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Development of switchable polymers to address the dilemma of stability and cargo release in polycationic nucleic acid carriers

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Abstract

Cationic polymer gene delivery vehicles that effectively resist premature serum degradation often have difficulty releasing their nucleic acid cargoes. In this work, we report a pH-sensitive polymer (SP), poly(oligo(ethylene glycol) monomethyl ether methacrylate)-*co*-poly(2-(dimethylamino)ethyl methacrylate)-*block*-poly(propargyl methacrylate)-*graft*-propyl-(4-methoxybenzylidene)-amine) (p(PMA-PMBA)-*b*-(p(OEGMA-DMAEMA))), for successful *in vitro* and *in vivo* gene transfer. In the physiological condition, the hydrophobization of p(OEGMA-DMAEMA) polycations by p(PMA-PMBA) significantly enhanced the stability of its polyplexes counterpart. In endosomes, the polymer undergoes an acid-triggered hydrophilic transition through the cleavage of benzoic imines, thus allowing the vector to quickly release nucleic acid cargo due to the loss of hydrophobic functionalization. Compared to a pH-insensitive polymer (IP), SP exhibited more significant luciferase plasmid delivery efficiency with HeLa cells *in vitro* and with *in vivo* intraventricular brain injections. Therefore, the polymer designed here is a good solution to address the dilemma of stability and cargo release in gene delivery, and may have broad potential applications in therapeutic agent delivery.

Keywords

gene delivery; hydrophobization; pH sensitive; benzoic imine

1. Introduction

Polycations have been investigated extensively for nucleic acid delivery due to their ability to condense nucleic acids into nanoparticles *via* electrostatic interaction, thereby preventing undesirable enzymatic degradation as well as facilitating cellular uptake [1]. Cationic

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polymers-based non-viral vectors feature numerous advantages compared to viral vectors, including facile modification for tailored applications, reduced susceptibility to neutralizing antibodies and more affordable large-scale production [2-5]. As such, polycations have been used to deliver plasmid DNA (pDNA) [6, 7], micro RNA (miRNA) [8, 9], small interfering RNA (siRNA) [10, 11], and messenger RNA (mRNA) [12, 13], indicating the broad potential for therapeutic interventions for this delivery approach.

Although there are several examples of effective polycation-mediated nucleic acid delivery to cell lines *in vitro*, the *in vivo* delivery profile of polycations is typically significantly compromised. One unique challenge faced *in vivo* by polycation-based complexes (“polyplex”) is poor stability and aggregation in the blood circulation and upon encountering extracellular matrix in tissues [14-16]. Several approaches have been implemented to improve polyplex stability *in vivo*, including polyplex crosslinking [17-19], nucleic acid affinity enhancement or conjugation [20, 21], hydrophilic modification with poly(ethylene glycol) (PEG) (“PEGylation”) [22, 23] and hydrophobization of polycations [24]. Hydrophobization of polycation has been shown to reduce cytotoxicity and improve stability of polyplexes, thus also improving transfection efficiency and circulation time of polyplexes *in vivo* [25]. For example, our previous work showed that hydrophobic functionalization of oligoamine tetraethylenepentamine (TEPA)-decorated poly(glycidyl methacrylate) (PGMA) with poly(ϵ -caprolactone) (PCL) significantly improved the stability of polyplexes against salt and serum-induced aggregation, and also led to promising *in vivo* delivery efficiency [26]. However, enhanced stability of polyplexes conversely impedes the unpacking of cargoes in the cell cytoplasm. Hence, selective de-hydrophobization after endocytosis is needed for cargo release and activity. A reduction-sensitive disulfide bond was thus placed between polycation and hydrophobic polymer blocks, resulting in high extracellular stability and triggered release in reducing environment of the cell cytoplasm.

One potential drawback of this approach is the release of hydrophobic polymer chains in the cell that may interact with cellular proteins and compromise cell viability. We therefore decided to develop reversibly hydrophobized polycations based on releasable small molecule capping agents. We selected the benzoic imine bond as a pH sensitive linkage that is stable under physiological conditions and cleaved in slightly acid environments [27-31]. Polymers with pendant benzoic imine bonds produce primary amine groups after hydrolysis, enabling a switch from hydrophobic to hydrophilic properties. Thus, we hypothesized that benzoic imine-containing polymers would exhibit intracellularly-triggered hydrophobization and be useful for *in vitro* and *in vivo* gene transfer. In this work, we therefore synthesized a pH-sensitive block copolymer, composed of a hydrophilic block for nucleic acid condensation and colloidal stabilization and a second benzoic imine-functionalized block for hydrophobization and extracellular stability. We demonstrate that this block copolymer exhibited significantly increased gene transfer efficiency both *in vitro* and *in vivo* compared to a control pH-insensitive hydrophobized control polymer.

2. Materials and methods

2.1 Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA) and poly(ethylene glycol) methyl ether methacrylate (OEGMA, average $M_n = 300$) were ordered from Sigma-Aldrich, followed by purification using a basic alumina column before polymerization. 4-Cyanopentanoic acid dithio-benzoate (CPADB), N,N' -Azobisisobutyronitrile (AIBN), propargyl glycol, methacryloyl chloride, benzyl bromide, sodium azide, 3-chloropropylamine hydrochloride, copper (I) bromide (CuBr), N,N,N',N'',N''' -pentamethyldiethylenetriamine (PMDETA), 4-methoxybenzaldehyde, toluene and dioxane were obtained from Sigma-Aldrich for use without further purification. pCMV-Luc plasmid (Endotoxin-free) was prepared as described before [26]. Branched polyethylenimine (bPEI, $M_w \sim 25,000$, $M_n \sim 10,000$, Sigma) was dissolved in PBS (10 mg/mL), and the pH was adjusted to ~ 6 through the addition of 1 M HCl solution, which was diluted with water to get the final concentration. The pmaxGFP[®] plasmid (green fluorescent protein from the copepod *Pontellina p.*) was purchased from Lonza. YOYO-1 iodide was purchased from Invitrogen (Carlsbad, CA).

2.2 Characterization

^1H and ^{13}C NMR spectra were recorded on a Bruker AV 300 (Bruker Corporation, Billerica, MA) nuclear magnetic resonance (NMR) instrument in deuterium oxide (D_2O) or deuterated chloroform (CDCl_3). The molecular weight and polydispersity index (PDI) were tested by size exclusion chromatography (SEC) [5]. The buffering capacity (BC) of all the polymers were tested by acid-based titration followed the protocol of our previous work [26].

2.3 Polymer synthesis

2.3.1 Synthesis of (trimethylsilyl)propargyl methacrylate (TMS-PMA)—Propargyl alcohol (12.1 g, 0.21 mol) and triethylamine (TEA, 44 mL, 0.31 mol) were dissolved in 100 mL dichloromethane, and the mixed solution was cooled down to 0 °C. Methacryloyl chloride (26 mL, 0.26 mol) was added dropwise over 30 min, then continued at ambient temperature overnight. The reaction solution was filtered to remove ammonium salt, and the filtrate was washed sequentially by water, 0.5 % NaHCO_3 solution and water, and then was dried with magnesium sulfate. After evaporating the solvent, propargyl methacrylate was collected by passing a silica column with the mixture of hexane and ethyl acetate as elution (1/25, v/v). Yield: 80%.

Next, the alkynyl group was protected by chlorotrimethylsilane. Silver chloride (0.61g, 4.5 mmol), propargyl methacrylate (4.5 g, 36.3 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (8.8 mL, 58.1 mmol) was suspended in 50 mL of dichloromethane. The reaction was placed in an oil bath (40 °C), followed by the addition of chlorotrimethylsilane (8.4 mL, 64.5 mmol). After stirring for 24 hours, the mixture was cooled to room temperature and diluted with 200 mL of hexane. The organic phase was washed consecutively with saturated NaHCO_3 aqueous solution, hydrochloric acid (1 %) and water, and was dried over MgSO_4 . After removal of hexane, the crude product was purified by column chromatography eluting with a 25:1 mixture of hexane and diethyl ether. Yield: 90%. ^1H and ^{13}C NMR spectra are shown in Fig. S1.

2.3.2 RAFT polymerization of TMS-PMA—TMS-PMA (2.1 g, 10.7 mmol), CPADB (50.0 mg, 0.214 mmol) and AIBN (5.8 mg, 0.035 mmol) were first dissolved in 2.1 mL toluene. The oxygen in the solution was removed through purging with nitrogen for 10 min, and then the polymerization was initiated through heating to 60 °C. After 8 h, the polymerization was quenched by liquid nitrogen, and the polymer solution was precipitated into a methanol/water mixture (5/1, v/v). The polymer was collected by centrifugation under 6000 rpm for 10 min, followed by washing with methanol/water mixture for three times. The targeting polymer, poly(trimethylsilyl)propargyl methacrylate (p(TMS-PMA)), was received as a pink-color powder after freeze-drying for 24 h. Yield: 75%.

2.3.3 Copolymerization of OEGMA and DMAEMA—The chain transfer agent, p(TMS-PMA) (0.785 g, 0.073 mmol) (macro CTA), OEGMA (0.87 g, 2.9 mmol), DMAEMA (2.28 g, 14.6 mmol) and AIBN (1.2 mg, 0.0073 mmol) was dissolved in 10 mL dioxane in a 25 mL flask. The oxygen in the vessel was removed by nitrogen purging for 10 min. The polymerization was initiated by placing the reaction in an oil bath at 60 °C and then progressed for 20 h. The block copolymer, poly((2-dimethylamino)ethylmethacrylate-*co*-oligo(ethylene glycol) -*block*-poly(trimethylsilyl)propargyl methacrylate (p(TMS-PMA)-*b*-p(OEGMA-DMAEMA))), was purified by dialysis against water using dialysis membrane (MWCO: 3.5 kDa, Fisher Scientific) for three days, followed by freeze-drying for 48 h. Yield: 70%.

2.3.4 Deprotection of p(TMS-PMA)-*b*-p(OEGMA-DMAEMA)—The alkynyl-protected polymer, p(TMS-PMA)-*b*-p(OEGMA-DMAEMA) (1.2 g, 2.44 mmol alkynyl group) was first dissolved in 40 mL tetrahydrofuran (THF) with a small amount of acetic acid (300 µL). The mixture was placed in a salted ice bath (-15 °C), and purged with nitrogen for 10 min. TBAF solution (5 mL, 1 M in THF) was added dropwise with an automatic pump during 3 min. The reaction was placed in a salted ice bath for 30 min, and then the reaction was kept stirring at room temperature overnight. After passing through a short silica column, the polymer solution was concentrated, and was subjected to dialysis against distilled water. The final product, poly(propargyl methacrylate)-*block*-poly((2-dimethylamino)ethylmethacrylate-*co*-oligo(ethylene glycol), pPMA-*b*-p(OEGMA-DMAEMA), was harvested by freeze-drying. Yield: 60%.

2.3.5 Synthesis of (3-azido-propyl)-(4-methoxy-benzylidene)-amine (APMBA)—(3-Azido-propyl)-(4-methoxy-benzylidene)-amine was synthesized in two steps. First, 3-azido-propylamine was synthesized by azidation reaction in the presence of sodium azide. 3-Chloropropylamine hydrochloride (3.9 g, 30 mmol) was dissolved in 50 mL distilled water, followed by addition of sodium azide (5.85 g, 90 mmol). The mixture was placed in an oil bath (80 °C) for 24 h, and then pH was adjusted to 10 by potassium hydroxide, followed by extracting with ether for three times. The extracts were dried with MgSO₄ overnight. 3-Azido-propylamine was obtained as colorless oil after removal of solvent. Yield: 40%.

3-Azido-propylamine (1 g, 10 mmol) and 4-methoxybenzaldehyde (1 g, 8 mmol) were mixed in 100 mL anhydrous ethanol, and the solution was gently refluxed overnight. The solvent and excess 3-azido-propylamine were removed by vacuum evaporation, resulting in

(3-azido-propyl)-(4-methoxy-benzylidene)-amine as yellow oil. Yield: 95%. ^1H and ^{13}C NMR spectra are shown in Fig. S2.

2.3.6 Synthesis of azidomethyl benzene (AB)—Benzyl bromine (3.1 g, 18 mmol) and sodium azide (3.5 g, 54 mmol) was dissolved in 30 mL DMF under nitrogen and the mixture was stirred at room temperature for 24 h. Then, the solution was diluted by 200 mL dichloromethane, and the organic phase was washed by water twice and then dried by MgSO_4 overnight. After removal of the solvent, azidomethyl benzene was received as yellow oil. ^1H and ^{13}C NMR spectra are shown in Fig. S2. Yield: 50%.

2.3.7 Click coupling of APMBA and AB into block copolymer—APMBA and AB were conjugated to the polymer by click chemistry separately with the same procedure. The coupling reaction of APMBA to polymer was listed as an example. pPMA-*b*-p(OEGMA-DMAEMA) (20 mg, 0.05 mmol alkynyl group), APMBA (22 mg, 0.1 mmol) and PMDETA (20 μL) were first dissolved in 2 mL DMF. After completely dissolving the reagents, the oxygen in the flask was removed through purging with nitrogen for 10 min. Then CuBr (12 mg) was added quickly under inert atmosphere condition. The click reaction was progressed in the oil bath at 40 $^\circ\text{C}$ for 24 h. Next, the solution was directly subjected to dialysis against methanol for three days. Then the targeting polymer was obtained after evaporation of methanol. Yield: 80%.

2.4 Sizes of polymer nanoparticles at different pHs

The size of nanoparticles at different pH values was measured by dynamic light scattering (DLS) using a ZetaPLUS instrument (Brookhaven Instruments Corporation, Holtsville, NY). The stock solutions of polymers except p(OEGMA₁₃-DMAEMA₆₈) were prepared in anhydrous DMSO (20 mg/mL). Afterwards, 1 mg/mL polymer solutions were prepared through dilution with either 15 mM PBS to pH 7.4 or HCl solution to pH 5.5, and sizes were measured at different time points at room temperature. The surface charge of different formulations was also recorded at N/P ratio of 5.

2.5 DNA polyplexes characterization

For different N/P ratio, the polymer stock solution was diluted with 15 mM PBS, and it was stored at ambient condition for 30 min before mixing with plasmid DNA. For polyplexes preparation, the polymer solution with different concentrations was added to the luciferase plasmid solution with an equal volume (0.1 mg/mL), and the formed polyplexes solution were vortexed for 10 seconds and incubated at room temperature for 10 min. The polyplexes solution (10 μL) with different N/P ratios was subjected to electrophoresis on agarose gel (1%) at 100 V for 40 min to test the DNA condensing ability. For the size testing, polyplexes with 1 μg DNA at N/P ratio of 5 (20 μL) were diluted with ddH₂O or optiMEM (800 μL), and subjected to DLS measurement. The measurements were performed at different time points.

2.6 Transmission electron microscope (TEM)

The morphology of polymers and polyplexes in dried state were imaged on a JEOL 1140 TEM. For the morphology of polymer assemblies at different pHs, the polymer stock

solutions were diluted with either 15 mM PBS to pH 7.4 or HCl solution to pH 5.5. After incubation for 4 hours, the solutions (10 μ L) were deposited on the top of the copper grids (400-mesh) and left at room temperature for 30 minutes. After staining with uranyl acetate, the grids were subjected to air dry overnight. The polyplexes were obtained as shown before, and the TEM samples were prepared as for the polymer samples.

2.7 Polyplex unpacking

Plasmid DNA was labeled with YOYO-1 iodide through bis-intercalation (one dye to 100 base pair) and incubated under darkness for 1 hour. Polyplexes were prepared as before using YOYO-1 labeled DNA (N/P = 5), and then were treated with distilled water or HCl solution (pH = 5.5) for 1 h. Thereafter, PBS, fetal bovine serum (FBS) and heparin sulfate were further added to some samples to achieve a final concentration of 10% for FBS and 5 μ g/mL for heparin sulfate, respectively. After 1 hour, the fluorescence was measured on a plate reader (ex: 491 nm, em: 509 nm). The percentage of unpackaged DNA was defined as the fluorescence intensity in polyplexes groups under different conditions compared to that in DNA only group.

2.8 In vitro testing

2.8.1 Gene transfection in HeLa, Z310 cells and primary neural progenitor cells

—HeLa cells (ATCC CCL-2™) were plated in a 24-well plate (20,000 cells/well) in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (AbAm) (100 IU of penicillin, 100 μ g/mL of streptomycin, and 0.25 μ g/mL of amphotericin B). After incubation at 37 °C (5% CO₂) overnight, the cells were washed with PBS, and polyplexes with different N/P ratios (1 μ g pDNA) were added on the top of the cell layer with 200 μ L of complete cell culture media. 4 hours later, the transfection solution was removed and cells were washed twice with PBS followed by the addition of 500 μ L fresh media. Luciferase activity was analyzed after two days with the method we reported before [26]. The transfection study with green fluorescence plasmid (GFP) was the same as that using luciferase plasmid. The transfected cells were detected by flow cytometry [7].

Z310 cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated FBS, 10% penicillin/streptomycin, 40 mg/mL gentamicin, and 10 ng/mL nerve growth factor (NGF). The transfection study was performed under the same condition as HeLa cells.

Primary neural progenitor cells (NPCs) were cultured in DMEM/F12 supplemented with N-2 supplement (1%), heparin (5 mg/mL), L-glutamine (2 mM) and endothelial growth factor and NGF (20 ng/mL). NPCs were seed at a density of 30,000 cells per well (24 well plate) in growth medium 72 h prior to transfection. Polyplexes (1 μ g) in 100 μ L were added into wells, and incubated with cells for 48 h. Luciferase activity in cell lysates was measured as described for other cell lysates.

2.8.2 Intracellular trafficking of polyplexes by confocal microscope—To evaluate the endosomal escape ability of IP and SP polyplexes, DNA was firstly labeled with YOYO-1. The cells were incubated with polyplexes for 4 h. The acidic vesicles and the

nuclei of cells were stained with LysoTracker Red, DND-99 and 4',6-diamidino-2-phenylindole (DAPI), respectively. To quantify the endosomal escape ratio of polyplexes, the colocalization ratio between DNA and LysoTracker Red was quantified as follows using Image J software:

$$\text{Colocalization ratio}(\%) = \frac{\text{YOYO} - 1 \text{ pixels}_{\text{colocalization}}}{\text{YOYO} - 1 \text{ pixels}_{\text{total}}} \times 100\%$$

Where YOYO-1 pixels_{colocalization} represents the number of YOYO-1 pixels colocalizing with LysoTracker Red, and YOYO-1 pixels_{total} represents the number of all YOYO-1 pixels in the confocal images. Results were presented as the mean of 15 individual cells.

2.8.3 Polyplex uptake assay—Cellular uptake of polyplexes was evaluated *via* flow cytometry using fluorescently-labeled DNA. Cells were prepared as listed in transfection study. Polyplexes with YOYO-1 labeled DNA (1 µg) were added into the well, and incubated with cells for 60 min at 37 °C. Then cells were washed twice with PBS, detached by treatment with trypsin, pelleted, and resuspended in 300 µL PBS with 0.5% BSA, and analyzed using flow cytometry.

2.9 In vivo plasmid delivery of polymers

All animal work was completed using approved procedures and following the strict guidelines from the University of Washington IACUC. The injection procedure was followed as described before [32]. Different formulations were prepared in 5% glucose solution containing 2.5 mg of DNA at desired N/P. Female C57/B16 mice (7-9 week old) were housed for 1 week before the treatment. Mice were anesthetized by an intraperitoneal injection of Avertin (0.23 mL/20 gram). A craniotomy (1 mm diameter) was made on the right-side of the skull using a dental drill, and 10 µL of different formulations were stereotactically injected at 1 mm lateral, 0.5 mm caudal to bregma, and 1.75 mm deep from the dura using a 33 gauge 10 µL Hamilton syringe. The injection was made at 1-2 µL/min and the syringe was kept in the injection site for 2 min to prevent backflow. After two days, the brains (left, right and hind brain) were collected in lysis buffer with protease inhibitors, and then mechanically homogenized. Luciferase activity was tested with the method we used for *in vitro* transfection, and reported as relative light units (RLU) per mg brain protein.

3. Results and discussion

3.1 Polymer synthesis and characterization

The pH-sensitive block copolymer, comprised of a polycationic block, poly(oligo(ethylene glycol) monomethyl ether methacrylate)-*co*-poly(2-(dimethylamino)ethyl methacrylate) (p(OEGMA-DMAEMA)), and a pH-sensitive hydrophobized block, poly(propargyl methacrylate-*graft*-propyl-(4-methoxy-benzylidene)-amine) (p(PMA-PMBA)), was synthesized by a combination of RAFT (reversible addition-fragmentation chain transfer) polymerization and click chemistry (Scheme 1). This polymer contains a block functionalized with pH-sensitive benzoic imine groups. A pH-insensitive control polymer

was also synthesized by RAFT polymerization for comparison. This control polymer shares the same cationic p(OEGMA-DMAEMA) block but has a benzyl decorated pH insensitive block, poly(propargyl methacrylate-graft-methylene benzene) (p(PMA-MB)).

To synthesize the polymers, the macro CTA, poly(trimethylsilyl)propargyl methacrylate (p(TMS-PMA)), was first prepared *via* RAFT polymerization, followed by consecutive RAFT polymerization with OEGMA and DMAEMA to obtain the block copolymer, p(TMS-PMA)-*b*-p(OEGMA-DMAEMA). The trimethylsilyl protecting group was then removed to obtain pPMA-*b*-p(OEGMA-DMAEMA). Each step was characterized by ^1H NMR and GPC. As shown in Fig. S3, the disappearance of the signals at 0.5 ppm assigned to the methyl group of TMS and the emergence of a new peak at 2.5 ppm attributed to the alkynyl group revealed successfully removal of the protecting group. Moreover, the GPC trace of pPMA-*b*-p(OEGMA-DMAEMA) exhibited distinct right shift toward low molecular weight compared to p(TMS-PMA)-*b*-p(OEGMA-DMAEMA) (Fig. S4). The molecular weights of different polymers were listed in Table S1.

It is reported that the benzoic imine bond is relatively stable in neutral pH solution and can be cleaved in low pH and produce a free amine group [28]. We therefore introduced the benzoic imine moiety to the side chain of pPMA-*b*-p(OEGMA-DMAEMA). At physiological pH condition, the modified polymer is expected to assemble into micelles with the benzoic imine-containing block restricted in the core. In acidic conditions, such as in endosome/lysosome, the imine bond should be cleaved, triggering a hydrophilic phase transition for the polymer. In this work, a pH sensitive moiety, (3-azido-propyl)-(4-methoxy-benzylidene)-amine (APMBA), was conjugated to the pPMA-*b*-p(OEGMA-DMAEMA) to produce the pH-sensitive polymer denoted as SP (pH sensitive polymer); for comparison, a pH insensitive moiety, azidomethyl benzene (AB), was also conjugated to pPMA-*b*-p(OEGMA-DMAEMA) to produce the pH-insensitive control polymer denoted as IP (pH insensitive polymer). Both polymers were characterized by ^1H NMR as shown in Fig. S5. We donated pPMA-*b*-p(OEGMA-DMAEMA) as precursor polymer.

In order to validate the pH triggered cleavage of benzoic imines, the ^1H NMR spectrum of SP at various pH values in D_2O were first obtained. Due to its amphiphilic structure, SP assembles into micellar nanoparticles with p(OEGMA-DMAEMA) as the shell and p(PMA-PMBA) as the core. Therefore, at pH 7.4, we only observed the proton signals (1.9 and 3.8 ppm) of p(OEGMA-DMAEMA) while the signals for p(PMA-PMBA) were suppressed due to shielding by the hydrophilic shell (Fig. 1). The signals attributed to protons in the benzyl ring of PMBA emerged only after incubation in pH 5.5 for 1 h, and the intensity increased with incubation time, indicating cleavage of the benzoic imine bond. Moreover, a signal around 9.8 ppm assigned to benzaldehyde also appeared, further confirming pH triggered-cleavage.

We next tested the assembly behaviors of IP and SP at neutral and acidic pH by DLS and TEM (Fig. 2). Light scattering analysis showed that the size of the assemblies formed by IP was consistent at both pH values and stable with time at around 80 nm in diameter (Fig. 2A). These results were verified by TEM imaging, which revealed condensed structures with diameters around 50 nm, likely due to shrinking upon dehydration (Fig. 2B). However, the

size of the assemblies formed by SP decreased from 83 to 45 nm after 20 h incubated in pH 7.4 accompanied with the increasing of PDIs from 0.133 to 0.471 (Table S2), demonstrating the instability of the assembly nanostructure even in physiological conditions with longer time. At pH 5.5, the average hydrodynamic size decreased significantly from 81 nm to 34 nm within 4 h, and the PDIs also increased from 0.136 to 0.523. Furthermore, while condensed structures were observed at pH 7.4 by TEM, no structures were observed for polymer solutions at pH 5.5. Cumulatively, these results confirm the pH sensitivity of SP.

3.2 Polyplexes characterization

The DNA binding ability of SP and IP at various polymers to DNA ratios was determined by agarose gel electrophoresis assay (Fig. S6), which showed that complete DNA retardation was achieved when the N/P ratio (amine to phosphate) was higher than 2, indicating efficient DNA condensation. There was no obvious difference in DNA binding ability between SP and IP. Moreover, the surface charge of all polyplexes was positive (~ 35 mV), beneficial for cell membrane binding (Fig. S7).

The poor resistance of polyplexes to physiological salt concentrations and polyanionic biomacromolecules has been recognized as the significant obstacle for *in vivo* nucleic acid delivery. We therefore monitored stability of the polyplexes in 150 mM PBS or optiMEM by DLS (Fig. 3A). The size of polyplexes formed by IP and DNA were stable (~ 200 nm) regardless of media even after 20 h incubation. For polyplexes formed using SP, particle size increased by ~ 100 nm after incubating for 20 h in PBS and optiMEM. This size increase may be attributed to the gradual hydrolysis of the benzoic imine bond and resulting loosening of the inner core, which was validated by the fast size increase of SP polyplexes in pH 5.5 (Fig. S8). These results demonstrate the excellent colloidal stability of both IP and SP polyplexes. Conversely, large aggregates with diameter > 1000 nm were detected by the polyplexes formed by p(OEGMA₁₃-DMAEMA₆₈) in optiMEM, which lacks the hydrophobic block to stabilize the resulting polyplexes. These results suggest that incorporation of a hydrophobic block improves the stability of resulting polyplexes. Finally, the morphologies of the polyplexes formed by IP and SP were observed by TEM. Both condense DNA into compact spherical nanoparticles (Fig. 3B).

3.3 In vitro transfection

In vitro gene delivery efficiency of IP and SP were screened in HeLa cells in serum-containing media using both luciferase and green fluorescence reporter plasmids and compared to precursor polymer, p(OEGMA-DMAEMA) and branched poly(ethylenimine) (bPEI, 25 kDa). The N/P ratio used for bPEI was 5 because the optimal condition for bPEI polyplexes was previously determined to be at this N/P ratio [15]. As shown in Fig. 4A, the transfection efficiency of polyplexes increased with N/P ratio, and the polyplexes formed by SP showed the highest luciferase transgene expression among the tested groups. However, the cell viability was reduced when cells were treated with SP polyplexes formulated at N/P ratio higher than 5, likely due to the excess positive charge generated after cleavage of the benzoic imine bond (Fig. S9). Thus, the optimized N/P ratio of 5 was used for all remaining *in vitro* studies.

Several observations are worth noting from this experiment. First, the luciferase activity mediated by SP polyplexes with different N/P ratios were 50 ~ 500 fold and 15 ~ 30 fold higher than that by IP and CP polyplexes, respectively. Under the optimized N/P ratio of 5, the transfection efficiency of SP was higher than that of the gold standard, bPEI. Second, the precursor polymer polyplexes displayed improved transfection efficiency compared to that by IP, suggesting that the benzyl group introduced into side chains of pPMA block reduced transfection efficiency. The benzyl side groups could stack tightly based on π - π interactions, leading to enhanced stability of the formed polyplexes that prevents DNA release and compromises delivery efficiency, which was confirmed by the reduced decomplexation from IP polyplexes in the presence of heparin (Fig. S10). Third, the hydrophobization of polycations by pH insensitive blocks did not lead to the increase of transfection efficiency. Although IP and precursor polymer based polyplexes showed enhanced salt and serum resistance compared to that of CP, the improved stability conversely impaired the unpacking of DNA when the polyplexes trafficked into cytoplasm. The introduction of stimuli-triggered de-hydrophobization after entry into cells of polyplexes is the key factor to successful delivery of nucleic acids.

To determine the percentage of transfected cells by the different polyplex formulations, the green fluorescence protein (GFP) plasmid was employed as a reporter gene for transfection to HeLa cells. The percentage of live cells expressing GFP was determined by flow cytometry using PI as a cell viability marker (GFP+/PI- population). Around 30% cells are transfected using SP based polyplexes, significantly higher than that mediated by IP (5%), precursor polymer (8%), CP (10%) and even bPEI (17%) (Fig.4B). Representative flow analysis data is shown in Fig. S11. Fluorescence microscopy was also used to observe the transfected cells. Representative images of GFP gene expression mediated by IP, SP, precursor polymer, CP and bPEI are shown in Fig. 4C. Cells treated with SP polyplexes showed the strongest green fluorescence, indicating the highest percentage of cells transfected. These results were in accord with that by luciferase activity and flow cytometry analysis, confirming the superior transfection efficiency of SP.

In addition, to test the intraventricular gene transfer capability to the brain, we evaluated the transfection efficiency of the polyplexes to Z310 cells (an immortalized rodent choroid plexus cell line) and primary murine neural progenitor cells (NPCs). At the optimal N/P ratio of 5, SP mediated increased luciferase activity compared to that by IP, p(OEGMA-DMAEMA) and precursor polymer in Z310 cells, and the transfection efficiency was in the same level as bPEI (Fig. 5A). Hence, we moved forward to transfect the primary NPCs in the same condition. However, only slight increase in transfection efficiency was observed in NPCs by SP compared to other polymers (Fig. 5B). Moreover, all the formulations were tolerant in both cell lines (Fig. S12).

3.4 Elucidating the mechanism of enhanced gene delivery by SP

Successful gene delivery based on polycations requires efficient entry into cell, endosomal escape and cargo release. To better understand the advantages of SP as a gene carrier, we compared the efficiencies of SP versus IP polyplexes for each of these steps. First, polyplex uptake was determined by fluorescently-labeled DNA with YOYO-1 and monitoring cells by

flow cytometry. YOYO-1 fluorescence is a function of packaging state so that the cellular uptake was measured at short time (1 h) before benzoic imine bond cleavage. As shown in Fig. 6A, there was no statistical difference of cellular uptake between IP and SP polyplexes. This is not surprising, since the IP and SP polyplexes possess the same polycation part p(OEGMA-DMAEMA) that mediates cellular uptake through charge interactions with the cell membrane.

Second, we tracked both YOYO-1 labeled SP and IP polyplexes in HeLa cells by triple fluorescence confocal microscopy. As shown in Fig. 6B, the fraction of the green fluorescence (YOYO-1 DNA) separated from the red fluorescence (LysoTracker Red) from SP polyplexes was higher than that from IP polyplexes, indicating improved endo/lysosomal escape capability of SP/DNA polyplexes. The additional images with more cells were listed in Fig. S13. The colocalization ratio of DNA with endo/lysosomes with SP polyplexes calculated by Image J software was 50.8% compared with IP polyplexes (80.6%) (Fig. 6C). Moreover, the buffering capability by SP was stronger than other polymers (Fig. S14). The cleavage of the benzoic imine bond also consumed H^+ and increased the endosomal osmotic pressure followed by membrane rupture. Thus, the higher transfection efficiency achieved by SP may be partially attributed to increased endosomal escape capability.

Third, polyplex unpackaging was evaluated by YOYO-1 assay under various conditions [32]. Ideal gene carriers should strongly condense pDNA avoiding undesired degradation before cellular uptake while also releasing the cargo intracellularly, *e.g.* by competitive displacement with anionic biomacromolecules, for plasmid expression. As shown in Fig. 5D, polyplexes formed in water showed very low fluorescence, indicating efficient condensation of DNA by SP and IP. In addition, although increased release of DNA was observed in the presence of salts and anionic biomolecules, there is no difference observed between SP and IP without acid pretreatment. However, the release of DNA by SP polyplexes after acid trigger was accelerated compared to that by IP polyplexes. The hydrophilic transition of the p(PMA-PMBA) block after the cleavage of benzoic imine bonds compromised the stability of SP polyplexes, facilitating cargo release. Although the cleavage of benzoic imine bonds generates additional cationic charge that may increase binding to DNA, the electrostatic interaction between polymer and DNA is readily reversed by competition with heparin and serum components. In summary, the improved transfection efficiency by SP can be attributed to its efficient endosomal escape and improved cargo release in cytoplasm.

3.5 In vivo delivery

The promising *in vitro* transfection efficiency obtained using SP suggests its utility for *in vivo* gene delivery. We therefore evaluated gene transfer of SP, IP, and CP polyplexes to the brains of C57/BL6 mice through intraventricular injection. Polyplexes formed by IP, SP and CP with DNA at N/P = 5 and N/P = 10 were injected stereotactically into the right lateral ventricle of mice. After 2 days, different parts of the brain were harvested and analyzed for luciferase activity. As shown in Fig. 7, SP polyplexes-treated mice had the highest luciferase expression in the hindbrain regardless of N/P ratios; 3.2- and 7.7-fold higher luciferase activity were mediated by SP polyplexes than IP at N/P ratio of 10 and 5, respectively; 4.6

and 4.9-fold higher luciferase activity were mediated by SP polyplexes than IP at N/P ratio of 10 and 5, respectively. In the left and right brain, we also found the higher luciferase activity mediated by SP than that by IP with N/P ratio of 5 (Fig. S15). However, compared to the delivery efficiency of bPEI, the luciferase activity in the brain tissue was reduced, probably due to the stronger DNA condensing capability of bPEI [33]. We also tested the transgene expression the lung and liver, and no obvious was detected (Fig. S16). Moreover, no acute mortalities were observed during the treatment with different formulations.

4. Conclusions

In summary, we report here the synthesis of a pH sensitive polymer, p(PMA-PMBA)-*b*-p(OEGMA-DMAEMA) (SP), and evaluated it as carrier for *in vitro* and *in vivo* gene transfer. SP could efficiently condense DNA into nanoparticles, and endow high stability of the resulting polyplexes through hydrophobic modification by p(PMA-PMBA) block. Conversely, the hydrophilic transition through the cleavage of benzoic imine bonds accelerates the unpacking of the DNA cargoes, leading to enhanced transfection efficiency compared to control polymers IP and CP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. ^1H NMR spectra of p(PMA-PMBA)-*b*-p(OEGMA-DMAEMA) in D_2O at pH 7.4 and 5.5 with different incubation times.

Fig. 2.

A). Hydrodynamic diameters of IP and SP assemblies in aqueous solution at pH 7.4 and 5.5 for different incubation time tested by dynamic light scattering (DLS). B). TEM images of IP and SP assemblies at pH 7.4 and 5.5. Scale bar: 100 nm.

Fig. 3.

A). Average hydrodynamic diameter of IP and SP polyplexes formed at N/P ratio of 5 with in PBS and OptiMEM at various incubation times. B). TEM images of IP and SP polyplexes with N/P ratio of 5. Scale bar: 100 nm.

Fig. 4. (A) *In vitro* transfection of HeLa cells with different formulations. (B) GFP expression mediated by different polyplexes at N/P ratio of 5 analyzed by flow cytometry. (C). Representative images of GFP expression mediated by various polyplexes in HeLa cells. Scale bar: 50 μ m. Data are shown as mean $\pm$ SD (n = 3; student's t-test, *p < 0.05, **p < 0.01).

Fig. 5.
 (A) *In vitro* transfection of Z310 cells and (B) neural progenitor cells with different formulations at N/P ratio of 5. Data are shown as mean $\pm$ SD (n = 3; student's t-test, *p < 0.05).

Fig. 6.

(A). *In vitro* cellular uptake of YOYO-1 labeled DNA delivered by IP and SP. Data are shown as mean $\pm$ SD (n=3). (B). Confocal images of HeLa cells treated with polyplexes containing YOYO-1 labeled DNA for 4 h and stained with LysoTracker Red. DAPI (4',6-diamidino-2-phenylindole, blue) was used to stain cell nuclei. Scale bar: 10 μ m. (C). Colocalization ratio of YOYO-1-DNA with LysoTracker Red stained endosomes. Results were presented as the mean of 15 individual cells. Data are shown as mean $\pm$ SD (n=15; student's t test, *p<0.001). (D). Unpacking study of IP and SP polyplexes in different conditions. Data are shown as mean $\pm$ SD (n=3; student's t test, *p<0.001).

Fig. 7. Luciferase expression in the mouse hindbrain two days after intraventricular injection using glucose, polyplexes formed from IP, SP and p(OEGMA-DMAEMA) at N/P 5 and 10 (n=5; student's t-test, *p<0.05, **p<0.01).

Scheme 1.

Scheme of synthetic routes of p(PMA-PMBA)-*b*-p(OEGMA-DMAEMA) and p(PMA-MB)-*b*-p(OEGMA-DMAEMA).