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Engineering biodegradable micelles of polyethylenimine-based amphiphilic block copolymers for efficient DNA and siRNA delivery

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ABSTRACT

Polycationic micelles have shown advantageous properties as nucleic acid delivery vectors both in vitro and in vivo. In contrast to polycationic micelles reported so far, we designed particles integrating a sufficient nucleic acid condensation capability by polycationic polyethylenimine (PEI) segments as well as only a mild cytotoxic behavior. The micelles composed of a hydrophobic oligoester core with glycolide units resulting in fast degradation after cellular internalization in combination with PEG moieties acting as shielding agents. By grafting branched 25 kDa polyethylenimine (PEI25) and poly(ethylene glycol) (PEG) on poly[(ε-caprolactone)-*co*-glycolide] (CG), amphiphilic PEI-CG-PEI and PEG-CG block copolymers were used to form a series of micelles via self-assembly of PEI-CG-PEI or co-assembly of both copolymers for DNA and siRNA delivery. This modular system enabled a systematic investigation of different parameters and their synergetic effects as different functions were introduced. The polyplex formation and serum stability, cytotoxicity, and transfection activity could be tailored by changing the CG chain length in PEI-based copolymer, incorporating PEG-CG, and varying the N/P ratio. All micelle-based polyplex compositions showed high DNA transfection activity according to reporter gene-expression and an exceptionally high knockdown in siRNA delivery experiments. Remarkably, the GFP expression of >99% cells was successfully knocked down by micelle-mediated siRNA interference, resulting in a decrease of two orders of magnitude in fluorescence intensity. Incorporation of PEG-CG in the micelles reduced the PEI-related cytotoxicity, and markedly enhanced the serum stability of both DNA and siRNA polyplexes. Compared with homo-PEI25, these micelles showed several advantages including the lower toxicity, higher siRNA transfection efficiency and higher polyplex stability in the presence of serum. This study therefore provides an effective approach to tune the structure, property and function of polycationic micelles for efficient DNA and siRNA delivery, which could contribute to the design and development of novel non-viral transfection vectors with superb functionality.

1. Introduction

The transfer of nucleic acids with therapeutic functions into cells to modify defined cellular processes is a promising strategy for disease treatment [1,2]. There is a demand to apply different types of nucleic acids depending on the treatment strategy, including DNA [3], siRNA [4], miRNA [5], mRNA [6], and antisense oligonucleotide [7]. However, a series of extracellular and intracellular barriers must be conquered to achieve an efficient delivery of nucleic acids to the desired intracellular sites to fulfill their functions. For example, DNA delivered via systemic administration can be rapidly degraded by nucleases in the plasma [8]. The recognition and uptake of DNA complexes by the reticuloendothelial system (RES) might lead to a rapid clearance of DNA from the circulation [9]. DNA may be susceptible to nucleases when passing through the extracellular matrix (ECM) [10]. After reaching the target cells, DNA needs to overcome several barriers for their cellular internalization, endosomal escape, cytosolic transport and nuclear entry to finally arrive in the cell nucleus [11]. Although transit across the nuclear membrane is not necessary in case of siRNA and mRNA, their intrinsic properties including hydrophilic nature, negative charge and high molecular weight still limit their permeability across biological barriers [12].

By now, various delivery techniques and carriers have been developed to enhance the transfection efficiency of nucleic acids. Notably, polyethylenimine (PEI) has been most widely applied and studied due to its high effectivity for condensing and delivering nucleic acids [11,13,14]. This complexation of PEI with nucleic acid materials could accelerate the process of polyplex endocytosis. Intracellularly, PEI with a branched structure might induce a so-called proton sponge effect, thereby triggering the rupture of endosomes and facilitating the

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endosomal escape of the delivered nucleic acid [15]. The branched PEI of 25 kDa (PEI25) has shown a superior transfection activity and has become a gold standard for gene therapy by polymeric carriers [16,17]. Similarly, high siRNA delivery capacity was reported for PEI25 compared to 22 kDa linear PEI and 800 Da branched PEI [14]. However, the relatively high toxicity of PEI, which has been attributed to both necrotic and apoptotic mechanisms resulting from cell membrane damage [18,19], is one of the major factors limiting its use, especially for in vivo applications. An effective strategy to reduce the toxicity of PEI has been demonstrated by covalent attachment of poly(ethylene glycol) (PEG) to PEI polymers [20,21]. As a nonionic hydrophilic polymer, PEG could shield the surface charge of polyplexes and reduce the intermolecular interactions by forming a hydration shell around the polyplexes, and hence decrease their toxicity. Importantly, PEGylation (i.e., PEG modification) has been shown to hinder the interaction of polyplexes with blood components. This feature is critical to enhance the serum stability of polyplexes, reduce the clearance by RES and prolong their blood circulation lifetime after intravenous administration [9,22–24].

In recent years, polymeric micelles have attracted interest due to their potential applications in nanomedicine [25–27]. They are usually formed by self-assembly of amphiphilic block- or graft-copolymers with a typical core-shell morphology. Compared to homo-polymers like PEI, polymeric micelles might offer several unique advantageous features for nucleic acid delivery such as the capacity to condense and protect the nucleic acid segment, while showing a higher colloidal stability, longer in vivo circulation time, improved cell association and internalization, enhanced transfection efficiency as well as lower toxicity [26,28]. Importantly, their physical and biological properties can be easily tuned by using multiple copolymers with different shell-forming blocks to form co-assembled micellar structures [27,29,30]. Considering potentially strong effects of micelle composition on biological processes, an enhanced penetration of (intra-)cellular barriers may be achieved by incorporation of different functions, namely in the most important aspects (i) PEI as polycation for nucleic acid condensation, (ii) hydrophobic segments to enable the formation of a micellar structure, (iii) repetitive units that serve as weak links in the hydrophobic block for intracellular degradation, and (iv) mixed micelle concepts that allow to add additional features such as PEG to reduce cytotoxicity and increase serum stability via a shielding effect.

In this context, the aim of this study was to investigate the branched PEI-based micelles with tunable composition and structure as non-viral transfection vectors. Two amphiphilic block copolymers were utilized for micelle formation: (i) triblock PEI-CG-PEI as polycationic copolymer for nucleic acid condensation synthesized by grafting branched PEI25 on poly[(ε-caprolactone)-*co*-glycolide] (CG) with different molecular weights (6 kDa (CG6) and 10 kDa (CG10)); (ii) diblock copolymer PEG-CG for micelle PEGylation, which was prepared by grafting PEG (5 kDa) on CG (5 kDa). The series of micelles were prepared via self-assembly of PEI-CG-PEI or co-assembly of both copolymers (1:1 wt%) in PBS (pH = 7.4), whereby a hydrophobic CG core providing hydrolytic degradable bonds and a hydrophilic PEI shell (or mixed shell with PEG segments to enable the shielding effect) can be formed (Scheme 1). The two PEI molecules conjugated at both ends of CG are supposed to provide high density of amino groups for nucleic acid condensation. Although the PEG-CG contains only one PEG molecule at one end of CG, the PEGylation degree could be easily controlled by varying the ratio of PEG-CG to PEI-CG-PEI. The glycolide units in the CG block (20 mol% for CG6 and CG10, 46 mol% for CG conjugated with PEG) were expected to promote the copolymer degradation. The higher amount of glycolide units in PEG-CG than in PEI-CG-PEI was expected to induce faster degradation of the CG block in PEG-CG in acidic pH of the early endosome and thereby eliminate the shielding effect from PEG after cellular internalization of the polyplexes. This might contribute to efficient transgene expression, as the wrapping of PEG chains on DNA could block the intracellular release of DNA and hence reduce the transfection efficiency [31,32]. The PEI-CG-PEI with different CG molecular weight might affect the size of initial micelles and consequently endow the micelles with different nucleic acid loading capacity as well as transfection efficiency. The optionally incorporated PEG segments could form a PEG corona on the micelle surface, leading to a micellar structure with mixed hydrophilic shell. These micelles might have different condensation capacity for nucleic acids with respect to the polyplex size and zeta potential, both of which are critical for cellular binding of polyplexes and internalization via endocytosis [33–36]. Importantly, these PEG-containing micelles were expected to have reduced cytotoxicity and enhanced serum stability with high potential for in vivo applications. By using such a modular system, we were able to systematically investigate multiple parameters that are critical for micelle-mediated nucleic acid delivery including the chain length of hydrophobic segment, incorporation of shielding molecules, copolymer biodegradability and their synergetic effects on micelle characteristics, nucleic acid condensation, cytotoxicity, serum stability and transfection efficiency. In addition, as we used branched PEI25 for the hydrophilic segments of the cationic copolymers, we could compare these micelles with branched PEI25 gold standard to study the influence of micellar structure on transfection. Even more, we speculated that the transfection activity of the micelle-based polyplexes could be optimized by varying these parameters. Instructive knowledge could be gained from this modular system, with high potential for design and development of safe and efficient polymeric gene transfer agents for both in vitro and in vivo applications. Therefore, the prepared micelles were evaluated with respect to their condensation capacity, cytotoxicity, transfection efficiency for both DNA and siRNA as well as the stability of the polyplexes in the presence of serum.

2. Materials and methods

2.1. Synthesis of block copolymers and formation of polymeric micelles

The triblock PEI-CG-PEI copolymers were synthesized in a three step procedure. The hydrophobic CG oligoester was obtained by ring-opening polymerization of ε-caprolactone and diglycolide using octanediol as initiator. The resulting hydroxyl end groups were functionalized with succinic anhydride to create carboxylic acid moieties, which were subsequently modified with branched PEI25. The diblock copolymer PEG-CG was achieved by ring-opening polymerization of ε-caprolactone and diglycolide initiated by monohydroxy PEG-OH.

Polymeric micelles were prepared by dissolving PEI-CG-PEI copolymers (or mixture of PEI-CG-PEI and PEG-CG) in DMSO and the resulting solution was added dropwise to the PBS solution to enable the formation of micelles by self-assembly. DMSO was removed by dialysis with PBS buffer. The sizes of micelles ranged between 19 ± 1 nm and 43 ± 2 nm as detected by Dynamic Light Scattering (DLS). The zeta potential was between 11.4 ± 0.3 mV and 17.8 ± 0.9 mV.

2.2. Amplification and purification of plasmid DNA

Luciferase plasmid (pcDNA3.1-Luc, Invitrogen, Carlsbad, CA, USA) was transformed into *Escherichia coli* DH5α strain and propagated in selective Luria-Bertani medium at 37 °C by shaking overnight at 200 rpm. The purification of amplified plasmid DNA was performed using the plasmid DNA purification kit (Macherey-Nagel, Düren, Germany). The purified plasmid DNA was dissolved in Tris-EDTA (TE) buffer and concentrated in a lyophilizer. The concentration and purity of plasmid were determined by loading the DNA onto the NanoQuant plate followed by measuring the ultraviolet (UV) absorbance at 260 and 280 nm with a microplate reader (Infinite 200 PRO®, Tecan Group Ltd., Mannedorf, Switzerland).