Construction of Polycation-Based Non-Viral DNA Nanoparticles and Polyanion Multilayers via Layer-by-Layer Self-Assembly

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Introduction

Gene therapy, which can be defined as a method to efficiently transfer genetic material into the somatic cells of individual patients in order to producing specific therapeutic proteins to prevent, diagnose, correct, or modulate disease, has been extensively researched worldwide. The main objective of gene therapy is to develop effective DNA delivery systems. More recently, polycation-based non-viral DNA nanoparticles, which proved to be effective both in vitro and in vivo, are receiving increasing attention because of several advantages, such as ease of manipulation, stability, low cost, safety, and high flexibility. Nanoparticles of poly(ethyleneimine) (PEI), poly(L-lysine) (PLL), or chitosan with DNA have been developed.

Although several delivery methods, including injection, systemic, intramuscular, and oral administration, have been developed for polycation-based DNA nanoparticles, both biological and physical barriers in the body have inhibited the use of these traditional methods. For example, in the case of systemic administration, the availability of nanoparticles to the targeted cells would be reduced if non-target cells take up the nanoparticles. The nanoparticles in the body would also be eliminated or destroyed by the

Summary: The multilayers of polycation-based non-viral DNA nanoparticles and biodegradable poly(L-glutamic acid) (PGA) were constructed by a layer-by-layer (LbL) technique. Poly(ethyleneimine) (PEI) was used to condense DNA to develop non-viral DNA nanoparticles. AFM, UV-visible spectrometry, and TEM measurements revealed that the PEI-DNA nanoparticles were successfully incorporated into the multilayers. The well-structured, easily processed multilayers with the non-viral DNA nanoparticles may provide a novel approach to precisely control the delivery of DNA, which may have great potential for gene therapy applications in tissue engineering, medical implants, etc.

A TEM image of the cross section of a (PGA/PEI-DNA nanoparticle)_20 multilayer.
reticuloendothelial cells and serum proteins. In the case of gene therapy targeting tumours in the central nervous system, the blood–brain barrier will be the main obstacle. Therefore, the development of new systems that can deliver the DNA nanoparticles directly to the focus in the patients’ body will accelerate the application of these DNA nanoparticles in the field of gene therapy.

The layer-by-layer (LbL) self-assembly technique, introduced by Decher et al. in 1991, is a powerful tool to construct biomacromolecule-loaded films on a variety of surfaces. This technique allows nanoscale control over the deposition of polyanions and polycations from aqueous solutions through electrostatic interactions. By controlling parameters such as the ionic strength and pH value in solution, one can engineer a variety of properties of the films, including film thickness, roughness, molecular conformation, surface wettability, and surface charge.

Since polycation-based DNA nanoparticles are positively charged, we hypothesized that they can be deposited with a polyanion by a LbL self-assembly technique. The objective of this paper is to assess the possibility of the construction of (polymer-based DNA nanoparticle/polyanion)_n multilayers via an LbL technique. Poly(ethyleneimine) (PEI)-DNA nanoparticles are selected as model DNA nanoparticles. PEI-DNA nanoparticles exhibit remarkable transfection efficiency, and an overview of the nanoparticles' characterization and transfection issues has been covered in previous papers.

A DNA solution was prepared at a concentration of 100 μg · mL⁻¹ (0.3 × 10⁻⁶ m in amine nitrogen) in HEPES buffer [molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) was 5]. The nanoparticles were formed by adding an equal volume of DNA solution to the PEI solution, followed by intense stirring and allowing to equilibrate at room temperature for ~20 min before being characterized or used for LbL deposition. The nanoparticles were formed in HEPES buffer (20 × 10⁻³ m HEPES with 20 × 10⁻³ m NaCl, pH 7.4) unless otherwise noted.

**Characterization of the PEI-DNA Nanoparticles**

The DNA nanoparticles were investigated by transmission electron microscopy (TEM, Jem-1230, JEOL, Japan, at 80 kV). Briefly, a small drop of nanoparticle-containing solution was deposited onto a carbon-coated copper grid. The sample was then fully dried for measurements. The zeta potential of the nanoparticles was determined by laser Doppler anemometry (LDA) (Zetasizer 3000HS, Malvern Instruments, Malvern, UK). All measurements were carried out at room temperature.

**Experimental Part**

**Materials**

PGA (M_w 17,000) and PEI (branched, M_w 25,000) were purchased from Sigma. DNA (Fish Sperm, Sodium Salt) and N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES, free acid, high purity grade) were purchased from AMRESCO. The water used was distilled three times. All reagents were used as received.

**PEI-DNA Nanoparticles Formation**

A PEI solution was prepared at a concentration of 65 μg · mL⁻¹ (1.5 × 10⁻⁶ m in amine nitrogen) in HEPES buffer [molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) was 5]. The nanoparticles were formed by adding an equal volume of DNA solution to the PEI solution, followed by intense stirring and allowing to equilibrate at room temperature for ~20 min before being characterized or used for LbL deposition. The nanoparticles were formed in HEPES buffer (20 × 10⁻³ m HEPES with 20 × 10⁻³ m NaCl, pH 7.4) unless otherwise noted.

**Characterization of the (PEI-DNA nanoparticle/PGA)_n Multilayer Construction**

The (PEI-DNA nanoparticle/PGA)_n multilayers were deposited LbL onto PEI-coated quartz substrates. A PGA solution (1 mg · mL⁻¹) was prepared by dissolving PGA in HEPES buffer. First, a layer of PGA was adsorbed onto the surface of the substrate by immersing in the PGA solution for 10 min. The substrates were then washed in water, followed by drying under a stream of N₂. The PEI-DNA nanoparticles were then attached to the PGA layer by subsequently immersing the quartz substrate in the nanoparticle solution for 10 min followed by a water wash. The process was repeated until a desired number of bilayers had been deposited. Experiments were carried out at room temperature.

**Characterization of the (PEI-DNA nanoparticle/PGA)_n Multilayers**

The growth of the PGA and PEI-DNA nanoparticle bilayer was followed by UV-vis spectrometry on a UV-vis spectrophotometer (CARY 100 BIO, America). A multilayer of 20 bilayers was constructed and embedded in an epoxy resin for microtoming, and then the cross section of the multilayers was investigated by TEM. The surface topographic feature of the films was monitored by atomic force microscopy (AFM) in tapping mode in air. The root mean square (RMS) roughness values were obtained from the software. All measurements were carried out at room temperature.

**Results and Discussion**

**Characterization of the PEI-DNA Nanoparticles**

Previous papers reported that the condensation of PEI with DNA is almost complete at an N/P ratio of 5. A further increase in the N/P ratio does not alter the properties of the PEI-DNA nanoparticles. Therefore, in our experiments,
the PEI-DNA nanoparticles formed at an N/P ratio of 5 are used for LDA and TEM characterization and LbL construction.

Branched PEI has a zeta potential of 37 mV in solution. After the condensation of PEI with DNA, the value should decrease. In our case, the zeta potential of the PEI-DNA nanoparticles is 31.6 ± 6.09 mV. Previous papers reported similar results. This value indicates that PEI has mainly condensed the DNA molecules and have conferred an excess cationic charge. The diameter of the nanoparticles is found to be 10–30 nm by TEM characterization (see Supporting Information Figure 1). It is reported that PEI-DNA nanoparticles have several ordered structures such as spheroids, toroids, and rods. In our case, as can be seen in the TEM micrograph, the nanoparticles are spheroids and toroids. Dunlap et al. demonstrated that PEI coats DNA and then folds it into forms of loops or globules. These structural morphologies might influence the transfection efficiency. Small dimensions and a highly positive zeta potential could stabilize the nanoparticles and guarantee the LbL construction via electrostatic interactions.

Characterization of the LbL Deposition of PGA and the PEI-DNA Nanoparticles

The construction of the first (PGA/PEI-DNA nanoparticle) bilayer, which is defined as the alternative deposition of PGA and PEI-DNA nanoparticle layers onto the PEI-coated quartz substrates, is monitored to determine if the DNA nanoparticles could adsorb onto the PGA layer. Figure 1 presents micrographs of the PGA layer and the PEI-DNA nanoparticle layer. When the first PGA layer is introduced onto the substrates, as shown in Figure 1(a), the surface of the film is very flat with an RMS roughness value of 0.173 nm, and no granules can be observed. However, after PEI-DNA nanoparticles are then introduced onto the PGA layer, the surface of the film is significantly different. As shown in Figure 1(b), many nanoparticles are adsorbed onto the PGA layer. The RMS roughness accordingly increases to 0.893 nm. There are some relatively large nanoparticles observed on the film. They could by attributed to the aggregation or accumulation of the PEI-DNA nanoparticles during the process of adsorption. The AFM micrographs suggest that the PEI-DNA nanoparticles are successfully adsorbed onto the surface of the PGA layer. Although we cannot obtain the absolute value of percentage, the PGA layer is not fully covered with the DNA nanoparticles. Under our experimental conditions, the constructions are carried out in HEPES buffer (20 mM HEPES with 10 mM NaCl, pH 7.4). It is known that PEI and PGA are weak polyelectrolytes with pKa values of 8.4 and 5.1, respectively. Therefore, the polyelectrolytes are partially charged at pH 7.4. The electrostatic interaction between the PGA molecules and the PEI-DNA nanoparticles might be not strong enough to develop fully covered deposition. In addition, since the PEI-DNA nanoparticles are a kind of biomacromolecular particle, the different charge density, the spheriform morphology, and unstable figuration of the nanoparticles may also hinder their adsorption and cause assembly problems, as was suggested for a somewhat related protein/polyelectrolyte system.

After (PGA/PEI-DNA nanoparticle)_1 is successfully deposited, the multilayering is pursued by further deposition of PGA and PEI-DNA nanoparticles up to 20 bilayers. UV-Vis spectrometry is employed to follow the construction of the multilayers. The UV-vis absorption spectra of the (PGA/PEI-DNA nanoparticle)_n multilayers of 5, 10, 15, and
20 bilayers are shown in Figure 2. A typical linear LbL growth of the (PGA/PEI-DNA nanoparticle)\textsubscript{n} multilayers is observed, which means that the PEI-DNA nanoparticles have been successively incorporated into the multilayers and show a linear increase with the bilayer number. To further verify the build-up of multilayers, TEM is employed to investigate the multilayers of (PGA/PEI-DNA nanoparticle)\textsubscript{20}. Figure 3 presents the TEM image of the cross section of the multilayers. The micrograph shows that the multilayer is not homogeneous. Small and large complexes are found in the multilayers, which suggest that the PEI-DNA nanoparticles are incorporated into the multilayers with slight aggregation. These relatively large complexes are observed by AFM (Figure 1). The TEM micrographs also indicate that the thickness of the (PGA/PEI-DNA nanoparticle)\textsubscript{20} multilayers is about 120 nm, from which the bilayer thickness of (PGA/PEI-DNA nanoparticle)\textsubscript{1} is about 6 nm. However, the diameter of the PEI-DNA nanoparticles is 10–30 nm. The diameter of the nanoparticles is higher than the bilayer thickness of the (PGA/PEI-DNA nanoparticle)\textsubscript{1}. The discrepancy between the bilayer thickness and the diameter of the PEI-DNA nanoparticles might be explained mainly by two reasons. First, the PEI-DNA nanoparticles are a kind of biomacromolecular particle. The ‘soft’ PEI-DNA nanoparticles would shrink during the drying process as well as change into a ‘flattened’ image in the multilayers. Second, as mentioned above, the coverage is incomplete when one layer of PEI-DNA nanoparticles are deposited onto the PGA layer (Figure 1), which means that there is not a full coverage of nanoparticles on the substrates until several bilayers of (PGA/PEI-DNA nanoparticles)\textsubscript{1} have been assembled.

The surface topographic features of the multilayers are characterized by AFM. The AFM micrographs of multilayers of 5, 10, 15, and 20 bilayers are shown in Figure 4. Many particles are observed on the film surface. Worm-like features are observed, and the dimensions of the ‘worm’ grow with the LbL deposition. Correspondingly, the RMS roughness value increases from 2.59 [(PGA/PEI-DNA nanoparticle)\textsubscript{5} film] to 8.74 nm [(PGA/PEI-DNA nanoparticle)\textsubscript{20} film]. Furthermore, as can be seen in Figure 4(d), the surface of the film is covered by the nanoparticles. We constructed a (PGA/PEI)\textsubscript{20} film (the PGA and PEI solutions are also prepared in HEPES buffer) to enable comparison with the (PGA/PEI-DNA nanoparticle)\textsubscript{n} films, as can been seen in Figure 4(e). The surface of the (PGA/PEI)\textsubscript{20} film is found to be very flat with an RMS roughness value of 1.44 nm [8.74 nm for the (PGA/PEI-DNA nanoparticle)\textsubscript{20} film]. This observation indicates that the PEI-DNA nanoparticles are incorporated into the films and significantly change their structure.
Figure 4. Typical topographical AFM micrographs of a) 5 bilayers, b) 10 bilayers, c) 15 bilayers, d) 20 bilayers (PGA/PEI-DNA nanoparticle) films, and e) 20 bilayers (PGA/PEI) films.
Conclusion

We have demonstrated that polycation-based non-viral DNA nanoparticles, such as PEI-DNA nanoparticles, can be incorporated into ultrathin multilayers by an LbL self-assembly technique. TEM characterization shows that the PEI-DNA nanoparticles formed at an N/P ratio of 5 are spheroids and toroids with diameters of 10–30 nm. PEI confers an excess positive charge onto the nanoparticles, which have a zeta potential of 31.6 ± 6.09 mV. The AFM, UV-vis spectrometry, and TEM data show that the nanoparticles are adsorbed onto the PGA layer and the multilayers can be constructed successfully up to 20 bilayers. By taking advantage of the LbL technique, such multilayers could be easily deposited onto a variety of surfaces of medical implants, such as vascular stents, intervention catheters, and tissue engineering scaffolds. The (PGA/PEI-non-viral DNA nanoparticle)$_n$ multilayers may provide a new approach to load and deliver non-viral DNA nanoparticles to targets in vivo, which may advance the application of the non-viral DNA nanoparticles in the field of gene therapy.

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