Control of DNA incorporation into nanoparticles with poly(L-lysine) multilayers

Annie C. Dorris, Kimberly L. Douglas, Maryam Tabrizian, and Christopher J. Barrett

Abstract: Nanoparticles coated with DNA and poly(L-lysine) (PLL) were prepared using a layer-by-layer assembly technique under various solution conditions. Both the level of DNA loading into the materials, the rate and the extent of DNA released from films upon degradation were controlled by varying the pH of polyelectrolyte assembly solutions. To determine optimal conditions for DNA loading, analogous films assembled on flat surfaces were prepared under various pH conditions. Using UV–vis spectroscopy and thin-film ellipsometry, it was found that the extent of DNA incorporation could be varied by more than a factor of two, and that the highest loading was obtained for films built using a combination of DNA and poly(L-lysine) solutions of pH = 4 and pH = 7, respectively. The layers coated onto the silica nanoparticles permitted the surface charge to be characterized by zeta potential electrophoresis. Furthermore, the acid–base dissociation constant measured for PLL on the outermost layer of the DNA/PLL film showed that the pK_a of PLL can be shifted by more than three units. Film degradability was investigated via the exposure of films assembled under different pH conditions to α-chymotrypsin. The fraction of DNA released from degraded films can also be increased by a factor of three when films are built under conditions of pH = 4 for the DNA solution. The resultant effect on the transfection ability of pEGFP-N1/PLL coated particles was then measured, and results suggest that the control achieved over the bulk film properties also extends to a strong influence on cell uptake and transfection.

Key words: polyelectrolyte multilayer, DNA incorporation, enzymatic degradation, nanoparticles, gene therapy.

Résumé : Des nanoparticules enrobées d'ADN et de poly(L-lysine) ont été preparées sous différentes conditions par la technique d'assemblage de multicouches. Le degré d'incorporation d'ADN dans le matériau ainsi que la vitesse et la quantité d'ADN libérée du film, préalablement soumis à une degradation enzymatique, peuvent être contrôlés par la variation du pH des solutions de polyélectrolytes utilisés lors de l'assemblage. Des films analogues assemblés sur des surfaces planes ont aussi été préparés sous des combinaisons de pH différentes afin de caractériser par ellipsométrie et spectroscopie UV–vis les conditions d'incorporation d'ADN optimales. Il a été démontré que l'incorporation d'ADN peut être multiplié par un facteur supérieur à deux et que son incorporation est maximale lorsque l'assemblage d'ADN et de poly(L-lysine) s'effectue à des pH respectifs de 4 et 7. Afin de permettre la caractérisation des charges de surface par electrophorèse du potentiel zeta, des multicouches ont été deposées sur des nanoparticules de silica. La mesure de la constante de dissociation acide-base du poly(L-lysine), formant la couche terminale des films d'ADN/poly(L-lysine), a démontrée que la valeur de pKa du polymère peut varier de trois unités. L'étude de dégradation des films par l'enzyme α-chymotrypsin a aussi demontrée que la fraction d'ADN libérée des films peut être augmentée d'un facteur de trois lorsque les films sont construits à partir de la solution d'ADN à pH 4. Finalement, les résultats de l'étude sur la capacité de transfection des particules enrobées de pEGFP/poly(L-lysine) suggèrent que le contrôle obtenu sur les propriétés du film d'origine influence fortement l'ingestion cellulaire et la transfection.

Introduction

Gene delivery systems based on polymer nanoparticles have received much recent attention due to improved thera-

A.C. Dorris and C.J. Barrett.[1] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A 2K6, Canada.
K.L. Douglas and M. Tabrizian. Department of Biomedical Engineering, McGill University, 3775 University Street, Montreal, QC H3A 2B4, Canada.

[1]Corresponding author (e-mail: christopher.barrett@mcgill.ca).

peutic gene transfection and the potential to tune properties chemically. Inter-polyelectrolyte complexes (IPECs) formed between DNA and polycations, such as PEI (1), poly(L-lysine) (PLL) (2), and chitosan (3), are now widely used as simple and effective vectors for the delivery of DNA into cells. In recent years, the use of polyelectrolyte multilayers (PEMs) in many biomedical applications has generated great interest, in particular as drug delivery systems, as they offer more precise control over structure and release properties (4). Layer-by-layer (l-b-l) deposition method is especially convenient for incorporating natural polymers into thin films and nanostructures. This self-assembly technique relies on the sequential adsorption of oppositely charged polymers onto a charged substrate or particle (5); film thickness, layer interpenetration, and water content can be tailored quite pre-

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cisely by varying several parameters, such as the number of adsorption cycles, the pH, and the ionic strength of the assembly baths. The method allows for gentle ionic adhesion permitting subsequent facile release and good retention of the desired biological activity.

Most recently, the incorporation of DNA into multilayered films has been achieved (4, 6–18), and has been successfully applied to both targeted release (9, 10, 12) and as sensor devices (6, 7). Trubetskoy et al. in particular have reported coating poly(L-lysine) (PLL) and succinylated PLL on a condensed DNA core using the l-b-l method (13). Following their demonstration that these layered structures could be used as non-viral gene delivery vectors, several groups performed further studies on these potential vectors (10, 14, 19–22). Since DNA transport across the cell membrane is a fundamental requirement for successful gene delivery, localized delivery and controlled released of DNA incorporated into PLL multilayered films have been one of the most investigated systems (9, 10). Viral vehicles have also been used previously to carry DNA, but these approaches suffer limitations, such as high cost, toxicity, potential infection, and limited DNA carrying capacity, leading to interest in the development of non-viral gene vectors. Cationic polymers including PEI and PLL appear most promising because they can transport DNA efficiently across the cell membrane (2, 23–26). PLL especially has the significant advantage of offering the possibility of covalently attaching target ligands to the polymer backbone so that receptor-mediated uptake can be localized. In addition to the complex formation between DNA and the polycation, the layer-by-layer fabrication procedure offers a good way to incorporate the material in a controlled fashion by varying assembly parameters, such as the pH, the ionic strength, and the number of polymer layers. Lynn and co-workers were the first to study the sustained release of functional DNA from multilayered polyelectrolytes assemblies (15), and to enhance the localized transfection of cells in contact with the DNA multilayered film (4).

A key feature of these films is their variable composition, which influences the final transfection efficiency, the most crucial requirement for a system to be competitive. The ability of a gene to be transfected depends both on the shape and the size of the gene carrier, as well as its capacity to carry a significant amount of DNA to target cells. In addition to the degree of loading, two other factors can also be important to overall transfection efficiency: the conformation and density of the polyelectrolyte in the assembly (related to the water content), and its acid–base equilibria (surface charge). Both of these effects have been studied previously and extensively by our group with synthetic polyelectrolytes (27–31). By varying the l-b-l assembly pH (and to a lesser extent the ionic strength), good control over both morphology and charge could be achieved. Fluctuations by more than two units in the pK_as of weak-acid and weak-base functional groups can also result from pH variations. It is of great interest then to investigate whether it is possible to extend this pH control of parameters to DNA to optimize DNA loading conditions. Salt effects have been investigated previously by Nguyen (32), who studied the adsorption of supercoiled DNA to a natural organic matter-coated silica surface, and by Zhou and co-workers, who studied the extent of DNA in-

corporation in PLL/DNA films under the influence of different ionic strength assembly solutions (10). They have identified ionic strength conditions that favoured DNA incorporation but they did not investigate the effect of pH of the assembly, which has been shown to play a key role in DNA/dye film formation, and represents a more powerful parameter tool for tuning properties (33). Binding interactions between a cationic polymer and DNA are influenced by the degree of ionization of polyelectrolytes, molecular weight, and type of cationic polymer used; thus, it should be possible to control the degree of ionization of weak polyelectrolytes by varying the pH of the solution. Several groups have reported a pH influence on the formation of polycation/DNA complexes (34, 35), where they observed that under certain pH conditions polyelectrolyte affinities were greater and more favourable to complex stability.

Poly(L-lysine) is a weak polycation with an amino group on each lysine residue. In the pH range where amino groups are protonated (pK_a = 10.5–10.7), these functional groups can undergo strong ionic interaction with DNA. In dilute aqueous solution, PLL can adopt one of three significantly different conformations, depending on pH and temperature (36). Even though below its pK_a, PLL behaves as a random coil at room temperature; it has been found that when PLL is adsorbed on a surface the polymer can adopt a β-sheet structure (37). In this paper, we study the degree of DNA incorporation into PLL/DNA multilayered films submitted to different pH assembly solutions. As necessary for film characterization, such as the extent of polymer adsorption upon exposure to different ionic strength and pH conditions, analogous films were first coated on flat surfaces of silicon with an oxide layer. Multilayered films were then coated on nanoparticles (NPs) of silica from which surface charge analysis could be performed directly over an extended pH range. Degradation of films with different polyelectrolyte composition was then studied, and finally, the resultant transfection properties of films coated on silica nanoparticles were measured, demonstrating how the pH control of physico-chemical properties can be extended to control transfection efficiency by more than a factor of two.

Materials and methods

Materials

Poly(L-lysine) hydrobromide (MW 70 000–90 000) and salmon testes DNA (MW 1 300 000) were purchased from Sigma. MES and TRIS used to prepare buffer solutions, and α-chymotrypsin (from bovine pancreas 60 U/mg) were also obtained from Sigma. Plasmid DNA (pEGPF-N1) was provided by Clontech. Milli-Q water of 18.2 MΩ resistivity was prepared with a Millipore MiliQ purification system. Silica nanoparticles (Snowtex-zl, 40 wt) with a diameter ranging between 70 and 100 nm were provided by Nissan chemical as a suspension in water.

Preparation of DNA/PLL multilayer films assembled on flat surfaces

Alternate layers of PLL and DNA were assembled onto silicon wafers (Wafernet Inc.) and quartz slides (Canadawide Scientific). Prior to their use, both substrates were first immersed for 30 min in a boiling piranha solution (sulfuric

acid – hydrogen peroxide mixture, 1:3 ratio) and then rinsed with large amounts of Milli-Q water. The first layer of PLL was adsorbed onto the two substrates by dipping them for 20 min in a 1 mg/mL PLL solution at fixed pH and ionic strength. Then, the substrates were rinsed for 2 min in two consecutive water baths, whose pH and ionic strength were adjusted to those of the PLL solution. Dipping the substrate in a 1 mg/mL DNA solution allowed the formation of the DNA layer onto the pre-adsorbed PLL layer. Substrates were rinsed two times in water, and the whole deposition process was repeated until the desired number of layers was reached. Films were then rinsed with Milli-Q water and dried under a stream of argon gas. Citric acid (25 mmol/L, pH 4), MES (25 mmol/L, pH 5.5), MES/TRIS (25 mmol/L, 25 mmol/L, pH 7), and TRIS (25 mmol/L, pH 9) buffers were used to adjust pH of solutions. Ionic strength of all solutions was adjusted with NaCl. Ionic contribution of buffers to ionic strength was considered in the calculations.

Film characterization

Film thickness was measured using a Gaertner ellipsometer at 633 nm, and the absorbance of DNA incorporated into films was monitored by means of a UV–vis spectrophotometer (Varian) at the DNA characteristic absorbance wavelength of 260 nm. Both techniques were used to investigate the influence of pH conditions on DNA loading into films. Each reported value was the average of 20 measurements taken at various points on over the film.

Preparation of coated silica nanoparticles

Positively charged silica nanoparticles (0.25 g of suspension) were exposed to 20 ml of 1mg/mL PLL (pH 7, containing 0.1 mol/L NaCl) solution for 1 h. Sonication was performed on particles during the adsorption process. Suspensions were then centrifuged, rinsed twice with water, and redispersed in a minimal amount of water. Afterwards, a 20 mL aliquot of 1 mg/mL DNA solution (pH 7, 0.1 mol/L NaCl) was added to the suspension. The mixture was sonicated for 1 h, centrifuged, and washed. The PLL and DNA adsorption steps were repeated until the desired numbers of layers were coated on the particles.

Nanoparticle characterization

Layer adsorption was monitored using zeta potential electrophoresis (Zeta Plus, Brookhaven Instrument Corporation). A small sample of suspension (1 ml) was used for characterization. Samples were redispersed in aqueous solution of different pHs and ionic strengths. Dissociation constants of this particular system were determined by plotting the zeta potential of particles over a wide pH interval.

Film degradation

Enzymatic degradation of PLL/DNA multilayered films was monitored during the hydrolysis of poly(L-lysine) with the endopeptidase α-chymotrypsin. Selected multilayers were exposed to 5 U/mL enzyme solution under physiological pH (50 mmol/L TRIS, pH 7.4) at 37 °C. As controls, films of PLL and DNA were incubated in TRIS buffer alone. At pre-determined times, films were removed from these solutions, rinsed, and dried for analysis. Absorbance of the films on quartz surfaces was recorded with UV spectrophotometry, and the thickness of films deposited on silicon was measured by ellipsometry.

In vitro transfection

Three types of DNA carrier were assembled for transfection studies. Four layers of PEGFP-1 (plasmid DNA) and PLL were coated on silica nanoparticles (snowtex-zl) using the same layer-by-layer technique described previously, but with smaller aliquots of 200 μL, so that vortex mixing and microcentrifugation replaced the sonication and centrifugation steps, respectively. Particle suspensions (50 μg/mL) were prepared from DNA solutions (0.125 mg/mL) at pH values of 4, 7, and 9, while the pH of PLL (0.1 mg/mL) solution was fixed to pH = 7. The ionic strength of the assembly solutions was kept low, and autoclaved water was used for solution preparation. The ρEGFP-N1 plasmid (Clontech, Inc.) encoding for green fluorescent protein (GFP) was used to monitor gene transfer and transgene expression after transfection. The plasmid was amplified and isolated using the Plasmid Maxi Kit (QIAGEN). The concentration of the plasmid was measured by UV absorption at 260 nm and the purity was determined using agarose gel eletrophoresis following linearization using an appropriate enzyme. COS7 cells, African green-monkey kidney cells, were cultured in DMEM (Gibco) supplemented with 10% (*v/v*) FBS (ATCC) and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were sub-cultured prior to confluence using trypsin–EDTA. Cells were seeded in 12-well plates at a density of 6×10^4 cells/well 18 h prior to transfection in DMEM/10% FBS. For transfection, the culture medium was replaced with 600 μL serum-free medium containing either 4, 8, or 16 μL of PLL–pDNA coated nanoparticles. Lipofectamine™ (Invitrogen) was used as a positive control, with each well receiving 4 μL Lipofectamine™ and 1 μg pEGFP–N1. The medium was increased to 1000 μL with DMEM/10%FBS after 4 h, and completely replaced with fresh complete medium after 24 h. Transfection was assessed 72 h post-transfection using flow cytometry. Briefly, cells were removed from the wells using cold EDTA (0.6 mmol/L in PBS), transferred to FACS tubes and analyzed directly using a FACSCalibur. Appropriate controls and gates were used for analysis of the results. All experiments and protocols described here were carried out under ethics guidelines and approval of McGill University.

Results and discussion

Multilayers on flat surfaces

Before the study of DNA loading onto the NPs, analogous films were prepared on flat substrates of Si or quartz where precise thickness and DNA density measurements could be taken. A thin native oxide layer of 1–2 nm on top of the cleaved Silicon provided a comparable surface chemistry of amorphous SiO_2 to that of the quartz and the silica NPs. Properties of DNA/PLL multilayers were studied in the pH range of 4 to 9; under these conditions, the negatively charged double helix of DNA is stable. At both pH extremes, the secondary structure of DNA can be somewhat perturbed due to base protonation; however, this process is reversible. Since DNA and PLL are oppositely charged

within this pH range, it was possible to assemble multilayers of alternate polyelectrolyte layers. The ionic strength was fixed to either 1 mmol/L, 100 mmol/L, 150 mmol/L, and 500 mmol/L NaCl during the multilayer buildup to find the ionic strength conditions under which the pH will have the most pronounced effect on DNA loading.

As depicted in Figs. 1*a* and 1*b*, both the DNA incorporation (*a*) and film thickness (*b*) increase with increasing salt concentration. This phenomenon is attributed to the screening of the electrostatic repulsive forces between DNA segments by ions (16). Consequently, DNA can adopt a less rigid conformation, which in turn produced thicker layers, and further reduced repulsion at the polymer interface facilitating greater polyelectrolyte adsorption.

Similar results for DNA incorporation in multilayers have been reported by Shi and co-workers (10), and Zhou and Li (33). Salts will have the same effect on PLL, and within this salt concentration range, there is a non-uniform bonding between oppositely charged polymers, which is due to a non-cooperative and irreversible binding between poly(L-lysine) and DNA (38). At 0.5 mol/L NaCl, polyelectrolyte adsorption decreased as a result of reduced electrostatic attraction. To estimate a film's capacity to incorporate DNA (the DNA loading level), the relative ratio of DNA to PLL in the multilayers was determined. This simple ratio is an indicator of film density and is defined as the DNA absorbance per thickness. Qualitatively, this ratio provides a comparison between films built under different ionic strength and pH conditions.

Figure 2 shows that at very low salt concentrations, the variation of pH of the assembly solution has a higher impact in DNA incorporation. At higher salt concentrations, the double layer does not contribute significantly to the total potential of interaction due to charge screening by ions. Thus, the charges on polyelectrolytes will have a less significant effect in polymer interactions.

Using low ionic strength conditions, which were found to be favourable to DNA loading, the effect of pH was studied in greater detail. To determine optimal DNA incorporation, the pH of both PLL and DNA were varied. Figure 3 depicts that the best DNA loading is obtained at low pH of the DNA solution. This is because the cytosine (pK_a of 4.3) (39) and adenosine (pK_a of 3.8) (40) bases are partially protonated near pH 4 where they neutralize almost 40% of the negative charge on the DNA phosphate backbone. This can have a significant effect on DNA conformation in solution in reducing repulsion between segments, thus allowing DNA to adopt a more coiled conformation. This screening effect will increase the excluded volume of the molecule and produce thicker layers. This thickening phenomenon may also be attributed to the increase of DNA loading on PLL layers owing to charge equilibrium. Moreover, since DNA partial denaturation occurs at low pH, the absorbance increase can also arise from a hypochromic effect. At pH 9, the DNA solution DNA loading is not favoured because the guanine bases are partially deprotonated. It is likely that the creation of negative charges increases the repulsion with negatively charged phosphate groups on the backbone and reduces stability of the DNA helices.

The results presented in Fig. 3 also show that the pH of the PLL solution has a considerable effect on film composi-

Fig. 1. PLL/DNA films (10 layers) built at different salt concentration whilst varying the pH of the DNA solution. The pH of the PLL solution was fixed to 7. (*a*) Absorbance measurements at 260 nm on a quartz substrate. (*b*) Film Thickness measurements on silica substrates via ellipsometry.
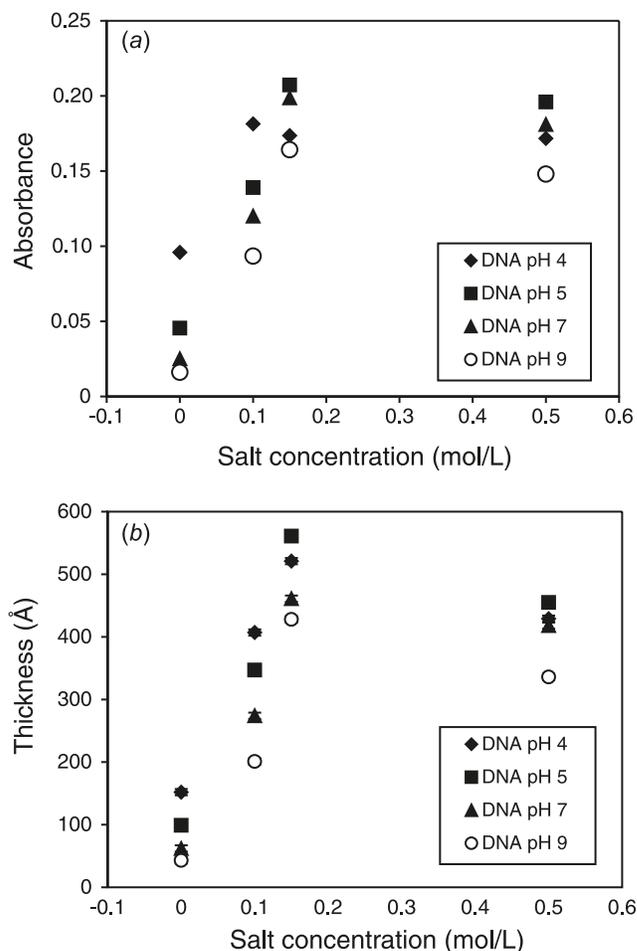

Fig. 2. Effect of DNA solution pH on the relative ratio calculated for DNA/PLL films built under different ionic strength conditions and a fixed pH of 7.
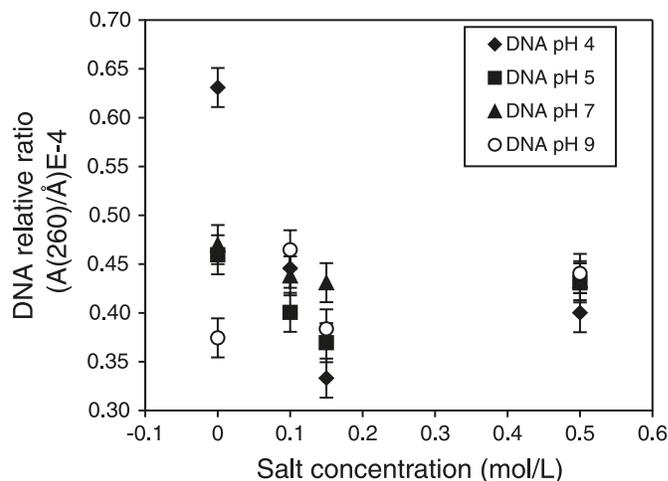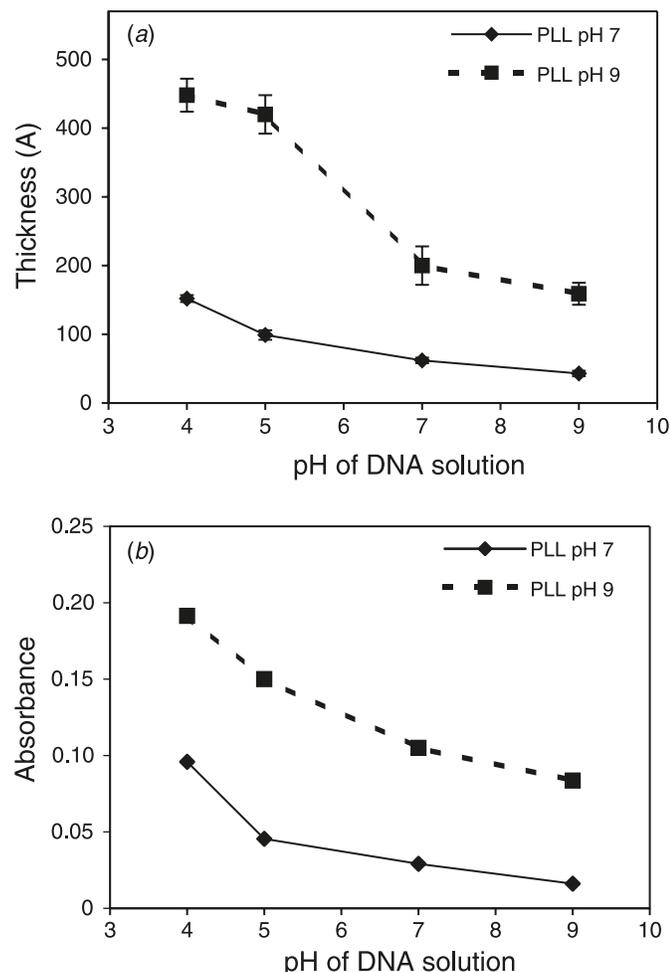
Fig. 3. Effect of pH of PLL and DNA assembly solutions on film composition assembled at low ionic strength. (*a*) Thickness measurements on silica substrates via ellipsometry. (*b*) Absorbance measurements at 260 nm on a quartz substrate.
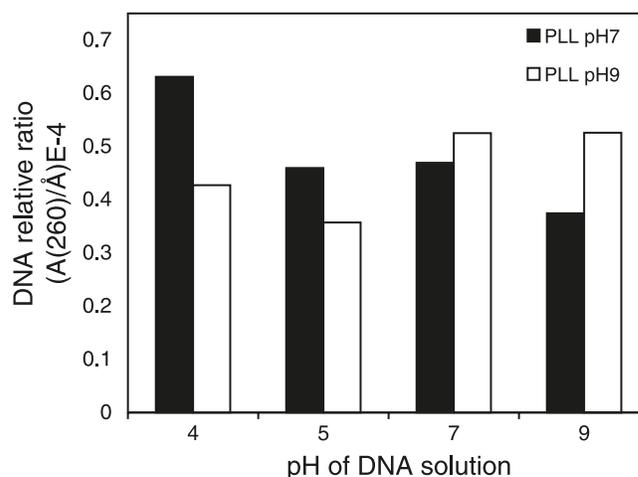

Fig. 4. Comparative pattern of DNA composition in films built at different pH values of assembly solution.

tion. When the pH of the PLL solution reaches nine, twice as much DNA is incorporated into the films and film thickness is more than doubled. As the degree of protonation of PLL decreases when the pH of the solution approaches its pK_a, greater PLL adsorption is promoted by a charge equilibrium effect. The reason why DNA adsorption may also increase at pH 9 lies in the fact that when PLL is less protonated, the adsorbed weak polyelectrolyte on film surface will adopt a daisy-looped shape (41, 42), producing a rougher surface that creates more sites for DNA to adsorb and hence a greater incorporation of DNA into the films.

Figure 4 reveals that DNA loading is optimal when both assembly pH of PLL and DNA is low (near pH 4). Under these pH conditions, PLL is fully charged and DNA adopts a coil conformation.

Multilayers coated on silica nanoparticles

On the analogous flat surfaces, pH and salt effects were clearly shown to affect film architecture via a change in polymer conformation. Surface charge measurements could be made directly via zeta potential electrophoresis following polyelectrolyte adsorption on the surface of nanoparticles.
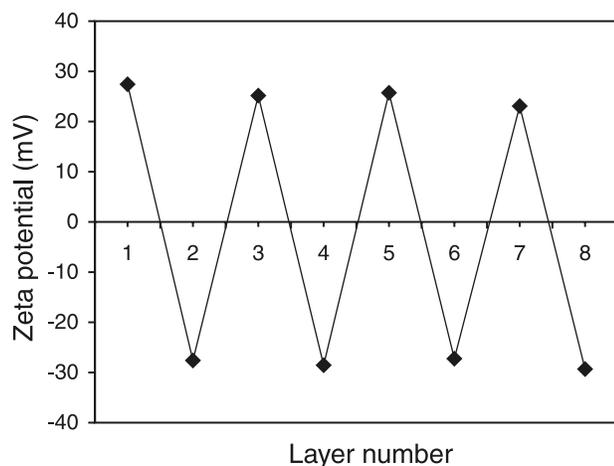
Surface charge density on film surfaces can be affected by changes in secondary structure, intra-molecular repulsions, as well as the concentration of counterions in the film. The charge on the surface of the previous layer has also a known effect on the adsorption behaviour of the next polyelectrolyte layer (27, 28). Microelectrophoresis is a commonly used technique to determine the effect of various parameters (salt, pH) on surface charge properties of colloidal particles, and to study these surface properties, multilayers of PLL and DNA were coated onto the surface of silica NPs of 100 nm diameter. In some respects, these DNA-loaded NPs are still model systems, and in that end, pharmaceutical applications would more comprise DNA-filled or hollow capsules (obtained by dissolving the core). However, the Si particles used here as templates are ideal for the precise surface charge studies required, and also for solid state NMR studies that are being carried out in parallel by our group. Furthermore, the Si NPs possess acceptable non-toxicity so that exactly the same particles characterized here electrophoretically could be used for the in vitro transfection studies described presently.

Alternation of the zeta potential measured after every adsorption step is reported in Fig. 5. A potential of +30 mV is obtained when PLL is the outer layer, while the potential switches to –30 mV when DNA is the outermost layer. Plots of zeta potential vs. the pH of the solution for particle containing acid–base functional groups display similarities to titration curves used to determine acid–base dissociation constants. It is therefore possible to estimate the effective pK_a of polyelectrolytes coated on particle surfaces because the inflection point of zeta curves corresponds to an apparent pK_a (27).

Figure 6 shows the surface charge of PLL on the surface of the ninth outer layer obtained by alternate deposition of DNA/PLL layers exposed to a range of pH between 4 and 12 at two salt concentrations of 1 mmol/L and 100 mmol/L, respectively. Corresponding apparent pK_a values are shown on the graph, and these are compared to pK_a of PLL measured free in solution, as measured by potentiometric titrations. These bulk solution pK_a values are in good agreement with those reported in the literature (43). At both salt concentra-

Fig. 5. Zeta potential of the surface of silica nanoparticles coated with PLL/DNA after each deposition step.
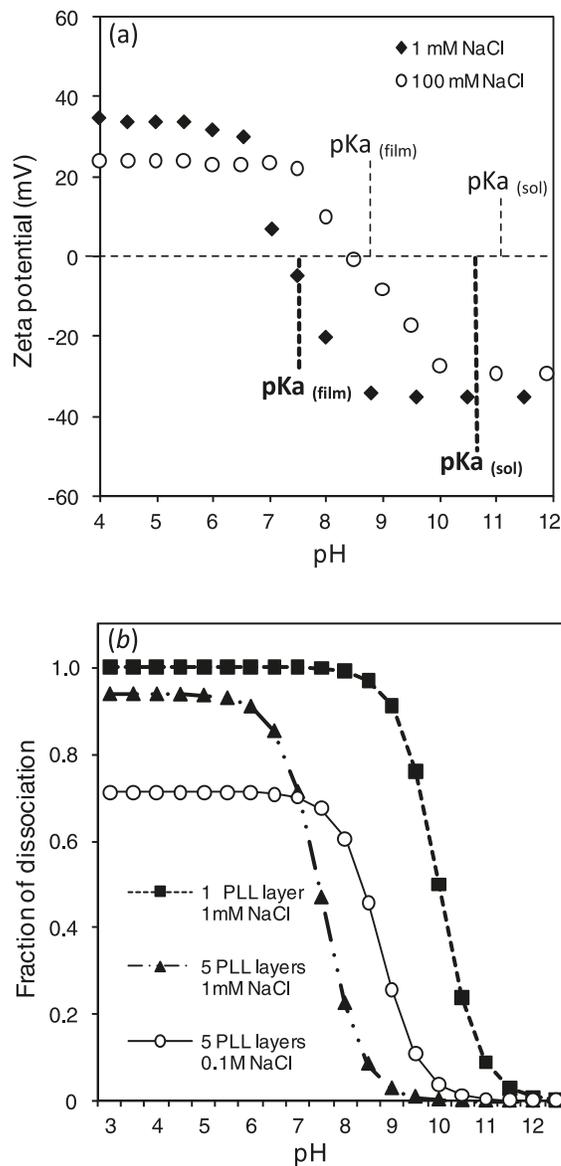

Fig. 6. Surface charge of PLL forming the terminal layer of a nine-layer film of PLL/DNA deposited on silica nanoparticles (snowtex-zl, 100 nm diameter). (*a*) A pK_a value on the PLL film surface corresponds to the inflexion point, which is then compared to the pK_a of PLL in solution obtained by a potentiometric titration. (*b*) Degree of dissociation of PLL adsorbed on PLL/DNA film surface coated on silica nanoparticles.

tions, a shift of approximately three pK_a units is observed when PLL is adsorbed on top of multilayers. Several groups have reported that in self-assembled polymer systems, the acid–base equilibria deviates substantially from ideal behaviour (44–47). Our group has previously reported a pK_a shift of up to a few units in PLL and HA multilayer system as a result of layer accumulation (27), which perturbs the local environment of the outer layer of PLL. The ionic network created between PLL and DNA upon polymer adsorption leaves participating functional groups unavailable for proton exchange, which in turn decreases the degree of ionization of polymer on film surface, which results in a decrease of pK_a. Increasing the ionic strength has the opposite effect on apparent pK_a because ions in solution can stabilize the charged amino groups on PLL.

The fraction of dissociation was calculated for PLL at the terminal layer of a nine-layer film (Fig. 6*b*), assuming a degree of dissociation of one for one layer of PLL coated on a silica nanoparticles. At low salt concentration, the degree of ionization of PLL on top of the DNA multilayer is very high below pH 6.5. Above this pH, PLL dissociates, which reduces PLL net charge, hence lowering the attraction to the previous PLL layer, which can explain why higher DNA incorporation is observed under low pH conditions (Fig. 3). At pH 9, only 1% of PLL molecules are charged, which leads to a relatively poor DNA adsorption. When salt concentration is increased, the pK_a of PLL is higher. Within the pH range of 4 to 9, the charge density is constant except at pH 9 where the degree of dissociation decreases. This could explain why the pH has less of an effect on polymer adsorption at higher salt concentrations.
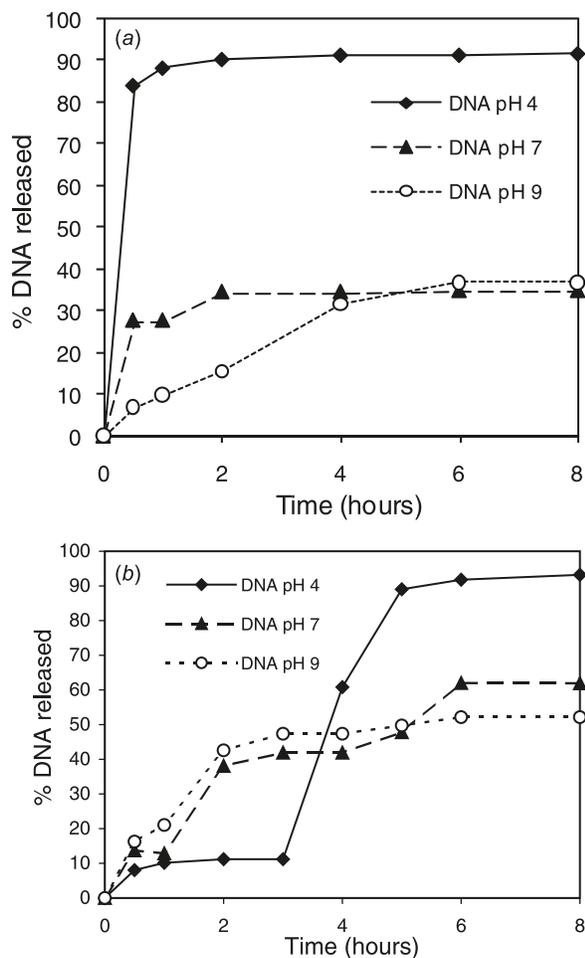
Degradation studies

In gene therapy, it is important to exert precise control over DNA release, and polymers and small molecules can be released from films by changing pH (29, 48), or salt concentration (12), or by using an enzyme (4, 9, 15), if the material is biodegradable. In a therapeutic context, physiological conditions restrict the layer degradation to a constant ionic strength and pH. This leaves biodegradation as the method of choice and indeed several proteolytic enzymes have been used for material degradation. It has been reported that

films, which contain polyamino acid groups can be degraded by chymotrypsin, an endopeptidase widely used in in vitro polypeptide degradation (49). Although trypsin is the specific enzyme for lysine residue degradation, it has been observed that chymotrypsin shows similar degradation activity for PLL membranes (50). Moreover, DNA/PLL multilayered films have recently been successfully degraded with this enzyme (14). Since the architecture of the film affects the affinity of the polymer chain for the binding site of the enzyme molecule, and thus controls the rate at which the polymer is degraded, several PLL/DNA films built under different pH and salt conditions were exposed to the enzyme

Fig. 7. Level of DNA release from PLL/DNA films built under different pH conditions and at low ionic strength calculated from absorbance measurements on quartz slide. (*a*) PLL is fully charged. (*b*) PLL is partially charged.

Fig. 8. The effect of film composition on the degradation of films of same thickness. Film 1: PLL pH 9 / DNA pH 4, 1 mmol/L NaCl. Film 2: PLL pH 7 / DNA pH 7, 0.15 mol/L NaCl. Film 3: PLL pH 7 / DNA pH 7, 0.5 mol/L NaCl. (*a*) Thickness variation of film exposed to α-chymotrypsin. (*b*) Percent of DNA released from films.
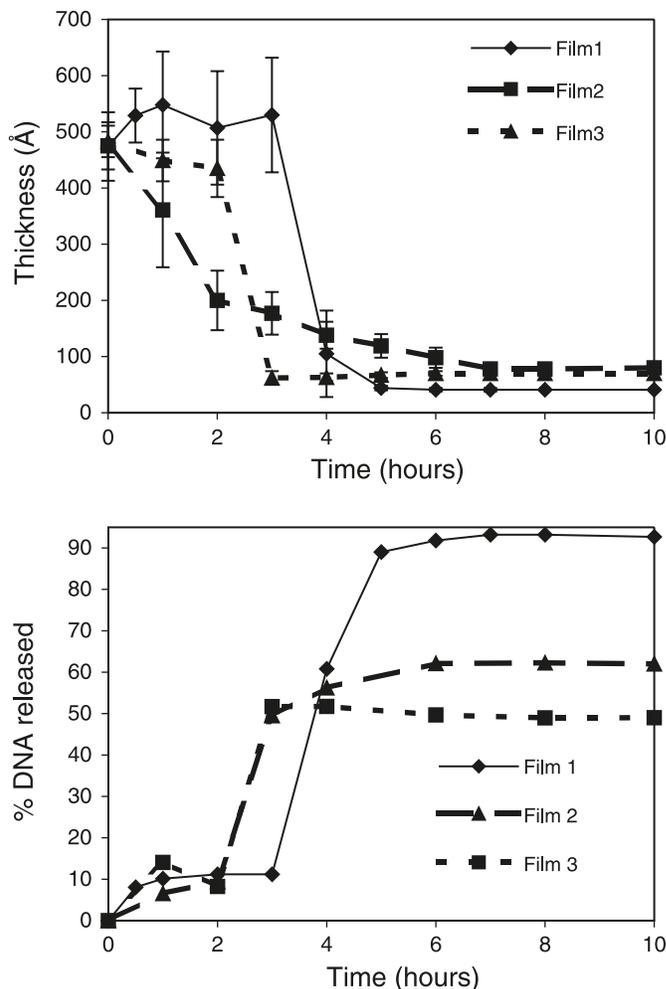

to track film deconstruction and hence study how film architecture influences degradation behaviour. Film thickness and accessibility of the bonds to be cleaved both influence the mechanism and rate of enzyme action. Indeed, it was noticed that film degradation varies strongly with the pH and salt conditions at which the films were built.

Figure 7*a* shows that less than 40% of the DNA is liberated after 2 h of enzyme treatment when the pH of DNA solution is 7 or higher. With films built with pH 4 DNA solution, DNA release is 1.5 to 3 times greater, with over 90% of DNA released. We note that control tests have shown that no DNA is lost when the same films were exposed to a buffered solution for the same period of time.

It was noticed that degradation of PLL/DNA films depends greatly on the film thickness. Ji and co-workers observed a progressive deconstruction of their PLL/DNA films of 170 nm thickness (14). After 35 h of enzyme exposition, 90% of DNA was released from their films, whereas the same release was obtained after only 2 h with a film of less than 10 nm thickness. Prolonged enzymatic treatments did not further degrade the film because as fragments depolymerize the effect of polymer end groups becomes

more pronounced, and eventually, the polymer molecule is too short to form a sufficiently stable complex with the enzyme active site (51, 52). In our films built with a pH 4 DNA solution, DNA is more globular and less charged, and thus the interaction with PLL is weakened, and the reduced steric hindrance allows the enzyme to bind more easily to the PLL substrate. The same phenomenon is observed with films built with a pH 9 PLL solution (Fig. 7*b*). However, in this case, the percent of DNA release is more important for each film. At higher pH values, PLL is weakly bound to DNA, which leaves more amino groups available for degradation. Moreover, the loopy PLL conformation is more favourable for film degradation and DNA release. Since the thickness of these films was not constant however, it is difficult to make a critical comparison. As shown in Fig. 8, degradation follows a similar scheme for each film. Although films have been coated on two different substrates, it is worth mentioning that an early study had demonstrated that an equal extent DNA was released from a film deposited

onto silicon or quartz (15). Early in the enzymatic treatment, Fig. 8*a* shows that film thickness and hence roughness increase early in the enzymatic treatment, as peptide bond cleavage loosens polymer linkages non-uniformly over the film surface.

When enough bonds are broken, clusters of polymer fragments are released, which lead to an abrupt thickness decrease. Further degradation occurred more slowly until polymer fragments are short enough to hinder the hydrolysis process. DNA release differs between films, and it appears that films built with pH 4 DNA and pH 9 PLL solutions have the best ability to release DNA from films exposed to the enzyme. Early studies demonstrated that even though compact complexation between DNA and the polycation is required to protect DNA from degradation, the DNA and the polymer must eventually decomplex to allow efficient transfection (53). Concurrent reduction of the positive PLL charge and the DNA negative phosphate net charge decrease the level of electrostatic interaction between polymers, which may favour dissociation of the complexes inside the cell. This is clearly revealed later in the degradation studies. Thus, we demonstrate that it is possible to tailor decomposition behaviour simply by changing film architecture and composition. Optimal DNA release is obtained when the DNA and PLL polyelectrolyte incorporated into the film are less charged, which in consequence weakens mutual attraction and allows DNA to be liberated more easily.

Transfection studies

To measure the transfection ability, PLL/DNA nanoparticles coated with pEGFP–N1 and PLL were prepared under different pH conditions and at low ionic strength. The salmon DNA used previously for film assembly was replaced by plasmid DNA (pEGPF–N1) that encodes for enhanced green fluorescent protein. However, it was not certain that the pH will have the same effect on the level of DNA incorporation into multilayered films, since DNA morphology has an impact on the structure of the formed complex. The multilayer support in this case is a silica spherical particle having a diameter between 70 and 100 nm. The main motivation for using silica NPs as a support was facility of zeta potential characterization, but silica is also an acceptable support for a DNA delivery system based on previously reported results, which demonstrated that silica nanoparticles with surface-modified aminosilane were an effective carrier for gene delivery (54). Five layers of alternated pEGFP–N1 and PLL were coated onto these silica nanoparticles, and the terminal layer was PLL layer to provide a positive outer charge to bind to the negatively charged cell membrane. Immediately after their preparation, particles were tested for transfection.

Fluorescence microscopy demonstrated that the COS-7 cell line remained healthy when treated with nanoparticles (Fig. 9). No dead or dying cells, which experienced changes in their cytoplasm size, were detected by microscopy. Compared with the positive Lipofectamine™ control (40% fluorescence events), coated nanoparticles were found to transfect cell lines relatively poorly, with the transfection efficiency of these un-optimized particles less than 30% relative to Lipofectamine™. However, it is important to note that tested nanoparticles had only two layers of DNA, and it

Fig. 9. Transfection of COS-7 cells by PLL/pDNA coated silica nanoparticles assembled with DNA solutions adjusted to three different pH values. The pH of the PLL solution during the assembly was maintained at neutral pH.
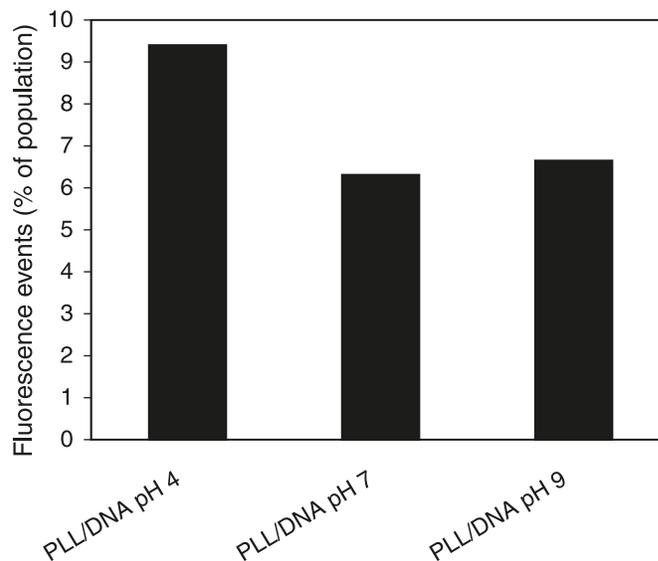
would probably be possible to enhance cell transfection significantly by increasing the number of DNA layers. These results suggest that particles assembled with a pH 4 DNA solution transfect 30% more cells than other nanoparticles. This may be attributed to a combination of the high DNA density, and efficient DNA release specific to these particles as characterized independently (Figs. 4. and 7). To verify the reproducibility of the transfection results, these same particles were tested 2 month after their storage. Very little transfection was obtained this time (less than 4% relative to the control), and this could be due to particle aggregation that occurs with time. However, a difference in transfection is still observable between samples, showing that the transfection is still increased with particles assembled at pH 4 DNA solution. Our results thus suggest that these particles have a potential for good and facile control over transfection, and that the pH of assembly has a significant impact on the degree of transfection.

Conclusion

Multilayers constructed from PLL and DNA have been successfully optimized by the variation of the pH of the polyelectrolyte assembly solutions. The optimization of DNA incorporation into multilayered films was achieved when the pH of both the PLL solution and the DNA solution was lowered to pH = 7 and pH = 4, respectively. Under these conditions, the reduced negative charge on DNA phosphate backbone allows DNA to adopt a more globular form that favours its incorporation into the multilayer. Degradation studies revealed that the film composition resulting from these pH conditions was more easily degraded by chymotrypsin and that more DNA was released during the film degradation. It appears that film architecture and composition play a crucial role in film degradability behaviour and that the pH of multilayer assembly alters the way polymers are degraded

and released from these films. Transfection tests performed on the COS-7 line showed that NPs coated with DNA and PLL were non-toxic to living cells, and that particles assembled under optimal DNA-loading pH conditions were transfected more effectively than the ones that were built under other pH conditions. Although the efficiency of transfection was still poor compared with that of other DNA delivery vectors, these results demonstrate the possibility of creating a vector for DNA made of PLL multilayers that could eventually lead to better gene-therapy delivery systems. In summary, we demonstrated that DNA loading and release from polyelectrolytes films can be controlled well simply by varying the pH of assembly solutions used in film formation, and we determined how these variations in polymer conformation and surface charge affect film composition of this system.

Acknowledgements

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