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Ternary complexes of amphiphilic polycaprolactone-graft-poly (N,N-dimethylaminoethyl methacrylate), DNA and polyglutamic acid-graft-poly (ethylene glycol) for gene delivery

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ABSTRACT

Binary complexes of cationic polymers and DNA were used commonly for DNA delivery, whereas, the excess cationic charge of the binary complexes mainly leads to high toxicity and unstability *in vivo*. In this paper, ternary complexes by coating polyglutamic acid-graft-poly(ethylene glycol)(PGA-g-mPEG) onto binary complexes of polycaprolactone-graft-poly(N,N-dimethylaminoethyl methacrylate) (PCL-g-PDMAEMA) nanoparticles (NPs)/DNA were firstly developed for effective and targeted gene delivery. The coating of PGA-g-mPEG was able to decrease the zeta potential of the nano-sized DNA complexes nearly to electroneutrality without interferring with DNA condensation ability. As a result, the stability, the escape ability from endosomes and the transfection efficiency of the complexes were enhanced. The ternary complexes of PCL-g-PDMAEMA NPs/DNA/PGA-g-mPEG demonstrated lower cytotoxicity in CCK-8 measurements and higher gene transfection efficiency than the binary complexes *in vitro*. In addition, Lactate dehydrogenase (LDH) assay was performed to quantify the membrane-damaging effects of the complexes, which is consistent with the conclusion of CCK-8 measurement for cytotoxicity assay. The *in vivo* imaging measurement and histochemical analysis of tumor sessions confirmed that the intravenous administration of the ternary complexes with red fluorescent protein (RFP) as payload led to protein expression in tumor, which was further enhanced by the targeted coating of PGA-g-PEG-folate.
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1. Introduction

Gene therapy holds great promise to treat cancers, viral infection and cardiovascular diseases [1–3]. The success of gene therapy requires efficient and safe carriers. Viral vector systems have generally been used, owing to their superior ability to deliver and express genes to target cells. However, the shortcomings (such as safety, immunogenicity, low transgene size and high cost) limit the application of viruses for gene delivery [4]. In order to overcome the drawbacks of viral carrier, non-viral carriers have been designed as alternative systems.

Synthetic cationic polymers as a main group of non-viral carriers have been widely researched, including polyethylenimine (PEI) and its derivatives [5–7], poly (L-lysine) (PLL) [8], polyamidoamine (PAMAM) [9], and poly(2-(N,N-dimethylamino)ethyl methacrylate) (PDMAEMA) [10] etc. These cationic polymers interact with negatively charged DNA through electrostatic interactions to form complexes. Among the many non-viral delivery systems, poly(N, N-dimethylaminoethyl methacrylate) (PDMAEMA) has shown great potential to construct ideal carrier because of its versatile synthesis strategies using atom transfer radical polymerization (ATRP) or reversible addition fragmentation chain transfer (RAFT) polymerization methods [11–17]. Recently, a group of amphiphilic cationic polymers was developed for gene delivery or codelivery of gene and anticancer drugs [11,16–24]. Whereas, the excess cationic charge of the cationic polymer/DNA complexes mainly leads to the high toxicity and unstability *in vivo*, due to protein adsorption. It is reported that introduction of PEG into the carriers can significantly reduce the cytotoxicity and improve the stability of the delivery

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systems *in vivo* by preventing protein adsorption into large aggregates [25–27]. However, conjugating hydrophilic PEG segments into the delivery system markedly reduced the gene transfection efficiency since the inserted PEG segments inhibit the binding of nucleic acid and reduce its association with cells [16,28–32]. Therefore, we designed ternary complexes for gene delivery to transfect cells with low cytotoxicity and high efficiency.

Our previous work [24] had indicated that the cationic amphiphilic poly(ϵ-caprolactone)-graft-poly(N, N-dimethylaminoethyl methacrylate) (PCL-g-PDMAEMA) nanoparticles (NPs) had the ability to entrap hydrophobic drug and nucleic acid payloads simultaneously, which also performed excellent gene transfection efficiency and pH responsive sensitivity to release encapsulated drug payload. Unfortunately, the binary complexes of PCL-g-PDMAEMA NPs/DNA showed high cytotoxicity, and they are not stable because of aggregating into large particles. In order to resolve the problems, we tried to conjugate PEG on PCL-g-PDMAEMA [16,17], but the cytotoxicity was improved slightly with sacrifice of the transfection efficiency and DNA-binding capacity, which were supposed as the mutual interference between PEG and PDMAEMA segments. Here, we introduced polyglutamic acid-graft-polyethylene glycol (PGA-g-mPEG and PGA-g-PEG-folate), a PEGylated anionic copolymer, on the surface of the binary complexes to form non-covalent post-PEGylated ternary complexes (PCL-g-PDMAEMA NPs/DNA/PGA-g-PEG) by electrostatic assembling (as shown in Scheme 1). In our expectation, polyglutamic acid should reduce the surface cationic charge and the hydrophilic PEG layer will reduce the cytotoxicity and improve the stability of the complexes, thereby prolonging the circulation, and at the same time the post-PEGylation on the cationic polymer NPs/DNA complexes is able to avoid affecting the bind of nucleic acid and the transfect efficiency. Physicochemical properties of the ternary complexes, including hydrodynamic diameter, zeta potential and DNA retarding ability, were characterized. *In vitro* gene transfection, intracellular uptake, and cytotoxicity were evaluated. Furthermore, we also investigated *in vivo* gene expression using tumor-bearing mice by tail intravenous injection.

2. Materials and methods

2.1. Materials

Branched PEI ($M_w = 25$ kDa) and ethidium bromide were purchased from Sigma Aldrich (St. Louis, MO). Agarose was purchased from GEN TECH (Shanghai, China). PGA ($M_w = 46$ kDa) was purchased from Vedan (Taichung, Taiwan). mPEG-NH_2 and folate-PEG-NH_2 ($M_w = 5$ kDa) were provided by JenKem Technology (Beijing, China). Opti-MEM, penicillin/streptomycin, fetal bovine serum (FBS) and trypsin were obtained from Gibco (Grand Island, NY). pGL3.0 (Promega, Madison, WI, USA), RFP and EGFP-N1 pDNA encoding EGFP (Clontech, Palo Alto, CA, USA) were amplified in the *Escherichia coli* DH5α, and isolated and purified by PureYield™ Plasmid Maxiprep System (Promega, Madison, WI, USA). The absorbance at the wavelength of 260 and 280 nm was measured by UV Spectrophotometer (Lambda 950 UV/VIS/NIR spectrophotometer, Perkin Elmer, MA, USA). BCA protein assay kit and ECL kit were bought from Pierce (Rockford, IL). Ripa buffer was purchased from Beyotime (Shenzhen, China). Rabbit EGFP (Abcam, Cambridge, MA) and antibody horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (Bio-Rad Laboratories, Hercules, CA) were used for Western blot.

2.2. Synthesis of PCL-g-PDMAEMA, PGA-g-mPEG and PGA-g-PEG-folate

PCL-g-PDMAEMA was synthesized as described previously [18,24]. Firstly, macroinitiator, poly(caprolactone-co-γ-(2-bromo-2-methylpropionate)-ϵ-caprolactone) (P(CL-co-BMPCL)), was synthesized by copolymerization of CL and BMPCL using aluminum isopropoxide as initiator in toluene solution at room temperature. The molecular weight of P(CL-co-BMPCL) calculated from ^{1}H NMR spectra was 9500 g/mol and P(CL-co-BMPCL) had about 3 BMPCL units. The molecular weight of P(CL-co-BMPCL) measured by gel permeation chromatography (GPC) was 2.03×10^4 g/mol, and its polydispersity index (PDI = M_w/M_n) was 1.29. Then, PCL-g-PDMAEMA was synthesized by ATRP of DMAEMA using P(CL-co-BMPCL) as macroinitiator. The bulk polymerization was carried out at 60 °C for 14 h and then the product was dissolved in THF. The polymer solution was purified by dialysis against double-distilled water for 24 h, and then freeze-dried to obtain the product.

In accordance with the approach in the reference [33], PGA-g-mPEG and PGA-g-PEG-folate were prepared. Briefly, PGA, mPEG-NH_2 or folate-PEG-NH_2 and NHS were dissolved in sodium tetraborate buffer (pH = 8.5). EDC was then added into the mixture with stirring. The reaction was allowed to proceed for 6 h at room temperature. After filtration, the reaction mixture was dialyzed (Spectra/Por, cut-off at MW 10,000) for 24 h, first against phosphate buffer (2 l, 0.1 M, pH = 7.4, Na_2HPO_4/NaH_2PO_4) and subsequently against deionized water (2 l). The product was frozen-dried and stored at −20 °C. The structure of PGA-g-mPEG and PGA-g-PEG-folate was characterized by ^{1}H NMR. The grafting percentage (X) is calculated as follows:

$$\frac{454 \times X}{2 \times 10} = \frac{A_{3.55}}{A_{2.13}}$$

$A_{3.55}$ means the characteristic proton peak area of PEG at $\delta = 3.55$ ppm; $A_{2.13}$ means the characteristic proton peak area of PGA at $\delta = 2.13$ ppm.

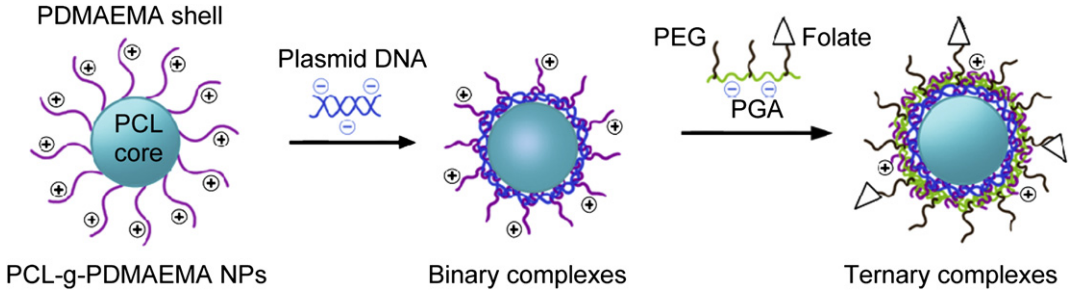
The grafting percentage is 6.7% and 5.4% for PGA-g-mPEG and PGA-g-PEG-folate, respectively.

2.3. Preparation of binary and ternary complexes

The charge ratio (N/P, N/P/C) of binary and ternary complexes was calculated as the moles ratio of moles of the amino groups (N) on PCL-g-PDMAEMA to the phosphate groups (P) on plasmid and the carboxyl groups (C) on PGA-g-PEG. Ternary complexes were prepared as follows. Briefly, plasmid (1 μg/50 μl) was mixed with 50 μl of PCL-g-PDMAEMA NPs at different N/P ratios to form binary complexes using a pipette. After incubation for 15 min, ternary complexes were obtained by the addition of 50 μl of PGA-g-mPEG into the binary complexes and then completely mixing the reaction mixture using pipette and incubating for another 15 min at room temperature before transfection and further characterization. For the N/P of 10, the concentration of PCL-g-PDMAEMA in binary complexes was 0.0633 g/l. For the N/P/C of 10/1/10, the concentration of PCL-g-PDMAEMA and PGA-g-mPEG in ternary complexes was 0.0422 g/l and 0.0992 g/l, respectively. The samples of ternary complexes for physiochemical characterization and *in vitro* experiments were constructed by PGA-g-mPEG and did not contain folate. PGA-g-PEG-Folate was only used in *in vivo* experiments.

2.4. Agarose gel electrophoresis

To assess DNA condensation ability of the NPs, agarose gel electrophoresis was performed. The binary and ternary complexes were formed as described above. Complexes (containing 0.2 μg DNA) were mixed with 4 μl 6× loading buffer and loaded into a 0.8 wt% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was set up in 1× TAE buffer at 120 V and kept for 40 min. DNA retardation was analyzed on UV illuminator to show the location of the DNA.



Scheme 1. Preparation of binary and ternary complexes by self-assembly method.

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