

Decreased Binding to Proteins and Cells of Polymeric Gene Delivery Vectors Surface Modified with a Multivalent Hydrophilic Polymer and Retargeting through Attachment of Transferrin*

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Binding of serum proteins to polyelectrolyte gene delivery complexes is thought to be an important factor limiting bloodstream circulation and restricting access to target tissues. Protein binding can also inhibit transfection activity *in vitro*. In this study a multivalent reactive hydrophilic polymer has been used to inhibit protein binding. This polymer is based on poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) bearing pendent oligopeptide (Gly-Phe-Leu-Gly) side chains terminated in reactive 4-nitrophenoxy groups (8.6 mol%). The polymer reacts with the primary amino groups of poly(L-lysine) (pLL) and produces a hydrophilic coating on the surface of pLL-DNA complexes (as measured by fluorescamine). The resulting pHPMA-coated complexes show a decreased surface charge (from +14 mV for pLL-DNA complexes to -25 mV for pHPMA-modified complexes) as measured by ζ potential analysis. The pHPMA-coated complexes also show a slightly increased average diameter (approximately 90 nm compared with 60 nm for pLL-DNA complexes) as viewed by atomic force and transmission electron microscopy and around 100 nm as viewed by photon correlation spectroscopy. They are completely resistant to protein interaction, as determined by turbidometry and SDS-polyacrylamide gel electrophoresis analysis of complexes isolated from plasma, and show significantly decreased nonspecific uptake into cells *in vitro*. Spare reactive ester groups can be used to conjugate targeting ligands (e.g. transferrin) on to the surface of the complex to provide a means of tissue-specific targeting and transfection. The properties of these complexes therefore make them promising candidates for targeted gene delivery, both *in vitro* and potentially *in vivo*.

Successful introduction of DNA into target cells *in vivo* could unleash many strategies of gene therapy that promise considerable therapeutic advances against a wide range of diseases (1–6). Presently, however, nearly all of these strategies are severely limited by inefficient delivery and expression of ther-

apeutic DNA (7). There are two broad problems; first, the efficiency of transgene expression in target cells and tissues is usually low or of inadequate duration. Second, possibilities for delivery of genes to target cells *in vivo* are limited to local administration either by direct injection into the target tissue or its blood supply (8–10) or by introduction into an appropriate body compartment (11). There are presently no plausible options for systemic delivery of genes following intravenous injection.

Viruses comprise the most efficient means to introduce transgenes into cells, but often their immunogenic and inflammatory properties combine with safety concerns to limit their realistic clinical application (12, 13). We are therefore seeking to develop nonviral systems, with limited immunogenicity, suitable for targeted delivery of genes *in vivo*. Distribution kinetics of nonviral vectors based on cationic lipids or on cationic polymers have previously been examined *in vivo*; however, they are invariably cleared quickly from the bloodstream and often accumulate in the lungs (14). This is thought to result from binding and aggregation of serum proteins, leading to selective retention within the first capillary bed encountered, usually the pulmonary bed. Consequently, several studies have examined various strategies to inhibit the binding of serum proteins to DNA complexes (15). For example, self-assembly of DNA with block copolymers containing cationic segments (e.g. poly(L-lysine) (pLL)¹ or poly(trimethylammonio ethylmethacrylate) linked to hydrophilic segments (poly(ethylene glycol), dextran, or poly-N-(2-hydroxypropyl)methacrylamide) can produce small particles that are enshrouded, to some extent, with hydrophilic polymers (16, 17). Analogous to stealth liposomes, the resulting complexes show a marked fall in their interaction with serum proteins, becoming up to 100-fold less prone to aggregation by albumin *in vitro*. However, following intravenous administration to mice, such block copolymer-DNA complexes still show rapid bloodclearance.²

One difference between the technology of polyethylene glycol-shielded liposomes (which show circulatory half lives of several hours in mice (18)) and polyethylene glycol-pLL-DNA complexes is that the former has lateral stabilization of its surface by bonding between adjacent lipid groups. The polyelectrolyte particles, on the other hand, are condensed into a hydrophobic core that has no such lateral stabilizing force. Although it is protected, to some extent, by polyethylene glycol

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¹ The abbreviations used are: pLL, poly(L-lysine); pHPMA, poly-[N-(2-hydroxypropyl)methacrylamide]; ONp, 4-nitrophenoxy; PCS, photon correlation spectroscopy; AFM, atomic force microscopy; TEM, transmission electron microscopy.

² P. R. Dash, unpublished results.

chains, protein molecules that penetrate the polyethylene glycol layer may be able to undergo hydrophobic interactions with the core of the complex and gradually destabilize it. This appears to happen at high protein concentrations *in vitro* and probably underlies the rapid blood clearance observed *in vivo*. Consequently, our attention has been focused recently on developing a system capable both of protecting the surface of polyelectrolyte DNA particles and simultaneously endowing the complexes with a lateral stabilizing force to resist destabilization by even physiological concentrations of proteins. Such a system is described here. It is based on hydrophilic polymers that carry several identical 4-nitrophenoxy groups capable of binding to lysine amino groups exposed on the surface of pLL-DNA complexes. These polymers are thought to undergo a cooperative binding reaction that results in cross-linking of the surface of the polyelectrolyte particles and protects them completely against even physiological concentrations of serum proteins. To permit activation of the DNA following its arrival within target cells, the coating polymer can be designed to contain linkages that can be hydrolyzed or cleaved by target-associated enzymes to release DNA in a form suitable for transcription.

MATERIALS AND METHODS

Sources of DNA—The 5.5-kilobase pair expression vector DNA used (JL1132) contains the β -gal gene driven by the cytomegalovirus promoter. The plasmid was grown in *Escherichia coli* and purified using Qiagen Gigaprep Kits (Crawley, W. Sussex, UK). Calf thymus DNA was obtained from Sigma and purified using phenol/chloroform extraction and ethanol precipitation. Concentration and purity of the DNA was checked on a spectrophotometer at A_{260}/A_{280} absorbance wavelengths. Unless otherwise stated expression vector DNA was used throughout this study.

Radiolabeling of Linearized DNA Expression Vectors—The expression vector was linearized using *Hind*III restriction enzyme and was then radiolabeled using [32 P]dCTP and the Ready-to-Go DNA labeling beads from Amersham Pharmacia Biotech. Unincorporated nucleotides were removed using MicroSpin S-300 Sephacryl columns (Amersham Pharmacia Biotech), and the purity of the labeled DNA was checked by agarose gel electrophoresis and PhosphorImager analysis (Molecular Dynamics). Less than 1% of the radioactivity was found to be due to the presence of free nucleotides.

Formation of pLL-DNA Complexes—DNA was added to a polypropylene microcentrifuge tube at a final concentration of 20 μ g/ml in ultra-pure water and mixed thoroughly. A small volume of pLL (5 mg/ml stock in water, average molecular weight 20,000) was added to the DNA solution to give an N:P ratio of 2.0 (defined as the ratio of lysine ϵ amino groups to DNA phosphates), unless otherwise stated, and gently mixed. The complexes were allowed to form for at least 30 min at room temperature prior to use. The N:P ratio of 2.0 was selected in order to combine efficient complex formation with minimal residual-free pLL (19).

Synthesis of Multivalent Reactive Polymer—A hydrophilic polymer was synthesized based on poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) bearing pendent tetrapeptide (Gly-Phe-Leu-Gly) side chains (8.6 mol %) terminated in reactive 4-nitrophenoxy groups, as described previously (20). The material (pHPMA-ONp) was characterized by fast protein liquid chromatography (calibrated with pHPMA standards) and showed a weight average molecular weight of 35,000 (Fig. 1).

Reaction of Multivalent Reactive Polymer with pLL-DNA Complexes—To introduce the multivalent polymer coating, reactive hydrophilic polymer pHPMA-ONp (various amounts in water) was added to the preformed pLL-DNA complexes following the addition of 5% v/v Hepes buffer (1 M, pH 7.4) to give a final Hepes concentration of 50 mM. The reaction between pHPMA-ONp and lysine amino groups results in the loss of 4-nitrophenoxy groups and a corresponding fall in absorbance at 274 nm, which can be monitored spectrophotometrically (molar extinction coefficient 9500 cm^{-1}). Unless otherwise stated the coating reaction was allowed to proceed overnight with remaining ONp groups aminolysed with aminoethanol (0.1 μ l/ml) added just prior to use.

Determination of Free Amino Groups Using Fluorescamine—Polyelectrolyte complexes were formed by mixing calf thymus DNA (40 μ g/ml) with pLL (20 kDa) in water at a N:P ratio of 2.0 for at least 30 min. The pHPMA-ONp polymer and an equal volume of 200 mM sodium

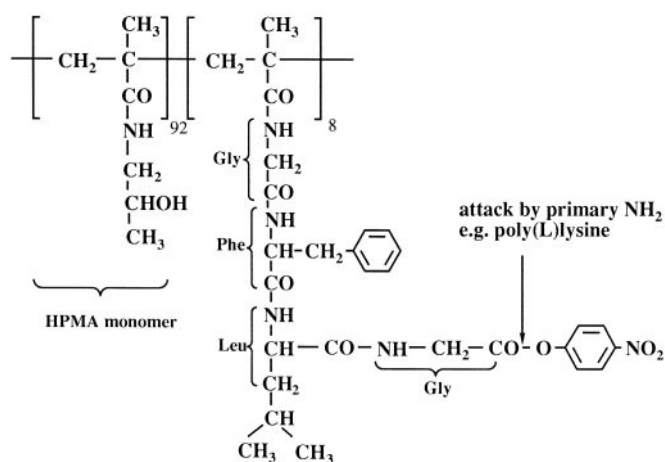


FIG. 1. Structure of the pHPMA-ONp polymer.

borate, pH 7.3, were added to the preformed complexes, and the coating reaction continued overnight at room temperature. The final concentration of coating polymer used ranged from 0 to 200 μ g/ml. To vary the pH, alternative solvents were used, including sodium citrate buffer (200 mM, pH 6.0) and sodium borate (200 mM, pH 8.2). To assay for amino group availability, 50- μ l aliquots of samples were removed and diluted with 1.5 ml of assay buffer (100 mM sodium borate, pH 7.0). 500 μ l of fluorescamine (0.01% in acetone) was then quickly added, and the reaction was incubated at room temperature for 10 min. Fluorescence was measured using a Perkin-Elmer LS 50B fluorimeter, λ_{ex} 392 and λ_{em} 480 nm with 5-nm slit widths, with buffer-only background deducted. In control experiments, the amine availability of free DNA, poly(L-lysine), and coating polymer alone were also examined.

Analysis of the Surface Charge of Polymer-DNA Complexes—Complexes were formed at a DNA concentration of 20 μ g/ml in water and reacted with pHPMA-ONp as described above. Surface charge was measured using a Zetamaster (Malvern Instruments, Malvern, UK) equipped with a 5-milliwatt internal laser. Sampling time was set to automatic, and the zeta range was set to unknown. All ζ potentials were determined in 50 mM Hepes buffer.

Analysis of Particle Size—The size of complexes was determined using a Malvern Instruments Zetasizer with a 70-milliwatt external laser (wavelength 488 nm). Complexes were formed in water at a DNA concentration of 20 μ g/ml as described above. Photon correlation spectroscopy (PCS) was performed at 25 $^{\circ}$ C in Hepes buffer (50 mM, pH 7.4) in triplicate with sampling time and analysis method set to automatic.

Atomic Force Microscopy (AFM)—Samples (1–2 μ l) containing complexes formed in water at a DNA concentration of 20 μ g/ml were deposited onto the surface of a freshly split untreated mica disc (Agar Scientific, Stansted, Essex, UK). Following adsorption (1–2 min at room temperature) excess fluid was removed by adsorption onto filter paper. The mica surface was then dried at room temperature before imaging using an AFM-2, part of the NanoScope II system (Digital Instruments, Santa Barbara, CA). A 200- μ m-long Si_3N_4 cantilever with a spring constant of 0.12 N/m and a D scanner head (AFM 351) with a 12- μ m scan range were used. The image mode was set to constant force, hence the images obtained are height images of the sample surface. The scanning speed varied between 2.48 and 8.68 Hz.

Transmission Electron Microscopy (TEM)—pLL-DNA complexes (10 μ l, 20 μ g of DNA/ml) were added to a formvar/carbon-coated copper grid and allowed to air dry. Uranyl acetate (10 μ l of a filtered 2% solution) was added for 2 min before the excess was drawn off. The grid was allowed to air dry, washed with water, and visualized with a Joel 1200 EX transmission electron microscope.

Turbidity Analysis—Turbidity of polyelectrolyte complexes is known to increase in the presence of serum albumin. Effects of albumin on turbidity of the complexes prepared here were measured as light reflection in a Perkin-Elmer fluorimeter with both excitation and emission wavelengths set to 600 nm and slit widths set to 3 nm. Complexes were prepared in water at a calf thymus DNA concentration of 20 μ g/ml; albumin was added in 1- μ l aliquots from a 35 mg/ml stock solution; and changes in turbidity were noted.

Isolation of Complexes from Plasma and Identification of Bound Proteins—Complexes containing 32 P-labeled DNA were coated with 200 μ g/ml of pHPMA-ONp as described above or left uncoated. Complexes were then incubated in filtered human plasma (2 ml, 50% solution, 1 h,

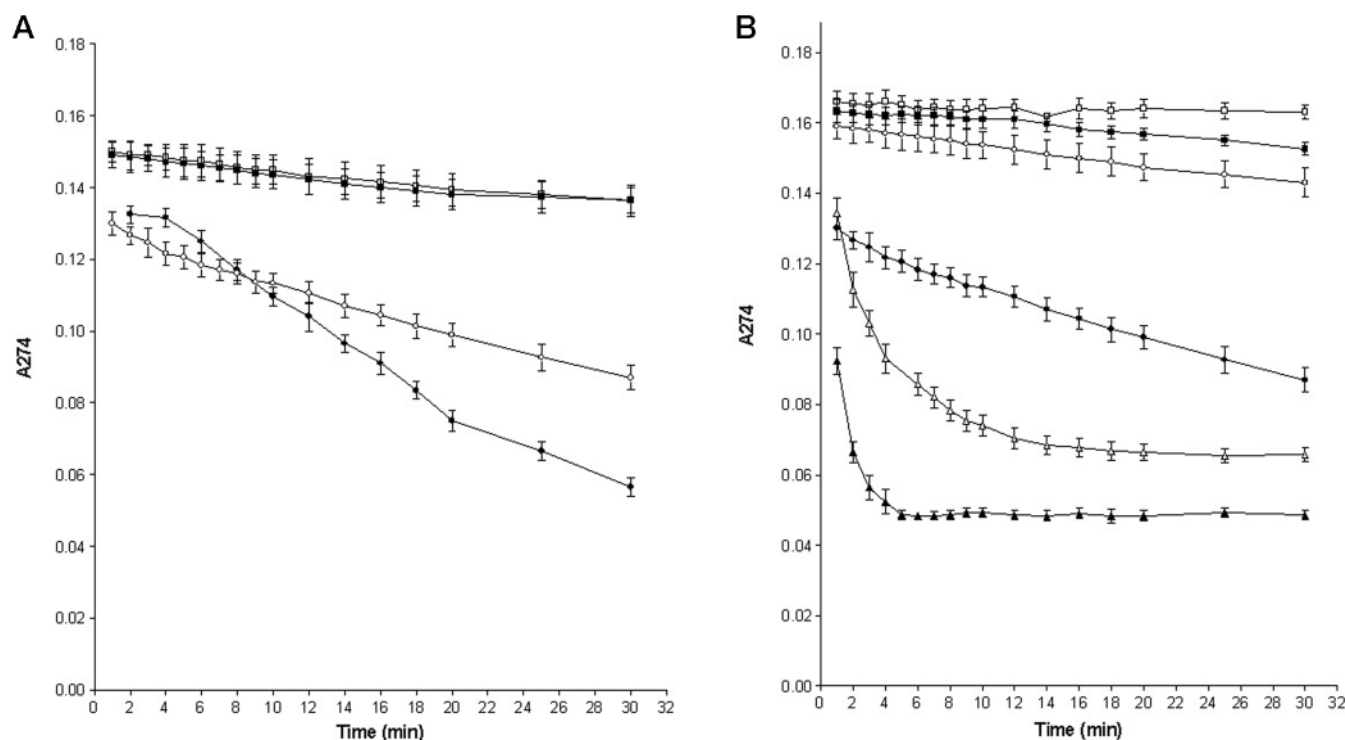


FIG. 2. A, reaction between pHPMA-ONp and free pLL (closed circles), 2:1 N:P pLL-DNA complexes (open circles), and free DNA (open squares). The rate of background hydrolysis is also shown (closed squares). Complexes were formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water, and the reaction was monitored by disappearance of 4-nitrophenoxy groups. B, reaction between pHPMA-ONp and pLL-DNA complexes (N:P ratio 2.0) at different pH values. Triangular symbols represent pH 8.2, circles, pH 7.3; and squares, pH 6.0. In each case open symbols represent the reaction in the absence of pLL-DNA complexes (i.e. represents the rate of hydrolysis of the ester groups), and closed symbols represent the rate of reaction in the presence of pLL-DNA complexes.

37 °C). Samples (1 ml) were then loaded carefully into a water layer (8 ml) above a simple sucrose cushion (45% (w/v) 5 ml). Samples were then centrifuged in a Beckman Ultracentrifuge L₂65B (30,000 rpm, 12 h) SW40Ti rotor to pellet the complexes. Tubes were then frozen in liquid nitrogen, and the bottom part of the tube (containing approximately 1 ml of sucrose solution) was removed with a razor blade. The sample was then allowed to thaw; supernatant sucrose was removed, and the ³²P-labeled pellet was resuspended in 500 μl of water. Recovery of the DNA was determined by measuring the radioactivity retrieved. More than 99% of the DNA was shown to be in the resuspended pellet. The complexes were then dissociated and denatured by boiling in mercaptoethanol/SDS (Laemmli buffer) and proteins resolved by electrophoresis on a 12.5% polyacrylamide gel (Hoeffer Minigel, 25 mA, 80 min) with visualization using diamine silver. To permit comparison of bound protein a standard amount of DNA (120 ng) was loaded onto each lane.

Transfection and Uptake Studies in K562 Cells—The human leukemia cell line K562 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cellular uptake studies were performed with complexes formed with a trace amount of ³²P-radiolabeled DNA and 20 $\mu\text{g}/\text{ml}$ plasmid DNA. Complexes were coated with pHPMA as described above, and transferrin was added after 2 h. Cells were placed into 12-well plates at a concentration of 10⁵ cells/well containing fresh Dulbecco's modified Eagle's medium with 10% serum, and complexes were added to a final DNA concentration of 2 $\mu\text{g}/\text{ml}$. Media were removed at set timepoints, and cells were washed with phosphate-buffered saline and dissolved with lysis solution. The cell lysates, media, and cell washings were placed in scintillation vials; scintillant was added, and the radioactivity was counted in a Packard (1900TR) scintillation counter. The percentage of radioactivity associated with the cells was then calculated.

Transfection studies were also performed in K562 cells in the presence of serum. Complexes were formed with a β -Gal plasmid expression vector. The complexes were then formulated as described above and were incubated with the cells for 4 h before the media were replaced. The cells were then left for 48 h before assaying for β -galactosidase using the Galactolight-Plus kit (Tropix, Cambridge, UK).

RESULTS

Reaction between pHPMA-ONp and Polylysine-DNA Complexes—Reactivity of pHPMA-ONp esters toward nucleophilic

centers is highly dependent on pH. Examination of the hydrolytic stability of the reactive polymer pHPMA-ONp showed that there was little time-dependent decrease in bound 4-nitrophenoxy groups at pH 6.0. Conversely, at pH 8.2, virtually all of the ester bonds were hydrolyzed within 5 min of dissolution, indicating rapid reaction at this higher pH. When hydrolysis was examined at pH 7.4, an intermediate rate of time-dependent hydrolysis was determined (Fig. 2A).

In the presence of pLL-DNA complexes there was only a very slow reaction at pH 6.0, suggesting stability of the ester at this pH. At pH 8.2, in the presence of pLL-DNA complexes, the reaction was very fast although the profile of disappearance of reactive esters was very similar to that due simply to hydrolysis, reflecting the high reactivity of the ester at this pH. At pH 7.4 the reaction observed in the presence of pLL-DNA complexes was appreciably faster than that due to hydrolysis alone, and this pH was selected as optimal to promote covalent linkage of the reactive polymer to the complexes.

The reactivity of pHPMA-ONp reacting with pLL-DNA complexes at pH 7.4 was compared with reactivity to free pLL alone, and also to DNA alone (Fig. 2B). Rates of reaction in the presence of pLL alone were similar to those generated in the presence of pLL-DNA complexes, indicating appreciable reactivity of pHPMA-ONp with the cationic component of the complexes. When mixed with DNA alone the loss of reactive esters from pHPMA-ONp was similar to or less than that produced by hydrolysis. This indicates that there was no reaction occurring between the activated esters and the amino groups present in DNA.

Determination of the Efficiency of Modification of pLL-DNA with pHPMA-ONp—Covalent reaction between the reactive esters of pHPMA-ONp and the pLL-DNA complexes would be expected to decrease the number of free amine groups on the surface of the complex. This hypothesis was tested using fluo-

rescamine to determine the number of free amine groups before and after the addition of varying amounts of pHPMA-ONp to the complexes (Fig. 3A). In every case the number of free amino groups measured using fluorescamine was represented as a percentage of the number determined using the equivalent quantity of free pLL. Addition of 200 $\mu\text{g/ml}$ pHPMA-ONp (corresponding to 85.5 μM reactive esters) to free pLL led to a fall of 45% in available amino groups (corresponding to 59.9 μM amines). This suggests that the reactive esters are binding to amino groups in pLL with an efficiency of about 70% with remaining reactive esters presumably hydrolyzing in water. Binding of pLL to DNA physically protects approximately 30% of the free amino groups of pLL from reactivity with fluorescamine. Reaction with increasing amounts of pHPMA-ONp results in a further decrease in the number of amino groups available for derivatization.

The availability of amino groups in pLL and pLL-DNA complexes for reaction with fluorescamine was examined at different pH values, including the effects of reaction with pHPMA-ONp. Unexpectedly over 20% of amino groups of free pLL were masked following reaction with pHPMA-ONp at pH 6.0 (Fig. 3B), perhaps reflecting a noncovalent association and subsequent masking of the amino groups by the intact polymer. As expected a greater quantity (nearly 50%) became masked at pH 7.3 and the reaction was slightly more efficient at pH 8.2, with over 50% of amino groups becoming masked by pHPMA-ONp. Precisely the same trends were seen for availability of amino groups present in pHPMA-modified pLL-DNA complexes using corresponding pLL-DNA complexes at appropriate pH values to supply the appropriate 100% figures for calibration. The pH also affects the availability of pLL amino groups within pLL-DNA complexes for reaction with fluorescamine. At lower pH values (e.g. pH 6.0), fewer amino groups are available for reaction compared with unconjugated pLL at the same pH values, presumably reflecting stronger electrostatic binding of pLL to DNA. The overall result confirms that covalent modification of pLL-DNA complexes using pHPMA-ONp is substantially more efficient at pH 7.3 and 8.2 than it is at 6.0.

Characterization of pHPMA-coated Complexes—pHPMA-modified and simple pLL-DNA complexes were characterized by TEM and AFM to determine their overall structure. Both TEM and AFM showed that simple pLL-DNA complexes were discrete, apparently spherical complexes with an average diameter of around 60 nm (Fig. 4, A and C). Analysis of pHPMA-coated complexes by AFM suggested a greater tendency to adhere to the mica surface, because there were many more images in the field of view than observed with unmodified complexes (Fig. 4B). Analysis of the pHPMA-coated complexes by TEM showed an area of less dense staining surrounding the complex, possibly due to the presence of the coating polymer (Fig. 4D). Both AFM and TEM showed that pHPMA-coated complexes maintain a discrete, apparently spherical morphology, with a slightly larger size than uncoated pLL-DNA complexes (90–100 nm approximately).

The surface charge of particles is known to be one of the major factors influencing their biodistribution (21) and transfection activity (22). For this reason the surface charge on coated and uncoated complexes was determined using ζ potential analysis. At an N:P ratio of 2, pLL-DNA complexes were positively charged with a ζ potential of around +14 mV (Table I). The addition of increasing concentrations of pHPMA-ONp led to corresponding decreases in surface charge, falling to around neutral (+1 mV) with 50 $\mu\text{g/ml}$ of pHPMA-ONp and reaching as low as -25 mV when 200 $\mu\text{g/ml}$ coating polymer was used.

Determination of particle size was also performed using

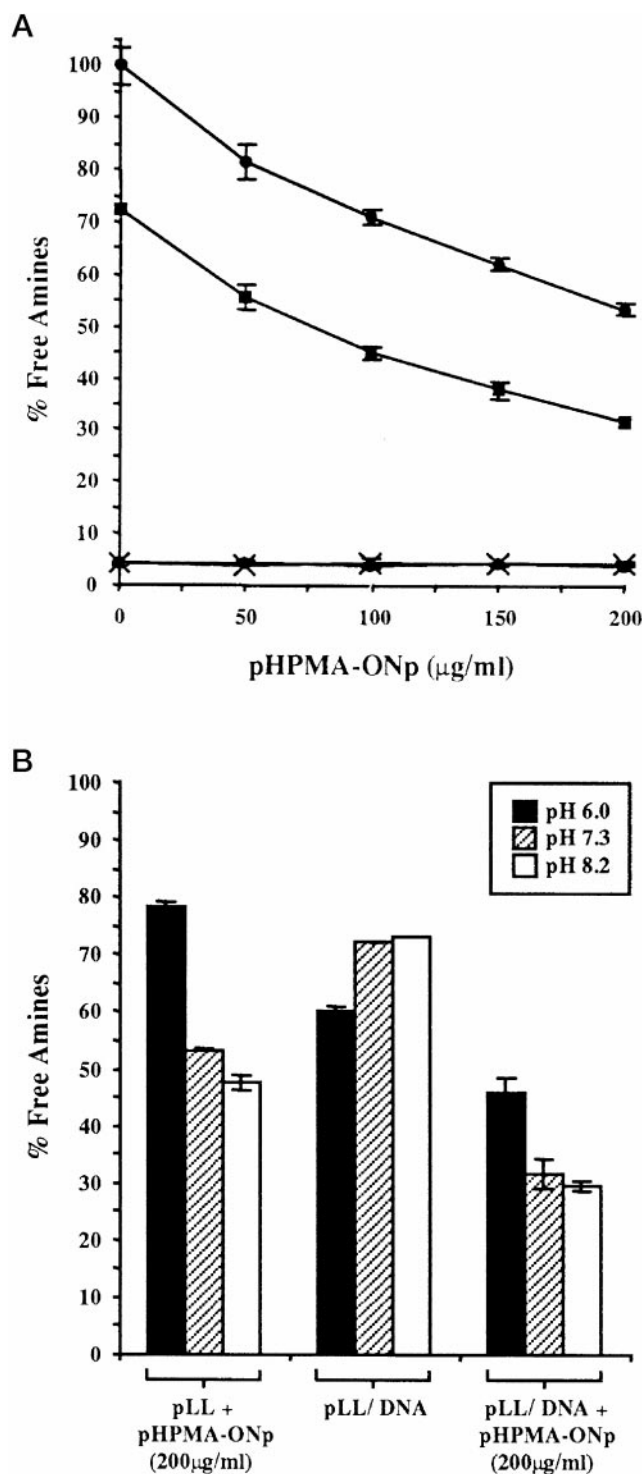


FIG. 3. Fluorescamine determination of free amine groups before and after reaction with pHPMA-ONp (A) pLL-DNA complexes formed at a DNA concentration of 20 $\mu\text{g/ml}$ in water (squares), pLL (circles), DNA (crosses), and buffer alone (diamonds, indistinguishable from DNA line) were incubated overnight with increasing amounts of pHPMA-ONp (0–200 $\mu\text{g/ml}$), and the availability of free amines was determined by the fluorescamine assay. Results are expressed as percent of free amines available in pLL alone (12.8 $\mu\text{g/ml}$ in 100 mM sodium borate, pH 7.3). B, effect of pH on coating reaction. pLL-DNA complexes and pLL alone were incubated overnight with 200 $\mu\text{g/ml}$ pHPMA-ONp at pH 6, 7.3, and 8.2 as indicated. Results are expressed as percent of free amines available in pLL alone at pH 6, 7.3, and 8.2 accordingly. The data represent an average of three determinations.

PCS. The results show that uncoated pLL-DNA complexes have an average diameter of 50 nm (Fig. 5A). Coating the complexes

FIG. 4. Microscopy analysis of pHPMA-coated and -uncoated pLL-DNA complexes formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water. A, AFM images of uncoated pLL-DNA complexes; B, AFM images of coated complexes; C, TEM images of uncoated pLL-DNA complexes; D, TEM images of coated complexes. Bar, 100 nm.

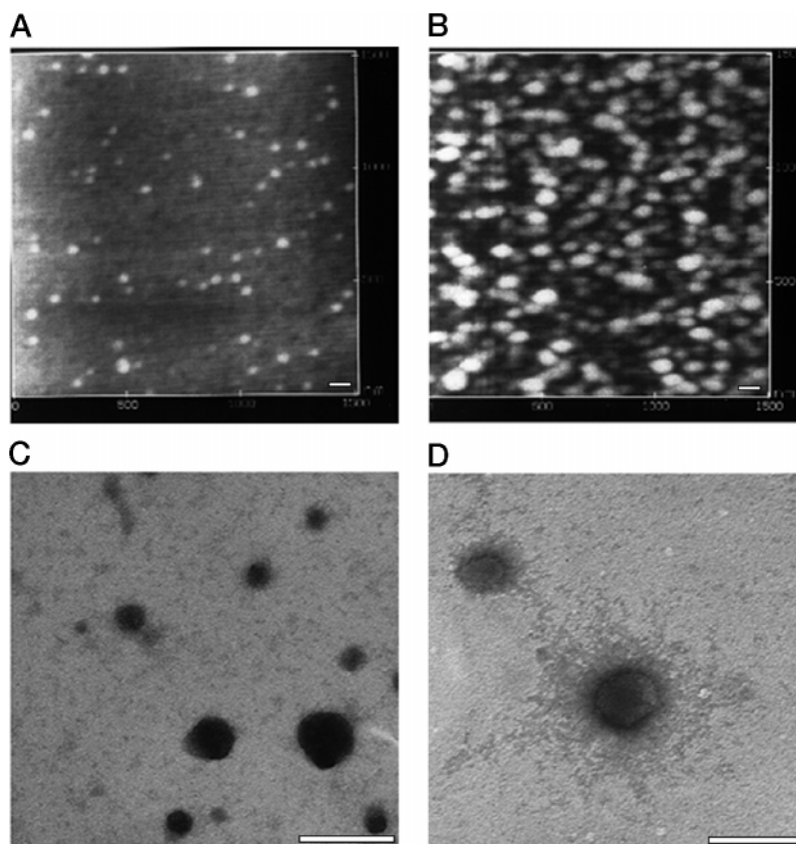


TABLE I

ζ Potential of pLL-DNA complexes (N:P ratio 2.0, formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water) coated with different concentrations of pHPMA-ONp

pHPMA-ONp concentration	ζ potential	Standard deviation
$\mu\text{g}/\text{ml}$	mV	
0	+13.7	5.10
50	+0.88	0.37
100	-7.78	0.15
150	-21.2	0.64
200	-25.1	0.29

with pHPMA produces a population of particles, as measured by PCS, with a slightly greater average diameter of around 90 nm, possibly due to incorporation of polymer on the surface of the particles (Fig. 5B). Combined with the microscopic analysis (Fig. 4) these results confirm that the complexes have not become noticeably cross-linked by reaction with pHPMA-ONp, instead remaining predominantly as discrete complexes.

Physicochemical Changes Induced in Complexes Following Binding of Proteins—Polymer-coated and -uncoated pLL-DNA complexes were incubated in the presence of 5% serum overnight, and their size was determined by PCS. It was found that the addition of serum to uncoated pLL-DNA complexes resulted in an increase in particle diameter from around 50 nm to over 1000 nm (Fig. 5, A and C). In comparison, pHPMA-coated complexes remained at a constant 90-nm diameter even in the presence of 10% serum (Fig. 5, B and D), suggesting that the complexes are resistant to serum-induced aggregation. An alternative assay to monitor protein binding relies on changes in turbidity measured as light reflection in a fluorimeter. Following the addition of aliquot portions of albumin solution to pLL-DNA complexes in water there was an obvious increase in the turbidity of the solution, probably due to an aggregation of the complexes (Fig. 6). pLL-DNA complexes premodified with 50 $\mu\text{g}/\text{ml}$ of pHPMA-ONp displayed a substantial reduction in

albumin-induced turbidity. A further fall in turbidity was achieved using 100 $\mu\text{g}/\text{ml}$ pHPMA-ONp, whereas 200 $\mu\text{g}/\text{ml}$ completely prevented albumin-mediated increases in turbidity.

Influence of Surface Charge of Coated Complexes on Protein Binding—It was not clear whether the decreased protein binding following reaction with pHPMA-ONp was due to decreased availability of electrostatic sites within the pLL-DNA complex for protein binding or was perhaps mediated by the strong negative surface charge endowed by hydrolysis of unreacted ester groups present on the coating polymer. Consequently pLL-DNA complexes surface modified with pHPMA-ONp (200 $\mu\text{g}/\text{ml}$) were aminolysed with aminoethanol at various times following the addition of the reactive polymer to remove unreacted 4-nitrophenoxy groups. This prevented subsequent hydrolysis and formation of carboxylic acid groups and led to the formation of complexes with a surface charge much closer to neutrality (Fig. 7A). This modified charge did not affect the ability of the complexes to withstand albumin disruption, according to determination of albumin-induced turbidity (Fig. 7B). This suggests that it is the surface masking or stabilization effect of the pHPMA, not the surface charge, that is important in preventing an interaction with albumin. It was also demonstrated that the presence of free pHPMA in solution is not responsible for the improved stability to serum albumin (Fig. 7B).

Characterization of Plasma Proteins Binding to pLL-DNA and pHPMA-coated Complexes—To elucidate the interaction of pLL-DNA complexes with plasma proteins, both simple and pHPMA-coated complexes were incubated in 50% fresh plasma. The complexes were then recovered from the plasma by centrifugation through a sucrose cushion and analyzed by polyacrylamide gel electrophoresis to determine which proteins were associated with the complexes (Fig. 8). Silver staining revealed a number of proteins binding to uncoated pLL-DNA complexes, particularly a 66-kDa protein that co-electrophoreses with serum albumin. There were also several other proteins

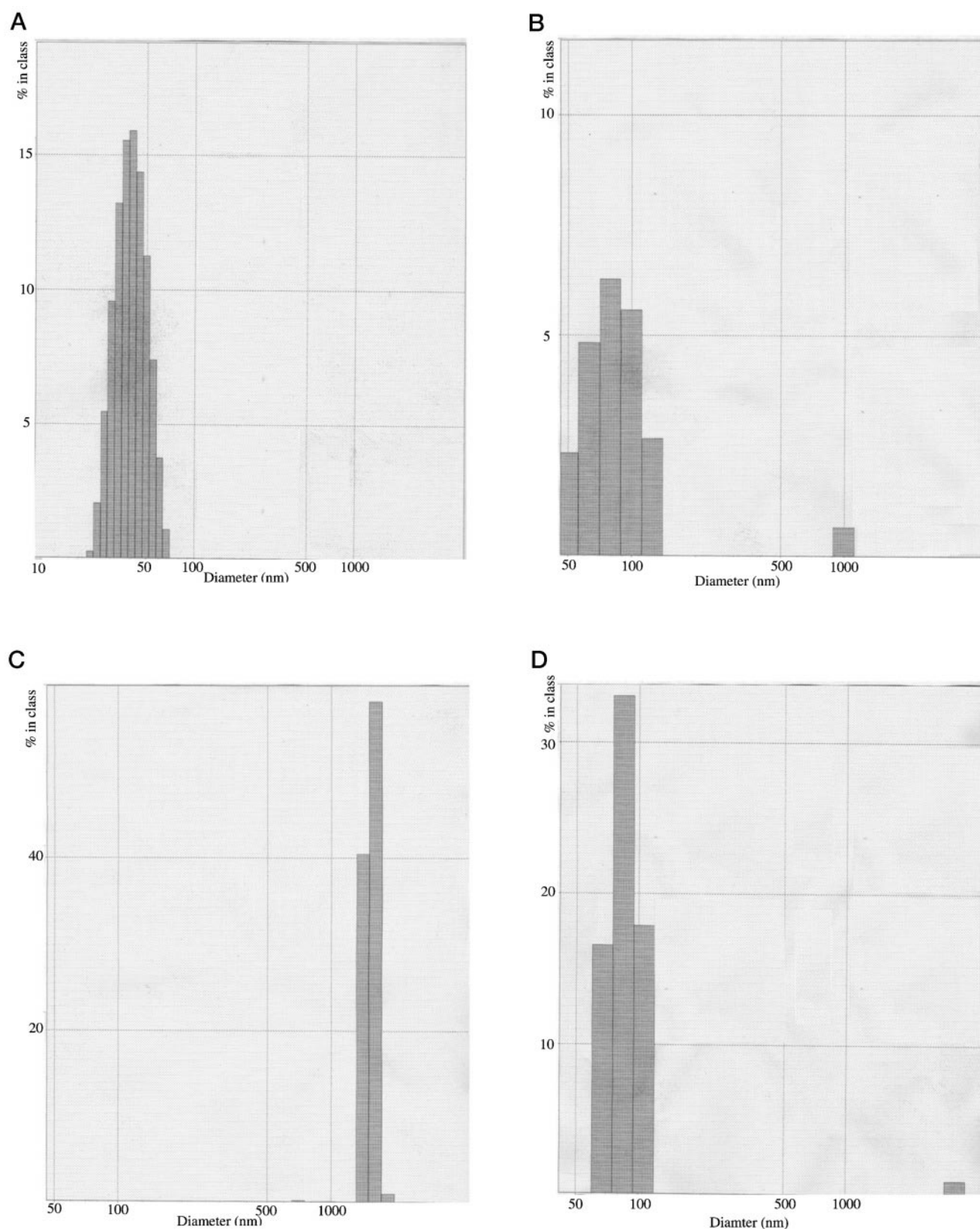
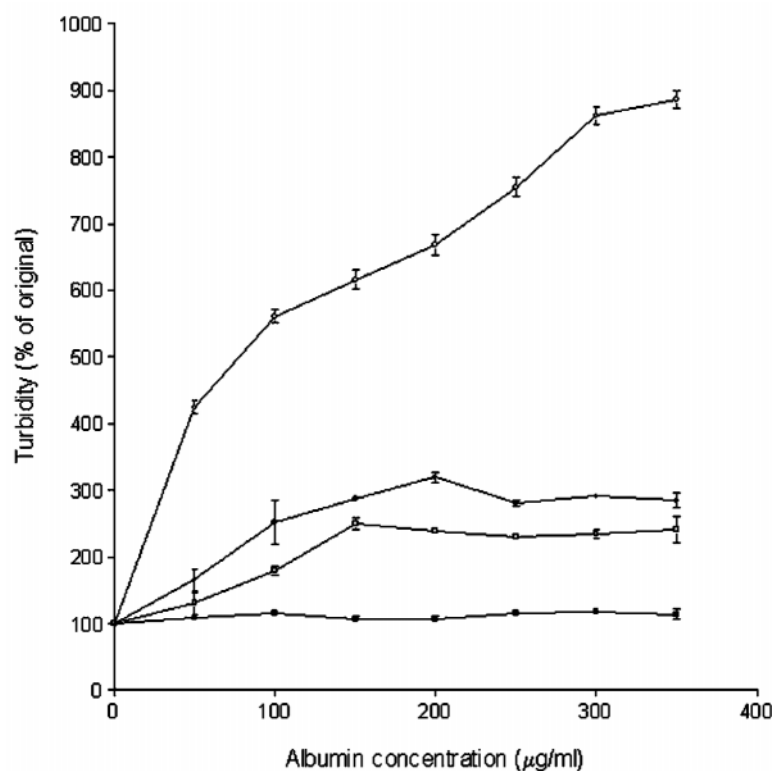


FIG. 5. PCS analysis of particle size (A) pLL-DNA complexes, (B) pLL-DNA complexes reacted with pHPMA-ONp, (C) pLL-DNA complexes + 10% serum, and (D) pLL-DNA complexes reacted with pHPMA-ONp + 10% serum. All complexes were formed at a DNA concentration of 20 $\mu\text{g/ml}$ in water

associated with pLL-DNA complexes, notably bands with molecular masses of 66 and 120 kDa, approximately. In contrast, no proteins were detected bound to the pHPMA-coated complexes.

Cell Association and Transfection Studies—Rates of association of coated and uncoated pLL-DNA complexes with K562 cells were determined *in vitro* using pLL-DNA complexes trace labeled with [^{32}P]DNA (Fig. 9A). After 30 min around 33% of

FIG. 6. Stability of simple pLL-DNA complexes (formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water) and complexes reacted with pHPMA-ONp to disruption by bovine serum albumin. Turbidity of complexes following addition of bovine serum albumin was measured in a fluorimeter with excitation and emission wavelengths set to 600 nm. pLL-DNA complexes (open circles), complexes reacted with 50 $\mu\text{g}/\text{ml}$ pHPMA-ONp (closed circles), complexes reacted with 100 $\mu\text{g}/\text{ml}$ pHPMA-ONp (open squares), complexes reacted with 200 $\mu\text{g}/\text{ml}$ pHPMA-ONp (closed squares).



simple pLL-DNA complexes have been internalized by the cells, whereas only around 15% of the pHPMA coated complexes have been internalized over the same time point. After 5 h approximately 50% of the pLL-DNA complexes have been taken up by the cells compared with around 25% for the pHPMA-coated complexes. This suggests that coating the complexes with pHPMA can reduce the nonspecific cell interactions observed with simple pLL-DNA complexes by approximately 2-fold. In the absence of serum the reduction in nonspecific cell uptake is even greater, with around a 10-fold reduction in cell uptake of pHPMA-coated complexes (data not shown). However, targeting the complexes to cell surface receptors using transferrin restored the uptake of pHPMA-coated complexes. Transferrin-modified pHPMA-coated complexes produce a much greater and more rapid cellular uptake of the complexes. After 30 min around 85% of the transferrin-targeted complexes have been internalized by the cells, an approximately 6-fold increase over untargeted coated complexes. This figure then remains fairly constant over the 5-h time course of the experiment, probably reflecting depletion of the complexes from the media. Fig. 9B shows the transfection activity, as measured by β -galactosidase activity of K562 cells transfected with complexes containing the β -Gal plasmid. This shows a 15-fold increase in transfection activity of transferrin-targeted pHPMA-coated complexes over either simple pLL-DNA complexes or untargeted pHPMA-coated complexes, reflecting the increased rate of uptake determined above.

DISCUSSION

Self-assembling vectors for gene therapy, containing DNA and cationic polymers, have considerable promise and already several useful applications in transfection science and gene therapy. They are easy to prepare and characterize, have low toxicity and immunogenicity, and their tropisms can be easily manipulated to enable them to introduce transgenes into a range of target cells. However, both polyelectrolyte components (DNA and cationic polymers) are known to bind serum proteins, and the complexes they form also show avid protein

binding. In plasma there is evidence that complexes bind several proteins, including albumin and high molecular weight components of the complement cascade (23, 24). These proteins may serve to opsonize the complexes and perhaps promote the rapid clearance into phagocytic cells that is often observed following intravenous administration. This rapid clearance effectively limits useful systemic application of such polyelectrolyte vectors, because their short plasma dwell time restricts access to peripheral target cells and tissues. Hence an important goal in this field is to identify means to prevent protein binding and subsequent opsonization. Following unsuccessful attempts to prevent protein binding using self assembling cationic-hydrophilic block copolymer structures (16, 17), in this study we have tried to overcome this problem by using a multivalent reactive hydrophilic polymer (pHPMA-ONp) to engineer a cross-linked hydrophilic shield on the surface of polycation-DNA complexes.

pHPMA-ONp shows rapid reaction with both free pLL and with pLL-DNA complexes, and it is estimated that about 70% of the ester groups can become linked to amines. The presence of several reactive esters/molecule (8.6 mol %) means that for each pHPMA molecule there are an average of 15 reactive esters, and therefore about 10 covalent links formed to pLL. Several pHPMA molecules become linked to each pLL-DNA complex, and hence the polymer coating is expected to endow the complexes with a significant level of surface cross-linking that may prevent binding and disruption by proteins.

Analysis of the coated complexes by AFM shows a slight increase in overall size compared with uncoated complexes. In addition, coated complexes show a higher affinity for the mica surface, and many more images are generated in each field of view. The reasons for this are not clear but could involve increases in density or perhaps a slight hydrophobic association mediated through the tetrapeptide sequences of the reactive polymer. Analysis by TEM generated similar conclusions, although the greater resolution permitted visualization of the surface layer of polymer. These results indicate that modifica-

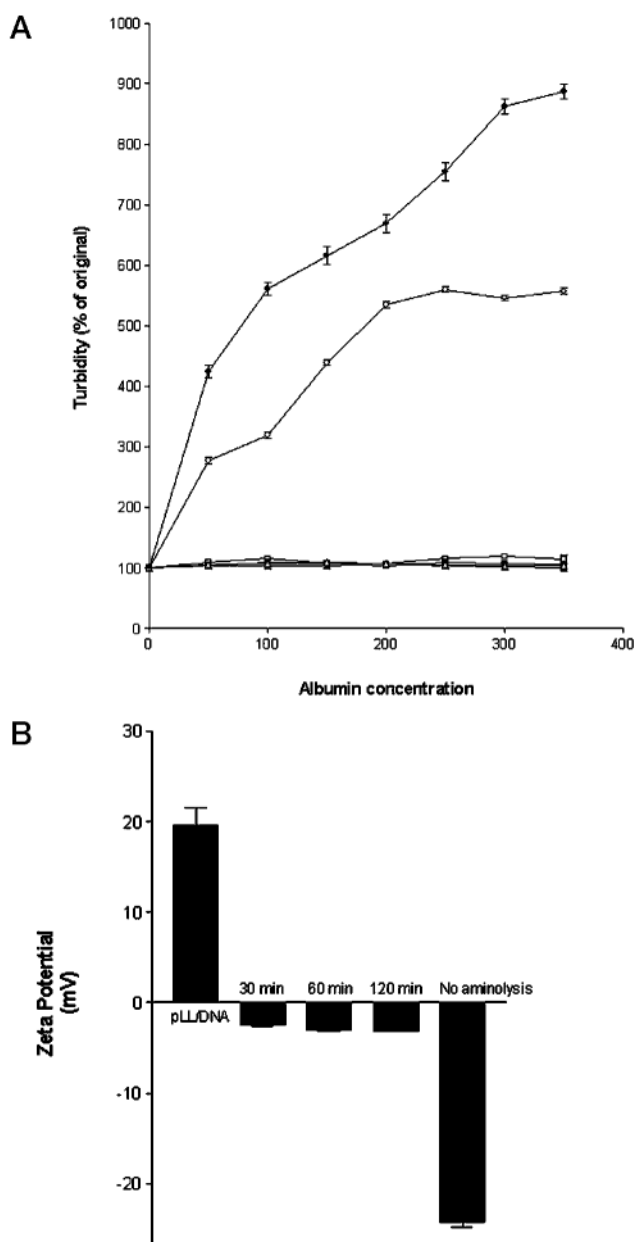


FIG. 7. Effect of aminolysis of excess ester groups on surface charge and albumin-induced turbidity of pLL-DNA complexes (formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water) reacted with pHPMA-ONp. A, albumin-induced turbidity; and B, surface charge (ζ potential) of simple pLL-DNA complexes and complexes reacted with pHPMA-ONp following aminolysis of excess reactive ester groups with aminoethanol after different times. Aminoethanol was added 30 min (open triangles), 60 min (closed triangles), and 120 min (open squares) after the addition of pHPMA-ONp. Alternatively the reaction was allowed to proceed overnight without the addition of aminoethanol at any stage (open circles). In one case pHPMA-ONp was aminolysed before reaction with pLL-DNA complexes (open circles). Properties of uncoated pLL-DNA complexes are also shown (closed circles).

tion of pLL-DNA complexes with multivalent pHPMA-ONp did not result in aggregation or cross-linking of the complexes, perhaps instead endowing the complexes individually with a thin polymer coating.

The average diameter of the polymer-coated complexes was estimated as 60–90 nm by both AFM and TEM. However both techniques rely on the particles adhering to a surface, which automatically results in analysis of a subpopulation of particles, and the diameter of particles increases due to flattening of

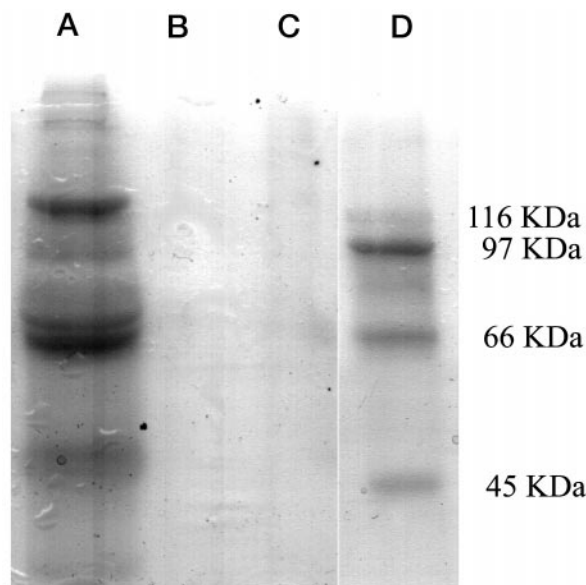


FIG. 8. SDS-polyacrylamide gel electrophoresis analysis of proteins bound to pHPMA-ONp-coated complexes (formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water) re-isolated following incubation in the presence of plasma. Uncoated pLL-DNA complexes (lane A); blank (lane B); 200 $\mu\text{g}/\text{ml}$ pHPMA-coated pLL-DNA complexes (lane C); and molecular weight markers (lane D).

their structure. In addition, other artifacts may be introduced during the sample handling process (e.g. dehydration). In comparison PCS permits analysis of a whole population of particles in solution. Examination of complexes by PCS showed that complexes coated with pHPMA had a diameter approximately 40 nm larger than uncoated complexes (90 nm compared with around 50 nm), probably reflecting attachment of pHPMA onto the surface of the complexes (visualized by TEM in Fig. 4D). There was no indication of any aggregation or cross-linking due to the polymer modification.

Surface charge changes significantly after coating. Simple pLL-DNA complexes bear a net positive charge, influenced to some extent by the loose association of excess free pLL with the complexes. Complexes coated with pHPMA show a reduced ζ potential, proportional to the amount of pHPMA-ONp applied. Hydrolysis of the polymer-bound reactive esters that fail to bind amino groups in pLL yields carboxylic acid groups on the surface of the complex. Whereas this may be useful to decrease a positive charge, a strong negative charge may also be undesired for various applications. This can be modulated by termination of the reaction at the appropriate time. Alternatively different chemistry using, for example, reactive esters based on carbonate esters can produce neutral groups on hydrolysis (e.g. hydroxyl groups), and the use of such polymers is currently being explored to produce less negatively charged particles. Mixed chemistry can also be applied if appropriate to suit the requirements of individual applications.

The binding of small amounts of serum albumin to complexes results in their increased size and can lead to aggregation and precipitation (25). This can be measured experimentally by an increase in the turbidity of the solution. In the presence of relatively small amounts of albumin (1 mg/ml) the solution of complexes becomes very cloudy, and the turbidity rises to over 300 times the original level. Increasing the concentration of albumin to physiological levels removes the turbidity completely, probably by saturating the pLL-DNA complexes and inhibiting their cross-linking. Alternatively, low concentrations of albumin may partially modulate the surface charge of the complexes making them less positively charged and more likely

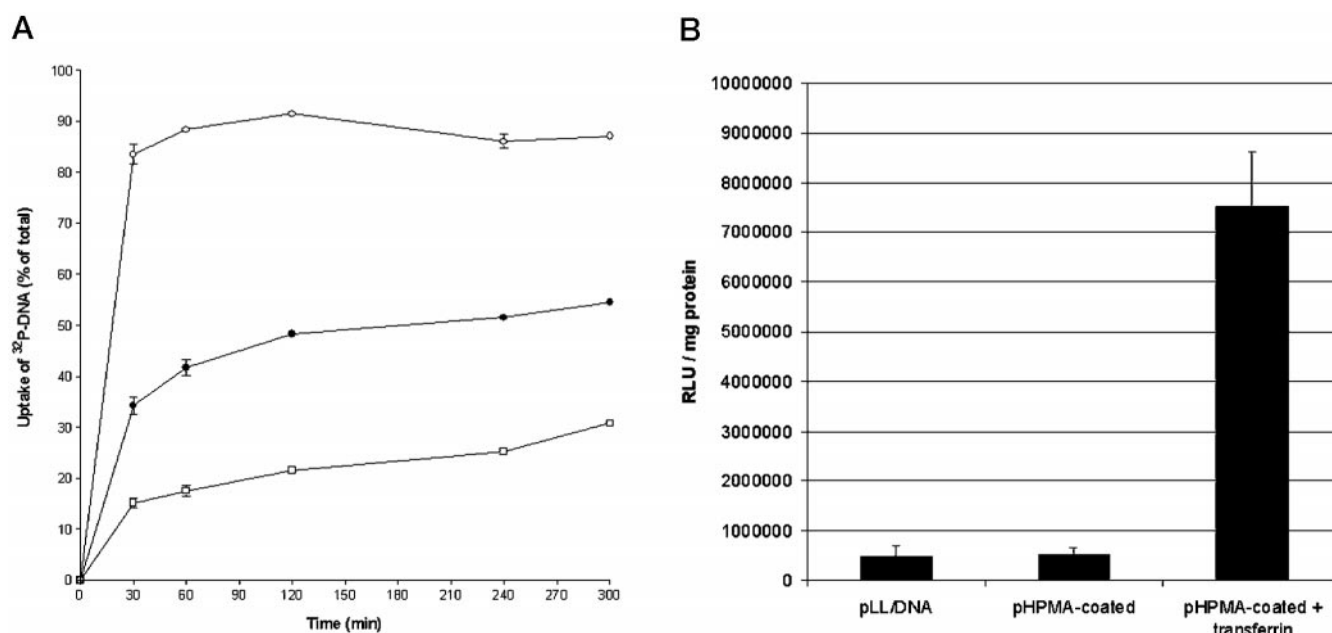


FIG. 9. Cellular uptake and transfection activity of untargeted pHPMA-coated and -uncoated pLL-DNA complexes compared with transferrin-targeted pHPMA-coated complexes. A, uptake of ^{32}P -labeled complexes (formed at a DNA concentration of 20 $\mu\text{g}/\text{ml}$ in water) in K562 cells; simple pLL-DNA complexes (closed circles), pHPMA-coated complexes (open squares), and pHPMA-coated complexes reacted with transferrin (open circles). B, transfection of K562 cells with pLL-DNA complexes and pHPMA-coated complexes \pm transferrin.

to aggregate due to their hydrophobic nature. It is known that physiological concentrations of albumin cause a sharp decrease in the surface charge of complexes (from +15 mV to -15 mV, data not shown), and it is possible that the strong negative surface charge on complexes under these conditions will prevent them from cross-linking. In comparison, complexes coated with pHPMA-ONp (200 $\mu\text{g}/\text{ml}$) show no increase in turbidity at any concentration of albumin, indicating that albumin is unable to bind to the complexes.

One possible explanation for the inability of albumin to interact with the coated complexes could be that the pHPMA-modified complexes possess a strong negative charge on their surface that might repel albumin from binding to the surface. This was examined by aminolysing the surface of the complexes with aminoethanol at different times after coating. Although this significantly changed their resulting overall surface charge, there was no effect on albumin binding. Hence it is clear that albumin binding is inhibited by the shielding effect of the pHPMA chains, not by an electrostatic mechanism.

When complexes were incubated in serum, reisolated, and analyzed by SDS-polyacrylamide gel electrophoresis, it became clear that unmodified pLL-DNA complexes bind a range of serum proteins, the major one corresponding to the molecular weight of albumin. In contrast, very little protein was detected bound to the pHPMA-modified complexes. The reduced serum protein binding of pHPMA-modified complexes is potentially important in terms of circulatory half-life, because it is known that just a 2-fold reduction in the amount of protein is associated with a substantial increase in the half-life of the liposomes in the bloodstream (26).

Reducing the amount of protein bound to the complexes is not the only requirement of a successful systemic delivery system, it is also important to reduce nonspecific interactions with cells. Interaction of the complexes with erythrocytes, monocytes, and other circulating cells will reduce the ability of the complexes to reach their target site. Similarly, nonspecific interactions with cell membranes are likely to lead to either rapid clearance by the reticulo-endothelial system or a widespread distribution of the complexes throughout the body. Cellular uptake studies in the human leukemia cell line K562 have

shown that pHPMA-coated complexes have a much lower rate of cell association than do unmodified pLL-DNA complexes, probably reflecting decreased nonspecific binding to cell membranes.

In the ideal system, transfection of cells should only occur following binding of the delivery vector to specific target-associated receptors. Indeed, the incorporation of the Gly-Phe-Leu-Gly sequence into the pHPMA-ONp is designed to permit enzymatic removal of the polymer and activation of the complex by cathepsins B, H, and L only following endocytic internalization into target cells. Clearly other sequences, including pH-sensitive or hydrolytically unstable sequences, could be incorporated to regulate activation of the complexes as required.

One major advantage to the use of multivalent polymers for coating polycation-DNA complexes is that unreacted ester groups can be exploited for the attachment of targeting ligands to the surface of the complexes. In this paper we have shown that attaching transferrin to the surface of pHPMA-coated complexes significantly enhances both cell uptake (~ 6 -fold) and transfection activity (~ 15 -fold) compared with either the simple pLL-DNA complexes or the untargeted pHPMA-coated complexes. The attachment of transferrin to the complexes enhances transfection activity beyond what would be expected through a simple increase in the cellular uptake of complexes. It may be that transferrin-targeted complexes show an improved cellular routing that is responsible for the enhanced efficiency of gene expression. The attachment of targeting ligands onto the surface of gene delivery vectors in this way is both novel and extremely flexible. Any primary amine-bearing ligand can be covalently attached through simple mixing with the complexes during the coating reaction. Studies in our laboratory have shown that it is possible to attach fibroblast growth factor-2, vascular endothelial growth factor, asialofetuin, and various antibodies to pHPMA-coated pLL-DNA complexes.³ Furthermore the presence of the pHPMA coating reduces nonspecific interactions of the complexes with cell

³ K. D. Fisher, K. Ulbrich, V. Subr, C. M. Ward, V. Mautner, D. Blakey, and L. M. Seymour, submitted for publication.

membranes, thereby enhancing the selectivity of delivery to the target cell.

We consider that this class of polymer-modified DNA prodrugs should find several applications in the fields of gene delivery. The well defined nature of the complexes means that their surface properties can be regulated for specific purposes, they can be programmed for activation by selected biological conditions, and targeting groups can be incorporated easily to promote selectivity of gene delivery.

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Decreased Binding to Proteins and Cells of Polymeric Gene Delivery Vectors Surface Modified with a Multivalent Hydrophilic Polymer and Retargeting through Attachment of Transferrin

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