

Importance of Lateral and Steric Stabilization of Polyelectrolyte Gene Delivery Vectors for Extended Systemic Circulation

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Gene therapy for systemic diseases requires intravenous administration, but existing vectors are not suitable for systemic delivery, often showing rapid elimination from the bloodstream that restricts potential transfection sites to "first-pass" organs. To develop long-circulating vectors, here we have compared polyplexes containing DNA and poly-L-lysine (PLL) or polyethylenimine (PEI), surface-modified with either monovalent polyethylene glycol (PEG) or multivalent copolymers of *N*-(2-hydroxypropyl)methacrylamide (PHPMA), correlating their biophysical properties with their distribution following intravenous injection. A key difference between the two types of coating is the introduction of lateral stabilization by surface attachment of multivalent PHPMA, in addition to the steric stabilization provided by both types of polymers. The α -half-life for bloodstream clearance of polycation/DNA polyplexes (typically < 5 minutes in mice) could be extended using multivalent PHPMA coating to > 90 minutes. We found that the dose administered, as well as the amount and molecular weight of the coating PHPMA, had important effects on circulation properties. Multivalent PHPMA coating allows, for the first time, considerably extended circulation time using polyplex systems—a prerequisite for systemic gene delivery.

Key Words: *in vivo*, pharmacokinetics, polyplexes, polylysine, polyethylenimine, steric stabilization, lateral stabilization, PEG, HPMA, gene delivery

INTRODUCTION

Systemic delivery of nucleic acids following intravenous injection is a major goal in gene therapy, permitting access of therapeutic genes to disseminated targets such as cells of the bloodstream or vasculature, widespread organs such as skin, muscles, and joints, as well as dispersed and often occult metastatic cancer cells. Unfortunately, most of the existing vectors are unsuitable for systemic delivery, invariably showing rapid elimination from the bloodstream coupled with nonspecific accumulation in organs and tissues. In the case of viral vectors, relatively few studies of systemic distribution kinetics have been done, although adenovirus is thought to be sequestered rapidly from the circulation into the liver [1]. Cationic lipid formulations (lipoplexes), on the other hand, often show accumulation in the pulmonary capillary bed [2,3], probably reflecting a tendency of the lipoplexes to aggregate

in the presence of serum and become lodged in the first vascular bed encountered. In all cases the area under the plasma concentration/time curve (AUC) is small, usually too small to permit quantitative delivery or targeting to specific peripheral targets. Polycation-based vectors (polyplexes) are also presently unsuitable for systemic administration. Poor solubility combined with nonspecific charge-mediated interactions with proteins and cells, notably components of the reticuloendothelial system (RES), result in rapid bloodstream clearance into liver [4].

We have focused on the development of long-circulating polyplexes using hydrophilic polymers to provide a stealthlike shield around nanoparticles of self-assembling polycation/DNA complexes to reduce protein and cellular interactions. Previous attempts to achieve stealthlike coating included the use of cationic-hydrophilic block copolymers, designed to undergo oriented self-assembly with

TABLE 1: Biophysical properties of the studied polyplexes

Polycation	Polycation/DNA		PEG-polycation/DNA			PHPMA(0.2)-polycation/DNA			PHPMA(2)-polycation/DNA		
	<i>Size</i> ^a nm	<i>ZP</i> ^b mV	<i>Size</i> ^a nm	<i>ZP</i> ^b mV	% reacted ^c NH ₂	<i>Size</i> ^a nm	<i>ZP</i> ^b mV	% reacted ^c NH ₂	<i>Size</i> ^a nm	<i>ZP</i> ^b mV	% reacted ^c NH ₂
PLL(29k)	84 ± 2 (0.15)	39.8 ± 1.2	93 ± 2 (0.16)	2.0 ± 1.3	29	92 ± 2 (0.15)	-4.1 ± 1.8	11	90 ± 1 (0.20)	-8.9 ± 1.0	45
	1253 ± 112 (0.7)		95 ± 1 (0.14)			105 ± 1 (0.15)			105 ± 1 (0.24)		
PLL(205k)	84 ± 2 (0.16)	39.3 ± 0.4	95 ± 1 (0.14)	0.2 ± 0.9	24	93 ± 2 (0.16)	-4.5 ± 0.1	9	95 ± 2 (0.23)	-8.1 ± 0.3	42
	1126 ± 30 (0.99)		97 ± 1 (0.16)			104 ± 1 (0.19)			105 ± 3 (0.24)		
PEI(25k)	77 ± 1 (0.10)	36.2 ± 0.5	93 ± 2 (0.08)	3.2 ± 0.4	19	93 ± 1 (0.09)	-3.9 ± 0.1	10	94 ± 1 (0.11)	-10.6 ± 0.7	63
	1315 ± 67 (0.21)		94 ± 1 (0.06)			104 ± 1 (0.15)			117 ± 1 (0.14)		
PEI(800k)	85 ± 1 (0.08)	34.6 ± 0.9	98 ± 1 (0.09)	3.5 ± 0.7	17	104 ± 2 (0.09)	-3.5 ± 0.1	5	111 ± 2 (0.12)	-10.0 ± 0.5	68
	1437 ± 4 (1.00)		100 ± 1 (0.06)			113 ± 1 (0.13)			122 ± 3 (0.15)		

^aSize and polydispersity (in brackets) of the complexes as measured by DLS in Hepes buffer (first line) and in 0.15 M NaCl (second line).

^bZeta-potential of the complexes.

^c% of amino groups of the complexes that reacted in coating reaction determined by TNBS assay.

plasmid DNA [5]. The resulting polyplexes showed improved biophysical properties *in vitro*, with decreased surface charge and less nonspecific binding to cells; however, an interference of hydrophilic nonionic blocks with the DNA condensation led to instability at serum concentrations of proteins and rapid elimination from the bloodstream following intravenous injection [6,7].

Covalent attachment of the monovalent hydrophilic polymers to the surface of the preformed complexes was suggested as a more efficient method of forming stealth-like shielded polyplex particles while keeping stable and compact the hydrophobic polycation/DNA core. It was implied that this approach could avoid the possible incorporation of hydrophilic polymer into the hydrophobic core of the vector during the self-assembly reaction, resulting in more stable complexes. Ogris *et al.* used this strategy with monovalent PEG and produced a stealth complex (additionally containing transferrin) [8]. Following intravenous administration to mice, this vector showed some accumulation and transgene expression within subcutaneous B16 melanoma. Formal pharmacokinetics studies were not done, although the low recovery of DNA from

organs sampled (determined by Southern blot) indicated that the complexes were still rapidly eliminated from the bloodstream and degraded. We carried out related experiments using a monovalent end-reactive poly-[N-(2-hydroxypropyl)methacrylamide] to modify the surface of polyplex particles. Despite encouraging improvements in resistance to protein binding and reduced phagocytic uptake *in vitro*, the complexes were cleared from the mouse bloodstream even more rapidly than nonmodified complexes [9].

We reasoned that the steric shield provided by attachment of monovalent polymers, while improving solubility and providing effective steric stabilization of the polyplex particles against aggregation, might still lead to disruption of the polycation/DNA core of the particles by intracomplex reorganization and allow the penetration of proteins and polyelectrolytes between the polymer chains, particularly at the high concentrations encountered *in vivo*. In this way, biological molecules might be able to gain access to the core of the polyplex, and perhaps mediate its disruption. To counter this, it was desirable to increase the stability of the polycation/DNA core by

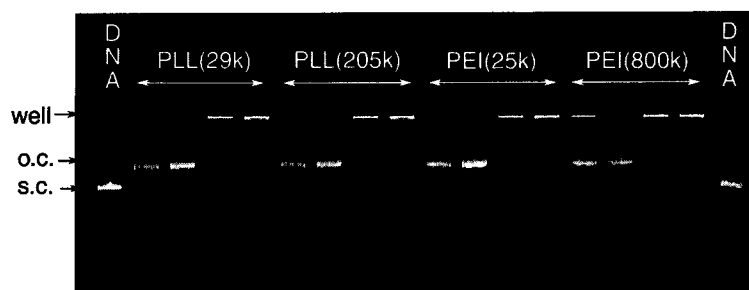


FIG. 1. Resistance of polyplexes to polyelectrolyte exchange reactions. All polyplex samples were incubated with 2 mg/ml of poly-L-aspartic acid and subjected to agarose gel electrophoresis (0.8% agarose, 0.5% ethidium bromide, 60 minutes, 110 V, TBE). Samples in each group (PLL(29k), PLL(205k), PEI(25k), and PEI(800k)) from left to right: polycation/DNA; PEG-polycation/DNA; PHPMA(0.2)-polycation/DNA; PHPMA(2)-polycation/DNA (o.c., open circular; s.c., supercoiled form of plasmid DNA).

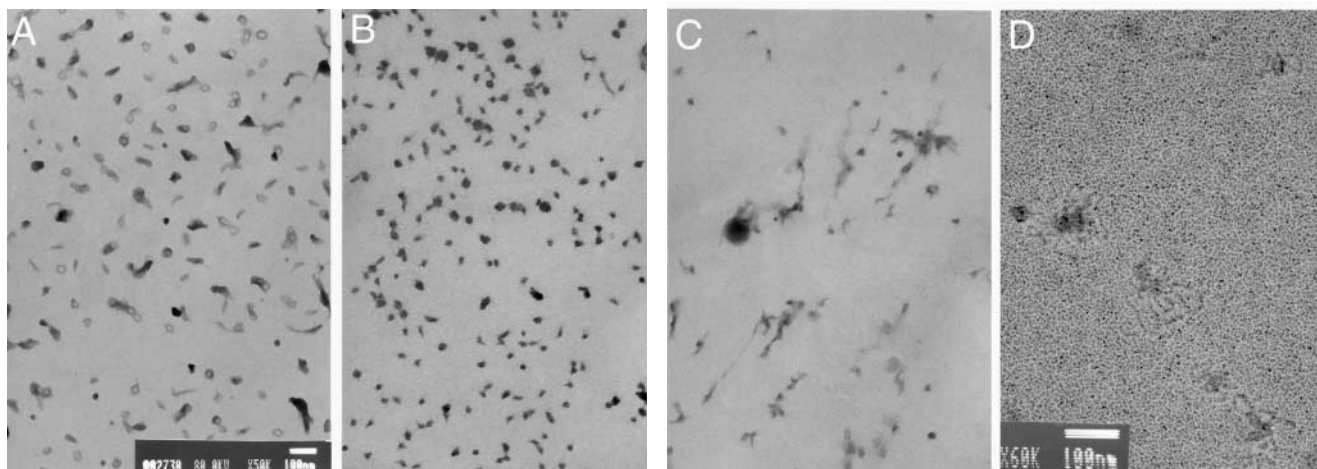


FIG. 2. Effect of surface coating on the morphology of polyplexes. Transmission electron micrographs of PLL(205k)/DNA (A), PHPMA(0.2)-PLL(205k)/DNA (B), and PEG-PLL(205k)/DNA (C) polyplexes (negative staining, $\times 50,000$ magnification, bar indicates 100 nm). (D) Detailed representation of the loose structures induced by coating of PLL(205k)/DNA polyplexes with monovalent PEG (rotary shadowing, $\times 60,000$ magnification, bar indicates 100 nm).

lateral stabilization, linking together molecules at the surface of the polyplex to prevent its dissociation by molecules that otherwise penetrate through the polymer shielding layer. Recently, multivalent reactive polymers based on PHPMA have been developed to bind around the surface of the polyplex particles, linking together surface amino groups and providing lateral stabilization against disruption [10,11]. No *in vivo* data have previously been reported for these polymer-stabilized vectors.

In this study we have compared the biophysical and *in vivo* circulatory properties of polymer-coated nanoparticles prepared by self-assembly of DNA with PLL or PEI and surface-modified with either monovalent PEG or multivalent PHPMA.

RESULTS

Biophysical Properties of Polymer-Coated Complexes

In this study we have evaluated the influence of surface modification of polyelectrolyte DNA complex nanoparticles on their physical properties, and correlated the effects with modified behavior in biological environments. Table 1 summarizes the basic physical characteristics of the polyplexes studied, based on PLL(29k), PLL(205k), PEI(25k), and PEI(800k). Although complexes based on different molecular weight polycations are reported to show significant variation in biological properties [12,13], the influences of the polycation on the physical properties of the polyplex particles are relatively minor. For example, all polyplexes based on PLL and PEI form highly positively charged (35–40 mV) particles of approximately the same size (77–85 nm) in 10 mM Hepes (pH 7.4). Similarly, despite some variation in aggregation kinetics, all the polyplex particles exhibit poor colloidal stability in salt solutions, with their z-average diameters increasing into the

micron range after 30 minutes incubation in 150 mM NaCl. A way to improve the solubility of the complexes, and simultaneously to decrease the effective surface charge, is to modify the surface of polyplex particles with reactive hydrophilic polymers. Modification with either PEG or PHPMA leads to a steric stabilization that is manifested by increased resistance to salt-induced aggregation (Table 1)—achieved using both mono- and multivalent coating polymers. The polymer coating also decreases the ζ -potential of polyplex particles, with monovalent PEG producing almost neutral surface charge while the coating with PHPMA leaves the particles negatively charged. The negative charge arises from a partial hydrolysis of reactive 4-nitrophenyl groups during the coating, yielding negatively charged carboxylate groups, and use of greater concentrations of PHPMA results in higher negative ζ -potential values. Noticeably, the properties of the polyplex particles formed are predominantly determined by the coating polymer, and are not significantly affected by the specific polycation used.

Although both monovalent and multivalent types of surface modification can endow polyplex particles with steric stability, the key difference between the two approaches is the possibility of using multivalent coating to introduce also lateral stabilization. This is most obviously shown by the resistance of polyplexes modified with multivalent PHPMA to polyelectrolyte exchange reactions. Whereas typical polycation/DNA polyplexes release free DNA following incubation with polyanions (for example, poly-L-aspartic acid (PAA); Fig. 1), polyplexes coated with PHPMA (0.2 and 2 mg/ml) are laterally stabilized and resistant to polyelectrolyte exchange reactions. In contrast, polyplexes coated with monovalent PEG show no increased resistance to polyelectrolyte exchange reactions; in fact a slight decrease in stability was observed, shown

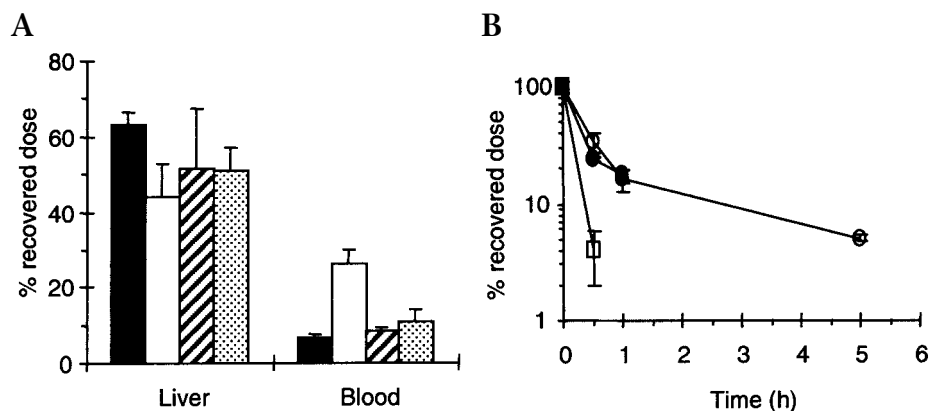


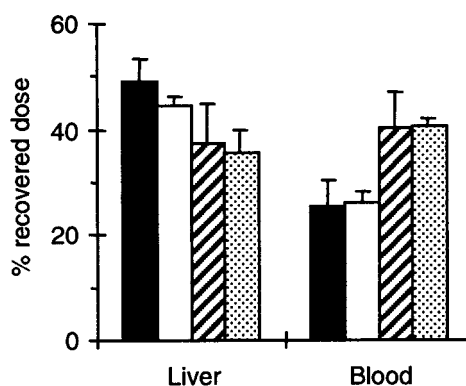
FIG. 3. Biodistribution of polycation/DNA polyplexes coated with monovalent PEG. (A) Influence of the type of the polycation on hepatic uptake and the amount of polyplexes remaining in the bloodstream 30 minutes post-injection (50 μ g DNA/mouse). PEG-PLL(29k)/DNA (filled bars), PEG-PLL(205k)/DNA (empty bars), PEG-PEI(25k)/DNA (hatched bars), PEG-PEI(800k)/DNA (dotted bars). (B) Influence of the injected dose on the blood clearance of PEG-PLL(205k)/DNA (6 μ g DNA/mouse (empty squares); 50 μ g DNA/mouse (empty circles); 100 μ g DNA/mouse (filled circles)).

by the disappearance of all residual well-associated fluorescence. Compatible with these observations, electron-microscopic analysis showed that multivalent PHPMA-coated complexes retained the compact spherical/toroidal structure of the original polycation/DNA, in contrast to the monovalent PEG-coated complexes showing looser extended structures (Fig. 2).

In Vivo Pharmacokinetics of Coated Complexes

We determined the blood clearance and biodistribution of polyplexes by including radiolabeled DNA in the polyplexes. Because of the typically very fast blood clearance of polyplexes, we have routinely compared biodistributions 30 minutes following intravenous injection. Hepatic uptake is the primary route for removal of polyplexes from the circulation, and therefore we present only the amounts of polyplexes found in the liver and blood. We routinely measured radioactivity also in other organs (spleen, kidney, lung, intestine), but these have always been < 10% of the total. It is nevertheless worth noting that uptake by the spleen was often comparable to that of the liver when expressed per gram of tissue. Analysis of the distribution between blood plasma and cellular fractions showed both PEG- and PHPMA-coated polyplexes to be present predominantly (> 95%) in the plasma.

FIG. 4. Biodistribution of polycation/DNA polyplexes coated with multivalent PHPMA. Influence of the type of the polycation on the hepatic uptake and the amount of polyplexes remaining in the bloodstream 30 minutes after intravenous injection (80 μ g DNA/mouse) for polyplexes coated with multivalent PHPMA at 0.2 mg/ml. PHPMA(0.2)-PLL(29k)/DNA (filled bars), PHPMA(0.2)-PLL(205k)/DNA (empty bars), PHPMA(0.2)-PEI(25k)/DNA (hatched bars), PHPMA(0.2)-PEI(800k)/DNA (dotted bars).



We have maintained the injection volume at 100 μ l/mouse in accordance with UK Home Office guidelines, and thus avoided possible volume-related effects on the pharmacokinetics observed. In other studies, strong volume effects have been observed in hydrodynamics-based protocols for delivery of naked DNA (injection volumes up to 2 ml/mouse [14]), and even in more conventional protocols involving PEI/DNA complexes, injection volumes of 400 μ l were required to achieve high levels of gene expression [15].

For longer-term studies of biodistribution, use of 32 P-labeled DNA may be inappropriate because of possible DNA degradation and re-metabolism of

the 32 P label giving misleading results. However, 32 P-labeled DNA can be used reliably for short-term studies, and the technique was validated here for assessment of 30-minute biodistribution studies because the results were the same as those obtained using polyplexes containing 125 I-labeled DNA (data not shown).

Polyplexes Coated with Monovalent PEG

Our previous studies have shown that polycation/DNA complexes coated with monovalent PEG are quickly cleared from the bloodstream when applied intravenously at a dose of 2 μ g DNA per mouse (~3% remaining after 30 minutes; data not shown). However, the results of Ogris *et al.* raise the possibility that improved circulation times of PEG-coated DNA complexes can be achieved by increasing the DNA dose [8]. Accordingly, we have evaluated biodistributions at a DNA dose of 50 μ g/mouse using monovalent PEG-coated polyplex particles containing each of four polycations (Fig. 3A). The

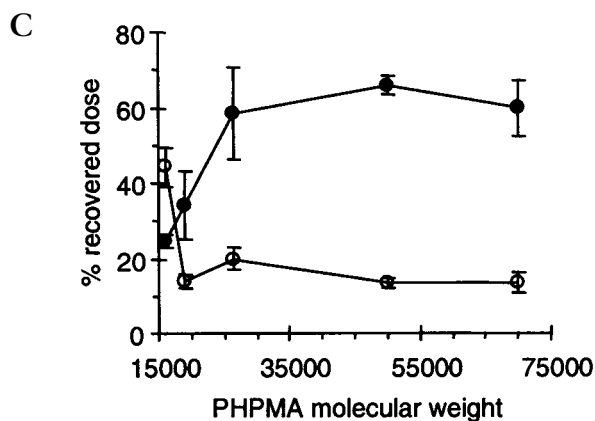
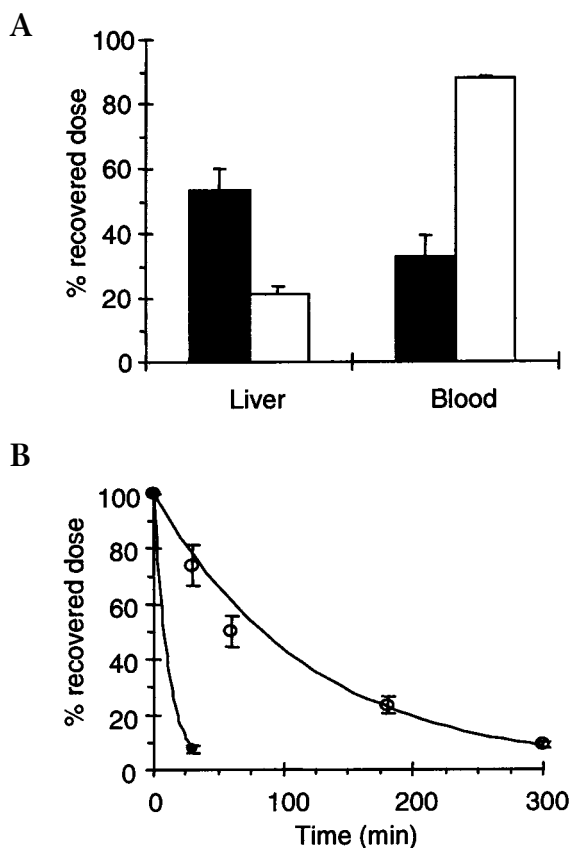


FIG. 5. *In vivo* circulation properties of multivalent PHPMA-coated PEI(25k)/DNA polyplexes. (A) Effect of concentration of multivalent PHPMA in the coating reaction on hepatic uptake and the amount of polyplexes remaining in the bloodstream 30 minutes after intravenous injection for PHPMA-PEI(25k)/DNA (80 μ g DNA/mouse); PHPMA(0.2)-PEI(25k)/DNA (filled bars), and PHPMA(2)-PEI(25k)/DNA (empty bars). (B) Time course of blood clearance of PEI(25k)/DNA polyplexes coated with multivalent PHPMA (2 mg/ml) (empty circles) and monovalent PHPMA (M_n 55K) (filled circles). (C) Effect of PHPMA molecular weight on *in vivo* distribution of PEI(25k)-DNA polyplexes. The hepatic uptake (empty circles) and the amount of multivalent PHPMA-coated PEI(25k)/DNA polyplexes remaining in the bloodstream (filled circles) were determined 30 minutes after intravenous injection (100 μ g DNA/mouse, 2 mg/ml PHPMA).

data show that simply increasing the dose of most PEG-coated polyplexes has only a limited effect on extending the circulation, showing 7–11% remaining in the bloodstream after 30 minutes for PEG-coated PEI(25k)-, PEI(800k)-, and PLL(29k)-based polyplexes, compared with ~3% at low doses. The high-molecular-weight PLL-based complex (PEG-PLL(205k)/DNA) shows considerably better plasma circulation than complexes formed using the other polycations; however, this is likely to be related to the unusual distribution kinetics of the unmodified PLL/DNA complex, which shows ~45% of the dose (1 μ g DNA/mouse) still in the bloodstream after 30 minutes, albeit bound to erythrocytes [16]. In fact a similar dose of PEG-PLL(205k)/DNA (6 μ g DNA/mouse) was cleared quickly from circulation, with only 3.9% remaining in the blood circulation after 30 minutes (Fig. 3B); hence the linkage of monovalent PEG seems actually to accelerate the clearance of this complex. This figure rose to 35% at the higher dose of 50 μ g DNA/mouse, representing a > 70-fold increase in the concentration of polyplexes in the circulation and corresponding to a DNA concentration of > 8 μ g/ml blood. Still higher doses (100 μ g DNA/mouse) led to a further increase in the absolute levels of DNA in the circulation (up to ~11 μ g DNA/ml blood) (Fig. 3B).

Polyplexes Coated with Multivalent PHPMA

In vivo application (80 μ g DNA/mouse) of PHPMA (0.2 mg/ml)-coated polyplex particles based on PLL(29k), PLL(205k), PEI(25k), and PEI(800k) produced biodistribution data shown in Fig. 4. In contrast to the use of monovalent PEG, the multivalent coating significantly improved the circulation properties of all studied polyplexes, with PEI-based polyplexes showing superior extended circulation performance to the PLL-based ones. We therefore performed more detailed studies of the relationship between structure and biological properties using PHPMA-coated complexes based on PEI(25k).

The data in Table 1 showed that increasing the coating concentration of PHPMA from 0.2 to 2 mg/ml further modifies the biophysical properties of the polyplexes (ζ -potential); accordingly we have examined whether this is reflected in the distribution kinetics of PHPMA-PEI(25k)/DNA polyplexes. Increasing the coating concentration of PHPMA from 0.2 to 2 mg/ml leads to dramatic increases in levels of polyplexes found in the circulation 30 minutes postinjection, up to 88% of the administered dose (Fig. 5A). The blood clearance profile of PHPMA-coated polyplex particles followed biexponential kinetics, with the α -half-life of > 90 minutes (Fig. 5B).

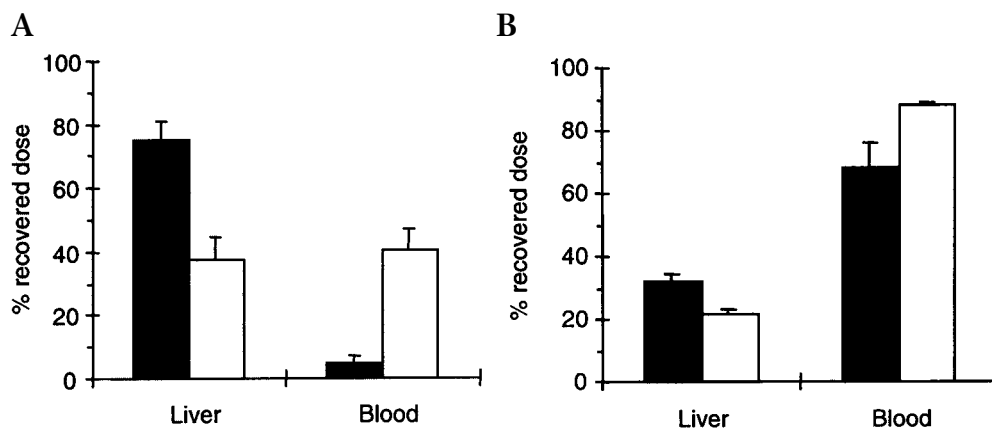


FIG. 6. Effect of injected dose on *in vivo* distribution of multivalent PHPMA-coated PEI(25k)/DNA polyplexes. The hepatic uptake and the amount of PHPMA-coated PEI(25k)/DNA polyplexes remaining in the bloodstream were determined 30 minutes after intravenous injection. (A) Polyplexes coated at 0.2 mg/ml PHPMA (PHPMA(0.2)–PEI(25k)/DNA) administered at 5 µg DNA/mouse (filled bars) and 100 µg DNA/mouse (empty bars). (B) Polyplexes coated at 2 mg/ml PHPMA (PHPMA(2)–PEI(25k)/DNA) administered at 5 µg DNA/mouse (filled bars) and 100 µg DNA/mouse (empty bars).

Apart from the concentration, the molecular weight of the coating PHPMA also significantly influenced the distribution kinetics of PHPMA-coated complexes (Fig. 5C). We tested multivalent PHPMA copolymers with molecular weights (M_r) ranging from 16,000 (16K) to 70,000 (70K) for their ability to improve pharmacokinetics, each containing 8 mol% of 4-nitrophenyl groups. We have found that PHPMA with $M_r > 30K$ is required to provide polyplexes with circulation times extended to similar levels to those shown in Fig. 5A.

We used monovalent PHPMA (similar to the PEG used, having one reactive end group) to examine the possibility that the different kinetics of polyplexes coated with multivalent PHPMA and monovalent PEG (Figs. 3 and 4) was caused by differences in chemical structures of the two coating polymers. Previously we have used monovalent PHPMA (M_r 16K) for coating PLL/DNA complexes and found no improvement in distribution kinetics [9]. However, in view of the molecular weight effect of multivalent PHPMA on biodistribution, here we have also examined whether increasing the molecular weight of monovalent PHPMA could influence distribution kinetics. Coating PEI(25k)/DNA polyplexes with monovalent PHPMA (M_r 55K) resulted in ~8% of the injected dose remaining in the blood circulation 30 minutes after injection (100 µg DNA/mouse dose; Fig. 5B), similar to the levels observed for polyplexes coated with monovalent PEG (Fig. 3A) and earlier reported for monovalent PHPMA of lower molecular weight [9]. This was a strong indication that modification with monovalent polymer is not adequate to promote extended plasma circulation, and emphasized the requirement for lateral stabilization.

The strong effect of dose administered on blood circulation for PEG–PLL(205k)/DNA (Fig. 3B) raised the possibility that similar effects might apply for multivalent PHPMA-coated polyplexes. Even in the case of PEI(25k)/DNA polyplexes coated at 0.2 mg/ml PHPMA, a high dose (100 µg DNA/mouse) is required to achieve extended circulation (Fig. 6A). However, this dose dependence was dramatically decreased when the polyplexes were

coated at the higher PHPMA concentration of 2 mg/ml. In that case even a low-dose injection (5 µg DNA/mouse) resulted in almost 70% of the dose remaining in the blood circulation 30 minutes post-injection (Fig. 6B).

Prolonged circulation times of liposomal delivery vectors are known to be an important prerequisite enabling their passive accumulation into solid tumors [17,18]. We have therefore evaluated whether long-circulating polyplexes (PHPMA(2)–PEI(25k)/DNA) can accumulate in subcutaneously inoculated B16F10 tumors in C57BL/6 mice. The time course of tumor accumulation (Fig. 7) documents significant levels of complexes accumulated in the tumor over the period of 24 hours, although it is likely that the majority of accumulation occurs at earlier times, when there are high amounts of polyplex particles circulating in the bloodstream. The observed levels of accumulation (6.5% per g tumor) are similar to those observed for non-targeted liposomal DNA vectors [19], and are thought to result from hyperpermeability of tumor-associated vasculature coupled with ineffective drainage of tissue fluid from the tumor interstitium [20].

DISCUSSION

Delivery through the bloodstream represents the only means to gain access to disseminated and widespread disease targets. The development of long-circulating nonviral vectors for gene delivery can therefore facilitate a number of therapeutic strategies. However, the biophysical characteristics of polyplex particles like those based on PLL and PEI [12,21] make them unsuitable for systemic intravenous application, despite their usefulness for *in vitro* transfection. Their poor solubility in physiological salts, coupled with nonspecific charge-mediated interactions with proteins and cells, leads to rapid clearance from the bloodstream and precludes the possibility of targeting to specific organs [4,22,23]. Some success has been achieved in obtaining transgene expression following intravenous administration, although most of the activity has been localized to sites of first clearance (notably

the first capillary bed encountered) [15,23–26]. To achieve the possibility of systemic targeting, rapid elimination from the bloodstream must be prevented and the low AUC values typical for polyplexes must be increased.

Plasma pharmacokinetics of particulate delivery vectors are known to be influenced by their surface characteristics [17,18]; hence, design of polyplexes capable of efficient systemic gene delivery requires precise control of their surface properties to regulate their interactions with proteins and cells. The main objective of this study was therefore to modify the surface of PEI/DNA and PLL/DNA polyplex particles, stabilizing them against unwanted interactions with cells and proteins, and to correlate these biophysical properties with extended systemic circulation.

Several previous studies have sought to improve the surface properties of polyplex particles by incorporating shielding molecules during the initial self-assembly with DNA. However, despite showing promising results *in vitro*, these approaches have not achieved better distribution kinetics *in vivo* [6,8,27–31]. An alternative approach involves coating preformed polyplex particles with reactive polymers such as monovalent PEG, to avoid trapping the polymer during the condensation process. However, this procedure also does not result in extended plasma circulation of polyplexes. In this study we have addressed the hypothesis that the introduction of lateral stabilization, using multivalent coating polymers, provides better stability to polyplex particles than simple steric stabilization, and could better promote extended circulation in the bloodstream. This hypothesis has been addressed by comparing properties of polyplexes coated with monovalent and multivalent reactive polymers encompassing a broad range of polymer molecular weights, evaluated using both PLL/DNA and PEI/DNA polyplexes applied intravenously to mice at high and low doses.

Table 1 shows that the positive zeta potential of the polyplex particles can be suppressed by coating with either monovalent PEG or multivalent PHPMA, and that both approaches lead to the steric stabilization against salt-induced aggregation. However, electron-microscopic study of the PEG-coated polyplexes indicated the presence of relatively poorly condensed structures, a property that may be reflected also in their decreased stability against polyelectrolyte exchange reactions—that is, decreased strength of the polycation/DNA interaction (Fig. 1). This is reminiscent of the weakened polycation/DNA interaction based on graft copolymers of PEG–PLL, compared to PLL-based complexes [23], raising the possibility that internal reorganization of the polyplex particles after coating with monovalent polymers leads to production of similar, relatively unstable, polyplex structures in both cases.

In contrast, surface modification of preformed polyplex particles with multivalent polymer PHPMA maintains the improved properties introduced by monovalent coating and extends them by providing the complexes with lateral stability. This is achieved by individual multivalent

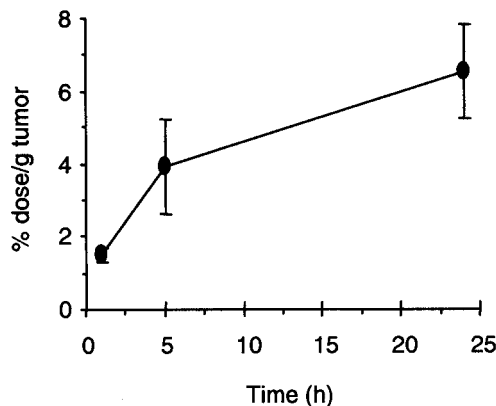


FIG. 7. Time course of tumor accumulation of intravenously injected PHPMA(2)–PEI(25k)/DNA in C57BL/6 mice bearing subcutaneous B16 melanoma (100 μ g DNA/mouse).

reactive polymers binding cooperatively to amino groups on the surface of the preformed polyplex particles, effectively crosslinking the surface. One result is a considerable increase in resistance against polyelectrolyte exchange reactions (Fig. 1) that cannot be achieved using monovalent polymers.

Modified biodistribution properties of nonextravasating polyplexes *in vivo* can be used as a direct measure of their interactions with components of the bloodstream [32] and are therefore an important complement to *in vitro* tests. Polyplex particles coated with monovalent PEG display steric stability and neutral charge that decreases non-specific interactions *in vitro*; however, their rapid elimination from the bloodstream *in vivo* indicates that other variables have a major influence on biodistribution. Only monovalent PEG-coated polyplexes of PLL(205k) show increased blood circulation *in vivo*; however, these polyplexes show good circulatory properties even when not coated, for reasons that are not completely clear [16], and the presence of the PEG actually accelerates their clearance from the bloodstream. The observation that PEG–PLL(205k)/DNA circulates significantly better at higher doses raises the hypothesis that the elimination mechanism may be saturable. This idea is also supported by results of several groups working in related fields who found that liposomal circulation times improve at higher doses [33,34].

Our observation of the rapid blood clearance of PEG–PEI(800k)/DNA contrasts with extended plasma circulation times already published for similar polyplexes [8]; in these studies the authors hypothesized that the circulatory properties are due to the presence of PEG decreasing the surface charge. In a later report self-assembling polyplexes based on transferrin–PEI conjugates (without PEG) gave similar circulatory properties [35], although the results were again explained by the reduction of positive

surface charge due to the transferrin. However, in the present study a number of neutral PEG-coated polyplex particles still showed very fast clearance, even at relatively high doses. This challenges the hypothesis that surface charge is the only determinant of circulatory properties.

In contrast to coating with monovalent PEG, coating with multivalent PHPMA results in improved circulatory properties for all polyplexes examined, irrespective of the type of polycation used and its molecular weight. In a series of control experiments we have confirmed that the improvement in circulatory properties of the PHPMA-coated polyplexes is not primarily due to their negative charge or the chemical nature of the coating polymer but instead is a consequence of the lateral stabilization endowed by the coating polymer. Indeed we have demonstrated the importance of lateral stability in two other studies: In the first we have shown that crosslinking amino groups on the surface of polyplex particles before coating with monovalent PEG significantly prolongs their circulation times *in vivo* [36]; in the second study, we have shown that when polyplex particles are surface-modified with a multivalent PEG [37], similarly improved circulatory properties are obtained as demonstrated here with multivalent PHPMA (C. M. Ward, M. Pechar, D.O., K.U., and L.W.S., manuscript submitted). We assume that the low stability of polyplexes modified with monovalent PEG against polyelectrolyte exchange reactions makes their integrity easily compromised in the bloodstream, leading to a partial loss of the PEG coating and subsequent recognition and fast removal by the RES.

Because PEI/DNA polyplexes coated with multivalent PHPMA reproducibly performed better than PLL-based ones we have selected PEI(25k)-based polyplexes for further optimization of distribution properties. We have shown that efficient lateral stabilization and subsequent prolonged circulation of the coated polyplexes demands the use of PHPMA with minimal molecular weight of 30K, and that the concentration of PHPMA employed plays a vital role in enabling prolonged circulation. Using 2 mg/ml concentration of PHPMA leads not only to significantly increased circulatory half-life compared with 0.2 mg/ml, but most importantly these polyplexes exhibit long-circulating properties even at low administered doses. The precise reasons for this effect are not yet fully understood. The increased amount of PHPMA bound to the polyplexes at 2 mg/ml, combined with possibly further improved lateral stability, are most likely one of the important reasons.

Having achieved stability of polyplexes in biological fluids, allowing extended circulation and receptor-mediated targeting, it is necessary that the vectors can undergo efficient transcription following their entry into target cells. To satisfy the contradictory requirements for high stability in the extracellular environment and easy intracellular availability of DNA [38], the design of the vector should incorporate a specific intracellular trigger mechanism that would reverse the lateral stability induced by

multivalent PHPMA. We have recently developed such an approach in our group, exploiting the dependence of the lateral stabilization on the molecular weight of the polycation complexed with DNA in the core of the polyplex particle. The strategy of reversible stabilization utilizes the increased reductive capacity of the intracellular environment, whereby following entry into cells, a newly synthesized degradable polycation undergoes reductive degradation to lower-molecular-weight species and the stabilizing effect of the polymer coating is lost, enabling efficient transcription of the delivered DNA [39].

Vectors based on laterally and sterically stabilized polyplex particles provide a promising platform for targeted systemic delivery of genes. The possibility of combining extended plasma circulation with easy attachment of targeting ligands [10,11], and the use of trigger mechanisms based on activation of the vectors following arrival within target cells, provides an enticing means to achieve systemically targeted transfection that could translate into therapeutic activity against disseminated targets.

MATERIALS AND METHODS

Chemicals and DNA. A circular 5.2-kb plasmid vector pGL3-control (Promega UK, Southampton) containing the simian virus-40 (SV40) promoter-driven luciferase reporter and ampicillin resistance genes was used throughout the study. This was prepared by growth in *Escherichia coli* DH5 α and purified using Qiagen EndoFree Plasmid Giga kit (Qiagen, Dorking, UK).

Poly-L-lysine hydrobromide was from Sigma Chemical Co. (Poole, UK). PEI and O-[2-(N-succinimidylloxycarbonyl)-ethyl]-O'-methylpolyethylene glycol (5000) (PEG) were from Fluka (Dorset, UK). Azo-bis-isobutyronitrile (AIBN), 4-nitrophenol, and dicyclohexylcarbodiimide (DCCI) were from Fluka AG (Buchs, Switzerland).

Copolymer of N-(2-hydroxypropyl)methacrylamide with 8 mol% of methacryloylglycylglycine 4-nitrophenyl ester (M_n 76K) (PHPMA) was a gift from Polymer Laboratories (Church Stretton, UK). We used this copolymer for all the experiments except the study of the influence of PHPMA molecular weight on the biodistribution.

We prepared polymer precursors (copolymers of HPMA (prepared as described in [40]) with methacryloylglycylglycine (MAGlyGlyOH)) differing in molecular weights in methanol containing 7 vol% dimethylsulfoxide (DMSO) by radical solution polymerization in sealed ampoules at 58°C. The monomer concentration was 18 wt%, with 9 mol% molar content of MAGlyGlyOH. We prepared the sample with M_n 70K in the same way, but in 100% DMSO. Polymerization time was 24 hours. We controlled the molecular weight of the copolymers by changing the initiator (AIBN) concentration in the range 0.1–1 wt%. We isolated the polymers by precipitation into acetone–diethylether mixture and further purified by reprecipitation into acetone. We prepared polymer 4-nitrophenyl esters by esterification of carboxylic groups of the polymer precursors with 4-nitrophenol in *N,N*-dimethylformamide using the DCCI esterification method (sixfold excess of 4-nitrophenol and DCCI). We isolated the reactive polymers by precipitation into acetone–diethylether mixture and purified by reprecipitation into acetone.

We characterized the polymer precursors and reactive polymers by the content of COOH groups (titration using an automatic titration system Radiometer Copenhagen TIM900), content of 4-nitrophenoxy groups (UV spectrophotometry at 274 nm, DMSO, ϵ = 9500 M $^{-1}$ cm $^{-1}$), and molecular weight (AKTA-Pharmacia equipped with Superose 6 or TSK 5000 PW columns, RI (refractive index) and multiangle light scattering DAWN-DSP-F (Wyatt Technology Corp.) detectors). We prepared copolymers with number average molecular weights of 16K, 19K, 26.3K, 50K, and 70K.

Radiolabeling of DNA expression vectors. We used two methods for radiolabeling DNA. To prepare ^{32}P -labeled DNA, we linearized plasmid DNA with *Hind*III and then radiolabeled with [^{32}P]dCTP using the Ready-to-Go Oligolabeling kit (Pharmacia Biotech, St. Albans, UK). We removed the unincorporated nucleotides using Sephacryl S-200 MicroSpin columns (Pharmacia Biotech) and checked the purity of the labeled linear DNA following agarose gel electrophoresis and quantitative analysis with a PhosphorImager (Molecular Dynamics, Chesham, UK). Alternatively, we labeled DNA with ^{125}I using a modified Commerford method, allowing preservation of the supercoiled and circular structure of plasmid DNA [41]. In this method, typically, 20 μg plasmid DNA were mixed with 100 μCi of sodium iodide-25 in the presence of thallium trichloride in ammonium acetate buffer (pH 5). The radiolabeled DNA was purified using Tip20 anion-exchange column (Qiagen). We determined the yield and specific activity of iodinated DNA from 260 nm absorbance, γ -counting, and agarose gel electrophoresis analyzed by PhosphorImager.

Formation of polyplexes. We prepared polyplexes by mixing equal volumes of the polycation and DNA solutions in 10 mM Hepes (pH 7.4) to achieve a final DNA concentration of 20 $\mu\text{g}/\text{ml}$. We used PLL of M_r 29K and 205K (PLL(29K) and PLL(205K)) and PEI of M_r 25K and 800K (PEI(25K) and PEI(800K)). We prepared PLL–DNA complexes at an N:P ratio (the ratio of polycation amino groups to DNA phosphates) of 2.0 and PEI–DNA complexes at an N:P ratio of 5.0. We allowed the complexes to form for at least 30 minutes at room temperature before use.

Coating polyplexes with monofunctional PEG and multifunctional PHPMA. To introduce the multivalent polymer coating, we added multivalent PHPMA (20 mg/ml in water) to the preformed polycation/DNA complexes followed by the addition of 1 M Hepes buffer (pH 7.8) to give a final HEPES concentration of 50 mM and PHPMA 0.2 or 2 mg/ml (corresponding polyplexes abbreviated: PHPMA(0.2)–polycation/DNA and PHPMA(2)–polycation/DNA). We monitored the reaction by measuring decrease of absorbance of polymer-bound 4-nitrophenoxy groups at 274 nm (combined contribution of coating reaction, hydrolysis, and reaction with free polycation). The coating reaction was allowed to proceed overnight at room temperature, with remaining reactive groups aminolyzed with twofold excess of aminoethanol, added 2 hours before the use of the polyplexes.

We carried out coating with PEG by addition of *N*-hydroxysuccinimidyl ester of PEG(5000) (20 mg/ml in water) to the complexes in 10 mM Hepes, pH 7.4, applying a 30-fold weight excess (PEG to polycation).

We quantified the content of reactive amino groups in the polyplexes before and after coating reactions by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described [36]. In the case of PHPMA coating, we made a correction for the absorbance of free 4-nitrophenolate produced during the coating reaction.

Measurement of hydrodynamic size of polyplexes. We determined the size of polyplexes by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer-1000 with a 50-mW solid-phase internal laser (wavelength 488 nm) (Malvern Instruments Ltd., Malvern, UK). We performed the analysis at 25°C in 50 mM Hepes buffer in triplicate with sampling time set to automatic and analyzed using CONTIN algorithm. The results are reported as *z*-average diameters of the complexes obtained from three measurements \pm SD.

Measurement of ζ -potential of polyplexes. We measured zeta potentials of polyplexes (in 50 mM HEPES buffer) using a Zetamaster (Malvern Instruments Ltd.) equipped with a 5-mW internal laser.

Determination of morphology of polyplexes by transmission electron microscopy. We dispensed solutions of polyplexes (10 μl) onto paraffin and placed a piece of freshly cleaved mica (Agar Scientific, Stanstead, UK) on top for 2 minutes. We removed the mica and placed it on a drop of 2% solution of uranyl acetate for an additional 2 minutes, then washed twice in water and allowed to dry. We carried out the rotary shadowing of some samples with platinum in a High-Vacuum Coating Unit (5° angle, 6 cm distance, rotation at 60 rpm) (Edwards, Crawley, UK). We cut the sheet of mica into sections and floated these on water to release the sample, which we then picked up using sticky grids (carbon 200 mesh), and allowed to

dry. We viewed the samples using a Joel 1200 EX transmission electron microscope.

Evaluation of the stability of polyplexes to polyelectrolyte exchange reactions. We evaluated the stability of complexes against polyelectrolyte exchange reactions with PAA (M_r 35K) by monitoring the release of free DNA using agarose gel electrophoresis. We incubated the polyplexes (20- μl aliquots) with PAA (5 μl , 10 mg/ml) at 37°C for 30 minutes and analyzed them on the gel (0.8% agarose, 0.5% ethidium bromide, 60 minutes, 110 V, TBE). We then visualized and analyzed the gel on a Typhoon fluorescence scanner (Pharmacia, UK; 50 μm resolution, λ_{ex} 530 nm, λ_{em} 610 nm).

Determination of plasma clearance and body distribution. We used 6-week-old female BALB/c mice (20–25 g) for routine pharmacokinetic studies, whereas we used female C57BL/6 mice for tumor accumulation experiments. In the latter case we inoculated the mice subcutaneously with 1 million B16F10 cells, and used them for experiments 8–10 days later when the tumor weights reached 0.5–1 g. We examined body distributions and plasma clearance kinetics using plasmid DNA (pGL3-control) spiked with either ^{32}P - or ^{125}I -labeled plasmid before addition of a polycation. We then formed and coated the polyplexes as described above. In addition, we then concentrated the polyplexes several hours before use to 1 mg DNA/ml using VivaSpin20 centrifugal concentrators (M_r cutoff 100K). We checked the size of the polyplexes after concentration by DLS and observed only minimal increase in sizes, while recovery was typically > 95% as measured by radioactivity content. We then diluted the coated complexes with 10% (vol/vol) of 50% glucose and administered them intravenously, via the tail vein, to mice briefly anesthetized with fluorothane.

For measurement of blood clearance, we isolated serial blood samples (20 μl) at different times from the tail vein using heparinized glass capillaries. In most cases we killed the animals 30 minutes post-injection and determined the body distribution of radioactivity. We dissolved the isolated organs in 10 M sodium hydroxide. For determination of ^{32}P content, we added scintillation medium Ultima-Flo (20 ml) to each blood (20 or 50 μl) or tissue (1 ml) sample before geometry-corrected analysis in a Packard scintillation counter. We measured the radioactivity content in tissue samples containing ^{125}I by γ -counter using a standard 1-ml sample volume.

We separated red blood cells by centrifuging EDTA-treated blood samples (isolated post mortem) at 5000 rpm for 1 minute and measured the distribution of radioactivity between blood plasma and cellular fraction.

We used a blood volume of 8.6 ml/100 g body weight for calculating the total amount of radioactivity in the bloodstream. We present the amount of radioactivity in each tissue as the mean and the standard deviation of the total recovered dose obtained in triplicate injections.

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