Multifunctional oligomer incorporation: a potent strategy to enhance the transfection activity of poly(L-lysine)

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Multifunctional oligomer incorporation: a potent strategy to enhance the transfection activity of poly(L-lysine)

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Natural polycations, such as poly(L-lysine) (PLL) and chitosan (CS), have inherent superiority as non-viral vectors due to their unparalleled biocompatibility and biodegradability. However, the application was constrained by poor transfection efficiency and safety concerns. Since previous modification strategies greatly weakened the inherent advantages of natural polycations, developing a strategy for functional group introduction with broad applicability to enhance the transfection efficiency of natural polycations without compromising their cationic properties is imperative. Herein, two uncharged functional diblock oligomers P(DMAEL-b-NIPAM) and P(DMAEL-b-Vlm) were prepared from a lactose derivative, *N*-iso-propyl acrylamide (NIPAM) as well as 1-Vinylimidazole (Vlm) and further functionalized with four small ligands folate, glutathione, cysteine and arginine, respectively, aiming to enhance the interactions of complexes with cells, which was quantified utilizing quartz crystal microbalance (QCM) biosensor, circumventing the tedious material screening process of cell transfection. Upon incorporated with PLL and DNA, the multifunctional oligomers endow the formulated ternary complexes great properties suitable for transfection, such as anti-aggregation in serum, destabilized endosome membrane, numerous functional sites for promoted endocytosis and therefore robust transfection activity. Furthermore, different from conventional strategy to decrease cytotoxicity by reducing the charge density, the multifunctional oligomer incorporation strategy maintains the highly positive charge density, which is essential for efficient cellular uptake. This system develops a new platform to modify natural polycations towards clinical gene therapy.

1. Introduction

Despite great potential and rapid advance in genome engineering technology, gene therapy is still plagued by poor transfection efficacy,[1-3] and numerous efforts have been made to explore efficient gene vectors.[4-6] Cationic polymers, as a major class of non-viral vectors, have been widely developed to mediate gene delivery,[7, 8] among which natural polycations, such as PLL[9] and CS[10], have inherent superior properties of biocompatibility, biodegradability and availability compared to chemically synthesized cationic polymers. Nonetheless, an excess of positive charges on the formed complex surface may bind negatively charged serum proteins, leading to rapid clearance by the reticuloendothelial system (RES).[11] Worse still, this further results in increased cytotoxicity through interactions with negatively charged biological membranes, leading to inhibition of crucial cellular processes.[12] To navigate this obstacle, various modification strategies, such as PEGylation[13] and polyanions shielding[14], were developed, however the performance was usually constrained by the compromise between transfection efficacy and cytotoxicity.[15, 16]

Considering these, Zhou *et al.*[17] combined low charge density (thereupon low cytotoxicity) along with hydrophobility into poly(amine-co-ester) terpolymers for DNA condensation, differing from the traditional polycation condensation mechanism. Furthermore, a synthetic negatively charged peptide containing arginine-glycine-aspartic acid (RGD) sequence was coated on the complexes to improve the stability and provide targeting through interactions with integrins. Undoubtedly, this is a great attempt, especially in decreasing the cytotoxicity of the gene vector and tailoring the balance of transfection efficacy and cytotoxicity. Adjustable sequences afford peptides desirable properties, such as hydrophobicity, stability, cell-penetrating and targeting to specific cells.[18, 19] Despite these encouraging advantages, peptides are complicated to synthesize and of high cost. As such, to develop chemically synthesized polymers from readily available chemicals mimicking the functions of peptides is of great significance. In addition, polyanions shielding may reduce the charge density and harm the cellular uptake. Therefore, exploring new strategies to collaboratively combine the synthesized polymers and natural polycations possessing the key attributes of the negatively charged targeting peptides whilst preserving the inherent superior properties of natural polycations is imperative.

To this end, previously our group has proposed a ternary strategy by incorporating P(His-co-DMAEL) (M_w = 16,769) into the PLL/DNA binary complexes *via* a non-electrostatic assembly, and results showed that the transfection activity of PLL was substantially

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enhanced.[20] Although PLL is biodegradable, the degradation and clearance of the incorporated polymers post transfection should be considered carefully since the long-term accumulation in body would induce cytotoxicity and inflammation.[21] From this point of view, incorporation of smaller oligomers instead of polymers would have its own superiority in improving the safety profile. Besides, the previous system lacks functional groups for targeting or increasing the interactions of complexes with cells, key parameter that has been repeatedly reported for facilitating the cellular uptake efficiency and ultimately resulting in higher gene transfection efficiency.[21, 22] Modification on PLL will weaken the inherent advantages of the natural properties, such as reduced positive charge density (thus reduced DNA packaging capability) and compromised degradability, especially when large amounts of functional groups are intended to be introduced.[23, 24] Since oligomers have the superiority of low cost, ease of structure tailoring and chemical functionalization, modification on oligomers mimicking the peptides would be more favorable: introducing plenty of functional groups, whilst preserving the unparalleled inherent performance of the natural polycations. Particularly, the preserved highly positive charge density in this ternary system would induce minimal cytotoxicity, substantially different from the long-held view that high charge density usually leads to high cytotoxicity.[12, 17] This multifunctional oligomer incorporation strategy would endow natural polycations high flexibility as well as broad applicability to enhance the transfection efficiency without compromising their inherent cationic advantages.

Another significant challenge in developing non-viral gene delivery vector is to correlate how molecular structures influence the transfection activity, which is in general, through meticulous cell transfection experiments. Obviously, this is a time and resource consuming process, especially when a large number of materials are needed to be evaluated, and sensitive or passage limited cells (such as primary cells) need to be used. However, so far there is no alternative option to identify, at least partially, efficient vectors without massive gene transfection. Therefore, to develop a new method to screen vectors prior to tedious cell transfection is highly desired. QCM is a versatile biosensor, as oscillation frequency shift (Δf) of QCM is directly proportional to the subtle mass or energy change on the surface of electrode,[25] and this feature makes it an ideal candidate for investigating the interactions of materials with cells. After grafting to the electrode, efficient vectors can be screened out through quantitatively comparing the interaction strength between the gene vectors and cells, circumventing the tedious and massive screening process of cell transfection.

Herein, to combine the advantages derived from the natural characteristics and non-covalent modification strategy to enhance the transfection activity of PLL, two uncharged functional diblock oligomers P(DMAEL-b-NIPAM) and P(DMAEL-b-Vlm) were prepared from a lactose derivative and NIPAM or Vlm by RAFT polymerization, which were then terminally functionalized with low cost folic acid (FA), reduced glutathione (GSH), cysteine (Cys) and poly-*L*-arginine (Arg), respectively. We hypothesize that i) cellular uptake efficiency of complexes can be enhanced through receptor-mediated pathway[26, 27]; disulfide linkage formation with mucin glycoproteins on cell membranes[10, 28] or cell-penetrating property[18, 29, 30]; ii) deprotected lactose segment (PDMAEL) is highly biocompatible and hydrophilic, thus can promote the anti-aggregating ability in serum; iii) PNIPAM has a low critical solution temperature (LCST) below 37 ^{o}C, while at the cell incubation environment the hydrophobicity of PNIPAM can enhance the interaction with cytomembrane and destabilized endosome membrane[31, 32]; iv) PVlm possesses many imidazole groups with a pK_a around 6.0 and can induce endosomal escape *via* the ''proton sponge effect'', thus resulting in improved gene delivery efficiency.[33] Prior to transfection, the effectiveness of the diverse oligomer ligands was assessed with QCM by quantifying the interaction strength between the functionalized oligomers and cells, aiming to contribute to ligands screening and optimization. Oligomer ligands were then assembled with DNA and PLL to form the P(DMAEL-b-NIPAM or Vlm)-R/DNA/PLL ternary complexes, which introduced massive functional groups, whilst preserved the suprior cationic performance of PLL, meanwhile not only was the cytotoxicity substantially decreased, but also the highly positive charges were retained, making this ideal for cellular uptake. Briefly, in this ternary system: this is the first time to evaluate target effects by the ligand modified oligomer assembly, forming complexes with polycation; the oligomer ligands perfectly mimicked the functions of the above mentioned peptide[17], resulting in robust transfection activity; the cytotoxicity was decreased in an entirely different mechanism. Our multifunctional oligomer incorporation strategy therefore provides a new reliable approach for modifying natural polycations to mediate robust and safe gene transfection.

2. Experimental

2.1. Materials

PLL (M_w = 150-300 kDa), folate (FA), glutathione (GSH), arginine (Arg), cysteine (Cys), dithiothreitol (DTT), ethidium bromide (EB), agarose, bovine serum albumin (BSA, Mw = 69 kDa, pI = 4.8), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein isothiocyanate (FITC) and phosphate buffer solution (PBS, 0.01 M, pH = 7.2-7.4) were purchased from Dingguo Biotech. Co. Ltd. (Tianjin, China). Branched polyethyleneimine (PEI, M_w = 25 kDa) and 1-Vinylimidazole (Vlm) were obtained from Sigma (St. Louis, MO). 4,4'-Azo-bis(4-cyanopentanoic acid) (ACVA, 98%) was purchased from Fluka (Shanghai, China). (4-Cyanopentanoic acid) dithiobenzoate (CPADB) as a chain transfer agent was synthesized as per the literature.[34] N,N-dimethylformamide (DMF), anhydrous methanol, anhydrous ethyl ether, N-isopropyl acrylamide (NIPAM) were purchased from the Sixth Chemical Reagent Company (Tianjin, China). Glycomonomer 2-*O*-meth-acryloyloxyethoxyl-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyr-anosyl)-(1-4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (MAEL) was prepared according to our previous work.[35] 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were purchased from Aladdin (Shanghai, China). Green fluorescent protein plasmid pCMV-GFP and cell secreted Gaussia princeps luciferase plasmid (pCMV-GLuc) (EGFP) were driven by immediate early promoter CMV, and their expansion, isolation and purification were performed using Endfree plasmid kit (Tiangen, China) as per

protocol. BioLux™ Gaussia Luciferase Assay Kit (New England Biolabs) was used according to protocol.

2.2. Instrumentation

^{1}H NMR spectra was performed on a Varian UNITY-plus 400 spectrometer operated at 400 MHz and was analyzed using MestReNova processing software.

The molecular weight (M_n) and polydispersity index (PDI) of the polymers were measured by gel permeation chromatograph (GPC) equipped with a Waters 2414 refractive index detector and a Waters 1525 Binary HPLC Pump. Columns were eluted by THF and calibrated with polystyrene.

The QCM200 from Stanford Research Systems Inc. (USA) was composed of an oscillator module, crystal holder, frequency counter and PC interface connection for signal output visualization. Microgravimetric assays were performed by using a 5 MHz, AT-cut quartz crystal (2.54 cm in diameter) with gold electrodes (0.40 cm^2 active oscillation area) on both sides.

Complex sizes and zeta potentials were measured using a Malvern Zetasizer Nano ZS. Complex sizes were determined by dynamic light scattering (DLS), and the zeta potentials were analyzed by electrophoretic light scattering.

2.3. P(DMAEL-b-NIPAM) and P(DMAEL-b-Vlm) synthesis

The glycopolymer PMAEL was synthesized by RAFT polymerization first as the macromolecular transfer reagent. MAEL (4.0 mmol), CPADB (0.26 mmol) and ACVA (0.086 mmol) were dissolved in 10 mL of DMF. After degassing by three freeze-pump-thaw cycles, the glass tube was sealed and transferred to a oil bath at 70 ^oC for 48 h. The reaction mixture was then quenched with ice water. Afterwards, PMAEL was precipitated in cold ethyl ether three times and dried under vacuum for 24 h prior to use (1.9 g, 63% yield). Then PMAEL (0.15 mmol), NIPAM (2.65 mmol) and ACVA (0.05 mmol) were dissolved in 10 mL of DMF. After degassing by three freeze-pump-thaw cycles, the glass tube was sealed and transferred to a oil bath at 70 ^oC for 48 h. After quenching the polymerization with ice water, the diblock oligomers were precipitated in cold ethyl ether three times and dried under vacuum for 24 h to give pure P(MAEL-b-NIPAM) with a yield of 89%. The synthesis of P(MAEL-b-Vlm) followed the same protocol. Briefly, PMAEL (0.15 mmol), Vlm (42.6 mmol) and ACVA (0.075mmol) were dissolved in 10 mL of DMF and other procedures were performed as above. Yellow solid (0.8 g) was obtained with a yield of 17%.

Afterwards, the glycopolymer segments of the copolymers were deprotected. P(MAEL-b-NIPAM) and P(MAEL-b-Vlm) were dissolved in anhydrous methanol, afterwards sodium methoxide (30%, mass concentration) was added and the solutions were stirred for 1 h at room temperature. Finally, the mixtures were dialyzed with deionized water for 3 days (cutoff M_w = 3.5 kDa) and lyophilized to give P(DMAEL-b-NIPAM) (92% yield) and P(DMAEL-b-Vlm) (94% yield).

2.4. Synthesis of oligomer ligands

FA-NH_2 was synthesized first. Briefly, FA (2.27 mmol) was dissolved in 10 mL of anhydrous DMSO (in dark), then EDC·HCl (2.72 mmol) and NHS (2.72 mmol) were added to react for 3 h. Afterwards, ethanediamine (1 mL) and pyridine (0.5 mL) were added and stirred for 48 h in the dark. The product was precipitated in acetone and dialyzed with deionized water for 1 day (cutoff M_w = 1 kDa). Finally, 0.63 g of FA-NH_2 was obtained (63% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.65 (s, 1H), 8.18 (m, 1H; NH), 8.02 (m, 1H; NH), 7.68 (d, J = 7.2 Hz, 2H; Ar H), 6.66 (d, J = 6.8 Hz, 2H; Ar H), 4.50 (s, 2H; CH_2), 4.38-4.25 (m, 1H; CH), 3.15 (td, 2H; CH_2), 2.72 (m, 2H; CH_2), 2.38-1.90 (m, 4H; CH_2).

P(DMAEL-b-NIPAM) (0.007 mmol) was dissolved in 10 mL deionized water, and EDC·HCl (0.035 mmol) and NHS (0.035 mmol) were added. After stirring for 0.5 h, Arg (0.10 mmol), GSH (0.10 mmol), or Cys (0.10 mmol) was added respectively, and pH value was adjusted to 5.5. 48 h later, DTT was added (final concentration 20 mM) to cleave the disulfide bonds for 3 h. The solutions were then dialyzed with deionized water for 2 days (cutoff M_w = 3.5 kDa) and lyophilized to give P(DMAEL-b-NIPAM)-Arg (89% yield), GSH (95% yield) or Cys (90% yield). For P(DMAEL-b-NIPAM)-FA synthesis, pH value was adjusted to 10.0 after FA (20 mg) addition and the product was dialyzed in basic solution for 2 days, deionized water for 1 day and then lyophilized to give 92% yield. The functionalized oligomers were termed as P(DMAEL-b-NIPAM)-R (R = Arg, GSH, Cys or FA). The procedures were the same for P(DMAEL-b-Vlm)-R preparation. All reactions containing FA were in the dark.

2.5. QCM assays

Prior to use, the quartz wafer was first immersed in piranha etching solution (1:3, v/v 30% H_2O_2/concentrated H_2SO_4) for 3 min, then rinsed with deionized water and ethanol three times respectively, and lastly dried under gentle nitrogen flow atmosphere. Afterwards, the electrode was immersed in the DMF/H_2O (v/v 1:1) solution of P(DMAEL-b-NIPAM)-R or P(DMAEL-b-Vlm)-R (0.1 mM). 48 h later, the gold electrode was rinsed with ethanol and deionized water to remove unreacted polymers and then transferred to the QCM chamber. Before each test, PBS was injected into the chamber to equilibrate until stabilizing within ± 2 Hz in 30 min was achieved. 5× 10^5 cells (Hela and 293T) in PBS were then injected, and as the cells adsorbed, the frequency decreased. The oscillation frequency shift (Δf) is directly proportional to the mass change on the surface of electrode, making the QCM convenient to measure interactions between oligomer ligands and cells.

2.6. Preparation of oligomer/DNA/PLL ternary complexes

For the formation of ternary complexes, required amount of P(DMAEL-b-NIPAM) or P(DMAEL-b-Vlm) was first mixed with DNA. After 15 min incubation, calculated PLL was added into the mixture, and the mixture was left for another 15 min. The assembly process was shown in Figure 1. The N/P ratios were designed at 8 and 16 according to our previous work. [20] The weight of P(DMAEL-b-NIPAM) or P(DMAEL-b-Vlm) was 0, 1, 2, 3 or 4 times that of PLL and the corresponding ternary complexes were termed as P(DMAEL-b-NIPAM)-x/DNA/PLL or P(DMAEL-b-Vlm)-x/DNA/PLL, where the "x" represented the multiple of the weight of P(DMAEL-b-NIPAM) or P(DMAEL-b-Vlm) to that of PLL.

2.7. Gel retardation studies

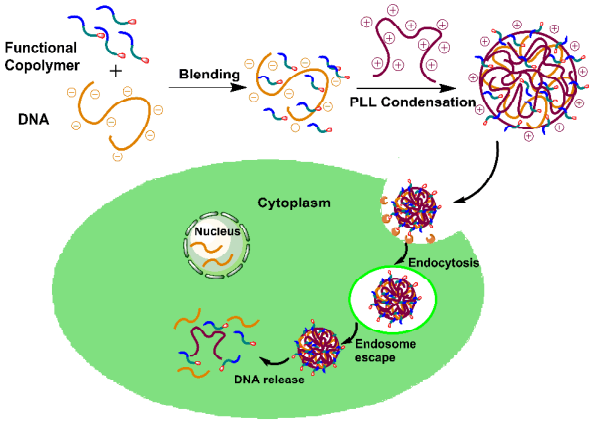

Figure 1. Illustration of the gene delivery process of the functionalized ternary complexes.

Packaging ability of DNA by PLL was evaluated by agarose gel electrophoresis. 120 ng of DNA was utilized for each sample. Complexes were prepared as mentioned above and transferred to a 1.0% agarose gel well (Tris-boric acid-EDTA, pH = 8.0), with gels being run for 45 min at 100 V. Afterwards, DNA bands were stained with ethidium bromide and visualized with an UV illuminator.

2.8. Complex size and zeta potential assays

5μg of DNA was utilized for each measurement and the complexes were prepared as mentioned above. To investigate the complex stability in protein solutions, complexes were prepared at N/P ratio of 16 and complex sizes were measured. BSA was then added to the complexes (0.5% final concentration) and complex sizes were measured again 6 h later.

2.9. Cell culture

Human embryonic kidney cell line (293T) and human cervical cancer cell line (HeLa) were purchased from ATCC (Teddington, UK). All cells were cultured in DMEM containing 10% FBS at 37 ^{o}C, 5% CO_2 in a humid incubator under standard cell culture techniques.

2.10. *In vitro* transfection

293T or Hela cells were seeded in 96-well plates at a density of 1 × 10^4 cells per well and cultured until 70-80% confluence. Afterwards, complexes were prepared with 0.25 μg DNA per well and added to the cells. For commercial transfection reagents, PEI was used at a N/P ratio of 10:1 as a positive control. After 48 hours of incubation, GFP positive cells were monitored and imaged on an inverted fluorescence microscope. Analysis of the secreted Gluciferase activity was performed as per the provided protocol, with Gluciferase activity directly detected in the cell supernatant and plotted in terms of relative light units (RLU).

2.11. Complex cellular uptake and trafficking

FITC labeled PLL (PLL-FITC) was used to prepare the complexes. 293T cells were seeded at a density of 5 × 10^4 cells per well in a 24-well plate and transfection was conducted in serum conditions at the N/P ratio of 16. 4 h post transfection, the culture media were aspirated and cells were washed with PBS, followed by fixing in 4% paraformaldehyde and observing with an inverted fluorescence microscope.

2.12. Cytotoxicity assays

Cytotoxicity analysis was performed on all cells using the MTT assay. 48 h after initial transfection, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well. After 4 hours of further incubation, the media were replaced with 150 μL of DMSO and after complete dissolution of formazan, absorbances were measured using a microplate reader (Labsystem, Multiskan, Ascent, Model 354 Finland) at a wavelength of 570 nm. Cells without any treatment were plotted as 100% viability.

2.13. Statistics

All data were expressed as average ± standard deviation (SD), with SD represented by error bars. Statistical comparisons between control and treated groups were performed using Student's T-tests. Average values and SDs were calculated for each sample examined, with all samples ran in triplicate. P-values of < 0.05 (*) were considered statistically significant.

3. Results and discussion

3.1. Synthesis of the oligomer ligands

As outlined in Figure 2, to synthesize P(DMAEL-b-NIPAM)-R, PMAEL was first prepared as the chain transfer agent (CTA). The molecular weight (M_n) and PDI of PMAEL were 3881 and 1.07 respectively, indicating roughly 5 glycomonomer units present in each PMAEL molecule. The ^{1}H NMR spectrum of PMAEL was shown in Figure S1. Following this, the diblock copolymers were synthesized by

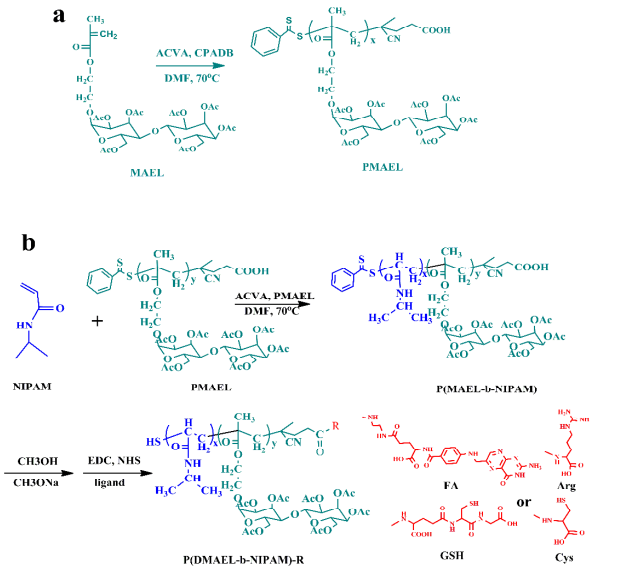

Figure 2. Synthetic approach to PMAEL (a) and the multifunctional block oligomer P(DMAEL-b-NIPAM)-R (b).

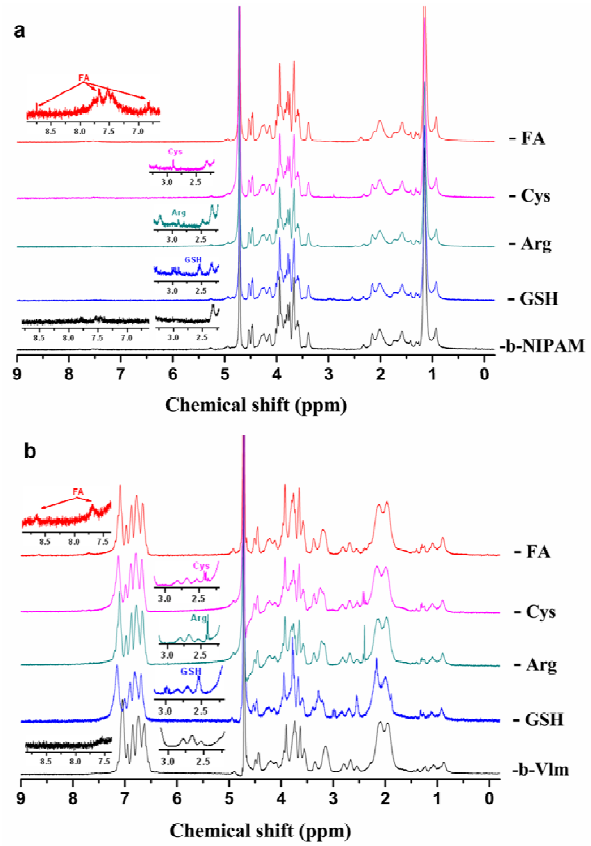

Figure 3. ^{1}H NMR spectra of P(DMAEL-b-NIPAM)-R (D_2O, 400 MHz) (a) and P(DMAEL-b-Vlm)-R (D_2O, 400 MHz) (b).

copolymerizing NIPAM or Vlm with PMAEL. The M_n of P(MAEL-b-NIPAM) and P(MAEL-b-Vlm) were 6071 and 3465 with a PDI of 1.19 and 1.06, respectively (Table 1 and Figure S2). The relative decrease in M_n was due to different solubility of P(DMAEL-b-Vlm) in THF compared to standard polystyrene. Finally, the block oligomers were deprotected by sodium methoxide and further functionalized with ligand molecules (FA, Arg, GSH and Cys) by amidation between carboxyl groups of copolymers and amino groups of ligands. The modified diblock copolymers were characterized by ^{1}H NMR spectra as shown in Figure 3a and 3b. The methyl groups of DMAEL and NIPAM were located in the region of 0.8-1.3 ppm and methylene groups on the backbones were located in the region of 1.3-2.2 ppm.

Table 1. Molecular weight and polydispersity (PDI) of the polymers

	M_n	M_w	PDI	M_{NMR}
PMAEL	3881	4151	1.07	3787
P(MAEL-b-NIPAM)	6071	7219	1.19	/
P(MAEL-b-Vlm)	3465	3656	1.06	/
P(DMAEL-b-NIPAM)	/	/	/	4615
P(DMAEL-b-Vlm)	/	/	/	5278

GPC measurements were conducted at 40 °C with THF as elution at the flow rate of 1 mL/min. $CDCl_3$ was used for ^{1}H NMR analysis of PMAEL, while D_2O was utilized for that of P(DMAEL-b-NIPAM) and P(DMAEL-b-Vlm).

By comparing the integral area of the methyl groups with that of the methylene groups, the molar ratio of DMAEL units to NIPAM units in P(DMAEL-b-NIPAM) was calculated to be 1:3.9. The molecular weight of P(DMAEL-b-NIPAM) was 4615 from the ^{1}H NMR spectra, as the protected PDMAEL has a molecular weight of 2411. For P(DMAEL-b-Vlm), the imidazole groups can be identified from the region of 6.5-7.2 ppm and the molar ratio of DMAEL and Vlm was calculated as 1:6.1 by comparing the integral area of the methyl groups in DMAEL with imidazole groups in Vlm. As such, the molecular weight of P(DMAEL-b-Vlm) was calculated to be 5278 from the ^{1}H NMR spectra.

The successful functionalization of copolymers was also confirmed by ^{1}H NMR spectra. As shown in Figure 3a, FA can be identified at 8.75, 7.7 and 6.8 ppm, methylene groups of Cys can be identified at 2.9 ppm, and methylene groups of Arg and GSH can be both identified at 2.5 and 2.9 ppm. The characteristic peaks of Cys and Arg shifted to lower ppm due to slightly basic environment caused by protonation of Vlm (Figure 3b). By comparing the integral area of characteristic peaks, FA, Cys, Arg and GSH modified on one P(DMAEL-b-NIPAM) molecule were caluculated to be 0.8, 1.6, 2.0 and 2.5, reapectively. While the ligands modified on one P(DMAEL-b-Vlm) molecule were 0.9, 2.7, 2.8 and 4.1 in sequence.

3.2. Formation and stability of the ternary complexes

Sizes and zeta potentials of the ternary complexes were first assessed. As shown in Figure 4, P(DMAEL-b-NIPAM)/DNA/PLL ternary complexes had average sizes below 200 nm with zeta potentials around + 20 mV, which were both in favour of cellular uptake. Additionally, the sizes and zeta potentials of the complexes were not significantly altered after P(DMAEL-b-NIPAM) incorporation. And after FA or GSH modification, P(DMAEL-b-NIPAM)-R/DNA/PLL complexes exhibited no increase in complex sizes compared to that of the P(DMAEL-b-NIPAM) counterparts. The similar trend was also observed in P(DMAEL-b-Vlm)/DNA/PLL complexes (Figure S3). Anti-aggregation ability of the ternary complexes in protein solutions was also investigated. In 0.5 mg/mL BSA solutions, the sizes of P(DMAEL-b-NIPAM or Vlm)-2/DNA/PLL and P(DMAEL-b-NIPAM or Vlm)-4/DNA/PLL ternary complexes increased slightly, which however were still below 250 nm and suitable for cellular uptake. By contrast, the size of DNA/PLL binary complexes increased remarkably from 150 nm to 800 nm (Figure 4c). These results indicate that multiple hydrophilic lactose moieties introduction can effectively protect the complexes from aggregating with negatively charged serum proteins. The gel retardation assays were utilized to assess the packaging ability of PLL incorporated with P(DMAEL-b-NIPAM) and P(DMAEL-b-Vlm). Figure S4 showed that PLL maintained its high capacity to condense DNA after the diblock oligomers incorporation at the N/P ratios of 8 and 16. Moreover, terminal functionalization (FA or GSH) on the oligomers did not affect the DNA condensation ability.

3.3 Interactions between oligomer ligands and cells probed by QCM

Prior to transfection experiments, QCM was utilized to assess the interactions between the P(DMAEL-b-NIPAM)-R (R = Arg, GSH, Cys

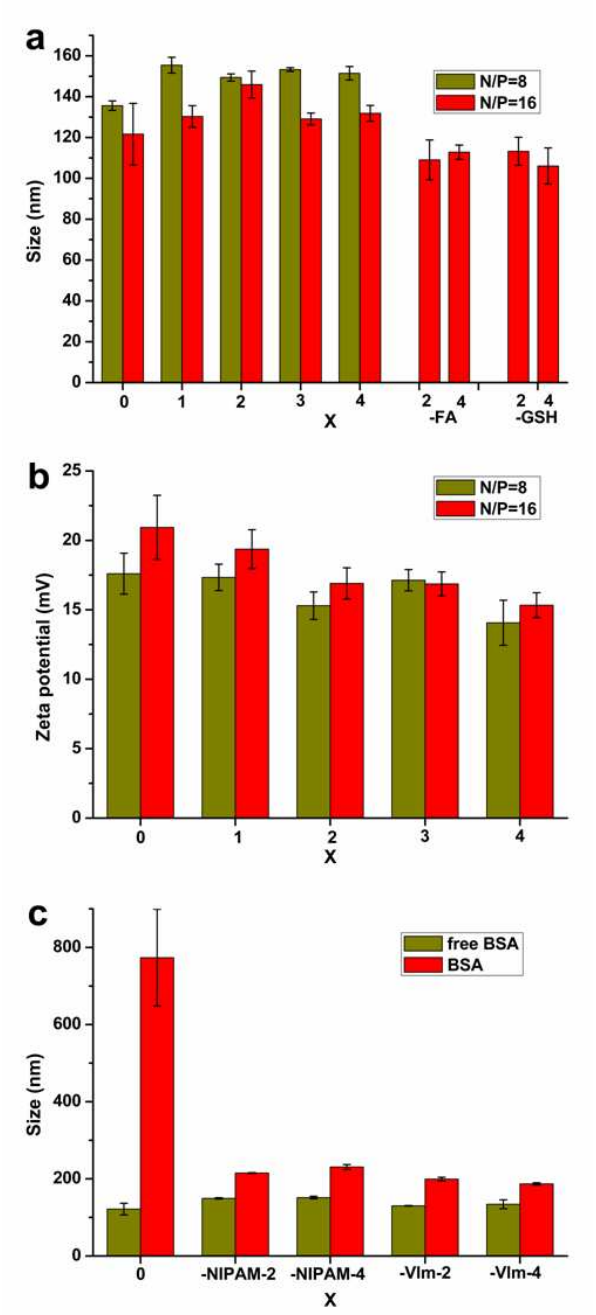

Figure 4. (a) Complex sizes of P(DMAEL-b-NIPAM)-x/DNA/PLL ternary complexes. (b) Zeta potentials of P(DMAEL-b-NIPAM)-x/DNA/PLL ternary complexes. (c) Sizes of P(DMAEL-b-NIPAM)-x/DNA/PLL and P(DMAEL-b-Vlm)-x/DNA/PLL ternary complexes in 0.5 mg mL^{-1} BSA solution at an N/P ratio of 16. "x" represents the weight multiple of P(DMAEL-b-NIPAM) or P(DMAEL-b-Vlm) to that of PLL. "0" represents PLL/DNA binary complexes.

or FA) oligomers and cells. After deprotection by sodium methoxide, the phenyl dithio esters of CTA were also reduced to sulfydryl groups, which could be grafted onto the QCM electrode by coupling reaction between sulfydryl groups and gold. As sulfydryl groups of P(DMAEL-b-NIPAM)-GSH and P(DMAEL-b-NIPAM)-Cys could also be grafted onto the gold electrode, the interaction enhancement between oligomer ligands and cells due to sulfydryl introduction could not be probed here. The oscillation frequency shift is directly proportional to the mass change on the surface of quartz crystal, and the frequency of the gold electrode decreased by about 50 Hz after immersion in copolymer solutions, indicating that the copolymers were grafted onto the electrode surface successfully. With the changed frequency shifts, the interaction strength between oligomer ligands and cells was compared. Gold can attract cells strongly, and the frequency of naked gold electrode decreased by 26 Hz after 293T cells adsorption as shown in Figure 5a. However, the frequency of P(DMAEL-b-NIPAM) grafted electrode decreased by 14 Hz. The reduced frequency shift can be attributed to the spatial repulsive effect of the hydrophilic glycopolymer segments, as a result P(DMAEL-b-NIPAM) grafting weakened the cell adsorption. After terminal functionalization of the copolymers, P(DMAEL-b-NIPAM)-FA and P(DMAEL-b-NIPAM)-Arg had larger acting forces to 293T cells due to FA-receptor interaction or enhanced cell-penetrating property. Eventually, the frequency of P(DMAEL-b-NIPAM)-FA and P(DMAEL-b-NIPAM)-Arg grafted electrodes decreased by 22 Hz and 18 Hz respectively, indicating the enhanced interactions between the oligomer ligands and 293T cells. These results were also consistently seen in Hela cells (Figure 5b). The holistic smaller frequency shifts may be

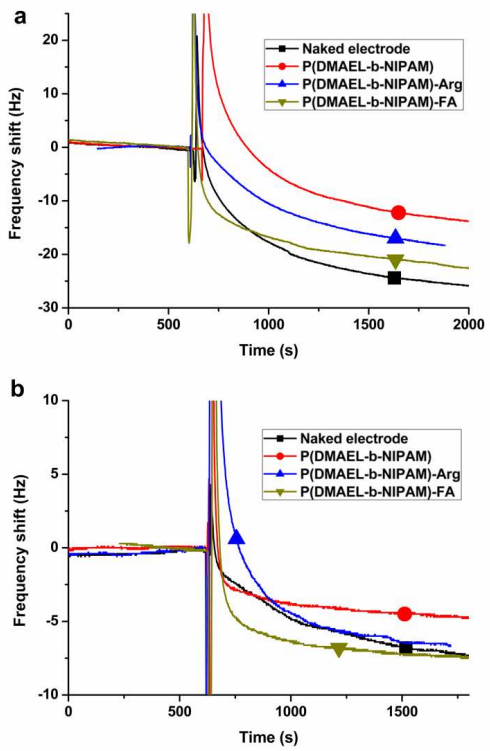

Figure 5. Adsorption behaviour of 293T (a) and Hela (b) cells to naked gold electrodes and P(DMAEL-b-NIPAM)-R modified electrodes.

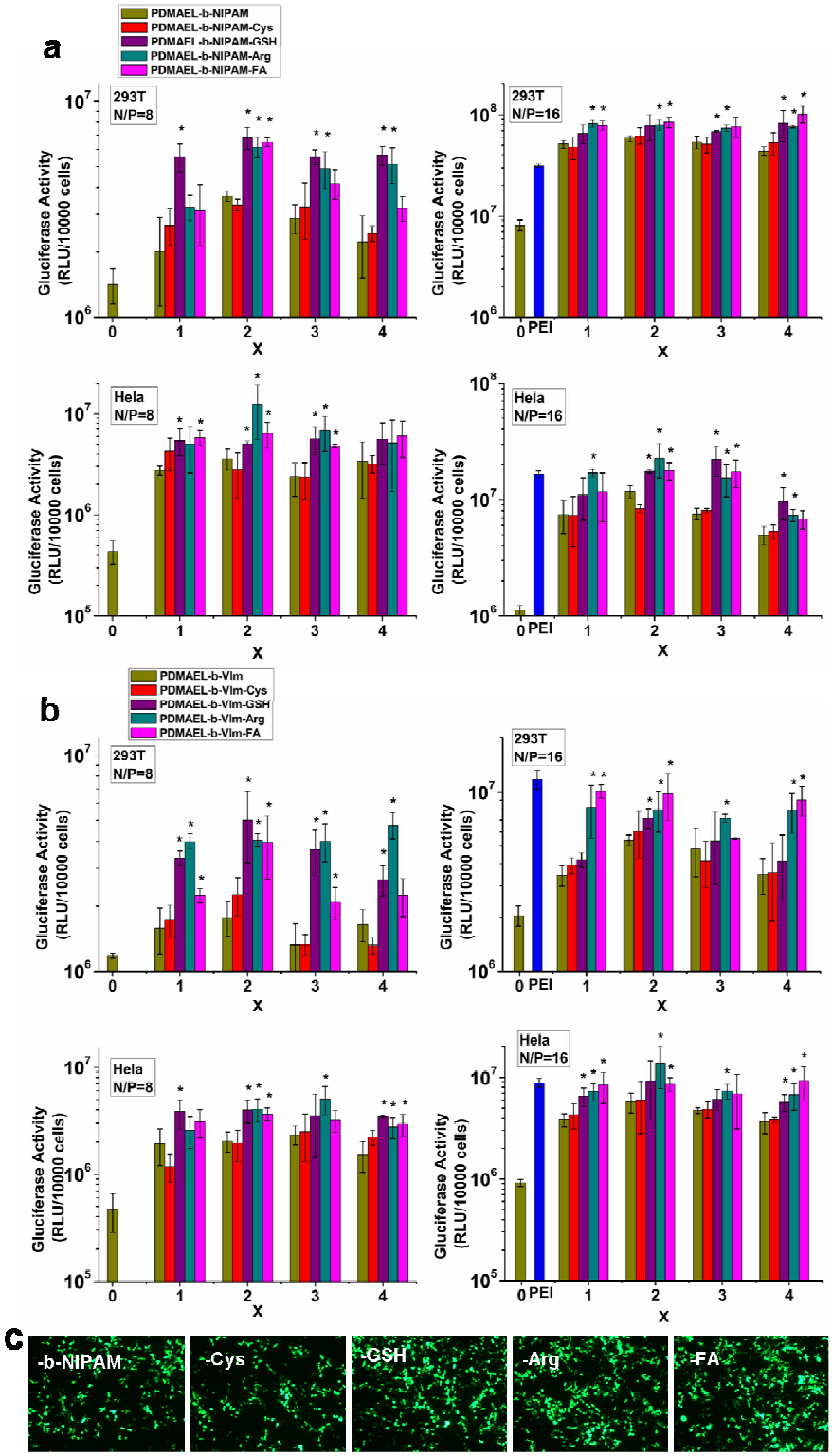

Figure 6. Gluciferase activity of P(DMAEL-b-NIPAM)-R/DNA/PLL (a) and P(DMAEL-b-Vlm)-R/DNA/PLL (b) ternary complexes in 293T and HeLa cells in the presence of serum. Commercial PEI (25 k) was utilized as a positive control at the N/P ratio of 10. *P < 0.05 superior Gluciferase activity compared to P(DMAEL-b-NIPAM or Vlm)/DNA/PLL complexes at the corresponding x values. (c) Fluorescence images of 293T cells transfected by P(DMAEL-b-NIPAM)-R-2/DNA/PLL complexes at the N/P ratio of 16:1. "x" represents the weight multiple of oligomer ligands to that of PLL. "R" represents Cys, GSH, Arg or FA. "0" represents PLL/DNA binary complexes.

attributed to weaker interactions between Hela cells and the gold electrode compared to that of 293T counterpart. Through QCM assays, terminal functionalizing agents effective for enhancing interactions between cells and copolymers can be identified, making this an excellent approach for selecting oligomer ligands prior to cell transfection studies.

3.4. *In vitro* transfection

As a biocompatible and biodegradable polycation, PLL has remarkable DNA condensing capacity, making it suitable for gene delivery. However, its high levels of binding to serum proteins and ultra-low transfection efficiency have limited its application. Here, multifunctional oligomers were designed and incorporated into binary PLL/DNA complexes to improve on these shortcomings and incorporate beneficial properties for gene delivery. Lactose was designated a segment of the copolymers, as its hydrophilicity could shield the positive charges and protect the complexes from protein adsorption.[20] Also, it has been proved previously that hydrophobicity can enhance the interactions between complexes and cell membranes, resulting in promoted endocytosis.[31, 32] Thus NIPAM was selected and copolymerized into the oligomer. To further improve the cellular uptake efficiency, ligand molecules were designed to functionalize the oligomers. RGD peptide is an efficient targeting ligand, nevertheless it usually functions in a peptide chain, which is of high cost.[19, 36] Thus pursuing effective ligands of low cost is of great significance. FA can bind to folate receptors (FR) that are over-expressed in many cancer cells.[26, 27] GSH can act as specific peptide ligand to promote cellular uptake, as its free sulfydryl groups can form disulfide linkages with mucin glycoproteins on cell membranes.[10, 28] Acting as cell-penetrating peptide (CCP), poly-*L*-arginine can translocate through cell membranes and show efficient internalization.[18] Previous works reported that simple guanidinylation could also transport complexes into cells efficiently.[29, 30] As such, the oligomer was then functionalized with FA, GSH, Cys or Arg, respectively, and the product were termed as P(DMAEL-b-NIPAM)-R (R = FA, GSH, Cys or Arg). Herein, the ternary complexes own the properties of DNA protection, anti-aggregation in serum and high endocytosis efficiency, which will synergistically result in high transfection efficiency. To add further scope to this study, another copolymer containing lactose and Vlm, which has “proton sponge effect” was prepared, followed by terminal functionalization, donating P(DMAEL-b-Vlm)-R.

Gene expressions of P(DMAEL-b-NIPAM)-R/DNA/PLL and P(DMAEL-b-Vlm)-R/DNA/PLL ternary complexes under serum conditions were evaluated and compared with that of PLL/DNA counterparts and PEI (25 k)/DNA complex. P(DMAEL-b-NIPAM)/DNA/PLL complexes exhibited excellent gene delivery efficiency, better than the “gold standard” PEI/DNA complex as shown in Figure 6a. Moreover, complexes at the N/P ratio of 16 showed higher transfection efficiency than that of N/P ratio of 8, which might be attributed to smaller particle sizes. P(DMAEL-b-NIPAM or Vlm) incorporation highly increased the gene transfection efficiency, however it was worth noting that the gene expression of the ternary complexes did not always increase as the oligomer contents increased, while P(DMAEL-b-NIPAM or Vlm)-2/DNA/PLL complex showed the best result compared to other counterparts.

To bring in functions, the diblock oligomers were further terminally modified. As shown in Figure 6a and 6b, P(DMAEL-b-NIPAM or Vlm)-GSH/DNA/PLL complexes showed higher Gluciferase activity than that of their unmodified counterparts, although Cys modification had no effect on the efficiency. The enhancement can be explained by way of free sulfydryl groups of reduced GSH forming disulfide linkages with mucin glycoproteins on cell membranes, enhancing the cellular uptake. This result also indicated that not all sulfydryl groups could work as this function and sulfydryl groups of GSH might have their own advantage compared to that of Cys. Next, Arg was introduced, aiming to improve the cell-penetrating property of the complexes. As expected, P(DMAEL-b-NIPAM or Vlm)-Arg/DNA/PLL complexes exhibited higher transfection efficiency than that of the unmodified counterparts in Figure 6. Also, P(DMAEL-b-NIPAM or Vlm) with FA target ligands had higher cellular uptake efficiency *via* the folate-receptor mediated endocytosis, leading to higher transfection efficiency in FR-positive 293T and Hela cells. Fluorescence images of GFP positive 293T transfected with P(DMAEL-b-NIPAM)/DNA/PLL and its modified counterparts also indicated that GSH, Arg and FA modification efficiently improved the gene expression, while Cys modification was not the case (Figure 6c). These results of Arg and FA functionalizations are consistent with the QCM data that Arg and FA modified diblock copolymers have stronger interactions with cell membranes, leading to higher gene delivery efficiency. This further highlights the potential QCM assays has for selecting oligomer ligands prior to *in vitro* experiments.

3.5. Cellular uptake assays

As observed in Figure S5, there were very few PLL-FITC/DNA binary complexes observed inside the cells at 4 h post-transfection under serum conditions. By contrast, massive P(DMAEL-b-NIPAM)-R/DNA/PLL-FITC ternary complexes were observed inside the cells, due to the shielding effect from serum proteins by lactose segments and enhanced interactions between the ternary complexes and cells by hydrophobic effect of P(DMAEL-b-NIPAM). Additionally, GSH, Arg and FA functionalizations induced higher cellular uptake, mainly because of the formed disulfide linkages with cell membranes, enhanced cell-penetrating property and folate-receptor mediated endocytosis, respectively.

3.6. Cytotoxicity assays

Cell viability of the complexes was evaluated by MTT assays and the results were shown in Figure 7. In both 293T and Hela cells, P(DMAEL-b-NIPAM or Vlm)/DNA/PLL ternary complexes showed no obvious cytotoxicity, while PLL/DNA binary complexes exhibited slight cytotoxicity. Furthermore, terminal functionalization (Cys, GSH, Arg or FA) did not decrease the cell viability.

4. Conclusion

Natural PLL has inherent superiority as the non-viral vector due to

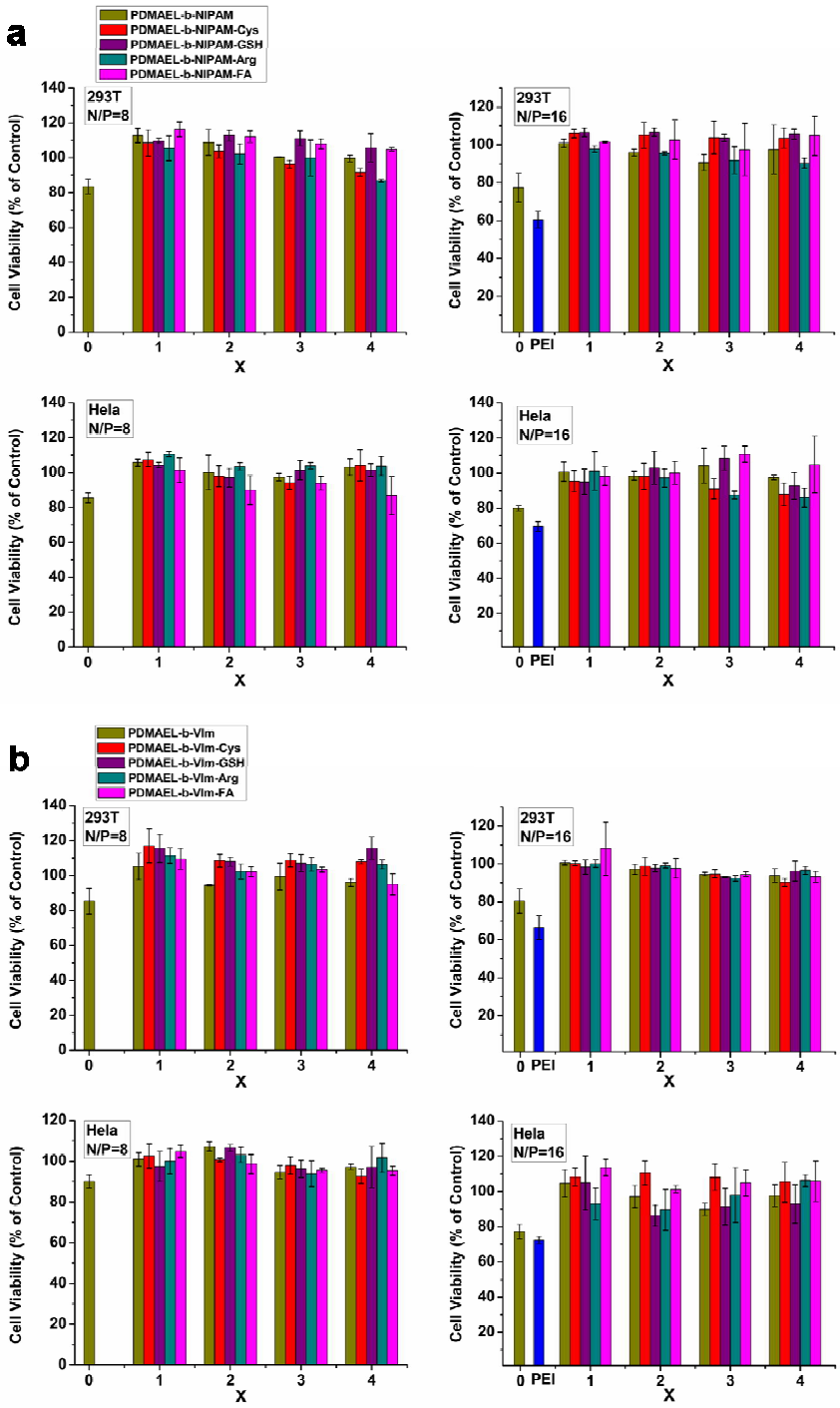

Figure 7. Cell viability of P(DMAEL-b-NIPAM)-R/DNA/PLL (a) and P(DMAEL-b-Vlm)-R/DNA/PLL (b) ternary complexes at the N/P ratio of 8 and 16 in 293T and HeLa cells.

its biocompatibility and biodegradability, however transfection efficiency and cytotoxicity still need to navigate. In this work, two oligomer ligands, P(DMAEL-b-NIPAM)-R and P(DMAEL-b-Vlm)-R were synthesized through RAFT polymerization and further functionalization. The prepared oligomer ligands were then incorporated into PLL/DNA complexes *via* non-electrostatic assembly to obtain P(DMAEL-b-NIPAM)-R/DNA/PLL and P(DMAEL-b-Vlm)-R/DNA/PLL ternary complexes. The effect of functionalization was determined by quantifying interaction strength between oligomer ligands and cells with QCM assays. This circumvented the tedious screening process of transfection for ligands selection and *in vitro* transfection results later confirmed the validity of this method that P(DMAEL-b-NIPAM or Vlm)-Arg or FA/DNA/PLL complexes exhibited higher transfection efficiency than that of the unmodified counterparts. Results indicated that this ternary system simultaneously exhibited great properties for gene delivery, such as

stability in serum conditions, high cellular uptake efficiency, low cytotoxicity, high transfection efficiency and so on. The multifunctional oligomer incorporation strategy proposed here broadens the application and gives great hope to the development of natural polycations towards clinical gene transfection.

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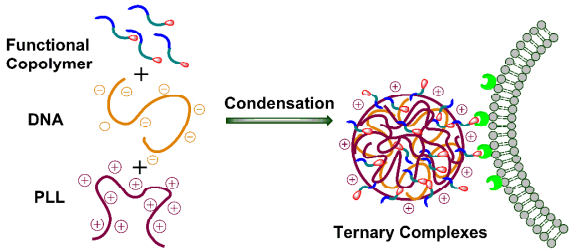
TOC Graphic



The multifunctional oligomer incorporation strategy is the first time to evaluate target effects by the ligand modified oligomer assembly, forming complexes with DNA and polycation.