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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 μl^{-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 (λ_{ex} 485 nm, λ_{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.

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