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Self-assembling nanocomposites for protein delivery: Supramolecular interactions between PEG-cholane and rh-G-CSF

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ABSTRACT

$PEG_{5\ kDa}$-cholane, $PEG_{10\ kDa}$-cholane and $PEG_{20\ kDa}$-cholane self-assembling polymers have been synthesised by the end-functionalisation of 5, 10 and 20 kDa linear amino-terminating monomethoxy-poly(ethylene glycol) (PEG-NH_2) with 5β-cholanic acid. Spectroscopic studies and isothermal titration calorimetry showed that the CMC of the PEG-cholane derivatives increased from 23.5 ± 1.8 to 60.2 ± 2.4 μM as the PEG molecular weight increased. Similarly, light scattering analysis showed that the micelle size increased from 15.8 ± 4.9 to 23.2 ± 11.1 nm with the PEG molecular weight. Gel permeation studies showed that the polymer bioconjugates associate with recombinant human granulocyte colony stimulating factor (rh-G-CSF) to form supramolecular nanocomposites according to multi-modal association profiles. The protein loadings obtained with $PEG_{5\ kDa}$-cholane, $PEG_{10\ kDa}$-cholane and $PEG_{20\ kDa}$-cholane were 7.4 ± 1.1, 2.7 ± 0.3 and 2.1 ± 0.4% (protein/polymer, w/w %), respectively. Scatchard and Klotz analyses showed that the protein/polymer affinity constant increased and that the number of PEG-cholane molecules associated to rh-G-CSF decreased as the PEG molecular weight increased. Isothermal titration calorimetry confirmed the protein/polymer multi-modal association. Circular dichroism analyses showed that the polymer association alters the secondary structure of the protein. Nevertheless, *in vitro* studies performed with NFS-60 cells showed that the polymer interaction does not impair the biological activity of the cytokine. *In vivo* studies performed by intravenous and subcutaneous administrations of rh-G-CSF to rats showed that the association with $PEG_{5\ kDa}$-cholane prolongs the body exposure of the protein. After subcutaneous administration, the protein t_{max} values obtained with rh-G-CSF and 1:14 and 1:21 rh-G-CSF/$PEG_{5\ kDa}$-cholane (w/w ratio) nanocomplexes were 2, 8 and 24 h, respectively. The 1:21 (w/w) rh-G-CSF/$PEG_{5\ kDa}$-cholane formulation resulted in 149% relative bioavailability, and the pharmacokinetic behaviour was similar to that obtained with an equivalent protein dose of rh-G-CSF chemically conjugated with one linear 20-kDa PEG. A single administration of a 1.5 mg/kg dose of a 1:21 (w/w) rh-G-CSF/$PEG_{5\ kDa}$-cholane formulation induced a high production of white blood cells for 96 h.

1. Introduction

In recent decades, a variety of water-based nanovehicles, obtained by either conjugation or physical assembly of soluble polymers, have been developed to enhance the therapeutic profile of proteins [1–5].

The conjugation of hydrophilic macromolecules, including polysaccharides, polyacrylates, polyvinyls and polyoxyalkanes, has been found to ameliorate the biopharmaceutical properties of proteins [4]. PEGylation [poly(ethylene glycol) bioconjugation] in particular has been successfully applied to produce second- and third-generation biotech drugs, a few of which have reached the pharmaceutical market or are under advanced development [6,7]. Nevertheless, polymer conjugation may induce alterations in the protein structure or mask the active site from recognition of the pharmacological target, which may result in a dramatic reduction of bioactivity [8,9].

Delivery systems relying on physical protein/polymer complexation represent an attractive alternative to polymer bioconjugation, as they can prevent possible activity loss due to chemical modifications while prolonging body exposure [4]. Therefore, the rational design of amphiphilic macromolecules may yield nanocarriers for the optimised delivery of diverse therapeutic proteins that impact the treatment of multiple pathologies.

Micelle-forming amphiphilic polymers have been found to bind non-covalently to proteins [10]. Hyperbranched polyglycerol end-functionalised with lactic acid oligomers has been found to associate with BSA through surface-exposed hydrophobic structures [11]. Channel proteins can interact with self-assembling triblock copolymers

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[poly(2-methyloxazoline)-b-poly(dimethylsyloxane)-b-poly(2-methyloxalozyne)] without loss of activity [12]. Insulin/polymer nanocomplexes have been obtained with a variety of amphiphilic macromolecules, including polyoxyethylene, polyvinyl and polyacrylate derivatives as well as hydrophobised polysaccharides [13–16]. Comb-like hydrophobically modified poly(glutamate)s have been used to obtain slow protein release *in vivo* [17,18]. Recently, we demonstrated that polyhydroxyethylaspartamide (PHEA) derivatised with alkyl chains can associate with human growth hormone to provide sustained protein release without loss of activity [19].

The surface properties of natural steroidal molecules, such as cholesterol and other polycyclic surfactants secreted in mammalian bile have been largely exploited to bestow amphiphilic characteristics to polysaccharides and synthetic polymers. Chitosan derivatised with cholanic acid yields nanoassemblies that have been investigated for drug delivery [20]. Cholesteryl end-capped thermosensitive poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide) was used for cyclosporin and indomenthacin delivery [21]. Amphiphilic macromolecules obtained by the end-functionalisation of linear or dendritic PEGs with polycyclic moieties, namely cholesterol, have been used for protein brain targeting [22]. Cholesterol-bearing pullulan was found to associate with several proteins to form nanocomplexes [14].

Aimed at investigating the carrier properties of simply structured amphiphilic macromolecules, we synthesised bioconjugates by the end-derivatisation of linear 5-, 10- and 20-kDa monoamino-monomethoxy-poly(ethylene glycol) with 5β-cholane acid. β-cholanic acid was selected because with respect to other low molecular weight surfactants it was found to form thermodynamically stable supramolecular assemblies [20]. The saturated structure and the lack of double bonds and hydroxyl functions bestow this molecule with high chemical stability and hydrophobic character that can be properly exploited to establish interactions with hydrophobic pockets of proteins [20,23,24]. Importantly, as compared to other steroidal polycyclic molecules, it does not display specific biological activity [25–27] and presents very low toxicity [28]. Finally, the presence of a carboxyl function on the side chain represents an excellent site for the polymer conjugation through simple and effective chemical processes. The self-assembling properties of the bioconjugates as well as their ability to form nanocomplexes with recombinant human colony stimulating factor (rh-G-CSF) were investigated by chromatographic, spectrometric and calorimetric analyses. *In vitro* and *in vivo* studies were carried out to evaluate the biopharmaceutical, pharmacokinetic and pharmacodynamic behaviour of the cytokine formulated with the amphiphilic vehicles.

2. Materials and methods

Recombinant human granulocyte colony stimulating factor (rh-G-CSF) was a kind gift of Bio-Ker (Pula, Italy). Linear 5, 10 and 20 kDa monoamino-monomethoxy-poly(ethylene glycol)s ($PEG_{5\ kDa}$-NH_2, $PEG_{10\ kDa}$-NH_2 and $PEG_{20\ kDa}$-NH_2, respectively) were obtained from Nektar (Huntsville, AL, USA). 5β-cholanic acid was purchased from Fluka Chemika (Buchs, Switzerland). Triethylamine (TEA) and trinitrobenzenesulfonic acid (TNBS) were furnished by Aldrich (Milwaukee, WI, USA).

In vivo studies were carried out using male Sprague–Dawley rats weighing 200–220 g and male C3H/HeN mice weighing 20–25 g. The animals were fed *ad libitum*. The care and handling of animals used for the pharmacokinetic studies were in accordance with the provisions of EU Council Directive 86/209 and NIH publication no. 85-23, revised in 1985.

2.1. Synthesis of PEG-cholane

5β-cholanic acid (600 mg, 1.66 mmol) was dissolved in a 50:50 CH_2Cl_2/DMF V/V ratio (6 mL). The organic solution was added of thionyl chloride (1.45 mL, 16.6 mmol) and the reaction mixture was refluxed under a nitrogen atmosphere. After 3 h, the unreacted thionyl chloride was eliminated by distillation and the cholanic chloride was recovered from the reaction solution by desiccation in a vacuum. The degree of conversion of cholanic acid to cholanyl chloride was spectrophotometrically estimated by reaction with 5-kDa PEG-NH_2 and determination of the unreacted amino groups by the colorimetric TNBS assay [29].

Cholanyl chloride (34 mg, 0.08 mmol) was dissolved in methylene chloride (1 mL) and TEA (30 μL, 0.21 mmol), and then added to 4 mL of methylene chloride containing either 400 mg (0.02 mmol) of $PEG_{5\ kDa}$-NH_2 or equimolar amounts of $PEG_{10\ kDa}$-NH_2 or $PEG_{20\ kDa}$-NH_2. After the overnight reaction, the organic solution was added dropwise to 100 mL of ethyl ether. The precipitate was washed three times with 50 mL of ethyl ether and desiccated in a vacuum. The product was then dissolved in 10 mL of water, and the aqueous solution was centrifuged. The PEG concentration in the supernatant was determined using the iodine test [30]. The degree of PEG modification by cholanyl chloride was determined by the amount of free amino groups of PEG-NH_2 according to the TNBS assay. An aliquot of the final product was dissolved in $CDCl_3$ and analysed by ^{1}H NMR spectrometry using a Bruker Spectrospin 300 spectrometer (Fallanden, Switzerland). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.64 [s, 3H, C**H**$_3$ (C19) of cholane], δ 0.91–0.93 [s + d, 6H, C**H**$_3$ (C18) + C**H**$_3$ (C21) of cholane], δ 3.38 [s, 3H, C**H**$_3$O-PEG], δ 3.64 [s, 4nH, –(C**H**$_2$C**H**$_2$-O)$_n$– of PEG].

2.2. Critical micelle concentration analysis

Aliquots of 20 μL of 5 mg/mL pyrene solutions in acetone were desiccated in a vacuum and then added to 1 mL of 0.4–400 μM PEG-cholane solutions in distilled water. After overnight shaking at 37 °C, the samples were centrifuged at 5000 rpm for 5 min and the supernatants were analysed by spectrofluorimetry. The excitation spectra were registered in the range of 300–360 nm with an emission wavelength of 390 nm. The intensity ratio at 339/334 nm was elaborated according to the method reported in the literature [31]. Each experimental point was repeated five times.

2.3. Light scattering analysis

Photon correlation spectroscopic studies were carried out using a Malvern Zetasizer Nano S Photon Correlation Spectrometer (Worcestershire, UK) equipped with a 633 nm laser and 173° backscattering. Polymer solutions (0.23 and 1.17 mg/mL of $PEG_{5\ kDa}$-cholane, 0.73 and 3.6 mg/mL of $PEG_{10\ kDa}$-cholane and 1.2 and 6 mg/mL of $PEG_{20\ kDa}$-cholane) in 20 mM phosphate buffer, 0.15 M NaCl, H 7.4, were filtered with 1 μm cut-off filters and then analysed.

2.4. Rheological analysis

Viscosimetric analyses were performed using a Thermo Haake 7R (Victoria, Australia) supplied with an R2 sensor at 200 rpm. $PEG_{5\ kDa}$-cholane and $PEG_{5\ kDa}$–NH_2 solutions (10 mL) in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4, from 20 to 160 mg/mL were analysed in triplicate.

2.5. rh-G-CSF/PEG-cholane associations

Twenty μL of 2 mg/mL rh-G-CSF solution in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4, was added to 0, 10, 20, 40, 60, 80, 100, 120, 140 or 160 μL of 20 mg/mL PEG-cholane solution in the same buffer. Phosphate buffer was added to reach final volumes of 200 μL. After overnight mild top-down mixing at room temperature, the solutions were centrifuged at 5000 rpm for 3 min and analysed using HPLC gel permeation chromatography using a Bio-Gel SEC 40XL (300 × 7.8 mm) column (Bio