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Photothermal Nanomaterial Mediated Photoporation

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CONSPECTUS:

Delivering biological effector molecules in cultured cells is of fundamental importance to any study or application in which modulation of gene expression is required. Examples range from generating engineered cell lines for studying gene function, to the engineering of cells for cell-based therapies, such as CAR-T cells and gene-corrected stem cells for regenerative medicine. It remains a great challenge, however, to deliver biological effector molecules across the cell membrane with minimal adverse effects on cell viability and functionality. While viral vectors have been frequently used to introduce foreign nucleic acids into cells, their use is associated with safety concerns like immunogenicity, high manufacturing cost and limited cargo capacity.

For photoporation, depending on the laser energy, membrane permeabilization either happens by local heating, or by laser-induced water vapor nanobubbles (VNB). In our first study on this topic we demonstrated that the physical force exerted by suddenly formed VNB leads to more efficient intracellular delivery as compared to mere heating. Next, we explored the use of different photothermal nanomaterials, finding that graphene quantum dots display enhanced thermal stability compared to the more traditionally used gold nanoparticles, hence providing the possibility to increase the delivery efficiency by repeated laser activation. To enable its use for the production of engineered therapeutic cells, it would be better if contact of cells with non-degradable nanoparticles is avoided as it poses toxicity and regulatory concerns. Therefore, we recently demonstrated that photoporation can be performed with biodegradable polydopamine nanoparticles as well. Alternatively, we demonstrated that nanoparticle contact can be avoided by embedding the photothermal nanoparticles in a substrate made from biocompatible electrospun nanofibers. With this variety of photoporation approaches, over the years we demonstrated successful delivery of a broad variety of biologics (mRNA, siRNA, Cas9 ribonucleoproteins, nanobodies …) in many different cell types, including hard-to-transfect cells such as T cells, embryonic stem cells, neurons and macrophages.

In this Account, we will first start with a brief introduction on the general concept and historical development of photoporation. In the next two sections, we will extensively discuss the various types of photothermal nanomaterials which have been used for photoporation. We discriminate two types of photothermal nanomaterials: single nanostructures and composite nanostructures. The first one includes examples like gold nanoparticles, graphene quantum dots, polydopamine nanoparticles etc. The second type includes polymeric films and nanofibers containing photothermal nanoparticles, as well as composite nanoscale biolistic nanostructures. A thorough discussion will be given for each type of photothermal nanomaterial, from its synthesis and characterization to its application in photoporation, with its advantages and disadvantages. In the final section we will provide an overall discussion and elaborate on future perspectives.

KEY REFERENCES

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- Harizaj, A.; Wels, M.; Raes, L.; Stremersch, S.; Goetgeluk, G.; Brans, T.; Vandekerckhove, B.; Sauvage, F.; De Smedt, S. C.; Lentacker, I.; et al. Photoporation with Biodegradable Polydopamine Nanosensitizers Enables Safe and Efficient Delivery of mRNA in Human T Cells. *Advanced Functional Materials* **2021**, *31* (28). DOI: 10.1002/adfm.202102472.[2] *This work shows for the first time that biocompatible and biodegradable polymeric nanoparticles can be used as sensitizers for photoporation.*
- Xiong, R.; Hua, D.; Van Hoeck, J.; Berdecka, D.; Leger, L.; De Munter, S.; Fraire, J. C.; Raes, L.; Harizaj, A.; Sauvage, F.; et al. Photothermal nanofibres enable safe engineering of therapeutic cells. *Nature Nanotechnology* **2021**, *16* (11), 1281-+. DOI: 10.1038/s41565-021-00976-3.[3] *This work reports on photoporation of cells with electrospun polymeric nanofibers containing iron oxide nanoparticles. This system has the advantage of avoiding direct contact of cells with the sensitizing nanoparticles, offering benefits towards the safe use of photoporation for engineering of therapeutic cells.*
- Fraire, J. C.; Shaabani, E.; Sharifiaghdam, M.; Rombaut, M.; Hinnekens, C.; Hua, D.; Ramon, J.; Raes, L.; Bolea-Fernandez, E.; Brans, T.; et al. Light triggered nanoscale biolistics for efficient intracellular delivery of functional macromolecules in mammalian cells. *Nature Communications* **2022**, *13* (1). DOI: 10.1038/s41467-022-29713-7.[4] *This work reports onlight-triggered self-assembled nanobombs capable of make large pores in the cell membrane and enhancing intracellular delivery of large nucleic acids like mRNA and pDNA.*

1. INTRODUCTION

Delivering biological effector molecules in cultured cells is of fundamental importance to any study or application in which modulation of gene expression is required[5, 6]. Examples range from generating engineered cell lines for studying gene function, to the engineering of cells for cell-based therapies, such as CAR-T cells and gene-corrected stem cells for regenerative medicine. Viral vectors are often selected to introduce foreign nucleic acids into primary and difficult-to-transfect cells. Nevertheless, their use comes with safety concerns like immunogenicity, insertional mutagenesis, costly production and limited cargo capacity. Non-viral carriers, in this context also sometimes referred to as transfection reagents, offer an attractive alternative option[7]. They are typically composed of synthetic lipids or polymers which are easier to produce and straightforward to use, but often perform less well on primary cell types. A third option which is gaining in interest, is the use of cell membrane permeabilization methods, allowing direct influx of external molecules in the cytosol. Enhancing cell membrane permeability can be achieved with (bio)chemical reagents, such as by bacterial toxins or solvents, but more often a physical force is applied, such as ultrasound (sonoporation), electrical fields (electroporation), or mechanical forces (mechanoporation)[8].

Thanks to the high level of control of laser light, also the use of light has emerged as an attractive physical trigger to permeabilize cells[9, 10]. This is referred to as photoporation or optoporation, although other names have been used as well, such as optoinjection or laserfection. In its original form, a pulsed laser was directly focused to a small spot on the cell membrane to create a pore (**Figure 1a**). While cells could be successfully transfected this way, throughput was very low being essentially a single cell technology. In the last two decades, thanks to the emergence of photothermal nanomaterials, photoporation throughput could be substantially enhanced. For 'photothermal nanomaterial mediated photoporation', which we will simply refer to as photoporation from here on (**Figure 1b**), cells are incubated first with the photothermal nanomaterials to let them adsorb to the cell membrane. Next, pulsed laser irradiation is applied to produce photothermal effects which induce pore formation in the cell membrane. Depending on the laser energy, membrane permeabilization either happens by photochemical reactions, local heating, or by laser-induced water vapor nanobubbles (VNB)[9,

[11, 12]. When a fs (femtosecond) ultra-fast pulsed laser is applied below the laser fluence threshold of bubble formation (in our work, the VNB threshold fluence was defined as the laser fluence at which 90% of the particles generate VNBs in the laser spot[3, 9]), the reactive free electrons can be generated by multiphoton ionization, resulting in highly reactive oxygen species which can increase the permeability of the cell membrane. The use of plasma-mediated nanobubbles was first proposed by the Meunier group, using fs pulsed laser irradiation of 100 nm AuNPs[13]. When instead ns (nanosecond) or ps (picosecond) laser pulses are used below the laser fluence threshold of VNB formation, direct heat transfer from the membrane associated photothermal nanoparticles can also create pores. Above the VNB threshold, the water surrounding the nanoparticles will start to evaporate, resulting in expanding VNB which implode when the thermal energy from the nanoparticle is consumed. The emerging pressure waves and liquid jets can then create pores in the cell membrane[14]. For a more in depth understanding of the interaction of (plasmonic) nanomaterials with pulsed laser light, the interested readers is referred elsewhere[9].

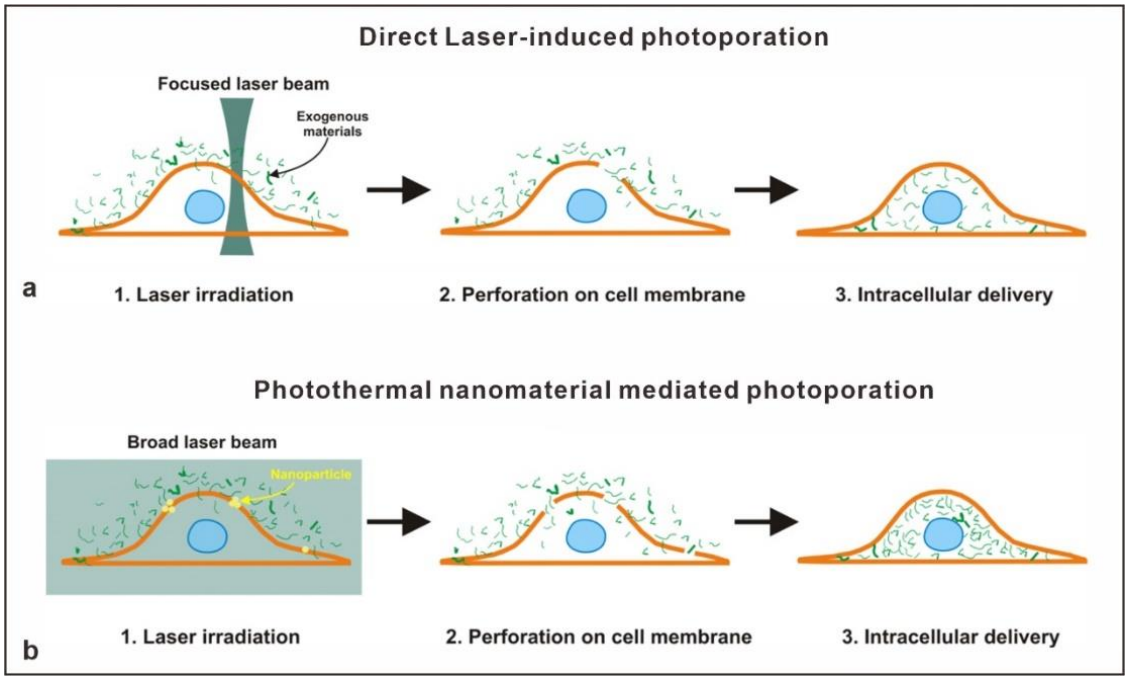

Figure 1. Direct laser-induced photoporation and photothermal nanomaterial mediated photoporation. **a**. In direct photoporation, the laser beam is focused precisely on the cell membrane to achieve a locally high photon density for photoporation of the cell. **b.** In photothermal nanomaterial mediated photoporation, the nanomaterials are first incubated with the cells to allow them to adhere to the cell membrane. Following laser irradiation, exogenous compounds in the cell medium can diffuse

through the membrane pore(s) into the cell's cytoplasm. Reproduced with permission from ref 9. Copyright 2016, the Authors, published by Informa UK Limited, trading as Taylor & Francis Group.

In the past 10 years, our group has made substantial contributions to the development of photoporation, driven by the quest for ever more efficient, flexible and safe intracellular delivery technologies. In our first report we demonstrated that the physical force exerted by suddenly formed VNB from 70 nm gold nanoparticles (AuNPs) leads to more efficient intracellular delivery as compared to mere heating [1, 15-18]. Next, we explored the use of different photothermal nanomaterials, finding that graphene quantum dots display enhanced thermal stability compared to the more traditionally used AuNPs, hence providing the possibility to increase the delivery efficiency by repeated laser activation[19-21]. We also investigated black phosphorus quantum dots (BPQDs) which have a broad and uniform absorption spectrum from visible to near infra-red (NIR) wavelengths, enhancing the photoporation efficiency in thick light scattering tissues[22]. To enable its use for the production of engineered therapeutic cells, for which contact of cells with non-degradable nanoparticles like AuNPs poses toxicity and regulatory concerns[3], we recently demonstrated that photoporation can be performed with biodegradable polydopamine nanoparticles as well[2]. Alternatively, we also demonstrated that nanoparticle contact can be avoided by embedding the photothermal nanoparticles in a substrate made from biocompatible electrospun nanofibers[3, 23]. With this variety of photoporation approaches, over the years we demonstrated successful delivery of a broad variety of effector molecules (mRNA, siRNA, Cas9 ribonucleoproteins, nanobodies …) in many different cell types, including hard-to-transfect cells such as T cells, embryonic stem cells, neurons and macrophages. In addition, apart from intracellular delivery, we have explored the use of laser-induced VNB for other applications as well, such as improving antibiotics diffusion in bacterial biofilms, spatially controlled cell killing, or ablation of human vitreous opacities and non-cellular membranes in the eye[24-29].

Since the success of photoporation very much depends on the type of photothermal nanomaterial that is used as a sensitizer, in this Account we will discuss in detail which nanomaterials we have used over the years with a focus on efficiency and safety. We discriminate two types of photothermal nanomaterials: single nanostructures and composite

nanostructures. The first one includes gold nanoparticles, graphene quantum dots, black phosphorus quantum dots and polydopamine nanoparticles. The second type includes polymeric films and nanofibers containing photothermal nanoparticles, as well as drug loaded nanoparticles and nanobombs. We will discuss the synthesis and characterization of these nanomaterials and their application in photoporation, with their advantages and disadvantages. In the final section we will elaborate on future perspectives in this area.

2. SINGLE PHOTOTHERMAL NANOSTRUCTURES

2.1 Gold nanoparticles

Gold nanoparticles (AuNPs) have been used by far the most as photothermal nanomaterials for photoporation as they can efficiently absorb light by Localized Surface Plasmon Resonance (LSPR). Additional benefits are ease of synthesis and flexible surface functionalization. As indicated in **Figure 2a**, we commonly used 60-80 nm AuNPs which have a plasmon extinction peak around 530-560 nm. These AuNPs can be easily synthesized using the Turkevich method to generate gold nanoparticle seeds, followed by an overgrowth step to reach the desired size by adding gold ions and ascorbate solution in equimolar concentrations. Finally, a coating with the cationic polymer diallyldimethylammonium chloride (PDDAC) is applied onto the AuNPs (PDDAC@AuNPs) to facilitate electrostatic adsorption to the negatively charged cell membrane. Standard characterization includes determining the size and zeta potential by dynamic light scattering, the extinction spectrum by UV-VIS spectrometry and the morphology by Transmission/Scanning Electron Microscopy (T/SEM).

Next, VNB formation can be detected by dark field microscopy as the nanobubbles efficiently scatter light, as shown in **Figure 2b**. By counting the number of VNB for increasing laser pulse fluences, this allows to determine the VNB threshold, which is defined as the laser fluence at which VNBs are formed within the illuminated sample region with 90% certainty, as indicated by the dashed lines in **Figure 2c**. For the above mentioned AuNPs, for instance, the VNB threshold was ~0.5 J/cm^2 using 7 ns laser pulses at a wavelength of 561 nm. It is an observational fact that AuNPs become fragmented after already one laser pulse at or above the VNB threshold, as confirmed by TEM images (**Figure 2d**). This is caused by laser pulses ionizing and melting the gold nanoparticle, causing it to eject a highly charged progeny droplet

and following with the creation of the complex fragmentation patterns under prolonged irradiation[30]. Due to AuNP fragmentation, typically only one VNB can be formed per AuNP.

Photoporation of cells with AuNP sensitizers happens as schematically shown in **Figure 2e.** (1) AuNPs are added to cells in the cell culture medium to let them associate with the cell membrane. (2) The remaining free AuNPs are washed away and fresh cell culture medium is added containing the effector molecules of interest. (3) Laser irradiation is applied to permeabilize the cell membrane, allowing the effector molecules to penetrate into the cytosol. (4) Finally, cells are washed again and fresh culture medium is added to let the cells continue to grow. In order to achieve the best balance between delivery efficiency and cell viability for a given cell type, the concentration of AuNPs, their incubation time and laser pulse fluence is typically optimized. For convenience, often a fluorescent macromolecule like FITC-dextran is used as a model cargo molecule, allowing easy read-out of delivery efficiency e.g. by flow cytometry. AuNP concentrations typically range from 10^7 to 10^9 NPs/mL, while the laser fluence is varied from about half to several-fold the VNB threshold. It is to be noted that the AuNP incubation time influences the required AuNP concentration. Longer incubation times usually require lower AuNP concentrations to let a sufficient number of AuNPs adsorb to the cells. However, the AuNP incubation time shouldn't be too long since AuNPs will gradually become endocytosed and trafficked deeper into the cell, which may lead to excessive internal damage upon laser irradiation.

As an example, the efficiency of delivering 10 kDa Alexa-Fluor 647 dextran (RD10) as a model macromolecule in H1299-eGFP by photoporation was determined for increasing AuNP concentrations and laser pulse fluence. It was found that a laser fluence of 1.6 J/cm^2 (561 nm, 7 ns) with a AuNP concentration of 1.6 X10^7 NPs/mL was most optimal, with more than 90% RD10+ cells and more than 80% cell viability. Continuing with those conditions, eGFP expression in H1299-eGFP cells could be silenced in 80% of the cells by delivering eGFP-siRNA (**Figure 3a**)[1]. When a five-fold lower laser pulse fluence was used to generate pores by heating instead of VNB formation, the silencing efficiency was markedly reduced. This led us to conclude that delivery is more efficient when pores are created by VNB rather than by mere heating of the AuNPs, while cell viability remained virtually the same.

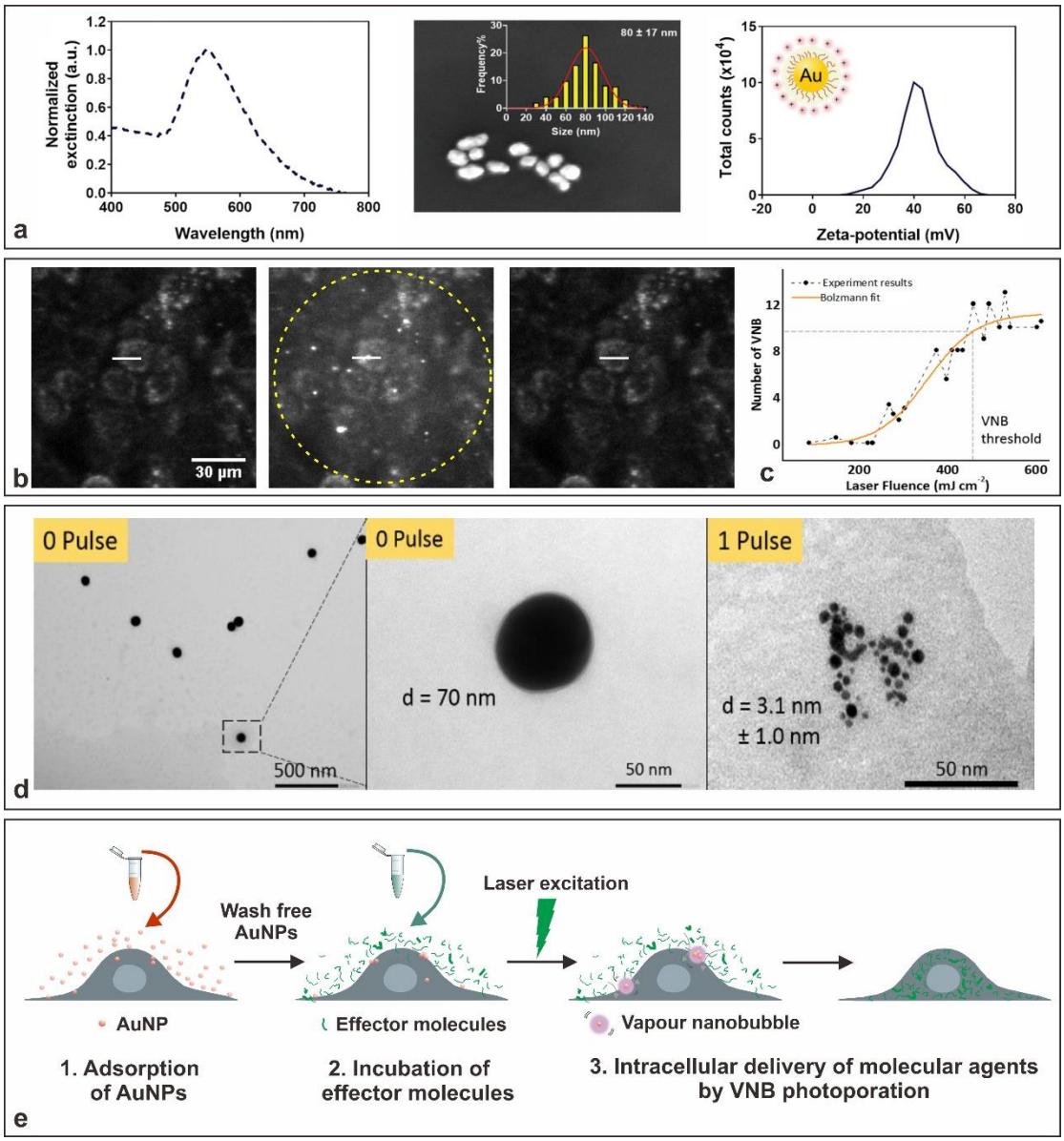

Figure 2. AuNP mediated photoporation. **a.** An example of a UV/VIS extinction spectrum of ~80 nm PDDAC@AuNPs, a SEM image with size distribution, and the ζ-potential. **b.** VNBs detected by dark field microscopy after incubating cells with PDDAC@AuNPs. Cells are shown before, during (VNBs visible as bright spots) and post laser illumination. The dashed circle marks the laser illumination area **c.** Determination of the VNB threshold of PDDAC@AuNPs. **d.** TEM images of PDDAC@AuNPs before (0 pulse) and after 1 laser pulse (0.94 J/cm^2, 7 ns, 561 nm), showing that AuNPs become fragmented after laser irradiation. **e.** Schematic overview of the experimental procedure of AuNP mediated photoporation. Reproduced with permission from refs 1, 18, 19. Copyright 2014 American Chemical Society, Copyright 2021 Elsevier, Copyright 2018 Nature Publishing Group.

In a follow-up study, we investigated if AuNP mediated photoporation can be used to transfect cells with larger nucleic acids, like mRNA[17]. As mRNA becomes easily degraded by nucleases,

we initially found that mRNA quickly degraded in a matter of 5-10 minutes after addition to the cell culture medium of cultured HeLa cells. However, by first washing the cells with Opti-MEM before adding the mRNA in fresh cell culture medium, mRNA degradation could be avoided for up to 10 min, which is sufficient to carry out the photoporation procedure. We also found that mRNA transfections were best if transfection was performed in PBS buffer supplemented with magnesium and calcium. Using an mRNA concentration of 0.1 mg/mL, a transfection efficiency of ~40% was obtained on HeLa cells with a cell viability >80% (**Figure 3b**). Also a suspension of Jurkat T cells was photoporated with mRNA, achieving 45% transfection efficiency. Most interestingly, we found that the number of mRNA transfected viable Jurkat T cells was five-fold more as compared to electroporation, which was primarily the result of photoporation being much more gentle to cells and keeping much more cells alive compared to electroporation. While in our hands AuNP photoporation has not proven effective to deliver large plasmid DNA in cells, others have published that it was possible in 'hard-to-transfect' T-cells [31].

We also demonstrated successful delivery of functional proteins in cells by AuNP mediated photoporation. We used, for instance, AuNP mediated photoporation to deliver Cas9 ribonucleoprotein (RNP) complexes in cells. In a H1299-eGFP cell line, eGFP expression could be knocked out in 80% of the cells with a cell viability >80% as shown in **Figure 3c**.[32]. In another application, the mixed-lineage kinase domain like (MLKL) protein, the most terminal mediator of necroptosis, was delivered in murine B16 melanoma cells, next to caspase-8 and -3 protein. Functional delivery was evident from enhanced cell death in all three cases[33]. Besides the delivery of organic biological molecules, we also delivered inorganic contrast agents in cells for long term *in vivo* cell tracking experiments. CdSe/ZnS quantum dots were delivered in INS-1E cells (insulin producing cell line) for fluorescence imaging while we delivered gadolinium complexes into SK-OV-3 IP1 cells for MRI (Magnetic Resonance Imaging) studies[15, 18]. Direct delivery of labels in the cytosol prevents sequestration of labels in endosomes, which otherwise can lead to quenching and degradation of the labels, resulting in poor contrast and fast fading of the signal.

While the studies mentioned so far were performed on cell lines, we also applied AuNP photoporation to hard-to-transfect primary cells. For instance, FD10 was successfully delivered

in primary human CD4$^+$ T cells reaching ~40% FD10+ cells with ~70% cell viability. Murine CD8$^+$ T cells could be transfected with siRNA in order to downregulate CD45 expression. As show in **Figure 3d**, the yield of living transfected murine CD8$^+$ T cells obtained with photoporation was three times higher as compared to electroporation[16, 34]. In another study, primary hippocampal neurons in dense cultured networks were labeled with a fluorescent contrast agent using AuNP mediated photoporation in order to accurately quantify dendritic spine density and morphology in mature neuronal networks[35, 36]. Labelling by photoporation produced better quality confocal images as compared to traditionally used lipophilic dyes, as shown in **Figure 3e.**[36]. Finally, as shown in **Figure 3f**, we also demonstrated that AuNP mediated photoporation is suited to deliver macromolecules into the cytosol of primary macrophages[37].

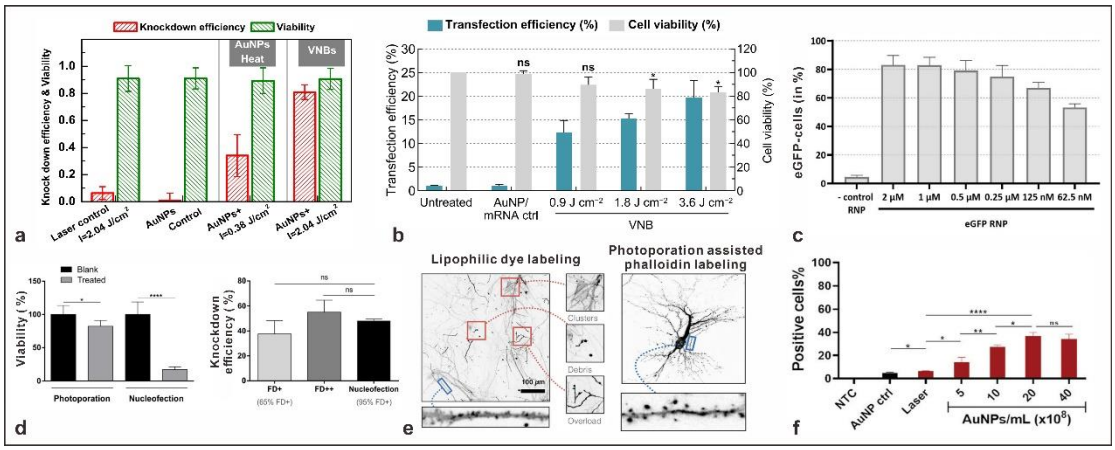

Figure 3. Intracellular delivery of effector molecules in various cell types by AuNP mediated photoporation. **a.** AuNP mediated photoporation of H1299 eGFP cells with varying laser intensity for siRNA gene silencing. eGFP expression was quantified with flow cytometry after 24h. **b.** eGFP-mRNA transfection in HeLa cells by AuNP mediated photoporation with 0.3 µM eGFP-mRNA using increasing laser fluence and a fixed AuNP concentration of 8×10^7 NPs/mL. **c.** H1299-eGFP knock-out levels after photoporation in the presence of decreasing concentrations of eGFP RNPs. **d.** Cell viability and gene silencing in cytotoxic T lymphocytes (CTLs) by AuNP mediated photoporation versus electroporation (nucleofection). **e.** Labeling primary neurons by incubation with the lipophilic dye DiI results leads to staining artefacts in microscopy images, such as the presence of debris and uneven staining across the cells. Instead, intracellular delivery of phalloidin by photoporation resulted in high quality staining of selected neuronal cells. **f.** AuNP mediated photoporation of macrophages with FITC-dextran of 150 kDa at a laser fluence of 1.6 J/cm^2 for increasing AuNP concentration. Reproduced with permission from refs 1, 17, 32, 16, 36, 37. Copyright 2014 American Chemical Society, Copyright 2020 the Authors published

While photoporation traditionally refers to delivering effector molecules in cells by permeabilization of the outer cell membrane, we have shown that VNB formation by AuNPs can be leveraged to permeabilize the nuclear envelope as well. Upon incubating HeLa cells with AuNPs for extended periods of time (8-24h), AuNPs became endocytosed and were trafficked to the perinuclear area. Upon laser irradiation and VNB formation, the nearby nuclear envelope could be controllably ruptured. This resulted in efflux of nuclear content in the cytoplasm and, vice versa, influx of cytosol content in the nucleus. In many cases, damage of the nuclear envelope was restored in less than an hour and cells continued to grow. Although the method needs further optimization to improve efficiency, it shows that photoporation could possibly be used to enhance intranuclear transport of macromolecules as well[38, 39]. In conclusion, AuNP mediated photoporation offers the possibility to deliver a broad variety of cargo molecules in many different cell types, adherent or suspension cells, cell lines and primary cells.

2.2 Carbon-based nanoparticles

As discussed above, AuNPs become easily fragmented upon pulsed laser irradiation. Instead, carbon-based nanoparticles, such as reduced graphene oxide (rGO) nanoparticles and graphene quantum dots (GQD), have better thermal stability and can be irradiated multiple times without being destroyed. To obtain rGO NPs, GO was first synthesized by a modified Hummer's method and oxygen functional groups were reduced with hydrazine monohydrate to obtain rGO[21]. Upon ultrasonication of rGO NPs, smaller GQD nanoparticles can be obtained. **Figure 4a-d** shows the UV-NIR extinction spectra, the zeta potential, hydrodynamic size and SEM images of pristine and PEI (polyethyleneimine) or PEG (polyethyleneglycol) functionalized GQD and rGO nanoparticles.

The possibility to repeatedly form VNB from GQDs was confirmed by dark field microscopy (**Figure 4e**). It was found that GQDs can form VNBs for up to 5-6 times, although the probability decreases for increasing number of laser pulses (**Figure 4f**). This is due to the GQDs gradually becoming smaller after each laser pulse, likely due to removal of CO_2^-/CO_2H groups. The ability to form VNB repeatedly allowed us to permeabilize cells repeatedly with the same

GQDs, which proved useful to carefully tune the amount of extrinsic fluorescent labels that could be delivered in cells for live cell microscopy studies. We furthermore demonstrated that the labeled cells could be imaged by internal reflection fluorescence (TIRF) microscopy and super-resolution microscopy[19].

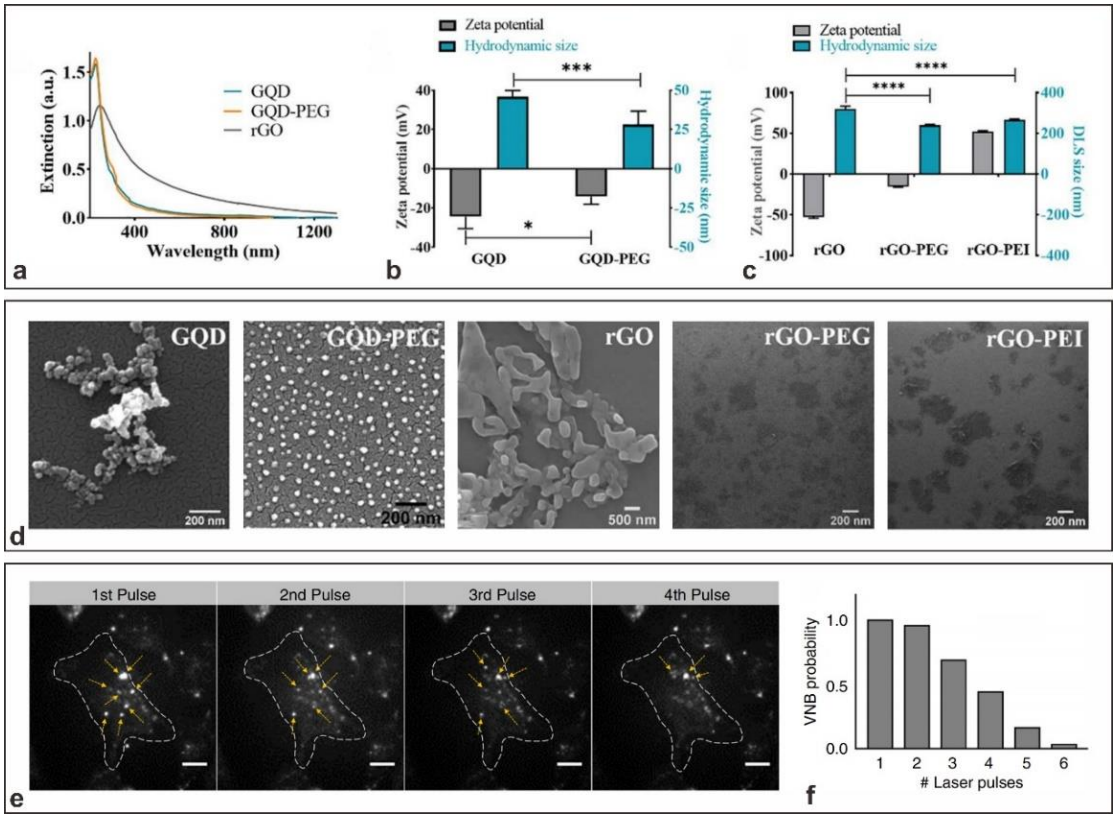

Figure 4. Physicochemical properties of GQD and rGO and repeated VNB formation. **a**. UV-NIR extinction spectra of GQD, GQD-PEG and rGO. **b**. Zeta potential and hydrodynamic size of GQD and GQD-PEG as measured by DLS. **c.** Zeta potential and hydrodynamic size of rGO, rGO-PEG, and rGO-PEI. **d**. SEM images of GQD, GQD-PEG, rGO, rGO-PEG and rGO-PEI. **e**. Dark-field images showing GQDs on HeLa cells and irradiated with 4 discrete laser pulses at twice the VNB generation threshold (7 ns, 561 nm). Repeated formation of VNBs can be observed (yellow arrows) for each subsequent laser pulse; the cell boundaries highlighted in the dashed lines and scale bar 20 μm. **f**. Probability for GQDs to repeatedly form VNB upon repeated exposure to laser pulses. Reproduced with permission from refs 21, 19. Copyright 2020 the authors published by MDPI, Copyright 2018 Nature Publishing Group.

In this initial study it was noticed that aggregates of GQDs were often formed upon addition to cell culture medium, leading to excessive cell damage upon laser irradiation by the formation of very large vapour bubbles. Therefore, to improve the colloidal stability of GQDs and better control VNB formation, bare rGO and GQD were coated with stabilizing polymers. Coating

with polyethylene glycol (PEG) was performed via a covalent bond between the terminal amino group on PEG and carboxyl groups on the nanoparticles. Coating of the negatively charged rGO nanoparticles with the cationic polymer polyethyleneimine (PEI) was achieved based on electrostatic adsorption. The optical and physicochemical properties of GQD and rGO before and after functionalization with PEG or PEI are shown in **Figure 4a-d**. The coated rGO nanoparticles had a size of 200-350 nm, while GQDs were much smaller at ~30 nm. As expected, only in the case of PEI coated rGO a positive zeta potential was obtained, while for all other nanoparticles the zeta potential was negative. Setting 80% viability as a minimum, the delivery efficiency of FITC dextran 10 kDa in HeLa cells was ~50% and~40% for bare rGO and GQDs, respectively. This increased to 81% for PEG@GQDs, 74% for PEG@rGO and 90% for PEI@rGO[21]. Using PEI@rGO as the best performing particles, fluorescently labeled nanobodies were delivered in cells for high-quality long-term microscopic investigations[20, 40]. It is worth noting that also others have made use of carbon-based materials for photoporation, such as carbon black nanoparticles and carbon nanotubes[41, 42].

2.3 Biodegradable nanoparticles

When it comes to clinical translation of photoporation, for instance for the production of engineered therapeutic cells, there is concern about potential toxic effects of AuNPs or carbon-based NPs which do not, or only very slowly, degrade over time. Also, the fact that AuNPs fragment upon pulsed laser irradiation poses additional safety risks as small nanoparticles may cause genotoxic effects by intercalating with intracellular DNA[43-45]. Therefore, we explored the possibility of performing photoporation with biodegradable photosensitizers, such as iron oxide NPs (IONPs), black phosphorus quantum dots BPQDs or organic polydopamine NPs.

IONPs have already been clinically approved as a magnetic resonance imaging (MRI) contrast agent. After uptake in cells, IONPs can be degraded in endosomes *via* a wide variety of hydrolytic enzymes such as lysosomal cathepsin L. In our work, we used commercially available magnetic nanoparticles (MagPs) of 500 nm in size, consisting of a polystyrene core surrounded by a shell of small IONP. The MagPs were functionalized with a PEI coating via electrostatic adsorption. As shown in **Figure 5a-b,** photoporation with MagPs was compared to AuNPs by delivering FD150 in macrophages. For the most optimal concentration of AuNPs (20

× 10^8 NPs/mL) and optimized laser fluence of 1.6 J/cm^2, 37% positive cells were obtained with an rMFI of 2.2 and 90% viable cells. Using an optimized MagP concentration of 10 × 10^7 MagPs per/mL and laser fluence fo 0.84 J/cm^2, 51% positive cells were obtained with an rMFI of 9 and cell viability of ~80%. This showed that IONPs represent a viable alternative to AuNPs, with even better performance on macrophages.

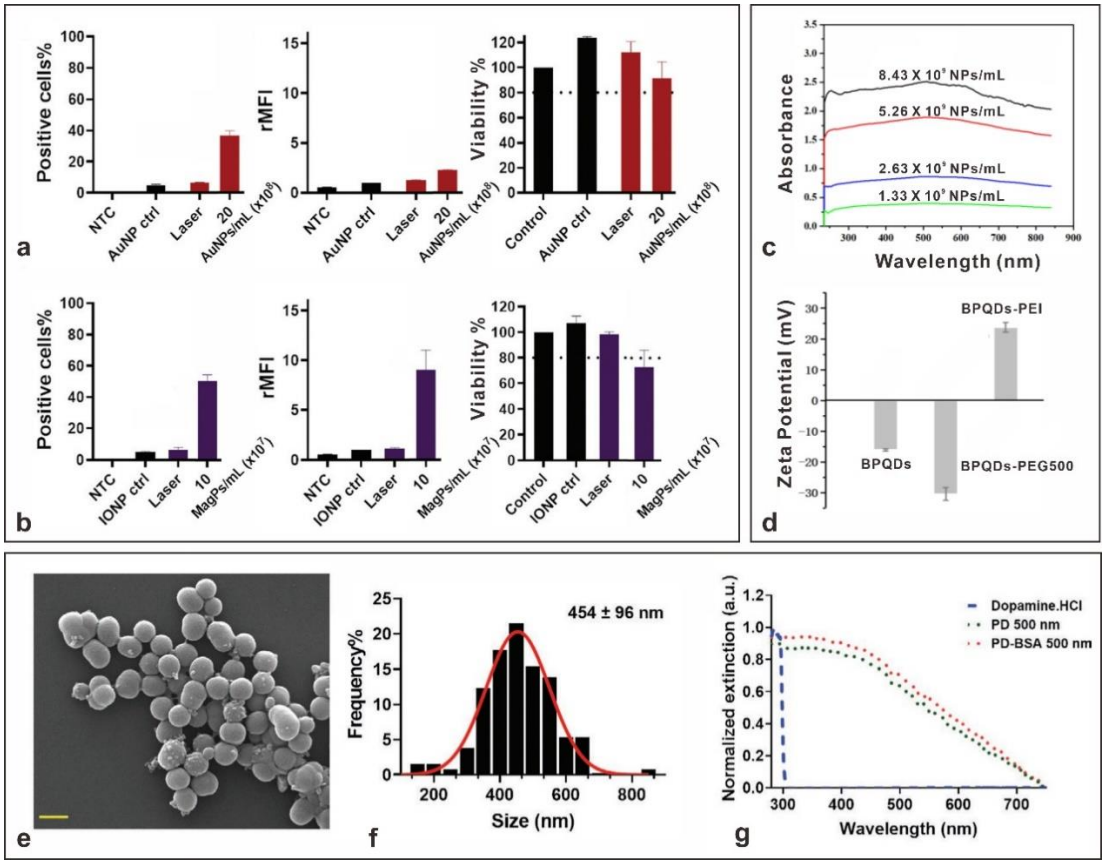

Figure 5. Photoporation with biodegradable nanoparticles, including MagPs, BPQDs and polydopamine nanoparticles. a-b, FITC-dextran of 150 kDa (FD150) is delivered in macrophages by photoporation with AuNPs (**a**) and MagPs (**b**). Delivery efficiency is expressed as the percentage of FD150+ cells. The relative Mean Fluorescence Intensity (rMFI) expresses the average fluorescence per cell compared to untreated cells. Cell viability is measured by the metabolic WST-1 cell viability assay 6 h after photoporation. **c-d**, BPQD UV-Vis absorption spectra at several concentrations in water (**c**), and the zeta potential of pristine and functionalized BPQDs with 500 kDa polyethyleneglycol (BPQD-PEG500) or 25 kDa polyethyleneimine (BPQD-PEI) (**d**). **e-g**, Physicochemical characterization of bare or BSA coated PD NPs. SEM image of bare ~500nm PD NPs (scale bar 500 nm) (**e**) and the corresponding size distribution determined by image processing (**f**); UV-VIS extinction spectrum of respectively dopamine.HCl, bare PD NPs and PD NPs coated with bovine serum albumin (PD-BSA) (**g**). Reproduced with permission from refs 37, 22, 2. Copyright 2021 Royal

In another study we used BPQDs which can be degraded into biocompatible substances such as PO_2^{3-},PO_3^{3-} and PO_4^{3-} upon laser irradiation[46].Their broad near-uniform absorption spectrum from 250 nm to 850 nm (**Figure 5c**) and high photothermal conversion efficiency makes them interesting nanosensitizers for photoporation[22]. BPQDs of ~20 nm were synthesized by liquid stripping of bulk BPQDs using sonication and functionalized with 25 kDa branched polyethyleneimine (PEI) or 500 kDa PEG (**Figure 5d**). Thanks to the broad absorption spectrum, photoporation was successful when using both visible (532 nm) and NIR (800 nm) laser irradiation, reaching 53% mRNA transfected HeLa cells when using an mRNA concentration of 0.4 μg/mL. Interestingly, BPQD mediated photoporation was used to deliver macromolecules in cells with thick phantom tissue in the optical path, achieving ~50% better delivery efficiency when using NIR laser irradiation instead of visible light (561 nm).

Besides inorganic nanoparticles, we also explored biodegradable organic polydopamine nanoparticles (PD NPs) as photoporation nanosensitizers[2]. As only dopamine-HCl is required as a precursor (a clinically approved compound), and because it has been shown that polydopamine can be degraded under acidic (lysosomes) or reducing (cytosol) conditions, these nanoparticles are attractive for clinical translation[47, 48]. First, we synthesized ~0.5 μm PD NPs via spontaneous oxidation of dopamine hydrochloride followed by polymerization, after which they were coated with bovine serum albumin (BSA) to improve colloidal stability and cell attachment (**Figure 5e-f**). UV/VIS measurements of BSA@PD NPs showed a decreasing yet broad extinction spectrum ranging from the UV up to the near-infrared region (**Figure 5g**). Next, using BSA@PD NPs as photoporation sensitizers, the intracellular delivery of 500 kDa FITC-dextran as well as eGFP-mRNA were tested in adherent HeLa cells and suspension Jurkat cells. An mRNA transfection efficiency of more than 45% was achieved with a cell viability of >85% for HeLa cells. For Jurkat cells, a frequently used model cell line for T cells, a transfection efficiency of ~45% was obtained with a viability of ~50 %. On activated primary human T cells, the mRNA transfection efficiency was 29%±3% for a cell viability of 63%±2%. This means that the overall yield of living transfected T cells was ~20%, being 2.5-fold more than what could be achieved with electroporation as a benchmark technology. In a follow-up

study we investigated delivery of 500 kDa FITC-dextran in non-activated human T cells, finding that smaller BSA@PD NPs of 150 or 250 nm have less impact on T cell functionality rather than 400 nm particles[49]. These findings show that photoporation with BSA@PD NPs can be used to deliver compounds in quiescent T cells as well, but that the nanoparticles should be small enough to avoid damaging cells too much. Recently, we also reported on the use of Response Surface Methodology to facilitate finding the optimal parameters for photoporation of cells with BSA@PD NPs (size, concentration, laser fluence)[50]. Compared to the more typical 'one factor at a time' optimization strategy, Response Surface Methodology allowed to find optimal conditions 5 to 8-fold more efficiently, thus proving to be a valuable approach to efficiently optimize photoporation conditions for a particular cell type.

3. COMPOSITE PHOTOTHERMAL NANOSTRUCTURES

Intracellular delivery by nanosensitizer photoporation depends on passive influx of external molecules in cells through the created membrane pores. Intracellular delivery of macromolecules becomes increasingly difficult for increasing size due to slower diffusional mobility and steric hindrance at the pores. To increase the delivery efficiency of large cargo molecules like mRNA and pDNA, we developed a composite nanostructure, called nanobombs, to create bigger pores in the cell membrane[4]. The light-triggered nanobombs were made of a photothermal core particle coated with smaller nanoparticles which function as nanoprojectiles (**Figure 6a**). Upon irradiation with pulsed laser light, the photothermal core particle will quickly heat up, resulting in vapor bubble formation whose mechanical forces will propel the outer nanoprojectiles through the cell membrane of nearby cells (**Figure 6a**). This allows to tune the size of the created pores by controlling the size of the nanoprojectiles that are used. As the photothermal core particle should be relatively large, we opted to work with MagP particles of 0.5 μm diameter. The MagP particles were functionalized with streptavidin, allowing straightforward coupling of biotinylated nanoprojectiles, including fluorescent polystyrene nanospheres, poly(lactic-co-glycolic acid) (PLGA) NPs and TiO_2 NPs. Photoporation with nanobombs happens according to a similar procedure as for traditional nanosensitizer photoporation. Cells are incubated with nanobombs and cargo and irradiated with pulsed laser light, after which cargo molecules will enter into the cells via the created membrane pores. We

first evaluated the light-triggered NBs have the capabilities of penetration to cell-membrane, showing the successful penetration of nanoprojectiles in the cell's cytoplasm. Next, both mRNA and pDNA were successful intracellular delivery in adherent HeLa and suspension Jurkat cells by light-triggered NBs. Finally, we were able to show that nanobomb photoporation was able to transfect both adherent and non-adherent cells with mRNA and pDNA, outperforming electroporation by a factor of 5.5–7.6 in transfection yield.

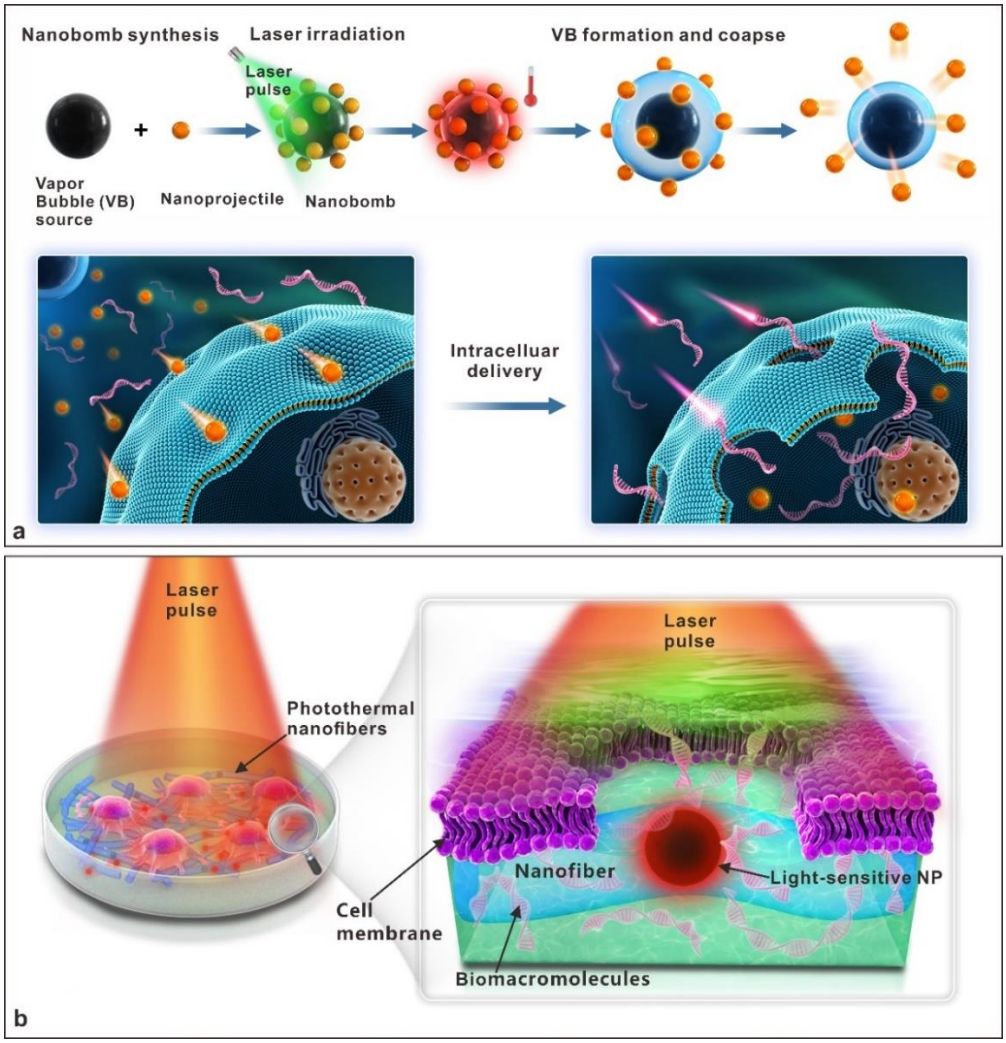

Figure 6. Photoporation with composite photothermal nanostructures, including nanobombs and photothermal nanofibers. a. Optically triggered nanobombs are composed of a photothermal core particle (vapor bubble source) to which nanoprojectiles are conjugated. Upon irradiation with an intense laser pulse, a VB is formed whose mechanical forces will propel the nanoprojectiles across the plasma membrane of nearby cells. Foreign molecules, such as mRNA, present in the cell medium can then migrate into the cell's cytoplasm. **b.** Schematic overview of intracellular delivery by membrane permeabilization with photothermal nanofibres. Reproduced with permission from refs 4, 3. Copyright 2021 and 2022 Nature Publishing Group.

As mentioned before, when it comes to engineering cells for therapeutic applications, long-term toxicity of nanoparticles is an area of concern. While working with biodegradable nanoparticles alleviates these concerns to some extent, it would be even simpler in case photoporation could be performed without direct contact between cells and nanoparticles. Therefore, we decided to embed iron oxide nanoparticles (IONPs) as photosensitizers in poly (lactic acid) (PLA) polymeric films which can serve as a substrate onto which cells can be cultured. Irradiation of the films with pulsed laser light generates heat or water vapor bubbles at the surface of the films, creating pores in cells cultured on top of the films[23]. Importantly, after treatment the cells can be collected from the substrate while the photothermal nanoparticles remain safely embedded in the substrate. However, due to the narrow space between the cells and the substrate, diffusion of molecules into the cells proved to be limited in this concept, resulting in low intracellular delivery efficiency. Therefore, we exchanged the flat films by electrospun nanofibers which form a 3D porous structure below the cells, allowing better diffusion and influx of cargo molecules in cells[3]. As shown in **Figure 6b**, by incorporating IONPs within biocompatible electrospun nanofibers, photothermal effects can still be transferred to nearby cells as in traditional photoporation, but without direct contact between cells and NPs. With these laser-irradiated photothermal nanofibers model macromolecules up to 500 kDa could be delivered in cells. Importantly, ICP-MS/MS (tandem ICP-mass spectrometry) analysis confirmed that no detectable amounts of IONPs were released from the fibers upon laser irradiation. The photothermal nanofibers were successfully used to deliver effector molecules, like CRISPR/Cas9 ribonucleoprotein complexes and siRNA, in adherent and suspension cells, including embryonic stem cells and hard-to-transfect human T-cells. *In vivo* experiments furthermore demonstrated successful tumor regression in mice treated with CAR-T cells in which expression of PD1 was downregulated after nanofiber photoporation with siPD1. In conclusion, photothermal nanofibers are a promising concept towards the safe and more efficient production of engineered cells by photoporation for therapeutic applications.

4. SUMMARY AND PERSPECTIVES

Photothermal nanomaterial mediated photoporation is a relatively new method that is receiving more and more attention in recent years to deliver effector molecules into live cells. In the last ten years, our group extensively explored various photothermal nanomaterials as sensitizers for photoporation, each with their own specific benefits. A summary of which nanosensitizers we applied to which cell types and cargo molecules is shown in **Table 1**. In our opinion, the following research directions can be further explored in the future.

Table 1. A summary of cell types used in connection with certain nanosensitizers and cargos.

Photothermal Nanomaterials	**Cell types**	**Cargos**
AuNPs	HeLa	FD10[1], mRNA[17]
	H1299	RD10[1], CRISPR/Cas9[32]
	Jurkat	mRNA[17]
	Murine B16	MLKL[33]
	INS-1E	QDs[15]
	SK-OV-3 IP1	Gadolinium complexes[18]
	Human CD4+ T cells	FD10 and siRNA[16, 34]
	Murine CD8+ T cells	FD10 and siRNA[16]
	Primary hippocampal neurons	Phalloidin[36]
	Primary bone-marrow derived macrophages	FD150[37]
rGO/GQDs	HeLa	FD10 or Nanobody[19-21]
IONPs	Primary bone-marrow derived macrophages	FD150[37]
BPQDs	HeLa, Jurkat	FD150 and mRNA[22]

PD NPs	HeLa, Jurkat, T cells	FD500 and mRNA[2]
Nanobombs	HeLa, Jurkat	mRNA and pDNA[4]
IONPs@Nanofibers	HeLa, Jurkat, H1299, Human embryonic stem cells, Human	FD10, siRNA, CRISPR/Cas9[3]

(a) Although we are convinced that photoporation can be used to efficiently deliver effector molecules into cells with acceptable toxicity, further fundamental research is required to fully understand the underlying pore forming mechanisms, like heat transfer from photothermal nanoparticles to the environment, nanobubble dynamics and cell repair mechanisms. A better understanding of these fundamental aspects will enable to better control and predict the influx of molecules into the cytoplasm.

(b) As photoporation matures as an in vitro transfection technology, it is to be expected that it will find its way into clinical applications as well, similar to what has happened for electroporation. Especially with the booming field of engineered cell based therapies, photoporation makes a good case to be considered as the transfection technology of choice to engineer cells ex vivo. This is supported by the emerging evidence that photoporation is quite gentle to cells while offering broad applicability in terms of types of cargo and cells. Direct application of photoporation in vivo may be considered as well, especially in those cases where the light and sensitizing nanoparticles can relatively easily reach the target cells. A nice example of this is the in vivo transfection of retinal ganglion cells by the Meunier group[51]. The Lapotko group even demonstrated the possibility of applying photoporation intraoperatively to eliminate microscopic residual disease in cancer[52].

(c) While delivery in mammalian cells has seen great progress in recent years, intracellular delivery in plant cells is lagging behind. This can be mainly attributed to the multilayer rigid plant cell wall, which has a size exclusion limit to external compounds of ~5 – ~20 nm. While a formidable barrier, adapting photoporation so that it can breach plant cell walls would constitute a major advance in the field. Compared to the golden standard of Agrobacterium transformation, as a general cell wall permeabilization method photoporation could offer broader applicability in terms of types of cargo that can be

delivered and types of plants that can be transfected.

(d) Besides using laser-induced VNBs to form pores in cell membranes, it will be of interest to explore this phenomenon to locally perturb other biological tissues. For instance, VNBs have been used to loosen up the structure of bacterial biofilms, resulting in improved diffusion and effectivity of antimicrobial agents[24]. As another example, we explored the use of laser-induced VNB photoporation to safely ablate vitreous opacities *in vitro* or *vivo*[25, 29, 53] and permeabilize the eye's inner limiting membrane for enhanced retinal drug delivery after intravitreal administration[54, 55]. These further successful applications demonstrate that it has great potential to other biological membranes or tissues.

Taken together we can conclude that photoporation is a promising technique for unprecedented flexible intracellular delivery of membrane impermeable substances or other applications to locally and precisely disturb biological tissues.

Biographies

Ranhua Xiong obtained a Ph.D. in pharmaceutics science from Ghent University in 2017. Since 2017 he has been a FWO post-doctoral fellow at the Department of Pharmacy of Ghent University. His doctoral and post-doctoral research was focused on photoporation as a novel strategy for intracellular delivery. In parallel he was also developing a microscopy-based method to perform sizing measurements of nanomaterials. Since 2021, he has been a professor at Nanjing Forestry University where he is co-directing Joint Laboratory of Advanced Biomedical Materials (NFU-UGent) with Prof. C. Huang. Recent research interest is at the interface between biophonic, drug delivery, and material sciences.

Félix Sauvage earned his pharmacy degree (Pharm. D) from the University of Rouen Normandie in 2013 and later completed a PhD at the Institut-Galien Paris-Saclay, focusing on delivering heat shock protein inhibitors to tumours. Since 2017, as a postdoctoral research scientist at Ghent University, he has focused on using pulsed-lasers and light absorbing nanoparticles for the treatment of eye diseases. He was recently appointed as an associate professor at Ghent University. His research is situated at the interface between drug delivery, material sciences and photonics with a major focus on ophthalmology.

Juan C. Fraire studied chemistry at the National University of Cordoba and obtained his degree in 2011. He obtained his PhD in Chemical Sciences from the same university in 2016 for his work focused on the development of plasmonic nanobiosensors. In 2016 he joined as a postdoctoral researcher at Ghent University. He is currently senior scientist at the Institute for Bioengineering of Catalonia - IBEC (Spain). His current research interests are at the interface of drug delivery, material science and active matter, mainly focused on the design of advanced drug delivery systems combining light-triggered effects and self-propulsion (nanomotors).

Chaobo Huang studied pharmaceutical sciences at Ghent University (Belgium) and graduated in 2011. Since 2011 he has been a post-doctoral fellow at EPFL (Switzerland). In 2013 he became Professor at College of Chemical Engineering of Nanjing Forestry University (NFU). The research of Prof. Dr. Huang explores the potential of micro- and nanosized fibers and particles for different applications. Prof. Dr. Huang is the first or corresponding author of more than 90 manuscripts including contributions to Chemical Society Reviews, Advanced Materials, Advanced Functional Materials, Biomaterials, Small and ACS Applied Materials & Interfaces.

Stefaan C. De Smedt studied pharmacy. He became Professor at Ghent University in 1999. He served as dean of his Faculty (2010-2014) and is a member of the Board of Directors of Ghent University. He has been a Guest Professor at various universities in Belgium and China. Since 2004 he serves as the European Editor of the JCR, currently as Editor-in-Chief. His research is at the interface between drug delivery, biophysics and material sciences. He is the (co-) author of > 400 manuscripts. He is as member of the Belgian Royal Academy of Medicine and the Académie Nationale de Pharmacie of France.

Kevin Braeckmans obtained a Master's Degree in Physics 1999 and a PhD in Pharmaceutical Sciences in 2004 at Ghent University, Belgium. Having performed research on advanced optical microscopy methods for pharmaceutical applications during his PhD studies and postdoc years, he was appointed Professor at Ghent University in 2008. At present he is leading the Biophotonics Research Group as part of the Lab. of General Biochemistry and Physical Pharmacy (director: prof. Stefaan De Smedt; co-director: prof. K. Braeckmans). His research is primarily focused on the development of biophotonics technologies to better understand and enhance the efficiency of drug delivery through nanotechnology and light-based methods.

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