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Evaluation for the effects of amphiphilic oligomer in PEI based ternary complexes on the improvement of pDNA delivery

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Abstract

Two kinds of novel oligomers were prepared by reversible addition fragmentation chain transfer (RAFT) polymerization and incorporated into the polyethyleneimine (PEI) gene delivery system through non-electrostatic assembly to improve gene transfection efficiency. The non-electrostatic assembly process was first investigated via probing the interactions of the oligomers with plasmid DNA (pDNA), PEI and AD-293 cells by quartz crystal microbalance (QCM). The results show that the prepared oligomers almost had no interaction with pDNA while they had much stronger interactions with PEI and AD-293 cells. Meanwhile, we found that the two kinds of oligomers had different interactions with AD-193 cells, which caused different effects on gene transfection. The data of QCM tests combining with the in vitro transfection results can be used to explain what effects of the oligomers have on improving gene transfection. The results also indicate that the strategy of detecting the interactions of oligomers with pDNA, polycations and cells will contribute to predetermine whether the prepared oligomer is efficient to improve gene transfection.

1. Introduction

Gene therapy holds great potential in treatment of various diseases, however it is largely dependent on the development of gene delivery vectors. In recent years, since viral vectors have limitations of immunogenicity, oncogenicity and high cost, non-viral vectors have been considered to be more suitable for gene delivery.[1-5] Among the numerous non-viral gene vector materials, polyethyleneimine (PEI) as a polycation is widely used. The gene transfection

efficiency and cell cytotoxicity of PEI are proved to be closely related to its molecular weight (MW).[6-9] Both the transfection efficiency and cell cytotoxicity increase with the increasing of molecular weight of PEI. To obtain the ideal PEI gene vector with both high transfection efficiency and low cytotoxicity, covalent and conjugated grafting strategy are usually adopted.[10-12] However, one of the problems with the covalent modification is that it will consume amino groups of polycations, which would change the characteristics of polycations and the corresponding polycation/pDNA complexes.[13] In order to conquer this drawback, our group recently has proposed a new strategy based on incorporating oligomers into ternary complexes through non-electrostatic assembly to improve gene transfection. The synthesized functional oligomers were incorporated into Poly (L-lysine) (PLL) or PEI gene delivery systems through non-electrostatic assembly to enhance gene transfection efficiency and reduce cell cytotoxicity,[14-16] which could prove that the incorporation of oligomers to obtain ternary complexes through non-electrostatic assembly is a promising strategy to modify cheap and common polycations into ideal gene vectors. The reason why the oligomers could be condensed together with pDNA by polycations we supposed was due to the synergetic hydrophilic and hydrophobic interactions of the oligomers with polycations. The hydrophilic group of the oligomers can maintain the stability of the complexes in serum and the hydrophobic group can help to be condensed by the polycations through hydrophobic interactions. But whether the synthesized oligomers could improve gene transfection can only be seen through complicated and tedious cell experiments. So there is vital importance to find a certain way which can be used beforehand to detect whether the prepared oligomer is efficient in improving gene transfection.

QCM has been extensively used in detection or recognition of biomacromolecules as a mass-sensitive device in the last several decades. It can be introduced to test the interactions between materials, nucleic acids and cells by detecting the mass change due to the adsorption behavior.[17-19]

Here we synthesized two kinds of novel hydrophobicity and hydrophilicity balanced oligomers [denoted as P(HEMA-b-NIPAM) and P(PEGMA-co-NIPAM)] which were incorporated into PEI/pDNA complexes to improve gene transfection. QCM was firstly used to measure the interactions of the oligomers with pDNA, polycation PEI and AD-293 cells. The

combination of QCM results with preliminary transfection results can be used to clarify how the oligomers are incorporated into the ternary complexes to enhance gene transfection efficiency. As we all know, 2-hydroxyethyl methacrylate (HEMA) is a water soluble monomer, and the solubility of the polymer of HEMA is molecular weight dependent.[20] Poly(ethylene glycol) methyl ether methacrylate (PEGMA) is a kind of water soluble macromolecular monomer. Although the polymer of N-isopropyl acrylamide (NIPAM) is water soluble, it also has a lower critical solution temperature (LCST).[21] It is believed that the hydrophobicity of the polymer of NIPAM at 37 ˚C above its LCST can enhance gene transfection.[22, 23] So the copolymerization of HEMA and PEGMA with NIPAM may reach a balance on hydrophobicity and hydrophilicity, which can improve the gene transfection. The hydrophilic segment of gene vectors can maintain the stability of gene complexes in serum, meanwhile the hydrophobic segment of gene vectors can increase the transfection efficiency.[24-26] Based on those considerations, we designed the hydrophobicity and hydrophilicity balanced block oligomer P(HEMA-b-NIPAM) and copolymer P(PEGMA-co-NIPAM). Reckon that PEGMA is a macromolecular monomer, we used easy random copolymerization to prepare comb-like oligomer P(PEGMA-co-NIPAM). And in contrast, block copolymerization was adopted to prepare P(HEMA-b-NIPAM) to guarantee the balance on hydrophobicity and hydrophilicity of the oligomer. These two kinds of oligomers were incorporated into PEI/pDNA complexes to obtain the ternary complexes respectively. Electrophoresis and dynamic light scattering (DLS) were used to evaluate the pDNA condensation capacity and stability PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes. The in vitro gene transfection efficiency and cytotoxicty of the ternary complexes were tested using human cervical cancer cells (HeLa) and human hepatoma cells (HepG2).

2. Experimental

2.1. Materials and reagents

Polyethylenimine (branched, PEI, average M_W = 10 kDa; PEI-25k, average M_W = 25 kDa), poly(ethylene glycol) methyl ether methacrylate (PEGMA, average Mn = 475) were purchased from Sigma Aldrich (St. Louis, MO). The molecular weight of PEI we used in this work is 10 kDa, positive control of PEI is 25 kDa. 2, 2′- Azoisobutyronitrile (AIBN) was recrystallized from methanol. 4-Cyanopentanoic acid dithiobenzoate (CPADB) as chain transfer agent was prepared according to literature.[27] 2-Hydroxyethyl methacrylate (HEMA), N-isopropyl

acrylamide (NIPAM), sodium tetrahydroborate ($NaBH_4$) and N,N-dimethylformamide (DMF) were obtained from The Sixth Chemical Reagent Company (Tianjin, China). Trypsin–ethylenediaminetetraacetic acid (EDTA), agarose, ethidium bromide, bovine serum albumin (BSA), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), phosphate buffer solution (PBS, 0.01 M, pH=7.2-7.4), fluorescein isothiocyanate (FITC) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were obtained from Dingguo Biotech. Co. Ltd. (Tianjin, China). Plasmid pEGFP-N1 (4.7 kbp, Clontech, Palo Alto, CA, USA) encoding enhanced green fluorescent protein (EGFP) was driven by immediate early promoter of CMV. The plasmid DNA (pDNA) was propagated in DH5α strain of *E.coli* and purified by use of Endfree plasmid kit (Tiangen, China). The purity and concentration were confirmed by spectrophotometry (A260/A280). Other agents used were all of the highest purity available.

2.2. Instrumentation

A Varian UNITY-plus 400 spectrometer operated at 400 MHz was used to record the ^1H NMR spectra of the chemical structures of the synthesized oligomers.

The molecular weight and polydispersity index (PDI) of the prepared oligomers were determined with a gel permeation chromatograph (GPC) instrument (equipped with Waters 2414 refractive index detector and Waters 1525 Binary HPLC Pump, using Waters styragel HT2, HT3, HT4, Tetrahydrofuran (THF) 7.8 *300 mm^2 columns). THF was used as an eluent solvent and polystyrene standard samples were used to calibrate the results.

The morphology of the surface of the QCM gold electrode was characterized by a multimode IIIa AFM (Veeco Metrology, USA) under ambient conditions. The samples were prepared as follows: firstly, the gold electrode was first immersed in the Piranha etching solution (v/v 30% H_2O_2/concentrated H_2SO_4) for 5 min, and then rinsed with deionized water and ethanol for three times respectively, finally dried under gentle nitrogen flow atmosphere. After that the electrode was immersed in DMF solution of R-P(HEMA-b-NIPAM) or R-P(PEGMA-co-NIPAM) (concentration was 0.1 mM). 48h later, the electrode was washed three times by ethanol. Then the AFM samples were prepared by breaking the QCM gold electrode into small pieces and put on mica.

The quartz crystal microbalance (QCM200) from Stanford Research Systems Inc. (USA) is composed of crystal holder, oscillator module, frequency counter and PC interface connection

for signal output visualization. A 5 MHz AT-cut piezoelectric quartz wafer (2.54 cm in diameter) attached with two gold electrodes (0.40 cm^2 active oscillation area) on both sides was used as the transducer of the QCM sensor of this study.

The gel shift assays were conducted on 1.5% agarose gel in Tris–boric acid–EDTA buffer (pH=8.0) at 100 V for 45 min.

The transmission electron microscope (TEM; FEI Tecnai G2 F20) was used to observe the morphologies of prepared complexes. The complexes were suspended in PBS and then placed on the copper grid. The excess liquid was air dried and the samples were not stained.

The complexes sizes and zeta potentials were determined with a Zetasizer Nano Series Nano-ZS (Malvern) instrument equipped with a 4.0 mW He-Ne laser at a scattering angle of 90° producing a wavelength of 633 nm.

2.3 Preparation of macromolecular transfer reagent

In a typical RAFT living polymerization, HEMA (25.6 mmol), AIBN (0.335 mmol), CPADB (1.67 mmol) were dissolved in 10ml of DMF. After degassing three freeze-pump-thaw cycles, the polymerization was conducted in a sealing glass tube at 65°C for 24h. The reaction was quenched in ice water when the polymerization finished. P(HEMA) (Fig. 1(a)) was obtained after precipitation in cold ether and washed three times. The product was dried in the vacuum for 24h before use.

2.4 Preparation of P(HEMA-b-NIPAM) copolymer

P(HEMA) (1.08 g, 1.84 mmol), NIPAM (24.0 mmol), AIBN (0.0817 mmol) were dissolved in 3ml of DMF. After degassing three freeze-pump-thaw cycles, the polymerization was conducted in a sealing glass tube at 70°C for 48h. When the polymerization finished, the reaction was quenched in ice water. The product was precipitated in cold ether and washed three times. Then the copolymer (Fig. 1 (a)) was purified by dialysis against deionized water for 2 days (cutoff Mw = 0.8 kDa) and lyophilized.

2.5 Preparation of P(PEGMA-co-NIPAM)

In a typical RAFT polymerization, PEGMA (5 mmol), NIPAM (25 mmol), AIBN (0.494 mmol), CPADB (0.871 mmol) were dissolved in 5ml of DMF. The polymerization was conducted in a sealing glass tube at 70°C for 24h after degassing three freeze-pump-thaw cycles. Then the reaction was quenched in ice water. The product (Fig. 1 (b)) was precipitated in cold ether and

washed three times. The copolymer was further purified by dialysis against deionized water for 2 days (cutoff Mw = 3.5k) and lyophilized.

2.6 Reduction of the ending groups of oligomers

In a typical reaction, P(HEMA-b-NIPAM) (30mg) was dissolved in deionized water, to which, 3ml of aqueous solution of $NaBH_4$ (concentration of $NaBH_4$ is 1.0M) was added.[28] The mixture was then stirred for 4days at room temperature. When the reaction finished, the mixture was dialyzed against deionized water for 2days (cutoff Mw = 0.8kDa) and lyophilized. The obtained reduced copolymer was denoted as R-P(HEMA-b-NIPAM) (Fig. 1(a)).

P(PEGMA-co-NIPAM) was treated the same way to give the reduced copolymer, denoted as R-P(PEGMA-co-NIPAM) (Fig.1 (b)).

2.7 Preparation of PEI/oligomer/pDNA ternary complexes

To obtain the ternary complexes, calculated pDNA and P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) were first mixed and left for 15 min at room temperature. Then according to the N/P ratio, calculated PEI was added to the mixture. The assembly process was schemed in Fig. 2. The molecular weight of PEI we used in this work is 10 kDa, positive control of PEI is 25 kDa. The N/P ratios were 10 and 15. The weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) was 0, 1, 2, 3, 4 times that of PEI and the corresponding ternary complexes were denoted PEI/P(HEMA-b-NIPAM)-x/pDNA or PEI/P(PEGMA-co-NIPAM)-x/pDNA in which the ‘‘x’’ represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI.

2.8 QCM measurement

The gold electrode was first immersed in the Piranha etching solution (v/v 30% H_2O_2/concentrated H_2SO_4) for 5 min, then rinsed with deionized water and ethanol for three times respectively, finally dried under gentle nitrogen flow atmosphere. After that the electrode was immersed in DMF solution of R-P(HEMA-b-NIPAM) or R-P(PEGMA-co-NIPAM) (concentration was 0.1 mM). 48h later, the electrode was washed three times by ethanol and transferred to the chamber of the 5MHz quartz crystal microbalance. Before each measurement, PBS was injected into the chamber to equilibrate. For the resonance tests of oligomer modified electrode with PEI, the basic oscillation frequency was first recorded (f_0) while it was stabilizing within ± 2Hz in 30min in PBS, and then 150μL of PEI solution sample in

different initial concentrations (10^{-6}g/ml, 10^{-5}g/ml and $5*10^{-5}$ g/ml) was injected into the flow cell. 20 min of each injection, the oscillation frequency was recorded again (f_i) and the frequency shift could be calculated ($\Delta f_i=f_i-f_0$). Then 10ml of NaOH (concentration was 0.5M) was injected into the chamber to elute the PEI binded to the oligomer modified electrode, followed with the injection of PBS until the oscillation frequency was back to the basic frequency. And the resonance tests of oligomer modified electrode and pDNA, AD-293 cells were carried out in the same way.

2.9 Gel shift assays

The condensation ability of pDNA by PEI in PEI/P(HEMA-b-NIPAM)/pDNA complexes was investigated by gel shift assays. The amount of pDNA employed for each sample was 120 ng. PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes were prepared as mentioned above. Electrophoresis was conducted on 1.2% agarose gel at 100 V for 45 min (trypsin-EDTA, pH= 8.0). Then pDNA bands were stained with ethidium bromide at 1.0 μg/ml and visualized with an UV illuminator.

2.10 Complex size and zeta potential

To determine the complex sizes and zeta potentials, dynamic light scattering (DLS) was employed. The PEI/P(HEMA-b-NIPAM)/pDNA complexes were prepared as mentioned above and 5 μg pDNA was used for each sample.

2.11 Cell culture

The AD-293 adenovirus-transformed human embryonic kidney cell line (AD-293), human cervical cancer cell line (HeLa) and human hepatoma cell line (HepG2) were purchased from ATCC (Teddington, UK) and maintained in DMEM containing 10% FBS without any antibiotics at 37 °C, 5% CO_2 atmosphere.

2.12 In vitro transfection

For in vitro transfection, AD-293 cells were seeded in 24-well plates at a density of $5*10^4$ cells per well in 500μl of complete DMEM media and transfected the next day at 70~80% confluence. Then the PBS solution of PEI/P(HEMA-b-NIPAM)/pDNA or PEI/P(PEGMA-co-NIPAM)/pDNA ternary complexes at a concentration of 1μg pDNA per well was added. The cells were further incubated for 48 h. According to our previous work the cells transfected with PEI-25k/pDNA complexes at the N/P ratio of 10 were used as the positive

controls.[29] The GFP positive cells were observed under an inverted fluorescence microscope and images were recorded with a CCD camera (Nikon ECLIPSE TE2000-U, Japan). The gene transfection efficiency was determined with a flow cytofluorometer (BD FACS Calibur, USA) equipped with an argon laser exciting at 488 nm, at least 5,000 cells were counted.

2.13 Cytotoxicity assays

Complexes with 0.5μg pDNA per well were prepared. AD-293 cells were seeded in 96-well plate at a density of 8,000 cells/well in 100 μl complete medium. PBS solution of PEI/P(HEMA-b-NIPAM)/pDNA or PEI/P(PEGMA-co-NIPAM)/pDNA ternary complexes or PEI-25k/pDNA binary complexes were added after 24h. The cells were cultured for additional 24 h. Then the media were replaced with 100 μl of MTT solution (1 mg/ml, diluted with complete media). The cells were further cultured for 4 h. After that the MTT solution was replaced with 100 μl of DMSO and the plate was shaken for 10 min in dark to dissolve the formed formazan. Absorbance of the dissolved formazan was measured with a microplate reader (Labsystem, Multiskan, Ascent, Model 354 Finland) at 490 nm.

2.14 Cellular uptake of complexes

To investigate the cellular uptake of complexes, PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes were prepared and transfection was carried out under serum conditions with AD293 cells as mentioned above. At 6 h post-transfection, the cells were washed three times with 500μl of PBS, fixed with 4% paraformaldehyde and observed under the fluorescence microscope. The fluorescence images were recorded with a CCD camera.

2.15 Statistics

Data were expressed as mean ± standard deviation. Significance between the control and treated groups were performed with the Student's t-test. Experiments were performed at least in triplicate. The levels of statistical significance were set at $P < 0.05$ (*) or $P<0.01$ (**).

3. Results and discussion

3.1 Synthesis of P(HEMA-b-NIPAM) and P(PEGMA-co-NIPAM) oligomers

P(HEMA-b-NIPAM) was prepared in two steps. First, HEMA was polymerized by reversible addition fragmentation transfer (RAFT) polymerization with 4-cyanopentanoic acid dithiobenzoate (CPADB) as the chain transfer agent (CTA) to get the macromolecule CTA, P(HEMA). The chemical structure of P(HEMA) was characterized by ^{1}H NMR (Fig. S1 (a)).

Then P(HEMA-b-NIPAM) was synthesized by polymerizing NIPAM with P(HEMA) as CTA. The chemical structure of P(HEMA-b-NIPAM) was also characterized by ^{1}H NMR (Fig. S1 (b)). The molecular weight (Mn) and PDI of P(HEMA) were 1587 and 1.03 (Table 1). The molecular weight (Mn) and polydispersity index (PDI) of P(HEMA-b-NIPAM) were 2131 and 1.14 (Table 1). The molar ratio of HEMA and NIPAM in P(HEMA-b-NIPAM) was calculated as 1.8:1 by the integration of HEMA and NIPAM in the ^{1}H NMR spectrum of P(HEMA-b-NIPAM) respectively. P(PEGMA-co-NIPAM) was synthesized by copolymerizing PEGMA and NIPAM with CPADB as CTA through RAFT polymerization. ^{1}H NMR was used to characterize the structure of P(PEGMA-co-NIPAM) (Fig. S2 (a)). The molecular weight (Mn) and PDI of P(PEGMA-co-NIPAM) were 6811 and 1.18 (Table 1). The molar ratio of PEGMA and NIPAM in P(PEGMA-co-NIPAM) was calculated as 1.2:1 by the integration of PEGMA and NIPAM in the ^{1}H NMR spectrum of P(PEGMA-co-NIPAM) respectively.

3.2 Detection of interactions among oligomers, PEI, DNA and AD-293 cells

Before the in vitro transfection, QCM was introduced to measure the interactions of the oligomers with pDNA, PEI and AD-293 cells. Firstly, the phenyl dithio esters of CTA agent residues of P(HEMA-b-NIPAM) was reduced to mercapto group to prepare the reduced oligomer (denoted as R-P(HEMA-b-NIPAM)) (Fig. 1 (a)). P(PEGMA-co-NIPAM) was treated in the same way to obtain the reduced oligomer (denoted as R-P(PEGMA-co-NIPAM)) (Fig. 1 (b)). The chemical structures of R-P(HEMA-b-NIPAM) and R-P(PEGMA-co-NIPAM) were determined by ^{1}H NMR spectra (Fig. S1 (c) and Fig. S2 (b)). Then the reduced oligomers were grafted onto the gold electrode of QCM via the coupling reaction between the mercapto group and gold. From the three-dimensional images of AFM of Fig. 3 we can see that the sizes of the gold particle on the electrode surface became larger after the modification of oligomers, which indicates that the oligomers were successfully grafted onto the surface of gold nanoparticles of the electrode. It is known that the mass change of adsorption will cause the frequency shift change of QCM. And the mass of adsorption is consistent with the strongness or weakness between interactions of the oligomers grafted on the electrode with DNA or PEI. That is, the values of frequency shift can express that whether the interaction of the oligomers with DNA and PEI is strong or not. It can be inferred from the QCM results (Fig. 4 (a, b)) that there was almost no interaction of the two kinds of oligomers with pDNA respectively. So both oligomers

incorporated into the PEI/oligomer/pDNA ternary complexes would not affect the release of pDNA inside the cells. In contrast, the two oligomers all had strong interactions with PEI (Fig. 4 (c, d)). That can be used to explain why the oligomers can be condensed into the ternary complexes together with pDNA. Though the two kinds of oligomers have similar interactions with PEI and pDNA, the interactions of oligomers with AD-293 cells are totally different. The results of Fig. 5 show that P(HEMA-b-NIPAM) grafted on electrode has stronger interactions with AD-293 cells than naked electrode with about 7 Hz frequency shift higher, while the frequency shift of AD-293 cells with P(PEGMA-co-NIPAM) was almost the same with naked electrode. So it can be inferred that the oligomer of P(HEMA-b-NIPAM) could enhance the interactions between the formed ternary complexes and cellular membranes, while P(PEGMA-co-NIPAM) could not. Thus we presume that the two kinds of oligomers would have different effects on the gene transfection efficiency.

3.3 In vitro transfection tests

For in vitro transfection, the representative fluorescence images of GFP positive AD-293 cells transfected with PEI/P(HEMA-b-NIPAM)/pDNA or PEI/P(PEGMA-co-NIPAM)/pDNA ternary complexes were shown in Fig. S3. It can be seen that there are distinctly different effects on the gene trasfection after incorporating the two kinds of oligomers into the PEI/pDNA binary complexes. The incorporation of P(HEMA-b-NIPAM) can significantly improve the gene transfection. In comparison, P(PEGMA-co-NIPAM) did not have much improvement on the gene transfection. The results coincided with the prediction based on QCM of the effects which the oligomers would have on gene transfection.

It has been widely proved that the hydrophobic groups employed into PEI gene delivery system can facilitate the interactions of PEI/pDNA complexes with cellular membranes to increase the adsorptive endocytosis.[30, 31] Also the hydrophobic interactions between the complexes and endosomal membranes will be helpful to enhance the defusion of the complexes with endosomal membranes.[32] Thus the hydrophobic modification is expected to improve the gene transfection through enhancing cellular uptake and endosomal escape.[33-36] Meanwhile, the hydrophilic modifications like grafting PEI with polyethylene glycol (PEG) or saccharides can both protect the nano particles from the digestion of enzymes and adsorption of proteins to maintain the stability in serum conditions.[37-41] As is known that PEGMA is more

hydrophilic than PHEMA, thus the oligomer P(HEMA-b-NIPAM) is more hydrophobic than P(PEGMA-co-NIPAM). So the relatively more hydrophobic P(HEMA-b-NIPAM) bolck oligomer incorporated into the complexes could enhance the interaction between the complexes and cellular/endosomal membranes leading better improvement in the gene transfection.

3.4 Cell cytotoxicity

The cytotoxicity assays (Fig 6) indicate that both the PEI/P(PEGMA-co-NIPAM)/pDNA and PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes have no obvious cell cytotoxicity in comparison with PEI-25k/pDNA binary complexes. Since PEGMA, HEMA and NIPAM are all biocompatible monomers, the prepared oligomers are also biocompatible.[42-44] Consequently, the incorporation of the oligomers with relativelly low molecular weight PEI did not cause the increase of cytotoxicity.

3.5 On the morphology and stability of PEI/oligomer/pDNA ternary complexes

The morphology and stability of PEI/P(HEMA-b-NIPAM)/pDNA complexes were further investigated. Firstly, from the TEM pictures of the complexes in Fig. 7 we can see that with the incorporation of P(HEMA-b-NIPAM) the sphere morphologies of PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes are more structured than PEI/pDNA counterparts. That is mainly because the hydrophobic interactions of P(HEMA-b-NIPAM) oligomer incorporated into the ternary complexes. Also the ternary complexes are larger than PEI/pDNA counterparts. The sizes and zeta potentials of PEI/P(HEMA-b-NIPAM)/pDNA complexes were measured by dynamic light scattering (DLS). The results of Fig. 8 show that the sizes of the ternary complexes were around 200 nm to 300 nm and zeta potentials are around 10 mV to 15 mV. Additionally, the sizes of the complexes did not increase much but the zeta potentials decreased from larger than 25 mV to about 10 mV after being incorporated with oligomers. The fact of the decrease of zeta potentials indicates that the oligomers distributing on the surface of the complexes causes the positive charge shielding effect. The gel shift assays were used to test the condensation ability of PEI incorporated with P(HEMA-b-NIPAM). The results of Fig. S4 show that PEI maintains its capacity to condense pDNA after being incorporated with P(HEMA-b-NIPAM). Due to the great DNA condensation capacity of PEI might result in the exclusion of ethidium bromide, the intensity of DNA staining was so low that the DNA band was almost invisible. Also we can see that the ternary complexes can maintain good

integration in the conditions of DNase .

3.6 Further in vitro transfection of PEI/P(HEMA-b-NIPAM)/pDNA complexes

The representative fluorescence images of GFP positive AD-293 cells transfected with PEI/P(HEMA-b-NIPAM)/pDNA at different N/P and oligomer/PEI ratios were shown in Fig. 9. The results showed that with the increase of oligomer/PEI ratios gene transfection was better improved. And the transfection results showed that the gene transfection efficiencies (Fig. 10) of PEI/P(HEMA-b-NIPAM)-1/pDNA, PEI/P(HEMA-b-NIPAM)-2/pDNA, PEI/P(HEMA-b-NIPAM)-3/pDNA were 35%, 49%, 56% respectively at N/P of 15, much higher than that of PEI/pDNA binary complexes (5.8%), also even higher than the gene transfection efficiency of the 'gold standard' PEI-25K/pDNA complexes (at the optimized N/P ratio of 10). With HeLa and HepG2 cells, the gene transfection efficiencies are relatively lower than that of with AD293 cells, which is probably because of the different biological activities of different cell lines. Still the transfection efficiencies of ternary complexes are much higher than that of PEI/pDNA countertparts.

3.7 Cellular uptake assays

In cell uptake assays with AD293 cells, at 6 h post-transfection, there are very few FITC-PEI/pDNA counterparts which can be observed inside the cells (Fig. S5 (a, b)). In comparison, there are massive FITC-PEI/P(HEMA-b-NIPAM)-3/pDNA ternary complexes observed inside the cells (Fig. S5 (c, d)). The cellular uptake results indicate that the relatively stronger hydrophobic effect of P(HEMA-b-NIPAM) can increase the interactions between the ternary complexes and cellular membranes, then enhancing the cellular uptake efficiencies.[31, 45, 46]

4. Conclusion

In this work, two kinds of hydrophobicity and hydrophility balanced oligomers of P(HEMA-b-NIPAM) and P(PEGMA-co-NIPAM) were synthesized through RAFT polymerization. The prepared oligomers were incorporated into PEI/pDNA complexes via non-electrostatic assembly to obtain PEI/P(HEMA-b-NIPAM)/pDNA and P(PEGMA-co-NIPAM)/pDNA ternary complexes. Before the in vitro transfection, the interactions of the oligomers with PEI, pDNA and AD-293 cells were detected by QCM. The results showed that both oliomers had almost no interaction with pDNA but had strong

interactions with PEI, which could prove that the oligomers can be effectively condensed together with pDNA by PEI without affecting the release of pDNA inside the cells. Also it can be seen from the results that P(HEMA-b-NIPAM) had strong interactions with AD-293 cells, while P(PEGMA-co-NIPAM) almost had no interaction with AD-293 cells. So it can be inferred that the two kinds of oligomers might have different effects in improving gene transfection. The in vitro transfection verified that only P(HEMA-b-NIPAM) incorporated into the complexes could greatly improve the gene transfection via enhancing interactions between the complexes and cellular/endosomal membranes. Thus the developed strategy of beforehand detecting the interactions of oligomers, pDNA, polycations and cells can be an efficient way to predetermine whether the prepared oligomer is efficient in improving gene transfection.

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Figure captions:

Fig. 1 Synthesis of process of the oligomers, P(HEMA-b-NIPAM) and R-P(HEMA-b-NIPAM) (a), P(PEGMA-co-NIPAM) and R-P(PEGMA-co-NIPAM) (b).

Fig. 2 Assembly and transfection process of PEI/P(HEMA-b-NIPAM)/pDNA ternary complexes.

Fig. 3 Three-dimensional images of Atomic force microscopy of naked gold electrode (a) and gold electrode grafted with R-P(HEMA-b-NIPAM) (b).

Fig. 4 Adsorption behaviour of oligomers modified electrodes to pDNA and PEI. Frequency response curve of P(HEMA-b-NIPAM) grafted electrode with pDNA (concentration of pDNA was 10^{-6}, $5*10^{-6}$, 10^{-5} g/μl), P(PEGMA-co-NIPAM) grafted electrode (b). Frequency response curve of P(HEMA-b-NIPAM) grafted electrode with PEI of different concentrations (c), P(PEGMA-co-NIPAM) grafted electrode (d).

Fig. 5 Adsorption behaviour of oligomers modified electrodes to AD-293 cells. Frequency

response curve of AD-293 cells (concentration of cells was 10^5/ml) with naked gold electrode P(HEMA-b-NIPAM) grafted gold electrode and P(PEGMA-co-NIPAM) grafted gold electrode.

Fig. 6 Viability of AD-293 cells treated with PEI/P(HEMA-b-NIPAM)-x/pDNA complexes (a), PEI/P(PEGMA-b-NIPAM)-x/pDNA complexes (b) at 24 post transfection. Cells treated with PEI-25K/pDNA and without any treatment were used as a positive and negative control. “X” represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI.

Fig. 7 TEM images of PEI/pDNA ternary complexes at N/P=10 (a), PEI/P(HEMA-b-NIPAM)-3/pDNA ternary complexes at N/P=10 (b). “3” represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI.

Fig. 8 (a) Sizes of PEI/P(HEMA-b-NIPAM)-x/pDNA ternary complexes. (b) Zeta potential of PEI/P(HEMA-b-NIPAM)-x/pDNA ternary complexes. “X” represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI. “0” represented PEI/ pDNA binary complexes.

Fig. 9 Fluorescence images of green fluorescence positive (GFP positive) AD293 cells transfected with PEI/P(HEMA-b-NIPAM)-1/pDNA at N/P=10 (a), PEI/P(HEMA-b-NIPAM)-1/pDNA at N/P=15 (b), PEI/P(HEMA-b-NIPAM)-3/pDNA at N/P=10 (c) and PEI/P(HEMA-b-NIPAM)-3/pDNA at N/P=15 (d). The magnification is 200. “1” and “3” represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI.

Fig. 10 Gene transfection efficiency of PEI/P(HEMA-b-NIPAM)-x/pDNA ternary complexes with AD-293, HeLa, HepG2 cells under serum conditions at N/P=10 (a) and N/P=15 (b). PEI-25k was used as positive control at N/P=10. $P < 0.05$ (*) or 0.01 (**) compared to PEI/pDNA complexes at N/P= 10. “X” represented the multiple of the weight of P(HEMA-b-NIPAM) or P(PEGMA-co-NIPAM) to that of PEI. “0” represented PEI/ pDNA binary complexes. All the transfection were conducted under the same serum condition.

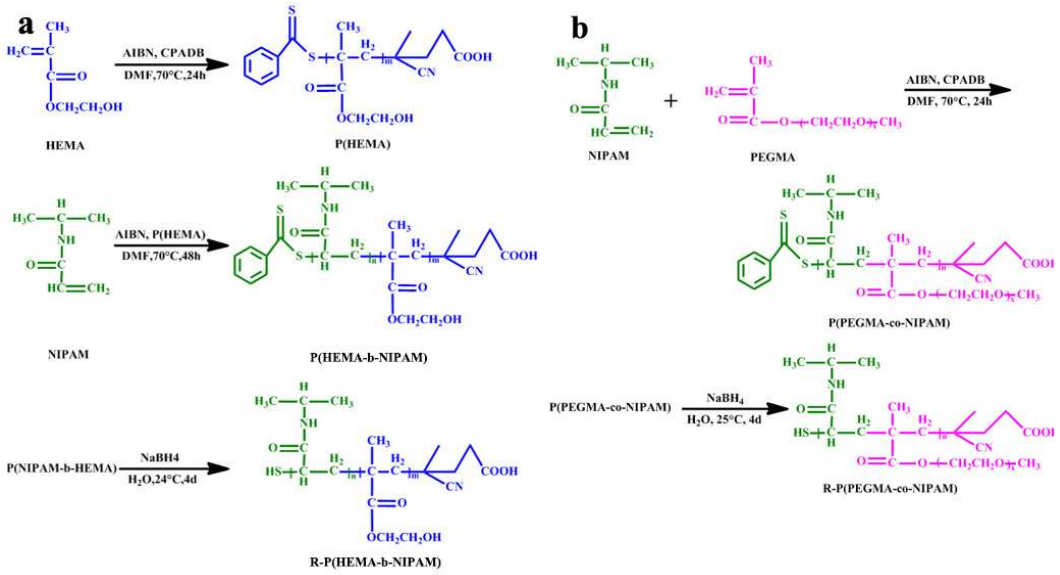


Fig. 1

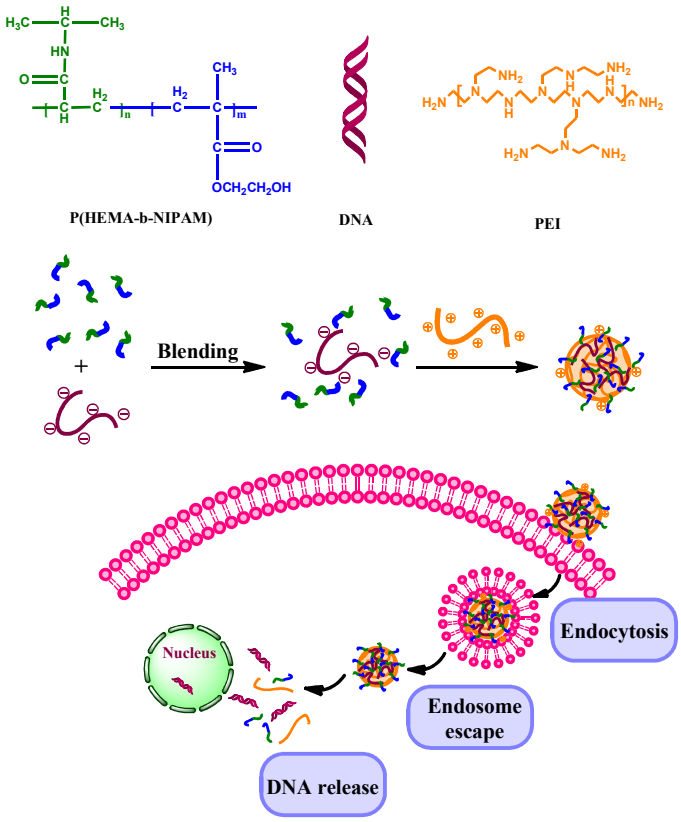
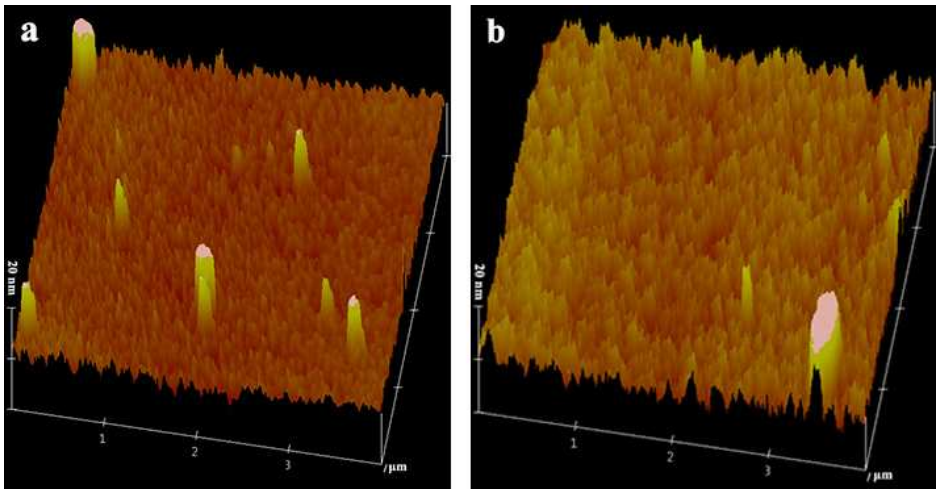


Fig. 2



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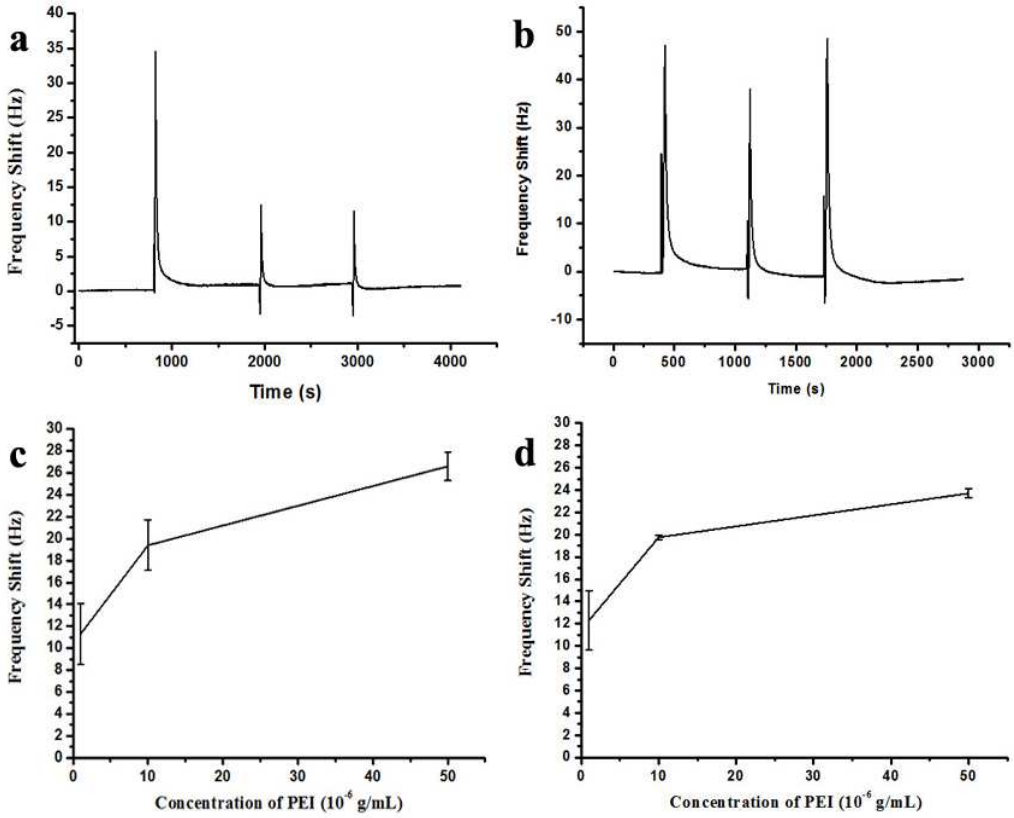

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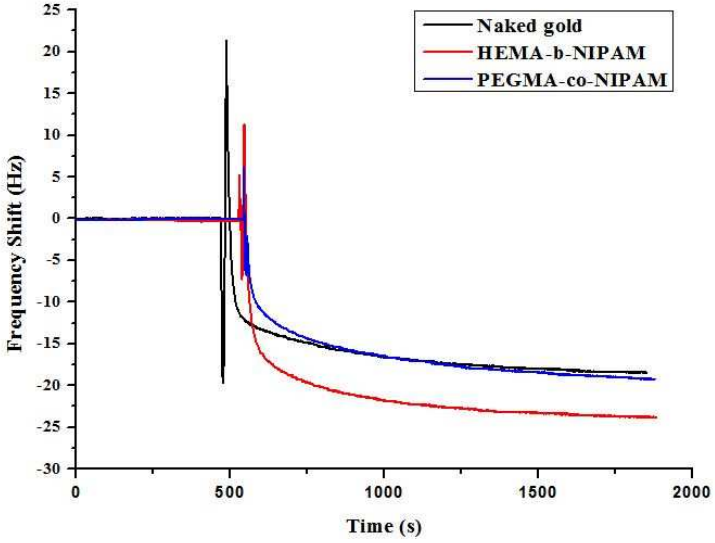

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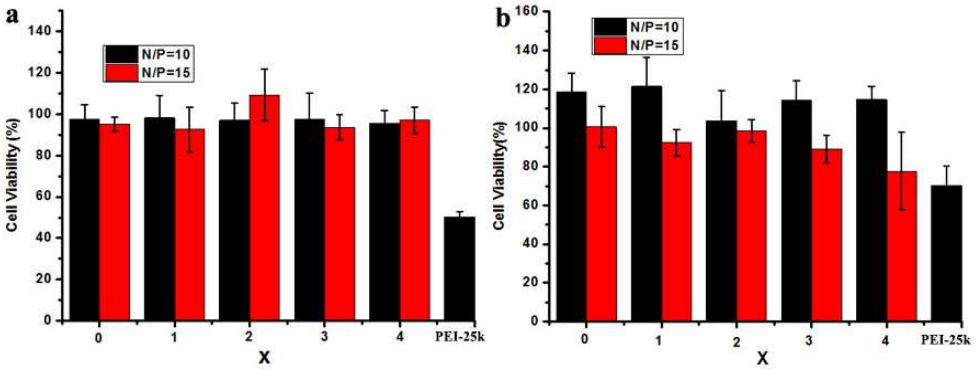

Fig. 6

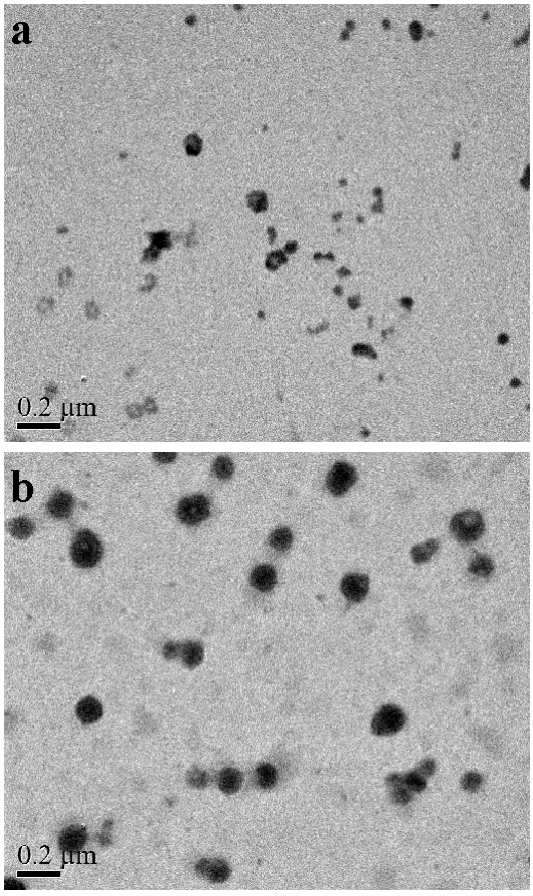

Fig. 7

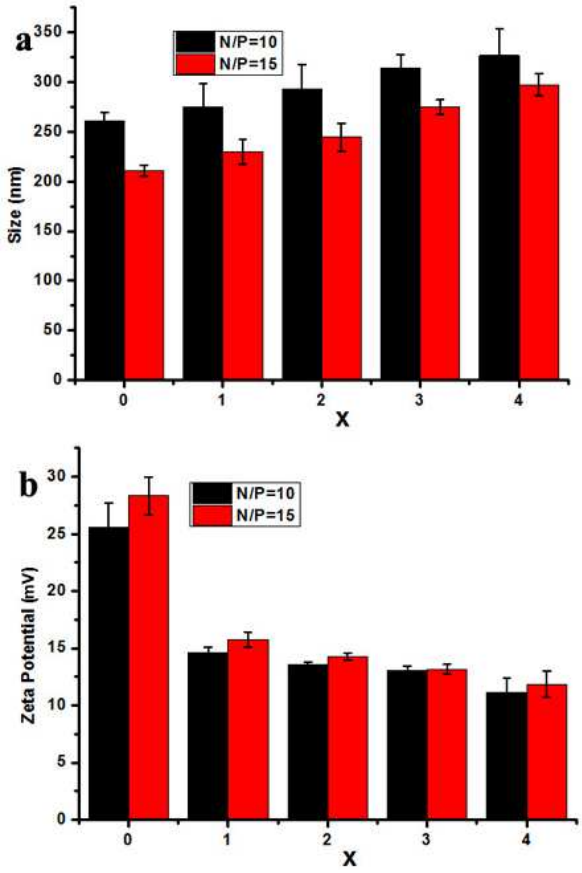

Fig. 8

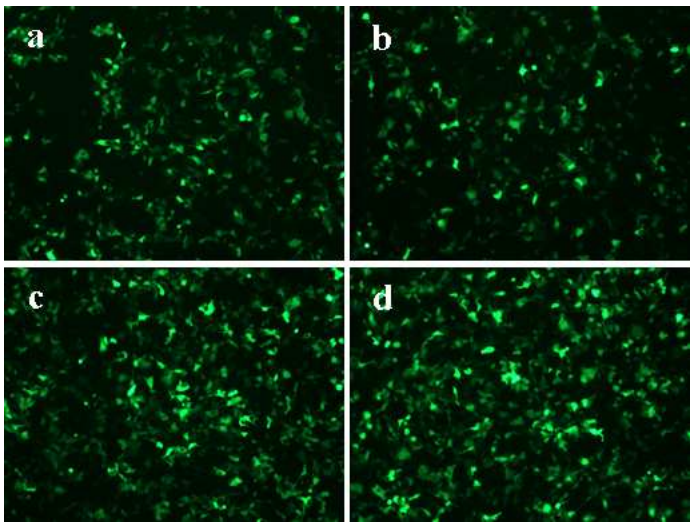

Fig. 9

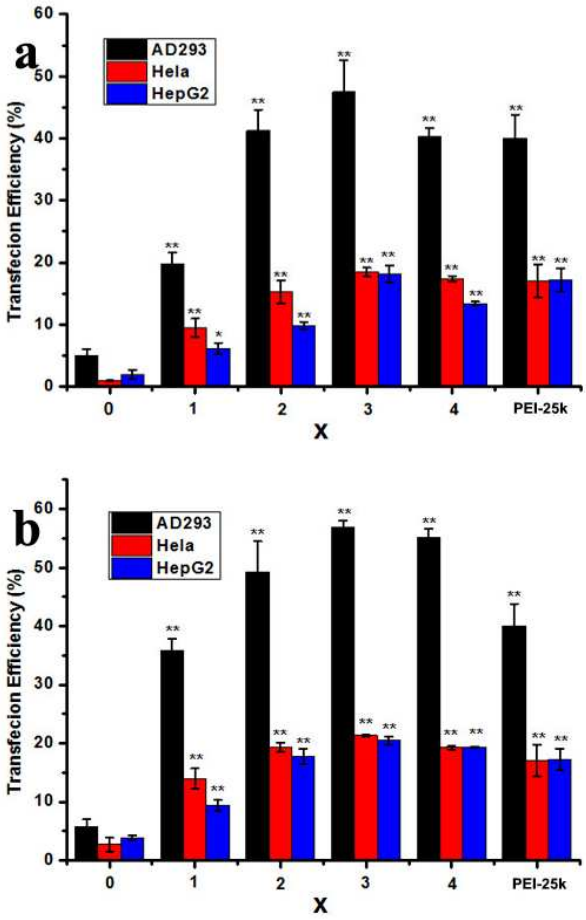

Fig. 10

Table 1 Molecular weight and polydispersity (PDI) of P(HEMA), P(HEMA-b-NIPAM) and P(PEGMA-co-NIPAM).

	M_n	M_w	PDI
P(HEMA)	1587	1630	1.027
P(HEMA-b-NIPAM)	2131	2434	1.142
P(PEGMA-co-NIPAM)	6811	8037	1.18

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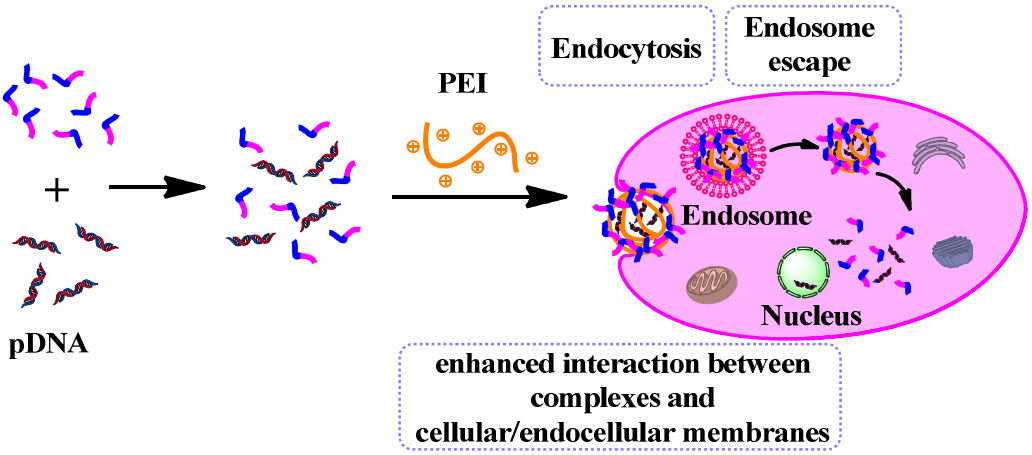

(HEMA-b-NIPAM) was incorporated into PEI/P(HEMA-b-NIPAM)/pDNA ternery complexes through non-electrostatic assembly to enhance interaction between complexes and cellular/endocellular membranes to improve gene transfection.