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Photoporation with biodegradable polydopamine nanosensitizers enables safe and efficient delivery of mRNA in human T cells

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Abstract

Safe and efficient production of chimeric antigen receptor (CAR)-T cells is of crucial importance for cell-based cancer immunotherapy. Physical transfection methods have quickly gained in importance to transfect T-cells, being readily compatible with different cell types and a broad variety of cargo molecules. In particular, nanoparticle-sensitized photoporation has been introduced in recent years as a gentle yet efficient method to transiently permeabilize cells, allowing subsequent entry of external cargo molecules into the cells. Gold nanoparticles (AuNPs) have been used the most as photothermal sensitizers because they can easily form laser-induced vapor nanobubbles, a photothermal phenomenon that was shown to be particularly efficient for permeabilizing cells. However, as AuNPs are not biodegradable, clinical translation is hampered. Here we report for the first time on the possibility to form laser-induced vapor nanobubbles from biocompatible polymeric nanoparticles. Compared to electroporation as the most used physical transfection method for T cells, 2.5 times more living mRNA transfected human T cells were obtained via photoporation sensitized by polydopamine nanoparticles. Together it shows that photoporation is a viable approach for efficiently producing therapeutic engineered T-cells at a throughput easily exceeding 10^5 cells/second.

1. Introduction

Cell engineering of human T cells with a transmembrane chimeric antigen receptor (CAR) has shown outstanding clinical outcomes in cancer immunotherapy.^[1,2] Viral transduction is in the majority of cases being used for the production of such CAR-T cells.^[3] However, viral vectors are associated with several drawbacks such as a high cost, labor intensive production and safety issues.^[4,5] In addition they can only incorporate nucleic acids, with a vector-dependent size limit. More flexible physical transfection techniques are, therefore, gaining in interest, including for the delivery of CAR encoding genetic constructs into human T cells.^[6] Transient CAR transfection with messenger RNA (mRNA) is of particular interest as it enables better control over the duration of expression without any risks for unintended mutations by genomic integration.^[7,8]

At present electroporation is the most explored physical transfection technique for the production of CAR-T cells with mRNA.^[8–10] However, electroporation is often associated with high levels of cell death, which is unfavorable since patient-derived T cells can be limited in number.^[11–13] Hence, many expansion cycles are required to compensate for cell loss by the electroporation process, but that in turn leads to T cell exhaustion and reduced therapeutic potency.^[14] Indeed, recently it has been shown that human T cells that survive electroporation suffer from reduced functionality and dysregulation of gene expression, resulting in reduced anti-tumoral activity.^[15–17] Therefore, alternative physical transfection techniques which combine efficiency with safety have been avidly sought for in the past decade.^[6]

In particular, nanoparticle-sensitized photoporation has been suggested as a gentler alternative to electroporation for engineering of T cells. The procedure typically starts with incubating cationic 60–70 nm gold nanoparticles (AuNPs) with cells which adsorb electrostatically to the

cell membrane.^[14,18–21] Upon the application of a nano- or picosecond laser pulse, the AuNPs quickly heat up. When the laser pulse fluence is sufficiently high, the AuNP's temperature can exceed the critical temperature of water, leading to the formation of water vapor nanobubbles (VNBs) around those AuNPs.^[22] The mechanical energy which is created due to the rapid expansion and collapse of VNBs results in the creation of transient pores in the cell membrane.^[18,19] In the past decade, photoporation with AuNPs has been successfully used to deliver a wide variety of molecules in many different cell types, including in primary murine and human T cells^[14,18,23,24]. However, clinical translation of this technology for the production of engineered therapeutic cells is still a bottleneck due to the fact that AuNPs are not biodegradable. In addition, it has been repeatedly shown that AuNPs fragment into small pieces of several nanometers (<10 nm) after being exposed to pulsed laser irradiation.^[25–29] This poses additional safety risks as small gold nanoparticles may cause genotoxic effects by intercalating with intracellular DNA.^[30,31]

Therefore, to enable clinical use of photoporation for the production of engineered therapeutic cells, biocompatible and biodegradable photosensitizers would be of great interest. However, formation of laser-induced VNB has so-far only been shown to be possible from nanoparticles composed of metals, metal oxides or carbon allotropes, which do not or only very slowly degrade over time. Interestingly, it has been found that melanosomes from retinal pigment epithelial cells have the ability to generate vapor microbubbles when irradiated with pulsed laser light.^[32] Moreover, hemozoin crystals, formed by the malaria parasite, have also been shown to produce vapor nanobubbles upon irradiation with a pulsed laser.^[33] These observations suggest that VNBs could perhaps be formed from synthetic photo-responsive polymeric nanoparticles as well. The use of synthetic (melanin-like) polydopamine nanoparticles (PD NPs) seemed particularly interesting considering that PD NPs have photothermal properties and that only dopamine-HCl is required as a precursor, which is a clinically approved compound.^[34,35] Moreover, it has been shown that polydopamine is

biocompatible and biodegradable which is a favorable trait for clinical translation.^[34,35] Indeed, it has been suggested already that polydopamine could be degraded via lysosomal degradation.^[35,36] For instance, polydopamine has been shown to be degraded in the presence of an acidic environment.^[37–39] The degradation can also occur through oxidizing agents such as hydrogen peroxide which is produced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases which are expressed by different cells, including T cells.^[34,40] Glutathione has also been shown to degrade polydopamine and is present in different cell types.^[41] Degradability of polydopamine has been explicitly demonstrate in *in vivo* experiments as well.^[39,42] In particular, intravenous injection of polydopamine was shown before to have an excellent safety profile.^[39] In addition, degradation products of polydopamine have been also shown to have anti-inflammatory properties.^[43]

In this work we will synthesize 0.5 μm PD NPs as a biocompatible replacement for the traditionally used gold nanoparticles for photoporation. PD NPs will be coated with bovine serum albumin (BSA) to enhance colloidal stability and cell attachment. Dark-field imaging will be used to verify successful VNB formation upon nanosecond pulsed laser irradiation. Using BSA-coated PD NPs as photoporation sensitizers, the intracellular delivery of 500 kDa FITC-dextran as well as eGFP-mRNA will be tested in both adherent (HeLa cells) and suspension cells (Jurkat and human T cells). mRNA transfection in human T cells by photoporation with PD NPs will be compared to nucleofection as a state-of-the-art electroporation platform.

2. Results and discussion

2.1. Synthesis, functionalization and physicochemical characterization of polydopamine nanoparticles

PD NPs were synthesized according to an adapted previously described protocol.^[44] A relatively large particle size of 0.5 μm was aimed for aimed at generating large enough VNBs and therefore membrane pores for efficient entry of large macromolecules, such as mRNA, into cells (**Figure 1**).

Figure 1. Schematic figure of polydopamine-sensitized photoporation for the delivery of mRNA into cells. First, PD NPs and mRNA are added to cells after which an intense nanosecond laser pulse is applied. This leads to the creation of VNBs around the PD NPs, which eventually collapse and transiently permeabilize the cells, allowing mRNA molecules to enter into the cells. mRNA transfected cells are indicated in green since eGFP-mRNA was used in this work.

The synthesis of the PD NPs involves spontaneous oxidation of dopamine hydrochloride (dopamine.HCl) followed by polymerization. When a fixed ratio of dopamine.HCl : NaOH

(1:0.8) and temperature (45-50 °C) were used, a gradual growth of the nanoparticles could be observed over time (mixture becomes yellow and eventually turns into a dark-brown solution). After 7 hours of stirring, PD NPs with a hydrodynamic diameter (Z-average) of 636 ± 41 nm were obtained (**Figure 2a, Figure S1**). Upon sonication with a tip sonicator for 30 seconds to remove any agglomerates, PD NPs with a final hydrodynamic diameter of 465 ± 6 nm were obtained (**Figure 2a, red arrow, Figure S1**). The synthesis of spherical PD NPs was confirmed by scanning electron microscopy (SEM) (**Figure 2b**). The corresponding size distribution showed a core size of 454 ± 96 nm (**Figure 2c**) in correspondence to DLS. The PD NPs had a slightly positive zeta-potential of $+17.9 \pm 2.1$ mV in HyClone water which is likely due to the protonation of amine groups (**Figure S2, blue bar**). Next, the colloidal stability of these uncoated PD NPs was assessed in Opti-MEM, which was shown before to be a suitable medium for mRNA transfections with limited mRNA degradation.^[11] A fast size increase upon dispersion of PD NPs in Opti-MEM medium (≤ 10 minutes incubation time) was observed (**Figure 2d**). This is in line with previous reports where uncoated PD NPs were found to have an excellent colloidal stability in water but not in phosphate buffered saline (PBS).^[44] Therefore, to maintain colloidal stability of the PD NPs in Opti-MEM as transfection medium, an extra functionalization step with bovine serum albumin (BSA) was performed. The functionalization is based on a Schiff base type or Michael addition reaction between amine groups of BSA and catechol/quinine of polydopamine, which can be achieved through overnight incubation at room temperature.^[45–47] After several washing steps with HyClone water, the particle's hydrodynamic diameter slightly increased to 513 ± 6 nm which gave a first indication that coating with BSA was successful. In addition, the zeta-potential turned slightly negative after the coating procedure (-19.4 ± 0.8 mV in HyClone water), providing further proof that the BSA coating was successful (**Figure S2, violet bar**). Most importantly, BSA-coated PD NPs did no longer aggregate in Opti-MEM (**Figure 2e, red curve**) thanks to the electrostatic and steric repulsion of the BSA molecules.

To analyze the stability of uncoated and BSA-coated PD NPs over time, we measured the hydrodynamic diameter of both an uncoated and a BSA-coated polydopamine batch after 7 months of preparation via DLS in HyClone water. Uncoated PD NPs slightly agglomerated from 504 ± 8 nm to 723 ± 39 nm according to DLS (**Figure S3a**). BSA-coated PD NPs on the other hand remained perfectly stable (**Figure S3b**). This was expected as the BSA coating provides an additional steric repulsion next to the electrostatic repulsion.

We additionally analyzed if (partial) degradation may have occurred during this 7 month storage of PD NPs in HyClone water (4°C), which should lead to the release of dopamine monomers in solution. Therefore, we first pelleted all the PD NPs from the 7 month-old batches via centrifugation (10.000 rcf for 10 minutes) and measured the UV/VIS spectrum of the supernatant. Release of dopamine should be visible in the UV region as is also observed for the Dopamine.HCl precursor (**Figure S4**, green line). While the supernatant of freshly prepared PD NPs did not show any noticeable absorption, this was different for both the uncoated and BSA-coated PD NPs in which case the two distinct UV peaks of Dopamine.HCl could be seen. The spectral broadening into the visible range is likely stemming from larger polydopamine chains that are also released.

As the PD-BSA NPs will be used for photoporation, for which laser light absorption is an important requirement, we additionally performed UV/VIS measurements. The starting solution of dopamine.HCl had a sharp extinction peak in the UV region (**Figure 2f**, blue striped line). Instead, 500 nm PD NPs had a broad extinction spectrum ranging from the UV up to the near-infrared (NIR) region (**Figure 2f**, green dots), which remained virtually unaltered after BSA coating (**Figure 2f**, orange dots).

In a next step, we tested the possibility to create VNBs by irradiation of the PD-BSA NPs with pulsed laser light (7 ns pulse duration, 561 nm wavelength). The occurrence of VNBs was visualized by dark field microscopy based on the fact that they intensely scatter light during their lifetime.^[48] While before laser irradiation no VNBs can be seen (**Figure 2g**), several VNBs

appeared upon illumination with a laser pulse (**Figure 2h**). By counting the number of VNBs as a function of the applied laser pulse fluence and by fitting the plotted data with a Boltzmann sigmoidal function, the bubble threshold was determined, which is defined as the laser fluence at which 90% of the particles in the irradiated region generate VNBs. This resulted in a VNB laser fluence threshold of 1.1 J/cm^2 (**Figure 2i, dashed line on the left of the blue area**), which falls within the range of fluences that have been reported before to generate VNBs from AuNP.^[18,18,26,49] Upon decreasing the size of BSA-coated PD NPs down to respectively 300 nm (i.e., $282 \pm 1 \text{ nm}$) and 100 nm (i.e., $134 \pm 2 \text{ nm}$) (**Figure S5**), it could be seen that the VNB threshold increased. The 300 nm NPs had a VNB threshold of around 3.1 J/cm^2 (**Figure S6**), while for the 100 PD BSA NPs hardly any VNBs could be generated even at the highest possible laser fluence (4 J/cm^2) of our optical setup. Therefore, 500 nm was considered to be a suitable minimal size for further photoporation experiments. For cell experiments it is preferred that all PD NPs create VNBs so that a sufficiently high fluence of 1.60 J/cm^2 was selected for further photoporation experiments.

Figure 2. Physicochemical characterization of bare and BSA coated PD NPs. (a) The Hydrodynamic diameter of PD NPs in function of time during the synthesis as measured with DLS up to 7 hours. Sonication was finally applied to remove any remaining agglomerates which may have formed during the synthesis. (b) Representative SEM image of bare 500 nm PD NPs (scale bar = 500 nm). (c) Corresponding size distribution of bare 500 nm PD NPs showing a mean size of 454 ± 96 nm. (d) Hydrodynamic diameter of bare PD NPs as measured in respectively water (blue curve) and Opti-MEM (red curve) by DLS (incubation time ≤ 10 minutes), showing a clear size increase in Opti-MEM. (e) Hydrodynamic diameter of BSA coated PD NPs as measured in respectively water (blue curve) and Opti-MEM (red curve) by DLS (incubation time ≤ 10 minutes). (f) UV/VIS spectrum of respectively dopamine.HCl (blue striped line), bare PD NPs (green dotted line), and BSA coated PD NPs (orange dotted line). (g-h) Dark field microscopy images of PD NPs (g) before and (h) after the application of a laser pulse (7 ns pulse duration, 561 nm wavelength, $1.6 J/cm^2$) (scale bar = 50 μ m). (i) Graph showing the quantification of the number of VNBs from dark field microscopy images as a function of laser fluence. The dotted line to the left of the blue filled area represents the laser fluence threshold above which 90% of the irradiated PD NPs will form vapor bubbles.

2.2. Photoporation of HeLa cells: optimization of PD NP incubation protocol

Next, we investigated the potential of PD-BSA NPs to be used as sensitizers for the delivery of macromolecules into cells by photoporation. To deliver proof-of-concept, this was first done on HeLa cells as an often used model for photoporation studies^[19,20,50], in which FITC-dextran of 500 kDa (FD500) was delivered as a large model macromolecule with a comparable molecular weight as eGFP-mRNA (353 kDa, 996 nucleotides)^[51]. We tested two types of protocols to expose cells to the nanoparticles. In the first protocol the nanosensitizers were incubated for 30 minutes with HeLa cells followed by a washing step, adding of FD500 and applying laser irradiation (**Figure 3a**). We will refer to this protocol as the ‘traditional method’ because it is identical to how photoporation with AuNPs is normally performed. In the second protocol, PD NPs were simply added to the cell medium mixed with FD500 and irradiated immediately (i.e. without further incubation or washing) (**Figure 3b**). We will refer to this protocol as the ‘mixing method’. The ‘mixing method’ was tested on both adherent HeLa’s and HeLa’s in suspension after trypsinization since the final aim is to transfect T-cells, which are suspension cells (**Figure 3c**).

The percentage of FD500 positive cells, determined by flow cytometry after gating for viable cells (i.e. negative for TO-PRO-3 iodide) (‘% Efficiency’), gradually increased for increasing concentrations of PD-BSA NPs in all three cases (**Figure 3d-f**). This was accompanied by a gradual decrease in cell viability, measured two hours after treatment with the CellTiter-Glo assay. By multiplying the percentage of viable cells with the percentage of FD500 positive cells the delivery yield can be calculated, which is the percentage of living FD500 positive cells relative to the starting population (**Figure S9**). From this we see that the highest yield values were obtained for 8×10^7 NPs/mL, 8×10^7 NPs/mL and 16×10^7 NPs/mL for the traditional method, mixing method on adherent cells and mixing method on suspension cells, respectively. The differences between the corresponding yield values were, however, not statistically significant (**Figure 3f**). Also the relative mean fluorescence per cell was virtually the same for those conditions, with an rMFI value of about 15 (**Figure S10**). Together these results indicate that

incubating cells for 30 minutes with the PD-BSA NPs gives no additional advantage and that the simpler and faster ‘mixing method’ works equally well for BSA-PD NPs, irrespective if the cells are adherent or in suspension.

As there is no washing step included in the mixing method to remove unbound PD-BSA NPs, we wanted to clarify to which extent bound vs. unbound nanoparticles contributed to the delivery effect. To test this, 8×10^7 NPs/mL PD-BSA NPs were added to adherent HeLa cells similar to the mixing protocol. Next, cells were immediately washed twice with Opti-MEM to remove any unbound PD-BSA NPs so that the PD NPs that remain present are the ones adsorbed to the cells. Upon laser irradiation the percentage of FD500 positive cells decreased only slightly, while there was no significant difference in rMFI (**Figure S11**). This points to the fact that the cell permeabilizing effect in the mixing method still comes primarily from cell-associated BSA-coated PD NPs with little or no contribution from the PD NPs that are still free in the solution. Nevertheless we decided to continue with the mixing method as it is the most straight-forward while it is equally performant.

Next we wanted to verify successful mRNA delivery in HeLa’s. Since the final aim is to apply PD NP photoporation to suspension cells, HeLa cells were trypsinized and transferred to Opti-MEM containing $0.3 \mu\text{M}$ eGFP-mRNA and 16×10^7 NPs/mL PD-BSA NPs. Next, the sample was immediately irradiated with pulsed laser light, after which the cells were supplemented with complete cell culture medium for 24 hours. The eGFP-mRNA transfection efficiency of was $46 \pm 1\%$ (**Figure 3h and Figure 3i,II**) with a cell viability of $88 \pm 13\%$ (**Figure 3h, red bar**), leading to a transfection yield percentage of $41 \pm 7\%$ living transfected cells (**Figure 3h, green bar**). Confocal microscopy images gave further support that photoporation of HeLa cells resulted in bright eGFP expressing cells (**Figure 3j**). This result is markedly better as compared to our previous report where AuNPs were used as sensitizers resulting in an eGFP-mRNA transfection yield in HeLa cells of 16% (20% transfection efficiency with a cell viability of 80%).^[11] The fact that PD-BSA NPs perform better than AuNPs could potentially be explained

by a difference in size (i.e., $\approx 0.5 \mu\text{m}$ PD-BSA NPs versus $\approx 70 \text{ nm}$ AuNPs), which may result in bigger vapor bubbles and larger membrane pores, hence facilitating easier entry of large mRNA molecules. In addition, larger pores may take a longer time to repair, so that the molecules have more time to enter the cells.^[52] Another reason that could explain the enhanced transfection efficiency could be the generation of liquid jets from large PD NP induced vapor bubbles, as has previously been proposed for clusters of AuNPs as well.^[53,54]

To exclude if BSA or BSA in combination with laser light could induce permeabilization of the cellular membrane, we performed a FD500 and mRNA (eGFP) delivery experiment according to the mixing protocol on adherent HeLa cells. Cells were incubated with OptiMEM containing an excess of the BSA (0.1 mg/mL) in combination with either FD500 (1 mg/mL) or eGFP-mRNA. The viability was assessed after respectively 2 hours (FD500) or 24 hours (mRNA) with CellTiter Glo assay. As can be seen from figure S7, no FD500 nor eGFP fluorescence could be detected, confirming that neither BSA nor BSA in combination with pulsed laser irradiation (1.6 J/cm^2) could permeabilize the cellular membrane (**Figure S7**). Since incubation with PD-BSA NPs alone (i.e. without laser irradiation) did not induce any permeabilization either (**Figure 3d-f**), it can be concluded that only laser-irradiated PD NPs cause permeabilization of the cell membrane.

Finally, we verified if larger particle sizes ($> 500 \text{ nm}$) would have any advantage for the delivery of mRNA. First, PD NPs of 750 and 1000 nm were synthesized using the same protocol as for the 500 nm particles. The hydrodynamic diameter of those particles as measured in HyClone water via DLS is shown in Figure S5. We performed a screening experiment for the delivery of mRNA via the mixing photoporation method in adherent HeLa cells (**Figure S8**). To find the most optimal concentration for every size, we first optimized the photoporation experiments on HeLa cells for the delivery of FD500. Next, the concentration which generated the highest delivery yield were selected for the mRNA delivery experiment (i.e., $500 \text{ nm} \approx 8 \times 10^7 \text{ NPs/mL}$, $750 \text{ nm} \approx 8 \times 10^7 \text{ NPs/mL}$, $1000 \text{ nm} \approx 3.2 \times 10^7 \text{ NPs/mL}$). To reduce mRNA consumption, we

used a reduced concentration of 0.025 mg/mL for this screening experiment (instead of 0.1 mg/mL for the main experiments in the manuscript). Based on the photoporation results (**Figure S8a and S8b**), it could be observed that bigger PD-BSA NPs did not result in a better transfection yield (**Figure S8b**). Therefore, we finally continued our experiments with 500 nm PD-BSA NPs.

Figure 3. Comparison of the ‘traditional’ and ‘mixing’ incubation method for 500 nm PD-BSA NPs on HeLa’s. (a) Schematic illustration of traditional photoporation on adherent cells. (b) Schematic illustration of mixing photoporation on adherent cells. (c) Schematic illustration of mixing photoporation on suspension cells. (d) Delivery efficiency of 500 kDa FITC-dextran

(FD500) (left y-axis, blue bars) and cell viability (2 hours after photoporation, right y-axis, red bars) via the traditional method in adherent HeLa cells. UTC: untreated control, Ctrl: mock FD500 control, PD ctrl: PD-BSA NPs and FD500 control, Laser ctrl: laser and FD500 control, Photo ctrl: mock photoporation control (32×10^7 PD-BSA NPs/mL). e) Delivery efficiency of FD500 (left y-axis, blue bars) and viability (2 hours, right y-axis, red bars) via the mixing method in adherent HeLa cells. (f) Delivery efficiency of FD500 (left y-axis, blue bars) and viability (2 hours, right y-axis, red bars) via the mixing method in suspension HeLa cells (i.e. after trypsinization). (g) The optimal yield percentage of viable FD500 positive cells for the different methods (i.e. traditional method on adherent HeLa cells (left bar with diagonal yellow stripes), mixing method on adherent HeLa cells (middle bar with horizontal yellow dots) and mixing method on suspension HeLa cells (right bar with horizontal yellow lines). (h) Transfection efficiency of mRNA, encoding for eGFP, (left y-axis, blue bars) and corresponding viability (24 hours, left y-axis, red bars) with the mixing method (8×10^7 NPs/mL) in suspension HeLa cells. The transfection yield (right y-axis, green bars) is the percentage of viable eGFP positive cells relative to the entire initial cell population. (i) Flow cytometry density plots of HeLa cells transfected with eGFP (I) mock transfected HeLa cells mRNA control and (II) photoporation in the presence of mRNA. (j) Confocal microscopy images of HeLa cells transfected with eGFP-mRNA for respectively (I) mRNA control and (II) photoporation in the presence of eGFP-mRNA. Scale bar = 100 μm . Data are shown as mean $\pm$ SD. Statistical significance: One-way ANOVA, n.s. refers to non-significant.

2.3. PD NP sensitized photoporation of Jurkat cells

Following successful proof-of-concept experiments on HeLa cells, we moved on to Jurkat cells as an immortalized human lymphocyte suspension cell-line which is frequently used as a model for primary human T cells.^[55] Jurkat cells dispersed in Opti-MEM were mixed with an increasing concentration of PD-BSA NPs together with FD500 and immediately (i.e. < 1 minute) photoporated with a laser fluence of 1.60 J/cm² (3 ns pulse duration, 532 nm wavelength), similar to the mixing procedure performed before on HeLa cells in suspension. It was found that both the percentage of FD500 positive cells ('% Efficiency') as well as the relative mean fluorescence intensity (rMFI) increased with an increasing concentration of PD-BSA NPs (**Figure 4a, dark green bars = delivery efficiency, blue bars = rMFI**). As usual this was accompanied by a gradual drop in cell viability (**Figure 4a, red dots**). Starting from 16×10^7 PD-BSA NPs/mL the delivery yield remained rather constant at 30-35% (**Figure S12**).

This is again substantially higher than what was previously obtained with AuNP photoporation of Jurkats in which case the yield was limited to ~20% (delivery efficiency of ~50% with a 2 hours viability of ~40%).^[18] Even more remarkably, the rMFI (± 38) obtained with PD-BSA NPs is about 8-fold higher compared to the rMFI with AuNP (± 5), which can again be explained by the fact that bigger pores are created, allowing more efficient influx of large macromolecules in the cytosol.

Next, the potential to deliver eGFP-mRNA into Jurkat cells was assessed. Jurkat cells (250.000 cells/well) dispersed in Opti-MEM were mixed with PD-BSA NPs (64×10^7 NPs/mL) together with mRNA (0.3 μ M). Photoporation resulted in a transfection efficiency of $45 \pm 14\%$ (**Figure 4b, dark green bars**) with a viability of $50 \pm 15\%$ (**Figure 4b, red bars**), amounting to a cell transfection yield of about 21% (**Figure 4b, green bars**). Exemplary flow cytometry density plots for the controls and photoporated samples are shown in **Figure 4c** while confocal microscopy images of transfected cells are shown in **Figure 4d**. A transfection yield of 21% is slightly better than what was previously reported for eGFP-mRNA photoporation of Jurkats with AuNP for which a transfection yield of 16% was obtained.^[11] Most strikingly, it is much better as compared to nucleofection, i.e. a state-of-the-art electroporation platform, for which a eGFP-mRNA transfection yield of only 4% could be obtained in Jurkat cells.^[11]

Figure 4. Delivery of FD500 and eGFP-mRNA into Jurkat cells by the mixing photoporation method with PD-BSA NPs as sensitizers. (a) Delivery efficiency (left y-axis, dark green bars) and relative mean fluorescence intensity (right y-axis, blue bars) of FD500 into Jurkat cells after photoporation for an increasing concentration of PD500-BSA NPs. The corresponding viability was measured after 24 hours with the metabolic CellTiter Glo assay (left y-axis, red dots). UTC: untreated control, Ctrl: mock FD500 control, Photo ctrl: mock photoporation control (i.e. only laser irradiation with PD-BSA NPs). (b) Transfection efficiency for eGFP-mRNA (left y-axis, dark green bars) by photoporation with a fixed concentration of PD-BSA NPs (64×10^7 PD-BSA NPs/mL). The corresponding viability was measured after 24 hours with the CellTiter Glo assay (left y-axis, red bars). The transfection yield (right y-axis, green bars) expresses the percentage of living and transfected cells compared to the initial population of cells. (c) Corresponding flow cytometry density plots of mRNA transfection in Jurkat cells for respectively (I) untreated control (UTC), (II) mRNA control, (III) photoporation control (64×10^7 NPs/mL) and (IV) photoporation in the presence of mRNA (64×10^7 NPs/mL). (d) Confocal microscopy images of Jurkat cells transfected with eGFP-mRNA by respectively (I) untreated control (UTC), (II) mRNA control, (III) mock photoporation control (64×10^7 NPs/mL) and (IV) photoporation in the presence of mRNA (64×10^7 NPs/mL). Scale bar = 100 μ m. Data are shown as mean $\pm$ SD.

2.4. Photoporation of human T cells

Finally, we proceeded to the *ex vivo* modification of hard-to-transfect primary human T cells using PD NP sensitized photoporation with the mixing method. Experiments were performed in an identical manner as for Jurkat cells, except that 1 million T cells were seeded per well. First, the optimal concentration of PD-BSA NPs was determined by FD500 delivery (**Figure 5a, dark green bars and blue bars**). As expected, the corresponding viability after 24 hours decreased with an increasing concentration of PD-BSA NPs (**Figure 5a, red dots**). Both 250×10^7 and 500×10^7 NPs/mL performed equally well in terms of delivery yield, reaching ~30% living and FD500 positive cells, quite similar to what was obtained for Jurkats (**Figure S13**). For 1000×10^7 NPs/mL the delivery yield was slightly but significantly less (~25%), mainly due to higher cytotoxicity. Confocal microscopy images confirmed successful delivery of FD500 into human T cells for increasing PD-BSA NP concentrations (**Figure 5b, II-IV**) in comparison to the control (**Figure 5b, I**).

Next, the potential to transfect primary human T cells with eGFP-mRNA was assessed. An increased transfection efficiency could again be observed for an increasing concentration of PD-BSA NPs. Indeed, transfection efficiencies of $22 \pm 2\%$, $29 \pm 3\%$ and $30 \pm 6\%$ could be obtained for a concentration of respectively 250×10^7 , 500×10^7 and 1000×10^7 PD-BSA NPs/mL (**Figure 5c, dark green bars**) with corresponding cell viabilities of $87 \pm 4\%$, $63 \pm 2\%$ and $44 \pm 9\%$ (**Figure 5c, red dots**). Exemplary flow cytometry plots and confocal images are shown in **Figure 5d** for a PD NP concentration of 250×10^7 PD-BSA NPs/mL. Exemplary flow cytometry plots for the other two concentrations are shown in **Figure S14**. The transfection yield was the highest at ~19% for a concentration of 250×10^7 and 500×10^7 PD-BSA NPs/mL, while it was slightly lower for 1000×10^7 PD-BSA NPs/mL (**Figure S15**).

To put these findings into perspective, mRNA was also delivered into human T cells via nucleofection as a benchmarking technology. Nucleofection of human T cells (using the optimized pulse program EO-115) in the presence of $0.3 \mu\text{M}$ mRNA resulted in a transfection

efficiency of 62.4 ± 27.9 % (**Figure 5e, dark green bar**). However, this was accompanied by a strong decrease in cell viability to 11.5 ± 3.6 % (**Figure 5e, red bar**), as also others have reported.^[14,15] With a corresponding transfection yield of only 7.5 ± 4.8 % (**Figure 5f**), this is 2.5 times less as for photoporation with PD NPs. In addition it was observed that the transfection results were much more variable in case of nucleofection with transfection yields ranging from 1.8% to 14.9% between biological repeats, while this was much less for photoporation with yields ranging from 16.6% to 19.9%.

As our data have shown that PD NPs have to be associated with the cells in order to be photoporated (**Figure S11**), it cannot be excluded that they may become internalized by endocytosis. However, it is important to note that the number of particles per cell is very limited in photoporation, since too many pores will else kill the cells. For instance, using 70 nm gold nanoparticles as sensitizers for photoporation, we determined before that on average 8 particles are present on HeLa cells^[20], while this was only 3 particles for murine T cells.^[14] Considering that 500 nm PD NPs likely make bigger pores than 70 nm AuNPs, the number of cell-associated PD NPs is probably even less than that. Therefore, the amount of PD NPs that are eventually endocytosed into cells is very small in any case, especially when considering that human T cells are known to have a very low endosomal uptake. Combined with the fact that PD NPs can be degraded in endolysosomes, uptake of a small number of PD NPs is not expected to give any adverse long term effects.

Figure 5. Delivery of FD500 and eGFP-mRNA into human T cells by the mixing photoporation protocol with PD-BSA NPs. (a) Delivery efficiency (left y-axis, dark green bars) and relative mean fluorescence intensity (right y-axis, blue bars) of FD500 into human T cells after photoporation for an increasing concentration of PD-BSA NPs. The corresponding viability was measured after 24 hours with the CellTiter Glo assay (left y-axis, red dots). UTC: untreated control, Ctrl: mock FD500 control, Photo ctrl: Mock photoporation control. (b) Representative confocal microscopy images of FD500 ‘loaded’ human T cells without (I) and with (II-IV) photoporation for a PD-BSA NP concentration of respectively (II) 250×10^7 , (III) 500×10^7 and (IV) 1000×10^7 NPs/mL (scale bar = 100 μ m). (c) Transfection efficiency for eGFP-mRNA (left y-axis, dark green bars) and corresponding relative mean fluorescence intensity (right y-axis, blue bars) after photoporation with an increasing concentration of PD-BSA NPs. The corresponding viability was measured after 24 hours with the CellTiter Glo assay (left y-axis, red dots). UTC: untreated control, Ctrl: mock mRNA control, Photo ctrl: mock photoporation control. (d, top images) Representative flow cytometry density plots for photoporation without mRNA encoding for eGFP (photoporation control) and with mRNA encoding for eGFP (photoporation mRNA) into human T cells (PD-BSA NP concentration of respectively 250×10^7 NPs/mL). (d, images below) Confocal microscopy images for photoporation without mRNA encoding for eGFP (photoporation control) and with mRNA encoding for eGFP (photoporation mRNA) into human T cells (PD-BSA NP concentration of respectively 250×10^7 NPs/mL). scale bar = 100 μ m. (e) Transfection efficiency for eGFP-mRNA (left y-axis, dark green bars) and corresponding relative mean fluorescence intensity (right y-axis, blue bars) after nucleofection of human T cells. The corresponding viability was measured after 24 hours with the metabolic Glo assay (left y-axis, red bars). (f) Comparison of the transfection yield (right y-axis, green bars) for respectively nucleofection (5 biological donors; 2 technical duplicates per donor)

and PD500-BSA NP-sensitized photoporation (4 biological donors; 2 technical duplicates per donor). Data are shown as mean $\pm$ SD. Statistical significance: Student's t-test, *** $p \leq 0.001$.

3. Conclusion

In this study we successfully synthesized polydopamine nanoparticles with a size of around 0.5 μm . These PD NPs were further functionalized with BSA to improve colloidal stability without interfering with the NP's ability to adhere to the cell membrane. We demonstrated successful vapor bubble formation from PD NPs upon irradiation with ns pulsed laser light, which is to our knowledge the first that this is demonstration for synthetic organic nanoparticles. Interestingly, polydopamine-sensitized laser-assisted photoporation could be applied on both adherent and suspension cells by simply mixing the nanoparticles with the cells and immediately applying laser irradiation. With this method we successfully delivered FD500 and eGFP encoding mRNA into HeLa cells, as well as in hard-to-transfect Jurkat and human T cells. Importantly, we found that about 2.5 times more living transfected primary human T cells were obtained after photoporation as compared to nucleofection. Considering that PD-BSA NPs are prepared from a clinically approved precursor, they open up the possibility for photoporation to be used for the production of engineered therapeutic cell products, such as CAR-T cells.

4. Experimental section

Synthesis of polydopamine NPs:

The synthesis of polydopamine nanoparticles was performed according to a previously described protocol with some slight modifications.^[44] Briefly, 70 mg of Dopamine.HCl (Sigma-Aldrich) was dissolved into 20 mL HyClone water (VWR, HyPure, Cell Culture Grade) of 45-50 °C. Next, 295 μL of a 1 M NaOH solution was added under vigorous stirring (molar ratio:

1/0.8). The colour of the solution immediately turned yellowish and then changed after some time into a dark brown colour. The solution was allowed to stir for about 7 hours. The size of the particles was carefully measured every hour by DLS. The solution was collected and washed for several times with HyClone water by centrifugation (4.000 rcf, 10 minutes). Next, to reduce potential aggregation, tip sonication was applied for 30 seconds (10% A, Branson digital sonifier, Danbury, USA).

Functionalization with bovine serum albumin:

To increase the colloidal stability of 500 nm PD NPs, functionalization with a bovine serum albumin (BSA, VWR Chemicals, Biotechnology grade, USA) layer was performed. Briefly, PD NPs were mixed with a solution prepared from mixing DPBS with 10 mg/mL BSA solution at a volume ratio of 1:1. The mixture was then allowed to react by vigorous stirring overnight and the remaining unbound BSA was removed by several washing steps with HyClone water (4000 rcf, 5 minutes). Finally, the BSA-coated 500 nm PD NP dispersed in HyClone water were stored at 4°C.

The synthesis and functionalization of BSA-coated PD NPs of 100, 300, 750 and 1000 nm was performed similarly as described above, except for using adapted reaction times (longer reaction times lead to larger particles).

Physicochemical characterization:

SEM images were acquired by using an FEI Quanta 200F (Thermo Scientific), operating at an accelerating voltage of 20 kV. The PD NPs were dried on a silicon wafer one day prior to the SEM measurements. The dried samples were additionally coated with a gold layer.

Dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern instruments Co., Ltd.) and Nanoparticle tracking analysis (NTA, NanoSight LM10, Malvern Panalytical, UK) were used to measure the hydrodynamic diameter. NTA was performed in scattering mode with a 488 nm laser. NTA was also used to determine the nanoparticle concentration. The UV/VIS spectrum was measured with a Nanodrop 2000c spectrophotometer (Thermofischer, Rockford, USA).

Vapor nanobubble generation and threshold:

The VNB generation from PD NPs was visualized by dark field microscopy upon the application of a pulsed nanosecond laser (OpoletteTM HE 355 LD, OPOTEK Inc, CA, 7 ns pulse duration, 561 nm wavelength). The laser pulse energy was measured with an energy meter (J-25MBHE&LE, Energy Max-USB/RS sensors, Coherent) which was synchronized with the pulsed laser. An EMCCD camera (Cascade II: 512, Photometrics, Tucson, USA) was used to record short movies which were processed with NIS elements software (Nikon, Japan). The bubble threshold, which is the 90% probability that the PD NPs in the irradiated area will form VNBs, was determined after fitting the data with a Boltzmann sigmoid function.

Cell cultures:

HeLa cells (ATTC[®] CCL-2TM) were cultured in DMEM/F-12 medium (Gibco-Invitrogen) which was further supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin/Streptomycin (Gibco-Invitrogen) and 2 mM L-glutamine (Gibco-invirogen). The cells were frequently passaged upon reaching confluency and kept in a humidified incubator (37 °C, 5% CO₂).

Jurkat cells (American Type Culture Collection, ATCC[®] TIB-152) were cultured in RPMI 1640 medium which was additionally supplemented with 10% fetal bovine serum (FBS), 100 U/mL

Penicillin/Streptomycin (Gibco-Invitrogen) and 2 mM L-glutamine (Gibco-invirogen). During the experiments, Jurkat cells were kept in culture in a humidified incubator (37 °C, 5% CO₂) and frequently passaged.

The human T cells were generated from buffy coats from blood donors after obtaining informed consent (Biobank Red Cross Flanders) and were used following the guidelines of the Medical Ethical Committee of the Ghent University Hospital (Belgium, Ghent). First, the isolation of peripheral blood mononuclear cells (PBMCs) from buffy coats was performed via density centrifugation using Lymphoprep (Alere Technologies AS, Oslo, Norway). The isolated PBMCs were then stimulated with Immunocult Human CD3/CD28 T cell Activator (Stemcell Technologies, Vancouver, Canada). The PBMCs were kept in culture for 7 days in IMDM medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS, Bovogen, Melbourne, Australia), 100U/ml penicillin (Invitrogen, Merelbeke, Belgium), 100 µg/ml streptomycin, 2 mM glutamine and 5 ng/ml IL-2 (Roche) for 7 days. Next, the expanded T cells were collected and restimulated with 1 µg/ml phytohemagglutinin (Remel Europe, KENT, UK) in the presence of a feeder mixture consisting of irradiated PBMCs (40 Gy) and JY cells (50Gy). After 4 days, 5 ng/ml IL-2 (Roche Diagnostics, Mannheim, Germany) was added. Ten to 14 days later, these culture expanded T cells were used and referred to as human T cells in the manuscript.

Traditional and mixing photoporation of adherent and suspension HeLa cells:

Traditional photoporation on adherent HeLa cells:

HeLa cells were seeded in a 96-well plate at 20.000 cells per well and incubated overnight in an incubator (37 °C, 5% CO₂). Next, the adherent HeLa cells were washed once with DPBS and new HeLa cell culture medium containing PD-BSA NPs with an increasing concentration was added which were allowed to incubate for 30 minutes. After 30 minutes of incubation, the

cells were washed once with Opti-MEM medium to remove the excess of unbound PD-BSA NPs. After washing, 50 μ L of fresh Opti-MEM medium containing 1 mg/mL FD500 was added to the cells. Photoporation was then performed with a fluence of 1.6 J/cm² on a custom built photoporation setup (pulse duration: 3 ns, 532 nm wavelength) similar to a previously reported design.^[20] Mock photoporation was performed with the PD-BSA nanosensitizers but without any cargo. The cells were then washed several times with cell culture medium to remove the excess of FD500 so as to avoid further spontaneous uptake of the marker by pinocytosis.

Mixing photoporation on adherent HeLa cells:

HeLa cells were seeded in a 96-well plate at 20.000 cells per well and incubated overnight in an incubator (37 °C, 5% CO₂). Next, the adherent HeLa cells were washed once with Opti-MEM medium and 25 μ L of Opti-MEM medium containing an increasing concentration of PD500-BSA NPs was added. An additional 25 μ L of Opti-MEM containing FD500 was added such that the final concentration of FD500 in the mixture was 1 mg/mL. Photoporation was then performed with a fluence of 1.6 J/cm² on the homemade photoporation setup (pulse duration: 3 ns, 532 nm wavelength). The cells were then washed several times with cell culture medium to remove the excess of FD500.

Mixing photoporation on suspension HeLa cells:

The suspension of HeLa cells was first collected after performing trypsinization. The cells were dispersed in cell culture medium, counted and washed twice with Opti-MEM medium. Next, 24 μ L of Opti-MEM medium containing 40.000 cells (doubling time of HeLa cells $\approx$ 24 hour) was added per well in a 96-well plate. The cells were then supplemented with another 25 μ L of Opti-MEM medium containing an increasing concentration of PD500-BSA NPs and FD500. The final concentration of FD500 in the 50 μ L mixture was 1 mg/mL. All the conditions had a final volume of 50 μ L and a quick spin-down (5-10 seconds, 500 rcf) of the 96-well plate was

performed such that the cells were sedimented on the bottom of the 96-well plate. Note: a quick spin-down was performed for all the different methods. The cells were then washed several times with cell culture medium to remove the excess of FD500.

Mixing photoporation of suspension HeLa cells for mRNA transfection:

A similar protocol as described above was followed. Briefly, 20 μL of Opti-MEM medium containing 40.000 cells was added per well in a 96-well plate. The cells were then supplemented with another 25 μL of Opti-MEM medium containing 16×10^7 NPs/mL. Next, the mixture was supplemented with 5 μL of mRNA, encoding for eGFP, resulting in a final mRNA concentration of 0.3 μM or 0.1 $\mu\text{g}/\mu\text{L}$. All the conditions had a final volume of 50 μL and a quick spin-down (5-10 seconds, 500 rcf) of the 96-well plate was performed such that the cells were on the bottom of the 96-well plate. Photoporation was performed at a fluence of 1.6 J/cm² and the cells were supplemented with 200 μL complete DMEM/F-12 medium. After 24 hours of incubation the cells were ready to be harvested for eGFP read-out (by flow cytometry or confocal microscopy) and viability measurement after 24 hours (see details below).

Photoporation of Jurkat cells and human T cells for the delivery of FD500:

Photoporation of Jurkat and human T cells was performed in a similar manner as mentioned before. Briefly, the cells were first washed several times with Opti-MEM (3 min, 500 rcf) in order to remove the medium. Next, 24 μL of Opti-MEM containing 250.000 Jurkat cells or 1 million human T cells was put into a single well of a 96-well plate (VWR, plastic bottom). Next, 25 μL of Opti-MEM containing the PD-BSA NPs at the desired concentration was immediately added to the cells. Finally, 1 μL of Opti-MEM containing FD500 (stock concentration 50 mg/mL) was added to the 49 μL mixture. For the untreated cells (UTC) which served as a

negative control, cells were dispersed in a total volume of 50 μ L Opti-MEM medium. An additional 'FD500 control' was included to correct for any spontaneous uptake of FD500 by cells, for which cells were first dispersed in 49 μ L of Opti-MEM and 1 μ L of FD500 (stock concentration of 50 mg/mL) was added to the mixture. Also a 'photoporation control' condition was included to check the effect of performing the photoporation procedure in the absence of cargo. Here, cells were dispersed in 25 μ L of Opti-MEM and supplemented with another 25 μ L of Opti-MEM containing PD-BSA NPs of the desired concentration. The photoporation control was performed for every tested PD-BSA NPs concentration such that FD500 delivery could be corrected for a shift in the background fluorescence in the presence of PD-BSA NPs. All the conditions had a final volume of 50 μ L and a quick spin-down (5-10 seconds, 500 rcf) of the 96-well plate was performed such that the cells were on the bottom of the 96-well plate. Photoporation was then performed operating at a fluence of 1.6 J/cm² and only took 3-4 seconds per well. The wells were then supplemented with 200 μ L fresh complete cell culture medium. In order to assess the cytotoxicity of the photoporation procedure, the exact same steps were performed but with the exception that FD500 was left out during the photoporation process. After the photoporation, the cells were supplemented with 200 μ L cell culture medium and left to incubate for the indicated amount of time (i.e. Jurkat cells: 24 hours, Human T cells: 4 hours, 24 hours and 48 hours).

Photoporation of Jurkat cells and human T cells for the mRNA transfection:

Transfection with mRNA was performed similarly to the photoporation of FD500s. Briefly, cells were first washed several times with Opti-MEM medium. Both Jurkat (250.000 cells) and human T cells (1 million cells), suspended in 20 μ L Opti-MEM, were transferred into a single well of a 96-well plate and were mixed with 25 μ L of Opti-MEM containing PD-BSA NPs at the desired concentration. Next 5 μ L of eGFP-mRNA (stock concentration of 1 μ g/ μ L,

CleanCap, Trilink Biotechnologies, San Diego, CA, USA) was quickly added to the 45 μ L mixture and the cells were quickly spun-down (5 seconds centrifugation at 500 rcf) such that the cells were at the bottom of the 96-well plate. Photoporation was then performed as mentioned before. For the untreated cells (UTC), the cells were dispersed in a total volume of 50 μ L Opti-MEM medium. For the mRNA control, the cells were first dispersed in 45 μ L of Opti-MEM and 5 μ L of mRNA eGFP (stock concentration of 1 μ g/ μ L) was added to the cells. For the photoporation control, the cells were dispersed in 25 μ L of Opti-MEM and supplemented with another 25 μ L of Opti-MEM containing PD-BSA NPs at the desired concentration. Following photoporation treatment, another 200 μ L of full cell culture medium was added to the cells and incubated for 24 hours in an incubator (37 $^{\circ}$ C, 5% CO₂).

Flow cytometry:

Flow cytometry was used to assess the delivery efficiency (FD500) or transfection efficiency (eGFP-mRNA) of the photoporation procedure. Following photoporation, the cells were washed several times (3 minutes, 500 rcf) with DPBS (-/-) to remove the excess of FD500 or cell debris. After the washing steps, flow buffer containing DPBS, 1% bovine serum albumin (BSA) and 0.1% NaN₃ was added to the cells. Furthermore, 0.5 μ M TO-PRO-3 iodide was used to stain the dead cells. The samples were then measured with a CytoFLEX flow cytometry (Beckman Coulter, Krefeld, Germany) using respectively a 488 nm and 637 nm excitation laser. eGFP fluorescence was detected with a 525/40 nm bandpass filter, while the signal of the TO-PRO-3 probe was detected with a 660/20 nm bandpass filter. The TO-PRO-3 positive cells (i.e. dead cells) were excluded for quantification of the FD500 and eGFP signal.

Confocal microscopy:

The Nikon A1R HD laser scanning confocal microscope, operating at room temperature, was used to record confocal images with respectively a 10x (CFI Plan Apo Lambda 10X, 0.45 NA), 20x (CFI Plan Apochromat VC 20X, 0.75 NA) and 60x (CFI SR Plan Apo IR 60XAC WI, NA 1.27) objective. Excitation was performed with a 488 nm laser and the fluorescent signal was detected at 525/50nm (MHE57030, A1 Filter Cube 525/50) by A1-DUG-2 GaAsP Multi Detector Unit (this means following detectors/channel: GaAsP PMT for 488 nm).

Viability assay – CellTiter Glo assay:

Cell viability was determined by using the CellTiter Glo[®] metabolic viability assay according to the manufacturer's recommendations. Briefly, for both the adherent and suspension (i.e. trypsinized) HeLa cells, 100 μ L of HeLa cell culture medium was supplemented with 100 μ L CellTiter Glo solution in each well of the 96-well plate. For the suspension cells (i.e. Jurkat and human T cells), 50.000 cells were diluted in 100 μ L cell culture medium and another 100 μ L of CellTiter Glo solution was added to the 96-well plate. The plates were then allowed to stir on a shaker for about 10 minutes (100 rpm) and 100 μ L of the mixture was transferred into a white opaque 96-well plate (Greiner Bio-One, Belgium). The read-out of the luminescent signal was performed by a luminometer (plate reader, GloMax, Promega).

mRNA transfection by nucleofection:

Activated primary human T cells were transfected with eGFP-mRNA by a 4D-Nucleofector[™] according to the manufacturer's instructions with a P3 Primary Cell 4D-Nucleofector[™] X Kit (V4XP-3032) (Lonza, Breda, The Netherlands). Briefly, 1×10^6 cells were resuspended in P3 nucleofector solution, combined with 2 μ g eGFP-mRNA (total concentration: 0.3 μ M) and transferred a 16-well Nucleocuvette[™] strip. Transfection was obtained using pulse program

EO-115 (High functionality). Immediately after transfection, the cells were supplemented with 80 μ L pre-heated culture medium and 40 μ L was transferred to a 96-well plate containing 160 μ L pre-heated culture medium (total mRNA concentration: 0.1 μ g/ μ L or 0.3 μ M). Cells were incubated at 37°C, 5% CO₂ for 24 hours prior analysis of cell viability and mRNA expression by flow cytometry.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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The table of contents entry should be 50–60 words long and should be written in the present tense and impersonal style (i.e., avoid we). The text should be different from the abstract text.

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Photoporation with biodegradable polydopamine nanosensitizers enables safe and efficient delivery of mRNA in human T cells

ToC figure ((Please choose one size: 55 mm broad × 50 mm high **or** 110 mm broad × 20 mm high. Please do not use any other dimensions))

‘Bovine serum albumin (BSA)-functionalized polydopamine nanoparticles, with a size of around 500 nm, are shown to generate vapor nanobubbles (VNBs) upon the application of a nanosecond pulsed laser. Polydopamine-sensitized VNB-induced photoporation is shown to deliver macromolecules (including fitc-dextran of 500 kDa and mRNA) into hard-to-transfect Jurkat and human T cells after a quick mixing.’

Supporting Information

Photoporation with biodegradable polydopamine nanosensitizers enables safe and efficient delivery of mRNA in human T cells

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Figure S1. Size distributions of PD NPs during the synthesis as measured with DLS up to 7 hours. As a final step sonication was used to remove remaining agglomerates which have been formed during the synthesis.

Figure S2. Zeta-potential characterization of bare (blue bar) and BSA (violet bar) coated 500 nm PD NPs as measured in HyClone water by the DLS.

a **b**

Figure S3. Long-term stability of uncoated and BSA-coated PD NPs as measured with DLS. (a) Size distribution (Intensity %) of freshly prepared (blue line) and 7 month-old (green line) uncoated PD500 NPs dispersed in HyClone water. (b) Size distribution (Intensity %) of freshly prepared (blue line) and 7 month-old (green line) BSA-coated PD500 NPs dispersed in HyClone water.

Figure S4. UV/VIS spectrum of respectively dopamine.HCl (0.09 mg/mL, green line), supernatant of bare freshly prepared PD NPs (red line), supernatant of 7 month-old uncoated PD NPs (black line) and supernatant of 7 month-old BSA-coated PD NPs (blue line).

Figure S5. Size distributions of BSA-coated PD NPs of different sizes as measured by DLS in HyClone water.

Figure S6. Quantification of the number of VNBs from dark field microscopy images as a function of laser fluence for 300 nm BSA-coated PD NPs. The dotted line to the left of the blue filled area represents the laser fluence threshold above which 90% of the irradiated 300 nm BSA-coated PD NPs will form vapor bubbles.

a **b**

Figure S7. (a) Delivery efficiency of FD500 (left y-axis, blue bars) and viability (2 hours, right y-axis, red bars) using a concentration of 0.1 mg/mL BSA (with or without laser) in adherent HeLa cells. Note that the blue bars are virtually invisible due to the fact that the delivery efficiency was near zero. (b) Transfection efficiency of mRNA (0.1 mg/mL), encoding for eGFP, (left y-axis, blue bars) and corresponding viability (24 hours, right y-axis, red bars) using a concentration of 0.1 mg/mL BSA (with or without laser) in adherent HeLa cells. Note that the blue bars are virtually invisible due to the fact that the transfection efficiency was near zero.

Figure S8. (a) Transfection efficiency of mRNA (0.025 mg/mL), encoding for eGFP, (left y-axis, blue bars) and corresponding viability (24 hours, right y-axis, red bars) with the mixing method in adherent HeLa cells for PD-BSA NPs with different sizes. (b) The transfection yield for PD-BSA NPs with different sizes (concentration used; 500 nm and 750 nm $\approx 8 \times 10^7$ NPs/mL; 1000 nm $\approx 3.2 \times 10^7$ NPs/mL). Statistical significance: Student's t-test, n.s. refers to non-significant.

Figure S9. The yield of viable FD500 positive HeLa cells for the different methods of PD-BSA NP incubation. (a) Delivery yield percentage for FD500 'loaded' HeLa cells after performing the traditional incubation method on adherent HeLa cells with an increasing concentration of PD-BSA NPs. (b) Delivery yield percentage for FD500 'loaded' HeLa cells after performing the mixing method on adherent HeLa cells with an increasing concentration of PD-BSA NPs. (c) Delivery yield percentage for FD500 'loaded' HeLa cells after performing the mixing method on suspension HeLa cells with an increasing concentration of PD-BSA NPs. Data are shown as mean $\pm$ SD.

Figure S10. Comparison between the traditional and mixing method for photoporation of HeLa cells with 500 nm BSA coated PD NPs as sensitizing agents for the cytosolic delivery of FD500. (a) Relative mean fluorescence intensity of FD500 'loaded' HeLa cells after performing the traditional incubation method on adherent HeLa cells with an increasing concentration of PD-BSA NPs. (b) Relative mean fluorescence intensity of FD500 'loaded' HeLa cells after performing the mixing method on adherent HeLa cells with an increasing concentration of PD-BSA NPs. (c) Relative mean fluorescence intensity of FD500 'loaded' HeLa cells after performing the mixing method on suspension HeLa cells with an increasing concentration of PD-BSA NPs. Data are shown as mean $\pm$ SD.

Figure S11. Elucidating the contribution by cell-associated and unbound PD-BSA NPs in the mixing procedure. FD500 delivery into adherent HeLa cells was compared with and without performing an additional washing step to remove unbound PD-BSA NPs. (UTC: untreated control, Control: mock FD500 control, Photo: photoporation). Data are shown as mean $\pm$ SD. Data are shown as mean $\pm$ SD. Statistical significance: Student's t-test, ** $p \leq 0.01$.

Mixing method, jurkat

Figure S12. Delivery yield for FD500 in Jurkat cells for an increasing concentration of PD-BSA NPs. Data are shown as mean $\pm$ SD. Statistical significance: Student's t-test, * $p \leq 0.05$.

Mixing method, human T cells

Figure S13. Delivery yield percentage for FD500 'loaded' human T cells for increasing PD-BSA NP concentrations. Data are shown as mean $\pm$ SD. Statistical significance: Student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$.

Figure S14. Flow cytometry density plots for mRNA transfected human T cells. (a) Representative flow cytometry density plots for photoporation without eGFP-mRNA (top) and with eGFP-mRNA (bottom) in human T cells (PD-BSA NP concentration of 500×10^7 NPs/mL). (b) Representative flow cytometry density plots for photoporation without eGFP-mRNA (top) and with eGFP-mRNA (bottom) in human T cells (PD-BSA NP concentration of 1000×10^7 NPs/mL).

Mixing method,
mRNA, human T cells

Figure S15. Transfection yield percentage for mRNA eGFP 'loaded' human T cells for increasing PD-BSA NP concentrations.

Data are shown as mean ± SD. Statistical significance: Student's t-test, * $p \leq 0.05$.