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The highly efficient delivery of exogenous proteins into cells mediated by biodegradable chimaeric polymersomes

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ABSTRACT

Biodegradable chimaeric polymersomes based on asymmetric PEG-PCL-PDEA triblock copolymers were prepared and investigated for delivery of exogenous proteins into cells. PEG-PCL-PDEA copolymers with $M_{n\ PEG} = 5$ kg/mol, $M_{n\ PCL} = 18.2$ kg/mol, and short PDEA blocks ranging from 1.1, 2.7 to 4.1 kg/mol (denoted as copolymer **1**, **2** and **3**, respectively) were obtained by controlled reversible addition-fragmentation chain transfer (RAFT) polymerization. The direct hydration of copolymer thin films in MES buffer (pH 5.3) yielded uniform polymersomes with sizes of 130–175 nm. These polymersomes had close to neutral zeta potentials (−2 ∼ +2.7 mV) at pH 7.4. The polymersomal structures were confirmed by confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), and catalytic activity experiment on 3,3′,3″-phosphinidyne(trisbenzenesulfonic acid)-loaded polymersomes. MTT assays showed that these polymersomes were non-toxic up to a concentration of 0.5 mg/mL. These chimaeric polymersomes, in particular polymersome **2**, showed remarkably high protein loading efficiencies and loading contents for bovine serum albumin (BSA), cytochrome C (CC), lysozyme (Lys), ovalbumin (OVA) and immunoglobulin G (IgG). The encapsulation of proteins did not significantly alter the polymersome size distributions and zeta potentials. The protein release studies showed that both BSA and CC were released in a controlled manner. Importantly, the released CC fully maintained its activity. Notably, CLSM studies showed that FITC-CC loaded polymersomes efficiently delivered and released proteins into the cytoplasm of RAW 264.7 cells. Moreover, these chimaeric polymersomes were able to simultaneously load and transport proteins and doxorubicin into the cytoplasm as well as the cell nucleus. We are convinced that these biodegradable chimaeric polymersomes have great potentials in protein therapy.

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1. Introduction

Protein drugs have emerged as potent medicines for various types of human diseases such as autoimmune diseases, certain cardiovascular and metabolic disorders, and cancers [1–4]. To elicit therapeutic effects, many proteins (e.g. BAX, cytochrome C, caspase-3, etc.) have to be directed to a proper cellular compartment like the cytoplasm [5,6]. Protein-based biotherapeutics encounter several challenges such as rapid elimination from the circulation, poor bioavailability, low cell permeability, and inefficient endosomal escape [7]. The clinical success of protein drugs is critically dependent on the advancement of non-toxic, efficient and economically viable delivery systems. In the past decades, hydrogels [8,9], microparticles [10,11], liposomes [12], polyion complex micelles [13], and carbon nanotubes [14] have been investigated as carriers for controlled protein delivery. These delivery approaches, however, require the use of organic solvents which may possibly lead to protein denaturation, involve chemical modifications of proteins, have low protein loading levels, and/or are not applicable for systemic applications.

Polymersomes are polymeric vesicles made of amphiphilic block copolymers [15–18]. Like liposomes, polymersomes contain an aqueous interior that is separated from the outer fluids by hydrophobic membranes. In the past decade, polymersomes, either degradable or non-degradable, with better stability and easier to modify as compared to liposomes have been explored for programmed delivery of hydrophobic and hydrophilic drugs [19,20]. Notably, several biodegradable polymersomes have been developed [21–24]. Recently, several groups have studied the encapsulation of proteins including myoglobin and TNF-α into polymersomes

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[25–27]. However, due to inefficient loading the protein contents were low.

In this paper, we report on biodegradable chimaeric polymersomes based on asymmetric poly(ethylene glycol)-*b*-poly(ϵ-caprolactone)-*b*-poly(2-(diethylamino) ethyl methacrylate) (PEG-PCL-PDEA) triblock copolymers for highly efficient encapsulation and delivery of exogenous proteins into cells (Scheme 1). These chimaeric polymersomes were designed on the basis of the following reasons: (1) the PEG block ($M_n = 5$ kg/mol), which is longer than the PDEA block ($M_n = 1.1$–4.1 kg/mol), will be preferentially oriented at the polymersome outlayer, thereby offering excellent biocompatibility and stability in the circulation; (2) the shorter cationic PDEA block will be preferentially located inside the polymersomes, which on one hand facilitates efficient encapsulation and stabilization of proteins and on the other hand may assist polymersomes escaping from endosomes via the "proton sponge effect" [28], resulting in efficient cytoplasmic delivery of proteins; (3) the hydrophobic PCL block is non-toxic and biodegradable; and (4) these chimaeric polymersomes may be capable of simultaneously delivering proteins and hydrophobic anticancer drugs such as doxorubicin (DOX). The combination cancer therapy has shown tremendous potentials in cancer treatment [29–32].

2. Materials and methods

2.1. Materials

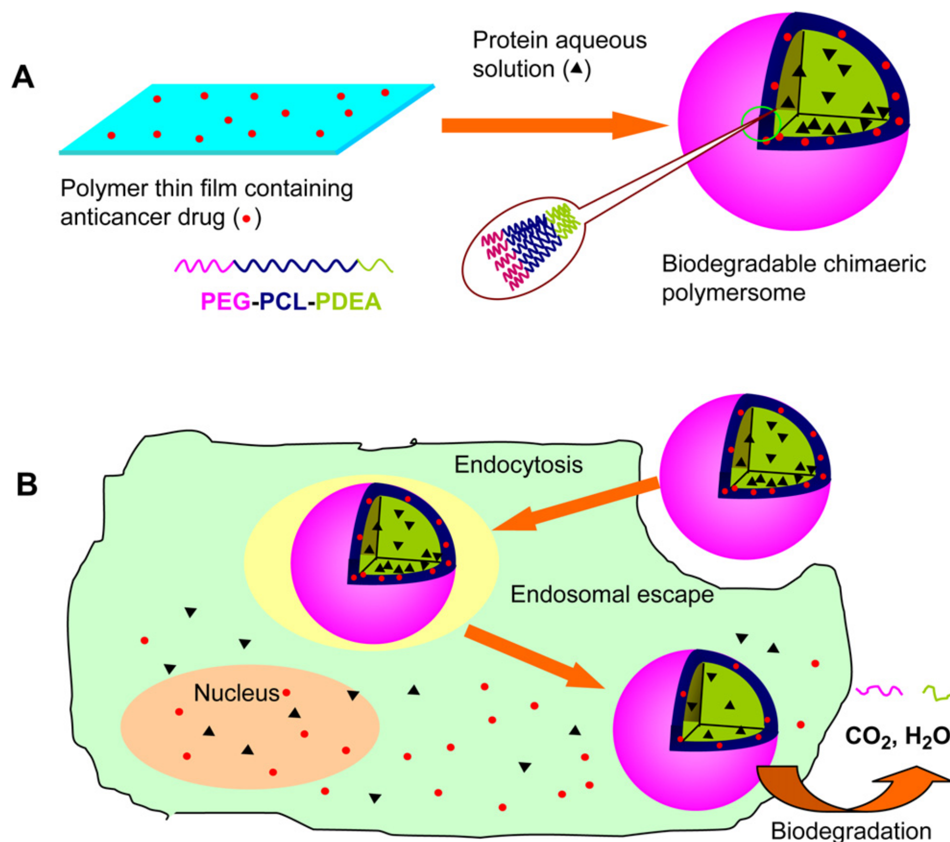
2-(Diethylamino)ethyl methacrylate) (DEA, 99%, Aldrich) was purified by passing through a basic alumina column. Poly(ethylene glycol) monomethyl ether (CH_3O-PEG, $M_n = 5000$, Fluka) was dried by azeotropic distillation from dry toluene. Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from methanol. ϵ-Caprolactone (ϵ-CL, 99%, Alfa Aesar) and dioxane were dried over CaH_2 and distilled under reduced pressure prior to use. Toluene and tetrahydrofuran (THF) were dried by refluxing over sodium wire and distilled prior to use. Stannous octoate (95%, Sigma), nile red (99%, Sigma), fluorescein isothiocyanate (95%, Fluka), pyrene (97%, Fluka), cytochrome C from equine heart (Sigma), bovine serum albumin V fraction (>98%, Roche), ovalbumin (Sigma), γ-IgG from bovine (Sigma), lysozyme (Amresco), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS, Amresco), 3,3′,3″-phosphinidynetris-benzenesulfonic acid (PH, 95%, J&K), 5,5′-dithiobis(2-nitrobenzonic acid) (DTNB, 99%, Alfa Aesar), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), 4-(dimethylamino) pyridine (DMAP, 99%, Alfa Aesar) and doxorubicin hydrochloride (99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.) were used as received. 4-cyanopentanoic acid dithiobenzoate (CPADN) was synthesized according to literature [33].

2.2. Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using $CDCl_3$ or D_2O as solvent. The chemical shifts were calibrated against residual solvent signals of $CDCl_3$ or D_2O. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of polymersomes was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. The zeta potential of the polymersomes was determined with a Zetasizer Nano-ZS from Malvern Instruments. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg/mL polymersome suspension on the copper grid followed by staining with phosphotungstic acid (1 wt.%).

2.3. Synthesis of PEG-PCL-CPADN

The macro-RAFT agent was synthesized by coupling 4-cyanopentanoic acid dithiobenzoate (CPADN) to PEG-PCL-OH. PEG-PCL-OH was prepared by ring-opening polymerization of ϵ-caprolactone (3.79 g, 0.026 mol) using monomethoxy



Scheme 1. Schematic presentation of biodegradable chimaeric polymersomes. (A) PEG-PCL-PDEA forms nano-sized polymersomes directly in aqueous solution via the film rehydration method; both proteins and DOX can be readily loaded into polymersomes with high loading efficiency; (B) these polymersomes after being taken up by cancer cells, can escape from endosomes through the buffer effect of PDEA; proteins and DOX are released into the cytoplasm and/or cell nucleus.

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