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PEG-*b*-PPA/DNA micelles improve transgene expression in rat liver through intrabiliary infusion

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Abstract

We have developed a new block copolymer gene carrier that comprises of a polyethylene glycol segment and a degradable cationic polyphosphoramidate (PPA) segment. This PEG-*b*-PPA copolymer carrier formed micelles upon condensation with plasmid DNA in aqueous solution. PEG-*b*-PPA/DNA micelles exhibited uniform and reduced particle size ranging from 80 to 100 nm and lowered surface charge, compared with complexes of DNA with the corresponding cationic PPA carrier. PEG-*b*-PPA/DNA micelles maintained similar transfection efficiency as PPA/DNA complexes, which was comparable to that of PEI/DNA complexes in HepG2 cells, but yielded about 16-fold lower transgene expression in primary rat hepatocytes than PPA/DNA complexes. Following bile duct infusion in Wistar rats, PEG-*b*-PPA/DNA micelles mediated 4-fold higher and more uniform gene expression in the liver than PPA/DNA complexes. Liver function tests and histopathological examination indicated that PEG-*b*-PPA/DNA micelles showed low toxicity and good biocompatibility in the liver. This study demonstrated the potential of PEG-*b*-PPA/DNA micelles as an efficient carrier for liver-targeted gene delivery.

Keywords

Polymeric micelles; gene delivery; liver-targeted; polyphosphoramidate; block copolymer; intrabiliary infusion

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1. INTRODUCTION

Liver is one of the most important targets for gene medicine applications because of its susceptibility to many metabolic genetic disorders, viral infection, and various malignancies. In addition, transgene products produced in the liver have ready access to systemic circulation making this organ attractive for *in situ* production of recombinant therapeutics in treating “off-site” diseases [1-3]. A number of gene vectors have been studied for liver-targeted gene delivery, including viral and non-viral vectors. Viral vectors, particularly adenovirus [4,5], and lentivirus [6], have demonstrated high level of transgene expression. Significant barriers for clinical applications of viral vectors include host immune response against the vectors that leads to the rapid clearance of transduced cells [7]; intrinsic toxicity of the viral proteins; and limited packaging size. Non-viral gene carriers offer the advantages of versatile design, ease of synthesis and scale-up, better storage stability, flexibility on transgene size, and most importantly, the low toxicity and minimal host immune response [8]. Various non-viral vectors have been evaluated for delivering genes to the liver, including liposomes and cationic polymers carriers, but failed to show significant transgene expression in the liver [9-11]. A major problem associated with these delivery systems is their instability in physiological medium. For example, DNA/carrier complexes could aggregate severely in serum, and therefore would be trapped in lung capillaries and scavenged by macrophages (e.g. alveolar macrophages and Kupffer cells) [9].

We have developed a new class of polyphosphoramidates (PPAs) as non-viral gene carriers [12]. This series of water soluble polycationic gene carriers possess the same hydrophilic backbone but different side chain structures carrying various types of charge groups. These carriers exhibited less cytotoxicity than PEI and PLL *in vitro* and *in vivo* [13,14]. Among the most effective carriers in this series is PPA with N,N-bis(aminopropyl) phosphamido side chains (Fig. 1), based on transfection activities in cell lines and primary rat hepatocytes [14]. However, these PPA/DNA complexes suffer from the common aggregation problem upon contacting physiological media, e.g. medium containing serum or bile, leading to low transfection efficiency *in vivo*.

Polymer/DNA micelles have been developed recently as a particularly attractive approach to enhance the colloidal stability of polymer/DNA complexes in physiological medium. Polymer/DNA micelles comprised of a characteristic core-shell structure with polycation/DNA complexes surrounded by a protective and stabilizing hydrophilic corona [15-19]. The high mobility and hydrophilicity of the PEG corona prevent protein adsorption and confer stability of the micelles in physiological media. This shell layer also likely provides additional protection to plasmid DNA against enzyme degradation [17]. In this report, we will describe the synthesis and characterization of new PEG-*b*-PPA block copolymer and PEG-*b*-PPA/DNA micelles. We will compare the transfection efficiency of PEG-*b*-PPA/DNA micelles with PPA/DNA and PEI/DNA complexes in cell culture. More importantly, we will evaluate the transfection efficiency in rat liver after injecting these micelles and complexes through intrabiliary infusion.

2. MATERIALS AND METHODS

2.1. Materials

Polyethylenimine (PEI, branched, MW 25 kDa), methoxy polyethylene glycol (PEG, Mw 2 kDa), sodium, carbon tetrachloride (CCl₄), N,N-dimethylformamide (DMF), tetrahydrofuran (THF), triethylamine, triisobutylaluminum, linoleic acid, glutamine, ZnSO₄, dexamethasone, CuSO₄, Na₂SeO₃, Dulbecco's Modified Eagle's Medium (DMEM), Williams' E medium and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO), if not specified. Fetal calf serum and fetal bovine serum were purchased from HyClone (Logan, UT). Epidermal

growth factor, penicillin–streptomycin, insulin and HEPES buffer were purchased from Invitrogen (Carlsbad, CA). N^1,N^9 -bis(trifluoroacetyl) dipropyltriamine was synthesized following the procedure reported by O'Sullivan et al [20]. The cyclic monomer 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane was prepared as reported previously [21].

2.2. Amplification and purification of plasmid DNA

VR1255C is a 6.4 Kb plasmid DNA encoding firefly luciferase driven by the cytomegalovirus (CMV) promoter (a gift from Carl Wheeler, Vical, San Diego, CA). The plasmid was amplified in *Escherichia coli* DH5 α and purified by an endotoxin free QIAGEN Giga plasmid purification kit (QIAGEN, Valencia, CA). Purified plasmid DNA was dissolved in distilled water.

2.3. Synthesis and characterization of PEG-*b*-PPA copolymer

The synthesis of PEG-*b*-PPA is summarized in Fig. 1. PEG- O^-K^+ macroinitiator (**1**) was prepared by reacting 1.0 g of methoxy PEG with potassium granules (over stoichiometry) in 50 mL of anhydrous THF for 8 h under refluxing [22]. The concentration of PEG- O^-K^+ was determined by titration using 50 mM HCl. The polymerization of 4-alkyl-2-oxo-2-hydro-1,3,2-dioxaphospholane was initiated by adding PEG- O^-K^+ solution into the reaction vessel at a molar ratio of 1:500. The mixture was stirred at room temperature for 48 h. The precursor polymer (**2**) was obtained by precipitation into anhydrous toluene followed by vacuum drying. The precursor polymer (**2**) (3.0 g) was then dissolved in 20 mL of anhydrous DMF under argon. To this solution was added 27.2 g of N^1,N^9 -bis(trifluoroacetyl)dipropyltriamine, followed by addition of 10 mL of anhydrous triethylamine and 10 mL of anhydrous CCl_4 . The mixture was stirred at 0°C for 30 min then at room temperature for 24 h. The reaction mixture was then precipitated into ether and dried under vacuum to yield polymer (**3**). The residue was suspended in 25% ammonia solution and stirred at 60°C for 16 h. The solution was concentrated and dialyzed in dialysis tubing (MWCO 3500, Spectrapor, Spectrum Labs, CA) against distilled water for 2 days with frequent water change. The PEG-*b*-PPA (**4**) was obtained after lyophilization (0.6 g, yield 12%). PPA control ($M_n = 24.8$ KDa, polydispersity = 1.64) was prepared by a method we reported previously [14]. The molecular weight of PEG-*b*-PPA and PPA control was determined using a Waters 2690 HPLC module equipped with a Phenomenex PolySep-GFCP 4000 HPLC column (Torrance, CA), which was connected to a multiangle light scattering detector (MiniDawn, Wyatt Technology, Santa Barbara, CA) and a differential refractive index detector (Optilab DSP interferometric refractometer, Wyatt Technology). Phosphate buffer (0.1M, pH 7.4) with 0.15 M NaCl was used as the mobile phase (flow rate = 0.5 mL/min).

2.4. Compaction ability of PEG-*b*-PPA polymer

VR1255 DNA solution (25 μ L) in DI water containing at a concentration of 20 μ g/mL was added to an equal volume of PEG-*b*-PPA solution in DI water at various concentrations. The mixture was vortexed for 20 sec, incubated for 30 min at room temperature, and then electrophoresed on a 0.8% (w/v) agarose gel for 40 min at 80 V. The gel was stained with ethidium bromide and visualized on an UV illuminator (Eagle Eye II, Stratagene, La Jolla, CA).

2.5. Size and zeta potential of PEG-*b*-PPA/DNA micelles

Nanoparticle size and zeta potential were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizers 3000 (Malvern Instruments, Southborough, MA, USA). Size measurement was performed at 25°C at a 90° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential measurements were performed using an aqueous dip cell in the automatic mode.

2.6. Transmission electron microscopy

Ten μL of PEG-*b*-PPA/DNA micelle solution (N/P = 8, containing 25 $\mu\text{g/mL}$ of DNA) and PPA/DNA complex solution (N/P = 8, containing 25 $\mu\text{g/mL}$ of DNA) were added to a holey carbon TEM grid (SPI, West Chester, PA) and incubated for 5 min at 25°C, followed by washing with deionized water. The grid was further stained with uranyl acetate (1% solution) and washed with deionized water twice.

2.7. *In vitro* gene transfection

In vitro gene transfection was performed in HepG2 (human hepatocellular carcinoma) cells and primary rat hepatocytes. HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2 . Cells were seeded in 24-well plates at a density of 6×10^4 cells per well and incubated for one day. Primary rat hepatocytes were harvested from male Wistar rats weighing from 250 to 300 g by a two-step *in situ* collagenase perfusion method [23]. The concentration and viability of hepatocytes were determined by Trypan blue exclusion test. Rat hepatocytes were plated in Type I collagen-coated 24-well plates (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) at a density of 3×10^5 cells per well, and allowed to incubate overnight before washing off the unattached cells. Hepatocytes were cultured in Williams' E medium supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 100 $\mu\text{g/mL}$ penicillin–streptomycin, 5 nM dexamethasone, 0.5 $\mu\text{g/mL}$ insulin, 15 mM HEPES, 50 ng/mL linoleic acid, 2 mM glutamine, 0.5 mg/mL bovine serum albumin, 50 pM ZnSO_4 , 0.1 μM CuSO_4 and 3 nM Na_2SeO_3 . PEG-*b*-PPA/DNA micelles or PPA/DNA complexes were added to each well at a dose of 2 μg of plasmid DNA. After 4 h of incubation, the culture media were refreshed. Two days later, the culture media were removed, and cells were washed with 0.5 mL of phosphate buffered saline (pH 7.4). Cells were then lysed with a reporter lysis buffer (0.2 mL/well, Promega, Madison, WI), and subjected to two freeze-thaw cycles. The suspensions were centrifuged at 14,000 rpm for 5 min. Twenty μL of cell lysate supernatant was mixed with 100 μL of luciferase substrate (Promega), and the light units were measured on a luminometer (Lumat LB9507, Berthold, Germany). The luciferase activity was converted to the amount of luciferase using recombinant luciferase (Promega) as the standard, and normalized against protein content using the BCA protein assay (Bio-Rad Laboratories, Hercules, CA).

2.8. Gene delivery in rats through intrabiliary infusion

The animal protocols were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine. Male Wistar rats aged 6–8 weeks (200–300 g) were grouped randomly. The rat was first anesthetized with a mixture of Ketamine (60 mg/mL) and Xylazine (8 mg/mL), and the liver was separated from the surrounding tissue. A 33 gauge needle was inserted into the common bile duct and a tie was used to secure the needle. Four mL of PEG-*b*-PPA/DNA micelles (N/P = 8) or PPA/DNA complexes containing 20 μg VR1255 DNA in 5% glucose solution were administered through the needle over 20 min (0.2 mL/min) using a syringe pump. A tie was then placed around the bile duct between the liver and the point of infusion to prevent back flow before the needle was withdrawn. After 30 min, all ties were removed. Stitches with 10-O nylon (Ethicon, Somerville, NJ) were used to repair the needle hole in the bile duct to prevent bile leakage, whenever necessary.

Three days after administration, three rats from each group were sacrificed, and major organs (liver, heart, lung, spleen and kidney) were harvested, weighed and homogenized with a tissue homogenizer (Heidolph, Schwabach, Germany). The homogenate was subjected to two freeze-thaw cycles and centrifuged at 14,000 rpm for 5 min at 4°C. Luciferase activity was analyzed as described above and normalized against the weight of whole tissue.

2.9. Evaluation of liver function and toxicity

Blood samples were collected from rat tail vein on Days 1, 2 and 3 after bile duct infusion, incubated at 4°C for 4 h and centrifuged at 2,000 rpm for 15 min. Serum was isolated and stored at -80°C. Liver functions including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were tested at the Clinical Chemistry Lab at the Department of Pathology, Johns Hopkins School of Medicine.

For histochemical analysis, liver tissue was isolated three days after injection, fixed in phosphate buffered formalin (4%), washed, and embedded in paraffin. H&E staining of sectioned tissues was performed by the Pathology Lab at Department of Comparative Medicine, Johns Hopkins School of Medicine.

3. RESULTS AND DISCUSSION

3.1. Synthesis of PEG-*b*-PPA copolymer

The most commonly used methods for synthesizing PEG cationic block copolymers include sequential polymerization and macromolecular reaction [15]. The use of anionic PEG macroinitiator has obvious advantages due to the availability of a wide range of PEG with defined molecular weights and narrow distribution. The terminal groups can be easily converted to reactive center to initiate the polymerization of the second monomer. Poly(ethylene glycol)ate (PEGate) anion prepared by reacting with potassium has been used to initiate ring-opening polymerization of poly(ϵ -caprolactone) and polylactides [22,24,25]. In our study, this PEGate was used to initiate ring-opening polymerization of the cyclic phosphite monomer, 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane. Due to the highly sensitive nature of the precursor polymer (**2**), we could not analyze its molecular weight. Judging from the molecular weight distribution of the final polymer (**4**), we concluded that the molecular weight distribution of (**2**) was narrow.

The structure of PEG-*b*-PPA (**4**) was characterized by ¹H-NMR. The percentage of phosphoramidate groups in PEG-*b*-PPA was 77.5 mol%, as calculated from the ¹H-NMR spectrum using the integration of peaks assigned to methylidyne protons associated with phosphoramidate and phosphate groups (δ 4.30~4.48 ppm and δ 4.15~4.25 ppm, respectively). The incorporation of PEG segment was evidenced by peaks at δ 3.40~3.65 ppm, which were attributed to the methylene protons and terminal methoxy protons. The number average molecular weight (*M_n*) of PEG-*b*-PPA was determined to be 24,400 with a polydispersity of 1.5 by GPC using a combination of multiangle light scattering and refractive index detectors. The number average degree of polymerization (*DP_n*) of the PPA segment was 97.5. This corresponds to an average of 151 primary amine groups and 22 phosphate ions per polymer chain.

3.2. Preparation and characterization of PEG-*b*-PPA/DNA micelles

DNA compaction ability of PEG-*b*-PPA copolymer to plasmid DNA was evaluated by the electrophoretic mobility of DNA and complexes on an agarose gel. As shown in Fig. 2, PEG-*b*-PPA and PPA achieved complete retardation of DNA at an N/P ratio of 3 and 2 respectively, suggesting a slightly weaker DNA binding ability of PEG-*b*-PPA.

As shown in Fig. 3a, the average particle size of PEG-*b*-PPA/DNA micelles was independent of N/P ratio as shown by dynamic light scattering measurement. The average size of PPA/DNA complexes varied with N/P ratios. PEG-*b*-PPA/DNA micelles showed a narrow size distribution and a nearly constant average size of 80 to 100 nm when N/P ratio increased from 1 to 20. In contrast, severe aggregation occurred in PPA/DNA complexes at low N/P ratios; the average size of the complexes was 100 to 300 nm at N/P ratio of 5 or higher. PEG-*b*-PPA/

DNA micelles displayed lower surface charge compared to PPA/DNA complexes at N/P ratio of 3 and higher (Fig. 3b), presumably due to the shielding effect of the PEG corona. Transmission electron microscopic examination of PEG-*b*-PPA/DNA micelles revealed spherical and rod like morphology with diameters ranging from 70 to 80 nm (Fig. 4b), which is consistent with dynamic light scattering measurement, whereas PPA/DNA complexes showed irregular morphology (Fig. 4a).

3.3. In vitro transfection efficiency

Several polyelectrolyte complex micelle systems have been developed to improve the colloidal stability of the polymeric gene delivery systems. For example, block copolymers of PEG and polylysine (PLL) or poly (2-(dimethylamino)ethyl methacrylate) (PAMA) were reported by Kataoka *et al.* [16-19]. However, these micelles typically showed poor transfection efficiency *in vitro* without the aid of lysosomolytic agent chloroquine. Interestingly, our PEG-*b*-PPA/DNA micelles did not show reduced transfection efficiency in HepG2 cells compared with PPA/DNA complexes and PEI. As shown in Fig. 5, the transfection efficiency of both PPA/DNA complexes and PEG-*b*-PPA/DNA micelles increased with N/P ratio and peaked at N/P ratio of 10 in both HepG2 and primary rat hepatocytes. The highest gene expression level mediated by PEG-*b*-PPA/DNA micelles was 3-fold higher than PPA/DNA complexes in HepG2 cells ($p < 0.05$, Fig. 5a). Nevertheless, in primary rat hepatocytes, reduced transfection efficiency of PEG-*b*-PPA/DNA micelles compared with PPA/DNA complexes was observed at all N/P ratios and the highest transfection efficiency of PEG-*b*-PPA/DNA micelles was about 16-fold lower than that of PPA/DNA complexes at N/P ratio of 10 ($p < 0.05$, Fig. 5b). It remains to be investigated whether the discrepancy between the two types of cells is related to the difference in cell uptake, division or intracellular trafficking steps.

3.4. Transfection efficiency of PEG-*b*-PPA/DNA micelles in liver after intrabiliary infusion

To evaluate the *in vivo* transfection efficiency, PEG-*b*-PPA/DNA micelles were administered through bile duct infusion to rat liver. The advantage of bile duct infusion is the direct access to hepatocytes, bypassing Kupffer cells lining endothelium. The large surface area of biliary tree and wide distribution of the biliary system throughout the liver provide ready access to hepatocytes in liver parenchyma via bile canaliculi [26]. More importantly, intrabiliary infusion can be readily applied in clinical settings through endoscopic retrograde cholangiopancreatography (ERCP), a routine bile duct cannulation procedure. A number of non-viral gene delivery vectors have been successfully administered through bile duct and demonstrated much higher gene transfection efficiency in liver compared with that achieved with intraportal infusion or tail vein injection [26-29].

In our study, PEG-*b*-PPA/DNA micelles and PPA/DNA complexes were infused with a syringe pump with a steady control of the flow rate and pressure to achieve reproducible results and minimize the potential damage to the bile duct and liver. In various reported intrabiliary infusion studies, including those unrelated to gene delivery, infusion rates ranging from 0.02 to 2.7 mL/min have been used in rats without indication of liver damage [26,30-32]. No systematic study has been reported on examining the effect of infusion rate and infusion volume on transfection efficiency, it is assumed that the tolerated infusion volume and infusion rate are interdependent [26,31,32]. It has been shown that rats do not tolerate high infusion rate (0.4–2.25 mL/min) and high infusion volume (> 4 mL) well [30,32]. There should be a balanced set of conditions that need to be identified that correlate with a high transfection and low hepatic injury and toxicity. Our pilot study suggested that higher infusion volume correlates with higher transgene expression; an infusion rate up to 0.25 min/mL and a total infusion volume of 2–4 mL could be well tolerated by Wistar rats [29]. For the purpose of evaluating the delivery efficiency of micelle carrier and PPA/DNA complexes, we fixed the infusion rate at 0.2 mL/min and infusion volume at 4 mL for intrabiliary infusion in this study.

The complexes and micelles were infused to the common bile duct, which leads to all parts of liver parenchymal tissue, resembling the clinical situation for ERCP procedure. Infusion of PPA/DNA complexes resulted in transgene expression in only one lobe of the liver three days after infusion (Fig. 6). This was likely due to the aggregation of complexes in bile, which limits the diffusion and transport of complexes through the biliary tree and bile ductules. The bile canaliculi measure 1 – 1.25 μm in diameter in the region of portal triads and 0.5 – 1 μm in the region of central veins. Therefore, any aggregation of the particles will severely impede the transport of the particles throughout the biliary tree. In contrast, infusion of PEG-*b*-PPA/DNA micelles resulted in a more uniform expression throughout the whole liver, showing relatively similar expression in all lobes of the liver (Fig. 6). The overall luciferase expression was 4-fold higher than that mediated by PPA/DNA complexes. This is likely the result of improved colloidal stability of PEG-*b*-PPA/DNA micelles in the bile, leading to uniform distribution of transgene expression. No expression was detected in other major organs (lung, kidney, heart and spleen) in both groups transfected with micelles and PPA/DNA complexes.

It is worth noting that, despite of the relatively high infusion volume (4 mL), this volume and the infusion rate (0.2 mL/min) are much lower than that of the hydrodynamic injection conditions, which typically adopt an injection volume of about 25 mL (100 mL/kg) and an injection rate of about 100 mL/min in rats [33,34]. Therefore the infusion pressure is much lower compared with that for the hydrodynamic injection. Under this condition, we have shown that naked DNA (VR1255 plasmid) at a dose of 200 μg per rat yielded about 0.6 pg luciferase per gram of liver tissue [29], which is 3-fold lower than that of PEG-*b*-PPA/DNA micelles at 20 μg DNA dose. We have not directly compared the transgene expression levels of naked DNA and micelles at the same dose; it is unlikely that naked DNA at 20 μg dose would yield higher gene transfection efficiency than that at 200 μg dose.

The design and synthesis of PEG-*b*-PPA copolymers is versatile, allowing for incorporation of other functional units without significantly compromising DNA condensation and the ability to self-assemble. Several recent study demonstrated that a built-in segment with buffering capacity in copolymer (e.g. polysilamine [35] or poly[(3-morpholinopropyl) aspartamide] [36] segments in PEG-PLL or PEG-PAMA carrier), these block copolymer/DNA micelles showed improved transfection activities in cell lines. PEG-*b*-PPA was synthesized by a living polymerization scheme; hence we can readily introduce additional blocks to improve the endosomal escape ability.

Liver function tests were performed to monitor toxicity and potential damage of intrabiliary infused complexes and micelles. As we reported previously [29], intrabiliary infusion of PEI/DNA complexes induced a high level of serum alanine transaminase (ALT) and aspartate transaminase (AST) activities in serum samples 1 to 3 days following infusion, although both returned to normal levels by day 5. Infusion of naked DNA and saline using this same protocol yielded mild and transient elevation of ALT and AST. In contrast, infusion of PPA/DNA complexes and PEG-*b*-PPA/DNA micelles caused a moderate increase of ALT and AST on days 1 through 3 (Fig. 7). These enzyme activities subsided to normal levels gradually. This short-term increase of ALT and AST could also be compounded by some backflow of bile during infusion.

Consistent with the liver enzyme activity analyses, histopathological examination performed on day 3 showed minimal inflammation and damage to liver tissue that received either PPA/DNA complexes or PEG-*b*-PPA/DNA micelles (Fig. 8), at a level similar to naked DNA infusion [29]. Only patchy mild bile ductular proliferation and reactive changes in biliary epithelium were apparent. This is in contrast to the strong biliary tract changes observed in PEI/DNA complex-infused rat liver, where bile ductular proliferation, reactive epithelial changes, significant portal edema and inflammation were observed [29].

Long term morphological changes and toxicity to liver remain to be evaluated. However, biochemical analyses of liver enzymes in serum suggested that these reactions to complexes and micelles infusion were transient.

4. CONCLUSIONS

This study highlighted the promise of PEG-*b*-PPA/DNA micelles as a new carrier for liver-targeted gene delivery. The PEG-*b*-PPA copolymer exhibited comparable DNA compaction capacity as the corresponding PPA, and self-assembled into micelles with uniform and smaller particle size (80 – 100 nm) upon complexation with DNA. Unlike other reported DNA micelle systems that required the aid of chloroquine to show transfection activity, PEG-*b*-PPA/DNA micelles mediated high transfection efficiency in HepG2 cells and primary rat hepatocytes in the absence of chloroquine. After intrabiliary infusion in rats, PEG-*b*-PPA/DNA micelles mediated more uniform transgene expression throughout the liver and 4 times higher of overall transgene expression than PPA/DNA complexes. In addition, due to the intrinsically lower cytotoxicity of PPA, both PEG-*b*-PPA/DNA micelles and PPA/DNA complexes did not induce any significant level of liver toxicity.

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Figure 1.
Synthetic scheme of PEG-*b*-PPA copolymer.

Figure 2. Gel electrophoresis mobility of DNA in PPA/DNA complexes and PEG-*b*-PPA/DNA micelles. Plasmid DNA (500 ng) and various amounts of PPAs or PEG-*b*-PPA were mixed at the indicated N/P ratio (primary amino groups in polymers to phosphate groups in DNA), followed by electrophoresis on an agarose gel (0.8% w/v) and stained with ethidium bromide.

Figure 3. Average particle size (a) and average zeta potential (b) of PEG-*b*-PPA/DNA micelles and PPA-DPA/DNA complexes.

Figure 4. TEM images of (a) PPA/DNA complexes ($N/P = 10$) and (b) PEG-*b*-PPA/DNA micelles ($N/P = 10$).

Figure 5. Transfection efficiency of PEI/DNA complexes (N/P = 10), PPA/DNA complexes and PEG-*b*-PPA/DNA micelles at various N/P ratios in (a) HepG2 cells and (b) primary rat hepatocytes 48 h after transfection. Each bar represents average $\pm$ standard deviation (n = 4). * denotes statistically significant comparison ($p < 0.05$) by the Student's *t* test.

Figure 6. Luciferase expression in rats on day 3 following intrabiliary infusion of PPA/DNA complexes (N/P = 10) and PEG-*b*-PPA/DNA micelles (N/P=10) at a DNA dose of 20 μ g per rat. Each bar represents average $\pm$ standard division (n = 3).

Figure 7. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels in rats following intrabiliary infusion of PPA/DNA complexes (N/P = 10) and PEG-*b*-PPA/DNA micelles (N/P = 10) at a DNA dose of 20 μ g per rat. Each bar represents average $\pm$ standard deviation (n = 3).

Figure 8. H&E stained tissue sections of the portal triad region of liver tissue harvested on day 3 from rats received (a) PPA/DNA complexes (N/P = 10) or (b) PEG-*b*-PPA/DNA micelles (N/P = 10).