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A family of cationic polyamides for *in vitro* and *in vivo* gene transfection

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ABSTRACT

The purpose of this study is to develop biodegradable cationic polyamides for non-viral gene delivery and elucidate their structural effects on gene transfection activity. To this end, a group of novel cationic polyamides were synthesized by polycondensation reaction between different di-*p*-nitrophenyl esters and tertiary amine-containing primary diamines. These linear polyamides have flexible alkylene group (ethylene or propylene), protonable amino group and bioreducible disulfide linkage in the polyamide main chain. The alkylene group and disulfide linkage in these polyamides have a distinct effect on their gene delivery properties including buffering capacity, gene binding ability and intracellular gene release profile. Those cationic polyamides containing disulfide linkage and 1,4-bis(3-aminopropyl)piperazine (BAP) residue exhibited high buffering capacity (endosomal escape ability), high gene binding ability, and intracellular gene release ability, thus inducing fast gene nucleus translocation and robust gene transfection *in vitro* against different cell lines and rat bone marrow mesenchymal stem cells. Moreover, the transfection efficiencies *in vitro* were comparable or higher than those of 25 kDa branched polyethylenimine and Lipofectamine 2000 transfection agent as positive controls. These cationic polyamides and their polyplexes were of low cytotoxicity when an optimal transfection efficacy was achieved. *In vivo* transfection tests showed that bioreducible BAP-based polyamides were applicable for intravenous gene delivery in a mouse model, leading to higher level of transgene expression in the liver as compared to 22 kDa linear polyethylenimine as a positive control. These cationic polyamides provide a useful platform to elucidate the relationship between chemical functionalities and gene transfection activity.

1. Introduction

Gene therapy has been considered as an innovative method for the treatment of human being diseases such as cancer [1]. However, for successful gene therapy, the availability of low-toxic and highly efficient gene delivery vectors remains a huge challenge [2]. Over the past two decades, much effort has been made to develop functionalized cationic polymers as non-viral gene delivery vectors [3]. Compared to potent viral vectors [4], cationic polymers gain additional advantages including low immunogenicity, non-oncogenicity, relatively high gene-carrying capacity, large-scale production and low cost. Thus, a large number of cationic polymers such as 25 kDa branched polyethylenimine (BPEI) and poly(amido amine)s have been studied as polymeric gene delivery vectors recently [5]. These polymers can bind gene to form nanosized polyplexes and mediate their endosomal escape, thereby inducing detectable *in vitro* and *in vivo* transfection efficiency. Typically, 25 kDa BPEI has been regarded as one of the most potent polymeric gene carriers *in vitro*, being a gold standard when assessing *in vitro* transfection ability of cationic polymers. Although linear polyethylenimine (LPEI) has been evaluated in clinical gene therapy trails, most of cationic polymer systems reported have not been advanced for clinical trials mainly because of their relatively low transfection ability.

In order to develop cationic polymers with stronger transfection ability than 25 kDa BPEI, over the past decade a lot of researches have been focused on the development of cationic polymers with different chemical functionalities, thereby optimizing gene delivery properties and obtaining enhanced transfection efficacy [6]. A typical work is the library of poly(amino ester)s prepared by Michael-type addition reaction [7]. By screening the poly(amino ester)s with different functional side groups, aminobutanol was found as a hit structure which offered cationic polyesters with superior *in vitro* gene transfection activity to 25 kDa BPEI [8,9]. In the other work [10], poly(amido amine)s with the aminobutanol

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side group were also able to induce higher transfection efficacy than the BPEI in COS-7 cells. Furthermore, the transfection efficacy of these cationic polymers like poly(amido amine)s can be markedly augmented by integrating disulfide linkage into aliphatic segment because disulfide-based (bioreducible) poly(amido amine)s may facilitate intracellular gene release by the cleavage of disulfide bond inside the cytoplasm and nucleus which typically maintain a highly reducing condition [9,11]. Moreover, the biodegradability of bioreducible cationic polymers inside the cells endows their lower cytotoxicity compared to their counterparts without disulfide linkage. In addition to aminobutanol and disulfide linkage, other hit functionalities used for the design of potent polymeric gene delivery vectors largely encompass oligoamine, low-molecular weight BPEI and imidazole group [12]. For example, bioreducible BPEIs obtained by disulfide-crosslinking of low-molecular weight BPEI were found to be highly potent for *in vitro* gene delivery followed by low cytotoxicity [13,14]. Bioreducible poly(amido amine)s with imidazole side chain displayed superior transfection activity to 25 kDa BPEI [15,16]. However, these hit functionalities reported to date are regularly applied as side groups for the design of efficient polymeric gene delivery vectors [17,18]. It is not yet elucidated as to the effect of chemical functionalities in polymeric main chain on gene transfection activity [18]. This context may be one major reason why most of current polymeric gene delivery vectors usually possess similar transfection ability with 25 kDa BPEI but much lower ability than viral vectors. Thus, there is a fundamental need to develop cationic polymers with flexible structures in polymeric main chain, thereby reaching new hit structures for enhanced transfection efficacy.

We are interested in the design of cationic polyamides as non-viral gene delivery vectors because polyamides are peptidomimetic materials with good biocompatibility. Engbersen and we previously presented a lot of poly(amide amine)s for non-viral gene delivery *in vitro* [19–21]. This polyamide system offers a useful platform to understand the effect of polymeric side groups on gene transfection activity. As a result, aminobutanol or imidazole as the side group was identified as a hit functional group and corresponding poly(amido amine)s thus caused superior transfection activity to BPEI against cell lines. However, a clear relationship between chemical functionalities in polymeric main chain and transfection activity is not thoroughly elucidated. In this work, a group of new cationic polyamides were synthesized by stepwise polycondensation reaction between di-*p*-nitrophenyl esters and tertiary amino-containing primary diamines (Scheme 1). These cationic polyamides are designed with different alkylene (ethylene/propylene) group, protonable tertiary amine and disulfide linkage in polymeric main chain. We hypothesized that a hit functional group could be identified from these new cationic polyamides. Herein, we describe the synthesis and characterization of these polyamides and evaluation on their gene delivery properties such as buffering capacity, gene binding ability, endocytic pathway and intracellular gene release behavior. Structural effects of these cationic polyamides on their gene transfection activity were investigated in different cells. Moreover, *in vivo* gene transfection of these polyamides was evaluated by intravenous injection of their polyplexes in a mouse model.

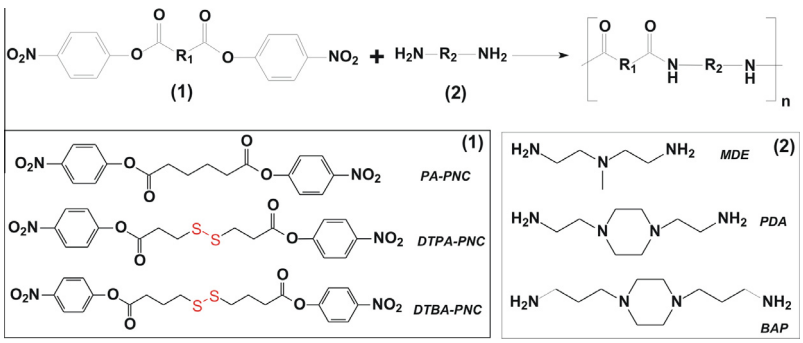
2. Materials and methods

2.1. Materials

All the reagents were used without further purification. 3, 3′-dithiodipropionic acid (DTPA), 4, 4′-dithiodibutyric acid (DTBA), *p*-nitrophenol, dicyclohexylcarbodiimide (DCC), 1,4-bis(3-aminopropyl)piperazine (BAP), pyridine, 4-dimethylamino pyridine, adipic acid, dichloride methylene (DCM), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), rhodamine B isocyanate, dithiothreitol (DTT), branched polyethylenimine (BPEI, M_w = 25 kDa) were purchased from Sigma–Aldrich. Piperazine-1, 4-diethylamine (PDA), N-methyl-2, 2′-diaminodiethylamine (MDE), wortmannin, chlorpromazine, methyl β-cyclodextrin (mCD) and genistein were ordered from J&K. Linear polyethylenimine (LPEI, M_w = 22 kDa) was ordered from Polysciences (USA). DMEM, Mccoy's-5α, RPMI1640, α-MEM, LysoTracker Green DND26 and penicillin/streptomycin were ordered from Life Technology (USA). The plasmids, pCMV-GFP and pCMV-Luc, encoding green fluorescent protein (GFP) and Luciferase (Luc), respectively, under the control of a SV40-CMV promoter, were purchased from Plasmid Factory (Germany). The chemicals, di-*p*-nitrophenyl-3, 3'-dithiodipropanoate (DTPA–PNC) and di-*p*-nitrophenyl adipate (PA–PNC) were obtained according to the reference [22].

2.2. Synthesis of di-p-nitrophenyl-4, 4'-dithiodibutanoate (DTBA–PNC)

Di-*p*-nitrophenyl-4, 4′-dithiodibutanoate (DTBA–PNC) was synthesized by the conjugation of 4, 4'-dithiodibutyric acid and *p*-nitrophenol in the presence of dicyclohexylcarbodiimide (DCC). As a typical experiment, DTBA (1.0 g, 4.2 mmol), 4-dimethylamino pyridine (122 mg, 0.92 mmol), DCC (1.91 mg, 9.23 mmol) and *p*-nitrophenol (1.28 g, 9.23 mmol) were added into a round-bottom flask and suspended in DCM (50 mL). After stirring for 48 h, the solid (urea) was removed by vacuum filtration. The organic phase was washed with an aqueous solution of sodium bicarbonate (4 × 25 mL), saturated sodium chloride (50 mL), and finally dried with anhydrous sodium sulfate. The crude oil was obtained by rotary evaporation and purified in a silica (#60741, Sigma) chromatography with DCM as a mobile phase, yielding orange oil (2.0 g). After re-crystallization in diethyl ether, DTBA–PNC was obtained as yellow solid (1.1 g). ^{1}H NMR (CCl_3): δ 2.18



Scheme 1. Synthesis of linear cationic polyamides by stepwise condensation reaction between di-*p*-nitrophenyl esters and primary diamines.