

Polyglutamic Acid Based Polyanionic Shielding System for Polycationic Gene Carriers^{*}

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Abstract For the purpose of increasing the *in vivo* stability of polycation gene carriers, we prepared a kind of pH-sensitive poly(ethylene glycol)-poly (γ -benzyl-L-glutamate-co-glutamic acid) (PEG-PGA(65), 65 denotes the molar ratio of glutamic acid in poly(γ -benzyl-L-glutamate-co-glutamic acid)). PEG-PGA(65) showed low cytotoxicity and could shield the positive charge of DNA/PEI (1:1) polyplexes efficiently. The transfection was enhanced due to the partially charge shielding in HeLa cell line at pH of 7.4. There was almost no transfection efficiency when the surface charge of the ternary particles turned to negative at pH of 7.4. However, the transfection efficiency recovered a lot by culturing at pH of 6.0 at the beginning of transfection. Confocal microscopic observation and flow cytometry results showed DNA/PEI polyplexes should be efficiently released and endocytosized at pH 6.0, because of the pH triggered deshielding action of PEG-PGA(65). Due to the good biocompatibility and suitable pH triggered shielding/deshielding property, PEG-PGA(65) could be a potential shielding system for polycationic gene carriers used *in vivo*.

Keywords: Gene delivery; Shielding system; PEGylation; pH Sensitive.

INTRODUCTION

Gene therapy was defined as a promising strategy for treating genetic disorders, especially cancers^[1–3]. The bottleneck of gene therapy is how to transfer the gene drug to the lesion site safely and effectively^[4]. Non-immunogenicity and potential for large-scale production make the nonviral cationic polymer vectors increasingly attractive for gene therapy^[5–10]. However, the positive surface charge of polyplexes which can cause nonspecific interaction with blood cells and plasma proteins may lead to exclusion of the polyplexes from systemic circulation. PEGylation^[11–14] or shielding of positive charges with polyanions^[15–18] of cationic polymers can stabilize the complexes, minimize nonspecific interactions, and prolong circulation time *in vivo*, thereby increasing tumoral accumulation *via* enhanced permeation and retention effect.

The pH of tumor microenvironment is estimated to be 6.5, which is significantly lower than that of blood and normal tissues^[19, 20]. On the basis of the pH difference, substantial research involving pH-sensitive gene

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carriers has been designed extensively^[21]. Walker *et al.*^[22, 23] developed PEG-shielded polyplexes with pH-labile hydrazone bond and thus the PEG shield could be removed at the lower pH within endosome. Bae^[24] and Kataoka^[25, 26] have developed two kinds of pH-sensitive shielding systems, which could construct stable ternary particles with DNA/polycation complexes by electrostatic interaction at tissue pH, and deshield to release the DNA/polycation for transfection at lower pH when the ternary complexes reach the acid tumor site. These strategies were taken fully into account that the positive charge could be shielded during transmission and deshielded when arriving to the acid tumor tissue.

Our previous work indicated that pH sensitive polyanionic PGA could effectively shield the positive charge of polyplex^[18]. In the present study, PEG segment was introduced to PGA segment, PEG-PGA(65) was synthesized as pH sensitive shielding system. The shielding ability of PEG-PGA(65) was investigated here and distinct transfection behaviors with various PEG-PGA(65) weight ratios were also found using ternary complexes.

EXPERIMENTAL

Chemicals

Methoxypolyethylene glycol amine (mPEG-NH₂, $M_w = 2000$) and hydrobromic acid in acetic acid 33% (*V/V*) were purchased from ACROS. Branched PEI ($M_w = 25$ kDa, PEI-25k), ethidium bromide and calf thymus DNA were purchased from Sigma. *N*-Carboxyanhydride of γ -benzyl-L-glutamate (BLG-NCA) was prepared according to the reported method^[27]. Trifluoroacetic acid was purchased from GL Biochem Ltd. (Shanghai, China). Luciferase plasmid (pGL3-control), cell lysate and luciferase reporter gene assay kit were purchased from Promega (Mannheim, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, Ohio, USA). BCA protein assay kit was purchased from Pierce (Rockford, USA).

Syntheses of PEG-PGA(65)

In a flame-dried flask, 0.4 g of mPEG-NH₂ and 2.1 g of BLG-NCA (8.0 mmol) were dissolved in 100 mL of dried chloroform and stirred for 72 h at 30 °C. Then the product mixture was precipitated with an excess amount of ethyl ether under vigorous stirring. The purified PEG-PBLG was obtained as white solid.

PEG-PGA(65) was then synthesized by deprotection of the benzyl groups in the PEG-PBLG. Typically, 0.2 g of PEG-PBLG was dissolved in 2 mL of dichloroacetic acid. Then 0.6 mL of hydrobromic acid in acetic acid 33% (*V/V*) was added. Samples were dropped into excess ethyl ether after 10 min reaction to yield slight yellow solid. The end white products were obtained by lyophilization after dialyzing against deionized water.

Preparation of DNA/PEI/PEG-PGA(65) Ternary Complexes

DNA/PEI polyplex solutions were prepared at the DNA/PEI ratio of 1:1 (wt/wt). The DNA concentration was 10 μ g/mL. After 30 min incubation at room temperature, various amount of PEG-PGA(65) was added to gain DNA/PEI/PEG-PGA(65) ternary complexes with diverse weight ratios (wt/wt/wt).

Zeta Potential and Particle Size Analysis

The zeta potential and particle size of the ternary complexes in aqueous solution were measured by a zeta potential/BI-90Plus particle size analyzer (ZetaPALS, Brookhaven, USA) at room temperature.

Gel Retardation Assay

After incubating for 20 min, 10 μ L of ternary complex solutions with different weight ratios were analyzed by 1% agarose gel electrophoresis (100 V, 45 min). pGL3-control was used as the DNA model.

Cell Culture

Cells were cultured in DMEM medium with high glucose concentration and supplemented with 10% (*V/V*) heat-inactivated FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin in a 5% CO₂ incubator at 37 °C under 95% humidity.

Cytotoxicity Assay

The cytotoxicity of PEG-PGA(65) and ternary complexes were assessed using MTT assay. In brief, HeLa cells were seeded at 1.0×10^4 cells/well in 96-well plates and then cultured for 24 h as described above. The culture medium was aspirated and replaced with 180 μ L of DMEM, 20 μ L PEG-PGA(65) at different concentrations were added to each well and the plate was returned to the incubator for an additional 48 h. The absorbance of each well at 492 nm was recorded by an ELISA microplate reader (Bio-Rad) after treated with MTT. The cell viability (%) was calculated according to the following equation: Cell viability(%) = $(A_{\text{sample}}/A_{\text{control}}) \times 100$ where A_{sample} is the absorbance of the PEG-PGA(65) treated cells and A_{control} is the absorbance of the untreated cells.

Transfection at Different pH

To study the transfection activity of the ternary complexes, HeLa cells were seeded and cultured for 24 h. Then the culture medium was aspirated and replaced with DMEM at pH 7.4 or 6.0 containing pGL3-control/PEI or pGL3-control/PEI/PEG-PGA(65) complexes. The cells were incubated for 4 h at either pH 7.4 or 6.0. Then the medium was replaced with fresh DMEM medium of pH 7.4 and incubated for further 44 h. The gene transfection efficiency was expressed as luciferase expression/mg protein.

Cellular Uptake Efficiency at Different pH

To determine the intracellular translocation, the complexes were evaluated in HeLa cells using CY5-labeled DNA by confocal microscopic observation. HeLa cells (2.0×10^5 cells/well) were seeded on coverslips in 6-well plates and incubated for 24 h. The medium was replaced with a complete DMEM medium (pH 7.4 or 6.0) containing DNA/PEI, DNA/PEI/PEG-PGA(65) complexes. The final concentration of CY5-pDNA in the culture medium was 1 μ g mL⁻¹ in all the experimental groups. The cells were incubated with the complexes for 2 h at either pH 7.4 or 6.0, and then fixed by paraformaldehyde (4% in PBS) for 10 min at room temperature. The cellular uptake of complexes was visualized by a confocal laser scanning microscope (LSM 780, Carl Zeiss Inc., Jena, Germany) after the nucleus was stained with DAPI.

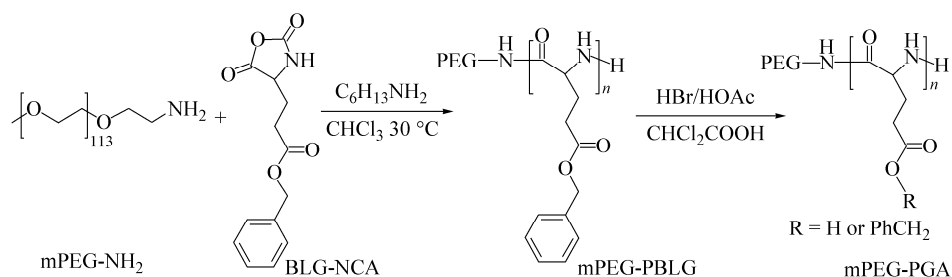
For flow cytometric analysis, HeLa cells were seeded in 6-well plates at 2.0×10^5 cells per well in 2 mL complete DMEM medium and cultured for 24 h. The medium was replaced with DMEM medium (pH 7.4 or 6.0) containing DNA/PEI or DNA/PEI/PEG-PGA(65) complexes. The cells were incubated with the complexes for 2 h at either pH 7.4 or 6.0 and washed twice with PBS (pH 7.4). The cells were detached with 0.25% trypsin and resuspended in 300 μ L PBS. Finally, the cellular uptake efficiency was evaluated using a BD FACS Calibur flow cytometer (BD Bioscience, USA).

RESULTS AND DISCUSSION

Synthesis and Characterization of PEG-PGA(65)

As our previous work demonstrated that PGA(60) showed suitable pH transition area^[18], here we designed PEG-PGA with similar glutamic acid ratio of PGA(60).

Take PEG-NH₂ as the macromolecular initiator, the synthetic approach of PEG-PGA is shown in Scheme 1. The ¹H-NMR spectrum of PEG-PBLG and PEG-PGA are shown in Fig. 1. The resultant PEG-PBLG molecular weight calculated based on ¹H-NMR is about 11.2 k, which is consistent with what we have designed, 10.7 K. After treating with hydrobromic acid, 65% of benzyl groups were removed from PBLG segment and produced PEG-PGA(65), which contains similar glutamic acid ratio with PGA(60). Since the PGA(60) shows pH transition starting at around pH 6.5 and ending at around pH 4.0, the PEG-PGA(65) should be negative and soluble at tissue pH 7.4, but neutral and insoluble when pH down to 4.0. It's expected that the PEG-PGA(65) can shield DNA/PEI polyplexes at normal tissue pH and release it at tumor pH (about pH 5.5).



Scheme 1 Synthesis of PEG-PGA(65)

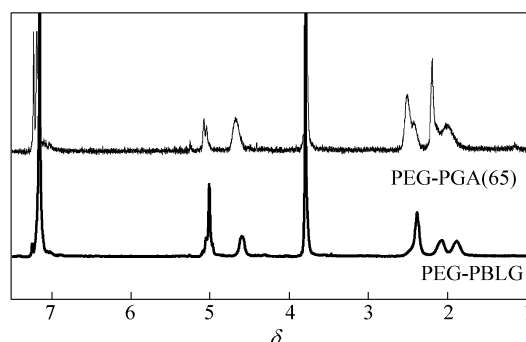


Fig. 1 ^1H -NMR spectra of PEG-PBLG and PEG-PGA(65) in TFA-d

Zeta Potential and Particle Size Analysis

The surface charge and particle size of gene carriers have significant influence on the transfection efficiency. Hence, these properties have been investigated and the results are shown in Fig. 2. The surface charge of DNA/PEI polyplex is about +19 mV and particle size is about 145 nm.

As shown in Fig. 2(a), the surface charge of the ternary complexes gradually changed from positive to neutral and finally to negative with the addition of PEG-PGA(65). The surface charge of the DNA/PEI complexes were reversed to negative charge when the feed ratio of PEG-PGA(65) increased to 2.0:1.0 (wt/wt) versus DNA/PEI complexes. These results indicated that the DNA/PEI complexes could be effectively shielded by PEG-PGA(65). Particle size evaluation (Fig. 2b) revealed that the particle size increased a bit to about 190 nm when PEG-PGA(65) was added.

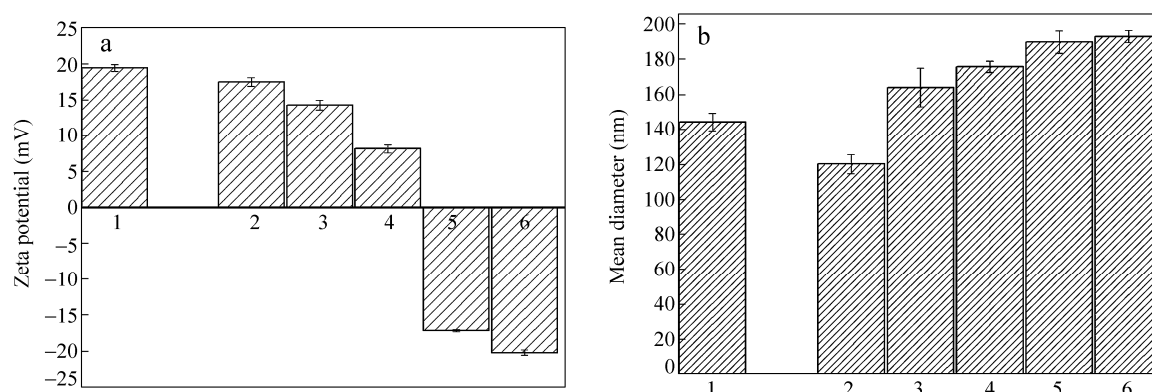


Fig. 2 Zeta potential and particle size of ternary complexes: (a) Surface charge of ternary complexes (25 °C, three parallel samples, 30 circles for each sample); (b) Particle sizes of ternary complexes (25 °C, three parallel samples, cumulate 3 min for each sample)

Lane 1, DNA/PEI (wt/wt) 1:1; lanes 2 to 6, DNA/PEI (1:1) shielded by various ratio (wt/wt) of PEG-PGA(65): 0.25, 0.5, 1.0, 2.0, 4.0, respectively

Gel Retardation Assay

Gel electrophoresis was used to monitor the stability of the newly formed ternary complexes (Fig. 3). It was clearly observed that all the ternary complexes were retarded and no ethidium bromide-intercalated DNA was detected in all lanes. It means that the PEG-PGA(65) did not destroy the structure of DNA/PEI polyplex; there was no chain exchanging occurred between PEG-PGA(65) and DNA molecules.

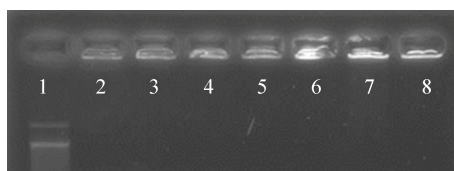


Fig. 3 Gel retardation assay for DNA/PEI/PEG-PGA(65) ternary complexes: (1) DNA; (2) DNA/PEI (1:1); (3 to 8) DNA/PEI (1:1) shielded by various ratios (wt/wt) of PEG-PGA(65): 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, respectively

Cytotoxicity Assay

The cytotoxicity of PEG-PGA(65) was investigated on HeLa cell by MTT-based assay. As shown in Fig. 4, the cell viability is at least 85% at the test concentrations for PEG-PGA(65). The result shows good biocompatibility of the synthesized PEG-PGA(65).

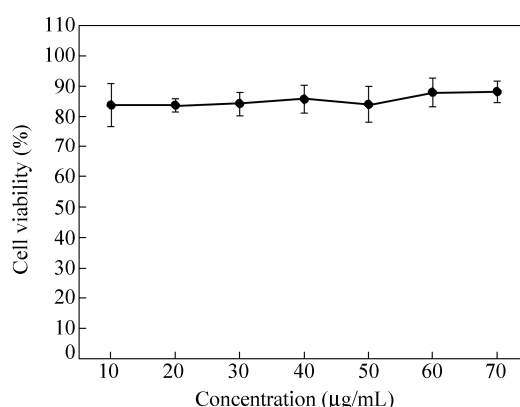


Fig. 4 Viability of HeLa cells exposed to various PEG-PGA(65) concentrations

In vitro Transfection and Cellular Uptaken

The potential use of PEG-PGA(65) as the shielding system for nonviral nucleic acid delivery vehicle was investigated by *in vitro* transfection study of PEI based polyplexes using HeLa cell line, at both tissue pH of 7.4 and acidic pH of 6.0. Transfection of the ternary complexes was compared to that of PEI/DNA polyplexes. Ternary complexes demonstrated different transfection efficiencies depending on the pH and the amount of PEG-PGA(65). As shown in Fig. 5, at pH of 7.4, the transfection was enhanced when the PEG-PGA(65) ratio was less than 1. There was almost no transfection observed when the ternary complexes surface changed to neutral or negative. However, treating the ternary complex at pH 6.0 at the beginning of transfection, the transfection efficiency recovered a lot to the no-shielding level. So, we can conclude that positive charges on these ternary complexes surface may be necessary during the gene transfection, and PEG-PGA(65) should shield/deshield DNA/PEI polyplex with changing the surrounding pH values.

To investigate the influence of introducing PEG-PGA(65) shielding system on the transfection efficiency of the DNA/PEI complexes, the intracellular uptake of CY-5 labeled DNA formulations by HeLa cells at pH 7.4 or 6.0 was monitored by confocal laser scanning microscopy (Fig. 6). The fluorescence images showed that the DNA/PEI/PEG-PGA(65) ternary complexes at the ratio of 1:1:2 can effectively internalize CY5-pDNA into the

cells at pH 6.0. In comparison, only slight fluorescence was observed at pH 7.4. Nanoparticles with positive charges can adhere to the negatively charged cell membrane and be internalized by cells more easily than negative nanoparticles^[2]. The invariable lower internalization effect of the DNA/PEI/PEG-PGA(65) complexes were achieved at pH 7.4, which was obviously caused by the negatively charged shielding of PLG segments. The enhanced intracellular uptake phenomenon at pH 6.0 was mainly attributed to the pH triggered deshielding behavior of PEG-PGA(65). The positively charged surface of the DNA/PEI complexes were re-exposed in the acid environment, thus enhancing the intracellular uptake of the complexes. Overall, cellular uptake studies clearly demonstrated that the negatively charged nanoparticles reduced internalization at pH 7.4, whereas the positively charged particles sharply enhanced internalization at pH 6.0.

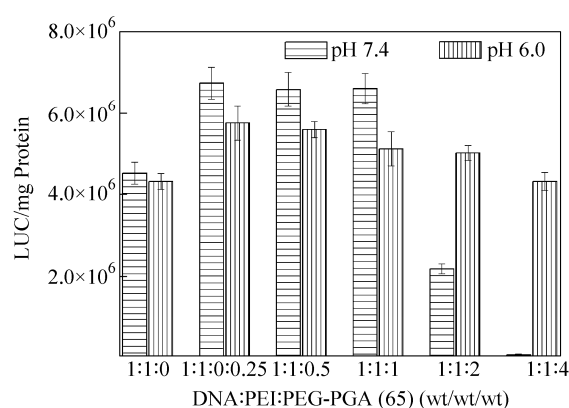


Fig. 5 Transfection efficiency of DNA/PEI/PEG-PGA(65) at various weight ratios in HeLa cells

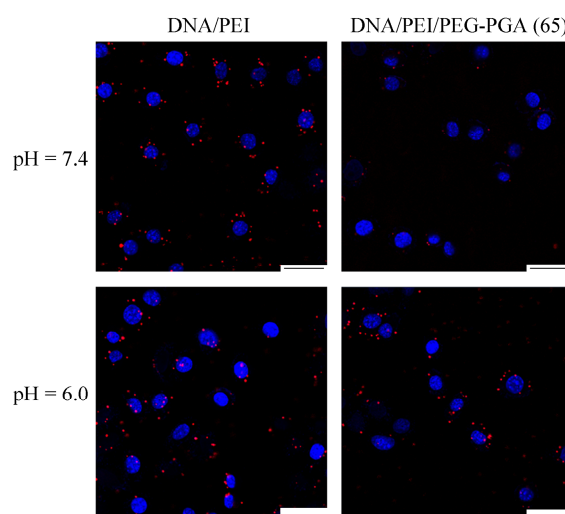


Fig. 6 Confocal laser scanning microscope (CLSM) images of HeLa cells incubated with DNA/PEI (wt/wt = 1:1), DNA/PEI/PEG-PGA(65) (wt/wt/wt = 1:1:2) at pH 7.4 or 6.0 (DAPI, cell nucleus (blue); CY5-pDNA (red). All complexing ratios are expressed as weight ratios. Scale bars = 20 μ m.)

Intracellular uptake was further monitored by flow cytometric analysis in HeLa cells. These cells were cultured with DNA/PEI or DNA/PEI/PEG-PGA(65) complexes for 4 h in either pH 7.4 or 6.0. As shown in Fig. 7, the fluorescence peaks for the DNA/PEI/PEG-PGA(65) complex remarkably shifted to the right at pH 6.8, indicating that the internalization efficiency improved significantly in comparison with cells cultured at pH 7.4. The differences in endocytosis efficiency may indicate that PEG-PGA(65) shielding can achieve significantly enhanced gene transfection efficiency in an acidic tumor microenvironment.

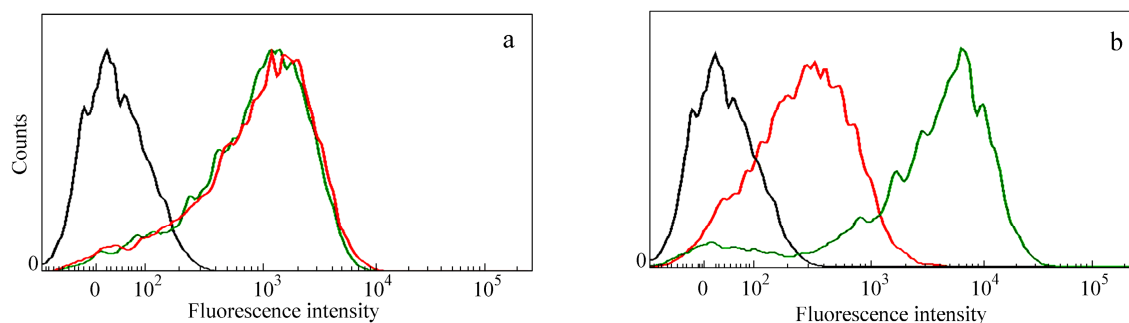


Fig. 7 Cellular uptake of DNA/PEI (1/1) (a) and DNA/PEI/PEG-PGA(65) (1/1/2) (b) at different pH values in HeLa cells (gray, control; red, pH 7.4; green, pH 6.8) (All complexing ratios are expressed as weight ratios.)

These findings suggest that PEG-PGA(65) should shield the positive charge of DNA/PEI, stabilize the complexes, minimize nonspecific interactions, and prolong circulation time during circulation *in vivo*. And the pH triggered deshielding should effectively mediate DNA to achieve considerably improved acidic tumor cell endocytic and improve the gene transfection efficiency.

CONCLUSIONS

Taking PEI-25k as the gene carrier model, PEG-PGA(65) was synthesized and characterized as shielding system. The PEG-PGA(65) could shield the DNA/PEI complexes efficiently. Gene transfection, CLSM and flow cytometric analysis at different pH values indicated that PEG-PGA(65) could deshield DNA/PEI polyplex and enhance gene transfection efficiency in acidic tumor microenvironment. We would like to believe that the PEG-PGA(65) would be promising to be used as the shielding system for polycationic gene carriers applied in *in vivo* gene transfection.

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