Achieving High Gene Delivery Performance with Caveolae-mediated Endocytosis Pathway by (l)-Arginine/(l)-Histidine Co-modified Cationic Gene Carriers

Hui Li¹, Ting Luo¹, Ruilong Sheng^{*1,2}, Jingjing Sun¹, Zhao Wang¹, Amin Cao^{*1}

1. *CAS Key Laboratory for Organic Functional Materials, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China*
2. *Department of Chemistry, Université de Montréal, C.P.6128, Succursale Centre-ville, Montréal, Québec, H3C3J7, Canada*

*Author for correspondence

Phone: +86-21-5492-5535, +1-5143405121-7422

Email: rlsheng@mail.sioc.ac.cn

Abstract

Developing new amphiphilic polymers with natural product moieties has been regarded as a promising way to achieve biocompatibility and certain biological functions. In prior work, we developed some natural (*L*)-arginine modified cationic polymers (PAHMAA-Rs) as cationic gene carriers. For the sake of continuing optimize the gene delivery performance, herein, a new series of (*L*)-arginine and (*L*)-histidine co-modified cationic poly (ω -aminohexyl methacrylamide)s (PAHMAA-R-H) were synthesized and characterized with ^1H NMR, GPC-SLS and FT-IR. Their proton buffering capacities were studied by acid-base titration assay. pDNA binding affinity and self-assembly properties of the polyplexes were analyzed by agarose gel retardation assay, DLS and AFM, respectively. In vitro cytotoxicity of the PAHMAA-R-H was determined by MTT and LDH assays in H1299 cells, the gene transfection efficacy and intracellular uptake capability were evaluated by luciferase assay and FACS, respectively. Moreover, the endocytosis pathways and intracellular distribution of the polyplexes were investigated by using specific endocytic inhibitors and fluorescent co-localization techniques. The results demonstrated that co-modification of (*L*)-arginine and (*L*)-histidine onto the PAHMAA polymer could enhance proton buffering capacity, shield surface charge, decrease cytotoxicity, and improve gene transfection efficiency and serum-compatibility. Moreover, the gene transfection and intracellular uptake behaviors were disclosed strongly rely on the (*L*)-arginine/(*L*)-histidine modification ratios. The polyplexes tend to be internalized through caveolae-mediated endocytosis gateway and localized with endosomes/lysosomes in H1299 cells. Notably, among the polymers, the PAHMAA-R₁₈-H₆ exhibited remarkable gene delivery efficiency and serum compatibility, which made it promising gene transfection agent for practical application.

Key words: co-modification, (*L*)-arginine, (*L*)-histidine, endocytosis pathway, serum compatibility

1. Introduction

In recent years, constructing new cationic polymer gene/drug carriers based on renewable natural products (such as amino acids [1]) has been regarded as a promising way to achieve high biocompatibility and certain biological/pharmacological functions.[2-7] Although many natural-based polymers were explored as non-viral gene vectors, their gene delivery performance was still far below that of virus-based gene carriers. For practical application, developing natural-based gene delivery systems with the merit of low cytotoxicity, high transfection efficiency and good serum compatibility is need to be highly focused.[8]

To achieve high gene tranfection efficiency, endosomes/lysosomes were essential intracellular barriers need to breakthrough.[9] The endosomes/lysosomes are acidic (pH 4.5~6.5) subcellular organelles acidified by ATPase-driven proton transportation, and which containing various hydrolases for digest endocytic substances including the gene/drug payloads [10, 11]. Thus, enhancing the “endosome/lysosome escape” capability of gene carrier complexes to avoid pDNA-payload degradation inside endosomes/lysosomes, is regarded as one of the effective ways to improve the gene transfection efficiency of non-viral polymeric gene carriers[12]. Some chemical/biochemical substances with "proton sponge effect"[13] such as synthetic polyethylenimine (PEI) polymers, cationic lysosome fusion peptides, were employed to induce endosome/lysosome destruction and realize the endosomal/lysosomal “escape” of gene/drug payloads. Leong et al [14] found the addition of proton scavenger chloroquine to chitosan-based cationic polymer/pDNA polyplexes could improve lysosomal escape effect and enhance gene transfection efficiency, while high toxicity of the chloroquine restricted its application in gene transfection. Polyethylenimine (PEI) is a typical series of "proton sponge" polymers with multi-types of amino groups (1° , 2° and 3° , $pK_a=7.2\sim5.1$), which demonstrated remarkable gene transfection efficiency but very high cytotoxicity due to the charge-induced cell membrane disruption[15]. Regarding the use of lysosome fusion peptides, Pun et al [16] recently reported Melittin peptide conjugated cationic polymer carriers can lead to a 35-fold improvement of gene transfection efficiency. Considering the complicated architecture and comparatively high cost of functional lysosome fusion peptides, alternatively, (*l*)-histidine/imidazolyl were used as "proton sponge" moieties to build biocompatible and efficient cationic polymer gene carriers. e.g. Midoux et al^[17] reported a series of imidazolyl-bearing cationic polymers with improved gene transfection efficiency through lysosomal escape effect. Kim et al [18] prepared some imidazole-containing cationic chitosan polymers with low cytotoxicity and enhanced gene transfection efficiency. Hashida et al [19] reported that (*l*)-histidine modified chitosan polymers with

enhanced buffering capacity could lead to efficient “lysosomal escape” and improve the gene transfection efficiency. Lin et al²² prepared some (*L*)-Histidine pendent cationic polymers via Michael addition, which showed low cytotoxicity and high gene transfection efficiency above bPEI-25K reference.

It has been reported the (*L*)-histidine/imidazolyl-modified cationic polymer gene carriers could achieve high gene transfection capability under the presence of serum. Midoux et al[20] disclosed that (*L*)-histidine modification on pendent primary ω -amino groups of poly-(*L*)-lysines could reduce the surface positive charges, which led to high serum-stability of the pDNA-loaded polyplexes and thus enhanced the gene transfection efficiency under serum conditions. Pan [21] et al modified 4th-generation-dendritic PAMAM (PG4) polymer with natural (*L*)-histidine moiety to obtain HPG4 polymers, compare to the PG4/pDNA polyplexes, HPG4/pDNA polyplexes found not only have enhanced “lysosomal escape” capability, but also with remarkable intracellular uptake and gene transfection efficiency under serum conditions in MDA-MB-231 cells. Thus, it could be expected that (*L*)-histidine/imidazolyl-modification on cationic polymers maybe a promising approach for developing serum compatible and high efficient polymer gene carriers.

On the other hand, endocytosis pathway has been known played important roles in the gene transfection processes of nanoscale gene-payloads [22, 23]. The endocytosis pathways [24] mainly including clathrin-mediated endocytosis, caveolae-/lipid raft-mediated endocytosis, macropinocytosis and phagocytosis. Sung et al^[25] found some N-palmitoyl chitosan nanoparticles were uptaken through the lipid raft-mediated endocytic route, and which could be turned to caveolae-mediated endocytosis upon increasing the degree of N-palmitoyl substitution. Jeong et al^[26] revealed the clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis were spontaneously involved in the uptake of hydrophobic 5 β -cholanolic acid modified glycolchitosan (HGC) nanoparticles. Chen et al^[27] disclosed some cholesterol-grafted poly(amido amine)s/SiRNA payloads were presumably undergo the cholesterol-related caveolae-mediated endocytic pathway and followed with lysosomal localization. It has been revealed that caveolae-mediated endocytosis pathway maybe more favorable for high efficient non-viral gene carriers than other pathways [28, 29]. In fact, natural simian virus (SV40) vectors entered into mammalian cells by caveolae/lipid raft-mediated endocytosis after binding to ganglioside cell surface receptors [30]. Noteworthy, up to date, the correlation between the gene vector/carrier structures and endocytosis pathways for the most of the natural-based gene delivery systems still remains unclear.

In our previous work, we have prepared a series of natural (*L*)-arginine grafted polymers as R8 cationic peptide [31] mimicking gene carriers, among them, PAHMAA-R₁₆ possessed the optimized cellular uptake and gene delivery efficiency. However, the PAHMAA-R₁₆ has moderate cytotoxicity, its gene transfection and poor serum-compatibility still need to be further optimized. Besides, the correlation between the modification groups/moieties on PAHMAA-R polymers and the related endocytosis pathways is still unknown. It has been known that introducing (*L*)-histidine to arginine-rich peptide could improve the lysosome escape capability [32]. Therefore, in order to further improve the gene transfection efficiency and serum compatibility of the PAHMAA-R polymers, in this work, a series of natural (*L*)-arginine and (*L*)-histidine co-modified cationic poly (ω -aminohexyl methacrylamide)s (PAHMAA-R-H) were synthesized and characterized. The physico-chemical properties such as proton buffering capacity and pDNA binding affinity of the PAHMAA-R-H polymers, average particle size, surface zeta potential and morphology of the PAHMAA-R-H/pDNA polyplexes were studied. *In vitro* cytotoxicity of the PAHMAA-R-H polymers was determined by MTT and LDH assays in H1299 cells and the *in vitro* gene transfection efficiency were evaluated in the absence/presence of 10% FBS with luciferase assay kit. Intracellular uptake capability of the polyplexes was analyzed by FACS. Endocytosis pathway and intracellular distribution of the polyplexes in H1299 cells were investigated with the luciferase assay by using endocytosis-specific inhibitors and fluorescent co-localization, respectively.

2. Experimental section

2.1. Materials

O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU, 98 %), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 98 %), Boc-N ω -(2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl)-L-arginine (Boc-Arg(Pbf)-OH, 99%), di-isopropyl-ethylamine (DIPEA, 97%), N α -N $_{im}$ -di-*t*-Boc-L-histidine dicyclohexyl ammonium salt (Boc-His (Boc)-OH·DCHA, 99%), and trifluoroacetic acid (TFA, 99%) were purchased from *Gill Biochemical Co. Ltd* (Shanghai, China). pCMV-Luc DNA was provided by Prof. Yuhong Xu of *Shanghai Jiaotong University, China*. pEGFP DNA and H1299 cell-line were generously gifted by Dr. Bo Wan of *Fudan University, Shanghai, China*. Luciferase assay and bicinchoninic acid (BCA) protein quantification kits were supplied by *Promega* (Cat#E1500, USA) and *Biomiga* (Cat#PW0104, USA), respectively. 0.01 M phosphate buffer solution (1×PBS), Tris-buffered saline and Tween 20 (1×TBST), RPMI-1640 medium and Dulbecco's modified eagle media (DMEM) were received from *Hangzhou Genom Co. Ltd*,

China. Fetal bovine serum (FBS) was supplied by GIBCO (Cat#10099-141, USA) and ExCell Bio (Cat#FSP500, China). Lactate dehydrogenase release (LDH) assay kit II (Cat#ab65393) was provided by Abcam Corporation (UK). Lysosomal labeling reagents anti-Lamp-1 antibody (H4A3) (mouse monoclonal IgG1, Cat#-20011) were purchased from *Santa Cruz Biotechnology, USA*. Secondary antibodies Alexa Fluor® 488 labeled rabbit anti mouse IgG (Cat#A11059) was purchased from *Life Technologies Corporation, USA*. DAPI was purchased from Roche (Cat#70217321, Switzerland). Label IT-Cy3 Nucleic acid labeling kit was purchased from *Mirus Bio Corporation* (Cat#MIR7010, USA). In addition, all other chemicals were of analytical grade and were used as received.

2.2 Syntheses of the cationic polymers

2.2.1. Series PAHMAA-R-H The synthesis of macromolecules

The N-(tert-butoxycarbonyl)aminohexyl methacrylamide (Boc-AHMAA) monomer was synthesized and characterized according to the method which had been described in our prior report[33].

2.2.2. Synthesis of the Boc-AHMA (PAHMAA-Boc) by RAFT polymerization and preparation of the cationic polymer (PAHMAA-TFA) by Boc-deprotection

In a 25 ml Schlenk flask added RAFT agent 4-cyano-4-(dodecylsulfanyl thiocarbonyl) sulfanyl pentanoic acid (78.0 mg, 0.28 mmol), Boc-AHMAA monomer (2.86 g, 10.0 mmol) and initiator AIBN (23.4 mg, 0.060 mmol) dissolved in 10.0 ml of THF and subjected to three freeze-pump-thaw cycles. Then the RAFT polymerization was conducted at 70 °C for 12 h and was concentrated and precipitated with cold dry diethyl ether, filtered and dried in vacuum to give the PAHMAA-Boc products (1.9 g, yield: 67%). After that, the Boc protecting groups of the PAHMAA-Boc were removed in TFA/CH₂Cl₂ (1/1, v/v) at room temperature for 2 h and was precipitated with anhydrous diethyl ether, then filtrated and dried in vacuum oven to obtain the final product PAHMAA-TFA as yellowish solid.

PAHMAA-TFA: ¹H NMR (CDCl₃, δ in ppm): 3.09-2.80 (CH₂NH₂, CH₂NHCOCCH₃), 2.15-1.02 (COC (CH₃) CH₂, CH₃, C (CH₃)₃). FT-IR (in cm⁻¹): 3290, 3079, 2946, 1680, 1632, 1553, 1209, 1132.

2.2.2. Synthesis of the PAHMAA-R-H cationic polymers

Predetermined amount of Boc-Arg(Pbf)-OH, Boc-His(Boc)-OH·DCHA, HBTU and DIPEA were dissolve in N,N-dimethylformide (DMF), the mixture was stirred at room

temperature for 30 min to prepare the activated esters. Afterward, the above-prepared cationic polymer p(AHMAA-TFA) was added and the reaction mixture was continuing stirred at room temperature for 3 days. Then the organic solvent was removed by a vacuum rotary evaporator, added a mixture solution of TFA/CH₂Cl₂ (1/1, v/v) and stirred at room temperature for 2 h. The residue was concentrated and re-dissolved in small amount of distilled water and dialysis (MWCO 3500) in distilled water for 2 days and the water was replaced every 6 hours, the dialysis solution was further freeze dried to obtain the final products PAHMAA-R-H cationic polymers as white solid. (Yield: 50%~70%).

¹H NMR (CDCl₃, δ in ppm): 7.73-7.60 (NCHNH), 6.95-6.82 (CCHNCH), 3.94-3.78 (CHNH₂CH₂C), 3.77-3.67 (CH NH₂CH₂CH₂), 3.27-2.69 (CH₂NH₂, CH₂NHCOCH, CH₂NHCOCCH₃, CNHCH₂CH₂, CCH₂CHNH₂), 2.50-0.46 (NHCHCH₂(CH₂)₂), COC (CH₃) CH₂, CH₃, CH₂)

2.3. Instrumental characterization of the synthesized polymers

¹H NMR spectra were recorded on a Varian-300 FT-NMR spectrometer at 300.0 MHz for proton nuclei, and ¹³C NMR structural characterization was implemented on a Bruker Avance-400 NMR spectrometer, operating at 100.0 MHz for ¹³C nuclei. Measurements of all NMR spectra were conducted at room temperature with tetramethylsilane (TMS) as an internal chemical shift reference.

~~ESI-MS were measured on a Varian SATURN 2000 mass spectroscopy instrument.~~

FT-IR spectra were recorded on a Bio-Rad FTS-185 spectrometer at room temperature in the wavenumbers ranging from 4000 to 500 cm⁻¹ with 4 cm⁻¹ spectral resolution (64 times scanning).

Gel permeation chromatography (GPC) was utilized to measure the average molecular weights (M_n , M_w) and polydispersity index (M_w/M_n) of the synthesized P(AHMAA-Boc) polymers at 35°C on a PerkinElmer 200 GPC instrument, which equipped with two TSK gel Multipore HXL-M (300×7.5mm) (TOSOH, Japan) gel chromatographic columns and a refractive index detector (RI), with THF was utilized as the eluent at a flowing rate of 1.0 mL/min, and a series of commercial polystyrene standards (*Polymer laboratories, UK*) with narrow molecular weight distribution were applied to calibrate the GPC elution traces.

2.4. Buffering capacity analysis by acid-base titration

The proton buffering capacity for the synthesized cationic polymers was measured by acid-base titration on a Schott Titroline Easy instrument at room temperature [34]. Each cationic polymer was dissolved in distilled water to prepare the solution with total amine concentration of 0.01 M. The solution pH was adjusted by 1.0 M HCl to the pH value of 3. Then the solution was gradually titrated with 0.01 M NaOH solution to the final pH value of 10~11. The buffering capacity of the cationic polymer is defined as the percentage of the protonated amine in the pH range of 5.1~7.2, and the formula is shown as follows: Buffer capacity (%) = $100 (\Delta V_{\text{NaOH}} \times 0.01 \text{ M}) / N \text{ mol}$. Here, ΔV_{NaOH} is the consumed volume of 0.01 M NaOH solution when the pH value change from 5.1 to 7.2, N means the molar of total protonated amine groups (0.01 mmol).

2.5. pDNA binding affinity of the cationic polymers by agarose-gel retardation assay

The pDNA binding affinity of the synthesized cationic polymers was analyzed by agarose gel retardation assay [35]. The cationic polymers/pDNA polyplex samples were firstly prepared by mixing the cationic polymers with 2.0 μg pDNA in 1 $\times$ PBS solution (0.01 M, pH=7.4) under various N/P charge ratios and were further incubated for 30 min at 37°C. Each complex solution was then loaded onto 1% agarose gel (w/v) containing 0.5 $\mu\text{g/mL}$ ethidium bromide (EB) dye in TAE buffer solution (pH=7.4), the cationic polymers/pDNA polyplex samples were run on agarose gel in 1 $\times$ TAE buffer running at the electric tension of 100 V for 1 h. the migration bands of the plasmid DNA was illuminated and photographed on a UV transilluminator (UVP, USA).

2.6. Average particle sizes and zeta potentials measured by Dynamic light scattering (DLS)

Average particle sizes and zeta potentials of the cationic polymers/pDNA polyplex samples were analyzed on a Malvern Zetasizer Nano ZS90 dynamic light scattering instrument at room temperature, with laser beam wavelength at $\lambda=633 \text{ nm}$ and a fixed scattering angle of 90°. All analyses were conducted in triplicate (n=3).

2.7. Morphology of the DNA-loaded polyplexes measured by atomic force microscopy (AFM)

Morphology of the DNA-loaded polyplexes were observed and recorded on a Nanoscope IVa atomic force microscopy[34] (AFM, Veeco Instrument) at ambient temperature in tapping mode with Olympus-AC160TS probe cantilever (Frequency: 300 kHz, stiffness: 42 N/m) Prior to AFM measurements, the solution samples of the polyplexes were deposited onto fresh mica, and air-dried at room temperature.

2.8. Cytotoxicity of the cationic polymers by MTT and LDH assays

For MTT assay[36], H1299 cells were seeded into 96-well microplates at 5×10^3 cells/well with 100 μ L of RPML-1640 culture medium (with 10% FBS) and cultured at 37°C for 24 h, the RPML-1640 medium was replaced with fresh medium (with 10% FBS), and a predetermined dose of the synthesized cationic polymers were added and incubated with the H1299 cells for 24 h. After that, 20 μ L of MTT solution (5.0 mg/mL) was added into each well and further incubated at 37°C for 2 h for formazan formation. Then the RPML-1640 medium was discarded and DMSO (100 μ L /well) was added to dissolve the MTT formazan. Absorbance of the solution at the wavelength of 490 nm was measured on an ELX800 microplate reader (BioTek, USA) with the wavelength of 630 nm as the references. The cell viability of blank samples was set as 100% and commercially available bPEI-25K was used as the reference, relative cell viability (%) of the cationic polymers were measure in six-replicates (n=6).

LDH assay [35] was used to evaluate the cytotoxicity of plasma membrane disruption induced by the synthesized cationic polymers. First, H1299 cells at 1×10^5 cells/well were seeded into 24-well microplates with 100 μ L of RPML-1640 culture medium (with 10% FBS) and cultured at 37°C for 24 h, predetermined amounts of cationic polymers were added and incubated for 4 h. Afterward, 100.0 μ L LDH buffer solution was added into each well and incubated for 30 min. Then the cells in each well was took out and centrifuged at 600 rpm for 10 min to remove the cell fragments, the supernatant (10.0 μ L/well) was transferred into an optically clear 96-well microplate. Light absorbance of all control and testing samples at the wavelength of 450 nm were measured on microplate reader the wavelength of 630 nm as the references. Finally, cytotoxicities were evaluated as the subtracted absorbance ($I_{450} - I_{630}$) according to the manufacture's LDH assay protocol.

2.9. Luciferase reporter gene transfection assays in the absence/presence of serum

H1299 cells at 1×10^5 cells/well were seeded into 24-well microplates with 500 μ L of RPML-1640 culture medium (with 10% FBS) and cultured at 37 °C for 24 h, when the cell density in each reaches about 70~80%, the RPML-1640 medium was replaced with 400 μ L of fresh medium (FBS-free or 10% FBS), then the cationic polymers/pDNA polyplexes (pDNA 1.0 μ g/well) with preset N/P ratios was added, with naked pDNA and bPEI-25K/pDNA polyplexes (N/P=10) was used as the references. For the tranfection assays in the absence of serum, after the incubation of the cationic polymers/pDNA polyplexes for 4 h, the culture medium were carefully removed and replaced with fresh RPML-1640 medium containing 10%

FBS and further incubated for 24 h. For the transfection assays in the presence of serum, the cationic polymers/pDNA polyplexes were added and incubated in RPMI-1640 medium containing 10% FBS within 24 h. Then follow the Luciferase Assay System instructions for detection of Luciferase expression of luciferase gene. Firstly 200 μ L of cell lysates was added to lyses cells at room temperature with gently shake for 10 min, 50 μ L of lysis buffer transfer to 1.5 mL centrifuge tube then added 20 μ L of luciferase substrate solution, after 10 s mixing quickly put the centrifuge tubes into Glomax 20/20 Illuminometer (Promega) for determination of relative chemoluminescence value (RLU). Followed by BCA method to determine the concentration of proteins in cell lysates, the data are expressed as per mg protein corresponds to the relative light units (RLU/mg protein).

2.10. EGFP gene transfection assay in the absence of serum

H1299 cells were seeded into 6-well microplates at 1×10^5 cells/well with 1.0 mL/well RPMI-1640 medium containing 10% FBS and kept incubation at 37°C for 24 h. Then, the culture medium was replaced with FBS-free medium, and the cationic polymers/pEGFP-N1 DNA polyplexes (2.0 mg pDNA/well, N/P=5) were added and further incubated for 4 h, then the culture medium was replaced by fresh RPMI-1640 medium (with 10% FBS) and kept incubation for 24 h. Finally, the H1299 cells were observed and recorded on a Nikon Ti-S invert fluorescence microscopy for EGFP imaging.

2.11. Cell uptake capability of the polyplexes measured by FACS

To evaluate the cell uptake capability of the polyplexes in H1299 cells, Cy3-labeled pEGFP-N1 DNA was firstly prepared in accordance to the protocol of Label-IT-Tracker Cy3 Kit [37]. The PAHMAA-R, PAHMAA-H and PAHMAA-R-H/Cy3-pDNA polyplexes were prepared by mixing the cationic polymers with Cy3-labeled pDNA and incubated at room temperature for 30 min. Cell uptake capability of the Cy3-pDNA payloads were determined on a flow cytometer (BD FACS Calibur, USA)[38]. H1299 cells were seeded into 6-well microplates (3×10^5 cells/well) and were cultured overnight in RPMI-1640 medium with 10% FBS. The polyplexes were added and incubated for 4 h, then the cells were washed with $1 \times$ PBS for three times, followed by trypsinization and centrifugation at 1200 rpm for 5 min to harvest the cells, which were re-suspended in $1 \times$ PBS and the fluorescent intensity were measured by flow cytometry. In FACS measurement, the H1299 cells were gated by sideward scatter versus forward scatter (SSC/FSC) plots, and fluorescence measurements were conducted in the FL2-H channel.

2.12. Luciferase transfection assays under various endocytosis pathway inhibitors and lysosome acidification inhibitor nigericin

Herein, endocytosis pathway analysis was conducted by luciferase transfection assays under various endocytosis pathway inhibitors [39]. H1299 cells were seeded in 24-well plates (3×10^5 cells/well) and incubated in RPMI-1640 medium (with 10% FBS) at 37°C for 24 h. The endocytosis pathway-specific inhibitors, including: Chlorpromazine (CPZ, clathrin-mediated endocytosis pathway inhibitor, 3.0 $\mu\text{g/mL}$), Genistein (GENI, caveolae-mediated endocytosis pathway inhibitor, 50.0 $\mu\text{g/mL}$), Amiloride (AMIL, micropinocytosis pathway inhibitor, 2.3 $\mu\text{g/mL}$), Nocodazole (NOCO, microtubule-assisted phagocytosis inhibitor, 2.0 $\mu\text{g/mL}$) and methyl- β -cyclodextrin (MBCD, lipid-raft pathway inhibitor, 10.0 $\mu\text{g/mL}$) were used for the endocytosis pathway analysis. For study the role of lysosome in the gene transfection process, a lysosome acidification inhibitor nigericin (1.0 mM) was employed. The cytotoxicity of the endocytosis pathway inhibitors and nigericin at the working dosages were measured by MTT assay (Figure S4). The inhibitors and nigericin were respectively added into the H1299 cells and further incubated for 1 h before transfection [39], then the RPMI-1640 medium was replaced with fresh FBS-free RPMI-1640 medium and the polyplexes (pDNA 1.0 $\mu\text{g/well}$, N/P=10) were added and incubated at 37 °C for 4 h. Afterward, the medium was replaced with fresh RPMI-1640 medium with 10% FBS and kept incubation for 24 h. Luciferase transfection assays were conducted according to the protocol of the Luciferase assay system (Promega), then total protein was determined by BCA assay kit (Applygen Technologies Inc). The luciferase relative light unit (RLU) was analyzed and expressed as mean and SD. (n=3)

2.13. Intracellular localization of the cationic polymers/Cy3-pDNA polyplexes observed by confocal microscopy

H1299 cells were seeded in 6-well plates (2×10^5 cells/well) and incubated in RPMI-1640 medium (with 10% FBS) at 37°C for 24 h. Then the medium was removed and fresh serum-free medium with the PAHMAA- R_{18} -H₆/Cy3-pDNA polyplexes (N/P=5) was added and incubated for 6 h, the H1299 cells were then washed with 1×PBS and 1×TBST buffer for three times, then fixed with 4% paraformaldehyde in 1×PBS for 30 min and washed with 1×PBS for three times. Thereafter, the cells were dealt with 0.5% Triton X-100 in 1×PBS for 15 min at 4°C. Non-specific binding of antibody was prevented with 5% bovine serum albumin in PBS for 1 h at room temperature. The plate were incubated with the Lamp-1 primary antibodies (1:1000) in TBS with 5% BSA at 4°C overnight, then washed with

1×TBST buffer three times and incubated with Alex 488 labeling second antibodies (1:500) in 1×TBST with 5% BSA for 40 min. DAPI stain was added (1:1000) and incubated for 45 min, washed with 1×PBS buffer. Afterward, cell images were observed and recorded by confocal microscope system (Olympus fluoview FV1000).

3. Results and discussion

3.1 Preparation and characterization of the (*l*)-arginine and (*l*)-histidine co-modified cationic polymers (PAHMAA-R-H)

According to our previous report[33], macromolecular precursor P(AHMAA-Boc) has been prepared by RAFT polymerization and characterized (Figure S1) by NMR ($M_{n, NMR} = 9300$) and gel permeation chromatography-static laser scattering (GPC-SLS, absolute molecular weight $M_{w, GPC-SLS} = 10600$, $M_w/M_n = 1.16$, Figure S2). The Boc protecting groups on the P(AHMAA-Boc) precursor were removed in TFA/CH₂Cl₂ to afford a water-soluble cationic macromolecular P(AHMAA-TFA). Thereafter, a predetermined ratio of protected (*l*)-arginine (Boc-Arg(Pbf)-OH) and (*l*)-Histidine (Boc-His(Boc)-OH) were coupled to the pendent terminal primary amino groups of cationic P(AHMAA-TFA) polymers by HBTU/DIPEA activation, followed by the deprotection of Boc/Pbf groups in TFA/CH₂Cl₂ solution to prepare the (*l*)-arginine and (*l*)-histidine co-modified PAHMAA-R-H (R represents Arg and H represents His) cationic polymers as the final products. The related synthetic routes were shown in Scheme 1. ¹H NMR characterization of the PAHMAA-R-H cationic polymers was shown in Figure S3. The proton signals (single peaks) at δ 7.75~7.60 ppm and 6.95~6.80 ppm belong to the imidazole groups on (*l*)-histidine residues, and the signal peaked at δ 3.76~3.67 ppm was identified as the α -H of the pendent (*l*)-arginine residues. By comparing the proton signals of the PAHMAA-R-H polymers, proton signals of the (*l*)-histidine residues found shift to high-field due to statistically insertion of (*l*)-arginine residues decreased π - π stacking interactions of imidazole rings of the (*l*)-histidine moieties [40]. To study the impact of (*l*)-histidine on gene transfection, the polymers of PAHMAA-R₁₈-H₃, PAHMAA-R₁₈-H₆ and PAHMAA-R₁₈-H₉ with same (*l*)-arginine ratio but different (*l*)-histidine ratios were designed and synthesized, the modification ratios of (*l*)-arginine and (*l*)-histidine residues on PHAMM-R-H polymers were calculated by comparing their individual proton signal integration intensities (Table S1). The (*l*)-arginine modification ratios were around 60% with various (*l*)-histidine ratios of about 10%, 20% and 32%. Moreover, to elucidate the structure-property relationships, a (*l*)-arginine-rich (80%) polymer PAHMAA-R₂₅-H₆ and two cationic polymers

modified with only (*l*)-arginine (PAHMAA-R₁₈) or only (*l*)-Histidine (PAHMAA-H₂₂) were prepared and employed as references.

3.2. Proton buffering properties of the PAHMAA-R-H polymers

It has been revealed that improving the proton buffering effect of gene carriers would enhance the envisaged “lysosomal escape” effect and promote the subsequent dissociation of polymer carriers/pDNA polyplexes, which may bring about better gene transfection efficiencies [41]. (*l*)-histidine is an effective moiety (imidazole, pK_a~6.0) which was employed to obtain high proton buffering capacity, since the imidazole moieties could be easily protonated under acidic lysosomal environment. Herein, proton buffering effect of the synthesized cationic polymers was measured by acid-base titration assay and the titration curves were shown in Figure 1. The proton buffering effect was obviously improved by increasing of the (*l*)-histidine modification ratios, which due to the “proton sponge” effect of the (*l*)-histidine moieties. Moreover, the proton buffering capacity was quantitatively calculated according to literature [42] by counting the protonation percentage of amino groups in polymers within the pH range of 5.1~7.2, the corresponding formula is shown as follows: Buffer capacity (%) = 100 ($\Delta V_{\text{NaOH}} \times 0.01 \text{ M}$)/N mol, the ΔV_{NaOH} is the NaOH volume used to adjust pH value of the polymer solution from 5.1 to 7.2, N was the total amount of the amines (0.06 mmol). As shown in Table S2, It could be seen that the proton buffering capacity increased from 23.4% to 47.5% when the (*l*)-histidine modification ratios increased from 10% to 32%, whereas the (*l*)-arginine moieties showed less impact on the proton buffering effect. The results indicated the proton buffering capacity of the cationic PAHMAA-R-H polymers could be tuned by controlling the (*l*)-histidine modification ratios. Moreover, it was expected that the PAHMAA-R-H polymers with higher proton buffering capacity may result in stronger “lysosomal escape” effect and may subsequently lead to higher gene transfection efficiency.

3.3. pDNA binding affinity of the PAHMAA-R-H polymers

pDNA binding affinity of the cationic polymer gene carriers is an essential parameter used to determine their gene binding/loading efficiency [43]. By agarose gel electrophoresis assay [44], the pDNA binding affinity of the PAHMAA-R₁₈, PAHMAA-R-H and PAHMAA-H₂₂ were measured and showed in Figure 2, PAHMAA-R₁₈ showed the highest pDNA binding affinity (N/P≤0.5) among the polymers, due to the strong electrostatic interactions between (*l*)-arginine moieties and pDNA [33]. The PAHMAA-R-H polymers showed slightly less but still high pDNA binding affinity (N/P≤2) and the PAHMAA-H₂₂ showed weaker

pDNA binding affinity (N/P~5). The results indicated that the strong basic (*l*)-arginine (guanidine, pKa~12.4) moieties played essential roles in pDNA binding. Moreover, the co-modification of the proton buffering (*l*)-histidine moieties (modification ratios of 10~32%) with (*l*)-arginine cationic moieties would not significantly change the pDNA binding affinity[45], which made the PAHMAA-R-H polymers as efficient carriers for pDNA loading.

3.4. Average particle size, surface potential and morphology of the cationic polymers/pDNA polyplexes

It was known that the gene/drug delivery behaviors largely depend on the average particle size, surface zeta potential and morphology of nanoscale gene/drug payloads. Herein, the average particle size and surface zeta potential of the cationic polymers were measured by DLS. As shown in Figure 3A, average particle sizes of the cationic polymers/pDNA polyplexes drastically decreased while the N/P ratio increasing from 1 to 10, then decreased slowly and finally reached a platform. Notably, the (*l*)-arginine-containing (PAHMAA-R₁₈ and PAHMAA-R-H) polymers showed comparatively small average particle size (220~390 nm, N/P=5) and which tend to decrease with the increasing of (*l*)-arginine ratios, indicating the pDNA condensation effect of (*l*)-arginine, which due to the strong +/- charge interactions between (*l*)-arginine moieties and pDNA. Whereas the only (*l*)-histidine-containing PAHMAA-H₂₂/pDNA polyplexes showed much larger particle sizes than the (*l*)-arginine-containing polymers (N/P 1~20), due to their comparatively lower pDNA binding affinity and comparatively loose polyplex architectures[46]. Noteworthy, within the (*l*)-histidine modifying ratio of 10~32%, the average particle size of PAHMAA-R-H/pDNA polyplexes were around 200 nm (N/P≥10), which was regarded as the suitable size for cellular uptake [47]. Furthermore, surface zeta potential of the as-prepared polyplexes was measured by DLS (Figure 3B), it could be seen that the zeta potential of PAHMAA-R₁₈/pDNA polyplexes increased rapidly to around +35 mV with the N/P ratio increased from 1 to 10, and then tend to reach a platform at around +40 mV (N/P>10). The slowest zeta potential increasing was observed on the PAHMAA-H₂₂/pDNA polyplexes, due to low protonation capability of the (*l*)-histidine moieties (pKa~6.0) under neutral conditions (pH 7.4) [36]. The PAHMAA-R-H/pDNA polyplexes showed increasing trends of zeta potential between that of the PAHMAA-R₁₈/pDNA and PAHMAA-H₂₂/pDNA polyplexes (N/P 1~10) and the zeta potential slightly decreased with the increasing of (*l*)-histidine ratios. The results indicated that the surface potential of the PAHMAA-R-H/pDNA polyplexes could be tuned by changing the (*l*)-arginine and (*l*)-histidine co-modification ratios. Moreover, the lowered

surface zeta potential was expected to result in lower cytotoxicity and higher serum compatibility.

Morphology of the pDNA polyplexes (N/P=5) were further observed by AFM (Figure 3C, D and E). It could be seen that all of the three polyplexes are near-spherical nanoparticles and the particle size of PAHMAA-R₁₈/pDNA and PAHMAA-R₁₈-H₆/pDNA polyplexes were comparatively small (diameter: 100~250 nm), whereas much larger particle size was observed on the PAHMAA-H₂₂/pDNA polyplexes (300~950 nm). The results may be due to the lower pDNA binding affinity of PAHMAA-H₂₂ result in the formation of loose and larger polyplexes. Moreover, the trend of particle sizes observed by AFM is in coincidence with the DLS results.

3.5. *In vitro* cytotoxicity of the cationic polymers

Low cytotoxicity is a prerequisite for employing cationic polymeric gene carriers in practical gene transfection and/or clinical gene therapy [48]. Hereby, cytotoxicity of the synthesized polymer carriers were studied by MTT assay in human lung cancer (H1299) cells, with the “gold standard” bPEI-25K as a reference [49]. As shown in Figure S4A, the cells incubated with bPEI-25K showed relatively lower cell viability (higher cytotoxicity) around 40%, while much higher cell viability around 81% (incubated with PAHMAA-R₁₈) and around 97% (incubated with PAHMAA-H₂₂) was observed (dose: 20 µg/mL). The cells with (*l*)-arginine and (*l*)-histidine co-modified PAHMAA-R-H polymers showed relatively high cell viability (89%~98%) at the same dose range. The MTT results demonstrated that the (*l*)-arginine and (*l*)-histidine co-modified PAHMAA-R-H polymers have comparatively low cytotoxicity. To further understand the mechanism of toxicity, LDH assay was carried out for evaluate the cell membrane disruption effect of the cationic polymers (dose: 20 µg/mL). In Figure S4B, bPEI-25K reference showed a high lactate dehydrogenase release (high cytotoxicity) up to 70%, indicating its high cell membrane damage effect, which due to the high surface positive charge density of bPEI-25K [50]. Similarly, PAHMAA-R₁₈ also showed high cytotoxicity near 70% by positive charge induced cell membrane damage. In contrast, PAHMAA-H₂₂ showed much lower cytotoxicity (<5%), due to its lower surface positive charge. Moreover, the (*l*)-arginine and (*l*)-histidine co-modified PAHMAA-R-H polymers showed moderate cytotoxicity of 30%~48%, much lower than that of bPEI-25K and PAHMAA-R₁₈. The results revealed that proper introducing of (*l*)-histidine moieties onto the (*l*)-arginine-containing polymers could decrease the cell membrane damage effect and lower the cytotoxicity. The trend of the LDH results is in accordance with the MTT results, indicating

the charge-induced cell membrane damage played dominant roles in the *in-vitro* cytotoxicity of the cationic polymers. In addition, the results suggested that the PAHMAA-R-H cationic polymers may serve as comparatively low toxic and safe carriers for gene delivery application.

3.6. *In vitro* gene transfection efficiency of the cationic polymers

Gene transfection properties of the cationic polymer carriers were investigated under serum-free and serum conditions in H1299 cells. As shown in Figure 4, under serum-free conditions, PAHMAA-H₂₂ showed negligible gene transfection efficiency as the same level as that of naked pDNA. Similarly, in our prior works, we disclosed that some cholesterol-based cationic lipids with (*l*)-histidine headgroup have much lower gene transfection efficiency [36]. PAHMAA-R₁₈ showed much higher transfection efficiency at the N/P ratio of 2~10, the PAHMAA-R₁₈-H₃ showed 2~4 fold transfection improvement than PAHMAA-R₁₈. Notably, PAHMAA-R₁₈-H₆ showed remarkable gene transfection efficiency, with almost 45-fold than that of PAHMAA-R₁₈ and 3-fold than that of "gold standard" bPEI-25K at the optimum N/P ratio of 5~10. The PAHMAA-R₂₆-H₆ showed as high transfection efficiency as PAHMAA-R₁₈-H₆ at the optimum N/P ratio of 5, while PAHMAA-R₁₈-H₉ showed much lower gene transfection efficiency at the same N/P range. The results indicated that proper co-modification of (*l*)-arginine-containing polymer PAHMAA-R by (*l*)-histidine moieties could effectively improve the gene transfection efficiency, and which could be further optimized by adjusting the (*l*)-arginine and (*l*)-histidine modification ratios. Similarly, Ganguli et al [51, 52] reported that insertion of (*l*)-histidine moieties to some arginine-rich pVEC peptides could significantly improve the gene transfection efficiency, due to the enhanced cellular uptake and subsequent "lysosomal escape" process. Thus, it could be expected that the PAHMAA-R-H cationic polymers may possess enhanced cellular uptake and lysosomal localization/escape behaviors. Furthermore, in order to visualize the gene transfection efficiency of the cationic gene carriers in H1299 cells, enhanced green fluorescent protein (EGFP) encoded-DNA was used as a reporter gene and the pEGFP DNA transfection was performed at the optimized N/P ratio of 5 (Figure S5), the GFP expression in transfected cells by PAHMAA-H₂₂ carriers showed negligible green fluorescence, while the cells transfected by PAHMAA-R₁₈-H₆ and PAHMAA-R₂₅-H₆ showed the strongest green fluorescence, which were in coincidence with the luciferase gene transfection results.

Generally, cationic carrier/pDNA complexes were positively-charged nanoparticles, which tend to absorb/bind negatively-charged serum proteins (such as albumin) to form aggregates. The low serum compatibility of pDNA-loaded complexes made them be easily removed by

endothelial reticulum systems and further cause low gene transfection efficiency [53]. Therefore, the serum compatibility/stability of pDNA-loaded complexes are regarded as important factors that could directly affect the gene transfection efficiency. Herein, Luciferase gene transfection efficiency of the cationic polymer carriers under the presence of serum (10% FBS) were shown in Figure 5. The transfection efficiency of “gold standard” bPEI-25K and PAHMAA-R₁₈ drastically dropped to around 0.6% of that under serum-free conditions, indicating their low serum compatibility/stability, which may be caused by their high surface positive charge induced serum protein adsorption/interaction and resulted in the decreasing of gene transfection efficiency [54]. Notably, the gene transfection efficiency of PAHMAA-R₁₈-H₆ showed slightly rising (129.6%) under 10%FBS than that of serum-free conditions, whereas the PAHMAA-R₂₅-H₆ with higher (*l*)-arginine modification ratios showed much lower transfection efficiency (~3.7%) under the same conditions. The results indicated that the introduction of (*l*)-histidine moieties could enhance the serum compatibility of the PAHMAA-R carriers. Moreover, it could be expected that better serum compatibility might be achieved by properly controlling the (*l*)-arginine/(*l*)-histidine modification ratios. Likewise, Kichler et al [55] found some (*l*)-arginine and (*l*)-histidine-rich peptide (LAH4) derivatives with high serum-resistance properties. Nevertheless, it needs to be noted that, the mechanism study and rational design of serum compatible gene carriers are not very clear so far.

3.7. Cellular uptake properties of the polymer carriers/pDNA polyplexes

Cellular uptake properties of the polymer carriers/pDNA polyplexes was investigated by flow cytometry in H1299 cells (the original data was shown in Figure S6A). Cy3-labeled pDNA was firstly prepared and mixed with cationic polymers at N/P of 5 to prepare the carriers/Cy3-pDNA polyplexes, the fluorescent intensity of the PAHMAA-R₁₈/pDNA polyplexes was set as 100% reference [37]. As shown in Figure S6B, the cellular uptake of PAHMAA-R₁₈/pDNA was a bit lower than that of PAHMAA-H₂₂/pDNA and PAHMAA-R₁₈-H₃/pDNA polyplexes, due to strong interactions of the (*l*)-arginine moieties with cell membrane that induced high cellular uptake efficacy. Notably, the PAHMAA-R₁₈-H₆/pDNA and PAHMAA-R₂₅-H₆/pDNA polyplexes showed relatively high uptake efficiency and reached around 5.02 and 4.87-fold higher than that of PAHMAA-R₁₈/pDNA, respectively. These results indicated that proper introducing (*l*)-histidine moieties to the PAHMAA-R could enhance the cellular uptake efficiency. On the contrary, early literatures reported that (*l*)-histidine modification on cationic polymer carriers may reduce the surface positive charge of their polyplexes and lower the uptake efficacy [56]. Similar to our results, recently Pan [21]

and Hanes [57] reported some (*L*)-histidine-modified cationic polymer carriers with enhanced cellular uptake efficiency. It had been revealed that the interactions of the cationic polymers with cell membrane (such as electrostatic forces, hydrogen bonding and hydrophobic interactions) could lead to efficient cellular uptake and endocytosis [58]. Thus, we deduced that the introduction of (*L*)-histidine moieties may increase the hydrophobicity of the polymers/polyplexes and then lead to the enhanced cellular uptake efficiency.

3.8. Intracellular mechanisms of the polymer carriers/pDNA polyplexes

In order to get deeper insight of the endocytosis mechanisms for the polyplexes, the luciferase gene transfection assay in the presence of endocytosis pathway specific inhibitors was conducted [33]. First, MTT cytotoxicity assay was performed to evaluate the cytotoxicity under comparatively high concentration of the inhibitors (Figure S7). It could be seen that all inhibitors showed low cytotoxicity at the desired dose, indicating the cytotoxicity-reduced gene transfection could be neglected. Gene transfection efficiency of the PAHMAA-R₁₈, PAHMAA-R₁₈-H₆ and PAHMAA-R₂₅-H₆ carriers under the inhibitors were shown in Figure 6, gene transfection efficiency of the PAHMAA-R₁₈/pDNA, PAHMAA-R₁₈-H₆/pDNA and PAHMAA-R₂₅-H₆/pDNA polyplexes declined in some extent (26%~75%) under the addition of methyl- β -Cyclodextrin (m- β -CD), genistein and nocodazole, while the incubation of chlorpromazine (clathrin-mediated endocytosis inhibitor) led to a dramatic arising (1.2~2.6-fold), the addition of amiloride (pinocytosis inhibitor) showed no significant effect on the gene transfection. The results revealed that the polyplexes inclined to enter H1299 cells via caveolae-mediated endocytosis pathway and microtubules played some roles in the endocytosis. Similarly, in our prior work, we found PAHMAA-R₁₆/pDNA polyplexes could be uptaken into COS-7 cells via caveolae/lipid raft-related endocytosis pathway [33]. The results indicated that (*L*)-arginine moieties played essential roles in the caveolae-mediated endocytosis pathway. Moreover, it seems that the modification of (*L*)-histidine moieties on PAHMAA-R would not greatly change/shift the endocytosis pathways/mechanisms.

It had been reported that (*L*)-histidine-based gene transfection carriers have high buffering efficiency and related “lysosomal escape” capability. [21, 32] To further investigate the roles that endosomes/lysosomes played in the intracellular trafficking mechanisms of the polyplexes, an endosome/lysosome acidification inhibitor nigericin [56] was utilized in the gene transfection experiments and bPEI-25K (N/P=10) was used as a reference. As shown in Figure S8, the luciferase gene transfection efficiency of all the polyplexes were significantly

decreased in the presence of nigericin inhibitor (1.0 μ M), indicating that gene transfection of the polyplexes was closely related to the protonation process inside endosomes/lysosomes.

3.9. Intracellular localization of the polymer carriers/pDNA polyplexes

In order to investigate the intracellular trafficking of the PAHMAA-R-H/pDNA polyplexes in H299 cells, intracellular localization of the PAHMAA-R₁₈-H₆/Cy3-labeled pDNA polyplexes [37] was observed by confocal fluorescence microscopy combined with immunofluorescence labeling technique. Figure 7 showed the intracellular localization of the polyplexes after 6 h transfection in H1299 cells, the lysosome organelles were labeled with lysosome-specific LAMP-1 antibody [59] (green fluorescence) and the nuclei were stained with Hoechst 33342 (blue fluorescence). There are some yellow fluorescent points could be observed in the merge pictures (points with arrows), indicating that a part of PAHMAA-R₁₈-H₆/Cy3-labeled pDNA polyplex co-localized with lysosomes. Moreover, it could be seen that many red fluorescence points are not co-localized with green fluorescent points, indicating that the Cy3-labeled polyplexes may escape from the lysosome. The “lysosomal escape” results were in coincidence with the nigericin inhibition assays, indicating the lysosomes played important roles in the intracellular localization of the PAHMAA-R-H/pDNA polyplexes.

4. Conclusion

In summary, a new series of natural (*l*)-arginine and (*l*)-histidine co-modified cationic polymers (PAHMAA-R-H) were successfully synthesized and characterized. The PAHMAA-R-H polymers showed high proton buffering capacity and high pDNA binding affinity, and could form nano-scaled (100~250 nm) polyplexes with lowered surface positive charge. The proper introduction of (*l*)-histidine moieties could reduce cytotoxicity by reducing the cell membrane damage and could enhance high intracellular uptake efficiency and gene transfection efficiency in the presence/absence of 10% FBS. The optimized transfection efficiency could reach to 10-fold of that of the “gold standard” bPEI-25K in H1299 cells. The PAHMAA-R/pDNA and PAHMAA-R-H/pDNA polyplexes tend to be internalized through caveolae-mediated endocytosis pathway and strongly relied on the microtubule and endosome/lysosome organelles. Moreover, the gene transfection related properties could be tuned by controlling the (*l*)-arginine/(*l*)-histidine modification ratios. Notably, among the PAHMAA-R-H polymers, PAHMAA-R₁₈-H₆ exhibited low cytotoxicity, remarkable gene

transfection efficiency and serum compatibility, made it a promising gene transfection agent for practical application.

Acknowledgement

The authors are indebted to the financial supports partially from the national science foundation of China (21174160, 21002116 and 21372251). The authors are also grateful to Prof. Xiangyang Shi and Dr. Xueyan Cao of Donghua University for his kind assistances in FACS measurements. Dr. Ruilong Sheng appreciates the Chinese Academy of Sciences (CAS) for the CAS-Canada Young Visiting Scientist Scholarship and Youth Innovation Promotion Association (YIPA, NO2012204) for the sponsorship.

Reference:

- [1] R.K. O'Reilly, *Polym. Int.* 59 (2010) 568-573.
- [2] M.B. Oliveira, J.F. Mano, *Polymers in Regenerative Medicine: Biomedical Applications from Nano-to Macro-Structures.* (2015) 49-90.
- [3] C. Helary, M. F Desimone, *Curr. Pharm. Biotech.* 16 (2015) 635-645.
- [4] S.G. Roy, K. Bauri, S. Pal, P. De, *Polym. Chem.* 5 (2014) 3624-3633.
- [5] S. Kumar, R. Acharya, U. Chatterji, P. De, *Langmuir* 29 (2013) 15375-15385.
- [6] S. Kumar, P. De, *Polymer* 55 (2014) 824-832.
- [7] K. Bauri, S.G. Roy, P. De, *Macromol. Chem. Phys.* 217 (2016) 365-379.
- [8] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, *Nat. Rev. Genetics.* 15 (2014) 541-555.
- [9] K. Remaut, V. Oorschot, K. Braeckmans, J. Klumperman, S.C. De Smedt, *J. Controlled Release.* 195 (2014) 29-36.
- [10] B. Levine, D.J. Klionsky, *Develop. Cell* 6 (2004) 463-477.
- [11] K. Miyata, N. Nishiyama, K. Kataoka, *Chem. Soc. Rev.* 41 (2012) 2562-2574.
- [12] S. Barua, S. Mitragotri, *Nano Today* 9 (2014) 223-243.
- [13] D. Lechardeur, A. Verkman, G.L. Lukacs, *Adv. Drug Deliv. Rev.* 57 (2005) 755-767.

- [14] H.Q. Mao, K. Roy, V.L. Troung-Le, K.A. Janes, K.Y. Lin, Y. Wang, J.T. August, K.W. Leong, J. Controlled Release 70 (2001) 399-421.
- [15] R. Kircheis, L. Wightman, E. Wagner, Adv. Drug Deliv. Rev. 53 (2001) 341-358.
- [16] J.G. Schellinger, J.A. Pahang, R.N. Johnson, D.S. Chu, D.L. Sellers, D.O. Maris, A.J. Convertine, P.S. Stayton, P.J. Horner, S.H. Pun, Biomaterials 34 (2013) 2318-2326.
- [17] P. Midoux, C. Pichon, J.J. Yaouanc, P.A. Jaffres, Brit. J. Pharm. 157 (2009) 166-178.
- [18] T.H. Kim, J.E. Ihm, Y.J. Choi, J.W. Nah, C.S. Cho, J. Controlled release 93 (2003) 389-402.
- [19] K.L. Chang, Y. Higuchi, S. Kawakami, F. Yamashita, M. Hashida, Bioconjug. Chem. 21 (2010) 1087-1095.
- [20] M. Bello Roufai, P. Midoux, Bioconjug. Chem. 12 (2001) 92-99.
- [21] Y. Wen, Z. Guo, Z. Du, R. Fang, H. Wu, X. Zeng, C. Wang, M. Feng, S. Pan, Biomaterials 33 (2012) 8111-8121.
- [22] C.W. Pouton, L.W. Seymour, Adv. Drug Deliv. Rev. 34 (1998) 3-19.
- [23] M.A. Mintzer, E.E. Simanek, Chem. Rev. 109 (2009) 259-302.
- [24] Y. Yan, G.K. Such, A.P. Johnston, J.P. Best, F. Caruso, ACS Nano 6 (2012) 3663-3669.
- [25] Y.L. Chiu, Y.C. Ho, Y.M. Chen, S.F. Peng, C.J. Ke, K.J. Chen, F.L. Mi, H.W. Sung, J. Controlled Release 146 (2010) 152-159.
- [26] H.Y. Nam, S.M. Kwon, H. Chung, S.Y. Lee, S.H. Kwon, H. Jeon, Y. Kim, J.H. Park, J. Kim, S. Her, Y.K. Oh, I.C. Kwon, K. Kim, S.Y. Jeong, J. Controlled Release. 135 (2009) 259-267.
- [27] C.J. Chen, J.C. Wang, E.Y. Zhao, L.Y. Gao, Q. Feng, X.Y. Liu, Z.X. Zhao, X.F. Ma, W.J. Hou, L.R. Zhang, Biomaterials 34 (2013) 5303-5316.
- [28] Q.P. Luu, J.Y. Shin, Y.K. Kim, M.A. Islam, S.K. Kang, M.H. Cho, Y.J. Choi, C.S. Cho, Mol. Pharm. 9 (2012) 2206-2218.
- [29] Y.C. Chung, T.Y. Cheng, T.H. Young, Biomaterials 32 (2011) 4471-4480.

- [30] S. Engel, T. Heger, R. Mancini, F. Herzog, J. Kartenbeck, A. Hayer, A. Helenius, J. Virol. 85 (2011) 4198-4211.
- [31] K. Han, J. Yang, S. Chen, J.X. Chen, C.W. Liu, C. Li, H. Cheng, R.X. Zhuo, X.Z. Zhang, Int. J. Pharm. 436 (2012) 555-563.
- [32] G.S. Yu, Y.M. Bae, H. Choi, B. Kong, I.S. Choi, J.S. Choi, Bioconjug. Chem. 22 (2011) 1046-1055.
- [33] H. Li, T. Luo, R. Sheng, J. Sun, Z. Wang, A. Cao, Biomaterials 34 (2013) 7923-7938.
- [34] R. Sheng, F. An, Z. Wang, M. Li, A. Cao, Rsc Adv. 5 (2015) 12338-12345.
- [35] R. Sheng, T. Luo, H. Li, J. Sun, Z. Wang, A. Cao, Bioorg. Med. Chem. 21 (2013) 6366-6377.
- [36] R. Sheng, T. Luo, H. Li, J. Sun, Z. Wang, A. Cao, Colloid. Surf. B. 116 (2014) 32-40.
- [37] R. Sheng, T. Luo, Y. Zhu, H. Li, J. Sun, S. Chen, W. Sun, A. Cao, Biomaterials 32 (2011) 3507-3519.
- [38] J. Sun, T. Luo, R. Sheng, H. Li, S. Chen, F. Hu, A. Cao, Macromol. Biosci. 13 (2013) 35-47.
- [39] H. Li, T. Luo, R. Sheng, J. Sun, Z. Wang, A. Cao, Biomaterials 34 (2013) 7923-7938.
- [40] C.D.M. Churchill, S.D. Wetmore, J. Phys. Chem. B 113 (2009) 16046-16058.
- [41] I. Richard, M. Thibault, G. De Crescenzo, M.D. Buschmann, M. Lavertu, Biomacromolecules 14 (2013) 1732-1740.
- [42] M. Ou, X.L. Wang, R. Xu, C.W. Chang, D.A. Bull, S.W. Kim, Bioconjug. Chem. 19 (2008) 626-633.
- [43] R. Sheng, K. Xia, J. Chen, Y. Xu, A. Cao, J. Biomater. Sci. Polym. Ed. 24 (2013) 1935-1951.
- [44] R. Sheng, T. Luo, Y. Zhu, H. Li, A. Cao, Macromol. Biosci. 10 (2010) 974-982.
- [45] C. Pichon, C. Goncalves, P. Midoux, Adv. Drug Deliv. Rev. 53 (2001) 75-94.

- [46] J. Chang, X. Xu, H. Li, Y. Jian, G. Wang, B. He, Z. Gu, *Adv. Func. Mater.* 23 (2013) 2691-2699.
- [47] M. Zhu, G. Nie, H. Meng, T. Xia, A. Nel, Y. Zhao, *Acc. Chem. Res.* 46 (2013) 622-631.
- [48] B. Shi, H. Zhang, Z. Shen, J. Bi, S. Dai, *Polym. Chem.* 4 (2013) 840-850.
- [49] J. Dai, S. Zou, Y. Pei, D. Cheng, H. Ai, X. Shuai, *Biomaterials* 32 (2011) 1694-1705.
- [50] F.W. Huang, H.Y. Wang, C. Li, H.F. Wang, Y.X. Sun, J. Feng, X.Z. Zhang, R.X. Zhuo, *Acta Biomater.* 6 (2010) 4285-4295.
- [51] Rajpal, A. Mann, R. Khanduri, R.J. Naik, M. Ganguli, *J. Controlled Release* 157 (2012) 260-271.
- [52] R. Sharma, S. Shivpuri, A. Anand, A. Kulshreshtha, M. Ganguli, *Mol. Pharm.* 10 (2013) 2588-2600.
- [53] H.M. Wu, S.R. Pan, M.W. Chen, Y. Wu, C. Wang, Y.T. Wen, X. Zeng, C.B. Wu, *Biomaterials* 32 (2011) 1619-1634.
- [54] J.A. Roth, R.J. Cristiano, *J. Natl. Cancer. Inst.* 89 (1997) 21-39.
- [55] A.J. Mason, C. Leborgne, G. Moulay, A. Martinez, O. Danos, B. Bechinger, A. Kichler, *J. Controlled Release* 118 (2007) 95-104.
- [56] T.I. Kim, T. Rothmund, T. Kissel, S.W. Kim, *J. Controlled Release* 152 (2011) 110-119.
- [57] N.J. Boylan, A.J. Kim, J.S. Suk, P. Adstamongkonkul, B.W. Simons, S.K. Lai, M.J. Cooper, J. Hanes, *Biomaterials* 33 (2012) 2361-2371.
- [58] K.L. Chang, Y. Higuchi, S. Kawakami, F. Yamashita, M. Hashida, *J. Controlled Release* 156 (2011) 195-202.
- [59] Z. Kadlecova, Y. Rajendra, M. Matasci, L. Baldi, D.L. Hacker, F.M. Wurm, H.A. Klok, *J. Controlled Release* 169 (2013) 276-288.

Scheme 1. Synthesis approach to polycationic poly(ω -aminoethyl methacrylamide)s co-modified with (*L*)-arginines and (*L*)-histidines (PAHMAA-R-H)

Figure 1. Acid-base titration of polycationic poly(ω -aminohexyl methacrylamide)s conjugated with *(L)*-arginines and *(L)*-histidines (PAHMAA-R-H) in 150 mmol L⁻¹ NaCl aqueous solution

Figure 2. Agarose gel retardant assay of plasmid DNA affinity under various N/P ratios for the polycationic (*l*)-arginine and (*l*)-histidines conjugated PAHMAA-R-H. Lane 1: Mock; lane 2: Naked pDNA; lanes 3-9: N/P ratios of polymer/DNA=0.1, 0.5, 1, 2, 3, 5, and 10, respectively

Figure 3. Mean particle sizes (A) and zeta potentials (B) of the PAHMAA-R-H/pDNA polyplexes under various N/P ratios and AFM morphologies recorded in the tapping mode for the PAHMAA-R₁₈/pDNA (C) PAHMAA-R₁₈-H₆/pDNA (D) and PAHMAA-H₂₂/pDNA (E) polyplexes under N/P ratio of 5

Figure 4. Luciferase gene transfection assay by the PAHMAA-R-H at various N/P ratios with H1299 under serum-free conditions. Gene transfection assay was performed under a dosage of 1.0 μ g pDNA per well (n=3, ***P<0.001)

Figure 5. Effect of serum (10% FBS) on luciferase gene transfection efficiency by the PAHMAA-R-H vectors

Figure 6. Luciferase gene transfection assays in H1299 cells by the PAHMAA-R₁₈, PAHMAA-R₁₈-H₆ and PAHMAA-R₂₅-H₆ vector in the presence of various endocytic inhibitors (*P<0.05, **P<0.01, ***P<0.001)

Figure 7. CLSM images of the localization of Cy3-labeled pDNA in H1299 cells recorded after 6 h gene transfection by PAHMAA-R₁₈-H₆ (lysosomes was visualized in green and Cy3 labeled pDNA in red; Blue represents Hoechst 33342 stained cell nuclei)

Table 1. Molecular characteristics of water-soluble poly(ω -aminohexyl methacrylamide)s conjugated with (*l*)-arginine and (*l*)-histidine (PAHMAA-R-H)

Sample	Molar percentage of the ω -amino groups conjugated with (<i>l</i>)-arginines (mol%) ^{<i>l</i>}	Molar percentage of the ω -amino groups conjugated with (<i>l</i>)-histidine (mol%) ^{<i>m</i>}	Polymerization degree of the free ω -amino groups (n-x-y) ^{<i>u</i>}	Number of the ω -amino groups conjugated with (<i>l</i>)-arginines (x) ^{<i>v</i>}	Number of the ω -amino groups conjugated with (<i>l</i>)-histidines (y) ^{<i>w</i>}
PAHMAA-R₁₈	60%	0%	12.4	18.6	0
PAHMAA-R₁₈-H₃	58%	10%	10	17.9	3.1
PAHMAA-R₁₈-H₆	61%	20%	7	18.9	6.2
PAHMAA-R₁₈-H₉	60%	32%	2.5	18.6	9.9
PAHMAA-R₂₅-H₆	80%	20%	0	24.8	6.2
PAHMAA-H₂₂	0%	72%	8.7	0	22.3

Notes: ^{*l,m*} Molar percentages of the ω -amino groups conjugated with (*l*)-arginines and (*l*)-histidines were evaluated as $4I_a / [(I_d + I_{d'} + I_{d''} + I_e + I_{e'} + I_{e''} + I_c + I_g) - 2(I_a + I_b)] \times 100\%$ and $4I_b / [(I_d + I_{d'} + I_{d''} + I_e + I_{e'} + I_{e''} + I_c + I_g) - 2(I_a + I_b)]$ by ¹H NMR with the intensities of ¹H signals as assigned in Figure S3, respectively. ^{*u,v,w*} Data express average free ω -amino groups, the ω -amino groups conjugated with (*l*)-histidines in a PAHMAA-R-H polymer chain, respectively.

Table 2. Buffer capacity of the (*l*-)-arginine and (*l*-)-histidine modified polymers

Sample	Buffer capacity (%)
PAHMAA-R ₁₈	23.4
PAHMAA-R ₁₈ -H ₃	29.7
PAHMAA-R ₁₈ -H ₆	34.8
PAHMAA-R ₁₈ -H ₉	39.3
PAHMAA-R ₂₅ -H ₆	34.0
PAHMAA-H ₂₂	47.5

Graphical Abstract