Degradable Cell-Adhesive Hybrid Hydrogels by Crosslinking of Gelatin with Poly(2-isopropenyl-2-oxazoline)

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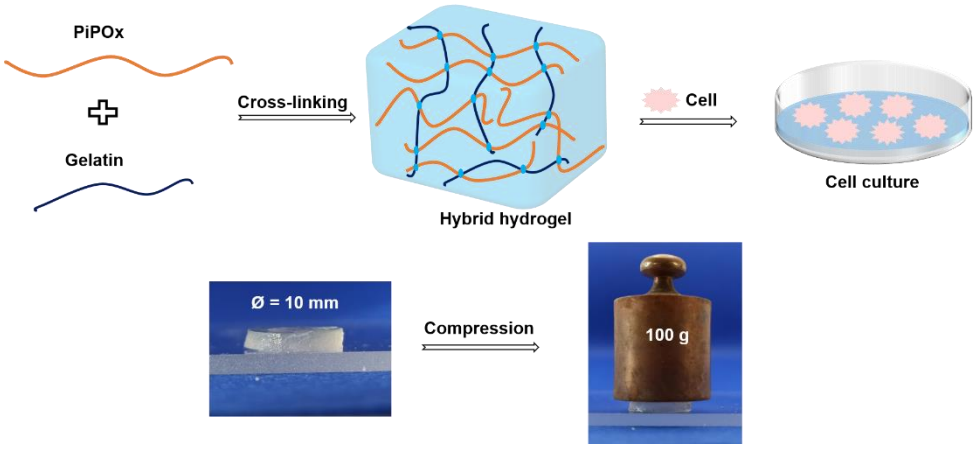
Graphical abstract:



ABSTRACT

This study focused on cross-linking of poly(2-isopropenyl-2-oxazoline) (PiPOx) with gelatin to obtain strong, degradable hybrid hydrogels with good cell adhesion. The molecular weight and concentration of PiPOx, and the PiPOx-to-gelatin ratio were varied to adjust the mechanical and swelling properties of the hybrid hydrogels. The swelling degree of PiPOx-gelatin hydrogels in water ranged between 1260 % and 810 %, with corresponding Young's compressive moduli ranging from 77 to 215 kPa. Rheological measurements demonstrated the mechanical stability of hydrogels. The hydrogels exhibited substantial degradation in Dulbecco's phosphate-buffered saline (DPBS) and cell culture medium within several weeks, indicating their degradability and responsiveness. The cell adhesion assay with primary human foreskin fibroblasts revealed the hybrid hydrogels are non-cytotoxic and support cell attachment and proliferation. These strong hydrogels thus show excellent potential as biomedical cell scaffolds, combining the tunability and strength of PiPOx hydrogels with gelatin’s cell-interactive properties while the ester-containing crosslinks provide tunable degradability.

1. INTRODUCTION

Hydrogels are an important class of soft materials composed of a polymer network structure that can absorb and retain substantial amounts of water or biological fluids.[1] Natural and synthetic hydrogels are ubiquitous in our daily lives and have been extensively studied from basic scientific principles to practical applications.[2] In particular, hydrogels can mimic extracellular matrices, making them one of the most promising scaffold materials for tissue engineering.[3-6]

Gelatin, as a natural polymer material derived from the hydrolysis of collagen, is biocompatible, bioactive, and biodegradable. Gelatin undergoes an upper critical solution temperature phase transition from solid to liquid in aqueous media resulting from structural changes from a helical to a coiled form.[7] Therefore, the use of physically crosslinked gelatin hydrogels in tissue engineering is limited by the sol-gel transition of gelatin solutions at physiological temperatures.[8] Another limitation for applications is that physical gelatin hydrogels barely exist at body temperature.

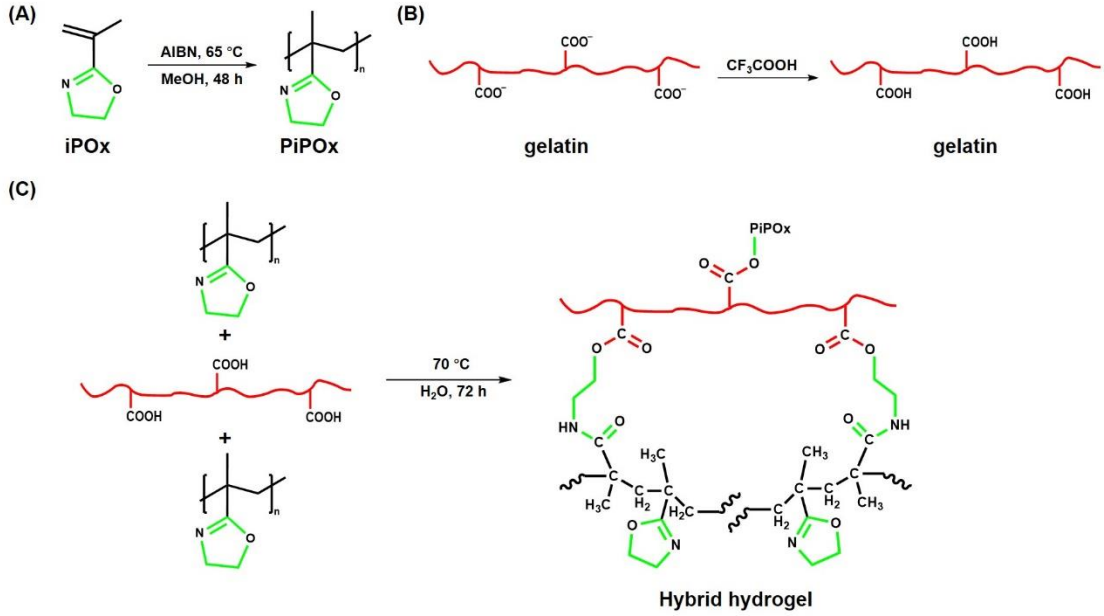
Chemical crosslinking can be used to increase the stability of gelatin hydrogels at higher temperatures. For example, the functionalization of gelatin with methacrylamide and methacrylate residues provides methacryloylated gelatin (also known as GelMA or GelMod) that retains cell adhesion capability and biodegradability.[9,10] However, chemically crosslinked gelatin hydrogels that are stable at body temperature usually still have relatively poor mechanical properties, easily deform in response to relatively small external forces, and show stiffness (Young's modulus) well below 100 kPa.[11,12] Therefore, developing gelatin hydrogels that have strong at body temperature represents an important research challenge in the biomedical field.

Poly(2-isopropenyl-2-oxazoline) (PiPOx) is a hydrophilic and highly biocompatible synthetic polymer.[13] In contrast to other poly(2-oxazoline)s that are obtained by cationic ring-opening polymerization of the corresponding monomers, such as 2-ethyl-2-oxazoline or 2-

methyl-2-oxazoline, 2-isopropenyl-2-oxazoline (iPOx) is polymerized through its alkenyl side-chain substituent, thus retaining the 2-oxazoline rings in the resulting polymer structure (**Scheme 1A**). Free radical or controlled polymerization of iPOx has been reported *via* anionic polymerization, reversible addition−fragmentation chain-transfer, atom transfer radical polymerization, and metal-mediated group transfer polymerization.[14-19] The pendant 2-oxazoline side groups of PiPOx are available for post-polymerization reactions with carboxylic acids, allowing for modification and crosslinking.[15,20-25] The versatility of PiPOx platform has been explored for the synthesis of many polymeric networks with various applications including water purification,[26] drug delivery,[27] antibacterial properties,[22] detection and sensing.[28,29] Nevertheless, synthetic polymers such as PiPOx lack cell-adhesive properties limiting their biomedical use. Thus, combining the PiPOx with gelatin would result in hydrogels with increased mechanical stability, degradability, and cell-interactive properties as novel responsive materials. Moreover, because gelatin contains carboxylate groups that can be converted to carboxylic groups by protonation, PiPOx may be used directly as a crosslinker avoiding additional synthetic steps for the modification of gelatin and usage and/or usage of toxic low molar mass crosslinkers.

Herein, we report a facile strategy to prepare degradable strong gelatin-based hybrid hydrogels in water as solvent. PiPOx of different molecular weights were synthesized by free radical polymerization (**Scheme 1A**) to be used as a high-molecular-weight crosslinker of gelatin. Gelatin (type B) was first pretreated with trifluoroacetic acid (TFA) to protonate its carboxylate groups and make them susceptible to reacting with the pendant 2-oxazoline rings. TFA was selected as strong acid, so that a small excess was sufficient to protonate all carboxylate groups. When mixing with PiPOx with protonated gelatin, the 2-oxazoline rings are protonated by the carboxylic groups followed by attack of the resulting carboxylate onto the 2-oxazolinium cation, (**Scheme 1B**). Hence, the PiPOx-gelatin hydrogels were synthesized *via* crosslinking between the 2-oxazoline side groups of PiPOx and the carboxylic acid groups of gelatin (**Scheme 1C**) at elevated temperature. The hybrid hydrogels were characterized in

terms of swelling and mechanical properties, degradability and cultured with primary human skin fibroblasts to evaluate their potential in biomedical applications. The proposed strategy can offer a simple and straightforward method for the synthesis of strong gelatin-based hydrogels that alleviate the problems related to gelatin modification.



Scheme 1. Preparation of the PiPOx-gelatin hydrogels: (A) PiPOx synthesis by free radical polymerization with 2,2′-azobis (2-methylpropionitrile) (AIBN) as initiator in methanol, (B) Protonation of gelatin with trifluoroacetic acid, (C) Synthesis of the hybrid hydrogel by thermal crosslinking at 70 °C.

2. EXPERIMENTAL SECTION

2.1 Materials

Chloroform (99.8 %), diethyl ether (DEE, 99.8 %), glutaric acid (98.0 %), and Sigmacote® were purchased from Sigma-Aldrich. Methanol (99.9 %) was purchased from Thermo Fisher Scientific. 2,2′-Azobis (2-methylpropionitrile) (AIBN, 98.0 %) was obtained from Sigma-Aldrich and recrystallized from methanol. Trifluoroacetic acid (TFA, peptide grade) was bought from IRIS-biotech. 2-Isopropenyl-2-oxazoline (98.0 %) was purchased from Sigma-Aldrich and was distilled under reduced pressure over CaH_2 before use. Sodium carbonate

(Na_2CO_3, 99.5 %) was obtained from Sigma-Aldrich. Dulbecco's phosphate-buffered saline (DPBS, 1×) was purchased from Life Technologies Europe B.V. Cell culture medium (MEM Alpha Medium (1×) + GlutaMAX^{TM}-I) was received from Thermo Fisher Scientific. Gelatin type B (gelatin, composition shown as **Table S1**), isolated from bovine skin through an alkaline process, was supplied by Rousselot (Ghent, Belgium). The Human foreskin fibroblasts were obtained from American Type Culture Collection (ATCC).

2.2 Instrumentation

Size exclusion chromatography (SEC) was performed with an Agilent 1260 system equipped with a multi-angle light scattering detector miniDawn Treos II. The column set was 2x PLGEL MIXED-D (300×75 mm) columns and a guard column (50×7.5mm MIXED-D), and the eluent *N*,*N*-dimethylacetamide with 50 mM LiCl. Samples were filtered before injection (0.2 μm PTFE filter). The specific refractive index increment value was determined as dn/dc = 0.0902 mL/g. The polymer sample concentration was 5 mg/mL.

^{1}H NMR spectra were recorded at 25 °C on an instrument operating at 300 MHz (Bruker Corporation, USA). Chemical shifts (δ) are referenced to $CDCl_3$ (δ 7.24 ppm) or D_2O (δ 4.73 ppm). Infrared spectra were measured on a Perkin-Elmer Spectrum1000 FT-IR spectrometer. pH was measured with Mettler Toledo FiveEasy pH F20 pH-meter.

Compression tests were performed using a Tinius-Olsen H10KT compression tester equipped with a 100 N load cell. For all compression experiments, hydrogels were cut by a puncher into disk shapes with a diameter of 8 mm and the height of ~2.5 mm (depending on swelling degree) and the compression rate of samples was taken as (sample height / 10) mm/min for all the compression experiments. At least, three samples were used to obtain average data. The compressive modulus was calculated as the slope in the initial linear region of the stress-strain curve corresponding to around 5-15 % strain.

Rheological measurements were performed on an Anton Paar MCR 302 rheometer equipped with a CTD 180 oven. Measurements were performed with a gap of 1 mm, a temperature of

22 °C, a strain of $\gamma = 0.1$ % and an angular frequency in the range of $\omega = 0.01$-100 rad/s. First, a frequency sweep measurement was performed with a fixed strain of 0.1 %, followed by a strain sweep at a fixed strain of 10 rad/s. The hydrogel disks were prepared as described above.

2.3 Synthesis of PiPOx

The synthesis of PiPOx was adapted from a literature procedure.[14,16] To obtain PiPOx with different number average molecular weights, free-radical polymerization was performed using different ratio of monomer to initiator [M]/[I]: 50, 100, and 200 by dissolving the respective amount of AIBN (e.g. 1.7995, 0.8898, 0.4499 mmol) in 10 g (90.09 mmol) of iPOx and 13 mL of methanol (4 mol/L of iPOx). The Schlenk flask was placed in an ice bath and the reaction mixture was deoxygenated by bubbling with argon for 30 min. The reaction vessel was heated to 65 °C for 48 h to allow for high conversion. Then, the reaction mixture was cooled to room temperature, diluted with chloroform (~50 mL), and precipitated into diethyl ether. After filtration and vacuum drying at 45 °C, the product was obtained as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 4.10 (m, 2H, –O–CH_2–), 3.70 (t, 2H, =N–CH_2–), 2.0−1.53 (m, 2H, –CH_2–, backbone), 1.33−1.12 (m, 3H, –CH_3, backbone). The absolute number average molecular weight (M_n) of the polymers was determined by SEC-MALS (**Table 1, Figure S1**). The conversions of the monomers were calculated from ^{1}H NMR spectroscopy (300 MHz, $CDCl_3$).

2.4 Preparation of PiPOx-gelatin hydrogels

2.4.1 Gelatin protonation

In a typical experiment, 0.5 g of gelatin was dissolved in 9.5 g of deionized water at 45 °C, and then TFA was added in 0.05-µL steps. The gelatin solution was kept at 45 °C in a water bath to avoid physical gelation during the pH measurement. The pH of the gelatin was measured immediately and after 10, 30, 60, and 720 min, respectively.

2.4.2 Preparation of PiPOx-gelatin hydrogels

PiPOx solutions with different molecular weight and concentrations of 15 and 20 wt% were prepared at room temperature using deionized water as solvent. At the same time, gelatin solutions of 15 and 20 wt% were prepared by dissolving gelatin in deionized water at 45 °C. 0.078 mmol of TFA per 1g of gelatin was used to protonate the gelatin (pH about 3.15). PiPOx and gelatin solutions of equal concentrations (20 wt% and/or 15 wt%) were mixed at mass ratios of 2.0, 1.8, 1.6, 1.4 and 1.0 (**Table 2**). The reaction mixture was vortexed at least 10 min, allowed to stand for 2 hours in the heating block at 45 °C to eliminate air bubbles, and then transferred using a syringe and needle into the space between two parallel rectangular glasses, separated by a 2 mm silicone spacer, that were in advance hydrophobized by Sigmacote® to obtain hydrogels of uniform thickness. The mould was then placed in an oven and kept at 70 °C for 72 h. The mould was then cooled down to room temperature, carefully opened and the hydrogel was taken out. The hydrogels were immersed in deionized water and extracted for 6 days to remove unreacted polymers and TFA. The water was changed daily throughout the extraction. The hydrogels were dried at room temperature until a constant mass was reached.

For mechanical testing, the hydrogels were placed in deionized water for 6 days to reach the equilibrium swelling state. The equilibrated hydrogel was further placed in DPBS and cell culture medium for 3 days, respectively, to investigate the compressive and rheological properties of the hydrogels in different biologically relevant buffer systems.

In addition, a hydrogel without gelatin crosslinked with glutaric acid instead of gelatin was synthesized as a control sample for the cell culture assay. A 15 wt% PiPOx (M_n= 23.1 kg·mol^{-1}) solution and glutaric acid was prepared in deionized water and vortexed for 10 min. The molar ratio between glutaric acid and iPOx units was 0.1. Then, the reaction mixture was injected into the above-described mould and was placed in an oven at 70 °C for 24 h to obtain the control hydrogel. The hydrogel was extracted in deionized water and equilibrated in the cell culture media in the same way as the hybrid hydrogel samples.

2.5 Swelling experiment and gel fraction determination

The swelling behavior of the hydrogels was determined by placing weighed dry polymer network disk (W_x) in deionized water or DPBS solutions at room temperature (25 °C), respectively. The swollen disks were removed from the aqueous solution at specific time intervals, the surface was wiped with filter paper, weighed (W_y) and sunk back into the aqueous solution. The measurements were carried out for 6 days in DW and for 3 days in DPBS, the weight of these swollen gel reaches a constant value, corresponding to the equilibrium swelling degree (ESD). Experiments were performed in triplicate and the mean of the measurements are reported. The degree of swelling at different time intervals, expressed as the amount of water absorbed by 1 g of dry polymer network, was calculated using the equation S-1:

$$ESD\ (\%) = \frac{W_y - W_x}{W_x} \times 100 \quad \text{(S-1)}$$

The gel fraction, expressing the degree of crosslinking was determined as follows: a portion of the as prepared hydrogel disk was first dried to constant weight (W_0), then the disk was swollen in an excess of deionized water to extract unreacted compounds, and dried. The time needed for extraction was typically 15 days with daily water exchange. The final dry weight (W_{ext}) of the extracted hydrogel disk was recorded. Gel fraction (GF) was then calculated according to the following equation (S-2):

$$GF = \frac{W_{ext}}{W_0} \cdot 100 \quad \text{(S-2)}$$

Experiments were performed in triplicate and the average value measurements were reported.

2.6 Degradability analysis

Degradability experiments were performed in water, DPBS, and the cell culture medium at 37 °C. Disk-shaped hydrogels (8 mm in diameter and ~2.5 mm in height) that reached swelling equilibrium in deionized water were weighed (m_0), and then put in 20 mL vials containing 3 mL of buffer heated at 37 °C. The hydrogels were taken out at specific time intervals, a filter paper was used to remove the liquid excess on the sample surface, and the samples were

weighed (m_t). The buffer was changed every week. The remaining mass of the hydrogel sample was calculated by comparing the mass at given time point with the initial mass according to equation (S-3):

$$\text{Mass remaining (\%)} = \frac{m_t}{m_0} \times 100 \quad \text{(S-3)}$$

Where m_0 is the mass of the swollen hydrogel in deionized water prior to immersion in buffer.

2.7 Cell adhesion assay using primary human foreskin fibroblasts

For the cell adhesion assay, the hybrid hydrogel (PiPOx-gelatin) was first neutralized with a solution of the weak base Na_2CO_3 (pH of ~8.1) in an excess. As control hydrogel, the PiPOx material (M_n: 23.1 kg·mol^{-1}) was prepared with glutaric acid as crosslinker instead of cell-interactive gelatin and underwent the same neutralization procedure. After the hydrogels were swollen in the Na_2CO_3 solution with a pH of ~8.1 overnight, the hydrogels were re-swollen with excess of deionized water (~100 mL) for 3 days with daily water replacement. The successful neutralization was proven as follows: After the neutralization procedure, ~0.5 mL of culture medium (DMEM + 10 v% FBS + 1 v% P/S) was administered on top of the hydrogel. When the color of the phenol red indicator present within the culture medium did not change, the hydrogels were considered neutral and suitable for *in vitro* cell testing. Furthermore, the hydrogel dialysis water after 3 days was measured and should fall in a range of pH of ~7.4 - 7.6. Next, the neutralized hydrogel sheets were swollen in DPBS buffer for 3 days with daily buffer changes. The hydrogel sheets were punched out into disks with 6 mm diameter and height of ~2.5 mm (depending on the sample swelling properties) which were placed in a 96-well plate to cultivate primary human foreskin fibroblast cells. The samples were subjected to a 70 v% ethanol solution for 24 hours with a change after 12 hours. Then, they were re-swollen in PBS buffer at 37 °C for 3 days.

Human foreskin fibroblasts were cultured at standard incubator conditions (37 °C, 5% CO_2) in cell culture Dulbecco's modified eagle medium (DMEM) with the extra addition of 10 v% fetal bovine serum (FBS) and 1 v% penicillin/streptomycin (P/S). Twice a week, the medium was

changed up to a confluency of 80-90% upon which the cells were split. Fibroblasts of passage 12 were used for cell seeding. 20,000 fibroblasts were drop-seeded on top of the prepared sterile substrates. After an incubation period of 5 minutes at 37°C, cell culture medium was added and the cell-seeded substrates were cultured for 1, 3 or 7 days. To assess viability, at the designated time points, a Live/Dead (2 v% calcein-acetoxymethyl / 2 v% propidium iodide in PBS) solution was applied on the cell-seeded substrates followed by a 10-minute incubation at 37°C in the dark. The morphology of the seeded cells was then captured with a confocal scanning microscope (Zeiss LSM710) equipped with a green fluorescent protein (GFP) filter for living cells and a texas red (TxRed) filter for dead cells. The metabolic activity of the seeded cells was evaluated through the addition of a 17 v% 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) in cell culture medium solution followed by a 2-hour incubation at 37 °C in the dark. Next, the absorbance of the supernatant was read at 490 nm with a spectrophotometer (Tecan Infinite M200 Pro). Each experimental group was evaluated in triplicate (3 biological replicates per experimental group).

2.8 Statistical analysis

All data are expressed as mean ± standard deviation. The p values were generated by analyzing data with a one-way ANOVA and Tukey's test using OriginLab. Differences were taken to be significant for $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of PiPOx

To assess the influence of the PiPOx molar mass on the preparation and properties of PiPOx-gelatin hydrogels, a series of PiPOx polymers were synthesized by free-radical polymerization under argon atmosphere using 2,2′-azobis (2-methylpropionitrile) (AIBN) as the initiator. Depending on the monomer to initiator ratio to control the number of formed radicals by dissociation of AIBN, polymers with different average molar masses of 14.5, 23.1, and 45.5

kg·mol^{-1} and dispersity ranging from 1.36 to 1.40, as determined by size exclusion chromatography (SEC) (**Table 1**; **Figure S1**), were obtained. The free-radical polymerization thus provides rather good control over the molar mass and dispersity of PiPOx for hydrogel synthesis, albeit the apparent correlation between [M]/[I] ratio and obtained molar mass seems to be a coincidence. The molecular structure of PiPOx was confirmed by ^{1}H NMR spectroscopy (**Figure S2**). The monomer conversion was dependent on the amount of initiator used varying between 69 % and 98 % after 2 days of polymerization at 65 °C. The signals corresponding to the monomer are not present in the ^{1}H-NMR spectrum after precipitation of the polymer in diethyl ether and subsequent drying.

Table 1. The synthesis of PiPOx hydrogel precursors

Sample code	$\overline{M}_n$ (kg·mol^{-1}) [a]	$Đ$ [b]	[M]/[I]	Conversion (%) [c]	DP [d]
PiPOx-01	**14.5**	**1.36**	**50**	**98**	**130**
PiPOx-02	**23.1**	**1.40**	**100**	**79**	**207**
PiPOx-03	**43.5**	**1.36**	**200**	**69**	**391**

[a] Obtained by SEC in DMA with LS detection as described in methods;

[b] Polymer dispersity;

[c] Determined by ^{1}H NMR spectroscopy, 300 MHz in $CDCl_3$ (see section 2.3 Synthesis of PiPOx);

[d] Degree of polymerization (DP) was calculated as $\overline{M}_n$ divided by the molar mass of iPOx monomer unit (M = 111.14 g·mol^{-1}).

3.2 Preparation and characterization of PiPOx–gelatin hydrogels

PiPOx-gelatin hydrogels were prepared according to **Scheme 1**. Gelatin was pretreated with TFA prior to the hydrogel synthesis (section 2.4.1 Gelatin protonation). Subsequently, the

PiPOx solution was mixed with TFA-pretreated gelatin solution at different ratios leading to the formation of hydrogels (**Table 2**). When attempting the preparation of hydrogels by reacting native, non-protonated (i.e. not treated by TFA) gelatin with PiPOx no hydrogels were obtained, indicating the importance of protonating the carboxylate side chains. Hydrogels with tunable water uptake and mechanical properties should be obtained by varying the gelatin amount. Decreasing the PiPOx/gelatin ratio increases the number of carboxylic acid groups available for the crosslinking reaction, potentially leading to a higher degree of crosslinking. The solution obtained after mixing both polymers was heated to 70 °C for 3 days to achieve the crosslinking reaction. Afterward, the hydrogels were extracted in deionized water (referred to as water) to remove the unreacted polymers and dried prior to characterization. The sample codes start with H standing for 'hydrogel' and contain three numbers referring to the molecular weight of PiPOx in kg·mol^{-1}, followed by the total polymer concentration in wt% and the mass ratio of PiPOx to gelatin. As example: H23-15-1.4 refers to a hydrogel synthesized using PiPOx of 23.1 kg·mol^{-1} where the combined total concentration of PiPOx and gelatin is 15 wt% and the mass ratio of PiPOx:gelatin is 1:1.4.

Table 2. Overview of the synthesis of the hybrid PiPOx-gelatin hydrogels

Sample code	$\overline{M}_n$ (kg·mol^{-1})[a]	C (wt%)[b]	R[c]	*GF*[d] *(%)*	Handling resistance	Shape stability
H23-15-2.0	**23.1**	**15**	**2.0**	**96.2 ± 0.8**	**poor**	**bending**
H23-15-1.8	**23.1**	**15**	**1.8**	**93.5 ± 1.4**	**poor**	**bending**
H23-15-1.6	**23.1**	**15**	**1.6**	**93.9 ± 1.1**	**poor**	**bending**
H23-15-1.4	**23.1**	**15**	**1.4**	**95.8 ± 0.2**	**strong**	**stable**
H23-15-1.0	**23.1**	**15**	**1.0**	**96.5 ± 2.3**	**strong**	**stable**
H23-20-1.4	**23.1**	**20**	**1.4**	**98.7 ± 0.8**	**strong**	**stable**
H14-15-1.4	**14.5**	**15**	**1.4**	**94.1 ± 1.2**	**poor**	**bending**
H14-20-1.4	**14.5**	**20**	**1.4**	**97.8 ± 0.9**	**strong**	**stable**

H43-15-1.4	**43.5**	**15**	**1.4**	**94.9 ± 0.6**	**strong**	**stable**

[a] The number average molar mass ($\overline{M}_n$) of the PiPOx as determined by SEC-LS;

[b] Total polymer concentration;

[c] Mass ratio of PiPOx and gelatin: m_{PiPOx}:$m_{gelatin}$;

[d] Gel fraction.

From the ^{1}H NMR spectrum of the hydrogel after extraction, drying and reswelling in D_2O (**Figure S3**), it can be seen that the peak of the phenylalanine (Phe) of gelatin, at a chemical shift of 7.3 ppm is observed in the hybrid hydrogel besides the signals of PiPOx indicating the presence of both components.[30] FTIR analysis was used to investigate the chemical structure of the extracted dried xerogels. The FTIR spectrum (**Figure 1**) of the hybrid hydrogel displayed the characteristic bands of gelatin at 3305-3271 cm^{-1} (amide A, N-H groups, stretching) and of the 2-oxazoline ring at 987, and 958 cm^{-1} (ring skeletal vibrations).[31,32] The band at 1650-1580 cm^{-1} can be attributed to the overlapping amide I ($\upsilon_{C=O}$) band from the gelatin, −C=N vibration of the 2-oxazoline ring and amide I ($\upsilon_{C=O}$) band from the newly generated ester-amide bond. The signal corresponding to the carbonyl stretching vibration of the newly generated ester can be found at 1732 cm^{-1} ($\upsilon_{C=O}$) as a small broad shoulder of the 1650 cm^{-1} band. The characteristic amide II band typically found in PiPOx-modified copolymers with carboxylic acids overlaps with the amide II band from gelatine. The FTIR data prove the successful synthesis of the hybrid PiPOx-gelatin hydrogels *via* crosslinking reaction between 2-oxazoline rings of PiPOx and the carboxylic acid groups of the protonated gelatin.

All hydrogels were optically clear following synthesis, suggesting that no phase separation occurred during the crosslinking reaction. The gel fraction (GF) of the obtained hybrid hydrogels was higher than 93 % in all cases (**Table 2**), indicating the highly efficient crosslinking reaction under the used conditions.

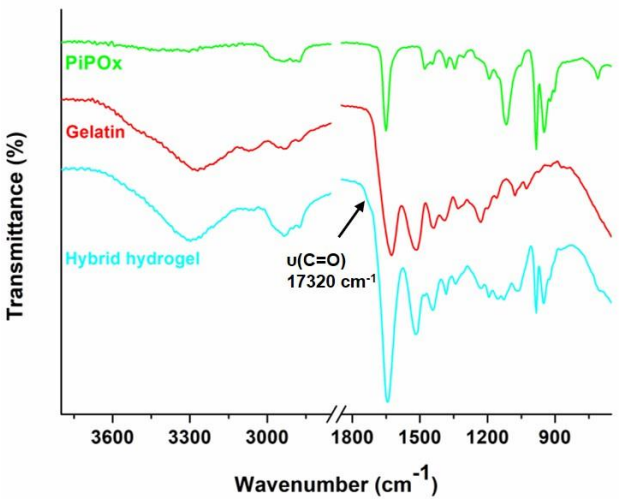


Figure 1. FTIR spectra of PiPOx 23.1 g·mol^{-1}, gelatin and the hybrid PiPOx-gelatin H23-15-1.4.

3.3 The effect of composition on stability of PiPOx-gelatin hydrogels

Crosslinking gelatin with PiPOx to prepare hybrid hydrogels can offer an attractive strategy to improve the poor mechanical properties and instability of gelatin hydrogels. The reported method has the advantage of direct crosslinking of gelatin with PiPOx under mild reaction conditions, using water as the solvent and avoiding the need for catalysts and without generation of by-products. A synthetic screening was performed to obtain an entire library of hydrogels with a broad range of mechanical properties. The influence of the following synthetic parameters was investigated: i) the molar mass of PiPOx, ii) the total polymer concentration, and iii) the mass ratio of PiPOx to gelatin (**Table 2**).

PiPOx with a M_n of 23.1 kg·mol^{-1} was used to investigate the influence of the gelatine content on the crosslinking process, keeping the overall polymer concentration constant that is 15 wt%. Higher PiPOx/gelatine ratio (i.e., lower carboxylic acid group content) leads to mechanical unstable hydrogels due to inefficient crosslinking. The hydrogels bend from the corner edges along Y axis (**Table 2** and **Figure S5**) under the internal stress suggesting a swelling mismatch most probably due to the presence of high swelling domains (uncrosslinked or less crosslinked

gelatin) and relatively low swelling domains (crosslinked gelatine). Increasing the amount of gelatin (i.e., lower PiPOx:gelatin ratio), resulted in stiffer and stronger hydrogels. A PiPOx to gelatin mass ratio of 1:1.4 was chosen as optimal and further experiments were performed using this ratio including cell culture studies.

Next, the influence of the M_n of PiPOx on the crosslinking process was investigated. The ratio of PiPOx:gelatin was fixed at 1.4 keeping a total polymer concentration of 15 wt%. The hydrogel prepared with shorter PiPOx (14.5 $kg \cdot mol^{-1}$) had poor mechanical properties and bent upon swelling to equilibrium (H14-15-1.4 in **Figure S5**), whereas the hydrogels obtained using intermediate (23.1 $kg \cdot mol^{-1}$) and higher (43.5 $kg \cdot mol^{-1}$) molar mass PiPOx were stable upon swelling and did not bend.

Finally, the influence of polymer concentration was tested. The hydrogels were synthesized at two different polymer concentrations in water, namely 15 wt% and 20 wt%. The hydrogel mechanical stability was, dependent on the polymer concentration in the feed as expected. In the case of PiPOx-01 15 wt% polymer concentration, the hydrogel showed poor mechanical properties whereas a shape stable hydrogel could be obtained when using PiPOx-02. However, increasing the polymer concentration to 20 wt% led to hydrogels that maintained their shape and could be manipulated without breaking, for PiPOx-01 and PiPOx-02. Thus, it can be assumed that the intermolecular coupling is preferred at higher concentrations, resulting in mechanically stronger hydrogels. However, in the case of PiPOx-03 with the highest DP a phase separation was observed at 20 wt%, precluding the synthesis of homogeneous PiPOx-gelatin hybrid hydrogels.

Overall, the screening study proved that hybrid hydrogels can be successfully obtained, and their mechanical stability can be easily enhanced by controlling the molar mass of PiPOx, the total polymer centration, and the PiPOx:gelatin ratio making it possible to target different applications.[33-35]

3.4 The effect of reaction parameters on the swelling properties of PiPOx-gelatin hydrogels

Biomedical applications are predetermined by the swelling behavior of hydrogels that reflects the ability of the hydrogel crosslinked network to absorb and retain the water.[36]

The effect of the composition of the hybrid PiPOx-gelatin hydrogels on its equilibrium swelling degree (ESD) in water was investigated. The ESD was determined from kinetic swelling experiments to be 6 days. The ESD decreased with decreasing PiPOx to gelatin ratio from 1:1.6 to 1:1.0, due to the formation of a more crosslinked network, which lowers the water uptake (**Figure 2**). Furthermore, the ESD decreased from 1260 % to 810 % (**Figure 2b**) with increasing polymer concentration from 15 wt% to 20 wt% at a fixed PiPOx:gelatin ratio of 1:1.4 which can be attributed to a higher crosslinking density. Lower polymer concentration favors the formation of intramolecular loops, leading to a lower effective crosslinking density and hence a larger ESD of the hydrogel.[37] The molecular weight of PiPOx strongly affected the ESD. The ESD decreased with increasing the M_n of PiPOx due to increased chain entanglements leading to more efficient crosslinking (**Figure 2a**).

As the hybrid PiPOx-gelatin hydrogels are intended to be used in cell culture, the effect of buffer on the swelling behavior of hydrogels was also investigated. The ESD of the hybrid hydrogels in Dulbecco's phosphate-buffered saline (DPBS) is significantly lower than that in water (**Figure 2b**)**,** regardless of the composition of the hydrogel. DPBS is a buffered saline solution containing 137 mM of NaCl, which has a salting-out effect on the hydrogel.[38] Therefore, the water absorption of the hydrogels is reduced. Higher crosslinking density seems to minimize the salting-out effect of the DPBS for the H23-20-1.4 hydrogel as compared to the H23-15-1.4 hydrogel.

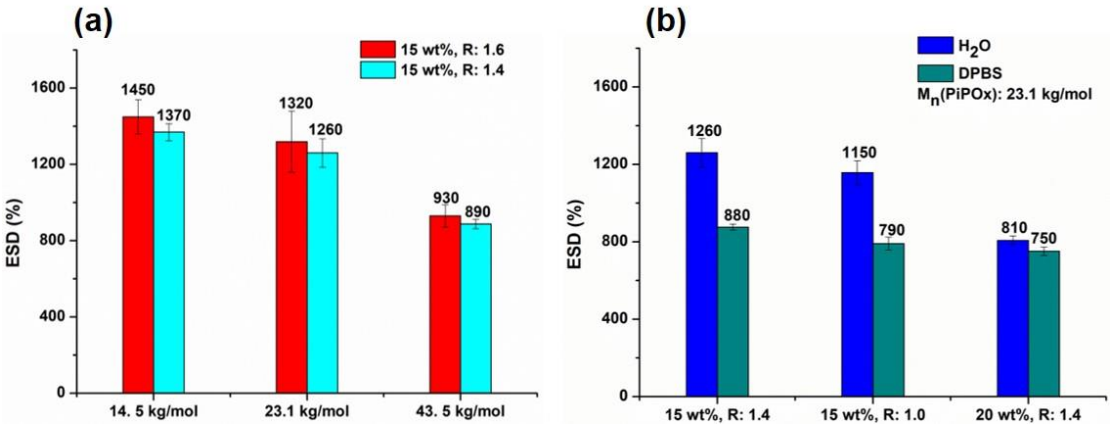


Figure 2. The equilibrium swelling degree (ESD) dependence of PiPOx-gelatin hydrogels on: (a) the molecular weight of PiPOx and the PiPOx:gelatin ratio, in deionized water, (b) the PiPOx:gelatin ratio and total polymer concentration in deionized water and in Dulbecco's phosphate-buffered saline DPBS. The measurements were performed in triplicate at room temperature and the average values were reported.

3.5 Compression analysis of PiPOx–gelatin hydrogels in water and different buffers

Hydrogels H23-15-1.4, H23-15-1.0, and H23-20-1.4, showing increased dimensional stability, were selected to further study their mechanical behavior in water and different buffer systems. Compression tests (**Figure 3** and **Figure S6)** reveal that the compression strength and elastic compression modulus (E_c) could be controlled by varying the gelatin amount and/or the polymer concentration. As expected, the E_c of the H23-15-1.4 hydrogel was lower than that of the H23-15-1.0 and H23-15-1.4 hydrogels (**Figure 3b**), indicating higher crosslinking density for these two hydrogels in agreement with the ESD results. The strain failure decreased with increasing polymer concentration and/or decreasing PiPOx/gelatin ratio due to reduced elasticity in more highly crosslinked structures. The H23-20-1.4 hydrogel showed a E_c of 215 kPa, a strength of 192 kPa, and a strain failure of 45% (**Figure 3**). Notably, its E_c is higher than the E_c reported for other gelatin hydrogels in the literature.[11,12] In addition, a sharp decrease in failure strains is frequently observed with increasing crosslinking in hydrogel networks.[39]

However, the decrease in strain failure of the hybrid PiPOx-gelatin hydrogels is moderate as the crosslinking degree increases.

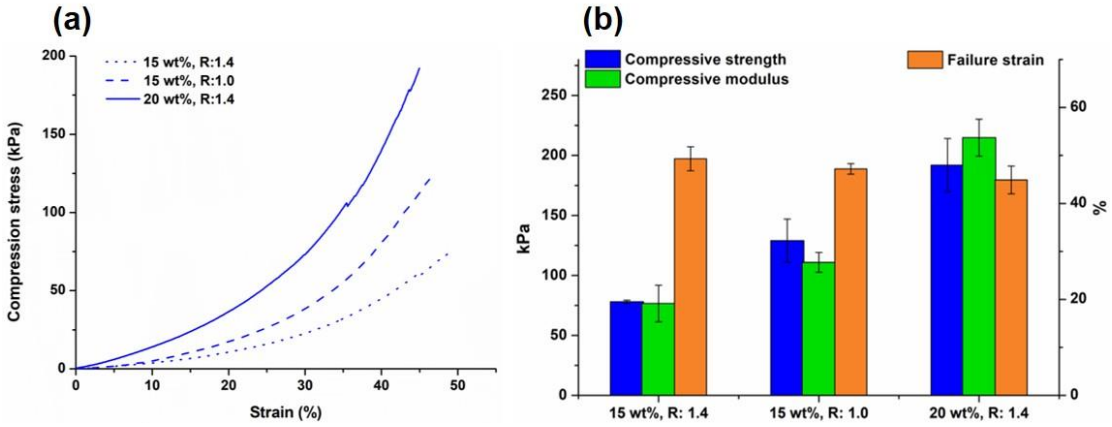


Figure 3. Compression analysis of H23 hydrogels: (a) Representative stress-strain curves of hydrogels swollen in deionized water; (b) compression strength, average elastic compression modulus (E_c), and failure strain of hydrogels swollen in deionized water. The measurements were performed in triplicate on separate hydrogels.

Swelling the hydrogels in DPBS buffer led to a decrease of the E_c and an increase of the compressive strength (**Figure S6)**. The reduction of the E_c can occur due to the hydrolysis of the ester bond from the ester amide junction points leading to lower crosslinking density. DPBS might promote the hydrolysis of some ester groups by facilitating water access due to the coordination of the ester bond by sodium and/or potassium cations present in the DPBS.[40] The increase of the compression strength can be explained by the salting-out effect on the hydrogels leading to lower water absorption and making the hydrogel more resistant to deformation.[38]

The mechanical properties of the hydrogels swollen in the cell culture medium (MEM Alpha Medium (1×) + GlutaMAX^{TM}-I) markedly decreased as compared to when swollen in water or DPBS. This increased degradation can be assigned to a faster hydrolysis of the ester bonds facilitated by the presence of small molecules such as amino acids and sodium pyruvate, lipoic acid, and ascorbic acid. The degradability of the hybrid hydrogels will be further discussed in section 3.7.

3.6 Rheological characterization of PiPOx–gelatin hydrogels in water and different buffers

Rheological investigations were carried out on H23-15-1.4, H23-15-1.0 and H23-20-1.4 hydrogels swollen in water, DPBS, and cell culture medium (MEM Alpha Medium (1×) + GlutaMAX^{TM}-I). The frequency sweep experiments showed that storage modulus (G′) remained larger than loss modulus (G″) and did not significantly change with the fixed 0.1 % strain (**Figure S7 a-c**), indicating the good stability of the hydrogels towards frequency oscillation. G′ and G″ increased with increasing polymer concentration and gelatin amount as expected (**Figure S7 a-c**), because of the higher crosslinking density. The hydrogels had a stable structure and consistent solid-like behavior in all three media after 3 days of incubation. The storage moduli of the investigated hydrogels in DPBS and cell culture medium are lower than that in water (**Figure S7d-f)**, which is in accordance with compression experiments and supporting the crosslinks hydrolysis.

Dynamic strain sweep experiments showed that the G' was always higher than G" with strain varying from 0.01 % to 1 % (**Figure 4** and **Figure S8**), indicating that the hydrogels have solid-like properties in linear viscoelastic (LVE) region. The LVE was dependent on the polymer concentration and gelatin amount. For H23-15-1.4 hydrogel the LVE region ranges from 0.01 % to 1.5 % shear strain, whereas for H23-15-1.0 and H23-20-1.4, their LVE regions range from 0.01 % to 1.0 % and 0.01 % to 0.4 %, respectively. The LVE region of the hybrid hydrogels swollen in DPBS and cell culture medium (**Figure 4b-c**) are extended to larger strains suggesting a looser network structure due to hydrolysis of the crosslinking points.

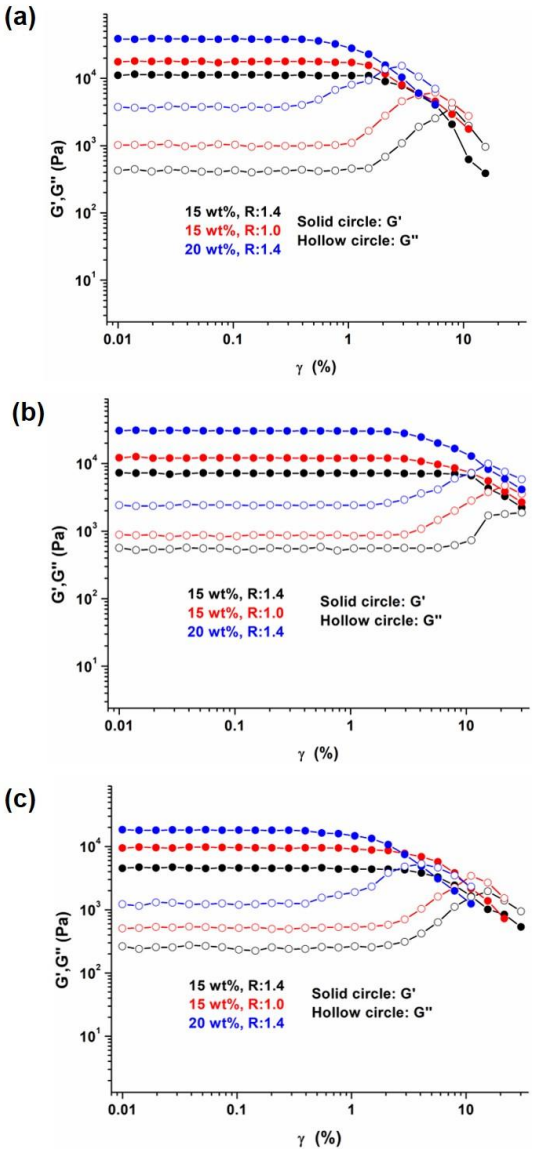


Figure 4. Rheological characterization of PiPOx-gelatin hydrogels by strain sweep measurements: hydrogels swollen in (a) water, (b) DPBS, and (c) cell culture medium. DPBS: Dulbecco's phosphate-buffered saline; Medium: MEM Alpha Medium (1×) + GlutaMAX^{TM}-I.

3.7 Degradability of PiPOx-gelatin hydrogels

Hydrogel H23-20-1.4 was selected as a representative sample to study its degradability in water, DPBS, and cell culture medium (MEM Alpha Medium (1×) + GlutaMAX^{TM}-I) at 37 °C (**Figure 5a**). The sample swollen at equilibrium in water at room temperature was immersed in the respective aqueous media. After one day of incubation at 37 °C, the mass of the swollen

hydrogel was reduced to 80-90 % of its original swollen mass in all three systems, which is associated with establishing a new equilibrium. Especially in DPBS, the hydrogel shrunk to ~80%, which can be ascribed to the salting-out effect of DPBS on PiPOx hydrogels due to the presence of NaCl in the buffer.[38] In comparison to DPBS (~137 mM of NaCl), the cell culture media contains much lower amounts of salts (26 mM of Na_2CO_3) hence, the salting-out effect is negligible. After the initial decrease, the mass of the hydrogels, especially in DPBS and cell culture medium revealed a continuous increase in the following days. This mass increase can be attributed to the increase in mesh size due to partial hydrolysis of the ester crosslinks as also discussed and speculated in section 3.5.[40,41] After 9 days, the mass of H23-20-1.4 in the cell culture medium reached its maximum. Subsequently, the sample started to fall apart due to decrease in the mechanical stability and excessive handling during the experiment, reflected in a sharp decrease in the swollen mass after day 9. After 11 days, the sample fell apart (**Figure 5b-3**), and its mass decreased sharply to ~60 % and after 33 days, the hydrogel was completely degraded. In DPBS, a similar behavior was observed spread out over a longer timeframe. The mass gradually increased from 3 to 26 days, whereas after 30 days, the sample became mechanically unstable, reaching a 75 % degradation after 35 days (**Figure 5b-2**). In contrast, the hydrogels incubated in water were hydrolyzed relatively slowly, and a more gradual decrease in mass was observed compared to cell culture medium and DPBS (**Figure 5b-1**). These results are in line with the recently reported stability of soluble PiPOx in different media.[42] The tunable degradation behavior of hydrogels into PiPOx chains and gelatin fragments in different buffers confirmed the high potential of these hybrid hydrogels as future biomaterial for further research. It should be noted that the degradation is based on gelatin degradation and the PiPOx chains will remain intact and are released as hydrophilic chains. These released PiPOx chains should be small enough for renal clearance when considering *in vivo* applications, which is likely the case for the PiPOx with 14.5 kg/mol and 23.1 kg/mol.

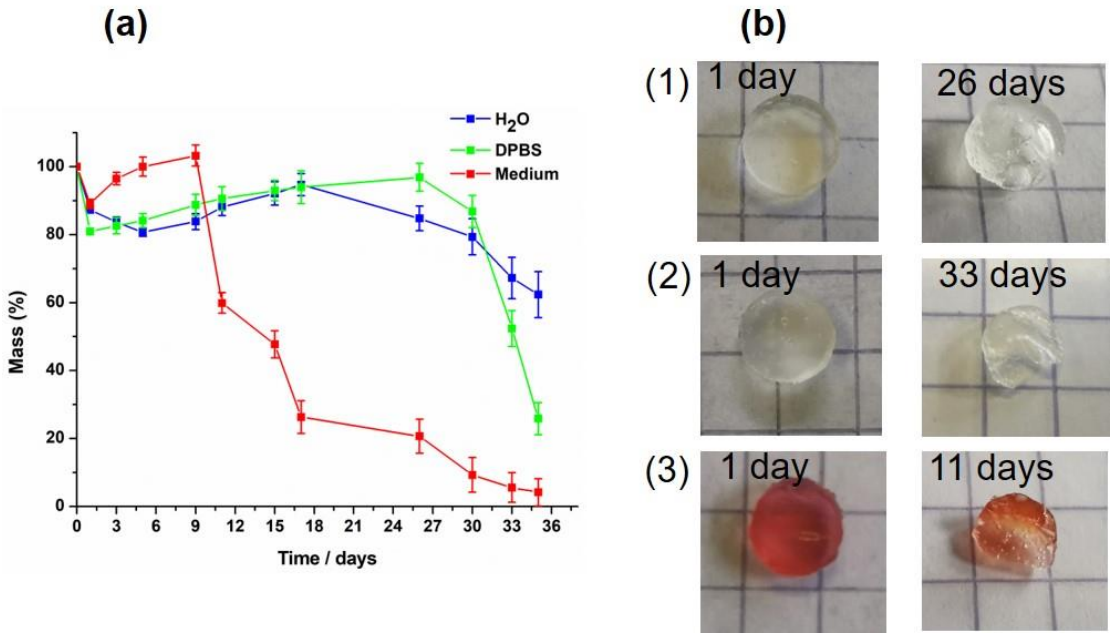


Figure 5. The degradation of PiPOx-gelatin hydrogel H23-20-1.4: (a) The relative change in mass of swollen hydrogel with time in water, DPBS, and cell culture medium at 37 °C. Lines are to guide the eye and do not represent data points. (b) The appearance of hydrogels during the degradation experiment (1 – water, 2 – DPBS, 3 – cell culture medium). DPBS: Dulbecco's phosphate-buffered saline; Cell culture medium: MEM Alpha Medium (1×) + GlutaMAX^{TM}-I. The measurements were performed in triplicate on separate hydrogel samples.

3.8 Cell adhesion assay on PiPOx-gelatin hydrogels

To determine the cell adhesion on the PiPOx-gelatin hydrogels, hybrid hydrogels H23-15-1.4 and H23-15-1.0 with good mechanical properties and different gelatin contents were selected as representative samples. Primary fibroblasts were seeded and cultured on top of the H23-15-1.4 and H23-15-1.0 samples and compared against a control hydrogel which was crosslinked with glutaric acid instead of gelatin (**Figure 6**). Cell-interactive recognition sequences such as Arg-Gly-Asp (RGD) in gelatin bind to various integrin proteins, thus aiding in cell attachment, migration, and survival.[43-45] At day 1, the cells adhered to both hybrid hydrogels but not to the control hydrogel since there were no cell-interactive domains present within the used low-molecular-weight crosslinker. More cells adhered to the hybrid sample containing more gelatin

(H23-15-1.0), hence having more cell-interactive domains resulting from gelatin. Also, an increase in metabolic activity (displayed relative to the metabolic activity of the tissue culture plate control) was observed when the gelatin concentration was increased. On days 3 and 7, the observed differences between the hydrogels became even larger hence indicating the need for cell-interactive domains for efficient cell proliferation. In summary, the hybrid PiPOx-gelatin hydrogels were found to be non-cytotoxic and had an increased cell attachment and cell metabolic activity when the gelatin content was higher.

Compared to PEG hydrogels, which are commonly used as synthetic extracellular matrices (ECMs) for cell culture but require modification with adhesive ligands to enable cell adhesion. The swelling behavior of PEG hydrogels affects ligand density and mechanical stability.[46,47] In contrast, hybrid PiPOx-gelatin hydrogels retain the natural cell-interactive domains of gelatin, providing good cell adhesion.

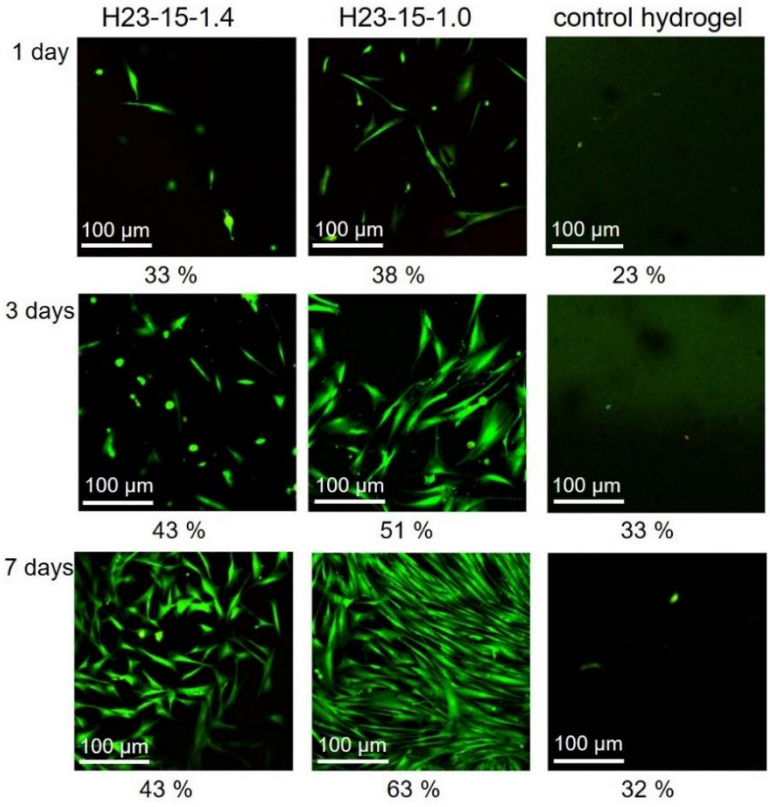


Figure 6. Cell viability and metabolic activity up to 7 days of primary human foreskin fibroblasts on PiPOx-gelatin hydrogels, the PiPOx hydrogel crosslinked with glutaric acid instead of gelatin was used as a control sample (scale bar indicates 100 µm). The percentages

correspond to the metabolic activity of the seeded cells. The reduced product can be quantitatively evaluated through UV-VIS spectrophotometry. The obtained absorbance value from the H23-15-1.4, H23-15-1.0 and negative control samples is then normalized by the values of the positive tissue culture plate control which consists of a monolayer of fibroblasts which are seeded out in a cell-interactive well.

4. CONCLUSIONS

Hybrid hydrogels based on gelatin and PiPOx, a synthetic biocompatible polymer, were developed. Gelatin was protonated to activate free carboxylic groups for crosslinking with the pendant 2-oxazoline groups of PiPOx. The swelling and mechanical properties of the hybrid hydrogel were controlled by varying the molecular weight of PiPOx, the total polymer concentration, and the ratio of PiPOx to gelatin. The equilibrium swelling degree of the hydrogels ranged from 1260 % to 810 %. The compressive strength of hydrogels ranged from 78 kPa to 192 kPa, and the corresponding elastic compression moduli from 77 kPa to 215 kPa. Rheological tests showed that the hybrid hydrogels have a stable solid-like structure, and it is demonstrated that the hydrolysis of some ester groups by DPBS and cell culture medium (MEM Alpha Medium (1×) + GlutaMAX^{TM}-I) does not affect the stability of its polymer cross-linked structure after 3 days of swelling. The degradability test of a PiPOx-gelatin hydrogel in water, DPBS, and cell culture medium at 37 °C revealed that the hydrogel is substantially degraded after about 5 weeks in the cell culture medium, and that degradation is slower in DPBS and water. The hybrid hydrogels exhibited good cell adhesion properties to primary human foreskin fibroblasts, with cell proliferation ability increasing with the increasing amount of gelatin in the hybrid hydrogel.

These hybrid PiPOx-gelatin hydrogels exhibit a unique combination of strong mechanical properties, cell-interactivity, and degradability. This makes them excellent potential as biomedical cell scaffold materials by combining the tunability and strength of PiPOx hydrogels

with the cell-interactive properties of gelatin while the ester-containing crosslinks provide tunable degradability.

ACKNOWLEDGEMENTS

P.Y. acknowledges the Chinese Scholarship Council (CSC, File No. 202108440160) for the financial support (Ph.D. program); R.H. and S.V.V. thank the Research Foundation – Flanders (FWO) and Ghent University for continuous financial support. L.P. acknowledges Research Foundation – Flanders (FWO) for the awarded Ph.D. fellowship.

Notes

The authors declare to have no conflict of interest.

Supporting information

The supporting information files contain further details on experimental materials (SI S1), the determination of the amount of TFA used for gelatin protonation, the SEC-LS traces and ^{1}H NMR spectrum of PiPOx, and the ^{1}H NMR spectrum of the hybrid hydrogels (SI S2). Photographs illustrating the composition of the hybrid hydrogels and their mechanical stability after swelling in water are also included (SI S3). The SI file also contains Figures showing the mechanical performance of hybrid hydrogels in three different buffers (SI S4) and rheological properties derived from frequency sweep tests (SI S5).

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