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Trigger-responsive, fast-degradable poly(β-amino ester)s for enhanced DNA unpackaging and reduced toxicity

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ABSTRACT

Poly(β-amino ester)s (PBAEs) represent an important class of cationic gene delivery materials which, however, suffer from uncontrolled DNA release due in part to the slow degradation of their polyester backbone. Additionally, PBAEs with high molecular weight (MW) also show considerable toxicities. In this study, we designed and developed PBAEs with trigger-responsive domains built-in polymer backbones that can be rapidly cleaved upon external UV light triggering to promote intracellular DNA release as well as reduce material toxicity. Photo-responsive PBAEs were prepared via polyaddition of (2-nitro-1,3-phenylene)bis(methylene) diacrylate and a bifunctional amine. The nitrobenzene moiety was placed in each repeating unit of the PBAE to allow fast response to external UV irradiation, and thus the ester linkers were cleaved and the polymers were degraded within several minutes upon UV irradiation. Cationic PBAEs with high MWs were able to mediate effective intracellular gene delivery, while upon UV irradiation post-transfection, enhanced DNA unpackaging and reduced material toxicity were observed, which collectively contributed to greatly improved transfection efficiencies in various mammalian cell types tested. This strategy allows precise manipulation of material toxicity and gene release profiles of PBAEs, and thus provides an effective design approach to address critical issues in non-viral gene delivery.

1. Introduction

Gene therapy holds great promise for the treatment of congenital or acquired diseases by delivering generic materials into target cells to promote or rectify specific cellular functions [1–5]. Up- or down-regulation of specific gene targets have also been used to direct stem cell differentiation, promote tissue repairing, and reprogram somatic cells [6–8]. Gene delivery has been largely achieved with the use of viral vectors which are known for their high gene delivery efficiencies. However, the viral approach is often associated with significant immunogenicity, insertional mutagenesis, and oncogenicity, which presents serious concerns for its clinical applications [9,10]. Non-viral gene delivery, known for its low immunogenicity and oncogenicity, has been intensively studied in the past two decades as a safer alternative to viral gene delivery [3,5,11–13]. Cationic polymers (or polycations), capable of condensing anionic nucleic acids to facilitate their intracellular delivery, are one of the most important classes of non-viral vectors. Polycations with higher molecular weight (MW) usually demonstrate stronger condensation capacity toward nucleic acids and mediate more efficient gene delivery than their lower MW analogues [14,15]. However, they also show appreciable cytotoxicities related to their high MWs and cationic charge densities [16,17]. Additionally, the excessive binding affinity of the high MW cationic polymer toward nucleic acids would also restrict the intracellular gene release, a critical roadblock toward effective gene transfection [18].

Poly(β-amino ester) (PBAE) is one of the few polycations that have been very intensively studied in non-viral gene delivery in the past decade. It is a class of biodegradable polymers designed by Langer and co-workers that can be easily obtained in large scale via elegantly simple yet extremely versatile polyaddition chemistry, which can be readily adapted to high-throughput processes to make a large library of structurally diverse materials [19–25]. Optimal structures have also been identified that lead to efficient gene delivery to a variety of mammalian cells *in vitro* and to eyes and tumours *in vivo* [26,27]. Because PBAEs are comprised of degradable ester linkers on the backbone, they are hydrolysable and therefore polymer degradation may trigger intracellular

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release of the gene cargos. However, the hydrolysis of the polyester backbone occurs on the time scale of several hours to a few days, and is largely affected by the polymer structure as well as the cellular condition [22,28,29]. As such, it is unlikely to control over when and where gene release will occur. For instance, excessively rapid polymer degradation may lead to undesired pre-release of the gene cargos in the extracellular compartment to hamper their cellular internalization, while slow degradation profile up to several days may retard the intracellular release to impair the transfection efficiency. Given the drawback of such uncontrolled release mechanism, it is of great interest to precisely control the polymer degradation to allow "on-demand" cargo release at a specific intracellular process (e.g. in the cytoplasm).

To realize this goal, we herein developed trigger-responsive PBAEs containing light-responsive 2-nitrobenzene moieties in the polymer backbone. We hypothesized that these trigger-responsive PBAEs with high MWs and cationic charge densities can efficiently condense and deliver genes intracellularly; while upon external triggering at the post-transfection stage, the polymeric backbone can be rapidly degraded into small fragments such that intracellular gene release can be facilitated and material toxicity associated with high MW and charge density can be reduced. To this end, a small library of trigger-responsive PBAEs was synthesized via condensation of (2-nitro-1,3-phenylene)bis(methylene) diacrylate (NPBMDA) and various bifunctional amines. The best-performing material with optimal structure was first identified toward gene transfection, and it was further subjected to evaluation on the trigger-responsive gene delivery properties, such as polymer degradation profiles, DNA release, intracellular kinetics, gene transfection efficiency, and cytotoxicity.

2. Materials and methods

2.1. Materials and cell lines

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), dichloromethane (DCM), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves before use. Pierce BCA assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Plasmid DNA (pDNA) encoding enhanced green fluorescence protein (EGFP) (pEGFP) was purchased from Elim Biopharm (Hayward, CA, USA). Lipofectamine™ 2000 (LPF), 3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA).

HeLa (human cervix adenocarcinoma cells), COS-7 (African green monkey kidney cells), and 3T3-L1 (mouse embryonic fibroblast) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (for HeLa and COS-7 cells) or 10% bovine calf serum (for 3T3-L1 cells).

2.2. Synthesis and characterization of monomers

2-Nitro-1,3-benzenedimethanol was synthesized as illustrated in Scheme 1A. 1,3-Dimethyl-2-nitrobenzene (15.0 g, 0.10 mol) was added to a stirred NaOH basic solution (0.2 M, 800 mL) at 95 °C. $KMnO_4$ (66 g, 0.418 mol) was then slowly added, and the resulting mixture was refluxed for 24 h. The mixture was then filtered after cooling to room temperature and the filtrate was acidified with HCl to pH 1 to obtain the product 2-nitro-1,3-benzenedicarboxylic acid as white solid (11.0 g, yield 52%). ^{1}H NMR (DMSO-d_6): δ 8.17 (m, 2H, ArH), 7.79 (m, 1H, ArH).

2-Nitro-1,3-benzenedicarboxylic acid (16.0 g, 76 mmol) was dissolved in anhydrous THF (100 mL) and cooled to 4 °C in an ice bath. Borane (1.0 M in THF complex solution, 400 mL) was slowly added by syringe over 1 h under N_2, and the reaction mixture was warmed to room temperature and stirred for another 48 h. Methanol (40 mL) was then added dropwise to the reaction mixture. The mixture was filtered and dried under vacuum. The residue was re-dissolved in EtOAc and washed with saturated NaCl solution (4 × 100 mL). The organic layer was dried with anhydrous $MgSO_4$ for 12 h before the solvent was removed under vacuum. The crude product was obtained as yellow solid, which was further purified by silica gel chromatography (hexane:EtOAc as eluent, 1:1, v/v) to obtain 2-nitro-1,3-benzenedimethanol (11.0 g, yield 80%). ^{1}H NMR (DMSO-d_6): δ 7.68 (m, 3H, ArH), 5.56 (t, 2H, −O*H*), 4.70 (d, 4H, −C*H*$_2$OH).

To synthesize (2-nitro-1,3-phenylene)bis(methylene) diacrylate (NPBMDA), the designated light-responsive monomer, triethylamine (100 mmol), was added dropwise into a solution of 2-nitro-1,3-benzenedimethanol (7.3 g, 40 mmol) in anhydrous DCM (50 mL) over 1 h under N_2. Acryloyl chloride was slowly added into the reaction mixture by syringe. The mixture was stirred for 18 h at room temperature and filtered. The filtrate was dried under vacuum and the residue was then re-dissolved in EtOAc. The resulting solution was washed with saturated NaCl solution (3 × 100 mL). The organic layer was dried with anhydrous $MgSO_4$ overnight before the solvent was removed under vacuum. The crude product was obtained as yellow solid which was further purified by silica gel chromatography (hexane:EtOAc as eluent, 1:1, v/v) to obtain NPBMDA as white crystal (8.1 g, yield 70%). ^{1}H NMR (DMSO-d_6): δ 7.65 (m, 3H, ArH), 6.28 (d, 2H, −CH=C*H*$_2$), 6.12 (dd, 2H, −C*H*=CH$_2$), 5.95 (d, 2H, −CH=C*H*$_2$), 5.23 (s, 4H, ArC*H*$_2$O−).

(1,3-Phenylene)bis(methylene) diacrylate (PBMDA) as the control, non-responsive monomer was synthesized by following the same method as described above with m-tolunitrile instead of 1,3-dimethyl-2-nitrobenzene as the substrate (Scheme 2). The final product PBMDA was obtained as colourless viscous liquid (yield 56%). ^{1}H NMR (DMSO-d_6): δ 7.36 (m, 3H, ArH), 6.34 (d, 2H, −CH=C*H*$_2$), 6.20 (dd, 2H, −C*H*=CH$_2$), 5.94 (d, 2H, −CH=C*H*$_2$), 5.16 (s, 4H, ArC*H*$_2$O−).

2.3. Synthesis and characterization of PBAEs

Light-responsive PBAEs were synthesized from NPBMDA and various amine-containing compounds via Michael addition reaction (Scheme 1B and Supplementary Table S1). Briefly, NPBMDA (0.4 mmol) and each individual amine-containing molecule (amine/diacrylate molar ratio = 1.05∼1.3) were dissolved in DCM (2 mL) and the reaction was allowed to proceed at 60 °C for 4 days. After removal of the solvent under vacuum, the polymers were purified by precipitation with methanol and isolated as yellow solid or viscous liquid (yield 31∼52%). DMSO was selected as the solvent for reactions using 2-(1H-imidazol-4-yl)ethanamine and 3-(1H-imidazol-1-yl)propan-1-amine that cannot dissolve in DCM, and the obtained polymers were purified by precipitation with ether. The MWs of the obtained polymers were measured by GPC. The obtained polymers were named as Pn-m, where n (1∼16) is the specific amine type as shown in Scheme 1B and m is the MW (Da).

To enable direct comparison on the light-responsiveness of PBAEs, P17, a non-responsive analogue of P1, was synthesized from the non-responsive monomer PBMDA and 4-amino-1-butanol at the starting feed ratio of 1.08 using the same method described above (Scheme 2).

2.4. UV-Triggered polymer degradation

Polymers were dissolved in DMF at 10 mg/mL, placed in a quartz vial, and irradiated with UV light (λ = 365 nm, 20 mW/cm^2) for different periods of time. The UV–Vis spectra were recorded to monitor the photo-triggered polymer degradation and the generation of nitrosobenzene derivatives. The MWs of the UV-irradiated polymers were determined by GPC to confirm the polymer degradation.

2.5. Preparation and characterization of polyplexes

Polymers were dissolved in DMSO at 100 mg/mL and diluted with sodium acetate buffer (25 mM, pH 5.2) to 1 mg/mL. The polymer solution was then added to DNA (0.2 mg/mL) at various pre-selected weight ratios, vortexed for 30 s, and incubated at room temperature for 20 min to allow polyplex formation. The polyplexes were subjected to electrophoresis in 1% agarose gel at 100 mV for 45 min to evaluate DNA condensation by the polymers in terms of DNA migration. To quantitatively monitor the DNA condensation level, the ethidium bromide (EB) exclusion assay was adopted [14]. Briefly, polyplexes were prepared and EB solution was added at the DNA/EB ratio of 10:1 (w/w). After incubation at room temperature for 1 h, the fluorescence intensity was monitored on a microplate reader (λ_{ex} = 510 nm, λ_{em} = 590 nm). A pure EB solution and the DNA/EB solution without any polymer served as negative and positive controls, respectively. The DNA condensation efficiency (%) was defined as:

$$\text{DNA condensation efficiency}(\%) = \left(1 - \frac{F - F_{EB}}{F_0 - F_{EB}}\right) \times 100$$

F_{EB}, F, and F_0 denote the fluorescence intensity of pure EB solution, DNA/EB solution with polymer, and DNA/EB solution without any polymer, respectively. The particle size and zeta potential of freshly prepared polyplexes were also evaluated by dynamic laser scanning (DLS) on a Malvern Zetasizer (Malvern Instruments Inc., Herrenberg, Germany).

2.6. UV-Triggered polyplex dissociation and DNA release

Freshly prepared polyplexes (polymer/DNA weight ratio of 10) were irradiated by UV light (λ = 365 nm, 20 mW/cm^2) for different periods of time and thereafter subjected to particle size analysis by DLS. The heparin replacement assay was adopted to evaluate the UV-triggered DNA release from the polyplexes [30]. Briefly, heparin was added to the non-irradiated and the UV-irradiated polyplexes (polymer/DNA weight ratio of 10) solution at various final concentrations and the solutions were incubated at 37 °C for 1 h. The DNA condensation level was quantified using the EB exclusion assay as described above.