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NONVIRAL TRANSFER TECHNOLOGY ***RESEARCH ARTICLE***

Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery

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Uncontrolled interactions of gene vectors and drug carriers in and with an in vivo environment pose serious limitations to their applicability. In order to reduce such interactions we have designed, synthesized and applied novel copolymers of poly(ethylene glycol) and reactive linkers which are derivatized with anionic peptides after copolymerization. The anionic copolymer derivatives are used to coat positively charged nonviral gene vectors by electrostatic interactions. The copolymer coat confers to polyelectrolyte colloids of DNA and polycations steric stabilization in their minimal size and prevents salt- and serum albumin-induced aggregation. Furthermore, complement activation and the interaction with serum proteins are drastically reduced or abolished in contrast to unprotected DNA complexes. The designed vectors are compatible with the intracellular steps of gene delivery and can even enhance transfection efficiency as demonstrated with various adherent and nonadherent cell lines in culture. The synthetic concept is amenable to the principles of combinatorial chemistry and the copolymeric products may be applicable beyond gene delivery in targeted drug delivery. Gene Therapy (2000) **7,** 1183–1192.

Keywords: *nonviral gene vectors; opsonization; protective copolymers; steric stabilization; complement activation; gene delivery*

Introduction

Although nonviral gene vectors are efficient *in vitro* as well as in some animal models, their uncontrolled and often undefined interactions under physiological conditions still represent a major obstacle to their application in gene therapy. In particular, it has been shown that nonviral gene vectors or their constituents interact strongly with blood components such as the complement system[1] and other blood proteins.[2] Such opsonization alters the physico-chemical characteristics of vectors, may interfere with vector targeting and is of concern if vectors are to be applied in humans. Consequently, one major objective in nonviral vector development is to devise vectors which are inert in the *in vivo* environment during the delivery phase. Gene delivery *in vivo* comprises an extracellular and an intracellular delivery problem where solutions to one part must be compatible with the requirements of the other. To overcome some of the intracellular barriers, nonviral vectors are designed to mimick important features of viral cell entry. Synthetic modules for receptor targeting, DNA binding and compaction and intracellular release are assembled with DNA in a stepwise manner by electrostatic interactions. In liposome[3] and nanoparticle technology,[4] poly(ethylene glycol) has been used to confer to these drug carriers the desired stability during the extracellular delivery phase. For the same purpose, PEG has been grafted covalently to preassembled polycation–DNA complexes.[2] It was the aim of the work presented here to develop a new class of protective copolymers (PROCOPs) based on PEG which are assembled with nonviral gene vectors by electrostatic interaction (Figure 1). Such polymers are derived from PEG by copolymerization with amino acids, amino acid derivatives or with peptides and optional subsequent derivatization. The synthesis can be carried out by estab-

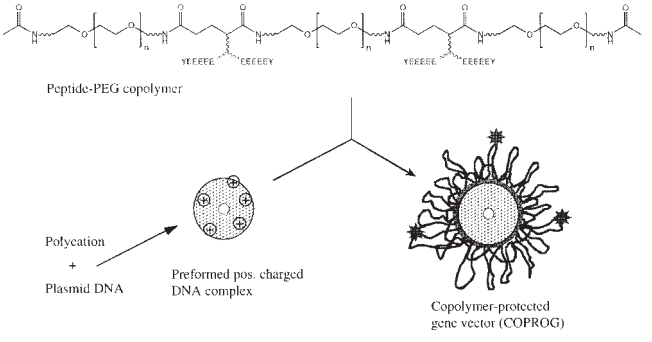

Figure 1 *Preparation of copolymer-protected gene vectors (COPROGs). A preformed lipoplex or polyplex with excess positive charge is incubated with a negatively charged peptide–PEG copolymer resulting in a surface layer of PEG loops. The copolymer backbone can be reacted with receptor ligands and other effector molecules (symbolized by asterisks).*

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lished methods of peptide chemistry. In this communication we describe one class of PROCOPs which is synthesized by coupling anionic peptides to a reactive copolymer backbone. We show that copolymers obtained with the peptides YE5C ($[Ac\text{-}YEEEEE]_2K$-ahx-C; ahx = 6-aminohexanoic acid, Ac = acetyl) and INF7 (GLFEA IEGFIENGWEGMIDGWYGC[5]), stabilize polyplexes at small size and protect from complement activation and opsonization. Finally, we demonstrate that protecting nonviral vectors with these copolymers is compatible with the cellular steps of gene delivery.

Results

Copolymer synthesis

Standard peptide chemistry was applied to synthesize 3-(2′-thio-pyridyl)-mercaptopropionyl-glutamic acid (compound 3, Figure 2a) as a building block for copolymerization with commercially available diamino-PEG of various molecular weights. The polymerization reaction yields a strictly alternating copolymer backbone with reactive side chains for further derivatization. The degrees of oligomerization of the main products (50% of total product) were 6 to 7 as calculated from the retention volumes on a calibrated Superdex 75 column. Minor higher molecular weight byproducts were separated using the same column. The calculated degrees of polymerization were consistent with the observed differences in retention volumes before and after coupling effector peptides to the reactive copolymers. The effector peptides in this study were YE5C, and INF7,[5] resulting in the products P3YE5C, P6YE5C and P3INF7, P6INF7, respectively (Figure 2b; P3 refers to PEG3400-derived, P6 refers to PEG6000-derived products).

The synthetic concept is amenable to combinatorial chemistry by systematic variation of the chain length of the PEG macromonomer, the type of linkage in the polymer backbone (eg amide *versus* ester), the overall degree of polymerization and the peptide type as well as its charge. Polymers appropriate for gene delivery can be selected using aggregation assays, complement activation and gene transfer assays in 96-well plate format such as described below.

PROCOPs P3INF7 and P6INF7 display pH-dependent membrane-destabilizing activity

The membrane-destabilizing activities of influenza and related peptides are examined using an erythrocyte lysis assay.[5] The purification of P3INF7 and P6INF7 by size exclusion chromatography yielded fractions that were substantially more active in the erythrocyte lysis assay than the starting peptide INF7 at pH 5. At pH 7.4 all products were inactive (Figure 3).

PROCOP-coated polyplexes are small and stable colloidal particles

Polymer-protected DNA complexes were assembled according to Figure 1. Binding of PROCOPs to preassembled polyplexes and lipoplexes was demonstrated in direct and indirect ways. Biospecific interaction analysis (BIAcore) experiments demonstrated that PROCOPs bind to lipoplexes and polyplexes (data not shown). Zeta potential determinations confirmed binding in that the highly positive zeta potentials of lipoplexes and polyplexes decreased as a function of the amount of PROCOPs added to preassembled DNA complexes (Figure 4). At no point of lipoplex or polyplex composition solubility problems were encountered such as observed with DNA complexes in the absence of PROCOP at electrostatic neutrality. PROCOP addition to preassembled polylysine or PEI polyplexes as well as DOTAP-cholesterol lipoplexes does not dissociate these gene vectors as judged by a gel retardation assay (data not shown). Polyplexes remain tightly condensed as is demonstrated by electron microscopy (Figure 5) and dynamic light scattering (Table 1). Both determination methods consistently indicated that copolymer-protected gene vectors (COPROGs)

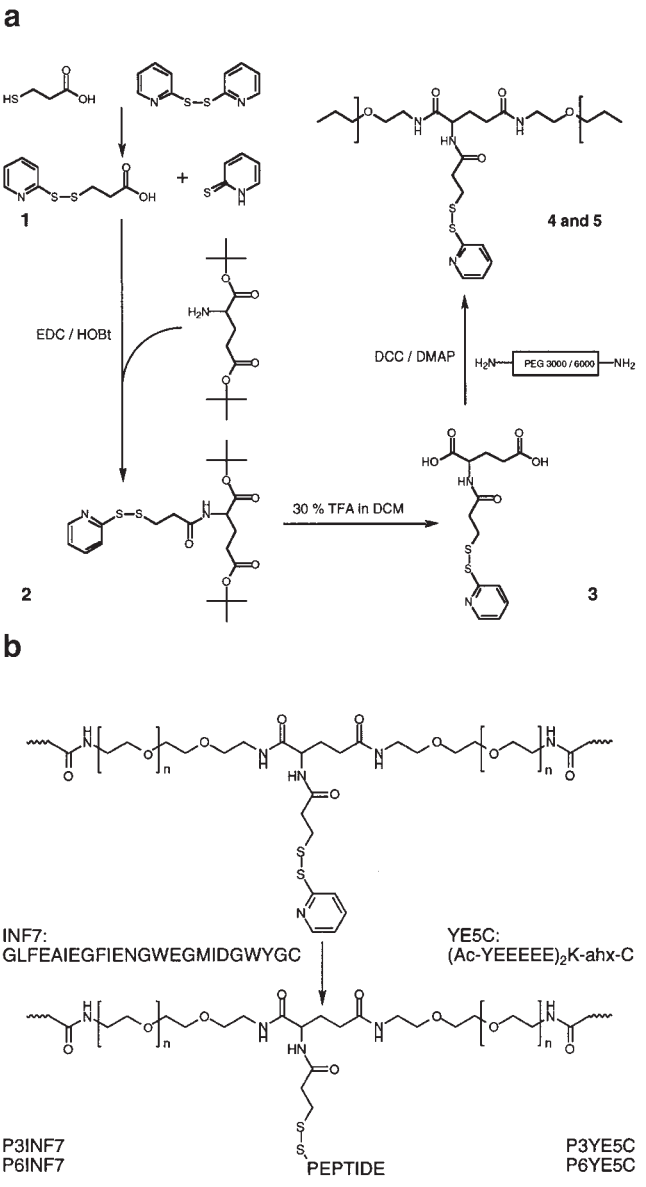

Figure 2 *(a) Synthesis of the reactive copolymer backbone. 3-(2′-thio-pyridyl)-mercaptopropionyl-glutamic acid (3) is derived in a standard carbodiimide-activated coupling reaction from t-butyl-protected glutamic acid and 3-(2′-thio-pyridyl)-mercaptopropionic acid (1). After deprotection, product (3) is copolymerized with diamino-PEG upon DCC activation in situ. (b) The purified thiol-reactive polymer backbone is reacted with negatively charged cystein-containing peptides. INF7, sequence GLFEAIEGFIENGWEGMIDGWYGC; YE5C, sequence* $(Ac\text{-}YEEEEE)_2$K-ahx-C, ahx = 6-aminohexanoic acid, Ac = acetyl.

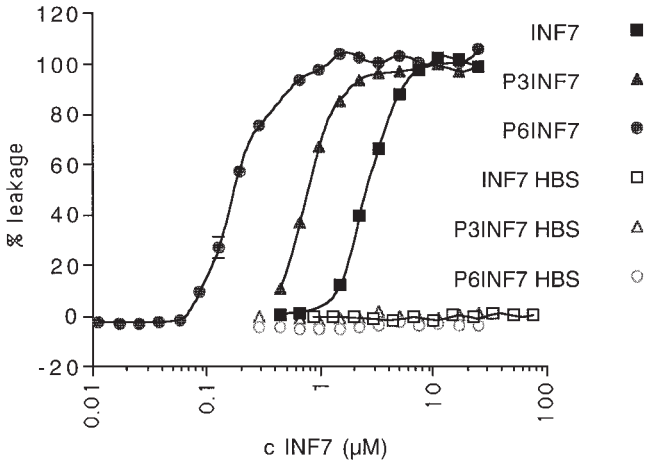

Figure 3 *Erythrocyte lysis assay (citrate buffer, pH 5, filled symbols). Quantities of polymers were applied so that equal amounts of peptide INF7 are compared. X-axis: μmolar concentration of peptide in the assay mix. No leakage is observed at pH 7.4 (open symbols).*

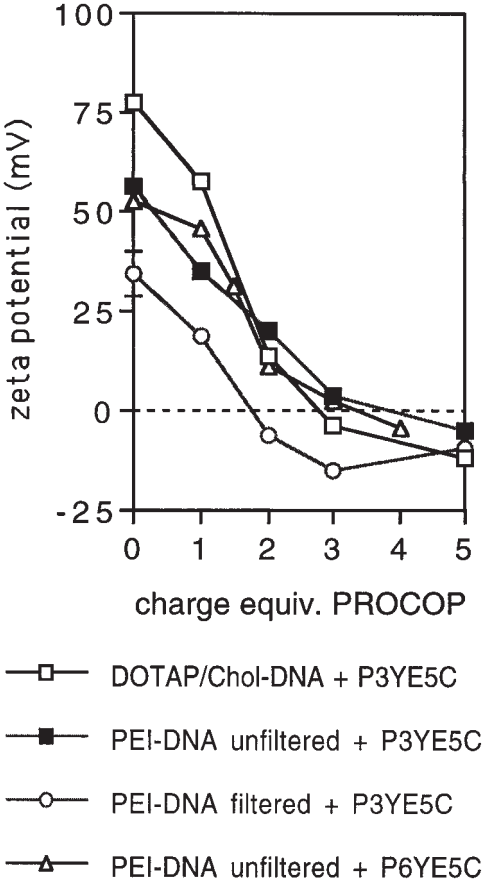

Figure 4 *Zeta potentials of COPROGs. Purified and unpurified PEI–DNA complexes (initial N/P = 8) and DOTAP/cholesterol (charge ratio 5) lipoplexes with increasing amounts of P3YE5C or P6YE5C added (amounts of PROCOP are given in equivalents of negative charges relative to the negative charges of DNA in the complex). Shown are mean values of at least four measurements with error bars too small to be seen.*

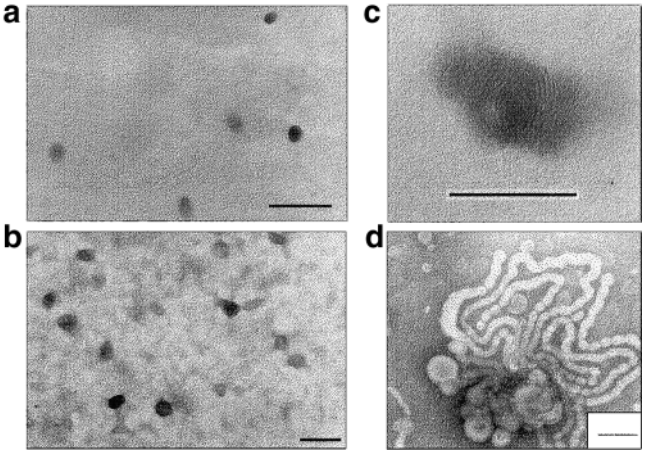

Figure 5 *Electron micrographs of COPROGs. Bars indicate 100 nm. (a) PEI–DNA, N/P = 8, in the presence of 3 charge equivalents of P3YE5C; (b) in the presence of 2 charge equivalents P3YE5C and 1 mg/ml BSA after overnight incubation; (c) DOTAP/cholesterol–DNA lipoplex charge ratio (±) 5; (d) the same in the presence of 5 equivalents P6YE5C. All complexes were prepared in 20 mM HEPES pH 7.4.*

Table 1 Particle sizes in nm determined by dynamic light scattering (DLS) in the absence and in the presence of 150 mM sodium chloride or 1 mg/ml BSA

	20 mM HEPES pH 7.4	*+150 mM NaCl*	*+BSA 1 mg/ml*
PEI–DNA	47.9 ± 3.8 (n = 9)	>2000 within 2 h	Precip
PEI–DNA filt	58.0 ± 7.0 (n = 3)	>2000 within 2 h	Precip
PEI–DNA + 2 equiv P3YE5C	35.3 (n = 2)	Slow aggreg 81.90 ± 6.2 (n = 11) 162.5 ± 1.8 (n = 5; o/n)	33.2 (n = 2)
PEI–DNA + 2 equiv P6YE5C	50.9 ± 5.3 (n = 10)	43.0 ± 4.0 (n = 8)	41.6 ± 4.6 (n = 4)
pL–DNA	50.0 ± 4.6 (n = 9)	>2000 within 1 h	ND
pL–DNA + 6 equiv P3INF7	72.5 ± 3.3 (n = 4)	86.3 ± 2.6 (n = 6)	ND
pL–DNA + 6 equiv P6INF7	61.7 ± 0.6 (n = 13)	ND	ND

ND, not determined; o/n, overnight.

prepared from PEI and pL are extraordinarily small particles with a remarkable monodispersity (22.7 ± 4.3 nm nm by electron microscopy of PEI–DNA, Figure 5a; 50–70 nm for pL–DNA by DLS, Table 1). According to an estimate published by Blessing *et al*[6] the observed structures are most likely monomolecular DNA complexes (ie one DNA molecule per particle) in the case of PEI-DNA. Very small particles (<50 nm) can easily be achieved with PEI-DNA in salt-free buffer of appropriate pH even in the absence of PROCOPs. However, these unprotected complexes aggregate immediately upon salt or BSA addition (sodium chloride addition to a final concentration of 150 mM; BSA final concentration 1 mg/ml, see below). In contrast, copolymer-protected polyplexes are sterically stabilized (37.0 ± 6.4 nm by electron microscopy, Figure 5b; 33.2 nm by DLS, Table 1). In salt-

free, as well as in salt-containing buffer and even in the presence of BSA the initial small and monodisperse particle size is retained upon storage of fully assembled COPROGs at 4°C for at least several days. Contrary to polyplexes, the shape of DOTAP/Chol lipoplexes changes dramatically upon PROCOP addition. Extended tubular structures are formed in salt-free buffer (Figure 5d) and the lipoplexes aggregate upon salt addition.

PROCOPs prevent opsonization of nonviral gene vectors

From previous studies it was known that during PEI–DNA complex formation excess PEI is only loosely associated with DNA so it can be removed by ultrafiltration using Centricon-100 filter devices (PE and J-SR, unpublished results). We examined ultrafiltrated and unpurified PEI–DNA complexes for their susceptibility to opsonization. In typical experiments 40–60% of the applied PEI was removed by ultrafiltration when complexes were formed at an N/P (nitrogen/phosphate) ratio of 8, while 80–90% of the initial amount of DNA were recovered as purified PEI–DNA complex in the retentate. As expected from the molecular weight cut-off of the filter membrane, no DNA was found in the filtrate. Particle sizing by electron microscopy and dynamic light scattering indicated a slight increase in particle size upon filtration (43.6 ± 4.4 nm by EM, not shown; 58.0 ± 7.0 nm by DLS, Table 1).

At concentrations below those prevailing in serum (which are 35–50 mg/ml), BSA can be used as a probe to assess opsonization of polyplexes. For both purified and unpurified PEI–DNA complexes binding of saturating amounts of bovine serum albumin manifests itself in a reduction of the zeta potential from around +50 mV to around −16 mV. Depending on the ratio (w/w) of polyplex to BSA, visible aggregation (opalescence) and precipitation are observed. For a PEI–DNA concentration of 40 μg of DNA per milliliter, the aggregation and precipitation range extends to about 5 mg/ml BSA, while at higher BSA concentrations the polyplex stays in solution (with BSA bound to its surface). If purified PEI polyplexes are coated with PROCOPs (3 charge equivalents of P3YE5C or P6YE5C), the zeta potential remains at around −6 mV to −7 mV upon BSA addition and aggregation and precipitation are prevented, indicating that no BSA binds to the vector. For unpurified PEI polyplexes, PROCOPs do prevent BSA-induced aggregation, however, the zeta potential is reduced to −26 mV upon BSA addition, indicating BSA binding due to incomplete protection.

Purified and unpurified (not shown) PEI polyplexes (N/P = 8) activate the complement system (Figure 6a and b). If purified polyplexes are coated with PROCOPs, complement activation is inhibited dependent on the amount of PROCOP added to the pre-assembled DNA complex. PROCOP P6YE5C (prepared from PEG6000, Figure 6b) is more efficient in this respect than P3YE5C (PEG3400) (Figure 6a). In the same concentration range unpurified PEI polyplexes are protected to some degree but still display complement activation according to the hemolytic assay (data not shown). Polylysine polyplexes are protected from complement activation by the influenza peptide PROCOP P6INF7, again dependent on the amount PROCOP present (Figure 6c).

Complement activation is just one aspect of opsonization. Therefore, surface plasmon resonance measurements[7] (BIAcore technology) were used to assess directly general serum protein binding to DNA complexes. Whole serum was coupled to a CM5 research grade sensor chip by EDC/NHS activation. This sensor chip was examined for binding of PEI–DNA complexes in the presence and in the absensce of PROCOPs (Figure 7). While unprotected PEI–DNA complexes bound avidly to the serum-loaded chip, PROCOP P3YE5C reduced binding considerably and even more so did PROCOP P6YE5C demonstrating the protecting capacity of both polymers consistent with the results of the complement activation assay where the same order of protecting capacity was found.

PROCOPs are compatible with transfection and promote vector targeting in cell culture

Transfections were carried out with adherent (NIH-3T3, HepG2 and MDA-MB435S) and non-adherent (K562) cell lines in serum-containing medium. The dependence of transfection efficiency on the presence of PROCOPs was examined with PEI[8] and pL polyfection as well as DOTAP/cholesterol[9] and lipofectamine lipofection. Parameters such as the presence or absence of a receptor ligand (transferrin; K562 cells), the charge ratio and salt concentration were varied. COPROGs are essentially as effective in *in vitro* gene delivery as their 'naked' analogues depending on COPROG formulation. For PEI polyplexes some inhibition is observed at the lower N/P ratios (4 and 6) while at the higher charge ratios PROCOPs can increase transfection efficiency by about two-fold, if complexes are prepared in salt-free buffer and up to 10-fold if prepared in HBS (Figures 8 and 9). If transferrin as a receptor ligand is present in the DNA complex, a pronounced synergistic enhancing effect of PROCOPs is observed (Figure 10a; five-fold enhancement in the case of hTf-PEI–DNA compared to PEI–DNA without PROCOP, up to 20-fold enhancement in the presence of P3YE5C). Purification of PEI polyplexes by ultracentrifugation hardly affected transfection efficiencies (slight enhancements were observed K562, HepG2 cells and NIH-3T3 cells, Figure 9). Complexes were generally more effective if prepared in salt-containing buffer (Figure 9, hatched bars). If the PROCOP is synthesized with the influenza peptide INF7 as the pending side chain it confers protection from aggregation and opsonization to a polyplex and due to the endosomolytic activity of this membrane-destabilizing peptide class,[5] transfecting activity to otherwise ineffective polyplexes. This is demonstrated by a transfection of the adherent cell line MDA-MB435S with polylysine–DNA that has been coated with P3INF7 (Figure 10b).

Discussion

Surface charge, particle size and colloidal stability are interdependent physical characteristics of nonviral gene vectors with a strong impact on their biological properties including their efficiencies in gene delivery. The excess positive charge of lipoplexes and polyplexes, which is required for full DNA compaction and nuclease resistance, implies strong interactions with solutes (eg blood proteins,[10] erythrocytes[2]) and the extracellular matrix[11,12] under *in vivo* conditions. The mostly undesired consequences can be unintentional vector targeting,

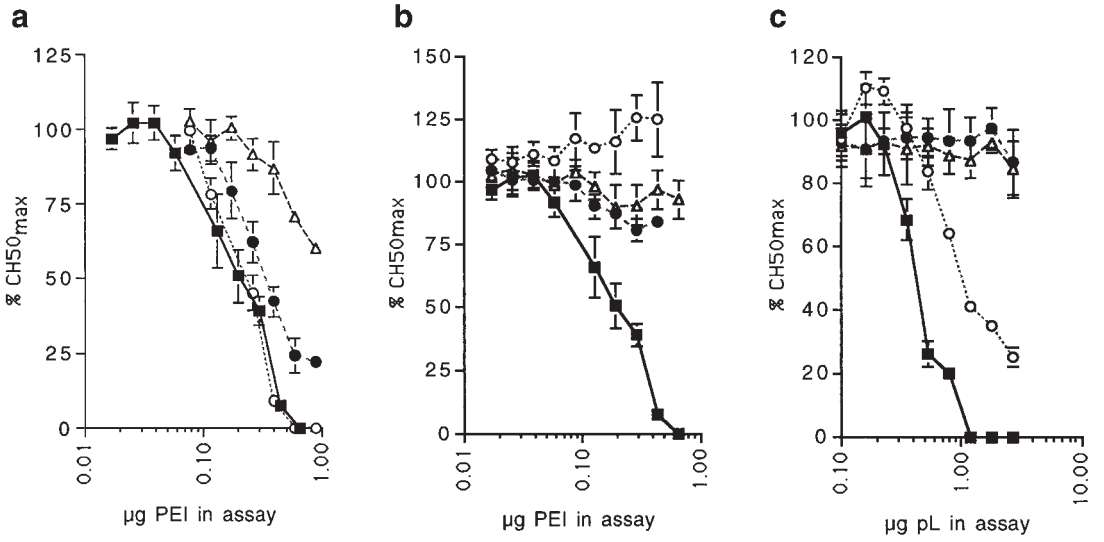

Figure 6 *Complement activation by COPROGs. Purified PEI–DNA (initial N/P = 8) with increasing amounts of P3YE5C (a) and P6YE5C (b). (c) pL170-DNA (charge ratio ± = 6.3) with increasing amounts of P6INF7. ■, 'naked' DNA complexes; ○, 1 charge equivalent P3YE5C (a), P6YE5C (b), 4.1 charge equiv. P6INF7 (c); ●, 2 charge equivalent P3YE5C (a), P6YE5C (b), 8.3 charge equivalents P6INF7 (c); △, 3 charge equivalents P3YE5C (a), P6YE5C (b), 12.4 charge equivalents P6INF7 (c). Complement serum is incubated with increasing amounts of the DNA complex under consideration (x-axis) resulting in consumption of complement proteins if complement activation occurs. As a consequence decreasing CH50 values are observed. The lower the percentage of $CH50_{max}$ the higher complement activation. CH50 values describe the particular dilution of serum which leads to lysis of 50% of the sheep red blood cells in the assay. For details see Ref. 1.*

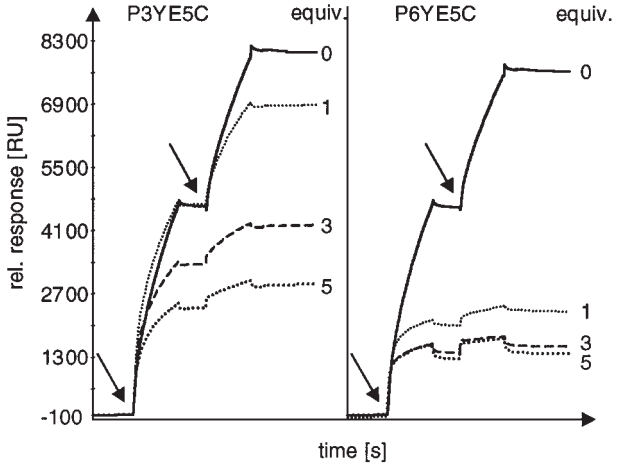

Figure 7 *Biospecific interaction analysis of PEI polyplex–serum interaction in the presence and absence of PROCOPs. PEI–DNA complexes (N/P = 8) coated with the indicated amounts of PROCOPs were injected twice for 4 min (arrows) followed by washes with running buffer until the signal stabilized (flow rate of 5 µl/min). Binding to the serum-loaded sensor chip results in increasing resonance units (RU, y-axis). The time scale over the total duration of each the experiment is 1200 s (x-axis). Left, P3YE5C-coated complexes; right, P6YE5C-coated complexes (overlay sensograms). Reduction of binding by increasing amounts of PROCOPs is evident from decreasing response signals. The loading signal for naked complexes ('0 equiv') does not reach a plateau due to continued aggregation in the presence of salt during injection.*

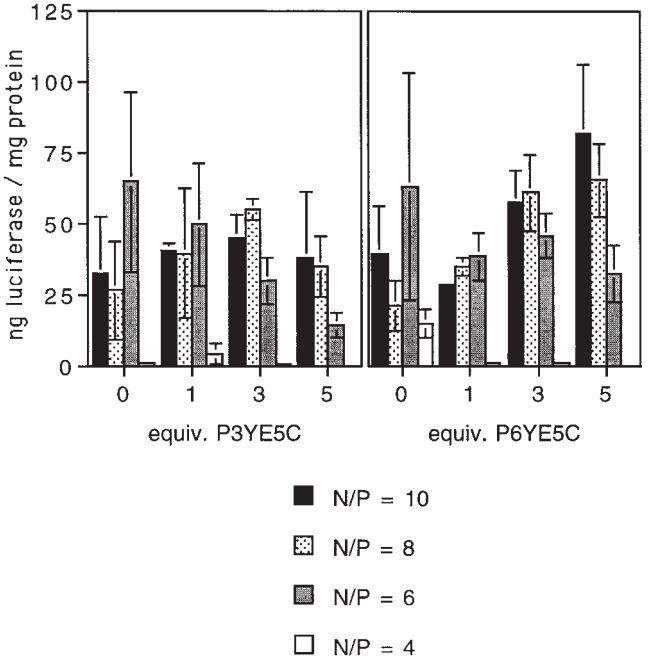

Figure 8 *Gene transfer studies, HepG2 cells. Dependence of PEI 25 kDa polyfection (unpurified complexes) on the amount of P3YE5C (a) and P6YE5C (b) at various charge ratios. Shown are mean values of transfections carried out in triplicates, error bars indicate standard deviation. Complexes were prepared in 20 mM HEPES pH 7.4 and adjusted to 5% glucose before addition to cells.*

complement activation,[1] vector inactivation[12] and clearance by the reticulo-endothelial system. Consequently, recent efforts in vector development have focussed on understanding and controlling the mentioned critical parameters. Analogous to what has been explored in drug delivery with nanoparticles[4] and liposomes,[3] poly(ethylene glycol) has been coupled covalently to pre-assembled vectors or their cationic component in order to confer the desired stability.[2,13]

As an alternative approach, we have developed a class of anionic copolymers which are attached to pre-

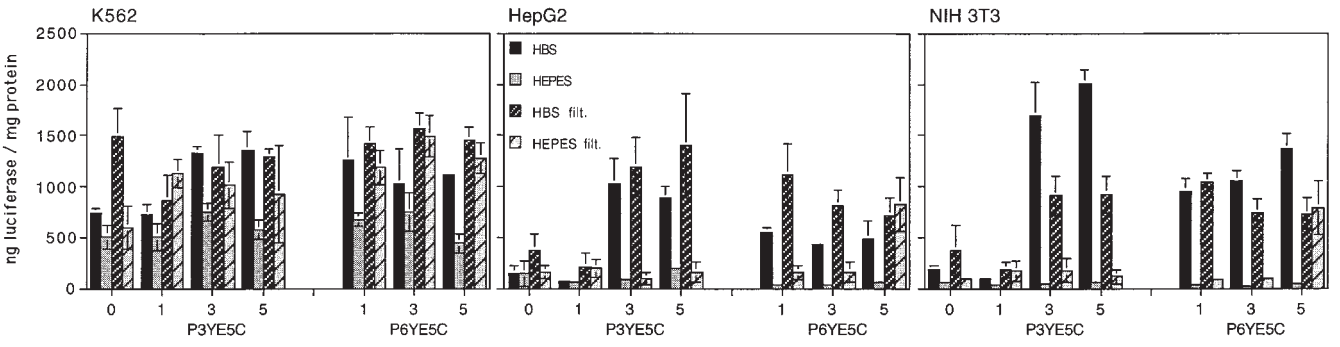

Figure 9 *Transfections of K-562, HepG2 and NIH 3T3 cells. Dependence of PEI 25 kDa polyfection (initial N/P = 8) on the amount of P3YE5C and P6YE5C, on complex purification by ultrafiltration (unpurified, solid bars; purified, hatched bars) and on the presence of salt during or after (for purified complexes) complex formation (black bars, 150 mM sodium chloride; gray bars, no salt). Left, K-562; middle, HepG2; right, NIH-3T3.*

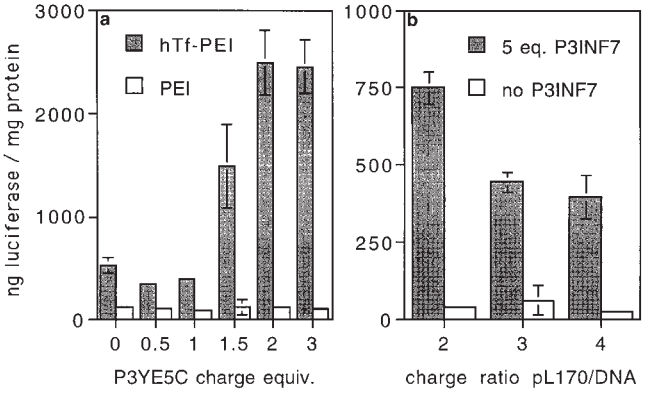

Figure 10 *(a) PEI-Transferrinfection of K562 cells in the presence of increasing amounts of P3YE5C. Complexes were prepared at N/P = 8 in 20 mM HEPES pH 7.4 containing 150 mM sodium chloride with or without transferrin–PEI conjugate (corresponding to 1 μg hTf per microgram DNA). (b) Polylysine polyfection (MDA-MB435S cells) in the presence or absence of the influenza peptide PROCOP P3INF7 at various pL/DNA charge ratios.*

(Figure 1). The work presented here comprised establishing a synthetic procedure to derive novel protective copolymers, the biophysical characterization of copolymer-protected gene vectors and their application in gene delivery to demonstrate the usefulness of the approach. The synthetic procedure to derive a reactive copolymer backbone intermediate (compounds 4 and 5, Figure 2a) was adapted from previous work by Nathan *et al*[14] who used copolymers of PEGs and lysine as carriers for 'small' molecule drugs. We derived the anionic copolymer end products for use in gene delivery by coupling the peptides YE5C and INF7 to the reactive backbone intermediates (Figure 2b). The sequence of the peptide YE5C ($[Ac\text{-}YE_5]_2K$-ahx-C) was based on previous work which demonstrated that at least eight positive charges are required to achieve efficient DNA binding with branched cationic peptides.[15] We reasoned that in turn at least the same number of negative charges may be required for an anionic peptide to bind to a preformed DNA complex efficiently and decided to use a peptide with 10 negative charges. For comparison and in order to derive endosomolytic copolymers, the membrane-destabilizing peptide INF7 (five negative charges) was grafted to the copolymer backbone.

The decrease of the zeta potentials as a function of the amount of PROCOP added to a pre-assembled gene vector (Figure 4) shows that the copolymers bind to vector particles. This is in agreement with the recent observations by Trubetskoy *et al*[16] who show that oppositely (negatively) charged polyelectrolytes can be deposited on the surface of condensed DNA particles. With respect to the results presented by this group, one concern was that PROCOPs may dissociate DNA complexes by competing with DNA for binding to the (poly)cation. According to a gel retardation assay this is not the case. This finding is further supported by particle size measurements using dynamic light scattering and electron microscopy which demonstrate that copolymer-protected polyplexes are stable particles of remarkably small size (Figure 5 and Table 1). Importantly, these particles do not aggregate or flocculate at electrostatic neutrality or upon addition of salt or serum albumin such as 'naked' polyplexes do. However, PROCOP addition induces a dramatic change in lipoplex structure (Figure 5d), although neither dissociation nor a reduction of transfection efficiency (data not shown) are observed. Certainly, these structural changes are not in favor of applications where a small particle size is required.

Apart from physical stability of vectors, inertness towards interaction with serum constituents during the delivery phase can be desirable, dependent on the target of gene transfer. While intravenous gene delivery to the lung with lipoplexes and polyplexes is feasible without further precautions, other targets such as distant tumors which require transvascular transport probably afford small and stable vector particles.[17] 'Naked' lipoplexes and polyplexes activate the complement system[1] (Figure 6) and bind a number of serum proteins.[2,10] While complement activation does not necessarily have an impact on biodistribution and efficiency of vectors,[18] it is certainly a matter of concern for applications in humans.

Therefore it is an important achievement that PROCOPs reduce or prevent opsonization and complement activation. This was independently demonstrated by determining BSA binding using zeta potential measurements (see Results), by examination of general serum protein binding using a biosensor approach (Figure 7) and by quantitating the extent of complement activation using the hemolytic assay (Figure 6). The stoichiometry of the polycation–DNA core and the molecular weight of

the PEG macromonomer of PROCOPs turned out to be important parameters governing the extent of opsonization and complement activation. Excess PEI is required for the formation of polyplexes optimized for transfection efficiency (Figure 8). Surprisingly, at an N/P ratio of 8, which was the standard composition chosen here, only a fraction of the PEI is tightly incorporated into the polyplex while about half of it can be removed by ultrafiltration. Important consequences of this procedure are decreased zeta potentials of the vector formulations (Figure 4) and complete inhibition of BSA binding and complement activation (Figure 6) upon addition of PROCOPs. Unpurified standard PEI polyplexes were not protected entirely from BSA binding and complement activation. Notably, the protective capacity of P6YE5C is superior to P3YE5C both in the complement assay (Figure 6a and b) and in biospecific interaction analysis of general serum protein binding (Figure 7). These experiments establish that the molecular weight of the PEG macromonomer in the PROCOP must be greater than 3400 in order to achieve the maximum protective effect. For comparison, polylysine polyplexes coated with the influenza peptide PROCOP P6INF7 were examined. Also here, PROCOP-dependent prevention of complement activation was observed (Figure 6c).

In order to be compatible with the intracellular steps of gene delivery, PROCOPs are designed to dissociate from the polycation–DNA core at the acidic pH of endosomes upon protonation of the glutamic acids. Also, cleavage of the disulfide bridge between the anionic peptide and the polymer backbone may play a role. Disulfide exchange reactions during endocytotic uptake of macromolecules[19] and Sindbis virus[20] have been described. Whatever mechanisms of intracellular vector processing apply, two opposing effects on transfection efficiency as a consequence of the effective shielding by PROCOPs could be envisaged for gene delivery: (1) an inhibitory influence due to the loss of cell binding capacity; (2) an enhancing effect due to reduced toxicity based on masking of excess positive charge of vectors and due to the integrity of vector particles towards opsonization by serum (all transfections were carried out in serum-containing medium).

Probably due to loss of cell binding capacity, an inhibitory influence of PROCOPs was observed at the lower N/P ratios (4 and 6; Figure 8) where toxicity of naked vectors is not limiting. Their toxicity becomes more pronounced at higher N/P ratios, hence the toxicity-reducing coating with PROCOPs counterbalances the loss of cell binding capacity, and transfection levels remain unchanged. Vectors prepared in salt-containing buffers aggregate and sediment on cells in culture, giving rise to increased transfection efficiency,[21] particularly with adherent cell lines (Figure 9). However, the toxicity of such aggregates is most striking, explaining the strong improvement in transfection by the toxicity-reducing PROCOPs (toxicity can be assessed from the protein content of cell extracts after transfection which in fact was up to three-fold higher when the polyplexes were coated with PROCOPs). If in addition the loss of cell binding capacity is compensated for by a receptor ligand as part of the vector, highest expression levels are achieved (Figure 10a). The versatility of the synthetic design of PROCOPs is again demonstrated with the compounds P3INF7 and P6INF7. These compounds display the desired membrane-destabilizing activity (Figure 3), inhibit particle aggregation and complement activation (examined for P6INF7, Figure 6c) and enhance the transfection efficiency of polylysine–DNA (examined for P3INF7, Figure 10b).

The data presented show that essential characteristics of nonviral gene vectors (particle size, charge, susceptibility to opsonization and toxicity) can be controlled with the help of PROCOPs without impairing gene transfer efficiency. The modular design of the synthetic procedure and of the vector assembly will greatly facilitate a systematic optimization towards successful gene delivery *in vivo*.

Materials and methods

Reagents

Chemicals were purchased from Sigma/Aldrich/Fluka (Deisenhofen, Germany) if not otherwise stated. Amino acid derivatives and resins for peptide synthesis were purchased from Bachem (Heidelberg, Germany). 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). LipofectAMINE was purchased from Life Technologies (Eggenstein, Germany). Cell culture media were purchased from Biochrom KG (Berlin, Germany). The plasmid pCMVLuc was a kind gift from Ernst Wagner (Boehringer Ingelheim, Vienna, Austria). PEI 25 kDa (Aldrich) was used throughout the study for PEI polyplex formation.

PROCOP synthesis

3-(2′-thio-pyridyl)-mercaptopropionyl-glutamic acid: One gram of 2,2′-dithiodipyridine (DTDP) dissolved in 4 ml ethanol was reacted with 87 μl (1 mmol) 3-mercaptopropionic acid in the presence of 100 μl triethylamine. After 1 h, excess DTDP was precipitated by the addition of water. The supernatant was separated by reverse phase HPLC (gradient elution 0.1% trifluoro acetic acid/0–40% acetonitrile in 24 min; flow rate 25 ml/min. Column: Vydac 218TP1022). Lyophilized product (0.5 mmol) was dissolved in 25 ml dichloromethane, cooled on ice followed by addition of 1 mmol each of glutamic acid-di-*t*-butyl ester, 1-hydroxybenzotriazole, N-ethyl-N′-(dimethylaminopropyl)-carbodiimide and diisopropylethylamine. After 48 h reaction at room temperature and removal of the solvent by rotary evaporation the oily residue was taken up in 20 ml ethyl acetate. This solution was extracted twice each with 0.5 M hydrochloric acid, saturated sodium bicarbonate solution and saturated sodium chloride solution. The organic phase was evaporated on a rotary evaporator, and the oily residue dried under high vacuum. Without further purification the product was treated for 2 h with 30 ml dichloromethane/TFA (2:1) for removal of the *t*-butyl protecting groups followed by removal of the solvent by rotary evaporation. The residue was treated with ice-cold ether and dried *in vacuo*. The product was dissolved in 20 mM HEPES pH 7.4 and purified by reverse phase HPLC (conditions as above). Pooled product fractions were lyophilized. Product identity was confirmed by mass spectroscopy (calculated 345.06; observed 345,0 (MH^+)). The overall yield was 27%.

Copolymerization of 3-(2′-thio-pyridyl)-mercaptopropionyl-glutamic acid (3) with O,O′-Bis(2-aminoethyl) poly(ethylene glycol) 3400 or -6000 (diamino-PEG-3400 or 6000): Product 3 was dissolved in 3 ml dimethyl formamide and diluted to 20 ml with dichloromethane. To 5 ml (67.5 μmol compound 3) of this solution 1.25 equivalents of diamino-PEG-3400 (or –6000, respectively), 30 mg dicyclohexylcarbodiimide (135 μmol, 2 equivalents) and 2 mg dimethylaminopyridine (0.25 equivalents) were added. After 2 h reaction at room temperature raw products (4) and (5) were obtained by precipitation with *t*-butyl-methyl-ether on ice and dried *in vacuo*. Aliquots of 20 mg were dissolved in HBS and purified by gel permeation chromatography after removal of unsoluble material by ultrafiltration (chromatography: Superdex 75 material filled in a Pharmacia XK 16/40 column (Freiburg, Germany)). Flow rate 1 ml/min with 20 mM HEPES pH 7.4 as eluent, optionally containing 150 mM sodium chloride). The main fractions eluted with an apparent molecular weight of 22.8 kDa (PEG-3400 polymer, 'P3-TP') and 40 kDa (PEG-6000 polymer, 'P6-TP'), respectively, preceded by minor overlapping higher molecular weight peaks that have not been used for subsequent studies (Figure 2).

Peptide synthesis

Peptide synthesis was carried out using a chlorotrityl chloride resin (Bachem, Heidelberg, Germany) and applying a modified FastMoc protocol on an Applied Biosystems (Weiterstadt, Germany) 431A automated peptide synthesizer. The purified branched peptide YE5C (sequence $(Ac\text{-}YE_5)_2K\text{-}ahx\text{-}C$; ahx = 6-aminohexanoic acid) was obtained by reverse phase chromatography as described above. Lyophilized product was dissolved in water upon dropwise addition of 1 M sodium carbonate. Peptide INF7 (GLFEAIE FGIENGWEGMIDGWYGC) was obtained in a similar manner and purified by gel filtration (Sephadex G10 filled in a Pharmacia HR 10/30 column, flow rate 1 ml/min with 20 mM ammonium bicarbonate pH 8 as eluent). Peptide identity and the absence of byproducts were confirmed by mass spectroscopy (electrospray/ion trap; Thermo Quest–LCQ, Egelsbach, Germany).

Coupling of peptides to copolymers P3-TP and P6-TP

Copolymer solutions were added to a 1.2-fold excess (thiol over thiopyridyl groups) of peptide solutions in 20 mM HEPES pH 7.4 and reacted overnight at room temperature. Completeness of reactions was confirmed by measuring the absorbance of released thiopyridone at 342 nm (extinction coefficient 8080 $M^{-1}\,cm^{-1}$). Reaction products were purified by size exclusion chromatography (Superdex 75 filled in a Pharmacia XK 16/40 column; conditions as described above). Copolymeric peptide concentrations were determined by measuring the absorbance at 280 nm in 6 M guanidinium hydrochloride/20 mM sodium phosphate buffer pH 6.5 using calculated extinction coefficients[22] of 2560 $M^{-1}\,cm^{-1}$ for YE5C and 12660 $M^{-1}\,cm^{-1}$ for INF7.

Erythrocyte lysis assay

Serial dilutions (1.5-fold) of the compound under consideration were carried out in U-bottom 96-well plates by transferring 100 μl out of 150 μl solution in column 1 to 50 μl buffer in column 2, mixing and so on, similarly as described previously[1] for the complement assay. The examined compounds were dissolved and dilutions were carried out in 20 mM HEPES/150 mM sodium chloride pH 7.4 or in 10 mM sodium citrate/150 mM sodium chloride pH 5, respectively. Human erythrocytes (3×10^6) in 150 μl of HEPES or citrate buffer, respectively, were added, and the plate was shaken at 400 r.p.m. in a Series 25 Incubator Shaker (New Brunswick Scientific Co, NJ, USA) for 1 h at 37°C. After centrifugation at 2000 *g*, 150 μl supernatant was transferred to flat-bottom 96-well plates, and relative hemoglobin concentrations were measured at 405 nm using an ELISA plate reader (Biolumin 960, Molecular Dynamics, Krefeld, Germany).

Assay for human complement activation

Stock solutions of 191.4 μg plasmid DNA (pCMVLuc) per milliliter were mixed with stock solutions of 200 μg PEI per milliliter in 20 mM HEPES pH 7.4. Aliquots of 610 μl of the resulting complexes were added to 305 μl solutions of 0, 1, 2 and 3 charge equivalents of P3YE5C and P6YE5C, respectively, in 20 mM HEPES pH 7.4. Charge equivalents refer to the amount of polymeric peptide in terms of negative charges as multiples of the negative charges of DNA in the complex. After addition of 101.7 μl of 50% glucose to each sample, 150 μl each (corresponding to 8.6 μg DNA and 9 μg PEI) were distributed to column 1 of a 96-well plate. A 1.5-fold dilution series and the complement assay were carried out as described.[1]

In the same manner purified PEI–DNA stock solutions of 21.7 μg DNA per ml were added to 0, 1, 2 and 3 charge equivalents of P6YE5C in 305 μl each, respectively. Before distribution of 150 μl aliquots to 96-well plates for the dilution series, samples were adjusted to 150 mM sodium chloride.

Also, a polylysine–DNA polyplex stock solution was prepared from pL170 (Sigma; average chain length 170; 256 μg in 800 μl HBS) and the plasmid pCMVLuc (64 μg in 800 μl HBS), corresponding to a calculated charge ratio of 6.3. Aliquots of 350 μl were incubated with 0, 35, 70 and 105 nmol of polymer-bound peptide INF7 ('P6INF7') and filled up to 1050 μl with GVB^{2+} buffer after 15 min of incubation. Aliquots of 150 μl each were added to column 1/A-F of a round-bottom 96-well plate. The complement activation assay was then carried out as described.

Biosensor studies of the interaction between PEI–DNA complexes and human serum proteins

To evaluate the interactions of DNA complexes and human serum proteins, surface plasmon resonance measurements were performed using a biosensor approach (BIAcore, Uppsala, Sweden). Human complement serum (Sigma) was covalently attached to the surface of a research grade carboxymethyl dextran chip (CM5) according to the standard protocol.[7] Briefly, after N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide/N-hydroxysuccinimide activation of the caboxyl groups, the serum dilution (100 μg/ml in 10 mM sodium acetate pH 4) was injected and residual N-hydroxysuccinimide esters were inactivated by injection of 1 M ethanolamine pH 8.5 in water. The immobilization procedure and the binding studies were performed at 25°C with 10 mM HEPES pH 7.4/150 mM sodium chloride/3.4 mM EDTA/0.05% Surfactant P20 as continuous flow buffer at

a constant flow rate of 5 μl/min. DNA complexes were prepared in 20 mM HEPES pH 7.4 at final DNA concentrations of 10 μg/ml as described below for transfections. Immediately before injection, sodium chloride was added automatically to a concentration of 150 mM. DNA complexes were injected twice for 4 min followed by washes with running buffer until the signal stabilized (flow rate of 5 μl/min).

Particle size and zeta potential measurements

PEI polyplexes (N/P = 8) were prepared by mixing 40 μg DNA (pCMVLuc) and 41.7 μg PEI (25 kDa, Aldrich) each dissolved in 333 μl 20 mM HEPES pH 7.4. After 15 min of incubation 0, 0.5, 1, 1.5, 2 or 3 charge equivalents of PROCOPs P3YE5C or P6YE5C in 333 μl HEPES were added (corresponding to 0, 152, 303, 455, 606 and 909 pmol of peptide). DOTAP/cholesterol lipoplexes were prepared similarly by mixing 333 μl each of 40 μg of DNA and 5 charge equivalents of a 5 mM DOTAP/cholesterol liposome suspension in 20 mM HEPES pH 7.4. PROCOPs were added as described for PEI–DNA. DOTAP/cholesterol (1:1 mol/mol) liposomes were prepared essentially as described.[23]

The particle sizes were determined by dynamic light scattering (using a Zetamaster 3000, Malvern Instruments, Herrenberg, Germany) at various time-points after complex formation and by electron microscopy as described previously.[24] Zeta potentials of the same samples were determined using the Malvern Zetamaster with refractive index, viscosity and dielectric constant parameters set to those of water as an approximation.

Purification of DNA complexes

Stock solutions of PEI polyplexes (N/P = 8; 100 μg DNA per milliliter in 20 mM HEPES pH 7.4) were centrifuged through Centricon-100 membranes (Millipore, Eschborn, Germany) four times 15 min at 500 *g* on a Beckman JA-20 fixed angle rotor. Between centrifugations the polyplex solutions were rediluted to the original concentrations. Care was taken not to exceed DNA concentrations of 500 μg per ml. The final centrifugation yielded polyplexes of 300–500 μg DNA per milliliter. The DNA concentration was determined by its absorbance at 260 nm (1 OD_{260} = 45 μg/ml for dsDNA in a PEI–DNA complex as determined from a standard dilution of a DNA complex of known DNA content). The amount of free, not DNA-associated PEI was determined in the combined filtrates by a trinitrobenzesulfonic acid assay.[25]

Cell culture and transfections

K-562 cells (human lymphoblasts; ATCC No. 45506), Hep G2 (human hepatoblastoma; ATCC No. HB-8065), the human breast cancer cell line MDA-MB435S (ATCC No. 45526) and NIH-3T3 mouse fibroblasts (DSMZ No. ACC 59) were grown at 37°C in an atmosphere of 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were seeded in 96-well plates at densities of 20 000–50 000 cells per well the day before transfection. For transfection, 150 μl of fresh medium were added and DNA complexes were added in a volume of 50 μl. Transfections were carried out in triplicates.

Preparation of polyplexes for transfections

For cell culture experiments polyplexes were prepared as stock solutions in 20 mM HEPES buffer pH 7.4 (with or without 150 mM sodium chloride or 5% glucose as indicated in the Figure legends), where each component (DNA, polycation, PROCOP) was dispensed in an equal volume to result in a final amount of 1 μg DNA per 50 μl which was the amount added to the cells in each well (same amounts of DNA complex in the same volume and buffer were also added in the case of purified polyplexes). First, DNA was added to the polycation with mild vortexing or mixing with the pipet, after 15 min PROCOP was added and after a further 30 min or more, polyplexes were added to the cells. A transferrin–PEI conjugate with one transferrin molecule per PEI molecule was prepared essentially as described.[26] For most studies, PEI polyplexes[8] were used at an N/P ratio of 8.

Luciferase assay

Twenty-four hours after transfection, cells were washed once with PBS and then incubated with 100 μl of lysis buffer (0.1% Triton X-100 in 250 mM Tris pH 7.8). Ten to 50 μl each of the cell lysates were transferred to black 96-well plates, mixed with 100 μl of luciferin buffer (60 mM dithiothreitol, 10 mM magnesium sulfate, 1 mM ATP, 30 μM D (–)-luciferin, in 25 mM glycyl-glycine pH 7.8) and assayed for bioluminescence using a TopCount instrument (Canberra Packard, Groningen, The Netherlands). The protein content of the cell lysates was determined using the BioRad protein assay adapted for use in a 96-well plate format. Specific luciferase activity in nanograms luciferase per milligram of protein were calculated from a calibration curve which was obtained from the luminescence of a serial dilution of luciferase (Roche, Mannheim, Germany).

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