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Endosomal-Escape Polymers Based on Multicomponent Reaction-Synthesized Monomers Integrating Alkyl and Imidazolyl Moieties for Efficient Gene Delivery

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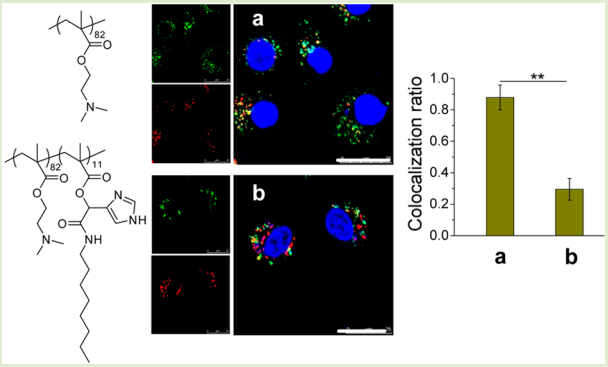
Supporting Information

ABSTRACT: As one of the toughest tasks in the course of intracellular therapeutics delivery, endosomal escape must be effectively achieved, particularly for intracellular gene transport. In this report, novel endosomal-escape polymers were designed and synthesized from monomers by integrating alkyl and imidazolyl via Passerini reaction and reversible addition−fragmentation chain transfer polymerization (RAFT). After introducing the endosomal-escape polymers with proper degrees of polymerization (DPs) into poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) as the gene delivery vectors, the block copolymers exhibited significantly enhanced hemolytic activity at endosomal pH, and the plasmid DNA (pDNA)-loaded polyplexes showed efficient endosomal escape compared with PDMAEMA, ultimately achieving dramatically increased gene transfection efficacy. These results suggest that the polymers that integrate alkyl and imidazolyl moieties for efficient endosomal escape have wide potential applications for intracellular gene delivery.



Nonviral gene vectors, particularly cationic polymers, have great advantages over viral ones, including favorable biological safety, facile large-scale manufacture, and simple structural design and modification.[1−5] However, nonviral gene carriers show relatively lower efficiency when they encounter a series of extra- and intracellular biological obstacles.[6,7] Notably, after endocytosis, the gene-loaded nanoparticles are entrapped in the endosome and degraded in the lysosomes if effective endosomal escape can not be achieved. Thus, efficient endosomal escape and transport of genes into the cytosol have been considered one of the primary tasks in the course of gene delivery. Great efforts have been made for high-efficiency endosomal escape. In general, three strategies have been proposed for cationic polymer-mediated endosomal escape. The "proton sponge" effect as the main mechanism is referred to as an osmotic swelling and endosomal rupture, which is induced when polymers become protonated upon endosomal acidification, with a subsequent massive influx of ions and additional water.[8−10] Another endosomal escape strategy is to utilize interactions between the polymer carriers and the endosomal membranes, which destabilize the membranes and induce their rupture. For example, cationic or hydrophobic moieties on the polymers can interact or fuse with the endosomal membranes and facilitate endosomal escape.[11−13] Moreover, "photochemical internalization" is a strategy of photoinduced endosomal escape that is mediated by a photosensitizer in the nanocarriers.[14−16] Even though great progress has been made, more efficient endosomal-escape molecules must be developed considering the significant functional role of endosomal escape in intracellular therapeutics transport.

Recently, various endosomal escape mechanisms were integrated into one system for more efficient endosomal escape through the synergistic effect.[11−13,17−19] For example, Stayton and co-workers prepared random copolymers of 2-(diethylamino)ethyl methacrylate (DEAEMA) and *n*-butyl methacrylate (BMA) at proper ratios.[11] In the endosomes, upon acidification, the DEAEMA segments can be protonated, which further increases the net positive charges of the polymers and improves the interactions between the carriers and membranes. In addition, the PBMA segments interacted with the membranes through hydrophobic interactions, which synergistically lead to endosomal destabilization and rupture. Monteiro and co-workers used the random copolymers consisting of imidazolyl and butyl groups to modify the gene delivery polymer, poly(2-dimethylaminoethyl acrylate) (PDMAEA), for higher endosomal escape efficiency.[18,19] However, endosomal-escape polymers with more precise structures that integrate multifunctionalities are more favorable for investigating mechanisms and have wide applications.

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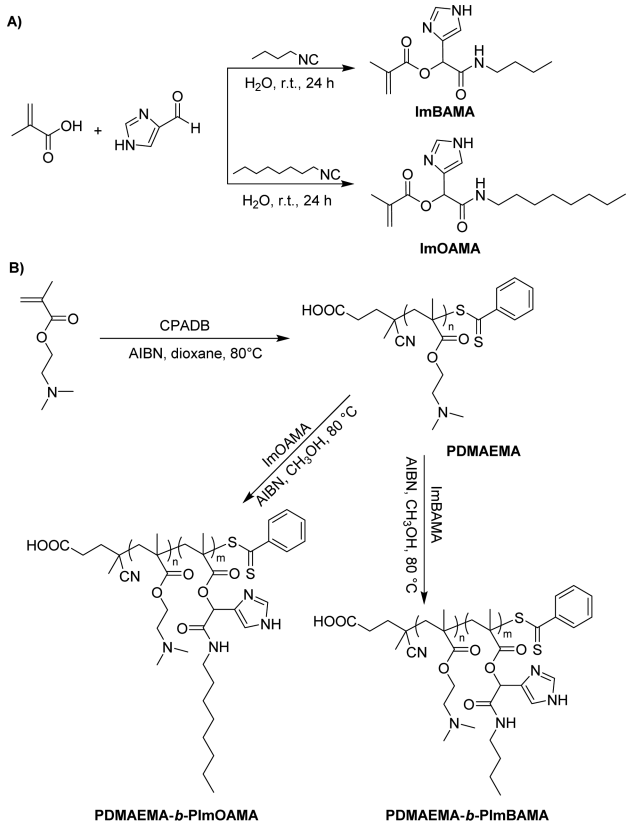
The multicomponent reaction generates the possibility that three or more molecules can be combined to produce a single product, which has been introduced into polymer synthesis and provides diverse polymer preparation strategies.[20−23] One method is to prepare the monomers that contain multiple functional groups to easily prepare polymers with pendant multiple functional groups. Meier et al.[20,24] prepared a series of functionalized olefin monomers through the Passerini three-component reaction (Passerini-3CR) and subsequent successful olefin metathesis polymerization. Additionally, they synthesized various acrylic monomers and obtained a series of unique thermoresponsive polymers via radical polymerization. Roth et al.[25] also prepared various styrenic and (meth)acrylic monomers with active functional groups and accomplished their controllable radical polymerization and postpolymerization modification. In this report, we synthesized methacrylic monomers that integrated alkyl and imidazolyl moieties and demonstrated their controllable radical polymerization through reversible addition−fragmentation chain transfer (RAFT) polymerization, thereby achieving the precise control over the structure of the polymers. Integration of the imidazolyl and alkyl groups was expected to efficiently facilitate endosomal escape using the proton sponge effect and fusion with endosomal membranes.[11,19,26] To further verify their endosomal escape capability, we prepared the block copolymers using poly(2-dimethylaminoethyl methacrylate) (PDMAEMA)-based macroRAFT chain transfer agent which showed remarkably improved gene transfection efficiency compared with the PDMAEMA precursor. The efficient endosomal escape accounted for the enhanced gene transfection efficiency which was further confirmed by the high hemolytic activity of the block copolymers at acidic pH (6.6 and 5.7).

As shown in Scheme 1, we selected methacrylic acid, 4-formylimidazole, and *n*-butyl/octyl isocyanide to synthesize monomers (ImBAMA and ImOAMA) that contain the functionalities for polymerization and endosomal escape through Passerini-3CR, which were clearly characterized using ^{1}H NMR (Figures 1A and S1). Subsequently, the RAFT polymerization kinetics of ImBAMA and ImOAMA were investigated using 4-cyanopentanoic acid dithiobenzoate (CPADB) as the chain transfer agent in methanol at the initial monomer concentration of 0.45 M (Figure S2). The linear relationship between the molecular weight and the monomer conversion indicates that both M_n and the polydispersity index (PDI) of molecular weight (M_w/M_n) can be well controlled. The linear kinetic plot of the polymerization shows a pseudo-first-order nature with a conversion below 70%. These results demonstrate that the RAFT polymerization of ImBAMA and ImOAMA can be performed in a controllable manner.

For PDMAEMA as a class of nonviral gene delivery polymers, the DNA complexation capability and gene transfection efficiency increase with increasing molecular weight.[27−29] Here, to highlight the effect of endosomal-escape polymers on the gene transfection efficiency, we synthesized PDMAEMA polymers with a relatively low M_n of ∼10000 g/mol via the RAFT polymerization of DMAEMA (Figure S3), which was further used as the macroRAFT chain transfer agent to polymerize ImBAMA or ImOAMA. We prepared the block copolymers PDMAEMA-*b*-PImBAMA and PDMAEMA-*b*-PImOAMA with low molecular weight distribution, and the degrees of polymerization (DPs) can be determined by comparing the signals of the imidazolyl groups and the known number of methylene groups of PDMAEMA (Figures 1B and S4). Relatively low DPs of the PImBAMA or PImOAMA blocks were incorporated to minimize the effect on the DNA complexation capability and physical properties of PDMAEMA (Table 1). At pH 7.4, $PDMAEMA_{82}$, $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$, $PDMAEMA_{82}$-*b*-$PImBAMA_{24}$, and $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ showed similar plasmid DNA (pDNA) complexation ability, as evidenced by gel electrophoresis evaluation (Figure S5). The block copolymers, $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$, $PDMAEMA_{82}$-*b*-$PImBAMA_{24}$, and $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$, were molecularly dissolved

Scheme 1. Synthetic Routes to Prepare Monomers, 1-(1*H*-Imidazol-4-yl)-2-(butylamino)-2-oxoethyl Methacrylate (ImBAMA) and 1-(1*H*-Imidazol-4-yl)-2-(octylamino)-2-oxoethyl Methacrylate (ImOAMA), and the Block Copolymers, PDMAEMA-*b*-PImBAMA and PDMAEMA-*b*-PImOAMA



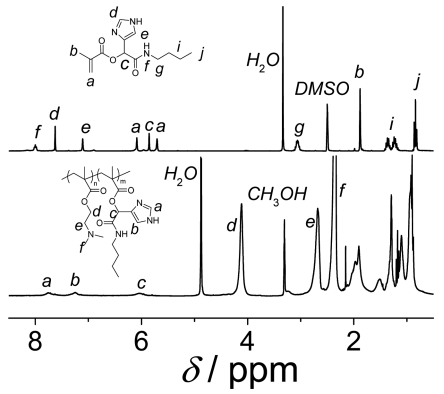


Figure 1. ^{1}H NMR spectra of the ImBAMA monomer in DMSO-d_6 and PDMAEMA-*b*-PImBAMA block copolymer in CD_3OD.

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Table 1. Characterization of the Polymers

polymer	$M_{n,\ GPC}$ [a]	PDI[a]	$M_{n,\ NMR}$ [b]
$PDMAEMA_{82}$	9600	1.05	12800
$PDMAEMA_{82}$-*b*-$PImBAMA_{12}$	14000	1.19	16000
$PDMAEMA_{82}$-*b*-$PImBAMA_{24}$	16100	1.20	19200
$PDMAEMA_{82}$-*b*-$PImOAMA_{11}$	13700	1.17	16400

[a]Determined by GPC analysis using DMF as the eluent. [b]Calculated from the ^{1}H NMR results.

in pH 7.4 phosphate-buffered saline (PBS) buffer at a concentration of 2 mg/mL, as evidenced by dynamic light scattering (DLS) characterization (Figure S6). Simultaneously, the promoted endosomal escape was expected by incorporating a proper length of PImBAMA or PImOAMA.

The hemolytic activity of the polymers was first evaluated using Triton X-100 as a positive control at various pH values, which mimicked endosomal trafficking (extracellular pH, 7.4; early endosome pH, 6.6; and late endosome pH, 5.7). First via titration analysis of the corresponding monomers, we estimated the pK_a values of the incorporated PImBAMA and PImOAMA segments to be 6.6 and 6.5, respectively (Figure S7). Moreover, as shown in Figure 2A, all polymers at pH 7.4 showed

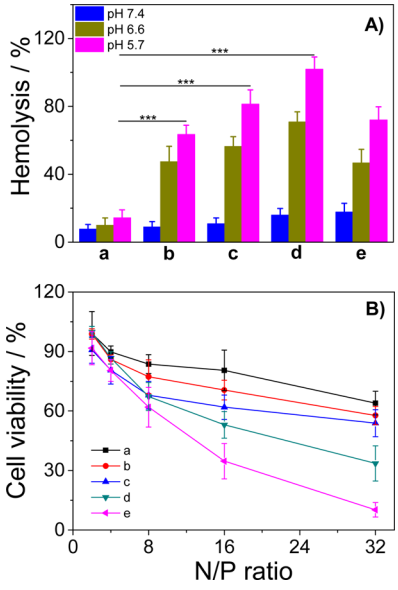

Figure 2. (A) Hemolysis activity of various polymers at a concentration of 10 μg/mL. The data are expressed as the mean ± s.d. (n = 3). (B) Cytotoxicity of the polyplexes from various polymers at various N/P ratios with pDNA concentration of 5 μg/mL. (a) $PDMAEMA_{82}$, (b) $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$, (c) $PDMAEMA_{82}$-*b*-$PImBAMA_{24}$, (d) $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$, and (e) branched polyethylenimine (bPEI, M_w = 25 kDa). The data are expressed as the mean ± s.d. (n = 4). ***P < 0.005 (t-test).

remarkably low hemolysis (<20%) at 10 μg/mL. In contrast, upon acidification to pH 6.6 or 5.7, the PDMAEMA polymer maintained relatively low hemolytic activity whereas the block copolymers PDMAEMA-*b*-PImBAMA and PDMAEMA-*b*-PImOAMA showed dramatically enhanced hemolysis (P < 0.005) compared with the PDMAEMA polymer. Notably, the block copolymers showed even stronger hemolysis than the branched polyethylenimine (bPEI) in acidic conditions. Thus, efficient endosomal escape can be anticipated in acidic conditions in late endosomes and lysosomes. Moreover, further cytotoxicity evaluation demonstrates the high cytotoxicity of $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ polyplexes at a high N/P ratio of 32 with ~25% cell viability. Presumably, the longer hydrophobic alkyl moiety and partially positively charged PDMAEMA segment of the free $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ block copolymer promote the interactions with cellular membranes and induce cytotoxicity. The other formulations exhibited relatively low toxicity with cell viability >50% in the N/P ratio range of 2−32 (Figure 2B).

Then, we investigated the gene transfection efficiency of the polymers by loading enhanced green fluorescent protein (EGFP) or luciferase (Luc)-encoded pDNA (pDNA-EGFP or pDNA-Luc). As shown in Figure 3A, PDMAEMA showed very

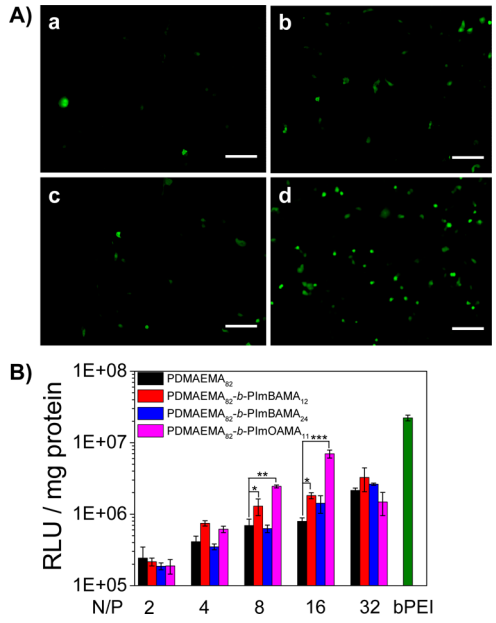

Figure 3. Transfection efficiencies of the polyplexes against HeLa cells. (A) Fluorescence images of EGFP expression by loading pDNA-EGFP at an N/P ratio of 8 (a, $PDMAEMA_{82}$; b, $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$; c, $PDMAEMA_{82}$-*b*-$PImBAMA_{24}$; d, $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$). The scale bars represent 200 μm. (B) Luciferase gene expression by loading pDNA-Luc, which was expressed in terms of relative light units per milligram of protein (RLU/mg protein). bPEI was used as the standard control at N/P 10. The data are expressed as the mean ± s.d. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.005 (t-test).

weak gene expression in HeLa cells at N/P 8. For polyplexes of block copolymers, in particular, PDMAEMA-*b*-PImOAMA, dramatically improved EGFP expression showed high gene transfection efficiency. Moreover, luciferase was also used as the reporter gene (Figure 3B). At N/P > 2, the block copolymer polyplexes of $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$ and $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ showed higher gene transfection efficiency than the PDMAEMA polyplex. At N/P ratios of 8 and 16, compared with PDMAEMA, $PDMAEMA_{82}$-*b*-$PImBAMA_{12}$ polyplexes showed 1.9- and 2.3-fold gene transfection efficacy, respectively, whereas $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ showed 2.4- and 8.9-fold enhancement, respectively. These results verify that the incorporation of PImBAMA or PImOAMA with a proper length can significantly increase the gene transfection efficiency, and the incorporation of longer alkyl moieties of octyl was more favorable for gene transfection than *n*-butyl. For the $PDMAEMA_{82}$-*b*-$PImOAMA_{11}$ polyplexes at N/P 32, the low gene transfection efficacy was presumably attributed to high

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cytotoxicity. For the PDMAEMA$_{82}$-*b*-PImBAMA$_{24}$ polyplexes, the gene expression was not significantly increased compared with PDMAEMA polyplex likely because the longer hydrophobic segment was unfavorable for unpacking the polyplexes in the cytosol, which consequently affected gene transfection.

To elucidate the mechanism by which the block copolymer polyplexes showed higher gene transfection efficiency than the PDMAEMA polyplex, we observed the intracellular distribution of the polyplexes after cellular internalization using confocal laser scanning microscopy (CLSM) to investigate the endosomal escape capability (Figure 4). After the polyplexes were incubated at N/P 8 against HeLa cells for 24 h and the colocalization ratios of late endosome/lysosome (green) and Cy5-pDNA (red) were quantified, the endosomal escape efficiency was estimated. The PDMAEMA polyplexes showed a colocalization ratio of 0.84, whereas approximately 0.6 of pDNA in PDMAEMA-*b*-PImBAMA polyplexes was localized in the late endosomes/lysosomes. PDMAEMA-*b*-PImOAMA polyplexes showed a lower colocalization ratio of 0.29, which was even lower than that of PEI. These results reveal that the incorporation of endosomal-escape polymers, particularly PImOAMA, achieves more effective endosomal escape, which is consistent with their corresponding hemolytic ability. Considering similar cellular internalization capability of PDMAEMA$_{82}$, PDMAEMA$_{82}$-*b*-PImOAMA$_{11}$, PDMAEMA$_{82}$-*b*-PImBAMA$_{12}$, and PDMAEMA$_{82}$-*b*-PImBAMA$_{24}$ polyplexes, as measured by cellular uptake evaluation by flow cytometry (Figure S8), the block copolymer polyplexes showed higher gene transfection efficiency presumably due to more efficient endosomal escape.

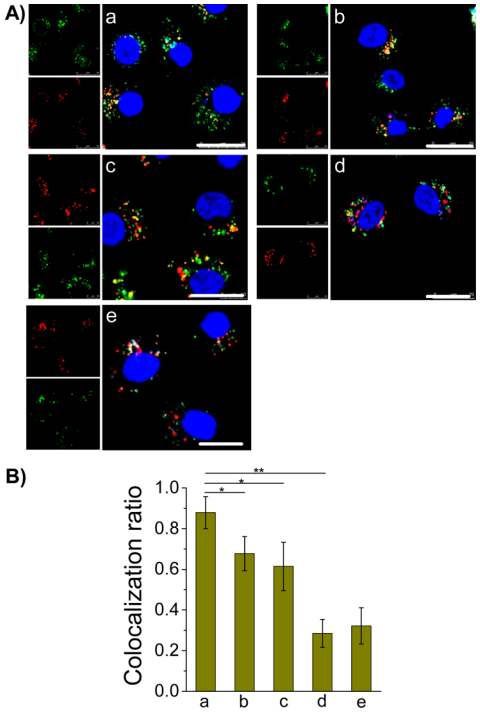

Figure 4. (A) CLSM images of the intracellular distribution of the polyplexes loading Cy5-pDNA (red) against HeLa cells at an N/P ratio of 8 (a, PDMAEMA$_{82}$; b, PDMAEMA$_{82}$-*b*-PImBAMA$_{12}$; c, PDMAEMA$_{82}$-*b*-PImBAMA$_{24}$; d, PDMAEMA$_{82}$-*b*-PImOAMA$_{11}$; e, bPEI). The late endosomes/lysosomes (green) and nuclei (blue) were stained with LysoTracker Green and DAPI, respectively. Scale bars represent 25 μm. (B) Quantification of Cy5-pDNA (red) colocalization with LysoTracker (green). The data are expressed as the mean ± s.d. (n = 10). *P < 0.05, **P < 0.01 (t-test).

In summary, we have reported a type of novel endosomal-escape polymers, PImBAMA and PImOAMA, based on the monomers that integrated alkyl and imidazolyl moieties prepared via Passerini-3CR and RAFT polymerization. After introducing into PDMAEMA polymer as gene delivery vectors, the hemolytic activity was enhanced dramatically at endosomal pH, which facilitated endosomal escape of PDMAEMA significantly, resulting in higher gene transfection efficacy. Precisely controllable structures ensure that this type of endosomal-escape polymers can be not only readily incorporated into gene vectors, but also applied to other intracellular delivery systems for endosomal escape preventing biodegradation in lysosomes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00615.

Synthetic methods, characterization of the monomers and polymers, polymerization kinetics, DNA complexation, DLS characterization, titration, and cellular uptake evaluation (PDF).

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Notes

The authors declare no competing financial interest.

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