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A poly(L-lysine)-based hydrophilic star block co-polymer as a protein nanocarrier with facile encapsulation and pH-responsive release

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A B S T R A C T

A hydrophilic star block co-polymer was synthesized, characterized, and evaluated as a protein nanocarrier. The star block co-polymer was composed of a hyperbranched polyethylenimine (PEI) core, a poly(L-lysine) (PLL) inner shell, and a poly(ethylene glycol) (PEG) outer shell. The model protein insulin can be rapidly and efficiently encapsulated by the synthesized polymer in aqueous phosphate buffer at physiological pH. Complexation between PEI–PLL–*b*-PEG and insulin was investigated using native polyacrylamide gel electrophoresis. The uptake of enhanced green fluorescent protein into Ad293 cells mediated by PEI–PLL–*b*-PEG was also investigated. The encapsulated insulin demonstrated sustained release at physiological pH and showed accelerated release when the pH was decreased. The insulin released from the star block co-polymer retained its chemical integrity and immunogenicity.

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1. Introduction

Protein drugs have emerged as potent medicines for various types of human diseases and have drawn significant interest due to their high specificity and activity at relatively low concentrations in comparison with small chemical drugs [1]. However, the use of protein-based biotherapeutics faces several challenges, such as their rapid elimination from the circulatory system, poor bioavailability, low cell permeability, and inefficient endosomal escape. The future success of proteins as therapeutic agents is critically dependent on the development of appropriate delivery systems. An ideal protein carrier should have a reasonably high protein encapsulation efficiency and loading capacity, and sustained release of the loaded protein while retaining bioactivity. In recent decades hydrogels [2–5], micro- and nano-particles [6–10], liposomes [11], polymersomes [12,13], polyelectrolyte microcapsules [14–17], and polyion complex micelles [18,19] have been investigated as carriers for controlled protein delivery. While many of these delivery approaches have shown the advantages of high loading capacities and stimuli-responsive release properties, some of them result in disassembly of the carriers under dilute conditions and/or require the use of organic solvents that may lead to protein denaturation. For example, the liposomal system is mechanically unstable in the infinitely dilute environment encountered after introduction into the bloodstream; disruption of the micellar structure leads to burst release of the entrapped protein molecules. The use of hydrophobic polymers and organic solvents involved in the microencapsulation procedure (e.g. the double emulsion–solvent extraction/evaporation method) in microparticle carrier systems frequently leads to the denaturation of proteins. A facile encapsulation of proteins under hydrophilic conditions with retained physical and chemical stability remains largely unexplored.

Here we report a hydrophilic star block co-polymer (PEI–PLL–*b*-PEG) with a hyperbranched polyethylenimine (PEI) core, a poly(L-lysine) (PLL) inner shell, and a poly(ethylene glycol) (PEG) outer shell (structure shown in Fig. 1) as a potential unimolecular protein nanocarrier. PEG is employed as the outer shell of the star block co-polymer owing to its good water solubility, high degree of biocompatibility, and prolonged circulation time in the blood. The star block co-polymer contains a biodegradable PLL inner shell. Therefore, at physiological pH, at which the PLL inner shell is positively charged while most proteins carry negative charges, these macromolecules could encapsulate protein molecules in aqueous conditions via electrostatic interactions. Furthermore, when the pH is decreased below the pI of the protein charge switching of the protein molecules may lead to their accelerated release from the star block co-polymer.

2. Materials and methods

2.1. Materials

Hyperbranched PEI (M_w 10 kDa) and poly(ethylene glycol) monomethylether (PEG) (M_w 2 kDa) were obtained from

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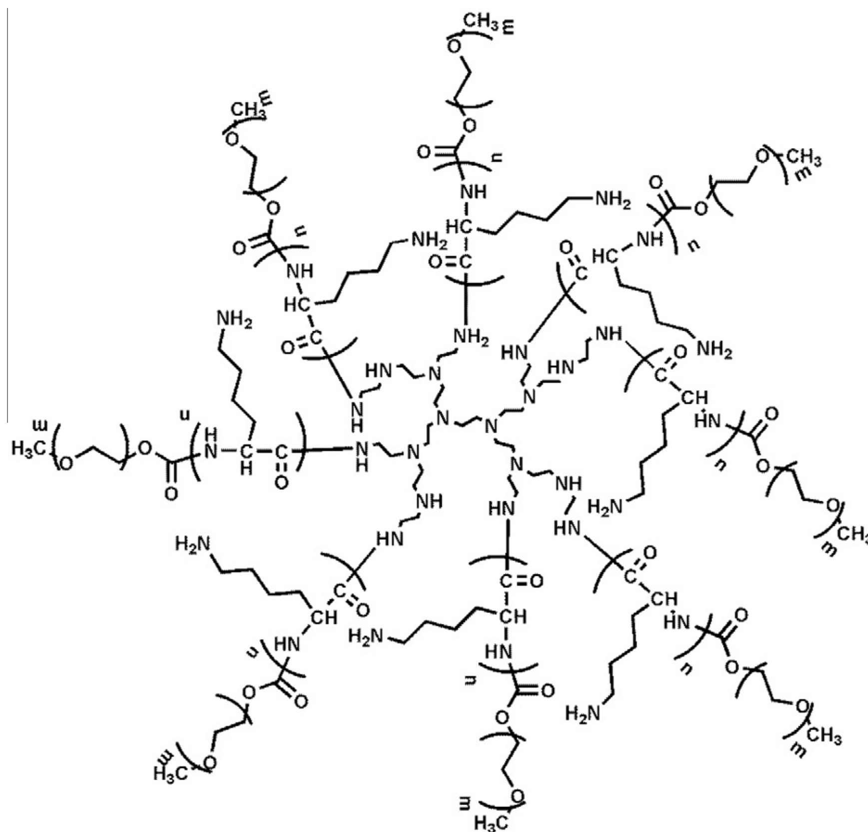

Fig. 1. Schematic structure of the synthesized star block co-polymer PEI–PLL–*b*-PEG. The depicted idealized polymer structure shows only eight star arms.

Sigma–Aldrich. ε-Benzyloxycarbonyl-L-lysine (ZLL) supplied by Sichuan Tongsheng Amino Acid Co. Ltd. (China) was used as received. Triphosgene, 4-nitrophenyl chloroformate (NPC), anisole, methane sulfonic acid, and trifluoroacetic acid (TFA) were obtained from Shanghai Darui Co. Ltd. (China). Bovine pancreas insulin was supplied by Shanghai Kenqiang Co. Ltd. (China). ELISA assay kits were supplied by Cusabio Co. Ltd. (China). Regenerated cellulose dialysis tubing (molecular weight cut-off 50 and 100 kDa) was obtained from Spectralab Co. Ltd. (USA). Dichloromethane (DCM), dimethylsulfoxide (DMSO), and ethyl acetate were dried over CaH_2. Petroleum ether and tetrahydrofuran (THF) were dried by refluxing over sodium.

2.2. Measurements

^{1}H NMR spectra were recorded in a Bruker DMX 400 MHz spectrometer using D_2O or $CDCl_3$ as the solvent. The molecular weight and molecular weight distributions were determined on a Waters 515 gel permeation chromatograph equipped with a 2414 RI detector. THF, DMF, or 0.50 mol l^{-1} HAc–NaAc buffer (pH 4.5) was used as the eluent at a flow rate of 1 ml min^{-1} at 30 °C. Molecular weights were calibrated on poly(styrene) (PS) or poly(ethylene glycol) (PEG) standards. The particle sizes of the PEI–PLL–*b*-PEG and PEI–PLL–*b*-PEG/insulin polyplexes were measured with a Brookhaven BI-200SM laser dynamic light scattering (DLS) system. Samples were dissolved in 0.020 mol l^{-1} phosphate buffer (pH 7.4), and five replicates were run. The scattered light was detected at a 90° angle at room temperature. The morphologies of the PEI–PLL–*b*-PEG and PEI–PLL–*b*-PEG/insulin polyplexes were visualized with a JEOL JEM-1400 transmission electron microscope at an operating voltage of 100 keV. A drop of an aqueous solution of PEI–PLL–*b*-PEG or PEI–PLL–*b*-PEG/insulin polyplex (0.5 mg ml^{-1}) was deposited onto a carbon-coated copper grid. The excess co-polymer solution was removed using filter paper, and the grid was dried under the ambient atmosphere for 1 h. The amount of insulin was measured using a reverse phase high performance liquid chromatography (RP-HPLC) (Agilent-1200) separation module equipped with a UV–visible detector. Chromatographic separation was performed at room temperature using a C18 (4.6 mm × 250 mm, 5 μm, Phenomenex, USA) chromatographic column. The mobile phase consisted of an acetonitrile:water mixture (30:70) with, pH 2.2, that was adjusted using TFA. The mobile phase was eluted at a flow rate of 1.0 ml min^{-1}, and the eluent was monitored at 214 nm. Samples were diluted with the mobile phase and incubated for 30 min. The injection volume was 100 μl. Standard insulin solutions were used to generate calibration curves. Fourier transform infrared (FTIR) spectrophotometry was used to investigate the complexation of insulin with PEI–PLL–*b*-PEG. Insulin, PEI–PLL–*b*-PEG, and insulin-loaded PEI–PLL–*b*-PEG (20%) were mixed with KBr and the samples pressed into disks. Infrared spectra of the samples were scanned in the range 400–4000 cm^{-1}, and recorded on an FTIR spectrometer (Thermo Fisher Nicolet iS5, USA). FTIR spectra were obtained at a resolution of 4 cm^{-1} with a minimum of 50 scans per spectrum. All measurements were taken at room temperature.

2.3. Synthesis of PEI–PLL–b-PEG

PEI–PLL–*b*-PEG was synthesized by the ring-opening polymerization of ε-benzyloxycarbonyl-L-lysine-*N*-carboxyanhydride (ZLL-NCA) initiated by the terminal amino groups of PEI, followed by peripheral PEGylation and subsequent deprotection of the side-chains of the poly(L-lysine) segments, as depicted in Scheme 1.

First, ZLL-NCA was synthesized by the phosgenation of ZLL in anhydrous ethyl acetate following the reported method [20,21],

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