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HPMA-oligolysine copolymers for gene delivery: Optimization of peptide length and polymer molecular weight

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ABSTRACT

Polycations are one of the most frequently used classes of materials for non-viral gene transfer *in vivo*. Several studies have demonstrated a sensitive relationship between polymer structure and delivery activity. In this work, we used reverse addition-fragmentation chain transfer (RAFT) polymerization to build a panel of *N*-(2-hydroxypropyl)methacrylamide (HPMA)-oligolysine copolymers with varying peptide length and polymer molecular weight. The panel was screened for optimal DNA-binding, colloidal stability in salt, high transfection efficiency, and low cytotoxicity. Increasing polyplex stability in PBS correlated with increasing polymer molecular weight and decreasing peptide length. Copolymers containing K_5 and K_{10} oligocations transfected cultured cells with significantly higher efficiencies than copolymers of K_{15}. Four HPMA-oligolysine copolymers were identified that met the desired criteria. Polyplexes formed with these copolymers demonstrated both salt stability and transfection efficiencies on-par with poly(ethylenimine) PEI in cultured cells.

1. Introduction

Synthetic gene transfer agents that are highly effective *in vitro* are often ineffective when administered *in vivo* or need substantial modification to maintain activity [1–4]. Many cationic lipid-based formulations, called lipoplexes, are not stable in serum and most cationic polymer-based formulations, called polyplexes, require steric stabilization by polymers such as poly(ethylene glycol) (PEG) to minimize salt-induced flocculation and non-specific protein adsorption that leads to opsonization [5–8]. However, PEG-modified polyplexes generally have reduced cellular uptake and transfection efficiency when compared with unmodified polyplexes [9,10].

We recently reported synthesis of an HPMA-oligolysine copolymer composed of peptide monomers of oligo-L-lysine (K_{12}) and *N*-(2-hydroxypropyl)methacrylamide (HPMA) [11]. These polymers were shown to efficiently deliver plasmid to cultured cells while forming salt-stable polyplexes. The high critical flocculation concentration of polyplexes formed with the HPMA-oligolysine copolymer was attributed to the HPMA backbone, as this hydrophilic polymer has demonstrated steric stabilization when grafted to polycations [12,13]. We further demonstrated that HPMA-oligolysine copolymers can be synthesized using reversible addition-fragmentation transfer (RAFT) copolymerization [14].

The efficient and statistical incorporation of peptides in copolymers by RAFT polymerization raises the possibility of efficient and well-defined synthesis of multifunctional peptide-based polymers that incorporate various monomers to facilitate delivery [15–17]. However, the properties of the base material, HPMA-oligolysine copolymer, should first be optimized before including peptides for cell targeting [18,19] or endosomal escape [20–23]. Therefore, the goal of this work was to determine the optimal oligolysine peptide monomer length and molecular weight of the copolymers. In this work, a series of 12 HPMA-oligolysine copolymers was synthesized and screened for salt stability, toxicity, and transfection efficiency in two cell lines. Several polymers that efficiently packaged DNA, maintained colloidal stability in physiological conditions, and transfected HeLa and NIH/3T3 cells with low cytotoxicity were identified.

2. Materials and methods

2.1. Materials

N-(2-hydroxypropyl)methacrylamide (HPMA) was purchased from Polysciences (Warrington, PA). The initiator VA-044 was purchased from Wako Chemicals USA (Richmond, VA). All reagents used in solid phase chemical synthesis were purchased from Merck Chemicals Int. (Darmstadt, Germany) except N-succinimidyl methacrylate which was purchased from TCI America (Portland, OR). All other materials including poly(ethylenimine) (PEI, 25,000 g/mol, branched) and poly (L-lysine) (PLL, 12,000–24,000 g/mol) were reagent grade or better and

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were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Endotoxin-free plasmid pCMV-Luc (Photinus pyralis luciferase under control of the cytomegalovirus (CMV) enhancer/promoter), described previously [24], was produced with the Qiagen Plasmid Giga kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

2.2. Synthesis of HPMA-oligolysine copolymer panel

2.2.1. Synthesis of peptide monomers

Peptides of repeating L-lysine, KKKKK (K_5), KKKKKKKKKK (K_{10}), and KKKKKKKKKKKKKKK (K_{15}), were synthesized on a solid support with Rink amide linker following the standard Fmoc/tBu chemistry on an automated PS3 peptide synthesizer (Protein Technologies, Phoenix, AZ). Prior to peptide cleavage from resin, the amino terminus of the peptides was deprotected and modified with Fmoc-protected 6-aminohexanoic acid (Ahx). Subsequent Fmoc deprotection and amine coupling to N-succinimidyl methacrylate provided a methacrylamido-functionality on each peptide. These functionalized peptide monomers are respectively referred to as $MaAhxK_5$, $MaAhxK_{10}$, and $MaAhxK_{15}$. Synthesized peptides were cleaved from the resin by treating the solid support with a solution of TFA/TIPS/1,3-dimethoxybenzene (92.5:2.5:5, v/v/v) for 2 h while gently mixing. Cleaved peptide monomers were then precipitated in cold ether, dissolved in methanol and reprecipitated in cold ether. Each peptide monomer was analyzed by RP-HPLC and MALDI-TOF MS and was shown to have greater than 95% purity after cleavage. MALDI-TOF MS calculated for $MaAhxK_5$ (MH^+) 839.12, found 839.38. MALDI-TOF MS calculated for $MaAhxK_{10}$ (MH^+) 1479.98, found 1479.85. MALDI-TOF MS calculated for $MaAhxK_{15}$ (MH^+) 2120.85, found 2120.19.

2.2.2. RAFT copolymerization of HPMA and oligo-L-lysine peptide monomers

Three sets of HPMA-*co*-oligolysine copolymers were synthesized: HPMA-*co*-$MaAhxK_5$, HPMA-*co*-$MaAhxK_{10}$, and HPMA-*co*-$MaAhxK_{15}$. Each set consisted of 4 copolymers with degree of polymerization (DP), or M_o/CTA_o, of 50, 100, 150, and 190 to yield polymers with targeted molecular weights of approximately 20 kDa, 40 kDa, 60 kDa, and 80 kDa. The weight fraction of lysine in all copolymers was kept constant at 0.63 mg lysine per mg of polymer. Thus, 40 mol% of $MaAhxK_5$, 20 mol% of $MaAhxK_{10}$, and 13.33 mol% of $MaAhxK_{15}$ were used in the respective copolymerization reaction cocktails. This translated to 0.425 mmol (357.1 mg) of $MaAhxK_5$ peptide monomer and 0.638 mmol (91.37 mg) of HPMA for HPMA-*co*-$MaAhxK_5$ copolymers; 0.204 mmol (302.6 mg) of $MaAhxK_{10}$ peptide monomers and 0.818 mmol (117.1 mg) of HPMA for HPMA-*co*-$MaAhxK_{10}$ copolymers; 0.405 mmol (858.3 mg) of $MaAhxK_{15}$ peptide monomers and 2.63 mmol (376.97 mg) of HPMA for HPMA-*co*-$MaAhxK_{15}$ copolymers. Monomers were dissolved in acetate buffer (1 M, pH 5.1) and 10% ethanol such that the final monomer concentration of the solution was 0.7 M. The solutions were divided equally among 4 round bottom flasks for each set of copolymers, after which the chain transfer agent and initiator were added to each individual flask. The RAFT chain transfer reagent (CTA) used was ethyl cyanovaleric trithiocarbonate (ECT, molecular weight 263.4 g/mol) [25] and the initiator (I) used was VA-044. The molar ratios of total $monomer_o$:CTA_o:I_o at the start of polymerization were 50:1:0.1 (DP50); 100:1:0.1 (DP100); 150:1:0.1 (DP150); 190:1:0.1 (DP190). The flasks were immediately capped with a rubber septum, purged with N_2 for 10 min and then submerged in an oil bath, equilibrated at 44 °C, to initiate copolymerization. The copolymerization reactions were allowed to proceed for 24 h. The round bottom flasks were then removed from the oil bath and the polymers were extensively dialyzed against distilled H_2O to remove unreacted monomers and buffer salts. The dialyzed polymers were then lyophilized producing a white, fluffy solid. The final yields after dialysis were between 72% and 84% of the theoretical yield.

The HPMA-oligolysine copolymers are referred to using the following convention: *pHKxxDPyy*, where the prefix "*pH*" indicates an HPMA copolymer; "*Kxx*" indicates oligo-L-lysine peptide monomer with *xx* number of lysine residues, and the suffix "*DPyy*" indicates the M_o/CTA_o (DP) in the RAFT copolymerization of the HPMA-oligolysine copolymer. Also, *pHKxx* is used to refer to a set of HPMA-oligolysine copolymers with the same oligolysine peptide monomer and DP of 50, 100, 150, and 190.

2.3. Characterization of HPMA-oligolysine copolymers

2.3.1. Size exclusion chromatography

Molecular weight analysis was carried out by size exclusion chromatography. The copolymers were dissolved at 10 mg/ml in running buffer (0.15 M sodium acetate buffer, pH 4.4) for analysis by size exclusion chromatography (SEC) as described by Hennink and coworkers [14]. Analysis was carried out on an OHpak SB-804 HQ column (Shodex) in line with a miniDAWN TREOS light scattering detector (Wyatt) and a Optilab rEX refractive index detector (Wyatt). Absolute molecular weight averages (M_n and M_w), and dn/dc were calculated using ASTRA software (Wyatt). The dn/dc for each copolymer was 0.133 ml/g.

2.3.2. Amino acid analysis

The relative amount of lysine to HPMA content in the final copolymers was determined through a modified amino acid analysis following the method of Bidlingmeyer and coworkers [26]. In this procedure, hydrolyzed lysine and HPMA (which results in 1-amino-2-propanol) were derivatized with o-phthalaldehyde/β-mercaptopropionic acid and run on a Poroshell 120 EC-C18 (Agilent Technologies, Santa Clara, CA) HPLC column with precolumn derivatization to label hydrolyzed lysine and 1-amino-2-propanol. Calibration curves were generated using serial dilutions of L-lysine and reagent grade 1-amino-2-propanol.

2.4. Characterization of HPMA-oligolysine polyplexes

2.4.1. Polyplex formulation

The pCMV-Luc plasmid (endotoxin free) was diluted in double distilled H_2O (ddH_2O) to a concentration of 0.1 mg/ml and mixed with an equal volume of polymer (in ddH_2O) by adding polymer solution to DNA solution at the desired lysine to phosphate (N/P) ratio. After mixing, the polyplexes were allowed to form for 5 min at room temperature.

2.4.2. Sizing of polyplexes by dynamic light scattering (DLS)

Polyplexes (1 μg DNA, 20 μl solution, N/P = 5) were mixed with either 80 μl of double distilled H_2O, PBS, OptiMEM (Invitrogen), or serum-containing media (DMEM with 10% FBS) and then used to determine the particle size of the polyplexes by dynamic light scattering (DLS, Brookhaven Instruments Corp ZetaPLUS). Particle sizing measurements were performed at a wavelength of 659.0 nm with a detection angle of 90° at RT.

2.4.3. Determination of DNA condensation using YOYO-1 fluorescence quenching

The pCMV-Luc plasmid was mixed with the bis-intercalating dye YOYO-1 iodide (Invitrogen) at a dye/base pair ratio of 1:50 and incubated at room temperature for 1 h. Polyplexes were formed at N/P ratios of 0, 0.5, 1, 2, 3, 4, 5, 7, and 10 by complexing YOYO-1-labeled DNA with pHK05DP150, pHK10DP150, pHK15DP150, PEI, or PLL. Ten microliters (containing 0.5 μg DNA) of polyplex was added to each well of a 96-wall plate, followed by 90 μl of either ddH_2O such that final salt concentration was 150 mM. The fluorescence from each well