International Journal of Pharmaceutics 434 (2012) 437–443

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Polymerized spermine as a novel polycationic nucleic acid carrier system

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ARTICLE INFO

Article history:
Received 9 January 2012
Received in revised form 20 May 2012
Accepted 24 May 2012
Available online 7 June 2012

Keywords:
Polyspermine
Gene delivery
Cytotoxicity
Transfection
Gene silencing

ABSTRACT

Spermine, an endogenous amino-group bearing monomer that condenses DNA in sperm, was used as the basic building block to form polycationic nucleic acid carriers via condensation with one of three linker molecules – bischloroformate, succinyl chloride, and glyoxal. The three cationic polymers, polyspermine carbamate (PSP-Carb), polyspermine amide (PSP-Amide) and polyspermine imine (PSP-Imine) were examined for their degradability, cytotoxicity, ability to condense nucleic acids to nanoparticles, and ability to transfect genes or siRNA to cells. PSP-Carb and PSP-Amide exhibited a half-life of more than 2 months when incubated in aqueous buffers at 37 °C, while the half-life of PSP-Imine was 11 h. Relative cytotoxicity of the polymers, as measured by COS-7 and HepG2 cell viability, was in the order of PSP-Carb > PSP-Amide > PSP-Imine. Each cationic polymer condensed the luciferase plasmid to nanoparticles of 150–200 nm diameters and with a zeta potential of +15–30 mV when the mass ratio of polymer-to-DNA was over 8/1. The three polycationic carriers showed similar luciferase transfection activity in COS-7 cells, while the transfection efficiency of PSP-Carb was significantly higher than that of the other two in HepG2 cells. PSP-Amide exhibited significantly higher gene silencing activity in COS-7 cells, suggesting the linkage structures play an important role in the activity of the polyspermine-based nucleic acid carriers.

1. Introduction

The lack of safe and effective carrier systems is preventing the use of DNA and siRNA as therapeutic drugs (Fire et al., 1998; Pack et al., 2005). To overcome this hurdle, extensive research has been performed on synthetic delivery systems because they are easily produced and formulated, and exhibit great capacity to condense nucleic acids (Wolff, 2002). To deliver siRNA to the site of therapy, a synthetic carrier must accomplish five tasks: (1) pack and protect nucleic acids into nanoparticulates; (2) direct the formed particles to diseased cells; (3) facilitate endosomal escape of nucleic acids; (4) release nucleic acids into the cytoplasm; and (5) metabolize itself to nontoxic fragments (Wolff and Rozema, 2008). These tasks may be accomplished if a well-designed cationic polymer is used as a component of the synthetic nucleic acid carrier. In our effort to form such a polymer, we used spermine as the basic building block. Spermine is an endogenous multi-amino group-bearing monomer that condenses DNA in sperm. Three linker molecules, bischloroformate, succinyl chloride and glyoxal, were selected to condense spermine to polymers of different degradability. In recent years, biodegradable cationic polymers have been well reported as nucleic acid carriers of reduced cytotoxicity. In most cases, a linker containing a cleavable structure, such as an ester or a disulfide bond, was used to link amino group-bearing monomers to form a polymer through non-degradable amine bonds (Christensen et al., 2006; Lim et al., 1999; Lin et al., 2007; Putnam and Langer, 1999; Wang et al., 2001; Zhao et al., 2011; Zugates et al., 2006). These linking strategies are less meaningful when endogenous monomers are used, as the building blocks since the degradation of the resulting polymers do not produce the monomers in their original endogenous states. For example, spermine was used as the repeating unit to form degradable polycationic gene carriers via Michael addition with an ester or disulfide-bearing linker (Jere et al., 2009, 2007; Shim and Kwon, 2011). Upon degradation, however, the linkers themselves were cleaved, leaving their fragments bonded to spermine. Thus, the advantage of using endogenous molecules as building blocks for degradable polycationic nucleic acids carriers was nullified.

In this study, we used ethylene bis-(chloroformate), succinyl chloride, and glyoxal as the linkers to polymerize spermine via degradable biscarbamate, amide, and π–π conjugated carbon–nitrogen double bond linkages, respectively. Upon degradation, these linkage structures may release spermine in its original state, while the linkers themselves turn into glycol, oxalic acid, and glyoxal (which can be oxidized to oxalic acid in vivo), respectively. In addition, the spermine released by the linkage degradation possesses two free amino groups, which may enhance the so-called "proton-sponge" effect (Boussif et al., 1995) and facilitate endosomal rupturing. The objective of this study is to examine the effect

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http://dx.doi.org/10.1016/j.ijpharm.2012.05.065

of the linkage structures of the spermine-based cationic polymers on cytotoxicity and transfection efficiency, as well as shed a light on the rational design of synthetic nucleic acid carriers.

2. Materials and methods

2.1. Materials

Polyethylenimine with a 25 kDa molecular weight (PEI 25 kDa), spermine, ethyl trifluoracetate (99%), ethylenebis-(chloroformate) (98%), succinyl chloride, and glyoxal solution (40%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). MicroBCA protein assay kits were obtained from Pierce (Rockford, IL, USA). Plasmids containing the green fluorescent protein (GFP) and luciferase assay kits were supplied by Promega (Madison, WI, USA). The siRNA used, which consisted of 21 nucleotides (sense: 5′-CUUACGCUGAGUACUUCGAtt-3′) and endotoxin free plasmid purification kits were purchased from Qiagen (Hilden, German). All other solvents and reagents were analytical grade. Triethylamine and chloroform were purified by refluxing over CaH_2 for 96 h, followed by distillation before use. The plasmids used in this study were pGL3-Control (Promega, Madison, WI, USA), which coded for luciferase.

COS-7 and HepG2 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. DMEM was obtained from Shanghai DOBIO Biotech CO., LTD (Shanghai, China). The medium used for transfection contained DMEM supplemented with 10% FBS (PAA, EU), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were maintained at 37 °C in a humid atmosphere containing 5% CO_2.

2.2. Methods

2.2.1. Synthesis of polyspermine carbamate (PSP-Carb) and polyspermine amide (PSP-Amide)

Spermine (420 mg, 2.08 mmol) was dissolved in methanol (30 ml) at −78 °C under a dry nitrogen atmosphere via Schlenk technique. Ethyl trifluoracetate (0.46 ml, 4.16 mmol) was then added dropwise and the resulting solution was magnetically stirred at 0 °C for 1 h (Geall and Blagbrough, 2000). Protected spermine, N^1,N^{14}-bis (trifluoracetyl) spermine, was isolated from this solution by evaporating the solvent and unreacted trifluoracetate. Then the product was recrystallized in ethyl acetate with over 98% yield.

Ethylenebis-(chloroformate) (144.0 mg, 0.77 mmol) or succinyl chloride(119.0 mg, 0.77 mmol) dissolved in chloroform (12 ml) was added dropwise to N^1,N^{14}-bis (trifluoroacetyl) spermine (303.4 mg, 0.77 mmol) dissolved in chloroform (15 ml) and triethylamine (5 ml) at 0 °C under a dry nitrogen atmosphere. The resulting solution was then continuously stirred at room temperature for 12 h (Xu et al., 2008). After the solvents evaporated, the reaction products were treated with a mixture of 30% ammonium water (1.4 ml) and methanol (8 ml) at 60 °C in a sealed vessel for 8 h to remove the trifluoroacetate protecting groups (Garrett et al., 2000). The de-protected polyspermine biscarbamate (PSP-Carb) and bisamide (PSP-Amide) linkages were obtained after evaporating their respective liquid residues and dialyzed using a membrane with molecular weight cut-off (MWCO) of 3500 (Fig. 1).

2.2.2. Synthesis of polyspermine imine (PSP-Imine)

Spermine (490 mg, 2.43 mmol) was dissolved in 18 ml anhydrous ethanol over molecular sieve (4 Å) and then continuously stirred while 40% glyoxal solution (0.28 ml, 2.43 mmol) was added slowly at 0 °C under nitrogen. Afterwards, the mixture was brought to room temperature and stirred overnight. The filtrate was then evaporated in vacuo, and the resulting polymer was transferred to dialysis membranes (MWCO = 3500). It was dialyzed against doubly distilled water and subsequently lyophilized.

2.2.3. Structural characterizations

^{1}H NMR spectra of N^1,N^{14}-bis (trifluoroacetyl) spermine, PSP-Carb, and PSP-Amide were recorded on a Varian Mercury-400 spectrometer, using $CDCl_3$ as the deuterated solvents of N^1,N^{14}-bis (trifluoroacetyl) spermine and D_2O as the deuterated solvents of the other three samples, respectively. The FT-IR spectrum of each synthetic product was recorded on a Perkin Elmer Paragon 1000 spectrometer ranging from 400 to 4000 cm^{-1}. The weight-average molecular weight ($\bar{M}_w$) and number-average molecular weight ($\overline{M}_n$) of the polymers were determined using a gel permeation chromatograph (GPC, Waters Ultrahydrogel Series 120 and 250) with a refractive index detector at 30 °C. The elution phase was 1% formic acid solution (elution rate: 0.6 ml/min) and PEG was used as the calibration standard.

2.2.4. Hydrolytic degradation of polymers

To examine the rate of hydrolytic degradation, each polymer was dissolved in water to obtain a final concentration of 2.0 g/dl. The pH was adjusted to 5.0, 5.8 or 7.4 by adding the appropriate amount of HCl and then incubated at 37 °C. The change in M_W for each sample was measured using GPC at predetermined time points.

2.2.5. Preparation and characterization of polyplexes

Polyplexes were prepared by adding the polymers to a 20 μg/ml solution of plasmid DNA for either enhanced green fluorescent protein (EGFP) or luciferase. Polymer quantity was incrementally varied such that a range of polymer-to-DNA ratios was obtained. The resulting polyplex dispersions were incubated for 30 min at the room temperature. The preparation of nanoparticles with siRNA was accomplished in the same manner. The particle size and zeta potential of the resulting polyplexes were determined using a Zetasizer 3000 HS (Malvern, Worcestershire, UK). The morphology of the polyplexes was examined using an atomic force microscope (Nanoscope IIIa, Digital Instruments/Veeco, Inc., Santa Barbara, CA). Electrophoresis of each polymer–DNA mixture was completed using 1% agarose gel containing 0.1 μg/ml ethidium bromide in a TAE (Tris–acetate–EDTA) buffer.

2.2.6. Transfection efficiency and cytotoxicity assay

For transfection assays, COS-7 and HepG2 cells were each seeded in 48-well plates (5×10^4 cells per well) in DMEM supplemented with 10% FBS and incubated at 37 °C for 24 h prior to luciferase transfection assay. The polyplexes with varying polymer-to-DNA ratios were added to the cell cultures. After incubating for 4 h, the cells were lysed via 1× lysis buffer and subsequent H_2O wash. The total protein contents were assayed using a BCA protein assay kit. In addition, EGFP expression was carried out at 10/1 ratio of amino-group of the polymers to the phosphate group of the nucleic acids, and viewed under a fluorescent microscope (Olympus, Tokyo, Japan) after 48 h of incubation. Luciferase activity was determined by luminary intensity recorded (as RLU/mg) with a Sirius Luminometer (Berthold, Germany).

The cytotoxicity of the polymers was measured by the viability of COS-7 and HepG2 cells as a function of concentration of the polycationic carrier molecules. To determine cell viability using the MTT assay, the COS-7 and HepG2 cells were seeded in a 96-well plate and incubated at 37 °C for 24 h. The cells were then treated with the polymer solution of each planned concentration. A well-reported polycationic transfection reagent, PEI 25 kDa, was also used to treat the cells as a reference. After 4 h of subsequent incubation, the supernatant (containing carrier molecules) was replaced by fresh DMEM supplemented with 25 μl of MTT solution (5 mg/ml in PBS

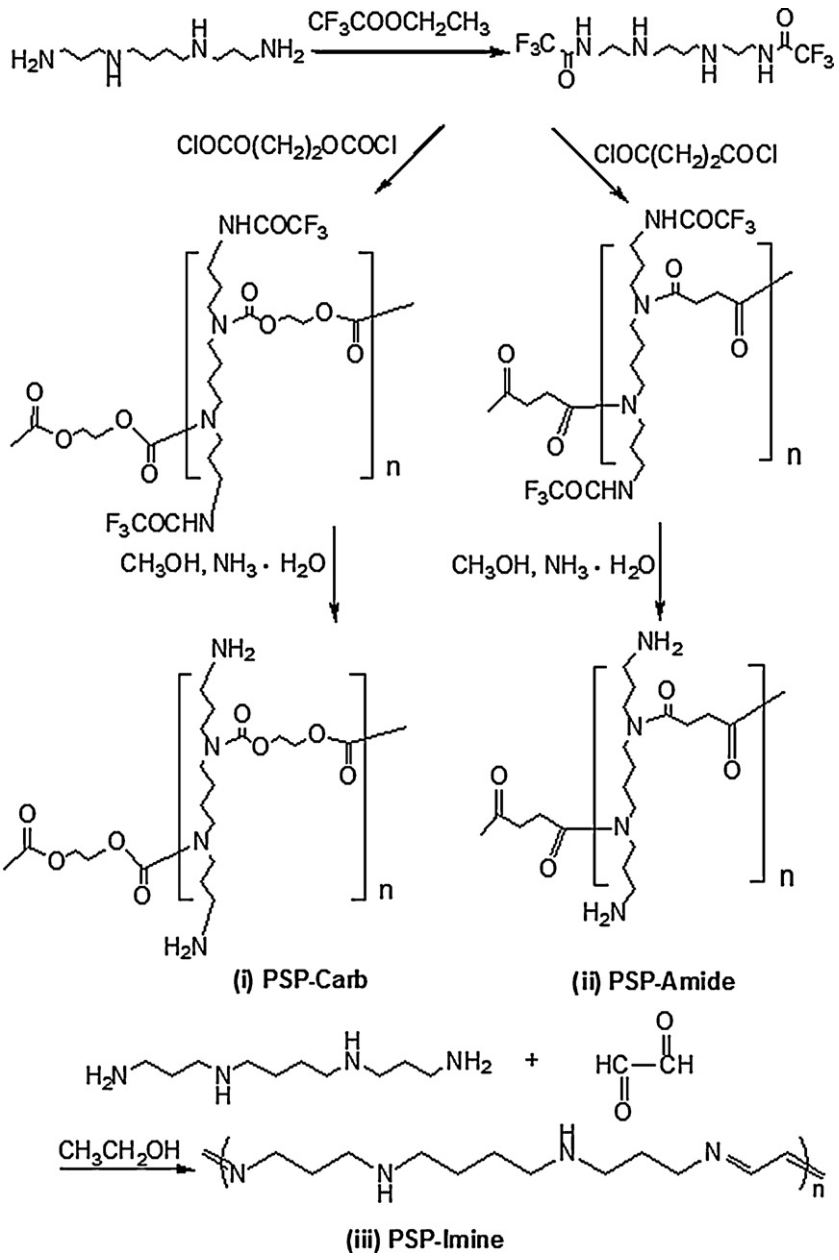

Fig. 1. Polymerization schemes of the three cationic polymers, (i) PSP-Carb; (ii) PSP-Amide and (iii) PSP-Imine.

buffer) in order to determine cell viability. Viable cells were determined by measuring absorbance of the samples at 570 nm (with 630 nm as the reference) using an ELISA reader (MK3 Thermo Labsystem, Finland). The percent cell viability was given by comparing the absorbance of each sample with the absorbance of cells not treated with carrier molecules.

2.2.7. Luciferase gene silencing by siRNA

COS-7 cells were seeded in a 48-well plate (5×10^4 cells per well) in DMEM containing 10% FBS and incubated at 37 °C for 24 h prior to gene transfection assay. The polyplex solution (50 μl) consisting of Lipofectamine 2000 and 300 ng luciferase plasmid at the mass ratio of 3 were added to the cell cultures. The cells were then incubated for 4 h at 37 °C and 5% CO_2. Silencing complexes of different mass ratios of our polycationic carriers to siRNA were prepared beforehand as described previously (Section 2.2.5). Following transfection by luciferase, the cells were incubated with the silencing complexes under normal cell growth conditions. Luciferase expression was measured 48 h after transfection. The media was changed as required.

2.3. Statistical analysis

Data were expressed as a mean ± SD. Statistical analysis was performed by unpaired *t*-test using IBM SPSS Statistics software. Differences were considered significant if $p < 0.05$.

3. Results and discussions

3.1. Characteristics of polymers

3.1.1. Structures of polymers

Because the four amino groups of spermine are reactive, its polymerization via ethylene bis-chloroformate or succinyl chloride linker molecules may result in a variety of products due to branching or intra-molecularly cross-linking. To achieve well-defined

Table 1
The weight-average molecular weight ($\overline{M}_w$), number-average molecular weight ($\overline{M}_n$), and molecular weight distribution ($\overline{M}_w/\overline{M}_n$) of the three polymers.

M_w	M_n	PDI	M_w/M_n
PSP-Carbam	5525	4135	1.33
PSP-Amide	6065	3158	1.92
PSP-Imine	4101	3417	1.20

linear polymers, the two primary amines of spermine were treated with ethyl trifluoroacetate, resulting in the protected spermine form, N^1,N^{14}-bis (trifluoroacetyl) spermine. This was confirmed by ^{1}H NMR and the FTIR spectra (Supplementary data, Figs. S1a and S1b, respectively). This protection allowed us to avoid intramolecular cross-linking and to obtain linear polymers, as confirmed by ^{1}H NMR and FTIR (Supplementary data, Figs. S2 and S3, respectively).

3.1.2. Molecular weight and hydrolytic degradation of spermine-backbone polymers

$\overline{M}_w$ and $\overline{M}_n$ of each polyspermine, as measured by GPC with PEG as the standard is summarized in Table 1. PSP-Carb showed the least polydispersity calculated from the values of $\overline{M}_w$ and $\overline{M}_n$ as 5525 and 4135, respectively. PSP-Amide topped the polydispersity value based on its $\overline{M}_w$ and $\overline{M}_n$ of 6065 and 3158, respectively. The polydispersity of PSP-Imine fell in between the other two, as its $\overline{M}_w$ and $\overline{M}_n$ were 4101 and 3407, respectively. Due to the poor structural similarity between the polyspermines and the polyethylene glycol (PEG) standard, the absolute values of molecular weights of the polymers could not be determined. Moreover, these spermine-based polymers are polyelectrolytes, which may interact with column packing materials and extend retention volume (Kim et al., 2005). To address this possible effect, a commercially available polyelectrolyte, PEI 25 kDa, was also used as a reference in GPC measurement (Petersen et al., 2002). Based on its use, the relative retention volume and the polydispersity calculated on the basis of relative $\overline{M}_w$ and $\overline{M}_n$ were valid.

Degradability of the three polyspermines was examined by incubating the polymers at 37 °C in aqueous buffers simulating the pH of various cellular environments (intercellular: 7.4, endosome: 5.8, and lysosome: 5.0). The changes in $\overline{M}_w$ of the polymers over time at these three pH levels are shown in Fig. 2(a). $\overline{M}_w$ of PSP-Carb was nearly unchanged when it was incubated in an aqueous buffer of pH 7.4 for 85 days. When incubated at pH 5.8 and 5.0, $\overline{M}_w$ of PSP-Carb dropped to 74.1% and 69.8%, respectively, of its original value in 85 days, suggesting a pH-responsive degradability of the carbamate linkage. PSP-Amide showed higher degradability under the same conditions in that $\overline{M}_w$ of which dropped to 66.3%, 59.6%, and 58.3% of the original $\overline{M}_w$ values over 85 days incubation at pH 7.4, 5.8 and 5.0, respectively (Fig. 2(b)). PSP-Imine degraded remarkably faster than the other two polymers. By incubating at 37 °C and pH 7.4, 5.8 and 5.0 for only 11 h, the $\overline{M}_w$ of PSP-Imine dropped to 57%, 48% and 42% of its original value, respectively. The rate of degradation of PSP-Carb, PSP-Amide and PSP-Imine accelerated with the dropping of pH.

3.2. Formation of polyplexes

Polyplex formation, as indicated by complete retardation of luciferase plasmid migration during electrophoresis, was accomplished by adding a polymer solution into a nucleic acid solution to reach an amino-group-to-phosphate ratio over 7. This ratio was equivalent to the polymer-to-DNA mass ratio of 3/1 for PSP-Carb and PSP-Amide and 2/1 for PSP-Imine (Fig. 3). This result is consistent with the AFM images of dried spherical polyplex particles, 60–100 nm in diameter (Fig. 4). Similarly, the diameters of the medium-suspended polyplexes measured by dynamic laser scattering ranged between 150 and 200 nm (Fig. 5(a)). Zeta potentials of the three types of polyplex particles varied from negative

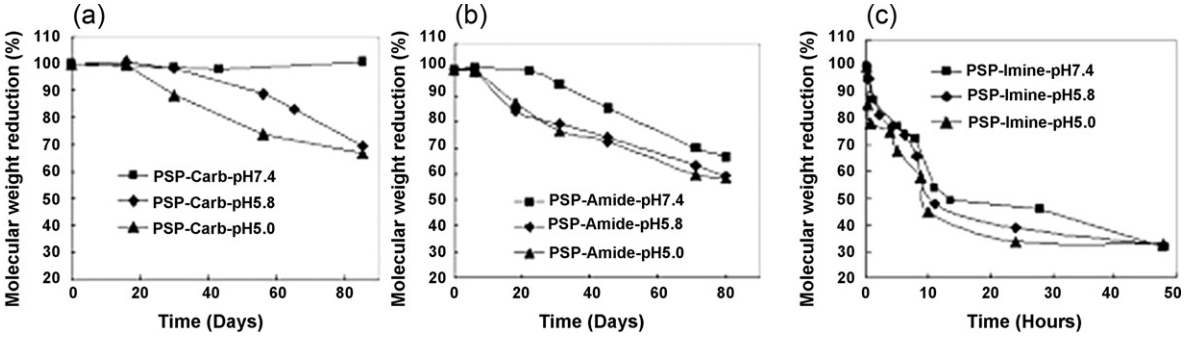

Fig. 2. Degradation of (a) PSP-Carb; (b) PSP-Amide and (c) PSP-Imine at different pH levels and 37 °C.

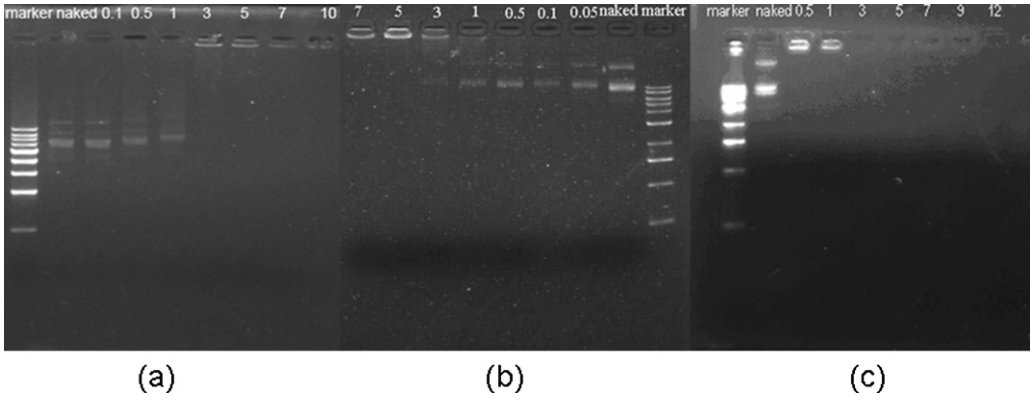

Fig. 3. Electrophoretic mobility in 1% agarose gel: (a) PSP-Carb; (b) PSP-Amide; (c) PSP-Imine with luciferase plasmids at 0.5, 1, 3, 5, and 7 polymer-to-DNA mass ratios.

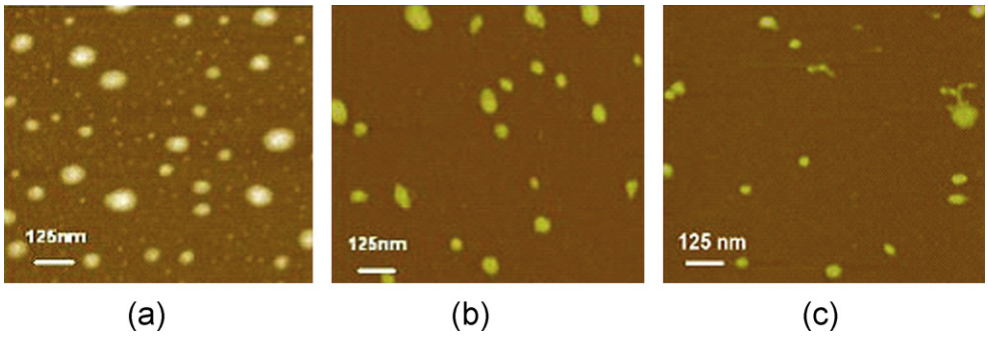

Fig. 4. Atomic force microscopic image of polyplexes composed of (a) PSP-Carb; (b) PSP-Amide and (c) PSP-Imine with DNA plasmids at the mass ratio of 3/1.

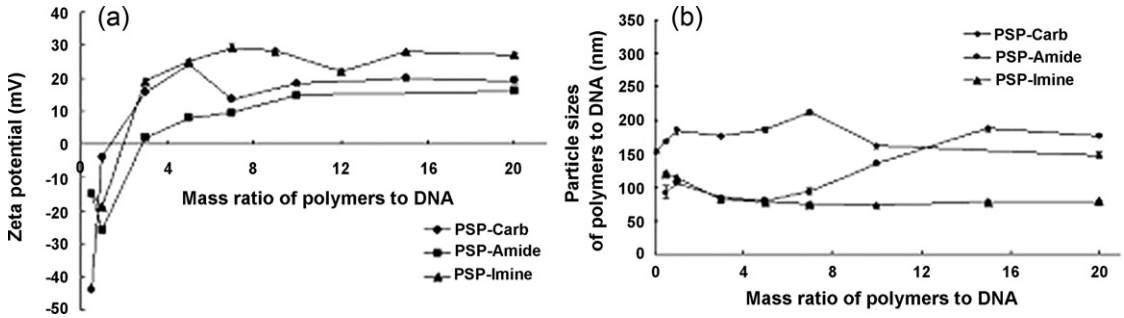

Fig. 5. (a) Zeta potential distribution and (b) particle size distribution of polyplexes composed of polymers and DNA plasmids at various polymer-to-DNA mass ratios.

(−44 to −25 mV) to positive (+15 to +30 mV) as the polymer-to-DNA ratio was increased from 0.5/1 to 20/1 (Fig. 5(b)).

The above results not only demonstrated the capability of the three spermine-based cationic polymers to pack nucleic acids into nanoparticles (including siRNA as shown in Supplementary data, Figs. S5 and S6), but also resolved, although partially, our concern regarding the instability of PSP-Imine. The polymer and its polyplexes remained sufficiently stable in water during the duration of our polyplex formation studies.

3.3. Cytotoxicity of spermine-based cationic polymers

As summarized in Fig. 6, cell viability dropped as a function of polymer concentration at different rates for our spermine-based cationic polymers. When PEI 25 kDa was added, the viability of COS-7 cells dropped immediately to 80% even though its concentration was only 1 μg/ml (Fig. 6(a)). The cell viability dropped gradually as polymer concentration was increased, ultimately reaching 20% of the original when PEI 25 kDa concentration was increased to 200 μg/ml. For PSP-Carb, viability of COS-7 cells began to decrease when the polymer concentration was exceeded 15 μg/ml, ultimately reaching 40% cell viability when the polymer concentration was 200 μg/ml. PSP-Amide showed less cytotoxicity than PSP-Carb; the viability of COS-7 cells remained at 80% when PSP-Amide concentration was 30 μg/ml (Fig. 6(a)). PSP-Imine exhibited the least cytotoxicity; the viability of COS-7 cells started to decline when the polymer concentration was exceeded 50 μg/ml and there still remained more than 50% viable cells when the polymer concentration reached 200 μg/ml (Fig. 6(a)). HepG-2 cells showed the same trend as COS-7 cells in the response to addition of the cationic polymers (Fig. 6(b)). The rates of cell viability declining as the function of polymer concentration were in the order of PEI 25 kDa, PSP-Carb, PSP-Amide and PSP-Imine. The cells treated with degradable PSP-Imine were remarkably viable (Fig. 6). These results indicate that cytotoxicity of the tested cationic polymers may be related to their amino-group density, molecular weight and, more significantly, their degradability.

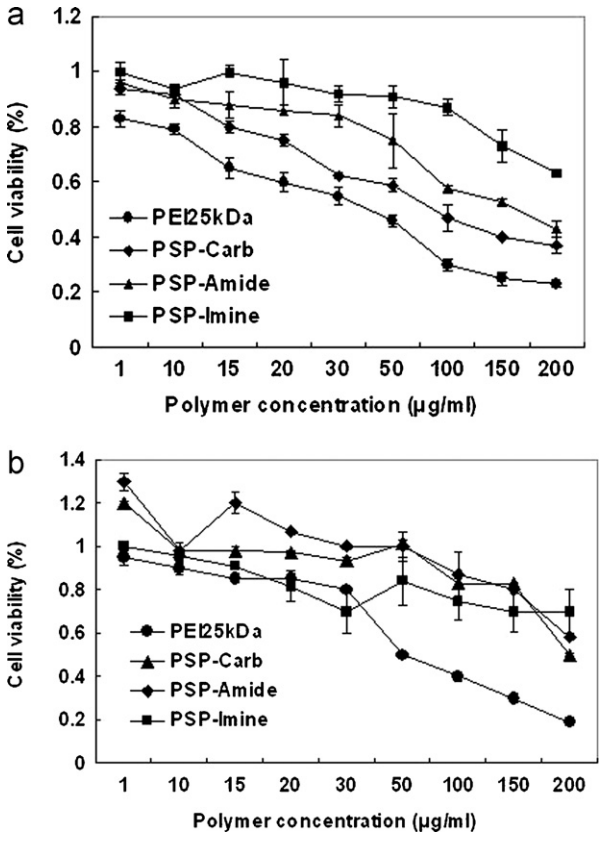

Fig. 6. Cytotoxicity of the three cationic polymers at various concentrations to (a) COS-7 and (b) HepG2 cells. Each bar represents the mean ± SD of three experiments.

3.4. In vitro transfection and silencing efficiency

As indicated in Fig. 7, all the cationic polymers were effective in delivering EGFP genes to cells. Quantitative comparison of the

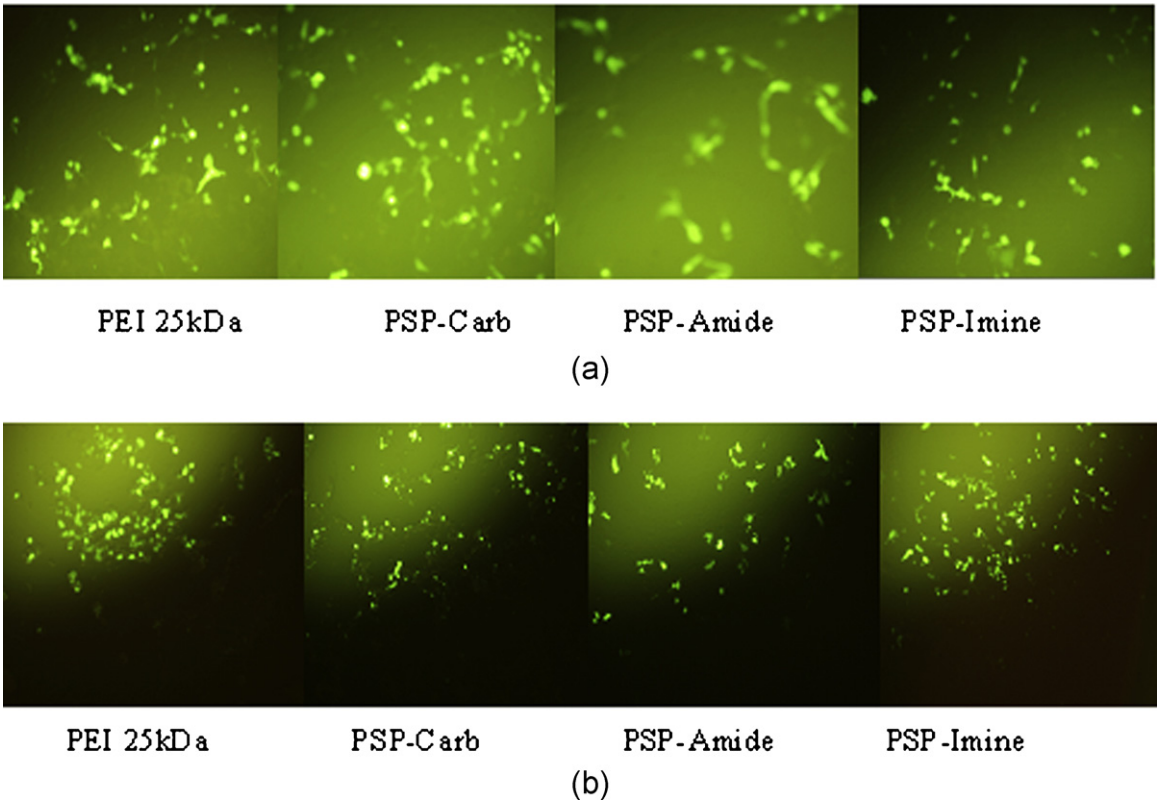

Fig. 7. Enhanced green fluorescent protein expression resulting from 48 h of (a) COS-7 and (b) HepG2 transfection via gene carriers at 10/1 ratio of amino-group of the polymers to the phosphate group of the nucleic acids.

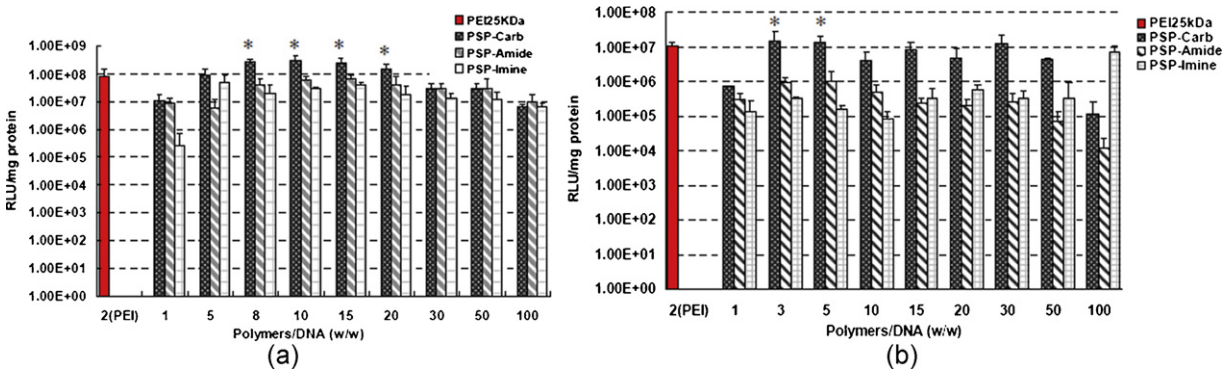

Fig. 8. Luciferase expression resulting from 48 h of (a) COS-7 and (b) HepG2 transfection via gene carriers at different polymer-to-DNA mass ratios. Each bar represents the mean ± SD of three experiments. $^{*,\#}p < 0.01$ vs PEI25 kDa.

delivery efficiency of the polymers was done using their activity in transfecting luciferase to the same cell lines, measured by the relative luminescence light units per mg total protein. For COS-7 cells, each spermine-based cationic polymer showed comparable efficiency to PEI 25 kDa within their optimized polymer-to-gene ratios of 8/1–15/1 (Fig. 8(a)).

For Hep2 cells, however, the transfection efficiency of PSP-Amide and PSP-Imine was over one order of magnitude lower than that of PEI 25 kDa and PSP-Carb (Fig. 8(b)). Surprisingly, despite the similar structure, molecular weight, and amino-group density, PSP-Carb and PSP-Amide were considerably different in luciferase gene transfection in Hep2 cells. The only difference between the two polymers was the linkage structures.

Moreover, the difference in luciferase transfection activity between PSP-Carb and PSP-Amide was reversed in the assay of luciferase gene silencing in COS-7 cells. As shown in Fig. 9, the efficiency of PSP-Amide-packed siRNA was remarkably higher than the other two spermine-based polymers; it induced close to 80% of luciferase knockdown at the mass ratio of 1 of PSP-Amide/siRNA. This luciferase inhibition by PSP-Amide was remarkable at higher polymer-to-siRNA ratios, exhibiting up to 90% of luciferase knockdown at the mass ratio of 10 of PSP-Amid/siRNA. As the polymer-to-siRNA ratio increased to 30/1, the activity of PSP-Imine caught up. The luciferase gene was mostly silenced for all the three polymers when the polymer-to-siRNA ratio was further increased to 50/1 (Fig. 9). This polymer-to-siRNA ratio was equivalent to the 15 μg/ml polymer concentration in the cytotoxicity assay in COS-7 cells (Fig. 6(a)). Thus, the reduced gene expression associated with PSP-Carb may be partially due to its cytotoxicity. For PSP-Imine, however, the cytotoxicity effect on gene silencing was likely minimal since it exhibited low toxicity at that concentration (Fig. 6(a)).

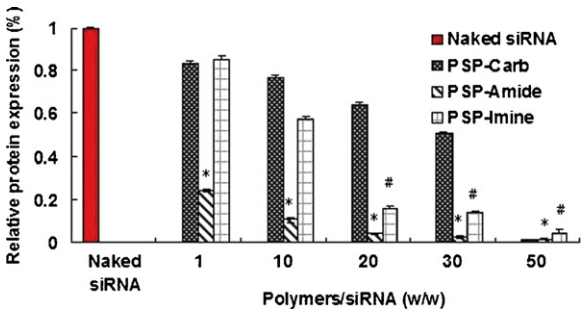

Fig. 9. Knockdown of luciferase expression in COS-7 cells after 48 h. Each bar represents the mean ± SD of three experiments. $^{*,\#}p < 0.01$ vs naked siRNA.

3.5. Discussion on structure-related behavior of spermine-based cationic polymers

While the three spermine-based cationic polymers possess similar molecular weight and charge density (Table 1), they behaved differently in cytotoxicity, gene expression, and gene silencing (Figs. 6, 8 and 9, respectively). In terms of cytotoxicity, PSP-Carb and PSP-Amide showed similar effects on cell viability, being less toxic than PEI 25 kDa and more toxic than PSP-Imine. Their lower charge density may make them less toxic than PEI 25 kDa, while their lower degradability may be why they are more toxic than PSP-Imine.

What is interesting but difficult to explain is their reversed behavior in luciferase gene transfection and silencing. These two spermine-based cationic polymers not only possess similar molecular weight, amino group density, and cytotoxicity, but also a similar structure, as well. They are both polymerized through the two secondary amino groups of spermine, leaving the two primary amino groups free. The difference in their degradability should not affect their transfection activity because no degradation was observed within the time frame of the cellular assays. The only structural difference between the two polymers is the linker molecule and the linkage structure (carbamate vs amide). It, therefore, seems that their observed reversed behavior in gene expression and silencing may be due to their linkage difference. Although a considerable number of examples may be required to figure out a predictive pattern between linkage structure and transfection activity of the spermine-based polycationic nucleic acid carriers, we have observed that selecting the linkage structure is a useful approach in designing cationic polymer nucleic acid carriers.

4. Conclusions

Spermine, an endogenous gene packaging monomer, may be used as a basic building block to form effective nucleic acid carriers. The type of linker molecules and linkage structures used for polymerization may be a significant design aspect in developing cationic polymers efficient in transfection and less toxic to cellular process.

Acknowledgments

This work was financially supported by the National Science Foundation of China (Grant No. 30901881), the Nanomedicine Grant of Shanghai Science and Technology Committee (1052nm03900), the Foundation of China Scholarship Council (2010831126), and BioPharm Solutions, Inc. The authors thank the Analytical Center of Shanghai Jiaotong University for AFM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.05.065.

References

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Christensen, L.V., Chang, C.W., Kim, W.J., Kim, S.W., Zhong, Z., Lin, C., Engbersen, J.F., Feijen, J., 2006. Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery. Bioconjug. Chem. 17, 1233–1240.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Garrett, S.W., Davies, O.R., Milroy, D.A., Wood, P.J., Pouton, C.W., Threadgill, M.D., 2000. Synthesis and characterisation of polyamine-poly(ethylene glycol) constructs for DNA binding and gene delivery. Bioorg. Med. Chem. 8, 1779–1797.

Geall, A.J., Blagbrough, I.S., 2000. Homologation of polyamines in the rapid synthesis of lipospermine conjugates and related lipoplexes. Tetrahedron 56, 2449–2460.

Jere, D., Kim, T.H., Arote, R., Jiang, H.L., Cho, M.H., Nah, J.W., Cho, C.S., 2007. A poly(beta-amino ester) of spermine and poly(ethylene glycol) diacrylate as a gene carrier. Key Eng. Mater. 342–343, 425–428.

Jere, D., Kim, J.E., Arote, R., Jiang, H.L., Kim, Y.K., Choi, Y.J., Yun, C.H., Cho, M.H., Cho, C.S., 2009. Akt1 silencing efficiencies in lung cancer cells by sh/si/ssiRNA transfection using a reductable polyspermine carrier. Biomaterials 30, 1635–1647.

Kim, Y.H., Park, J.H., Lee, M., Kim, Y.H., Park, T.G., Kim, S.W., 2005. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J. Control. Release 103, 209–219.

Lim, Y.B., Choi, Y.H., Park, J.S., 1999. A self-destroying polycationic polymer: biodegradable poly(4-hydroxy-L-proline ester). J. Am. Chem. Soc. 121, 5633–5639.

Lin, C., Zhong, Z., Lok, M.C., Jiang, X., Hennink, W.E., Feijen, J., Engbersen, J.F., 2007. Novel bioreducible poly(amido amine)s for highly efficient gene delivery. Bioconjug. Chem. 18, 138–145.

Pack, D.W., Hoffman, A.S., Pun, S., Stayton, P.S., 2005. Design and development of polymers for gene delivery. Nat. Rev. Drug Discov. 4, 581–593.

Petersen, H., Merdan, T., Kunath, K., Fischer, D., Kissel, T., 2002. Poly(ethylenimine-co-L-lactamide-co-succinamide): a biodegradable polyethylenimine derivative with an advantageous pH-dependent hydrolytic degradation for gene delivery. Bioconjug. Chem. 13, 812–821.

Putnam, D., Langer, R., 1999. Poly(4-hydroxy-L-proline ester): low-temperature polycondensation and plasmid DNA complexation. Macromolecules 32, 3658–3662.

Shim, M.S., Kwon, Y.J., 2011. Dual mode polyspermine with tunable degradability for plasmid DNA and siRNA delivery. Biomaterials 32, 4009–4020.

Wang, J., Mao, H.Q., Leong, K.W., 2001. A novel biodegradable gene carrier based on polyphosphoester. J. Am. Chem. Soc. 123, 9480–9481.

Wolff, J.A., 2002. The grand problem of synthetic delivery. Nat. Biotechnol. 20, 768–769.

Wolff, J.A., Rozema, D.B., 2008. Breaking the bonds: non-viral vectors become chemically dynamic. Mol. Ther. 16, 8–15.

Xu, S.L., Chen, M.Y., Yao, Y., Zhang, Z.Q., Jin, T., Huang, Y., Zhu, H., 2008. Novel poly(ethylene imine) biscarbamate conjugate as an efficient and nontoxic gene delivery system. J. Control. Release 130, 64–68.

Zhao, N., Roesler, S., Kissel, T., 2011. Synthesis of a new potential biodegradable disulfide containing poly(ethylene imine)–poly(ethylene glycol) copolymer cross-linked with click cluster for gene delivery. Int. J. Pharm. 411, 197–205.

Zugates, G.T., Anderson, D.G., Little, S.R., Lawhorn, I.E.B., Langer, R., 2006. Synthesis of poly(beta-amino ester)s with thiol-reactive side chains for DNA delivery. J. Am. Chem. Soc. 128, 12726–12734.