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Synthesis of intracellular reduction-sensitive amphiphilic polyethyleneimine and poly(ε-caprolactone) graft copolymer for on-demand release of doxorubicin and p53 plasmid DNA

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ABSTRACT

This study aims to present a new intelligent polymeric nano-system used for combining chemotherapy with non-viral gene therapy against human cancers. An amphiphilic copolymer synthesized through the conjugation of low molecular weight polyethyleneimine (LMw-PEI) and poly(ε-caprolactone) (PCL) via a bio-cleavable disulfide linkage was successfully employed for the simultaneous delivery of drug and gene molecules into target cells. Compared to the conventional PCL copolymerization pathway, this paper represents a straightforward and efficient reaction pathway including the activation of PCL-diol hydroxyl end groups, cystamine attachment and LMw-PEI conjugation which are successfully performed at mild conditions as confirmed by FTIR and ^{1}H NMR. Thermal, morphological characteristics as well as biocompatibility of the copolymer were investigated. The copolymer showed great tendency to form positively charged nanoparticles (~163.1 nm, +35.3 mV) with hydrophobic core and hydrophilic shell compartments implicating its potential for encapsulation of anti-cancer drug and plasmid DNA, respectively. The gel retardation assay confirmed that the nanoparticles could successfully inhibit the migration of pDNA at ~5 nanoparticle/pDNA w/w. The in vitro cytotoxicity tests and LDH assay revealed that the cationic amphiphilic copolymer was essentially non-toxic in different carcinoma cell lines in contrast to branched PEI 25K. Moreover, the presence of redox sensitive disulfide linkages provided smart nanoparticles with on-demand release behavior in response to reducing agents such as cytoplasmic glutathione (GSH). Importantly, confocal microscopy images revealed that in contrast to free Dox, the nanoparticles were capable of faster internalizing into the cells and accumulating in the perinuclear region or even in the nucleus. Finally, the co-delivery of Dox and p53-pDNA using the copolymer displayed greater cytotoxic effect compared with the Dox-loaded nanoparticle counterpart as revealed by cell viability and Caspase 3 expression assay. These results suggest the copolymer as a promising candidate for the development of smart delivery systems.

Statement of Significance

We employed cystamine dihydrochloride as a disulfide linkage for the conjugation of PCL diol and low molecular weight PEI segments through a straightforward and efficient reaction pathway at mild conditions. The new copolymer was essentially non-toxic in different carcinoma cell lines and showed great tendency to form positively charged nanoparticles. Therefore, it can be utilized as a promising platform for simultaneous drug and gene delivery to aggressive cancers. The results of drug and gene co-delivery experiments confirmed the pivotal role of disulfide linkage on the controlled release of both drug and gene molecules in response to glutathione concentration gradient between extracellular and intracellular microenvironments. In addition, the co-delivery of doxorubicin and p53 plasmid DNA using the new copolymer displayed greater cytotoxic effect compared with single agent (i.e. Dox) loaded counterpart, which indicated the significance of rapid dissociation of therapeutic agents from the carrier for synergistic cytotoxic effects on cancer cells.

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1. Introduction

The broad field of gene delivery holds great potential for the treatment of severe human diseases such as cancers [1,2], cardiovascular diseases [3,4], neurodegenerative disorders [5–7], and infectious diseases [8,9] in either preclinical or clinical trials [10–14]. Extensive research have be conducted for the design and synthesis of safe and effective non-viral vectors that could not only strive against viral counterparts in the level of gene expression and specificity, but also provide greater gene size flexibility and reduce or entirely eradicate acquired immune responses. Recently, the co-delivery of anti-cancer agents has been recognized as a promising strategy for the treatment of cancers with remarkable resistance to conventional therapies [12]. Such co-delivery can enhance treatment outcomes and mitigate the adverse side effects of cytotoxic drugs at high doses. Anticancer drugs such as paclitaxel (PTX) and doxorubicin (Dox) have demonstrated great promise to achieve greater cytotoxic effect via co-delivering with bio-active agents such as therapeutic plasmid DNA (pDNA) [12–14] and siRNA [15,16]. However, despite the successful fabrication of core-shell nanoparticles for drug and gene co-delivery [17,18], simultaneous and on-demand release of agents in cancer cells is still one of the main challenges of systemic cancer therapy.

The p53 protein is a powerful anti-tumor molecule, which plays a central role in the regulation of cellular pathways, leading to induction of cell cycle arrest, apoptosis, DNA repair, and senescence [19–22]. As demonstrated by previous studies, activated p53 is noxious to the development of cancers [23]. However, p53 is one of the most frequently mutated or deleted transcriptional factors in human tumors. In fact, approximately 50% of all human tumors show alterations in the p53 gene, which directly affects its performance within cells [21,24]. Therefore, the p53 delivery may increase the sensitivity of tumor cells to chemotherapeutic agents such as doxorubicin by providing fresh and healthy proteins for cells.

To date, developing an efficient and stable gene carrier is the major obstacle to cancer gene therapy. Therefore, many efforts have been devoted to improve the stability of genomic compounds upon administration into patient's body, improve pharmacokinetics as well as bio-distribution, enhance cell permeability within target tissue, and finally deliver the genes, either pDNA or small interfering RNA (siRNA), to specific subcellular locations [25]. Despite the great potential of cationic polymers such as PEI 25K in the encapsulation of genes, their inherent cytotoxicity attributed to their strong but destructive interaction with cell membrane has confined their practical applications for systemic gene therapy. Although low molecular weight polyethyleneimine (LMw-PEI) shows less toxic side effects compared to PEI 25K, the lower transfection efficiency has significantly delimited its applications for gene delivery. Two approaches have been proposed to address this issue: (i) crosslinking LMw-PEI by a stimuli-responsive linkage to form a high molecular weight bioconjugate [26,27]; (ii) grafting LMw-PEI to a biocompatible and biodegradable polymer chain to form a star-shaped nano carrier [27,28]. The conjugation of a hydrophobic polymer chain to LMw-PEI provides a dense hydrophobic nanostructure with lower toxicity, which can also be employed for the encapsulation of chemotherapeutic agents while the hydrophilic LMw-PEI heads formed the star-shaped spatial structure that enabled condensation of pDNA.

Over the past couple of decades, nanoparticulate systems, which provide a stimuli-responsive platform for the incorporation of multiple drugs, have gained considerable attention in the biomedical domain, particularly in drug delivery. These novel carriers express stimuli-responsive behavior to either tumor microenvironment triggers such as low pH, high redox tripeptide and enzyme concentrations and hyperthermia, or external stimuli such as magnetic field, ultrasound and radiation (e.g. Ultraviolet (UV), visible or near-infra red) [29–32]. According to this concept, the incorporation of cleavable linkage such as a covalent disulfide bond (—S—S—) to a biodegradable copolymer may result in the development of a sophisticated delivery system [31,33,34]. The resulting reduction-sensitive biodegradable copolymer is relatively oxidative (i.e. stable) in body fluids (e.g. blood, plasma) and in extracellular space; however, it undergoes rapid cleavage in a reductive environment inside cells [35–37]. The intracellular cleavage reaction is attributed to a considerable difference in the concentration of the abundant cellular free thiols, including glutathione tripeptide (γ-glutamyl-cysteinyl-glycine, GSH), between intracellular (~1–10 mM) [38] and extracellular (~20–40 μM) [39] milieu. Interestingly, the concentrations of reducing agents such as GSH are significantly higher (~4-fold) at tumor sites compared to normal tissues [40], which make it a versatile target for the use of disulfide-linked copolymers as efficient delivery vectors.

Based on the above considerations, we hypothesized that a block copolymer with hydrophobic and hydrophilic segments could successfully form nanoparticles to encapsulate drug and nucleic acid (pDNA), concomitantly (Scheme 1). Therefore, polycaprolactone-diol (PCL-diol) which is a chemo-synthetic, biocompatible and resorbable aliphatic polyester with a semi-crystalline structure was chosen to link to LMw-PEI through a disulfide-linker. However, compared to the conventional PCL copolymerization pathway (i.e. ring opening copolymerization, protection and deprotection of reactants and tedious acidification, basification and extraction steps [36,41,42]), we have suggested a straightforward and efficient reaction pathway including the activation of PCL-diol hydroxyl end groups, cystamine attachment and LMw-PEI conjugation which are successfully performed at mild conditions. 1,1′-Carbonyldiimidazole (CDI) was employed as a hydroxyl activating agent for the copolymerization of PCL-diol. The new synthesized block copolymer was characterized to confirm the progress of conjugation reactions as well as the chemical structure of the final product. The ability of the copolymer to form nanoparticles, encapsulate the anti-cancer drug and facilitate condensation of pDNA was subsequently investigated. In addition, the copolymer cytotoxicity was examined against three different cell lines.

2. Materials and methods

2.1. Materials

PCL-diol-2000, low molecular weight branched PEI (Mn ~ 1200, Mw ~ 1300), cystamine dihydrochloride (Cys·2HCl) (96%), succinic anhydride (⩾99% (GC)) and 1,1′-carbonyldiimidazole (CDI) (⩾97.0% (T)) were purchase from Sigma-Aldrich (Singapore). All other reagents and solvents were of analytical grade and used as received.

2.2. Synthesis procedures

The intermediates and the final products were synthesized as follows (Scheme 2).

2.2.1. Activation of PCL-diol hydroxyl groups

For activating the two hydroxyl (—OH) groups of PCL-diol, a solid state mixture of (1.0 g, 0.5 mmol) and 1,1′-carbonyldiimidazole (810.75 mg, 5 mmol) was dried under vacuum at 45 °C overnight, and subsequently was dissolved in 10 ml anhydrous dichloromethane. The reaction was carried out under nitrogen protection at room temperature for 24 h. The product (PCL-CDI) was concentrated and precipitated in 10-fold excess cold