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Bioresponsive poly(amidoamine)s designed for intracellular protein delivery

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ABSTRACT

Poly(amidoamine)s with bioreducible disulfide linkages in the main chain (SS-PAAs) and pH-responsive, negatively charged citraconate groups in the sidechain have been designed for effective intracellular delivery and release of proteins with a net positive charge at neutral pH. Using lysozyme as a cationic model protein these water soluble polymers efficiently self-assemble into nanocomplexes by charge attraction. At pH 5 (the endosomal pH) the amide linkages connecting the citraconate groups in the sidechains of the SS-PAAs are hydrolyzed by intramolecular catalysis, resulting in expulsion of the negative citraconate groups and formation of protonated amine groups, resulting in charge reversal of the polymeric carrier from negative to positive. The concomitant endosomal buffering effect and increased polymer–endosomal membrane interactions are considered to lead to increased protein delivery into the cytosol. Besides destabilization of the polymer–protein nanoparticles by the charge reversal effect, intracellular cleavage of disulfide linkages in the polymer ensure further unpacking of the protein in the cytosol. Cellinternalization and cytotoxicity experiments with primary human umbilical vein endothelial cells (HUVEC) showed that the SS-PAA-based nanocomplexes were essentially non-toxic, and that lysozyme is successfully internalized into HUVEC. The results indicate that these charge reversal SS-PAAs have excellent properties as non-toxic intracellular delivery systems for cationic proteins.

1. Introduction

Polymers that respond to external triggers, such as changes in the reduction potential, pH, or concentration of bioactive compounds, are of great interest for the development of carrier systems for selective and controlled drug delivery. In the application of pH-sensitive polymers as nanocarriers for intracellular delivery one of the functions can be that these polymers act as a buffer system after endosomal uptake and acidification and that their increasingly cationic charge facilitates endosomal escape by disruptive membrane interactions and/or an increase in the local osmotic pressure [1,2]. Another type of pH-sensitive polymer designed to enable polymer degradation in the endosome uses acetal, hydrazone or orthoester linkages in the backbone [3–5]. However, drug release from these systems in response to pH change is relatively slow. A more active and rapid response to a small pH decrease, such as the acidification occurring in the endosomal compartment after cellular uptake (pH decreases from ~7 to ~5), was recently obtained with polymers incorporating derivatives of cis-1,2-cyclohexanedicarboxylic anhydride or citraconic anhydride, providing specific release in early endosomes by charge reversal of the polymers [6,7].

Citraconic anhydride is an α-methyl derivative of maleic anhydride, which has been used to mask the positive charge of proteins [8–12]. After reaction of citraconic anhydride with a primary amine a citraconamide derivative is formed with a negative carboxylate group at the end. The citraconamide derivative is stable at neutral and basic pH, but it becomes unstable at acidic pH due to intramolecular catalyzed hydrolysis of the amide group, which promptly reverts back to its cationic primary amine group [13]. It was reported that the citraconamide function is almost completely hydrolyzed within less than 3 h around pH 5, which corresponds to the eventual endosomal pH [13]. The principle of charge reversal was recently exploited by Kataoka et al. [6,14–16] in the development of a block co-polymer with comb-like side groups of citraconamide, which functions as a protein nanocarrier that releases its cargo in the endosomes by repulsive electrostatic forces upon charge inversion of the polymer. The group of Park used citraconylated co-polymers for surface charge reversal of quantum dot and adenovirus nanoparticles to obtain enhanced intracellular delivery [17].

In this study we have utilized the principle of charge reversal in citraconamide functionalized poly(amidoamine)s (PAAs) to obtain

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efficient intracellular delivery of cationic proteins, using lysozyme as a model protein. PAAs can be easily synthesized by Michael-type polyaddition of primary or bis-secondary amines to bis(acrylamide)s. These polymers are water soluble, biodegradable and biocompatible, and show lower cytotoxicity than other usual polycationic vectors [1,18–20]. PAAs have the advantage of degrading to oligomeric products in aqueous media within days or weeks, depending on their structure [21,22]. These polymers have great potential in biomedical applications, as was previously shown by the groups of Ferruti and Duncan [23–25] for amphoteric PAAs carrying carboxyl groups as side substituents. PAAs have been found to be particularly suitable for use as intra-cytoplasmic and endosomolytic vectors for the delivery of anticancer drugs [26,27] and proteins [28,29]. Recently we developed novel linear PAAs containing repetitive disulfide linkages in their backbones (SS-PAAs) that worked as highly efficient intracellularly degradable protein delivery vectors. In previous work we have shown that the polycationic polymer p(CBA-ABOL), an SS-PAA polymer composed of cystamine bisacrylamide and 4-aminobutanol, efficiently condensed with negatively charged β-galactosidase into nanoscale, positively charged nanocomplexes. With these relatively stable and nontoxic nanocomplexes enzymatically active proteins could be successfully internalized into COS-7 cells [30,31]. The SS-PAAs have also been shown to be efficient vectors for gene delivery, resulting in transfection efficiencies much higher than those of their counterparts lacking disulfide linkages and the reference polymer polyethylenimine (PEI) [32–36]. Moreover, these water soluble, linear SS-PAAs are relatively stable in extracellular media but, due to the presence of the repetitive disulfide linkages, are prone to fast degradation in the reductive intracellular environment [37–40]. This property can be used in systems that need to be relatively stable during transport outside the cell but should disintegrate into fragments of low molecular weight after uptake into the target cells.

The favorable physico-chemical and biological properties of the SS-PAA polymers were further explored in this work to design and develop novel dual responsive carriers for efficient intracellular delivery of proteins that have a net positive charge at physiological pH (isoelectric point pI > 7.4). For this purpose two different SS-PAA polymers provided with charge reversible citraconamide sidechains, p(CBA-CA) and p(CBA/BAC-CA), were synthesized, together with an analogous reference polymer p(CBA-SA), possessing the relatively stable succinamide sidegroup (Fig. 1). At neutral pH charge interactions between the negative citraconic or succinic carboxylate moieties in the sidechains of the polymer and positively charged moieties in cationic proteins such as lysozyme induce the formation of nanoscale polymer/protein complexes with a net negative charge. Upon acidification of the complexes, as occurs in the endosomal compartment after cellular uptake, the citraconamide carboxylate derivatives on the sidechains become hydrolyzed with the formation of protonated amino moieties. Due to this charge reversal the complexes become destabilized, resulting in (partial) release of the protein cargo, as depicted in Scheme 1.

The different functionalities in these polymers are expected to make an important contribution to endosomal escape of the polymer and its cargo. The tertiary amino groups in the polymer backbone have pK_a values in the endosomal acidification range (pH 7.4–5.1), providing these groups with strong pH buffering properties [32–35]. Protonation of the citraconic carboxylate groups and the eventual formation of primary amino groups in the sidechains further contributes to the endosomal buffering capacity of polymers 1 and 2. The resulting proton sponge effect [1], in combination with increased interactions exerted by the newly formed positively charged ammonium groups with the negatively charged endosomal membrane, are expected to strongly promote endosomal disruption and escape of the cargo. In addition, after endosomal escape disulfide linkages present in the polymer backbone are rapidly cleaved by reductive agents in the cytoplasm (glutathione), resulting in rapid degradation of the polymer and a further decrease in protein–polymer interactions [33]. Reductive degradation of the polymer is also responsible for the low or no cytotoxicity of

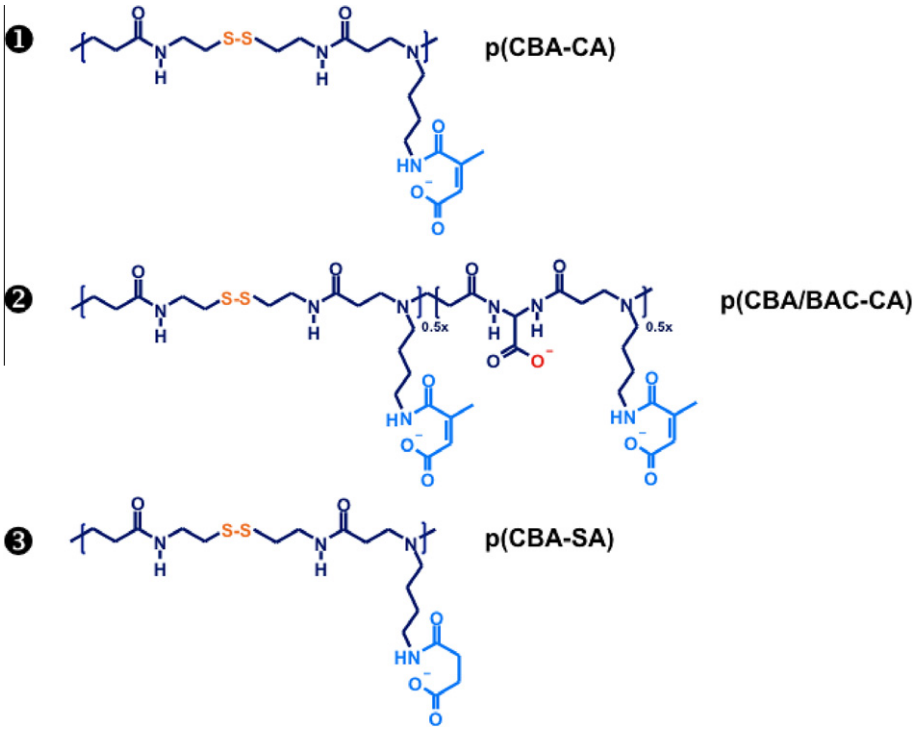

Fig. 1. Structure of the SS-PAAs. The polymers were coded in terms of the bisacrylamide and primary amine monomer used.