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RESEARCH PAPER

Promoted Transfection Efficiency of pDNA Polyplexes-Loaded Biodegradable Microparticles Containing Acid-Labile Segments and Galactose Grafts

Zhu Chen • Xiaojun Cai • Ye Yang • Guannan Wu • Yaowen Liu • Fang Chen • Xiaohong Li

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ABSTRACT

Purpose Targeting to antigen-presenting cells and efficient intracellular delivery of pDNA are essential for development of microsphere formulations of DNA vaccine.

Methods Biodegradable polymers containing acid-labile segments and galactose grafts were developed to entrap pDNA polyplexes into microspheres, which were proposed to promote transfection efficiency of pDNA.

Results Acid-labile characteristics were approved by the hemolysis capabilities of red blood cells and degradation behaviors of matrix polymers; release of pDNA polyplexes from microspheres was significantly accelerated after incubation in acid buffers. Presence of galactose moieties enhanced cellular uptake of microspheres and increased acid-lability due to hydrophilic grafts on acid-labile segments. There was no apparent cytotoxicity of blank microspheres; cytotoxicity of pDNA polyplexes was significantly decreased after encapsulation into and sustained release from microspheres. High transfection efficiency and a dose-dependent transfection were indicated for pDNA polyplex-loaded acid-labile microspheres when balancing with cytotoxicity.

Conclusions Integration of acid-lability, targeting effect into full biodegradable backbone represents an exciting approach to promote transfection efficiency through modulating release of pDNA polyplexes, targeting to antigen-presenting cells and intracellular delivery of pDNA.

KEY WORDS acid-labile biodegradable polymers · endosomal escape · pDNA polyplexes · targeting effect · transfection efficiency

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Z. Chen • X. Cai • Y. Yang • G. Wu • Y. Liu • F. Chen • X. Li (✉)
Key Laboratory of Advanced Technologies of Materials
Ministry of Education of China, School of Materials Science
& Engineering Southwest Jiaotong University
Chengdu 610031, People's Republic of China
e-mail: xhli@swjtu.edu.cn

Z. Chen
Research Institute of Tissue Engineering and Stem Cells
The Second Clinical Hospital of North Sichuan Medical College
Nanchong 637000, People's Republic of China

INTRODUCTION

The rapid development of biotechnology has led to the commercially availability of biomacromolecular therapeutics for the prevention, diagnosis and treating of human disease. DNA vaccination shows potentials in treating infectious disease, cancer, autoimmunity and allergy, while immunization strategies using proteins and peptides are associated with a short half-life of antigen presentation (1). But the clinical studies on DNA vaccines have suggested that several challenges need to be addressed, for example, multiple high dose immunizations are often required (2). One of the strategies focuses on the delivery by the use of biodegradable microspheres, which was supposed to achieve a sustained release of pDNA to prolong transgene expression and to reduce the number of doses in the immunization schedule (3). However, they are far from ideal for enhancing systematic and cellular immunity, and the major challenges are the protection of pDNA from losing the structural integrity and the delivery of pDNA into antigen presenting cells (APCs) for an efficient transfection.

One of the advantages of microsphere formulation of DNA vaccine is that microspheres are naturally taken up by APCs more efficiently than soluble antigen (4). However,

when biomolecules or the microspheres are taken up by targeted cells via endocytosis, they are often rapidly trafficked from early endosome to late endosome, and eventually to lysosome (5), where they are degraded by acid environment and enzymes, resulting in a decrease in the bioavailability or loss of bioactivity (6). So the escaping from lysosomes and endosomes was the key step for intracellular delivery of pDNA. There are pH gradients in the extracellular space and different intracellular compartments, for example, the pH values of extracellular environment and cytoplasm are 7.4, whereas those of lysosome and endosome are 5.0–6.5 (7). Therefore, many strategies have focused on the design and synthesis of polymeric drug carriers containing acid-labile linkage, weakly basic or acidic groups. Garripelli *et al.* connected pluronic-based multiblock copolymers via acid-labile acetal bonds, which formed gel at body temperature and allowed a responsive degradation at an acidic pH (8). Lin *et al.* synthesized acid-labile block copolymers containing both poly(ethylene glycol) and poly(2-dimethylaminoethyl methacrylate) segments connected by an acid-cleavable orthoester linkage, which were used as a carrier of DNA for endosomal delivery (9). Thus, most of the pH-dependent polymers were made from nonbiodegradable blocks connected by pH-sensitive linkages, and had a similar shortcoming that could not fully biodegrade after the delivery of their therapeutic cargo.

Another challenge of microsphere formulation of DNA vaccine is the low transfection efficiency, resulting from the less efficient nuclear localization and the degradation of DNA in the cytoplasm and extracellular space. In addition, during the formation of pDNA-loaded microspheres, DNA is exposed to high shear and an organic/aqueous interface, which cause the degradation of DNA molecules and the reduction in supercoiled DNA, resulting in low transfection efficiency (10). One of the strategies is to use cationic polymers, such as polyethylenimine (PEI) and polylysine, to condense DNA into compact polyplexes, proving to induce high transgene expression in target cells both *in vitro* and *in vivo* (11). Nguyen *et al.* blended PEI with matrix polymers to entrap DNA, and electrostatic complexes of DNA and PEI formed during the release process (12). But the depletion of PEI should be strong under *in vivo* conditions, causing only partial condensation with the released DNA and undesirable side effects of free PEI. Thus, an efficient transfection can be achieved by forming pDNA polyplexes with a cationic polymer prior to being entrapped into biodegradable microparticles (13). However, once entrapped into polymer matrix, DNA polyplexes do not diffuse appreciably into the release medium until the matrix polymers have been significantly degraded (14). The slow release rate limits the amount of DNA available for the transfection of target cells and induction of immune responses, due to the limited life span of APCs requiring the release in a reasonable time frame (15).

Macrophages are important professional APCs, and partially constitute the reticuloendothelial system, which phagocytizes most of the foreign particles carried by blood. Although liver macrophages (kupffer cells) make up less than 5% of the liver volume, they represent about 80% of the total fixed macrophage population in human body (16). There are various receptors on the surface of macrophages, and the galactosylation becomes a potential targeting strategy. Popielarski *et al.* indicated that about 90% of the galactose decorated nanoparticles were found within the liver 20 min after tail vein injections in mice (17). Dong *et al.* investigated the cellular localization of oligodeoxynucleotides delivered by galactosylated low-molecular-weight chitosan, which was inclined to enter into Kupffer cells rather than into liver parenchymal cells *in vivo* (18).

A novel drug carrier integrated the acid-lability, targeting capability and full biodegradable backbone were developed in current study. Acid-labile segments containing acetal groups were introduced into the biodegradable backbone of poly (DL-lactide), and galactose was conjugated as targeting moieties onto above polymers through click chemistry. pDNA polyplexes was encapsulated into above polymers, which was proposed to promote the transfection efficiency through directing the microspheres to target cells, initiating the escape of microspheres from endosome to cytoplasm and enhancing the release of pDNA polyplexes out of microspheres. The acid-lability was clarified from the release behaviors of pDNA, hemolysis activity of microspheres and degradation profiles of matrix polymers. The cytotoxicity and transfection efficiency were also determined on liver macrophages.

MATERIALS AND METHODS

Materials

Plasmid EGFP-N_2 (4737 bp), encoding green fluorescent protein (GFP) within a cytomegalovirus (CMV) promoter, were from FulenGen Co. (Guangzhou, China) and used a model pDNA. The plasmid was grown in *Escherichia coli.* DH5α bacterial culture using LB growth media, purified using Qiagen Giga kit (Hilden, Germany), and stored in Tris–EDTA buffer (TE, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 4°C. PEI (M_w = 25 kDa), agarose, Tris, glycine, ethidium bromide, Triton X-100, loading buffers for agarose electrophoresis, 4′,6-diamidino-2-phenylindole (DAPI) and Hochest 33258 were procured from Sigma (St. Louis, MO). Ultra-pure water was from a Milli-Q biocel purification system (UPI-IV-20, Shanghai UP Scientific Instrument Co., Shanghai, China) was used. All other chemicals and solvents were analytical grade

and received from Changzheng Regents Co. (Chengdu, China).

Preparation of Acid-Labile Biodegradable Polymers Bearing Galactose Groups

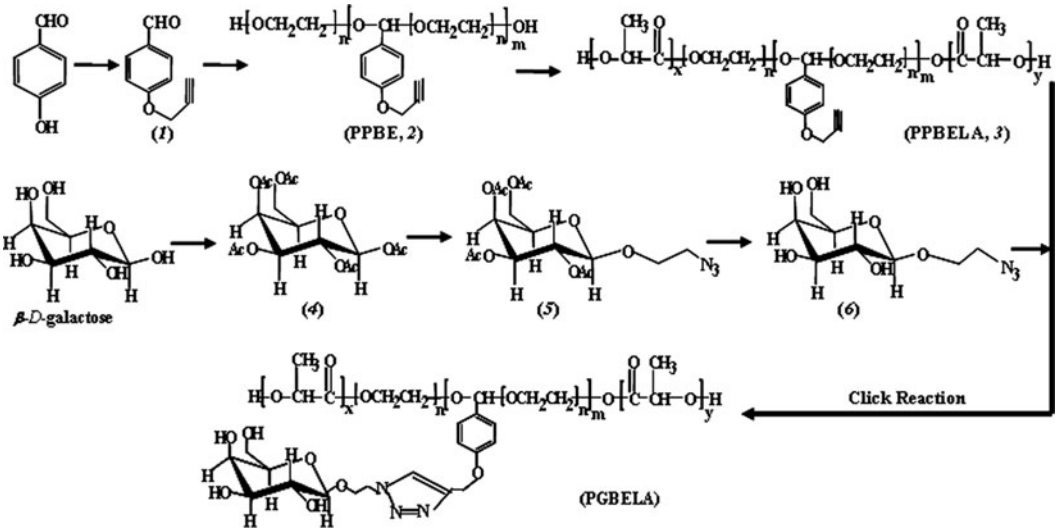
Galactose grafted poly(benzaldehyde-polyethylene glycol)-poly(DL-lactide) (PGBELA) was prepared through click reaction of 1-O-2-azidoethyl-*β-D*-glactose with acid-labile copolymer poly(4-propargyl-benzaldehyde-poly(ethylene glycol))-poly(DL-lactide) (PPBELA). Scheme 1 outlines the synthetic route, and the particular synthesis and characterization results were included in the supplementary materials. Copolymers PPBELA containing acetylene groups were synthesized through three steps. 4-Hydroxybenzaldehyde reacted with propargyl bromide in the presence of sodium alcoholate to obtain 4-propargyl-benzaldehyde (***1***). Acetal groups were introduced by reacting poly(ethylene glycol) (PEG) with (***1***) under dry nitrogen atmosphere to give poly (4-propargyl-benzaldehyde-poly(ethylene glycol)) (PPBE) (***2***). Copolymerization of PPBE with DL-lactide was carried out by bulk ring-opening polymerization to get copolymers PPBELA (***3***). As shown in Scheme 1, functionalized galactose with terminal azides was synthesized through three steps. *β-D*-Galactose reacted with acetic anhydride in the presence of anhydrous sodium acetate to obtain *β-D*-galactose pentaacetate (***4***). 2-Azidoethanol reacted with (***4***) in dried dichloromethane to prepare 1-O-2-azidoethyl-*β-D*-galactose pentaacetate (***5***), followed by deacetylating to yield 1-O-2-azidoethyl-*β-D*-galactose (***6***).

Copolymers without acid-labile segment or galactose graft were used as control. Acetal groups were introduced by reacting PEG with benzaldehyde to obtain poly (benzaldehyde-polyethylene glycol), which was further copolymerized with DL-Lactide to obtain poly(benzaldehyde-poly(ethylene glycol))-poly(D,L-lactide) (PBELA) (19). Copolymers poly(ethylene glycol)-poly(DL-lactide) (PELA) were prepared by bulk ring-opening polymerization of lactide/PEG using stannous chloride as initiator (20). Copolymers PGBELA, PBELA and PELA indicated weight-average molecular weight (M_w) of 24.6, 23.8 and 25.8 kDa, and polydispersity indices (M_w/M_n) of 1.38, 1.21 and 1.33, respectively.

Preparation of Microspheres with the Loading of pDNA Polyplexes

The pDNA-PEI polyplexes were prepared in phosphate buffer saline (PBS), followed by encapsulation into microspheres through double-emulsion/solvent evaporation method (21). Briefly, 500 μl PEI solution (22.3 mM in pH 7.0 PBS) and 500 μl naked pDNA solution (1 mg/ml in pH 7.0 PBS) were mixed together and vortexed for 30 s to form pDNA-PEI polyplexes. The pDNA polyplexes were centrifuged at 15,000 g for 30 min, re-suspended in 50 μl pH 7.0 PBS, and emulsified with 1 ml dichloromethane containing 40 mg PGBELA. The initial w/o emulsion was added into 20 ml 40% PEG (M_w = 20 kDa) solution containing 0.2% sodium oleate, and homogenized at 1400 rpm for 30 min (JJ-1, Shunhua Instrument Co., Jintan, China). The resulting pDNA/PGBELA microspheres were collected by centrifugation, washed three times by deionized water, and lyophilized. Similar procedures were utilized to prepare pDNA/PBELA and pDNA/PELA nanoparticles. Blank PELA, PBELA and PGBELA nanoparticles were prepared without the addition of pDNA polyplexes.

In order to visualize the pDNA encapsulation in microspheres, pDNA was labeled with DAPI through



Scheme 1 The synthesis routes of PGBELA.

combination with the guanine and thymine nucleotides of pDNA. Briefly, pDNA was added into DAPI solution (10 μg/ml in 10 mM PBS), and the mixture was kept in the dark for 5 min before dialyzing to remove any residual DAPI. Labeled DAPI-pDNA/PGBELA microspheres were prepared as above.

Characterization of pDNA-Loaded Microspheres

The size and the zeta potential of pDNA polyplexes were analyzed by a Zetasizer Nano ZS system (Malvern, UK). Microspheres were suspended in deionized water and the size of microspheres was examined by laser diffraction particle size analyzer (Horiba LA920, Japan). The microsphere dispersion was dropped on a metal stub, sputter-coated with gold for a period up to 120 s, and observed by scanning electron microscope (SEM, FEI Quanta200, The Netherlands) equipped with field-emission gun and Robinson detector. Labeled DAPI-pDNA/PGBELA microspheres were observed under fluorescence microscope (Leica DMR HCS, Germany) to indicate the encapsulation of pDNA polyplexes within microspheres. The fluorescence microscope was operated with a Cy2 filter with the excitation and emission wavelengths of 340/400 and 450/600 nm, respectively.

Characterization of pDNA Loaded in Microspheres

Core loading of pDNA in the microspheres was determined by extracting from microspheres, and quantifying with Hoechst 33258 dye (13). Briefly, a known amount of microspheres (around 30 mg) were dissolved in 250 μl dichloromethane, and extracted by 10 mM PBS containing 10 mg/ml heparin. To determine the encapsulation efficiency, extracted pDNA was mixed with Hoechst 33258 and the fluorescence intensity was measured by a fluorospectrophotometer (Hitachi F-7000, Japan) at an excitation wavelength of 347 nm and an emission wavelength of 450 nm. The presence of heparin was found no interference with the fluorescence measurement, and the pDNA concentration was obtained using a standard curve from known concentrations of pDNA solutions. The extraction efficiency was calibrated by adding a certain amount of pDNA polyplexes into polymer solution along with the same concentration as above and extracting using the above method.

Structural integrity of pDNA after the microsphere preparation was determined by agarose gel electrophoresis. The initial w/o emulsion was destabilized by the addition of excess buffer (10 mM PBS with 10 mg/ml heparin), followed by centrifugation at 4000 g for 15 min to separate the two phases. The water layer was subjected to 1% agarose gel electrophoresis (Power Pac^{TM} Universal, Bio-RAD, Hercules, CA). The gel was viewed by a gel documentation system and analyzed by the software Quantity One (Bio-RAD, Hercules, CA) to determine the ratio between the supercoiled and relaxed pDNA. The pDNA extracted from microspheres was also examined to detect the effect of the microsphere preparation process on the structural integrity.

In Vitro pDNA Release Under Different pH Environment

The *in vitro* release profiles of pDNA from microspheres were investigated in buffer solutions of different pH values to clarify the acid-sensitivity. Briefly, pDNA-loaded PELA, PBELA and PGBELA microspheres (around 30 mg) were incubated in 1.0 ml PBS (pH 7.4 and pH 6.0) and acetate buffer (pH 5.0) containing 10 mg/ml heparin. The suspensions were kept in a thermostated shaking water bath that was maintained at 37°C and 100 rpm. At predetermined time intervals, the samples were centrifuged and 100 μl of the release buffer was removed for analysis. An equal volume of fresh buffer was added back to the release media. The amounts of released pDNA were detected with Hoechst 33258 dye as described above.

In Vitro Degradation of pDNA-Loaded Microspheres Under Different pH Environment

The degradation of pDNA-loaded PELA, PBELA and PGBELA microspheres in buffer solutions were determined with respect to the mass loss and molecular weight reduction. Pre-weighted nanoparticles were placed into 5.0 ml buffer solutions of pH 7.4, 6.0 and 5.0, which were maintained at 37°C and 100 cycles/min. At predetermined intervals, triplicate samples were recovered, rinsed with distilled water to remove residual buffer salts, and dried to constant weight in a vacuum desiccator. The mass loss was determined gravimetrically by comparing the dry weight remaining at specific time with the initial weight. The molecular weight was determined by gel permeation chromatography (GPC, Waters 2695 and 2414, Milford, MA) using polystyrene as standard. The structure of recovered polymers was analyzed from proton nuclear magnetic spectra (^{1}H-NMR, Bruker Avance DPX 300, Faellanden, Switzerland), which were obtained in $CDCl_3$ with tetramethylsilane as the internal standard.

Hemolysis Ability of pDNA-Loaded Microspheres Under Different pH Environment

The capability of PGBELA, PBELA and PELA microparticles to induce pH-dependent membrane destabilization was measured using a red blood cell (RBC) hemolysis

assay described elsewhere (22). Briefly, approximately 15 ml whole human blood (Chengdu Blood Center, Chengdu, China) was poured into a centrifuge tube, and 0.15 mol/l sodium chloride solution was added to a final volume of 50 ml. After centrifugation at 10,000 rpm for 15 min at 4°C (Beckman Coulter Microfuge 22R, Altanta, GA), the erythrocyte pellet was washed three times with pH 7.4 PBS. The purified RBC pellet was diluted with buffer solutions of pH 7.4, 6.0 and 5.0 to obtain cell suspension of 10^9 RBCs/ml. Then 100 μl of the RBC suspension was added into 96-well tissue culture plate (TCP), followed by adding 100 μl of microspheres suspensions in each well, using PBS and Triton X-100 (1.0 vol%) as the negative and positive control, respectively. The microwell plate was incubated for 1 h at 37°C, and then centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was collected and the hemoglobin concentration was measured at 540 nm using μQuant microplate reader (Elx-800, Bio-Tek Instrument Inc., Winooski, VT). The results were expressed as percentage hemolysis with the assumption that Triton X-100 caused 100% hemolysis and pH 7.4 PBS, no hemolysis.

In Vitro Cytotoxicity of pDNA-Loaded Microspheres

Liver macrophages were isolated from rat liver according to the method of Nagelkerke *et al.* with some modifications (23), and were obtained from Huaxi Medical center of Sichuan University (Chengdu, China). Liver macrophages were cultured in RPMI1640 medium (Gibco BRL, Rockville, MD), supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD) at 37°C and 5% CO_2 in humidified atmosphere, and subcultivated according to standard protocols. The cells were plated in 96-well TCP at 10,000 cells per well, and incubated for 24 h at 37°C. Fresh media containing blank PGBELA, PBELA, PELA microspheres and pDNA-loaded microspheres were added with different microsphere concentrations, and incubated for 48 h at 37°C. MTT assays were utilized to evaluate the cell viability. To perform the assay, 20 μl of MTT stock solution (5 mg/ml in PBS) was added in each well and incubated for 4 h. Cell culture media was then removed and replaced with 100 μl of dimethyl sulfoxide, and absorbance was read at 570 nm using μQuant microplate reader. After subtracting background signal, cell viability was calculated as the ratio between the absorbance of cells incubated with microspheres and that of cells treated with culture media only.

In Vitro Transfection Efficiency of pDNA-Loaded Microspheres

To assess the transfection efficiency, liver macrophages were plated in 96-well TCP at 10,000 cells per well, and incubated for 24 h at 37°C. Fresh media containing pDNA-loaded PGBELA, PBELA and PELA microspheres were added with different concentrations, and incubated for 10 h at 37°C. Cells were washed with fresh media, incubated for another 48 h at 37°C, and then homogenized in the lysis buffer (0.1 M Tris–HCl, 2 mM EDTA, 0.1% Triton X-100). GFP Expression was quantified using a fluorospectrophotometer (Hitachi F-7000, Japan) at an excitation wavelength of 493 nm and an emission wavelength of 510 nm. Total protein of the cell lysate was determined using BCA protein assay kit (Pierce, Rockford, IL). Results of GFP expression were normalized as relative light units per mg of soluble protein (RLU/mg protein). To observe the GFP expression, liver macrophages were plated on microscope cover slides in a 24-well TCP, and incubated with fresh media containing pDNA-loaded microspheres for 10 h at 37°C. After incubation for another 48 h at 37°C, cells were washed with PBS twice, fixed with 4% glutaraldehyde for 2 h at 4°C, and then observed by a fluorescence microscope as indicated above.

Statistics Analysis

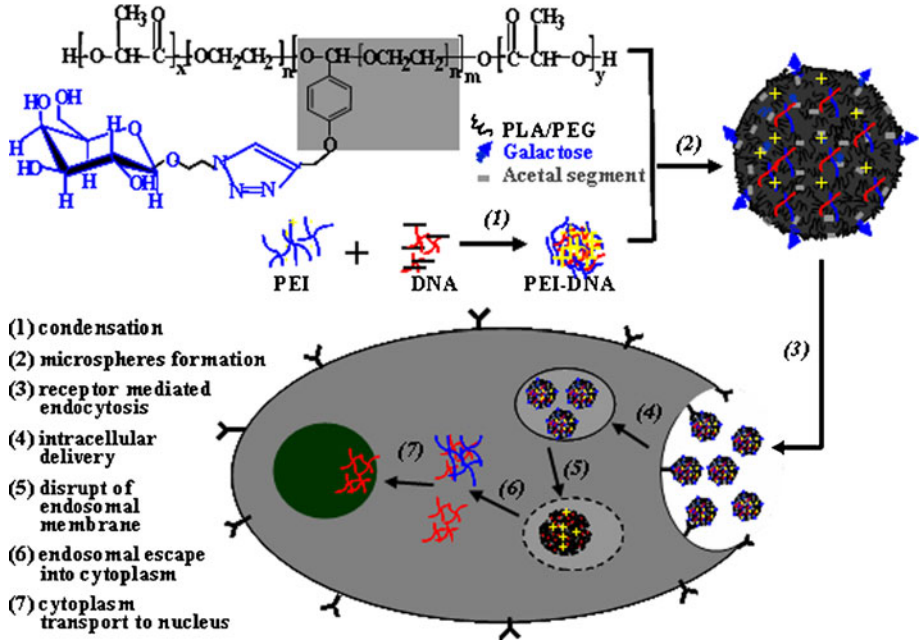
The values were expressed as means ± standard deviation (SD). Whenever appropriate, two-tailed Student's *t*-test was used to discern the statistical difference between groups. A probability value (p) of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Scheme 2 outlines the formation of acid-labile microspheres with pDNA polyplexes entrapped, and the subsequent cellular uptake, intracellular delivery, and pDNA release. The galactose moieties are supposed to enhance the uptake of the microspheres into target cells, and the low pH of endosome facilitates the release of pDNA polyplexes into cytoplasm. PEI is supposed to promote the nuclear localization of pDNA (24), and the dissociated pDNA readily enter the nucleus followed by expression of destined proteins.

Characterization of pDNA-Loaded Microspheres

The pDNA formed complexes with PEI before encapsulation into microspheres to achieve an efficient transfection. The formed pDNA polyplexes had average size of around 288 nm and zeta potential of about 4.2 mV. In order to determine the effect of matrix polymers, pDNA-loaded PGBELA, PBELA and PELA nanoparticles were prepared with close particle size and pDNA loading amounts, through optimizing the process parameters, such as the



Scheme 2 Schematic drawing of the formation of acid-labile PGBELA microspheres with entrapped pDNA polyplexes, and the subsequent cellular uptake, intracellular delivery, and pDNA release. The shaded area in the copolymer structure indicates the acid labile segment.

polymer concentration, the amounts of stabilizers in the outer aqueous phase (data not shown). Table I summarizes the characteristics of pDNA-loaded microspheres obtained, indicating the size of around 2–3 μm and pDNA loading amount of around 0.23%. It was indicated that the particle size of 1–5 μm was readily taken up by phagocytic cells (25). The encapsulation efficiency was related to the diffusion of pDNA polyplexes into the outer aqueous phase, and a high precipitation rate of matrix polymers during the 2nd emulsification process should enhance the encapsulation efficiency. As shown in Table I, higher pDNA encapsulation efficiency of 37.4% was obtained for PBELA, due to the more hydrophobic nature of PBELA than PELA and PGBELA.

Figure1a shows that the surface morphology of pDNA/PGBELA microspheres, indicating generally smooth surface and spherical shape. As shown in Fig. 1b, all the microspheres emitted fluorescent light, suggesting the presence of labeled pDNA polyplexes in the microspheres. Figure 1c showed the structural integrity of pDNA extracted from the initial emulsion and the resulting pDNA/PGBELA microspheres. Compared with purified pDNA, the supercoiled conformation of extracted pDNA decreased from 93% to 83%, indicating that the preparation process affected the pDNA structure. There was around 6% of loss in the supercoiled conformation during the initial emulsification process, indicating a higher impact on the structural integrity than other procedures. It may be resulted from the high shear force, exposure to organic solvents, and interfacial tensions at the oil/water interface.

In Vitro pDNA Release Profiles Under Different pH Environment

Figure 2 summarizes the release profiles of pDNA-loaded microspheres after incubation in buffer solutions of pH 7.4, 6.0 and 5.0. As shown in Fig. 2a, the initial burst release of pDNA from PELA microspheres was less than 5%, and the total amounts of release were around 35.2%, 38.5% and 42.1% after 35 d incubation in buffers of pH 7.4, 6.0 and 5.0, respectively. The entrapment of pDNA polyplexes with the size of around 300 nm led to a less significant of release until the formation of diffusion channels after significant degradation of the matrix polymers. Lei *et al.* explored the encapsulation of pDNA polyplexes inside PEG hydrogels cross-linked with matrix metalloproteinase-degradable

Table I Characteristics of pDNA-Loaded Microspheres ($n = 3$)

Microspheres	Diameter (μm)	Encapsulation efficiency (%)	pDNA loading amount (%)
pDNA/PELA	2.23 ± 0.21	20.9 ± 0.5	0.22 ± 0.06
pDNA/PBELA	2.74 ± 0.14	37.4 ± 0.7	0.25 ± 0.04
pDNA/PGBELA	2.54 ± 0.24	28.3 ± 0.4	0.23 ± 0.03

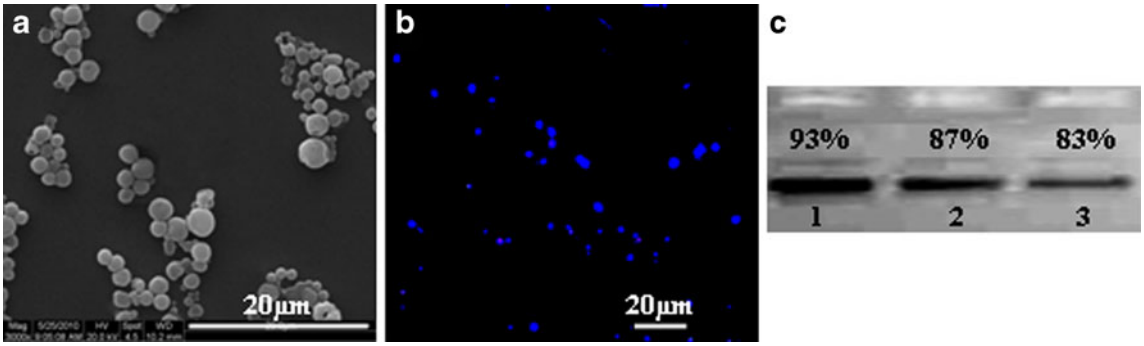


Fig. 1 SEM (**a**) and fluorescence microscope images (**b**) of pDNA/PGBELA microspheres; (**c**) Agarose gel electrophoresis analysis of purified pDNA (1), pDNA extracted from the initial emulsion (2) and the resulting pDNA/PGBELA microspheres (3). Numbers in percentage represent the percent of supercoiled pDNA.

peptides (26). Cumulative release in PBS indicated less than 15% of the total encapsulated DNA polyplexes for 14 d. In contrast, incubation in a trypsin solution resulted in breakdown of the hydrogel matrix and 74.4% release after 2.5 h of incubation. The release of pDNA polyplexes from microspheres formulation of DNA vaccine should be enhanced, due to the limited life span of APCs requiring the release of the encapsulated material in a reasonable time frame (14).

As shown in Fig. 2b and c, pDNA release from PBELA and PGBELA microspheres was significantly accelerated in acid buffer solutions compared with that in neutral environment. There was around 38.2% of pDNA release from PBELA microspheres after incubation in pH 7.4 PBS for 28 d, which was close to that from PELA microspheres. However, the initial burst release was around 15.8% and 16.8%, and the accumulated release during 28 d was around 93% and 100% after incubation PBELA microspheres in pH 6.0 and 5.0 buffers, respectively. The acceleration of pDNA release was more significant for PGBELA microspheres. There were around 19.3% and 23.4% of burst release from PGBELA microspheres after incubation in pH 6.0 and 5.0 buffers, respectively. This may be due to the microsphere erosion and the detachment of pDNA polyplexes from the microsphere matrix under acid buffer solutions. All the pDNA released out from PGBELA microspheres after incubation in pH 6.0 buffers for 11 d or in pH 5.0 buffers for 9 d, while there was around 48% of release in pH 7.4 PBS during 11 d. The galactose grafts of PGBELA improved the hydrophilicity of matrix polymers, accelerated the degradation of polymer backbone, and enhanced the water diffusion into and pDNA polyplexes diffusion out of the microspheres matrices. In conclusion, the initial burst release and accumulated release amount indicated a significant acid-lability of PGBELA microspheres.

In Vitro Degradation Profiles Under Different pH Environment

Degradation profiles of pDNA-loaded microspheres were assessed in buffer solutions of different pH values, and Fig. 3a summarizes the gravimetric evaluation results. There were about 18.9%, 22.2% and 22.6% of mass loss for pDNA/PELA microspheres after incubation for 35 d in buffer solutions of pH 7.4, 6.0 and 5.0, respectively, and no

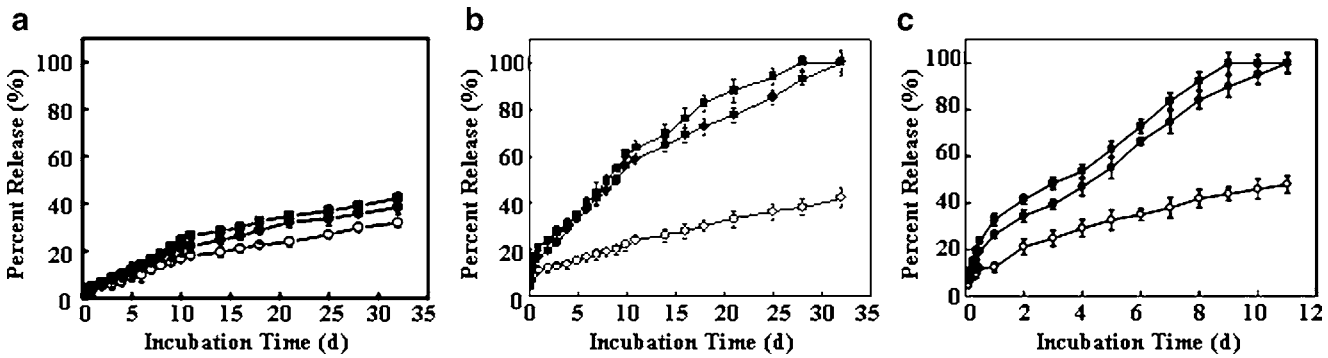


Fig. 2 Percent release of pDNA from (**a**) PELA, (**b**) PBELA and (**c**) PGBELA microspheres after incubation in buffer solutions of pH 7.4 (○), 6.0 (●) and 5.0 (■) at 37°C ($n = 3$).

significant difference was found among them ($p>0.05$). As shown in Fig. 3a2, the mass loss for pDNA/PBELA microspheres was found to be about 20.7% after incubation in pH 7.4 PBS for 35 d, while significantly higher mass loss of about 34.8% and 38.0% were detected after incubation in pH 6.0 and pH 5.0 buffers, respectively ($p<0.05$). A further enhanced mass loss was indicated for pDNA/PGBELA microspheres, and 28.3% and 35.3% of mass loss were observed during 12 d incubation in pH 6.0 and pH 5.0 buffers, respectively (Fig. 3a3).

Figure 3b summarizes the molecular weight reduction of pDNA-loaded microspheres. There were about 12.3%, 15.1% and 18.8% of molecular weight reduction for pDNA/PELA microspheres after incubation for 35 d in buffer solutions of pH 7.4, 6.0 and 5.0, respectively. Similar results were found for pDNA/PBELA and pDNA/PGBELA microspheres after incubation in pH 7.4 PBS. However, around 33.5% and 40.0% of molecular weight reduction were detected for pDNA/PBELA microspheres after incubation for 35 d in buffer solutions of pH 6.0 and 5.0, respectively (Fig. 3b2). The molecular weight loss was more significant for pDNA/PGBELA microspheres, and there were 34.1% and 40.0% after incubation for 12 d in pH 6.0 and 5.0 buffers, respectively (Fig. 3b3). As shown in Fig. 3b, the initial molecular weight loss was more significant for pDNA/PBELA and pDNA/PGBELA than pDNA/PELA microspheres during 1 d incubation in acid buffers. There was less than 5% of loss for pDNA/PELA microspheres in three buffers, and less than 10% for pDNA/PBELA and pDNA/PGBELA microspheres in pH 7.4 PBS. However, around 34% of molecular weight reduction was found for pDNA/PGBELA microspheres during 1 d incubation in pH 5.0 buffer (Fig. 3b3). The significant molecular weight loss was caused by the fast breakdown of the acid-labile segments of ABA copolymer backbone of matrix polymers PBELA and PGBELA.

As indicated above, all the microspheres have similar degradation behaviors in pH 7.4 PBS, but the degradation was enhanced for PBELA and PGBELA microspheres under acid conditions, indicating excellent acid-labile properties. In order to investigate the possible degradation mechanism, the degradation products of PGBELA microparticles were analyzed by ^{1}H-NMR (Fig. 4). Compared with PGBELA microspheres before degradation (Fig. 4a) and after degradation in pH 7.4 PBS (Fig. 4b), significantly smaller absorption peaks at 3.3–3.4 ppm, belonged to galactose, were found after degradation in pH 5.0 buffer for 12 d (Fig. 4c). This was related to the removal of galactose grafts after the degradation of acetal groups. A relatively higher absorption at 3.5–3.7 ppm, assigned to PEG, was due to the breakdown of polylactide segments. The absorption peaks at 5.1–5.2 ppm, assigned to methenyl protons of acetal groups and lactide, were broaden and

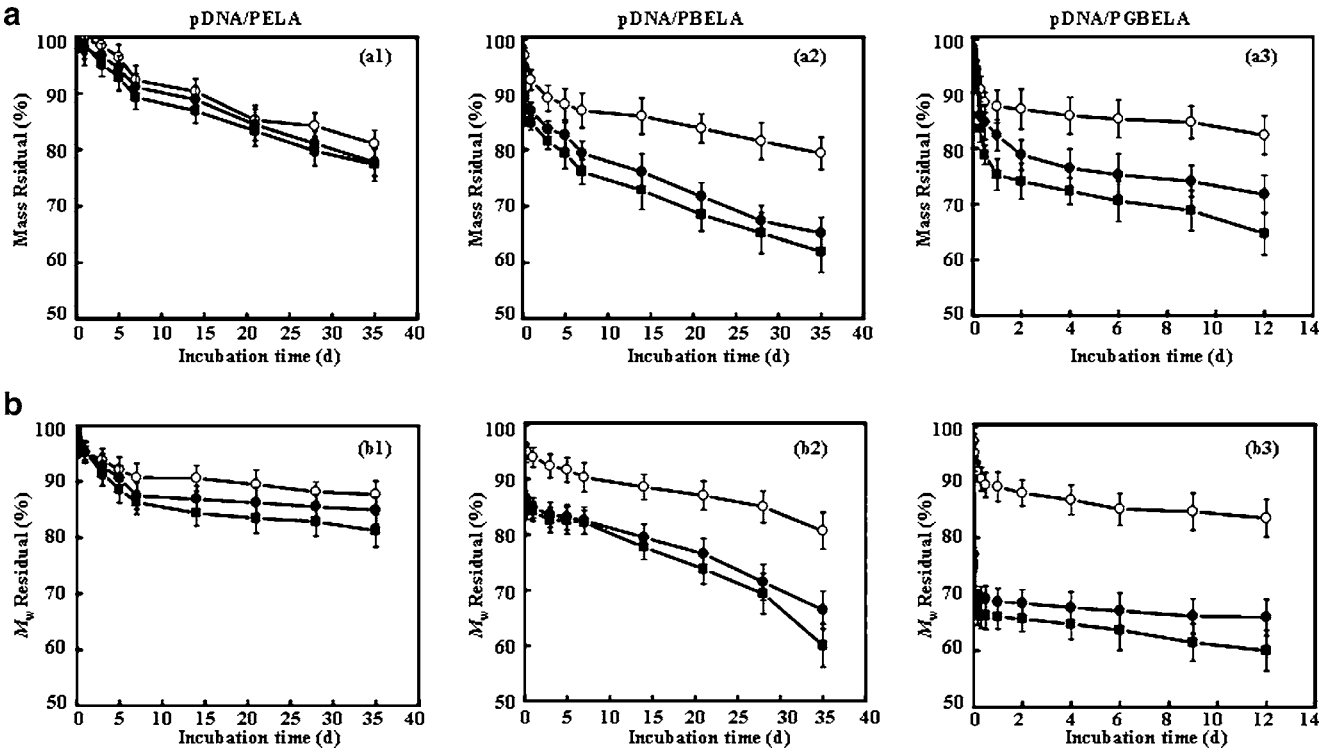


Fig. 3 (**a**) The mass residual and (**b**) molecular weight reduction of (a1 and b1) pDNA-loaded PELA, (a2 and b2) PBELA and (a3 and b3) PGBELA microspheres after incubation in buffer solutions of pH 7.4 (○), 6.0 (●) and 5.0 (■) at 37°C ($n=3$).

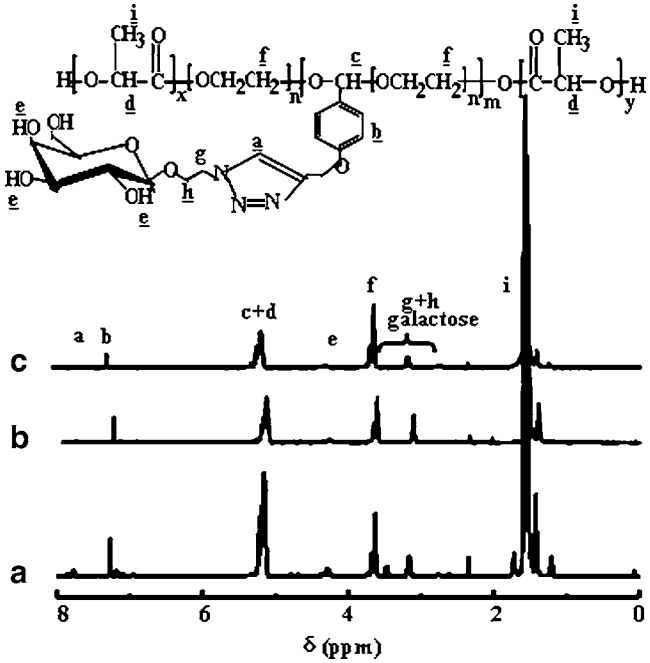


Fig. 4 ^{1}H-NMR spectra of pDNA-loaded PGBELA microspheres obtained (**a**) and after incubation in buffer solutions of pH 7.4 (**b**) and 5.0 (**c**) for 12 d.

shifted to higher region, which was associated with the degradation of acetal groups and the carbonyl groups of lactide. A shift of the absorption peak at 7.2–7.3 ppm, assigned to protons of the phenyl, was detected due to the degradation of the acetal groups as well as the formation of aldehyde groups. In conclusion, the degradation of PGBELA under acid buffers initially occurred on acetal groups of the polymer backbone, followed by the removal of galactose grafts and breakdown of polylactide segments.

Hemolysis Capability of pDNA-Loaded Microspheres Under Different pH Environment

Lysis of RBCs, i.e. hemolysis, has been shown to correlate with the endosome disruption and is therefore a suitable preliminary test to determine the endosomal membrane disrupting activity (27). Figure 5 shows the hemolysis profiles of PELA, PBELA and PGBELA microspheres in different pH buffers, ranging from 7.4 to 5.0, and the selected pH values were based on those in endosome (28). As shown in Fig. 5a, all the microspheres presented a hemolysis rate less than 5% at pH 7.4 under microsphere concentrations ranging from 0 to 2.0 mg/ml. Significantly higher hemolytic activity was determined after incubation of microspheres in acid buffer solutions ($p<0.05$). The hemolysis was gradually increased with the increasing of microsphere concentrations, and the hemolysis rates were up to 55% and 58% for PBELA and PGBELA microspheres, respectively, after incubation into pH 6.0 PBS at the concentration of 2.0 mg/ml, while significantly less hemolysis of about 30% was found for PELA nanoparticles ($p<0.05$). When the pH values further decreased to 5.0, around 60% and 65% hemolysis were detected for PBELA and PGBELA nanoparticles, respectively. They were significantly different from PELA nanoparticles with about 43% hemolysis at the same concentration ($p<0.05$). It should be mentioned that the acidic buffers also had a slight hemolytic activity, when the pH values decreasing from 7.4 to 5.0, the hemolysis increased to around 10%.

Generally speaking, the membrane disruption is caused by the electrostatic interaction with cell membrane and/or the changes of osmotic pressure (22). In the current study the breakdown of acid-labile acetal segments in the acid environment resulted in oligomers and small molecules solutes, leading to an osmotic imbalance that caused RBC lysis. In addition, after uptaken and transferred into cells, acid-labile PBELA and PGBELA microspheres reacted with the protons in the endosome, and the proton consumption indicated a phenomenon similar to the proton-sponge effect, which caused an increased flux of protons and their counterions into the endosome. The osmotic pressure buildup across the membrane was able to destabilize the endosomal membrane and promote endosomal escape (29). The above results sufficiently proved that PBELA and PGBELA microspheres were capable of significant membrane disruption at the endosomal pH, while the destabilization of cell membranes was limited at the physiological pH of 7.4. The higher hemolytic activity of PGBELA than PBELA microspheres can be attributed to the increase in the hydrophilicity of galactose grafts on the acid-labile segments, leading to a higher matrix degradation after incubation in acid environment.

Cytotoxicity of pDNA-Loaded Microspheres

MTT assay was used to measure the cellular activity following incubation of macrophages with blank microspheres and pDNA-loaded microspheres. Figure 6a shows the cell viability after incubation with PELA, PBELA and PGBELA microspheres with the dose ranging from 0.6 to 2.4 mg. The cell viabilities were comparable to that on TCP, indicating no apparent cytotoxicity. Higher cell viability was detected after incubation with above microspheres than TCP. This may be due to that the quantity and size of liver macrophages would be increased after the uptake of foreign microparticles (30).

The cytotoxicity of pDNA-loaded microspheres is summarized in Fig. 6b. The cell viability decreased with an increase in the dose of pDNA-loaded microspheres,

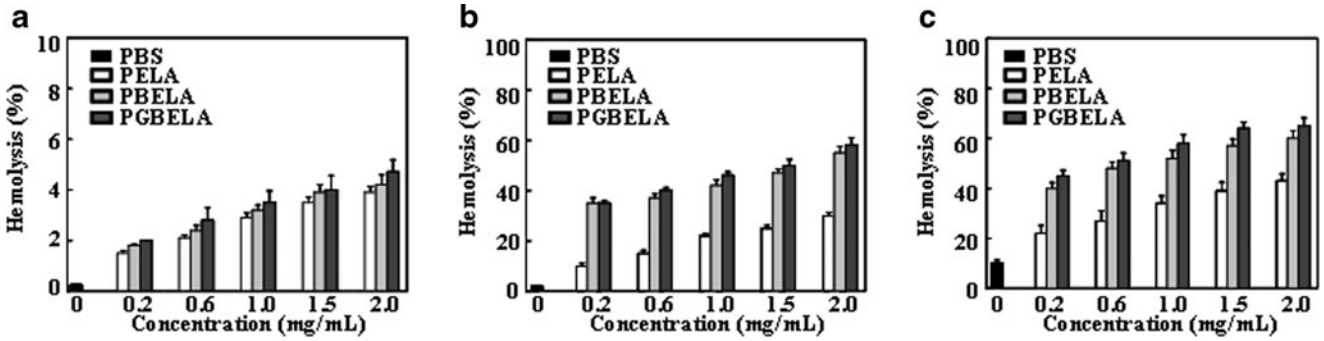


Fig. 5 Hemolysis of PELA, PBELA and PGBELA microspheres after incubation in buffer solutions of (**a**) pH 7.4, (**b**) 6.0 and (**c**) 5.0 ($n = 5$). Hemolysis activity was normalized relative to that observed from the positive control, 1.0% v/v Triton X-100.

indicating that the cytotoxicity was related with the amount of pDNA polyplexes released from microspheres. The relative cell viability after incubation with pDNA polyplexes containing 2.8 μg pDNA was around 30%, which was significantly lower than microspheres with the encapsulation of the same amount of polyplexes ($p < 0.05$), due to the gradual release of pDNA polyplexes from microspheres (31). The cytotoxicity of pDNA/PBELA microspheres was higher than that of pDNA/PELA microspheres at each dose level. This was due to the acid-lability of PBELA and the enhanced release of pDNA polyplexes under the acidic environment after uptake into endosome. As shown in Fig. 6b, lower cell viability was detected after incubation with pDNA/PGBELA than that with pDNA/PBELA microspheres at each dose level. This may be attributed to the increased release amount of pDNA polyplexes (Fig. 2) and enhanced cellular uptake, due to the targeting effects of galactose moieties of PGBELA microspheres.

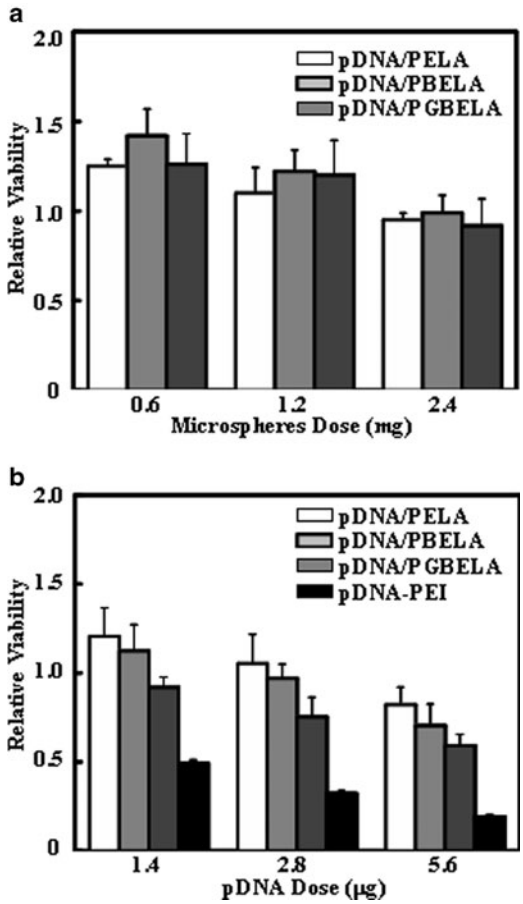


Fig. 6 The viability of liver macrophages after incubation with (**a**) PELA, PBELA and PGBELA microspheres, and (**b**) pDNA-loaded PELA, PBELA and PGBELA microspheres for 48 h.

Cellular Uptake and Transfection Efficiency of pDNA-Loaded Microspheres

The uptake of microspheres into macrophages could be detected through light microscope observation (25). Figure 7a shows the cellular uptake and targeting effect of pDNA/PGBELA nanoparticles with pDNA dose of 2.8 μg. The amount of pDNA/PGBELA microspheres uptaken into cells was higher than that of pDNA/PBELA microspheres. The enhanced cellular uptake efficiency was attributed to the galactose-receptor mediated endocytosis. In order to confirm this assumption, 100-fold molar excess of free galactose was added to the cell suspension 30 min prior to the addition of pDNA/PGBELA microspheres, the amount of microspheres uptaken into cells indicated a significant decrease (Fig. 7a).

The GFP expression of cells after incubation with pDNA-loaded microspheres is summarized in Fig. 7c. Significant higher transfection efficiency was obtained after incubation with pDNA/PBELA and pDNA/PGBELA than pDNA/PELA microspheres at each pDNA dose ($p < 0.05$). It was suggested that the acid-labile microspheres realized the escaping from endosome and released the pDNA polyplexes into cytoplasm. The transfection activity of pDNA/PBELA and pDNA/PGBELA microspheres containing 2.8 μg pDNA was 3.3×10^5 and 6.4×10^5 RLU/mg protein, respectively. The increased transfection was due to

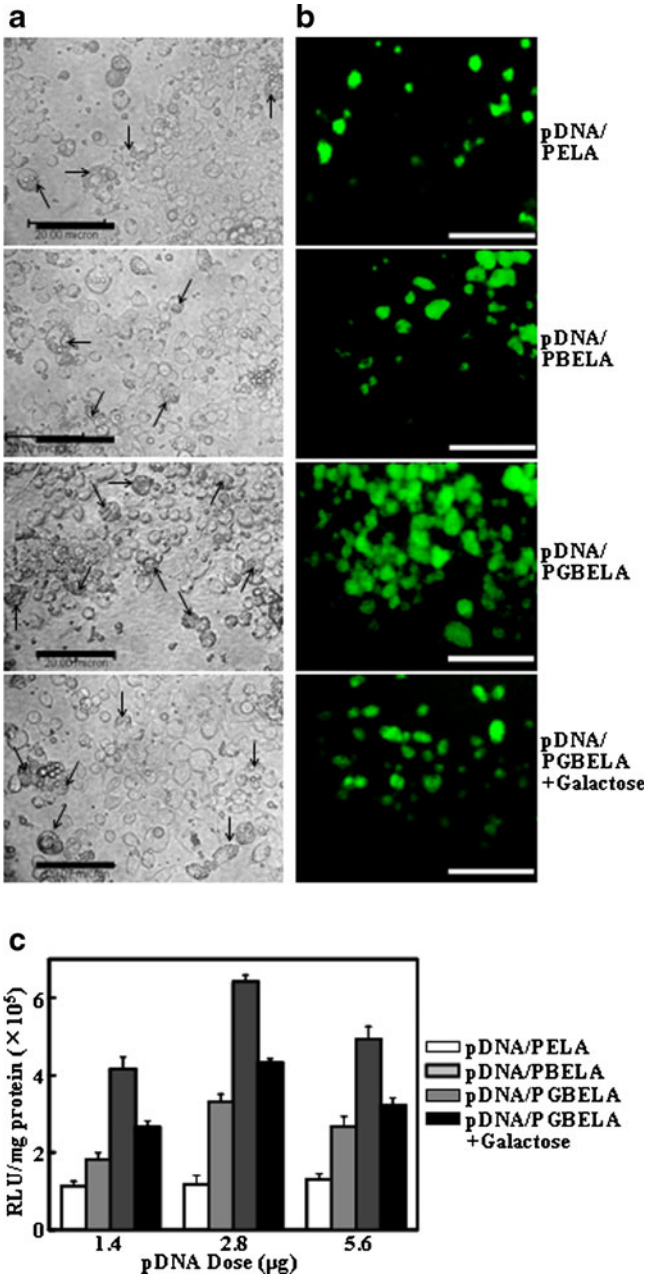


Fig. 7 (**a**) Transmission light microscope and (**b**) fluorescence microscope images of liver macrophages after incubation with pDNA-loaded PELA, PBELA and PGBELA microspheres containing 2.8 μg pDNA, and 100-fold molar excess of free galactose was added to the cell suspension 30 min prior to the addition of pDNA/PGBELA microspheres (pDNA/PGBELA + Galactose); Arrows indicate cells with microspheres entrapped, and scale bars represent 20 μm. (**c**) Transfection efficiency of liver macrophages after incubation with pDNA-loaded microspheres at pDNA doses of 1.4, 2.8 and 5.6 μg.

the enhanced uptake efficiency of galactose decorated microspheres (Fig. 7a) and the accelerated release of pDNA polyplexes from more hydrophilic matrix polymers (Fig. 2). This was also approved from the decrease in the transfection efficiency of pDNA/PGBELA microspheres after the addition of free galactose into the culture media (Fig. 7c), which competed with PGBELA microspheres to bind with cells (Fig. 7a). Figure 7b shows images of transfect cells, emitting great fluorescence. These images were coincident with the transfection efficiency as shown in Fig. 7c.

The life-span of tissue macrophages has been estimated to range from 6 to 15 d (32). As indicated in Fig. 2, around 70% and 100% of entrapped DNA was released out from PGBELA microspheres in acid buffers during 7 and 12 d, respectively. The pDNA polyplexes released from microspheres could alleviate the deactivation of pDNA, and there was no significant difference in the transfection efficiency of pDNA released over 10 d (14). Therefore, the targeting capability and time frame of pDNA release provided PGBELA microspheres as potential carriers for DNA vaccine. In addition, one criterion in designing a DNA delivery device is the balance between the transfection efficiency and cell viability (33). As shown in Fig. 7c, the highest transfection efficiency was detected for microspheres at the middle level of pDNA doses. A high dose of pDNA-loaded microspheres may promote the GFP expression, but a high cytotoxicity was indicated, leading a decrease in the transfection efficiency. It is suggested that an optimal dose of pDNA-loaded microspheres with acetal groups and galactose decorations should be achieved for specific cells and their applications.

CONCLUSION

Copolymers PGBELA containing acid-labile segments and galactose grafts were developed to entrap pDNA polyplexes into microspheres. The acid-lability of the microsphere matrices was clarified from the pDNA release profiles, hemolytic abilities and matrix degradation behaviors. Higher cellular uptake and promoted transfection efficiency were found for pDNA/PGBELA microspheres, and an optimal pDNA dose was obtained to achieve a balance between the transfection efficiency and cell viability. The integration of acid-lability, targeting effect, and full biodegradable backbone into microsphere matrices represents an exciting approach to target APCs, enhance the release of pDNA polyplexes and promote the transfection efficiency of pDNA.

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