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Bioreducible poly(amidoamine)s as carriers for intracellular protein delivery to intestinal cells

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ABSTRACT

An effective intracellular protein delivery system was developed based on linear poly(amidoamine)s (PAAs) that form self-assembled cationic nanocomplexes with oppositely charged proteins. Two differently functionalized PAAs were synthesized by Michael-type polyaddition of 4-amino-1-butanol (ABOL) to cystamine bisacrylamide (CBA) and to bisacryloylpiperazine (BAP), yielding p(CBA-ABOL) and p(BAP-ABOL), respectively. These water-soluble PAAs efficiently condense human serum albumin (HSA) by self-assembly into stable nanoscaled and positively-charged complexes. The disulfide-containing p(CBA-ABOL)/HSA nanocomplexes exhibited high mucoadhesive properties and, while stable under neutral (extracellular) conditions, rapidly destabilized in a reductive (intracellular) environment due to the cleavage of the repetitive disulfide linkages in the CBA units of the polymer. Human-derived intestinal Caco-2/TC7 cells and HT29-MTX mucus secreting cells were exposed to these PAAs/HSA nanoparticles and the extent of their uptake and the localization within endosomal compartments were examined. The higher uptake of p(CBA-ABOL)/HSA than that of p(BAP-ABOL)/HSA suggests that the mucoadhesive properties of the p(CBA-ABOL) are beneficial to the uptake process. The transported HSA was located within early endosomes, lysosomes and the cytosol. The enhanced uptake of the p(CBA-ABOL)/HSA nanoparticles, observed in the presence of Cyclosporin A, a non-specific Multi Drug Resistance (MDR) blocker, indicates the possible efflux of these nanoparticles through MDR transporters. The results show that bioreducible PAAs have excellent properties for intracellular protein delivery, and should be applicative in oral protein delivery.

1. Introduction

Peroral drug application is considered as the most convenient and preferred route for therapeutic protein administration, especially in long-term treatment. However, this route is associated with a great number of hurdles that have to be overcome before the drug can exert its therapeutic activity. The bioavailability of therapeutic proteins via the oral administration route is very low due to (i) physical and chemical instability and fast enzymatic degradation in the gastrointestinal tract [1,2], (ii) charge repulsion of proteins from negatively-charged cell membranes and mucosa cells, and (iii) slow and ineffective transport of large size and hydrophilic proteins through compartmental cellular barriers [3,4]. Encapsulation of proteins in polymeric nanocarriers that protect the proteins during transport to their intracellular destination is a possible strategy to overcome these hurdles. A promising approach to achieve improved stability and enhanced uptake compared to the free peptides and proteins is the use of appropriate biodegradable synthetic polymers that form self-assembled nanocomplexes with the peptide or protein drugs of interest.

For efficient delivery via the intestine, the mucoadhesion of the colloidal carriers has been reported to be one of the most important properties to improve the bioavailability of poorly absorptive drugs [3–7]. Mucoadhesive carriers which adhere to the mucus layer of intestinal mucosal membranes are expected to prolong the residence time at the local site of absorption, leading to increased drug absorption through the intestinal cell layer. An interesting possibility to increase the mucoadhesive properties of a polymeric carrier is the introduction of disulfide bonds in the polymer backbone since the repetitive disulfide linkages in the polymer can react with thiol groups and disulfide groups in the mucus [8–12].

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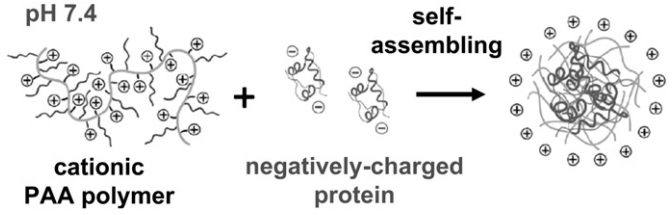
In this study we have evaluated two differently functionalized poly(amidoamine)s (PAAs), one PAA comprising repetitive disulfide linkages (SS-PAA), and one lacking disulfide linkages in their polymer backbone, for their efficacy to deliver the model protein human serum albumin (HSA) to human intestinal epithelial Caco-2/TC7 cells, and co-cultures Caco-2/TC7 and HT29-MTX mucus secreting cells. These cells are widely used in pharmaceutical research as a model for the human small intestinal mucosa to predict the uptake of orally administered drugs via the intestinal route. The PAAs have been chosen as carriers since these polymers can be synthesized with a great variety in structure by polyaddition of primary and secondary amines to bisacrylamides. These cationic polymers are water-soluble, biodegradable and have lower cytotoxicity than other usual polycationic vectors [13–16]. PAAs have shown high potential in biomedical applications [17,18], particularly for use as intracytoplasmic and endosomolytic vectors for the delivery of anticancer drugs [19,20], proteins [21–23] and nucleotides [24–28]. The tertiary amines in the main chain of the PAAs give these polymers high buffer capacity in the pH range 5.1–7.4 and this property facilitates PAA nanocomplexes once taken up by cells to escape from the endosomes by increasing polymer-membrane interaction and the proton sponge effect [13,29,30]. Consequently, degradation of the therapeutic cargo by lysosomal enzymes is prevented [31].

To evaluate the effect of the presence of repetitive disulfide groups in the main chain of SS-PAAs on the mucoadhesive criteria, we have synthesized the disulfide-containing polymer p(CBA-ABOL) and the reference polymer p(BAP-ABOL), lacking disulfide linkages, by Michael-type polyaddition of 4-amino-1-butanol (ABOL) to cystamine bisacrylamide (CBA) and to bisacryloylpiperazine (BAP), respectively. In addition, the p(CBA-ABOL) polymer is relatively stable in the extracellular medium but is prone to fast degradation in the intracellular environment due to reductive cleavage of the disulfide linkages, thereby releasing its therapeutic payload and diminishing potential cytotoxicity effects of the polymer [32–34]. By simply mixing negatively-charged HSA (pI = 5.3) and positively-charged PAA at neutral pH, self-assembled polyelectrolyte complexes (PECs) with nanosized dimensions are formed, as is schematically represented in Scheme 1. The PECs possess cationic surface charge, which makes them amenable to bind to negatively-charged cell membranes and internalize into the cells [35].

2. Materials and methods

2.1. Materials

Human serum albumin–fluorescein isothiocyanate conjugate (FITC-HSA, ~10 mol FITC per mol HSA) was obtained from Sigma. All monomers, 4-amino-1-butanol (ABOL, Aldrich), Mono-Boc-protected diaminobutane (MBDAB, Fluka), *N,N′*-cystamine bisacrylamide (CBA, Polysciences, USA), 1,4-bis(acryloyl)piperazine (BAP, Sigma–Aldrich) were purchased in the highest purity and used without further purification. HEPES (Sigma), dithiothreitol (DTT, Sigma–Aldrich), trifluoroacetic acid (TFA, Aldrich), porcine gastric mucus (Sigma), anhydrous dimethylsulfoxide (DMSO, Acros Organics), and methanol (MeOH, Biosolve) were used as received. The amine-reactive Alexa Fluor® 633 carboxylic acid, succinimidyl ester (AF633) was purchased from Invitrogen. Deionized water (DI water) was obtained from a MilliQ water purification system (Millipore, France).



Scheme 1. Self-assembling formation of nanocomplexes at neutral pH by charge attraction between a negatively-charged protein (such as human serum albumin) and a positively-charged polymer (PAA).

2.2. Synthesis of poly(amidoamine)s (PAAs)

The SS-PAA polymer, p(CBA-ABOL), was synthesized by Michael polyaddition of the primary amine monomer ABOL to CBA in equimolar monomeric ratios in MeOH/water 4/1, as described previously [27]. The PAA polymer analog lacking the disulfide moieties, p(BAP-ABOL), was synthesized similarly using BAP and ABOL as the monomers. In this case only DI water was used as the solvent [27]. The resulting polymers, collected in their HCl-salt form as white solid powder after freeze-drying, have a good solubility in water. For both polymers the yield was *ca.* 45% after ultrafiltration and lyophilization.

2.3. Synthesis of fluorescently labeled PAAs

The far-red fluorophore AF633 (λ_{em} = 647 nm) was chosen for labeling of the PAA polymers since the most common fluorescent label for detection in the red channel, Rhodamine B, is not usable as its fluorescence emission (λ_{em} = 610 nm) partly overlaps with the emission of fluorescein-HSA (λ_{em} = 525 nm) in the green channel. The AF633-labeled PAAs were prepared from copolymers having 10 mol% of the hydroxybutyl groups in the side chain substituted by aminobutyl groups. To synthesize these polymers, copolymers of (CBA-ABOL) and p(BAP-ABOL) were prepared by Michael addition of bisacrylamide (CBA or BAP, respectively) to a 9/1 mixture of amines, ABOL and MBDAB, followed by Boc-deprotection of the MBDAB units. Typically, p(CBA-ABOL/MBDAB) copolymer was synthesized by adding CBA (2.63 g, 10.75 mmol), ABOL (0.88 g, 9.67 mmol) and MBDAB (0.21 g, 1.08 mmol) into a brown reaction flask with 5 ml of a 4/1 (v/v) MeOH/DI water mixture as a solvent. The reaction mixture was allowed to proceed for 10 days at 45 °C in the dark under nitrogen atmosphere, yielding to a viscous solution. Subsequently, 10 mol% excess of ABOL (0.09 g, 0.11 mmol) was added to consume any unreacted acrylamide groups and stirring was continued for two days at 45 °C. The p(CBA-ABOL/MBDAB) polymer, containing Boc-protected amine in the side chain, was obtained in *ca.* 45% yield after isolation by exhaustive ultrafiltration (3 kg/mol cut-off) with acidified DI water (pH ~5), followed by freeze-drying. Deprotection of Boc-protected amino groups of the side chain of the polymer was performed in a mixture of TFA/MeOH overnight, yielding to the copolymers p(CBA-ABOL/DAB) with a 9/1 ratio of hydroxyl and primary amino groups in the side chains. Next, the reaction solution was diluted with DI water and adjusted to pH ~5 using a 1M NaOH solution and the resulting polymer solution was purified by ultrafiltration (3 kg/mol cut-off) with acidified DI water (pH ~5). The p(CBA-ABOL/DAB) polymer was recovered in its HCl-salt form as a white solid after lyophilization. The complete removal of the Boc protective groups was confirmed by the disappearance of the *tert*-butyl signal at 1.5 ppm in the ^{1}H NMR spectra after addition of TFA to the copolymer, yielding to the polymer with the free primary amine groups in the side chains. The primary amines were reacted with the amine-reactive AF633 carboxylic acid, succinimidyl ester, for fluorescent labeling of the polymer. In a typical example, for the synthesis of fluorescently-labeled AF633-p(CBA-ABOL), AF633 (2.5 mg, 2.08 μmol) in anhydrous DMSO was mixed with the p(CBA-ABOL/DAB) copolymer (146 mg, 41.60 μmol free NH_2) in 2 ml of sodium bicarbonate buffer (0.1 M, pH 8.3) and the solution was stirred overnight at room temperature. Then, the resultant solution was purified by ultrafiltration (1 kg/mol cut-off) with DI water. The labeled copolymer was isolated after freeze-drying. A similar experimental procedure was applied in the synthesis of AF633-p(BAP-ABOL).

2.4. Polymer characterization

The ^{1}H NMR spectra of the synthesized PAAs in D_2O were recorded on Varian Inova spectrometer operating at 300 MHz. The molecular weight and polydispersity (M_w/M_n) of the synthesized PAAs were determined by GPC relative to PEO standards (Polymer Labs) using a Viscotek GPCMax pump and autoinjector and two thermostated (30 °C) PL aquagel-OH 30 columns (8 μm, 300 × 7.5 mm, Polymer Labs, with a low-molar-mass separation range (200–40,000)). Data was collected using a TDA302 Tripledetector with RI, Visc and LS (7 and 90°). 0.3 M NaAc aqueous solution (pH 4.4) with 30% methanol was used as eluent at a flow rate of 0.7 ml/min.

2.5. Rheological studies

Rheological measurements were performed with a cone-plate (C35/28) rheometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). p(CBA-ABOL) and p(BAP-ABOL) were fully hydrated in 0.1 M phosphate buffer pH 6.8 to give a concentration of 6% (w/v). The polymer solutions were added to an equal volume of the 8% (w/v) solution of porcine gastric mucin. After an incubation period of 20 min at room temperature, the polymer–mucin incubates were transferred to the viscometer and allowed to equilibrate on the plate for 3 min at 25 °C. Dynamic oscillatory tests within the linear viscoelasticity region were performed at 1 Hz frequency. Frequency sweep measurements were also carried out with a frequency varying from 0.1 to 10 Hz. The storage modulus (G′) and the loss modulus (G″) of the disulfide-containing polymer p(CBA-ABOL) and the corresponding control polymer