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Self-Assembling Micelle-like Nanoparticles Based on Phospholipid-Polyethyleneimine Conjugates for Systemic Gene Delivery

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Abstract

With few exceptions, where local administration is feasible, progress towards broad clinical application of gene therapies requires the development of effective delivery systems. Here we report a novel non-viral gene delivery vector, 'micelle-like nanoparticle' (MNP) suitable for systemic application. MNP were engineered by condensing plasmid DNA with a chemical conjugate of phospholipid with polyethylenimine (PLPEI) and then coating the complexes with an envelope of lipid monolayer additionally containing polyethylene glycol-phosphatidyl ethanolamine (PEG-PE), resulting in spherical 'hard-core' nanoparticles loaded with DNA. MNP allowed for complete protection of the loaded DNA from enzymatic degradation, resistance to salt-induced aggregation, and reduced cytotoxicity. MNP also demonstrated prolonged blood circulation and low RES accumulation. Intravenous injection of MNP loaded with plasmid DNA encoding for the Green Fluorescence Protein (GFP) resulted in an effective transfection of a distal tumor. Thus, MNP provide a promising tool for systemic gene therapy.

Keywords

Gene delivery; Polyethylenimine (PEI); Polyethylene glycol (PEG); Self-assembly; Long-circulating nanoparticles

1. Introduction

In vivo gene therapy depends on the delivery of DNA-based drugs, either in the form of oligonucleotides (antisense ODN, siRNA) or entire genes (plasmid DNA) to their cellular site of action. With few exceptions, where local administration may be feasible, progress towards broad clinical application of gene therapies requires the development of effective non-invasive delivery strategies. Non-viral systems are desirable as DNA vectors because these are safer, simpler to handle, and less expensive than viral vectors. Among non-viral

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vectors, the cationic polymer, polyethylenimine (PEI), and its derivatives have been widely explored in gene delivery research[1–5]. PEI has the distinct advantage of the highest positive charge density among synthetic polycations that enables effective condensation of DNA by electrostatic interaction. PEI is also endowed with an intrinsic mechanism mediating "endosomal escape" by the so called "proton sponge" mechanism [1, 2] and nuclear localization [6], which allows for high transfection efficiency. While available in a wide range of molecular weights from approximately 1 to 800 kDa and in linear or branched forms, low molecular weight PEI has been shown to be well tolerated with low toxicity [7].

However, the PEI, in the form of PEI/DNA complexes, has not shown significant therapeutic efficacy *in vivo* due to its rapid clearance from the circulation and accumulation within RES (reticuloendothelial system) sites. This is attributed mainly to the overall positive charge of the complexes. Although the positive charges of the complexes interact with negatively charged components of cell membranes and thus trigger cellular uptake of the complexes, they also cause interaction with blood components and opsonization leading to rapid clearance from the blood circulation. As a result, the PEI/DNA complexes are cleared from circulation in a few minutes and accumulate mainly in RES organs such as liver and spleen[8]. When injected systemically, the PEI/DNA complexes are also a subject to DNA dissociation and aggregation in physiological environments[8]. The listed factors limit the *in vivo* application of the PEI/DNA complexes.

Several approaches have been tried to provide the PEI/DNA complexes with *in vivo* stability[3, 5, 9]. As with other nanoparticulate systems[10], poly(ethlylene glycol) (PEG) has been used to confer the *in vivo* stability to such complexes and prolong their circulation time. For this purpose, PEG has been covalently grafted to preformed PEI/DNA complexes[11], or PEG-grafted PEI has been used to form complexes with DNA[12]. Preformed PEI/DNA complexes were also coated with PEG using a copolymer of anionic peptide and PEG[13]. Still, steric stabilization of polyplexes by PEG did not provide a really high circulatory longevity and *in vivo* stability[8]. In combining PEI with the liposome technology, lipid-grafted PEI such as cetylated PEI[14] and cholesteryl-PEI[15] have been used to prepare polycationic liposomes (PCL) loaded with DNA. Water-soluble lipopolymer (WSLP) consisting of a low molecular weight PEI and cholesterol was employed for *in vivo* gene therapy for cancer or ischemic myocardium [16–19]. Preformed PEI/DNA complexes were encapsulated in PEG-stabilized liposomes resulting in the so-called "pre-condensed stable plasmid lipid particle" (pSPLP)[20].

Here, we report a novel micelle-like nanoparticle (MNP) loaded with plasmid DNA and based on a combination of a covalent conjugate between phospholipid and polyethylenimine (PLPEI), PEG and lipids. We demonstrate that PLPEI can self-assemble into monolayer-enveloped hard-core micelle-like nanoparticles in the presence of plasmid DNA along with unmodified lipids and PEG-PE, and that the resulting MNP have architecture and properties suitable for *in vivo* application. Finally, we demonstrated that MNP are non-toxic, long-circulating, and effective for the *in vivo* transfection of organs other than RES sites.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma-Aldrich unless otherwise stated. Plasmid DNA (pGFP) encoding Green Fluorescence Protein (GFP) was purchased at a final concentration of 1 μg/μl from Elim Biopharmaceuticals (Hayward, CA). Rhodamine labeled pGFP (pGeneGrip Rhodamine/GFP) was purchased from Genlantis (San Diego, CA). When necessary, the DNA was radioactively labeled with ^{111}In (PerkinElmer Life and Analytical Sciences, MA) to obtain 0.1 μCi/μg DNA according to methods described previously[21].

The concentration and purity were checked by 0.8% agarose gel electrophoresis. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-disrearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE), cholesterol and oxidized phospholipid, 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (AzPC Ester) were purchased from Avanti Polar Lipids (Alabaster, AL). Branched PEI (bPEI) with MW 1.8 kDa was purchased from Polysciences, Inc. (Warrington, PA) and dissolved in water to a final concentration of 1.0 μg/μl.

2.2. Methods

2.2.1. Synthesis of phospholipid-polyethylenimine conjugate (PLPEI)—Twelve miligrams of the branched PEI (7 μmole) were dissolved in 0.5 ml of chloroform and mixed with five miligrams of the oxidized PC (AzPC Ester, 7 μmole) dissolved in 1 ml of chloroform. Assuming that bPEI has 1:2:1 molar ratio of primary:secondary:tertiary amines, the reaction mixture corresponds to an acid-to-primary amine molar ratio of 1:10, i.e. contains an excess reactive amines. A half miligram of carbonyldiimidazole (CDI, 3 μmole) was added to the above solution for the activation of acid by forming an imidazolide derivative. The reaction mixture was incubated with 10 μl of TEA (triethylamine) at room temperature for 24 hrs with stirring. The chloroform was then removed under a stream of nitrogen gas and the residue was suspended with 2 ml of dH_2O. The products were purified by dialysis against dH_2O (MWCO 2,000 Da), lyophilized and their structure was confirmed by the ^{1}H-NMR (in $CDCl_3$, 300 MHz). The extent of conjugation was determined to be 1:1 molar ratio of PEI to lipid from the ratio of ethylene ($-CH_2CH_2-$) signal (2.4 – 2.8 ppm) of the PEI main chain to methyl ($-CH_3$) signal of the phospholipids head (3.4 ppm) on the NMR spectrum (δ 0.9:2.7 H, δ 1.3:17.6 H, δ 1.6:5.4 H, δ 2.4-2.8:96.0 H, δ 3.3:12.8 H, δ 3.6:1.58 H, δ 4.0-4.6:5.43 H). The PLPEI conjugate was dissolved in water to a concentration of 1.5μg/μl (1.0 μg/μl as of PEI).

2.2.2. Complexation of plasmid DNA with PLPEI—Constant amounts of plasmid DNA (100 μg) and varying amounts of PLPEI were separately diluted in HBG (10 mM HEPES, 5% d-Glucose, pH 7.4) to the final volume of 250 μl. The PLPEI solution was then transferred to the DNA solution by fast addition and vortexed. The resulting polyplexes were analyzed by agarose gel electrophoresis using the E-Gel elctrophoresis system (Invitrogen Life Technologies). A precast 0.8% E-Gel cartridge was pre-run for 2 min at 60 V and 500 mA followed by loading of 1 μg of pDNA. The desired amine/phosphate (N/P) ratio was calculated assuming that 43.1 g/mol corresponds to each repeating unit of PEI containing one amine, and 330 g/mol corresponds to each repeating unit of DNA containing one phosphate.

2.2.3. Preparation of micelle-like nanoparticles encapsulating plasmid DNA (MNP)—The MNP were constructed with PLPEI: POPC: Cholesterol: PEG-PE (4:3:3:0.3, mol/mol) and pDNA. First, PLPEI (130 μg as PEI) and plasmid DNA (100 μg) corresponding to N/P ratio of 10 were separately diluted in HBG to final volume of 250 μl. The PLPEI solution was transferred to the DNA solution by fast addition and vortexed. Dry lipid film was separately prepared from the mixture of POPC, cholesterol, and PEG-PE (42 μg, 21 μg, 15 μg, 3:3:0.3 mol/mol) and hydrated with 500 μl of HBG. The lipid suspension was incubated with the preformed PLPEI/DNA complexes for 24 hours at room temperature. Alternatively, the PLPEI/DNA complex was added directly to the lipid film. The resulting suspension of MNP was stored at 4°C until use.

2.2.4. Size and zeta potential—The MNP were diluted in HBG to obtain an optimal scattering intensity. Hydrodynamic diameter and zeta potential were measured by the quasi-electric light scattering (QELS) using a Zeta Plus Particle Analyzer (Brookhaven

Instruments Corp, Santa Barbara, CA). Scattered light was detected at 23°C at an angle of 90°. A viscosity value of 0.933 mPa and a refractive index of 1.333 were used for the data analysis. The instrument was routinely calibrated using a latex microsphere suspension (0.09 μm, 0.26 μm; Duke Scientific Corp, Palo Alto, CA, USA).

2.2.5. Freeze-fracture electron microscopy—The MNP were quenched using the sandwich technique and liquid nitrogen-cooled propane. At a cooling rate of 10,000 K/sec to avoid ice crystal formation and other artifacts of the cryofixation process. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment, and the exposed fracture planes were shadowed with platinum for 30 sec at an angle of 25 – 35 ° and with carbon for 35 sec [2 kV, 60 –70 mA, 1×10^{-5} torr (1 torr = 133 Pa)]. The replicas were cleaned with fuming HNO_3 for 24 – 36 h followed by repeated agitation with fresh chloroform/methanol [1:1 (vol/vol)] at least five times and examined with a JEOL 100 CX electron microscope.

2.2.6. Stability against salt-induced aggregation—Colloidal stability of the MNP particles against the salt-induced aggregation was determined by monitoring the MNP size (hydrodynamic diameter). NaCl (5 M) was added to the MNP in HBG to a final concentration of 0.15 M while measuring the size as described above.

2.2.7. Nuclease resistance—Nuclease resistance of the DNA molecules in MNP particles was determined by treating the samples with 50 units of DNase I (Promega Corp., Madison, WI) for 30 min at 37 °C. The reaction was terminated using EGTA and EDTA at a final concentration of 5 mM. The DNA molecules were dissociated using heparin (50 units/μg of DNA) at 37 °C for 30 min, and the products were analyzed on a 0.8 % precast agarose gel.

2.2.8. Cytotoxicity assay—The fibroblast NIH/3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) in 96-well plates. The cells were treated by replacing the media with serum-free media (100 μl) containing a serial dilution of each formulation up to 100 μg/ml of PEI. After 4 hrs incubation, the cells were washed twice with PBS and returned to complete media (100 μl). After 24 hrs incubation, 20 μl of CellTiter 96 Aqueous One solution (Promega, Madison, WI) was added to each well and the plates were re-incubated for 2 hrs. The absorbance at 490 nm was measured for each well using a 96-well plate reader (Multiscan MCC/340, Fisher Scientific Co). Relative cell viability was calculated with cells treated only with the medium as a control.

2.2.9. Pharmacokinetics and biodistribution—Male balb/c mice (20 –30 g) were maintained on anesthesia with ketamine/xylazine (1 mg/0.2 mg/animal) and catheterized with PE-10 in a retrograde direction via the right common carotid artery according to a protocol approved by the Institutional Animal Care and Use Committee at Northeastern University. The MNP loaded with ^{111}In-DNA (~ 2 μCi ^{111}In, 20 μg DNA) were injected through a tail vein. Blood samples (30 μl) were taken through the catheter in the common carotid artery at 1, 2, 5, 10, 30, 60 min after the intravenous bolus injection. The sample volume was replaced with PBS containing heparin (10U/ml). After the last blood sampling at 60 min. the animals were sacrificed by cervical dislocation and organ samples (lung, liver, spleen, kidney, muscle, and skin) were taken. Radioactivity of the blood and organ samples was measured by a γ-counter. The radioactivity was expressed as percentage of injected dose (%ID/g for organ, %ID/ml for blood). Organ distribution values were corrected for blood volume of the corresponding organs. Pharmacokinetic parameters were determined by fitting blood “concentration vs time” data to a biexponential equation ($C(t) = A*e^{-\alpha t} + B*e^{-\beta t}$).

2.2.10. In vivo gene expression—Male C57BL/6 mice (Charles River Laboratories) were inoculated subcutaneously in the left flank with 1×10^6 LLC tumor cells 14 days before treatment according to a protocol approved by the Institutional Animal Care and Use Committee at Northeastern University. MNP containing 40 μg pGFP in a 200 μl injection volume were administered by the tail vein injection. Noninjected mice with similar-sized tumors were used as negative controls. Anesthetized mice were sacrificed 48 hrs later by cervical dislocation, and excised tumors were immediately frozen in Tissue-Tek OCT 4583 compound (Sakura Finetek, CA) without fixation and 8 μm thick sections were prepared with a cryostat. GFP fluorescence was visualized with a fluorescence microscopy (Olympus BX51).

3. Results

3.1. Preparation of micelle-like nanoparticles (MNP)

The micelle-like nanoparticles (MNP) were prepared by complexing plasmid DNA with PLPEI and then enveloping the preformed complexes with a lipid layer containing also PEG-phosphatidyl ethanolamine conjugate (PEG-PE) (Fig. 1). As for the complexation, the optimal ratio of PLPEI to DNA was determined based on the amounts of amine required to completely inhibit DNA migration on an agarose gel, since the complex formation hinders the migration of DNA, retaining the DNA in the wells. Constant amounts of plasmid DNA were mixed with PLPEI at varying amine/phosphate (N/P) ratios and analyzed by agarose gel electrophoresis. The bound fraction of DNA was increased as the N/P ratio increased and the most DNA was bound at an N/P ratio higher than 6. The complexation profile of PLPEI was comparable to that of the unmodified PEI, indicating that the PEI capacity for DNA complexation was not diminished by lipid conjugation (Fig. 2a). An N/P ratio of 10, where all DNA is bound to the complexes, was chosen and used for the following steps.

For enveloping the PLPEI/DNA complexes, a mixture of free lipids comprising POPC, cholesterol, PEG-PE (3:3:0.3 mol/mol) was separately prepared as an aqueous suspension. The lipid suspension was then incubated with the preformed PLPEI/DNA complexes leading to spontaneous envelope formation, most probably a monolayer, driven by hydrophobic interaction between the lipid moieties of PLPEI and free lipids (post-insertion technique). The optimal amounts of the free lipid were estimated approximately from the number of lipid molecules that would provide a complete monolayer envelope to the preformed PLPEI/DNA complexes. It was calculated, assuming that a bilayer liposome with 50 nm diameter contains about 25,000 lipid molecules[22, 23] and PLPEI/DNA cores have a mass/volume ratio of 1 g/ml, that about 0.2 μmole of total lipids is required to cover the entire surface of the particulate cores with diameters of 50 nm and a total mass of 230 μg, i.e., one μmole of total lipids is required to cover completely the surface of the particulate cores with one milligram of the total mass. Thus, unless otherwise mentioned, 100 μg of DNA was complexed with 180 μg of PLPEI corresponding 131 μg (0.08 μmole) of PEI and 49 μg (0.08 μmole) of PL and then incubated with 42 μg (0.055 μmole) POPC, 21μg (0.055 μmole) cholesterol and 15μg (0.005 μmole) PEG-PE.

The interaction and incorporation of the free lipids into the PLPEI/DNA complexes was confirmed by the colocalization of the fluorescently-labeled free lipid (CF-PEG-PE) with the fluorescently-labeled DNA (Rh-DNA) under the fluorescence microscopy (Fig. 2b). The characteristic hard-core structure with monolayer envelope was clearly confirmed by the freeze-fracture transmission electron microscopy (ffTEM). ffTEM revealed well-developed spherical nanoparticles with a mean diameter of 50 nm (Fig. 2c). All particles displayed their shadow behind the structures which is typical for “hard-core” particles including micelles. This behavior is different from the fracture behavior of bilayer-structures such as

liposomes which display concave and convex fracture planes (shadow in front and behind the structure, respectively).

3.2. Physicochemical properties of MNP

Traditional PEI/DNA polyplexes tend to aggregate rapidly under physiological high salt conditions[8]. To demonstrate the stabilizing effect of the lipid envelope against the salt-induced aggregation, NaCl was added to complex formulations to a final concentration of 0.15M while monitoring the hydrodynamic diameter. As expected, PEI/DNA polyplexes aggregated immediately after adding NaCl with continuous increases in hydrodynamic diameter up to almost 20-folds over a 24 hour period. The intermediate PLPEI/DNA complexes without free lipids and PEG-PE showed a two-fold increase immediately after adding NaCl and then remained relatively constant over the 24 hours. At the same time, MNP remained stable with no significant aggregation upon salt addition for 24 hours (Fig. 3a).

Zeta potential measurement revealed that MNP have a favorable neutral surface charge of −2.1 ± 0.86 mV (mean ± s.e.m., n=5), while PEI/DNA polyplexes have a more toxic positive surface charge of 20.2 ± 1.38 mV (mean ± s.e.m., n=5). The neutral surface charge of MNP also suggested the presence of the lipid layer which provided a charge-shielding of the otherwise positive PEI/DNA core.

The presence and protective effect of the lipid/PEG-lipid layer was further demonstrated by the complete protection of the loaded DNA against the enzymatic degradation. The free DNA was completely degraded by the enzyme treatment while the DNA in MNP or, to a lesser extent, in PEI/DNA remains intact. The migration of the intact DNA was slightly retarded after the enzyme treatment probably due to interference with the enzyme. The quantification of the intact DNA (ImageJ, NIH) revealed that 93% of loaded DNA was recovered from MNP as compared to only 70% recovery from PEI/DNA, supporting the notion of complete encapsulation of DNA within the lipid membrane (Fig. 3b).

We have also evaluated the cytotoxicity of MNP towards the NIH/3T3 cells. MNP showed no toxicity at a PEI concentration of 100 μg/ml after 24 hrs of incubation that followed 4 hrs of treatment in striking contrast with PEI/DNA complexes, which were highly toxic at a PEI concentration of 15 μg/ml (Fig. 4). This result looks quite understandable in light of our data showing a neutral surface charge on MNP compared to the strong positive charge on the surface of PEI/DNA complexes.

3.3. In vivo biodistribution and gene expression

To demonstrate the prolonged circulation time of MNP in the blood and thus the feasibility of their enhanced delivery to target tissues such as tumors, pharmacokinetic and biodistribution studies were performed with MNP loaded with ^{111}In-DNA in mice. The radioactivity in major organs after i.v. bolus administration of MNP loaded with ^{111}In-DNA was measured and compared to that of control PEI/^{111}In-DNA complexes. After 10 min, as much as 30 % ID/ml of MNP remained in the blood compared to about 10% ID/ml for PEI/DNA polyplexes. At 1 hour post-injection, about 20 % ID/ml of MNP was still present in the blood, while only about 5 % ID/ml of PEI/DNA polyplexes was detected in the circulation (Fig. 5a).

The slower clearance and thus more prolonged circulation of DNA in MNP compared to PEI/DNA polyplexes were also confirmed by pharmacokinetic parameters. Earlier studies with PEGylated PEI-based polyplexes demonstrated some increase in circulation time, however such preparations still were suffering from limited longevity and stability [8,28]. Although, one can assume that the optimization of PEG-PEI-based polyplexes in terms of

PEG chains length and attachment density could result in better characteristics, we have still chosen more traditional and better investigated plain PEI-based polyplexes for comparison. The half-life ($t_{1/2\,\beta}$) was estimated by fitting the blood concentration data collected at 60 minutes to a two-compartment model and found to be approximately 239 minutes as compared to 33 minutes for PEI/DNA polyplexes. The area under the curve (AUC) obtained from the "concentration vs time" curves also revealed a significant increase in the systemic availability of plasmid DNA in MNP compared to the polyplexes of PEI (1404 %ID·min/ml vs. 530 %ID·min/ml). The extended circulation time was due to the reduced clearance by the RES uptake. While the DNA in the control polyplexes accumulated mainly in RES organs (40 %ID/g liver and 30 %ID/g spleen), the DNA in MNP bypassed the RES organs with significantly reduced accumulation (less than 5% ID/g for liver and spleen) (Fig. 5b). Taken together, the long circulation time along with low accumulation in RES sites makes MNP suitable for *in vivo* application.

The feasibility of the enhanced gene delivery and *in vivo* transfection of targets, such as tumors, suitable for the enhanced permeability and retention (EPR) effect-mediated passive accumulation of long-circulating pharmaceutical nanocarriers was demonstrated in mice bearing the LLC tumor. Gene expression at the tumor tissue was accessed following the i.v. administration of MNP loaded with the plasmid DNA encoding for the Green Fluorescence Protein (GFP). At 48 hours post-injection, bright GFP fluorescence was observed in tumors from the animals treated with MNP whereas no fluorescence was found in tumors from the control mice (Fig. 6). GFP expression in tumor tissues from the animals injected with PEI/DNA polyplexes was not accessed due to short survival of the animals. The intravenous administration of PEI/DNA polyplexes at a comparable dose caused death of the animals within 30 min from respiratory failure, additionally confirming significantly decreased toxicity of MNP.

Taken together, the prolonged circulation in the blood along with a low accumulation in RES sites allowed for a significant accumulation of MNP at the tumor site, leading to strong reporter gene expression. These qualities of MNP make them a promising candidate for the *in vivo* gene therapy.

4. Discussion

In this study, we developed a new gene delivery vector suitable for systemic application. The vector was constructed using a chemical conjugate of phospholipids and polyethylenimine (PLPEI) at the distal end of the alkyl chain. We hypothesized that the electrostatic interaction of polycationic PEI moieties with DNA would drive the formation of dense PEI/DNA polyplex cores while the amphiphilic phospholipid moieties, together with added free unmodified phospholipids and PEG-grafted phospholipids (PEG-PE) would form a lipid monolayer envelope around the polyplex cores and lead to the formation of DNA-loaded micelle-like nanoparticles (MNP) stabilized by a steric barrier of PEG chains and a membrane barrier of lipid envelope.

In contrast to dramatic successes with sterically stabilized liposomes[24], the steric stabilization of polyplexes by polyethylene glycol (PEG) has not successfully provided both circulatory longevity and *in vivo* stability[8]. Earlier studies with PEGylated PEI-based polyplexes demonstrated only a moderate increase in circulation time (by approx. 40% for non-crosslinked PEG-PEI polyplexes [28]). Significant improvement has been achieved only when steric stabilization is combined with the "lateral stabilization" by crosslinking the surface of the polyplexes[9]. This result indicates that steric stabilization plays only a limited role in the *in vivo* stability of polyplexes, and that additional stabilization

mechanisms (chemical and/or non-chemical) are necessary to confer added *in vivo* stability to polyplexes.

Among other possibilities, the additional stabilization is likely to be achieved by enveloping the polyplexes within lipid membrane barriers since the lipid membrane barrier could shield the polyplex core and thus protect it from the salt-induced instability. *In vivo* behavior of such systems is governed by the lipid membrane barrier, while the polyplex cores are shielded from the biological environment in the blood circulation. Steric stabilization of the lipid membrane barrier provides the loaded polyplexes with a prolonged circulation time and makes it possible to deliver the polyplexes to target organs other than RES sites via the EPR mechanism. Furthermore, upon the cellular uptake, PEI is still expected to exert its favorable functions, such as the endosomolytic activity and its protection from cytoplasmic nucleases to improve an intracellular pharmacokinetics of the DNA molecules.

MNP is additionally stabilized by the presence of the envelope of the lipid monolayer, which forms by a self-assembly process driven by the hydrophobic interactions between the lipid moieties of PLPEI and free lipids and PEG-PE. The strong resistance of the MNP against the salt-induced aggregation and enzymatic digestion confirms the presence of such a membrane barrier. The high salts in physiological conditions provide one of the mechanisms responsible for the poor *in vivo* stability of PEI/DNA polyplexes [8]. These polyplexes are formed by strong electrostatic interaction between polycationic PEI and polyanionic DNA molecules and colloidally stabilized by electrostatic repulsion between the particles. Under the physiological conditions, however, an increased salt concentration triggers the aggregation of polyplex particles as a result of screening of the electrostatic repulsion forces between the polyplex particles along with concurrent dissociation of the polyplex particles due to screening of attractive electrostatic interaction between polycations and polyanionic DNA[25].

Although steric stabilization by PEG chains resulted in a decreased sensitivity of the polyplexes of PEG-grafted PEI to the salt-induced aggregation, the moderate stability of the polyplexes suggests that steric stabilization provided by PEG alone is insufficient, and additional stabilization mechanisms are required to significantly prevent the aggregation of the polyplexes [12, 26–28]. The existence of the lipid membrane barrier shielding the positive charge of polyplex core contributes to the observed stability of the MNP in high salt conditions. The membrane barrier, as with liposomes, blocks the access of salts from the outer environment to the polyplex cores and thus provides protection against the salt-induced aggregation to the otherwise unstable polyplexes. Still noticeable aggregation of the intermediate PLPEI/DNA complexes without free lipids indicates that the phospholipid moieties of the PLPEI conjugates alone could not provide a complete membrane barrier.

The amount of PEG-PE was chosen to facilitate the incorporation of free lipids into the preformed complexes and also to provide steric stabilization of the final construct. Considering that mixtures of PEG-PE with phospholipids evolve from a micelle phase to lamellar phase as the PEG-PE content in the mixture increases with the onset of micelle formation at ~ 5 mol%[29, 30], the aqueous suspension of the free lipid mixture with a 10 mol% PEG-PE concentration favors the micelle phase transition to the lamellar phase. Upon the incubation with the preformed PLPEI/DNA complexes, the PEG-PE content of total lipids comprising the free and the conjugated lipids decreases to 4.3 mol%, at which a lamellar phase is favored. It has also been shown that PEG-PE molecules in a micelle phase spontaneously incorporate in the surface of preformed phospholipid vesicles by so called “micelle transfer”[31]. Taken all said together, free lipids are likely to interact with hydrophobic lipid domains of PLPEI/DNA polyplexes, leading to spontaneous incorporation of free lipids into the lipid layer of the preformed complexes following dissociation into

monomers and thus, along with the phospholipids moieties from PLPEI conjugates, form a lipid monolayer envelope surrounding the polyplex core. The final construct is a sterically stabilized micelle-like hard-core particle with a PEI/DNA polyplex core and lipid monolayer envelope. Certainly, the exact mechanism of such high stability of the hypothetical lipid barrier in MNP still has to be understood. One may assume, however, that the self-assembly process can results in such architecture of MNP that hydrophobic interactions of the non-polar groups of free barrier lipids with lipid moieties of PLPEI in the bulk of the polyplex core (not just in the monolayer) can impart to the system the thermodynamic stability similar to that of the lipid bilayer.

A similar hydrophobic interaction was proposed as a plausible stabilizing mechanism of Pluronic P123-grafted PEI/DNA polyplex systems [32, 33], in which the amphiphilic Pluronic P123 chains of Pluronic P123-grafted PEI form a micelle-like structure around the polyplex core and unmodified Pluronic P123 was incorporated into the polyplexes by the hydrophobic interaction with Pluronic P123-grafted PEI conjugates and thus filled in to optimize the stability of the micelle-like structure.

MNP, in a sense, resemble so called “liposome-entrapped polycation-condensed DNA particle” (LPD II) entrapping polylisine/DNA within folate-targeted anionic liposomes [34], or ‘artificial virus-like particles’ prepared by entrapping PEI/DNA polyplexes within preformed anionic liposomes[35–37], or “pre-condensed stable plasmid lipid particles” (pSPLP)[20] constructed by encapsulating PEI/ DNA polyplexes within a lipid bilayer stabilized by an external PEG layer. In particular, pSPLP demonstrate advantages of encapsulating polyplexes within stabilized liposomes, i.e. the effective systemic delivery of PEI/DNA polyplexes to tumors due to the prolonged circulation time and improved transfection potency due to the endosomolytic activity of PEI. However, the preparation of pSPLP involves a potentially damaging incubation of preformed polyplexes with lipids in ethanol (organic solvent) and thus requires multiple steps of concentration and dialysis.

With the MNP, we were able to achieve the same advantages of combining polyplexes with a sterically-stabilized lipid membrane, albeit a monolayer in this case. The PLPEI conjugate enabled a process of self-assembly of DNA-loaded MNP by simultaneous DNA condensation and lipid membrane formation. The MNP also provided a more convenient one-step DNA loading with 100% efficiency and allowed a loading capacity (530 μg DNA/ μmole total lipids, 30% of total mass), higher than any other method of DNA encapsulation into a liposomal formulation[38].

In conclusion, we have constructed a novel nanoparticle suitable for *in vivo* applications using a covalent conjugate of polyethylenimine and phospholipids. The final DNA-containing nanoparticle has a vesicular structure with a polyplex core surrounded by a mixed lipid/PEG monolayer envelope and combines desirable properties of a cationic polymer-based gene delivery system including simple preparation and high loading capacity with the *in vivo* stability of PEG-stabilized liposome. The MNP has a sufficient *in vivo* stability and a prolonged blood circulation time and effectively delivers a model gene therapeutics to tumor cells after i.v. administration. Such nanoparticles provide a promising tool for systemic gene therapy.

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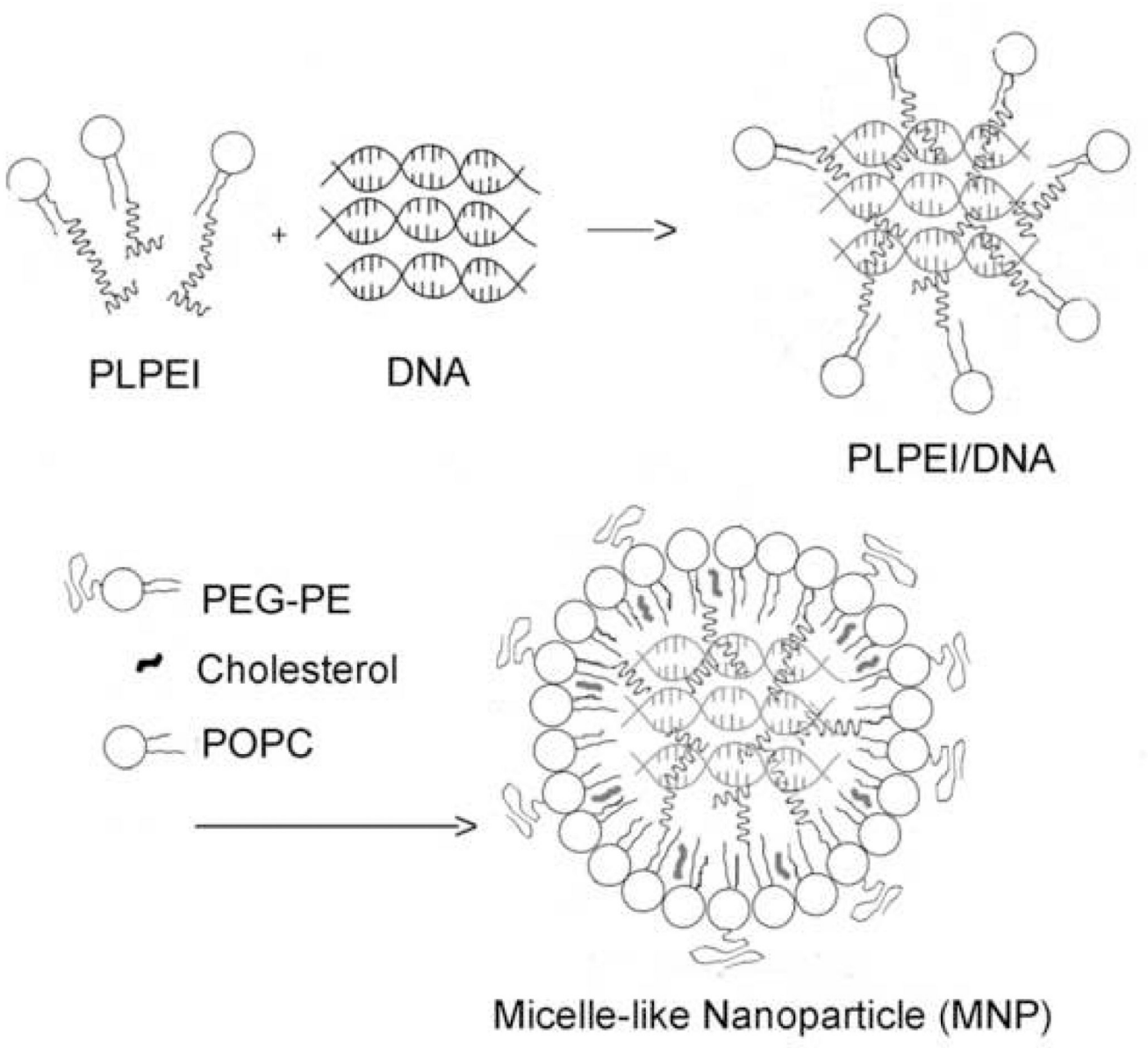
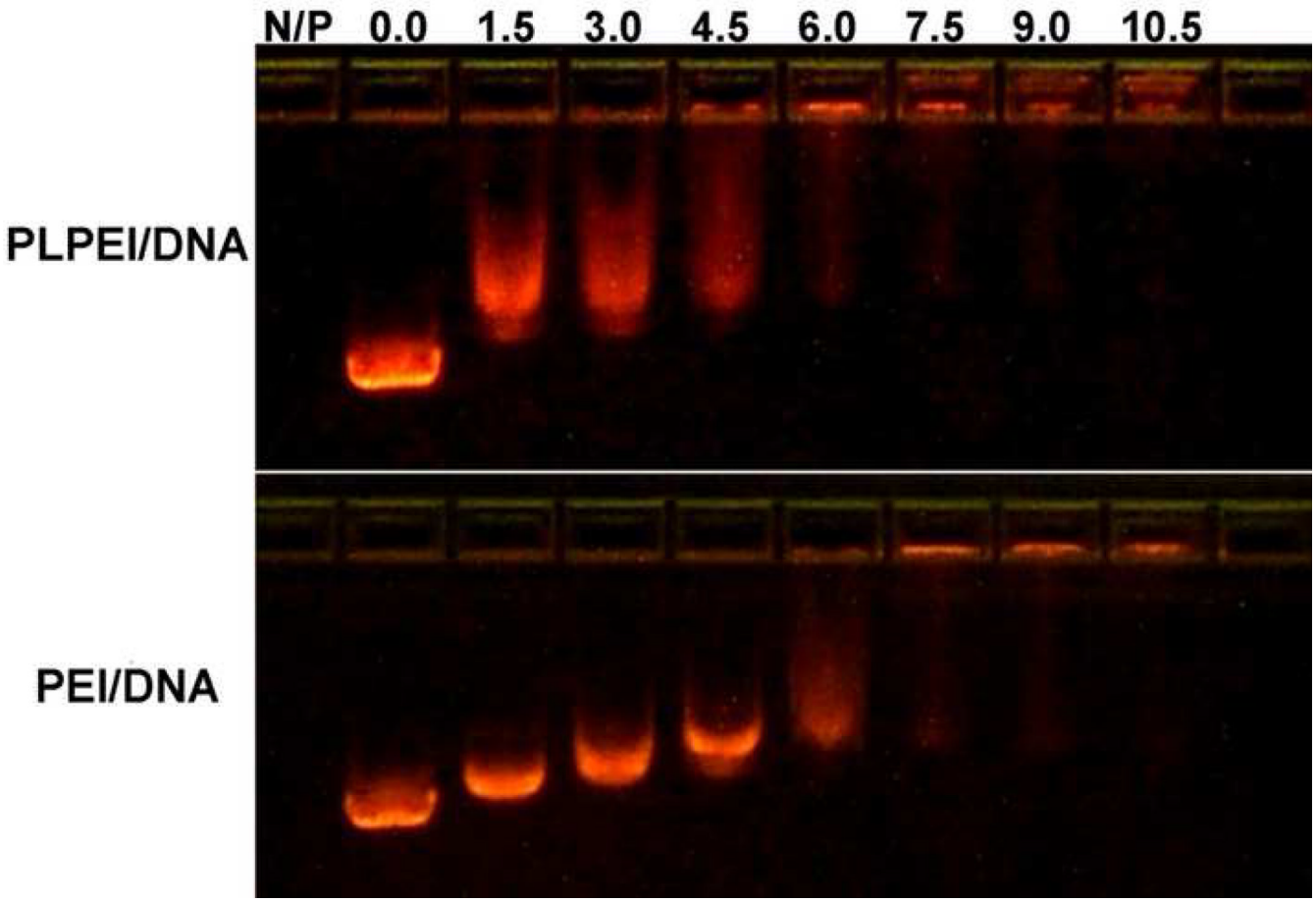


Fig. 1.

Schematic representation of the self-assembly process of micelle-like nanoparticles (MNP) with PEI/DNA core surrounded by the phospholipid monolayer. MNP form spontaneously in an aqueous media through the complexation of DNA with the phospholipid-polyethylenimine conjugate (PLPEI) followed by coating the complex with the lipid layer. The PEI moiety from PLPEI forms dense complexes with DNA resulting in a hydrophobic core, while the phospholipid moiety of PLPEI along with the unmodified lipids and PEG-PE forms the lipid monolayer that surrounds the PEI/DNA core. The lipid monolayer with incorporated PEG-PE provides also the *in vivo* stability.

2a



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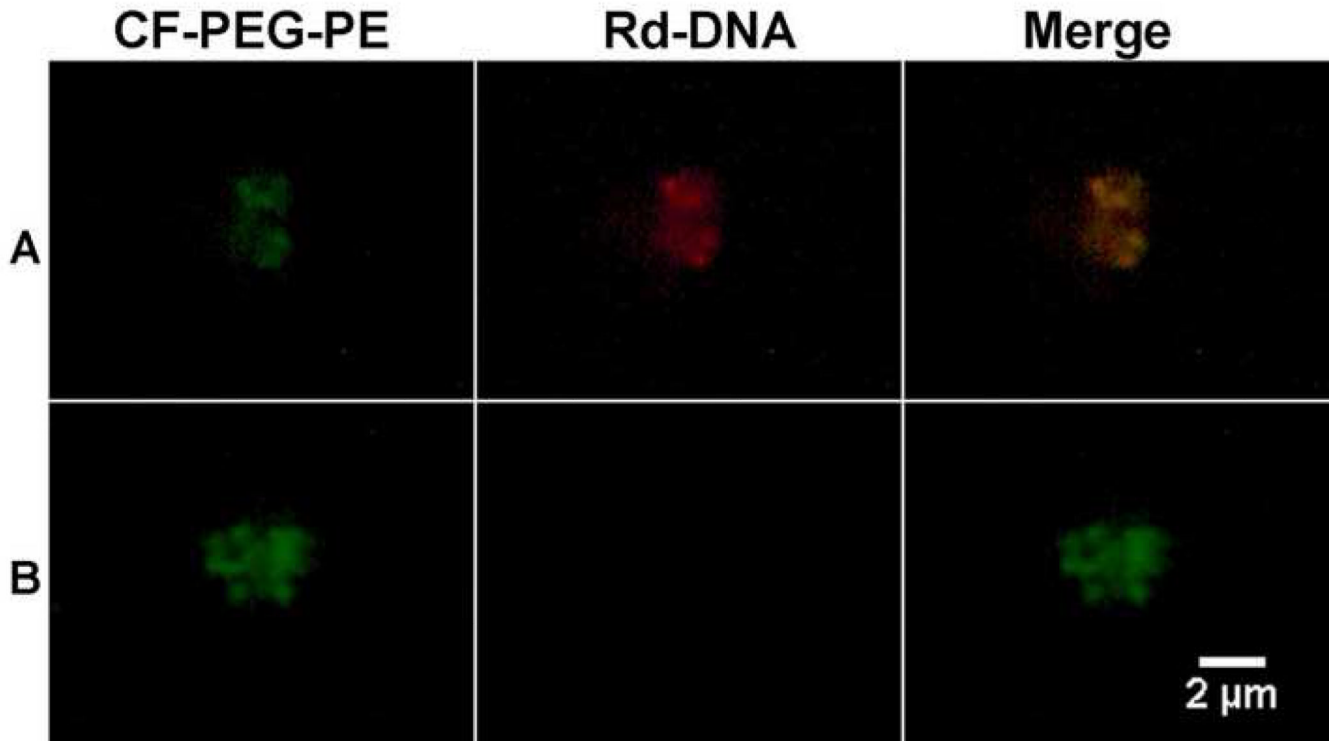

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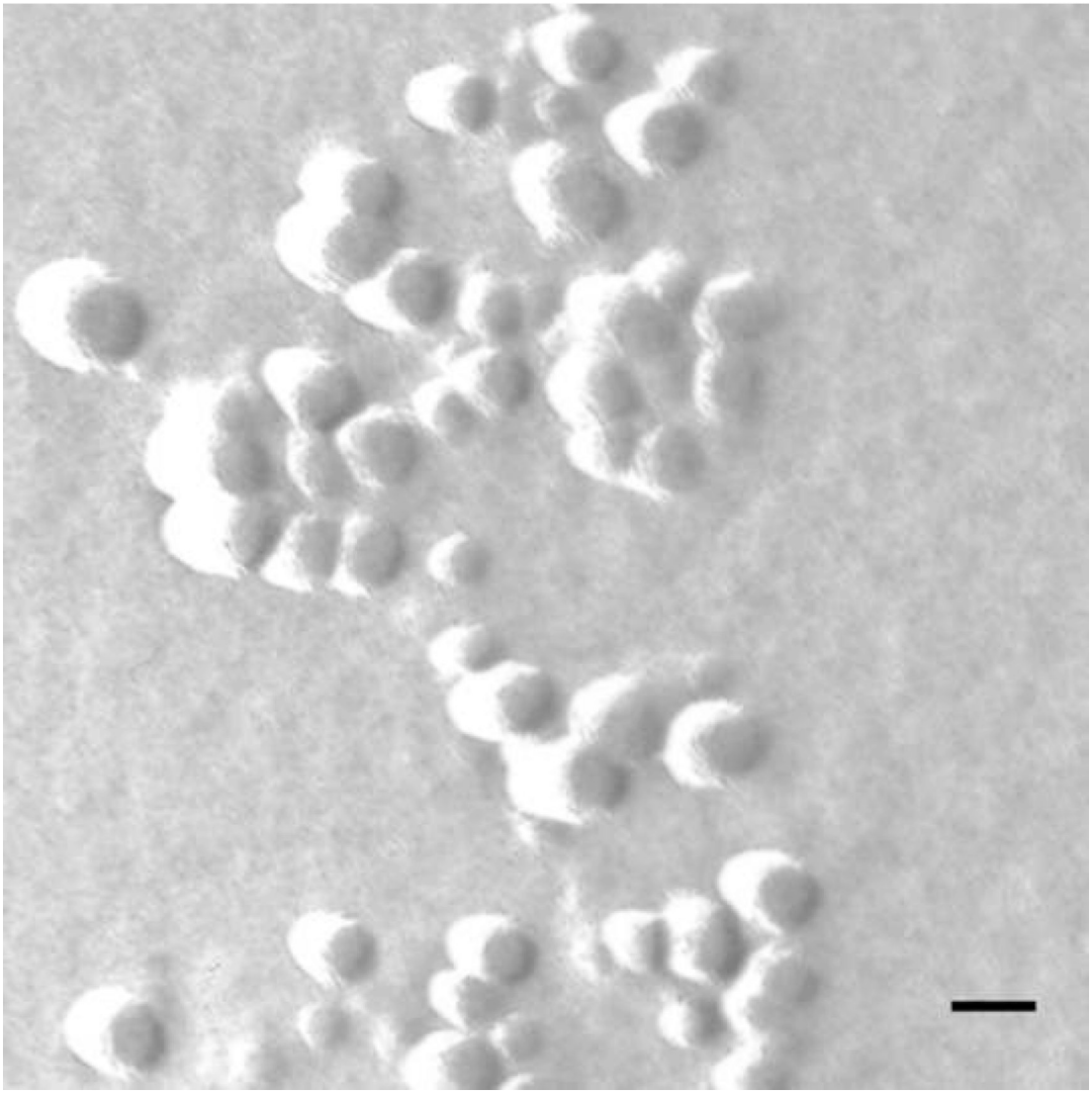
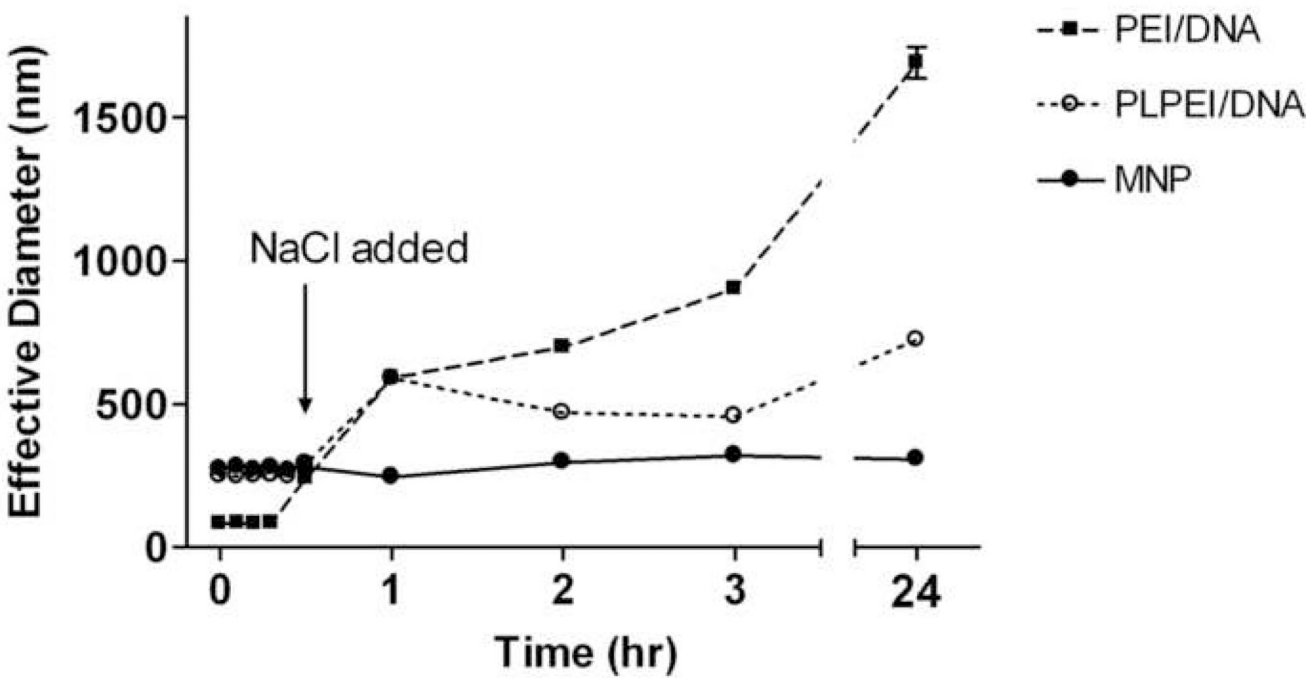


Fig. 2.

Analysis of MNP formation. (**a**) Agarose gel electrophoresis of PLPEI/DNA complexes in comparison to PEI/DNA complexes at varying N/P ratios. No migration of the DNA into the gel indicates the complex formation. DNA was completely complexed by PLPEI at N/P ≥ 6. The PLPEI showed complexation profile comparable to that of the unmodified PEI. (**b**) Analysis of double-labeled (A) and single-labeled MNP (B) by fluorescence microscopy. MNP were prepared using carboxyfluorescein (CF)-PEG-PE and rhodamine (Rh)-DNA and visualized by fluorescence microscopy. The co-localization of carboxyfluorescein (CF) and rhodamine (Rh) confirms the incorporation of PEG-PE into the preformed PLPEI/DNA. The bar is 2 μm. (**c**) Freeze-fracture electron microscopy (ffTEM) analysis of MNP. MNP appear

as well-developed spherical particles with an average diameter of 50 nm and a narrow size distribution. All particles display their shadow behind the structures, confirming micelle-like “hard-core” and “monolayer” structure. The bar is 50 nm.

3a



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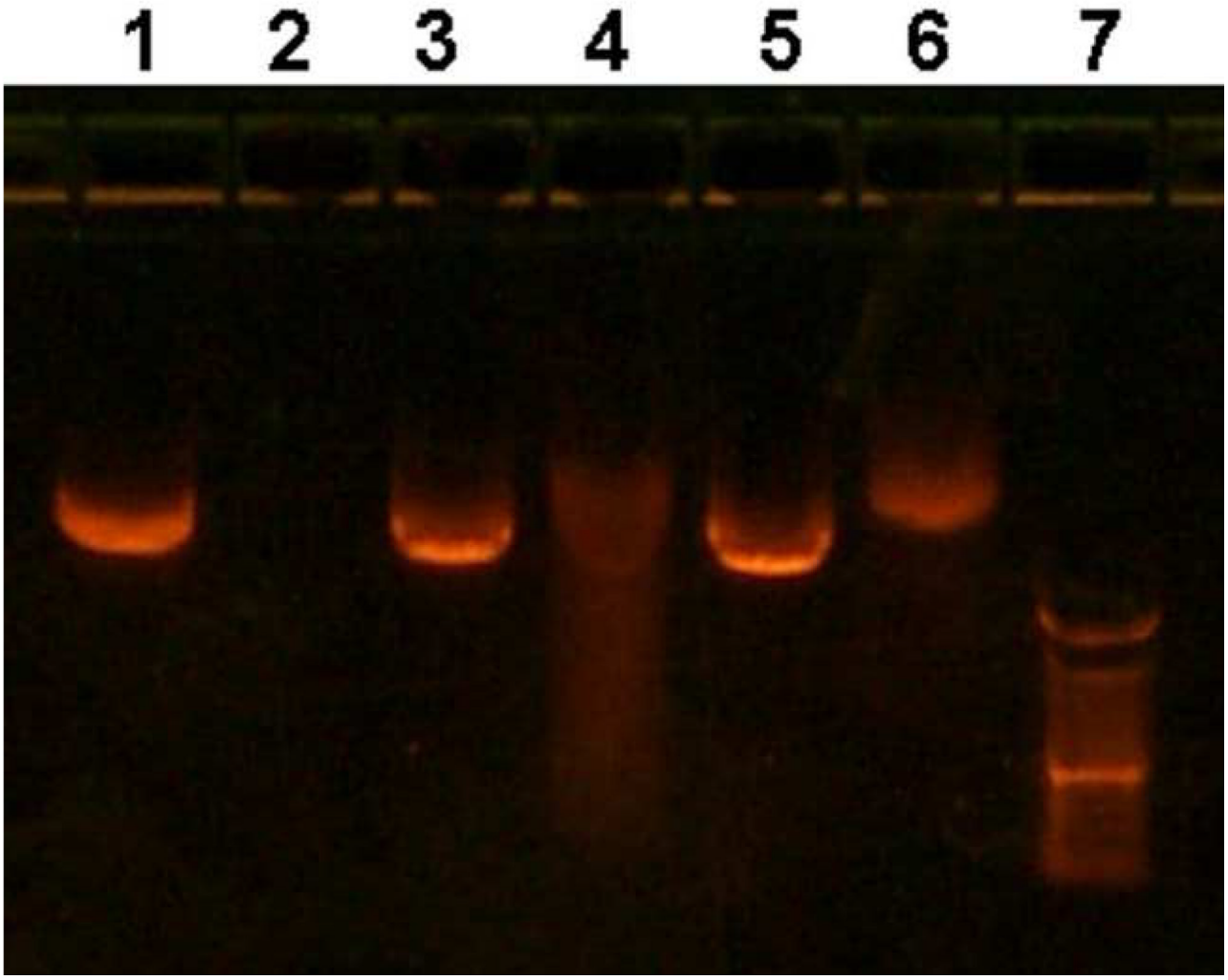


Fig. 3.

Stability of MNP. (**a**) Colloidal stability of NMP against salt-induced aggregation. Hydrodynamic diameters were monitored before and after adding salt (0.15 M NaCl). MNP remained stable while the PEI/DNA polyplexes showed rapid aggregation upon salt addition. Data represent mean ± s.e.m. (n = 3). (**b**) Protection of DNA loaded in MNP from the enzymatic degradation. MNP loaded with DNA and PEI/DNA polyplexes were analyzed on a 0.8% precast agarose gel after the treatment with DNAase I. DNA in MNP was completely protected from enzymatic degradation. *Lane 1*, DNA; *lane 2*, DNA, DNase; *lane 3*, PEI/DNA,; *lane 4*, PEI/DNA, DNAase; *lane 5*, MNP; *lane 6*, MNP, DNAase; *lane 7*, 100 base-pair ladder.

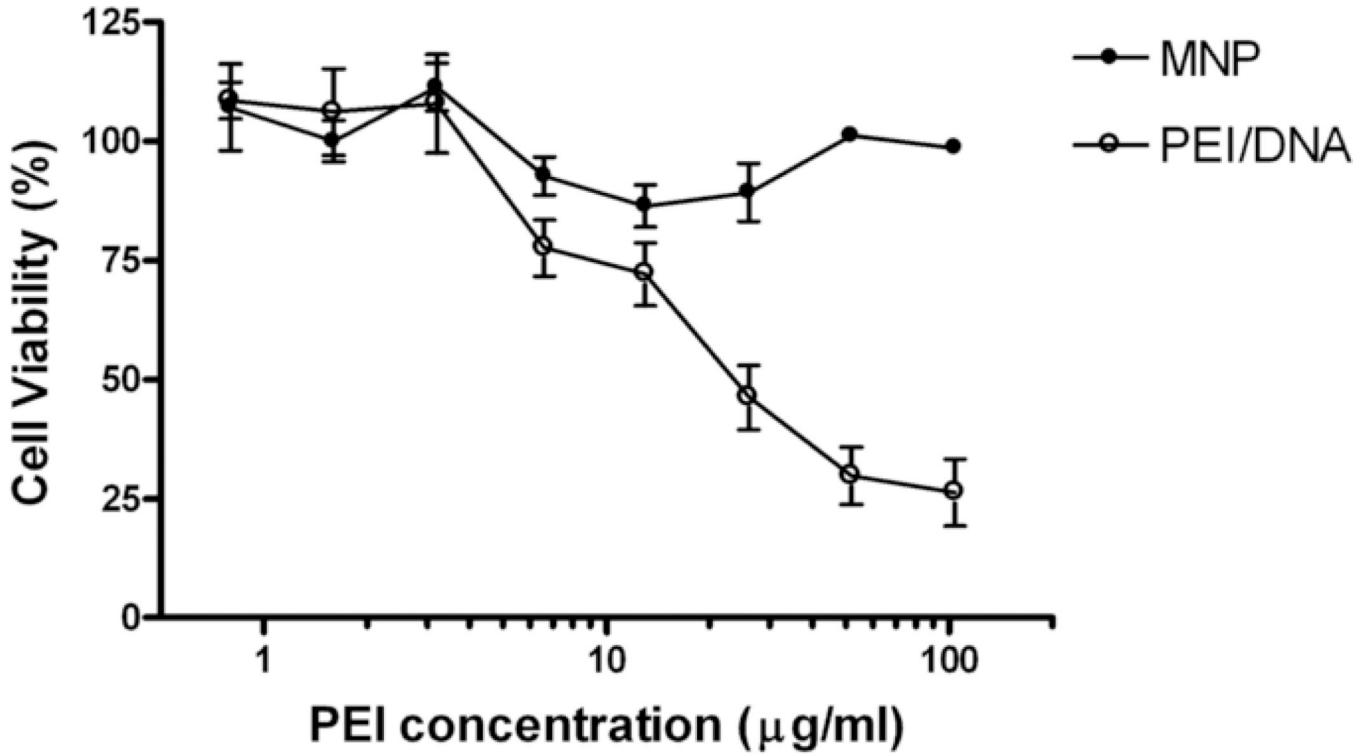
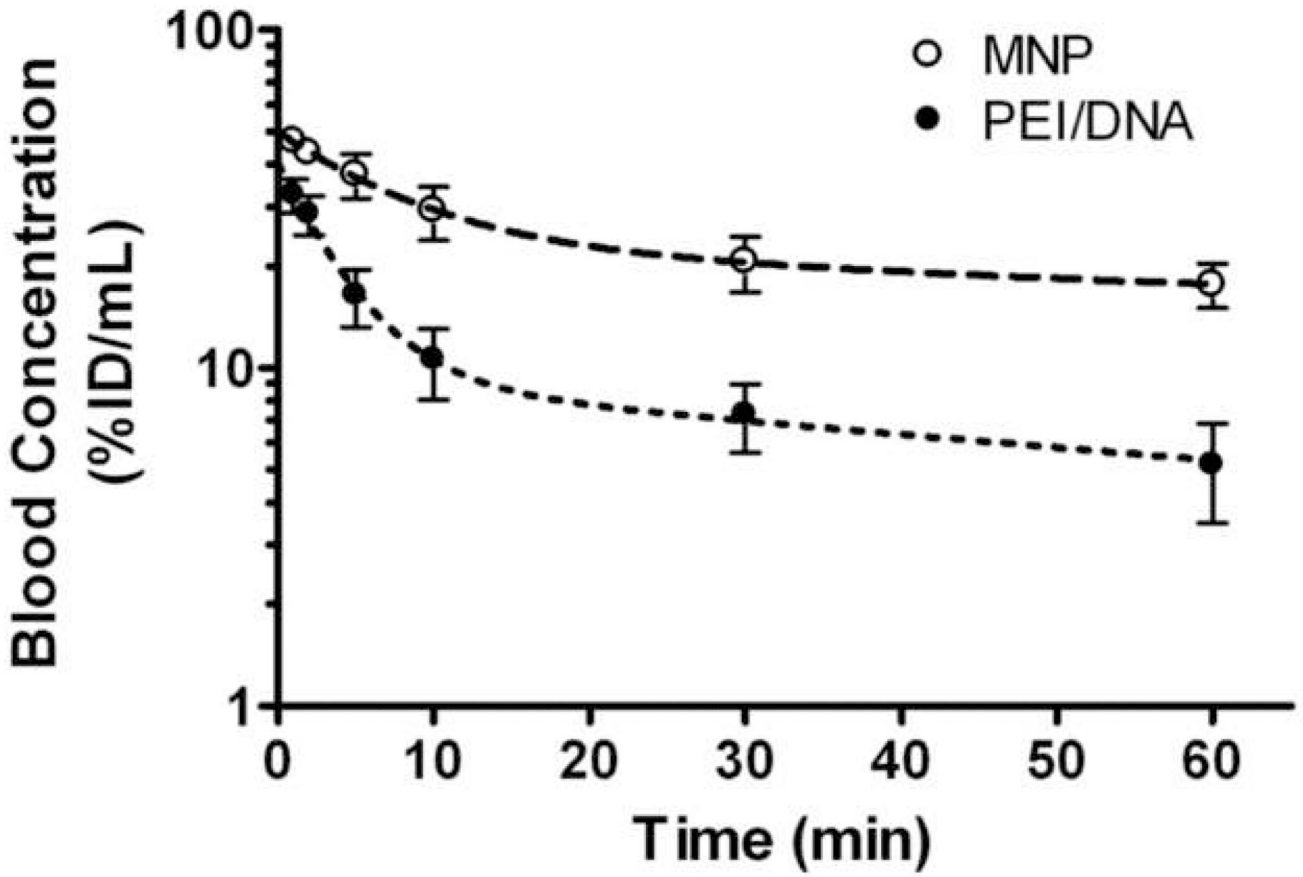


Fig. 4.

Cytotoxicity of MNP towards NIH/3T3 cells. The fibroblast NIH/3T3 cells were treated with DNA-loaded MNP or with PEI/DNA polyplexes at different PEI concentration. Relative cell viability was expressed as a percentage of control cells treated with the medium. In contrast to PEI/DNA polyplexes, MNP showed no cytotoxicity after 24 hrs incubation following 4 hrs of treatments.

5a



5b

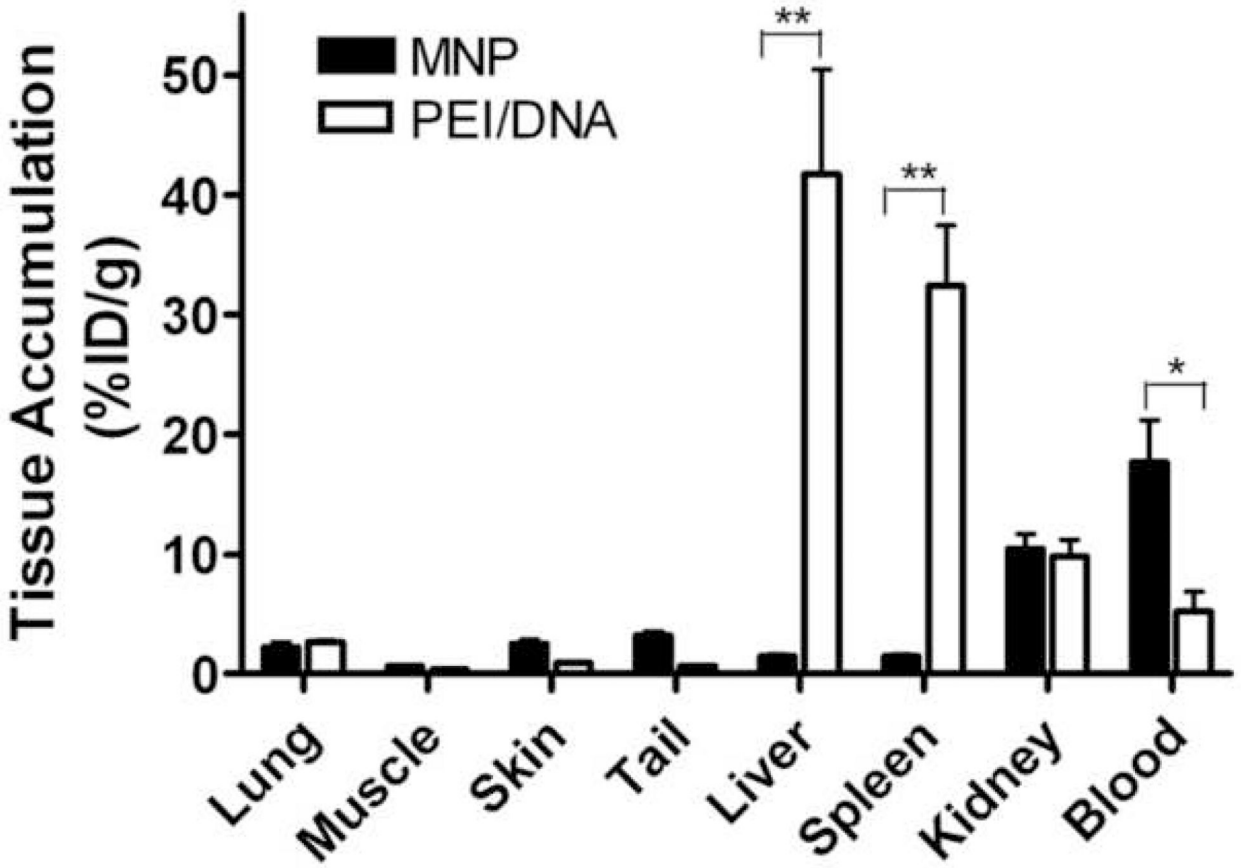


Fig. 5.

In vivo behavior of DNA-loaded MNP and PEI/DNA polyplexes in mice: (**a**) blood concentration-time curve (notice the logarithm scale), and (**b**) organ accumulation of DNA following the i.v. administration of the formulations carrying ^{111}In-labeled DNA. Blood was collected at different time points after the injection, and major organs were collected after the last blood sampling. Radioactivity of the blood and organ samples was measured by the gamma counter and expressed as a percentage of injected dose per ml blood or g tissue (%ID/ml or %ID/g). MNP showed a prolonged blood circulation and reduced RES uptake compared to PEI/DNA polyplexes. The p values were determined from the two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test.

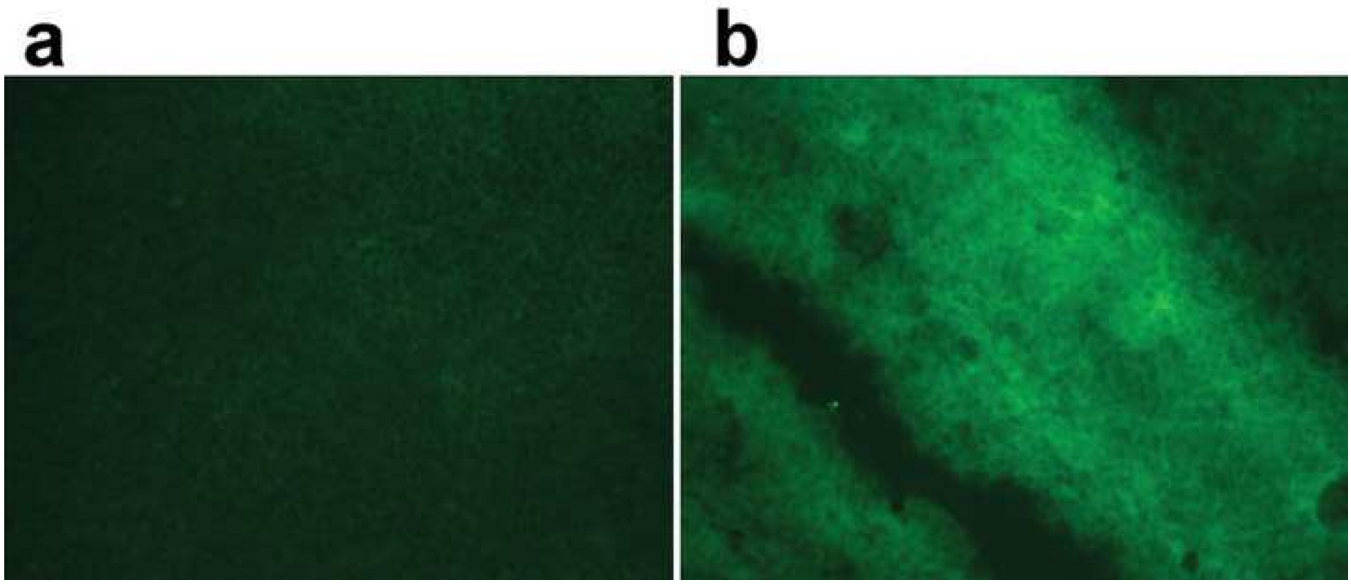


Fig. 6.

In vivo transfection with pGFP-loaded MNP in the mouse xenograft model. The mice bearing LLC tumors were intravenously injected with MNP loaded with pGFP. At 48 hours post-injection, GFP expression in tumors was accessed. The fluorescence microscopy of frozen tumor sections from *in vivo* grown-LLC tumors is shown. (**a**) Tumor section from a non-treated animal (background pattern); (**b**) Tumor section from the animal injected with MNP loaded with pGFP. Intravenous injection of pGFP-loaded MNP led to bright fluorescence in a distal tumor. GFP expression in tumor tissues from the animals injected with PEI/DNA polyplexes was not accessed due to immediate death of the animals following injections with plains polyplexes at the same DNA content (n = 3).