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Layer-by-layer deposition of oppositely charged polyelectrolytes on the surface of condensed DNA particles

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ABSTRACT

DNA can be condensed with an excess of polycations in aqueous solutions forming stable particles of submicron size with positive surface charge. This charge surplus can be used to deposit alternating layers of polyanions and polycations on the surface surrounding the core of condensed DNA. Using poly-L-lysine (PLL) and succinylated PLL (SPLL) as polycation and polyanion, respectively, we demonstrated layer-by-layer architecture of the particles. Polyanions with a shorter carboxyl/backbone distance tend to disassemble binary DNA/PLL complexes by displacing DNA while polyanions with a longer carboxyl/backbone distance effectively formed a tertiary complex. The ζ potential of such complexes became negative, indicating effective surface recharging. The charge stoichiometry of the DNA/PLL/SPLL complex was found to be close to 1:1:1, resembling polyelectrolyte complexes layered on macrosurfaces. Recharged particles containing condensed plasmid DNA may find applications as non-viral gene delivery vectors.

INTRODUCTION

DNA is condensed into compact structures by polycations (pC) that contain more than +2 charge (1). This phenomenon is critical to not only chromatin and viral assembly but also of importance in the construction of artificial gene delivery vectors. The DNA/pC complexes form a diverse set of structures dependent in part on the charge ratio of DNA/pC. When DNA is in excess (DNA:pC charge ratio >1), complexes assemble into 'daisy-shaped' particles with loops of uncondensed DNA that inhibit aggregation of the particles (2). When pC is in excess (DNA:pC ratio <1), DNA condenses completely within particles that customarily adopt a toroid or rod morphology (3). In low salt aqueous dilute DNA solutions, an excess of pC stabilizes these highly condensed structures and maintains them in a soluble state (4).

The ability of the DNA/pC complexes to function as non-viral gene transfer agents is influenced by their charge ratios and associated structures. Optimal transfection activity generally requires excess pC (5). The compact toroid morphology (which corresponds to the completely condensed DNA) was demonstrated to be associated with efficient gene transfer *in vitro* (3,6,7). However, the presence of a large excess of pC can be toxic to cells and tissues (8). Also, non-specific binding of cationic particles to all cells forestalls cell type specificity (9,10). Positive charge also adversely influences biodistribution of the complexes *in vivo* (9).

Previous efforts to avoid these unwanted effects have utilized anionic particles (DNA:pC charge ratio <1) containing an excess of DNA and ligands for cell receptor targeting (10,11). Also, pC-condensed DNA has been complexed with negatively charged liposomes (12,13). The present study describes the layer-by-layer deposition of oppositely charged polyelectrolytes on the surface of condensed DNA particles and the formation of negatively charged particles containing completely condensed DNA.

MATERIALS AND METHODS

Materials

The plasmid DNA (pCILuc) (14) used for the condensation studies was produced by a commercial supplier (Bayou Biolabs, Harahan, LA). Poly-L-lysine (PLL) (mol. wt 34 or 210 kDa), poly-L-aspartic acid (pAsp) (mol. wt 36 kDa), poly-L-glutamic acid (pGlu) (mol. wt 49 kDa) and rhodamine B isothiocyanate were products of Sigma (St Louis, MO). PLL of 34 kDa size was used unless otherwise indicated. Polymethacrylic acid (pMAA), metrizamide and fluorescein isothiocyanate were from Aldrich (Milwaukee, WI). *Label*IT kits (Mirus Corp., Madison, WI) were used for covalent labeling of DNA with fluorescein (Fl) and rhodamine (Rh) (15).

Synthesis of succinylated PLL (SPLL)

Succinic anhydride (30 mg) dissolved in 150 µl DMSO was added to PLL (20 mg) dissolved in 1 ml of 0.1 M sodium tetraborate solution, pH 9.0, in two equal portions (15 mg each). Reaction mixture pH was adjusted back to 9.0 with 4 M HCl after addition of the first portion. After 10 min incubation

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at room temperature, the polymer was precipitated with two volumes of isopropanol following subsequent reprecipitation and washing with the same solvent. The final preparation was reconstituted with deionized water.

Labeling of PLL and DNA with fluorescein and rhodamine

Fluorescein isothiocyanate (0.37 mg in 5 µl DMSO) was added to PLL (20 mg) in 1 ml of 0.1 M sodium tetraborate solution and incubated for 1 h. The resulting Fl–PLL was purified by isopropanol precipitation. Fl–PLL was also used for preparation of Fl–SPLL by succinylation as described above. For DNA labeling, DNA and *Label*IT reagent were mixed in HEPES buffer (25 mM HEPES, 1 mM EDTA, pH 7.5) in reagent/DNA weight ratios of 1:1 and incubated for 1 h at 37°C. Labeled DNA was precipitated twice with NaCl/ethanol mixture (final NaCl concentration 0.2 M, ethanol 66%) and immediately redissolved in deionized water.

The condensation state of DNA was monitored as previously described as self-quenching of fluorescein groups on the DNA upon its collapse (15).

DNA/polyion complex formation

Complexes were formed in 25 mM HEPES, pH 7.5, or in water at DNA concentrations of 20–100 µg/ml. The DNA/PLL/SPLL complexes with a DNA/PLL charge ratio of 1:3 were formed by sequential addition of PLL and various amounts of SPLL followed by vortexing for 30 s. Before addition of each sequential polyion, the complexes were centrifuged for 5 min at 13 000 *g* in a microfuge.

Light scattering and ζ potential measurements

The intensity of scattered light measured at a 90° angle (I_{90}, static light scattering) was estimated using a Shimadzu RF 1501 Spectrofluorometer at λ_{ex} = 600 nm, λ_{em} = 600 nm. Particle sizing and ζ potential measurements were performed using a Zeta Plus Particle Analyzer (Brookhaven Instruments Corp., Holtsville, NY), with a laser wavelength of 532 nm. For the particle sizing experiments depicted in Figure 3 the samples were prepared in water at a DNA concentration of 40 µg/ml. Series of samples were prepared where each consecutive layer was added to the complex with 1.25- to 3.5-fold charge excess. After addition of each layer, the samples were centrifuged in a microfuge for 5 min at 13 000 *g*.

Atomic force microscopy

Images of DNA particles were obtained using a BioProbe AFM microscope (Park Scientific Instruments, Sunnyvale, CA). Samples (DNA concentration 1 µg/ml in 25 mM HEPES, pH 7.5) were adsorbed onto freshly cleaved mica in the presence of 1 mM $NiCl_2$ for 5 min and then viewed in the buffer in non-contact mode.

Ultracentrifugation experiments

For stoichiometry studies, two types of tertiary complexes containing fluorescent dye-labeled DNA, PLL and SPLL (charge ratio 1:3:10) were formed in 25 mM HEPES, pH 7.5, with either Fl–PLL (Rh–DNA/Fl–PLL/SPLL complexes) or Fl–SPLL (Rh–DNA/PLL/Fl–SPLL complexes). The samples (1 ml) were layered on top of 10% sucrose solution (10 ml) with 1 ml of 40% metrizamide cushion on the bottom and were centrifuged in a SW-41 Beckman rotor in an Optima LE-80K ultracentrifuge (Beckman Instruments) at 30 000 r.p.m. for 20 min. DNA-containing complexes were retrieved from the sucrose/metrizamide boundary using a Pasteur pipette and were dissolved in 2.5 M NaCl solution. Visible spectra of the complexes and 1:1 charge ratio Rh–DNA/Fl–PLL and Rh–DNA/Fl–SPLL standards pre-mixed in the same medium were recorded using a Shimadzu UV 1601PC spectrophotometer in the 700–400 nm wavelength range.

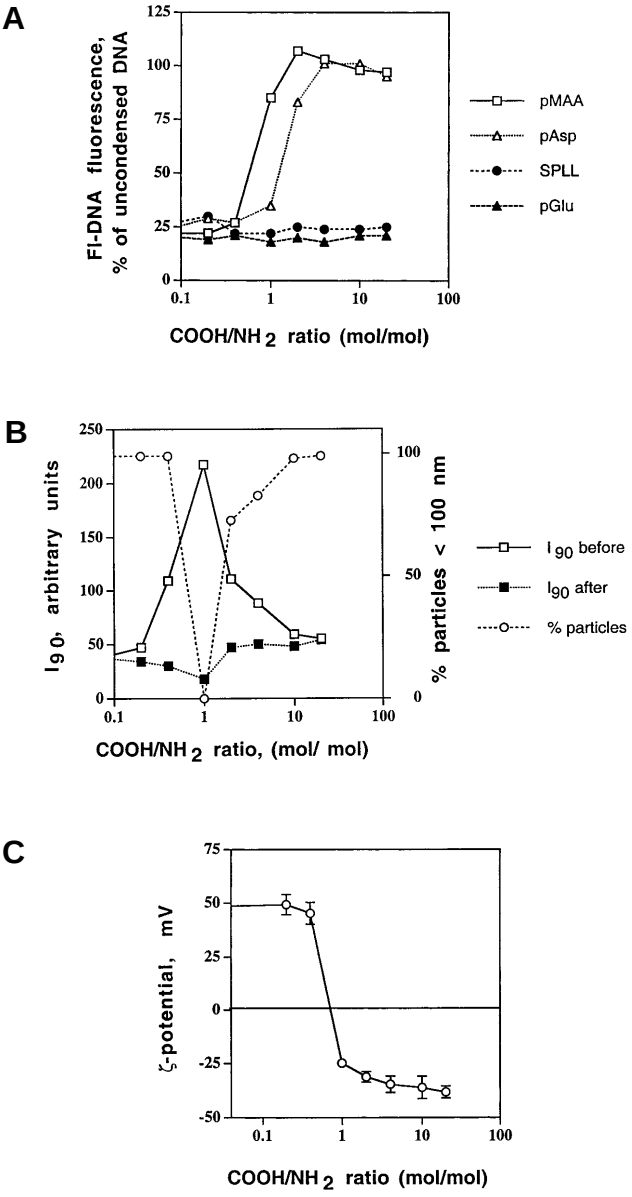

Figure 1. The effect of pA on the physical state of DNA/PLL complexes (1:3 charge ratio, [Fl–DNA] = 20 µg/ml) under low salt conditions (25 mM HEPES, pH 7.5). (**A**) Fl–DNA decondensation during titration of Fl–DNA/PLL complexes with different pA. (**B**) Titration of DNA/PLL complexes with SPLL as assessed by light scattering methods. Intensity of scattered light (I_{90}) was measured using a spectrofluorometer before and after centrifugation in a microfuge (5 min, 13 000 *g*). Percentage of particles <100 nm in diameter was measured using a particle size analyzer. (**C**) ζ Potential during titration of the DNA/PLL complex with SPLL.

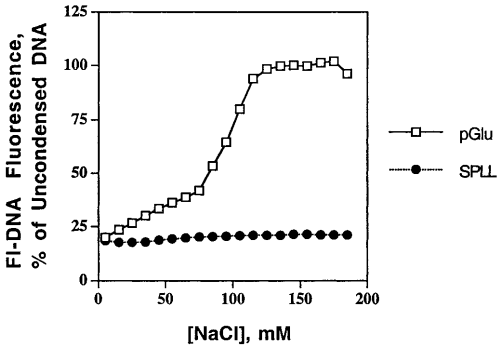

Figure 2. Decondensation of Fl–DNA/PLL/pA (1:3:20) complexes at increasing NaCl concentrations. Complexes (DNA concentration 20 μg/ml) were initially dissolved in 5 mM HEPES, pH 7.5. After addition of salt to the indicated concentration, the samples were allowed to stand for 5 min before fluorescence measurement.

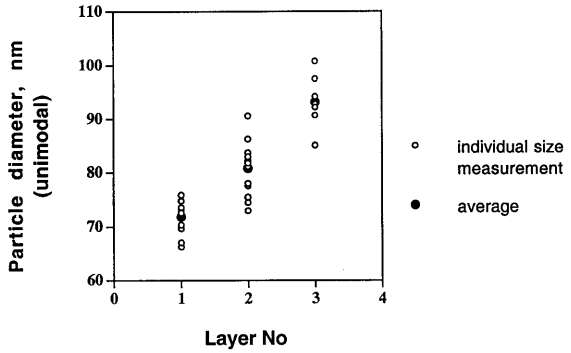

Figure 3. Sizing of DNA/PLL (layer 1), DNA/PLL/SPLL (layer 2) and DNA/PLL/SPLL/PLL (layer 3) complexes in water (dynamic light scattering, unimodal mode). Core DNA/PLL particles were prepared at a 1:1.2 charge ratio at a DNA concentration of 20 μg/ml in water. Each additional polyion was added in 1.5–4 charge excess. PLL and SPLL with mol. wt of 210 kDa were used for this experiment.

RESULTS

Recharging of pC-condensed DNA particles

The principal DNA/pC complex used in this study was DNA/PLL (1:3 charge ratio) formed in low salt aqueous buffer (25 mM HEPES). Under these conditions, plasmid DNA is completely condensed and compacted into toroid-shaped soluble particles stabilized with excess pC (3,4). The DNA particles were characterized after addition of a third polyion component to such a binary DNA/pC complex. Using a new DNA condensation assay, we found that certain polyanions (pA) such as pMAA and pAsp decondensed DNA within the DNA/PLL complexes (Fig. 1A). Surprisingly, pA such as SPLL and pGlu, even when added in 20-fold charge excess to the condensing pC (PLL), did not decondense DNA in DNA/PLL (1:3) complexes (Fig. 1A). It is interesting that pAsp and pGlu, although similar in structure, had such differing effects.

The extent of aggregation of condensed DNA particles was determined using both static and dynamic light scattering techniques. Upon titration of the DNA/PLL (1:3) complex with increasing amounts of SPLL, turbidity of the reaction mixture, an indication of flocculation, increases when the lysine to lysyl succinate (NH_2/COOH) ratio approaches 1:1 (Fig. 1B). A similar phenomenon was observed during earlier studies of DNA/pC interactions (16). With an excess of pA, the extent of aggregation decreases. After addition of SPLL the samples were centrifuged for 5 min at 13 000 *g* to remove flocculated material. The I_{90} value after centrifugation reflects the amount of small particles in solution. Note that with an excess of SPLL the I_{90} value is unaffected by centrifugation, indicating that a large number of particles avoid flocculation if the NH_2/COOH ratio is not 1. Correspondingly, assessment of particle size by dynamic light scattering shows that small DNA particles (<100 nm) exist before and after the charge equivalency point. Large aggregates are present only at a pC/pA charge ratio of 1:1.

Figure 1C demonstrates the change in particle surface charge (ζ potential) during titration of DNA/PLL (1:3) particles with SPLL. The particles become negatively charged and accordingly recharged at approximately the charge equivalency point (Fig. 1C).

Further studies explored the effect of ionic strength on the ability of pGlu or SPLL to decondense DNA/pC complexes (Fig. 2). While neither pA decondenses the DNA/PLL complex in 25 mM HEPES buffer, pGlu but not SPLL displaces DNA at higher salt concentrations.

The effect of the additional layers of polyions on particle size was determined using dynamic light scattering (Fig. 3). In this experiment, the next polyion of opposite charge was added at charge excess to underlying complexes in water. For example, excess SPLL (ranging from 1.25 to 3.5 ± ratio) was added to the DNA/PLL (1:1.5) complex in water. Consecutively, the same excess of PLL was added to the DNA/PLL/SPLL (1:1.5:2) complex. For DNA/PLL/SPLL/PLL particles, each layer increased particle diameter ~10 nm on average. After addition of the third layer polyion (PLL) DNA was still found condensed inside this quaternary complex (data not shown).

Atomic force microscopy was used to visualize the size and shape of the DNA/PLL/SPLL complexes (Fig. 4). It revealed the DNA/PLL/SPLL complexes to be non-aggregated spheroids, 50 nm in diameter on average. The slight discrepancy between the AFM and dynamic light scattering data may be the result of at least two factors: (i) dynamic light scattering data is based on unimodal particle size measurements which can be affected by a certain amount of cross-bridged aggregates inevitably present in any sample of polyelectrolyte complexes; (ii) the apparent size on AFM imaging can be affected by interaction of particles with the support.

Stoichiometry of purified DNA particles

In order to obtain a preparation of fully condensed DNA, DNA must be mixed with at least an equimolar amount of pC (in terms of phosphate/amine ratio). However, mixtures with a ratio close to 1:1 usually result in flocculation due to lack of charge stabilization of the DNA particles formed. The number of aggregates is dependent on the NaCl concentration. Hence, in order to obtain preparations with a maximum number of non-aggregated

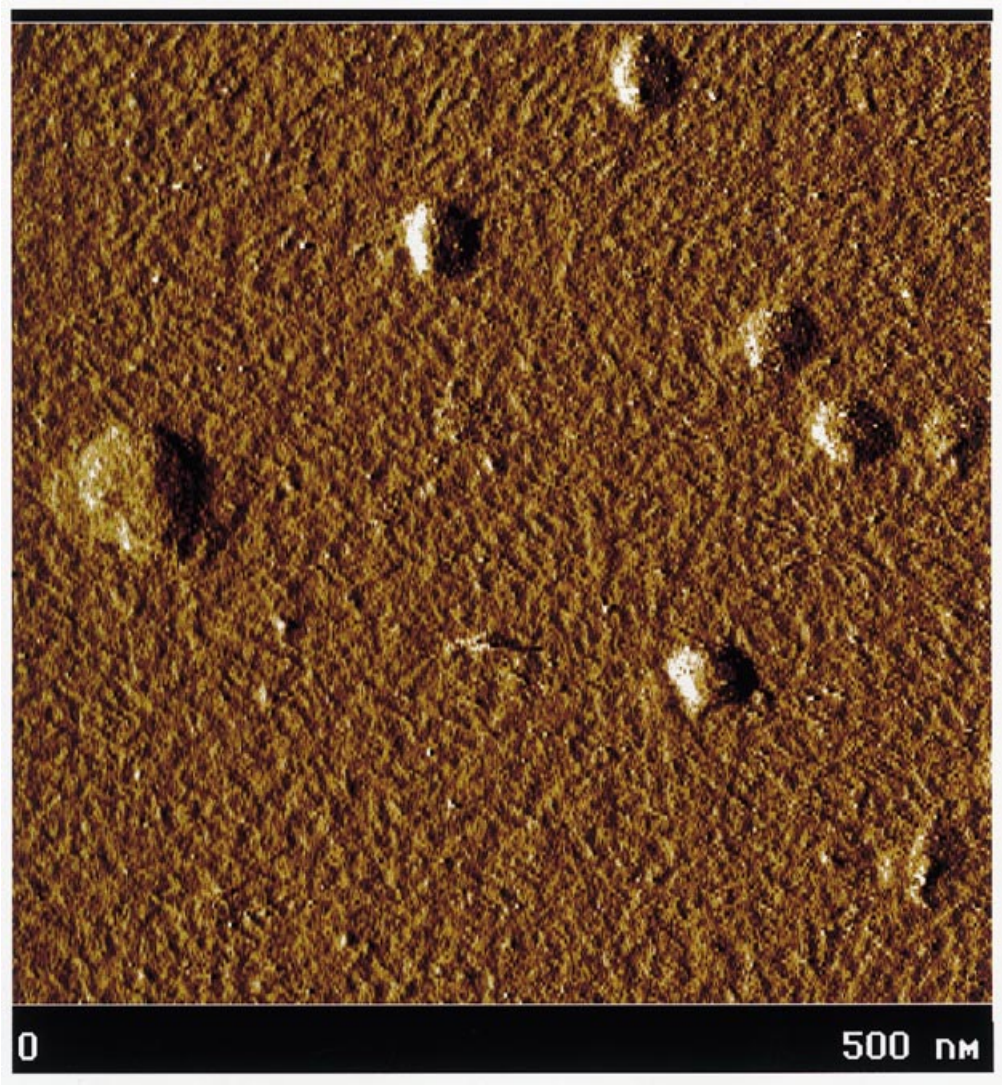

Figure 4. Atomic force microscopic images of DNA/PLL/SPLL complexes (1:3:10 initial ratio) absorbed on mica in 25 mM HEPES, 1 mM $NiCl_2$, pH 7.5.

particles of condensed DNA, one needs to add excess pC over DNA. Consequently, during recharging, an excess of recharging pA is required to obtain non-aggregated negatively charged particles of condensed DNA. Therefore, due to stoichiometric considerations, the recharged DNA/pC/pA tertiary complex contains some pC/pA or 'blank' particles, with the amount depending on the excess of pC used during formation of the first layer. To obtain only DNA-containing particles an ultracentrifugation method was used that separates DNA-containing particles from 'blank' ones.

DNA complexes were isolated from the DNA/PLL/SPLL (1:3:10 ratio) mixture using ultracentrifugation through a 10% sucrose solution to determine their polyelectrolyte stoichiometry. Rh–DNA and either Fl–PLL or Fl–SPLL were used to determine their relative amounts within DNA complexes. Optical densities at 495 and 595 nm indicated the amount of rhodamine and fluorescein label, respectively. After ultracentrifugation of Rh–DNA/Fl–PLL/SPLL (1:3:10) or Rh–DNA/PLL/Fl–SPLL (1:3:10) mixtures, all Rh–DNA entered the 10% sucrose solution. The top fraction contained no Rh–DNA but did contain PLL and SPLL. This indicates that, under these experimental conditions, no 'blank' particles entered the 10% sucrose solution, thereby allowing effective separation of DNA-containing and blank PLL/SPLL particles (Fig. 5A and B).

In order to determine the stoichiometric ratio of DNA/PLL/SPLL complexes, the ultracentrifugation experiments were further modified to include 1 ml of a 40% metrizamide cushion as a bottom layer. This dense solution aids in isolation of the DNA/PLL/SPLL complex at the boundary between the sucrose and metrizamide layers. After centrifugation of the preparations containing DNA/PLL/SPLL at 1:3:10 charge ratios, all of the Rh–DNA appeared at the sucrose/metrizamide border as precipitated complex. Precipitation apparently occurred due to loss of excess SPLL, which served as charge stabilizer to the whole complex. The recovered complexes were solubilized in 2.5 M NaCl and their visible spectra were compared to a 1:1

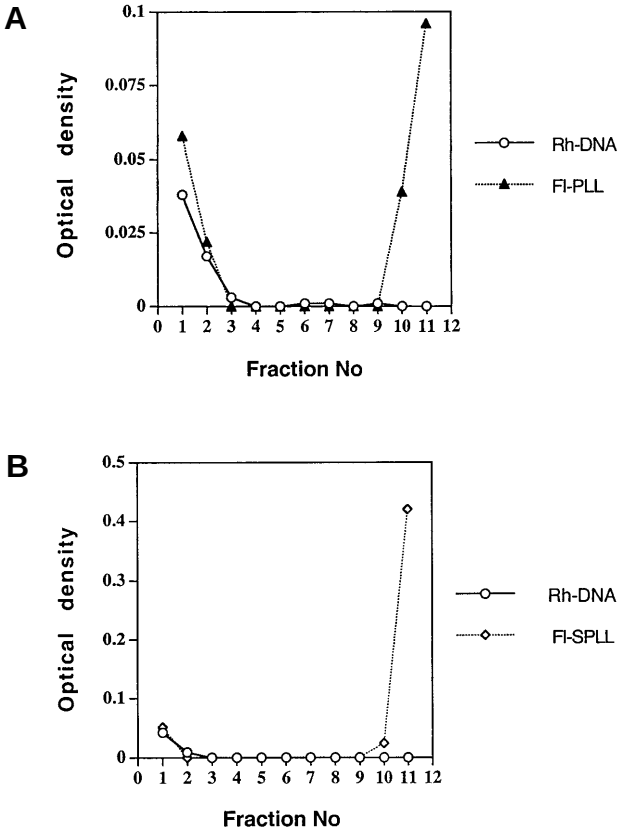

Figure 5. Fractionation of samples after ultracentrifugation of Rh–DNA/Fl–PLL/SPLL (**A**) and Rh–DNA/PLL/Fl–SPLL (**B**) complexes layered on top of 10% sucrose solution. Optical density indicates the relative amounts of Rh and Fl label in the fractions. Fraction 1 is at the bottom of the tube.

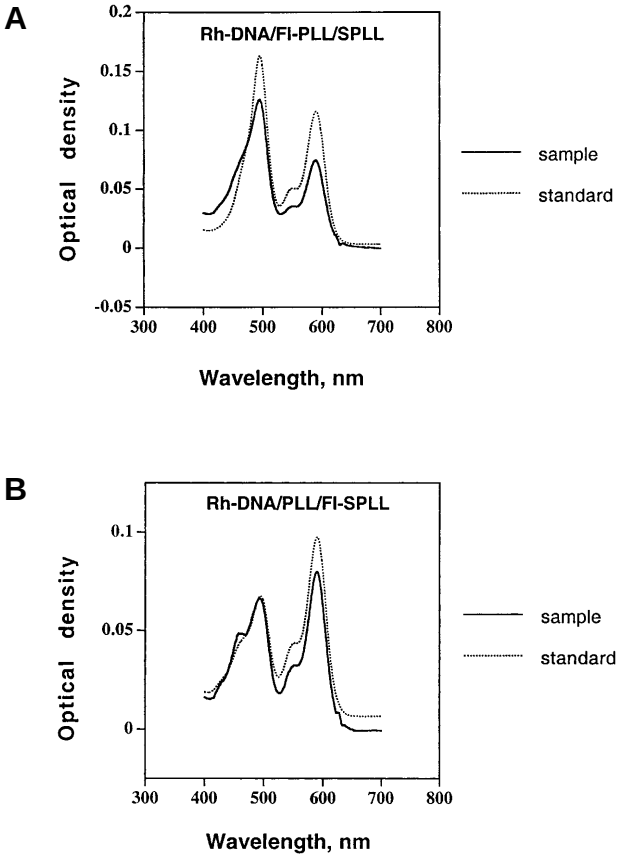

Figure 6. Visible spectra of ultracentrifuged DNA/PLL/SPLL complexes isolated from the sucrose–metrizamide border. (**A**) Rh–DNA/Fl–PLL/SPLL (1:3:10) after ultracentrifugation and Rh–DNA/Fl–PLL (1:1) standard dissolved in 2.5 M NaCl. (**B**) Rh–DNA/PLL/Fl–SPLL (1:3:10) after ultracentrifugation and Rh–DNA/Fl–SPLL (1:1) standard dissolved in 2.5 M NaCl.

standard mixture of Rh–DNA and Fl–PLL or Fl–SPLL in the same high salt solution (Fig. 6A and B). The data indicate that precipitated DNA/PLL/SPLL complexes contain all three polyelectrolytes with a stoichiometry close to a 1:1:1 charge ratio.

ζ Potential of purified, soluble particles

In a further modification of the ultracentrifugation studies, an attempt was made to isolate soluble triple complexes by placing 1 mg/ml SPLL solution in 15% sucrose immediately above the 40% metrizamide fraction. Since free PLL did not enter the SPLL–10% sucrose layer, only DNA/PLL particles were recharged with SPLL. The excess SPLL at the sucrose/metrizamide interface prevented the DNA complexes from aggregating. After centrifugation, sample retrieval and dialysis against 25 mM HEPES, pH 7.5, to remove sucrose and metrizamide, soluble DNA-containing particles were isolated and the size and ζ potential were found to be indistinguishable from non-separated DNA/PLL/SPLL complexes (data not shown).

DISCUSSION

This study demonstrates that negatively charged particles containing condensed DNA can be formed by recharging DNA/polylysine particles with certain pA. The condensed state of the DNA was ascertained by an assay that uses DNA covalently modified with fluorophores (Fig. 1A; 15). Polycarboxyls with a short carboxyl/backbone distance (pMAA and pAsp) decondensed DNA within PLL complexes while pA with a longer carboxyl/backbone distance (pGlu and SPLL) did not (Fig. 1A). These results are similar to the effect of pA on the release of DNA within cationic lipid complexes (17). Polyanions with a high linear charge density, such as dextran sulfate or heparin, release DNA from cationic lipid complexes while other pA, such as polyglutamic acid, with less charge density do not. Given that condensed DNA invariably compacts into particles, the condensed state of DNA in the DNA/PLL/SPLL mixture provides strong evidence that the DNA is within particles.

The ability of certain pA to release DNA from complexes with pC was found to be dependent on the salt concentration (Fig. 2). The higher the salt concentration the easier pA can release DNA from the complex. Under physiologic salt conditions, pGlu decondensed DNA/PLL particles but SPLL,with a slightly longer carboxyl/backbone distance, did not (Fig. 2). An accelerating effect of low molecular weight salt on reaching

equilibrium in ternary DNA/pC/pA complexes has recently been demonstrated (18). This result highlights the importance of the carboxyl/backbone distance for the pA in determining its interaction with DNA/PLL particles. It is also relevant to the preparation of complexes in pharmaceutical solutions.

The size of the DNA particles increased with each additional polyion (Fig. 3). Given that the DNA remains condensed, this suggests that more polyion assembles on the particles with each additional layer. Each additional polyion may be layered onto the preceding polyion of opposite charge without complete interpenetration, as has been observed for solid macrosurfaces (19) and latex particles (20).

ζ Potential measurements determined the surface charge of the DNA particles. As SPLL was added to DNA/PLL complexes, the ζ potential of the particles became negative when the ratio of SPLL carboxyls to PLL amines was equal to or greater than 1. Since the DNA/PLL/SPLL mixture presumably contained both DNA particles and 'blank' PLL/SPLL particles, DNA particles were isolated using an ultracentrifugation procedure in which DNA/PLL particles were separated from free PLL by spinning them into a 10% sucrose layer containing excess SPLL. The excess SPLL enabled non-aggregated DNA particles to be recovered. The ζ potential of these isolated DNA/PLL/SPLL particles free of contaminating 'blank' PLL/SPLL particles was negative.

The negative ζ potential of the DNA particles has to be considered in the light of the stoichiometry of their polyelectrolyte constituents. Our previous study showed that, contrary to a common assumption, DNA/PLL particles formed from mixtures containing excess PLL, have a ratio of DNA phosphate:PLL amine close to 1:1 even though their ζ potential is positive (21). Presumably, this results from an excess of DNA in the inner core and an excess of PLL in the outer layer of the particles. This would be similar to the partially overlapping ('fuzzy') layers of polyelectrolytes with opposite charge adsorbed onto solid macrosurfaces (19). Alternatively, the particles could contain a slight excess of pC (within the slight imprecision of the assays) that imbues the particles with a positive ζ potential. Concordant with our observations of a 1:1 ratio in particles is that when PLL is added to genomic or phage DNA solutions in high salt (~1 M NaCl) aggregates form that also contain a 1:1 ratio of DNA:PLL (22). The 1:1 DNA:PLL ratio is also congruous with the 1:1 ratio within complexes of oppositely charged strong polyelectrolyles (23,24).

Nonetheless, it is remarkable that DNA/PLL/SPLL complexes isolated by ultracentrifugation from excess PLL and SPLL contained a 1:1:1 ratio of DNA phosphate:PLL amine:SPLL carboxyl. There must be a major redistribution of charges to enable the initial DNA/PLL particle to bind an equimolar amount of SPLL carboxyls. The last added polyion (layer n) partially displaces the underlying polyion (layer $n-1$) from its complex with polyion $n-2$. The 1:1:1 polyion stoichiometry is consistent with a similar charge stoichiometry that occurs when oppositely charged polyions are deposited layer-by-layer on flat macrosurfaces (19).

The early studies on DNA condensation induced by pC provided a model system for better understanding of how DNA is compacted in viral particles (25,26). Given that most viruses are negatively charged, our study provides an advance for modeling viral assembly. In addition, the use of these novel, negatively charged DNA particles for gene transfer is currently under study. The preparation of negatively charged complexes containing condensed DNA opens up new opportunities in improving biodistribution of such complexes *in vivo* as well as reducing their toxicity and modulating their tissue tropism.

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