Polymer-based gene delivery system fabricated with the participation of a polyanion

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ABSTRACT

A polymer-based gene delivery system, denoted as "DNA/polycation/polyanion", is designed based on the concept of polyelectrolyte multilayer fabrication by the layer-by-layer polyelectrolyte self-assembly technology. The comparisons of the physicochemical properties between 'core' DNA/branched polyethylenimine (PEI) and DNA/chitosan complexes suggest that DNA/PEI complexes form as colloidally stable, well-dispersed, homogeneous and small around 100 nm (at pH 7.4) or 70 nm (at pH 4.0). When depositing the polyanion layer around the 'core', hyaluronic acid (HA) is found not to dissociate DNA/PEI complexes. DNA/PEI/HA complexes with increased size around 200~300 nm have thus been produced as the designed gene delivery system.

Keywords: gene delivery system, polyethylenimine, chitosan, hyaluronic acid, polyelectrolyte layer-by-layer self-assembly

1 INTRODUCTION

Currently polymer-based gene delivery systems generally consist of the therapeutic gene and a positively charged polymer (polycation). Various polycations, such as polylysine, polyethylenimine or dendrimers, have been investigated. However, the application of the DNA/polycation system is still limited due to their drawbacks including low transfection efficiency and the polycation toxicity. Chemical modifications of the structures of polycations to improve the transfection efficiency via introducing functionalities such as better endosomal escape or nuclear membrane transport are being investigated. In this study, the improvement is attempted from another direction, by creating a polymeric layer(s) around the condensed DNA 'core' and forming a multilayer system. It is expected that desired number of polyelectrolyte layers can be deposited, whereby the involvement of polymeric layers is expected to broaden the possibility of introducing the functional groups required for improved gene transfection to the delivery system. This concept of building DNA/polycation/polyanion system is generated from the polyelectrolyte multilayer (PEM) fabrication by layer-by-layer self-assembly technique. This technique was originally used to build PEM on planar substrate [1]; and then was developed to build PEM around spherical-like support [2].

2 METHODS

2.1 DNA Complex Preparation

To prepare DNA/polycation complexes, polyethylenimine (PEI; branched, Mw=25000) or chitosan chloride (Mw=340000, deacetylation degree 86%) was added to calf thymus DNA (ctDNA) solution respectively, at different polycation amino group to DNA phosphate group (N/P) ratios. The complexes were prepared in 10 mM Tris-HCl buffer of either pH 7.4 or pH 4.0.

To prepare DNA/PEI/polyanion complexes, poly(aspartic acid) (PAA; Mw=2000~10000), poly(styrene sulfonate) (PSS; polymerization degree=90, Mw/Mn=1.63) or hyaluronic acid (HA; Mw $\approx$ 300000) was added respectively to ctDNA/PEI complexes prepared at the N/P ratio 2.6 in pH 7.4 buffer or at the N/P ratio 2.0 in pH 4.0 buffer. The amounts of the added polyanions were calculated according to the polyanion monomer to N ratios.

2.2 Physicochemical Characterization

Ethidium Bromide Fluorescence Assay. The fluorescence after the subsequent titration of ethidium bromide (EtBr), DNA, the polycation and the polyanion into the buffer was measured using a Hitachi F4500 fluorescence spectrophotometer. The relative fluorescence was calculated designating the fluorescence of EtBr-DNA solution as the 100% relative fluorescence. **Gel Electrophoresis.** The optimal DNA/polycation ratios for the core formation were determined from gel electrophoretic studies in 0.8% Agarose gel. Components were visualized using appropriate dye stainings. **Particle Size Measurements.** The sizes in diameters of the DNA complexes were measured using a Malvern S4700 PCS system. **Zeta-Potential Measurements.** The surface charges shown as the zeta-potentials of the DNA complexes were measured using a Malvern Zetasizer 2000 system.

3 RESULTS AND DISCUSSION

3.1 DNA/polycation Complexes

The DNA/PEI and DNA/chitosan complexes have been extensively studied as gene delivery systems. This study mainly focuses on comparing their physicochemical properties in order to determine the optimized DNA/polycation ratios suitable for the "core" formation to

which the further layers can be deposited. EtBr fluorescence assay (see Fig. 1) shows that both PEI and chitosan can condense ctDNA. However, the lower fluorescence plateau levels suggest more efficient condensation by PEI. Interestingly, at pH 4.0 the polycations' condensation is more efficient than at pH 7.4, which is supposed to be related to the different ionization levels of the polycations at different pHs.

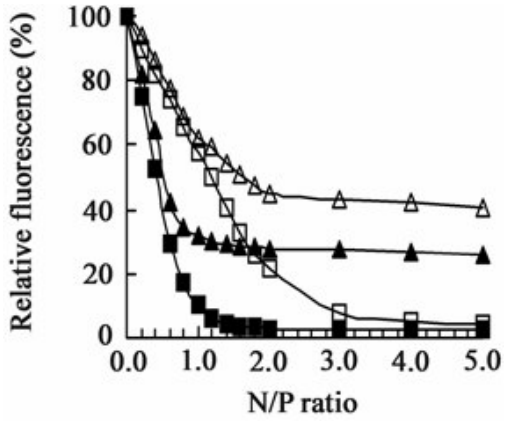

Figure 1: EtBr fluorescence assay of the binding abilities between ctDNA and PEI (□at pH 7.4 and ■at pH 4.0) or chitosan (△at pH 7.4 and ▲at pH 4.0) at different N/P ratios in 10 mM Tris-HCl buffer.

Gel electrophoresis study (see Fig. 2) shows that from the certain N/P ratios the free DNA smears in the gel cease, suggesting that the DNA has been complexed by the polycations. However, the fluorescence in the loading wells of DNA/chitosan complexes can be seen even at high N/P ratios. This is in accordance with the EtBr fluorescence assay (see Fig. 1), suggesting that DNA/chitosan complexes may have a looser structure containing free DNA segments that allow for the fluorescent probe intercalation.

The sizes and the surface charges of DNA/polycation complexes (see Fig. 3) show a similar pattern: the surface charges reverse from negative to positive with the increase of the N/P ratio. At either highly negative or highly positive surface charges, the DNA/polycation complexes have small sizes (except ctDNA/chitosan complexes at pH 7.4); while aggregation of the complexes occurs at the surface charges close to neutrality, attributable to the insufficient electrostatic repulsion, and/or the free DNA segments or the free polycation segments of the complexes are not long enough to provide electrostatic stabilization of the complexes [3]. Interestingly, DNA/chitosan complexes are more prone to aggregate, particularly at pH 7.4 where thread-like precipitates were observed in the samples beyond the N/P ratio 0.4 (accurate size measurements were not possible by PCS). At pH 4.0, however, with cautious mixing of the samples DNA/chitosan complexes in nanometer-range were prepared, although generally larger and less homogeneous than the corresponding DNA/PEI complexes. According to the above results, chitosan shows lower level of DNA complexation efficiency and forms larger and less colloidally stable complexes with looser structure, relative to PEI. This may be due to the lower charge density of chitosan [4] [5] and its folded conformation in solution [6]. Therefore, ctDNA/PEI complexes prepared at the N/P ratio 2.6 in pH 7.4 buffer and at the N/P ratio 2.0 in pH 4.0 buffer were selected as the "cores" for the polyanion deposition. These N/P ratios are selected because the following requirements can be fulfilled simultaneously: ctDNA is fully complexed; ctDNA/PEI complexes are positively charged, small (~100 nm at pH 7.4 and ~70 nm at pH 4.0), well dispersed and colloidally stable. Also importantly, there is no significant (measurable) excess of free PEI remaining in the system. Thus, 'washing of' ctDNA/PEI complexes to separate free polycation from the system before the addition of the polyanion can be avoided.

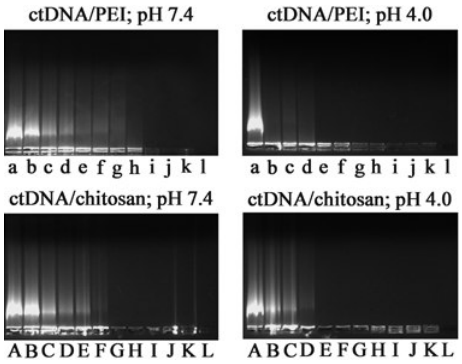

Figure 2: Gel electrophoresis images of ctDNA/polycation complexes. Lanes **a** and **A** represent free ctDNA; lanes **l** represents free PEI and lane **L** represents free chitosan; lanes from **b** to **k** represent ctDNA/PEI complexes prepared at the N/P ratios 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4 and 2.6; and lanes from **B** to **K** represent the ctDNA/chitosan complexes prepared at the N/P ratios 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.8, 2.0 and 2.2.

3.2 DNA/PEI/polyanion complexes

EtBr fluorescence assay (see Fig. 4) shows that the additions of PAA and PSS dissociate ctDNA/PEI complexes, and the presence of the free DNA consequently results in the increase of the fluorescence. Considering that DNA is a polyanion, this observation suggests that a competition between DNA and PAA, or PSS, for the interactions with PEI occurs, whereby PAA, or PSS, complexation with PEI appears to be occurring and thus releasing DNA. Similar competitive interactions among polyelectrolytes have been reported previously [7]. Interestingly, the addition of HA does not dissociate ctDNA/PEI complexes at both pH 7.4 and pH 4.0, as no increased fluorescence is observed. This is also confirmed by gel electrophoresis images (see Fig. 5), where no free ctDNA is detected in the gel after the addition of HA. The presence of free HA in the ternary system is detected by Stains-All staining. The staining demonstrates that free HA starts appearing in the gel from the low COO/N (HA

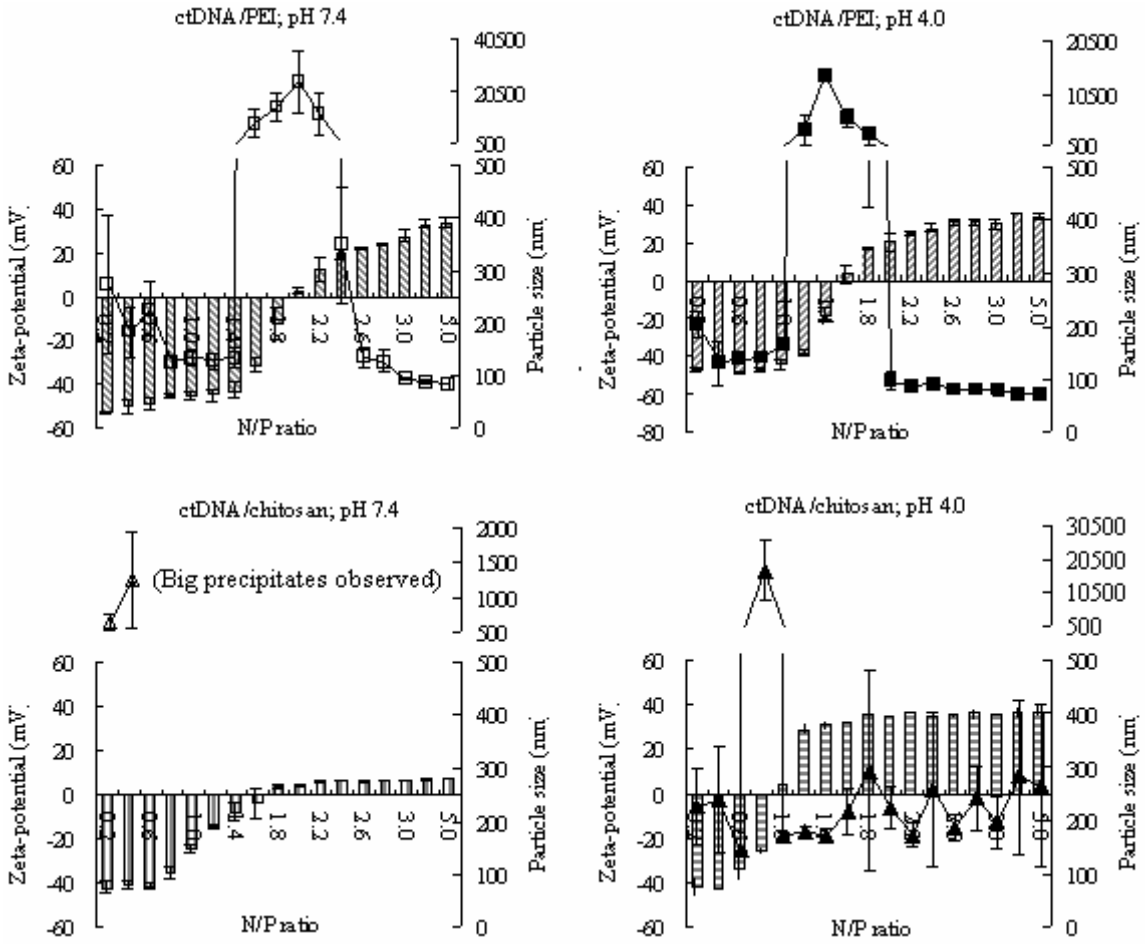

Figure 3: Surface charges and particle sizes of ctDNA/polycation complexes prepared at the N/P ratios 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 4.0 and 5.0.

carboxyl group to PEI amino group) ratio of 0.1. This is not unexpected, taking into account that PEI is complexed with DNA and at the chosen N/P ratio only the surface free amino groups from PEI are available for the interaction with HA. Following the addition of HA into DAN/PEI core the surface charge reverses from positive to negative starting from the COO/N ratio 0.1 (see Tab. 1), suggesting that HA has attached onto the ctDNA/PEI complexes and ctDNA/PEI/HA complexes have been formed. In Tab.1 it also indicates the formation of aggregates when the zeta potential values are relatively low (ctDNA/PEI/HA complexes of the COO/N ratio 0.1 in pH 7.4 buffer and of the COO/N ratio 0.1, 0.2 and 0.3 in pH 4.0 buffer). Interestingly agglomeration is more pronounced when the systems was prepared in the pH 4.0 buffer and may be associated with the level of HA ionization, although a pKa value of 2.9 has been reported.

The addition of increased amount of HA, from the ratios 0.2 in pH 7.4 buffer and 0.4 in 4.0 buffer, resulted in the formation of well-dispersed and colloidally stable complexes and in the range from 200 to 300 nm. The increased size comparing to ctDNA/PEI complexes further proves that ctDNA/PEI/HA complexes have been formed. HA layers are relatively thick, approximately 30 nm at pH 7.4 and 100 nm at pH 4.0. This may be the consequence of the adsorption of HA which retains a helical conformation even when it is highly ionized [8], and thus HA may occupy a large space in the complexes. Moreover, the level of polyanion ionization somewhat changes in the buffers used, at pH 7.4 PEI is nearly fully ionized (90.91 %, assumed pKa value 8.4) and HA is fully ionized (99.99%; assumed pKa value 2.9); while at pH 4.0 PEI is fully ionized (99.99%) and HA is nearly fully ionized (92.64%). It has been previously reported that the formation of polyelectrolyte multilayers from two weak polyelectrolytes when one of them is fully ionized and the other one is nearly fully ionized, tends to result in thick layers because the polyelectrolyte chain are not adsorbed in a flat conformation but with loops and tails [9]. It is also interesting to notice that for ctDNA/PEI/HA complexes the HA layer formed at pH 7.4 is thinner than the layer formed at pH 4.0. This may be because at pH 4.0 PEI is more ionized, and thus there are more positively charged groups on the surface of the 'core' of ctDNA/PEI complexes, which may promote HA deposition in a flat layer based on electrostatic interactions.

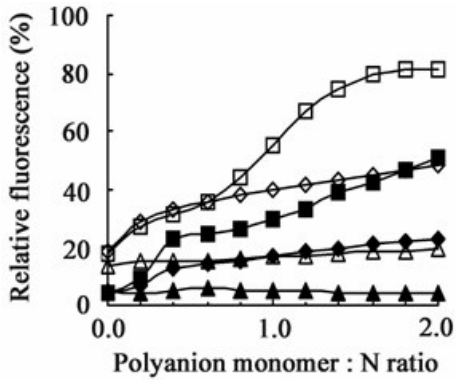

Figure 4: EtBr fluorescence assay of the interactions between ctDNA/PEI complexes and PAA (□at pH 7.4 and ■at pH 4.0), PSS (◇at pH 7.4 and ◆at pH 4.0) or HA (△ at pH 7.4 and ▲at pH 4.0) at different polyanion monomer to N ratio in 10 mM Tris-HCl buffer.

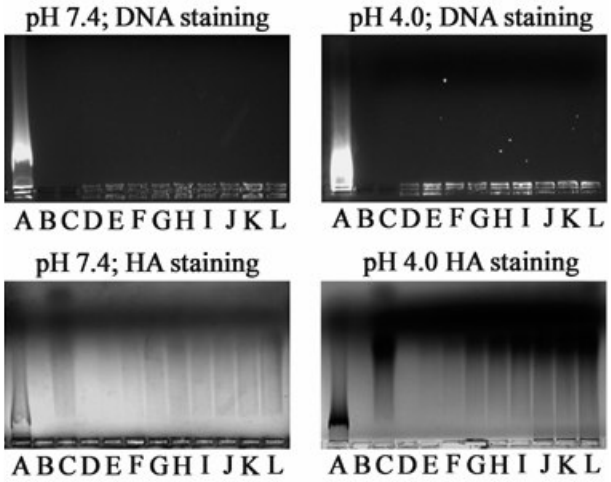

Figure 5: Gel electrophoresis images of ctDNA/PEI/HA complexes. Lanes **A**, **B**, **C** and **D** represent free DNA, free PEI, free HA and DNA/PEI complexes. Lanes from **E** to **L** represent DNA/PEI/HA complexes prepared at the COO/N ratios 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8. DNA was stained by EtBr. HA was stained by Stains-All.

4 CONCLUSION

A gene delivery system of DNA/polycation/polyanion composition is fabricated in this study by the layer-by-layer deposition. The formation of the system with optimal properties of gene delivery is highly dependent on the choice of the polyanions. ctDNA/PEI complexes were found to present the better "core" for polyanion layer deposition than ctDNA/chitosan complexes, mainly due to their better colloidal stability and smaller size. For the polyanion layer deposition, PAA and PSS were found to dissociate DNA/PEI complexes; while HA produces the ternary complexes with negatively charged surface and the increased size relative to the 'core', both indicating the formation of the HA surface layer. The fabrication of DNA/PEI/HA complexes opens a possibility for targeting the system to the cells over-expressing HA receptors [10].

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COO/N ratio	pH 7.4			pH 4.0		
	Zeta-potential (mV)	Diameter (nm)	Polydispersity	Zeta-potential (mV)	Diameter (nm)	Polydispersity
0.0	+23.2±2.3	136.4±24.0	0.352±0.158	+16.1±1.9	73.9±2.9	0.264±0.043
0.1	-6.2±3.4	10043.1±1625.3	0.991±0.015	-16.0±0.6	6337.0±1302.3	0.968±0.037
0.2	-22.5±0.3	245.2±18.7	0.317±0.064	-18.9±0.7	14690.0±6436.7	0.988±0.021
0.3	-21.1±0.6	188.6±19.1	0.199±0.090	-20.2±0.5	5851.1±1947.0	0.959±0.070
0.4	-21.5±2.1	209.0±18.2	0.391±0.113	-20.6±0.9	297.9±43.7	0.383±0.037
0.5	-23.1±1.0	164.0±1.1	0.205±0.006	-21.7±0.4	287.3±64.5	0.363±0.099

Table 1: Zeta-potentials and particles sizes of ctDNA/PEI/HA complexes prepared in pH 7.4 or pH 4.0 buffer.