International Journal of Pharmaceutics 370 (2009) 187–195

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Studies on bioadhesive PLGA nanoparticles: A promising gene delivery system for efficient gene therapy to lung cancer

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ARTICLE INFO

Article history:
Received 17 July 2008
Received in revised form 5 November 2008
Accepted 17 November 2008
Available online 27 November 2008

Keywords:
Bioadhesive nanoparticles
PLGA
Gene delivery
Carbopol (CP)
Lung cancer

ABSTRACT

The study aimed to design novel bioadhesive PLGA nanoparticles for efficient gene delivery to lung cancer cells. The bioadhesive agent and stabilizer, Carbopol 940 was chosen to establish bioadhesive PLGA nanoparticles and Pluronic F68, Pluronic F127 stabilized PLGA nanoparticles were formulated as control. The effects of different surfactants on the physicochemical and biological characterizations of PLGA nanoparticles were compared. All the obtained nanoparticles showed negative surface charge, similar spherical morphology, a relatively narrow particle size distribution, and lower cytotoxicity to A549 cells comparing with Lipofectamine 2000. Carbopol stabilized nanoparticles hold advantages in DNA-binding efficiency (>80%) at an optimal Carbopol concentration, DNA protection from enzymatic degradation *in vitro* release and better buffering capacity. Most importantly, higher transfection efficiency in A549 cells was observed comparing to Pluronics stabilized nanoparticles or naked DNA, similar to that of Lipofectamine 2000. These results revealed that the bioadhesive PLGA nanoparticles formulated with Carbopol might be a very attractive candidate as a non-viral vector for lung cancer gene therapy and might alleviate the drawbacks of the conventional cationic vectors/DNA complexes for gene delivery *in vivo*.

1. Introduction

At present, lung carcinoma is still a fatal malignancy in many cases due to the lack of major advancements in treatment strategy (Wu et al., 2007; Sasaki et al., 2008). Current treatments for lung cancer have shown little success because they cannot cure disseminated tumors with an acceptable level of toxicity. Thus, one alternative strategy that has shown promise in the treatment of lung cancer is gene therapy.

There are two main groups of vectors used in gene delivery: viral and non-viral vectors. Toxicity and immunogenicity concerns associated with viral vectors have led to an active interest in non-viral systems for gene delivery (Panyam and Labhasetwar, 2003). Among non-viral vectors, biodegradable nanoparticles have shown their advantage over other carriers by their increased stability and their controlled-release ability (Li and Huang, 2007; Mundargi et al., 2008). One of the most widely used polymers for nanoparticles is the biodegradable and biocompatible poly(D,L-lactide-co-glycolide) (PLGA), which has been approved by the FDA for certain human clinical uses. PLGA nanospheres have been suggested to be a good gene delivery carrier because of the safety and achieving sustained release (Prabha et al., 2002). The degradation time of PLGA can be altered from weeks to months by varying the molecular weight of the copolymer or the lactic acid to glycolic acid ratio in copolymer (Kim et al., 2005).

Typically, nanoparticles in gene delivery systems could be divided into two systems, cationic and anionic nanoparticles. Cationic nanoparticles systems utilize the ionic interaction between the cationic polymers and the anionic plasmid DNA, forming stable polymer/lipids-DNA complexes (Singh et al., 2000, 2006). Though many of these cationic vectors perform well *in vitro* in reduced serum conditions, most of them suffer from serious drawbacks or lose their efficiency when tested *in vivo* (Mahato et al., 1997). In systemic application, it tends to their aggregation and accumulation in the "first pass organs" such as lungs (consequently causing pulmonary embolism), liver and spleen, and finally opsonization and clearance by the reticuloendothelial system (RES), limiting their therapeutic applications (Fenske et al., 2001; Ogris and Wagner, 2002). These drawbacks of the conventional cationic nanoparticles for gene delivery *in vivo* might be alleviated in the case of anionic gene delivery, while it is difficult or inefficient to adsorb the plasmid DNA onto the surface of anionic nanoparticles. Therefore, plasmid DNA is usually encapsulated into the anionic nanoparticles using emulsification solvent evaporation method. Whereas this preparation process of nanospheres requires harsh manufacturing conditions such as sonication or high shear agitation by a homogenizer causing inactivation of the plasmid DNA (Tinsley-Bown et al., 2000; Oster and Kissel, 2005).

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doi:10.1016/j.ijpharm.2008.11.016

Taking all that into account, the aim of this study is to establish novel bioadhesive and anionic gene delivery system which might provide promise as an experimental strategy both in DNA binding and *in vivo* gene delivery. Bioadhesive drug delivery systems are attractive for the controlled delivery of hydrophilic macromolecules such as DNA since they provide high loading efficiency along with a protective environment (Haas and Lehr, 2002; de la Fuente et al., 2008). In addition, such systems can also interact with mucus to prolong the residence time of drug carriers at the drug absorption sites and protect the entrapped drugs from enzymatic degradation until they are absorbed (Cui et al., 2006). Thus, they could act as a depot of gene delivery vectors and provide a continuous supply of the vectors to the targeted cells over a period of time, circumventing their repeated administration. Recent studies showed that the adsorption of a hydrophilic polymer on the nanoparticle surface improves not only their transmucosal transport but also their efficiency as gene carriers (Munier et al., 2005; Prego et al., 2005). They protect the DNA against degradation in extra-cellular matrix (ECM) and maintain an elevated DNA concentration in the cellular microenvironment, increasing the transfection probability and generating prolonged gene expression (Agarwal et al., 2008). The use of muco- or bioadhesive agents in biodegradable and potentially biocompatible polymer-based particulate DNA delivery systems may offer excellent potential for the delivery of vaccines administration to the environment of the upper and lower respiratory tracts (Alpar et al., 2005). To establish bioadhesive gene delivery system for administration by pulmonary routes is not a strategy that has attracted a large volume of research. The advent of DNA vaccination, however, is still relatively new, and the development of new and effective vectors may be the key event in realizing the potential of this strategy.

Natural bioadhesive polymers like gelatin, chitosan, collagen and agarose, have been used as implantable matrices for sustained gene delivery (Dang and Leong, 2006). Carbopol, a high molecular weight poly(acrylic acid) loosely cross-linked with allyl sucrose copolymer, is suggested to be one of the promising stabilizers to fabricate nanoparticles (Vandervoort and Ludwig, 2002). Furthermore, it is a great bioadhesive agent which possesses very good bioadhesive properties and exhibits good retention both *in vitro* and *in vivo* (Kockisch et al., 2001; Grabovac et al., 2005). Carbopol has been used experimentally as a bioadhesive agent to enhance adhesion to mucosal surfaces in combination with other delivery systems (e.g., microspheres) and may facilitate enhanced protection of peptides and proteins against enzymatic degradation *in vivo* (Alpar et al., 2005). Additionally, low toxicity and biocompatibility make Carbopol ideal for inclusion in products intended for everyday use. Pluronics or poloxamers are block copolymers consisting of poly(oxyethylene) and poly(oxypropylene) units, which is one of the most widely used surfactants applied in gene delivery system because of the reported potential of polyoxyethylene derivatives for gene delivery, either alone (Lemieux et al., 2000; Riera et al., 2004; Sriadibhatla et al., 2006) or in combination with other polymeric materials (Jeon et al., 2003; Belenkov et al., 2004).

In this study, we aimed to establish bioadhesive PLGA nanoparticles using Carbopol as bioadhesive agent for efficient gene therapy to lung cancer, and Pluronics stabilized nanoparticles were investigated as a control. The formulations were investigated for physicochemical parameters such as particle size, zeta potential, DNA-binding ability, buffering capacity and *in vitro* release, and also for their biological properties such as cytotoxicity, resistance to nuclease degradation and gene transfection ability. Accordingly, formulation parameters including the type of surfactants and concentrations of Carbopol on the characterizations of nanoparticles were compared.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic) (PLGA, 50:50, Av.MW 25,000) with a carboxylic end group was purchased from Shandong Institute of Medical Instrument (Shandong, China). Pluronic F68 and Pluronic F127 were purchased from Sigma (China). Carbopol 940 (MW 1000,000) was provided by Shanghai Renmin Pharmaceutical Factory (Shanghai, China). pEGFP-N_1 was provided by Zhejiang University (China). SYBR Green I fluorescent dye was purchased from Molecular Probes (Eugene, OR, USA). Goldview was obtained from Beijing Saibaisheng Biological Engineering Co. (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). A549 lung cancer cell line was obtained from American Type Culture Collection (ATCC, USA). DNase I enzyme was obtained from Beijing Yinfeng Century Scientific Develop Co., Ltd. (Beijing, China).

2.2. Preparation of stabilizer solution

Exactly weighted Carbopol 940 (CP) was dispersed in distilled water at room temperature, placed overnight to be swollen, and neutralized by adding required amount of 1 M NaOH until pH 7.0 was reached. Afterwards the CP stock solution was diluted with distilled water into different concentrations (0.005%, 0.01%, 0.02%, 0.05%, 0.10%, w/v). 1% Pluronic F68 (F68) or 1% Pluronic F127 (F127) solution was obtained by dispersing the weighted polymer in distilled water under magnetic stirring at room temperature. The viscosity of the stabilizer solutions was measured using Ostwald's viscometer with distilled water as reference standard at room temperature.

2.3. Formation of blank nanoparticles

PLGA nanoparticles formation was performed under optimal conditions by solvent displacement technique (Fessi et al., 1989). Briefly, accurately weighted 50 mg PLGA was dissolved in 3 ml acetone. The organic phase was added drop-wise (0.5 ml/min) into 20 ml aqueous solution containing stabilizer either 1% F68 (w/v), 1% F127 or CP at different concentrations (w/v), and which was then stirred at 400 rpm by a laboratory magnetic stirrer at room temperature until complete evaporation of the organic solvent. The redundant stabilizers were removed from the nanoparticles by centrifugation at 15,000 rpm and 4 °C for 30 min (Cetra-MP4 centrifuge, International Equipment Company, Miami, USA). The pellet was resuspended in Milli-Q water and washed three times. The obtained nanoparticle suspensions (abbreviated as 1% F68-NP, 1% F127-NP, 0.005% CP-NP, 0.01% CP-NP, 0.02% CP-NP, 0.05% CP-NP, 0.10% CP-NP) were stored at 4 °C until use.

2.4. Morphology, particle size and zeta potential of the nanoparticles

The morphology of the nanoparticles was examined by transmission electronic microscopy (TEM) (JEM-1200EX, Japan). Samples were prepared by placing a drop of nanoparticles suspension onto a copper grid and air-dried, following negative staining with one drop of 3% aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then directly examined under the transmission electronic microscopy.

The mean particle size and zeta potential of the nanoparticles were analyzed by photon correlation spectroscopy (PCS) with a 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 ± S.D. ($n = 3$).

2.5. Fabrication of plasmid DNA loaded nanoparticles

The reporter gene encoding enhanced green fluorescence protein (EGFP) was adsorbed onto the nanoparticles by vortexing nanoparticles suspension with 1 mg/ml solution of DNA for 10 s at a theoretical loading of 1.0% (w/w), followed by incubating the mixture at 37 °C for 30 min. To measure the adsorption efficiency of the plasmid-loaded nanoparticles, the free drug was isolated from the nanoparticles by centrifugation at 15,000 rpm, 4 °C for 30 min and the supernatants were collected and analyzed using the SYBR Green I-fluorometry method (Ye et al., 2007). The concentration of plasmid DNA was assessed by fluorescence spectrophotometer (HITACHI F2500, Japan) according to the directions of the SYBR Green I Kit. The amount of DNA loaded in the nanoparticles was calculated according to the linear calibration curve of DNA.

2.6. DNA retardation assay

In order to further confirm the adsorption of DNA onto the surface of the bioadhesive nanoparticles and compare the adsorption capacity of all batches, the agarose gel electrophoresis was carried out. The DNA loaded nanoparticles were applied to a 0.8% (w/v) agarose gel (100 ng DNA per hole) in TAE buffer (40 mM Tris–HCl, 1% acetic acid, 1 mM EDTA, pH 8.0) containing 2 μl goldview. Electrophoresis (Electro-4, Thermo Electron, Waltham, MA, USA) was carried out at a constant voltage of 90 V for 30 min in TAE buffer. Images were obtained using a UV transilluminator and a digital imaging system (IS-2200, Alpha Innotech, USA).

2.7. Buffering ability evaluation of the bioadhesive nanoparticles

Buffering ability is one of the most important characteristics of gene nano-delivery systems. High buffering ability would introduce endo-lysosome escape and benefit the delivered gene (Munier et al., 2005). Ability of bioadhesive PLGA nanoparticles to resist acidification was tested using acid titration assay as described by Swami et al. (2007). Briefly, 10 ml PLGA nanoparticles suspensions stabilized by 1% F68, 1% F127, 0.02% CP or 0.05% CP were first adjusted to pH 9.0 and then titrated in small increments with 0.1 M HCl until a pH of 3.5 was reached. The slope of the pH versus HCl added graph provides an indication of the intrinsic buffering capability of the delivery vehicles.

2.8. In vitro release studies

The *in vitro* release studies of nanoparticles were performed in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 7.4). Typically, an aliquot of bioadhesive nanoparticles (equivalent to 2.5 μg DNA) were suspended in 1 ml TE buffer in Eppendorf® tubes at 37 °C shaking water bath at 100 rpm. Separate tubes were used for each data point. At predetermined time intervals, the nanoparticles suspensions were centrifuged (15,000 rpm, 30 min) and the amount of DNA released in the supernatant was analyzed by SYBR Green I-fluorometry method mentioned above. Background readings were obtained using the supernatants from blank nanoparticles.

2.9. Exposure of the nanoparticles to DNase I

Protection of plasmid DNA from nucleases is one of the most important properties for efficient gene delivery *in vitro* as well as *in vivo*. To test whether PLGA nanoparticles can protect adsorbed plasmid DNA from nucleases digestion, DNase I mediated digestion was evaluated using agarose gel electrophoresis. DNA loaded PLGA nanoparticles of different formulations and free DNA (1 μg) were respectively incubated with DNase I (1 U) in DNase I/Mg^{2+} digestion buffer (50 mM, Tris–HCl, pH 7.6, and 10 mM $MgCl_2$). The suspensions were incubated in shaking water bath at 37 °C and 100 rpm for 30 min. After that, the enzymatic digestion reaction was terminated with EDTA solution (0.5 M, pH 8.0). The nanoparticles in the system were collected by centrifugation and were dissolved in chloroform followed by adding an equal volume of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). And the mixture was rotated end-over-end to facilitate the extraction of DNA from the organic phase into the aqueous phase. The samples were then centrifuged at 15,000 rpm for 30 min. The configuration of plasmid DNA in the supernatant was analyzed by gel electrophoresis as described above. Untreated DNA was applied to the gel as control.

2.10. Structural integrity determination of plasmid DNA after release

Structural integrity of plasmid DNA released from nanoparticles at selective time points was also evaluated by agarose gel electrophoresis. To determine plasmid stability during release, the samples released at different time points (i.e. 0.5, 1, 2, 3, 5 and 7 days) along with control untreated plasmid DNA were applied to 0.8% agarose gel in TAE buffer. Band separation for supercoiled and open circular plasmid DNA was observed after gel electrophoresis at 90 V for 30 min.

2.11. Cytotoxicity/cell viability studies

Cytotoxicity of PLGA nanoparticles was assayed by MTT assay in A549 lung cancer cell line (Basarkar et al., 2007). The cells were seeded into a 96-well microtiter plates at a density of 8×10^3 cells per well in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 incubator at 37 °C. After 24 h, the culture medium was replaced by 200 μl fresh serum-free RPMI 1640 medium with different concentrations of the nanoparticles (expressed as PLGA concentration, 5, 25, 50, 100 and 250 μg/ml) and Lipofectamine 2000 in comparison. After incubating for 24 h, the effect of different treatments on cell viability was assessed by the MTT assay. Typically, 5 mg/ml of MTT in PBS were added to each well reaching a final concentration of 0.5 mg MTT/ml and incubated for 4 h. Then the supernatants were removed and the formazan crystals were dissolved in 100 μl DMSO. Aliquots were drawn from each well and the absorbance at 570 nm was determined by a microplate reader (Model 680, BIO-RAD, USA). Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells were calculated by $(A_{sample}/A_{control}) \times 100$. All treatments were done in quadruplicates and all experiments were repeated in triplicates. A paired t-test with $P < 0.05$ was used to establish statistically significant differences between treatments.

2.12. In vitro transfection experiments

The transfection efficiency of bioadhesive nanoparticles was evaluated on A549 cell lines using the plasmid EGFP. The A549 lung cancer cells were seeded into 24-well plates at a density of 8×10^4 cells/well in 1 ml of RPMI-1640 with 10% FBS, 24 h prior to transfection. When the cells were at about 80% confluence, the media were replaced with 500 μl serum-free media containing DNA loaded PLGA nanoparticles (1 μg DNA) at 37 °C. Lipofectamine 2000 was used as positive control, and the formulation of Lipofectamine/DNA complexes was carried out according to the manufacturer's protocol. Naked DNA was used as negative control. After incubation for 4 h at 37 °C in 5% CO_2 incubator, the origi-

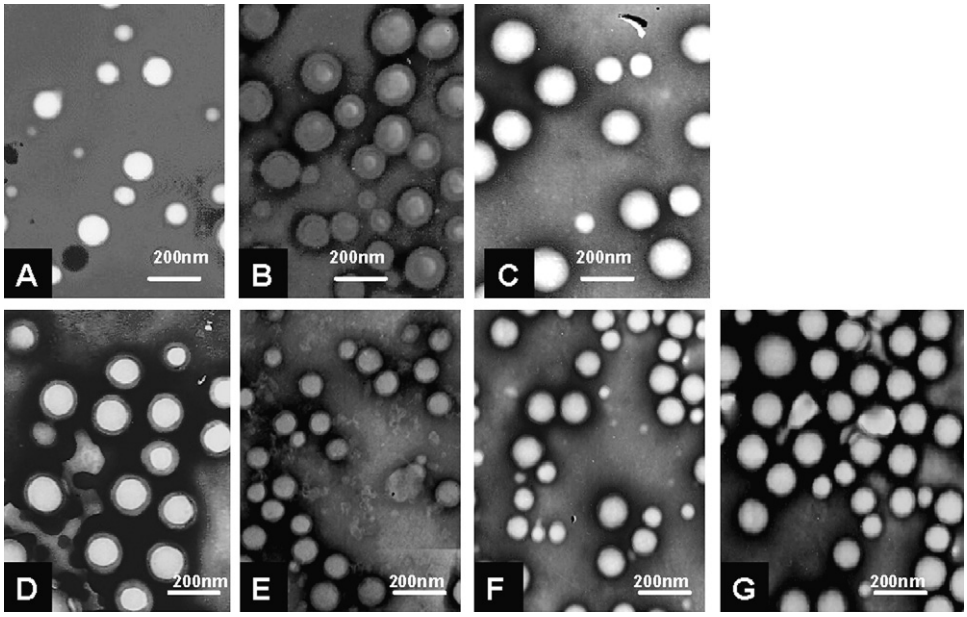

Fig. 1. TEM imaging of PLGA nanoparticles preparations: 1% F68-NP (A), 1% F127-NP (B), 0.005% CP-NP (C), 0.01% CP-NP (D), 0.02% CP-NP (E), 0.05% CP-NP (F), 0.1% CP-NP (G).

nal incubation media was replaced with 1 ml of complete medium and cells was incubated sequentially until 48 h post transfection. Fluorescent cells were observed using an inversion fluorescence microscope (OLYMPUS, ZX71, Japan) and the picture was captured. Transfection experiments were performed in triplicates.

3. Results and discussions

3.1. Formation of PLGA nanoparticles

The transmission electron micrograph of the PLGA nanoparticles of different formulations was shown in Fig. 1. It was shown that the obtained nanoparticles stabilized with different stabilizers appeared similar spherical in shape and separated from each other. The results of physicochemical characterization of PLGA nanoparticles were summarized in Table 1. The polydispersity index (PDI) of all samples ranged from 0.105 to 0.221, which demonstrated a relatively narrow particle size distribution. Particle size and surface charge of PLGA nanoparticles were found to be dependent on the concentration of CP. The mean particle size of nanoparticles decreased from 198 to 126 nm with raising the concentration of CP from 0.005 to 0.02% (w/v), while the mean size increased if furtherly increasing the concentration of CP from 0.02 to 0.1%. In terms of surface charge, when increasing the concentration of CP from 0.005 to 0.1%, zeta potential of nanoparticles reduced from −15.88 to −57.04 mV which were much more pronounced than in the case of nanoparticles using 1% F68 or 1% F127 as stabilizers (−10.65 mV or −7.34 mV). This could be explained that the non-ionic poloxamer at the surface of nanoparticles was possible to shield the surface negative charge of PLGA while the large active carboxylic groups present in anionic CP polymer could intensify the surface negative charge of PLGA nanoparticles. Thus the surface charge of the nanoparticles became even more negative with the increase of the concentration of CP used in the formulation. When concentration of CP was increased from 0.005 to 0.02% (w/v), the stabilizer would improve the protection of droplets against coalescence or interflow by increasing its surface charge repulsion or increasing surface hydrophilicity. However, even higher concentration of CP will accordingly increase aqueous phase viscosity (Table 1) which was possible to cause relatively larger emulsion droplets or larger particles due to the reduction of the stirring efficiency.

3.2. DNA retardation assay

The agarose gel electrophoresis was carried out to confirm whether the plasmid DNA could be associated to the formulated PLGA nanoparticles. The mobility of objects under the electric field is determined by combination of the charge, the size of themselves and the diffusion through the agarose matrix that hinders the big objects. It was found that sole plasmid DNA could migrate to the positive electrode under the electric field such as lane 1 in Fig. 2. Once DNA was associated with the PLGA nanoparticles which was too large to diffuse through the agarose matrix, the mobility of DNA was hindered and it was detained in the hole of the agarose gel. In addition, the part of DNA which did not bind onto the surface of PLGA nanoparticles migrate to the positive electrode in the same manner with the control DNA. Thus, different formulations of nanoparticles were incubated with constant amount of plasmid DNA to determine optimal formulation required for the most retardation of DNA. The fluorescent intensity in the channel 2 to channel

Table 1
The physical characterization of PLGA nanoparticles of all batches (mean ± S.D., $n = 3$).

Nanoparticle type	Relative viscosity of stabilizer solution	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
1% F68-NP	1.17 ± 0.05	185 ± 9	0.124 ± 0.005	−10.65 ± 0.48
1% F127-NP	1.21 ± 0.06	172 ± 5	0.203 ± 0.012	−7.34 ± 0.98
0.005% CP-NP	1.07 ± 0.04	198 ± 7	0.221 ± 0.016	−15.88 ± 2.30
0.01% CP-NP	1.14 ± 0.05	165 ± 5	0.155 ± 0.009	−23.62 ± 2.51
0.02% CP-NP	1.38 ± 0.08	126 ± 5	0.105 ± 0.004	−27.83 ± 3.27
0.05% CP-NP	2.85 ± 0.09	177 ± 6	0.168 ± 0.019	−35.10 ± 3.63
0.1% CP-NP	11.84 ± 0.53	205 ± 11	0.216 ± 0.021	−57.04 ± 4.75

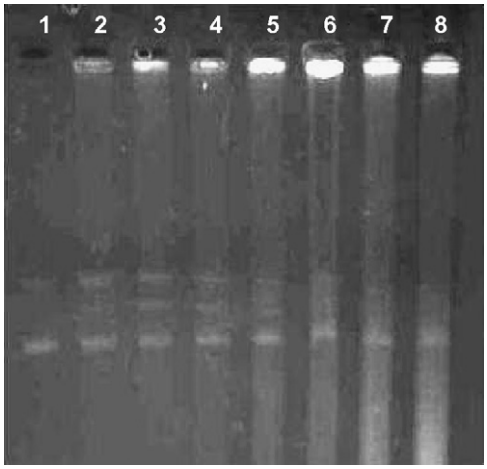

Fig. 2. Electrophoresis of the different formulations of DNA adsorbed nanoparticles on an agarose gel. The samples from the left to the right was as follows, lane1: Naked DNA control; lane 2: 1% F68-NP; lane 3: 1% F127-NP; lane 4: 0.005% CP-NP; lane 5: 0.01% CP-NP; lane 6: 0.02% CP-NP; lane 7: 0.05% CP-NP; lane 8: 0.1% CP-NP.

8 differentiated as shown in Fig. 2, meant that the engineered PLGA nanoparticles could adsorb DNA more or less onto its surface. The concentration of CP influenced the adsorption efficiency. When increasing the concentration of CP from 0.005 to 0.02%, the fluorescence in the channel became increasingly intensified, that was, there was more and more DNA adhered onto the nanoparticles. While with furtherly increased concentration, the fluorescence became weakened. Therefore the optimal concentration of CP for DNA-nanoparticles binding was 0.02% in our selective range. The exact value of adsorption efficiency of DNA-loaded nanoparticles was furtherly investigated quantitatively.

3.3. The plasmid DNA adsorption efficiency of the bioadhesive nanoparticles

Generally, cationic nanoparticles was formulated to efficiently adsorb anionic plasmid on the surface, primarily via ionic interaction, which depends on the charge attraction (Ravi Kumar et al., 2004; Kim et al., 2005). In theory, anionic nanoparticles has difficulty in binding DNA because of the net negative charges that both species carry, thus the combination of DNA and anionic nanoparticles seemed impossible. While in the present study, the plasmid DNA was successfully adsorbed onto the PLGA nanoparticles exhibiting negative zeta potentials, adsorption efficiencies differentiated in various formulations ranging from 50 to 90% (Fig. 3). Even for the nanoparticles stabilized with F68 or F127, the DNA adsorption efficiencies reached more than 50%, though which was contradicted with previous published findings (Taetz et al., 2008). The differential results might be mainly attributed to the differences in the molecular weight of the copolymer and the lactic acid to glycolic acid ratio in copolymer which determining the hydrophilicity of PLGA. The interaction between DNA and PLGA nanoparticles was probably based on the non-ionic interactions, such as van der Waals interactions or adhesive force between the bioadhesive nanoparticles and DNA. The concentration of CP affected the adsorption efficiency of the nanoparticles, and the plateau of the adsorption efficiency (87.45%) was reached at the CP concentration of 0.02%. This result was in accordance with the DNA retardation assay mentioned above. One possible explanation for that result was that the DNA adsorption efficiency was determined by the balance of non-ionic interactions and charge repulsion between the bioadhesive nanoparticles and DNA, increasing the concentration of CP from 0.005 to 0.02% was accompanied with the augmentation of viscosity and the surface negative charge of nanoparticles, the adsorption force was strong enough to overcome the charge repulsion between the nanoparticles and DNA, while when the concentration of CP was furtherly increased, the case was just contrary, that was, the charge repulsion overwhelmed the adsorption force between the nanoparticles and DNA. Another explanation was that the small nanoparticles stabilized with 0.02% CP owned larger surface area to volume, which benefited the adsorption of DNA, thus leading to higher adsorption efficiency. The formulation of 0.02% CP-NP was chosen for further investigation in comparison with 1% F68 and 1% F127-NP.

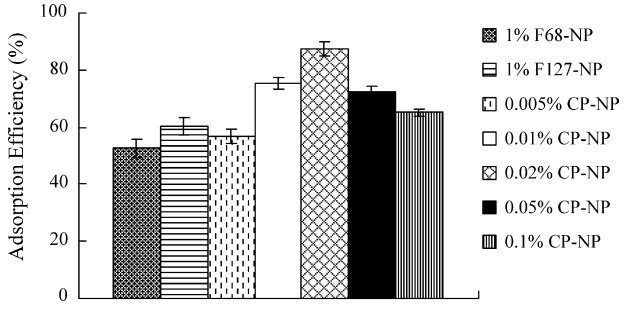

Fig. 3. The adsorption efficiency of plasmid-loaded PLGA nanoparticles of all batches ($n = 3$).

3.4. Buffering ability evaluation of the bioadhesive nanoparticles

High buffering ability would introduce endo-lysosome escape and benefit the delivered gene. The buffering ability of the 0.02% CP-NP was investigated in our study using 1% F68-NP and 1% F127-NP as control. In order to exclude the effect of nanoparticle size or the absolute number of particles to the result, 0.05% CP-NP with similar particle size and number to the control was also evaluated for its buffering ability. Significantly increased buffering ability of CP-NP compared to 1% F68-NP or 1% F127-NP was observed in our studies. As shown in Fig. 4, CP stabilized PLGA nanoparticles held advantages over the other two formulations in antiacid ability. And increasing CP concentration in the formulation led to slightly higher buffering capability to the nanoparticles. The CP stabilized PLGA nanoparticles with buffering capability have a potential to resist the acidic microenvironment of endosome and lysosome in cell (Panyam et al., 2002) and maintain the integrity of loaded DNA *in vivo*. On the other hand, it was known that the hydrolysis of PLGA usually leads to the accumulation of acidic monomers, lactic and glycolic acids within the drug delivery device, thereby resulting in a significant reduction of pH of the microenvironment and denaturation of the encapsulated proteins or gene. Therefore CP sta-

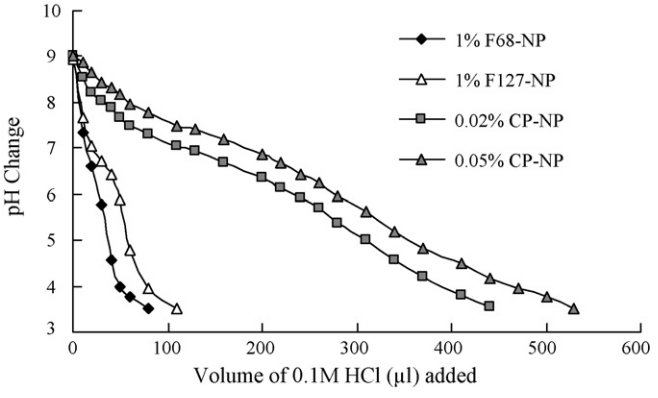

Fig. 4. Acid titration experiments with 0.1 M HCl to demonstrate the buffering ability of bioadhesive PLGA nanoparticles stabilized with different stabilizers.

bilized PLGA nanoparticles with buffering capacity was promising to prevent the loaded DNA from degradation when released from the nanoparticles both *in vitro* and *in vivo*. The buffering capability may be attributed to the presence of abundant ionized carboxyl groups of CP molecule applied in nanoparticle formulations, which could combine with hydronium ions in environment by hydrogen bonding and accordingly endow the nanoparticles with buffering ability.

3.5. In vitro release studies

The *in vitro* drug release property is one of the important characteristics of nanoparticles. Release profiles of different formulations in TE (pH 7.4) were illustrated in Fig. 5. The release profiles of all batches examined were similar and consisted of an initial rapid release phase in the first day, and followed by a sustained release period in 7 days. It could be explained that the adsorbed DNA bound weakly onto the nanoparticle surface caused initial rapid release, and the remained DNA which was adhered tightly with nanoparticles or inserted into the nanoparticles was released in a controlled fashion. In order to determine the release mechanism of DNA from PLGA nanoparticles, the release data were evaluated by model-dependent methods, and the model of different formulations all fitted into Higuchi model best, with a good correlation coefficient ($R \geq 0.99$). DNA released more rapidly from 0.02% CP-NP than the others, which might be ascribed to the smallest particle size of the 0.02% CP-NP because the smaller particle size with a larger surface area to volume was favorable to the drug release for the delivery system.

3.6. Protection effects from DNase I degradation

For effective gene expression, the DNA in the gene vehicle should be protected from degradation by enzymes (Park et al., 2005). To test whether bioadhesive PLGA nanoparticles could protect adsorbed plasmid DNA from nucleases digestion, the nanoparticles were exposed to DNase I for 30 min. Three different DNA conformations after the treatment with DNase I could be detected by the agarose gel electrophoresis, linear (after getting nicked), open circular (with only one strand nicked), or supercoiled as shown in Fig. 6. Non-treated free DNA (lane 1), which was not in contact with the DNase I, presented only two bands. The lower band corresponds to the supercoiled form and the upper band is equivalent to the open

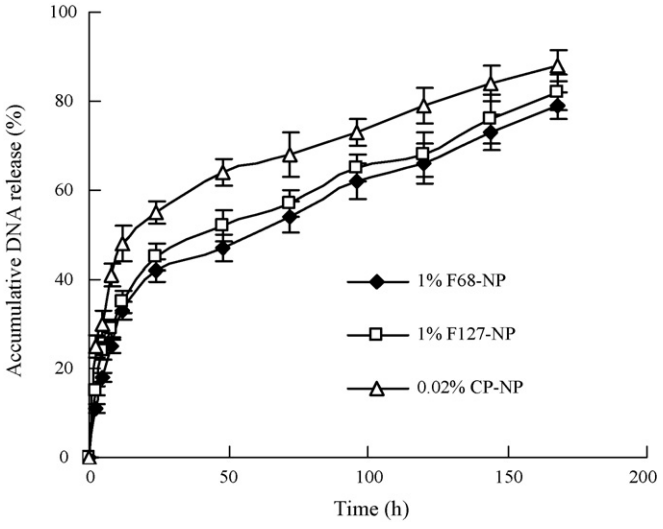

Fig. 5. The *in vitro* release of DNA-loaded PLGA nanoparticles stabilized by 1% F68, 1% F127, 0.02% CP ($n = 3$).

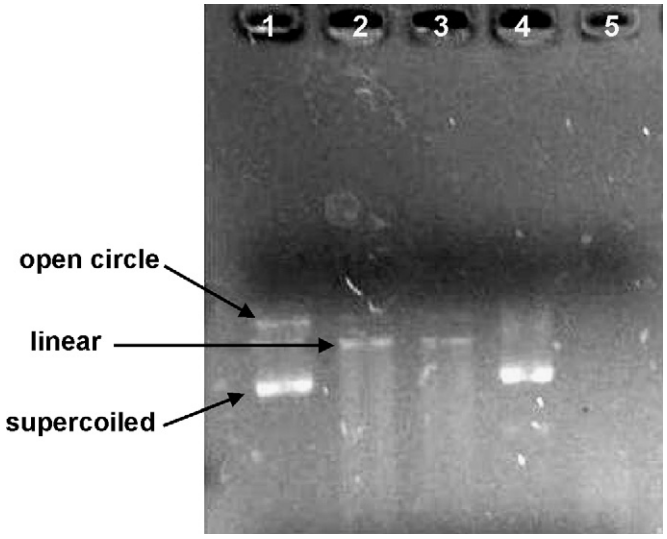

Fig. 6. Agarose gel electrophoresis for assessment of plasmid stabilization against DNase I degradation after the incubation for 30 min. Lane 1: untreated control DNA, lane 2: DNase I- treated 1% F68-NP, lane 3: DNase I-treated 1% F127-NP, lane 4: DNase I-treated 0.02% CP-NP, lane 5: DNase I-treated naked DNA.

circular form. When treated with DNase I, another intermediate band appeared, which equals to the linear form (lane 2 or 3). Naked plasmid DNA (lane 5) was completely digested within 30 min of incubation, confirming the activity of nucleases. In contrast, DNA in 0.02% CP-NP could be completely protected from the DNase I (lane 4), because of the supercoiled conformation was maintained and similar to control (lane 1). While in the case of 1% F68-NP or 1% F127-NP, plasmid DNA was mostly converted from the supercoiled into linear form (lane 2, lane 3), which meant minimal protection against nucleases degradation. It could be concluded that CP held advantage over F68 or F127 to protect DNA from enzymatic degradation. It might be explained by the stronger adhesion interaction between the CP-stabilized nanoparticles and DNA.

3.7. Structural integrity determination of plasmid DNA after release

Plasmid DNA released from PLGA nanoparticles was analyzed for structural integrity by agarose gel electrophoresis in comparison with control. In Fig. 7, it can be observed that the original plasmid DNA was predominantly supercoiled although a small amount of open circular plasmid DNA was visible. While, DNA released from 1% F68-NP or 1% F127-NP had almost transformed into liner and open circular conformation from supercoiled DNA (A and B). In contrast, the DNA released from 0.02% CP-NP maintained its integrity pattern (C), which was exactly the same as that of the control plasmid DNA. These results indicated that CP could protect the loaded plasmid from degradation during the release duration. This protective action might be explained by the high buffering ability of CP-NP. PLGA degradation governed by hydrolytic processes leads to acidic oligo and monomers and causes acidic microenvironment which was detrimental to the integrity of DNA. The use of CP could possibly prevent unwanted degradation of loaded DNA due to its intrinsic buffering ability.

3.8. Cytotoxicity evaluation

In vitro toxicity of different nanoparticles formulations was evaluated by MTT assay in A549 cells. The cytotoxicity of PLGA nanoparticles at various concentrations against A549 cell was shown in Fig. 8. Lipofactamine 2000, which is commonly used

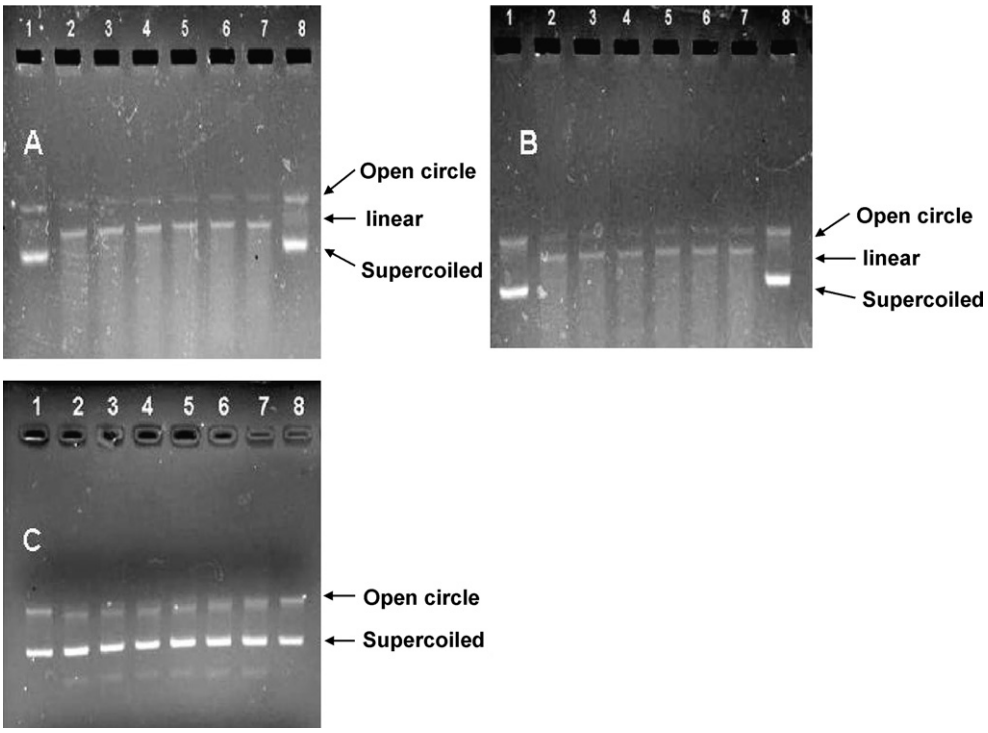

Fig. 7. Structural integrity of plasmid DNA released from 1% F68-NP (A), 1% F127-NP (B), 0.02% CP-NP (C) at selective time points evaluated by agarose gel electrophoresis. Lane 1, 8: DNA control; lanes 2–7, DNA released at 0.5, 1, 2, 3, 5 and 7 days.

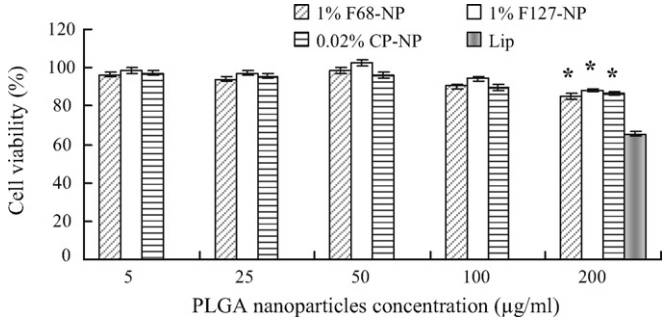

Fig. 8. Cell viability of PLGA nanoparticles of with different concentrations and Lipofectamine 2000 (Lip) against A549 cell line by MTT assay, 24 h post-treatment ($n = 3$). $^{*}P < 0.05$ compared with Lip.

in *in vitro* gene transfection, was evaluated in comparison with PLGA nanoparticles. As shown from Fig. 8, the A549 cell viabilities decreased slightly with the increased concentration of PLGA while the average cell viabilities of different formulations at the studied concentrations (5–200 μg/ml) were between 80 and 120% compared with control cells and no significant differences were observed ($P > 0.05$) among examined nanoparticles formulations at the same concentration. In addition, the cell viabilities of PLGA nanoparticles at the highest concentration (transfection doses, 200 μg/ml) were all significantly higher than that of Lipofectamine 2000 ($P < 0.05$), which indicated that the PLGA nanoparticles were safer than Lipofectamine 2000 to A549 cell. It might be attributed to the negative surface electrical potential of the nanoparticles used in our study, which could overcome the cytotoxicity of cationic non-viral vectors induced by excessive positive charge.

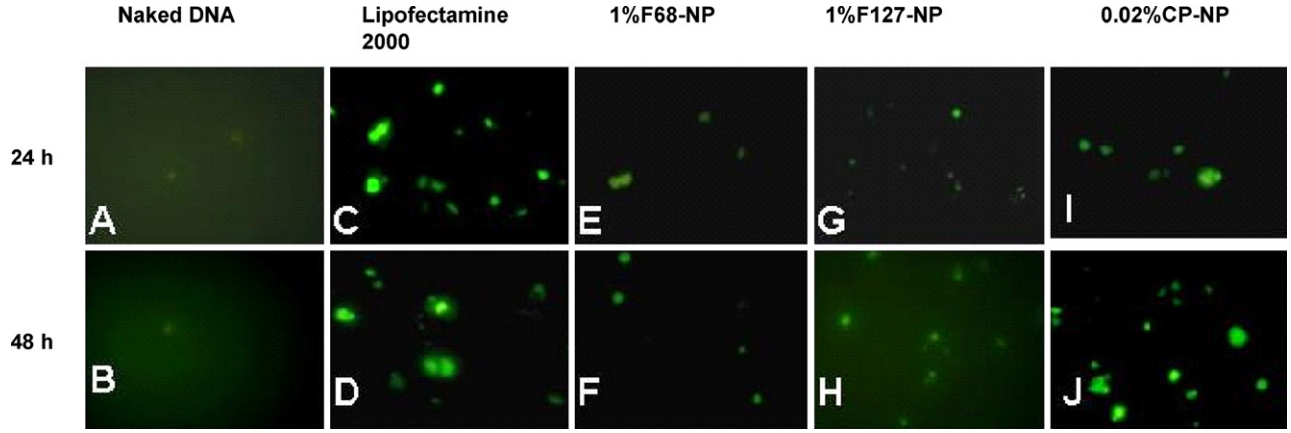

Fig. 9. Fluorescent micrograph of A549 cells transfected by plasmid EGFP with different carriers. Gene expression was examined after 24 and 48 h post-transfection, respectively.

3.9. 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 bioadhesive PLGA nanoparticles to transfer the reporter gene EGFP to lung cancer cell A549. The commercial cationic liposome based reagent, Lipofectamine 2000, well known to provide high transfection efficiency and high level of transgene expression in a range of mammalian cell types *in vitro* (Zhang et al., 2007) was chosen as positive control in our study. The pictures of inversion fluorescence microscope which presented the green fluorescence sent out by transfected A549 cells were shown in Fig. 9. It was shown that the naked DNA could barely transfected A549 cells. In comparison, all the selective gene carriers could realize the gene expression in A549 cells to some extent. Both 1% F68-NP and 1% F127-NP showed a relative lower transfection efficiency than Lipofectamine 2000 during 48 h. While in the case of 0.02% CP-NP, the transfection efficiency in 48 h was comparable to that of Lipofectamine 2000, although which was still lower in 24 h due to its sustained release property. That demonstrated that CP stabilized PLGA nanoparticles performed better in A549 cell transfection and it could be used as a potential delivery system for DNA in lung cancer gene therapy.

4. Conclusion

The current study has shown that a newly developed bioadhesive PLGA nanoparticles can be efficiently bind DNA onto its surface by non-ionic interaction and used for the production of PLGA-based particulate DNA carriers. The bioadhesive PLGA nanoparticles formulated with CP hold many advantages as gene delivery systems such as DNA-binding efficiency (>80%) at an optimal concentration, DNA protection from enzymatic degradation or DNA stability *in vitro* release, better buffering capacity, most importantly, a higher transfection efficiency in A549 cells than nanoparticles stabilized with F68 or F127. It could be concluded that CP stabilized bioadhesive PLGA nanoparticles can be used as promising gene delivery system but further optimization is necessary to exploit the full potential. Our future work will focus on the suitability and application of this gene carrier *in vivo* for lung cancer gene therapy.

Acknowledgements

The work was supported by the National Natural Science Foundation of China, no. 30572267. We thank Tao Wu (Shandong University) for the average diameter, polydispersity index and zeta potential measurements and Mr. Liu (Shandong University) for the transmission electron micrographs.

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