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Phenylboronic acid-sugar grafted polymer architecture as a dual stimuli-responsive gene carrier for targeted anti-angiogenic tumor therapy

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ABSTRACT

We present a cationic polymer architecture composed of phenylboronic acid (PBA), sugar-installed polyethylenimine (PEI), and polyethylene glycol (PEG). The chemical bonding of PBA with the diol in the sugar enabled the crosslinking of low-molecular-weight (MW) PEI to form high-MW PEI, resulting in strong interaction with anionic DNA for gene delivery. Inside the cell, the binding of PBA and sugar was disrupted by either acidic endosomal pH or intracellular ATP, so gene payloads were released effectively. This dual stimuli-responsive gene release drove the polymer to deliver DNA for high transfection efficiency with low cytotoxicity. In addition, PBA moiety with PEGylation facilitated the binding of polymer/DNA polyplexes to sialylated glycoprotein which is overexpressed on the tumor cell membrane, and thus provided high tumor targeting ability. Therapeutic application of our polymer was demonstrated as an anti-angiogenic gene delivery agent for tumor growth inhibition. Our judicious designed polymer structure based on PBA provides enormous potential as a gene delivery agent for effective gene therapy by stimuli-responsiveness and tumor targeting.

1. Introduction

Stimuli-responsive materials have shown immense potential for various applications because their behaviors can be modulated by specific signal [1–3]. Applications of stimuli-responsive polymers and nanomaterials to biomedical fields such as gene and drug delivery using their physicochemical variations towards specific triggers have been reported [4–7]. Especially, intracellular stimuli including the effects of biomolecules, redox potential, and pH have potential as precise triggers because they are responsive to specific intracellular sites [8–14].

Use of delivery vehicles raises the concern that they may have toxic side effects, so the vehicles must be eliminated after facilitating their mission to prevent long-term toxicity issues in body [14–18]. Therefore, the ideal delivery carrier should form a stable architecture when outside the target cell, but be degraded in response to intracellular triggers when inside the cell, thereby releasing their payloads for enhanced delivery efficiency and reduced non-specific toxicity.

Phenylboronic acid $C_6H_5B(OH)_2$ (PBA) selectively binds to *cis*-diol moieties of common sugars [19,20]. This unique property enables use of PBA as a ligand in several applications, such as glucose sensors, chromatographic separation of carbohydrates, and RNA affinity columns, etc [21–25]. Relevantly, the binding of PBA to a sugar moiety provides two modes of stimuli-responsive features [26,27]. First, the binding affinity of PBA with sugar depends on the pH; the affinity is strong at physiological and basic pH, but weak at acidic pH such as within endosomes; this pH-responsiveness has been exploited as a modulator for controlling the affinity of PBA to sugar [20,28]. Second, the diol moiety in the sugar easily exchanges its bond with PBA for one with another diols having better thermodynamic stability thereby rearranging the binding pair [29,30]. Adenosine triphosphate (ATP) which has a *cis*-diol moiety in its ribose ring, is one of the most abundant intracellular biomolecules inside the cell, and can therefore interfere with the PBA-sugar interaction, resulting in formation of PBA-ATP [9,25]. The

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concentration of ATP ([ATP]) is ~1–10 mM in the cell mainly providing energies for cellular metabolism, but only several micromolar outside the cells [31,32]. This huge difference in [ATP] at intra/extra cellular region enables the use of ATP as a intracellular-specific trigger. Therefore, a smart delivery system could be designed based on the interaction of PBA and sugar which is dependent on the dual stimuli of intracellular pH and [ATP].

Another important requirement for an effective delivery system is selective delivery of therapeutic molecules to target cells [33–35]. Tumorous regions, in particular, passively drain circulating nanocarriers and retain them when they are injected systemically because of enhanced permeability and retention (EPR) effect [34–36]. After reaching the tumorous region, the ligand on the surface of a nanocarrier recognizes cell-specific receptors, which conduct it into the target cell by receptor-mediated endocytosis [37,38]. For target-specific delivery, PBA could be used as a target ligand because it specifically binds to sialylated epitopes that are overexpressed on the surface of various types of tumor [39–41]. Compared to common targeting moieties like peptides, PBA has low immunogenicity and can be modified easily.

Using these multi-functionalities of PBA and PBA-sugar interaction, we developed a unique cationic polymer architecture composed mainly of polyethylenimine (PEI). PEI, the most well-known cationic polymer for gene delivery, was modified with PBA (PBA-PEI) or galactose (Gal-PEI). By exploiting the interaction of PBA and the diol of the sugar, low-molecular-weight (MW) PEI could be crosslinked to become high-MW PEI, which fell apart inside the cell in response to acidic pH and high [ATP] there. Previously, our group reported an intracellular-degradable cationic polymer that is responsive to the cellular redox condition for effective gene release with low cytotoxicity [11,42–44]. More recently, we published a polymeric self-assembled structure which has been obtained by multivalent polymer–polymer interaction to yield a stable nano-assembly [45,46]. Therefore, we expect that crosslinked PEI formed by PBA-sugar interaction should have high gene transfection efficiency due to stimuli-responsive gene release, but also have low cytotoxicity due to intracellular disassembly of high molecular weight polymer. In addition, the multivalent interaction between PBA-PEI and Gal-PEI offers strong binding of two polymers (CrossPEI), which leads to high stability in the blood. Besides, polyethylene glycol (PEG) was introduced to the CrossPEI to increase its blood compatibility (PEG-CrossPEI), then PEG-CrossPEI was further modified with PBA (PBA-PEG-CrossPEI) for tumor-targeting ability (Fig. 1). As a therapeutic gene against tumor growth, soluble vascular endothelial growth factor (VEGF) receptor (sFlt-1) plasmid was transfected using PBA-PEG-CrossPEI into the tumor site. Transfected sFlt-1 is expressed in the tumor cells and secreted out of cells, and it binds antagonistically to the VEGF receptor, thereby inhibiting signal transduction for angiogenesis and consequently inhibiting tumor growth [47–49]. We investigated the potential of PBA-PEG-CrossPEI carrier as a tumor-specific gene delivery carrier which is responsive to cellular environments in cellular-level and animal models.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI, MW = 25 kDa and 1.8 kDa) was obtained from Polysciences, Inc. (Warrington, UK). *N*-hydroxysuccinimide (NHS) was obtained from Fluka (China). The 6-arm polyethylene glycol succimidyl succinate (6-arm PEG, MW = 10 kDa) was purchased from SunBio, Inc. (Daejun, Korea). YOYO-3 iodide and mounting medium for fluorescence were purchased from Invitrogen, Inc. (Eugene, OR) and VECTOR (Burlingame, CA), respectively. All other chemical reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO) and used without further purification.

2.2. Synthesis of functionalized PEI with PBA or sugar

PEI (100 mg, MW = 1.8 kDa) was solubilized in deionized (D.I.) water and pH was adjusted to 7.4. Galacturonic acid (GA) and 3-fluoro-4-carboxyphenylboronic acid (PBA) were separately solubilized in D.I. water at 5 molar equivalence toward PEI. Then, 30 molar equivalent 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) and NHS were added to these solutions with stirring. After 30 min, PEI solution was separately added to the GA and PBA solution in a dropwise manner, followed by overnight incubation. The reaction solutions were dialyzed using cellulose membrane dialysis bag (MWCO = 1000 Da) against pure water to remove unreacted substances for 2 days and lyophilized. The degree of functionalization of each polymer was determined by back titiration from primary amine quantification based on fluorescamine assay.

2.3. Preparation of crosslinked polymers with PBA-PEI and GA-PEI

Gal-PEI solution (10 mg/mL) was mixed with PBA-PEI solution with vigorous stirring overnight. The reaction solution (CrossPEI) was purified by membrane centrifugation (MWCO = 3000 Da, 4000 rpm, 20 min, 5 times) against tris-acetate buffer (pH = 8.2 with 0.3 M NaCl) and used without freeze drying. The amount of CrossPEI was determined by copper acetate (CuOAc) amine quantification assay. For the PEGylation, 6-arm PEG with succimidyl succinate was mixed with CrossPEI at 1:5 ratio of succimidyl succinate in PEG to primary amine in PEI, resulted in PEG-CrossPEI. For further modification of PEG-CrossPEI with PBA, 3 molar equivalent 3-aminophenylboronic acid was added to 6-arm PEG succimidyl succinate, and the final products (PBA-PEG-CrossPEI and PEG-CrossPEI) were purified by membrane centrifugation (MWCO = 10 kDa, 4000 rpm) for 20 min at 5 times.

2.4. Instrumentation

Fourier Transform Infrared Spectroscopy (FT-IR) spectra were obtained by a Vertex 70 spectrophotometer (Bruker Optics, Germany). The molecular weight distribution of polymers was analyzed by gel permeation chromatography (GPC) with a refractive index detector (RID-10A, SHIMADZU, Tokyo, Japan). The hydrodynamic volumes and surface charges of materials were obtained by a Zetasizer Nano (Malvern Instruments, Malvern, UK). The fluorescence spectra were measured using a spectrofluorophotometer (RF-5301PC, SHIMADZU, Tokyo, Japan) and the absorbance spectra were recorded using UV–Vis spectrophotometry (UV 2550, SHIMADZU, Tokyo, Japan).

2.5. Cell culture

MCF-7 (human breast adenocarcinoma cells) and PC-3 (human prostate cancer cells) were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Hyclone). HeLa (human cervical cancer cells) and CT-26 (mouse colon cancer cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone). Each media contains 9% fetal bovine serum (FBS, Hyclone), 100 μg/mL streptomycin, and 100 U/mL penicillin. Each cell was incubated at 37 °C in a 5% CO_2 huidified incubator.