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TITLE:

Methionine Functionalized Biocompatible Block Copolymers for Targeted Plasmid DNA Delivery

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KEYWORDS:

Reversible addition-fragmentation chain transfer; RAFT; drug delivery system; DDS; methionine; guanidine; *N*-(3-aminopropyl)methacrylamide hydrochloride; APMA; *N,N-bis*(2-hydroxyethyl)methacrylamide; BNHEMA

SUMMARY:

This work presents the preparation of methionine functionalized biocompatible block copolymers (mBG) via the reversible addition-fragmentation chain transfer (RAFT) method. The plasmid DNA complexing ability of the obtained mBG and their transfection efficiency were also investigated. The RAFT method is very beneficial for polymerizing monomers containing special functional groups.

ABSTRACT:

Reversible addition-fragmentation chain transfer (RAFT) polymerization integrates the advantages of radical polymerization and living polymerization. This work presents the preparation of methionine functionalized biocompatible block copolymers via RAFT polymerization. Firstly, *N,N-bis*(2-hydroxyethyl)methacrylamide-*b*-*N*-(3-aminopropyl)methacrylamide (BNHEMA-*b*-APMA, BA) was synthesized via RAFT polymerization using 4,4'-azobis(4-cyanovaleric acid) (ACVA) as an initiating agent and 4-cyanopentanoic acid

dithiobenzoate (CTP) as the chain transfer agent. Subsequently, *N,N-bis*(2-hydroxyethyl)methacrylamide-*b*-*N*-(3-guanidinopropyl)methacrylamide (methionine grafted BNHEMA-*b*-GPMA, mBG) was prepared by modifying amine groups in APMA with methionine and guanidine groups. Three kinds of block polymers, mBG1, mBG2, and mBG3, were synthesized for comparison. A ninhydrin reaction was used to quantify the APMA content; mBG1, mBG2, and mBG3 had 21%, 37%, and 52% of APMA, respectively. Gel permeation chromatography (GPC) results showed that BA copolymers possess molecular weights of 16,200 (BA1), 20,900(BA2), and 27,200(BA3) g/mol. The plasmid DNA (pDNA) complexing ability of the obtained block copolymer gene carriers was also investigated. The charge ratios (N/P) were 8, 16, and 4 when pDNA was complexed completely with mBG1, mBG2, mBG3, respectively. When the N/P ratio of mBG/pDNA polyplexes was higher than 1, the Zeta potential of mBG was positive. At an N/P ratio between 16 and 32, the average particle size of mBG/pDNA polyplexes was between 100-200 nm. Overall, this work illustrates a simple and convenient protocol for the block copolymer carrier synthesis.

INTRODUCTION:

In recent years, gene therapy has emerged for the therapeutic delivery of nucleic acids as drugs to treat all kinds of diseases[1]. The development of gene drugs including plasmid DNA (pDNA) and small interfering RNA (siRNA) relies on the stability and efficiency of the drug delivery system (DDS)[2]. Among all DDS, cationic polymer carriers have the advantages of good stability, low immunogenicity, and facile preparation and modification, which give cationic polymer carriers broad application prospects[3,4]. For practical applications in biomedicine, researchers must find a cationic polymer carrier with high efficiency, low toxicity, and good targeting ability[5]. Among all polymer carriers, block copolymers are one of the most widely used drug delivery systems. Block copolymers are intensively studied for their self-assembly property and abilities to form micelles, microspheres, and nanoparticles in drug delivery[5]. Block copolymers can be synthesized via living polymerization or click chemistry methods.

In 1956, Szwarc et al. raised the topic of living polymerization, defining it as a reaction without chain-breaking reactions[6,7]. Since then, multiple techniques had been developed to synthesize polymers using this method; thus, living polymerization is viewed as a milestone of polymer science[8]. Living polymerization can be classified into living anionic polymerization, living cationic polymerization, and reversible deactivation radical polymerization (RDRP)[9]. Living anionic/cationic polymerizations have a limited scope of application due to their strict reaction conditions[10]. Controlled/living radical polymerization (CRP) has mild reaction conditions, convenient disposition, and good yield and has thus been a major research focus in recent years[11]. In CRP, active propagation chains are reversibly passivated into dormant ones to reduce the concentration of free radicals and avoid the bimolecular reaction of propagating chain radicals. The addition polymerization can continue only if the inactive dormant propagating chains are reversibly animated into chain radicals. As one of the most promising forms of living radical polymerization, reversible addition-fragmentation chain transfer (RAFT) polymerization is a method applicable to yield block polymers with controlled molecular weight and structure, narrow molecular weight distribution, and carrying functional groups[12]. The key to successful RAFT polymerization is the effect of chain transfer agents, usually dithioesters, which possess

very high chain transfer constant.

In this paper, a RAFT polymerization method was designed to prepare BNHEMA-*b*-APMA block polymer, taking 4,4'-azobis(4-cyanovaleric acid) (ACVA) as an initiating agent and 4-cyanopentanoic acid dithiobenzoate (CTP) as a chain transfer agent. RAFT polymerization was used twice to introduce BNHEMA into the cationic polymer carriers. Subsequently, the amine groups in the APMA chain were modified with methionine and the guanidinylation reagent 1-amidinopyrazole hydrochloride. Making the use of the positive charges of the guanidinylation reagent and methacrylamide polymer skeleton structure, the cellular uptake efficiency of the obtained block polymer carriers was improved.

PROTOCOL:

1. Synthesis of BNHEMA polymer (**PBNHEMA**)

1.1 Dissolve 1.87 g of *N, N-bis*(2-hydroxyethyl)methacrylamide (BNHEMA) in 1 mL of distilled water in a polymerization bottle.

NOTE: The polymerization bottle is a round-bottom flask with a rubber stopper and a magnetic stirrer.

1.2 Dissolve 0.03 g of 4-cyanopentanoic acid dithiobenzoate (CTP) and 0.02 g of 4,4'-azobis(4-cyanovalericacid) (ACVA) in 0.5 mL of 1,4-dioxane in a 5 mL beaker. Then, add the CTP and ACVA solution to the polymerization bottle from step 1.1.

1.3 Ventilate the reaction system in the polymerization bottle with nitrogen via three freeze-pump-thaw cycles.

1.3.1 In detail, freeze the solution in the polymerization bottle using a condensate trap, fix the polymerization bottle to the iron support, and vacuumize and inject nitrogen into the reaction mixture via a conduit tipped with a needle (#9 needle, inner diameter 0.65 mm, outer diameter 0.9 mm). Seal the polymerization bottle and thaw the solution at room temperature for 30 min.

1.3.2 Repeat the freeze-pump-thaw cycles three times.

1.4 Put the polymerization bottle into a 70 °C oil-bath and let the solution react for 24 h under the nitrogen atmosphere.

1.5 Chill the polymerization bottle at 0 °C and open the rubber stopper to terminate the polymerization process.

1.6 Precool acetone in a -20 °C fridge for 2 h and then mix it with the reaction solution of step 1.5 at 50:1 (v/v). After that, centrifuge at 8,200 x *g* for 10 min to remove acetone and collect the precipitate.

1.7 To purify the synthesized PBNHEMA, dissolve the collected precipitate in 2 mL of pure water and then mix it with 100 mL of precooled acetone, at the ratio of 1:50 (v/v). Centrifuge the solution at 8,200 x *g* for 10 min and collect the precipitate. Repeat this process three times.

1.8 Dry the produced PBNHEMA using a 50 °C vacuum drier. Once dried, weigh the powder with a balance. Calculate the yield rate according to **Equation 1.**

$$Yield\ (\%) = \frac{mass\ of\ PBNHEMA\ (g)}{mass\ of\ BNHEMA\ (g)} \times 100\% \quad (1)$$

NOTE: In this experiment, the yield obtained was 77.2%.

2. Synthesis of BNHEMA-*b*-APMA polymer (BA)

2.1 Dissolve 0.96 g of *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA) and 0.93 g PBNHEMA in 5 mL of distilled water in a 10 mL beaker.

2.2 Dissolve 0.01 g of 4,4'-azobis (4-cyanovaleric acid) (ACVA) in 0.5 mL of 1,4-dioxane and mix with the APMA-PBNHEMA solution from part 2.1.

2.3 Transfer the mixture into a polymerization bottle and ventilate with dry nitrogen for 1 h.

2.4 Put the polymerization bottle into a 70 °C oil-bath and let it react for 24 h under the nitrogen atmosphere.

2.5 Chill the polymerization bottle at 0 °C and open the rubber stopper to terminate the polymerization process.

2.6 Transfer the solution to the chilled acetone from step 1.6, and then centrifuge the solution at 8,200 x *g* for 10 min to precipitate the BA.

2.7 Dissolve the BA in 2 mL of distilled water and precipitate the polymer in chilled acetone. Repeat three times.

2.8 Dry the produced BA in a 50 °C vacuum drier and weigh the obtained powder. Calculate the yield rate according to **Equation 2**.

$$Yield\ (\%) = \frac{mass\ of\ purified\ BA\ (g)}{mass\ of\ PBNHEMA\ (g) + mass\ of\ APMA\ (g)} \times 100\% \quad (2)$$

NOTE: In this experiment, the yield rate was calculated to be 82.0%.

3. Determine the mole percent of APMA in BA copolymer via **the ninhydrin method**

NOTE: Spectrophotometry is used to determine the contents of multicomponent amino acids. The principle is a color reaction of ninhydrin and amino acid where the absorbance is correlated with the amino acid content to a certain extent[13,14].

3.1 Dissolve 5 g of ninhydrin in 125 mL of boiling distilled water. Also, dissolve 5 g of Vitamin C in 250 mL of warm distilled water. Add the 250 mL of Vitamin C solution dropwise to the ninhydrin solution under magnetic stirring. Continue to stir for 15 min and then chill the reaction solution in a 4 °C fridge.

3.2 Take the solution out of the fridge and filter by suction using a Buchner funnel to obtain reduced ninhydrin. Collect the precipitate and preserve it in a phosphorus pentoxide dehydrator.

3.3 Dissolve 85 mg of ninhydrin and 15 mg of reduced ninhydrin in 10 mL of ethylene glycol monomethyl ether to prepare the ninhydrin-coloring solution.

NOTE: Ninhydrin-coloring solution can react with α-amino in the APMA and form a violet compound with a structure as described in a previous study[15].

3.4 Dilute 1 mL of 0, 1, 10, 100, 1,000 mg/mL APMA monomer solutions with 1 mL of acetate buffer (2 M, pH 5.4), and then add 1 mL of ninhydrin-coloring solution, respectively.

3.5 Heat the mixtures for 15 min in a boiling water bath and then cool them using running water. Let the solutions sit for 5-10 min and dilute them with 3 mL of 60% ethyl alcohol and mix them thoroughly. Measure the absorbance at 570 nm using a spectrophotometer and draw the standard curve (**Equation 3**).

$$\text{Concentration of APMA (mg/mL)} = \frac{\text{Absorbance of solution at 570 nm (a.u)} - 0.0095}{0.615} \quad (3)$$

NOTE: Equation 3 was derived from the linear fitting of the absorbance at 570 nm versus the APMA concentration.

3.6 Dissolve 0.01 g of BA in 1 mL of distilled water; add 1 mL of acetate buffer (2 M, pH 5.4) and 1 mL of ninhydrin-coloring solution. Calculate the molar content of APMA according to the absorbance at 570 nm.

$$\text{Molar weight of APMA (mmol)} = \frac{\text{Concentrate of APMA (mg/mL)} \times \text{volume of APMA soultion (mL)}}{\text{molecular weight of APMA}} \quad (4)$$

$$\text{Molar weight of BNHEMA (mmol)} = \frac{\text{mass of BA (mg)} - \text{mass of APMA (mg)}}{\text{molecular weight of BNHEMA (g/mol)}} \quad (5)$$

$$\text{Molar ratio of APMA in BA (\%)} = \frac{\text{Molar weight of APMA (mmol)}}{\text{Molar weight of APMA (mmol)} + \text{Molar weight of BNHEMA (mmol)}} \times 100\% \quad (6)$$

NOTE: The calculation formulas are as follows (**Equations 3-6**).

4. Synthesis of methionine grafted BA polymer (**mBA**)

4.1 Dissolve 8.9 mg of Fomc-Methionine in 5 mL of DMSO in a recovery flask.

4.2 Add 6.92 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl) and 4.86 mg of 1-hydroxybenzotriazole (HOBT) to the recovery flask and react at 0 °C for 0.5 h.

4.3 Dissolve 2.59 g of BA in 5 mL of DMSO solution and then add 50 µL of trimethylamine; add this solution dropwise to the recovery flask (step 4.2) and let the solution react for 0.5 h at room temperature.

4.4 Dialyze to remove DMSO and trimethylamine from the BA solution in step 4.3 using a dialysis bag (MWCO 10 kDa) in a 2 L beaker for 24 h; replace the deionized water every 6 h.

4.5 Freeze-dry the obtained mBA and weigh to calculate the yield rate according to **Equation 7**.

$$Yield\ rate\ (\%) = \frac{mass\ of\ purified\ mBA\ (g)}{mass\ of\ BA\ (g) + \ mass\ of\ Fomc - Methionine\ (g)} \quad (7)$$

NOTE: In this case, the yield rate was determined to be 71%.

4.6 Quantify the NH_2 containing mBA by measuring the absorbance at 570 nm to calculate the amount of grafted methionine. Calculate the molar content of methionine according to **Equation 8**.

$$\text{Molar ratio of methionine in mBA } (\%) = \text{Molar ratio of APMA in BA } (\%) - \text{Molar ratio of APMA in mBA } (\%) \quad (8)$$

5. Synthesis of guanidinated and methionine conjugated BNHEMA-*b*-APMA polymer (**mBG**)

NOTE: Three different mBA1, mBA2 and mBA3 copolymer were synthesized. mBA3 copolymer is used as an example in the following steps.

5.1 Dissolve mBA3 containing 60 µmol of amino group in 5 mL of pure water.

NOTE: Amino group content was quantified using the ninhydrin method as described in step 3.5.

5.2 Dissolve 40.6 mg (300 µmol) of guanidinylation reagent 1-amidinopyrazole hydrochloride in mBA solutions.

5.3 Adjust the pH to 9.0 with the saturated solution of sodium carbonate and let it stabilize for

24 h at room temperature.

5.4 Dialyze the mBG product with deionized water using a dialysis bag in a beaker (MWCO 10 kDa, 2 L) and preserve it in the form of a freeze-dried powder.

The yield percentage was calculated to be 85% via **Equation 8**.

$$\text{Yield \%} = \frac{mass\ of\ purified\ mBG\ (g)}{mass\ of\ mBA\ (g) + mass\ of\ \text{guanidinylation reagent}\ (g)} \quad (8)$$

5.5 Dissolve mBG powder in D_2O in the NMR tubes and characterize it using ^1H nuclear magnetic resonance spectroscopy (^1H NMR)[16].

6. Preparation and characterization of mBG/pDNA polyplexes

6.1 Dissolve 50 µg of pDNA in 50 µL of RNase/DNase-free water.

6.2 Dissolve 1 mg of mBG copolymers in 1 mL of RNase/DNase-free water.

6.3 Add the mBG copolymers solution directly into the pDNA solution according to different feeding ratios, that is, different N/P ratios (1:1, 4:1, 8:1, 16:1, and 32:1).

NOTE: N/P ratio is defined as the molar ratio of the guanidine group in the polymer and the phosphate group in pDNA, namely the molar ratio of the GPMA chain in the polymer and the mononucleotide in pDNA. The N/P ratio is calculated according to the molecular weights of amino nitrogen (N) in mBG and phosphate group (P) in pDNA.

6.4 Mix the solutions with a vortex mixer and allow them to stand for 30 min at room temperature. After that, disperse the mixture in phosphate buffer solution (PBS, pH 7.4) and preserve the obtained mBG/pDNA polyplexes at 4 °C for the follow-up experiments.

NOTE: The average particle size and Zeta potential of mBG and the complexes were detected using dynamic light scattering (DLS)[17].

6.5 Dilute 10 µL of the mBG/pDNA polyplex solutions with 1 mL of PBS (pH 7.4) in the DLS and Zeta potential sample cells.

NOTE: Particle size and Zeta potential detection were performed three times and an average of the three values was taken.

7. Electrophoretic retardation experiment of mBG/pDNA polyplexes

NOTE: An electrophoretic retardation experiment was conducted to determine the minimum charge ratio.

7.1 Take five groups of the mBG/pDNA polyplexes with different N/P ratios (1:1, 4:1, 8:1, 16:1, and 32:1) containing 50 µg of pDNA.

7.2 Add 6x loading buffer to the mBG/pDNA polyplex samples to a final concentration of 1x.

7.3 Add the solutions to the 1.5% agarose gels and run the gel at 90 mV for 15 min, using pDNA as control.

7.4 Take pictures of the gels using a gel imager.

8. Cytotoxicity of mBG/pDNA polyplexes

8.1 Seed MCF-7 cells into the 96-well plates at a density of 10^4 cells per well. Then, culture the cells for 12 h using DMEM medium (10% FBS and 1% antibiotic) in a humidified 37 °C incubator supplied with 5% CO_2.

8.2 Replace the culture medium with antibiotic-free DMEM culture media containing 10% fetal bovine serum (FBS) and mBG/pDNA polyplexes of different charge ratios (N/P 4, 8, 16, and 32, n=6) for 6 h, taking cells added with equal volumes of PBS solution as the control. Then, replace the culture medium with 150 µL of fresh 1640 medium and further culture the cells for 24 h.

8.3 Add 5 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 µL/well) to the 96-well plates and further culture the cells for 4 h.

8.4 Remove the solution and add 150 µL of DMSO to each well and shake the 96-well plates 30 sec.

8.5 Measure the optical density (OD) at 490 nm with a microplate reader to show the cell viability. Calculate the cell viability percentage according to **Equation 9**.

$$\text{Cell viability \%} = \frac{OD\ values\ of\ mBG/pDNA\ polyplexes}{OD\ values\ of\ control\ cells} \times 100\% \quad (9)$$

9. Transfection efficiency of mBG/ GFP-pDNA polyplexes

9.1 Dissolve 50 µg of pDNA containing the reporter gene green fluorescent protein (GFP) pDNA (GFP-pDNA) in 50 µL of RNase/DNase-free water. Then dissolve 1 mg of mBG copolymers in 1 mL of RNase/DNase-free water. Mix pDNA and mBG solution at a charge ratio (N/P) of 1:1, 4:1, 8:1, 16:1, and 32:1 and incubate for 30 min at room temperature. Disperse the mBG/GFP-pDNA polyplexes solution using ultrasonic waves (30 s) and store at 4 °C for the follow-up experiments.

NOTE: The N/P ratio is calculated according to the molecular weights of amino nitrogen (N) in

mBG and phosphate group (P) in pDNA.

9.2 Seed MCF-7 cells at a density of 2×10^5 cells per well in a 6-well plate and culture them at 37 °C and 5% CO_2 in a humidified incubator for 12 h.

9.3 Replace the culture medium with the fresh culture medium containing mBG/GFP-pDNA polyplexes of different N/P ratios (4, 8, 16, and 32) for 6 h.

9.4 Replace the medium with 2 mL of fresh RPMI1640 medium and culture for 48 h.

9.5 Collect the cells and detect the green fluorescence with a flow cytometer.

REPRESENTATIVE RESULTS:

BNHEMA was fed according to the objective degree of polymerization shown in **Table 1**; the synthesis procedure of mBG is shown in **Figure 1**. Firstly, BNHEMA homopolymer was prepared via the reversible addition-fragmentation chain transfer (RAFT) method in the water-dioxane system, using 4-cyanopentanoic acid dithiobenzoate as a chain transfer agent. Secondly, PBNHEMA was used as a chain transfer agent to prepare BNHEMA-*b*-APMA block polymer. APMA monomer was fed according to the objective degree of polymerization shown in **Table 1**. Gel permeation chromatography (GPC) was carried out to detect the molecular weight of BA with different feeding ratios (**Table 1**). The actual molar content of APMA chains in BA with different feeding ratios was detected via the ninhydrin method (**Table 1**). According to our earlier study[5], we used mBG3 in the present study because it has the highest APMA content. Finally, methionine was grafted to the APMA chain and the residual amine groups were completely guanidinylated to prepare mBG. **Figure 2** is the ^{1}H NMR data of mBG. The average particle size and Zeta potential of mBG/pDNA polyplexes were 124 nm and +15.7 mV, respectively, at an N/P ratio of 16 (**Figure 3**).

Electrophoretic retardation is a rapid and simple technique that involves separation of unbound pDNA and copolymer/pDNA polyplex based on differences in their electrophoretic mobilities in agarose gels. The unbound pDNA band can move in the agarose gel under the action of the electric field. In the electrophoretic retardation experiment (**Figure 4**), pDNA (with an N/P ratio of 0) can move without restraint in the agarose gel and was observed as a stripe in the gel imager. When pDNA is complexed with mBG, the movement of pDNA is retarded and subsequently, the brightness of the band is reduced. **Figure 4** shows that mBG can completely complex the pDNA when N/P is higher than 4.

The cytotoxicity of the mBG/pDNA polyplexes was measured using the standard MTT assay (**Figure 5**). The results show that mBG/pDNA polyplexes have obviously lower cytotoxicity than PEI/pDNA polyplexes at the N/P ratios of 4, 8, 16, and 32 in the MCF-7 cell line ($p<0.05$, $n=3$, one-way ANOVA). Furthermore, the cytotoxicity of mBG/pDNA polyplexes increases with increased N/P ratio. The increase of the cytotoxicity of mBG/pDNA polyplexes with an increased N/P is a result of the positively charged GPMA component. mBG/pDNA polyplexes showed less cytotoxicity than the PEI/pDNA polyplexes, which may be attributed to the presence of

BNHEMA in the copolymers shielding the surface charge of cationic polymers.

The N/P ratio used in the pDNA transfection experiment was selected according to the cytotoxicity results. As shown in **Figure 6**, the transfection abilities of mBG1, mBG2, and mBG3 were compared by measuring the GFP fluorescence intensity and the results showed that mBG3 is the optimal gene carrier. Although PEI/pDNA polyplexes with an N/P ratio of 8 have similar transfection efficiency as mBG3/pDNA polyplexes with an N/P ratio of 32, PEI has higher cytotoxicity and has no functions such as drug loading, which restricts its application in the biomedical field. The results revealed that the increase of AMPA content is key to improving the transfection efficiency of mBG copolymer. As we mentioned in an earlier study[5], cell penetrating peptides (CPPs) are 9-35 mer cationic or amphipathic peptides with an ability to penetrate the cell membrane. The guanidine group is a cationic CPP that can be conjugated into a polymer to enhance the transfection of polymer/pDNA polyplex through a protein-independent transmembrane pathway. Therefore, mBG copolymer integrates advantages relating to guanidine groups for cell penetration and the BNHEMA component for shielding, showing great potential for effective gene delivery.

FIGURE AND TABLE LEGENDS:

Table 1: The composition of BNHEMA-*b*-APMA by RAFT polymerization.

Figure 1: Schematic synthesis procedure of mBG.

Figure 2: ^{1}H NMR spectra of mBG in D_2O.

Figure 3: Particle size and Zeta potential of the polyplexes. (**A**) The particle size of BA/pDNA. (**B**) The particle size of mBG/pDNA. (**C**) Zeta potential of BA/pDNA. (**D**) Zeta potential of mBG/pDNA.

Figure 4: Agarose gel electrophoresis images of BA3 (**a**)**,** mBA3 (**b**) and mBG3 (**c**) polyplexes at different charge ratios.

Figure 5: Cytotoxicity of mBG/pDNA polyplexes at different N/P ratio in MCF7 cell line. PEI/pDNA polyplex was used as the control. The data were shown as mean ± SD (n=3). * indicates $p<0.05$ as compared to the PEI group.

Figure 6: Transfection efficiency of mBG/pDNA polyplexes at N/P ratio of 4, 8, 16 and 32 were measured by flow cytometer. The data were showed as mean ± SD (n=3).

DISCUSSION:

This study introduced a series of BNHEMA-b-APMA block polymer cationic gene carriers. These block polymers were synthesized via the reversible addition-fragmentation chain transfer (RAFT) method. The hydrophilic segment BNHEMA was introduced to improve solubility. Methionine and guanidine groups were modified to improve the target ability and transfection

efficiency[5]. The APMA chain content increased and guanidinylation in mBG copolymer reduced the particle size of mBG/pDNA polyplexes. The particle size and surface potential make the complex easy to across the cell membrane and applicable to transfection. The critical points in the experiment lie in strictly controlling the molar ratio of the BNHEMA monomer and CTP to 45:1, the molar ratio of CTP and ACVA to 2:1, and the temperature to 70 °C. During the purifying process, the volume ratio of precooled acetone and polymer solution must be higher than 50:1.

With the same N/P value, along with the increase of the proportion of the APMA chain, the particle size of mBG/pDNA polyplexes decreases. These results indicate that APMA chain content increase and guanidinylation can reduce the particle size. The particle size and surface potential make the complex easy to transport across the cell membrane and applicable to transfection. Gel electrophoresis was used to investigate the relationship between complexing and N/P ratio and the data showed that the minimum N/P is 4. This study will provide valuable basic data for further study of cationic gene carriers. However, BNHEMA-*b*-APMA is not easily degraded and its metabolism process in vivo is not clear enough. Therefore, clinical application is still a great challenge for cationic polymer gene carriers.

High efficiency and low toxicity of gene carriers are the necessary conditions for their clinical use. Cationic polymer gene vectors have advantages of good stability and no immunogenicity and they can be prepared and modified easily.

RAFT polymerization is widely used in the polymerization of monomers including styrene, methacrylate, acrylonitrile, and acrylamide. This kind of polymerization process can be carried out at low temperature. Also, RAFT polymerization can be used to synthesize polymers of fine structures. RAFT polymerization described in this protocol is considered one of the most promising potential methods for the preparation of biomedical polymers.

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The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this article.

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14 Qin, Z., Liu, W., Guo, L. , Li, X. Studies on Guanidinated N-3-Aminopropyl Methacrylamide-N-2-Hydroxypropyl Methacrylamide Co-polymers as Gene Delivery Carrier. *Journal of Biomaterials Science, Polymer Edition.* **23**, 1-4 (2012).
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16 Habuchi, S., Yamamoto, T., Tezuka, Y. Synthesis of Cyclic Polymers and Characterization of Their Diffusive Motion in the Melt State at the Single Molecule Level. *Journal of Visualized Experiments.* **115**, 1-9 (2016).
17 Rao, D.A., Nguyen, D.X., Mishra, G.P., Doddapaneni, B.S., Alani, A.W. Preparation and Characterization of Individual and Multi-drug Loaded Physically Entrapped Polymeric Micelles. *Journal of Visualized Experiments.* **102**, 1-5 (2015).

Figure 1

Click here to access/download;Figure;Figure 1 scheme.jpg

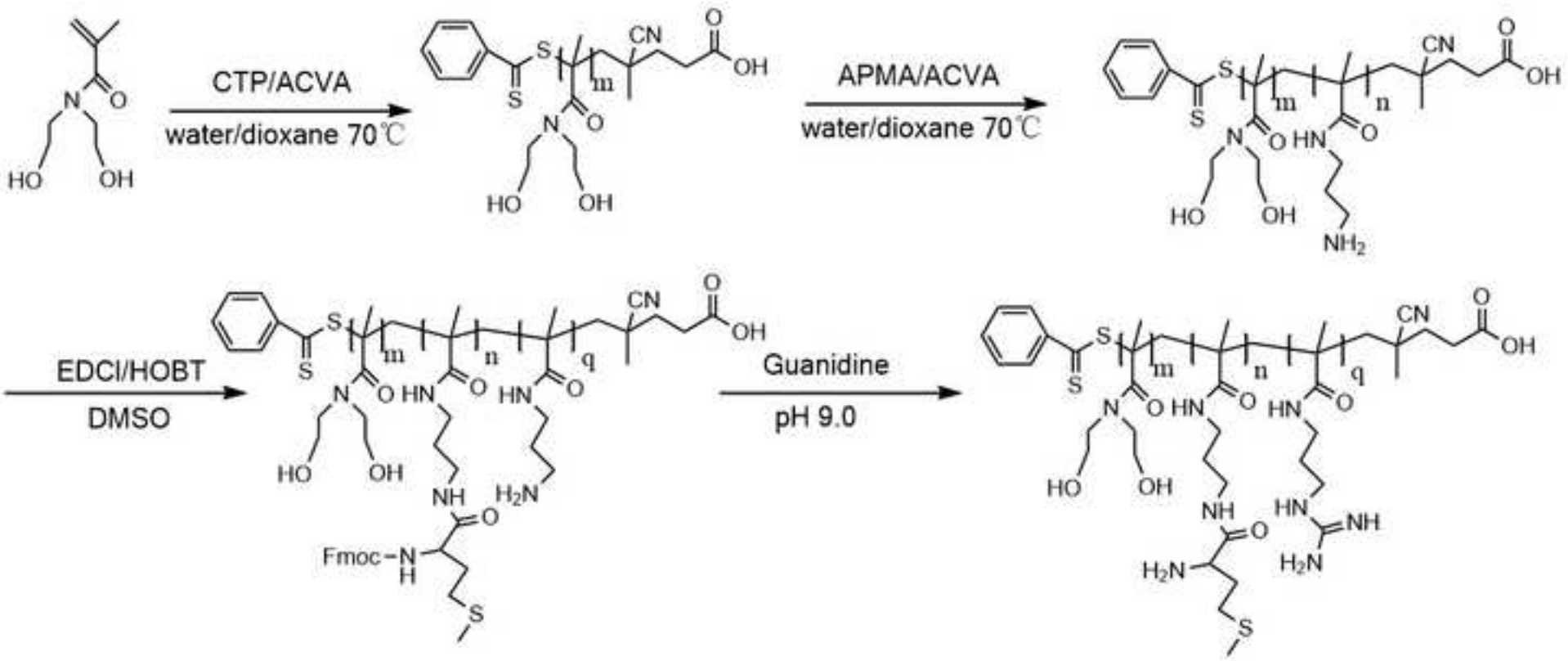

Figure 2

Click here to access/download;Figure;Figure 2 1H NMR spectra.jpg

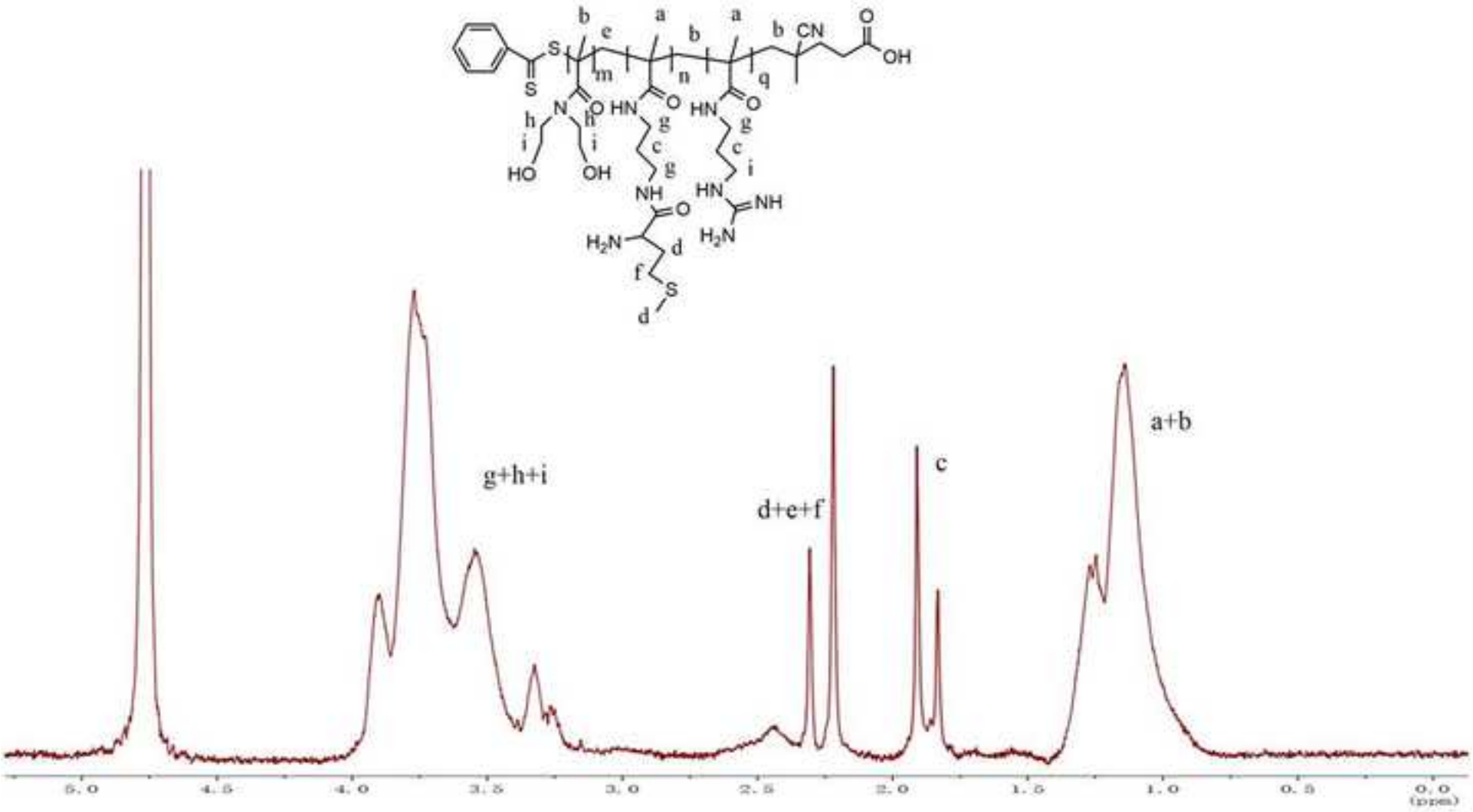

Figure 3

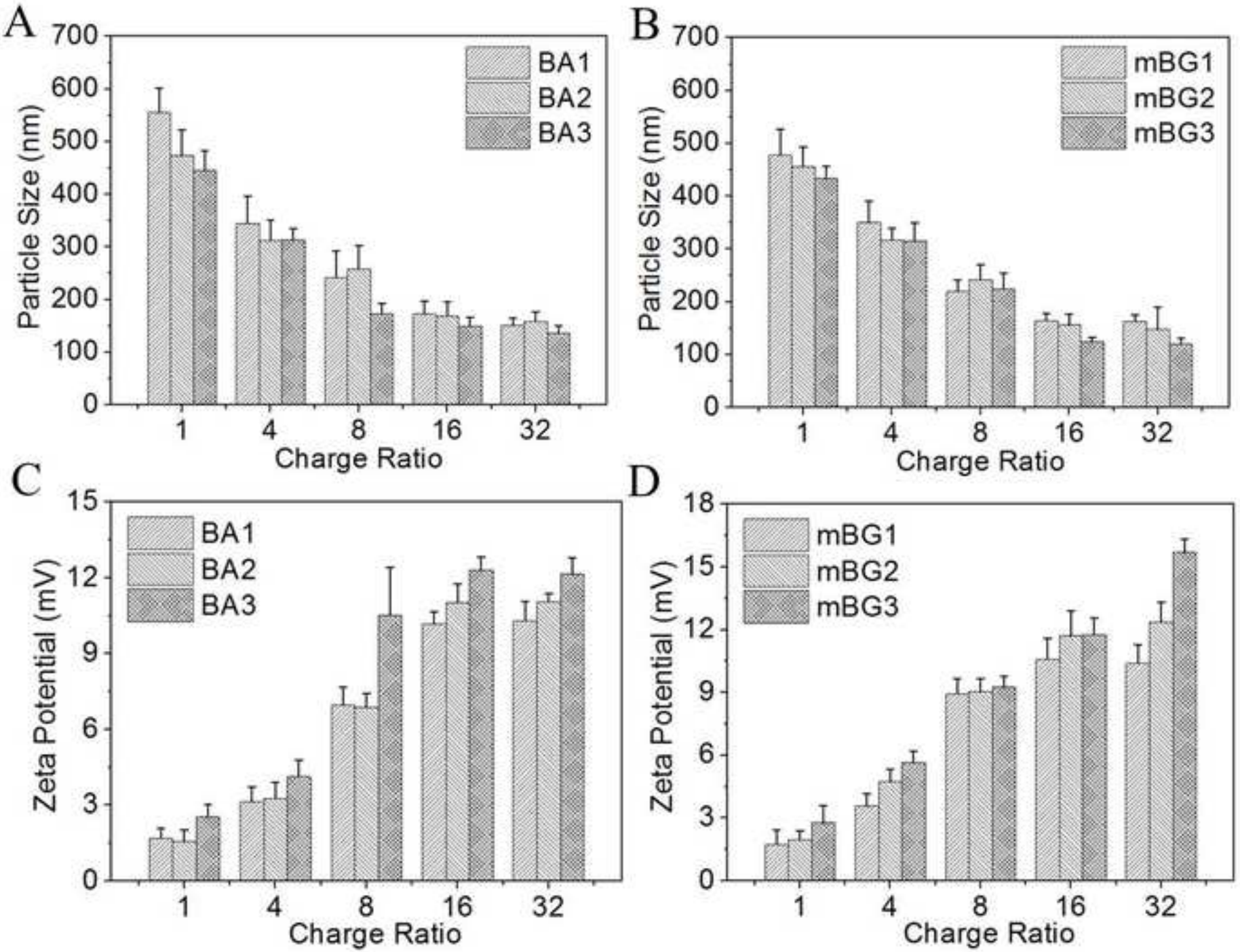

Figure 4

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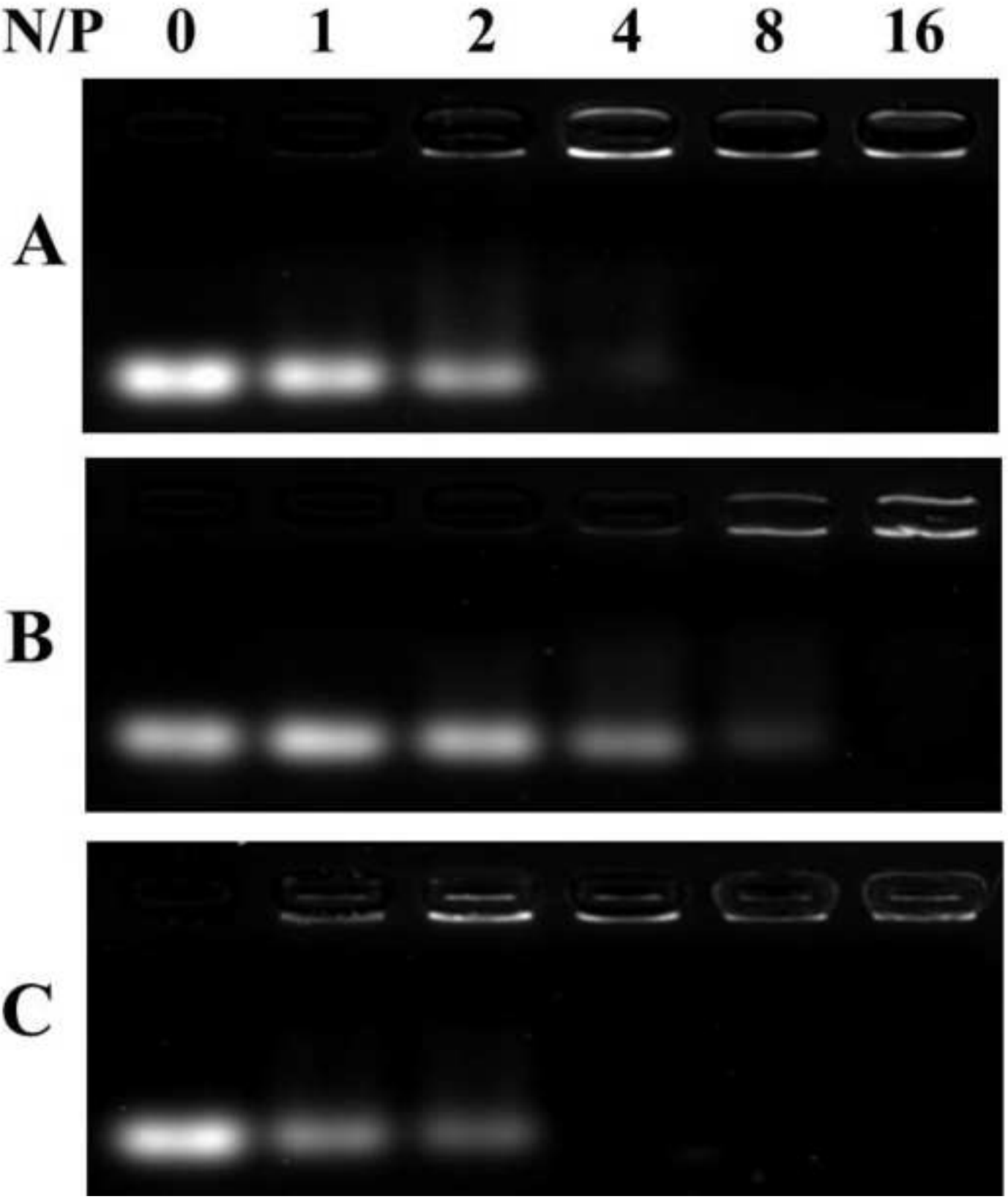


Figure 5

Click here to access/download;Figure;Figure 5 cell viability.tif

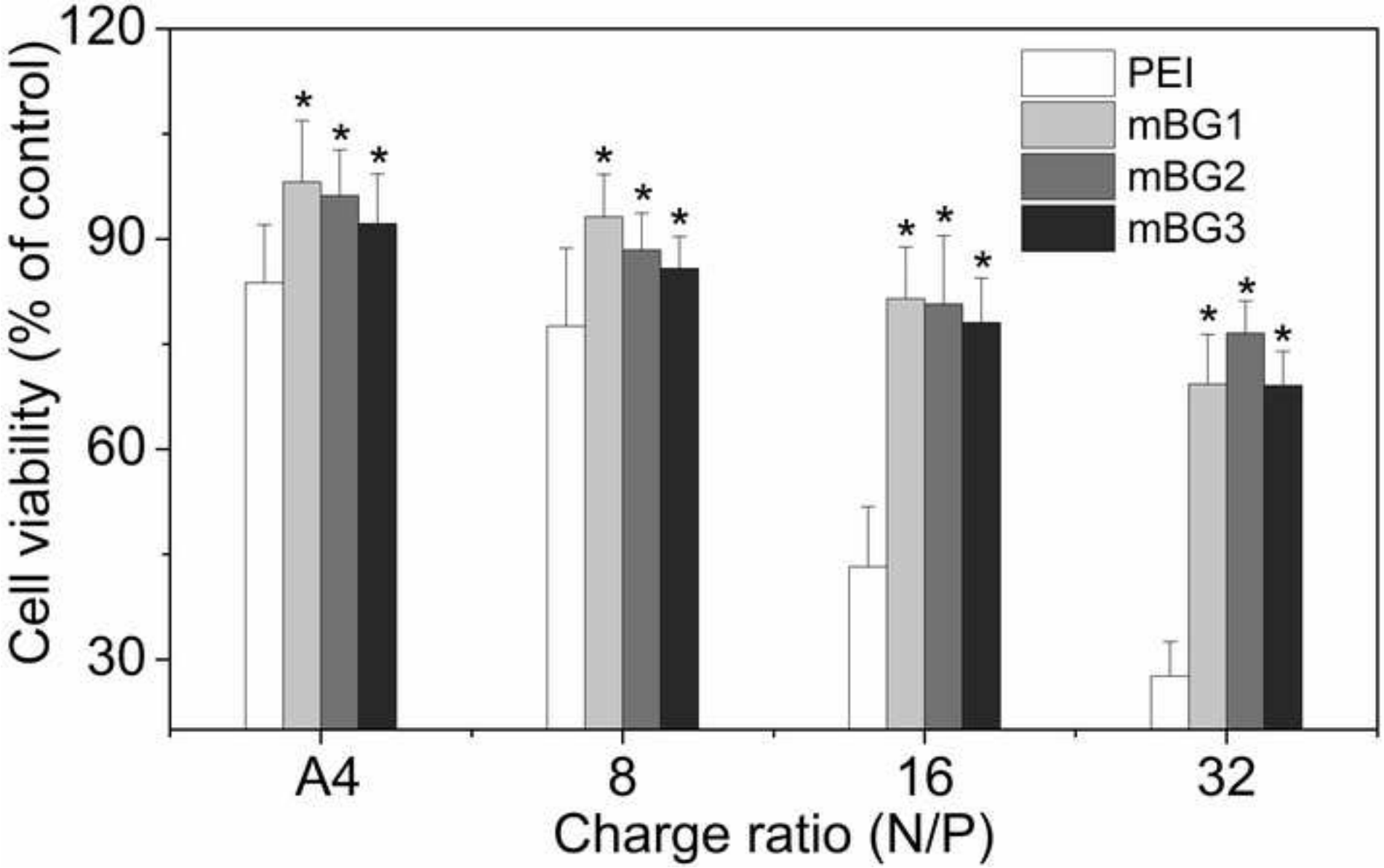
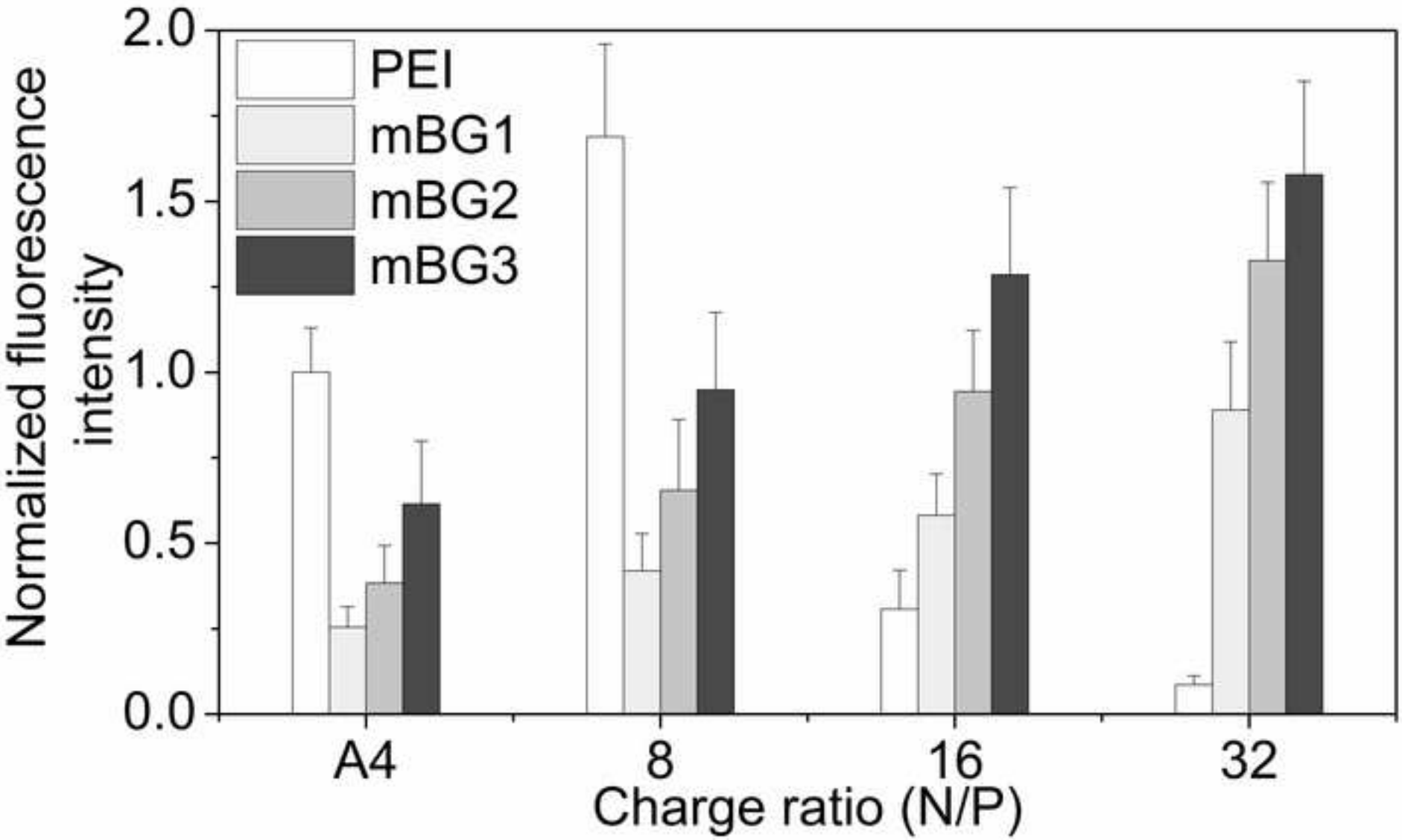


Figure 6



Table

Sample	BHEMA-b-APMA	
	Theoretical value of APMA content (%)	Actual value of APMA content (%)
$BNHEMA_{90}$-*b*-$APMA_{30}$(BA1)	25%	21%
$BNHEMA_{90}$-*b*-$APMA_{60}$(BA2)	40%	37%
$BNHEMA_{90}$-*b*-$APMA_{90}$(BA3)	50%	52%

Mw	PDI
16200	1.25
20900	1.21
27200	1.25

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-hydroxybenzotriazole	Macklin Biochemical Co., Ltd,China	H810970	≥97.0%
1,4-dioxane	Sinopharm chemical reagent Co., Ltd, China	10008918	AR
1-amidinopyrazole Hydrochloride	Aladdin Co., Ltd., China	A107935	98%
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride	Aladdin Co., Ltd., China	E106172	AR
4,4'-azobis(4-cyanovaleric acid)	Aladdin Co., Ltd., China	A106307	Analytical reagent (AR)
4-cyano-4-(phenylcarbonothioylthio)pentanoic Acid	Aladdin Co., Ltd., China	C132316	＞97%(HPLC)
Acetate	Sinopharm chemical reagent Co., Ltd, China	81014818	AR
Acetone	Sinopharm chemical reagent Co., Ltd, China	10000418	AR
Agarose	Aladdin Co., Ltd., China	A118881	High resolution
Ascorbic acid	Aladdin Co., Ltd., China	A103533	AR
DMSO	Aladdin Co., Ltd., China	D103272	AR
Ethylene glycol	Aladdin Co., Ltd., China	E103319	AR

N-(3-aminopropyl)methacrylamide hydrochloride	Aladdin Co., Ltd., China	N129096	⩾98.0%(HPLC)
N,N-bis(2-hydroxyethyl)methacrylamide	ZaiQi Bio-Tech Co.,Ltd, China	CF259748	⩾98.0%(HPLC)
Ninhydrin	Aladdin Co., Ltd., China	N105629	AR
PBS buffer	Aladdin Co., Ltd., China	P196986	pH 7.4
Plasmid DNA	BIOGOT Co., Ltd, China	pDNA-EGFP	pDNA-EGFP
Plasmid DNA	BIOGOT Co., Ltd, China	Pdna	pDNA
Sodium carbonate decahydrate	Aladdin Co., Ltd., China	S112589	AR
Trimethylamine	Aladdin Co., Ltd., China	T103285	AR

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Author(s): Wei Zhang, Jian Zhang, Zhi-Xiang Mao, Li Ding, Hao Li, Yang Wu, Jin-Hai Tang

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Dear editor,

Thanks for your kindles suggestions. We carefully reviewed this manuscript and revised all the comments you and reviewers mentioned.

Jin-Hai Tang
Department of General Surgery, the First Affiliated Hospital with Nanjing Medical University, Nanjing 210029, China

---

Editorial comments:

1. Please copyedit the manuscript carefully for any grammar or spelling issues.
A: We have polished the language of this manuscript.

2. Please address specific comments marked in the attached manuscript.
A: We have answered the specific comments in this manuscript.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.).
A: We have ensured all text in the protocol section is in imperative tense.

4. Please ensure you answer the “how” question, i.e., how is the step performed?
A: We have answered all the “how” questions.

5. After all the changes are made please ensure that the highlight is no more than 2.75 pages including headings and spacings.
A: We have checked the highlight in this manuscript.

6. Scheme 1: missing from the submission.
A: The missing Scheme1 was changed into Figure 1 and have been uploaded.

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A: All figures in this manuscript are original.

Reviewer #4:

Manuscript Summary:

This manuscript describes the RAFT polymerization of block copolymers for the complexation and cellular delivery of DNA. It covers polymer synthesis, some polymer characterization, complex formation/characterization, and cell culture experiments. Given the popularity of this research topic, it is certainly of interest. However, this manuscript severely lacks clarity, and many important experimental conditions are omitted. Furthermore, much of what is presented could be considered highly inconvenient at best and substandard or wrong at worst.

Major Concerns:

Outside the very poor descriptions, the biggest problem is that the described polymerization protocol will lead poor polymerization control and high polymer dispersities (as evidenced by all 3 of the representative results having dispersities near 1.5, the theoretical dispersity for uncontrolled radical polymerization).

A：The ^{1}H NMR result shows that the BA copolymers have been synthesized successfully. And the average particle size of BA/pDNA polyplexes was smaller than 200 nm and the PDI of the polyplexes was lower than 0.3. We think our GPC method had the problem of inaccuracies in measuring the polydispersity index of polymers, and we are conducting more detection for our samples.

Namely, the authors describe an oxygen removal strategy that is most assuredly inadequate. While bubbling nitrogen through the solution for 1 h prior to polymerization is a common practice, leaving the reaction vessel open and exposed to air during this time completely defeats the purpose of purging. Radical polymerizations should be septum-sealed prior to oxygen removal, and then should either be purged by bubbling nitrogen through the solution (along with a vent needle) or subjected to multiple freeze-pump-thaw cycles. Exposure to air at any point is unacceptable.

A：We have added freeze-pump-thaw cycles step in the manuscript (Section 1.3).

Furthermore, the authors describe the aqueous RAFT polymerization of primary amine-containing monomers in pure, distilled water. Such conditions will lead to rapid aminolysis of RAFT agent, resulting in uncontrolled polymerization. Aqueous RAFT of amine-containing monomers requires a buffered solution of pH = 5 or lower with a buffer concentration greater than or equal to the monomer concentration.

A：APMA (N-(3-Aminopropyl)methacrylamide hydrochloride) we used in this manuscript exists in the mode of hydrochloride. The method of polymerization was reported by our previous studies. [1-4]

1. Qin Z, Liu W, Li L, Guo L, Yao C, Li X: **Galactosylated N-2-Hydroxypropyl Methacrylamide-b-N-3-Guanidinopropyl Methacrylamide Block Copolymers as Hepatocyte-Targeting Gene Carriers**. *Bioconjugate Chemistry* 2011, **22**(8):1503-1512.

2. Wu Y, Ji J, Yang R, Zhang X, Li Y, Pu Y, Li X: **Galactosylated 2‐hydroxypropyl methacrylamide‐s‐3‐guanidinopropyl methacrylamide copolymer as a small hairpin RNA carrier for inhibiting human telomerase reverse transcriptase expression**. *The journal of gene medicine* 2014, **16**(5-6):109-121.

3. Wu Y, Qin Z, Ji J, Yang R, Zhang X, Li Y, Yin L, Pu Y, Li X: **Galactosylated poly (ethylene glycol) methacrylate-st-3-guanidinopropyl methacrylamide copolymers as siRNA carriers for inhibiting Survivin expression in vitro and in vivo**. *Journal of drug targeting* 2014, **22**(4):352-364.
4. Wu Y, Zhang W, Li T, Ma R, Chen D, Zhang J, Wu J, Tang J: **Multivalent methionine-functionalized biocompatible block copolymers for targeted small interfering RNA delivery and subsequent reversal effect on adriamycin resistance in human breast cancer cell line MCF-7/ADR**. *The Journal of Gene Medicine* 2017, **19**(8):e2969.

Additionally, the authors describe no polymer characterization other than an unnecessary ninhydrin assay. Common practice is to characterize all polymers (both macroCTA and final product) by size exclusion chromatography (SEC) and 1H NMR. Strangely, it appears that the authors performed these techniques, but they make no mention of either, other than off-handedly saying to characterize by NMR without further description. These techniques alone provide ample information to determine polymer molecular weights and compositions. The ninhydrin assay is tedious and redundant. If someone were using this protocol to learn how to polymerize block copolymers, not only would they be learning poor synthetic practices, they would also have an inaccurate understanding of the fundamentals of RAFT polymerization.

A: The ^{1}H NMR results of each step were added in this manuscript. Characterization results of mBG copolymer were show in Figure 2 and Table 1. The ninhydrin assay was used as additional experiments before synthesizing mBG copolymers. [1]
1. Qin Z, Liu W, Li L, Guo L, Yao C, Li X: **Galactosylated N-2-Hydroxypropyl Methacrylamide-b-N-3-Guanidinopropyl Methacrylamide Block Copolymers as Hepatocyte-Targeting Gene Carriers**. *Bioconjugate Chemistry* 2011, **22**(8):1503-1512.

Minor Concerns:
The introduction hardly provides significant background on the subject. The subject fundamentals are barely addressed, if at all, and virtually no literature examples are provided. Furthermore, the rationale for the describe approach is not discussed. Why block copolymers? Why these monomers? Why modify with methionine? Additionally, the title mentions that these complexes are cell-targeting, but no aspect of the polymers is described as having that property.
A: We modified the introduction and discussion sections.

General protocol comments: In addition to masses, it is generally preferred to list the number of moles used in a given step. The manuscript references synthesizing 3 different polymers, but only 1 is described. Grafting of methionine to the APMA units still results in a primary amine on those units. The quantification protocol does not account for this. Furthermore, it is likely that some of these later become guanidinylated. All of this drastically affects the calculated N:P.
A: In our previews study [4], we studied the relation between the APMA content and the DNA loading capacity of the polymers. (First paragraph in the REPRESENTATIVE RESULTS section)
4. Wu Y, Zhang W, Li T, Ma R, Chen D, Zhang J, Wu J, Tang J: **Multivalent methionine-functionalized biocompatible block copolymers for targeted small interfering**

RNA delivery and subsequent reversal effect on adriamycin resistance in human breast cancer cell line MCF-7/ADR. *The Journal of Gene Medicine* 2017, **19**(8):e2969.

Line by line issues:

71-72: Technically, only cationic polymerization is considered truly living, but the term "living" is highly discouraged regardless. Additionally, IUPAC recommends "Reversible deactivation radical polymerization (RDRP)" over "CRP."

A: We have modified these sentences (Introduction section).

82: By "two-disulfide esters," do the authors mean dithioesters? RAFT agents most commonly are dithioesters and trithiocarbonates, with the latter being by far and away the most popular in recent years.

A: We have modified “two-disulfide esters” with “dithioesters”.

98: As polymerization kinetics can change with reaction vessel shape and size, the "polymerization bottle" must be specified. Stir bars?

A: Polymerization bottle was descripted in this manuscript (Section 1.3 note).

104-11: I honestly have no idea what is being described here. See above comments about proper oxygen removal techniques.

A: We have modified the experimental steps here (Section 1.3).

113: Radical polymerizations are terminated by exposure to air (i.e. oxygen). Cooling the reaction slows it to a near halt, after which exposure to air terminates all chains consistently.

A: We modified the sentence in Section 1.5.

131-157: The same comments above apply here.

A: We have modified this section (Section 2.5).

133-134: APMA in distilled water? No wonder your dispersities are so high! This reaction has to be buffered at pH < 5 to prevent CTA aminolysis.

A：APMA (N-(3-Aminopropyl)methacrylamide hydrochloride) we used in this manuscript exists in the mode of hydrochloride.

159-198: See above comments regarding ninhydrin assay.

A: We have added the ^{1}H NMR data in this manuscript. The ninhydrin assay was used as an additional experiment.

207-211: Addition of polymer to the methionine solution is never mentioned.

A: We have modified this manuscript (Section 4.3).

243: Proton delay time? Relevant resonance shifts?
A: The proton delay time was 1.2s and the internal standard was TSP.

260-270: Complex formation is heavily dependent on ionic strength of the solution. Thus, complex size, polydispersity, and aggregation behavior will change drastically between pure water and PBS. I would highly advise against sonication as well.
A: We have modified mBG/pDNA polyplexes preparation in Section 6.4.

288: Media? Serum?
A: we have added the culturing conditions of MCF-7 cell line (Section 8.1).

301: Describe how cell viability is calculated. Also, controls and multiple replicates are necessary.
A: The computing method of cell viability was added in section 8.5.

402-403: In addition to the CTA:I ratio, the M:CTA ratio is even more important. M:CTA determines the polymer length, while CTA:I predominantly affects the polymerization rate.
A: The M:CTA ratio was added in this manuscript.

Reviewer #5:

The current version of the manuscript gave an interesting story of a novel in vitro transfection material, which has a similar transfection efficacy comparing to PEI. But as an article of protocol, it would be very helpful if the authors can add some detailed descriptions on the procedure of cell transfection part.

1. I am very confused, what is the relation between "mDG" and "mBG"? Why the authors sometimes show the result of mDG, and sometimes mBG, but there is no comparison between each other? Also, mBG in figure 4 legend, but mDG in figure 4. Is it a typo?

A：We are sorry, it is a typo and we have modified the abbreviation of the copolymer into “mBG” in the new manuscript.

2. In page 5 and 6, there are two "6.3".

A：We have carefully modified all the serial numbers in the manuscript.

3. In the section of "Preparation of mBG/pDNA polyplexes", the detailed procedure of the DNA polyplex preparation should be given. For example, how to mix pDNA and mBG, add which into which, whether vortex is needed, adding dropwise or directly add everything in. Also, the parameters of sonication, such as water bath or probe sonication, ultrasound power, temperature and interval…, were important, since over-sonication may break pDNA.

A: We have modified section of "Preparation of mBG/pDNA polyplexes". (Section 6.3 and 6.4)

4. In section 7, the preparation procedure is not entirely the same as the one in section 6. Why the sonication step is missing in this part?

A: We have simplified the description in section 7.

5. In the section 8 and 9, detailed procedure of in vitro transfection should be added, for example, why the sonication time is 30 sec here (1 min in 6.3 and no sonication in 7.1)? Do FBS and antibiotics in media affect transfection efficiency? How much pDNA/mBG was added into the cells, all the 50 ug pDNA in one well?

A: We have modified Section 8 and 9 and added some experimental details.

6. In line 350-351, the authors stated that "The results show that mBG/pDNA polyplexes have less cytotoxicity than PEI/pDNA polyplexes at the N/P of 4, 8, 16 and 32 in the MCF-7 cell line". But seems like it is not true from figure 4, at least at N/P=4 and 8, there may be no statistical difference between PEI and mBGs, if the authors do think so, please indicate the statistics method, n=? and P value. Actually, combined figure 4 and 5, at the optimal transfection condition (PEI: N/P=8, mBG3 or mDG3: N/P=32), the cell viabilities are very similar.

A: We have added the statistical data and modified the description (Paragraph 3, Section REPRESENTATIVE RESULTS).

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Ok

2. Please address specific comments marked in the manuscript.

I have done so for any comment that required a response or edit. I have also added new comments.

3. More results are needed for the validation of the polymer being synthesized. Please see my comments.

Ok. I think you added the required information. Please let me know if you have further concerns about this point.

4. Once done, please ensure that the protocol highlight is no more than 2.75 pages including headings and spacings.

If only the highlighted sections are put into a separate document it is less than 2 pages. Not all contiguous sections of the protocol were highlighted (for example 2.6-2.8); those sections were not considered.

5. Please proofread the manuscript carefully to ensure that there are no grammar or spelling issues.

I have done so. Please let me know if there are any questions about the edits or comments.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.).

Ok