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Preparation and Characterization of Cationic PLA-PEG Nanoparticles for Delivery of Plasmid DNA

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Abstract The purpose of the present work was to formulate and evaluate cationic poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) nanoparticles as novel non-viral gene delivery nano-device. Cationic PLA-PEG nanoparticles were prepared by nanoprecipitation method. The gene loaded nanoparticles were obtained by incubating the report gene pEGFP with cationic PLA-PEG nanoparticles. The physicochemical properties (e.g., morphology, particle size, surface charge, DNA binding efficiency) and biological properties (e.g., integrity of the released DNA, protection from nuclease degradation, plasma stability, in vitro cytotoxicity, and in vitro transfection ability in Hela cells) of the gene loaded PLA-PEG nanoparticles were evaluated, respectively. The obtained cationic PLA-PEG nanoparticles and gene loaded nanoparticles were both spherical in shape with average particle size of 89.7 and 128.9 nm, polydispersity index of 0.185 and 0.161, zeta potentials of +28.9 and +16.8 mV, respectively. The obtained cationic PLA-PEG nanoparticles with high binding efficiency (>95%) could protect the loaded DNA from the degradation by nuclease and plasma. The nanoparticles displayed sustained-release properties in vitro and the released DNA maintained its structural and functional integrity. It also showed lower cytotoxicity than Lipofectamine 2000 and could successfully transfect gene into Hela cells even in presence of serum. It could be concluded that the established gene loaded cationic PLA-PEG nanoparticles with excellent properties were promising non-viral nano-device, which had potential to make cancer gene therapy achievable.

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Introduction

Gene therapy is a rapidly advancing field with enormous potential to treat vital human diseases such as cancer and inherited genetic disorders fundamentally. The development of potent gene transfer systems that can deliver foreign genes efficiently and safely into target cells is of crucial importance for successful gene therapy. The non-viral vector, possessing significantly low safety risks and convenient preparation in large quantities easily and inexpensively, has been suggested as an alternative to viral vector [1]. Therefore, to develop a safe and effective nonviral vector system is an urgent matter. Among non-viral vectors, cationic gene delivery polymers include poly(ethylenimine) (pEI), poly(2-dimethylaminoethyl methacrylate) (pDMAEMA), and poly-L-lysine (pLL) have been frequently studied. Their gene binding and condensation capacities as well as their in vitro and in vivo transfection properties have been reported in recently ample literatures. These polymers are, however, non-degradable and there is consequently a risk that accumulation in the body occurs, in particular after repeated administration. Further, most of these cationic polymers show some cytotoxicity likely due to adverse interactions with membranes resulting in loss of cytoplasmic proteins, in permeabilization of cellular membranes and collapse of the membrane potential [2]. Consequently, there is a need for biodegradable gene delivery polymers. It's clear that the potential advantages of biodegradable carriers are their reduced toxicity (provided that degradation leads to non-toxic products) and avoidance of accumulation of the polymer in the cells. Moreover, the

degradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol. Recently, the use of nanoparticles prepared with biocompatible and biodegradable poly (D, L-lactide-co-glycolide) (PLGA) or poly (D, L-lactide) (PLA) polymers have attracted much attention due to their favorable physicochemical characteristics in terms of safety, stability, the relative ease of large-scale production, and lack of intrinsic immunogenicity that make them suitable candidates for gene delivery application [3, 4]. These biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent depending on the polymer molecular weight and copolymer [5]. The degradation products, lactic acid and glycolic acid, are removed from the body through citric acid cycle. The degradation time of PLGA/PLA can be altered from days to years by varying the polymer molecular weight, the lactic acid to glycolic acid ratio in copolymer. And recent studies demonstrated rapid escape of PLGA nanoparticles from the endo-lysosomal compartment into cytosol following their uptake [6]. Therefore, PLGA/PLA nanoparticles might be a promising and suitable candidate for gene delivery application.

Although all features makes PLGA/PLA attractive to many researchers involved in DNA delivery,it should be noticed that simple PLGA/PLA nanoparticles shows, indeed, several drawbacks as an ideal gene delivery system [7]. First, because of the large size and hydrophilic character of DNA, encapsulation of plasmid DNA in hydrophobic PLGA/PLA nanoparticles is a challenge. Second, the rate of DNA release from PLGA/PLA nanoparticles is often too slow, and thus also subsequent antigen production in case of DNA vaccination, which may prevent an optimal immune response. Third, PLGA/PLA nanoparticles tend to bind serum protein (such as albumin) in systemic application due to the hydrophobic surface of PLGA/PLA nanoparticles, which lead to opsonization and clearance by the reticuloendothelial system (RES), limiting their therapeutic applications. Based on these considerations, it's necessary to modify the structure of PLGA/PLA in order to improve its hydrophilicity, the gene loading efficiency, release behavior, and stability both in vitro and in vivo.

Polyethylene glycol (PEG) is a commonly used modifier, which is expected to be a good candidate as the soluble polymeric modifier in organic synthesis or a pharmacological polymer due to its high hydrophilicity, low cytotoxicity and high cell permeability [8]. Reportedly, the PLA and PLGA nanoparticles with either adsorbed or grafted PEG layers exhibit prolonged blood circulation times and reduced uptake by the reticuloendothelial system in comparison with their uncoated counterparts [9]. It has been proved that PEG corona could diminish non-specific interactions with serum proteins and decrease the uptake of cationic polymer/DNA complex by the macrophages in the liver and spleen leading to an increased blood circulation time, which ultimately improve the transfection efficiency of polycationic polymers. Furthermore, the hydrophilicity of PLA nanoparticles could be improved after modification with PEG, and the affinity between polymers and DNA would be accordingly enhanced which is favorable to load gene into the nanoparticles. In addition, the microenvironment formed by PEG is beneficial to the activity of protein or gene agents in storage or administration. In addition, although PEGylated PLA or PLGA polymer have come out for a while as drug delivery systems [10, 11], there are few reports on gene delivery systems [12, 13]. Therefore the feasibility and suitability of PEGylated PLA or PLGA polymer as gene delivery system remains to be adequately investigated and addressed.

Usually plasmid DNA is encapsulated into PLGA/PLA particles using the common water-in-oil-water (W/O/W) double emulsion/solvent evaporation method in order to achieve a better protection of plasmid and a more precise control of the release process [14, 15]. However, the double emulsion/solvent evaporation method can not guarantee the integrity of DNA under ultra-sonication or high speed homogenization, which is necessary in the encapsulation process to obtain smaller particle size [16, 17]. One strategy to solve the problem is to adsorb the plasmid DNA onto the surface of cationic PLA/PLGA particles which are modified to display a positively charged surface by inclusion of cationic surfactants such as cetyltrimethyl ammonium bromide (CTAB) in formulations [18, 19]. It was reported that the cationic gene loaded particles substantially improved the immune responses generated by DNA, both in mouse and macaque models [20].

Based on these considerations, the main goal of the present work was to develop a novel cationic PEGylated PLA (PLA-PEG) nanoparticles and explore its applicability and feasibility as a nonviral vector for gene transport. For this purpose, cationic PLA-PEG nanoparticles with appropriate positive charge and particle size was prepared applying an easily performed nanoprecipitation technique and using CTAB as a cationic surfactant and Tween 80 as a cosurfactant. The cationic gene loaded PLA-PEG nanoparticles was then obtained by adsorbing plasmid DNA onto the surface of cationic PLA-PEG nanoparticles through electrostatic interactions. The physicochemical properties and biological properties of the nanoparticles were investigated and characterized, respectively.

Materials and Methods

Materials

Poly(ethylene glycol)-block-polylactide copolymer (PLA-PEG, PEG average Mn 3,000 Da, PLA average Mn

50,000 Da, synthesized by school of chemistry of Shandong University). Cetyltrimethyl ammonium bromide (CTAB) was purchased from Amresco (Amresco, China). pEGFP-N1 was provided by Zhejiang University (China). PicoGreen® dsDNA reagent was obtained from Molecular Probes (Invitrogen, USA). Agarose was purchased from BIO-WEST (Spain). Goldview was obtained from Beijing Saibaisheng Biological Engineering Co. (Beijing, China). DNase I enzyme was obtained from Beijing Yinfeng Century Scientific Develop Co., Ltd (Beijing, China). MTT (3-[4, 5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H- tetrazolium bromide) were purchased from Sigma-Aldrich (China). Lipofectamine 2000 was from Invitrogen (USA). Hela cell line was obtained from American Type Culture Collection (ATCC, USA). All the other chemicals and reagents used were of analytical purity grade or higher, obtained commercially.

Preparation of Cationic PLA-PEG Nanoparticles

Cationic PLA-PEG nanoparticles (abbreviated as PLA-PEG-NPs below) was prepared by the nanoprecipitation method [21] according to the optimized formulation. Typically, accurately weighed (20 mg) PLA-PEG was dissolved into 5 mL acetone under sonication. The resulting polymer solution was slowly (30 mL/h) injected by a micro-syringe pump (KDS 100, USA) into 20 mL magnetically stirring (600 rpm, RCT basic stirrer, IKA, Germany) mixed surfactant solution containing CTAB (0.1%, w/v) and Tween 80 (0.2%, w/v) and agitated for 8 h at room temperature until complete evaporation of the organic solvent. The entire dispersed system was then centrifuged (Beckman, Fullerton, CA) at 15,000 rpm, 4 °C for 30 min. The pellet was re-suspended in Milli-Q water and washed three times to remove the redundant surfactant. Finally it was re-suspended in phosphate buffered saline (PBS, pH 7.4) solution followed by filtered through 0.45 μm nitrocellulose membrane (Millipore) filter and was stored at 4 °C until use.

Preparation and Optimization of Gene Loaded Cationic PLA-PEG Nanoparticles

Adsorption of Plasmid DNA onto PLA-PEG-NPs

The gene loaded cationic PLA-PEG nanoparticles (abbreviated as DNA-PLA-PEG-NPs below) were obtained by means of electrostatic attraction between the anionic plasmid DNA and the blank cationic nanoparticles. Briefly, the report gene pEGFP solution was added into PLA-PEG-NPs at a fixed weight ratio (PLA-PEG: DNA, w/w). The mixed liquor was kept for 20 min at room temperature and the resultant DNA-PLA-PEG-NPs were directly used for further study.

Agarose Gel Electrophoresis of DNA/Nanoparticles Complex

Complex formation between nanoparticles and DNA was analyzed by agarose gel electrophoresis. The gels were prepared with 0.8% (w/v) agarose in 20 mL TAE buffer (40 mM Tris, 40 mM Acetic acid, 1 mM EDTA, pH 8.5) containing 2 μL goldview as stains. A fixed amount (1 μg) of DNA was incubated with various amounts of PLA-PEG-NPs in 100 μL of PBS (pH = 7.4) (the weight ratio of PLA-PEG and DNA is 10:1, 50:1, 100:1, 150:1, 20:1, 500:1, respectively). The resultant DNA-PLA-PEG-NPs and control plasmid DNA were applied to gel electrophoresis at a constant 90 V for 25 min. After the electrophoresis, images were obtained using UV transilluminator and Multimage™ Light Cabinet (Alpha Imagers EC, Alpha Innotech Corporation) to show the location of DNA.

Determination of DNA Binding Efficiency

To measure the DNA binding efficiency of the DNA-PLA-PEG-NPs, the free DNA was separated from the nanoparticles by ultracentrifugation at 15000 rpm, 4 °C for 30 min and the free DNA in supernatants were collected and analyzed using fluorospectrophotometry with PicoGreenTM dsDNA quantitation reagent [22]. The fluorescence was measured by fluorescence spectrophotometer (HITACHI 850, Japan) at excitation and emission wavelengths of 480 and 520 nm, respectively. The amount of DNA was calculated according to the linear calibration curve of DNA ($F = 2 \times 10^{-4}\ C + 0.0437\ R = 0.999\ \ 4$). The binding efficiency was calculated from the following equation:

$$\text{DNA binding efficiency}\,(\%) = \frac{(\text{total DNA content} - \text{free DNA content}) \times 100}{\text{total DNA content}}.$$

Morphology, Particle Size and Zeta Potential of Nanoparticles

The morphology of PLA-PEG-NPs and DNA-PLA-PEG-NPs was examined under transmission electron microscope (TEM, JEM-1200EX, Japan). Samples were prepared by placing a drop of nanoparticle suspension onto a copper grid and air-dried, following negative staining with one drop of 2% aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then directly examined under the transmission electronic microscope.

The average particle size, size distribution, and zeta potential of the nanoparticles were measured by photon correlation spectroscopy (PCS) using Zetasizer 3000 (Malvern Instruments, Malvern, England). All measurements were carried out in triplicates. The average particle

size was expressed in volume mean diameter and the reported value was represented as mean ± SD ($n = 3$).

Stability Test of the DNA-PLA-PEG-NPs Against DNase I

Protection of plasmid DNA from nucleases is one of the most important properties for efficient gene delivery both in vitro and in vivo. To test whether DNA-PLA-PEG-NPs can protect the loaded plasmid DNA from nucleases digestion, the results of DNase I mediated digestion was evaluated using agarose gel electrophoresis [23]. In brief, 100 μL of DNA-PLA-PEG-NPs containing 1 μg of DNA were, respectively, incubated with different amounts of DNase I (0.1, 0.2 and 0.4 U/μg DNA) in DNase I /Mg^{2+} digestion buffer (50 mM, Tris–HCl, pH 7.6, and 10 mM $MgCl_2$). Naked DNA (1 μg) was treated with DNase I at 0.1 U/μg DNA as a reference. The suspension was incubated in shaking water bath (100 rpm) for 30 min at 37 °C. After that, the enzymatic digestion reaction was terminated with 5 μL EDTA solution (0.5 M, pH 8.0) for 10 min at room temperature. To asses the integrity of DNA loaded in the nanoparticles, it was dissociated from the cationic nanoparticles by adding heparin solution [24], an anionic glycosaminoglycan, at final concentration of 1% (w/v) and the suspension was then incubated in shaking water bath (100 rpm) for 3 h at 37 °C. The configuration of plasmid DNA in the nanoparticles after extraction was analyzed by gel electrophoresis with untreated naked DNA as a reference. The samples were applied to a 0.8% (w/v) agarose gel in TAE buffer as described above.

The Plasma Stability Investigation of DNA-PLA-PEG-NPs

To examine resistance of DNA-PLA-PEG-NPs to DNA degradation in plasma, 25 μL of plasmid DNA solution and DNA-PLA-PEG-NPs were, respectively, incubated with 25 μL of 20% human plasma for 1 h in a 37 °C incubator. Immediately following the incubation, nucleases were inactivated with 3 μL of EDTA solution (0.5 M, pH 8.0) for 10 min at room temperature. Thereafter, the plasma treated samples together with untreated DNA-PLA-PEG-NPs and DNA were all applied to 0.8% (w/v) agarose gel as described above.

The In Vitro Release of DNA-PLA-PEG-NPs

The Stability of Plasmid DNA in the Release Medium

During the prolonged in vitro drug release, the biomacromolecule drug may be degraded, and this may affect in vitro drug release studies [13, 25]. Therefore, it is essential to select a suitable drug release medium to protect the plasmid DNA from degradation during in vitro drug release period. Based on physiological environment and the result of preliminary tests, the mixed solution containing phosphate buffered saline solution at pH 7.4 (100 mM) and 150 mM NaCl solution was selected as the release medium for the DNA-PLA-PEG-NPs. The stability of plasmid DNA in the release medium in the duration of in vitro release was investigated at first. Briefly, 200 μL DNA solution (10 μg/mL) were diluted with isovolumic release medium mentioned above in Eppendorf® tubes and then shaken horizontally in water bath at 37 °C and 100 rpm. Separate tubes were used for each data point. At predetermined time intervals (2 h, 5 h, 8 h, 12 h, 24 h, 2 days, 4 days, 7 days, 10 days, 15 days, 20 days), the samples were withdrawn and the concentration of DNA was determined by the PicoGreen fluorimetric assay mentioned above. The experiments were repeated three times and all measurements were collected in triplicates. The profile of percent content of DNA versus time in release medium was drawn.

Measurement of In Vitro DNA Release

The profiles of in vitro DNA release from DNA-PLA-PEG-NPs were measured over 20 days using separate samples for each time point according to the following operations. Typically, 200 μL of DNA-PLA-PEG-NPs (DNA concentration: 10 μg/mL) were diluted with isovolumic release medium in Eppendorf® tubes and then shaken horizontally in water bath at 37 °C and 100 rpm. At predetermined time intervals, the tubes were withdrawn and centrifugated at 15,000 rpm for 30 min, the supernatants were collected for analysis. The amount of the released DNA was evaluated by the PicoGreen fluorimetric assay. Background readings were corrected using the centrifugation supernatants from blank nanoparticles.

Integrity Determination of Plasmid DNA After Release

The molecular form of DNA (often referred to as "topology") has been demonstrated to affect the transfection efficiency in vitro and in vivo [26]. Supercoiled plasmid was believed to be the most efficient form for cell transfection and it was established that the order of transfection efficiency was supercoiled > relaxed > linear [27]. Fabrication conditions such as sonication, lyophilization, and change of pH were reported previously to decrease the supercoiled content. It is well known that the hydrolysis of PLA may substantially decrease the pH in PLA nanoparticles, which potentially change the topology structure of DNA and decrease the biological activity of DNA accordingly. Therefore, it was crucial to examine the plasmid structural and functional integrity following release from nanoparticles.

Structural Integrity Determination

Structural integrity of plasmid DNA released from nanoparticles at selective time points was evaluated by agarose gel electrophoresis. To determine plasmid stability during release, the samples released at several time points (i.e. 2 h, 8 h, 24 h, 4 days, 10 days, 15 days and 20 days) along with control untreated plasmid DNA were applied to 0.8% agarose gel in TAE buffer. Band separation for topological structure of plasmid DNA was observed after gel electrophoresis.

Functional Integrity Determination

The in vitro transfection experiment was performed to investigate the functional integrity of released DNA. Hela cells (ATCC) were seeded into 24-well plates at a density of 1×10^5 cells/well 24 h prior to transfection and 1 μg of DNA of was used per well ($n = 6$ wells per formulation). An aliquot of untreated DNA solution and supernatants of nanoparticles in vitro release at different time points after moderate concentration (1 day, 10 days, 20 days, equivalent to 1 μg DNA) were complex with Lipofectamine 2000 according to the manufacturer's instructions [28]. After incubation for 4 h at 37 °C in 5% CO_2 incubator, the original incubation media was replaced with 1 mL of complete medium and cells were incubated sequentially until 24 h post transfection. The percentage of fluorescent cells (defined as cells having fluorescence levels above that of untreated control cells) presented in every 10,000 cells counted by the flow cytometry was calculated. Transfection experiments were performed in triplicates.

Cell Viability Test of DNA-PLA-PEG-NPs

The cytotoxicity of DNA-PLA-PEG-NPs was evaluated by MTT method in Hela cell line [19]. Briefly, the cells were seeded into a 96-well microtiter plates at a density of 1×10^4 cells per well in 0.2 mL of RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 incubator at 37 °C overnight. After that, the culture medium was replaced by 200 μL fresh serum-free RPMI 1640 medium with different concentrations of the nanoparticles (expressed as PLA-PEG concentration, 5, 10, 25, 50, 100 and 250 μg/mL) and Lipofectamine 2000 in comparison. The applied dosage of gene vectors (Lipofectamine 2000/DNA complex and DNA-PLA-PEG-NPs) were proportional to that used in transfection experiment. After incubation for 24 h, 20 μL of MTT stock solution in PBS (5 mg/mL, pH 7.4) was added into each well at a final concentration of 0.5 mg/mL MTT. The plate was then incubated at 37 °C in 5% CO_2 for 4 h. The medium was removed and 150 μL DMSO was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 570 nm by microplate reader (Model 680, BIO-RAD, USA). The cell viability (%) was calculated and compared with the untreated control (100%) according to the following equation:

$$\text{Cell viability}\ (\%) = \left(\text{OD570}_{(\text{samples})}/\text{OD570}_{(\text{control})}\right) \times 100$$

$\text{OD570}_{(\text{samples})}$ represented measurements from the cells treated with samples and $\text{OD570}_{(\text{control})}$ from the untreated cells.

Statistical Analysis

All measurements were collected in triplicates and experiments were repeated three times. Values were expressed as mean ± standard deviation. Unpaired Student's *t*-test was used to assess statistical significant differences ($p < 0.05$) between the group means.

In Vitro Transfection Assays of DNA-PLA-PEG-NPs

The transfection activity of DNA-PLA-PEG-NPs was evaluated in Hela cell lines using plasmid DNA, encoding enhanced green fluorescence protein (EGFP) as reporter gene in the transfection studies. The cells were seeded into 24-well plates at a density of about 1×10^5 cells per well in 1 mL of RPMI 1640 culture medium with 10% FBS, 24 h prior to transfection. At a confluence level of 70–80%, cells were washed twice with PBS, and, respectively, incubated with 500 μL of media (with or without 10% FBS) containing 1 μg of DNA in transfection vectors at 37 °C. Lipofectamine 2000 (Invitrogen) was used as positive control, and the formulation of Lipofectamine/DNA complex was carried out according to the manufacturer's protocols. 1 μg of plasmid DNA was used as negative control. The cells were incubated with the vectors for 4 h, in the presence or absence of serum medium. The transfection media was then replaced with 1 mL of fresh complete culture media, and the cells were incubated sequentially until 24 h post transfection. Detection of expression of EGFP was carried out using an inverted fluorescent microscope with an attachment for fluorescent observation (OLYMPUS, ZX71, Japan) and the picture was captured using a 400× objective. Transfection experiments were performed in triplicates.

Results and Discussions

Formation of PLA-PEG-NPs

In this study we have designed a novel cationic PLA-PEG nanoparticles modified by CTAB as a nonviral vector for

gene transport. Although PLA-PEG nanoparticles has several good qualities, such as good biodegradability, biocompatibility, and nonimmunogenicity, it takes on negative surface charges because of the carboxylic end groups of PLA. To prepare PLA-PEG mediated DNA complex, the cationic surface-active agent CTAB was used to modify the surface of PLA-PEG nanoparticles to make the surface take on positive charges. However, it was found that nanoparticles prepared with sole CTAB as surfactant (CTAB-NPs) revealed highly aggregated structures and heterogeneous size in TEM imaging (Fig. 1a), which was in accord with the other report [29]. In contrast, nanoparticles formulated with the popular non-ionic surfactant, Tween 80 (Tween 80-NPs) exhibited a uniform and smaller particle size without aggregation (Fig. 1b), which might be due to the superior emulsifying capacity of Tween 80. Moreover, it has some interesting characteristics useful for gene therapy because of the presence of poly(ethyleneglycol) (PEG) chains in its structure. It has been hypothesized that Tween 80 may have a similar fusogenic property with DOPE (Dioleylphosphatidylethanolamine) [30]. When a comparable concentration of Tween 80 is added to the formulation of nanoparticles, their transfection capacity will be improved significantly [31]. Besides, Tween 80 has another important characteristic for the transfection of these systems in vivo. It creates a steric barrier [32] which shields the excess of positive charges of the systems and reduces the interaction with blood components, such as serum proteins, which could limit the arrival of the gene therapy system to the cell surface. Taking these into consideration, Tween 80 was applied as a cosurfactant together with the cationic surfactant CTAB to formulate PLA-PEG nanoparticles in the present study.

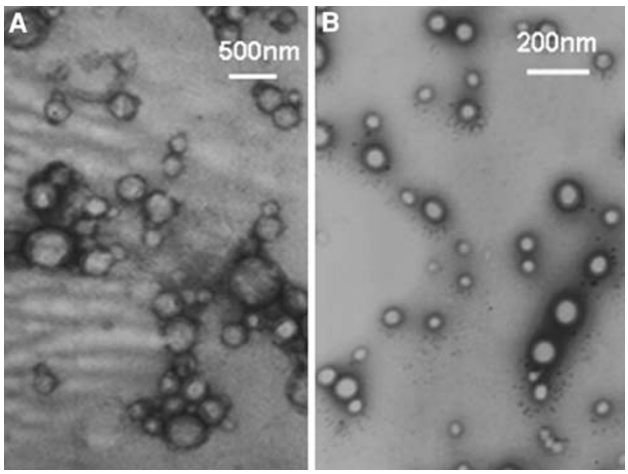

Fig 1 TEM imaging of CTAB-NPs (**a**) and Tween 80-NPs (**b**)

Formation of DNA-PLA-PEG-NPs

Agarose gel electrophoresis and Picogreen-fluorometry method were, respectively, performed to investigate the optimal ratio of NPs to DNA for the binding efficiency qualitatively and quantitatively. It was observed that sole plasmid DNA could migrate to the positive electrode under the electric field such as lane 1 and 8 in Fig. 2. Once DNA was associated with the nanoparticles which was too large to diffuse through the agarose matrix, the mobility of DNA was hindered and it was detained in the well of the agarose gel. In addition, the part of DNA which did not bind with PLA-PEG-NPs migrated to the positive electrode in the same manner with the control DNA. The results of agarose gel electrophoresis (Fig. 2) showed that PLA-PEG-NPs may bind with DNA to various degrees with different mass ratios of NPs to DNA in the complex. There was decreasing free DNA present in the lane with the increase of the ratio of NPs/DNA. When the mass ratio of NPs to DNA reached 100:1 or above, almost all DNA was combined with NPs without free DNA bands in the lane visible. Meanwhile, the results obtained by Picogreen-fluorometry analysis were consistent with that of agarose gel electrophoresis. As shown in Fig. 3, the binding efficiency was increasingly raised with the mass ratio of NPs to DNA from 10:1 to 100:1, and it was 95.36% when the ratio reached 100:1. Further increase of the ratio did not significantly improve the binding efficiency but will decrease the DNA loading efficiency of nanoparticles. Thus 100:1 was selected as the optimal mass ratio of NPs to DNA for further study.

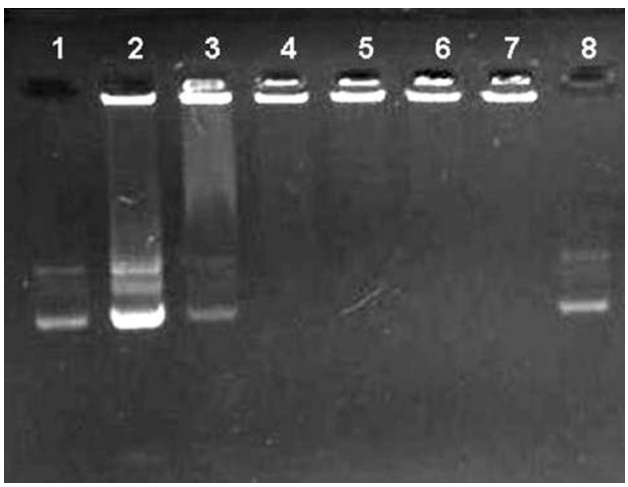

Fig. 2 Agarose gel electrophoresis analysis of PLA-PEG-NPs combining with DNA at different mass ratio. *Lane 1*, *8*: DNA Control; *Lane 2–7*: the mass ratio of NPs/DNA was 10:1, 50:1, 100:1, 150:1, 200:1, 500:1, respectively

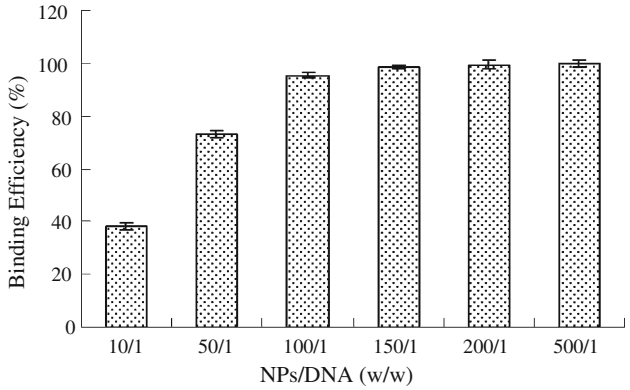

Fig. 3 Binding efficiency of PLA-PEG-NPs with DNA at different mass ratio ($n = 3$)

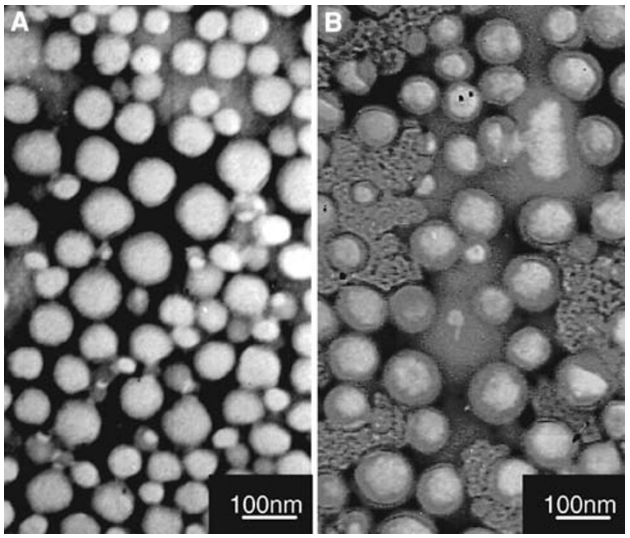

Fig. 4 TEM imaging of PLA-PEG-NPs (**a**) and DNA-PLA-PEG-NPs (**b**)

Physicochemical Characterization of PLA-PEG-NPs and DNA-PLA-PEG-NPs

The transmission electron micrograph of PLA-PEG-NPs and DNA-PLA-PEG-NPs was illustrated in Fig. 4. It was shown that the obtained nanoparticles appeared similar spherical in shape and separated from each other. Figure 5 showed a representative size distribution profile of the nanoparticles. The polydispersity index (PDI) of PLA-PEG-NPs and DNA-PLA-PEG-NPs were 0.185 and 0.161, respectively, demonstrating a relatively narrow particle size distribution. The resultant PLA-PEG-NPs and DNA-PLA-PEG-NPs were small in size with mean particle size of 89.7 and 128.9 nm, respectively, and positive surface charge with zeta potential of +28.9 and +16.8 mV, respectively. It could be explained that while DNA combined with PLA-PEG-NPs, the particles size increased and the absolute value of surface charge decreased due to the neutralization effect between the anionic DNA and the cationic nanoparticles.

Protection of DNA-PLA-PEG-NPs Against DNase I

Degradation of DNA by nuclease, such as DNase I, is a major barrier for gene delivery both in vitro and in vivo. To test whether the resultant nanoparticles can protect loaded plasmid DNA from nucleases digestion, the nanoparticles were exposed to DNase I. Figure 6 showed that naked plasmid DNA (lane 4) was completely digested by DNase I at 0.1 U/μg DNA within 30 min of incubation, confirming the activity of nucleases. While DNA extracted from DNA-PLA-PEG-NPs remained intact supercoiled form in the investigated concentrations of DNase I within 30 min of incubation (lane 2–4), similar to the DNA control in lane 5. These results demonstrated that DNA-PLA-PEG-NPs indeed could protect the loaded DNA from degradation by DNase I at determinate concentrations for 30 min, and maintained the structural integrity of gene.

Stability of DNA-PLA-PEG-NPs in Plasma

Figure 7 showed the stabilization of DNA-PLA-PEG-NPs in plasma. It could be observed that the original plasmid DNA bands in lane 1 disappeared at the exposure of 10% human plasma for 1 h in lane 2, suggesting that the plasmid DNA had been degraded completely by nucleases in plasma. However, DNA-PLA-PEG-NPs remained relatively stable in the presence of plasma, with most loaded DNA detained in the well. This confirmed that the DNA-PLA-PEG-NPs could not only bind DNA efficiently onto its surface but also partially protect the condensed DNA against degradation by plasma protein, which was promising to improve the in vivo stability of DNA and favored the in vivo delivery of theoretical gene.

In Vitro Release of DNA-PLA-PEG-NPs

The stability of plasmid DNA in the condition identical to in vitro release of DNA-PLA-PEG-NPs was investigated in the present study. As shown in Fig. 8, the DNA percent content at the predetermined time point maintained above 95% during 20 days. It was clearly indicated that the plasmid DNA could remain extremely stable in the adoptive condition during the monitoring time without substantial degradation, that was, the release medium has potential to improve the stability of plasmid DNA and prevent its degradation. This result could be explained that dsDNA has higher stability at high salt concentrations and physiological pH which was in agreement with other report [33]. Therefore, the content of DNA released from

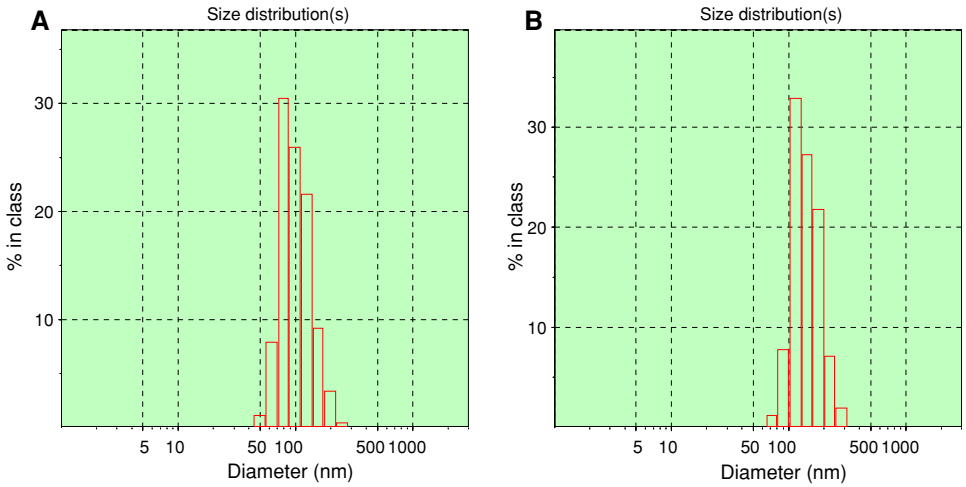

Fig. 5 Representative size distribution profile for PLA-PEG-NPs (**a**) and DNA-PLA-PEG-NPs (**b**)

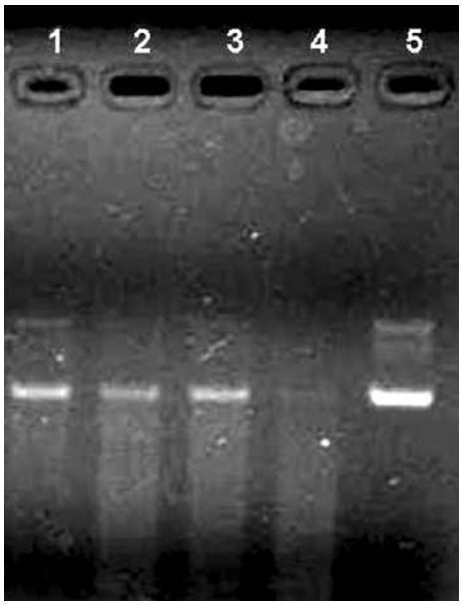

Fig. 6 Agarose gel electrophoresis of DNA-PLA-PEG-NPs after being incubated with DNase I at different concentrations. *Lane 1–3*: DNA-PLA-PEG-NPs incubated with different amount of DNase I at 0.1, 0.2, 0.4 U/μg DNA, respectively, for 30 min; *Lane 4*: Naked DNA incubated with DNase I at 0.1 U/μg DNA for 30 min; *Lane 5*: DNA control

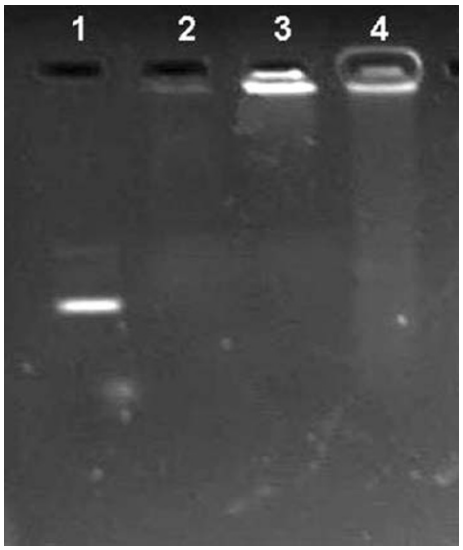

Fig. 7 Plasma stability of DNA-PLA-PEG-NPs. *1*: DNA control; *2*: Naked DNA incubated in 10% plasma for 1 h; *3*: DNA-PLA-PEG-NPs Control; *4*: DNA-PLA-PEG-NPs incubated in 10% plasma for 1 h

DNA-PLA-PEG-NPs in vitro could be determined directly and calculated without adjustment.

The in vitro drug release property is one of the important characteristics of nanoparticles.

Figure 9 showed the accumulative release (expressed as percentage of loaded DNA) versus time curve of DNA from DNA-PLA-PEG-NPs in vitro during 20 days. Release profile of DNA from DNA-PLA-PEG-NPs exhibited an initial burst of release of about 51.7% in the first 12 h, followed by slow and continuous release in 20 days. In order to determine the release mechanism of DNA from DNA-PLA-PEG-NPs, the release data were evaluated by

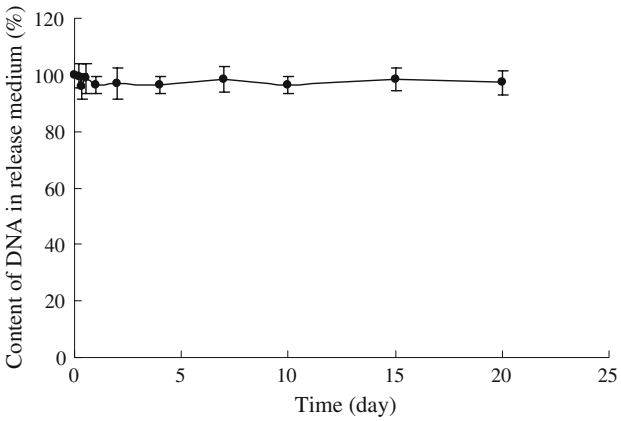

Fig. 8 The profile of content of DNA in release medium with time ($n = 3$)

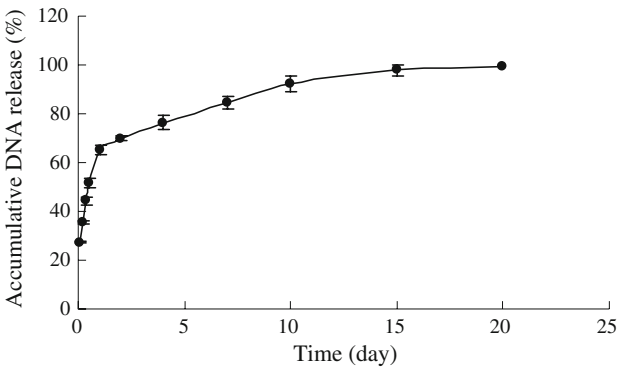

Fig. 9 Accumulative release of DNA from DNA-PLA-PEG-NPs ($n = 3$)

model-dependent methods, and the result showed that the release behavior of DNA from DNA-PLA-PEG-NPs fitted into bioexponential flow equation best: $100 - Q = 78.70e - 0.9919t + 51.37e - 0.2073t$ ($R_\alpha = 0.998$ 2, $R_\beta = 0.993$ 6). It could be predicted that the established DNA-PLA-PEG-NPs with a delayed release effect could keep the loaded DNA prolonged action in vivo.

Integrity Investigation of the DNA Released from DNA-PLA-PEG-NPs

Plasmid DNA released from DNA-PLA-PEG-NPs at various time points was evaluated for its structural integrity on 0.8% agarose gel in comparison with control DNA (Fig. 10). It can be observed that the DNA released from DNA-PLA-PEG-NPs was predominantly supercoiled (S.C.) in form and comparable with the native DNA. Although some evidence of gradual increase in the percentage of open circular plasmid DNA (O.C.) was visible at later time points, no linear configuration was observed during the process of in vitro release. These results indicated that DNA-PLA-PEG-NPs could protect the loaded plasmid DNA from degradation to some extent in the release duration.

The results of the functional integrity of released DNA based on in vitro transfection experiment were shown in Table 1. It was evident from the result that DNA after releasing from DNA-PLA-PEG-NPs still retained gene expression activity. In the prophase of in vitro release (1 day and 10 days), the released DNA from DNA-PLA-PEG-NPs completely maintained its bioactivity as the in

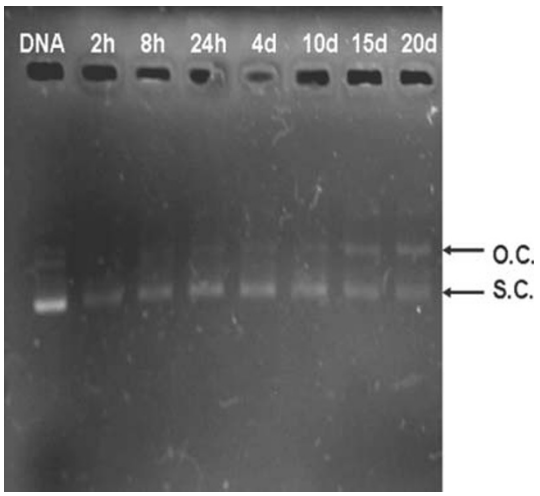

Fig. 10 Agarose gel electrophoresis of DNA released from DNA-PLA-PEG-NPs

vitro transfection efficiency of released DNA/lipofectamine complex was equivalent to the untreated DNA that was complexed with Lipofectamine 2000 ($p > 0.05$). While in the terminal in vitro release (20 days), the transfection efficiency of the released DNA/Lipofectamine complex was a little lower than the untreated DNA/Lipofectamine complex ($p < 0.05$). That results might be explained that more DNA had converted into open circular with release time as demonstrated by agarose gel electrophoresis described above, and the bioactivity of open circular DNA was reported to be less efficient than supercoiled configuration [34].

Evaluation of the Cytotoxicity of DNA-PLA-PEG-NPs

In vitro toxicity of DNA-PLA-PEG-NPs was evaluated by MTT assay in Hela cells. The cytotoxicity of DNA-PLA-PEG-NPs at various concentrations against Hela cell was shown in Fig. 11. Lipofectamine 2000, which is commonly used in in vitro gene transfection, was evaluated in comparison with DNA-PLA-PEG-NPs. As illustrated in Fig. 11, the Hela cell viabilities decreased with the increased concentration of DNA-PLA-PEG-NPs while the average cell viabilities of different formulations at the studied concentrations (5–250 μg/mL) were between 80 and 120% versus control cells and were all significantly higher than that of Lipofectamine 2000 ($p < 0.05$), which

Table 1 Biological activity of DNA released from DNA-PLA-PEG-NPs ($n = 3$)

	Untreated DNA/lipofectamine complex	Released DNA/lipofectamine complex		
		1 day	10 days	20 days
Transfection efficiency (%)	51.01 ± 2.43	49.36 ± 2.83	46.95 ± 1.68	43.41 ± 2.36*

* $p < 0.05$ compared with untreated DNA/lipofectamine complex

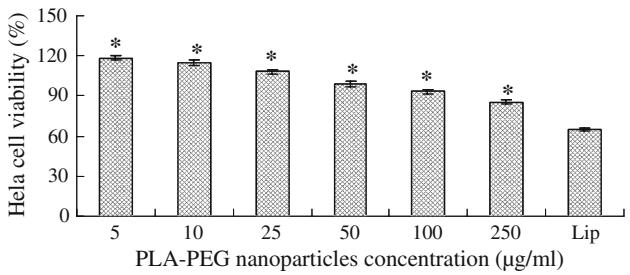

Fig. 11 Cell viability of DNA-PLA-PEG-NPs against Hela cell line by MTT assay ($n = 3$). $*p < 0.05$ compared with Lip

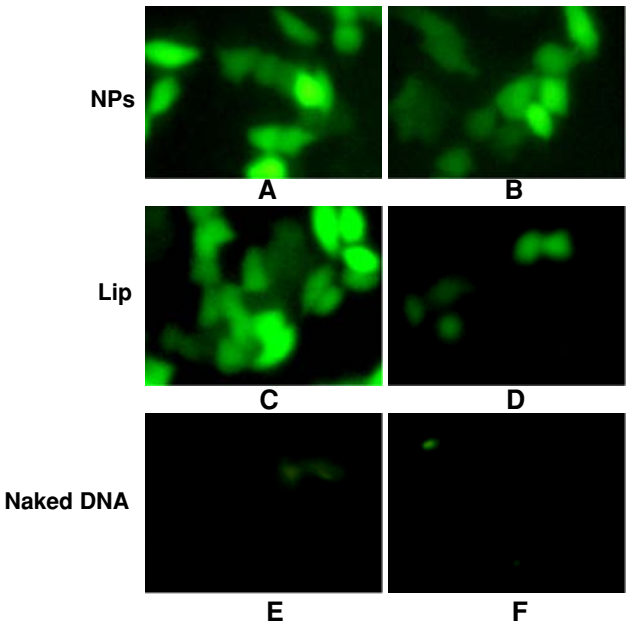

Fig. 12 Gene transfection of pEGFP in Hela cells (×400) (**a**, **b**) The transfection results of DNA-PLA-PEG-NPs in absence and presence of serum; (**c**, **d**): The transfection results of Lipofectamine 2000 in absence and presence of serum; (**e**, **f**): The transfection results of naked DNA in absence and presence of serum

indicated that the DNA-PLA-PEG-NPs were much safer than Lipofectamine 2000 to Hela cell.

In Vitro Transfection Studies

An efficient gene delivery system is required to transport the gene into cells and see to its eventual release, leading to gene expression and subsequent protein synthesis. The current study aimed to determine the ability of DNA-PLA-PEG-NPs to transfer the reporter gene EGFP to Hela cell. Transfection ability of our established vectors DNA-PLA-PEG-NPs was investigated in comparison with the commercial cationic liposome based reagent, Lipofectamine 2000 which was well known to provide high transfection efficiency and high level of transgene expression in a range of mammalian cell types in vitro. However, it was found that cationic liposomes tended to bind with some serum protein such as albumin in vivo, forming aggregation and eliminated quickly, which led to relative lower transfection efficiency. Thus it was meaningful to investigate the effects of serum in transfection medium on the in vitro transfection activity of gene vectors. In the present study, transfection activities of them were compared in the system with or without serum because serum was known to influence the transfection efficiency of gene vectors sometimes [35]. The results observed by inversion fluorescence microscope after transfection (Fig. 12) showed that DNA-PLA-PEG-NPs may successfully transfer plasmid DNA into Hela cells, and the gene can encode the green fluorescent protein. It was noticeable that DNA-PLA-PEG-NPs was a much more efficient carrier than the EGFP plasmid alone irrespective of serum in the medium, which could barely transfect Hela cell. In the absence of serum in the transfection medium, DNA-PLA-PEG-NPs produced slightly lower transfection efficiency than Lipofectamine 2000 during 24 h which might be due to the sustained release property of DNA-PLA-PEG-NPs. However, Lipofectamine 2000 showed apparently diminished transfection activities in the presence of serum whereas DNA-PLA-PEG-NPs still maintained high transfection activity in the presence of serum, which was visibly higher than that of Lipofectamine 2000. These results indicated that the serum in the transfection medium hardly influenced the transfection activity of DNA-PLA-PEG-NPs but weakened the transfection activity of Lipofectamine 2000 significantly. It might be explained that cationic liposomes tended to adsorb anionic serum protein and form a larger aggregate which could not get across the cell membrane and deliver the DNA payload to the nucleus. The DNA-PLA-PEG-NPs with PEGylation could prevent the non-specific interactions with serum protein and minimize particle aggregation in buffers especially in the transfection medium and accordingly improve the transfection efficiency of nanoparticles [36].

Conclusion

In the present work, cationic biodegradable nanoparticles based on PEGylated PLA copolymers was formulated using the mild and simple performed nanoprecipitation technique. Report gene, pEGFP was adsorbed onto the surface of the cationic PLA-PEG-NPs through electrostatic interactions, leading to the gene loaded nanoparticles (DNA-PLA-PEG-NPs). The obtained DNA-PLA-PEG-NPs with high binding efficiency (95.36%), positive surface charge (+16.8 mV) and small particle size (128.9 nm) showed sustained-release of DNA in vitro within 20 days. Adsorption and release from DNA-PLA-PEG-NPs did not alter structural and functional integrity of plasmid DNA.

Moreover, DNA adsorbed onto DNA-PLA-PEG-NPs was efficiently protected from nuclease degradation and could remain relatively stable in plasma. It was less toxic than commercial Lipofectamine 2000 and safer to Hela cell. And it could successfully transfer plasmid EGFP into Hela cells, what's more, the transfection activity was not diminished dramatically by the serum in transfection medium which surpassed the commercial cationic liposome based reagent, Lipofectamine 2000. It could be anticipated that the established DNA-PLA-PEG-NPs in current study was a promising nonviral gene delivery system used in gene therapy. It has a potential to alleviate the acute toxicity effect of other vectors and prolong the circulation time in vivo. Our further study will focus on the optimization of the formulation for higher transfection efficiency and the in vivo performance of the DNA-PLA-PEG-NPs as gene delivery system will also be conducted.

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