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# Gelatin coating to stabilize the transfection ability of nucleic acid polyplexes

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ABSTRACT

Amphiphilic polymers are effective in complexing and delivering therapeutic nucleic acids, such as plasmid DNA (pDNA) and short interfering RNA (siRNA). However, long-term stability of the complexes is not desirable, as it may have an impact on the transfection efficiency in vivo. To develop a method to preserve complex stability we first showed that pDNA complexes formed with the amphiphilic polymer linoleic acid-substituted polyethylenimine (PEI–LA) and incubated at 37 °C lost ~90% of their transfection efficiency after only 24 h of complex formation. Polyethyleneglycol modification of complexes to control the increase in complex size and incubation in scaffolds used for implantation did not preserve the transfection ability of the complexes. Among a variety of approaches explored, gelatin coating of complexes was found to be the best at maintaining the original transfection efficiency. Mechanistic studies suggested that improved complex uptake, not size stability, was responsible for retention of the transfection efficiency. Similarly to the results with pDNA, gelatin coating also prevented the decreases in uptake and silencing efficiency of siRNA complexes observed following incubation at 37 °C. Gelatin-stabilized complexes were, furthermore, effective in vivo and led to subcutaneous transgene expression with a low pDNA dose that was otherwise ineffective. We conclude that a simple gelatin coating approach offers an efficient means to preserve the transfection efficiency of polyplexes.



## 1. Introduction

Gene delivery is actively pursued for the treatment of a myriad of diseases due to its potential to eradiate the underlying cause of pathology, rather than alleviating the disease symptoms that are often targeted with drug therapy. Viral gene carriers are highly effective at introducing transgenes into human cells and have been used extensively in cell culture for modification of a wide variety of cells. For in vivo gene delivery, however, viral carriers display reduced or abolished efficacy [11,15,22,30] in immune-competent animals. The use of immune suppressors can restore the efficacy of viral carriers [10,22], but this is undesirable in a clinical setting. Viral carriers are also associated with potentially lethal consequences, such as insertional mutagenesis [13]. The poor safety profiles of viral carriers make non-viral carriers attractive alternatives for gene delivery. It has been well-established that plasmid DNA (pDNA) delivery without a carrier is inefficient and requires excessive doses for transgene expression [1,2,7]. Amphiphilic polymers incorporating cationic and lipophilic domains can bind, condense and neutralize anionic pDNA to facilitate transfer across the hydrophobic cell membrane and increase the efficiency of gene delivery. When administered in vivo the polymer/pDNA complexes are expected to remain stable and enable transfection, whether the complexes are administered freely or implanted along with biomaterial scaffolds. Encounters of complexes with cells is expected to take place after anywhere from minutes to days and the complexes must remain active during this period for effective internalization and expression.

Time-dependent changes in the transfection ability of complexes have been investigated in aqueous solution at room temperature and 4 °C [5,8,29], in the frozen state at −20 °C and −80 °C, in liquid $N_2$ [8], and as lyophilized formulations up to 40 °C [5,6]. These studies were aimed at investigating the stability of the complexes in vitro as a pharmaceutical formulation. A loss of transfection ability has routinely been observed for complexes incubated at room temperature [5,8,29]. Lowering the temperature to 4 °C [5,29] slows the loss of function, while freezing below −20 °C [8] and lyophilization [5,6] preserves the transfection ability of some complexes. Storage at low temperature or in lyophilized form, however, are not useful in predicting the performance of complexes under realistic transfection conditions, where the com-

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plexes come into contact with high concentrations of endogenous molecules at 37 °C for prolonged periods. Strategies that control complex size have been investigated to stabilize the complexes; one commonly used strategy is modification of polymeric carriers with hydrophilic polyethyleneglycol (PEG) [16]. PEG, due to enhanced hydration and steric repulsion, prevents pDNA association and subsequent aggregation. PEG modification of polyethyleneimine (PEI)-based complexes has been used, particularly smaller PEI complexes [24,27] that are prone to aggregation [16]. Despite good control over the particle size, the ability of PEG to preserve the transfection ability of the complexes over time was not investigated. Moreover, PEGylation of PEI abolished plasmid transfection efficiency in vivo [17], and increased the degradation of short interfering RNA (siRNA) in PEG-containing complexes [26], making this approach questionable for use in preserving complex stability.

In this study we explored the stability of polymer complexes prepared with nucleic acids and confirmed a significant reduction in transfection ability of the complexes in as little as 24 h after complexation. We then explored a method to preserve the transfection ability of the complexes and here report a simple approach to achieve this goal, based on gelatin coating of the complexes. A previously described amphiphilic polymer, 2 kDa PEI modified with linoleic acid (PEI2–LA) [19], was used as a prototypical polymeric carrier. This polymer was highly effective when the prepared pDNA and siRNA complexes were used immediately for transfection. We show that the proposed gelatin-coating method is applicable for both pDNA and siRNA complexes and that it improves the functional outcomes with both types of nucleic acids.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, SYBR Green I, and penicillin (10,000 U $ml^{-1}$)–streptomycin (10,000 μg $ml^{-1}$) solution were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from PAA Laboratories Inc. (Etobicoke, Canada) and Hank's balanced salt solution (HBSS) was from BioWhittaker (Walkersville, MD). Absorbable gelatin sponges (Gelfoam) were from Pharmacia & Upjohn (Kalamazoo, MI). An enzyme-linked immunosorbant assay (ELISA) to quantify bone morphogenetic protein-2 (BMP-2) in tissue culture supernatants was purchased from Peprotech (Rocky Hill, NJ). The gWiz-GFP and gWiz plasmids were purchased from Aldevron (Fargo, ND). The pCAG-dsRed2 plasmid was purchased from Addgene (Cambridge, MA). Heparin sodium salt, Type A 300 bloom gelatin from pork skin (catalog No. G1890), and thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma–Aldrich (St Louis, MO). The BMP-2 expressing plasmid BMP2-IRES-AcGFP was previously described [28]. The gWiz plasmid was labeled with the Cy3 fluorescent probe (gWIZ–Cy3) using a Label-IT Tracker Kit from Mirus (Piscataway, NJ). All siRNAs (unlabeled scrambled, FAM-labeled scrambled, and KSP-specific siRNA) were obtained from Ambion (Austin, TX). The 2 kDa PEI (PEI2) modified with linoleic acid (PEI2–LA) was prepared as described [19]. The extent of lipid modification was 1.2–1.6 linoleic acid molecules per PEI2 molecule. The PEI2 was also modified with polyethylene glycol (PEI–PEG) (~5 PEG per PEI2) as previously described [34].

### 2.2. Complex formation

The desired polymers and pDNA (gWIZ, gWIZ-GFP or BMP2-IRES-AcGFP) were mixed together in saline (150 mM NaCl) for complexation. For example, 9 μl of 1 mg $ml^{-1}$ PEI–LA was added to 4.5 μl of 0.4 μg $\mu l^{-1}$ pDNA in 22.5 μl of saline in Eppendorf tubes. After 30 min 264 μl of gelatin solution (0%, 0.01%, 0.1% or 1% in water) was added to the complexes and used either immediately (no incubation) or incubated in the Eppendorf tubes at 37 °C for periods of up to 48 h before addition to the cells. For complexes prepared with a combination of polymers (e.g. PEI–LA and PEI–PEG) the polymers were first mixed together before being added to pDNA in saline. For transfection in gelatin sponges complexes were formed as usual, incubated for 15 min at room temperature, and then added to the sponges for a further 15 min at room temperature, before the sponges are either incubated with cells or implanted. The polymer:pDNA weight ratio in the complexes was controlled at 5:1, and the concentration of pDNA during transfection was maintained at 2 μg $ml^{-1}$ pDNA in medium. The siRNA complexes were similarly prepared by replacing the pDNA with the desired siRNA. The polymer:siRNA weight ratio was 2:1 with a final siRNA concentration of 36 nM in cell culture medium.

### 2.3. Complex solubility, size, zeta potential, and dissociation

To assess the solubility of the complexes polymer/pDNA complexes in saline were centrifuged at 13,000 rpm for 10 min. The concentration of pDNA remaining in solution was measured at $A_{260}$ (Nanovue), and normalized against free pDNA (i.e. no polymers) in saline. The size and zeta potential of the polymer/pDNA complexes were evaluated using dynamic light scattering with a ZetaPlus-Zeta Potential Analyzer (Brookhaven Instrument Corp.) at room temperature. For size determination complexes were measured over a period of 60 min. at room temperature. The size of each complex was taken as the average of four readings. For these experiments 10 μg of gWiz was mixed with 50 μg of polymer in saline to give a polymer:pDNA weight ratio of 5:1. Particles were formed with PEI–LA alone, or mixtures of PEI–LA and unmodified PEI or PEI–PEG. For zeta potential determination complexes were made as usual in water with a polymer to pDNA weight ratio of 5:1. Complexes were measured after or not an additional 24 h incubation in either water or 0.1% gelatin. The viscosity of water was used for the measurements, and the zeta potential of each complex was taken as the average of 12 runs.

To assess dissociation the complexes were exposed to heparin (0–12 μg $ml^{-1}$) for 1 h at room temperature to induce dissociation. Free pDNA released from the complexes was measured using SYBR Green I in black 96-well plates with a fluorescent plate reader ($\lambda_{ex}$ 485 nm, $\lambda_{em}$ 527 nm). Complex dissociation was calculated by dividing the amount of pDNA released at a specific heparin concentration by the total amount of pDNA released without complex incubation. Experiments were performed in triplicate.

### 2.4. Cell culture

The 293T cell line was used to assess pDNA delivery in vitro. Cells were grown in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified incubator (37 °C, 5% $CO_2$). The 293T cells were seeded either directly on tissue culture plates (monolayer) or on gelatin sponges. Monolayer cultures were seeded the day prior to complex exposure (100 μl of complex per well in triplicate), whereas complexes were loaded onto the sponge immediately before cell seeding. Cells were seeded to achieve ~60% confluency for monolayer studies in multiwell plates. Sponges were seeded at approximately three times the density of monolayer cultures to account for the three-dimensional nature of the sponge. The human breast cancer cell line MDA-MB-435 was used for siRNA studies. Cells were grown in RPMI medium supplemented with 10% FBS and 1% penicillin–streptomycin under the conditions described above. Cells were seeded as a monolayer in 24-well plates the day before siRNA complex exposure.

### 2.5. Assessment of GFP and BMP expression

For the analysis of GFP expression 293T cells were exposed to the desired complexes for 24 h and then the medium changed to remove complexes. After 2 days monolayer cultures were washed with HBSS, trypsinized to create a single cell suspension, and fixed in 3.7% formalin in HBSS. Approximately 5000 cells per sample were analyzed for GFP expression with a Beckman Coulter Cell Lab Quanta SC flow cytometer using a 488 nm laser for excitation and the FL1 channel for emission. The "no treatment" control group was set at 1% for GFP-positive cells as a calibration. GFP expression in sponge culture was assessed with a fluorescent plate reader ($\lambda_{ex}$ 485 nm, $\lambda_{em}$ 527 nm), where the fluorescence of the "no treatment" group (sponges containing cells with no pDNA exposure) was subtracted from the fluorescence of the study groups. For the analysis of BMP-2 expression complexes containing plasmid BMP2-IRES-AcGFP were loaded onto gelatin sponges and 293T cells were seeded onto the sponges. Every 4 days sponges were transferred to new 48-well plates in lieu of passaging and supernatants were collected to detect BMP-2 secretion using a commercial ELISA kit. Each transfection was performed in triplicate.

### 2.6. Uptake of pDNA and siRNA

For pDNA uptake determination complexes were prepared as described in Section 2.2 with gWiz–Cy3. The fluorescence of the complexes was measured at formation and after 24 h at 37 °C to ensure that the Cy3 signal did not change significantly on polymer binding, gelatin addition or extended incubation. 293T cells were exposed to complexes for 24 h and harvested for flow cytometry as described in Section 2.5. Washing and trypsinization of cells has previously been found to effectively remove pDNA complexes that are absorbed on the surface but not internalized [25]. The Cy3 signal was measured in the FL2 channel, with the background (untreated cells) set at 1%. gWiz–Cy3 without polymeric carrier was included with cells as a control. Similarly, uptake of siRNA complexes was measured in MDA-MB-435 cells using FAM-labeled siRNA. The fluorescence of complexes was measured at formation and after 24 h at 37 °C to ensure that the FAM signal did not change significantly on polymer binding, gelatin addition or extended incubation. Complexes were prepared with FAM-labeled siRNA and incubated for 0 or 24 h at 37 °C. The siRNA–FAM complexes were then exposed to MDA-MB-435 cells for 24 h and similarly washed and trypsinized for flow cytometry. The FAM signal was measured in the FL1 channel, with the background (untreated cells) set at 1%. siRNA–FAM without polymeric carrier was included as a control. Each experiment was performed in triplicate.

### 2.7. KSP silencing with siRNA

The siRNA complexes were prepared with scrambled (control) or kinesin spindle protein (KSP)-specific siRNAs, and additionally incubated in saline or gelatin (1%, 0.1% or 0.01%) for 0 or 24 h at 37 °C. Selective KSP inhibition results in cell death in acute myeloid leukemia cells [4], and silencing similarly results in apoptosis and loss of viability in MDA-MB-435 breast cancer cells (unpublished data). Complexes at a 2:1 polymer:siRNA ratio were added to cells at 36 nM siRNA (in triplicate in 24-well plates), and an MTT assay performed after 72 h as described [19]. Cell viability after KSP silencing was normalized against control complexes and expressed as percent decrease in cell viability. Each experiment was performed in triplicate.

### 2.8. In vivo gene delivery

Gene delivery was assessed in vivo by expression of dsRed2 from the plasmid pCAG-dsRed2 [33] in a rat subcutaneous implantation model. All protocols involving rats were approved by the University of Alberta Animal Welfare Committee. Female Sprague-Dawley rats from Biosciences (Edmonton, Canada) were kept under standard laboratory conditions (12 h light/dark cycle) with free access to water and rat chow. Each rat received two sponges loaded with complex (duplicates of the same group) in the bilateral ventral pouches. The PEI–LA/pDNA complexes were prepared in microcentrifuge tubes for 15 min, and then saline or 0.1% gelatin was added to the tubes. Complexes containing 10 μg of pDNA and 50 μg of PEI–LA in 150 μl were then loaded onto sponges for 15 min prior to implantation. At designated time points rats were killed via $CO_2$ asphyxiation and the sponges harvested. Implants were imaged with a FujiFilm FLA-5000 scanner. Fluorescence of the implants was normalized against sponges containing only saline. Each study group contained 4 or 5 rats, giving 8–10 implants for assessment.

### 2.9. Statistical analysis

Statistical tests were applied to determine the significance of the study results. For comparison between two groups the results were analyzed using Student's *t*-test. Analysis of variance (ANOVA) was performed for comparisons among multiple groups. This was followed by a Dunnett test compared with a control group, as indicated in the figure captions. The results of the in vivo study were found not to follow a normal distribution, so a Kruskal–Wallis test (non-parametric ANOVA) was performed. Dunn's multiple comparison test was used to compare gene expression with controls, as described in the figure caption.

## 3. Results

### 3.1. Complex solubility, size, and transfection ability

We first investigated the aggregation of complexes after 30 min complex formation by centrifugation to determine whether pDNA complexes remain in solution. The PEI–LA complexes formed visible aggregates (i.e. cloudy solution), leaving <10% of the original pDNA in solution after centrifugation (Fig. 1A). Adding PEI2 to complexes at up to 50% by polymer weight gave a similar result, with 10–15% of pDNA remaining in solution after centrifugation. The PEI–PEG-containing complexes were more soluble under these conditions, with ~70% of pDNA remaining in solution when the complexes were formed at a 1:1 PEI–LA:PEI–PEG ratio. The size of the polymer/pDNA complexes followed a similar pattern (Fig. 1B), with both the PEI–LA and PEI–LA/PEI complexes increasing from ~380 nm after 5 min to ~1200 nm at 60 min, while the PEI–LA/PEI–PEG complexes remained at ~200 nm for the study duration.

The dissociation of pDNA complexes was investigated with heparin for complexes incubated for up to 24 h. Sigmoidal dissociation curves were observed for pDNA complexes of PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG (Fig. 1C) at the initial time point. However, after 24 h the PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG complexes all showed incomplete dissociation, whereas the PEI–LA and PEI–LA/PEI complexes released <20% of pDNA, while PEI–LA/PEI–PEG complexes gave ~60% pDNA release. The pDNA complexes formed with PEG–PEI gave the most robust dissociation at both time points, suggesting weaker binding of this agent.

The transfection ability of complexes were investigated in 293T monolayers and sponges, where the complexes were used either immediately after preparation or after 24 h incubation at 37 °C.

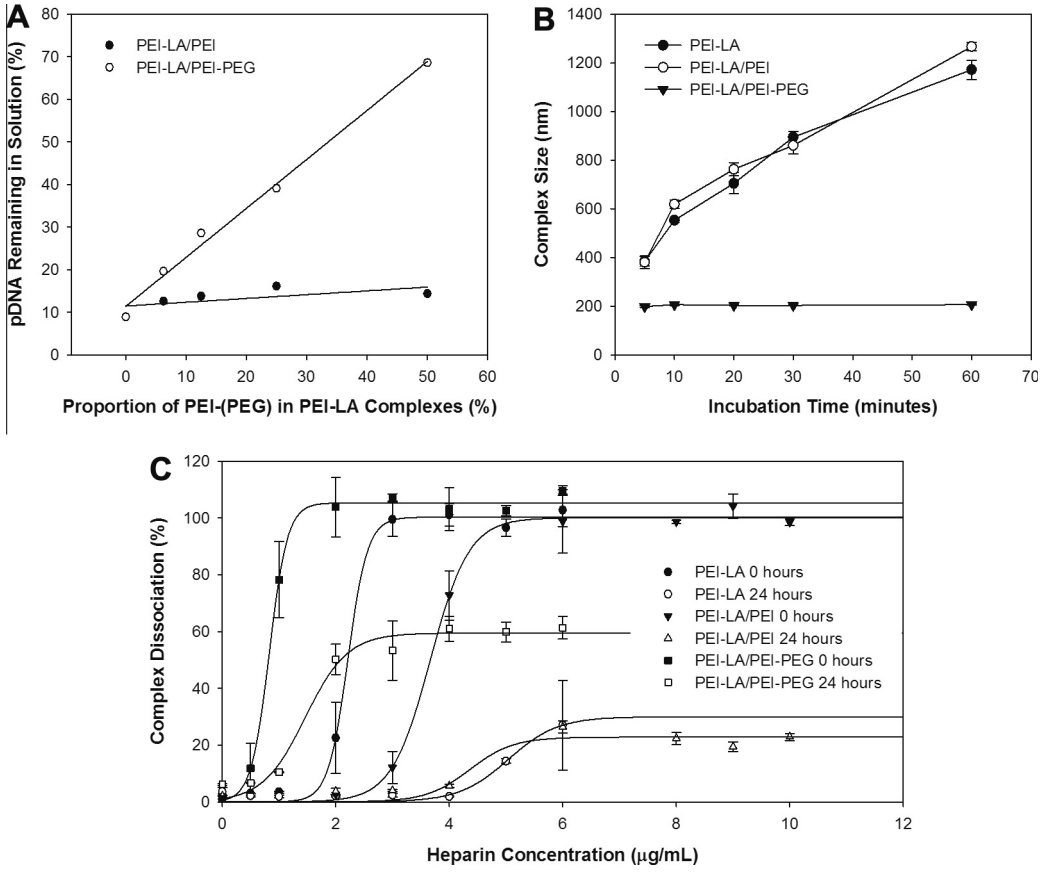


**Fig. 1.** Effect of incubation on size, solubility and transfection. (A) Changes in the solubility of PEI–LA complexes after 30 min complexation. The complexes were formed with PEI–LA/PEI and PEI–LA/PEI–PEG mixtures where the percentages of PEI and PEI–PEG were varied between 0 and 50%. (B) Size of PEI–LA, PEI–LA/PEI and PEI–LA/PEI–PEG complexes during 60 min complexation. (C) Heparin dissociation of PEI–LA, PEI–LA/PEI, or PEI–LA/PEI–PEG complexes was assessed after 0 or 24 h incubation at 37 °C.

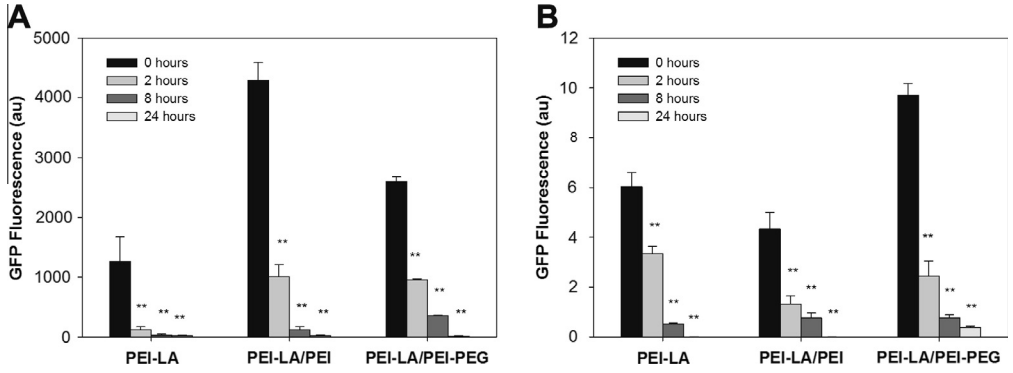


**Fig. 2.** Change in transfection efficiency with complex incubation. Transfection efficiency of complexes containing gWiz–GFP was determined after incubation of complexes (37 °C) for 0, 2, 8 and 24 h in 293T grown as (A) monolayers or (B) sponge cultures. GFP expression was assessed after 2 days by (A) flow cytometry or (B) using a fluorescent plate reader. $^{**}p < 0.01$ compared with 0 h for each complex type.

For fresh complexes high levels of GFP expression were observed for all groups in both the monolayer (Fig. 2A) and sponge cultures (Fig. 2B). The proportion of GFP-positive cells ranged from 40 to 80% for saline-incubated complexes (not shown). In both monolayer and sponge cultures the transfection efficiency decreased gradually when complexes were incubated for 24 h before addition to the cells, until GFP fluorescence was only slightly higher than the background when complexes were incubated for 48 h prior to transfection. This trend was observed for all PEI–LA, PEI–LA/PEI and PEI–LA/PEI–PEG complexes ($p < 0.01$). Similar decreases were observed even when complexes were incubated at room temperature or 4 °C, although the lower temperature helped slow the decline in transfection efficiency (Supplementary Fig. 1). Incubating the complexes in tissue culture medium containing serum led to a slight improvement compared with media without serum, but ultimately also led to minimal transfection ability after 48 h (Supplementary Fig. 2).

### 3.2. Effect of gelatin on transfection efficiency during extended incubation

The prepared complexes were incubated with a variety of buffers for 8 h to screen for formulations that retained transfection efficiency. HBSS, HEPES, Tris, and phosphate buffers in the pH

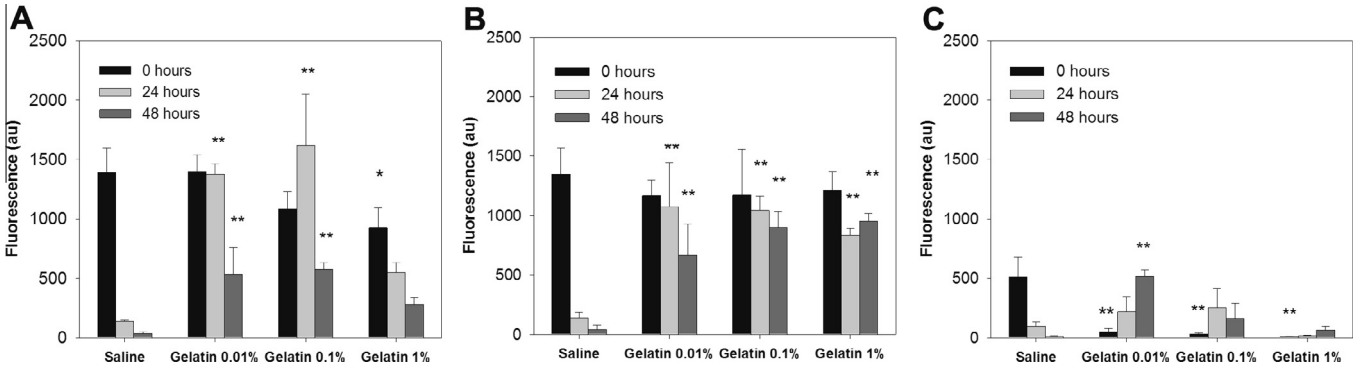


**Fig. 3.** Effect of gelatin during monolayer transfections. Transfection efficiencies of (A) PEI–LA, (B) PEI–LA/PEI and (C) PEI–LA/PEI–PEG complexes incubated at 37 °C with 0, 0.01, 0.1 and 1% gelatin. After 0, 24 and 48 h incubation the complexes were added to the cells and GFP expression analyzed using flow cytometry. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with complexes in saline for each time point.

range 5–8 did not alleviate the loss of transfection efficiency (not shown). Both dextrose and gelatin were found to improve transfection, but 0.1% gelatin was the only formulation to improve transfection with both PEI–LA and PEI–LA/PEI (Supplementary Fig. 3). Dextrose was subsequently found to be insufficient to maintain the transfection efficiency for longer periods (Supplementary Fig. 4). The addition of 0.01–1% gelatin to complexes during incubation at 37 °C maintained their transfection efficiencies, unlike the control conditions (150 mM NaCl, Fig. 3A–C). Addition of gelatin coatings led to higher transfection for PEI–LA (Fig. 3A) and PEI–LA/PEI (Fig. 3B) when the complexes were incubated for 24 or 48 h compared with complexes made in saline ($p < 0.05$–$0.01$), except for PEI–LA coated with 1% gelatin. For PEI–LA complexes incubated in gelatin no change in transfection efficiency was observed after 24 h but the transfection efficiency had decreased by ~50% after 48 h. For PEI–LA/PEI complexes no change in transfection efficiency was observed when complexes were incubated for up to 48 h. For both PEI–LA and PEI–LA/PEI complexes gelatin concentrations between 0.01% and 1% were similarly effective. The PEI–LA/PEI–PEG complexes incubated in 1% gelatin showed no improvement over saline incubation (Fig. 3C), whereas 0.01% gelatin improved transfection with increasing incubation time.

We next investigated the stability of complexes formed in the presence of gelatin (rather than gelatin coating). High GFP expression was observed with fresh PEI–LA and PEI–LA/PEI complexes prepared in saline (Supplementary Fig. 5). Complexes made in the presence of gelatin, however, showed only low (PEI–LA and PEI–LA/PEI) or no transfection efficiency (PEI–LA/PEI–PEG). Where some transfection was observed lower gelatin concentrations during complex formation gave higher GFP expression after transfection, indicating an adverse effect of gelatin when used during complex preparation. Two further screens on small molecules and polymeric materials were conducted to determine whether the stabilizing properties were unique to gelatin. The small molecule screen showed that incubation with cholesteryl hemisuccinate and glycerol gave improved transfection compared with saline (not shown). Cholesteryl hemisuccinate, however, was insoluble in water, while further studies with glycerol showed a limited effectiveness since higher concentrations impeded transfection (Supplementary Fig. 7). The polymeric screen demonstrated that Type B gelatin and free PEG helped to stabilize the particles (Supplementary Fig. 6). Further investigation of Type A and Type B gelatins demonstrated similar stabilizing properties (Supplementary Fig. 8).

The stability of complexes was next evaluated by adding the complexes to absorbable gelatin sponges as a model of implantable delivery of pDNA. Due to the difficulty of fully recovering cells from the sponges a BMP-2 expression plasmid was used for transgene expression and BMP-2 secretion was quantitated for 6 days in an initial study. Both PEI–LA and PEI–LA/PEI, but not PEI/PEI–PEG, led to some BMP-2 secretion from saline incubated complexes (Fig. 4A), but the addition of gelatin (0.01–1%) to the complexes increased BMP-2 expression ($p < 0.05$–$0.01$) by 2.1- to 5.1-fold. In a longer (12-day) study gelatin coating increased BMP-2 secretion ($p < 0.01$–$0.001$) from PEI–LA and PEI–LA/PEI complexes by 1.6- and 2.1-fold, respectively (Fig. 4B). As shown in Fig. 4A, PEI–LA/PEI–PEG complexes did not give appreciable BMP-2 secretion with or without gelatin.

### 3.3. Effect of gelatin on complex properties

Complex size, dissociation, and cellular uptake were investigated to determine the beneficial effect of gelatin on the transfection ability of complexes. The sizes of complexes incubated without and with gelatin were measured after 24 h incubation (Fig. 5A). The PEI–LA complexes in saline were ~9 μm while the PEI–LA/PEI complexes were ~1.5 μm. The PEI–LA/PEI–PEG complexes remained at ~300 nm, consistent with the expected PEG effect. In contrast, all three complexes incubated in 1% gelatin remained at ~300 nm. The size of PEI–LA and PEI–LA/PEI complexes increased with lower gelatin concentrations (0.1% and 0.01%). The zeta potential of the complexes was measured after 0 or 24 h incubation in either water or 0.1% gelatin (Fig. 5B). Initially the PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG complexes had zeta potentials of 48, 43, and 42 mV, respectively. The addition of gelatin decreased the zeta potential ($p < 0.011$) by approximately half, to 21, 19, and 16 mV for each of the three complexes. No change in zeta potential for any of the PEI–LA, PEI–LA/PEI, PEI–LA/PEI–PEG complexes was observed after 24 h incubation in either water or gelatin. After heparin-mediated dissociation PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG complexes prepared with 0.1% gelatin showed improved dissociation after 24 h incubation (Fig. 5). While the PEI–LA and PEI–LA/PEI complexes did not undergo complete dissociation (but still improved compared with the absence of gelatin; see Fig. 2), the PEI–LA/PEI–PEG complexes showed complete dissociation, reminiscent of freshly prepared complexes.

Finally, pDNA uptake was measured using complexes prepared with gWIZ–Cy3 (Fig. 6). Freshly prepared complexes with all three polymers gave high uptake (40–70% of cells) in the absence or presence of gelatin (0.01–1%). The uptake of PEI–LA and PEI–LA/PEI complexes incubated in the absence of gelatin was reduced after 24 h incubation (~5% of cells) (Fig. 6A). Gelatin-coated complexes of all three formulations (PEI–LA, PEI–LA/PEI and PEI–LA/PEI–PEG) gave significantly higher uptake ($p < 0.05$–$0.01$), except for PEI–LA/PEI with the lowest concentration (0.01%) gelatin coating. The PEI–LA/PEI–PEG complexes gave ~30% pDNA-positive

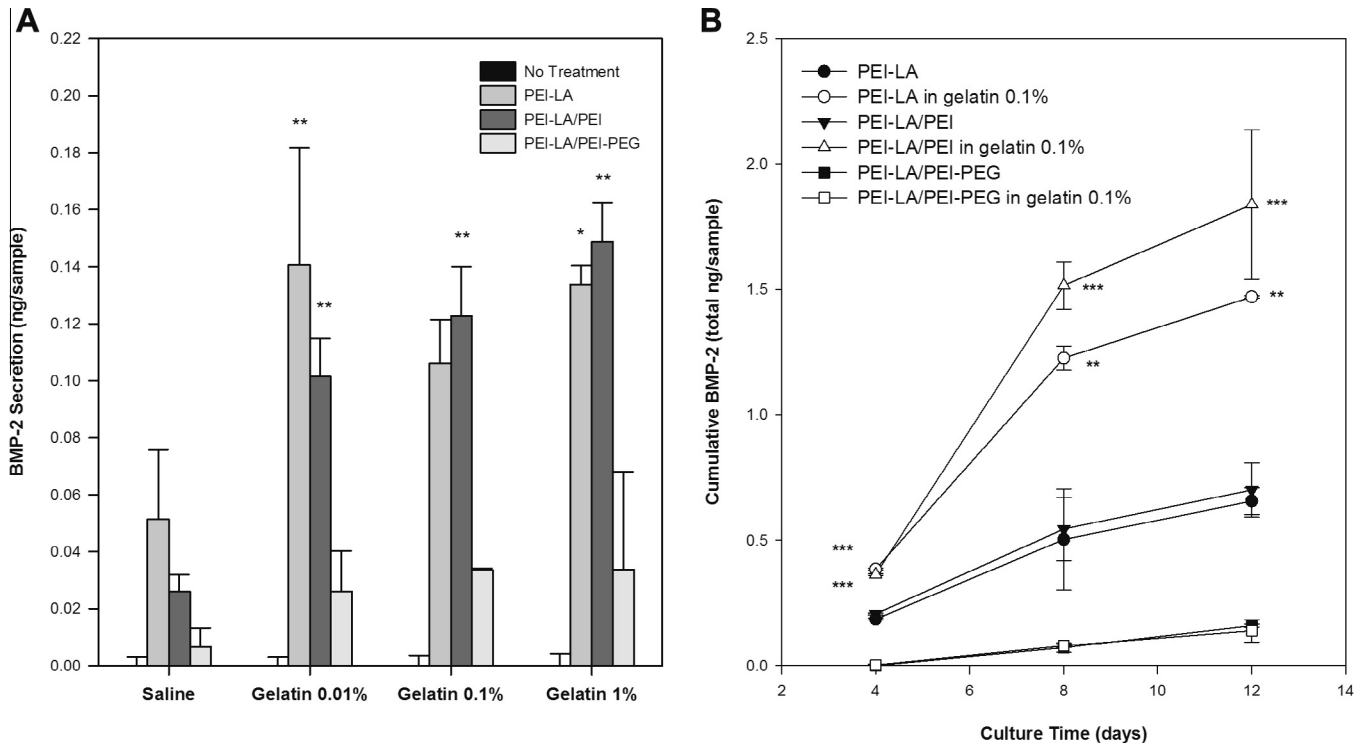


**Fig. 4.** Effect of gelatin during sponge transfections. Complexes were prepared with the BMP2-IRES-acGFP plasmid and polymers PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG and loaded onto gelatin sponges. The complexes were prepared in saline or with a gelatin coating (0.01–1%), added to the sponges after 30 min incubation, followed by the addition of cells. (A) The supernatant was collected for BMP-2 analysis after 6 days. (B) BMP-2 secretion was assessed over a 12 day period with complexes prepared in saline or 0.1% gelatin. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with complexes in saline for each time point.

cells, a decrease from 45% for freshly prepared complexes. All three complexes incubated in gelatin for 24 h displayed uptake profiles reminiscent of freshly prepared complexes.

### 3.4. Effect of the gelatin coating on siRNA delivery

We next investigated the effect of gelatin coating on the stability of siRNA complexes. A functional siRNA assay was employed, where the effect of KSP-specific siRNA on the viability of breast cancer cells was assessed. Cells treated with siRNA complexes containing scrambled siRNA showed no decrease in viability compared with untreated cells (data not shown). Treatment with fresh complexes containing KSP-specific siRNA led to a ~43% decrease in viability, but after 24 h incubation only a 3% decrease in viability was seen (Fig. 7A). Freshly made complexes with a gelatin coating showed a 34–55% decrease in cell viability depending on the gelatin concentration, which is equivalent to freshly made complexes. The complexes with a gelatin coating were more effective than complexes in saline after 24 h incubation at 37 °C ($p < 0.01$), showing a 32–45% decrease in cell viability.

The cellular uptake of siRNA complexes was similarly investigated to determine the beneficial effect of gelatin coating. As expected, cells treated with FAM-labeled siRNA alone (i.e. without a polymeric carrier) showed no uptake. Freshly prepared FAM-labeled complexes with and without a gelatin coating all showed 78–92% siRNA-positive cells (Fig. 7B), with no apparent differences among the study groups. The cellular uptake of complexes dropped to ~8% when the FAM-labeled complexes were incubated without gelatin for 24 h. The FAM-labeled complexes incubated in gelatin for 24 h, however, showed robust siRNA uptake ($p < 0.01$, 85–90% siRNA-positive cells), reminiscent of freshly prepared complexes.

### 3.5. Effect of gelatin on in vivo gene delivery

To determine whether gelatin coating was effective for in vivo gene delivery gelatin-coated complexes were delivered on an absorbable sponge and implanted subcutaneously. dsRed2 expression was investigated to confirm the effectiveness of gelatin, since dsRed2 protein is not secreted and would be localized at the implantation site longer than secreted BMP-2 protein. None of the sponges harvested 1 day after PEI–LA/pCAG-dsRed2 complex implantation showed any increase in dsRed2 fluorescence compared with control (no polymer) sponges (Fig. 8). Both uncoated and gelatin-coated PEI–LA complexes ($p < 0.05$ and $p < 0.01$, respectively) showed significant increases in dsRed2 fluorescence compared with control implants after 3 days, but only gelatin-coated PEI–LA complexes gave significant fluorescence ($p < 0.01$) 7 days after implantation.

## 4. Discussion

Non-viral carriers are considered to be the safest option for gene delivery. Among various non-viral carriers explored lipid-substituted polymers are beginning to attract wide interest [9] as they are able to create nucleic acid-containing nanoparticles with the optimal balance of cationic charge (essential for nucleic acid binding) and hydrophobicity (needed for cell membrane compatibility). In line with pharmaceutical studies investigating the stability of other non-viral formulations of DNA complexes [5,6,8,29], this study noted a significant decrease in the activity of complexes prepared with amphiphilic polymers with extended incubation at physiological temperature. Complexes loaded onto an absorbable gelatin sponge, which is commonly used to deliver complexes by implantation, similarly led to a decrease in transfection. This scaf-

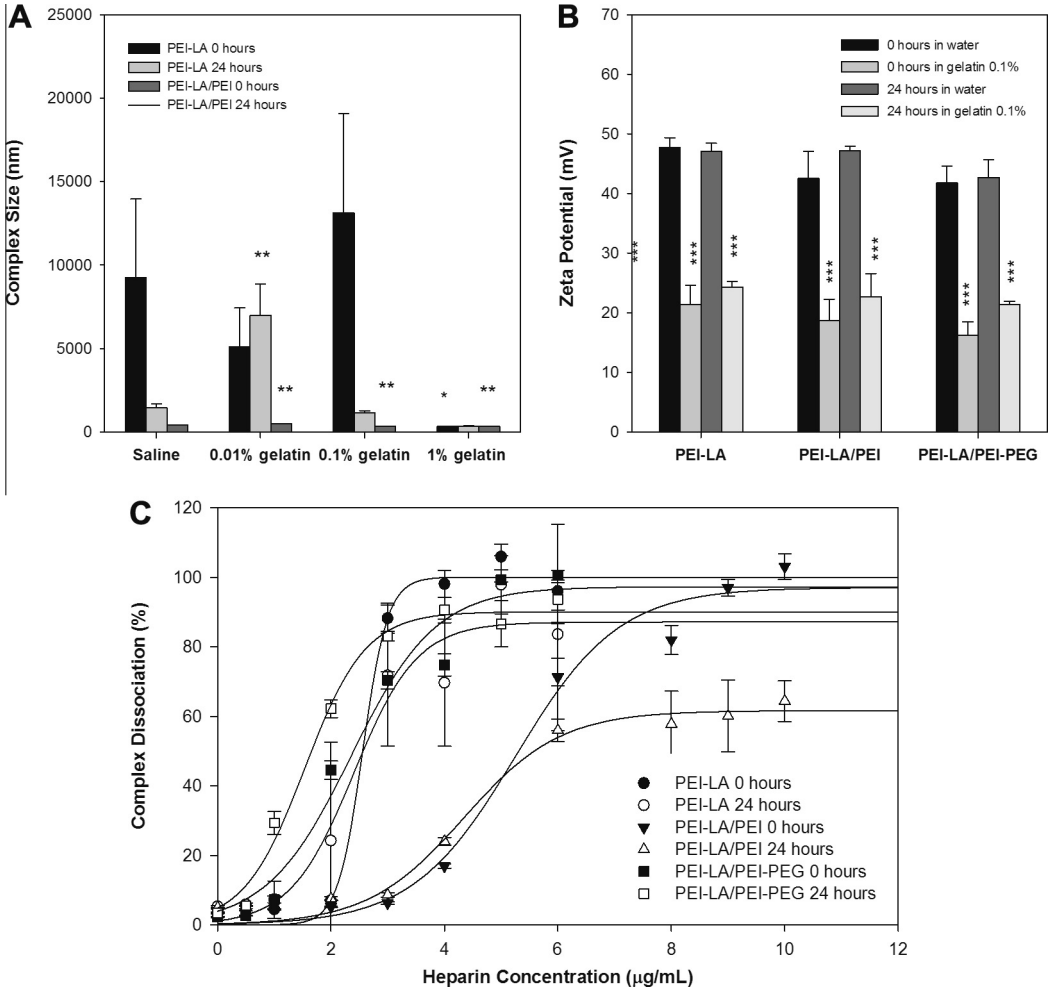


**Fig. 5.** Effect of gelatin on complex properties. Complexes were prepared with the gWIZ plasmid and polymers PEI–LA, PEI–LA/PEI and PEI–LA/PEI–PEG, and stabilized with gelatin (0.01–1%). (A) The complex size was measured after incubation at 37 °C for 24 h. (B) The zeta potential of complexes made in water was measured after a 0 or 24 h incubation in water or 0.1% gelatin. (C) Dissociation of PEI–LA, PEI–LA/PEI, and PEI–LA/PEI–PEG complexes with heparin after 0 or 24 h incubation at 37 °C in 0.1% gelatin. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with complexes in saline for each time point.

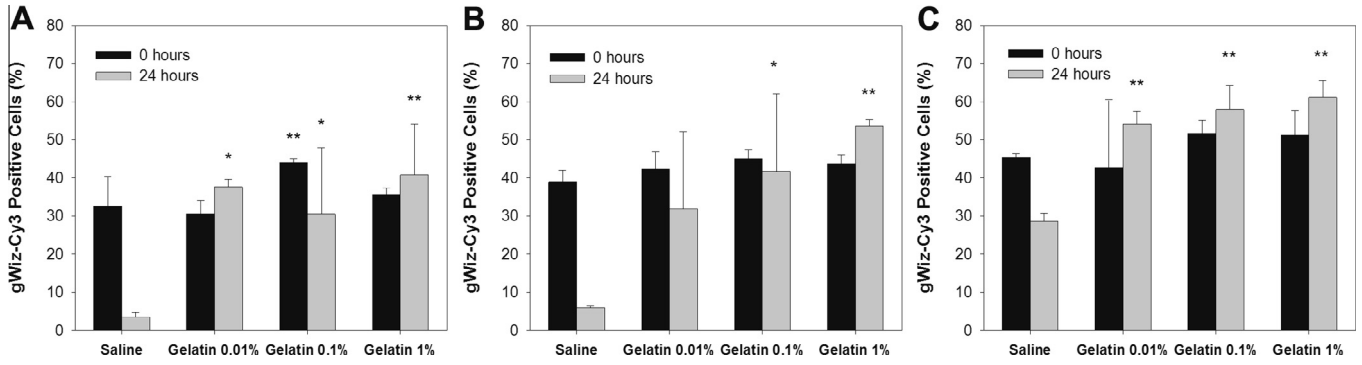


**Fig. 6.** Effect of gelatin on complex uptake. Cellular uptake of gWIZ–Cy3 complexes prepared with (A) PEI–LA, (B) PEI–LA/PEI and (C) PEI–LA/PEI–PEG and incubated for 0 or 24 h in saline and 0.01–1% gelatin. The cellular uptake of complexes summarized as the percentage of gWIZ–Cy3-positive cell population, as assessed by flow cytometry. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with complexes in saline.

fold, therefore, did not sufficiently stabilize the complexes, and suggests that the complexes could lose transfection efficiency with time until taken up by cells, a process that usually takes days. We had initially hypothesized that aggregation of hydrophobic complexes in aqueous buffers, resulting in an increase in complex size, was the reason behind the loss of transfection, but PEGylated PEI–LA complexes had a stable size and still lost transfection efficiency as quickly as unmodified complexes. This is a critical finding, since PEG is widely used to stabilize nanoparticles for systemic administration [18,23], however it does not seem to maintain the functional stability of nucleic acid-containing complexes despite retaining the initial complex size.

A gelatin coating on PEI–LA complexes of nucleic acids did not influence the transfection efficiency of freshly prepared complexes,

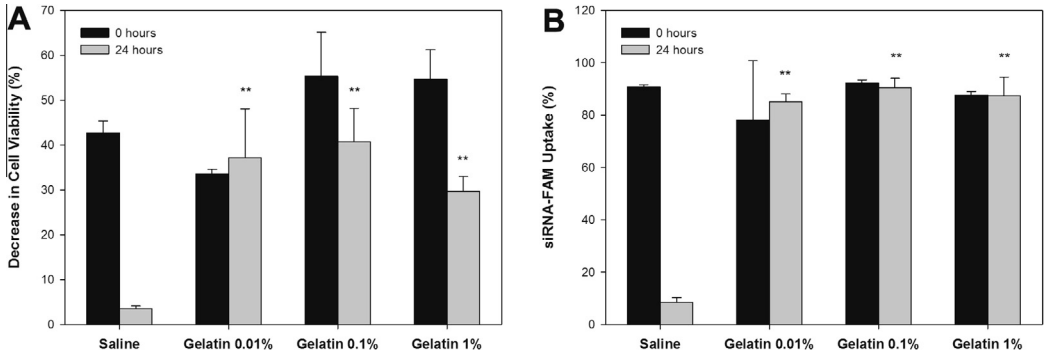


**Fig. 7.** Effect of gelatin on siRNA complexes. (A) The viability of MDA-MB-435 cells after treatment with KSP-specific siRNA. The complexes were formed with PEI–LA in saline and stabilized with gelatin (0.01–1%). Cells were treated with the complexes after 0 and 24 h incubation of the complexes at 37 °C. Viability was normalized against the cells treated with control complexes containing scrambled siRNA. (B) Uptake of complexes containing FAM-labeled siRNA. The siRNA–FAM complexes were formed with PEI–LA in saline and stabilized with gelatin (0.01–1%). The cells were treated with the complexes after 0 and 24 h incubation of the complexes at 37 °C. Cellular uptake was assessed by flow cytometry, where untreated cells were set to 1% siRNA–FAM positive population. $^{**}p < 0.01$ compared with complexes in saline.

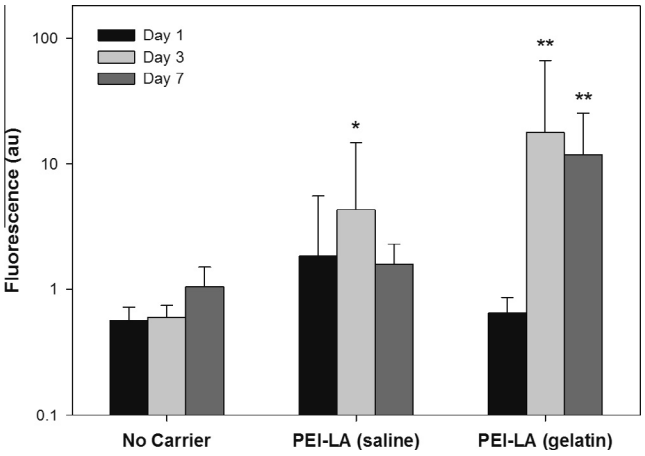


**Fig. 8.** In vivo application of gelatin-coated complexes. Sponges containing a BMP-2 plasmid without a carrier, a BMP-2 plasmid in uncoated (saline) PEI–LA complexes, or in gelatin-coated PEI–LA complexes, were implanted for up to 7 days and then assessed for ex vivo BMP-2 secretion. DsRed fluorescence was measured and normalized against saline (no treatment) sponges. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with complexes in saline.

but was highly effective at eliminating a decline in transfection activity when incubated at 37 °C. This was demonstrated with very different nucleic acids, the 5757 bp plasmid gWIZ-GFP, 5507 bp plasmid pCAG-dsRed2 and 22 bp KSP-specific siRNA that rely on different intracellular mechanisms of action. Gelatin also stabilized the transfection ability of complexes delivered on a sponge. Gelatin-coated complexes led to improved transgene expression compared with normal complexes, even with fresh complexes (see Fig. 3). It is likely that gelatin was able to stabilize the complexes during the time required for the trypsinized cells to recover and internalize the complexes, while the complexes in saline showed a decrease in activity during this time period. To determine the mechanism of improved stability we investigated complex size, zeta potential, dissociation, and uptake of complexes with a gelatin coating. Although 0.01–1% gelatin was effective in retaining activity, only the highest concentration stabilized the size of the PEI–LA/pDNA complexes, so that size stabilization alone was not the only reason for improved transfection. With respect to delivery, complexes incubated in saline showed a large decrease in the number of cells containing both pDNA and siRNA complexes. Complexes with a gelatin coating, however, showed minimal differences in cellular uptake in a 24 h study period. Given that many of the complexes were greater than 1 μm in size and are therefore prone to sedimentation, it is likely that the measurements underestimate the sizes of the complexes, particularly of the complexes in saline or low concentrations of gelatin. It appears that large complexes coated with a low gelatin concentrations were still readily taken up into the cells, in contrast to complexes in saline. This was despite the fact that the zeta potential of the complexes was reduced after gelatin coating. It must be pointed out that the zeta potentials were determined in water, and the actual measurements in saline could not be performed due to the high salt concentration. Gelatin was also found to rescue the impaired dissociation of complexes incubated in saline. Both pDNA and siRNA rely on effective dissociation from non-viral carriers to exert their action, so that better retention of this dissociation might provide a secondary mechanism (in addition to cellular uptake) for better functional activity.

To our knowledge no other studies have reported on gelatin maintaining the transfection efficiency of complexes. Other groups have employed layer by layer strategies similar to our approach [32], but the purpose of the individual layers was to improve those physico-chemical properties enhancing transfection rather than stabilizing the transfection activity of complexes with time. Cationized forms of gelatin containing amine groups have also been used as a polymeric carrier for pDNA [12,14] and siRNA [21], but there is no information on whether these complexes retain their original transfection ability after prolonged incubation at 37 °C. The fact that both Type A gelatin (pI range 7.0–9.0) and Type B gelatin (pI range 4.7–5.2) were equally effective indicates the possibility of using differently charged macromolecules to facilitate stabilization. We assume that the complexes interact with gelatin electrostatically, but did not control the pH of the medium in this study. It is likely that controlling the pH during the coating process will influence its effectiveness and may further improve the desired outcomes. Further details of this process need to be elucidated, but presumably the hydrophobic groups were retained in the right configuration for gelatin coating. Gelatin has also been modified to act as a hydrogel coating and release polymeric pDNA complexes [3], siRNA complexes [20], or naked pDNA [31]. In these cases the gelatin was cross-linked to form a hydrogel [3,31] or was dried as a thin film [20]. The gelatin in the microspheres and hydrogels were both extensively processed and modified and it is likely that they were unable to prevent a loss in transfection efficiency. Several methods have been employed to stabilize gene delivery complexes, but most studies only assessed the physico-chemical features, such as the size. Studies investigating how long the complexes remain active have only been undertaken from a pharmacological perspective, and no solutions to prevent the loss of transfection have been proposed.

A gelatin coating offers a practical solution to retain the original transfection efficiency, and was found to be effective at stabilizing PEI–LA/pDNA complexes for in vivo after implantation. No dsRed expression was observed 1 day after implantation in any group, and an intense signal was observed only with gelatin-coated particles at later time points. This strongly suggests that the delay between implantation and cell infiltration is long enough to cause the transfection efficiency of the complexes to decrease such that a robust transfection signal was not observed. The in vitro sponge model, furthermore, is a good indicator in testing the use of gelatin coatings, and correlated well with the results from the in vivo studies. Although earlier harvest time points were chosen in this study to better understand the dynamics of gene expression, the results obtained here agree with our previous in vivo gene delivery studies using GFP [28], in which transgene expression was observed with (uncoated) PEI–LA complexes after 2 and 3 weeks but not after 7 days. In the present study no transfection was detected after 7 days with uncoated particles, but significant transfection was observed for coated PEI–LA complexes. We hypothesize that the low transfection with uncoated particles may require extended periods of time before sufficient fluorescent protein accumulates to produce a detectable signal, and we further postulate that the higher transfection efficiency of the coated particles at early time points would be indicative of higher transgene expression at later time points as well.

Whether gelatin is the most ideal material for functional stabilization remains to be explored, and it might be possible to design more suitable and/or effective polymers for this end. Gelatin, however, is well characterized as a pharmaceutical excipient, generally regarded as biocompatible, easily obtained and inexpensive. Furthermore, it is easily applied as a stabilizing coating onto the complexes. Considering that gelatin was effective in stabilizing both pDNA and siRNA particles, we speculate that gelatin may prove useful for complexes made with other nucleic acids, such as antisense oligonucleotides and mRNA. Such coatings might prove useful for the administration of complexes where the complexes have to retain their transfection activity for a prolonged period of time.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.03.029.

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