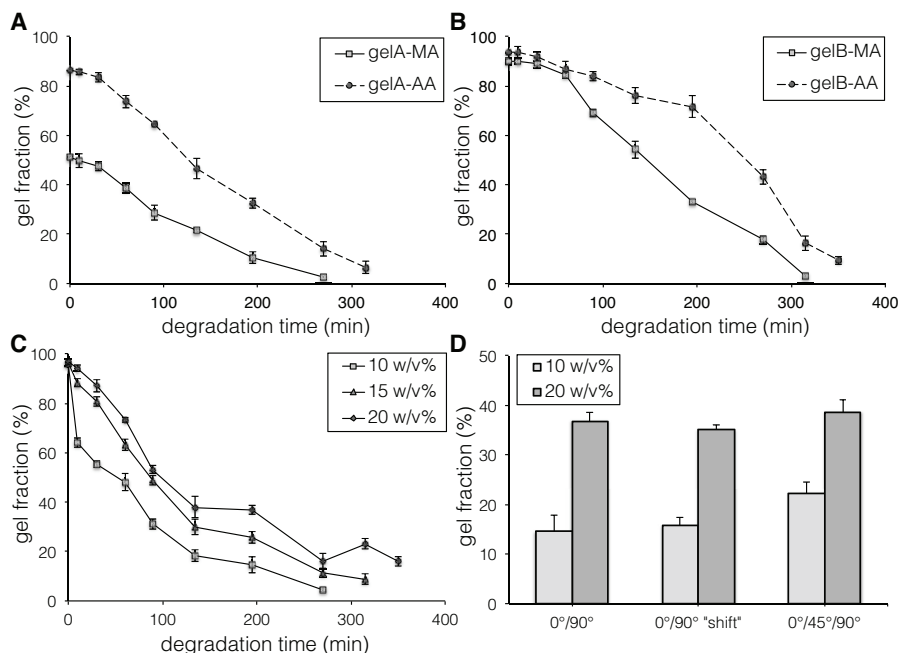


Figure 6.7: Enzymatic degradation behavior of 10 w/v % gel-MA and gel-AA 2D films (A,B) and 3D scaffolds as a function of gelB-MA concentration and scaffold design (C,D). For (D), values represent a degradation time of 195 min.

6.3.4 Release of polysaccharides and proteins: 2D films versus 3D constructs

The cumulative release of dextrans (DEX), as a model polysaccharide, and bovine serum albumin (BSA), as a model protein, from gelatin matrices was followed over a period of 5 days (Figure 6.8). The effects of release molecule, gelatin precursor type and concentration, and construct geometric shape (2D films vs. 3D scaffolds) were analyzed.

Dextrans having distinct molecular weights were released from 10 w/v % gelB-MA 2D films (A). With increasing molecular weight, the cumulative release is slowed

down, as would be expected due to increasing hydrodynamic radii of the release solutes. At a release time of 5 days, $> 90\%$ of the FITC-DEX₄₀₀₀ was released while only $\sim 40\%$ of the FITC-DEX₂₅₀₀₀₀ was released. The release of FITC-DEX₄₀₀₀₀ was also investigated for 3D constructs for two hydrogel building block concentrations (C). With increasing gelatin fraction, the release is inhibited substantially while only a slightly faster release was achieved compared to 2D sheets. The former could be attributed to the decrease in hydrogel network mesh size, while the latter could be explained by the increased surface area of 3D constructs.

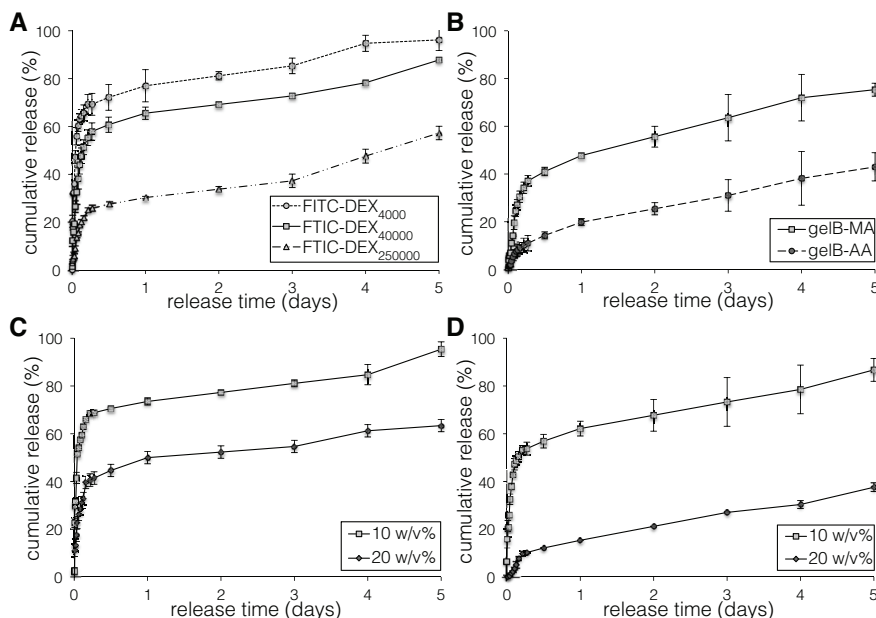


Figure 6.8: Release profiles of FITC-DEX (A,C) and FITC-BSA (B,D) in function of the release time. The effect of dextran molecular weight on the release behavior for 10 w/v % gelB-MA 2D films (A). The effect of gelatin precursor type (gelB-MA vs. gelB-AA, 10 w/v % 2D films) on the release of bovine serum albumin (B). The release profiles for gelB-MA 3D scaffolds as a function of the hydrogel building block concentration (C,D).

Apart from dextrans, BSA was released from 10 w/v % gelB-MA and gelB-AA 2D films (B). As would be expected, release from gelB-AA hydrogels occurs at a slower pace compared to gelB-MA hydrogels. Similar observations as for dextran release from 3D scaffolds were derived for BSA release from 3D scaffolds (D). However, the difference between 10 w/v % and 20 w/v % hydrogels was more pronounced. Generally, less complete release of BSA was observed compared to dextran release.

All release profiles displayed a rapid initial release rate followed by a gradual

increase over time. In chapter 4, the mesh sizes of 10 w/v % gel-MA and gel-AA, and 20 w/v % gel-MA were respectively determined to be 18.7, 13.9, and 9.4 nm (Table 4.3). Armstrong et al.⁴²⁰ determined the hydrodynamic radii of a series of macromolecules. The authors reported hydrodynamic radii of 1.86, 4.78, 11.46, and 3.51 nm for DEX_{9 500}, DEX_{40 000}, DEX_{250 000}, and BSA_{66 000}. Combining our mesh size determinations and their hydrodynamic radii calculations partially explain our release profiles. The lower release of BSA despite comparable hydrodynamic radius with DEX_{40 000} can logically be ascribed to increased electrostatic and hydrophobic interactions, leading to longer retention of the BSA molecules within the gelatin matrices.

Our results suggest that these systems could be used for sustained release of proteins and polysaccharides. The hydrophilicity of hydrogels has been shown to be favorable for preserving the native structure and functionality of the incorporated protein and the high water content and soft consistency of hydrogels minimizes mechanical irritation upon administration.¹⁵⁷

6.3.5 Towards co-culture systems: the use of micro-aggregates

6.3.5.1 Micro-aggregates improve hepatic phenotype

Micro-aggregates were fabricated based on agarose microwell technology. By varying parameters such as micro-well diameter, cell number and cell type, aggregates with different dimensions could be obtained and aggregate diameters varied between 100 and 300 μm for HepG2 cells and between 50 and 200 μm for primary hepatocytes. An example of HepG2 aggregates is illustrated in Figure 6.9.

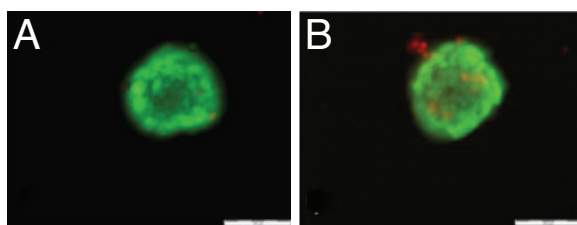


Figure 6.9: Live/dead staining of HepG2 micro-aggregates at day 3 (A) and day 7 (B) of cultivation. Scale bar represents 100 μm .

HepG2 and primary hepatocyte micro-aggregates demonstrated a clear and significant upregulation of hepatocyte functions, such as albumin, connexin32 (Cx32), hepatocyte nuclear factor 4 α (HNF4 α), E-cadherin, cyp3A4, and cytochrome 1A2 (cyp1A2), in comparison with two dimensionally cultured cells. In addition, hepatocyte performance is influenced by aggregate size and the number of cells/aggregate. Since an in depth discussion on these findings falls out of the

scope of this work, we refer to the PhD dissertation of Elien Gevaert from the group of Prof. Dr. R. Cornelissen.

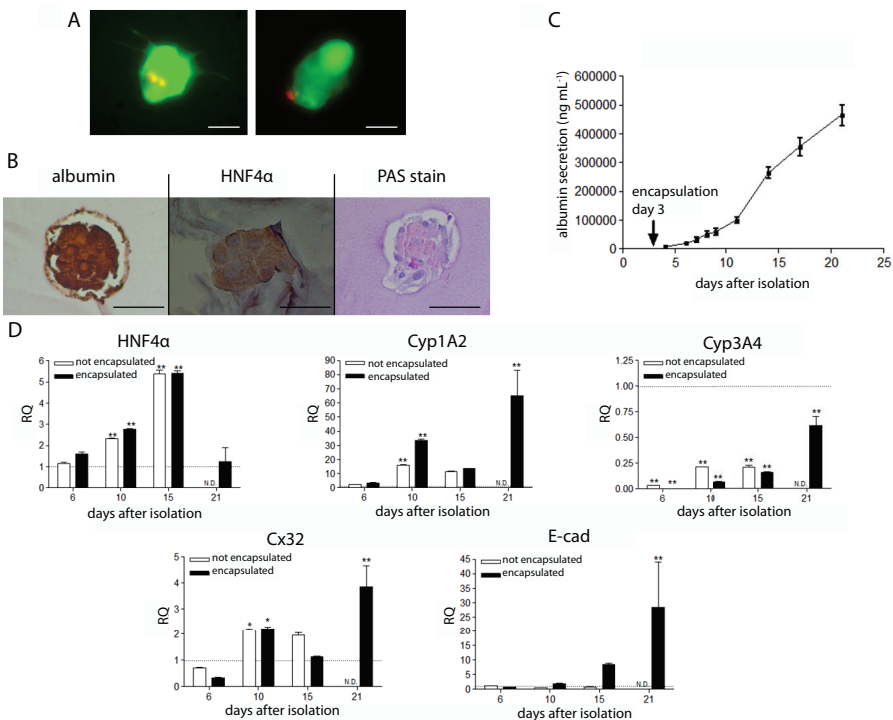


Figure 6.10: Evaluation of micro-aggregates after encapsulation in a gelatin hydrogel. (A) Live/dead stain of aggregates encapsulated in gelatin hydrogel at day 10. Scale bar = 50 μ m (B) Stainings of the immobilized micro-aggregates at day 10 after isolation. Scale bar = 50 μ m. (C) Cumulative albumin secretion determined by ELISA assay. Data are mean $\pm$ S.D., $n = 3$. (D) Real-time PCR for gene expression analysis for encapsulated and non-encapsulated aggregates. Data are mean RQ $\pm$ S.D., $n=3$. * $p < 0.05$; ** $p < 0.01$.

Aggregates ($\emptyset$ 100 μ m) were encapsulated and cultured in a 10 w/v % gel-MA hydrogel and gene expression and cell metabolism was compared with non-encapsulated aggregates (Figure 6.10). The viability of the immobilized aggregates compared to the non-encapsulated aggregates indicated that primary hepatocytes are not affected by the encapsulation procedure. Most of the aggregates maintained their rounded morphology while some cellular outgrowth was observed at day 10 of culture (A,B).

Similar gene expression profiles between encapsulated (black bars) and non-encapsulated (white bars) aggregates were found for HNF4 α , Cyp3A4, Cyp1A2,

E-cadherin and connexin 32 (Cx32) indicating that gelatin encapsulation did not affect aggregate performance. Stainings of encapsulated aggregates demonstrate that expression of albumin and HNF4 α protein was maintained during 10 days. Figure 6.10 C depicts the cumulative albumin secretion profile of the immobilized aggregates and indicates that albumin is secreted for the whole period under investigation (21 days). Altogether, these results indicate that encapsulation of the hepatocyte aggregates in a gelatin hydrogel is possible without adverse consequences for their characteristics.

6.3.5.2 3D printing of aggregate-laden constructs

To use aggregates as building blocks for modular tissue engineering, there is a need to organize and immobilize these small aggregate structures on a larger scale while cells remain alive and maintain their appropriate function.

Table 6.2: Processing conditions for the 3D fiber deposition of aggregate-laden 15 w/v % gelatin hydrogel precursors with conical needle types.

PARAMETER	15 w/v % gelB-MA	
	Experiment 1	Experiment 2
Aggregates [mL ⁻¹]	2300	2300
Needle gauge	G27	G25
$\emptyset$ [μ m]	200	250
Material temperature [°C]	27.5	27
Layer height [μ m]	170	200
Pressure [bar]	0.1	0.1
XY-speed [mm min ⁻¹]	500	750
Z-speed [mm min ⁻¹]	500	500
Preflow [sec]	0.05	0.05
Postflow [sec]	0.05	0.05
Corner delay [sec]	0.1	0.1
Pullback time [sec]	0.5	0.5
Interstrut distance [mm]	0.80	0.80

One such technique comprises encapsulation of the aggregates in a 2D sheet, as discussed above. However, internal macroscopic porosity is deficit in this approach. Another strategy capable of organizing cellular deposition in predefined patterns is the 3D bioplotter. Previously, we demonstrated the impact of cells on the plotting solution viscosity and therefore the plotting temperature (chapter 5). It was also shown that for single cell suspensions, gelatin hydrogel building blocks of 10 w/v % are preferred over higher concentrations (chapter 3). Initial encapsulation testing

with aggregates (HepG2 as well as primary mouse hepatocyte) on the other hand, revealed acceptable cell viability levels even up to 20 w/v % gelB-MA (data not shown). Altogether, a precursor solution of 15 w/v % was selected for the printing of HepG2 cell aggregates. Two sets of experiments were performed, of which the plotting parameters are presented in Table 6.2.

The plotting of aggregate-laden constructs applied conical needle types in order to limit the shear stress forces. As a first remark, it should be noted that the optimal plotting temperature for aggregate-laden solutions did not show the substantial drop as compared to single cell suspensions. Nonetheless, a critical plotting parameter appeared to be the needle internal diameter. Differences between the parameter sets of experiment 1 and 2 can be attributed to the difference in needle gauge. Constructs printed using the parameter set of “experiment 1” resulted in constructs with a higher cell loss and whereby the aggregate integrity was affected. This is illustrated by the live/dead staining in Figure 6.11.

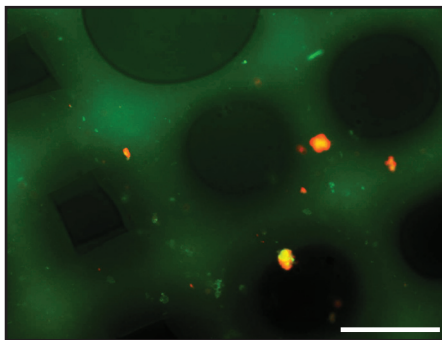


Figure 6.11: Live/dead staining of HepG2 micro-aggregates after 3D printing. Scale bar represents 500 μm .

It is clear that the aggregates were torn into smaller pieces, and the largest pieces had a reduced cell viability. The ratio of needle to aggregate diameter ratio was only a factor of two. Therefore, printing was repeated with a larger needle to aggregate diameter ratio (a factor of 2.5). This ratio proved capable of producing high viability aggregate-laden scaffolds with aggregate shapes intact. Figure 6.12 represents brightfield and live/dead stainings for these aggregate-laden constructs at day 6 (A,B), day 14 (C,D), and day 20 (E-H) of cultivation.

The micro-aggregates could be printed with high viability ($> 90\%$) and maintained their spherical morphology during the cultivation period (20 days). The brightfield image of day 20 (E) shows that some aggregates slowly migrate out of the scaffold. Once out the scaffold, the cells grow into a normal monolayer as illustrated by the brightfield images (G,H). These findings could be interesting when targeting cell

transplantation purposes.

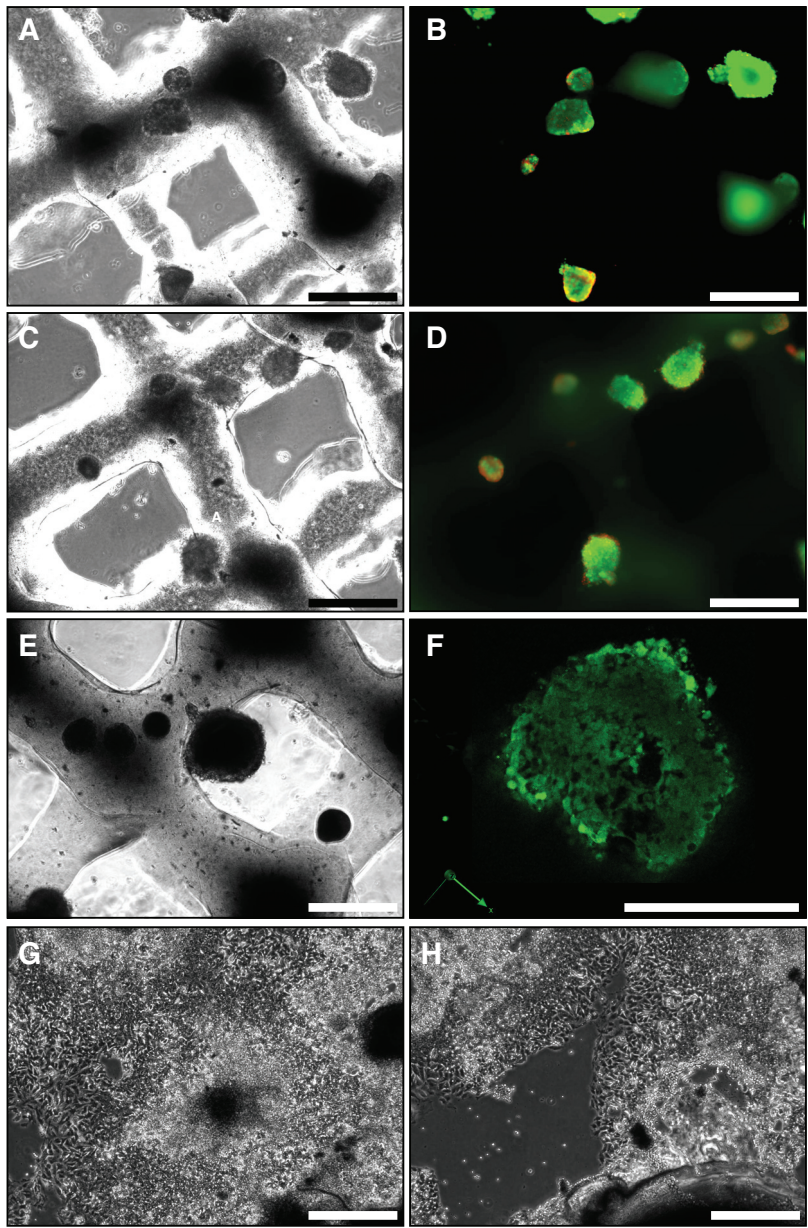


Figure 6.12: Images representing the printing and cultivation of aggregate-laden scaffolds at day 6 (A,B), day 14 (C,D), and day 20 (E-H) of cultivation. Brightfield images (A,C,E,G,H) and live/dead staining images (B,D). CFM image of a live/dead stain (F). Scale bar represents 500 μm (A-E,G,H) and 200 μm (F).

The principle was first introduced by Mironov in the framework of organ printing using spheroids as building blocks for macroscopic assembly.³²⁰ Very recently, Faulkner-Jones et al.⁴²¹ applied a valve-based rapid prototyping approach to print a stem cell suspension for the generation of micro-aggregates. In the previous chapter, single cell printed solutions proliferated into aggregate-like morphologies during cultivation as seen in Figure 5.16 (D, day 14 of cultivation). Our present results demonstrate the easy generation of cell-aggregates and subsequent assembly into 3D shapes, opening perspectives for modular tissue engineering.

6.3.5.3 HUVEC seeding on aggregate-laden scaffolds

Finally, in the last part of the present study, cell seeding on 3D scaffolds was performed. At day 20 of the aggregate-gel hybrid scaffold culture, HUVECs were seeded. Figure 6.13 represents live/dead stainings of the co-culture HUVEC seeded/HepG2 aggregate scaffolds at day 28 of culture.

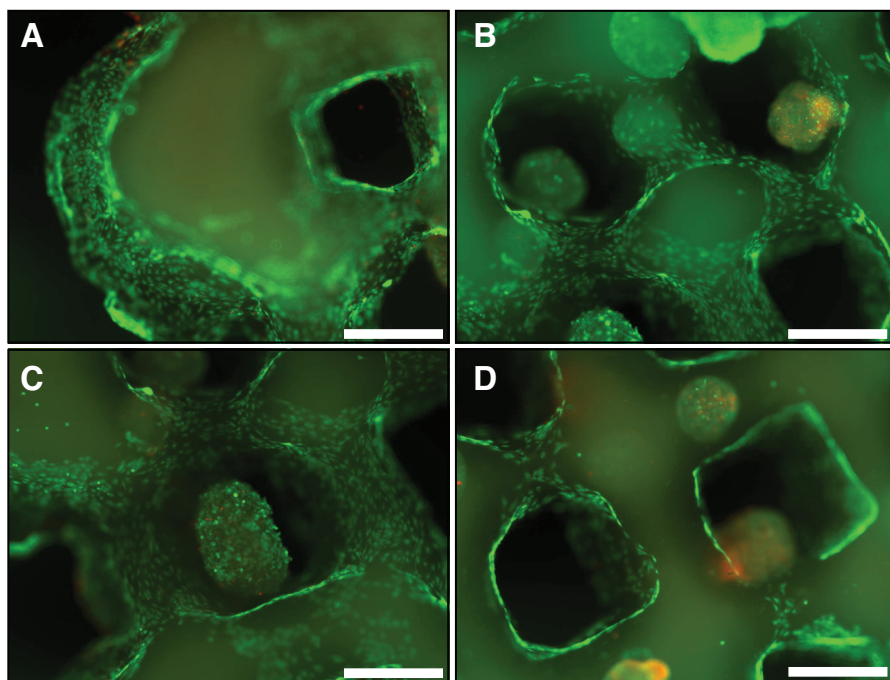


Figure 6.13: Live/dead staining of printed HepG2 micro-aggregates and seeded HUVECs at day 28 of cultivation. Image of the meander feature (A), the deposited fibers remain intact (B,C), cells are attached to the scaffold surface (D). Scale bar represents 500 μm .

The scaffold mechanical integrity remains up to 28 days of cultivation. The seeded cells colonized the scaffold surface. Some aggregates demonstrated a slight loss in cell viability. It should be noted that the micro-aggregates grew over the cultivation period, probably explaining the slight loss in viability due to hampered nutrient and metabolite waste/flow.

Confocal fluorescence microscopy (CFM) imaging was conducted to differentiate cells on the surface area and aggregates on the scaffold inside. Staining for the cell nuclei (DAPI staining, blue), the actin cytoskeleton (phalloidin staining, red), and the cells focal adhesion points (vinculin staining, green) are represented by Figure 6.14. Overlay images of the stainings with brightfield images illustrate the scaffold strand boundaries and location of the cells (A,B). The cells on the outer parts of the scaffold (HUVECs) demonstrate a spread morphology, whereas the aggregates reveal a more compact and rounded morphology as would be expected.

Hepatocytes tend to rapidly lose their phenotypic expression. Bhandari et al.⁴⁴ demonstrated a co-culture system with 3T3 fibroblast cells to modulate the function and viability of primary isolated rat hepatocytes. The hepatocytes within the co-cultures maintained their viability, possessed well-formed canalicular systems, and displayed functional markers. Using cell-sheet technology, Sasagawa et al.¹⁶ demonstrated the benefit of co-cultures. When sparsely cultured HUVECs were sandwiched between myoblast sheets, the inserted HUVECs sprouted and formed network structures *in vitro* and additionally, endothelial cell connections through the layers and capillary-like structures were found in a five-layer construct. Tuleuova et al.⁴²² demonstrated an improved hepatic differentiation when co-culturing hepatic stellate cells with mouse embryonic stem cells (mESCs). While the predictive value of these systems is still under investigation, these co-culture models can help to unravel complex cell-cell and cell-material interactions by developing more sophisticated *in vitro* systems.¹⁶⁰ Although our findings suggest that more complex 3D shaped constructs show potential for co-culture approaches thereby combining cell encapsulation, cell seeding, and rapid prototyping, future research (PCR assays) will need to be conducted to prove if a synergistic effect can be demonstrated.

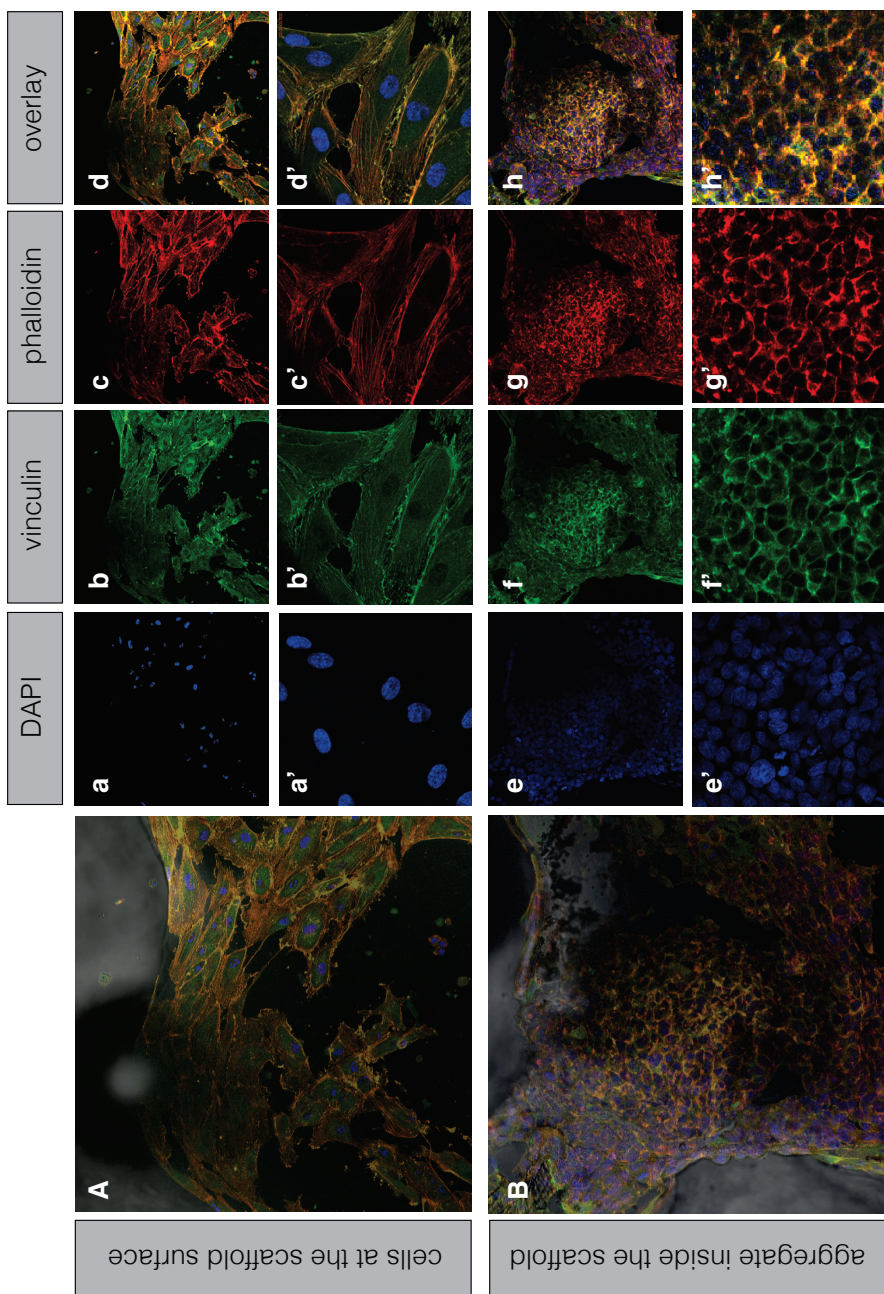


Figure 6.14: CFM imaging of HUVEC seeded Hepg2 aggregate-laden scaffolds. Overlay with brightfield images (A,B). Dapi, vinculin and phalloidin staining of HUVEC cells (a-c, a'-c') and overlay (d,d'). Dapi, vinculin and phalloidin staining of HepG2 aggregate (e-g, e'-g') and overlay (h,h'). Images recorded at 40 X magnification (a-d), and 63 X magnification (a'-d').

6.4 | CONCLUSION

It was demonstrated that using a combination of material properties and scaffold design, the mechanical properties, degradation behavior and polysaccharide/protein release can be tailored. The 3D printing of 3D models having distinct macroscopic internal pore networks proved successful with good to high shape fidelity. This parameter exerted its main influence on the scaffold mechanical properties.

The 3D assembly of cell micro-aggregates with high viability was feasible using the 3D bioplotter technology. For this, a crucial parameter revealed to be the needle internal diameter. Finally, a proof-of-principle experiment demonstrating the successful co-culture of aggregate-laden scaffolds and seeded HUVECs shows promising results for future research.

7 | GALACTOSYLATION OF PHOTSENSITIVE GELATIN HYDROGEL BUILDING BLOCKS IMPROVES THE FUNCTIONALITY OF ENCAPSULATED HEPG2 CELLS

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This chapter explores the applicability of liver-specific galactose moieties for enhancement of encapsulated cell performances. This chapter is based on an article published in Macromolecular Bioscience⁴²³ more related to the biological aspects, and a ‘to be submitted’ article more related to the synthesis and material characterization.

7.1 | INTRODUCTION

Currently, acute or chronic liver failure demands the development of alternatives to the gold standard, liver transplantation, to support, or take over (at least temporarily) liver function in order to overcome the organ shortage. One such alternative includes bio-artificial liver (BAL) systems, which can be defined as a bioreactor charged with a hepatoma cell line or isolated primary hepatocytes. As an extracorporeal device, the charged cells process blood or plasma and aim to support patients suffering from acute liver failure by assisting in failing detoxification, and synthetic and regulatory functionality.^{424–426} Low availability and difficulty to govern a suitable functionality for extended periods hampers the application of primary hepatocytes. Contrarily, hepatoma-derived cell lines, such as HepG2 cells, are more robust and can easily be upscaled and cultured for extended periods of time.^{425,426} On the downside, functionality comparable to primary hepatocytes of human origin is not reached, but may still be sufficient to bridge the time between disease and transplantation. In addition, genetic manipulation of the cells could bypass these problems, increasing their potential for the development of extracorporeal devices.^{425,427}

Various natural, synthetic and semi-synthetic ECMs have been employed as substrates for hepatocyte culture.^{38,428,429} For the most part, hepatocytes adhere well to natural ECMs (i.e. collagen, fibronectin, laminin and matrigel), however they have the tendency to exhibit low liver specific function levels typical for cell dedifferentiation.^{430,431} As such, they exhibit flattened and spread cell morphology, whereas a round morphology has been correlated with enhanced hepatocyte function.^{402,403} Furthermore, round morphologies are preferential in order to prolong hepatocyte function maintenance and have been observed using cell encapsulation strategies.^{404,432} Hydrogels, of which the hydrated states resemble that of the ECM, are suitable scaffold materials for cell encapsulation and allow for an easy exchange of gasses, nutrients and metabolites.^{1,7} *In vitro* culturing or *in vivo* transplantation then facilitates the repopulation of site defects or the production of growth factors and/or other molecules that will affect the targeted cell population.¹⁴⁰

Carbohydrates, on the other hand, can serve a key role in molecular recognition processes, including specific recognition of cells and intracellular communication.⁴³³ Therefore, the design of synthetic glycopolymers that maintain natural carbohydrate functions has drawn a lot of attention in recent years. The asialoglycoprotein receptor (ASGPR), a transmembrane receptor that recognizes galactose or N-acetyl- β -galactosamine as a specific ligand, was discovered on the surface of mammalian hepatocytes and characterized by Ashwell et al.⁴³⁴ In addition, more

abundant than the surface receptor, this receptor is also present in the internal membranes of rat hepatocytes.⁴³⁵ As a result, the development of synthetic glycopolymers with pendant galactose moieties has been investigated intensively for liver engineering or liver targeting.^{436,437} For example, galactose moieties were introduced onto gelatin,³⁵⁷ hyaluronic acid,¹³⁶ chitosan,⁴³⁶ alginate,⁴³⁸ and collagen.⁴³⁹ In general, the presence of galactose-containing moieties improved hepatocyte adhesion and hepatocyte specific functions by promoting the formation of round adherent cells.^{440,441} Cho et al.^{404,430} described the effects of galactose moieties for galactose-carrying synthetic ECMs investigating the geometry, type, density, and orientation of the galactose units on the hepatocyte attachment and function through receptor-mediated interactions between ASGPR and galactose moieties along glycopolymer chains. Hong et al.³⁵⁷ assessed a galactosylated gelatin cross-linked by carbodiimide chemistry for the seeding of primary hepatocytes. Nevertheless, galactose density was neither quantified nor varied. Usually, these studies are mainly focused on effects after cell seeding on a galactosylated biomaterial.^{136,439} To our knowledge, reports on galactose influence on encapsulated cells are limited.

In this chapter we report on the evaluation of cross-linkable galactosylated gelatin (gelGAL-MOD) as a substrate for cell encapsulation. The objective of this study was dual: first, the synthesis of galactosylated gelatin methacrylamide with varying galactose densities by modification of gelatin with (i) lactobionic acid, and (ii) methacrylic anhydride. Hereby, the influence of carbohydrate moieties on the radical polymerization process will be investigated. Secondly, using HepG2 cells as a model, viability and functionality of encapsulated cells using the previously synthesized galactosylated gelatin methacrylamides is compared to galactose-free gelatin methacrylamide.

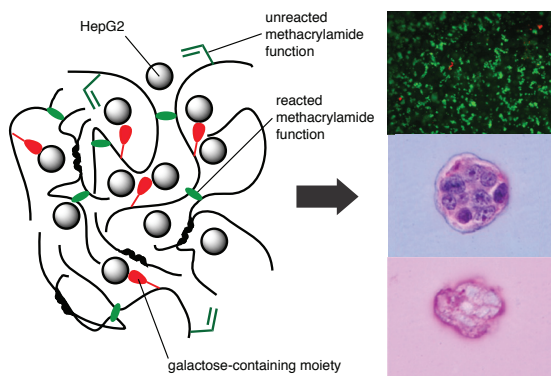


Figure 7.1: Schematic representation of the experimental setup of this chapter.

7.2 | EXPERIMENTAL PART

7.2.1 Synthesis and characterization of cross-linkable galactosylated gelatin (gelGAL-MOD)

7.2.1.1 Galactosylation of gelatin hydrogel building blocks

Bovine type B gelatin (approximate iso-electric point of 5 and Bloom strength of 257), isolated by an alkaline process, and porcine type A gelatin (approximate iso-electric point of 8.8 and Bloom strength of 202), isolated by an acid process, was supplied by Rousselot (Ghent, Belgium). The amount of free amines available for reaction was determined to be respectively 0.34 mmol g^{-1} gelA and 0.38 mmol g^{-1} gelB (see section 3.3.1). As shown in Figure 7.2, galactosylated gelatin methacrylamide (gelGAL-MOD) was developed by a two-step reaction. First, the free amines available on gelatin were derivatized with 4-O- β -D-galactopyranosyl-(1,4)-D-gluconic acid (lactobionic acid, LA) in the presence of EDC/NHS to produce amide bonds. Briefly, 10 g gelatin is dissolved in 100 mL phosphate buffer (0.1 M, pH 7.8) at 40°C . The carboxylic acid groups of 1.37 g LA (3.8 mmol, Sigma-Aldrich) were first activated using 1.1 g 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 5.7 mmol, Sigma-Aldrich) and 0.44 g N-hydroxysuccinimide (NHS, 3.8 mmol, Sigma-Aldrich) in 5 mL MES buffer (0.1 M, pH 6). The amount of LA is expressed as % LA added, relative to the amount of initially available free amines. After 15 min, the activated LA mixture was added drop-wise to the preheated gelatin solution. The reaction mixture was allowed to react for one hour to produce gelGAL.

Further functionalization with methacrylic anhydride, and subsequent dialysis and freeze-drying was performed according to the protocol described in section 4.2.2. Non-galactosylated gelatin methacrylamide (gel-MOD) was used as reference for further characterizations.

7.2.1.2 Direct method for determination of the galactosylation degree

A direct determination of the degree of galactosylation (DG) was performed with the Amplex Red Galactose/Galactose Oxidase Assay Kit (Invitrogen). First, a working solution of $100 \mu\text{M}$ Amplex Red reagent containing 0.2 U mL^{-1} horse radish peroxidase (HRP) and 4 U mL^{-1} galactose oxidase was prepared according to the manufacturer's protocol. A 50 mM Tris-HCl reaction buffer (pH 7.2, 1 mM CaCl_2) was used for the preparation of the gelGAL solutions (freeze-dried samples were applied). GelGAL-MOD suspensions (25 mg mL^{-1} Tris-HCl buffer) were heated for one hour at 37°C until complete dissolution. To $50 \mu\text{L}$ heated protein solution, $250 \mu\text{L}$ reaction buffer and $300 \mu\text{L}$ working solution were added.

The sample solution was incubated at 37°C for 30 min. Finally, the absorption was measured at 572 nm (BioTek, UVIKON XL) and 37°C. All measurements were performed in triplicate. Similar measurements were performed, in which the gelatin solutions were replaced by a series of solutions with known concentration of galactose (dilution series from 2 μ M to 250 μ M of LA). The degree of galactosylation (DG) is defined as the ratio of the amount of galactosylated amines to the amount of total amines present in unmodified gelatin.

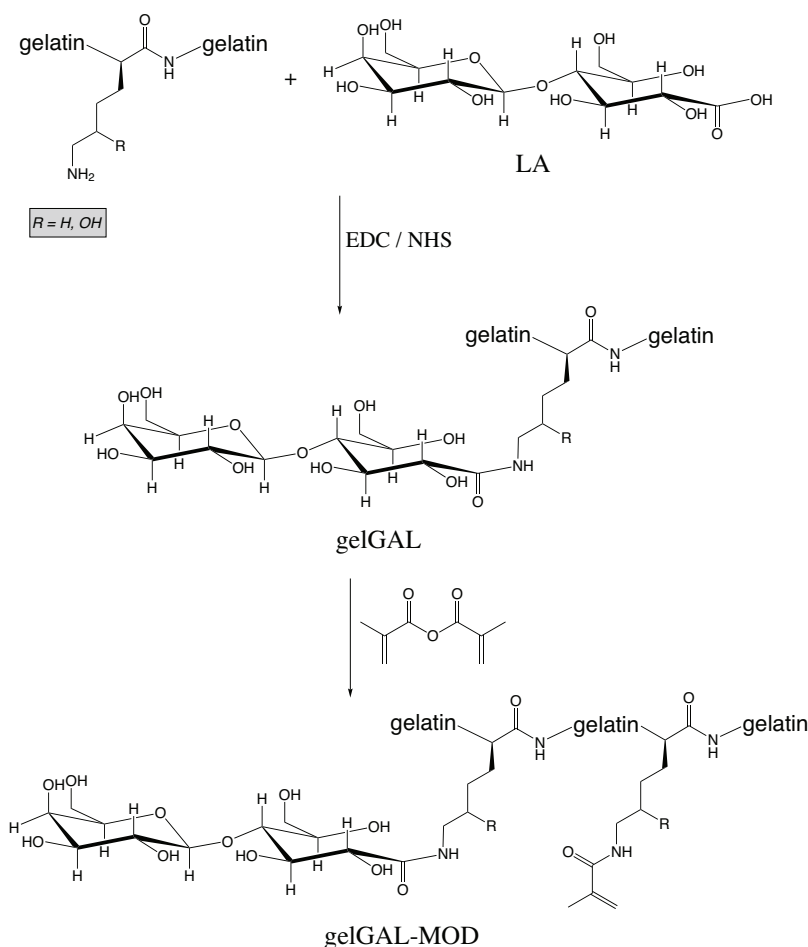


Figure 7.2: Chemical structure of gelGAL and gelGAL-MOD developed in this study for cell encapsulation of HepG2 cells. gelGAL is a galactosylated gelatin modified with lactobionic acid (LA), while gelGAL-MOD is a methacrylamide-functionalized gelGAL modified with methacrylic anhydride.

7.2.1.3 Indirect method for determination of the modification degrees

A second colorimetric method using o-phthalic dialdehyde (OPA, Acros, see section 11.4.2) was performed according to the protocol of Van Vlierberghe et al.³⁵⁹ Gelatin, gelGAL, gel-MOD and gelGAL-MOD suspensions (25 mg mL⁻¹ double distilled water at 37 °C) were prepared for measuring the amount of free amines remaining after each reaction step to determine the degrees of galactosylation (DG) and substitution (DS). The degree of substitution (DS) is defined as the ratio of the amount of methacrylated amines to the amount of total amines present in unmodified gelatin. A calibration curve was obtained by standards containing n-butylamine (5 µM to 250 µM, Acros). All measurements were performed in triplicate.

7.2.1.4 Fourier transform-infrared spectroscopy

Bulk changes were analyzed by means of Fourier Transform-Infrared spectroscopy (FT-IR) in attenuated total reflection (ATR) mode (Spotlight 400 Imaging System) according to the protocol described in section 4.2.3.1.

7.2.1.5 X-ray photoelectron spectrometry

Alterations in the chemical composition due to the modification steps, as well as differences between the gelatin types, were investigated by means of X-ray photoelectron spectrometry (XPS). Gelatin, gel-MOD and gelGAL-MOD samples were analyzed. Additional to survey scans recorded as described in section 4.2.3.1, the C 1s core signal was also collected (detail scan). The generated data are displayed as a plot of the electron counts versus electron binding energy in a fixed, small energy interval. *CasaXPS* software was used for data analysis.

7.2.1.6 Preparation of gelatin-based hydrogels

Sheets of gel-MOD and gelGAL-MOD solutions were prepared according to the protocol reported in section 4.2.2.2.

7.2.2 Characterization of the physico-chemical properties

7.2.2.1 High resolution-magic angle spinning NMR spectroscopy

The effective cross-linking efficiency was studied using high-resolution magic angle spinning (HR-MAS) NMR. Previously, this technique proved to be a powerful tool to characterize hydrogel cross-linking (Chapter 4). 1D ¹H HR-MAS NMR spectra of 10 – 20 w/v % type A (galactosylated) gelatin methacrylamide (gelA-MOD and gelAGAL-MOD) and type B (galactosylated) gelatin methacrylamide (gelB-MOD

and gelBGAL-MOD) were recorded as described in section 4.2.3.2. The generated data are expressed as the conversion in function of gelatin type and concentration. Each condition was measured in duplicate.

7.2.2.2 Equilibrium swelling studies

Equilibrium swelling experiments, according to section 4.2.3.2, were performed to evaluate the influence of galactosylation on the free-radical cross-linking properties of gelatin methacrylamides. Thus gel-MOD was used as reference condition for comparison to gelGAL-MOD cross-linking.

7.2.2.3 Rheological properties of hydrogels

Gel strength of gelA-MOD and gelB-MOD hydrogel films were compared by recordings of the storage modulus G' . The influence of the galactosylation on the cross-linking was investigated by *in situ* curing of 2D slaps. Rheometer setup and used protocols have been described in section 4.2.3.5.

7.2.3 Cell culture and encapsulation in the hydrogels

7.2.3.1 Cell encapsulation in gelatin-based hydrogels

HepG2 cells were encapsulated in modified gelatin derivates according to the protocol described in section 4.2.4. All gelatin products were sterilized by an ethylene oxide treatment (cold cycle, Hospital Maria Middelares, Ghent, Belgium). The chemically cross-linked gels were maintained in 500 μ L cultivation medium which was replenished every 24 hours.

7.2.3.2 Viability tests for encapsulated HepG2 cells

MTT assay: The cells were washed with PBS. A 5 mg mL⁻¹ solution of MTT (Calbiochem) in PBS was diluted 10 times with the cultivation medium and, subsequently, filter sterilized. The cells were incubated in the MTT solution (500 μ L/well) for 4 h, protected from light in a 5 % CO₂ incubator at 37 °C. The reagent was discarded by centrifugation (1000 rpm, 5 min), and the cells were treated for 30' with 500 μ L isopropanol-0.04 N HCL supplemented with 1 v % Triton X100 (Sigma) to rupture the cells and dissolve the formed formazan crystals. Subsequently, 200 μ L of each sample was transferred to a 96-well plate and the absorbance measured at 580 nm with an EL800 Universal microplate reader (BioTek instruments Inc.). Cells in suspension were considered to be a positive control. Accordingly, 300,000 cells/well were seeded in a 48-well suspension plate and kept in 500 μ L cultivation medium per well. At each time point, namely days 1, 3 and

7 after encapsulation, we calculated the relative cell number compared to the control. Within one experiment, three separate wells were sampled per time point for each condition, and each experiment was repeated three times, independently.

Live/Dead fluorescence staining: Cell viability imaging of HepG2 cells encapsulated in gelMOD and gelGAL-MOD was performed using Live/Dead fluorescence staining according to the protocol from section 4.2.4.

7.2.4 Functionality of encapsulated HepG2 cells

7.2.4.1 Enzyme-linked immunosorbent assay (ELISA) for detection of serum albumin secretion

Culture medium was collected at various time points and the amount of secreted albumin was determined using a human albumin ELISA quantitation kit (Bethyl Laboratories) according to the manufacturer's instructions. The specificity for human albumin was verified using unconditioned cultivation medium and no cross reactivity with bovine albumin was detected. The obtained values were normalized with the present cell numbers, determined using a standard curve and MTT assay.

7.2.4.2 Histochemistry and immunohistochemistry

Histochemistry and immunohistochemistry stainings were performed in a similar fashion as previously described in section 5.2.5.4.

7.2.4.3 Real-time PCR

On days 1, 3, 7, 14 and 21 of the experiment, RNA was extracted using the TRI Reagent (Sigma-aldrich). Potential genomic DNA contaminants were removed using a DNase digestion kit (Invitrogen), and cDNA was created using a RT Core Kit (Eurogentec) according to the manufacturer's instructions. A real-time PCR was performed using a SYBR Green PCR kit (Eurogentec) and a 7500 Fast Real-Time PCR system (Applied Biosystems), again according to the manufacturer's instructions. Primer efficiency was evaluated by means of standard curves, and the purity of the products was examined on the basis of melting curves. The RNA levels were normalized using GAPDH as a stable housekeeping marker and the relative gene fold changes. This can be compared to the gene expression of the control culture, which was determined using the $2^{-\Delta\Delta C_t}$ method. The primer sequences were previously described and taken from Roelandt et al.⁴¹⁸

7.2.5 Statistical analysis

All measurements were performed with $n \geq 3$, unless otherwise stated, and the experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical differences were explored by a one-way analysis of variance (ANOVA) followed by a Student t -test.

7.3 | RESULTS AND DISCUSSION

7.3.1 Characterization of galactosylated gelatin methacrylamide

Conversion of the gelatin free amines occurred according to the scheme from Figure 7.2. As illustrated, the modification scheme consists out of two subsequent steps. In the first part of this work, derivatization of gelatin free amines with LA was studied in depth. Two distinct methods were used to determine the degree of galactosylation. Applying the galactose oxidase assay (Amplex Red assay) determines directly the galactose content, while using ortho-phthalic dialdehyde (OPA) assay leads to an indirect estimation of the galactosylation degree. The indirect method involves determination of the free amine content before and after modification. Figure 7.3 depicts the degree of galactosylation for gelA and gelB.

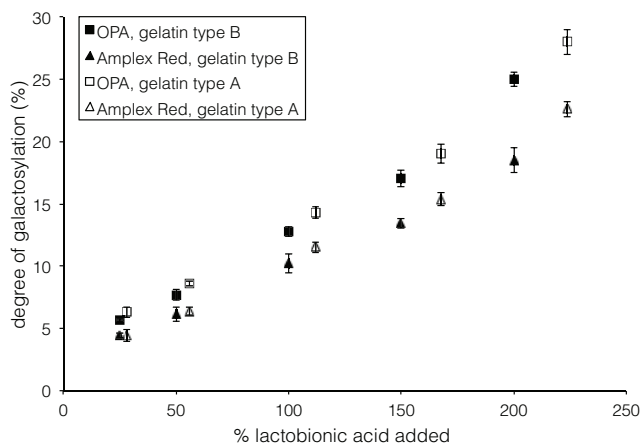


Figure 7.3: Synthesis of galactosylated gelatin methacrylamide: Degree of galactosylation (i.e. % amines initially present converted to galactose moieties) as a function of the amount of added lactobionic acid ($n = 3$).

The results demonstrate a significantly higher degree of galactosylation when using OPA. Hence less galactose-containing moieties were detected using galactose

oxidase. It is known that steric hindrance lowers the apparent activity of enzymes. For instance, Schumacher et al.⁴⁴² observed a significant decrease in galactose oxidase activity, comparing galactose to lactose quantification, due to steric hindrance of the β -glycosidic bond present in lactose. Our standard curve was based on LA concentrations, therefore limiting the mentioned effects. However, the covalent bonding to gelatin relatively increases steric hindrance, possibly explaining the underestimation of the amount of galactose-containing moieties using a direct quantification assay. Moreover, the OPA assay allows us to additionally quantify the amount of incorporated methacrylamide functionalities using a single determination method. The degree of galactosylation is proportional to the added amount of LA and comparable modification efficiencies for gelA and gelB were obtained. The absolute amount of incorporated galactose-containing units ranged from 20.9 – 92.4 $\mu\text{mol/g}^{-1}$ for type A and 21.6 – 95.5 $\mu\text{mol/g}^{-1}$ for type B gelatin. Hong et al.³⁵⁷ coupled lactobionic acid to gelatin by intermediate derivatization of gelatin carboxylic groups with ethylenediamine, followed by reaction of the terminal amines with lactobionic acid. However quantification of the reaction proceedings were not mentioned.

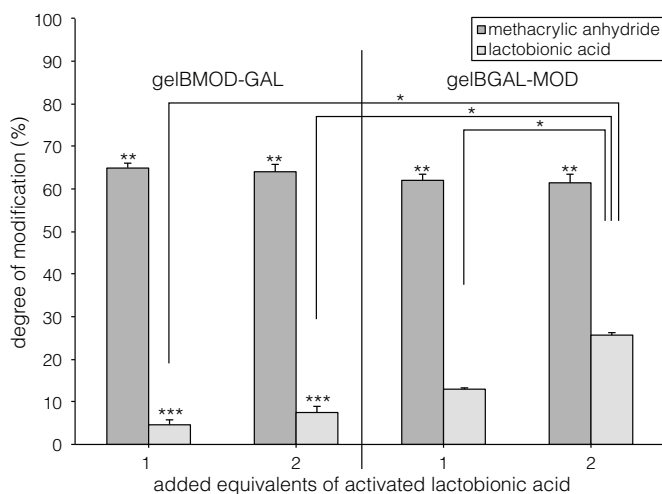


Figure 7.4: Synthesis of galactosylated gelatin methacrylamide: Influence of the modification sequence on the degrees of modification (i.e. % converted amines) as a function of the amount of lactobionic acid added. The amount of methacrylic anhydride added was kept at 1 equivalent with respect to the amount of initial free amines. * $p < 0.05$, **(*) $p > 0.05$

Secondly, methacrylamide functionalities were incorporated to allow the formation of chemically cross-linked networks to overcome the temperature dependent sol-to-gel transition of gelatins (Figure 7.2 step 2). It should be noted that the sequence of modification steps had an important influence on the overall galactosy-

lation degree as illustrated in Figure 7.4. Modification with methacrylic anhydride prior to galactosylation (gelMOD-GAL samples) yields relative galactosylation degrees similar to the above-mentioned results, i.e. when expressed relative to the amount of residual free amines after the first step. In other words, leading to lower absolute galactosylation degrees (approximate factor 3) relative to the native available free amines and thus lower absolute galactose content. On the other hand, the modification with methacrylic anhydride on the methacrylation degrees (DS) was not significant: for gelBMOD-GAL gels a DS of $64 \pm 1.3\%$ was reached, while for gelBGAL-MOD gels a DS of $61 \pm 1.5\%$ was quantified ($p < 0.05$). Non-galactosylated gelatin methacrylamide (gelB-MOD) had a DS of $65 \pm 0.9\%$. These values correlate well with previous work from our lab performed by Van Den Bulcke et al.,²²³ who originally established the synthesis of methacrylamide modified gelatin, and Van Vlierberghe et al.⁴⁴³, who reported a methacrylation degree of 65% . We obtained methacrylation degrees for both gelatin type A and gelatin type B within the range of $15 - 85\%$. Data on the DS as a function of the added amount of methacrylic anhydride was described in Chapter 4.

Table 7.1: Influence of gelatin type and gelatin modification on the surface composition determined by XPS analysis.

SAMPLE NAME	SURFACE COMPOSITION [%]			ATOMIC RATIO	
	O 1s	C 1s	N 1s	N/C	O/C
gelA	20.7 $\pm$ 0.1	64.2 $\pm$ 1.1	15.1 $\pm$ 0.9	0.23	0.32
gelB	18.4 $\pm$ 1.2	65.3 $\pm$ 1.3	16.3 $\pm$ 0.1	0.25	0.28
gelBGAL*	20.4 $\pm$ 0.6	64.4 $\pm$ 0.6	15.2 $\pm$ 0.6	0.24	0.32
gelB-MOD*	18.5 $\pm$ 0.6	66.6 $\pm$ 0.4	14.9 $\pm$ 0.2	0.22	0.27
gelBGAL-MOD*	20.5 $\pm$ 0.3	64.9 $\pm$ 0.2	14.6 $\pm$ 0.2	0.22	0.31

* modifications of type B gelatin

Alterations in the chemical composition due to the modification steps were studied using XPS spectroscopy. The results are summarized in Table 7.1. The covalent coupling of lactobionic acid increased both the oxygen content and the O/N atomic ratio respectively from 18.4% to 20.5% and from 0.28 to 0.32 . This would be expected after the introduction of a disaccharide structure. Additionally, the introduction of methacrylamide side groups results in a decrease of the N/C atomic ratio. Further insight was obtained by curve fitting of the high-resolution C 1s peaks. Generally, chemical identities are assigned to certain component peaks. However, it should be noted that proteins consist out of a broad spectrum of chemical functionalities.

Therefore, assignment of chemical identities to certain component peaks of the observed C 1s envelopes was deliberately over-simplified to decomposition into three peaks according to:⁴⁴⁴

1. A peak at 285.0 ± 0.1 eV corresponding to C-C and C-H bonds (peak 1);
2. A peak at 286.7 ± 0.1 eV attributed to C-O, C=O, C-N groups (peak 2), and;
3. A peak at 288.5 ± 0.1 eV due to O-C=O and N-C=O functional groups (peak 3).

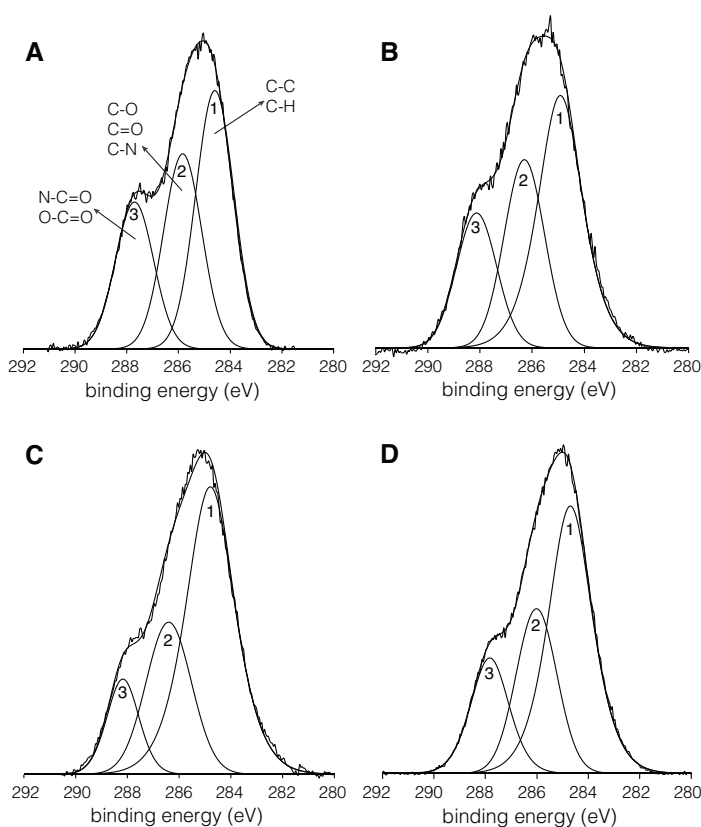


Figure 7.5: XPS C1s signals of gelB modifications: non-modified gelatin (A), gelBGAL (B), gelB-MOD (C) and gelBGAL-MOD (D).

The high-resolution carbon spectra are presented in Figure 7.5 and their values summarized in Table 7.2. The spectra demonstrate the changes in the chemical environment of the carbon atoms for each step in the modification process. In general, modification involved a decrease of peak 2 and 3 and an increase of peak 1

when compared to non-modified gelatin. On the other hand, comparing galactosylated gelatins with methacrylamide modified gelatins, peaks 2 and 3 are increased while peak 1 decreases.

Table 7.2: Decomposition of the gelatin type B C 1s envelope in different components (in percentages).

SAMPLE NAME	RELATIVE INTENSITIES OF C 1s [%]		
	285 eV	286.7 eV	288.5 eV
gelB	45.6±1.2	32.2±1.3	22.3±0.1
gelBGAL	48.6±1.6	31.3±0.9	20.1±0.6
gelB-MOD	54.4±0.6	27.9±0.4	17.7±0.2
gelBGAL-MOD	51.1±0.3	29.8±0.2	19.1±0.2

Figure 7.6 shows the FT-IR spectra of gelatin, LA and gelBGAL. Gelatin was found to show characteristic absorptions at 3280 cm^{-1} (amide A peak, N-H stretching vibration), at 1630 cm^{-1} (the amide I peak, C=O stretch), at 1547 cm^{-1} (the amide II peak, N-H bend and C-H stretch) and at 1240 cm^{-1} (the amide III peak, C-N stretch plus N-H in phase bending).³⁸⁷ The IR spectrum of LA exhibited a

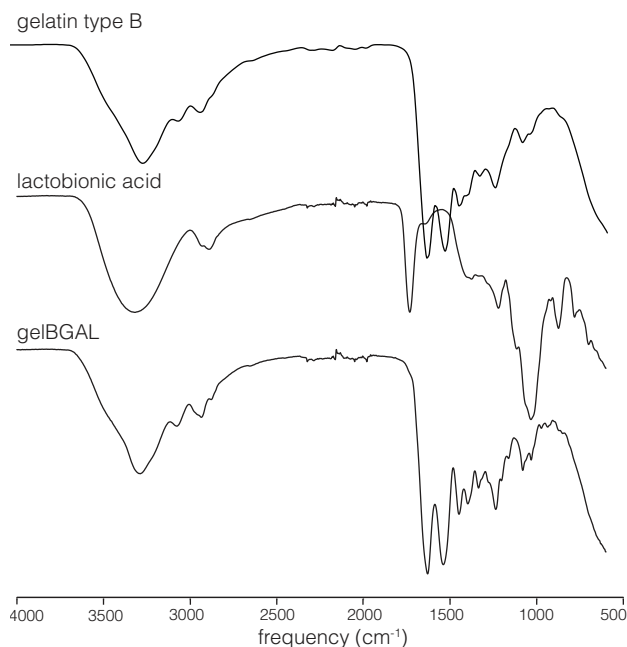


Figure 7.6: Changes in the FT-IR spectra before and after modification with lactobionic acid.

broad OH absorption occurring in the region between 3400 and 2400 cm^{-1} , and

a distinctive band at 1740 cm^{-1} due to the carbonyl stretching ($\text{C}=\text{O}$) of the carboxylic groups. In the IR spectrum of gelBGAL, the carbonyl stretching of LA disappeared due to the amide bond formation between carboxylic groups of LA and amine groups of gelatin. All peaks of amides A, I, II, and III of gelBGAL slightly shifted to 3292 , 1640 , 1559 and 1245 cm^{-1} , respectively, when compared with those of gelatin, an indication of the conformational change after reaction with LA.

7.3.2 Effect of galactosylation on the cross-linking kinetics of modified gelatins

As previously described, in this work we selected a chemical modification strategy of gelatins with methacrylamide functionalities in order to circumvent the limitations derived from the temperature dependent hydrogel properties. For this, I2959 was selected as biocompatible PI, which will generate free radicals upon UV irradiation. However, it has been described by some authors that the presence of carbohydrate moieties can hamper radical formation and propagation due to their antioxidant property as free-radical scavenger.^{445,446} Therefore, comparing gel-MOD with gelGAL-MOD for their cross-linking properties, we anticipated differences in the cross-linking degree and curing kinetics.

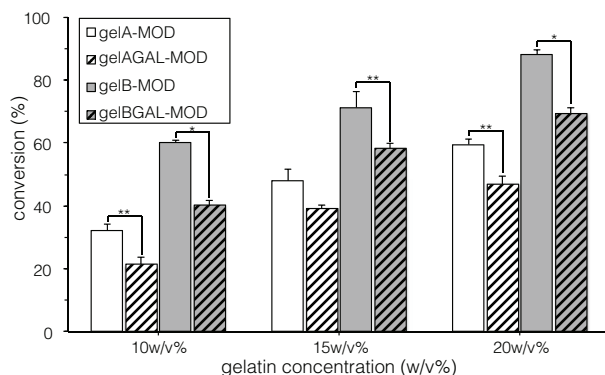


Figure 7.7: Effects of gelatin type on the curing properties of modified gelatins: Influence of gelatin type, modification, and concentration on the amount of reacted cross-linkable groups quantified by 1D ^1H HR-MAS NMR spectroscopy (* $p < 0.01$, ** $p < 0.05$).

7.3.2.1 Evaluation on the cross-linking efficiency by HR-MAS NMR

Initially, HR-MAS NMR spectroscopy was performed in order to derive the absolute cross-linking efficiency. The resonances of $\text{H}_2\text{C}=\text{C}(\text{CH}_3)-$ ($\delta = 5.7\text{ ppm}$ and

5.95 ppm) were used to quantitatively determine the conversion of the methacrylamide groups as described in more detail in section 4.3.2.3. The data are presented in Figure 7.7.

Gelatin type A methacrylamides (gelA-MOD and gelAGAL-MOD) and type B methacrylamides (gelB-MOD and gelBGAL-MOD) were compared in function of the gelatin building block concentration. In analogy to gel-MOD hydrogel building blocks, also for gelAGAL-MOD solutions increasing the concentration leads to an increased amount of reacted cross-linkable groups. As a first general remark, the reactivity of type A gelatins was significantly lower compared to type B gelatins, ultimately resulting in lower gel stiffness (Figure 7.8). Second, the introduction of galactose-containing units significantly lowered the amount of reacted methacrylamide functionalities confirming the hypothesis of galactose as free-radical scavenger. It should be commented that the obtained conversion values are depicted in reference to the initially available cross-linkable groups. When measuring hydrogels in fully swollen state (i.e. the sol fraction will be leached out), these values will increase since gelatin chains containing unreacted groups are leached out.

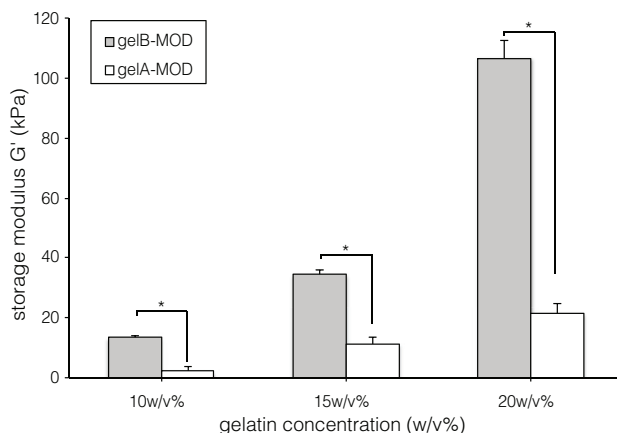


Figure 7.8: Effects of gelatin type on the curing properties of modified gelatins: Influence of gelatin type and concentration on the storage moduli (G') at 37 °C of fully cured gel-MOD hydrogels. Prior measurement, the hydrogels were swollen to equilibrium at 37 °C. Recordings occurred at a constant deformation of 0.1 % strain in the frequency range of 0.1 – 100 rad s⁻¹ at 37 °C.

7.3.2.2 Equilibrium swelling studies

To determine the relative degree of cross-linking in function of the irradiation dose, and to investigate the effect of galactose-content as an indicator of free-

radical scavenging, equilibrium swelling experiments were performed. The results are expressed as the gel fraction and the equilibrium mass swelling ratio q . The loss of reactivity/efficiency reflects upon the final gel fraction obtained. As seen in Figure 7.9 A, the gel fraction as a function of irradiation dose for 10 w/v % gelBGAL-MOD, containing $\pm 25\%$ galactose moieties, is reduced significantly compared to 10 w/v % gelB-MOD. Additionally, higher irradiation doses in the case of gelBGAL-MOD hydrogels were necessary before a substantial gel could be formed, i.e. 480 mJ cm^{-2} vs. 1200 mJ cm^{-2} . The plateau value, i.e. the irradiation dose at which point the gel fraction did not significantly increase anymore, shifted towards higher values. These data suggest not only a decreased cross-linking but also a slower one. Logically, the decreased gel yields led to greater swelling ratio q at equal curing conditions (Figure 7.9 B).

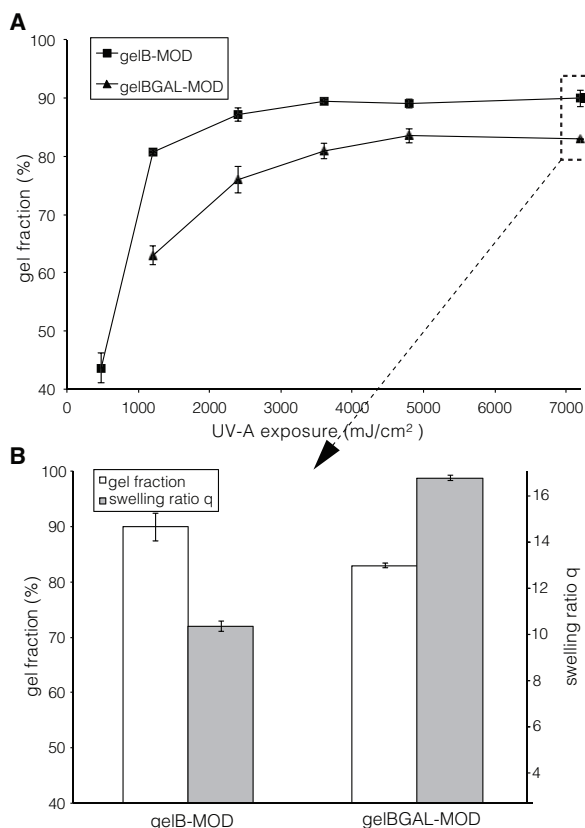


Figure 7.9: (A) Influence of galactose on the radical polymerization of 10 w/v % gelBGAL-MOD as a function of the UV-A exposure ($n = 3$) ($p < 0.05$). Gelatin methacrylamide (gelB-MOD, 10 w/v %) was selected as a reference. (B) Gel fraction and swelling ratio q at an irradiation dose of 7200 mJ cm^{-2} .

The scavenging effect was further investigated by increasing amounts of added (free) LA. The gel fractions and swelling ratios were monitored and are displayed in Figure 7.10. As anticipated, higher amounts of LA decrease the finally obtained gel fractions and increase the equilibrium swelling ratios. This can be attributed to an increase in scavenging activity upon increasing galactose-content. However, the effect on the swelling ratio is more pronounced compared to the effect on the gel fraction. For example, when incorporating 25 % of LA (with respect to the amount of initially free amines present) the gel fraction drops with 11 % while the swelling ratio increases with 74 % (Figure 7.9). The high swelling ratios up to 17 for the gelBGAL-MOD fully cured hydrogels illustrate the high water absorption capacity of these modified gelatins. Furthermore, the gel yield and swelling ratio can be manipulated by controlling the irradiation dose and galactosylation degree.

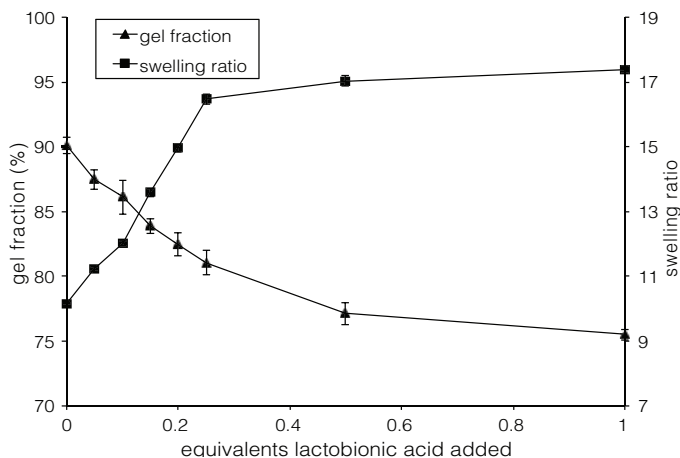


Figure 7.10: Influence of galactose presence on the radical polymerization of 10 w/v % gelatin methacrylamide (gelB-MOD) hydrogels as a function of the amount of added (free) lactobionic acid - expressed as equivalents to the amount of native gelatin free amines ($n = 3$).

Gel yields around 70 % for 10 w/v % methacrylamide modified gelatins (DS 68 %) and an irradiation dose of 9000 mJ cm^{-2} have been reported by Hoch et al.³⁸⁶ The mass swelling ratios in the work of Nichol et al.¹⁴³ were similar to our findings. Influences of gelatin galactosylation on the curing properties have not been found in the literature. The present work describes the changes in photo-initiated curing properties as a result of galactosylation and offers a more mechanistic insight in the hampered curing as a result of free-radical scavenging.

7.3.2.3 Rheological analysis of galactosylated gelating methacrylamide

Recordings of the storage moduli (G') as a function of the gelatin concentration have been provided in Figure 7.8. A broad range of moduli was obtained ranging between 13 and 110 kPa for the type B gelatins and between 1 and 25 kPa for the type A gelatins. It should be kept in mind that adjusting the amount of photo-sensitive groups enables to change the stiffness of methacrylamide modified gelatin hydrogels as has been demonstrated in the work of Hoch et al.³⁸⁶ and Nichol et al.¹⁴³ The mechanical spectra for both gelB-MOD and gelBGAL-MOD indicated a slight drop in gel strength ($G'_{gel-MOD} = 13.5 \pm 0.6$ kPa vs. $G'_{gelGAL-MOD} = 9.2 \pm 1.5$ kPa).

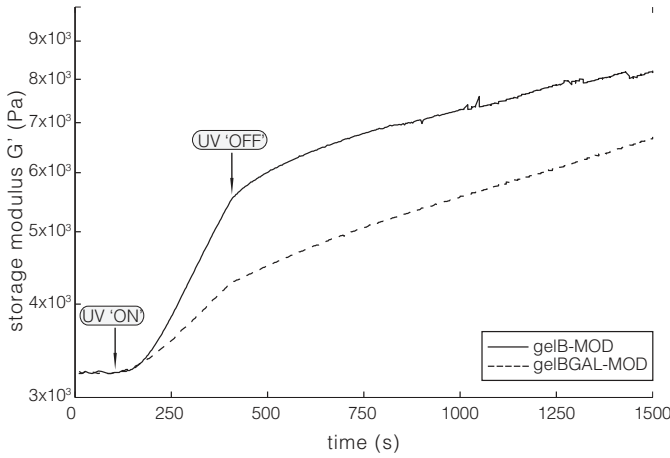


Figure 7.11: The cross-linking kinetics of type B gelatin methacrylamides monitored by rheological time-sweep recordings for the storage moduli (G') of 10 w/v % hydrogel precursors. Dashed line represents gelBGAL-MOD, full line represents gelB-MOD. The zone in between arrows indicates UV-A irradiation.

Rheological time-sweep tests coupled with *in situ* photocross-linking were performed to observe the increase in storage moduli (G') as the gel network is formed. Figure 7.11 depicts the observed effects for both gelB-MOD and gelBGAL-MOD using 10 w/v % hydrogels while keeping all other conditions fixed. Next to the previously described general properties of methacrylamides (the small lag phase and the post-curing after the beginning and ending of the irradiation), the gelB-MOD curve demonstrated a steeper slope and G' of gelBGAL-MOD hydrogels was lower at any given time point.

All of the above mentioned observations confirm the slower and less complete (i.e. less groups consumed) network formation in the case of the gelBGAL-MOD hydrogels.

7.3.3 Viability and functionality of the encapsulated cells

7.3.3.1 Galactosylation does not affect the viability of encapsulated cells

Comparing studies on gelatins remains challenging for researchers. Since gelatin is derived from the natural ECM constituent collagen, batch-to-batch variations as well as manufacturers extraction protocol differences remain plausible and could affect cell biology. Furthermore, as described in chapter 1 and 3, the extraction pre-treatment will highly impact the electrical nature, and as such the isoelectric point of the finally obtained gelatin. Type A gelatins will have isoelectric points between 7 and 9, while type B gelatins are situated around 5. In physiological conditions, gelA will carry a net positive charge, while gelB carries a net negative charge. Figure 7.12 A depicts live/dead stainings of HepG2 cells encapsulated in 10 w/v % gelA-MOD, gelB-MOD, and gelBGAL-MOD. The degree of substitution

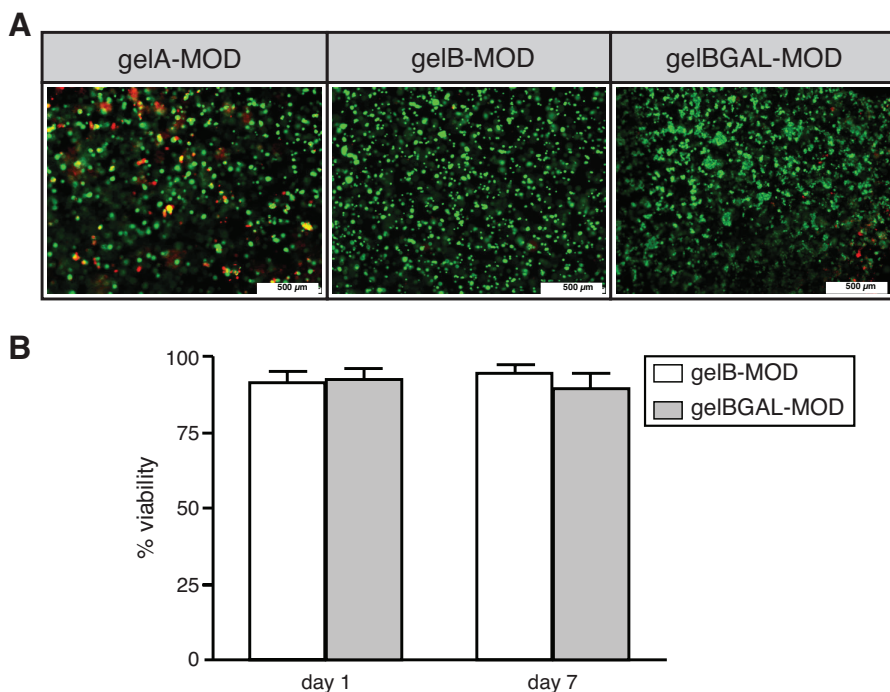


Figure 7.12: Viability assessment of encapsulated HepG2 cells in the hydrogels. (A) Live (green) and dead (red) fluorescence staining of encapsulated HepG2 cells in 10 w/v % gelatins: gelatin methacrylamide type A (gelA-MOD) and type B (gelB-MOD), galactosylated type B gelatin methacrylamide (gelBGAL-MOD). Scale bar represents 500 μm . (B) Live/dead assay of encapsulated HepG2 cells in 10 w/v % gelB-MOD and gelBGAL-MOD.

(methacrylamide functionality) was respectively $64 \pm 2.9\%$ (gelA-MOD), $65 \pm 0.9\%$ (gelB-MOD) and $61 \pm 1.5\%$ (gelBGAL-MOD). The galactosylation degree for the gelBGAL-MOD hydrogel was $25.2 \pm 0.6\%$. As can be seen, the photo-initiated free radical polymerization did not significantly alter cell survival during encapsulation. For gelA-MOD, cell survival was somewhat lower, in analogy to the titration experiments from chapter 3.

The cell viability was determined after the encapsulation and cross-linking of the hydrogel building blocks by applying UV-A light in the presence of I2959. Cell viabilities were compared for both the gelB-MOD and gelBGAL-MOD hydrogels (Figure 7.12 B). The data are mean percentages $\pm$ S.D. ($n = 3$). Viable cell fractions (relative to the total amount of encapsulated cells) of $91.5 \pm 3.4\%$ at day 1 and $94.2 \pm 2.5\%$ at day 7 for the gelB-MOD, and $91.6 \pm 4.3\%$ at day 1 and $89.2 \pm 5.2\%$ at day 7 for the gelBGAL-MOD. No significant ($p > 0.05$) improvement on the cell viability of encapsulated HepG2 could be observed for the galactosylated gelatin. In following experiments, gelA was excluded since both gelA (Section 3.3.5) and gelA-MOD showed lower viability levels.

As demonstrated by previous work, increasing the hydrogel precursor concentration leads to decreased mesh sizes (i.e. the distance between adjacent junction knots), resulting in increased network densities (i.e. tighter hydrogel networks). Tighter networks will, in turn, also have an impact on macroscopic properties, leading to increased mechanical strength and reduced swelling. Furthermore, the amount of reacted methacrylamide functions due to free-radical photocross-linking rises with increasing gelatin concentrations. As a consequence, cells will be subjected to fewer mechanical stresses, and might benefit from better nutrient/waste diffusion when encapsulated in lower concentration hydrogels. We can conclude that cell viability is, among other things, dependent on the hydrogel precursor concentration and, consequently, on network density, mechanical strength, and swelling properties. Based on these preliminary cell tests, the decision was made to perform all further evaluations only using the 10 w/v % hydrogels. Overall, good and stable cell survival was achieved. The functionalization of the methacrylamide modified gelatin hydrogel building blocks with galactose-containing side groups did not alter cell viability, suggesting that the introduction of other moieties (e.g. cell recognition motifs) might also be successful. The fraction of viable cells within both the galactosylated and non-galactosylated gelatin hydrogels (compared with the control) remained constant, suggesting that the hydrogels had no adverse effects on cellular proliferation.

7.3.3.2 Albumin secretion as a function of the galactosylation degree

The effect of galactosylation on the functionality was firstly evaluated by monitoring the albumin secretion of HepG2s encapsulated in gelBGAL-MOD hydrogels possessing varying galactosylation degrees (Figure 7.13). The DG was varied between 0 % (gelB-MOD, control condition) and 25 % in order to establish the effect of galactose pendant moieties. While no clear effect of galactose introduction was observed at day 1 and 3, a dose/concentration dependent effect of galactosylation was observed after a cultivation period of 7 days. It was clear that 25 % galactosylation significantly improved the albumin secretion of the encapsulated cells by a factor of 3, compared to the non-galactosylated gelatin derivate (gelB-MOD).

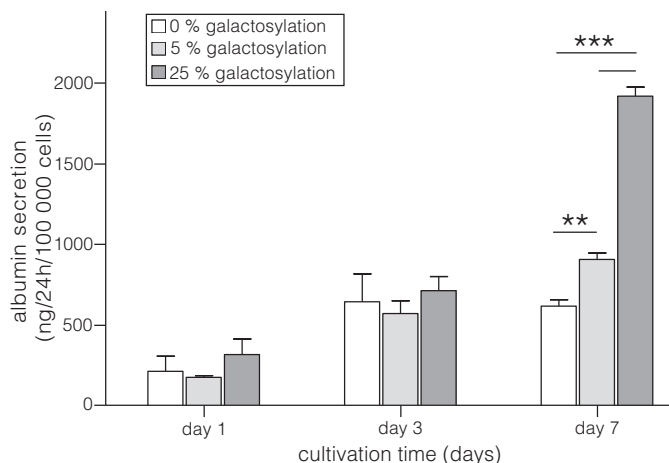


Figure 7.13: The effect of the galactosylation degree on the secretion of albumin by HepG2 cells encapsulated in 10 w/v % gelBGAL-MOD.

The rate of albumin secretion *in vitro* is 10 – 100times lower than the rate *in vivo* and has been reported to decrease rapidly over time for primary hepatocytes. Hong et al.³⁵⁷ compared the seeding of primary hepatocytes on collagen-coated 2D monolayer and on 3D galactosylated gelatin sponges. As a result of 3D cultivation, the formation of spheroids was promoted and the albumin secretion enhanced compared to the rate of the monolayer. Our findings indicate that encapsulation in a galactosylated gelatin matrix is able to significantly enhance the secretion of serum albumin, which offers potential for use as liver-specific encapsulation material since cell density can be increased.

7.3.3.3 Galactosylation improves the functionality of encapsulated HepG2 cells

Based on the albumin secretion results, gelBGAL-MOD possessing a degree of galactosylation of $\pm 25\%$ was selected for in depth immunohistochemistry and real-time PCR studies. Non-galactosylated gelatin methacrylamide (gelB-MOD) was selected as control condition. The IHC results revealed the expression of albumin (Figure 7.14 A-C) and HNF4 α (Figure 7.14 D-F) throughout the entire cultivation period in the modified hydrogels. The PAS staining (Figure 7.14 G-I) indicated the storage of glycogen granules in the cytoplasm at all of the investigated time points in the modified hydrogels. No differences in the staining of hepatocyte specific proteins (IHC albumin and HNF4 α) and the storage of glycogen (PAS staining) were found between the gelB-MOD and gelBGAL-MOD hydrogels.

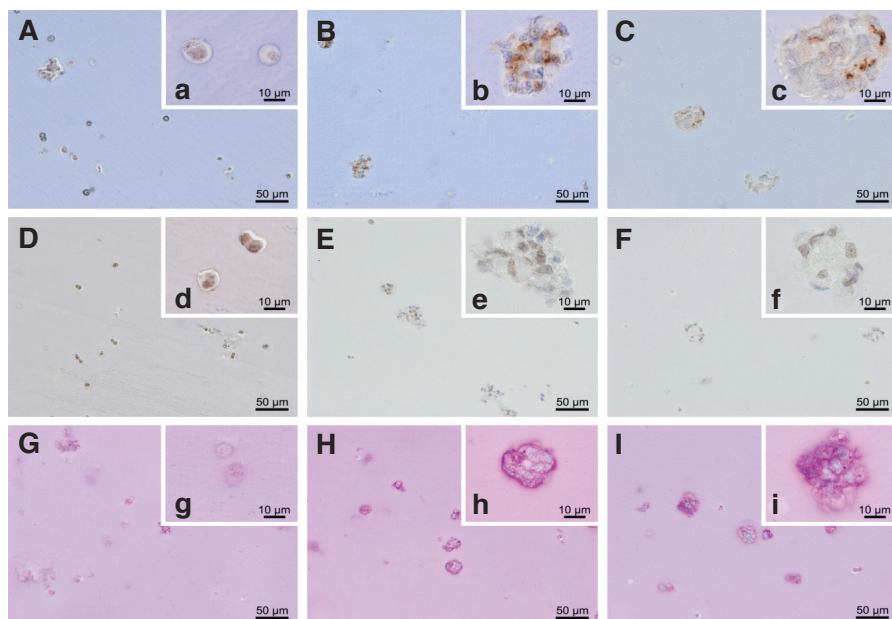


Figure 7.14: Maintenance of the hepatocyte phenotype of the HepG2 cells in the hydrogels: IHC and PAS staining of HepG2 cells in 10 w/v % gelBGAL-MOD. The pictures A-C represent staining for albumin at day 1 (A), day 3 (B), and day 7 (C). The pictures D-F represent staining for HNF4 α at day 1 (D), day 3 (E), and day 7 (F). The pictures G-H represent staining for glycogen at day 1 (G), day 3 (H), and day 7 (I). Pictures a-i are magnifications. Scale bar represents 50 μm (A-I) and 10 μm (a-i). The figure shows the pictures of one representative experiment from three.

Quantitative gene expression analyses of albumin, TTR and HNF4 α were performed using a real-time PCR analysis. The albumin ($p < 0.05$), HNF4 α ($p <$

0.01) and TTR ($p < 0.05$) expressions were significantly downregulated at day 1 after cell encapsulation in the gelB-MOD. However, at longer incubation times (day 3 to day 21), a gradual gene expression recovery towards the control levels (at day 7) was observed and maintained for all three of the genes studied (Figure 7.15). For the gelBGAL-MOD hydrogel, gene expression analyses of albumin, TTR and HNF4 α using a real-time PCR analysis revealed gene expression levels comparable to the control at day 1. Over time (up to day 21), the levels increased significantly ($p < 0.05$ for albumin and TTR and $p < 0.01$ for HNF4 α) compared to the control for all three of the genes tested.

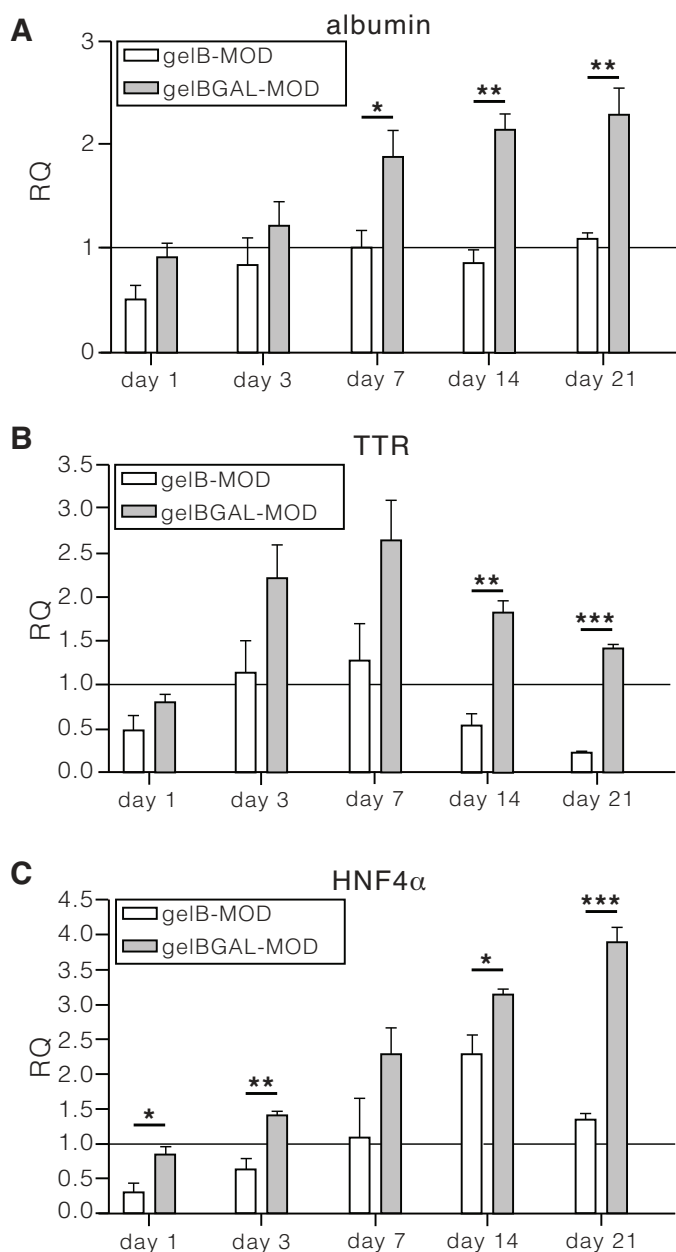


Figure 7.15: Real-time PCR analysis of HepG2 cells in a 10 w/v % gelB-MOD and gelBGAL-MOD hydrogels: gene expression levels of albumin (A), TTR (B) and HNF4α (C). The RNA levels were normalized using GAPDH as a stable housekeeping marker, while the relative gene fold changes, compared to the gene expression of the control culture, were determined using the $2^{-\Delta\Delta C_t}$ method. The data are the mean RQ $\pm$ S.D., $n = 3$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Many papers have reported on an improvement of hepatocyte functions via ASGPR-mediated interactions.^{357,438} Although the physiological function of the ASGPR is the receptor-mediated endocytosis of galactose/N-acetylgalactosamine-terminated ligands, it is also known to improve the hepatocyte phenotype. In most experimental setups (except in the work of Yang et al.⁴³⁸), cell seeding, and not cell encapsulation, was considered. Indeed, galactose functionalized gelatin sponges, chitosan, and polystyrene scaffolds have all been reported for the successful cultivation of hepatocytes.^{357,404,430,441,447,448} In the present work, we wanted to investigate whether the galactose-ASGPR interaction influences the functionality of encapsulated cells.

The positive IHC staining for albumin (considered to be an important synthesis product of hepatocytes) and HNF4 α (a transcription factor for the expression of hepatocyte specific genes), and the positive PAS staining, indicate the sustained functionality of the encapsulated cells. Importantly, quantification of the gene expression for albumin, HNF4 α and TTR, with the latter being another important synthesis product of hepatocytes, indicated the occurrence of a recovery period before the cells reached physiological gene expression levels in the non-galactosylated hydrogel. Very interestingly, for the galactosylated hydrogel, the gene expression of hepatocyte specific genes was even improved on and maintained over time. Furthermore, the hydrogels had no adverse effects on cellular proliferation, as supported by the positive Ki67 staining.

7.3.3.4 Characterization of the hepatocyte cell clusters

In a final part of the study, an examination of the general cell morphology after encapsulation was performed. The data (Figure 7.16 A, a-c) revealed cells with a round morphology and euchromatic viable nuclei, as indicated by the hematoxylin/eosin staining (Figure 7.16 A, a,a'). The cells formed clusters from day 3 onwards (Figure 7.16 A, b-c, b'-c'). In general, a better cell morphology was observed for the clusters compared to the individual cells, indicating the importance of cell-cell contact. The cluster formation and the constant viable cell fraction over time (Figure 7.12 B) suggest that the encapsulated cells proliferate at the same rate as the control. Proliferation was confirmed by positive Ki67 cell staining (Figure 7.16 A, d-f).

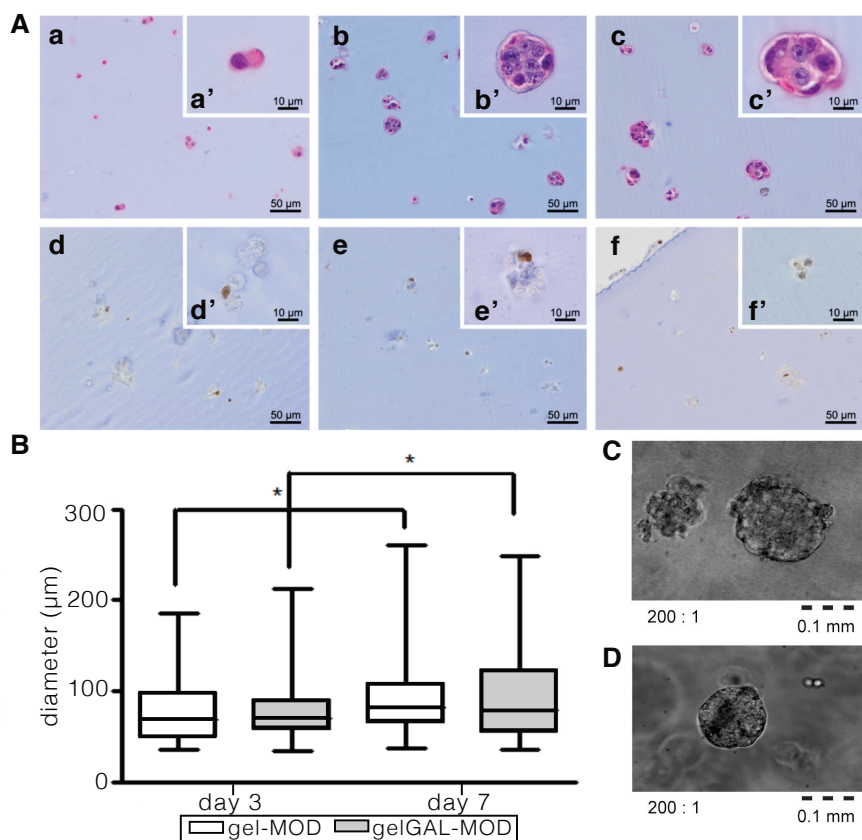


Figure 7.16: Cell morphology and spheroid characterization. (A) The hematoxylin/eosin and Ki67 IHC staining of HepG2 cells in 10 w/v % hydrogels. The pictures a-c represent hematoxylin/eosin stained cells at day 1 (a,a'), day 3 (b,b') and day 7 (c,c') after encapsulation in 10 w/v % gelB-MOD. The pictures d-f represent IHC staining for Ki67 at day 1 (d,d'), day 3 (e,e') and day 7 (f,f'), and show the proliferating cells. Scale bar represents 50 μm (a-f) and 10 μm (a'-f'). The figure shows pictures from one representative experiment of three. (B) Boxplot representing the spheroid diameter in gelB-MOD and gelB-GAL-MOD hydrogels at day 3 and day 7. (C-D) Representative brightfield images recorded at day 7 after encapsulation in gelB-MOD (C) and in gelB-GAL-MOD (D).

In both hydrogel types, spheroids were formed starting at day 3 post-encapsulation. The spheroids were characterized at days 3 and 7 with respect to their mean diameter and morphology. Although spheroids with highly variable diameters were observed, no significant differences in mean spheroid diameter were measured between the two hydrogel types. The mean spheroid diameter increased significantly ($p < 0.05$) between days 3 and 7, ranging from 76.73 μm to 91.20 μm for gelB-

MOD and from 79.26 μm to 96.09 μm for gelBGAL-MOD. This further confirms the ability of cells to proliferate upon encapsulation within the hydrogels under evaluation. Examination of the overall spheroid morphology revealed more pronounced spheroid delineation in the gelBGAL-MOD (Figure 7.16 D) than in the gelB-MOD (Figure 7.16 C).

As already mentioned, the interaction between sugar moieties (in this case galactose and ASGPR) is known to promote the formation of round cells and spheroids, thereby improving hepatocyte specific functions.^{438,440,441} In this study, the formation of spheroids with highly variable diameters was observed in both hydrogels, and no significant differences in mean spheroid diameter were observed. This is in contrast to the data described by Yang and co-workers,⁴³⁸ where a more pronounced spheroid formation could explain the improved hepatocyte function after encapsulation in a galactosylated alginate gel. However, the spheroid morphology in the two hydrogels utilized herein was different. A more pronounced delineation of the spheroids was observed in the galactosylated hydrogels, suggesting more established cell-cell contact. We concluded that the observed improvement in the hepatocyte functions must be related to the improved quality (better cell-cell contact) of the spheroids formed in the galactosylated hydrogel.

7.4 | CONCLUSION

In the present chapter, material properties as well as cell biology effects caused by the introduction of galactose-containing moieties was evaluated. First, the introduction of galactose pendent moieties on the backbone of photo-sensitive gelatins was investigated. Second, the effects of the galactosylation on (i) the physico-chemical properties of gelatins undergoing photopolymerization and (ii) the biological responses of encapsulated cells were examined.

A range of galactosylated gelatin methacrylamides was designed, having galactosylation degrees up to 25 % with respect to the free amines available on gelatin. The presence of galactose was shown to hamper the cross-linking efficiency related to the photopolymerization of the methacrylamide functions. As a result, conversion, gel yields en storage moduli decreased, while the equilibrium mass swelling ratios increased. Additionally, type A based gelatins exhibited lower hydrogel stiffness and decreased gel yields compared to type B gelatins.

For encapsulation in type A gelatin, cell survival levels were lower compared to type B gelatins. The photopolymerization triggered curing strategy did not significantly affect cytocompatibility, and it has been shown that highly viable ($> 90\%$) cell-laden constructs could be obtained for 10 w/v % gelB-MOD and gelBGAL-MOD hydrogels. The introduction of galactose moieties did not affect cell survival, however, albumin secretion was upregulated up to three-fold at day 7 for a galactosylation degree of 25 %. Additionally, galactosylated gelatin methacrylamides demonstrated significant improvement of hepatocyte specific function expression. Therefore, in combination with the tuneable hydrogel properties, the developed materials show promising potential for encapsulation of hepatocyte-like cells aiming for liver-like tissue engineering applications.

Building further on the obtained data, the possibility that the effects described above are only the consequence of transduction events taking place upon the ligand-receptor interaction must be taken into account. It has been reported that the interaction between glycopolymers and ASGPR can both regulate integrin-mediated signaling via the inhibition of FAK phosphorylation, and influence hepatocyte survival signaling. Moreover, the binding of the ASGPR to a galactosylated substrate may block receptor degradation via a post-translational mechanism.^{404,441} These reports indicate that different signal transduction events are triggered upon ASGPR binding. It is thus plausible that only the interaction between the immobilized ligand and its receptor is sufficient to improve hepatocyte functions via the modulation of intracellular pathways. The latter hypothesis could be a crucial subject for further investigation, since understanding the signal trans-

duction events taking place might, in general, contribute to the maintenance of *in vitro* hepatocyte functionality. Moreover, the in-depth evaluation of the galactosylated gelatin hydrogel with primary hepatocytes is an important topic for future research.

8 | SYNTHESIS OF 2-LACTOBIONAMIDOETHYL METHACRYLATE TO TAILOR THE GALACTOSE CONTENT OF GELATIN MATRICES

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The present chapter describes the bulk and surface galactosylation of gelatins via an intermediate photocross-linkable monomer. For this, plasma treatment was explored as a potential surface activation step.

8.1 | INTRODUCTION

Conventionally, bulk modification of gelatins occurs via either the free carboxylic or the free amine functionalities available on the backbone of the amino acid sequence.^{223,358,359,449} As a logical drawback, increasing the modification degree is limited to the amount of those free functional groups. Previously (chapter 7), we demonstrated the impact of bulk galactosylation on the functional behavior of HepG2 cells. By means of carbodiimide chemistry, galactose content could be tailored to a certain extend, limited not only by the amount of free amines but furthermore also by the necessary amount of photocurable methacrylamide groups that were also introduced via the free amines. Hong et al.³⁵⁷ increased the gelatin free amine content from 0.33 to 0.41 mmol g⁻¹ gelatin prior to galactosylation by reaction of gelatin free carboxylic groups with ethylene diamine. The final amount of galactose groups was not quantified. Our results indicated a relatively low reaction efficiency for the galactosylation of gelatins.

The question arises whether an inhibitory effect could occur with increasing galactose content, thus alternative strategies are desired in order to control the galactosylation over a broad range of concentrations. Narain et al.⁴⁵⁰ synthesized cyclic sugar methacrylate monomers for the atom transfer radical polymerization (ATRP) in methanol/water mixtures using PEO-based macroinitiators. The cyclic sugar ring was preserved, being an important advantage when aiming for molecular recognition applications. Via this pathway, bulk copolymerization of cyclic galactose methacrylate monomers with photocross-linkable hydrogel precursors should be possible and serve as an alternative bulk modification strategy.

Plasma techniques cover plasma-treatment, post-plasma irradiation grafting, plasma syn-irradiation grafting and plasma polymerization, and allow surface targeted modification.^{169,451} Applying Ar, O₂, N₂, or NH₄ plasmas results in the introduction of radicals, oxygen containing functional groups, or nitrogen containing functional groups on the surface.^{169,451} These functionalities can be applied for further modification with bioactive ligands. Our group grafted 2-aminoethyl methacrylate as intermediary step for the immobilization of proteins on PCL surfaces to increase their biocompatibility and cell interactions.¹⁴⁸ Ying et al.⁴⁵² reported the covalent coupling of galactose-containing molecules to post-plasma irradiation grafted acrylic acid on hydrophobic PET surfaces using carbodiimide chemistry. To our best knowledge, the post-plasma irradiation grafting of galactose-carrying molecules on hydrogel surfaces has not been reported so far. Altogether, limited studies are known about the use of plasma treatments on hydrogels for biocompatibility enhancements. Such studies focused mainly on the effect of the applied plasma treatment on hydrogel biocompatibility, instead of using plasma treatments

as an intermediary step for post-plasma grafting of bioactive ligands. For example, chitosan was treated with oxygen plasma to increase surface hydrophilicity by Wang et al.⁴⁵³ However, the effects of the plasma treatment were not permanent due to an aging process involving reorientation of polar functional groups. Nitrogen treated gelatin films increased mouse fibroblast cell adherence and proliferation.⁴⁵⁴ These examples can be regarded as non-cell specific surface modification approaches.

In this chapter we report on the evaluation of galactose bulk and surface functionalized gelatin methacrylamides. The synthesis of 2-lactobionamidoethyl methacrylate monomer and its subsequent copolymerization with or grafting on gelatin methacrylamide hydrogels is examined. For the grafting, a post-plasma UV-induced grafting process is introduced. The hypothesized grafting strategy applied is depicted in Figure 8.1. The objective was focused on gaining insight on the grafting conditions related to the resulting galactose densities, surface wettabilities, and topographical changes. Secondly, using primary hepatocytes, the effect of the grafting process is evaluated toward cell attachment morphology.

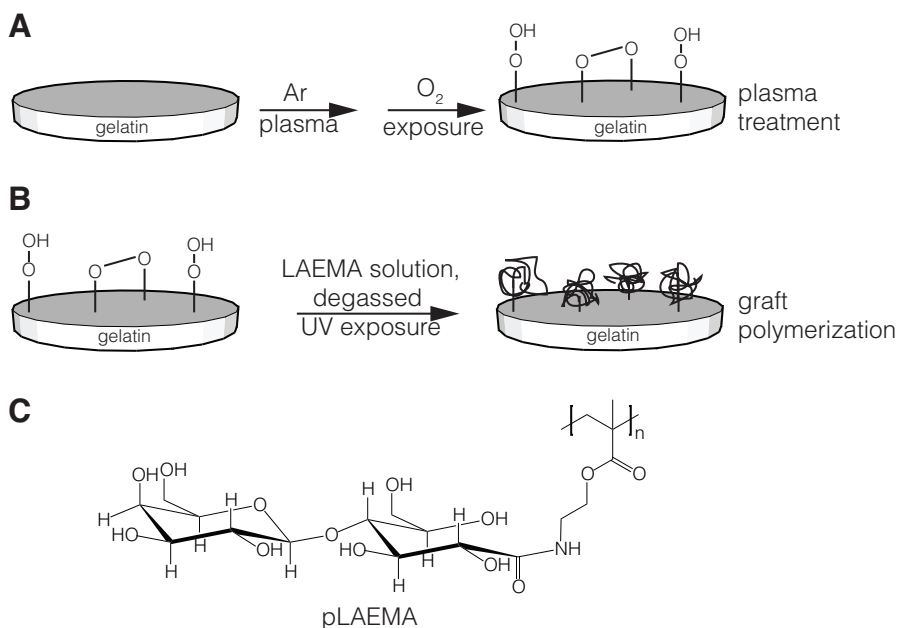


Figure 8.1: Post-plasma surface grafting of gelatin with LAEMA monomer. (A) Argon plasma treatment of gelatin films followed by exposure to air. (B) UV-induced graft polymerization of LAEMA monomer from gelatin surfaces. (C) Chemical structure of the grafted polymer chains (pLAEMA).

8.2 | EXPERIMENTAL PART

8.2.1 Synthesis of 2-lactobionamidoethyl methacrylate

2-lactobionamidoethyl methacrylate (LAEMA) was synthesized according to the method reported by Narain et al.⁴⁵⁰ with slight modification (Figure 8.2). Lactobionolactone was prepared by repeated vacuum distillations of 15 g lactobionic acid (LA, Sigma-Aldrich) in 150 mL of anhydrous methanol at 50 °C. Conversion was followed by thin layer chromatography (TLC) using 65:25:15 EtOH:H₂O:HAc ($R_{f\text{lactobionolactone}} = 0.57$, $R_{f\text{lactobionicacid}} = 0.8$). Freshly prepared lactobionolactone (10.0 g, 29.4 mmol) was immediately dissolved in 20.0 mL methanol at 50 °C and cooled to room temperature before the addition of 15.0 mL methanol solution of 2-aminoethyl methacrylate hydrochloride (10 g, 60.4 mmol, AEMA, Polysciences) and 10.0 mL of triethylamine (71.8 mmol). The mixture was kept stirring at room temperature overnight. The final product was precipitated in 2-propanol followed by dichloromethane washing and dried under vacuum. Final yield of LAEMA was 72%. Characterization of the LAEMA monomer by both ¹H and ¹³C NMR spectroscopy (Figure 8.3) confirmed its high purity (> 99%).

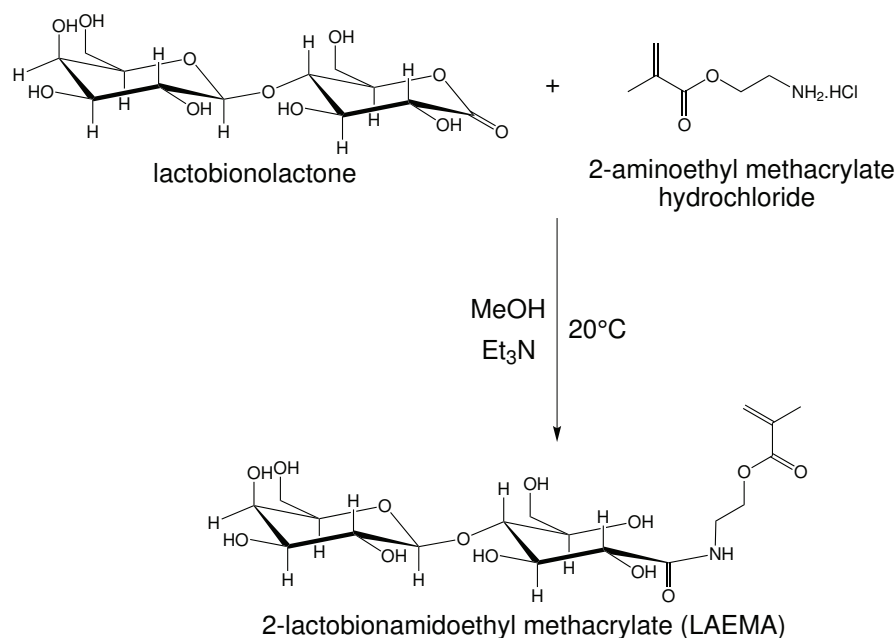


Figure 8.2: Synthesis of LAEMA monomer by the ring-opening reaction of lactobionolactone with 2-aminoethyl methacrylate at 20 °C.

8.2.2 Synthesis of gel-MOD and hydrogel film preparation

Bovine type B gelatin (Rousselot, Ghent) was chemically modified with methacrylamide side groups, as described in section 4.2.2. The degree of substitution, i.e. the amount of free amines converted to methacrylamide functionalities, was 63 % as determined by ^1H NMR spectroscopy.

gel-MOD-LAEMA - Gelatin methacrylamide solutions containing different concentrations of the LAEMA monomer were casted in films following the protocol from section 4.2.2.2. These samples will be referred to as gel-MOD-LAEMA.

gel-MOD - Substrates for LAEMA grafting were prepared by covalent coupling of the gel-MOD precursor mixtures to pre-activated glass cover slips, in order to prevent substrate floating. The glass cover slips were thoroughly rinsed with n-hexane and ethanol and dried. Subsequently, the clean cover slips were treated with a 2 v/v % solution of 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) in 95 % EtOH (pH 5 with acetic acid) for 3 min, rinsed with 100 % EtOH and baked at 110 °C for 10 min, leaving free methacrylate groups on the glass to react with gel-MOD during UV exposure. The gelatin hydrogel films were incubated overnight at 37 °C to remove the unreacted fraction. The films were dried under vacuum and stored at 5 °C prior to use.

8.2.3 Gel-MOD surface galactosylation

8.2.3.1 Argon plasma pre-treatment of gelatin films

Prior to the grafting process, (hydro)peroxides were introduced on the gelatin surfaces. Dry gelatin films were therefore subjected to an Argon plasma in a cylindric dielectrical discharge plasma reactor (Model Femto, version 3, Diener Electronic, Germany) for 60 s. More info can be found in section 11.1.3. The Argon pressure was kept at 0.8 mbar and the applied power was 100 W. In order to enable the reaction of free radicals on the surface with oxygen to generate (hydro)peroxides, the treated surfaces were exposed to the ambient atmosphere for 10 min prior to the UV grafting. The introduced active sites were subsequently used as initiating groups for the UV-induced graft polymerization.^{455,456} In this article, gel-Ar will be used to refer to these samples.

8.2.3.2 Grafting of LAEMA on gelatin surface

The pre-activated dry gelatin films were immersed in an aqueous LAEMA solution. The LAEMA solution was always freshly prepared and degassed at least 1 h prior to the grafting step. To investigate possible effects of the monomer concentration on the grafting efficiency, the LAEMA concentration was varied from 0.1 M to

0.2 M. The films were placed for different incubation times in a custom-made glass 6-well plate with a quartz cover. In this article, we will refer to these samples as gel-LAEMA x, where "x" indicates the grafting time (in minutes). Finally, the films were thoroughly rinsed with doubly distilled water, incubated overnight at 37 °C, and dried under vacuum.

8.2.4 Evaluation of the modification strategies

8.2.4.1 Equilibrium swelling experiments

gel-MOD-LAEMA samples with varying concentrations of copolymerized LAEMA were subjected to equilibrium swelling experiments as described in section 4.2.3.2.

8.2.4.2 Galactose oxidase assay

The Amplex Red Galactose/Galactose Oxidase Assay Kit (Invitrogen) was used for the quantitative determination of the copolymerized and grafted amounts of LAEMA monomer. The protocol has been described in section 11.4.3. For the quantification of the LAEMA amount copolymerized with gel-MOD (gel-MOD-LAEMA samples), the samples were first incubated overnight in a solution of collagenase (2 U mg⁻¹ gelatin, Sigma) in Tris-HCl buffer (0.1 M, pH 7.4). All measurements were performed in triplicate. Similar measurements were performed, in which the sample films were replaced by a series of solutions with known concentration of galactose (dilution series from 2 – 250 µM of LAEMA).

8.2.4.3 Infrared spectroscopy

Changes due to the copolymerization of LAEMA with gel-MOD were characterized using FT-IR spectroscopy in ATR mode, according to section 4.2.3.1.

8.2.4.4 Rheological characterization

Mechanical spectra of equilibrium swollen gel-MOD-LAEMA films and the *in situ* monitoring of the UV irradiation curing of gel-MOD-LAEMA mixtures were performed as described in section 4.2.3.5.

8.2.4.5 Static contact angle measurements

Static water contact angle measurements were performed to analyze the effect of the grafting procedure on the wettability of the substrate surface. Measurements of gelatin, gel-Ar, and gel-LAEMA surfaces were performed according to the protocol described in section 4.2.3.3. Each sample was measured at least in triplicate and each sample condition was analyzed in duplicate ($n \geq 6$).

8.2.4.6 X-ray photoelectron spectrometry

X-ray photoelectron spectrometry (XPS) was used to study alterations in the chemical composition of the surfaces caused by the argon plasma treatment and post-plasma grafting with LAEMA monomer. Gelatin, gel-Ar, and gel-LAEMA samples were analyzed. The setup is described in section 4.2.3.1. A survey scan spectrum was collected and from the peak-area ratios, the relative elemental composition of the material top layer was determined. The C 1s core signal was also collected. The generated data are displayed as a plot of the electron counts versus electron binding energy in a fixed, small energy interval.

8.2.4.7 Atomic force microscopy

Topographical changes induced by the argon plasma treatment and LAEMA grafting steps were studied by atomic force microscopy (AFM). AFM images were obtained in ambient conditions using the setup described in section 3.2.5. Surface scans of $15\text{ }\mu\text{m}^2$ were recorded in tapping mode with a silicon cantilever (OTESPA - Veeco).

8.2.5 Preliminary cell seeding experiments

Primary mouse hepatocytes were isolated and cultured as described in section 6.2.3.1. Preliminary cell seeding experiments were performed on gel-MOD, gelGAL-MOD, and gel-LAEMA samples (500 000 cells/sample), and brightfield images were recorded after 4 h post-seeding.

8.2.6 Statistical analysis

All measurements were performed with $n \geq 3$, unless otherwise stated, and the experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical analysis was performed with Student's t-test and significance was determined at $p < 0.05$.

8.3 | RESULTS AND DISCUSSION

8.3.1 Synthesis of LAEMA monomer

Both the bulk as well as the surface functionalization of the gel-MOD samples is based on the polymerization of a cyclic photocross-linkable galactose-containing monomer. As a disaccharide aldonic acid, lactobionic acid was selected as precursor to prepare a polymerizable glycomonomer. It comprises a galactose and a gluconic

acid linked via an enzymatically hydrolyzable glycosidic bond. The first step in the synthesis of LAEMA involved a conversion of lactobionic acid to lactobionolactone. This was performed by dissolving LA in dry methanol at 50 °C followed by vacuum distillation. Complete conversion was achieved by repeated distillation cycles and monitoring of the conversion by means of TLC. Next, dry lactobionolactone was reacted with AEMA to yield the LAEMA monomer. ^1H and ^{13}C NMR spectra of the achieved purified LAEMA monomer are presented in Figure 8.3.

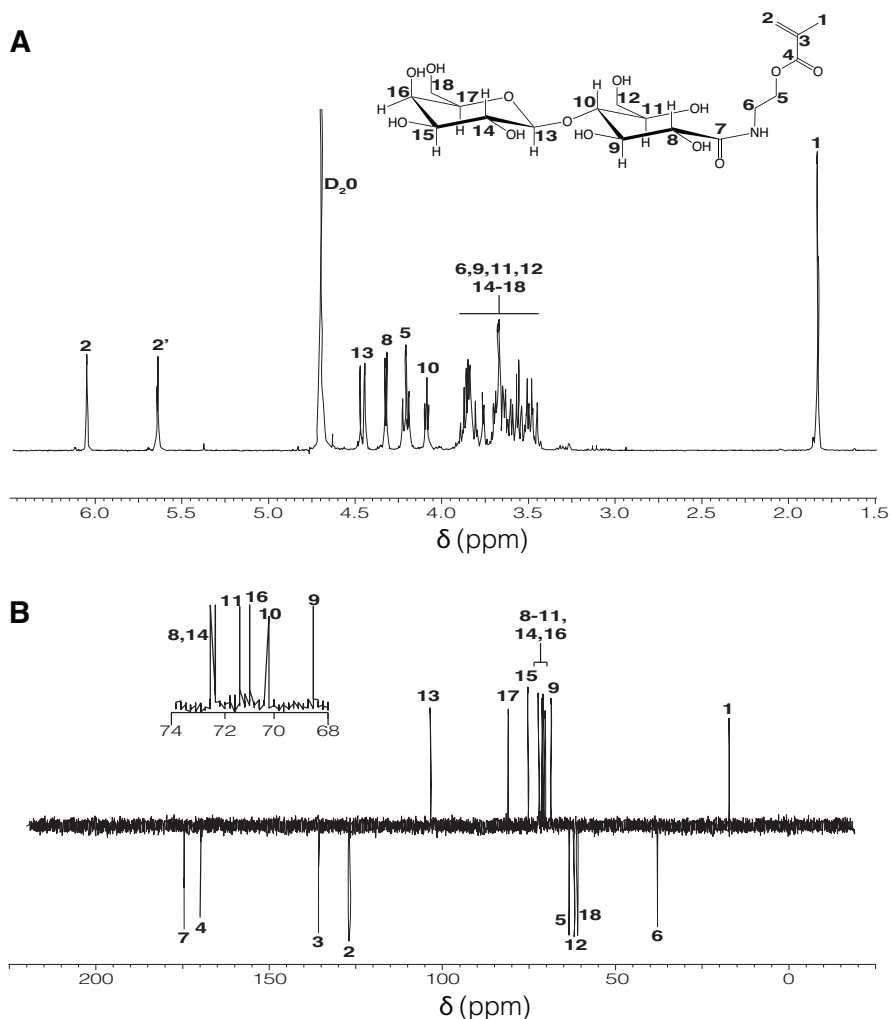


Figure 8.3: Assigned ^1H (A) and ^{13}C (B) NMR (D₂O) spectra for 2-lactobionamidoethyl methacrylate monomer.

Two-dimensional NMR spectroscopy measurements were performed in order to assign the peaks. The spectra confirm the successful synthesis of the LAEMA

monomer and can be assigned as in Figure 8.3. A reasonable yield ($\sim 72\%$) was reached (purity was $> 97\%$). Deng et al.⁴⁵⁷ synthesized a series of glycopolymers by RAFT homo- and copolymerization reactions of 2-aminoethyl methacrylamide. For the latter, a yield of $\sim 60\%$ was achieved. The slightly lower yield can be attributed to the slightly lower activity of the 2-aminoethyl methacrylamide primary amine compared to AEMA. Narain et al.⁴⁵⁰ achieved a yield of $\sim 78\%$ for the synthesis of LAEMA. Our yield is in good agreement with those reports.

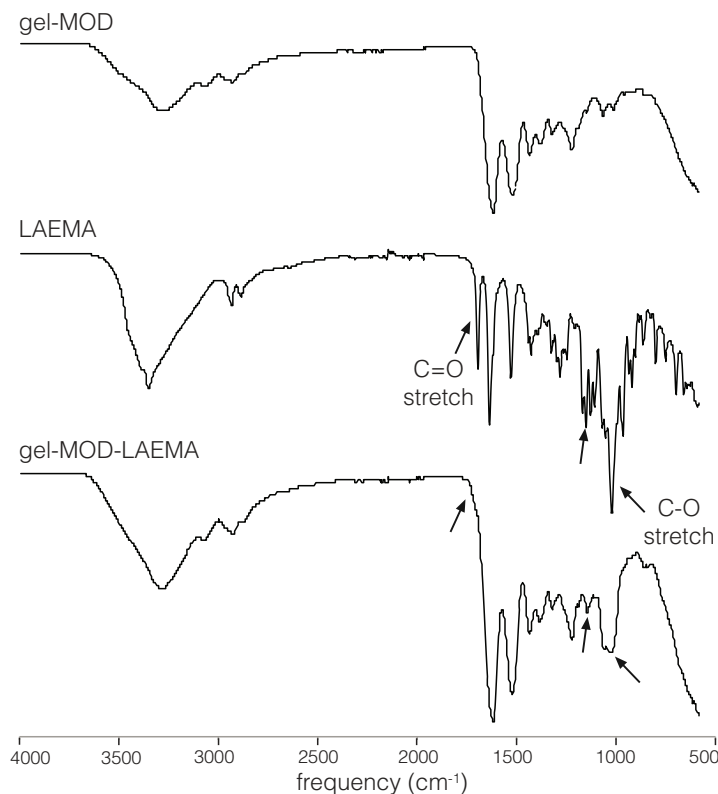


Figure 8.4: Changes in the infrared spectra of gel-MOD after copolymerization with LAEMA monomer to obtain gel-MOD-LAEMA hydrogels.

8.3.2 LAEMA can be copolymerized to increase galactose bulk content

In this chapter, two applications using LAEMA monomer for the galactosylation of gel-MOD are evaluated. In this part, the copolymerization of LAEMA monomer with gel-MOD macromonomer is evaluated. Prior to UV-induced free radical polymerization, varying amounts of LAEMA were mixed into gel-MOD

precursor solutions to result in gel-MOD-LAEMA hydrogels.

Typical infrared spectra of cross-linked gel-MOD hydrogel films, uncross-linked LAEMA monomer, and cross-linked gel-MOD-LAEMA hydrogels are displayed in Figure 8.4. The characteristic peaks of gelatin have been discussed previously. For LAEMA, a broad absorption in the region between 3400 and 2600 cm^{-1} (N-H, O-H stretch), the peak at 1705 cm^{-1} due to the carbonyl stretching ($\text{C}=\text{O}$), and the C-O stretch at 1035 cm^{-1} characterize the monomer. This is in close correlation to the infrared spectrum reported by Yoon et al.⁴⁵⁸ For gel-MOD-LAEMA, the characteristic gel-MOD peaks were more abundant compared to the peaks stemming from LAEMA copolymerization. However, it is clear that LAEMA was successfully incorporated in the hydrogel networks.

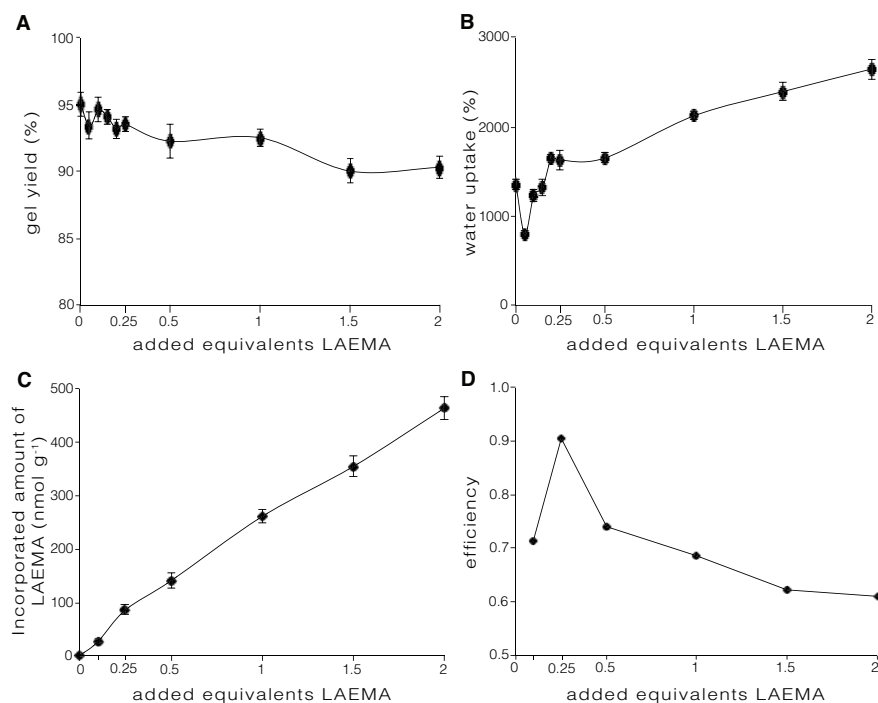


Figure 8.5: The copolymerization of 10 w/v % gel-MOD with LAEMA as a function of the added amount of LAEMA: equilibrium swelling studies (A,B), quantification of the copolymerized LAEMA (C), and efficiency of the LAEMA copolymerization (D). All samples received $14\,400\text{ mJ cm}^{-2}$ of UV-A irradiation.

The copolymerization of gel-MOD macromonomer with LAEMA monomer was initially studied by means of swelling experiments and galactose/galactose oxidase amplex red assay. Since sugar moieties (or even short chains) are introduced,

we anticipated some influence on gel yield and swelling behavior. As described in chapter 7, galactose can act as a free radical scavenger, thus inhibiting the photocross-linking to some extent while also increasing the swelling ratio. On the other hand, LAEMA will actively participate in the polymerization by its own methacrylate groups. Figure 8.5 summarizes the results for the equilibrium swelling studies (A,B) and the quantification of the incorporated LAEMA units (C,D) in function of the amount of LAEMA.

Varying the amount of LAEMA from 0, i.e. gel-MOD, to 0.5 equivalents (relative to the amount of free amines on gelB) did not significantly decrease the gel yield (Figure 8.5 A). Only for concentrations higher than 1 equivalent, a small decrease in gel yield could be observed ($p \leq 0.03$). On the other hand, the water uptake capacity did increase with increasing LAEMA concentration (Figure 8.5 B). This suggests that more LAEMA is effectively built-in into the hydrogel network, thereby increasing the swelling potential, as is confirmed by the galactose oxidase assay (Figure 8.5 C). The copolymerization efficiency was highest for 0.25 equivalents of added LAEMA (which is by and large similar to the degree of galactosylation of 25 % reported in chapter 7). For the other concentrations, the efficiency was situated $\sim 60 - 70\%$. Perhaps this is governed by the increased radical scavenging of the galactose moieties at higher concentrations, while for lower concentrations the increased cross-linkable moieties outweigh the scavenging effects, thereby reaching an optimal concentration.

Despite the more or less similar gel yields obtained, a clear increase in swelling potential could be observed. Therefore, the resulting equilibrium swollen gel-MOD-LAEMA hydrogels have decreased storage moduli G' with increasing LAEMA content (data not shown). More interesting however is the curing behavior *in situ*. Storage moduli G' recordings during UV-induced cross-linking are presented in Figure 8.6 in function of the LAEMA concentration.

In line with our anticipations, the increase in storage moduli of gel-MOD-LAEMA precursor solutions upon curing was influenced by the LAEMA monomer concentration. In general, it could be concluded that the curing is slowed down by the addition of LAEMA. For high concentrations, i.e. 1 and 2 equivalents, the LAEMA content also affected the starting value of G' prior to irradiation. On the other hand, for the 0.5 equivalents of added LAEMA, the after curing proved more pronounced. As a small remark, it should be noted that the intensity of the UV probe was higher compared to similar recordings in previous chapters. Therefore, the control condition (gel-MOD) has a steeper increase during UV irradiation when compared to previous figures. This however, does not affect the correctness of the observed trends.

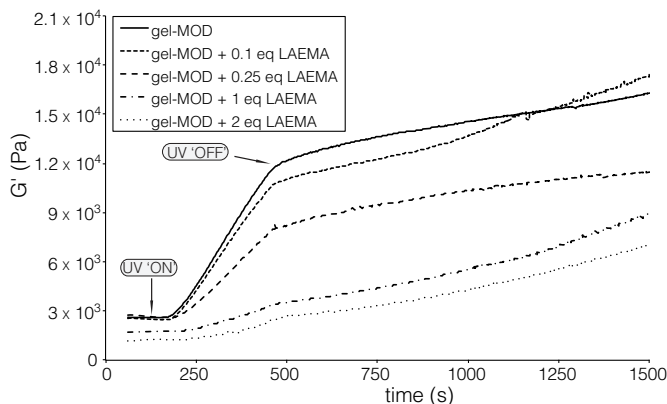


Figure 8.6: The copolymerization of 10 w/v % gel-MOD with LAEMA as a function of the added amount of LAEMA: *in situ* recordings of the increase in storage moduli G' upon UV-A irradiation.

Our findings demonstrate that tailoring of the bulk galactose content of photosensitive gelatins can be achieved by means of copolymerization with a polymerizable glycomer. It should be kept in mind that the polydispersity will be high and the influence of LAEMA incorporation on cell biology is still subject to future research.

8.3.3 Two-step LAEMA immobilization on gelB-MOD surfaces

As a second modification approach, LAEMA monomer was applied for the grafting on gel-MOD surfaces. In previous work, we reported on the grafting of AEMA monomer onto polyester substrates using plasma surface activation.¹⁴⁸ Post-plasma UV-induced irradiation grafting of monomers onto gelatin substrates could not be found in literature. Nonetheless, Yoon et al.⁴⁵⁸ successfully grafted LAEMA monomer on gold nanoparticles via ATRP. Lopez and coworkers⁴⁵⁹ functionalized PET surfaces with galactose moieties by NH_3 plasma treatment, conversion of the surface free amines to carboxyl functions and coupling with galactosamine using carbodiimide chemistry.

We selected a surface functionalization consisting out of two ensuing steps: (i) gel-MOD substrates were subjected to argon plasma, followed by oxygen exposure to introduce (hydro)peroxides, and; (ii) the introduced (hydro)peroxides consequently serve as initiating sites for the grafting of LAEMA monomer via exposure to UV irradiation. This is schematically presented in Figure 8.1.

In a first set of experiments, a suitable plasma treatment time was searched for. Initially, the static contact angle (SCA) advancement provided a first criterium.

The surface wettability has to be high enough to ensure good contact with the grafting medium later on. At the same time, the introduced (hydro)peroxides will increase the surface wettability (at the condition of similar roughnesses). Figure 8.7 A illustrates the decreasing SCA, i.e. increasing wettability, due to increasing plasma treatment times.

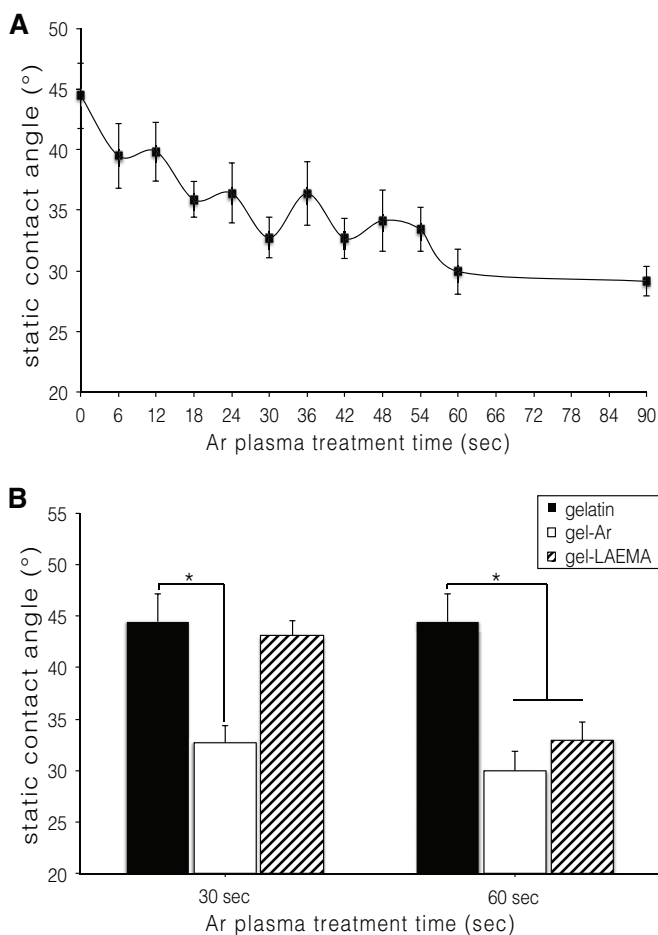


Figure 8.7: Influence of argon plasma treatment on gelatin surfaces (A) and recovery of the static contact angles after respective grafting with 0.2M LAEMA monomer ($*p < 0.05$).

The increasing wettability of gel-MOD surfaces after 30 s, 60 s, and 120 s was not statistically significant. Still, the wettability was improved when compared to non-treated samples. Figure 8.7 B summarizes the recovery of the SCA after the grafting step. LAEMA grafted samples will be referred to as gel-LAEMA. Two plasma treatment times were selected: 30 s and 60 s. A plasma treatment time

of 60 s resulted in a significantly decreased SCA after the grafting step, while for a treatment of 30 s, the SCA after grafting could not be distinguished from non-modified surfaces. As such, a treatment time of 60 s was selected for further investigation. As a small remark, it should be noted that at this point the reduced SCA could be attributed to either changes in the topographical roughness or due to a more efficient grafting step. However, this was not investigated in detail.

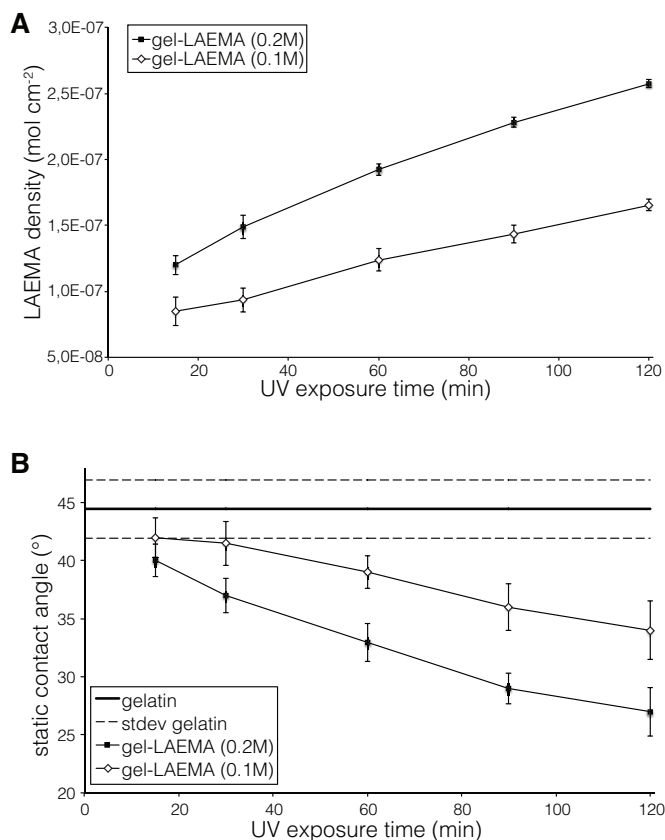


Figure 8.8: The effect of UV exposure time and LAEMA concentration on (A) the grafted concentration of galactose moieties on the gel-LAEMA surfaces as determined by the galactose oxidase assay, and (B) the wettability of the gel-LAEMA surfaces as determined by static contact angle measurements.

Next, the immobilization of LAEMA monomer onto gel-MOD surfaces as a function of the graft solution concentration and UV irradiation graft time was evaluated. The amount of grafted LAEMA units and the outcome on the resulting contact angles is given in Figure 8.8. Grafting times of 15 – 120 min and graft

solution concentrations of 0.1 and 0.2 M were evaluated. As expected, increasing both parameters results in increased amounts of grafted LAEMA monomer. As for the static contact angles, the wettability increased with increasing concentration and UV exposure times.

Due to the grafting, pLAEMA chains are ented on top of the gelatin surfaces, thereby altering the chemical composition of the surface top layer. Data collected by XPS survey scans (Figure 8.9 and Table 8.1) and C 1s detail scans (Figure 8.10 and Table 8.2) give insight in these compositional changes.

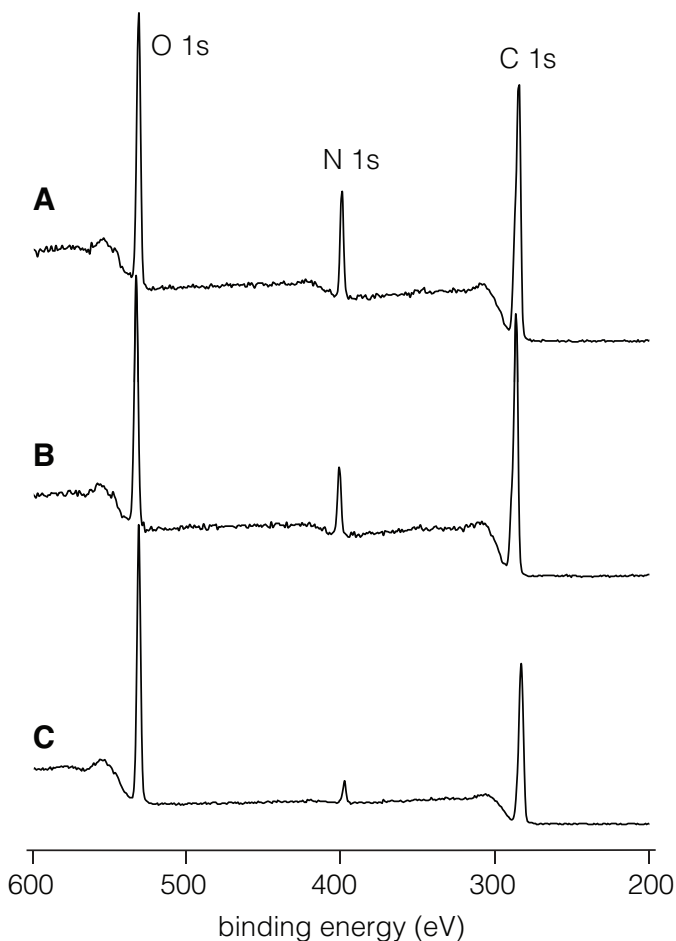


Figure 8.9: XPS survey spectra of (A) gel-MOD, (B) gel-Ar, and (C) gel-LAEMA120.

Due to the argon plasma treatment, the relative amount of oxygen is increased. It is believed that the radicals that are introduced by the argon plasma will readily react

Table 8.1: Influence of gelatin surface functionalization on the surface composition determined by XPS analysis.

SAMPLE NAME	SURFACE COMPOSITION [%]			ATOMIC RATIO	
	O 1s	C 1s	N 1s	N/C	O/C
gel-MOD	18.5±0.6	66.6±0.4	14.9±0.2	0.22	0.27
gel-Ar	22.8±1.2	69.1±1.2	8.6±2.3	0.12	0.33
gel-LAEMA30	30.7±0.5	64.7±0.1	4.7±0.6	0.07	0.48
gel-LAEMA60	31.2±0.7	65.8±1.1	3.2±0.5	0.05	0.47
gel-LAEMA120	33.4±1.4	63.4±1.8	3.2±0.5	0.05	0.53
pLAEMA*	40.6	56.3	3.1	0.05	0.72

* theoretical values

with oxygen from the air, thus forming oxygen-rich functionalities. Immobilization of LAEMA monomer alters the chemical composition of the surface, which will converge to the theoretical composition of pure pLAEMA (indicating a complete coverage with a layer thickness that equals or is larger than the XPS depth of analysis).

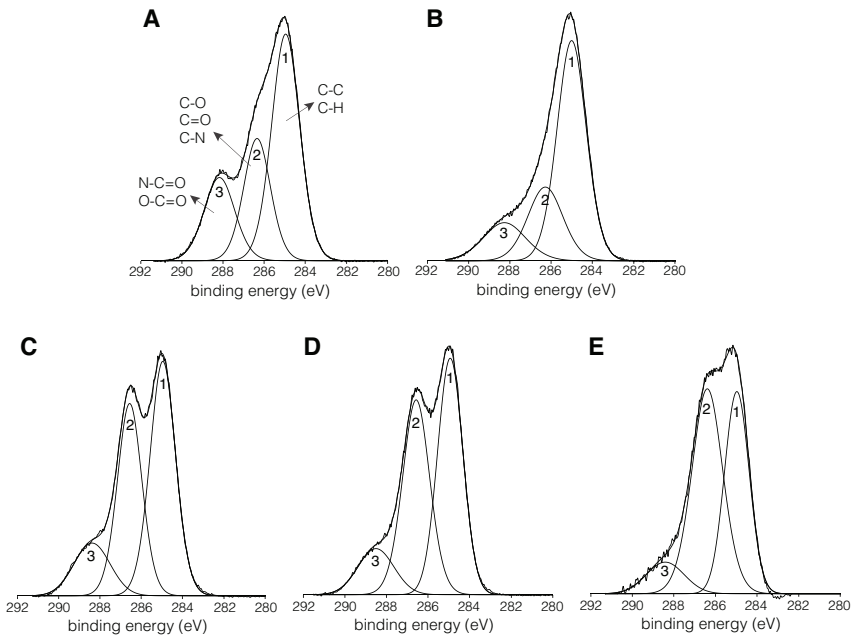


Figure 8.10: XPS C 1s signals of gelatin methacrylamide hydrogels: cross-linked gel-MOD (A), gel-Ar (B), and gel-LAEMA with grafting times of 30 min (C), 60 min (D), and 120 min (E).

Compositional changes as a result of the surface modification steps are demonstrated nicely in the decomposition of the C 1s envelopes (Figure 8.10). For simplicity, three peaks were assigned for the deconvolution, as has been described previously.

Table 8.2: Decomposition of the C 1s envelope in different components (in percentages).

SAMPLE NAME	RELATIVE INTENSITIES OF C 1s [%]		
	285 eV	286.7 eV	288.5 eV
gel-MOD	52.1±1.4	26.9±0.8	21.0±0.9
gel-Ar	60.4±1.6	24.3±1.0	15.3±1.2
gel-LAEMA30	47.6±0.8	37.5±1.3	14.9±2.1
gel-LAEMA60	46.1±1.1	41.2±1.0	12.7±1.6
gel-LAEMA120	44.5±0.3	47.4±1.7	8.1±1.9
pLAEMA*	43	50	7

* theoretical values

In correlation to the survey scan data, the C 1s peak attributions also converge to the theoretical value of pLAEMA. Since there still exist a discrepancy over a grafting time of 2 h, the results propose the idea of grafting clusters instead of a homogeneous coverage of the sample surface. This was further investigated by AFM mappings and roughness analysis (Figure 8.11).

The plasma treatment etched the surface of gel-MOD hydrogels, leading to a slight drop in surface roughness (not significant). Upon grafting, clusters of pLAEMA are formed on the surface. With increasing grafting time, these clusters grow bigger and surface roughness is increased. It can be seen that the dimensions of these clusters are polydispers and at a grafting time of 2 h, almost a complete coverage is reached. These data, together with the XPS measurements confirm the successful grafting of LAEMA monomer on gelatin hydrogel surfaces.

Previous work in our group demonstrated a sufficient argon plasma treatment time of 30 s for the grafting of AEMA on PCL surfaces.^{148,444} The work of Prasertsung on nitrogen and oxygen plasma treatments of gelatin surfaces advocated improved L929 mouse fibroblast adhesion and proliferation.^{454,460} However, the surface chemistry recovered over time. The present findings recommend longer argon plasma treatment times for gelatin hydrogels, in case grafting of monomers is targeted. For the immobilization of galactose moieties on several substrates, conventionally, plasma grafting of carboxyl or amine-containing monomers (e.g. acrylic acid) is applied in combination with a second step mostly involving carbodiimide coupling.^{404,430,448,459} In our setup, direct immobilization of (polydispers) clusters

containing galactose moieties is made possible. The UV irradiation time and graft solution concentration can be adjusted to obtain different grafting densities.

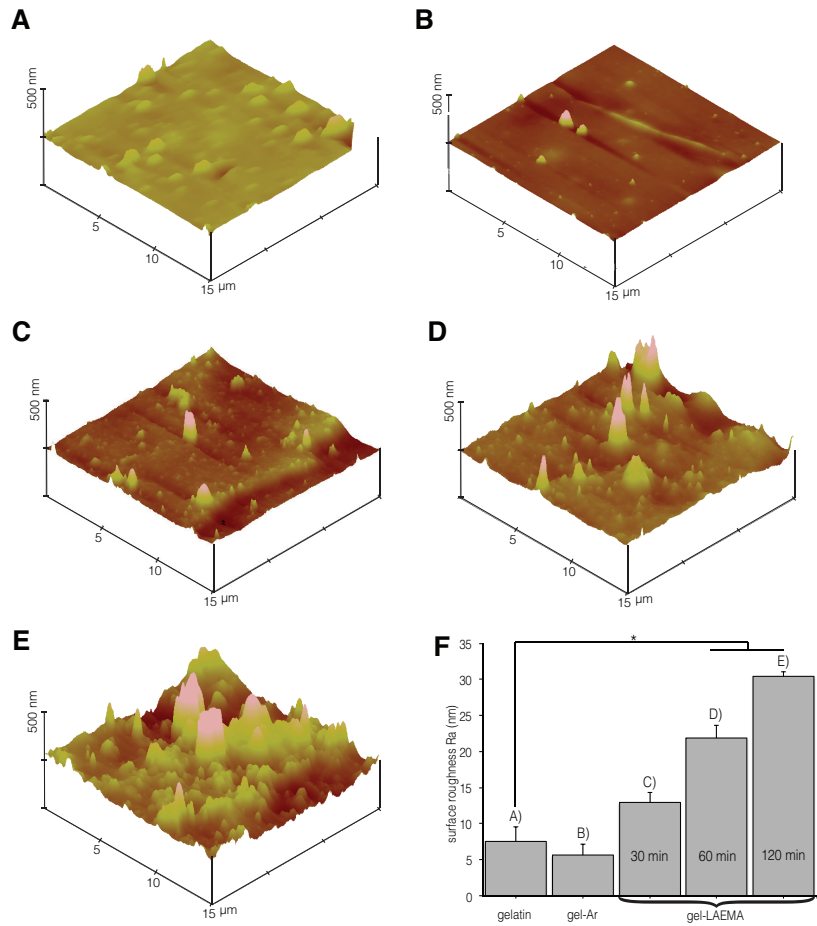


Figure 8.11: Evolution of the surface topography following the different modification steps as determined by AFM analysis: (A) cross-linked gel-MOD served as a reference, (B) gel-Ar after 60 sec plasma treatment, (C-D) gel-LAEMA samples with increasing UV-grafting times of 30 min (C), 60 min (D), and 120 min (E). (F) Represents the evolution of the surface roughness (Ra) as determined by AFM analysis on scan areas of $15 \mu\text{m}^2$ ($p < 0.05$).

8.3.4 Preliminary cell seeding

Primary mouse hepatocytes were seeded on gel-MOD, gelGAL-MOD (DG = 25 %), gel-LAEMA30 and gel-LAEMA60 to investigate hepatocyte morphology. Colla-

gen coated tissue culture plastic (TCP) was used as positive control condition. Brightfield images recorded 4 h post-seeding are given in Figure 8.12.

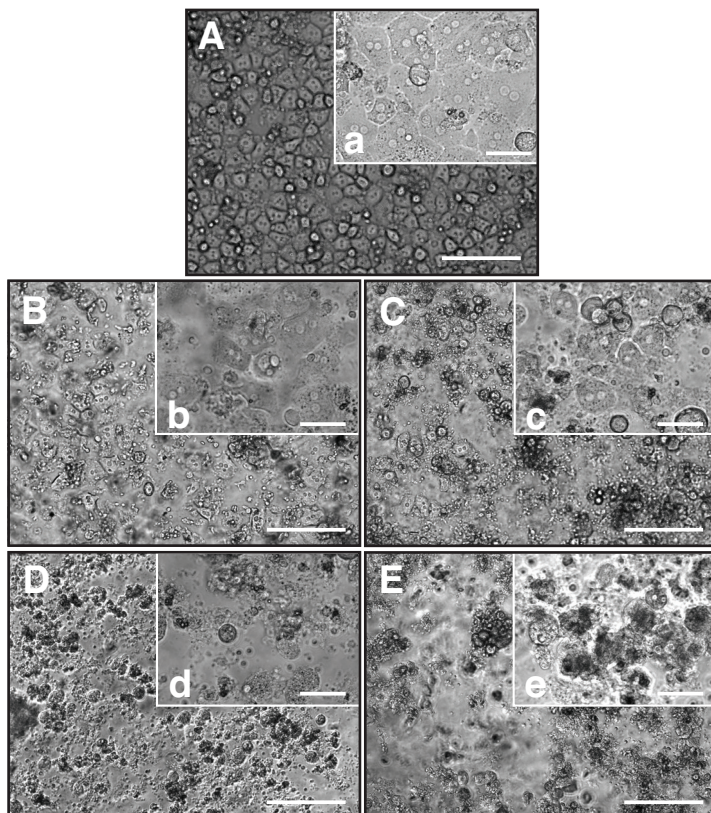


Figure 8.12: Brightfield images of primary mouse hepatocytes seeded on collagen (A,a), gel-MOD (B,b), gelGAL-MOD (C,c), gel-LAEMA30 (D,d) and gel-LAEMA60 (E,e). Scale bar represents 200 μm (A-E) and 50 μm (a-e).

It can be seen that hepatocytes attached to the surfaces, with highest cell attachment rates for gel-MOD and gelGAL-MOD and decreased attachment for the gel-LAEMA samples. Nevertheless, the latter samples promoted the formation of aggregates and attachment of aggregate-like clusters instead of the monolayer-type attachment observed with the gel-MOD and gelGAL-MOD samples. Increasing round morphology is demonstrated with the presence of galactose moieties compared to plain gel-MOD. As explained in the introduction of chapter 7, round morphologies are desired. Our findings are in correlation with reports from literature.^{448,452} Several authors have demonstrated that different cell attachment morphologies (HepG2 cells) can be achieved by plasma treatment with or without galactose immobilization (via carbodiimide chemistry) on PET and PNIPAAm

surfaces.^{448,459} Silva et al.⁴⁶¹ proved that plasma modification of chitosan surfaces improved the biocompatibility. Additional experiments have yet to be performed to investigate long-term cultivation influences on hepatic phenotypical expression, however, our first experiments suggest that surface grafted galactose moieties impair the attachment strength based on the observations during these preliminary screening.

8.4 | CONCLUSION

Liver-like cells can be targeted by means of the ASGPR receptor on the cell membrane. We demonstrated the potential of a cross-linkable glycomonomer, 2-lactobionamidoethyl methacrylate, for bulk as well as surface functionalization of gelatin matrices. Both strategies make use of UV energy. Manipulation of the UV irradiation dose and LAEMA concentration enables to control the amount of introduced galactose moieties.

The developed substrates can be regarded as an extension of the simpler bulk galactosylation strategy described in chapter 7. The modification strategies allow for easy preparation of galactosylated substrates whereby either the bulk or the surface can be functionalized starting from gel-MOD macromonomer and LAEMA monomer.

In depth biological characterization of the presented modification strategies still has to be performed. By this, it would be interesting to investigate if there is a certain barrier at which any more galactose units do not further improve the biological response, or if there even exists an adverse effect.

9 | CLEAN PRODUCTION OF GELATIN-BASED μ -PARTICLES USING ELECTROSPRAYING IN DRIPPING MODE: A PARAMETRIC STUDY TOWARDS MICROENCAPSULATION

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This chapter explores the production of hydrogel microparticles by means of electrospraying. This technique is theoretically not a rapid prototyping technique, however it is also not considered to be a conventional technique. Together with electrospinning, these techniques offer possibilities for the production of three-dimensional cell substrates. In this chapter we focussed on the potential as microencapsulation technique.

9.1 | INTRODUCTION

Microcarriers have been frequently used in a broad range of medical applications (e.g. for embolization therapy).⁴⁶² Most appealing are the applications as drug delivery vehicles^{463–466} or cell encapsulation matrices.^{467,468} Other applications could be aimed at cell expansion, delivery of bioactive agents via secondary encapsulation in hydrogel systems, and incorporation of microparticles to introduce porosity in TE matrices. However, the production of microcarriers with a homogeneous size distribution continues to be challenging. A variety of techniques have therefore been established as a fabrication route towards such carriers, including: droplet formation, desolvation, coacervation and emulsification methods.⁴⁶⁹ The most uncomplicated methods remain droplet formation techniques, established by simple gravitational forces, coaxial air/liquid flow, vibrating capillary, rotating systems and, more recently, electrostatic potential.

In electrospraying, a liquid precursor droplet is formed at the tip of a nozzle. When the nozzle is maintained at high electrical potential, a charge is induced on the surface of the droplet, forcing the liquid to be dispersed into fine droplets. Nearly monodisperse beads, with sizes ranging from hundreds of micrometers down to tens of nanometers can be fabricated, depending on the processing parameters.^{470,471} Furthermore, the biological activity of protein-based electrosprayed particles remains intact.⁴⁷² Several atomization modes can be established (see section 11.1.4), and are a function of the applied field strength and flow rate.⁴⁷³ The three most encountered modes are:

1. Dripping modes;
2. Cone-jet modes, and;
3. Multi-jet modes.

Dripping modes are characterized by a direct ejection of liquid fragments from the nozzle tip. The ejected fragments can take the shape of regular large drops, fine droplets, elongated spindles, or even irregular geometries. In the case of jet modes, the liquid ejects in the form of a continuous jet, which breaks into droplets only at some distance from the tip of the nozzle. Key parameters that determine the outcome of an electrohydrodynamic atomization (EHDA) process are: the applied voltage, the working distance, the feed rate, the nozzle diameter, and the physical/chemical properties of the precursors. Moreover, stable electrospraying processes are difficult to establish for high surface tension solutions (e.g. water solutions) because high nozzle-ground voltage differences are required for EHDA, while the corresponding electric field strength exceeds the

electric breakdown threshold of the surrounding gaseous medium (e.g. air) and triggers corona discharge.⁴⁷⁴ Poncelet et al.⁴⁷⁵ studied the effect of needle gauge, viscosity, and flow rate on alginate microbead size and droplet formation. Zhou et al.⁴⁷⁶ applied electrospraying in order to scale up hepatocyte encapsulation in collagen/terpolymer microbeads. Recently, Xie and Wang⁴⁷⁷ studied the effect of an ancillary electric field in order to achieve mono-dispersed microencapsulation of living cells in a 2 % sodium alginate aqueous solution. Wang et al.⁴⁷⁸ used a high-voltage electrostatic droplet generator to examine drug encapsulation properties in alginate/poly-L-arginine/chitosan ternary complex microcapsules.

Alginates are linear polysaccharides consisting out of 1-4 linked α -D-guluronic acid (G) blocks, β -D-mannuronic acid (M) blocks, and alternating MG blocks. Alginate hydrogels have been frequently applied in cell encapsulation strategies and in the preparation of drug delivery vehicles.^{83,84,102,194,476,477,479} Alginate gelation can be triggered with multivalent cations (Ca^{2+} , Ba^{2+} , Cu^{2+}), which induce the formation of a cross-linked gel network through ionic interactions with the carboxylic groups of the G blocks.⁴⁸⁰⁻⁴⁸² On the other hand, gelatin is regarded as a biocompatible, bioactive, biodegradable and low cost biomaterial.^{469,483} A combination of sodium alginate, for improved stability (however no cell interaction),^{484,485} and gelatin type B, for enhanced cell interactions, was selected as starting blend for this work.

To the best of our knowledge, up to this date, electrospraying of all-aqueous gelatin-sodium alginate blend solutions is not yet reported. The study in this chapter had as main objective to examine whether it is possible to obtain a stable electrospray process specifically for all-aqueous gelatin-sodium alginate blends. The influence of key electrospray parameters and material properties, on bead size, size distribution and bead morphology was investigated. Hydrogel beads obtained in the present study may find applications in cell microencapsulation, cell culture systems, and for controlled drug release. Finally, microencapsulation of HepG2 cells during electrospraying was evaluated.

9.2 | MATERIALS AND METHODS

9.2.1 Materials

Bovine type B gelatin (iso-electric point = 5, Bloom strength = 257), was supplied by Rousselot (Ghent, Belgium). Alginic acid sodium salt from brown algae (high M content, Aldrich) and calcium chloride dihydrate (ACS reagent, $\geq 99\%$, Sigma-Aldrich) were used without further purification. Dulbecco's Modified Eagle Medium (DMEM) was purchased from GIBCO Invitrogen co.

For a number of experiments, gelB was thiolated using Traut's reagent (Figure 9.1). An analogous reaction protocol as for obtaining gelatin (meth)acrylamides was applied. Briefly, 10 g gelB was dissolved in phosphate buffer (pH 7.8) at 40°C to obtain a 10 w/v % solution. The solution was thoroughly degassed. Subsequently, 523 mg (3.8 mmol, i.e. 1 equivalent with respect to the amount of gelatin free amines) of 2-iminothiolane hydrochloride (Traut's reagent, Sigma-Aldrich) was added. After one hour of reaction under constant degassing and stirring, the solution was dialyzed and freeze-dried as previously described (section 4.2.2). The degree of substitution was determined according to the OPA assay to be $\pm 70\%$.

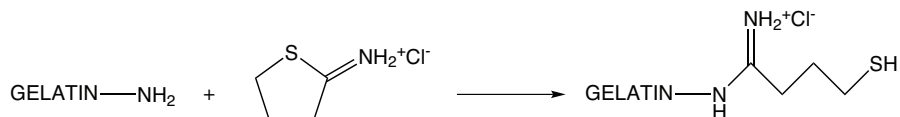


Figure 9.1: Traut's reagent reacts with gelatin free amines to obtain a thiolated gelatin derivate.

Rheological characteristics of the gelatin-sodium alginate solutions were measured at 35°C using a Physica MCR 350 (Anton Paar) with plate-plate geometry. The effective viscosity was determined over the shear rate range = $1 - 1000 \text{ s}^{-1}$. Five different measurements were performed on each solution. Mean averages ($\pm \text{SD}$) of shear viscosity at 100 s^{-1} were chosen for comparison between the different solutions.

9.2.2 Electrospraying: description of the setup

Gelatin-sodium alginate solutions with varying total concentrations ($2 \rightarrow 10 \text{ w/v \%}$) were prepared using distilled water at 40°C . A 0.1 M CaCl_2 -cross-linking medium in distilled water was prepared at room temperature. For electrospraying, the gelatin-sodium alginate solutions were loaded in a 10 mL syringe connected to a stainless steel needle using a Teflon tube (inner diameter of around 1.0 mm). The cross-linking medium was poured in glass petri dishes to obtain a CaCl_2 bath. A homemade ESP device was employed in a way that allowed the simple production

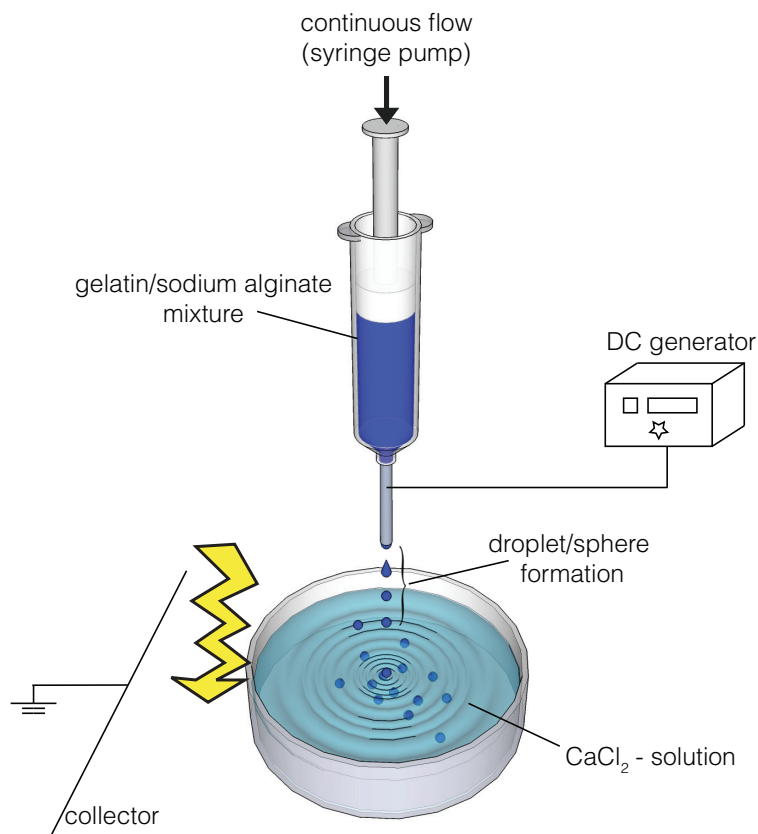


Figure 9.2: Schematic illustration of the experimental setup. A syringe continuously dispenses material at a given feed rate. The applied potential difference between the needle and the collector plate causes the material to be broken into a discontinuous droplet feed. The height difference allows droplet reshaping. Formed droplets were directly crosslinked in a CaCl₂ bath.

of microcarriers. Figure 9.2 represents a schematic illustration of the experimental setup. More information can be found in section 11.1.4. All experiments were conducted in triplicate at a temperature of 35 °C. Steady state flow of the different polymer solutions was obtained using a syringe pump (Model Multy Phaser NE 1600, New Era Pump Systems Inc), which controlled the flow rate of the polymer solution. The stainless steel needle protrudes from a metallic electrode, connected to an adjustable DC power supply (Inc-Model Series EL 50P00, Glassman High Voltage) capable of generating DC voltages in the range of 0 – 50 kV. A grounded counter/collector electrode (aluminum plate, 27 × 70 cm) was located at 10 cm apart from the metallic tip. Drop (re)formation was allowed over a distance of 9 cm prior to immersion and collection of the formed beads in the CaCl₂

bath. The whole device is located inside a wooden cupboard provided with a safety mechanism of total power-cut in case the doors open during the process.

9.2.3 Parametric study for the spraying of gelatin-sodium alginate beads

A parametric study was performed in order to study influences of typical electrospray parameters. These included: gelatin-sodium alginate ratio (9:1; 3:2; 6:1; 1:1), gelatin-sodium alginate total concentration (2 – 5 – 7 – 10 w/v %), needle gauge (G23, G25, G27, G30), applied voltage (4 → 20 kV), and flow rate (1 → 5 mL hour⁻¹). After bead formation, the beads were isolated using centrifugation. Half of the beads were used as such in order to perform analysis in wet conditions, the other half were dried and stored at room temperature until further analysis.

9.2.4 Characterization of the fabricated beads

9.2.4.1 Bead size and size distribution

Every condition from the parametric study setup was examined using light microscopy, to extract information regarding bead dimensions, size distributions, and bead shapes in the wet state. A Zeiss (KS 100, AxioTech) light microscope in combination with an Axiovision digital camera was used. Images were taken and measurements performed using Carl Zeiss AxioCam software. All images were taken at 5 times magnification. Beads of every condition were randomly selected. Matlab (2009b) was used to analyze the obtained results.

9.2.4.2 Bead morphology

Besides light microscopy images of the beads in wet condition, scanning electron microscopy (SEM) images were taken on dry bead samples using a tabletop SEM (PHENOMTM, FEI Company). Beads were analyzed for morphology and shrinkage after drying. Prior to SEM analysis, all samples were coated with a (approximately) 20 nm thin gold layer.

9.2.4.3 Bead composition and stability

Gelatin leaching behavior was studied using FT-IR in attenuated total reflection (ATR) mode (Spotlight 400 Imaging System). The infrared spectra of the samples were measured as described in section 4.2.3.1. Spectra of newly fabricated bead samples (blancs) were compared with the spectra of the same samples taken after immersion in DMEM culture medium for a period of 24 hours at 37 °C (300 rpm).

The obtained spectra were evaluated using Spectrum 100 software. ATR mapping was performed over an area of $40\text{ }\mu\text{m}^2$. The collected data were analyzed for correlation with the respective FT-IR spectra of pure gelatin type B and pure sodium alginate. The results are displayed as a surface mapping of the correlation coefficient.

Survey and C 1s detail XPS scans were collected as described previously in sections 4.2.3.1 and 7.2.1.5. The spectra recorded from sodium alginate and gelatin type B were chosen as references. Relative changes of the high-resolution carbon spectra were studied between both pure components and generated μ -particles. The C-C, C-H peak was calibrated at 285 eV and the high-resolution spectra were fitted with 3 peaks in order to achieve a good fitting. The generated data are displayed as a plot of the electron counts versus electron binding energy in a fixed, small energy interval.

9.2.5 Preliminary cytocompatibility tests of electrosprayed HepG2 cells

Some key electrospraying parameters were evaluated for their influence over HepG2 cell survival rates. Investigated parameters were the applied voltage, the feed mixture composition, the nozzle internal diameter, and the cell density. Gelatin-sodium alginate solutions were pasteurized three times at $70\text{ }^{\circ}\text{C}$, while the CaCl_2 -cross-linking solution was filtered ($0.22\text{ }\mu\text{m}$ Millex GS filter unit). To improve cell viability, hydrogel and CaCl_2 solutions were prepared using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) supplemented with 0.1% D-glucose (Merck). Hydrogel precursor solutions were subsequently loaded with HepG2 cells to obtain final concentrations of $1.5 \rightarrow 5 \times 10^6\text{ cells mL}^{-1}$.

9.2.6 Statistical analysis

All measurements were performed with $n \geq 50$, unless otherwise stated, and the experimental results are reported as mean $\pm$ standard deviation (S.D.). Statistical analysis was performed using a Student t -test and significance was determined at $p < 0.05$.

9.3 | RESULTS AND DISCUSSION

9.3.1 Viscosity measurements

Alginate beads, fabricated and cross-linked in aqueous media with divalent cations, are an established route for cell, drug and other biomolecules encapsulation pur-

poses. The simplest formation method for Ca^{2+} -alginate beads is based on drop-wise addition, using a syringe, of an aqueous alginate solution into a CaCl_2 bath.⁴⁸⁶ In this setup, the needle gauge, and the solution viscosity will control bead dimensions. Six gelatin-sodium alginate solutions were examined in the parametric study. Table 9.1 summarizes the used blend compositions and sample names that will be referred to in the following discussion.

Table 9.1: Compositions of the gelatin-sodium alginate solutions for electrospraying.

SAMPLE NAME	COMPOSITION w/v %			NEEDLE GAUGE
	Hydrogel Precursor		NaCl	
	Gelatin Type B	Sodium alginate		
G9A1	9	1	-	G27 (a), G30 (b)
G6A4	6	4	-	G30
G4.2A2.8a	4.2	2.8	-	G30
G4.2A2.8b	4.2	2.8	1	G30
G4.2A2.8c	4.2	2.8	2	G30
G6A1	6	1	-	G25 (a), G27 (b), G30 (c)
G3A2	3	2	-	G23 (a), G25 (b), G37 (c), G30 (d)
G1A1	1	1	-	G25 (a), G27 (b), G30 (c)

For viscid fluids, Weber extended the theory of Rayleigh and demonstrated that droplet breakup showed a direct proportionate with the viscosity, and an inverse proportionate with the density and surface tension.⁴⁸⁷ Thus, solution viscosity is regarded as a key electrospraying parameter. Viscosity sweeps for shear rates $1\text{ s}^{-1} < \dot{\gamma} < 1000\text{ s}^{-1}$ were performed. Higher viscosity solutions will lead to larger bead diameters produced.⁴⁸⁸ Temperature was kept constant at 35°C , since this is representative for the cabin temperature at which all solutions were electrosprayed. The results are presented in Figure 9.3 (shear viscosity) and Figure 9.4 (shear stress). At a shear rate of 100 s^{-1} , average shear viscosity was $0.021 (\pm 0.004)$ Pa.s, $0.073 (\pm 0.002)$ Pa.s, $0.085 (\pm 0.003)$ Pa.s, $0.85 (\pm 0.007)$ Pa.s, $0.33 (\pm 0.003)$ Pa.s, and $0.12 (\pm 0.001)$ Pa.s for the respective solutions G1A1, G6A1, G9A1, G6A4, G4.2A2.8a, and G3A2. As can be derived from Figure 9.3, difference intervals between the solutions remain stable over the shear rate range measured.

Shear viscosity gradually increased with increasing total concentration for solutions of the same gelatin-sodium alginate ratio (G3A2, G4.2A2.8, G6A4, 3:2 ratio). Comparing the values of G6A1 and G6A4 (increasing alginate content), and on the other hand G1A1, G6A1, and G9A1 (increasing gelatin content), demonstrated clearly the effect of viscosity increase attributed to increasing alginate contents. From literature, it is known that sodium alginate solutions with a concentration larger than $5\text{ w/v \%}$ are difficult to prepare, and drop formation will be reduced

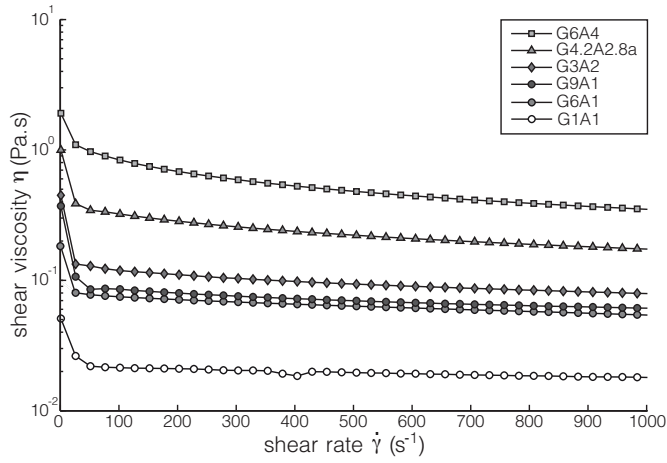


Figure 9.3: Shear viscosity at 35 °C as a function of gelatin and sodium alginate content.

with increasing viscosity of the liquids.⁴⁸⁷ These results were unmistakably translated into the manufacturability for electrospraying. Since G6A4 was too viscous, even at elevated temperatures of 35 °C, to result in steady state flow imposed by the syringe pump, this precursor solution will further not be taken into account.

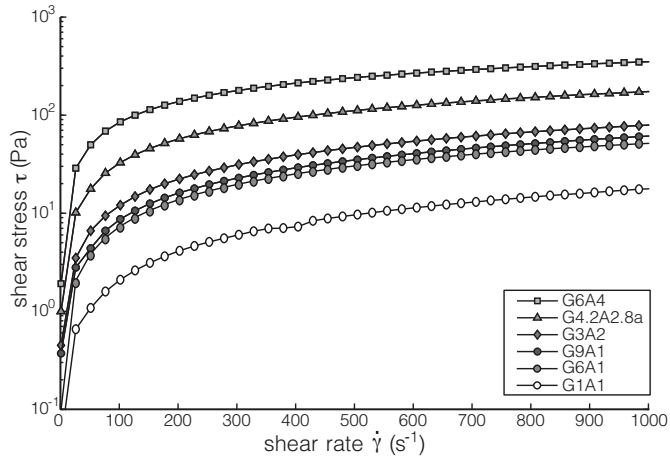


Figure 9.4: Influence of gelatin-sodium alginate ratio and concentration on the shear stress at 35 °C.

9.3.2 Droplet formation: The effect of electrospinning parameters on the size of the beads

Electrospraying, as well as electrospinning, is considered to be a cost-effective and versatile technique for the production of biomaterial matrices for numerous applications. When the needle is charged at a high DC voltage of positive polarity, the continuous flow of liquid gelatin-sodium alginate is broken into separate drops and pulled towards the ground (negative polarity), as illustrated in Figure 9.5. The polarity can also be exchanged, i.e. the solution at a negative polarity. Generally, the imposed surface charge density on pendant droplets is limited (termed Rayleigh limit), and beyond this limit, EHD forces overcome the surface tension forces of the droplets.⁴⁷¹

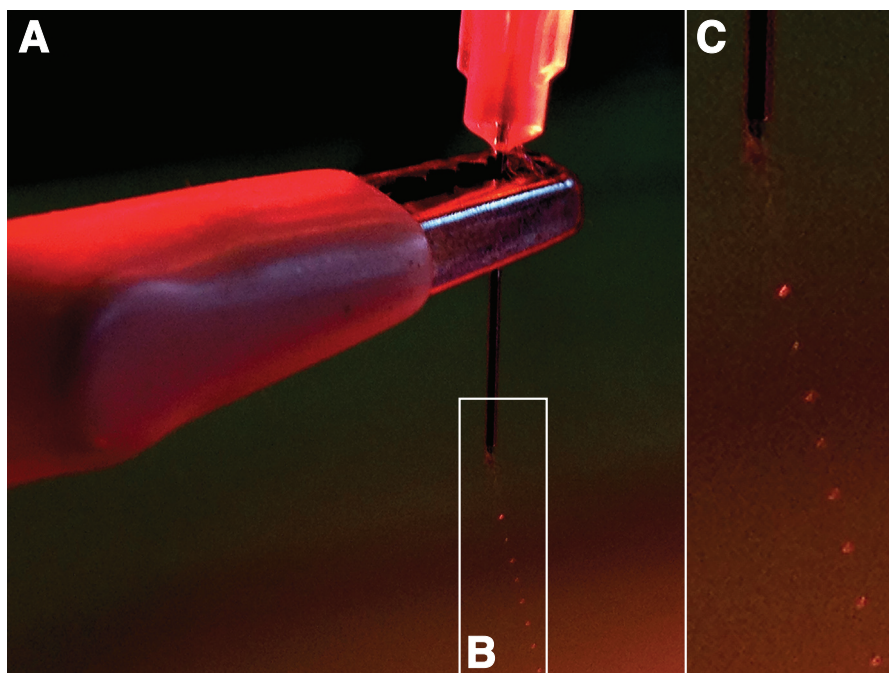


Figure 9.5: Electro spraying at work: (A) the voltage clamp connected to a G30 needle spraying a 7 w/v % gelatin-sodium alginate (6:1 ratio), (B) droplet formation, and (C) a close-up of the droplet formation.

In the first part of the present chapter, the influence of some key droplet formation parameters on the outcome of the spraying process was investigated. Gelatin will form a physical hydrogel at room temperature, complicating the electro spraying (and electrospinning) processes. In addition, gelatin can be regarded as a kind of polyelectrolyte, which possesses many ionizable groups. Acids can ionize the amine

and carboxylic functional groups. Therefore, the use of other than pure water-based solvents for the electrospinning of gelatin is often applied.^{483,489,490} There are few high polarity organic solvents available for the strong polarity biopolymer that gelatin is. Various toxic solvents, such as 2,2,2-trifluoroethanol (TFE), trifluoroacetic acid (TFA), 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and aqueous solutions of acetic acid, or ethyl acetate, are some examples. However, electrospayed gelatin remains water soluble, and therefore not suitable for longer-term applications at physiological conditions. In order to overcome this drawback, sodium alginate was blend in, which could be cross-linked by the simple addition of Ca^{2+} cations and allowed for a fast ionic cross-linking. Additionally, gelatin could be thiolated, which enables slow disulfide bridge formation, which can be broken under mild conditions such as cystein addition. This strategy was selected for the generation of insoluble (but reversible) hydrogel networks. Furthermore, the exclusion of toxic solvents, which would be difficult to remove after the process, was desired. Thus, in order to be successful, working at elevated temperatures was necessary to avoid the addition of fluorinated solvents or acetic acid, commonly used for the spinning of gelatin solutions. The device cabin was adapted so that a temperature of 35 °C was guaranteed, which allowed the processing of all-aqueous solutions.

9.3.2.1 Effect of the voltage and needle internal diameter on the bead dimensions

In Figure 9.6, the influence of the applied voltage and nozzle internal diameter as key processing parameters on the obtained bead dimensions is illustrated.

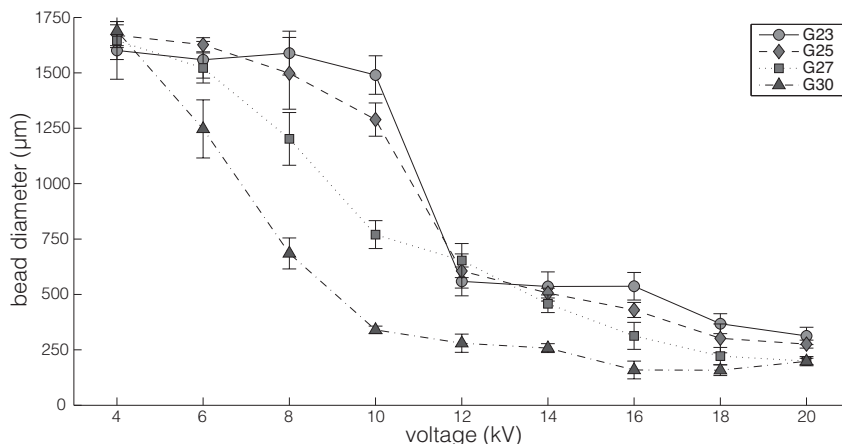


Figure 9.6: Bead sizes in function of the applied voltage and needle gauge for G3A2 condition. All samples were electrospayed at a constant flow rate of 1 mL hour⁻¹.

Comparable to the effect of voltage variation, needle gauge fulfills a pivotal role. A 3:2 gelatin to sodium alginate solution was selected for in depth examination of needle gauge and applied voltage effects: samples G3A2a-d. The solutions were sprayed at a feed rate of 1 mL hour^{-1} for all tested conditions. For the same solution, we observed three different behaviors for the needles investigated (Figure 9.6). For gauge 23 ($\emptyset = 0.33\text{ mm}$), an initial plateau value was observed till a voltage of 10 kV , from which point bead size rapidly decreased till a second plateau ($> 12\text{ kV}$). Gauge 25 and 27 ($\emptyset = 0.25\text{ mm}$ and 0.2 mm) on the other hand showed statistically significant effects over the whole voltage range investigated, with decreasing bead dimensions when increasing the applied voltage. However, decreasing the needle internal diameter even further (gauge 30, $\emptyset = 0.15\text{ mm}$) lessened the effect and showed only a significant influence for voltages $< 10\text{ kV}$. From this point on, bead sizes reached a plateau value, which converged to the smallest value for G27 spraying. Interestingly, standard deviations increased with increasing needle internal diameter for voltages higher than 8 kV , and the mean diameters were nearly the same size or larger than the needle internal diameter, correlating to the dripping mode. In other words, selection of the needle internal diameter and the applied voltage will impact the electrospray stability and bead size ranges that can be generated.

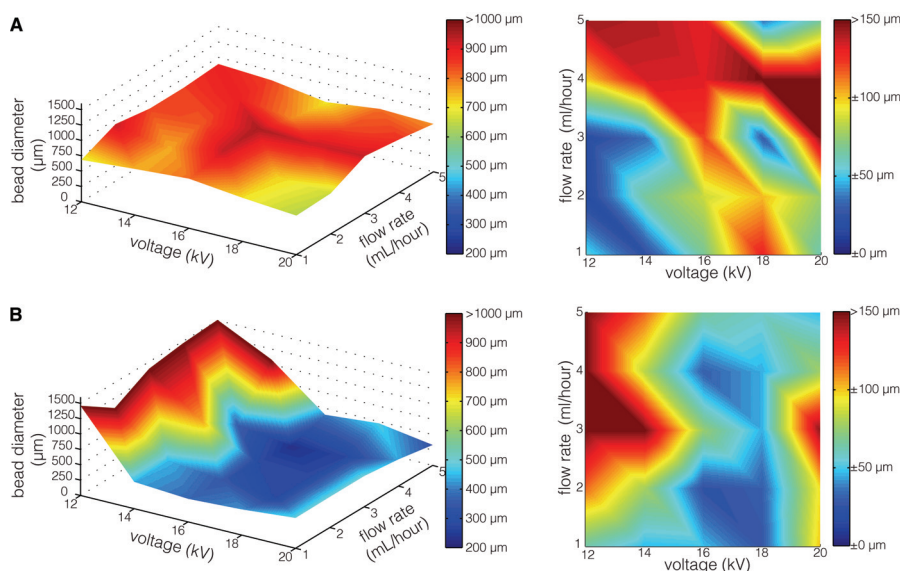


Figure 9.7: Bead size (left column) mapping for G9A1 samples as a function of the applied voltage and flow rate, and corresponding standard deviations (right column), sprayed using G27 (A) and G30 (B) needles.

9.3.2.2 The interplay of solution composition, applied voltage, and needle diameter

Comparing the bead sizes, and correlated standard deviations, obtained for sample G9A1 applying G27 needle (Figure 9.7 (A)) and G30 needle (Figure 9.7 (B)), gives an impression of the needle gauge, applied voltage, and flow rate effects on the process results. For this particular composition, needle gauge had a significant impact on the resulting bead sizes and stability of the process. The stability can be evaluated looking at the bead size standard deviations, which are presented on the right. Gauge 23 needle ($\emptyset = 0.33$ mm, G23) generated almost similar bead sizes produced over the voltage and flow rate ranges investigated. An example is shown in Figure 9.8 (A). In contrary, using gauge 30 needle ($\emptyset = 0.15$ mm, G30) enabled us to significantly manipulate the final sizes through variation of the remaining electrospraying parameters. Lowering the needle diameter generally resulted in the production of smaller particles, having smaller standard deviations. Only for the lowest applied voltage (i.e. 12 kV), bead sizes were larger compared to identical conditions using G27 needle. As can be clearly deduced from Figure 9.7 (B), optimal voltage conditions were located between 16 – 18 kV, with acceptable standard deviations for the flow rate range investigated. Nearly monodispers, homogeneous microcarriers were generated, as can be seen in Figure 9.8 (B, C).

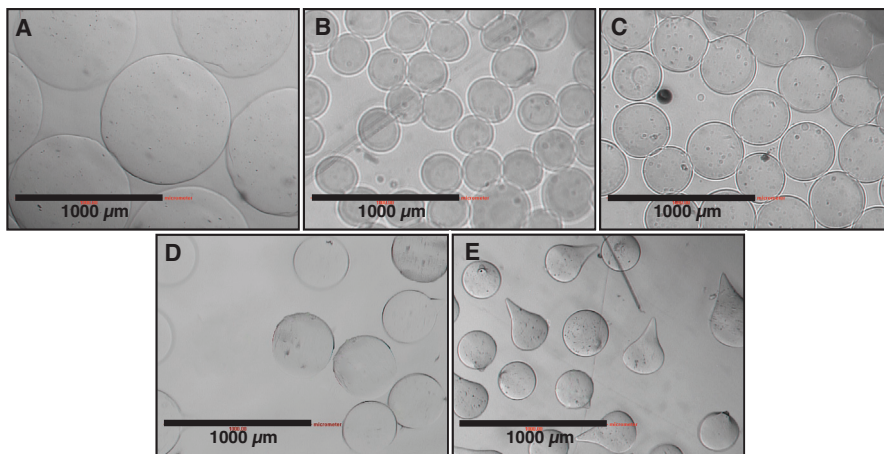


Figure 9.8: Light microscopy images of gelatin-sodium alginate beads. (A) G9A1a, 12 kV, 3 mL hour⁻¹ ($844 \pm 19 \mu\text{m}$); (B) G9A1b, 16 kV, 4 mL hour⁻¹ ($230 \pm 31 \mu\text{m}$); (C) G9A1b, 16 kV, 2 mL hour⁻¹ ($348 \pm 24 \mu\text{m}$); (D) G4.2A2.8a, 18 kV, 3 mL hour⁻¹ ($389 \pm 48 \mu\text{m}$); (E) G4.2A2.8a, 16 kV, 5 mL hour⁻¹ ($270 \pm 71 \mu\text{m}$); Scale bar indicates 1000 μm .

Moghadam et al.⁴⁸⁷ reported the increase in average bead size through increasing

needle gauge for electrosprayed sodium alginate solutions, however for larger nozzle internal diameters, this effect became negligible. As we have shown, larger needles limit the effect of flow rate and applied voltage variations for voltages > 12 kV on mean bead diameter. For gauge 30 needles, mean diameter could be controlled in the range of $200 - 1600$ μm , which opens perspectives when a certain bead size is demanded. A $1 \text{ w/v} \%$ sodium alginate solution fed through a 200 μm needle gave particles with sizes between $300 - 1900$ μm for voltages between $0 - 9$ kV.⁴⁸⁷ This means that mean bead size with simple gravitational droplet formation is around 2 mm when applying G27 needle. In our case, we obtained a composition whereby gelatin is incorporated, explaining the need for higher voltages in order to obtain a similar bead diameter range.

Electrospray precursor solutions containing the same amount of sodium alginate, however lower gelatin content were prepared to evaluate the effect of gelatin content on the spraying over a voltage range of $4 - 20$ kV for three needle diameters. Figure 9.9 gives the data points for the spraying at 12 kV.

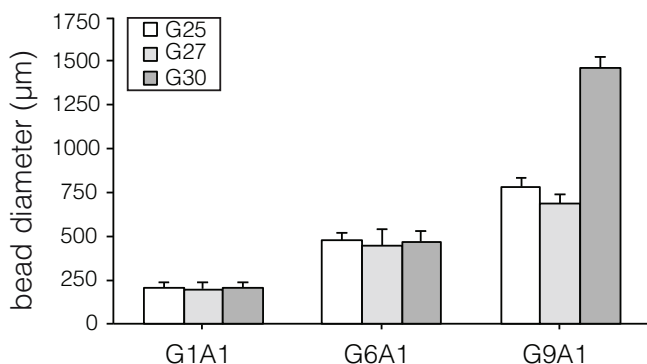


Figure 9.9: Influence of gelatin content on the bead diameter. Results represent data at 12 kV sprayed at a flow rate of 1 mL hour^{-1} .

As can be seen, decreasing the gelatin content enables to drastically decrease the average bead diameter. This can be attributed to the poor fiber-forming potential of gelatins due to the polyelectrolytic nature as well as the formation of hydrogen bonds. Furthermore, similar as the three different behaviors observed for different needle internal diameters, as discussed in the previous paragraph, the voltage exerted an analogous influence over the three precursor solutions (data not shown).

Two liquid precursors of higher viscosity than G9A1 sample were also subjected to the same voltage and flow rate range. Initially, a composition of equal total concentration ($10 \text{ w/v} \%$) but different gelatin to sodium alginate ratio ($3:2$) was prepared for electrospraying: sample G6A4. However, since G6A4 was too highly

viscous at the processing temperature, constraining microcarrier generation, investigation of this particular composition was postponed. As previously mentioned, viscosity was around ten times higher than G9A1 sample. In order to make electro-spraying possible, solutions with the same 3:2 ratio but lower total concentration were prepared. For the 7 w/v %, sample G4.2A2.8a, beads within the same size range could be obtained (Figure 9.10). However, for some of the conditions tested, tail formation appeared. The appearance of these so-called ‘tear-like’ microcarriers is illustrated in Figure 9.8 (E), and has been observed in literature with electro-spraying of pure alginate solutions. Suksamran et al.⁴⁸⁸ reported the formation of ‘large tail particles’, ‘long rod gel beads’, ‘small semi-circle particles’, and ‘small tail particles’ in their macroscopic evaluation of alginate particles produced by an electro-spraying process. Light microscopy images from 2 w/v % sodium alginate microcarriers, produced by electro-spraying in the dripping mode, showed similar tail-like macroscopic features for some of the beads.⁴⁷⁷ It is suggested that sodium alginate solution viscosity and cross-linking medium (Ca^{2+} concentration) influences the shape of the beads produced. In addition, since we worked at elevated temperatures, this effect is probably combined by a (more pronounced) partial dehydration, limiting drop sphere (re)formation. Adjusting the distance between the needle tip and the cross-linking bath can however also modify the sphericity of the beads. Since the relaxation time will be altered, this is especially the case for highly viscous solutions. Despite this, in our case doubling the distance had no significant impact on the macroscopic features of the tail (data not shown).

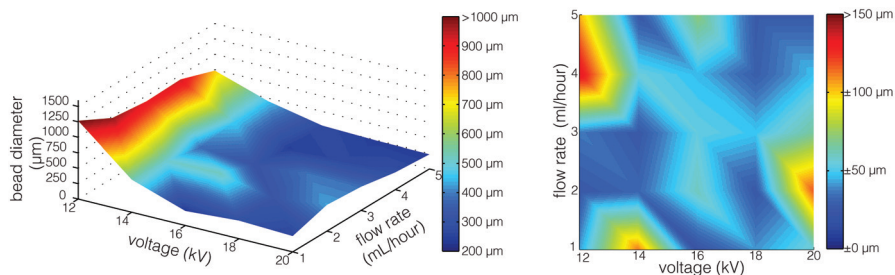


Figure 9.10: Bead size (left column) mapping as a function of applied voltage and flow rate, and corresponding standard deviations (right column) for G4.2A2.8a.

Interestingly, standard deviations for sample G4.2A2.8a tend to be smaller, indicating even lower dispersity levels or a more stabilized electro-spraying process as compared to G9A1 (Figure 9.10 right). In general, higher voltages lowered bead sizes, as has been reported in literature. This has been explained by Xie et al.⁴⁷⁷ the electrical field suppresses the surface tension since the electrical polarization forces act in the opposite direction of surface tension. This surface charge-electrical

field interaction will lead to an acceleration of the liquid precursor in the direction of the gravity, opposite from the nozzle, ultimately obtaining smaller droplets. For voltages between 0 – 9 kV, a flow rate of 50 mL hour⁻¹ and a sodium alginate concentration of 3 w/v %, mean bead sizes ranged between 1 – 2 mm. Since we employ larger voltages, and lower flow rates, it is expected to obtain much smaller bead sizes. However, in our case, lowering the flow rate in between the 1 – 5 mL hour⁻¹ range did not seem to significantly affect the bead sizes for sample G4.2A2.8a. This is somewhat in contrast to literature, where experimental results show how the feed rate can control the mean diameter sizes.⁴⁹¹ Normally, higher flow rates result in larger particles. Most likely, this contradiction can be explained by a combinatory effect of solution viscosity and syringe pump effectiveness: for high viscosity shear thinning samples, viscosity limits the flow rate decrease applied by the syringe pump. By this experimental setup we were able to demonstrate that altering the blend composition makes it possible to change the macroscopic shape of the generated beads.

Blend concentration demonstrated to influence the mean particle sizes. This can be deduced by comparing Figure 9.10 and 9.6. Lowering the hydrogel building block concentration, while keeping the blend ratio constant, resulted in a decrease of the mean particle diameters. A possible explanation is related to a reduction of the surface tension forces caused by decreasing polymer concentrations, ultimately leading to size reduction of the generated droplets. Liu et al.⁴⁹² confirmed in their work on controlling the numbers and sizes of beads, generated in electrospun fibers, that beads in fiber mats, were mainly caused by lower surface tensions. Lowering their polymer concentration, either many beads or many microspheres appeared and the process became electrospraying.

Nevertheless, among all the examined parameters, the applied voltage had the most pronounced effect on the size reduction of the beads. Increasing the voltage led to an increase in the droplet frequency, while the size was reduced. Concurrently, decreasing the flow rate can also increase the dripping frequency since the dispensed liquid will have more time to be charged, but this effect was less pronounced in the 1 – 5 mL hour⁻¹ range. On the other hand, needle gauge impacts on the electrospray stability that can be obtained.

9.3.3 Effect of NaCl on the bead macroscopic geometry

The formation of macroscopic ‘tear-like’ features has been mentioned in the former paragraph. This can be mostly contributed to the sodium alginate component, which impacts the viscosity and elasticity of the blend solution. Additionally, the sodium alginate compound is converted to calcium alginate upon immersion in

the cross-linking bath, which ‘freezes’ the macroscopic morphology of the network. Ideally however, spherical beads should be produced with a narrow size distribution in order to get homogeneous conditions favorable for cell culturing, drug delivery, etc. Proper solvent selection for improved ionization is a standard methodology. For instance, including acetic acid in the solvent mixture could be such an example strategy. We evaluated the incorporation of NaCl salt into the solution (samples G4.2A2.8b and c).

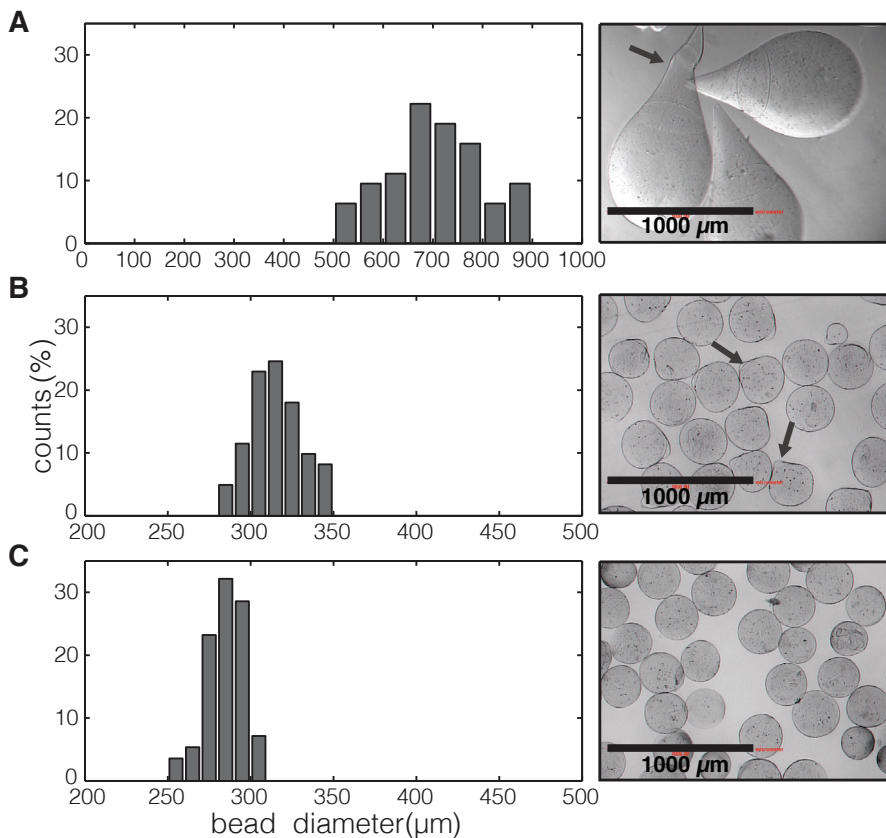


Figure 9.11: Bead diameter histograms for G4.2A2.8a (A), G4.2A2.8b (B), and G4.2A2.8c (C) accompanied by representative microscopy images. All samples were produced with a flow rate of 5 mL hour^{-1} at 12 kV . Respective mean diameters are $735(\pm 103) \mu\text{m}$, $316(\pm 17) \mu\text{m}$, and $286(\pm 13) \mu\text{m}$. Black arrows indicate ‘tear-like’ deformations. Scale bars indicate $1000 \mu\text{m}$.

Increasing the salt concentration from 0 to 2 % resulted in smaller, nearly monodisperse, and spherical beads. This effect is summarized with histograms and visualized with representative light microscopy images (Figure 9.11). Adding a minimal

amount of salt (1%) already evinced the significant effect on bead dimensions, which reduced mean diameter more than half of the former condition (0%). Additionally, size distribution was narrowed drastically and the deformation effect reduced. Further increasing the salt amount till 2% decreased these parameters to a lesser extent. Interestingly, spherical beads were obtained which were characterized by a very narrow size distribution, as can be seen in Figure 9.11 (C). Up to date, a study on the effect of salt addition was not yet reported. Nonetheless, we demonstrated a drastic impact on bead shape, size, and size distribution. As a possible hypothesis, the salt could be responsible for delaying the bead formation in the cross-linking medium. Therefore, the beads would have a longer time period to recover to spherical shape. On the other hand, it is likely that the rayleigh limit is lowered therefore smaller beads are obtained at equal field strength.

9.3.4 Bead morphology

Above mentioned results indicate that macroscopic features could be modified: spherical beads, tear-like beads, spindle-like beads (Figure 9.12 A-C). Next to bead size, size distribution, and shape, the microscopic features of microcarriers play a pivotal role. In order to characterize microscopic morphological properties, SEM images of dry bead samples were taken with different magnifications. As expected, the formation of a thin skin layer was observed. Indeed, when gelatin-sodium alginate solutions are exposed to a direct addition of bivalent calcium ions, nearly instant formation of a heterogeneous alginate gel will occur.⁴⁸² In our case, this meant a fast phase separation and the formation of an outer shell rich of Ca^{2+} -alginate.

Intriguingly, the skin configuration appeared different depending on the gelatin-sodium alginate composition. For samples G9A1, G6A1 and G1A1, a thin, but dense, film covering the complete beads was observed (Figure 9.12 (A,D)). When this thin layer was scraped off, a porous structure was revealed. It is hypothesized that this film is formed by phase separation and cross-linking of the alginate fraction, leaving an inner part mainly composed of (uncross-linked) gelatin. The outer microscopic morphology (skin) is comparable to that of pure alginate beads.⁴⁸⁸ As will be proven later on, this so-called skin consists primarily out of calcium-alginate for our beads. Large beads (i.e. larger than $500\text{ }\mu\text{m}$) demonstrated a non-complete covering, suggesting that in those cases the alginate content is insufficient to contain the whole bead or/and phase separation is hindered (data not shown). Contrary, sample G4.2A2.8a consisted of a similar dense skin, however, protrusions were observed. These are cluster-like areas of different material composition (i.e. gelatin protrusions) (Figure 9.12 (B,E)). As a result, the surface roughness is increased substantially. In addition, the drying process of these beads

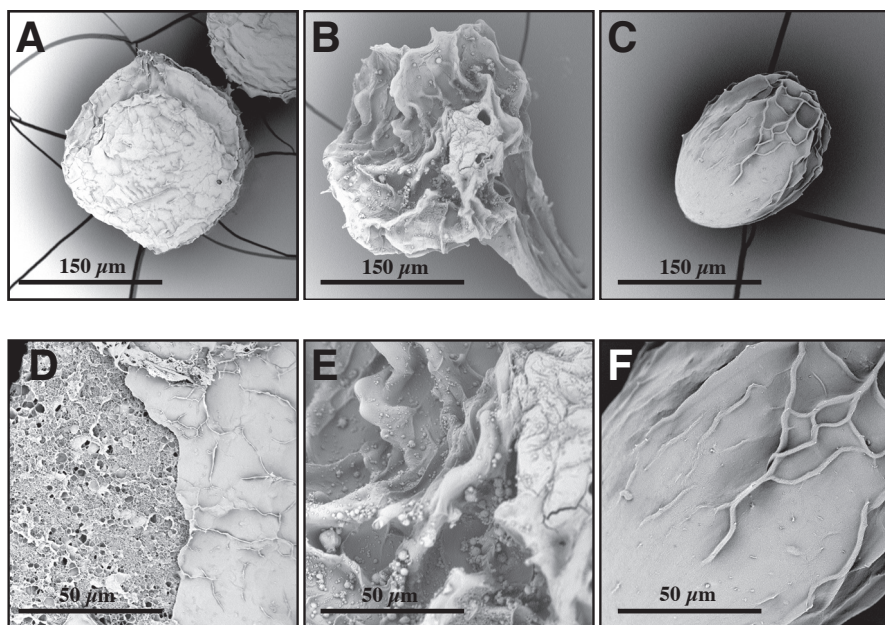


Figure 9.12: SEM images of some beads representative for conditions G9A1 (a,d), G4.2A2.8 (b,e), G3A2 (c,f).

resulted in beads having a heterogeneous shrinking. Even so, upon re-swelling, the initial macroscopic configuration was reformed. This appearance is in accordance to literature, for example it has been observed in SEM images of chitosan/gelatin composite microcarriers produced by an emulsification method.⁴⁶⁷ Finally, samples G3A2 demonstrated characteristics of both skin type formulations (Figure 9.12 (C,F)).

9.3.5 Bead composition and stability

FT-IR measurements (Figure 9.13 and 9.14) and XPS analysis (Figure 9.15) were performed in order to examine the ‘skin’ composition and to reveal the presence of the two blend components before and after swelling. In what follows, the results displayed confer to beads that were fully swollen at 37 °C for at least 4 h in distilled water.

The FT-IR spectrum of pure gelB was characterized by the amide A peak (N-H stretching vibration) at 3280 cm^{-1} , amide I peak (C=O stretch) at 1630 cm^{-1} , amide II peak (N-H bend and C-H stretch) at 1547 cm^{-1} , and amide III peak (C-N stretch plus N-H in phase bending) at 1240 cm^{-1} , which are the distinguishing features of gelatin. The spectrum of pure sodium alginate was characterized by

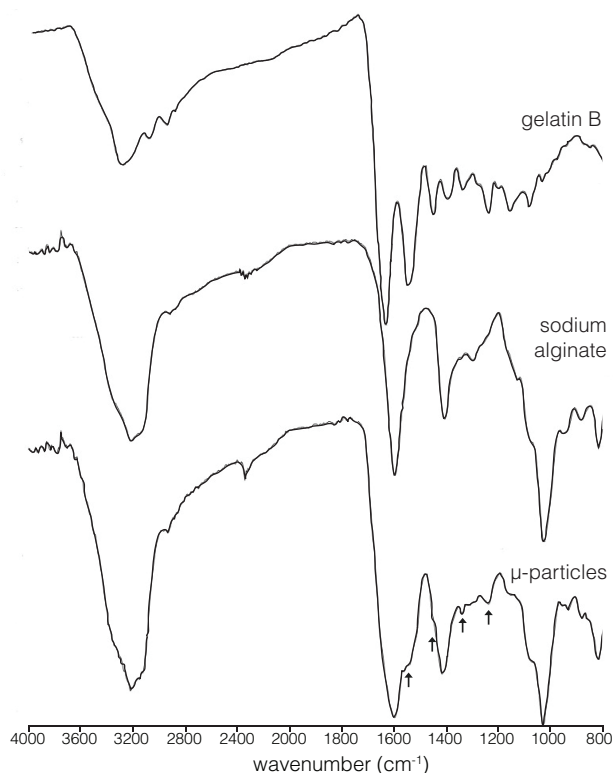


Figure 9.13: FT-IR spectra of gelB, sodium alginate and electrospayed beads after incubation at 37 °C for 24 h.

the peak at 3213 cm^{-1} (OH stretch), the peak at 1597 cm^{-1} (COOH stretch), and the peak at 1025 cm^{-1} (C-O-C stretch). The averaged (over an area of about $40\text{ }\mu\text{m}^2$) spectrum of the μ -particles revealed the presence of the major alginate peaks. However, some smaller peaks stem from the presence of gelatin as the second component (indicated by arrows).

From the average FT-IR signals displayed in Figure 9.13, it is clear that both components remain present in the μ -particles. However, it would be more interesting to have an indication about the localization of both components across the analyzed surface. Therefore, ATR mapping results were correlated with the spectra of the pure blend components (Figure 9.14). For all samples, the correlation with alginate (corr. coefficient > 0.9) was better than compared to gelatin ($0.75 < \text{corr. coefficient} < 0.88$), indicating a surface mainly composed of alginate. As can be derived from the correlation mapping, regions with lower alginate correlation coefficient corresponded to regions with higher gelatin correlation coefficient. These findings, which could not be derived from the averaged signals, confirm the

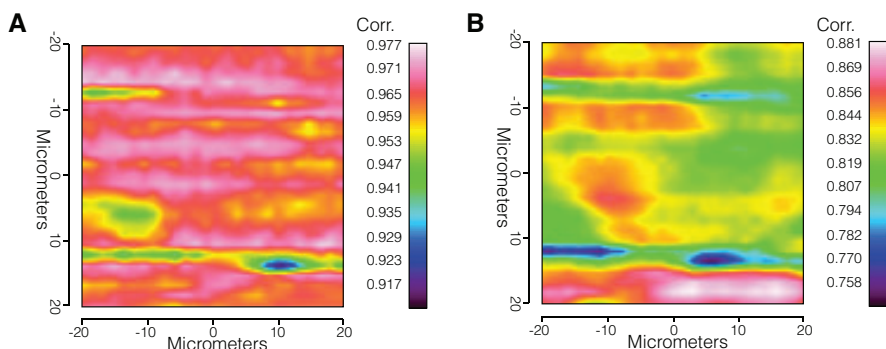


Figure 9.14: ATR mapping on microparticles. Correlation of the particle FT-IR spectrum with the spectra of pure sodium alginate (A) and pure gelatin type B (B). A surface area of $40\text{ }\mu\text{m}^2$ was scanned.

postulated hypothesis of a skin layer mainly composed of sodium alginate, with in some cases gelatin-rich protrusions.

More quantitative information concerning the surface composition can be obtained by XPS analysis. XPS survey spectra and high-resolution carbon 1s spectra were collected and are presented in Figure 9.15. In Table 9.2, the elemental composition of the different samples is given. The presence of the N 1s core signal can be used as an indication for the presence of gelatin in the skin that surrounds the particles. Pure alginate does not contain nitrogen, while pure gelatin contains 16 % nitrogen. The nitrogen content of the beads was situated around 4 %. On the other hand, the Ca signal, which is an indication for the ionic network formation of the alginate fraction, is about 3 %, while the sodium signal completely disappears. These findings altogether correlate with previous indications about the skin formation. A phase separation occurs during the Ca^{2+} cross-linking step with the formation of a skin layer (top nm range) mainly composed of a Ca^{2+} -alginate network, with some gelatin embedded.

Table 9.2: Elemental composition derived from XPS analysis.

SAMPLE NAME	SURFACE COMPOSITION [%]				
	O 1s	C 1s	N 1s	Na (Auger peak)	Ca 2p
Gelatin	18.4	65.3	16.3	-	-
Sodium alginate	29.2	63.8	-	7.3	-
μ -particles	30.9	62.9	3.5	-	2.7

Further insight can be gained comparing the high-resolution carbon 1s signals of the pure components (gelatin and alginate) with the blend (e.g. produced beads).

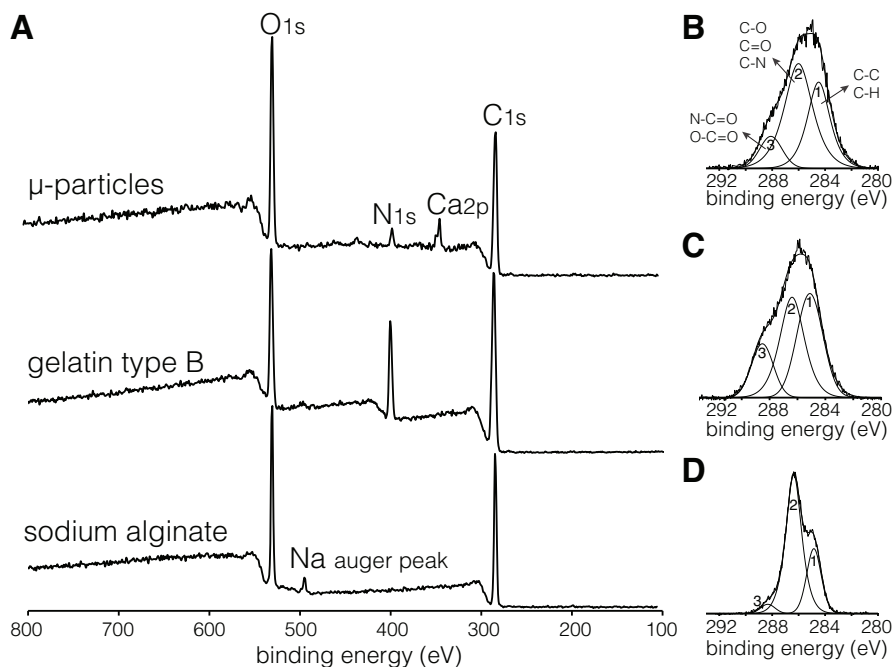


Figure 9.15: XPS surface scan spectra (A) and high-resolution carbon 1s spectra of the particles (B), gelatin type B (C), and sodium alginate (D).

It should be noted that assigning a chemical bond to certain component peaks is extremely difficult in the case of biomolecules, since a broad range of chemical functionalities is present. To simplify the quantitative comparison between the three examined samples, only 3 peaks were assigned (Figure 9.15 B-D), as previously mentioned.

The amide/carboxyl peak is most pronounced in the case of gelB ($\pm 20\%$), while almost not detected in the case of alginate ($\pm 4\%$). This is logical since gelatin is a protein (= polyamide) containing both amides and carboxyl functionalities, while the amount of carboxyl functionalities in alginate is rather low and amides are not present. In the case of the microcarriers, this component increased till $\pm 12\%$, further confirming that gelatin is also present on the microcarrier surface.

An important question that rises is related to the stability of the generated microcarriers. Since initially only cross-linking of the sodium alginate fraction occurs, is the sodium alginate skin layer sufficient in order to maintain integrity at physiological conditions? Therefore, dry beads were re-swollen in PBS or DMEM culture medium and incubated at 37°C for over 72 h. Light microscopy and SEM images were recorded, and their composition was evaluated with coomassie brilliant blue staining and infrared measurements. Staining of all beads revealed the prevalence

of gelatin inside the microcarriers. After incubation, a color intensity drop for samples from condition G9A1 was observed (Figure 9.16). Also the correlation of the recorded infrared spectra with that of pure gelB decreased. Some of the beads even lacked any coloring, indicating a total loss of the gelatin fraction. This was observed particularly for beads with sizes around 400 – 500 μm (intermediate sizes), and larger beads. This can be explained by the absence of a complete alginate layer for larger beads, while for beads with intermediate sizes, upon re-swelling the gelatin fraction swelled to such extent that the alginate skin layer was broken and the inner part was released: so-called ‘ghost’ beads. An example is given in Figure 9.16 A, and SEM analysis revealed that only the skin was left (Figure 9.16 B). For smaller beads, this was not observed, and beads remained intact. When gelatin content was decreased and sodium alginate content increased (conditions G4.2A2.8 and G3A2), swelling of the gelatin fraction was insufficient to break the alginate layer (Figure 9.16 C). Infrared analysis demonstrated a small decrease in correlation coefficient with gelatin after incubation, however, this was less pronounced compared to the decrease found with condition G9A1. This was further confirmed by XPS analysis whereby the N 1s signal dropped to 2 – 3 %.

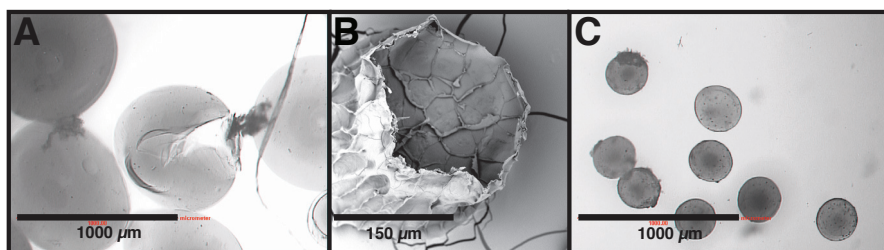


Figure 9.16: Images of coomassie stained microbeads: (A) G9A1b (18 kV, 1 mL h⁻¹), (C) G4.2A2.8c (12 kV, 5 mL h⁻¹). (B) Scanning electron microscopy of a so-called ghost bead for G9A1b.

Another strategy consisted out of a double cross-linking strategy whereby an initial fast cross-linking occurred due to ionic curing of the alginate fraction, followed by a slower curing of thiolated gelatin (e.g. adding H₂O₂ in the Ca²⁺-bath). By doing this, the fabricated beads can still be dissolved via non-enzymatical pathways, which could be beneficial for instance when trying to recollect attached or encapsulated cells. In addition, it is possible to increase gelatin content and decrease alginate content without the disadvantages described above. Thus, almost no decrease in gelatin content is observed. Examples are given in Figure 9.17.

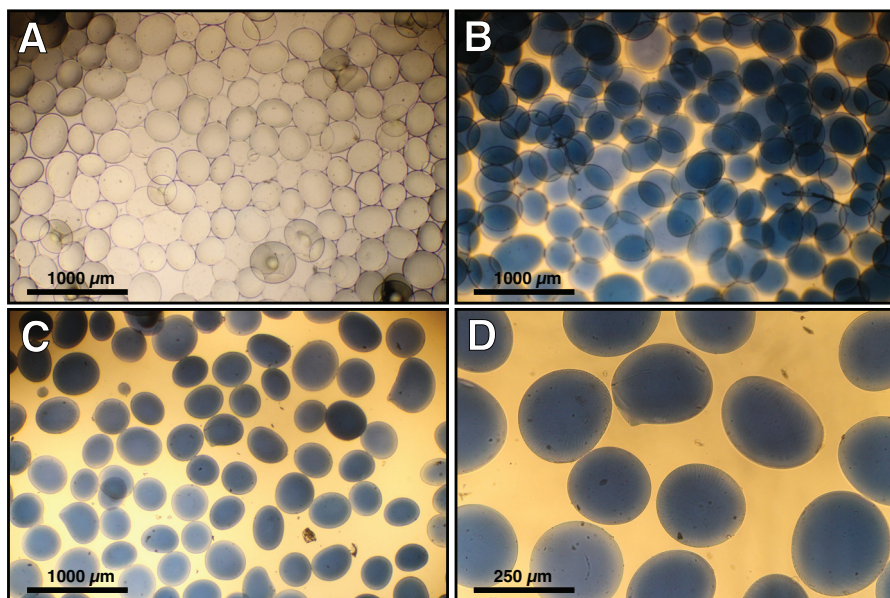


Figure 9.17: Images of G6A1b sample (with thiolated gelatin) sprayed at 16 kV and 3 mL h^{-1} before (A) and after (B) staining with coomassie. And after incubation at 37°C for 72 h (C,D).

9.3.6 Effect of electrospraying variables on cell survival

Next to potential applications, such as drug delivery vehicles, cell seeding matrices, ... electrospraying is steadily emerging as a cell encapsulation strategy. The cell-laden carriers can then, for example, further be used for the loading of BAL devices, or for embedding in other hydrogels aiming at large-geometry shaping (modular tissue engineering).

9.3.6.1 Influence of the blend composition on the HepG2 cell viability

At first, the influence of the hydrogel precursor concentration on the obtained viability was evaluated. In literature, good cell viability is reported for the spraying of cell-laden sodium alginate solutions. Therefore, a 2 w/v % sodium alginate solution was selected to serve as positive control condition. Figure 9.18 gives some live/dead stainings. In general, electrospraying parameters had to be drastically altered in order to result in a fraction of viable cells. Therefore, lower applied voltages and flow rates were used.

As a first observation, cells encapsulated during the spraying of alginate had a tendency of migrating toward the bead/fluid interface. In some cases this was relatively well pronounced (as seen for the 2 w/v % alginate condition). Gelatin

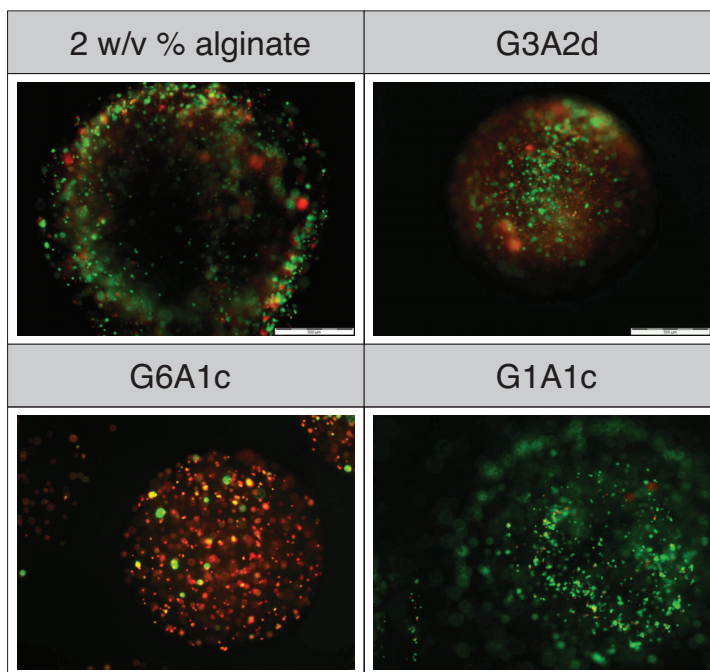


Figure 9.18: Live/dead fluorescence images of HepG2 cells encapsulated during the electrospraying process. For all conditions, a voltage of 6 kV, a flow rate of 1 mL h^{-1} , a G27 needle and a cell density of $2.5 \times 10^6 \text{ cells mL}^{-1}$ were applied. (4 X magnification)

containing solutions had a tendency to result in a more homogeneous encapsulation distribution of the loaded cells. On the downside, it appeared that with increasing gelatin content, cell survival rates decreased. For a gelatin concentration of 6 w/v % (G6A1) it is clear that almost all cells died during the process, while for a gelatin concentration of 1 w/v % (G1A1) acceptable viability could be reached ($> 80 \%$).

Electrospraying of sodium alginate solutions up to 2 w/v % have been reported to result in an acceptable viability.⁴⁷⁷ Zhang et al.⁴⁹³ proposed concentrations of $> 1.5 \text{ w/v } \%$ to obtain good results. Hepatocytes have successfully been encapsulated in other than alginate solutions using a modified collagen at a concentration of 1.5 mg mL^{-1} .⁴⁷⁶ No reports were found on polymer concentration effects for other polymers than alginate. In general, it is expected that the cell viability will drop with increasing concentration. Furthermore, the conductivity of gelatin is higher than that of alginate, which could explain the differences. However, the underlying mechanisms are still subject to further research and the experiments should be repeated for in depth characterization.

9.3.6.2 HepG2 cell density and needle internal diameter compete

In contrast with the plotting of cell-laden suspensions, increasing the cell density during electrospraying will not affect the process in a negative way. The viscosity reduction will even aid, potentially making the process easier. In Figure 9.19 A, the influence of increasing the cell density on the fractional cell survival is illustrated.

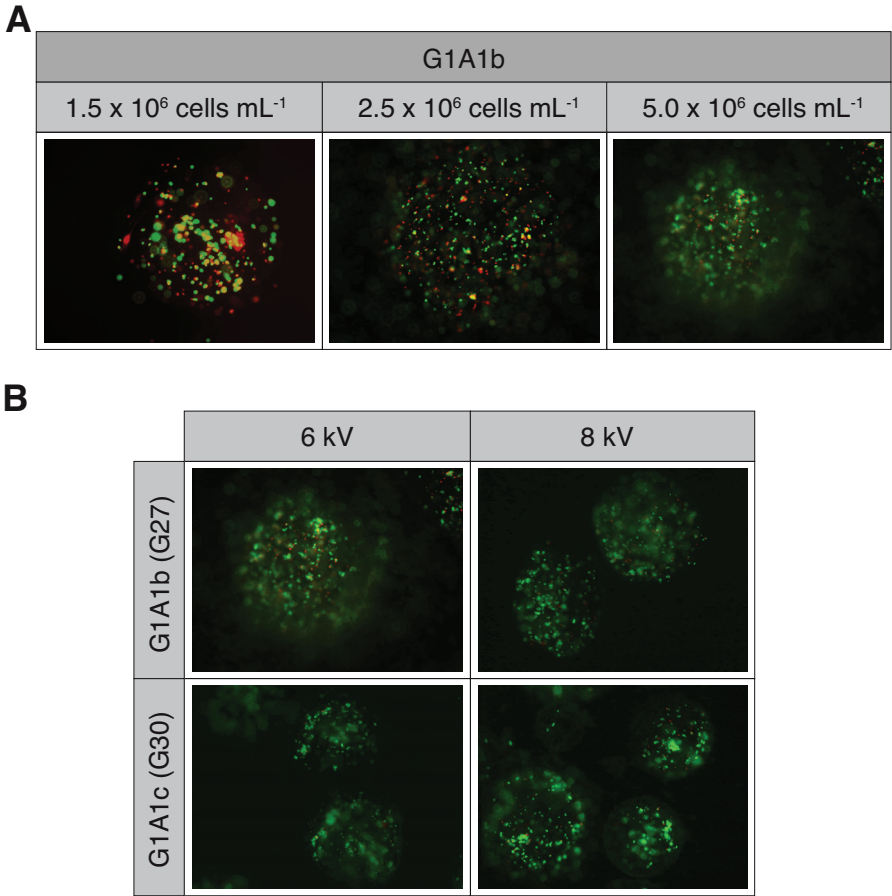


Figure 9.19: Live/dead fluorescence images of HepG2 cells encapsulated during the electrospraying of G1A1 solutions as a function of the cell density for G27 needle sprayed at 6 kV (A), and as a function of the needle internal diameter and voltage for a cell density of 5.0×10^6 cells mL⁻¹ (B). (4 X magnification)

Our first experiments suggest that an improved cell viability can be reached with increasing the cell density. In literature, cell densities from 1.0×10^6 cells mL⁻¹ and higher are common. One report suggests to work at elevated cell densities between $3.0 - 5.0 \times 10^6$ cells mL⁻¹ for the encapsulation of cells in alginate solutions, since

higher densities resulted in better encapsulation efficiency.⁴⁹³ In our experiments we did not evaluate the cell encapsulation efficiency as a function of the cell density, however, our findings demonstrate the influence on the obtained cell survival.

Furthermore, the effect is not absolute. The images represent spraying with G27 needle, whereby higher cell density results in a higher cell survival. However, when smaller needle internal diameters (i.e. G30) are used for the spraying, a lower cell density is preferred (i.e. $2.5 \times 10^6 \text{ cells mL}^{-1}$) to obtain optimal viability. This is illustrated in Figure 9.20. Most likely, shear stress induced effects play a role.

9.3.6.3 Influence of the applied voltage on the HepG2 viability

As previously discussed, increasing the voltage and decreasing the needle internal diameter will generally lead to smaller beads. This is illustrated in the live/dead stainings from Figure 9.19 B. Figure 9.20 gives a summary on the combined effects exerted by the applied voltage, needle internal diameter, and cell density for G1A1 samples.

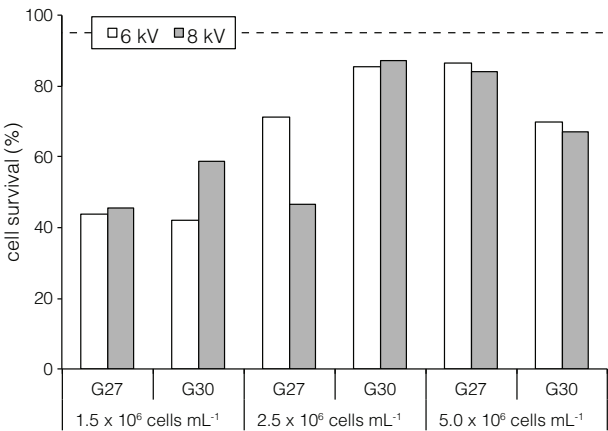


Figure 9.20: Summary of the influences of some key spraying parameters on the cell survival rate of encapsulated HepG2 cells directly after spraying.

In the case where no voltage is applied, cell survival is situated around 95 %, as indicated by the dashed line. It can be seen that by applying a voltage, cells are affected. The voltage put on the cell-laden suspension can either be positive or negative by inverting the polarity. Some experiments demonstrated that by applying a negative polarity instead of a positive polarity, cell viability dropped substantially at day 1 after microencapsulation (to $< 10\%$). Most likely, the inherent cell membrane polarity is disrupted resulting in apoptosis of the sprayed cells. This should be subject to future research.

Furthermore, the applied voltage had a more pronounced effect for lower cell density solutions, suggesting that perhaps cell-cell contact aids to overcome the induced electrical field. Between 6 and 8 kV, no major differences could be observed.

As an important remark, it should be stressed that the findings on the cell encapsulation during the electrospraying process are the result of initial experiments without multiple repetitions. Therefore, the experiments should be repeated and studied in more detail in order to confirm our hypotheses.

9.4 | CONCLUSION

In this work, a parametric study for the electrospraying of all-aqueous biopolymer solutions was performed. The possibility to electrospray all-aqueous gelatin-sodium alginate microcarriers proved successful due to processing at elevated temperatures. Nearly monodispers microcarriers with varying sizes could be fabricated in the 150 – 1600 μm range. The absence of any toxic components, such as fluorinated alcohols, makes it a clean fabrication technique suitable for numerous medical applications. Furthermore, macroscopic and microscopic features of the generated microcarriers could be varied. In this perspective, blend composition demonstrated its impact on the surface morphology. Solution viscosity appeared to have a substantial impact on the manufacturability of the electrospray process. Out of all the investigated parameters especially the applied voltage revealed to be a pivotal parameter for the size of the generated beads, while the addition of a salt drastically improved microcarrier shapes and sizes.

Finally, some initial experiments on microencapsulation of HepG2 cells were performed. From these results, decreasing the polymer concentration (especially the gelatin fraction), decreasing the needle internal diameter and increasing the cell-loading density led to an improvement of the final cell viability. Overall, applying a voltage decreased the cell survival. Despite the acceptable viability levels reached for certain conditions, these experiments should be subjected to repetition before final conclusions can be drawn.

10 | GENERAL CONCLUSIONS AND FUTURE OPPORTUNITIES

10.1 | GENERAL CONCLUSIONS

The present research was focussed on the application of gelatin-based hydrogel systems for liver tissue engineering purposes. Some questions were postulated and studied:

1. What is the effect of the gelatin composition, i.e. origin?
2. How does the selection of a chemical modification strategy in order to obtain irreversible networks affect the hydrogel network properties?
3. Is it possible to fabricate gelatin-only constructs using the 3D Bioplotter, with the aim at cell encapsulation?
4. How does the 3D printing affect mechanical strength, degradation and protein /polysaccharide release behavior?
5. Can gelatins be modified for specific targeting of hepatocyte-like cells?
6. Can electrospraying offer a suitable alternative for micro-encapsulation?

In chapter 3, four gelatins having different origin and extraction processes were investigated for their difference on the protein primary structure, MW, MWD, gelification, and *in vitro* cytocompatibility for HepG2 cells. It was demonstrated that they displayed different material properties. Cytocompatibility experiments revealed a preference for type B gelatins over type A gelatins for the encapsulation of HepG2 cells. In addition, increasing the gelatin hydrogel building block concentrations led to a reduction of the HepG2 cell viability.

Gelatins exhibit a reversible temperature dependent sol-to-gel transition (Upper Critical Solution Temperature (UCST) type of material), limiting its applications at physiological conditions. In order to overcome this drawback, a chemical modification strategy consisting out of the incorporation of photocross-linkable functionalities was explored. Two vinyl-based side groups (methacrylamides and acrylamides) were introduced and compared for their influence on the resulting hydrogel network properties. This topic was discussed in chapter 4. This study proved the capacity of the UV-A induced free radical polymerization strategy

to manipulate the hydrogel swelling/mechanical properties and the hydrogel network mesh sizes. Acrylamide-functionalized gelatins exhibited improved cross-linking efficiency, leading to faster curing kinetics, lower swelling ratios, higher mechanical strength, and smaller network mesh sizes compared to methacrylamide-functionalized gelatins. Other parameters, such as the hydrogel building block concentration, UV-A irradiation dose and gelatin type (A vs. B) can also be used in the tailoring towards desirable hydrogel network properties. Encapsulation of HepG2 cells inside 10 w/v % 2D sheets displayed high viability for both functionalized gelatins. However, at higher hydrogel building block concentrations, methacrylamide functionalized gelatins yielded higher cell viabilities.

Another major concern when working with hydrogels, and specifically with naturally derived hydrogels, is the difficulty to shape these hydrogels into predefined patterns. Nonetheless, in recent years a paradigm shift from 2D towards 3D cell culture models occurred. In chapters 5 and 6, this topic was addressed. Three-dimensional gelatin-only constructs were fabricated by means of the 3D Bioplotter technology. To this end, the device was adjusted to enhance the printing potential for gelatin solutions. It was proven that gelatin-only hydrogels could be printed in different patterns having a high shape fidelity over a broad range of hydrogel precursor concentrations. Post-fabrication construct curing could be translated from 2D films to these 3D scaffolds. Due to the introduction of a macroporous network, construct mechanical, degradation, and protein /polysaccharide release properties are altered compared to simple 2D sheets. Based on this knowledge, cell-laden constructs were generated whereby either a cell suspension or preformed cell micro-aggregates were printed. High cell viability levels could be reached due to an interdisciplinary approach studying chemistry, biology, and engineering feedback loops. These findings stipulate the potential of the technique for biomedical applications. Finally, a proof-of-principle co-culture experiment demonstrating the seeding of a second cell source on top of aggregate-laden scaffolds suggests promising results for future research on cell culture models of higher complexity.

In chapters 7 and 8, the specific recognition of gelatin substrates by hepatocyte-like cells was addressed by the immobilization of galactose-containing moieties. The asialoglycoprotein receptor (ASGPR) of hepatocytes specifically recognizes galactose units. Bulk galactosylation of methacrylamide-functionalized gelatins improved the functionality of encapsulated HepG2 cells. Moreover, the formation of round morphologies was promoted, which has been correlated to an enhanced hepatocyte function. In addition to the effect on the hepatocyte functionality, galactose-containing moieties were revealed to hamper the UV-A induced curing kinetics by means of their free radical scavenging property. This led to slightly less cross-linked gelatin methacrylamides having a greater swelling potential. A second strategy,

based on the synthesis of a cyclic galactose-containing cross-linkable monomer, demonstrated the possibility to selectively functionalize the gelatin surfaces and/or bulk material. The developed strategies were capable of controlling the amount of introduced biological ligands.

Finally, electrospraying was investigated as a processing technique for the production of microparticles. The electrospraying of gelatin-sodium alginate solutions is handled in chapter 9. Electrospraying was selected as a fabrication method capable of generating microparticles in all-aqueous conditions. This condition enabled us to spray cell-laden solutions. A parametric study was performed in order to perceive a mechanistic insight into the influence of the key electrospraying parameters on the spraying stabilization, particle dimensions, macro- and microscopic morphology, and cell viability of sprayed HepG2 cells.

In summary, the research from the present work was able to demonstrate the feasibility of modified gelatins for the fabrication of 3D geometries with high shape fidelity. In particular these systems were targeted at cell encapsulation purposes of hepatocyte-like cells. Future research should be conducted to investigate the possible translation towards other cell types, hereby broadening the application potential of the presented materials/fabrication techniques.

10.2 | FUTURE OPPORTUNITIES

A couple of interesting emerging trends tend to bring scaffold-based TE on the next level. In example, the implementation of gradient techniques, in the general sense, seems to provide some promising approaches. The utilization of blends laid down the foundation of working with material gradients. Utilizing gradient techniques, a more precise mimicking of the ECM composition and mechanical properties will be possible, with spatial alterations throughout the scaffold. Of course, this can be extended towards different and multiple cell types, biomolecules, growth factors, etc., deposited in predefined patterns throughout the scaffold. Furthermore, gradients applied on the deposited scaffold pattern in itself (the deposited strand configuration) offers interesting alternatives to alter the mechanical properties of the construct. Although some authors already performed some initial experiments,^{226,250} the applicability of such approaches needs to be investigated in depth.^{226,249}

Alternatively, the combination of different scaffolding techniques for the fabrication of a single scaffold is another topic which I believe to offer a variety of possibilities. Combining electrospinning ($\sim$ nanoporosity) and bioplotting ($\sim$ microporosity) for the production of a single scaffold was demonstrated by Kim et al.⁴⁹⁴ The combined

effect of different techniques will most likely exhibit positive cooperative effects on cell biology. Thus, instead of focusing on the exploitation of one single technique, it would be most fruitful to combine the positive effects of different techniques into one operation procedure. In particular when multiple materials are processed with different techniques. For instance, during this work we experimented with the combination of PCL and gelatin into one 3D scaffold as demonstrated in Figure 10.1. In the first example a 3D printed gelatin scaffold was combined with a PCL electrospun fiber network, in another PCL printed constructs were combined with a gelatin freeze-dried network.

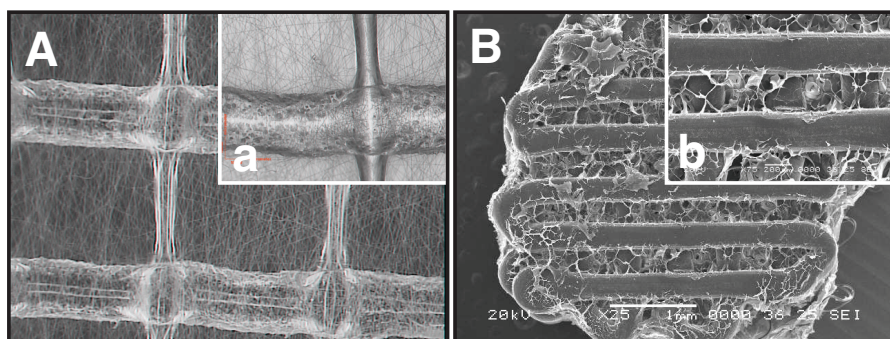


Figure 10.1: Combining multiple fabrication techniques. Gelatin printed scaffolds were combined with PCL electrospinning (A,a) and PCL printed scaffolds were filled with a freeze-dried gelatin matrix (B,b).

By doing this, 3D bioplotted structures could supply a highly porous structural framework with desired mechanical properties, while a second material could supply nanoporosity without impairing the mechanical integrity of the total construct.

As a very fascinating trend, material scientists should incorporate the knowledge of engineers into the designing step of the construct. This item is somewhat related to the previous one, with a clear, distinct focus on the mechanical properties. By means of finite element modeling, predicting the mechanical properties of the construct can be helpful. More importantly, adjusting the (predicted) mechanical properties of a model simply by varying the geometrical design of the material offers an interesting path to match its expected properties and the desired properties. The principles, advantages, and possible applications of this so-called Bio-CAD modeling in TE have been reviewed in 2005 by Sun et al.^{279,495} In the last couple of years, more and more, bone-engineering scientists follow this methodology.^{496–498} However, in the case of soft tissue engineering and/or tailored hydrogel scaffolds, this has not yet been intensively explored. In the next step, it would be helpful to develop models that relate the mechanical forces exerted on a scaffold to the viability of encapsulated cells and /or cell-aggregates.

The developed gelatin hydrogel scaffolds should be tested *in vivo* to explore revascularization of 3D printed constructs as well as inflammatory responses. As the liver is a highly vascularized tissue, this still poses one of the main difficulties in liver tissue engineering. Immobilization of vascular endothelial growth factor (VEGF) could offer opportunities. Additionally, *in vitro* cell culture models are still far from the targeted *in vivo* tissue performances. Cell culture models of higher complexity should be aimed at in order to converge to the *in vivo* situation. For instance, 3D plotting could be applied to generate a closed construct with flow in- and outlet adaptors to specifically design bioreactors with the scaffolds directly positioned *in situ*.

11 | TECHNIQUES

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This section contains additional information on some of the techniques that were used in the present studies. These techniques are classified into material processing, material characterization and imaging techniques.

11.1 | MATERIAL PROCESSING TECHNIQUES

11.1.1 Hydrogel film casting

2D hydrogel sheets were produced for a number of characterization techniques. Two casting methodologies were followed: (i) production of 2D sheets with a thickness of 300 – 1000 μm , and; (ii) spincoating of hydrogel building blocks on glass cover slips to produce thin films. In the former, spatial thickness is governed by using silicon spacers with the appropriate height (Figure 11.1).

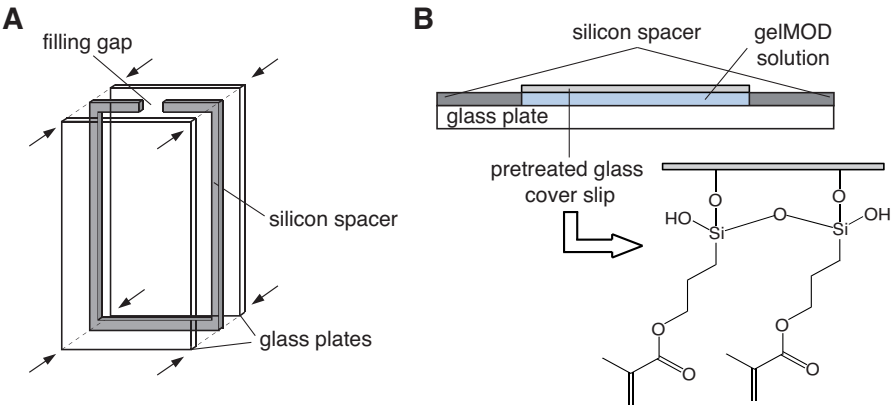


Figure 11.1: Casting of 2D gelatin hydrogel building block films: (A) the precursor solution is injected in between two glass plates separated by a silicon spacer of the appropriate height, or; (B) gelatin precursor solution is covalently attached onto pretreated glass cover slips whereby the film thickness is manipulated by silicon spacers.

Mostly, thick hydrogel films were prepared by simple casting in between two parallel glass plates (Figure 11.1 A). For some experiments, the potential floating behavior of gelatin films was countered by covalent coupling of the cross-linkable gelatins to pretreated glass cover slips (Figure 11.1 B). These cover slips were silanized using 3-(trimethoxysilyl)propyl methacrylate monomer, which enables further reaction with the (meth)acrylamide functionalities of gel-MOD upon UV irradiation.

11.1.2 The Bioscaffolder

As described previously in the introductory chapter, the 3D Bioplotter or Bioscaffolder is a rapid prototyping device suitable for the generation of 3D physical models generated from 3D virtual models. The Bioscaffolder used in this work is depicted in Figure 11.2. As can be seen, the device is designed to operate either

by the dispensing principle (I) or the extrusion principle (II). Dispensing is mainly preoccupied for low-viscosity liquids, such as hydrogels, and the dispensing head is equipped with a temperature control system for processing at 5 – 50 °C using a heating/cooling circuit (III). On the other hand, the extrusion head can be operated at elevated temperatures up to 250 °C using an electric heating circuit (IV) and finds its primary application in the processing of melt polymers, i.e. PCL.

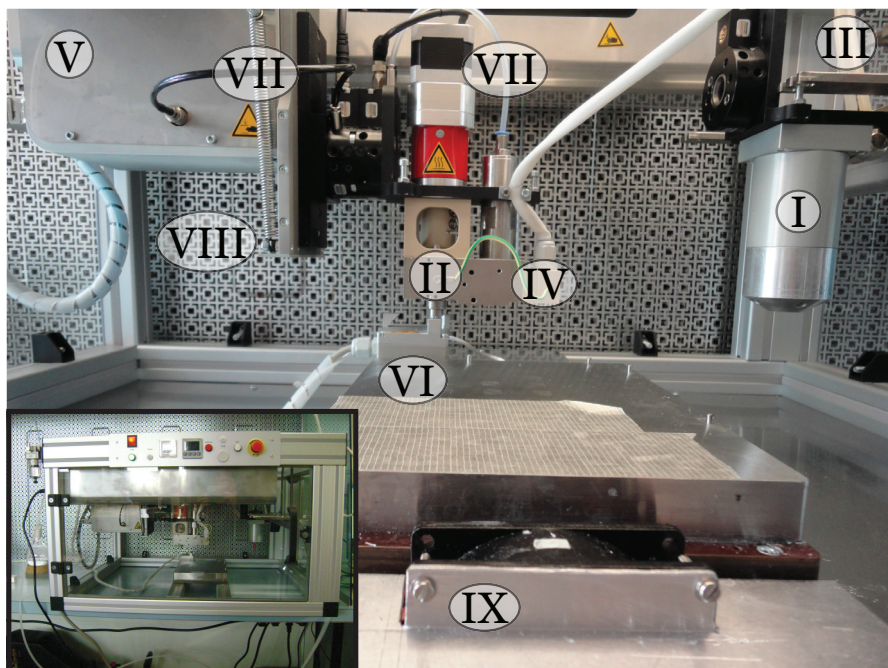
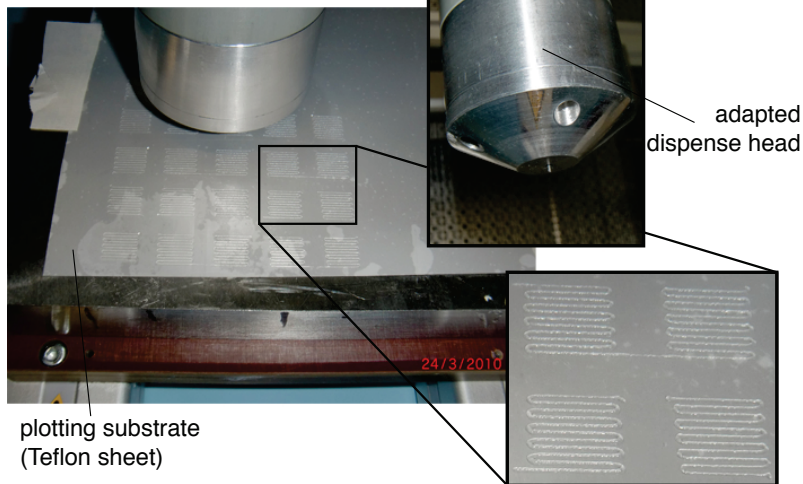


Figure 11.2: The 3D-Bioscaffolder used in this work consisting of a stationary built platform, a moveable dispensing head (typically applied for hydrogel processing), and a moveable extrusion head (typically used with melt polymers).

Both heads are moveable in the X-,Y-,and Z-directions (V) while the built platform (VI) remains stationary. Compressed air can supply the necessary processing pressure (VII), and the whole setup can easily be placed in a sterile, laminar flow environment (VIII). The built platform has a temperature control unit, which enables heating of the base plate. This was adapted and substituted to a Peltier element based system capable of heating as well as cooling. This is situated beneath the base plate, while a set of ventilators (IX) support removal of heated/cooled air.

CELL-FREE SCAFFOLD PRODUCTION



CELL-LADEN SCAFFOLD PRODUCTION

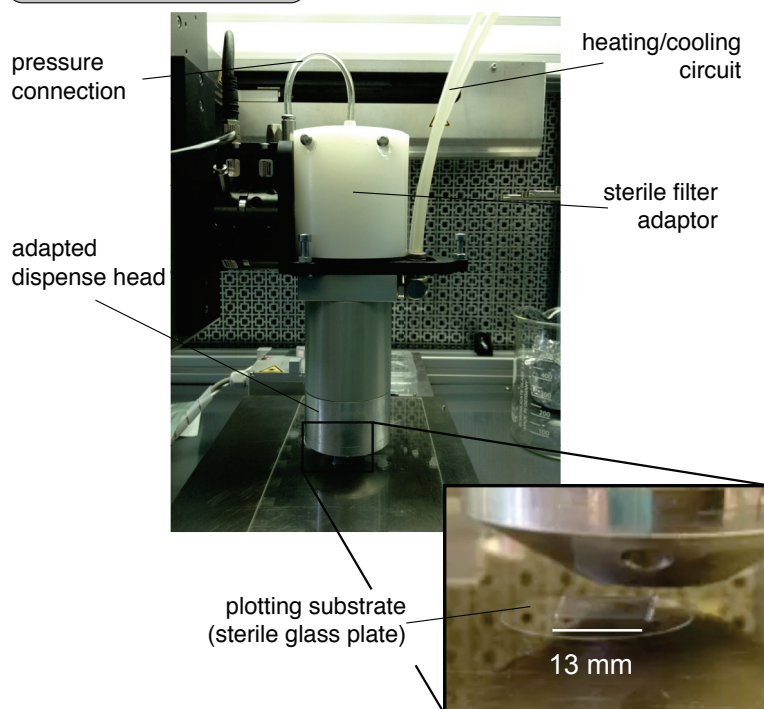


Figure 11.3: The dispensing unit operating while plotting gelatin solutions. The system was slightly changed for the production of cell-laden scaffolds, where an additional filter sterilized the incoming compressed air.

11.1.2.1 Dispensing of gelatin hydrogel building blocks

In this work, the main objective was the processing of hydrogel precursor solutions for the fabrication of 3D open porous constructs. For these type of materials, the dispensing principle was more suitable than the extrusion principle. A pressure-driven liquid ejection according to a layer-by-layer deposition of material through either a conical or cylindrical needle generates the 3D physical construct (Figure 11.3) . A couple of adaptation were specifically performed to improve the plotting of gelatin solutions, but also to guarantee sterile environments for cell-laden plotting:

- 1. Most importantly, temperature control over the mixture processing temperature was improved by adapting the bottom part of the dispensing head;
- 2. Equally important proved the be control over the substrate temperature to enhance the initial physical gelation. The importance increased at decreasing mixture concentrations;
- 3. Although the Bioscaffold was located in a laminar flow cabinet, the machine is often operational under non-sterile conditions. In order to reduce the risk for bacterial contaminations, a simple construct equipping the syringe inlet with a 0.2 μm sterile air filter was designed.

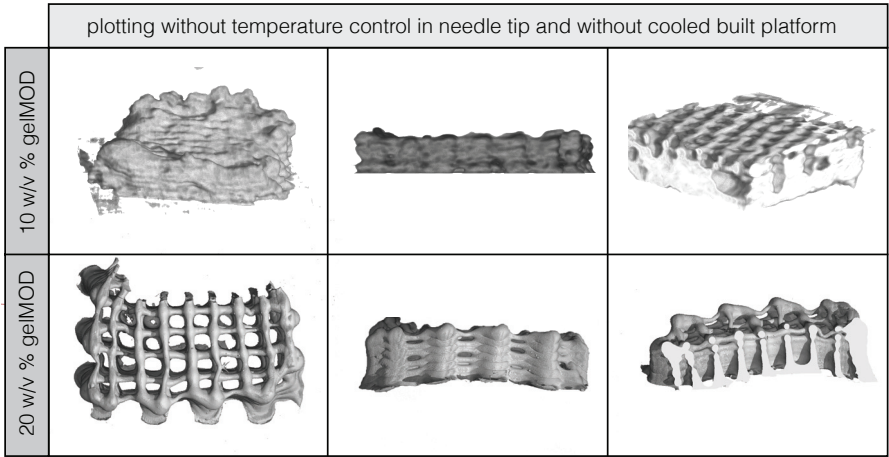


Figure 11.4: μ -CT images illustrating the plotting without precise temperature control over the gelatin mixture and built platform.

In Figure 11.4 the result of processing 10 and 20 w/v % gel-MOD mixtures before temperature control adaptations were implemented is presented. As can be seen, at lower concentration a complete collapse of the scaffold structure occurs, while at

higher concentrations some minor cross-sectional porosity occurs. However, even at high concentration, subsequent layers melt together due to the combinatory effect of a too high plotting temperature (to enable liquid flow) while triggering and rate of the physical gelation is insufficient.

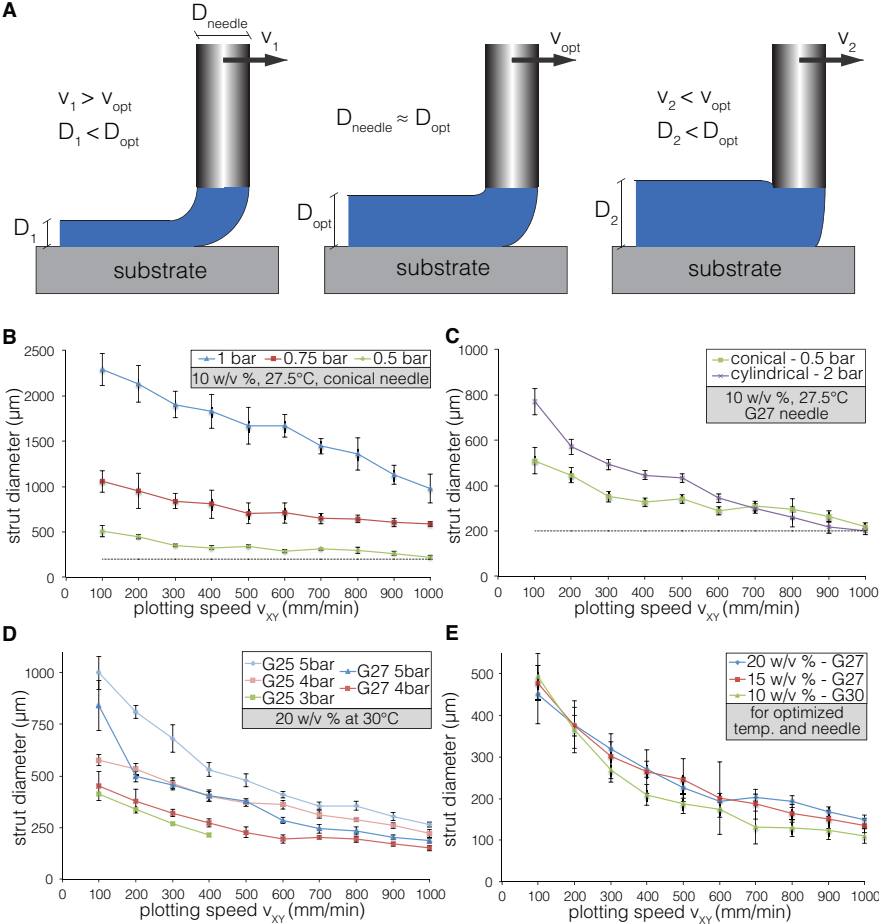


Figure 11.5: Data on optimization of the plotting process: hypothetical deposition patterns in relation to the deposition speed (A) and the evolution of the deposited strut diameters as a function of the applied plotting speed and dispensing inlet pressure (B-E). For 10 w/v % solutions applying a conically shaped needle (B) and the influence of needle design (C). For 20 w/v % (D), and for 10 – 15 – 20 w/v % at 4 bar inlet pressure (E) using cylindrically shaped needles.

As partially described in chapter 4, the plotting was optimized in order to plot (cell-laden) gelatin precursor solutions in the range of 10 – 20 w/v %. As the deposited strut diameter will impact scaffold properties such as porosity and mechanical

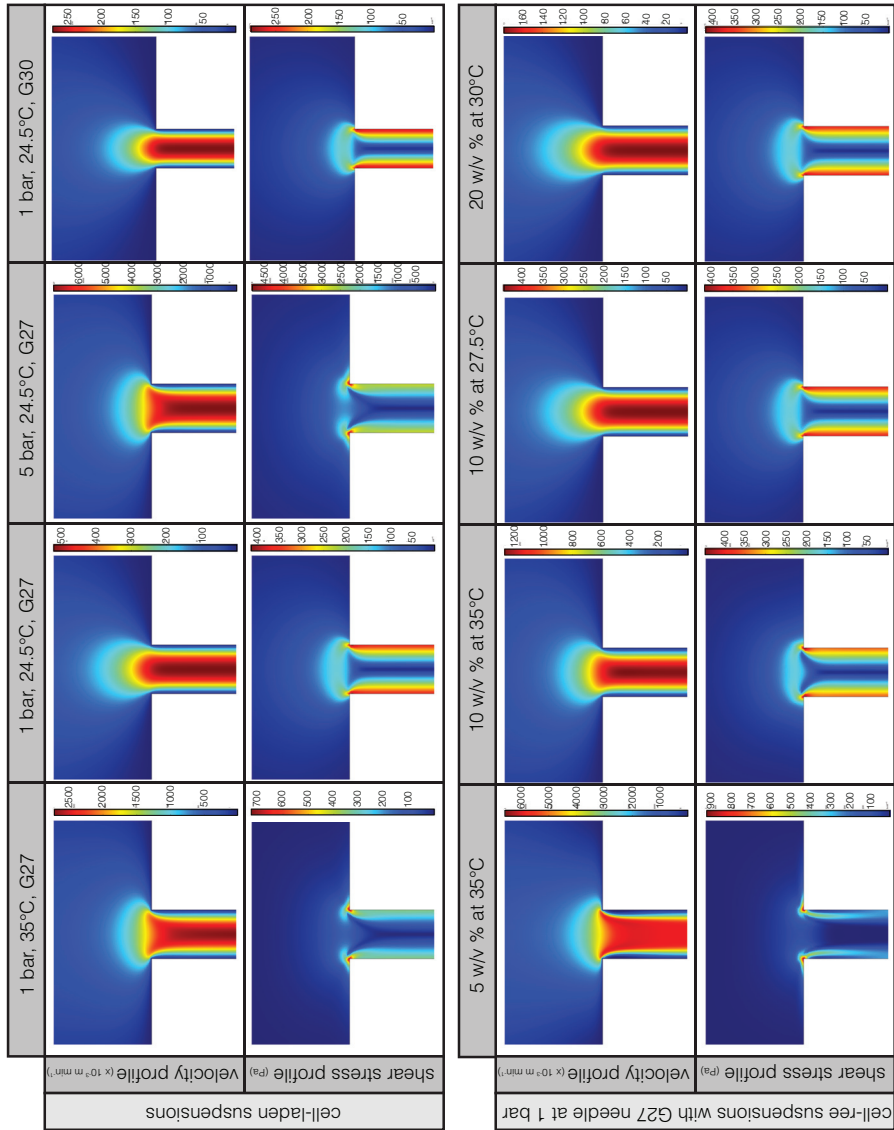


Figure 11.6: Shear stress and velocity profiles obtained by finite element simulations, illustrating effects such as: needle diameter, pressure, temperature, mixture composition, and mixture concentration.

strength, knowledge on its dimensions is desired. The influence of cells, dispensing pressure, temperature and needle internal diameter as a function of the deposition speed were discussed for (cell-laden) 10 w/v % gelatin building block mixtures using cylindrically shaped needles. In Figure 11.5, optimization data illustrating the influences of gelatin concentration, needle design (conical vs. cylindrical) and needle

internal diameter are depicted as function of the deposition speed. Additionally, a schematic representation explaining the relation between the deposition speed and deposited strut is presented in part A. Three hypothetical deposition speeds (v_1, v_{opt} and v_2) explain the importance of optimization: at the optimal nozzle traveling speed (v_{opt}), the nozzle internal diameter (D_{needle}) and the strut diameter (D_{opt}) converge leading to an optimal depositing height. For higher speeds (v_1), the depositing height (i.e. layer height) will be too big relative to the strand diameter (D_1), while for lower speeds (v_2) this will be too low. Furthermore, finite element simulations on the liquid flow behavior can help understand these effects and offer a way to relate flow behavior to cell survival rates. Some additional FEA profiles are presented in Figure 11.6.

In Figure 11.7, the influence of drying method is illustrated. Microporosity can be induced by freeze-drying. From the last μ -CT image, imperfections at the meander feature (as described in Chapter 5) are illustrated. One parameter has not yet been discussed: the scaffold porosity percentage. Considering a cube having an edge length l , the porosity percentage can be theoretically calculated based on the designed polymer volume ($V_{scaffold}$) and total volume of the scaffold, i.e. void and material volume (V_{cube}). For a lay-down pattern of $0^\circ/90^\circ$ following formulae can be used:

$$l = n_{xy} d_2 = n_z d_3 \quad (11.1)$$

$$V_{strut} = \left(\frac{\pi}{4} d_1^2 \right) l \quad (11.2)$$

$$V_{layer} = n_{xy} V_{strut} \quad (11.3)$$

$$V_{scaffold} = n_z V_{layer} \quad (11.4)$$

$$V_{cube} = l^3 \quad (11.5)$$

$$Porosity(\%) = 1 - \frac{V_{scaffold}}{V_{cube}} = \left(1 - \frac{\pi}{4} \frac{1}{\left(\frac{d_2}{d_1} \right)} \frac{1}{\left(\frac{d_3}{d_1} \right)} \right) \times 100 \quad (11.6)$$

However, these equations are not-valid for lay-down patterns where subsequent layers are rotated 45° since V_{strut} will be a function of the position and therefore differ in between adjacent struts. Also, n_{xy} can be different from layer-to-layer for “shifted” designs while for all actual scaffolds imaging revealed partial remelting at the strut knots of subsequent layers. Therefore, the calculated values of the porosity for 90° patterns are overestimated. Nevertheless, a porosity range can be calculated as presented in Table 11.1.

Although porosity by these calculations is probably overestimated, the calculated porosity ranges indicate the possibility to closely match theoretically designed porosity after optimization of the plotting process.

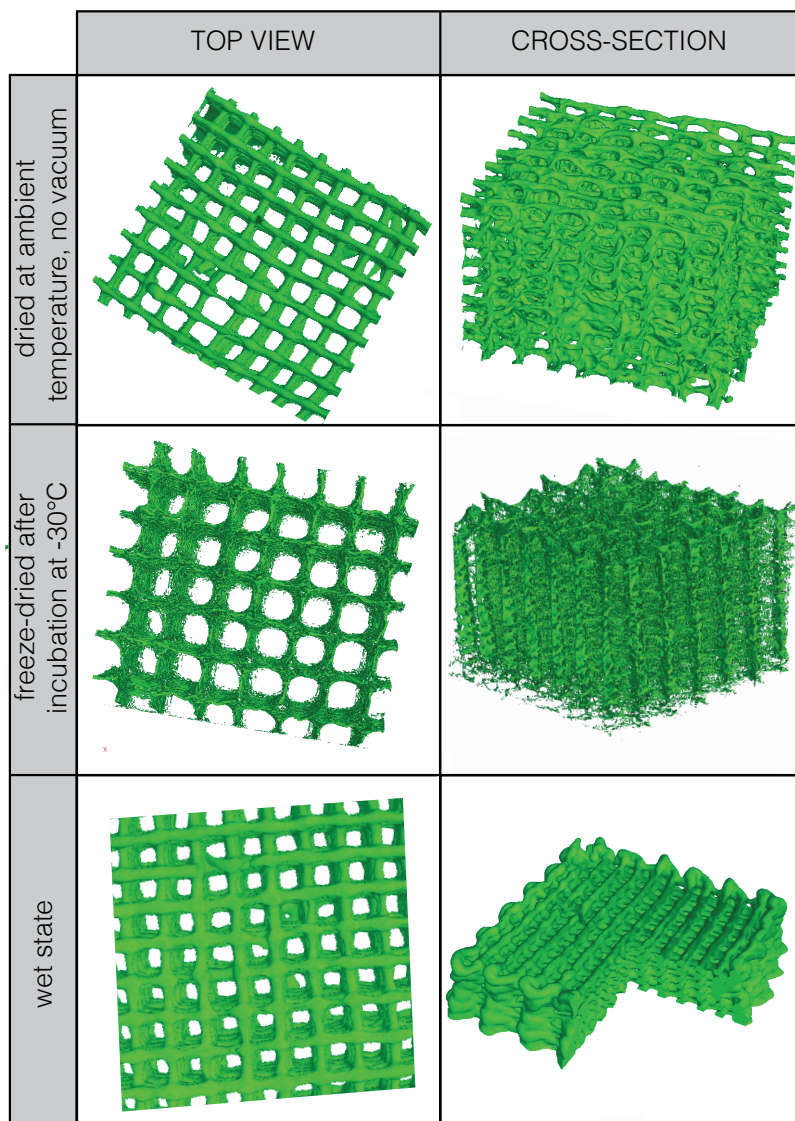


Figure 11.7: μ -CT images demonstrating the influence of the drying method on scaffold shrinkage as well as (micro)porosity.

Table 11.1: Porosity of gelatin scaffolds based on idealized geometries and for plotting with the optimized parameters. Dimension units represent μm .

SAMPLE	PATTERN	NEEDLE I.D.	DIMENSIONS _{theor.}		POROSITY _{theor.} (%)	DIMENSIONS*			POROSITY _{calc.} ** (%)
			$d_1 = d_3$	d_2		d_1	d_2	d_3	
20 w/v %	90°	200	240	500	62	219	511	212	59 - 70
	90°	200	240	600	68	238	624	224	65 - 72
	90°	200	240	700	73	245	729	234	62 - 76
	90°	200	240	800	77	246	827	242	67 - 81
	45°	200	240	600		237	638	222	
	90° + shift	200	240	700	74	234	733	229	66 - 77
10 w/v %	90°	150	180	500	72	190	520	164	61 - 71
15 w/v %	90°	200	240	700	73	254	708	233	64 - 73

* average values ** values based on upper and lower values of *

11.1.2.2 Extrusion of biodegradable polyesters

Another feature of the device is the plotting of melt polymers by means of the extrusion principle. Here, in addition to pressure, solid material is molten and an Auger screw transfers molten material to the extrusion needle. The setup is depicted in Figure 11.8.

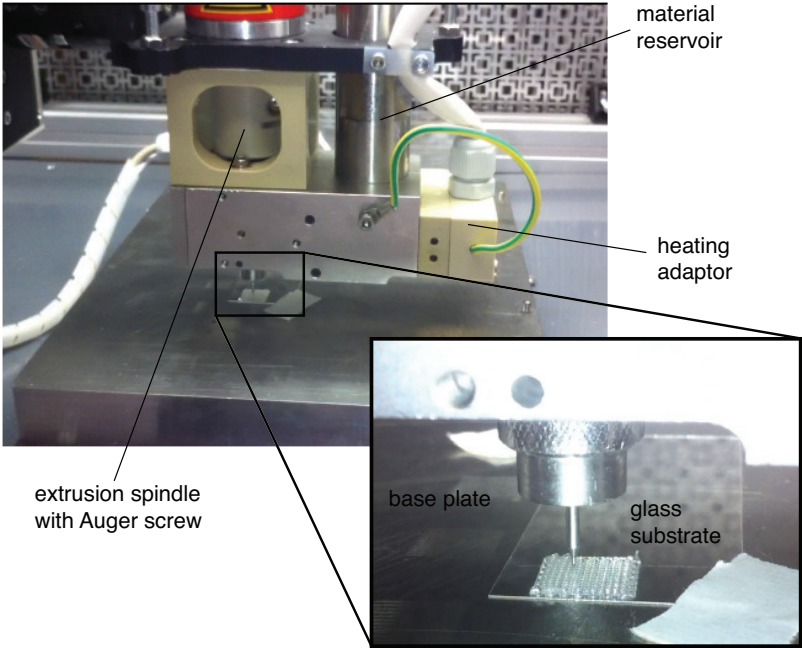


Figure 11.8: The extrusion unit operating while plotting p(D,L-LA-co-ε-CL). Glass cover slips were used as plotting substrate.

In this work, some preliminary testing on the plotting of elastic, biodegradable p(D,L-lactide-co- ϵ -CL) was performed. Glass cover slips were used as substrate. Due to continuous mixing and 100 % material deposition, the plotting conditions are more stable than in the case of dispensing and construct built-up closely resembles the virtual model as can be seen from the μ -CT images (Figure 11.9).

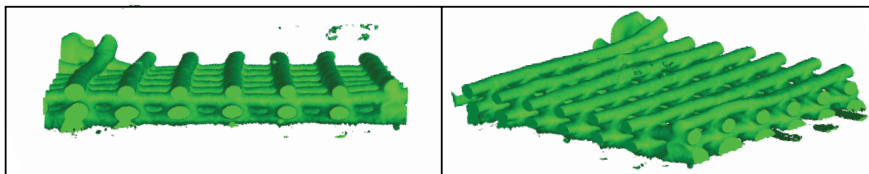


Figure 11.9: μ -CT images illustrating the extrusion of poly(D,L-lactide-co- ϵ -CL).

11.1.3 Surface plasma treatment

Other than the well-known liquid, gas, and solid aggregation states, plasma is regarded the fourth fundamental state of matter. Subjugation of materials to a plasma environment opens possibilities in modification of the materials surface.

Plasmas can be defined as quasi electrically neutral medium of positive and negative ions, electrons and radicals (also containing atoms and molecules). The large number of charge carriers make plasmas conductive so that it responds to induced electromagnetic fields. Thus, although the particles are unbound, they are not ‘free’ (i.e. moving charges generate magnetic fields which affect each other’s field, leading to a collective behavior). The typical luminosity (Figure 11.10 B) of a plasma stems from particles returning from an excited state back to their ground state.

As a result of their reactive environment, plasmas interact with material surfaces. Plasma polymerization, treatment and ablation are the main applications of plasmas. So-called inert gases, i.e. He, Ar, O₂ and N₂, will not form reactive polymerizable intermediates but create functional groups or radicals on the surface (= plasma treatment).

Typically, plasmas can be generated by excitation of gases using radio frequency (RF), microwave, or electrons from a hot filament discharge. A rough classification based on the plasma temperature can be performed: (i) non-equilibrium, i.e. non-thermal or low-temperature; (ii) equilibrium, i.e. thermal or high temperature plasmas. In this work, a cylindrical dielectric barrier discharge (DBD) setup at low pressure was applied for plasma treatment of biomaterial surfaces (Figure 11.10 A). For DBDs, the electrical discharge between both electrodes is separated by an insulating dielectric barrier.

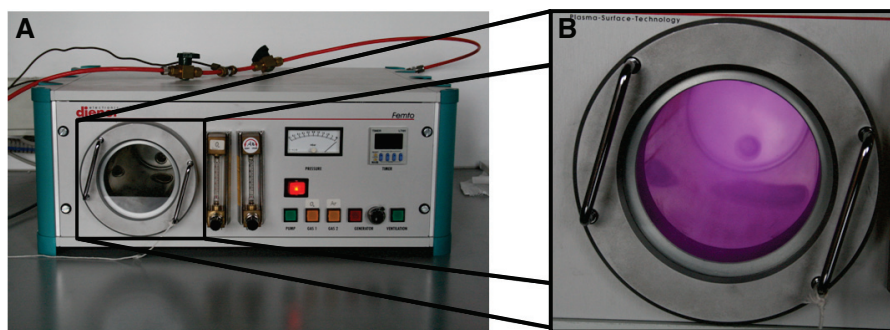


Figure 11.10: The Femto 3 (Diener Electronic) plasma reactor (A) with the plasma-discharge typical luminosity for Argon plasma (B).

The formation of plasmas by DBDs typically applies high voltage alternating currents ranging from RF to microwave frequencies. Following ionization at a certain location in the discharge gap, the dielectric surface accumulates the transported charge which generates an electrical field. Subsequently, the field in the gap is reduced, interrupting very briefly the current flow. At voltages sufficient for breakdown, microdischarges are induced. The dielectric barrier has a dual purpose: limiting charge transportation of single microdischarges, and spatial distribution of them over the entire electrode.

11.1.4 Electrospraying

Electrospraying is a technique, which can be assessed for the generation of particles from liquid solutions. The underlying principle is the physical process of inducing electric force to the liquid surface causing elongation of the liquid meniscus at the capillary outlet. The resulting cone and/or jet subsequently deforms and disrupts into small droplets. The principle was schematically presented in Figure 9.2. As a result, the droplets possess an electrical charge. The process was first reported by Bose in 1745, and later (1882) Rayleigh determined a stability criterion for the charged droplet. Zeleny (1992) established a criterion for disruption of the jet.⁴⁷⁰

Disintegration of the jet into droplets, while issuing high voltage to the capillary, can be established at many different modes. Typically, the modes are classified based on the geometry of the meniscus at needle outlet (drop, spindle, jet) and on the mechanism of disintegration (type of instability). Some typical spraying modes are illustrated in Figure 11.11. The process is governed by the physical properties of the liquid solution (surface tension, viscosity, density, and conductivity), and further also depends on the needle internal diameter, the applied voltage, and the liquid volume flow rate.

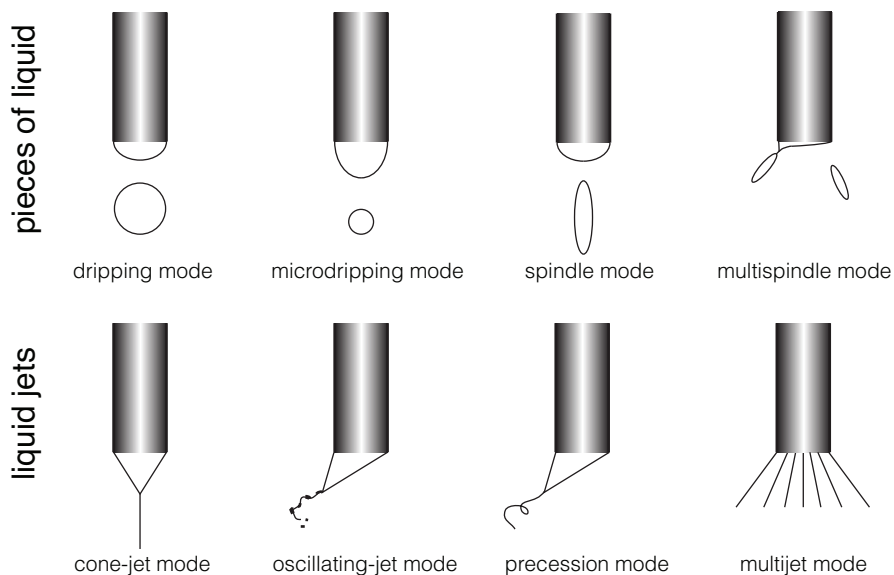


Figure 11.11: Illustration of some important spraying modes for the production of microparticles.

For an extended description on the different modes, readers are referred to the review of Jaworek and Krupa.⁴⁷⁰ In this work, non-newtonian liquids were subjected to high potential resulting mainly in dripping modes (dripping, microdripping, spindle) as illustrated by Figure 9.5.

Electrospraying and electrospinning are both techniques which are used to process polymers, based on the same underlying principle. If the formed polymer jet turns into very fine fibers instead of breaking into small droplets, the process is known as electrospinning (ESP). The main difference is based on the number of polymer chain entanglements and the concentration of the polymer solution. High concentrations are required to obtain spinning, while lower concentrations generally result in spraying. In this work, two experimental setups were used: (i) An adjusted stationary home-made ESP device, located in a wooden cabin (I, Figure 11.12). This setup was mainly used for the parametric study of the spraying process. The main components are the DC voltage generator (II) which provides a potential difference between an extrusion needle connected to a syringe pump (III) via an adjustable electrode (IV) and a stationary counterelectrode (V) on which the Ca_2^+ cross-linking bath is placed. (ii) A more mobile setup which could be constructed in a laminar flow cabin, but which consists out of the same basic components. This setup could be set up in a sterile environment and was therefore used for the spraying of cell-suspensions. In all cases, a ‘clean’ production setup was selected, i.e. no solvents other than water were applied.

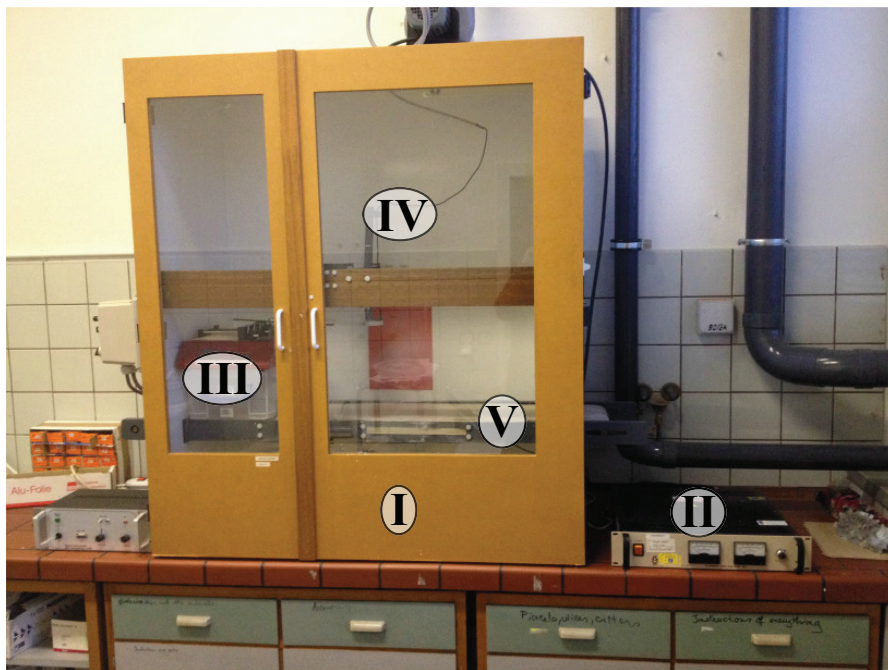


Figure 11.12: Electro spraying device used for the parametric study, located in a wooden cabin (I). Main components are the DC voltage generator (II), the syringe pump (III) and the electrodes (IV, V).

11.2 | MATERIAL CHARACTERIZATION TECHNIQUES

11.2.1 Gel yield and water uptake capacity

11.2.1.1 Equilibrium swelling studies

Swelling studies are a gravimetric sequence whereby cross-linked samples are dried directly after cross-linking (m_{di}), after swelling (to equilibrium, m_{he}) in a 100 % relative sorbate humidity, and again after drying (m_{de}). They reveal information on the gel yield and sorbate uptake of a cross-linkable hydrogel according to following formulae:

$$gel\ yield(\%) = \frac{m_{de}}{m_{di}} \times 100\% \quad (11.7)$$

$$sorbate\ uptake(\%) = \frac{(m_{he} - m_{di})}{m_{di}} \times 100\% \quad (11.8)$$

$$q = \frac{m_{he}}{m_{de}} \quad (11.9)$$

here, q is the swelling ratio. The loss of dry mass after equilibrium swelling, i.e. material leaching, is a measure for the gel yield. The relative error using Equation 11.8 when incomplete cross-linking is achieved can be overcome by expressing swelling through q , or substituting m_{di} by m_{de} .

For quantitative calculations, He-pycnometry (Accupyc 1330, Norcross, GA) was used for the determination of the volumetric changes during the experiments (Chapter 4). One cycle consisted out of 10 measurements to obtain an average volume.

11.2.1.2 Dynamic Vapor Sorption (DVS)

Dynamic Vapor Sorption (DVS) is a gravimetric technique that measures the absorption capacity of a sample by controlling the temperature and humidity of the environment as a result of varying the vapor concentration. Mass changes caused by sorption and/or desorption of the vapor molecules are accurately monitored at the hand of an ultra-microbalance. The principle is schematically presented in Figure 11.13.

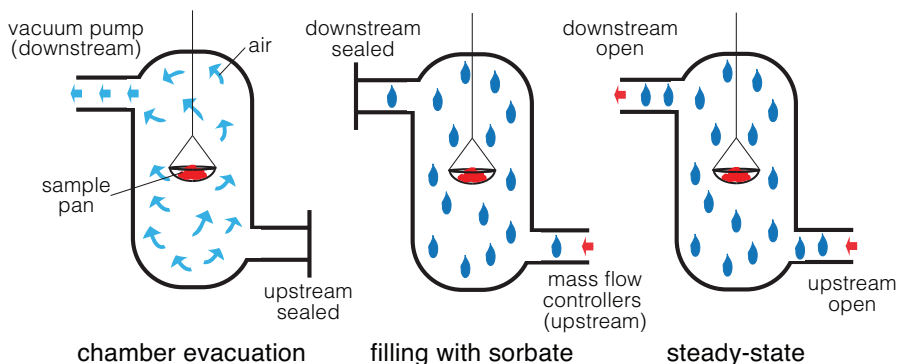


Figure 11.13: Schematic illustration of the subsequent phases during a dynamic vapor sorption measurement.

Generally, and also in this work, water vapor is exploited as sorbate, while a controlled mass flow of dry carrier gas (nitrogen) enables manipulation of the ratio of saturated to dry gas flow. The recording of water sorption isotherms, i.e. extracting data on the equilibrium vapor sorption as a function of steady-state relative humidity at a certain temperature, is the main application of DVS. For these experiments, time-dependent mass changes of a sample are monitored by cycling step changes in relative humidity. In between two steps, gravimetric equilibrium should be reached before advancing to the next step. The resulting isotherms can be divided in sorption (increasing humidity) and desorption (decreasing humidity).

The sorption part can further be divided into adsorption (surface bound sorbate) and absorption (bulk penetration of the sorbate).

The difference between 2D sheets and 3D Bioplotted structures on the water vapor isotherms was evaluated using a DVS-1 (Surface Measurement Systems). The apparatus consists of a Cahn microbalance placed in a temperature-controlled housing.

11.2.2 Rheology

The scientific domain concerning the study on the deformation behavior of solids and the flow behavior of liquids is called rheology (literally “flow science”). Large deformations resulting from shear forces cause many materials to flow, indicating the connection between both. Rheological data can be derived using rotational and oscillatory rheometers. In this work, a Physica MCR 350 (Anton Paar) rheometer consisting of a plate-plate geometry was applied (Figure 11.14). Viscous behavior is generally investigated by rotational tests, while oscillatory tests (also called dynamic mechanical analysis), relaxation tests and creep tests are typically performed in the study of viscoelastic behavior.

Shear behavior, as described by rheology, can be subdivided in between two extremes: (i) the flow of ideal viscous liquids following Newtons law (Equation 11.10), and; (ii) the deformation of ideal elastic solids, which obey Hooke’s law (Equation 11.11):

$$\tau = \eta \dot{\gamma} \quad (11.10)$$

$$\tau = G \gamma \quad (11.11)$$

with τ the shear stress, η the viscosity, $\dot{\gamma}$ the shear rate (or strain rate), G the shear modulus, and γ the shear strain. For ideal viscous liquids, the viscosity is independent from the applied deformation load (damper model). Upon removal of a deformational load, an ideal elastic solid will fully return to its initial position (spring model). The behavior of real materials however, is based on the combination of both the viscous and the elastic portion, therefore called viscoelastic behavior: viscoelastic fluids and/or solids show time-delayed (nearly complete) reformation. Oscillatory tests can be used to examine viscoelastic properties, whereby Newton’s law and Hooke’s law should be applied as follows:

$$\tau(t) = \eta^* \dot{\gamma}(t) \quad (11.12)$$

$$\tau(t) = G^* \gamma(t) \quad (11.13)$$

with η^* the complex viscosity, G^* the complex shear modulus, and the time-dependent values of the sine functions of $\tau(t)$, $\dot{\gamma}(t)$, and $\gamma(t)$. During oscillatory testing, a preset sinusoidal shear strain (Equations 11.14) or shear stress (Equations 11.15) function is applied. As a result of the viscoelastic behavior, the measured shear stress curve respectively shear strain curve is a phase-shifted sine function having the same frequency (Figure 11.14). The phase-shift is represented by the δ value and is called the loss angle. It is a measure of the time delayed reformation and is always $0^\circ < \delta < 90^\circ$.

$$\gamma(t) = \gamma_A \sin(\omega t) \rightarrow \tau(t) = \tau_A \sin(\omega t + \delta) \quad (11.14)$$

$$\tau(t) = \tau_A \sin(\omega t) \rightarrow \gamma(t) = \gamma_A \sin(\omega t + \delta) \quad (11.15)$$

here, γ_A and τ_A represent the shear strain (deformation) and shear stress amplitude, and w the frequency of the preset function.

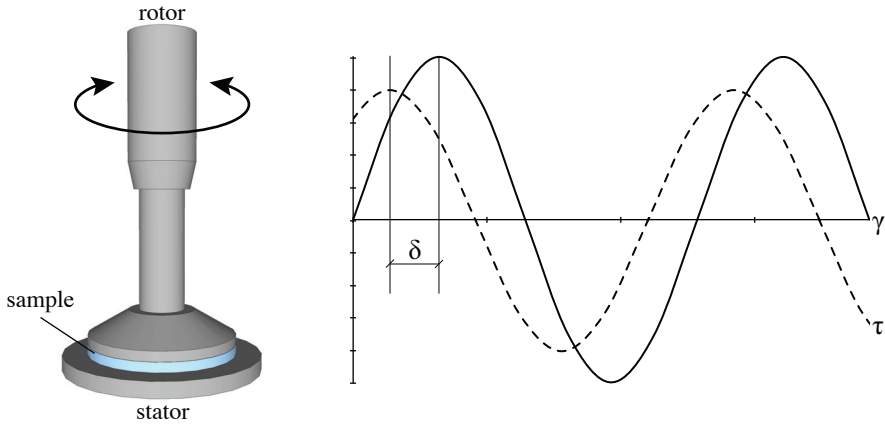


Figure 11.14: Schematic representation of the rheometer plate-plate geometry (left). Illustration of the time-delayed response $\tau(t)$ of viscoelastic materials upon a preset sinusoidal shear strain function $\gamma(t)$ (right).

In this work, controlled shear strain in the form of oscillatory sine functions were applied (Equations 11.14). Three important parameters describe the elastic and viscous behavior and the ratio of the viscous and elastic portion and characterize the viscoelastic behavior:

1. The storage modulus G' (G -prime) as a measure of the deformation energy stored during the shear process. Materials that completely store the deformation energy show reversible deformation behavior. Therefore, G' represents

the elastic behavior of a material:

$$G' = \frac{\tau_A}{\gamma_A} \cos \delta \quad (11.16)$$

2. The loss modulus G'' (*G-double-prime*) as a measure of deformation energy consumed during the shear process. Energy losing materials demonstrate irreversible deformation behavior. Thus, representing the viscous behavior of a material:

$$G'' = \frac{\tau_A}{\gamma_A} \sin \delta \quad (11.17)$$

3. The damping factor $\tan \delta$ (*loss factor*) as the ratio of the viscous and the elastic portion of a viscoelastic deformation:

$$\tan \delta = \frac{G''}{G'} \quad (11.18)$$

They relate to the complex shear modulus: $G^* = G' + i G''$. Characteristic values are represented in Table 11.2. Analogue, the complex viscosity η^* can be written as a complex form of η'' and η' , with the former specifying elastic behavior and the latter viscous behavior ($\tan \delta = \eta'/\eta''$).

Table 11.2: Overview of rheological behavior and their relation to oscillatory test variables.

ideal viscous flow behavior	behavior of a viscoelastic liquid	viscoelastic behavior showing 50/50 ratio of viscous and elastic portions	behavior of a viscoelastic gel or solid	ideal elastic deformation behavior
$\delta = 90^\circ$	$90^\circ > \delta > 45^\circ$	$\delta = 45^\circ$	$45^\circ > \delta > 0^\circ$	$\delta = 0^\circ$
$\tan \delta \rightarrow \infty$	$\tan \delta > 1$	$\tan \delta = 1$	$\tan \delta < 1$	$\tan \delta \rightarrow 0$
$(G' \rightarrow 0)$	$G'' > G'$	$G' = G''$	$G' > G''$	$(G'' \rightarrow 0)$

For hydrogels, the viscoelastic behavior was studied using dynamic oscillation measurements: (i) Deformation scans (amplitude sweep), i.e. isothermal measurements of G' and G'' as a function of shear strain at a constant frequency (1 Hz), were performed to determine the linear viscoelastic region. (ii) Mechanic spectra (frequency sweep), were recorded as a function of the angular frequency at a constant shear strain (0.1 %). (iii) Physical and chemical cross-linking was followed as a function of time at constant frequency (1 Hz) and shear strain (0.1 %) applying a normal force $F_N = 0.1$ N. (iv) Temperature sweeps at a constant frequency and shear strain were used to study the temperature dependance of G' and G'' . A linear ramp of 1°C min^{-1} was used for heating or cooling.

For liquid precursors, rotational tests were performed to determine the flow curve $\tau(\dot{\gamma})$ and viscosity function $\eta(\dot{\gamma})$ at constant temperature, illustrating shear load-dependent flow behavior (more specific shear thinning). The Ostwald-De Waele

function ($\tau = K \dot{\gamma}^n$) was used as model describing the shear thinning behavior. Here, n is the flow behavior index (< 1 for shear thinning behavior) and K the consistency coefficient. Additionally, the influence of temperature on the viscosity $\eta(T)$ was followed using rotational measurements at a constant shear rate.

11.2.3 Static Contact Angle (SCA)

Static contact angle measurements can be used as an indicator of substrate surface hydrophilic or hydrophobic properties. It measures the wettability of a solid surface by a liquid via the Young's equation based on the thermodynamic equilibrium between the three phases (solid (S), liquid (L) and gas (S)):

$$\gamma_{SG} - \gamma_{LG} \cos \theta_c = \gamma_{LS} \quad (11.19)$$

whereby γ_{SG} represents the solid-gas interfacial energy, γ_{LS} the liquid-solid interfacial energy, γ_{LG} the liquid-gas surface tension, and θ_c the Young's contact angle at equilibrium. Generally, the wettability characteristics can be subdivided based on the contact angle (Figure 11.15): (i) non-wetting ($\theta_c > 90^\circ$), (ii) partial wetting ($0^\circ \leq \theta_c \leq 90^\circ$), (iii) complete wetting ($\theta_c > 0^\circ$).

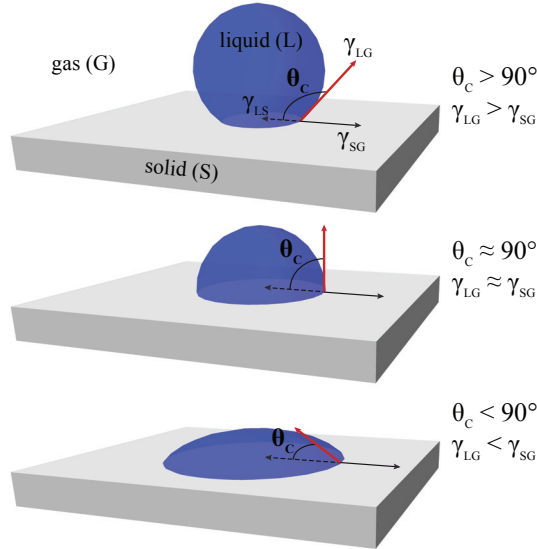


Figure 11.15: The physical principle behind static contact angle measurements with the interfacial energy between solid and gas (γ_{SG}), the surface tension between liquid and gas (γ_{LG}) and the interfacial energy between liquid and solid (γ_{LS}) acting as determinants for the contact angle (θ_c).

The sessile drop method was used for contact angle measurements in air on an

OCA 20 (Dataphysics) instrument, and Laplace-Young fitting on the collected movies for determination of the contact angle. Water was used as liquid phase, and substrates were prepared by spincoating on glass cover slips. Laplace-Young fitting presumes smooth surfaces subjected to uniaxial liquid spreading (so the drop is circular when viewed from above):

$$\Delta p = \frac{2\gamma_{LG} \cos \theta_c}{R} \quad (11.20)$$

with Δp the pressure drop across the liquid interface, and R the radius of the circular cross-section. For water-air, γ_{LG} is 0.0728 J m^{-2} . However, physical factors, especially surface roughness, impact on the resulting wettability of a substrate.

11.2.4 Atomic Force Microscopy (AFM)

The working principle behind Atomic Force Microscopy (AFM) is schematically presented in Figure 11.16. It is a technique that can be classified as material surface image mapping and material surface characterization and is based on the interaction forces between a very thin needle, the cantilever probe, and a material surface.

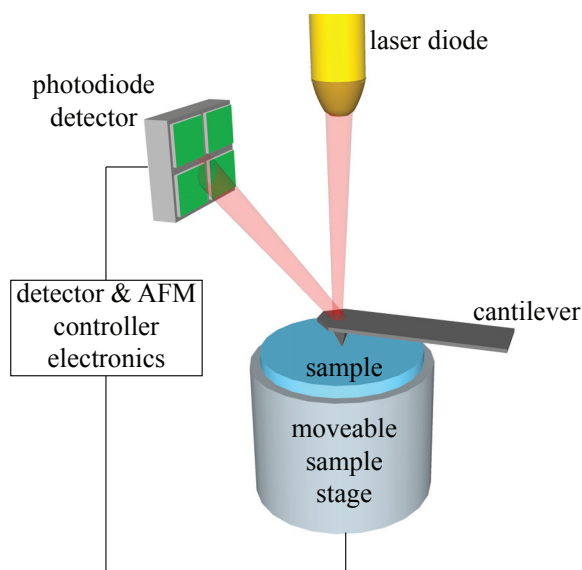


Figure 11.16: Illustration of the AFM working principle: a probe attached to the cantilever scans a sample surface area leading to variations in the cantilever deflection (due to interaction forces). The deflections are recorded by an array of photodiodes on which a reflecting laser spot is projected.

AFM is a type of scanning probe microscopy, whereby a mechanical probe (silicumnitride probe mounted onto the cantilever) gathers information by “feeling” the

surface of a material at a resolution in the nanoscale. By bringing the tip into proximity of a sample surface, the resulting varying interaction forces (ΔF) cause a cantilever deflection (Δz) according to Hooke's law ($\Delta F = k \Delta z$, whereby k represents a constant factor, the stiffness, of the probe). The imposed Δz deflection is typically recorded using a laser spot reflected from the top surface of the cantilever into an array of photodiodes (Figure 11.16). Gathering data by XY-translations makes spatial mapping of the interactions possible. The measured interaction forces can include, depending on the situation, mechanical contact force, Van der Waals forces, chemical bonding, electrostatic forces, ...

Three distinct modes of operation can be distinguished: direct contact mode, non-contact mode, and tapping mode. The first is a static mode whereby the interaction (deflection) between tip and surface is kept constant during data recording. Thus, the deflection is used as feedback signal and surface topology is directly measured resulting in high resolution but possibly destructive imaging. Therefore, contact mode is mostly performed when the resultant forces are repulsive.

For soft materials, both other modes are more suitable and dynamic of nature. The cantilever is oscillated by a piezoelectric element close to its resonance frequency and the oscillation amplitude, phase and frequency are altered by the interaction forces. These variations provide characteristic information on the sample topology. In non-contact mode, the oscillation amplitude or frequency is kept constant by adjustment of the average tip-to-sample distance. Typically, Van der Waals forces act as main interaction forces. The mode is non-destructive, however resolution is impaired. Tapping mode more or less combines the principles of both contact and non-contact mode. Thus, resulting in a higher resolution due to sample contact, while lessening the sample damage. The image is generated by measuring the interaction forces of the intermittent contact between the tip and the sample surface.

A multimode scanning probe microscope (Digital Instruments - USA) equipped with a Nanoscope IIIa controller was used for image recording in "tapping mode". Thin film samples were prepared by spincoating on glass cover slips.

11.2.5 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy is a quantitative spectroscopic technique allowing to evaluate the elemental surface composition of a sample (1 – 10 nm). XPS is also called ESCA (Electron Spectroscopy for Chemical Analysis), and is based on low-energy X-ray interaction with surface atoms: the photoelectric effect (Figure 11.17)

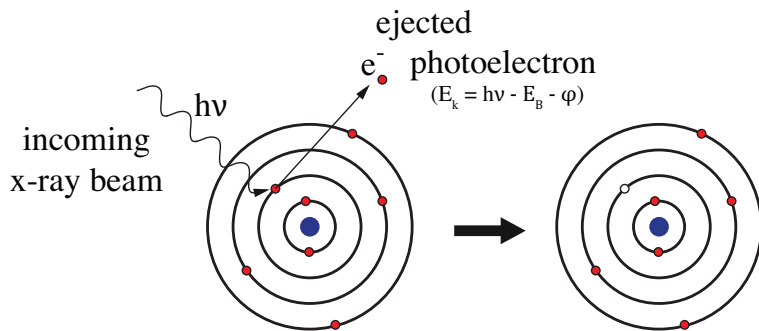


Figure 11.17: The principle of XPS (the photoelectric effect): emission of electrons occurs after absorption of an X-ray.

During an XPS measurement, the kinetic energy and number of emitted electrons (photoemission) escaping from the top 1 – 10 nm of the sample, due to irradiation with/absorption of an X-ray beam, are analyzed. The generated data are typically processed as a plot of the number of detected electrons in function of their binding energy, i.e. the survey scan. Since each element relates to a characteristic set of peaks at characteristic binding energies, corresponding to the electron configuration, and the detected number of electrons is directly related to the element amount, these spectra reveal information on the elemental composition of the surface layer. The technique requires ultra-high vacuum conditions to accurately count the electron numbers at each kinetic energy value.

For insulating sample materials (no well-defined Fermi level), the kinetic energy (E_K) can be calculated as a function of the X-ray photon energy ($h\nu$), the electron binding energy (E_B) and a factor that represents the spectrometer work function (φ , typically around 4.5 eV):

$$E_K = h\nu - E_B - \varphi \quad (11.21)$$

Additional spectra related to the chemical states of a detected element were retrieved: a detail scan. Deshielding of the nucleus positive charge due to electronegative effects of α - and β -neighboring atoms causes variations in the necessary energy to emit an electron, therefore revealing information on the chemical state of the element in consideration.

XPS spectra were collected on a ESCA S-probe VG monochromatized spectrometer with an Al $K\alpha$ X-ray source (1486 eV). In this work, the C-C/C-H signal was chosen as reference and set on 285 eV. Dr. Tim Desmet, Valérie Kodeck, Diana Giol, and dr. Els Vanderleyden were my colleagues responsible for XPS measurements.

11.2.6 High Resolution-Magic Angle Spinning (HR-MAS) NMR spectroscopy

Conventionally, NMR spectroscopy applying high resolution characterizations is limited to samples in solution, while Magic Angle Spinning (MAS) is reserved for solid materials. Spinning a sample (usually at 1 – 70 kHz) at the Magic Angle (θ_M , $\cos 2\theta_M = 1/3$) with respect to the direction of the magnetic field (B_0) improves the resolution of the normally broad lines, thus making identification and spectrum analysis better. The principle is illustrated in Figure 11.18

The dipole-dipole, chemical shift anisotropy and quadrupolar interactions lead to spectrum broadening. By utilizing MAS (θ_M), the time-dependent interactions are averaged: (i) the nuclear dipole-dipole interactions are averaged to zero; (ii) the chemical shift anisotropy to a non-zero value, and; (iii) the quadrupole partially averaged. For solutions, these interactions typically average out due to rapid time-averaged molecular motions. MAS mimicks this principle, leading to narrower signals.

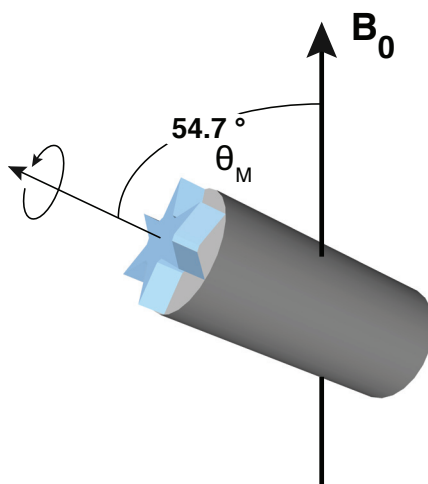


Figure 11.18: Magic Angle Spinning principle: an NMR rotor containing the sample is spinning at a high frequency, tilted by the magic angle (θ_M) relative to the principle axis of the imposed magnetic field (B_0).

High Resolution-Magic Angle Spinning is a variation on the MAS principle, especially suitable for materials in between both states, i.e. solutions and gels containing some internal molecular motion, but where the dipole-dipole interactions are insufficiently averaged by their internal molecular motion. Broadening caused by these dipole-dipole interaction is averaged out, resulting in the ability to apply most of the high resolution quantitative characterization techniques.

In this work, ^1H HR-MAS measurements were performed to quantify the cross-linking kinetics of photocross-linkable gelatin derivatives. The ^1H NMR spectra were recorded on a Bruker Avance II spectrometer operating at a ^1H frequency of 700.14 MHz and a spinning frequency of 6 kHz for 4 mm HR-MAS rotors. These measurements were performed at the NMR and Structure Analysis Unit (UGhent) in collaboration with dr. Björn Van Gasse and prof. dr. José Martins.

11.3 | IMAGING TECHNIQUES

11.3.1 Scanning Electron Microscopy (SEM)

A scanning electron microscope (SEM) scans samples with a high-energy beam of electrons to collect backscattered or secondary electrons for imaging (Figure 11.19). The captured electrons contain data on the sample surface topography as well as the composition, and magnification potential is orders of magnitude higher than that of optical microscopes.

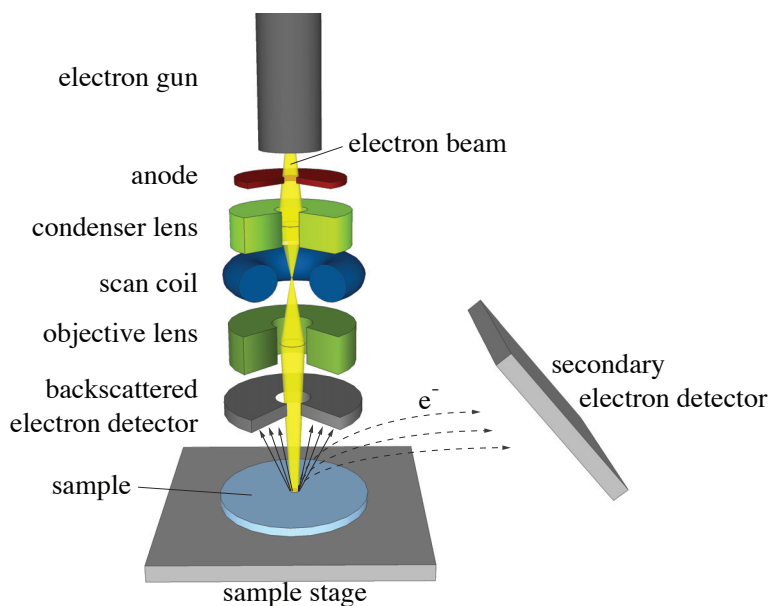


Figure 11.19: A schematic representation of the construction and working principle of a SEM device: a focussed electron beam scans the sample surface and the resultant emission of electrons is used to reconstitute the surface image.

The incident electron beam is focussed on the sample surface to a spot of 1 – 5 nm by the condenser and objective lenses. Between the lens systems, a magnetic field

from the scan coils deflect the beam to scan the surface. Impact of the primary electrons onto the sample surface results in the emission of secondary electrons, the backscattering of electrons (by elastic scattering), and the emission of characteristic X-rays. Collecting of the secondary electrons leads to high resolution reconstructed images, while using backscattered electrons for image reconstruction results in a deeper sampling depth and thus contains information on element distribution. The characteristic X-ray spectrum can be used to study the distribution of the chemical elements.

In this work, a FEI tabletop SEM instrument was used for imaging of hydrogel samples in Secondary Electron Impact (SEI) imaging mode. Prior to analysis, the samples were sputter coated with an approximately 20 nm gold layer.

11.3.2 Micro-Computed Tomography (μ -CT)

Micro-Computed Tomography (μ -CT) is a non-destructive 3D visualisation technique with resolution in the micrometer scale, useful for the visualization of pore network interconnectivity of plotted constructs. The principle is illustrated by Figure 11.20. X-rays create cross-sections of a 3D-sample, which can later be reconstructed to a virtual model.

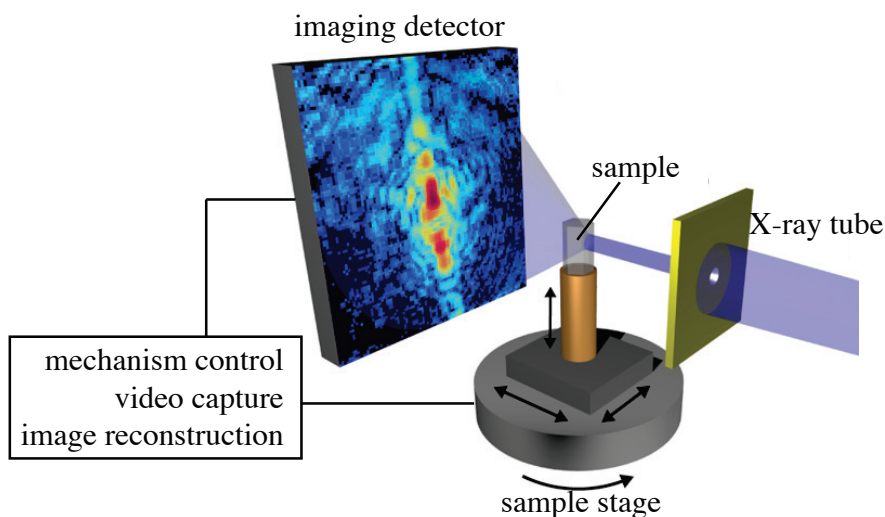


Figure 11.20: Schematic illustration of a static μ -CT setup whereby the sample is rotated during X-ray irradiation. The resulting projection images are subsequently reconstructed to form a three-dimensional image of the sample.

Using reconstruction software algorithms, the capture projection images are used to reconstruct the image cross-sections. The resultant slices can subsequently be

stacked and rendered to obtain a 3D virtual model. It is possible to determine the porosity of a 3D scaffold using this technique. However, due to the time consuming calculations necessary as well as the expensive technique as such, idealized design calculations were preferred.

μ -CT of 3D plotted scaffolds was performed using a VARIAN Paxscan 2520 V Flatpanel detector and a Feinfocus X-ray tube. The resulting tomography data has been reconstructed at a voxel size of $8\text{ }\mu\text{m}$ using *VGstudioMAX*. Images were taken at the UGhent Centre for X-ray Tomography (UGCT) in collaboration with Thomas De Schryver.

11.3.3 (Confocal) Fluorescence Microscopy

Next to optical light microscopy, cell biology frequently makes use of fluorescence microscopy (biological fluorescent stains, immunofluorescence). For fluorescent imaging, a sample is irradiated at a specific wavelength which gets absorbed by a fluorophore (from a fluorescent molecule within the sample) causing exciting of some electrons and leading them to transfer to an excited state (Figure 11.21).

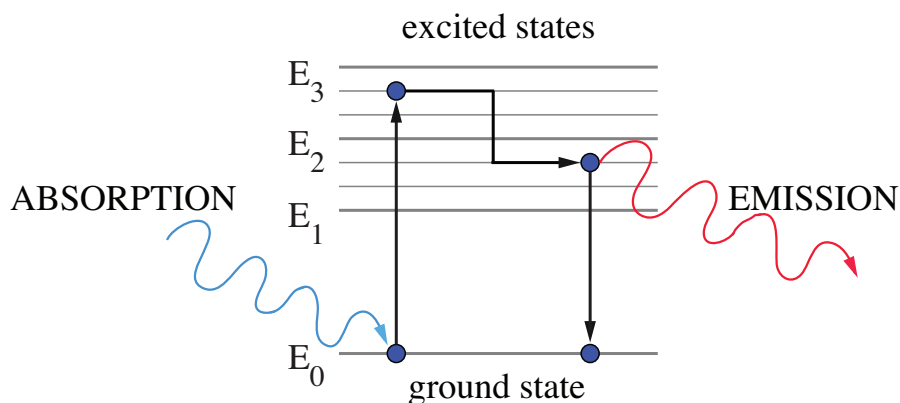


Figure 11.21: Fluorescence: absorption of energy of a certain wavelength by a fluorophore causes electron excitation which, upon relaxation to the ground state, dissipates longer wavelength energy.

Upon relaxation, the excited electron will dissipate part of its energy by emitting light at a lower wavelength than the incident beam.

The majority of these microscopes are of the epifluorescence design, i.e. excitation of the fluorophore and detection of the fluorescence occurs on the same light path. However, this design is subjected to high signal to noise ratio. A more advanced design, confocal (fluorescence) microscopes, aim to improve the optical resolution and contrast compared to the conventional design. Using point illumination

combined with a pinhole in an optically conjugate plane to eliminate out-of-focus signal, only fluorescence near the focal plain is detected enhancing the resolution. Long exposures are often required to overcome the decreased signal intensity due to pinhole blocking. As a result of the point illumination, 2D or 3D imaging is possible by scanning the sample. The focal plane thickness is a among other function of the optical properties of the specimen, but mainly dependent on the wavelength of the used light. The thin optical slicing is especially suitable for 3D imaging and surface profiling.

An inverted fluorescence microscope (Olympus IX81) equipped with *Xcellence* software (Olympus) was used for conventional fluorescence and phase-contrast imaging. The majority of cell culture and biological assays were a close collaboration with Elien Gevaert from the Tissue Engineering Group (UGhent) of prof. dr. Ria Cornelissen.

Confocal fluorescence microscopy (CFM) was performed on a Leica SP5 confocal laser scanning microscope with a 40X 1.25 oil immersion objective. Digital depth slices were reconstructed using *Velocity* image analysis software. Confocal imaging occurred at the Bio-imaging unit (VIB) in collaboration with dr. Eef Parthoens.

11.4 | OTHER TECHNIQUES

11.4.1 Amino acid analysis

For a number of characterizations, the amino acid composition of the used gelatins was desired. These analyses were performed by dr. Barbara Kerkaert from the nutriFOODchem group (UGhent). The technique is based on a HPLC quantitative detection of hydrolyzed gelatin samples.

11.4.2 OPA assay

Quantification of the primary amine (of gelatins) consumption during derivatization reactions were determined spectrophotometrically by orhto-phthalic dialdehyde (OPA) assay. The reaction scheme of OPA is given in Figure 11.22.

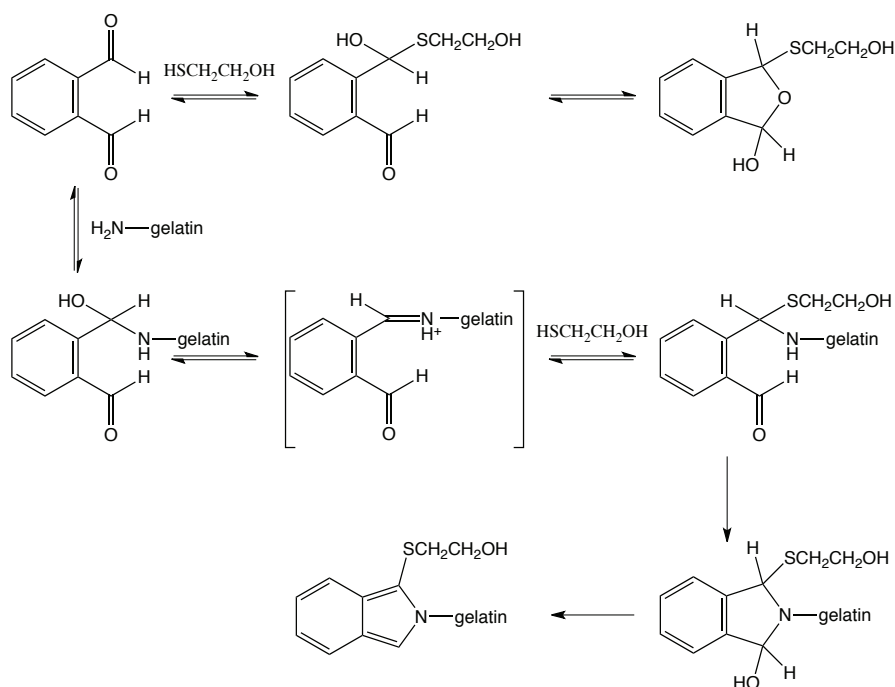


Figure 11.22: Reaction scheme of ortho-phthalic dialdehyde with the primary amines of gelatin.

Iso-indol chromophores are formed during the reaction, which possess an absorbance maximum at a wavelength of 335 nm ($\epsilon = \pm 104 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction proceeds fast and amine consumptions can be accurately obtained using calibration curves (in this work n-butylamine). A UVIKON XL (Biotek) UV-VIS spectrophotometer was used.

11.4.3 Galactose oxidase assay

In the case of gelatin derivatization with 2-lactobionic acid analogues (bulk grafting and surface grafting), a direct quantification of the incorporated galactose moieties was performed using the Amplex Red Galactose/Galactose Oxidase Assay Kit. During reaction of galactose units with galactose oxidase and amplex red reagent (Figure 11.23), the resultant resorufin chromophore shows an absorption maximum at a wavelength of 572 nm.

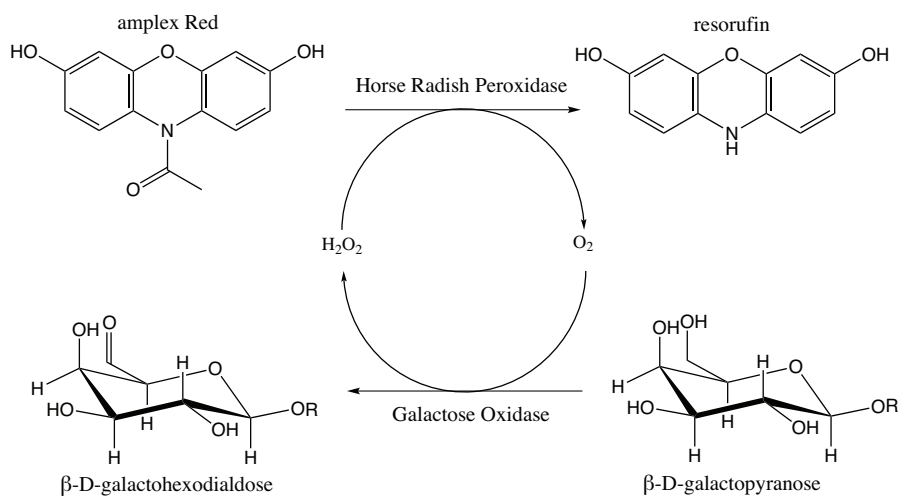


Figure 11.23: Reaction sequence of the Amplex Red Galactose/Galactose Oxidase assay.

LIST OF ABBREVIATIONS

μ-CT	Micro-computed tomography
μ-SLA	Micro-stereolithography
¹ H NMR	Proton nuclear magnetic resonance
2PP	Two-photon polymerization
3DP	3D printing
AA	Acrylic anhydride
	Gelatin acrylamide
ADSC	Adipose derived stem cell
AEMA	2-Aminoethyl methacrylate
AFM	Atomic force microscopy
ASGPR	Asioglycoprotein receptor
ATR	Attenuated total reflection
ATRP	Atom transfer radical polymerization
BAEC	Bovine aortic endothelial cell
BAL	Bioartificial liver device
BioLP	Biological laser printing
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
CAD	Computer assisted design
Calcein-AM	Calcein acetoxymethylester
CAM	Computer assisted modeling
cDNA	Complementary DNA
CFM	Confocal fluorescence microscopy
CHO	Chinese hamster ovary
CQ	Qamporquinone
Cx32	Connexin 32
Cyp1A2	Cytochroom P450 1A2
Cyp3A4	Cytochroom P450 3A4
DAPI	4',6-diamidino-2-fenylindool
DBD	Dielectric barrier discharge
DG	Degree of galactosylation
DIW	Direct ink writing
DLP	Digital light projection
DMD	Digital Micro-mirror Device

DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DS	Degree of substitution (for functionalization with (meth)acrylamides)
DTPH	dithiopropionylhydrazide
DVS	Dynamic vapor sorption
E-cad	E-cadherin
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EHDA	Electrohydrodynamic atomization
ELISA	Enzym-linked immunosorbent assay
EO	Ethylene oxide
ES	Embryonic stem cell
ESP	Electrospinning
FBS	Fetal bovine serum
FDM	Fused deposition modeling
FEA	Finite element analysis
FITC-BSA	Fluorescein isothiocyanate coupled bovine serum albumin
FITC-DEX	Fluorescein isothiocyanate coupled dextran
FITC-GAM	Fluorescein isothiocyanate conjugated goat anti-mouse IgG antibody
FMOC	Fluorenylmethyloxycarbonyl chloride
FT-IR	Fourier transform infrared spectroscopy
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GelA	Type A gelatin
GelB	Type B gelatin
GelGAL	Galactose-functionalized gelatin
GelGAL-MOD	Galactose-functionalized gelatin having methacrylamide moieties
Gel-MOD	Methacrylamide-functionalized gelatin
GelMOD-GAL	Methacrylamide-functionalized gelatin having galactose moieties
GRAS	Generally recognized as safe
HA	Hyaluronic acid
HDF	Human dermal fibroblast
HEMA	Hydroxyethylmethacrylate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Hepatocarcinoma cell line
HFP	1,1,1,3,3,3-Hexafluoro-2-propanol

HMVEC	Human microvasculature endothelial cells
HMW	High molecular weight
HPLC	High-performance liquid chromatography
HNF4 α	Hepatocyte nuclear factor 4 alpha
HR-MAS	High resolution-magic angle spinning
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cell
I2959	Irgacure 2959
IEP	Isoelectric point
IHC	Immunohistochemistry
ITX	isopropyl thioxanthone
LA	Lactobionic acid
LAEMA	2-Lactobionamidoethyl methacrylate
LAD	Liver assist device
LCD	Liquid crystal display
LCST	Lower critical solution temperature
LDM	Low-temperature deposition modeling
LMW	Low molecular weight
LWUV	Long wave ultraviolet
MA	Methacrylic anhydride Gelatin methacrylamide
MAPLE DW	Matrix assisted pulsed laser evaporation direct write
MAPS	Microwave assisted polymerization synthesis
M(-L)DM	Multi-nozzle (low-temperature) deposition modeling
MJS	Multiphase jet solidification
MRI	Magnetic resonance imaging
MSC	Multipotent stromal cell
mTGase	Microbial transglutaminase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
MWD	Molecular weight distribution
NC	Numerical code
NHS	N-hydroxysuccinimide
NVP	N-vinyl pyrrolidone
OPA	2-orthophtalic dialdehyde
OPTN	Organ procurement and transplantation network
PAA	Poly(acrylic acid)

PAH	Poly(allylamine) hydrochloride
PAM	pressure-assisted microsyringe
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCL	Poly(ϵ -caprolactone)
PCL-LPS	Poly(ϵ -caprolactone)-liposaccharide
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDI	Polydispersity index
PDL	Poly(D-lysine)
PED	Precision extrusion deposition
PEG	Poly(ethylene glycol)
PEG-D(M)A	Poly(ethylene glycol) di(meth)acrylate
PEI	Poly(ethylene imine)
PEM	Precise extrusion manufacturing
PEO	Poly(ethylene oxide)
PET	Polyethylene terephthalate
PGA	Poly(glycolic acid)
PI	Photo-initiator
PLA	Poly(lactic acid)
PLGA	Poly(D,L-lactide-co-glycolide)
Plu F127	Ploxamer 407
PMA	Poly(methyl acrylate)
PNIPAAm	Poly(N-isopropylacrylamide)
PNIPAM	Poly(N-isopropylacrylamide)
PO	Propylene oxide
PPO	Poly(propylene oxide)
PVA	Poly(vinyl alcohol)
PVP	Poly(vinyl pyrrolidone)
PZT	Piezoelectric transducer
RGD	Arginine-Glycine-Aspartic acid
RF	Radio frequency
RH	Relative humidity
RM	Rapid manufacturing
RNA	Ribonucleic acid
RP	Rapid prototyping
RPBOD	Rapid prototyping robotic dispensing
RT	Rapid tooling
SCA	Static contact angle

SEC	Size exclusion chromatography
sECM	Synthetic extracellular matrix
SEM	Scanning electron microscopy
SFF	Solid freeform fabrication
SGC	Solid ground curing
SLA	Stereolithography
SLS	Selective laser sintering
SRTR	Scientific registry of transplant recipients
STL	Stereolithography
TCP	Tissue culture plastic
TE	Tissue Engineering
TEMED	N,N,N',N'-tetramethylethylenediamine
TFE	2,2,2-Trifluoroethanol
TFA	Trifluoroacetic acid
TGase	Transglutaminase
TGF β	Transforming growth factor beta
TIA	Titanium diisopropoxide bisacetylacetonate
TLC	Thin layer chromatography
TMC	Trimethylane carbonate
TMP	Trimethylolpropane
TRITC	tetramethylrhodamine
TTR	Transthyretin
UCST	Upper critical solution temperature
UV(-A)	Ultraviolet (A)
UV-VIS	Ultraviolet-visible light spectrophotometry
VA-086	2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide]
VDI	Volume-driven injection
XPS	X-ray photoelectron spectroscopy

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