A pH Responsive Ternary Gene Carrier based on Branched-Poly(ethylenimine) and Poly(2-ethyl-2-oxazoline)-block-Poly(methacrylic acid)

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Abstract

This study demonstrates new ternary polyion gene carriers, based on the pH-responsive diblock copolymer poly(2-ethyl-oxazoline)-block-poly(methacrylic acid) (PEOz-b-PMAA), and the branched-poly (ethylenimine) (B-PEI). This study complexes the plasmid DNA with B-PEI and further with PEOz-b-PMAA to obtain ternary polyplexes (DNA/B-PEI/PEOz-b-PMAA). PMAA were partially dissociated under neutral pH with a negative charge, to attach to the positive charge surface of the B-PEI/DNA polyplex. The ternary polyplexes will also desorb and return to the original pre-poly complex to help gene release after cell uptake due to PMMA become neutral charge under an acid environment in endosome. The ternary polyplexes showed suitable mean particle size, low cytotoxicity, and acceptable transfection at pH 7.4. This ternary polyplex shows a suitable mean particle size and low cytotoxicity and acceptable transfection at pH 7.4. TEM morphology showed that the ternary complex micelles. The ternary polyplex constitutes a useful approach for gene carrier design.

Keywords: pH-Responsive; Ternary gene carrier; Poly (2-Ethyl-Oxazoline); Poly (Methacrylic Acid)

Introduction

Non-viral vectors, such as cationic polymers, peptides, and liposomes, have attracted interest because of their advantages compared to viral systems. For example, research suggests that non-viral vectors are safer, with more flexible structures and chemical properties [1-3]. Among the various types of polymer gene carriers, branched polyethyleneimine (B-PEI) is the most effective vector for delivering genes because the BPEI/DNA polyplexes exhibit great transgene expression in vitro and in vivo [4-5]. The high transfection efficiency of B-PEI is because of its high buffering capacity, which disrupts the endosomal membrane, and releases the polyplex into the cytoplasm [6]. The intrinsic high cytotoxicity of B-PEI limits its application in vivo. Excessive positive charge on the cationic polymer carriers’ surface is toxic to cells and tissues [7], and adversely influences biodistribution of the complexes in vivo [8]. Poly (2-ethyl-2-oxazoline) (PEOz) is a water-soluble polyelectrolyte, with low toxicity and high hydrophilicity similar to poly (ethylene glycol) (PEG) [9] and it is approved by the FDA as a biocompatibility polymer. The charge density in the linear polyethylenimine (LPEI) in our past research strongly affected cell viability, and the PEOz-coupled LPEI reduced cationic charge density and cytotoxicity [10].

Studies in recent years have examined the multilayer gene carriers made by “layer by layer self-assembly” [11-12]. Trubetskoy used the polyanion PMAA (poly(methacrylic acid)) and PEOz-coupled LPEI in our past research strongly affected cell viability, and the PEOz-coupled LPEI reduced cationic charge density and cytotoxicity [10].

Materials and Methods

2-ethyl-2-oxazoline (EOz) (Aldrich) and methyl p-toluenesulfo-
Synthesis of poly (2-ethyl-2-oxazoline)-poly (methacrylic acid), (PEOz-b-PMAA)

The diblock copolymer PEOz-b-PMAA was synthesized by free radical polymerization with the macroinitiator (PEOz) 2-ABCDA. The experiment dissolved (PEOz) 2-ABCDA in dichloromethane and heated it until 80°C, before adding MAA and stirring for 18 h. The product was purified by dialysis for 2 d using cutoff dialysis tubing with a molecular weight of 6000, and dried in a vacuum, obtaining the PEOz-b-PMAA product. The composition was analyzed by 1H NMR.

Preparation of B-PEI/DNA polyplex and PEOz-PMAA/B-PEI/DNA ternary polyplex

Polyplex preparation included diluting the required amount of polymer and 1 μg of luciferase encoded plasmid PUHC-13-3 in 100 μl of distilled water and gently mixing. The polyplex formulation was incubated at room temperature for 30 min before using. After preparing the B-PEI/DNA polyplex, the ternary polyplex was created by mixing the B-PEI/DNA solution and the PEOz-b-PMAA solution, and incubated for 30 m before using.

Measurement of particle size and zeta potential

To measure the particle size and zeta potential, various formulations of polyplexes were prepared. Dynamic light scattering was used to decide the particle size of polyplex particles in distilled water at 25°C (Zetasizer 3000HS, Malvern Instruments, Worcestershire, U.K.). Laser doppler micro-electrophoresis in the capillary electrophoresis cell of the Zetasizer 3000HS at 25°C determined the zeta potential of polyplexes prepared in distilled water. The mean values for these measurements were calculated from the data obtained in ten runs.

Cell culture and MTT assay

Human cervix carcinoma HeLa cells (1 x 10⁴ cells/well) were cultured onto a plate with ninety-six wells in DMEM media supplemented with 10% FBS in a humid atmosphere of 5% CO₂ at 37°C for 24 h. Then the growth medium was replaced with 200 μl medium that contained the desired amount of polymers and allowed to incubate for 72 h. The cytotoxicity of each sample was determined by measuring the cell viability using a tetrazolium dye (MTT) assay. 100 μl of the medium containing 10 μl of MTT PBS solution (5 mg/ml) was added. After incubation for 2 h, the formazan crystals were dissolved in 100 μl of DMSO/EtOH solution. The absorbance of each well was measured using a microplate reader (Stat Fax 2100, Awareness, Palm City, FL, USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. In vitro transfection and total protein assay

To evaluate the transfection potential of polyplexes, human cervix carcinoma HeLa cells (2 x 10⁴ cells/well) were incubated in DMEM media supplemented with 10% FBS and plated in six-well plates in a humid atmosphere of 5% CO₂ at 37°C for 24 h. The growth medium (2 mL) contained DNA or polyplexes that contained 5 μg of plasmid incubated at 37°C. After incubation for 48 h, the cells were washed with cold PBS and then lysed using 400 μL of the 1X cell lysis buffer. The cell lysate was centrifuged at 37°C for 24 h. The growth medium (2 mL) contained DNA or polyplexes that contained 5 μg of plasmid incubated at 37°C. After incubation for 24 h, the formazan crystals were dissolved in 100 μl of DMSO/EtOH solution. The absorbance of each well was measured using a microplate reader (Stat Fax 2100, Awareness, Palm City, FL, USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

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Morphology of the polyplex

The polyplex morphology prepared as described above was observed by transmission electron microscopy (TEM, Hitachi, H-7500, Tokyo, Japan). To prepare the TEM sample, 10 μl of polyplex solution was placed onto a copper grid coated with carbon, tapped with a filter paper to remove water, and air-dried for 5 m. Then, the grid was negatively stained with 2 wt% uranyl acetate solution for 30 s, tapped with a filter paper to remove water, and vacuum-dried for 24 h.

Cellular uptake of polyplexes

The amino end group of B-PEI was used to label the Cy5.5 dye to observe the cellular uptake behavior of polyplexes by a confocal laser-scanning microscope (CLSM). B-PEI was mixed with Cy5.5 NHS ester in DMSO at room temperature for 24 h. The product was purified by dialysis for 2 d in a dark bottle. Polyplex accumulated in HeLa cells was localized using LSM5 PASCAL CLSM (Carl Zeiss, USA). The HeLa cells were seeded on cover-slides for 24 h and then were treated with free DNA or polyplexes. After specific time, the cells were washed twice with PBS, and then LysoTracker was added to the culture medium without FBS and incubated for 20 m. Then the samples were stored at 4 °C refrigerator for 16 h, removed from the formaldehyde solution, and we added DAPI water solution (10μg/ml) for 5 m.

Result and Discussions

Polymer synthesis

Scheme 1 shows the synthesis of PEOz-b-PMAA. PEOz was coupled with ABCPA in DCC to form the macroinitiator (PEOz)-ABCPA. The catalyst, DPTS, prepared by equal mole PTSA and DMAP, incorporated with ABCPA in DCC to form the macroinitiator (PEOz).

| Polymer | Code     | Mn of PEOz | PDIa | Mn of PMAA | Total Mn |
|---------|----------|------------|------|------------|----------|
| PEOz2k-PMAA2k | E6M2    | 5300       | 1.15 | 2100       | 7400     |
| PEOz2k-PMAA4k | E6M4    | 5300       | 1.15 | 4100       | 9400     |
| PEOz10k-PMAA2K | E10M2   | 10500      | 1.06 | 1200       | 11700    |
| PEOz10k-PMAA4K | E10M4   | 10500      | 1.06 | 1400       | 11900    |
| PEOz15k-PMAA2K | E15M2   | 13900      | 1.11 | 1300       | 15200    |

Table 1: Compositions and characterization of diblock copolymer PEOz-b-PMAA.
value maintained an almost constant value of 30 mV with a weight ratio above 5. The previously mentioned results indicated that the B-PEI/DNA polyplex formed a most stable conformation with a weight ratio around 5. The following experiments adopted the B-PEI/DNA weight ratio of 5, and selected the B-PEI/DNA weight ratio of 1:3 as contrast forms. The code “B-PEI mx” represents m for the B-PEI/DNA weight ratio (μg/μg). For example, the code B-PEI 5X means the B-PEI/DNA weight ratio of 5, and selected the B-PEI/DNA weight ratio of 1:109.

**Ternary polyplexes:** This study prepared five compositions of PEOz-b-PMAA (E6M2, E6M4, E10M2, E10M4, and E15M2) to form ternary polyplexes by covering PEOz-b-PMAA polymers on the B-PEI/DNA core structure. The ternary polyplexes with a longer hydrophilic segment PEOz from PEOz-b-PMAA (E10M2, E10M4, and E15M2) showed higher particle size (above 200 nm) and zeta potential (around 30 mV). Increased positive charges and large size may be due to the higher hydrophilic force from the longer PEOz segment, exposing the positively charged PEI parts to the outer polyplex. The section below discusses the two kinds of PEOz-b-PMAA with a short PEOz segment (that is, E6M2 and E6M4). Figure 3 shows the results of particle size and zeta potential for the inner core, B-PEI 5X, and the outer shell, B-PEI 3X, B-PEI 1X, but zeta potential decreased to about -20 mV. It may be due to the ternary polyplexes having more negative charges from PMAA at the same weight ratio conditions.

The B-PEI parts are well-set inside the polyplex attracted by DNA, and the neutral PEOz parts are expected to come outside to provide less charge to the polyplex surface. The hydrophilic and neutral segment, PEOz, creates an optimal particle size and zeta potential to the polyplex for cell entry. The incoming negative charges from PMAA cover the positive charges of B-PEI/DNA. The greater the weight ratio of B-PEI/DNA is, the more PEOz-b-PMAA is needed to neutralize the B-PEI/DNA positive charge. In other words, the smaller the weight ratios of

**Figure 3:** Particle size and zeta potential of B-PEI 5X and ternary polyplex with B-PEI 5X inner core.

**Figure 4:** Particle size and zeta potential of B-PEI 3X and ternary polyplex with B-PEI 3X inner core.

**Figure 5:** Particle size and zeta potential of B-PEI 1X and ternary polyplex with B-PEI 1X inner core.
B-PEI/DNA are, the greater the negative trend for the zeta potential of the ternary polyplex. (Results of gel retardation assay are shown in supplementary information).

**Polyplexes in salt-containing media:** Figure 6 shows the results of ternary polyplexes prepared in an aqueous solution of 150 mM NaCl to discuss the stability of ternary polyplexes. The ternary polyplexes did not aggregate in salt-containing media. The results indicated that the neutral BPEI and LPEI polyplexes were prone to aggregate, because of the lack of electrostatic repulsion. The DNA/B-PEI/PEOz-b-PMAA ternary polyplexes were stable because they possessed a core-shell structure with a hydrophobic B-PEI/DNA core and a hydrophilic PEOz shell that prevented the polyplexes from aggregating.

**In vitro transfection and total protein assay**

This study evaluated *in vitro* transfection efficiencies of naked DNA, B-PEI/DNA polyplexes, and DNA/B-PEI/PEOz-b-PMAA (E6M2, E6M4) ternary polyplexes under various P/D ratios on the HeLa cell. The luciferase assay for the luciferase encoding plasmid determines transfection efficiency. This work used total protein production, an indirect measure of induced toxicity, to normalize the RLU. Figures 7 and Figures 8 present luciferase gene expressions of the naked DNA, B-PEI, E6M2, and E6M4 at optimum P/D weight ratios. The transfection using polyplexes resulted in greater efficiency compared to when using the naked DNA. The naked DNA with negative charges repelled the cell as it approached negatively charged cell membranes, resulting in poor transfection efficiency. The B-PEI polyplex transfection efficiency was approximately proportional to the weight ratio of B-PEI/DNA; the more B-PEI, the better the transfection result. Although the B-PEI polyplex has the highest gene expression, it has the lowest protein production, indicating the highest toxicity. A higher amount of B-PEI may kill the cell or retard physiological operation. Transfection decreased with a B-PEI/DNA weight ratio of 7.

This work compared the transfections of ternary polyplexes with B-PEI/DNA polyplexes. The ternary polyplex prepared from B-PEI 3X showed a decreasing tendency with increasing PEOz-b-PMAA. However, the ternary polyplex formed from B-PEI 5X showed better transition efficiency than the ternary polyplex formed from B-PEI 3X. The ternary polyplex formed from B-PEI 5X did not decline, but remained at the same level. The above phenomena match the results of zeta potential, the surplus negative charges from PMMA of ternary polyplex prepared from B-PEI 3X diminish the cell uptake outcome, but not in 5X.

**Cell toxicity and polyplex biocompatibility**

Figure 9 shows *in vitro* cytotoxicities of polymers, polycations B-PEI, as a typical polymer for gene transfer, and PEOz-b-PMAA (E6M2...
and E6M4 at various concentrations after 72 h incubation on HeLa cultures. This study treated the wells containing only media without polycations as positive controls and set them at a cell viability of 100%, calculating relative cell viability as [Abs] sample/[Abs] control x 100. The B-PEI exhibited stronger cytotoxicity with an IC_{50} = 2.5 μg/ml compared to PEOz-b-PMAA with IC_{50} = about 150 μg/ml for E6M2, and that of E6M4 was higher than 500 μg/ml. The cell viability of PEOz-b-PMAA declined as the PMMA percentage increased. It may be due to E6M4 has more negative charge from PMMA segment than E6M2 which make E6M4 hard to be absorbed by cell. Figure 10 shows polyplex cytotoxicities with B-PEI and PEOz-b-PMAA at various concentrations after 72 h incubation on HeLa cultures. To obtain the difference in biocompatibility between B-PEI/DNA and the PEOz-b-PMAA ternary polyplex, this study selected the ternary polyplex with a zeta potential of about 0~10mV for the HeLa cell viability test and compared it with the B-PEI/DNA polyplex. The cell culture incubated with DNA was the control. The results indicated cell viability: B-PEI 1X > B-PEI 3X > B-PEI 5X. After we covered it with PEOz-b-PMAA, the cell viability of these ternary polyplexes maintained high viability independent of the content composition of the core structure. Decreased toxicity resulted from shielding the positively charged polymer in the core by the PEOz hydrophilic shell structure. The previously mentioned results indicated a slight positive charge of the PEOz does not damage the cells as in B-PEI.

**Polyplex morphology by TEM observation**

TEM observed the polyplex morphology. The study incubated the polypexes in the phosphate buffer (pH=7) and succinate buffer (pH=5) solutions at room temperature for 1 h to observe the P^{+} -sensitive behavior. The TEM images in Figure 11(a) show that free DNA has a random coil structure. Figure 11(b), (c) and (d) show the B-PEI/DNA polyplex does not have a core-shell, but a global structure. In this case, when the B-PEI wraps the DNA to form a compact particle, the DNA cannot react with uranyl acid (UA) easily. The images of B-PEI/DNA demonstrate a white contrast in the particle center. Unlike B-PEI, E6M2/DNA and E6M4/DNA polyplexes form an obvious core-shell micelle structure as shown in Figures 12(a)–(d). The inner core, P(EOz/EI) complex with DNA, is negatively stained by UA and exhibits a dark image, where the nuclei acid from the DNA bond with UO_{2}^{+} due to UA dissociation [16]. The outer shell, the PEOz hydrophilic segment, provides less contrast. The hydrophilic and neutral charge segment PEOz of the shell may protect polyplexes from immune system destruction.

Figures 13(a) – (d) show the P^{+} -sensitivity of the ternary polyplex.
The pH response of the polyplex was carried out at pH 5. The random coil structure from DNA interconnected with the broken particles and the core-shell structures broke under acid conditions. The ruptured ternary polyplex formed a gel-like state and stuck together. The pH-responsive PEOz-b-PMAA produced by hydrogen bonding in an acidic environment may produce this state. The particle size showed the same results (Figure 14). Different from the B-PEI/DNA polyplex, the particle size of the ternary polyplex showed a great rise in the acidic environment. In short, the Pβ-sensitivity from PEOz-b-PMAA of the ternary polyplex may help DNA release from the polyplexes in the endosome after cell uptake.

**Cellular uptake of polyplexes**

Figure 15 shows the time-dependent fluorescence intensity of Cy5.5, LysoTracker, and DAPI after incubating B-PEI/DNA polyplexes and the ternary polyplex with HeLa cells. The current study used the LysoTracker and DAPI molecules as indicators in the acidic compartment and nucleus [17]. The CLSM traced the cell uptake process. The fluorescence signals of DAPI revealed a blue color, the signals of LysoTracker revealed a green color, and Cy5.5 was red. For B-PEI/DNA, the Cy5.5 signals intensified around the HeLa cell periphery. The B-PEI remained in the cell nucleus after 6 h. The cell uptake progress for the ternary polyplex was slower. Observations showed significant amounts of Cy5.5 signals at 6 h, and the ternary polyplex entered the cell nucleus at 12 h. The B-PEI/DNA polyplex showed a more obvious signal after 6 h, compared with the ternary polyplex because of the higher positive charge density on B-PBI for enhancing cell uptake.

**Conclusion**

This study synthesized the Pβ-responsive diblock copolymer PEOz-b-PMAA by polymerizing the MMA monomer with the macroinitiator (PEOz)-ABCPA. The study successfully prepared the ternary polyplex based on PEOz-b-PMAA and B-PEI, and evaluated the physicochemical and biological characteristics. The results showed the ternary polyplex efficiently covered up the positive charge of the B-PEI/DNA pre-complex and offered higher cell viability than the B-PEI/DNA polyplex. The ternary polyplex formed a clear core-shell structure by complexing with DNA under TEM observation. These polyplexes show Pβ-sensitivity to destroy core-shell structures and gene release in acidic buffers at a pH of 5. The non-viral gene carrier based on polyion PEOz-b-PMAA creates an optimal particle size and zeta potential for the polyplex, and

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**Figure 11:** TEM observation of (a) DNA and B-PEI/DNA (b) 1X, (c) 3X, (d) 5X.

**Figure 12:** TEM observation of ternary polyplex (a): BP(3X)/E6M2(2X), (b): BP(3X)/E6M4(2X), (c): BP(5X)/E6M2(2X), (d): BP(5X)/E6M4(2X).

**Figure 13:** TEM observation of polyplex under pH5, (a): B-PEI(5X), (b): BP(5X)/E6M2(5X), (c) - (d): BP(5X)/E6M2(5X).

**Figure 14:** Particle size of B-PEI/DNA and PEOz-b-PMAA/ B-PEI/DNA polyplexes for various pH.

**Figure 15:** Confocal images of HeLa cells incubated with LysoTracker, DAPI and ternary polyplex. Incubated time: 30 min, 1 hr, 6 hrs and 12 hrs. (Green fluorescence is associated with LysoTracker, blue fluorescence is associated with DAPI, and red fluorescence is associated with Cy5.5)
helps DNA release from polyplexes with pH-sensitivity. Accordingly, the ternary polyplex may have much potential in non-viral gene therapy.

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