Design of Amine-Modified Graft Polyesters for Effective Gene Delivery Using DNA-Loaded Nanoparticles

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Purpose. The purpose of this study was the design of a polymeric platform for effective gene delivery using DNA-loaded nanoparticles.
Methods. The polymers were synthesized by carbonyldiimidazole (CDI)-mediated coupling of diamines diethylaminopropylamine (DEAPA), dimethlyaminopropylamine (DMAPA) or diethylaminoethylamine (DEAEA) to poly(vinyl alcohol) (PVA) with subsequent grafting of D,L-lactide and glycolide (1:1) in the stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups/monomer units). The polymers were characterized by ^{1}H-NMR, gel permeation chromatography–multiple-angle laser-light-scattering, and differential scanning calorimetry. DNA-loaded nanoparticles prepared by a modified solvent displacement method were characterized with regard to their zeta (ζ)-potential and size. The transfection efficiency was assessed with the plasmid DNA pCMV-luc in L929 mouse fibroblasts.
Results. The polymers were composed of highly branched, biodegradable cationic polyesters exhibiting amphiphilic properties. The amine modification enhanced the rapid polymer degradation and resulted in the interaction with DNA during particle preparation. The nanoparticles exhibited positive ζ-potentials up to +42 mV and high transfection efficiencies, comparable to polyethlyenimine (PEI) 25kDa/DNA complexes at a nitrogen to phosphate ratio of 5.
Conclusions. The polymers combined amine-functions and short poly(D,L-lactic-co-glycolic acid) (PLGA) chains resulting in water-insoluble polymers capable of forming biodegradable DNA nanoparticles through coulombic interactions and polyester precipitation in aqueous medium. The high transfection efficiency was based on fast polymer degradation and the conservation of DNA bioactivity.

KEY WORDS: DNA vaccine; gene therapy; nanoparticles; polyester; transfection agent.

INTRODUCTION

DNA vaccines have been subject to intensive research efforts recently, and it has become increasingly clear that adjuvants are necessary to reduce the DNA doses used while reaching protective immune responses (1). Adjuvants, such as micro- and nanoparticles, have been studied intensively as DNA delivery systems providing (i) a sustained and predictable DNA release; (ii) targeting antigen-presenting cells using particles <10 μm, and (iii) stabilization of DNA in physiological environment (2). Several encapsulation techniques, mainly using biodegradable PLGA, have been reported, such as spray-drying (3) and modified double-emulsion methods (4), all of which use high-speed homogenization or sonication. These shear forces were found to compromise plasmid integrity and bioactivity (5,6). Additionally, DNA was damaged in the acidic environment created by PLGA degradation products (3).

Here we describe a gentle solvent displacement method for the encapsulation of DNA that relies on a new class of biodegradable polymers with rapid degradation properties (7). This method allows the encapsulation of DNA, without high speed/shear homogenization, by using amine-modified branched polyesters. These polymers interact with DNA by electrostatic interactions and facilitate nanoparticle formation due to their amphiphilic character. We systematically investigated these polymers to characterize the influence of polymer structure on functional properties such as nanoparticle size and charge and the protection of plasmid DNA by the transfection efficiency.

MATERIALS AND METHODS

Polymer Synthesis and Characterization

Biodegradable comb-branched polymers consisting of amine-modified poly(vinyl alcohol) (PVA) backbone grafted with PLGA side chains in ratio [n(OH)/n(monomer)] of 1:10 and 1:20 were synthesized and characterized as previously described (7). The amine modifications consisted either of 3-diethylamino-1-propylamine (DEAPA = P), 2-diethylamino-1-ethylamine (DEAEA = E), or 3-dimethylamino-1-propylamine (DMAPA = M). Briefly, after activation of the diamine component using carbonyl diimidazole (CDI) in tetrahydrofuran (Fig. 1), the activated components were added to PVA (Fluka, Neu-Ulm, Germany; degree of polymerization: p = 300) in *N*-methylpyrrolidone and reacted for 4 days at 80°C. Then, lactide and glycolide (1:1) were grafted in stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups/monomer units) by bulk polymerization onto the amine-modified PVA-backbones at 150°C using tin(II) 2-ethylhexanoate as catalyst.

The source-based IUPAC nomenclature for, for example, DEAPA modified polymers is the following: Poly[vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(DL-lactide-co-glycolide). As abbreviation, we use A(x)-y, where A indicates the type of amine substitution (P = DEAPA, M = DMAPA, E = DEAEA), x is the number of monomers in the backbone carrying amine substitutions, and y is the PLGA side-chain length calculated from feed. Resomer 502H (RG 502H) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

^{1}H-NMR spectra were generated in d_6-DMSO with a Jeol Eclipse+500 NMR Spectrometer (JEOL, Eching, Germany) at 50°C using 64 scans (500 MHz). Gel permeation chromatography–multiple-angle laser-light-scattering (GPC-MALLS) was carried out with a combination of DAWN EOS, Optilab DSP (Wyatt Europe GmbH, Woldert, Germany) and PSS SDV linear M column (PSS, Mainz, Germany) (flow rate 0.5 ml/min; solvent: dimethylacetamide + 2.5 g/L LiBr at 60°C). Differential scanning calorimetry (DSC) measurements were conducted with a Perkin-Elmer DSC 7 (Rodgau, Germany). Polymer degradation was measured gravimetrically after incubation of polymer films in PBS buffer at pH 7.4 (37°C) over 21 days according to Ref. 8.

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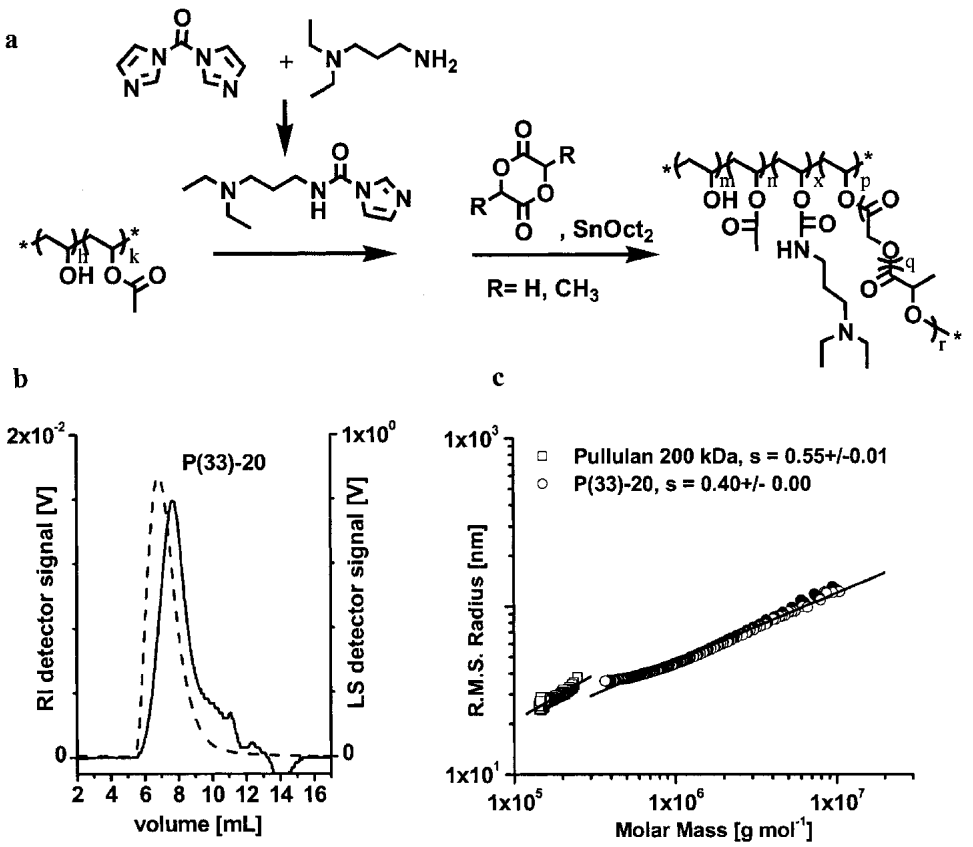

Fig. 1. (a) The synthesis of poly[vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide) using a three-step process with PVA, amine, and CDI as precursors. (b) GPC elution profile of P(33)-20 (straight line, signal of the refractive index detector; dashed line, light-scattering signal) indicating monomodal distribution of molar mass of the polyesters, but also a small, low-molecular-weight part. (c) Plot of the radius of gyration against MW. The reduced slope of the amine-modified polyester [P(33)-20] demonstrated the highly branched structure in comparison to random coil structured pullulan.

DNA Nanoparticle Preparation and Characterization

Nanoparticles were prepared by a modified solvent displacement method (9). Briefly, 500 μl of an aqueous solution containing 0.5 μg/μl plasmid DNA was added to 2.5 ml of an acetone solution containing 50 mg of the water-insoluble polymer. The product was injected into 10 ml stirred 0.1% Pluronic F68 (BASF, Ludwigshafen, Germany) in distilled water. The resulting nanoparticle suspension was stirred 3 h under constant laminar air flow to remove residual acetone. Particle size and ζ-potential measurements were carried out in a Malvern Zetasizer 4 (Malvern, Herrsching, Germany), according to Ref. 9 after calibration with a Malvern –50 mV transfer standard. Scanning electron microscopy (SEM) was performed with a CamScan 4 (Cambridge Instruments, Cambridge, UK) after gold sputter coating using a AUTO 306 (Edwards, UK). High-resolution transmission electron microscopy (HRTEM) imaging was performed after cryosectioning of the nanoparticles with a JEM 3010 (Jeol) on a collodium grid.

In vitro Transfection Efficiency

L929 mouse fibroblast (DSMZ, Braunschweig, Germany) cells were plated at a cell density of 50,000 cells/2 ml in 12-well dishes 24 h prior to transfection. Aliquots of the particle suspension containing 4 μg pCMV-luc theoretical load were added to 0.5 ml glucose 5% medium, pH 7.4. The cells were preincubated with the nanoparticle suspension for 5 min, after which 1.5 ml cell culture medium containing 10% fetal calf serum (FCS) was added. The nanoparticle suspension was dispersed in the glucose medium before the addition of the medium, as instabilities of nanoparticle suspension were observed in the medium. The nanoparticle suspension was removed after 4 h of incubation and replaced with fresh medium containing 10% FCS. Cells were harvested after 48 h, and luciferase transfection efficiency was assessed according to Ref. 10. Results were presented as luciferase/protein ratio (ng/mg).

RESULTS AND DISCUSSION

In this study, we present a new class of water-insoluble, amphiphilic polyesters developed specifically for the use of DNA encapsulation. We hypothesized that the loading efficiency of DNA nanoparticles could be greatly increased by three characteristics of the comb-branched polymers. First, electrostatic interactions are thought to stabilize and protect DNA during the encapsulation process. Second, fast polymer degradation rate should allow the release of bioactive DNA. Third, tertiary amino functions should facilitate gene delivery. We therefore developed polymers containing an amine-modified backbone for ionic interactions and possible "pro-

ton sponge" properties and relatively short but multiple, biodegradable PLGA side chains for fast polymer degradation. The unique properties of these polymers were confirmed in the nanoparticle formation process. DNA was solubilized by the polymer in the acetone solution due to the amphiphilic characteristics in the acetone/water mixtures used for the solvent displacement method, suggesting strong DNA/polymer interaction. The subsequent injection in aqueous medium resulted in nanoparticle formation. The biodegradable DNA nanoparticles exhibited effective gene delivery, demonstrated by high transfection efficiencies *in vitro*.

We synthesized 24 cationic and 2 neutral derivatives of amine-modified comb-branched polyesters and characterized their functional properties in relationship to their structure (Fig. 1). We grafted relatively short PLGA side chains consisting of approximately 10 or 20 repeating units on the amine-modified PVA backbone. Consequently, already a small number of hydrolytic cleavage events would result in water-soluble polymer fragments, thereby releasing the encapsulated DNA.

The total number of biodegradable PLGA side chains grafted on an amine-modified PVA backbone ranged from 150 to 240, resulting in a cationic and water-insoluble polyester. The general characteristics of the polymers' properties with different amine substitutions (DEAPA/DMAPA/DEAEA) were similar. The DEAPA-substituted polyesters were all soluble in acetone and thus suitable for the nanoparticles preparation process. Therefore, we selected this type of polymer for further study.

The brush-like structure of the graft-polymers was verified using ^{1}H-NMR spectroscopy, as well as GPC-MALLS depicted in Figs. 1b and 1c [P(33)-20]. The degree of PLGA side-chain substitution was calculated from the ^{1}H-NMR spectrum showing that only 5% to 35% hydroxyl groups of the PVA still remained free after reaction. The PLGA side-chain lengths (SCL) were calculated from these data, demonstrating good correspondence with the theoretical values (Table I). However, increasing amine substitution led to a decrease of SCL. A possible explanation could be an inhibitory effect of the amino-function on the tin catalyst, which competed with lactide/glycolide monomers. The molecular weights of the polymers were calculated from a combination of this data, based on the known amine substitution of the PVA backbones. The values for molecular weight (MW) were confirmed by GPC-MALLS (Fig. 1b). GPC measurements demonstrated the monomodal MW distribution of the polyesters. The molecular weights did not show an expected trend toward lower MW with increasing amine-substitution because of (i) the fast degradation of the polyesters, (ii) the resolution of GPC, and (iii) decreasing acetate content with increasing amine substitution. The nanostructure of the polymers in solution was characterized by the evaluation of the radius of gyration in a double logarithmic scale plotted against the molar mass of the polyesters (Fig. 1b). The resulting slope of the linear fit was compared to the slope of random coil structured pullulan (0.55). The flatter slope exhibited by the amine-modified polyesters [P(33)-20:0.40] indicated a compact, highly branched nanostructure of these polymers.

Polymer degradation at 37°C in PBS buffer at pH 7.4 was greatly accelerated as compared to common linear PLGA. NMR studies demonstrated the reduction of the SCL of P(12)-10 from originally 10.8 U to 8.6 U in 7 days and to 5.4

Table I. Characterization of the Amine-Modified Polyesters, Demonstrating the Low Glass Transition Temperatures, Extremely High Molecular Weights Combined with Fast Polymer Degradation at 37°C in PBS Buffer

Polyester	T_g (°C)[a]	MW (kg mol^{-1}) M_n^b	MW (kg mol^{-1}) M_n^c M_w^c	SCL[d]	Degradation half-life[e,f] (days)	Nanoparticle size[f] (nm)	Zeta potential[f] (mV)
P(6)-10	30.6	(107)	211 281	11.2	>21	n.d.	n.d.
P(12)-10	30.8	179	196 263	10.8	9	163 ± 1	22 ± 1
P(33)-10	27.7	179	195 367	9.4	1	152 ± 3	35 ± 3
P(68)-10	11.5	172	282 799	7.4	n.d.	309 ± 16	42 ± 2
P(12)-20	33.0	422	227 304	19.3	>21	n.d.	n.d.
P(33)-20	32.8	385	375 712	17.2	13	351 ± 7	31 ± 5
RG 502H[g]	36.5	6.1	6.6[g] 15[g]	84.6	16	602 ± 3	−55 ± 3

DNA nanoparticles exhibited smaller sizes and high ζ (zeta)-potentials compared to PLGA nanoparticles. I_g, glass transition temperature; MW, molecular weight; SCL, side-chain length.

[a] Glass transition temperature (heat rate: 10°C/min, –10 to 200°C, second run).

[b] MW calculated from the ^{1}H-NMR data.

[c] MW from GPC-MALLS (DAWN EOS, Optilab DSP, column PSS SDV linearM, solvent DMAc + 2.5 LiBr g/L, 60°C, 0.5 ml/min).

[d] PLGA side-chain length calculated from ^{1}H-NMR.

[e] Time at which 50% mass loss of a polymer film occurs (extrapolated from plot, n = 3).

[f] Mean of three independent measurements ± standard deviation.

[g] Commercial PLGA (1:1) lactic acid:glycolic acid subunits. MW: specifications supplied by the manufacturer (Boehringer Ingelheim).

U after another week. These measurements cannot be exclusively explained by physical erosion, as such an erosion would either not show decreased SCL or only a small SCL reduction. This behavior may substantially reduce the exposure time of an encapsulated substance to the detrimental effects of acidic degradation products generated by PLGA bulk erosion. This behavior is remarkable, as the molecular weights of the graft-polyesters are approximately 10-fold higher than the linear PLGA (RG 502H) (Table I). This property corresponded to our hypothesis of a substantial reduction in time for the drug release. An increase in the PLGA side-chain length from approximately 10 to 20 repeating units increased the degradation time as expected. P(33)-20, for example, showed a degradation half-life of 13 days, compared to 1 day for the P(33)-10 analog (Table I). The degradation rates increased more than proportionally with increasing amine substitutions of the polymer. For example, the degree of amine substitution in P(33)-10 was 3 times greater than in P(12)-10, however, P(33)-10 exhibited a 9-fold increase in the rate of degradation. This effect can be explained by the rapid, initial PLGA mass loss of the P(x)-10 polymer in comparison to the slower mass loss of P(x)-20 polymers, attributed to a catalytic effect of the amino-functions, promoting the acidic ester degradation, caused by their protonation. This would lead to new carboxyl-functions restarting the catalytic cycle. Furthermore, the protonated amino-functions will promote water uptake into the polymer effecting an increased rate of hydrolysis.

All polymers displayed glass transition temperatures near 30°C, implying they exist in the glassy state in physiological environment (Table I). In general, polymers with longer PLGA side chains and reduced amine substituents had higher transition temperatures. Thus, the amine groups were thought to have acted as a plasticizer in the polymer. The influence of the polymer chain motility has to be further investigated for possible interactions with cellular membranes and the influence on the gene delivery process.

An important feature of the polymer characteristics were the tertiary amine-modifications of the polymers, hypothesized to stabilize DNA within the polymer matrix and to facilitate the gene transfer. Ionic interactions with the polymer were presumably the reason for the solubilization of DNA in the acetone/water mixture. For example, DNA could be completely dissolved in an acetone/water 5:1 [v/v] solution of the polymer, whereas DNA alone precipitated. Therefore, no further homogenization process was necessary to disperse DNA before the subsequent coacervation of the water-insoluble polymer in the 0.1% poloxamer solution. Nanoparticles were only obtained with polymers exhibiting amine substitutions of 4% [P(12)-10] and higher, underlining the importance of the amphiphilicity induced by the amine substituents. The structure of the polymers was described to be brush-like, due to the short and numerous PLGA chains. Therefore, we did not expect a micellar assembly of the polymers neither in acetone, nor in the non-solvent water. In contrast, water soluble, poly(l-lysine)-g-PLGA polymers had a more distinct amphiphilic structure, containing a shorter hydrophilic backbone with few and long PLGA chains of approximately 210 monomers (11).

The nanoparticles exhibited hydrodynamic diameters ranging from 152.4 nm [P(12)-10] to 351.3 nm [P(33)-20], whereas PLGA (RG 502H) nanoparticles prepared by the same procedure were approximately 200 nm larger (Table I). Hence, despite a 33-fold higher molecular weight, the amphiphilic qualities of the polymers, influencing the viscosity, resulted in nanoparticles of reduced size.

Particle sizes measured by photon correlation spectroscopy were confirmed by scanning electron microscopy and transmission electron microscopy of nanoparticle cryosections. The particle morphology was examined by these methods as well [P(12)-10; Fig. 2]. Particles were uniform in size and had smooth surfaces.

ζ-potentials of all preparations were clearly positive, with the exception of the linear PLGA, arising from the DNA phosphate groups, which were inverted by the cationic polymers.

All DNA nanocarriers were used *in vitro* for transfection assays, as efficient gene delivery remains a prerequisite for subsequent *in vivo* immunization. By directly using the nanoparticles *in vitro*, we could detect the gene transfer properties of the amine-modified polymers, as well as the DNA bioac-

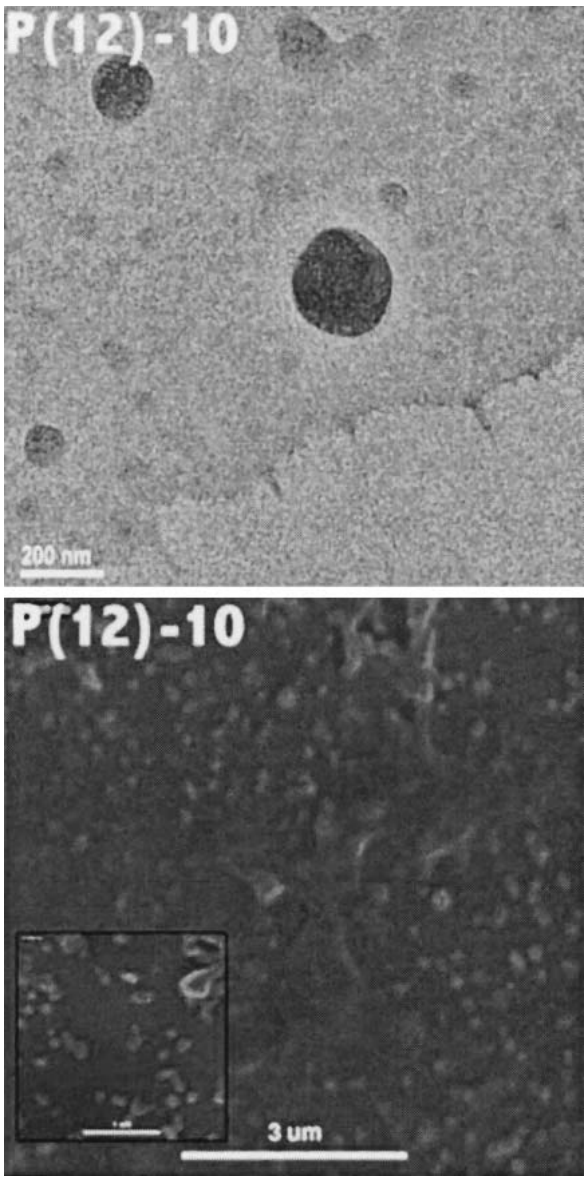

Fig. 2. TEM (top) and SEM (bottom, 1μm scale of the inlay) micrographs of DNA P(12)-10 nanoparticles confirm the particulate structure and the size measured by photon correlation spectroscopy.

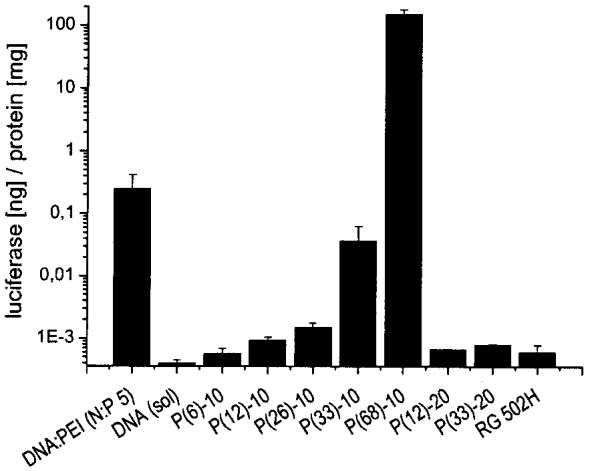

Fig. 3. Transfection efficiency of pCMV-luc DNA encapsulated in amine-modified nanoparticles was greatly enhanced compared to free DNA, DNA:PEI 25 kDa complexes (N/P 5), and a DNA RG 502H particle preparation.

tivity after nanoparticle preparation. Free plasmid and DNA complexes with PEI 25 kDa, a potent polymeric transfection agent, were used as references to compare the luciferase expression levels with other polymer types (12). On account of this, we could consider the nanoparticles as a potent transfection agent. All DNA nanocarrier formulations resulted in increased transfection efficiencies compared to free DNA (Fig. 3). The efficiency increased exponentially with the amount of amine substitution of the polymer. The 500,000-fold increase in transfection efficiency of the P(68)-10 plasmid nanoparticles, compared to free DNA, was remarkable, especially when considering the fact that the amount of polymer in relation to DNA was reduced by the factor 0.4 to avoid nocuous effects of an excess of cationic charges. Nanoparticles of P(x)-10 polymers clearly displayed higher efficiencies than their P(x)-20 analogs.

The transfection mechanism of the polymers is yet to be investigated; however, we assume that these findings do not depend on increased ζ-potentials or particle size effects, but must be dependent on the particle structure and DNA polymer interactions as well. The polymers consisted of dimethlyaminopropylamine substituents, representing tertiary amines, shown to be essential for the endosomal escape of polyplexes by the "proton sponge" effect (13). This effect could be intensified by the fast polymer degradation resulting in an increase of the osmotic pressure in the endosome, as proposed by Koping-Hoggard (14). However, other mechanisms of endosomal release have eventually to be considered, for example, fusogenic activities, taking into account the low glass transition temperatures and hydrophobic moieties of the polymer (15), or the "hydrogel effect" of swelling polymer in the endosome (16). Therefore, we concluded that the combination of different properties within one biodegradable polymer, resulting in a fast degradation, ionic interactions with DNA, and the formation of water-insoluble nanoparticles, provided considerable advantages with regard to the transfection efficiency *in vitro*. Further experiments investigating the transfection efficiency under *in vivo* conditions and the encapsulation with other compounds, such as peptides, susceptible to acid degradation are in progress.

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