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Polymer hydrogels for glutathione-mediated protein release

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ABSTRACT

The use of amine-terminated poly(ethylene glycol) star polymers as macroinitiators for the N-carboxyanhydride ring-opening polymerisation of S-*tert*-butylmercapto-L-cysteine N-carboxahydride is described to yield amphiphilic copolymers that are capable of forming discrete particles in aqueous solution. Poly(amino acid) deprotection liberates the pendant thiol groups that can then form covalent disulfide crosslinks with adjacent thiol groups and yield a crosslinked polymer that is capable of hydrogel formation. The model protein albumin–fluorescein isothiocyanate conjugate was encapsulated within the hydrogels produced, prior to its release upon hydrogel interaction with the reducing agent glutathione. Consequently, the stimuli-responsive polymers formed hold great promise as biomaterials capable of releasing a protein molecular cargo upon interaction with glutathione.

1. Introduction

Stimuli-responsive polymers are an important class of material that may readily be applied for the production of advanced biomaterials [1–3]. The controlled release of payload molecules from a polymeric carrier upon external stimulation enables the delivery of therapeutic agents on-demand, as part of a highly-effective drug delivery system [4,5]. In addition, stimuli-responsive polymers may be utilised as scaffolds for tissue regeneration [6–8]. Consequently, polymers that are susceptible to a controlled response and/or degradation upon interaction with stimuli including alterations in environmental temperature [9], pH [10–12], and the presence of particular enzymes, are highly sought [13].

Polymers that undergo physical/chemical alterations upon interaction with reducing agents are also of significance for use within a biomedical context [14–16]. Glutathione (GSH) is a natural tripeptide that is abundant in the majority of animal cells. The thiol group of the cysteine unit of glutathione dictates that glutathione is a reducing agent; for instance the disulfide bonds that are formed within cytoplasmic proteins are reduced to cysteine units due to glutathione being an electron donor [17]. Micromolar concentrations of GSH are found within the blood plasma, compared to intracellular GSH concentrations of between 0.5 mM and 10 mM. This renders glutathione to be a particularly valid target for therapeutic treatment as extracellular drug release is minimised [18].

Employing a polymeric hydrogel as a carrier vehicle permits the controlled delivery of biomolecules, such as the protein drugs Trastuzumab, Bevacizumab and Rituximab, *in vivo* [19]. Polymer hydrogels are well-suited to the controlled delivery of proteins as they present an aqueous environment that prevents protein denaturation. The hydrogel enables the efficient encapsulation of the protein cargo, restricting its metabolisation whilst maintaining its bioactivity, and enables protein release at a pre-programmed rate upon stimulation. Such materials have been utilised for the delivery of growth factors as part of tissue regeneration, and the controlled delivery of proteins that act as therapeutic agents [20,21]. The relative ease

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of polymer hydrogel synthesis, coupled with the feasibility of creating materials that possess general biocompatibility, adds to their suitability for employment as drug delivery vehicles and scaffolds to promote tissue regeneration.

Hereon in we report the generation of poly(ethylene glycol) (PEG) star polymers that are terminated with cysteine oligomers for use as protein delivery vehicles. Cysteine is a particularly useful component of functional materials due to its pendant thiol group that may be utilised for thiol-ene 'click' reactions [22], or participate in the formation of disulfide bridges [23]. The terminal cysteine units were grafted by N-carboxyanhydride ring-opening polymerisation (NCA ROP) and possessed tertiary butyl protecting units to protect the thiol groups of the cysteine repeat units, thus producing an amphiphilic structure that possessed the capability to form discrete particles in aqueous solution. Cysteine deprotection liberated the thiol groups, enabling the polymers to undergo chemical crosslinking, *via* the formation of disulfide bridges, to yield a polymeric network. This polymeric network was able to uptake appreciable amounts of water, due to the considerable PEG content, and form hydrogels that were susceptible to reduction upon incubation with glutathione. The polymer hydrogels formed were able to encapsulate and selectively release the fluorescently labelled model protein bovine serum albumin, deeming them highly-applicable for the controlled release of protein payloads within reductive environments. It is envisaged that the hydrogels formed may act as scaffolds for tissue engineering that permit the release of a protein growth factors, and/or as protein delivery vehicles that perform payload release upon interaction with a reducing agent that is either present, or injected to the intended site of action, in both instances.

1.1. Materials and general methods

Triphosgene (⩾98%), anhydrous ethyl acetate (⩾99.8%), *n*-hexane (⩾98%), anhydrous dimethylformamide (DMF) (⩾99.8%), diethyl ether (⩾99.8%), 4-arm amine-terminated poly(ethylene glycol) (PEG star polymer; 10,000 Da), cobalt phthalocyanine (>97%), S-*tert*-butylmercapto-L-cysteine, 1,4 Dithiothreitol (⩾97%), albumin–fluorescein isothiocyanate conjugate (FITC-albumin) and L-glutathione (⩾99.8%) were all acquired from Sigma Aldrich. α-Pinene (⩾98%) and phosphate buffered saline (PBS) tablets (Dulbecco 'A' tablets) were supplied by Thermo Fisher Scientific Laboratories. HPLC grade water (18.2 MΩ cm) was supplied by VWR International. All chemicals were used as received unless stated otherwise.

^{1}H NMR and ^{13}C NMR spectra were recorded at 25 °C on a Bruker Avance 500 MHz spectrometer and analysed using MestreNova® Research Lab software. Elemental analyses were conducted using a Thermo FlashEA Analyzer 1112 Series instrument. Fourier Transform-Infrared Spectroscopy (FTIR) measurements were performed on a Bruckner Alpha-P spectrometer, equipped with a diamond ATR crystal and the data was processed using Opus 7.2 software. Scanning electron microscopy (SEM) studies were performed on a JEOL JSM-6610LV microscope (Oxford Instruments) equipped with a field emission electron gun as an electron source, using a working distance of 11 mm. The accelerating voltage was varied between 5 kV and 15 kV. Fluorescence imaging was carried out using an Axio observer Z1 microscope (Zeiss Instruments) equipped with an AxioCam IC camera and an LD A-plan 5 × 0.15 Ph1 objective lens (excitation; 492 nm, emission; 515 nm, exposure time; 445 ms). Differential scanning calorimetry (DSC) analyses were carried out on a DSC Q20 unit (TA instruments) calibrated with indium and a nitrogen sample purge flow of 50 mL/min. A heating rate of 10 °C per minute was adopted for all samples. A TGA Auto Q20 unit (TA Instruments) was used to carry out thermogravimetric analysis (TGA). Samples were heated from room temperature at 10 °C per minute. A balance purge flow of 40 mL/min and sample purge flow of 60 mL/min were used. Dynamic light scattering (DLS) was conducted using a Malvern ZetaSizer NS. Sample equilibration was conducted for 1 min prior to analysis.

2. Experimental procedures

2.1. Synthesis of S-tert-butylmercapto-L-cysteine NCA

NCA synthesis was conducted using triphosgene for amino acid cyclisation [24]. S-*tert*-butylmercapto (STBM)-L-cysteine (5.00 g, 23.89 mmol) was dissolved in anhydrous ethyl acetate (60 mL) and injected under nitrogen flow into a three neck round bottom flask equipped with a magnetic stirrer bar, condenser and dropping funnel. α-pinene (7.17 g, 52.62 mmol) was degassed and then injected into the reaction flask and the suspension was heated to reflux. Then, triphosgene (4.90 g, 16.5 mmol) dissolved in anhydrous ethyl acetate (20 mL) was added dropwise into the refluxing suspension over a period of 30 min. The reaction was then left to reflux for 4 h, at which point all the suspension had dissolved. After cooling, the crude solution was concentrated by removing 75% of the ethyl acetate using rotary evaporation. The concentrated crude solution was precipitated from cold ethyl acetate/n-hexane (1:9 v/v) and left to stand at −18 °C for 24 h. The precipitated product was isolated by gravitational filtration, purified further by repeated precipitation from ethyl acetate and *n*-hexane (1:9 v/v) and subsequently dried under vacuum to obtain the NCA as a white powder. Yield: 3.57 g, 15.17 mmol, 64%.

^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm): 6.37 (s, 1H, NH), 4.63 (dd, J = 9.20, 2.9 Hz, 1H, αCH), 2.99 (ddd, J = 23.3, 14.0, 6.2 Hz, 2H), 1.30 (s, 9H, *tert*-Butyl). FTIR: ν_{max}/cm^{-1} (solid): 3232 (NH), 2959 (CH), 1845 and 1805 (asymmetric anhydride C=O). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm):

2.2. Synthesis of poly[(STBM-L-cysteine$_n$)$_4$-b-(StarPEG$_{10k}$)] macromolecules

A representative procedure for the syntheses of poly[(STBM-L-cysteine$_n$)$_4$-*b*-(StarPEG$_{10k}$)] macromolecules is given for a monomer:macroinitiator feed ratio of 10:1. STBM-L-cysteine NCA (0.19 g, 0.81 mmol) was dissolved in anhydrous DMF

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