Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal Name

ARTICLE

Received 00th January 20xx,

Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Dual functionalized amino poly(glycerol methacrylate) with guanidine and Schiff-base linked imidazole for enhanced gene transfection and minimized cytotoxicity

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Efficient transfection activity and minimal toxicity are crucial importance to development of gene delivery systems to practical applications. In this work, guanidine and Schiff-base linked imidazole dual functionalized poly(glycerol methacrylate) (IGEP) was firstly synthesized. Subsequent investigations revealed this new biomedical material capable of sufficiently binding to plasmid DNA (pDNA) and formulating optimal sized 100 nm nanoparticle with positive ζ potentials of 10-30 mV. Biological evaluations demonstrated strategic use of guanidine resulting in enhanced cellular uptake and nuclear localization activities by virtue of its favorable affinity to the cellular membrane, and Schiff-base linked imidazole resulting in promoted endosome escape and DNA cargo releasing behaviors, consequently leading to better transfection efficacy compared to PEI_{25K} in the targeted cells. Another noteworthy was guanidine and imidazole for minimized cytotoxicity, hence, these advantageous features provided substantial information to construct safe and efficient gene delivery carrier towards practical development.

Introduction

Gene therapy, for either DNA or RNA, has been deemed as a useful curative approach to treat both inherited and acquired genetic diseases.[1] Successful gene therapies, however, strongly relies on the development of effective and safe gene delivery systems. Viral vectors have dominated as the delivery vehicles used in clinical trials but their progress has been hampered by restricted encapsulation, severe safety risks, immunogenicity, potential carcinogenicity and high manufacturing cost.[2] Therefore, non-viral delivery alternatives, in particular cationic polymers based systems, have been developed to circumvent drawbacks of natural delivery vectors.[3] A variety of cationic polymer based systems were used as non-viral vectors for delivering nucleic acids, including polyethylenimines,[4] poly-(L-lysine),[5] poly(amino amine) dendrimers,[6] polyaminoesters,[7] poly-(aspartic acid),[8] chitosans[9] and cyclodextrin oligomers.[10] Although the complexes formed with cationic polymers are considered to be the most promising candidate for nucleic acid delivery, these systems still encountered substantial challenges with respect to the multiple hurdles during the delivery process, such as low cellular uptake, inefficient endosome escape, reluctant DNA unpacking and release, and poor nuclear import.[11,12] Therefore, it requests multi-functionalized delivery systems to overcome these ensemble of defined hurdles to achieve high transfection efficiencies while maintain low toxicity.

Cell penetrating peptides (CPP), intensively investigated peptide consisting of a large number of positively charged amino acids, has been demonstrated to entitle the various linked molecular cargos uptaken into cells.[13] These tempting properties spurred intense interest in drug and gene delivery field. Further research suggested that the vital role of guanidinium group in CPPs in promoting cellular uptake. Guanidine groups are highly basic, capable of forming bifurcated hydrogen bonds with nucleic acid phosphates, thus eliciting additional affinity with DNA. In addition, they can also form a specific bidentate hydrogen-bonding with the cell membrane, which are anticipated to facilitate endocytosis and cellular uptake, resulting in enhanced transfection efficiency.[14] Also, the cell toxicity could also be anticipated to be relieved through guanidinylation with respect to the delocalization of the cationic charge and good biocompatibility of guanidine groups. On the other hand, imidazole as a functional moiety of several biomolecules (such as histidine), once introduced to delivery system, could render strong endosomolytic activity in the endosomal compartments (endosomal escape), consequently resulting in enhanced the transfection efficiency and gene expression.[15,16] According to the previous report, the imidazole-chitosan prepared through the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chemistry has been developed, and used to deliver small interfering RNA (si-RNA) for potential treatment of lung diseases and genes in nervous system.[17] However, the amide linkage between the imidazole

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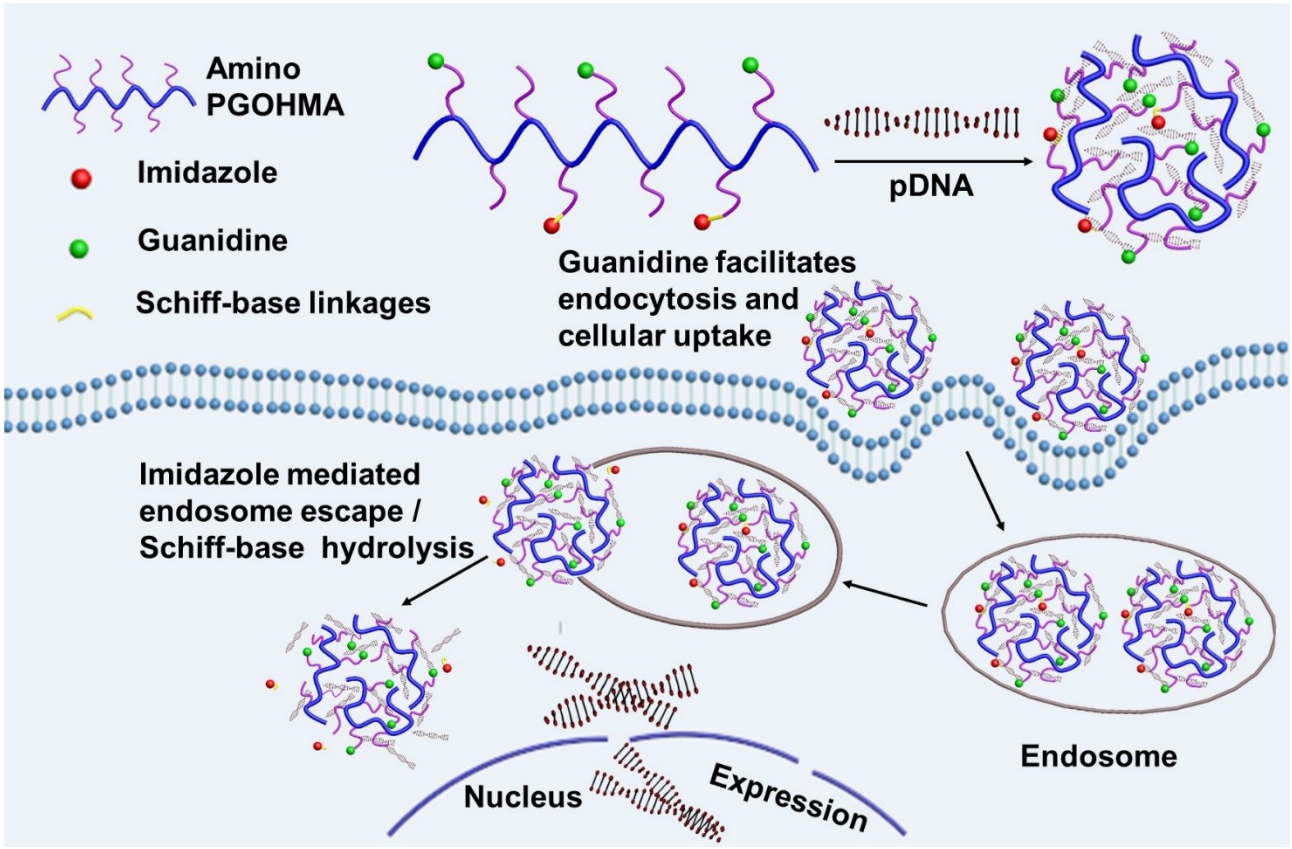


Scheme 1 Schematic illustration of IGEP/pDNA complexes formation, subsequently guanidine facilitates endocytosis and cellular uptake, imidazole mediates endosome escape, Schiff-base regulates DNA unpacking and release.

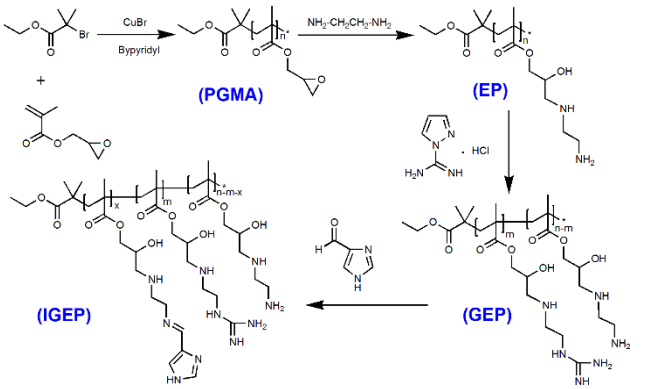
ring and chitosan might be easily digested by enzymes *in vivo*.[18] Schiff-bases are widely investigated as one of the imine type compounds because of their structural variety and specific molecular properties.[19] The Schiff-base linker is particular interesting due to its endosomal pH sensitive. It preserves intact at physiological pH while hydrolysis in the weakly acidic endosomal microenvironment (pH 6.5 drops to 5.0 from early to late endosome), thus able to facilitate intracellular DNA unpacking and release. [20] In addition, it has been reported that Schiff-base can enhance gene-binding ability due to its fully protonation at physiological pH.[21] Based on these findings, we conjectured that imidazole functionalized polymer based on Schiff-bases linker should be a good candidate as the gene carrier in gene delivery.

Poly(glycerol methacrylate) (PGMA) is a versatile polymer because it has pendant epoxide groups, which can react readily and irreversibly with nucleophilic groups to generate polyglycerol methacrylate (PGOHMA) derivatives.[22, 23] In our previous work, amino PGOHMAs were found to be an excellent gene vector because it can condense antisense oligonucleotide (AON), forming spherical nanoparticles of polymer/AON complexes and interact efficiently with cells to deliver it. Then, thiourea groups were introduced into the side-chain of amino PGMA, and amino PGOHMA was partially quaternary ammonium salt-functionalized, resulting in a reduced cytotoxicity, and enhanced transfection efficiency.[24-26] In our present study, we first synthesized ethylenediamine-functionalized PGMA (EP), followed by synthesis of guanidine modified EP (GEP). To further facilitate intracellular DNA unpacking and enhance the transfection efficiency of PGOHMA derivatives, an imidazole group was introduced to GEP through Schiff-base linker (IGEP) (**Scheme 1**). The combination of dual functionalization of amino PGOHMA is expected to obtain a promising pDNA vector with enhanced gene transfection and minimum cellular toxicity.

Experimental

Materials and methods

Materials. Glycidyl methacrylate (GMA), 2-bromoisobutyryl bromide, bipyridyl, CuBr, 1H-pyrazole-1-carboxamidine hydrochloride (HPC), N,N-diisopropylethylamine (DIPEA) and 4-imidazolecarboxaldehyde were purchased from Shanghai Adamas Reagent Co., Ltd (Shanghai, China). Polyethylenimine [average Mw ~25,000 Da by LS, number average molecular weight (Mn ~10,000 Da) by GPC, branched] was purchased from Sigma-Aldrich (Shanghai, China). Prior to use, tetrahydrofuran (THF) was dried by refluxing over sodium, in the presence of benzophenone as an indicator. A plasmid vector coding for luciferase with a CAG promoter was used in the DLS measurement, the *in vitro* cell viability and transfection



Scheme 2 Synthetic scheme of preparation of PGMA derivatives contain diverse functional components.

experiments (RIKEN, Japan). Cell Counting Kit-8 (CCK8) was used in the cell cytotoxicity assay (Dojindo, Japan). The Micro BCA Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL) and the luciferase assay kit was a product of Promega (USA). All other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China).

Preparation of complexes. The charge ratio (N/P) of polymers and pDNA was calculated as the moles ratio of the amino/guanidino/imidazole groups (N) from polymer to the phosphate groups (P) from pDNA. Briefly, all polymers were dissolved in distilled water. The solutions were sterilized by passing through 0.22 μm filter prior to complexation. Polymer/pDNA complexes were created by adding filtered polymer solutions of prescribed concentrations to an equal volume of a defined pDNA solution (25 μg mL^{-1}) to obtain the N/P ratio of 0.5-10.0. The two solutions were mixed and vortexed for 15 s, followed by incubation at room temperature for 30 min to obtain the polymer/pDNA complexes.

Cell viability assay. To determine the polymer cytotoxicity in vitro, a CCK8-based cell viability test was performed in 96-well plates. A549 cells were seeded at a density of 5×10^3 cells per well and the cells were cultured for 24 h in serum containing culture medium (100 μL of medium per well). The cells were then exposed to complexes at pDNA concentration of 50 μg mL^{-1} with increased N/P ratio. After incubation at 37 °C for 48 h, the cells in the culture medium of 0.1 mL were treated with CCK8 solution (10 μL) to measure cell survival. The absorbance of the solution was monitored at 450 nm using a microplate reader (Epoch, BioTek, Gene company Limited) after 2 h incubation. The cell viability in each well was calculated from the obtained values as a percentage of control wells. The results were presented as a mean and standard deviation obtained from three samples.

***In vitro* transfection.** Transfection assays were performed using plasmid CAG-Luc as the reporter gene in A549 cell lines. In brief, cells were seeded in 24-well plates at a density of 1×10^4 cells per well and cultured with 400 μL of culture medium containing 10% FBS for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 until the cells reached 70% confluency. The polymer/pDNA complexes at different N/P ratios were added into the culture plate (2.5 μg mL^{-1} pDNA), followed by a further incubation for 48 h to express the luciferase reporter gene. Cells were washed twice with PBS and harvested in reporter lysis buffer. Luciferase activity was measured by detecting the light emission from an aliquot of cell lysate incubated with 100 μL of a luciferin substrate (Promega) in a luminometer (FLX800, BioTek, Gene company Limited). The results were expressed as relative light units per milligram of cell protein (RLU per mg protein), and the protein concentration of each well was measured using protein assay kits (Pierce). The results were presented as a mean and standard deviation obtained from three samples.

Flow cytometric assay. Cy5-labled DNA (50 μg mL^{-1}) was used to form complexes at the N/P ratio of 10.0. A549 cells were seeded into 24-well culture plates overnight at a density of 1×10^5 cells per well in serum containing culture medium (400 μL of medium per well). Then the medium was replaced with fresh medium and 40 μL of complexes solution (N/P = 10.0) was applied to each well (n = 4). After 24 h incubation, the media was removed and the cells were washed with 0.5 mL of PBS. The cells were treated with a trypsin-EDTA solution for 2 min and suspended in PBS, then analyzed using the flow cytometer (BDLSR II, BD, Franklin Lakes, NJ).

Confocal laser scanning microscopy (CLSM). For intracellular translocation, A549 cells were plated in a 35 mm glass base dish at an initial density of 1×10^5 cells per well and incubated overnight in 1 mL of culture media (RPMI 1640, 10% FBS). At the end of incubation, 100 μl of complex solutions (50 μg mL^{-1} pDNA) were suspended in serum-free medium for each well. After 1 h incubation at 37 °C in a humidified atmosphere with 5% (*v*/*v*) CO_2, the cells were washed twice with complete RPMI 1640 medium and the serum-free medium was replaced with fresh complete RPMI 1640 medium. After 23 h post-incubation, the old medium was removed and the cells were washed twice with PBS. Then the intracellular distribution of the complexes was observed by CLSM after staining acidic late endosomes/lysosomes with LysoTracker Green DND-26 (YEASEN, Shanghai, China), and nuclei with DAPI (Dojindo Laboratories, Kumamoto, Japan). CLSM observation was performed using an LSM510 (Nikon 108, Japan) equipped with a 40 × objective at the excitation wavelengths of 488 nm for LysoTracker Green, 633 nm for Cy5, and 402 nm for DAPI.

Statistical analysis. Significant differences in cell viability and transfection efficiency between the obtained polymers and PEI were evaluated using Student's t-test.

Results and discussion

Synthesis and characterization of the polymers.

Polymerization to obtain PGMA bearing epoxy residues as pendants can be achieved by ATRP, followed by primary amine-containing molecules introduced into the pendants through amine/epoxy reaction, yielding diverse PGMA derivatives characterized with flanking cationic amine and non-ionic hydrophilic hydroxyl groups.[27] Following this reaction scheme, PGMA with approximate polymerization degree of 85 units [Mn: 12,000 Da; polydispersity index (PDI): 1.32] was synthesized as precursor by ATRP. Furthermore, introduction of amine-containing molecules into the side chain of PGMA was performed via the ring-opening reactions with the epoxy groups from PGMA. The obtained products followed further

functionalization with guanidine to yield GEP, and ultimate functionalization with 4-imidazolecarboxaldehyde to yield IGEP (**Scheme 2**).

The resulting products of EP, GEP and IGEP were characterized by ^{1}H NMR spectra (**Fig. S1, ESI†**). The peaks assigning the methylene groups linked to amino group in EP were located at 2.6-2.9 ppm. Post functionalization by guanidinylation, the emerging peaks were identified at 3.2-3.4 ppm, corresponding to protons of guanidine groups and two methylene groups in proximity to guanidines. In addition to distinct resonance signals from ethanediamine and guanidine components, the occurrence of proton signal at 9.6 ppm indicates the formation of Schiff-base (-**H**C=N-) linkages. The successful synthesis of IGEP can be confirmed by the presence of imidazole rings with their characterized proton chemical shifts at 7.5-8.0 ppm. According to post integral analysis of the obtained ^{1}H NMR spectra, the average number of imidazole residues per IGEP was calculated to be approximate 13, through comparing the integration intensities of proton peak of Schiff-base linkages (9.6 ppm) and PGMA (1.6-2.25 ppm) (**Table S1, ESI†**). Post analysis on the resulting product based on FTIR spectra also confirmed the targeted chemical structures (**Fig. S2, ESI†**), the distinct peak of EP at 1725 cm^{-1} implied additional C=O stretch band, and the peaks at 1570 cm^{-1} and 1476 cm^{-1} are attributed to the distortion vibration and bending vibration of N-H of EP. Guanidinylation of EP to yield GEP elicited distinguishable consequence in FTIR spectra. As compared to EP, new peak from GEP appeared at 1676 cm^{-1} corresponding to the stretching vibration of C=N, demonstrating the introduction of guanidine to EP precursor. IGEP presents moderate absorption at 1634 cm^{-1} corresponding to the C=N vibration of the formation of Schiff-base between GEP and imidazole, while the peak at 1405 cm^{-1} is attributable to the N-H bending of the imidazole ring.

The conversion ratio of amino of all polymers was calculated to be 63.8% and 81.6% respectively, according to the nitrogen percent determined by elemental analysis. Based on the nitrogen content in EP and GEP, the number of guanidine groups per GEP was consequently calculated to be 25.5 (**Table S1, ESI†**).

Characterization of polymer/DNA complexes

Agarose gel electrophoresis assay on complexation of polymer/pDNA. A primary requirement for gene delivery system is the adequate capacity to strongly bind to pDNA, neutralize its negative charge and condense it into nanosized particulate in order to pursue colloidal stabilities to withstand dissociation in the biological milieu and circumvent the inherent nature of naked pDNA for facilitated cellular uptake.[28] Herein, the capabilities of the PGMA derivatives in comparison with that of branched PEI_{25K} were investigated by agarose gel electrophoresis assay. As shown in **Fig. 1(A)-(C)**, both PEI and all cationic PGMA derivatives could fully neutralize the negative charge of pDNA starting from N/P ratio of 1.5. This result indicated all of amino, guanidino and imidazole groups were capable of binding to, via electrostatic interaction, thereby neutralize the negative charge of pDNA to make complexes.

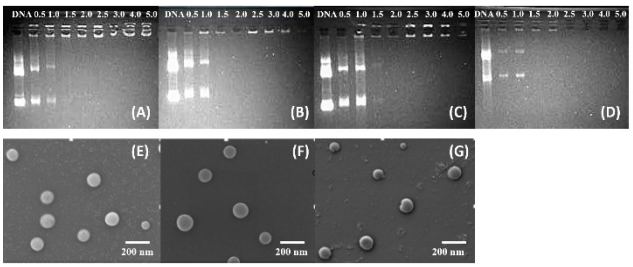


Fig. 1 Gel electrophoresis profile of (A) EP/pDNA; (B) GEP/pDNA; (C) IGEP/pDNA and (D) PEI_{25K}/pDNA complexes at varying N/P ratios. *Notes*: samples of naked pDNA (lane 1); samples of polymer/pDNA complexes with N/P ratio of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0, respectively (lanes 2-9). SEM images of polymer/DNA complexes at N/P =5.0. (E) EP/pDNA; (F) GEP/pDNA; (G) IGEP/pDNA.

Particle size and zeta potential measurements. As demonstrated in gel electrostatic assay, all cationic PGMA derivatives can electrostatically complex with negatively charged pDNA. Furthermore, the particle size and surface charge subsequence to this electrostatic complexation was investigated, because these characters were reported to essentially correlate to their activities in cellular uptake and gene transfection.[29] DLS measurements revealed an overall trend that the particle size appeared to decrease along a rising N/P ratio for classes of EP/pDNA complexes and PEI/pDNA (**Fig. S3 (A), ESI†**). The size of PEI/pDNA is over than 200 nm at all N/P ratios. While the DLS sizes of the GEP/pDNA and IGEP/pDNA systems were observed to be less than 120 nm for those at N/P ratio exceeding 5.0, which may be benefited from additional positive charge from guanidine to induce a higher degree of condensation. The obtained compacted complexes implied their potentials in pursuit of facilitated cellular uptake.[30]

The zeta-potentials of all complexes were investigated and determined to be ranging from -45 to +30 mV (**Fig. S3 (B), ESI†**). Negative zeta potentials, consistent with gel electrostatic assay, were observed for the complexes at N/P ratios of 0.5-1.0 for all complexes, except for IGEP/pDNA complexes (negative zeta-potentials up to N/P ratio of 1.5). Post functionalization of guanidinylation, additional positive charge could be identified for the sample of GEP/pDNA as compared to EP/pDNA, which is likely to be as a consequence of extra positive charge gained from the conversion of primary amines into guanidine groups. These results revealed the constructed formulation with appropriate particle size and zeta potential, possibly utilized as gene delivery system for cell transfection.

Morphology of complexes by SEM. Morphology of diverse PGMA derivatives and pDNA complexes at an N/P ratio of 5.0 were observed by SEM (**Fig. 1(E)-(G) and Fig. S4, ESI†**). The observations revealed uniform compacted structures ranging from 100-150 nm. This value was relatively less than the sizes obtained by DLS measurement, and the shrinkage of complexes was feasible with regard to SEM conducted in dehydrated condition as opposed to DLS measurement in aqueous solution.[31]

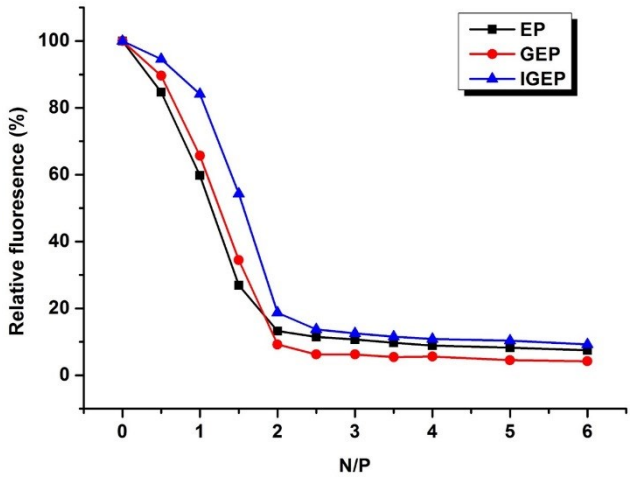


Fig. 2 Ethidium bromide displacement for the systems of diverse PGMA derivatives and pDNA at varying N/P ratios.

EB displacement assay. As aforementioned, an important requirement to construct efficient gene delivery system is to provide adequate binding affinity to pDNA so as to withstand dissociation from competing species in the biological milieu. In this regard, the binding potency of diverse PGMA derivatives were investigated according to an EB displacement assay. Herein, the binding potency of polymer and pDNA can be characterized by the decrease of the fluorescence of EB due to the dissociation of the DNA/EB complexes.[32] Once EB displacement is completed, the fluorescence can be anticipated to reach a plateau. As shown in **Fig. 2**, the fluorescence of the all complexes reached a plateau at N/P ratio of 2.0 and quenched to be 7-15% as compared to the initial fluorescence of the EB/pDNA complexes. These results suggests that all PGMA derivatives were capable of efficiently bind to pDNA. Noteworthy was that guanidine-containing PGMA derivatives displayed slightly higher pDNA binding potencies. A plausible reason for this may be the guanidine residues which could impart bidentate hydrogen bonding with phosphates of nucleic acid so as to provide additional binding affinities.[33]

Buffering capacity. Subsequence to cellular uptake, the polymer/pDNA complexes were transported into endosome and lysosome to undergo degradation. Hence, efficient escape from endosomes is one of the most important factors to be considered for the design of gene delivery vehicles. This event is associated with the buffering capacity of gene delivery vehicles within the pH range of 7.4-5.0, where entrapped delivery vehicles undergo pH gradient from the extracellular neutral environment to the endosomal acidic environment.[34] To pursue efficient endosome escape, imidazole residues were chemically conjugated to the side chains of GEP via the Schiff-base reaction. The buffering capacity of GEP and IGEP was confirmed according to a conventional acid-base titration method. As shown in **Fig. S5** (**ESI†**), slight enhanced buffering capacity was observed for the IGEP polymer, suggesting the utility of incorporation of imidazole residues to pursue proton sponge effect in response to pH gradient associated with endosomal/lysosomal entrapment.[35]

In vitro evaluation of cell viability. The cytotoxicity of gene delivery vehicle is also one of the critical concerns that may restrict the progress into practical applications. A CCK-8 assay was followed to evaluate cell viability. Herein, the samples prepared from diverse PGMA derivatives and pDNA at varying N/P ratios were incubated with A549 cells. As shown in **Fig. 3(A)**, all PGMA derivatives exhibited substantially lower toxicity than PEI (Mw ~ 25,000 Da, branched). Most likely, the flanking non-ionic hydrophilic hydroxyl groups in the PGMA derivatives not only conduced to lower density of amino groups, but also shield effect on the deleterious cationic charges, provided that acute cytotoxicity was generally associated with high charge density of amino groups of cationic species.[36, 37]. On the other hand, post guanidinylation led to delocalization of the cationic charge served another strategy to reduce the cytotoxicity. [38, 39] In particular, minimal cytotoxicity of IGEP is above 100% at the entire N/P ratios validated the strategic use of guanidine and imidazole substitution of primary amino groups to seek safety profile. As documented, protonated imidazole ring is markedly less cytotoxic than protonated amines.[40]

In vitro transfection. The *in vitro* gene transfection efficiencies of all PGMA derivatives formulated samples were evaludated in A549 cells using CAG-Luc plasmid as a reporter gene. **Fig. 3(B)** shows the gene transfection efficiencies mediated by the functioned PGMA system at varying N/P ratios in the presence of serum in comparison with that of "gold standard" PEI (M_w~25,000 Da, branched) at the N/P ratio of 10.0, where branched PEI usually exhibited the exceedingly high transfection efficiency.[41] The transfection efficacy of EP appeared to be lower than that of PEI at N/P ratios of 5.0 and 10.0. Guanidine functionalized GEP showed markedly enhanced transfection efficiency than aminated PGMA and its transfection efficiency of GEP at N/P ratio of 10.0 appeared to be even higher (~3.55 times) than that of PEI, suggesting the functional role of guanidine groups in assisting gene transfection. Presumably, advantageous bidentate hydrogen bonding between the guanidine group and the phosphate of the phospholipid conduced to the favorable affinity of complexes to the cellular membrane, thus resulting in enhanced cellular uptake, ultimately accounting for improved gene transfection.[42] At N/P ratio of 5.0 and 10.0, 2.7-fold higher gene expression was observed with IGEP/pDNA than GEP. These results indicated that, subsequence to cellular internalization, the imidazole mediated endosome escape as well as Schiff-base linkage regulated DNA unpacking and release, synergistically, dictate an appreciable intracellular trafficking behaviors, therefore resulting in the efficient gene transfection activity.

Cellular uptake of complexes. Flow cytometric analysis was used to quantify the cellular uptake activity of diverse complexes with respect to pDNA prior labeled by Cy5. Overall, all the samples showed excellent cellular uptake activities (**Fig. 4**). Close examination observed guanidine functionalization resulted in improvement of cellular uptake, which should be attributable to hydrogen bonds existing between guanidino groups and the phospholipids in the lipid bilayers, accordingly

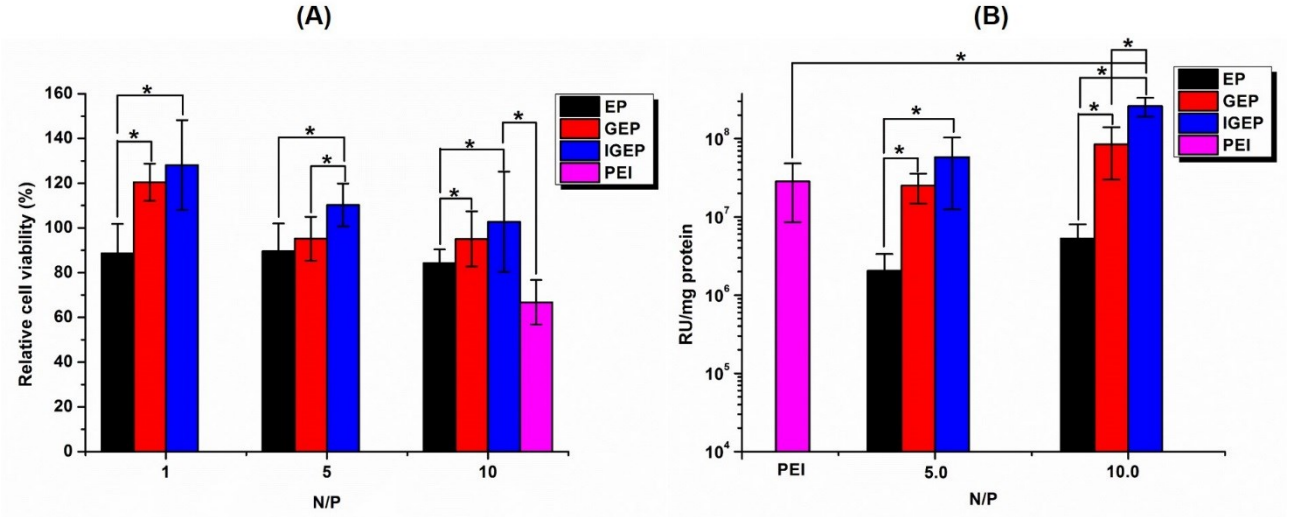


Fig. 3 (A) Cytotoxicity of the PGMA derivatives/pDNA complexes at varying N/P ratios against A549 cells. Percentage of cell viability was expressed relative to the blank control cells. PEI (M_w~25,000 Da, branched) was used for comparison (n=8) Asterisk (*) indicates significant differences ($^*p < 0.05$); (B) Transfection efficiencies of PGMA derivatives/pDNA complexes at varying N/P ratios in A549 cells, PEI (M_w~25,000 Da, branched, N/P = 10.0) was used for comparison (n=4). Asterisk (*) indicates significant differences ($^*p < 0.05$).

contributing to facilitated cellular uptake. This result also demonstrated that guanidine functionalization resembling cell-penetrating properties as conventional CPP molecules could be possibly employed as a facile chemistry to promote cellular uptake and transfection activity of synthetic delivery system. As for GEP and IGEP, Schiff-base linked imidazole functionalization didn't enhance the cellular uptake. Similar results were found in Schiff-base linked imidazole polylysine with reversible PEGylation.[43, 44]

Intracellular localization of complexes. In order to distinguish the functional role of guanidine and imidazole component in gene delivery, the cellular uptake efficiencies and intracellular trafficking behavior of EP, GEP and IGEP were studied by CLSM with Cy5-labeled pDNA (red) and recorded by CLSM. Lyso Tracker Green (green) and DAPI were used to label late endosomes/lysosomes and nuclei, respectively. As shown in **Fig. 5**, Cy5-labeled pDNA signals (red fluorescent dots) are observed abundantly in the entire cells. The Cy5-labeled pDNA delivered by GEP and IGEP are observed to be more than that by EP, supporting functional role of guanidine components for improved cellular uptake. Of note, considerable fraction of Cy5-labeled pDNA is observed inside the nuclei by GEP and IGEP, as opposed to the limited Cy5-labeled pDNA by EP, which can be interpreted as that guanidine mediated nuclear trafficking of pDNAs and Schiff-base linkages regulated DNA unpacking and release synergistically results in the efficient nucleus transport of pDNA. In addition, less Cy5-labeled pDNA was colocalized with green lysotracker for IGEP, as compared with GEP, which implies the functional role of imidazole residue to promote endosome escape. To this end, we could determine the strategic use of guanidine and imidazole in pursuit of efficient gene transfection: guanidine serves as a membrane associating moiety to promote cellular uptake and nuclear trafficking; and imidazole to GEP by Schiff-base linkages can further elevate the transfection efficiency by facilitate endosome escape and DNA cargo releasing.

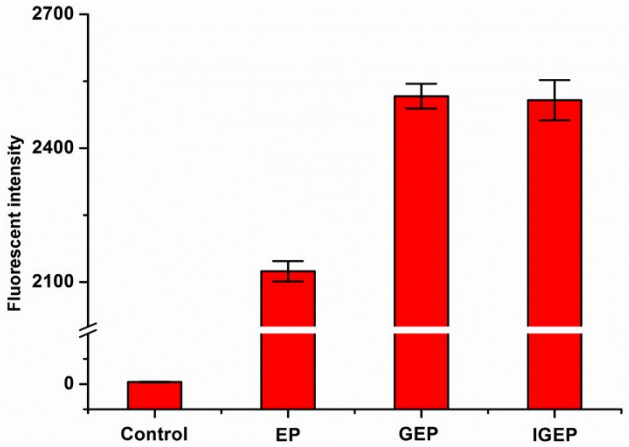


Fig. 4 Mean fluorescence intensity of PGMA derivatives/Cy5-labeled pDNA complexes at N/P ratio of 10.0 obtained from flow cytometry of A549 cells. Results are shown as mean ± standard deviation (n = 4).

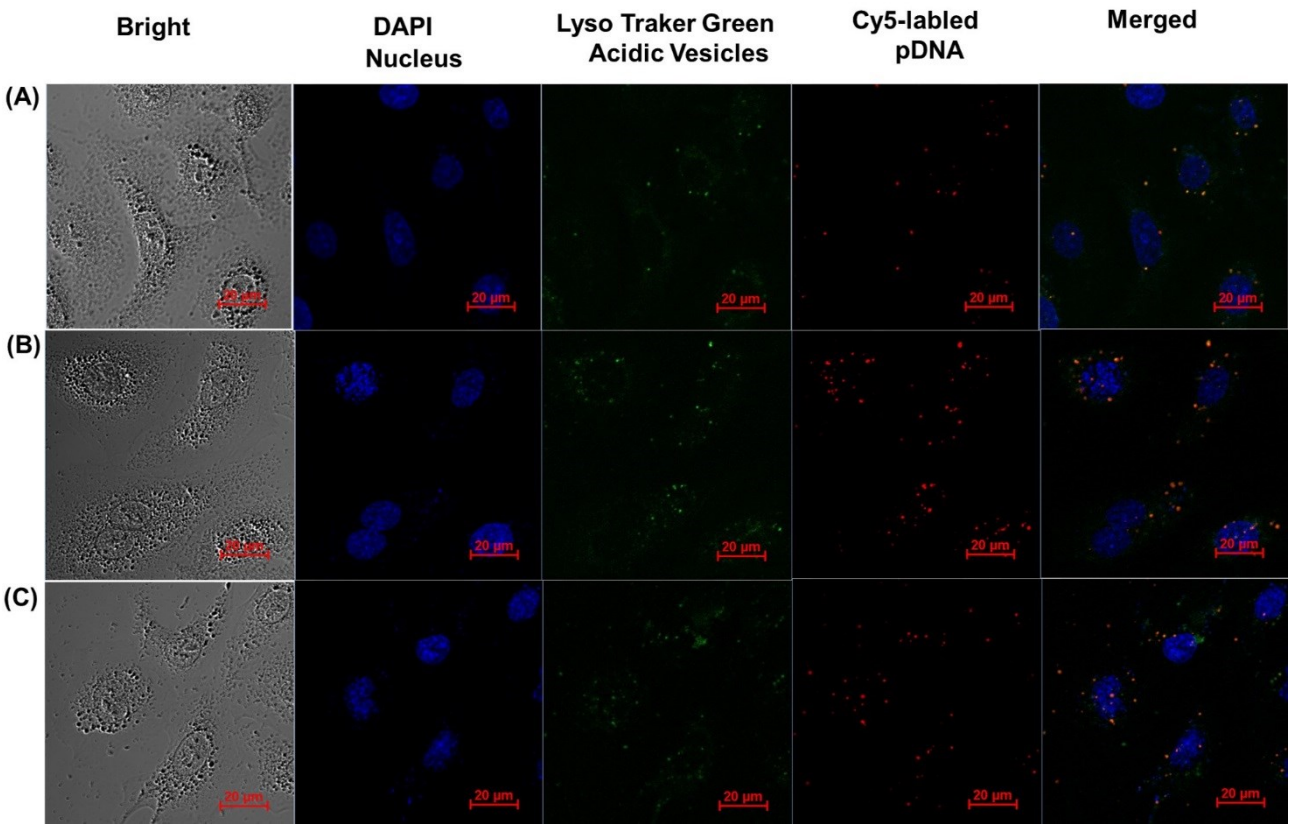


Fig. 5 CLSM observation of the intracellular distribution of (A) EP/CAG-Luc (Cy5-labeled, red) complexes; (B) GEP/CAG-Luc complexes; (C) IGEP/CAG-Luc complexes. Late endosomes and lysosomes were stained with Lyso Tracker Green (green). The nuclei was stained with DAPI (blue).

Conclusions

In the present study, a series of functionalized amino PGOHMAs have been successfully synthesized and their physicochemical properties have been evaluated. Despite the transfection efficacy of precursor EP was lower than that of commercial PEI, their appreciable safety profile, substantially lower than that of PEI, implies its promising prosperity. Post guanidine functionalization, GEP led to remarkable enhancement in transfection efficiency and further reduced cytotoxicity as compared to the precursor EP. According to subsequent flow cytometry and confocal observation, it was determined that its higher transfection efficiency was attributable to enhanced cellular uptake and nuclear localization by virtue of guanidine to induce readily affinity to the cellular membrane. Furthermore, imidazole contained IGEP afforded anther worthy strategy to entitle efficient endosome escape and readily DNA releasing functions, eventually giving rising to efficient gene expression at the targeted cells. Therefore, cationic PGOHMAs post-modified with guanidine and imidazole provided substantial information in strategically overcoming the barriers presented in cell transfection, thus should be emphasized in design and development of synthetic gene delivery system.

Acknowledgements

The authors thank National Natural Science Foundation of China (21374079, 21244004), Program for New Century Excellent Talents in University (NCET-11-1063), and Program for Prominent Young College Teachers of Tianjin Educational Committee for financial support.

Notes and references

1 Z. H. Liu, Z. Y. Zhang, C. R. Zhou, Y. P. Jiao, *Prog. Polym. Sci.*, 2010, **35**, 1144-1162.
2 H. Wei, L. R. Volpatti, D. L. Sellers, D. O. Maris, I. W. Andrews, A. S. Hemphill, L. W. Chan, D. S. H. Chu, P. J. Horner, S. H. Pun, *Angew. Chem. Int. Edit.*, 2013, **52**, 5377-5381.
3 Y. Nie, D. Schaffert, W. Rödl, M. Ogris, E. Wagner, M. Günther, *J. Controlled Release*, 2011, **152**, 127-134.
4 M. Arif, S. K. Tripathi, K. C. Gupta, P. Kumar, *J. Mater. Chem. B*, 2013, **1**, 4020-4031.
5 M. Dimitrova, Y. Arntz, P. Lavalle, F. Meyer, M. Wolf, C. Schuster, Y. J. C. Haïkel, J. Ogier, *Adv. Funct. Mater.*, 2007, **17**, 233-245.
6 M. A. Mintzer, M. W. Grinstaff, *Chem. Soc. Rev.*, 2011, **40**, 173-190.
7 J. T. Zhang, D. M. Lynn, *Macromolecules*, 2006, **39**, 8928-8935.

8 J. J. Nie, X. B. Dou, H. Hu, B. Yu, D. F. Chen, R. X. Wang, F. J. Xu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 553-562.
9 Z. Chen, L. Zhang, Y. He, Y. Li, *ACS Appl. Mater. Interfaces*, 2014, **6**, 14196-14206.
10 S. Srinivasachari, K. M. Fichter, T. M. Reineke, *J. Am. Chem. Soc.* 2008, **130**, 4618-4627.
11 N. Sakaguchi, C. Kojima, A. Harada, K. Koiwai, K. Shimizu, N. Emi, K. Kono, *Biomaterials*, 2008, **29**, 1262-1272.
12 Y. Yue, C. Wu, *Biomater. Sci.*, 2013, **1**, 152-170.
13 L. Qian, H. Xiao, G. Zhao, B. He, *ACS Appl. Mater. Interfaces*, 2011, **3**, 1895-1901.
14 T. A. Theodossiou, A. Pantos, I. Tsogas, C. M. Paleos, *ChemMedChem.*, 2008, **3**, 1635-1643.
15 D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discov.*, 2005, **4**, 581-593.
16 S. Boeckle, J. Fahrmeir, W. Roedl, M. Ogris, E. Wagner, *J. Controlled Release*, 2006, **112**, 240-248.
17 B. Shi, H. Zhang, J. Bi, S. Dai, *Colloids and Surf. B-Biointerfaces* 2014, **119**, 55-65.
18 R. Chandra, R. Rustgi, *Prog. Polym. Sci.*, 1998, **23**, 1273-1335.
19 B. Shi, H. Zhang, Z. Shen, J. Bi, S. Dai, *Polym. Chem.* 2013, **4**, 840-850.
20 B. Shi, H. Zhang, S. Dai, X. Du, J. Bi, S. Z. Qiao, *Small*, 2014, **10**, 871-877.
21 J. Liang, G. Steinberg, N. Livnah, M. Sheves, T. G. Ebrey, M. Tsuda, *Biophys. J.*, 1994, **67**, 848-854.
22 Q. L. Li, W. X. Gu, H. Gao, Y. W. Yang, *Chem. Commun.*, 2014, **50**, 13201-13215.
23 H. Gao, X. Lu, Y. Ma, Y. W. Yang, J. Li, G. Wu, Y. Wang, Y. Fan, J. Ma, *Soft Matter*, 2011, **7**, 9239-9247.
24 C. Li,; Y. W. Yang, Z. X. Liang, G. L. Wu, H. Gao, *Polym. Chem.*, 2013, **4**, 4366-4374.
25 H. Gao, M. Elsabahy, E. V. Giger, D. Li, R. E. Prud'homme, J. C. Leroux, *Biomacromolecules*, 2010, **11**, 889-895.
26 Z. X. Liang, X. S. Wu, Y. W. Yang, C. Li, G. L. Wu, H. Gao, *Polym. Chem.*, 2013, **4**, 3514-3523.
27 F. J. Xu, M. Y. Chai, W. B. Li, Y. Ping, G. P. Tang, W. T. Yang, J. Ma, F. S. Liu, *Biomacromolecules*, 2010, **11**, 1437-1442.
28 X. C. Yang, M. Y. Chai, Y. Zhu, W. T. Yang, F. J. Xu, *Bioconjugate Chem.*, 2012, **23**, 618-626.
29 R. Qi, S. Wu, H. Xiao, L. Yan, W. Li, X. Hu, Y. Huang, X. Jing, *J. Mater. Chem.*, 2012, **22**, 18915-18922.
30 W. Zauner, M. Ogris, E. Wagner, *Adv. Drug Deliv. Rev.*, 1998, **30**, 97-113.
31 C. Volcke, S. Pirotton, C. Grandfils, C. Humbert, P. A. Thiry, I. Ydens, P. Dubois, M. Raes, *J. Biotechnol.*, 2006, **125**, 11-21.
32 P. Dubruel, B. Christiaens, B. Vanloo, K. Bracke, M. Rosseneu, J. Vandekerckhove, E. Schacht, *Eur. J. Pharm. Sci.*, 2003, **18**, 211-220.
33 R. Zhang, N. Zheng, Z. Song, L. Yin, J. Cheng, *Biomaterials*, 2014, **35**, 3443-3454.
34 X. B. Dou, M. Y. Chai, Y. Zhu, W. T. Yang, F. J. Xu, *ACS Appl. Mater. Interfaces*, 2013, **5**, 3212-3218.
35 H. S. Hwang, J. Hu, K. Na, Y. H. Bae, *Biomacromolecules* 2014, **15**, 3577-3586.
36 F. J. Xu, Y. Zhu, M. Y. Chai, F. S. Liu, *Acta Biomater.*, 2011, **7**, 3131-3140.
37 M. A. Mintzer, E. E. Simanek, *Chem. Rev.*, 2009, **109,** 259-302.
38 S. J. Hwang, N. C. Bellocq, M. E. Davis, *Bioconjugate Chem.*, 2001, **12**, 280-290.
39 D. Lee, Y. M. Lee, C. Jeong, J, Lee, W. J. Kim, *ChemMedChem*, 2014, **9**, 2718-2724.
40 R. Goyal, R. Bansal, S. Tyagi, Y. Shukla, P. Kumar, K. C. Gupta, *Mol. BioSyst.*, 2011, **7**, 2055-2065.
41 M. Ma, F. Li, F. J. Chen, S. X. Cheng, R. X. Zhuo, *Macromol. Biosci.*, 2010, **10**, 183-191.
42 D. W. Hwang, S. Son, J. Jang, H. Youn, S. Lee, D. Lee, Y. S. Lee, J. M. Jeong, W. J. Kim, D. S. Lee, *Biomaterials*, 2011, **32**, 4968-4975.
43 Q. Cheng, Y. Huang, H. Zheng, T. Wei, S. Zheng, S. Huo, X. Wang, Q. Du, X. Zhang, H. Y. Zhang, X. J. Liang, C. Wang, R. Tang, Z. Liang, *Biomaterials*, 2013, **34**, 3120-3131.
44 X. Cai, Y. Li, D. Yue, Q. Yi, S. Li, D. Shi, Z. Gu, *J. Mat. Chem. B*, 2015, **3**, 1507-1517.

Table of Contents

for

Dual functionalized amino poly(glycerol methacrylate) with guanidine and Schiff-base linked imidazole for enhanced gene transfection and minimized cytotoxicity

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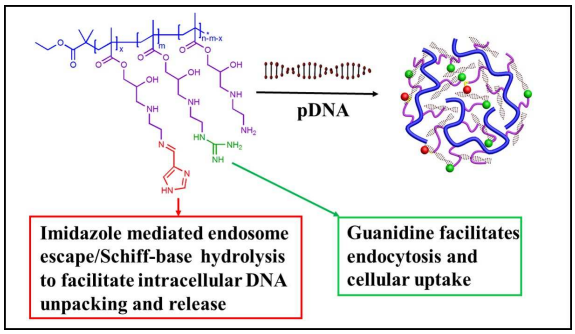

Guanidine and Schiff-base linked imidazole dual functionalized poly(glycerol methacrylate) (IGEP) leads to minimized cytotoxicity and better transfection efficacy than PEI_{25K}.