International Journal of Pharmaceutics 505 (2016) 24–34

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Highly stable polyglutamate derivatives/siRNA polyplex efficiently down-relegate survivin expression and augment the efficacy of cisplatin

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ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 28 March 2016
Accepted 30 March 2016
Available online 31 March 2016

Keywords:
siRNA delivery
Survivin
Stability
Cisplatin

ABSTRACT

RNA interfere (RNAi)-based technology holds great promise in cancer treatment. The use of small interfering RNA (siRNA), however, is hampered by its low delivery efficiency *in vivo* when they are diluted in blood biofluids and in the presence of serum and salt. In this study, we developed the polyglutamate derivative polymer brush, poly(ethyleneglycol) monomethyl ether-b-polyglutamate-g-spermine (mPEG-b-PG-g-spermine, PPGS), which could efficiently deliver survivin-siRNA under ultra-high dilution and in the presence of salt (NaCl 150 mM) and serum (10% FBS), most likely due to its PEG-shelled polymer brush structure. On the contrary, aggregation occurred when PEI/siRNA polyplex dispersed in saline and serum-containing media and PEI polyplex dissociated after making a 256-fold dilution. PPGS/si-survivin polyplex exhibited high cellular uptake efficiency and efficiently down-regulated the expression of survivin mRNA in the cisplatin-resistance of non-small cell human lung adenocarcinoma (A549/DDP) cells in the presence of serum. However, either PEI polyplex or Lipofectmine 2000 complex was unstable in serum and salt-containing media and at high dilution rates, which resulted in their dramatical decrease of cellular uptake and gene-silencing efficiency in these conditions. The PPGS/si-survivin polyplex also exhibited synergistic effects of killing the cancer cells by combination treatment with cisplatin. Therefore, the PPGS gene carrier showed great potential in systemic siRNA delivery, and its combination with chemotherapeutic drug is promising in treating drug resistant cancers.

1. Introduction

Lung cancer continues to be the leading cause of cancer-related death in both men and women (Spiro et al., 2010). The most common type of lung cancer is non-small cell lung cancer representing more than 80% of lung cancer diagnoses (Walker, 2008). Five year survival rates for lung cancer are poor when compared to other high incidence cancers. Cisplatin (DDP) is one of the first-line chemotherapies in non-small cell lung cancer, however, the nephrotoxicity of DDP limit its maximum tolerated dose and clinical application (Yao et al., 2007). Therefore, the current treatments urgently need to be improved.

Combination of gene therapy and chemotherapy can reduce the dosage of drugs and improve their therapeutic efficacy, thus, hold substantial promise for the treatment of cancer (He et al., 2015; Lin et al., 2003; Tang et al., 2015). In recent years, gene therapy such as using small interfering RNA (siRNA) to down regulation target gene in body have achieved successful advances in some clinical trials (de Fougerolles et al., 2007). Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is an apoptosis inhibitor and cell-cycle regulator. Survivin is highly expressed in a variety of human malignancies including non-small cell lung cancer cells A549, but lacks expression in differentiated adult tissues. This characteristic enables it as a potential new target for cancer treatments (Falleni et al., 2003).

Gene therapy such as RNA interfere (RNAi) requires efficient carriers to delivery siRNA to the target site. Over the past decade, cationic gene carriers have been widely investigated and achieved efficient gene transfer into cell (Huang et al., 2014; Kullberg et al., 2013). However, results based on non-viral gene delivery of gene

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http://dx.doi.org/10.1016/j.ijpharm.2016.03.062
0378-5173/

therapy clinical trials revealed there is a large gap between clinical and preclinical studies (Ginn et al., 2013). There are many reasons for this problem. Firstly, the dilution stability of siRNA/polycation polyplex is low. SiRNA/polycation polyplex is commonly prepared through electrostatic interactions between negatively charged siRNA and positively charged polycation. The self-assembled polyplex tends to loosen and further disassociate at high dilution. In our early work, we observed the particle size of DNA/PEI 25KDa polyplex significantly increased at a 16-fold dilution (Huang et al., 2014). The disassembly of polyplex weakens carriers' ability to protect siRNA, and thus, the therapeutic effect of siRNA will be compromised. Although the dilution stability of siRNA/polycation polyplex is often neglected because the dilution ratios is low in transfection experiments *in vitro*, it is a prerequisite for application *in vivo*. Once polyplex enter the systemic circulation, they will encounter the problem of ultra-high dilutons. Therefore, the dilution stability is necessary for siRNA/polycation polyplex to achieve high delivery efficiency in clinical trials.

Another important reason that leads to low efficiency of gene transfer *in vivo* is the low stability of polyplex in the presence of serum and ion. Negatively charged plasma proteins are nonspecific adsorbed by polycation, which induce aggregates and disassociation of siRNA/polycation polyplex (Li et al., 1999; Zelphati et al., 1998). The polyplex also tend to aggregate and coalesce in the presence of various ions contained in biological fluids, and consequently, the low stability of polyplex affects strongly the biodistribution, cellular uptake and cytoplasmic release of the siRNA (Mahato et al., 1997). Therefore, a highly stable gene carrier in physiological environments is the important prerequisite for efficient gene delivery *in vivo*, but it remains a challenge.

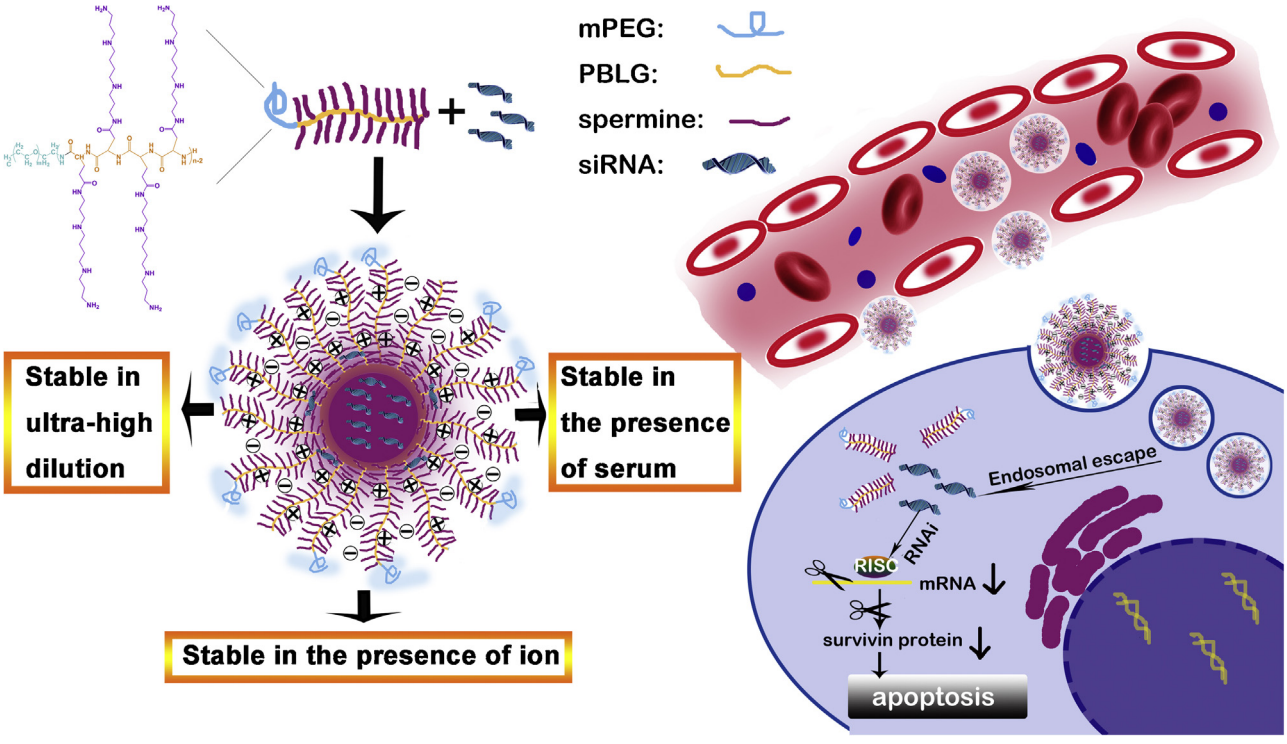
Polymer brush with unique properties including highly colloidal stabilization and tunable surface properties have attracted an increasing interest in recent years (Ballauff and Borisov, 2006; Kobayashi et al., 2012; Kou et al., 2015). It is consists in densely grafting chains to planar substrate or to strongly curve surfaces of colloidal particles. The distance between grafting points is smaller than the chain end-to-end distance. The limited space leads to a strong extension of the grafting chains, leading to high osmotic pressure in polymer brush. The high osmotic pressure of polymer brush spontaneously expels proteins and ions in media (Blattler et al., 2006; Nowak et al., 2003). Surface modification with poly(ethyleneglycol) (PEG) to form dense PEG brush adlayers is a well-known strategy for rendering surfaces protein resistant (Du et al., 1997; Hansson et al., 2005; Zhang et al., 2002). The polymer brush technique offers exciting strategies to significantly improve colloidal stability of polyplex in biological fluids for meeting the requirements for successful *in vivo* applications.

In the present study, we aimed to develop the polyglutamate derivative polymer brush, poly(ethyleneglycol) monomethyl ether-b-polyglutamate-g-spermine (mPEG-b-PG-g-spermine, PPGS) as an efficient and stable gene delivery carrier (Scheme 1). The colloidal stability of PPGS/siRNA polyplex in ultra-high dilution and in the presence of saline (NaCl 150 mM) and serum was intensively investigated. Whether the PPGS could efficiently deliver survivin-siRNA in the presence of serum to down-regulate the expression of survivin mRNA and improve the anti-tumor efficiency of DDP on the cisplatin-resistance of non-small cell human lung adenocarcinoma (A549/DDP) cells has been evaluated.

2. Materials and methods

2.1. Materials

PEG-monomethyl ether (mPEG) 2k, triphosgene, spermine and cisplatin (DDP) were purchased from Aladdin Chemistry Co., Ltd. Glutamic acid was obtained from Sinopharm Chemical Reagent Co. Branched polyethylenimine (PEI 25KDa), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and 4′,



Scheme 1. Schematic illustrations of the formation of PPGS/siRNA polymer brush and their endocytosis by A549/DDP cells.

6-Diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma-Aldrich. RPMI-1640 medium, fetal bovine serum (FBS) and trypsin were obtained from Gibco. Lipofectamine2000 and TRIzol reagent were purchased from Invitrogen. Annexin V-FITC apoptosis detection kit was purchased from shanghai BestBio biology co. Reverse Transcription System and Go Taq qPCR Mastermix were purchased from Promega (Madison, WI, USA).

The siRNA targeting survivin mRNA was synthesized by Ribobio Co. FAM-labeled siRNA and negative control siRNA were purchased from Gene PharmCo. Ltd. Survivin and GAPDH primer were synthesized by Invitrogen. The sequences are as follows: si-survivin: sense: 5′-UCCGGUUGCGCUUUCCUUUdTdT-3′; antisense: 5′-AAAGGAAAGCGCAACCGGAdTdT-3′. Negative control siRNA: sense: 5′ -UUCUCCGAACGUGUCACGUTT-3′; antisense: 5′-ACGUGACACGUUCGGAGAATT-3′. Survivin primer: forward: 5′- CAACCGGACGAATGCTTTT-3′; reverse: 5′-AAGAACTGGCCCTTCTTGGA-3′. GAPDH primer: forward: 5′-GAACGGGAAGCTCACTGG-3′; reverse: 5′-GCCTGCTTCACCACCTTCT-3′.

2.2. Synthesis of mPEG-b-PG-g-spermine

Polyglutamate derivative polymer brush, mPEG-b-PG-g-spermine (PPGS), was synthesized as follows. Firstly, the synthesis of poly(ethyleneglycol) monomethyl ether-b-poly(benzyl-glutamate)(mPEG-b-PBLG) was according to a similar procedure (Deng et al., 2004; Tian et al., 2005; Wen et al., 2009). In brief, γ-benzyl-L-glutamic (BLG) was synthesized through esterification reaction of L-glutamic acid and benzyl alcohol. Then 10 g BLG was dissolved in 80 ml anhydrous tetrahydrofuran, and 6 g triphosgene was added to the reaction mixture. After stirring at 60 °C for 40 min the reaction solution was precipitated using cold petroleum ether and γ-benzyl-L-glutamic-*N*-carboxyanhydride (BLG-NCA) was obtained. Secondly, mPEG-b-PBLG was prepared by ring opening polymerization of BLG-NCA in anhydrous chloroform using PEG-monomethyl ether 2k as a macromolecule initiator with the molar ratio of Monomer to Initiator = 100:1. Thirdly, 1 g mPEG-b-PBLG was dissolved in dimethylformamide (DMF) (w/v 1/20). Spermine (10-fold mol of the ester groups of mPEG-b-PBLG) and 2-hydroxypyridine (5-fold mol of the ester groups of mPEG-b-PBLG) were added to the reaction mixture, and then the solution was stirred at 40 °C for 48 h. Finally, PPGS was obstained by precipitation in cold diethyl ether, followed by dialyzed for 48 h in 6–8 kD MWCO dialysis bag to remove unreacted spermine. The final product PPGS was lyophilized and characterized by ^{1}H NMR.

2.3. Preparation of PPGS/siRNA polyplex

PPGS polymer brush/siRNA polyplex was prepared by adding 20 μM of siRNA in DEPC-treated water to equal volumes of PPGS by gentle vortexing. The working concentration of siRNA was 100 nM, and the amounts of PPGS based on the molar ratio of amino groups of PPGS to phosphate groups of siRNA (N/P). The mixture was incubated for 30 min at room temperature to form PPGS/siRNA polyplex.

2.4. Particle size of PPGS/siRNA polyplex in ultra-high dilution, saline and in the presence of serum

PPGS/siRNA polyplex was prepared as described above with the N/P ratio of 40:1. To evaluate the particle size of the PPGS/siRNA polyplex in the presence of salt and serum, media containing series concentration of NaCl and 10% final concentration of serum were chosen as model dispersion media. The PPGS/siRNA polyplex dispersion were balanced in room temperature for 10 min, then their particle size was measured by Malvern Zetasizer NS90 (Malvern Instruments, U.K.). To evaluate the changes in particle size of the PPGS/siRNA polyplex when it was diluted, serial dilutions of polyplex were carried out and polyplex dispersion were balanced in room temperature for 10 min, then their particle size was measured by Malvern Zetasizer NS90 (Malvern Instruments, U.K.). PEI 25KDa/siRNA polyplex at the N/P ratio of 10/1 was used as a control.

2.5. Cytotoxicity of PPGS and PPGS/siRNA polyplex

The cytotoxicity of PPGS and PPGS/siRNA was measured by using MTT assay. A549/DDP cells were seeded at 5000 cells per well in 96-well plates, with 100 μL RPMI 1640 containing 10% FBS. After further cultured for 24 h, the culture media were replaced by fresh medium containing 10% FBS with various amounts of PPGS or PPGS/siRNA. Following incubation for 4 h, the medium was replaced by fresh medium containing 10% FBS and further cultured for 48 h. MTT was added to each well at a final concentration of 0.5 mg/mL, and following incubation for 4 h, the supernatant was removed, and the resulting formazan was dissolved in 150 μL DMSO. The absorbance at 490 nm was measured by microplate reader (ELX800, Bio-Tek, USA.). The cell viability was expressed as the percentage of the absorbance of sample to that of the untreated cells.

2.6. Effects of serum concentration on cellular uptake

Flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) assays were used to evaluate the effects of serum concentration on cellular uptake. A549/DDP cells were seeded onto 12-well plates at a density of 1×10^5cells per well, with 1 mL RPMI 1640 medium containing 10% FBS. After the cells reaching 70–80% confluence, the culture medium were replaced with fresh complete medium with varying concentration of serum (0–50%) and PPGS/FAM-siRNA polyplex at varying N/P ratios were added to the cells. PEI 25KDa/FAM-siRNA polyplex (N/P = 10/1) and Lipofectamine2000/FAM-siRNA complex (w/w = 2/1) were used as positive controls according to manufacturer's instruction. Naked FAM-siRNA served as a negative control. After 4 h incubation, the media were removed and the cells were washed with PBS for three times. Subsequently, transfected cells were treated with trypsin/EDTA for 2 min, collected by centrifugation and suspended in 500 μL PBS. The cell samples were measured by FCM using a FACS-Calibur Instrument (Beckman, USA.) and data was analyzed with Winmdi.

CLSM was used to observe the intracellular distribution and the changes of fluorescence intensity with the increase of serum concentrations (0–50%) in media. Coverslips putting in the 6-well plates were seeded with A549/DDP cells at the density of 1×10^5 cells per well. After incubated for 24 h, the medium were replaced by fresh culture media with varying concentration of serum (0–50%). PPGS/FAM-siRNA polyplex (N/P = 40/1) and Lipofectamine2000/FAM-siRNA complex (w/w = 2/1) were added to the cells, respectively. After 4 h incubation, the media were removed and the cells were washed with PBS for three times. The extracellular fluorescence was quenched with 0.4% trypan blue for 3 min. Then the cells were fixed with 4% paraformaldehyde for 30 min and stained with 10 mg/mL of DAPI for 20 min at 37 °C in dark. After that, cells were observed using Zeiss LSM710CLSM (Carl Zeiss, DE) at excitation wavelength of 405 nm and 488 nm to visualize nuclei (blue fluorescence) and FAM- labeled siRNA, respectively.

2.7. Gene silencing experiment

The RNAi efficiency of PPGS/si-survivin polyplex was determined by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). A549/DDP cells were seeded in 6-well plates at a density of 2×10^5cells per well, with 2 mL RPMI 1640 medium containing 10% FBS. After the cells reaching 50% confluence, the culture medium were changed with fresh complete medium containing 10% FBS, PPGS/si-survivin polyplex (N/P = 40/1), PPGS/siNC polyplex (N/P = 40/1), naked si-survivin, PEI 25KDa/si-survivin polyplex used as a positive control (N/P = 10/1) and Lipofectamine 2000/si-survivin used as a positive control (w/w = 2/1) were added to the cells, respectively. After 4 h incubation, the medium contained samples were replaced with fresh complete medium, and the cells were allowed further cultured for 48 hThen the cells were washed with PBS and lysed by TRIzol reagent (Introvigen). The isolated total RNA were quantified by UV–vis Spectrophotometer NanoDrop 2000 (Thermo Scientific, USA.) at 260 nm. 1 μg RNA was reverse transcribed to complementary DNA (cDNA) by using a Reverse Transcription System kit (Promega, USA) according to the manufacturer's instructions. Quantitative RT-PCR analysis were performed using Go Taq qPCR Mastermix (Promega, USA), and detected with LightCycler 480II (Roche, Swit). The data was analyzed using the comparative CT ($2^{-\Delta\Delta CT}$) method.

2.8. Cell proliferation and apoptosis analysis

Apoptosis was quantified by AnnexinV-FITC/PI assay. After 48 h transfection to effectively down-regulate the activity of survivin, 25 μg/mL cisplatin was added and the cells were incubated for another 24 h. Then the cells were harvested and stained using the AnnexinV-FITC apoptosis detection kit according to the manufacturer's protocol prior to flow cytometry measurements. Data analysis was performed by CellQuest software (Becton-Dickinson).

For cell proliferation assay, A549/DDP cells were seeded in 96-well plates at a density of 5000 cells per well, after 48 h transfection to down-regulate the survivin, varying concentration of cisplatin was added and the cells were incubated for another 24 h. The cell viability was measured by MTT assay as described before.

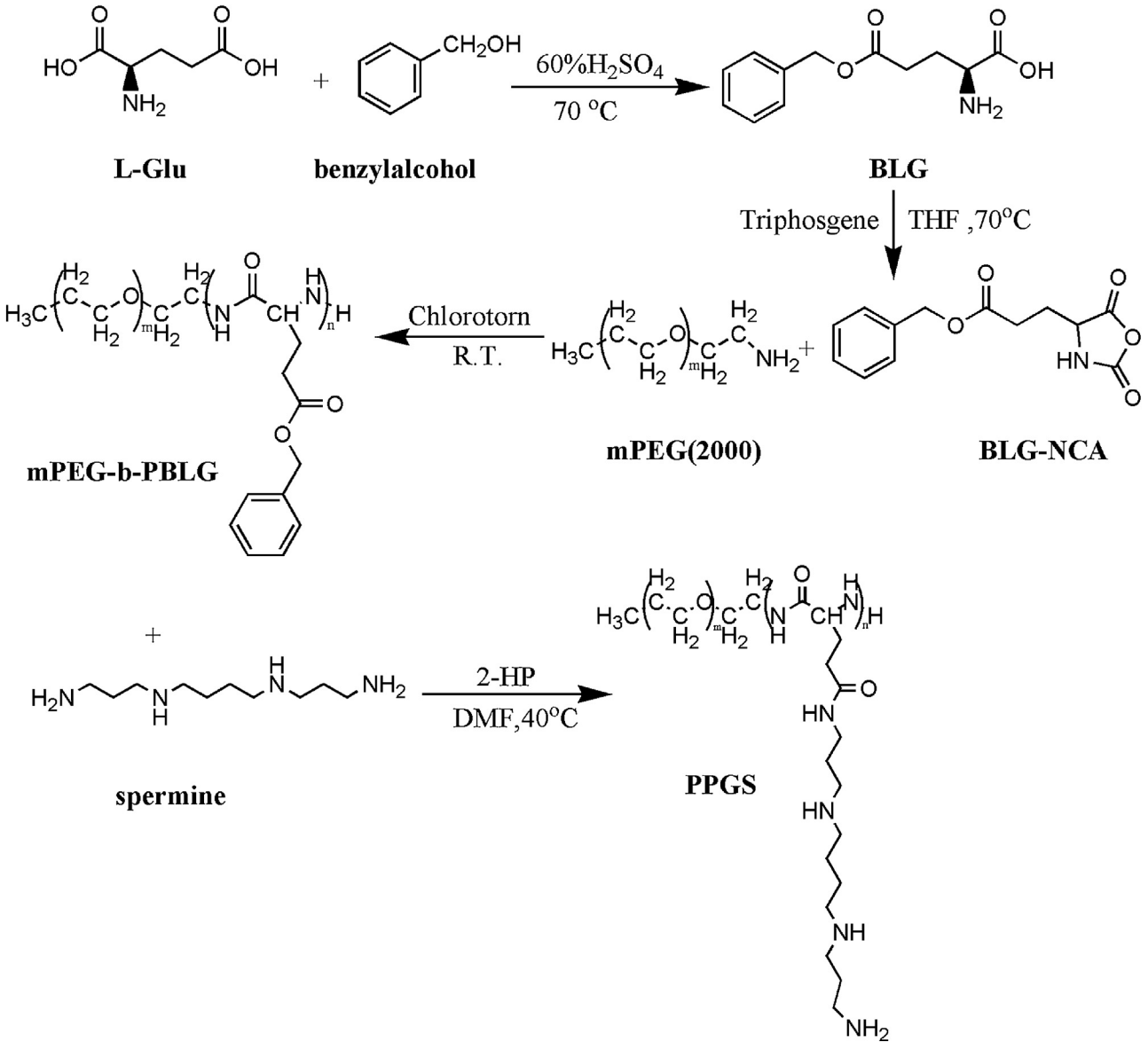


Fig. 1. Synthesis route of mPEG-b-PG-g-spermine polymer.

2.9. Statistical analysis

Data were expressed as mean ± SEM. The analysis of variance is completed using a one-way ANOVA. $P < 0.05$ and $P < 0.01$ were considered statistically significant and highly statistically significant, respectively.

3. Results and discussion

3.1. Synthesis and characterization of PPGS polymer bursh

To develop a stable and efficient gene carrier for siRNA delivery, mPEG-b-PG-g-spermine (PPGS) was synthesized as show in Fig. 1. Firstly, mPEG-b-PBLG was synthesized by ring-opening polymerization using mPEG-NH2 as an initiator. Then, polymer brush PPGS was synthesized by densely grafting spermine to macromolecules mPEG-b-PBLG through aminolysis reaction. Synthesized PPGS was characterized using ^{1}H NMR and results were showed in Table 1 and Fig.S1. The grafting ratio of spermine was high (>80%) indicated that the densely grafted spermine chain with high charge density could form a polymer brush structure. It has been found that polymer brush is a novel antifouling system because its high osmotic pressure in polymer brush could spontaneously expel proteins and ions in media (Blattler et al., 2006; Nowak et al., 2003). The colloidal stability of gene/polycation polyplex in biological fluids is a prerequisite for application *in vivo*, but it often neglected in earlier studies. We designed the PPGS polymer brush anticipated that it could be both stable and efficient gene carrier in biological fluids.

3.2. Particle size of PPGS/siRNA polyplex in ultra-high dilution, saline and in the presence of serum

Diluted by body fluids and transported in the presence of salt and serum are inevitable for gene/polycation polyplex in systemic delivery. These factors must be considered when designing a gene carrier to reduce the gap between clinical trials and preclinical studies for gene therapy. The importance of serum resistance for gene delivery carrier have been reported by several research groups (Garcia et al., 2007; Han et al., 2008; Takahashi et al., 2005), but the dilution stability and salt stability as the equally important factors, were seldom investigated. Here, we intensively investigated the colloidal stability of PPGS/siRNA polyplex in ultra-high dilution and in the presence of saline (NaCl 150 mM) and serum. Fig. 2(A) showed that the particle size of PPGS/siRNA polyplex kept in approximately 150 nm when polyplex was diluted up to 256 times. There was no obvious change compared with its initial particle size. Meanwhile, the polydispersity index (PDI) of PPGS/siRNA polyplex is below 0.3 when a set of serial dilutions is made (Fig. 2(B)). However, the particle size of PEI 25KDa/siRNA polyplex undergoing 256-times dilution sharply increased to about three times bigger than its initial particle size, and its PDI rose to 0.65 suggesting the aggregation occurred.

The salt stability of PPGS/siRNA polyplex was evaluated by measuring its particle size and size distribution in physiological saline. The particle size of PPGS/siRNA incubating in physiological saline for 24 h exhibited no obvious change compared with that incubating in deionized water, while the particle size of PEI 25KDa/siRNA polyplex sharply increased to 2.5 μm under same conditions (Fig. 2(C)). We also challenged the PPGS/siRNA polyplex and PEI 25KDa/siRNA polyplex used as a control with increasing concentration of NaCl and compared the concentrations that led to complex aggregation. The results were shown as Fig. 2(D), the particle size of PPGS/siRNA polyplex was stable and less than 200 nm when the concentration of NaCl less than 200 mM. The PPGS/siRNA polyplexes slightly aggregated when the concentrations of NaCl were above 300 mM. However, the particle size of PEI 25KDa/siRNA sharply increased when the concentrations of NaCl were above 100 mM. The results indicate that PPGS/siRNA had a stronger salt tolerance ability than PEI 25KDa/siRNA polyplex.

Similarly, PEI 25KDa/siRNA polyplex exhibited larger particle size than that of PPGS/siRNA polyplex incubating in media containing 10% serum (Fig. 2(E)). The results demonstrated that PPGS/siRNA polyplex was highly stable in the medium containing salt and serum, and ongoing ultra-high dilution. Whereas, PEI 25KDa/siRNA polyplex is not stable in these conditions (Fig. 2(F), Fig. 2(G)). We believed that the colloidal stability characteristics of PPGS/siRNA polyplex under physiological condition were likely due to their unique polymer brush structure. After densely grafted spermine chains onto polyglutamate, a strong electrostatic interaction in spermine brushes induced the counterions flowing into the PPGS polymer brush, resulting in a high osmotic pressure and swelling of brushes. The stretching of brush chains enhancede the stability of PPGS/siRNA polyplex in the physiological fluids and at ultrahigh dilution.

3.3. Cytotoxicity of PPGS polymer brush and PPGS/siRNA polyplex

To estimate the safety profile for the possible applications of PPGS, cytotoxicity of PPGS polymer brush and PPGS/siRNA polyplex were evaluated on A549/DDP cells by using the MTT assay, as shown in Fig. 3. Fig. 3(A) showed that the cell viabilities of PPGS were significantly higher at all investigated levels compared with PEI 25KDa. The IC_{50} of PPGS polymer brush was 91.58 μg/ml, it was approximately 12 times higher than the IC50 of PEI 25KDa (Fig. 3(A)). Similarly, cell viabilities of PPGS/siRNA polyplex with the largest N/P ratio tested was >90%, while cell viability of PEI 25KDa/siRNA polyplex was less than 60% at the N/P ratio above 80:1 (Fig. 3(B)). The relatively low cytotoxicity of PPGS polymer brush allowed for its further biomedical applications.

3.4. Effect of serum resistant PPGS/siRNA polyplex on cellular uptake

The most widely used commercial gene transfection reagents, Lipofectamine 2000 and PEI 25 KDa are not serum resistant reagents, leading to their cellular uptake and transfection efficiency decrease dramatically in the presence of serum and their application *in vivo* is limited. Cellular uptake of PPGS/FAM-siRNA in serum-containing media was evaluated by flow cytometry. PEI 25KDa and Lipofectamine 2000 were used as controls. After 4 h incubation in 10% serum media, the percentage of FAM positive cells of PPGS with various N/P ratios (20–50) was nearly 100% that was statistically significant higher than PEI 25KDa (56.4%) and comparable to Lipofectamine 2000 (Fig. 4(A) and (B)). The fluorescence intensity of PPGS (522.1 a.u. at the optimal N/P ratio of 40:1 was 18-fold and 5-fold higher than PEI 25KDa (29.2 a.

Table 1
Characterization of PPG polymer brush.

Polymer	Molecular weights of each block			Mn	Polymerization degree	Grafting ratio
	PEG	PLG	spermine			
PPGS	2000	13030	16749	31779	102	81.7%

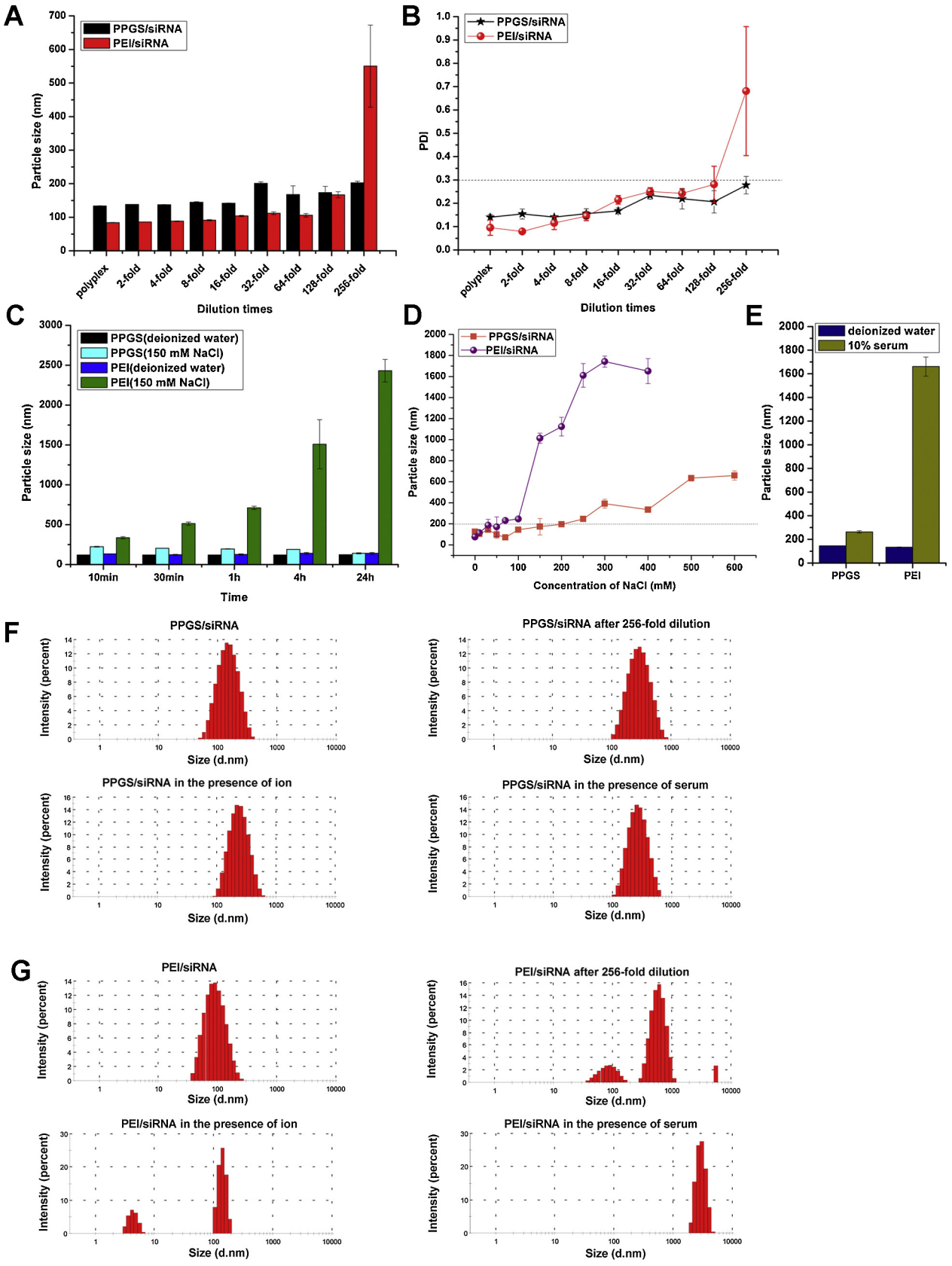


Fig. 2. (A) Particle size and (B) the polydispersity index (PDI) of polycation/siRNA polyplex at a serial dilution. Both PPGS/siRNA and PEI/siRNA were dispersed in deionized water and the initial concentration of siRNA in polyolex was 60 μg/ml. (C) Particle size of polycation/siRNA polyplex in the deionized water and 150 mM NaCl after incubation for various periods. The concentration of siRNA in polyolex was 20 μg/ml. (D) Particle size of polycation/siRNA polyplex with the increased concentration of NaCl. (E) Particle size of polycation/siRNA polyplex polyplex in the presence of 10% serum. Representative size distribution of PPGS/siRNA polyplex (F) and PEI 25KDa/siRNA polyplex (G) in the presence of 150 mM NaCl, 10% serum and undergoing 256-fold dilution. The concentration of siRNA in polyolex was 20 μg/ml and 20 μg/ml for saline and serum incubation, respectively. The initial concentration of siRNA in polyolex was 60 μg/ml for 256-fold dilution. Data represent the mean ± SD, n = 3.

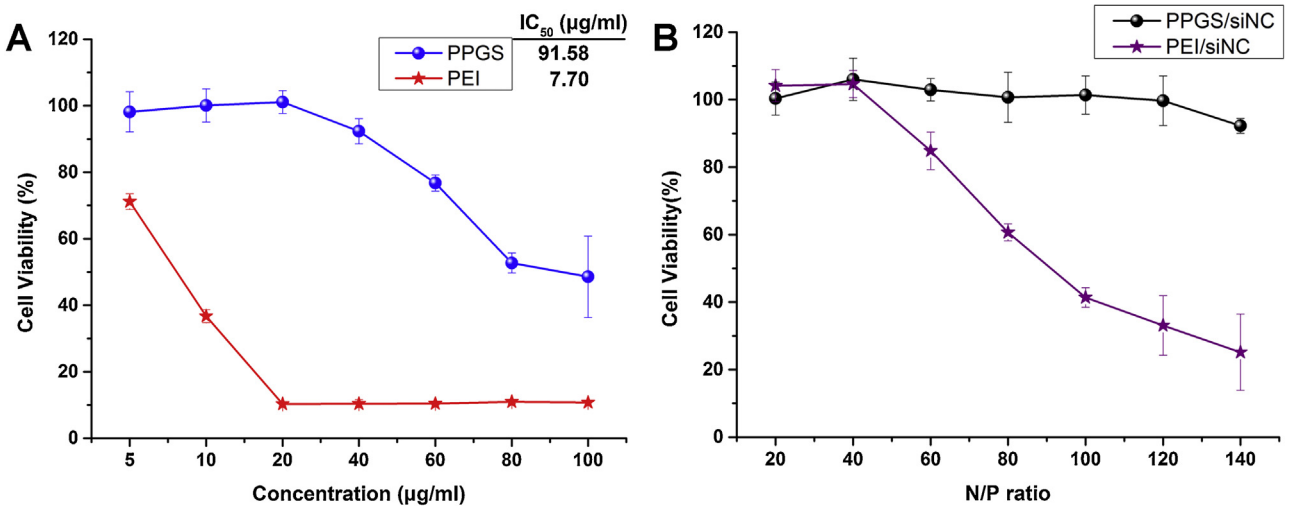


Fig. 3. Cell viabilities of (A) polymers at various concentrations and (B) polymer/siRNA complexes at different N/P ratios. The concentration of siRNA was 100 nM. Data represent the mean ± SD, n = 3.

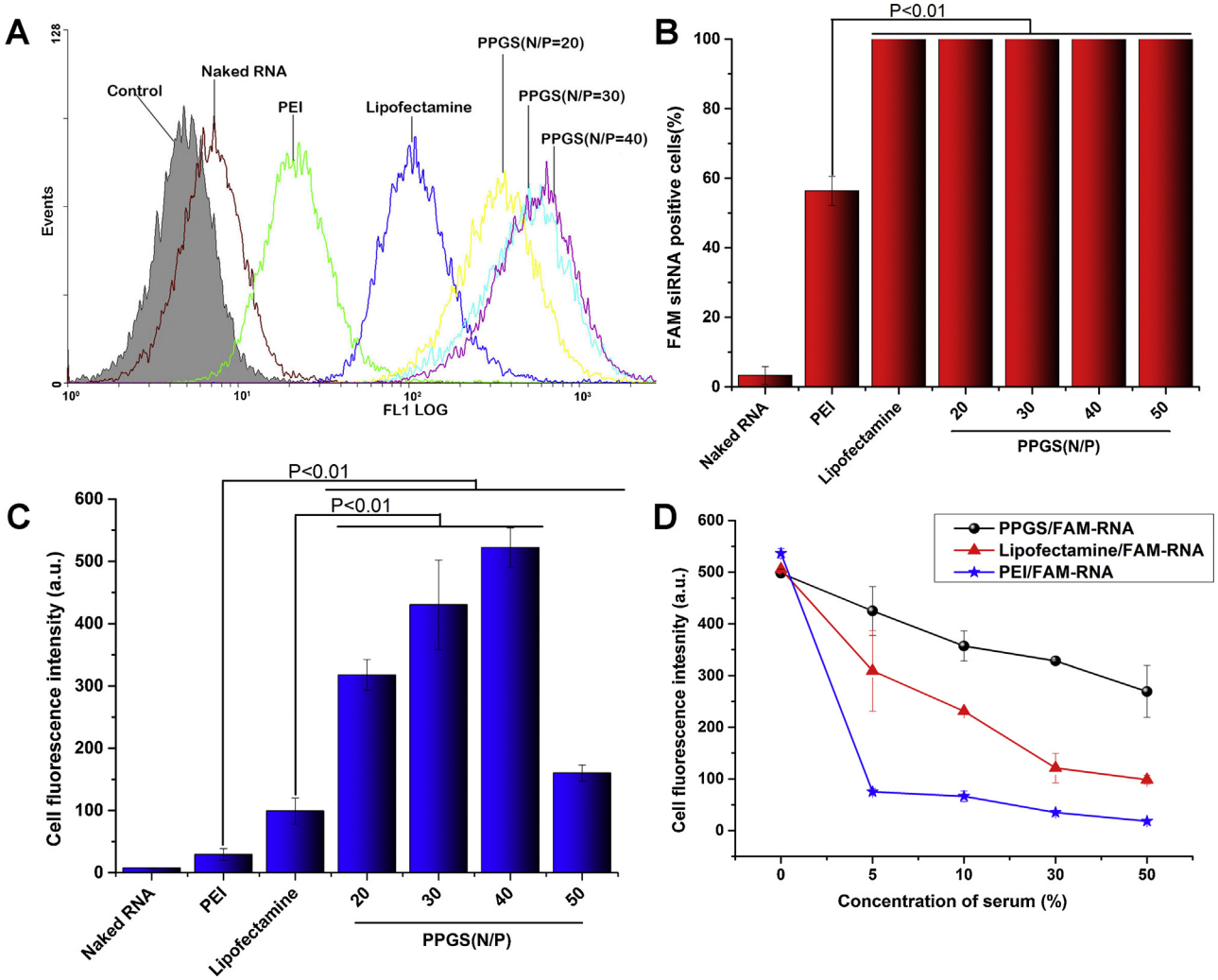


Fig. 4. Cellular uptake of FAM-siRNA polyplex. (A) Representative flow cytometric histogram, (B) percentages of FAM-siRNA cells and (C) mean florescence intensity of A549/DDP cells after 4 h incubated with polyplex at various N/P ratios in 10% serum conditions. (D) Mean fluorescence intensity of cells after 4 h incubated with PPGS/FAM-siRNA (N/P = 40) at various serum conditions. The concentration of FAM-siRNA was 100 nM. Data represent the mean ± SD, n = 3.

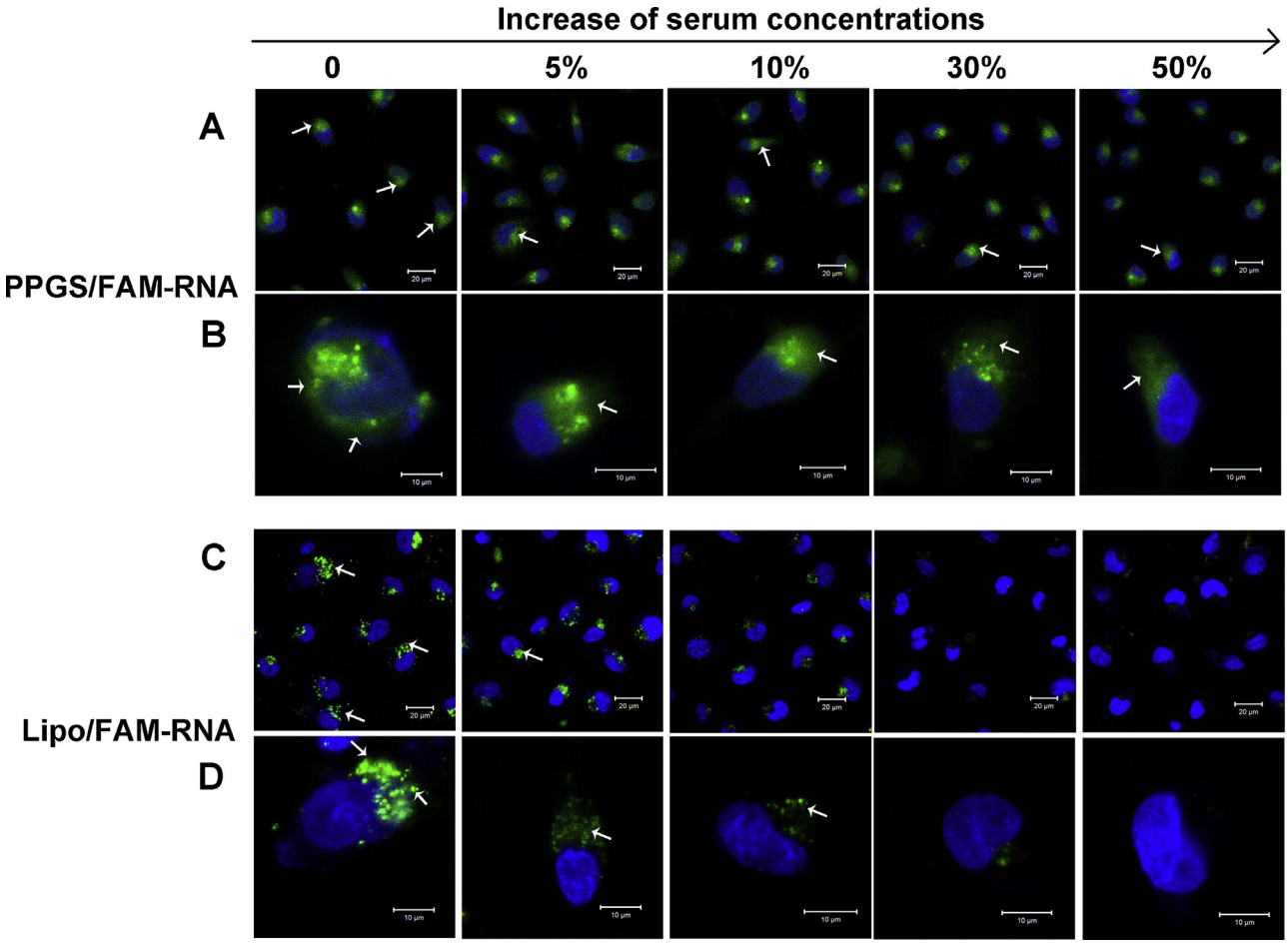


Fig. 5. Intracellular distribution of FAM-siRNA polyplex in A549/DDP cells after 4 h incubated with (A) (B) PPGS/FAM-siRNA (N/P = 40), (C) (D) Lipofectamine 2000/FAM-siRNA (w/w = 2). Cell nuclei were stained blue with DAPI. The concentration of FAM-siRNA was 100 nM. Scale bars represent (A) (C) 20 μm, (B) (D) 10 μm.

u.) and Lipofectamine 2000 (99.1 a.u.), respectively, as shown in Fig. 4(A) and (C). However, the fluorescence intensity of cells transfected with PPGS was reduced at the N/P ratio of 50 (Fig. 4(C)). It was speculated that PPGS at high N/P ratios might damage the cells although did not kill the cells, leading to decreasing the cellular uptake. Taken together, these results demonstrated that the amounts of siRNA internalized into cells delivered by PPGS were much higher than PEI 25KDa and Lipofectamine 2000. The high levels of cellular uptake of PPGS could provide benefits in terms of the transfection efficiency.

We also investigated the influence of serum concentrations on cellular uptake. The experiments were performed in a medium containing 0, 5, 10, 30 and 50% serum, respectively. Fig. 4(D) showed that the presence of serum inhibited the cellular uptake of PEI 25KDa and Lipofectamine 2000 significantly. Both their cellular uptake efficiencies were sharply decreased with the increase of the serum concentration. In the presence of 50% serum, the cellular uptake efficiency decreased by 30-fold of PEI 25KDa and 5.3-fold of Lipofectamine 2000 comparing to in the absence of serum, respectively. The results were consistent with previous studies and indicated that PEI 25KDa and Lipofectamine 2000 were not stable in serum containing media, most likely due to that positively charged carriers nonspecifically adsorbed the negatively charged serum proteins. However, the cellular uptake efficiency of PPGS marginally decreased by 1.8-fold with the increase of serum concentrations. The serum resistant PPGS/siRNA polyplex was

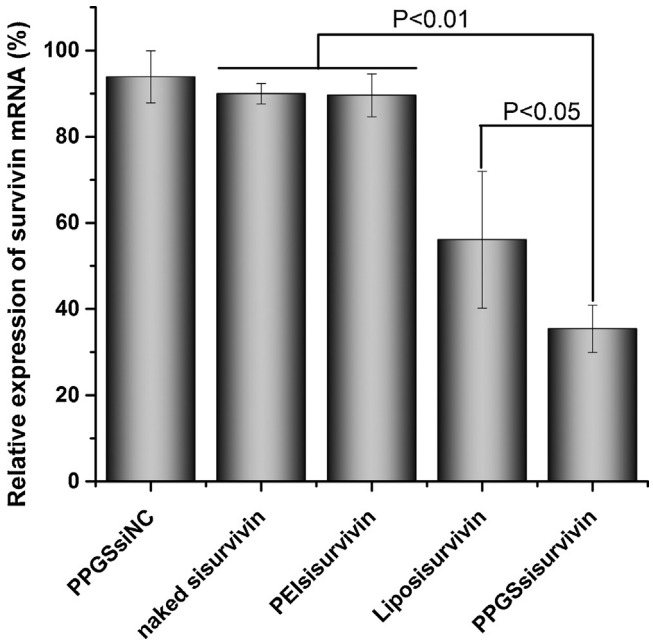


Fig. 6. Silencing of survivin mRNA in A549/DDP cells. The concentration of polycation/si-survivin was 100 nM. Data represent the mean ± SD, n = 3.

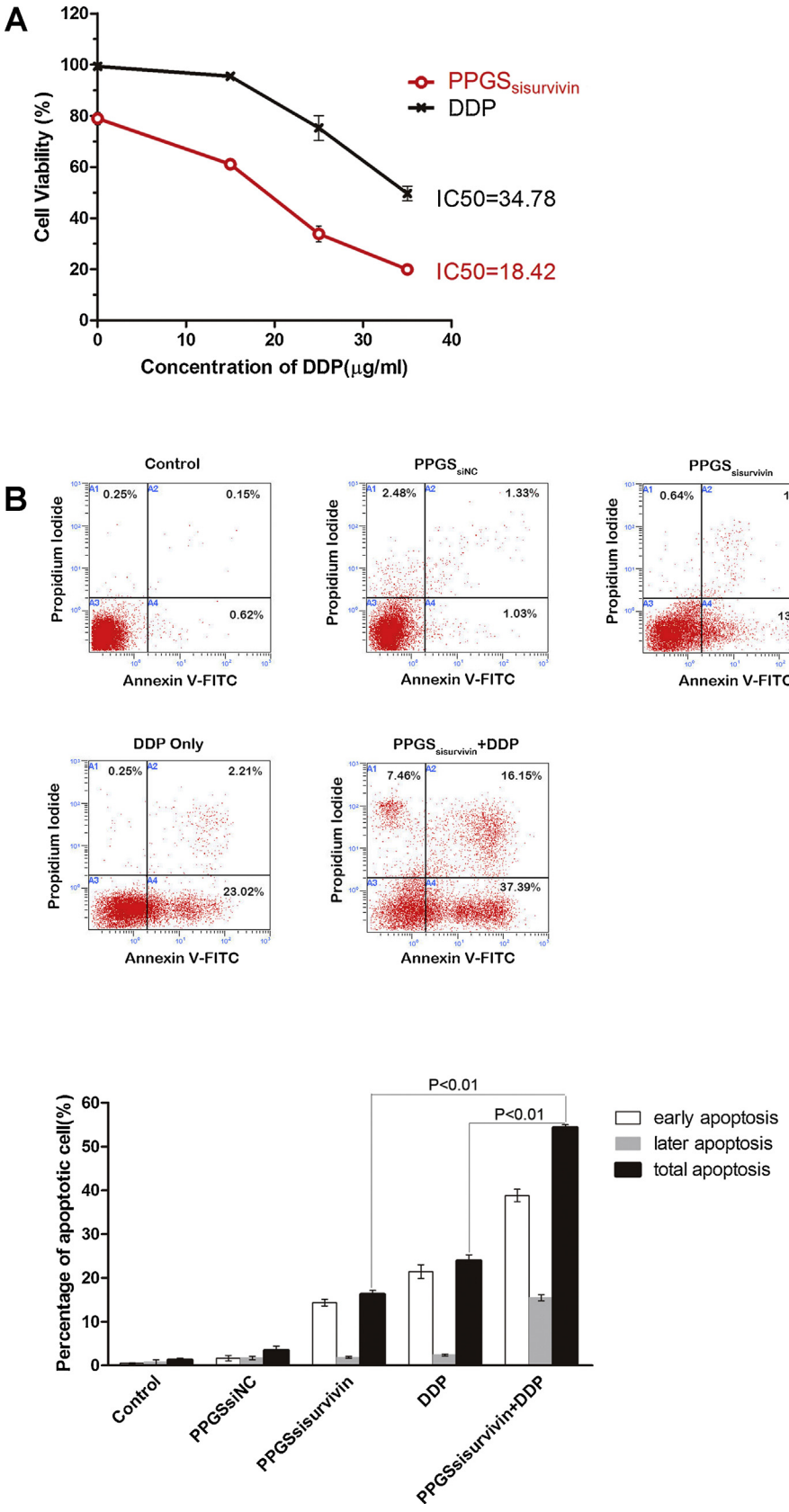


Fig. 7. (A) Cytotoxicity of A549/DDP cells and (B) apoptosis analysis of A549/DDP cells treated with 25 μg/mL of DDP after transfection with or without PPGS/si-survivin polyplex. The concentration of FAM-siRNA was 100 nM. Cells well stained with Annexin V-FITC and propidium iodide.

benefit by polymer brush to keep the PPGS stable in serum. Therefore, PPGS having good stability in presence of serum is unambiguously proven the feasibility for its further *in vivo* applications.

Confocal microscope was also used to investigate the cellular uptake in the presence of different serum concentrations and visualize the intracellular distribution of polyplexs. In the absence of serum condition, the florescence intensity in cells transfected by PPGS and Lipofectamine 2000 were strong, and almost all the cells in the field of vision could be transfected (Fig. 5), indicating that both PPGS and Lipofectamine 2000 were able to efficiently deliver siRNA into cells in the absence of serum. The results were consistent with those conducted by flow cytometry. When serum concentrations were increased, the florescence intensity in the cells treated by the PPGS was not significantly reduced, while weakened obviously in the cells treated by Lipofectamine 2000. Furthermore, in the images the arrows indicated that FAM-labeled siRNA delivered by PPGS was evenly spread throughout the cytoplasm of A549 cells (Fig. 5(A) and (B)). On the contrary, the punctate distribution of siRNA delivered by Lipofectamine 2000, as shown in (Fig. 5(C) and (D)). These results implied that PPGS could efficiently assist siRNA escape form endosomal and released them into the cytoplasm, which offered benefits to enhance RNAi effectiveness (Li et al., 2012).

3.5. Gene silencing efficiency

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is found up-regulated in malignant tumors, especially in drug resistant cells, enabling it a potential new target for cancer treatment (Ambrosini et al., 1997). In the present study, an anti-survivin siRNA was complexed with PPGS and delivered into A549/DDP cells in the presence of serum and salt. The silence efficiency of PPGS/si-survivin polyplex was evaluated by using qRT-PCR and compared to PEI 25KDa/si-survivin polyplex and Lipofectamine2000/si-survivin complex. As shown in Fig. 6, the PPGS polyplex containing 100 nM si-survivin reduced the level of survivin mRNA to 35.4%, which were statistical significantly superior to Lipofectamine2000/si-survivin complex ($p < 0.05$) and PEI 25KDa/si-survivin polyplex ($p < 0.01$). It was noted that PEI 25KDa in the presence of serum showed very low silencing efficiency, comparable to naked si-survivin. These results suggested that the gene carriers were stable in serum and salt-containing media played an important role in achieving efficient gene silencing in physiological fluids.

3.6. Enhancement of cytotoxicity and apoptosis of DDP combined with PPGS/si-survivin

Cisplatin (DDP) is one of the common chemotherapy drugs for NSCLC treatments, but the maximum tolerated dose of DDP is limited by nephrotoxicity. DDP combined with down-regulation apoptosis protein survivin is a potential strategy to reduce the dose of DDP and improve therapeutic effects. To further examine whether the efficient down-regulation of survivin can effectively increase the chemotherapy efficacy of cisplatin, the cytotoxicity of DDP and DDP with PPGS/si-survivin polyplex pretreatment were measured. A549/DDP cancer cells which over-express survivin were used as cell model. The results were present in Fig. 7(A), DDP with or without pretreatment of PPGS/si-survivin polyplex exhibited dose-dependent cytotoxic effects. The IC_{50} of DDP was significantly decreased from 34.78 (μg/mL) to 18.42 (μg/mL) by pretreating the cells with PPGS/si-survivin polyplex ($p < 0.01$).

AnnexinV-FITC/PI assay was performed to further evaluate the apoptosis enhancement of DDP combined with PPGS/si-survivin polyplex, as shown in Fig. 7(B). The cytogram of cells undergoing apoptosis should show the early apoptotic cells in the lower right quadrant being Annexin V positive and PI negative; late apoptotic are in the upper right quadrant being PI positive and Annexin V positive; live cells in the lower left quadrant being negative for both fluorescent probes. The total number of apoptotic cells (early apoptosis and later apoptosis) treated with DDP and pretreated PPGS/si-survivin polyplex was 53.5% that was significantly increased compared with the same dosage of DDP alone (25.23%) ($p < 0.01$). Only use of PPGS/si-survivin polyplex could induce 15.2% of total apoptosis. These results were consistent with that of cytotoxicity. It was noted that later apoptosis of DDP pretreated by PPGS/si-survivin polyplex was 7.3-fold and 9.8-fold higher comparing to treatments with DDP only and PPGS/si-survivin polyplex only, respectively. These results demonstrated that combination of DDP and PPGS/si-survivin polyplex could enhance the killing effect on A549/DDP cancer cells.

4. Conclusions

In vitro studies on gene delivery often neglected the stability of gene/polycation polyplex under physiological conditions that is a prerequisite for application *in vivo*. In this study, we developed the polyglutamate derivative polymer brush PPGS as a stable and efficient gene delivery carrier. The PPGS/siRNA polyplex with the structural characteristics of PEG-shelled polymer brushes achieved highly stable in ultra-high dilution and in the presence of saline (NaCl 150 mM) and serum. Whereas aggregation occurred when PEI 25KDa/siRNA polyplex dispersed in saline and serum-containing media and a 256-fold dilution induced PEI 25KDa polyplex dissociation. PPGS/si-survivin polyplex exhibited high cellular uptake efficiency and efficiently down-regulated the expression of survivin mRNA in A549/DDP cells in the presence of serum and salt. However, either PEI 25KDa polyplex or Lipofectmine 2000 complex was unstable in serum and salt-containing media and at high dilution rates, resulting in both their cellular uptake and gene-silencing efficiency were dramatically decreased in these conditions. Furthermore, PPGS/si-survivin polyplex also demonstrated synergistic effects of killing the cancer cells by combination treatment with cisplatin. Therefore, PPGS polymer brushes exhibited significant advantages in delivering siRNA and held great potential in systemic gene delivery. Its combination with chemotherapeutic drug is promising in treating drug resistant cancers.

Acknowledgment

This work is supported by National Natural Science Foundation of China (Project No. 81373332) and Guangdong Natural Science Fund (Project No. 2014A030313155).

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.2016.03.062.

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