Colloids and Surfaces B: Biointerfaces 99 (2012) 38–44

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Dual crosslinked hydrogel nanoparticles by nanogel bottom-up method for sustained-release delivery

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ARTICLE INFO

Article history:
Available online 19 September 2011

Keywords:
Nanogel
Polysaccharide
Sustained drug delivery
Hydrogel
Biodegradable
Asymmetrical flow field-flow fractionation

ABSTRACT

Polysaccharide–PEG hybrid nanogels (CHPOA–PEGSH) crosslinked by both covalent ester bonds and physical interactions were prepared by the reaction of a thiol-modified poly(ethylene glycol) (PEGSH) with acryloyl-modified cholesterol-bearing pullulan (CHPOA). Experimental parameters, including CHPOA concentration, the degree of acryloyl substitution of CHPOA, and the initial amounts of CHPOA and PEGSH, were modified in order to assess their effect on the size of the nanogels (50–150 nm) and on their degradation kinetics, monitored by dynamic light scattering (DLS) and asymmetrical flow field-flow fractionation (AF4) chromatography. Rhodamine-labeled nanogels were injected intravenously into mice and their concentration in blood was determined by a fluorescence assay as a function of post-injection time. The elimination half-life ($t_{1/2}$) of CHPOA–PEGSH nanoparticles was about 15-fold longer (18 h) than that of CHP nanogels (1.2 h). The half-life enhancement of CHPOA–PEGSH was attributed to the presence of the crosslinker PEG chains, which prevent non-specific protein adsorption, and to the slow hydrolysis kinetics of the crosslinking esters in the biological milieu. The hybrid CHPOA–PEGSH nanogels are expected to be useful as injectable nanocarriers for drugs and proteins, in view of their low surface fouling and slow hydrolysis rate.

1. Introduction

Nanometer-sized polymer hydrogel particles (nanogels) have recently received much attention for biomedical applications such as drug delivery systems [1–4]. Chemically crosslinked nanogels are usually prepared by nanoemulsion polymerization or by chemical crosslinking of the hydrophilic or hydrophobic polymer chains in polymer micelles [5–7]. We reported a new method of preparation of physically crosslinked nanogels formed by amphiphilic derivatives of the neutral polysaccharide pullulan, such as pullulans bearing low levels of cholesteryl groups (CHP), upon simple self-assembly in dilute aqueous solution [8]. The nanogels were shown to prevent the massive aggregation of proteins that typically occurs upon rapid removal of urea from solution of unfolded proteins. The enhanced yield of correct protein folding was ascribed to the hydrophobic binding of CHP with proteins, which in turn slows down the kinetics of protein aggregation [9,10].

In this fashion, CHP nanogels have been used to encapsulate in a transient fashion proteins, such as insulin [11], interleukin-12 (IL-12) [12], antigen proteins for cancer vaccine [13,14], and nasal vaccine [15]. Recombinant murine IL-12 (rmIL-12) was successfully incorporated in CHP nanogel simply by incubating rmIL-12 with nanogels. Subcutaneous injection into mice of CHP/rmIL-12/nanogel complexes resulted in a prolonged elevation of rmIL-12 concentration in the serum. However, the kinetics of drug level in serum was the same following intravenous or intraperitoneal injections of CHP/rmIL-12 nanogels as those recorded upon injection of a rmIL-12 solution used as a control. This observation was taken as an indication of the poor stability of CHP nanogels in the bloodstream, presumably due to the fact that nanogels are held together exclusively by physical forces without any covalent crosslinkers. Consequently, they rapidly release their cargo in blood following displacement of the therapeutic protein by plasma proteins.

To overcome the instability of physically crosslinked nanogel in vivo, we devised a means to strengthen nanogels by chemical crosslinking of the nanogels with polyethylene glycol (PEG)

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doi:10.1016/j.colsurfb.2011.09.025

derivatives and coating the surface of nanogels with PEG. Acryloyl group-modified CHP nanogels were crosslinked by thiol group-modified four-armed polyethylene glycol (PEGSH) in water, thus forming nanogel-assembly like "raspberry-like" nanoparticles [16]. In the previous article, we reported the stability and sustained release of the nanogels assemblies upon subcutaneous injection in mice. We assess here the stability of PEG-crosslinked nanogels in blood serum and blood both in vitro and in vivo, following intravenous injection in mice. The nanogels assemblies were analyzed by asymmetrical flow field-flow fractionation with combined multiangle light scattering and dynamic light scattering detection coupled to UV–vis absorption detection, to assess, respectively, the concentration and the hydrodynamic size of the nanogels.

2. Experimental

2.1. Materials

Water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA). Pentaerythritol tetra(mercaptoethyl) polyoxyethylene (PEGSH, Mw = 1.0×10^4 g mol^{-1}) was purchased from NOF Co. (Tokyo, Japan). Di-*n*-butyltin (IV) dilaurate (DBTDL), cysteine hydrochloride monohydrate, and 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and minimum essential medium (MEM) were obtained from Invitrogen (Carlsbad, CA). 2-(Acryloyloxy) ethyl isocyanate (AOI) was purchased from Showa Denko Co (Tokyo, Japan). The cholesteryl pullulan nanogel sample (CHP, 1.2 cholesteryl groups per 100 glucose units) was synthesized as previously reported [17].

2.2. Dynamic light scattering (DLS)

The hydrodynamic diameters of the nanogels were determined by dynamic light scattering (DLS; Zetasizer Nano; Malvern, UK). The scattering angle was kept at 137° and the wavelength was set at 633 nm. The CHPOA nanogel concentration ranged from 1.0 to 5.0 mg mL^{-1}.

2.3. Asymmetrical flow field-flow fractionation (AF4)

Asymmetric flow field-flow fractionation was performed using an AF4 system (AFx2000MT, Postnova Analytics, Landsberg, Germany) combined with an UV/vis detector (SPD-20A, Postnova Analytics), a multiangle light scattering detector (MALS, Dawn 8+, Wyatt Technology), and a dynamic light scattering detector (Wyatt-QELS, Wyatt Technology). The channel had a thickness of 350 μm and was fitted with a regenerated cellulose membrane (10 kDa cut off, Z-MEM-AQU-631, RC, Postnova Analytics). The carrier medium was prefiltered (0.1 μm) phosphate buffered saline (pH 7.4). The sample was injected with a flow rate of 0.2 mL/min, followed by a 4 min-focusing with a cross-flow rate of 1.8 mL/min and a detector flow rate of 0.52 mL/min. Following a 1 min transition, a three-step cross-flow rate gradient was initiated for the elution mode. The starting cross-flow rate (1.8 mL/min) was kept constant for 8 min. It was decreased linearly to 0 mL/min within 10 min, and kept constant at 0 mL/min for 15 min to allow elution of the nanogels or assemblies of nanogels. The detector flow rate was kept at 0.52 mL/min throughout. The detection of the eluted nanogels was performed sequentially by UV absorbance at 556 nm (ε_{556} = 1411 mmol g^{-1} cm^{-1}, determined using a calibration curve), fluorescence with λ_{ex} 468 nm and λ_{em} 581 nm, multiangle light scattering (MALS) and DLS. Each fractogram presented is representative of a triplicate sample.

2.4. Synthesis of the nanogels

The CHPOA nanogels having 5, 19 or 23 acryloyl groups per 100 glucose units were prepared starting from a CHP sample (1.0 g, 6.2 mmol of anhydrous glucose units) dried under vacuum for 2 days at 70 °C before use. The CHP was dissolved under nitrogen in 50 mL of anhydrous dimethyl sulfoxide (50 mL, DMSO) at 45 °C. DBTDL (53, 201 or 242 μL; 90, 340 or 410 μmol) and AOI (42, 155 or 181 μL; 0.3, 1.2 or 1.4 mmol) were added to the CHP solution. The resulting mixture was kept in the dark for 24 h at 45 °C. The reaction mixture was subjected to repeated precipitations into ether/ethanol solution (ether >80% v/v). The isolated solid material was suspended in DMSO, dialyzed against deionized water, and isolated by lyophilization. The degree of substitution of acryloyl groups was determined from the ^{1}H NMR (500 MHz, DMSO-d6/D_2O: δ (ppm) 0.6–2.4 (cholesterol); 3.1–4.0 (glucose 2H, 3H, 4H, 5H, 6H); 4.7 (glucose 1H (1 → 6)); 4.9–5.1 (glucose 1H (1 → 4)); 5.9–6.3 (olefinic protons of the acryloyl group $-CH{=}CH_2-$). Rhodamine-labeled CHPOA (CHPOA–Rh) nanogels were synthesized as reported previously [18]. The degree of labeling was determined by UV/vis analysis at 556 nm (UV-1650PC, Shimadzu, Japan).

2.5. Preparation of CHPOA–PEGSH nanoparticles

To prepare CHPOA–PEGSH nanoparticles, samples of CHPOA nanogel (8 mg) in a PBS buffer (1 mL, pH 7.4) were treated with PEGSH in amounts such that the acryloyl group:thiol group molar ratios were 1:1, 2:1 and 4:1. The mixtures were kept at 37 °C for 24 h. Micrographs of CHPOA nanogels and CHPOA–PEGSH nanoparticles were obtained by freeze–fracture TEM (FF-TEM, JEM-1011, JEOL, Tokyo, Japan) at an accelerating voltage 100 kV. The sample was prepared using the aqueous solution of CHPOA nanogel or CHPOA–PEGSH nanoparticles containing 30% glycerol. The concentration of nanogel was 4 mg mL^{-1}, and the acryloyl:thiol molar ratio was 2:1 or 4:1. CHPOA–PEGSH nanogels were isolated in the dry form by lyophilization after 24 h incubation in PBS. Freeze-dried samples were resuspended in deionized water.

The thiol content of CHPOA–PEGSH nanoparticles was determined using Ellman's reagent [19]. CHPOA–PEGSH (4.0 mg mL^{-1}, obtained from acryloyl group:thiol group molar ratios of 1:1, 2:1 and 4:1) were dissolved in PBS. The nanoparticle solution (250 μL) was added to a mixture of the reaction buffer (2.5 mL, 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA) and of Ellman's reagent solution (50 μL, 4.0 mg Ellman's Reagent in 1 mL reaction buffer). The mixture was kept at room temperature for 15 min prior to analysis. The SH concentration of the solutions was determined from their absorbance at 412 nm using a calibration curve obtained from cysteine–HCl. The degradation of CHPOA–PEGSH nanoparticles was monitored by ^{1}H NMR spectroscopy for samples incubated in PBS containing D_2O for 24 h at 37 °C. The level of nanoparticle degradation was estimated from the increase in the signal at 2.36 ppm, attributed to the protons in the Fig. S2 [20]. The stability of the nanogels in serum was monitored using 500 μL of CHPOA nanogels or CHPOA–PEGSH nanoparticles (acryloyl:thiol = 1:1, 2:1 or 4:1) solutions in MEM (2.0 mg mL^{-1}, 500 μL). The mixtures were incubated at 37 °C with 5% (v/v) FBS for different times. The resulting solution was analyzed by DLS in order to determine the hydrodynamic sizes of the nanoparticles.

2.6. Blood clearance measurements

The animal experiments were carried out under the guidance of the Animal Care and Use Committee, Kyushu, University. Seven-week-old Balb/c mice purchased from Kyudo Co., Ltd. (Tosu, Japan) were treated with suspensions of CHP–Rh or CHPOA–Rh–PEGSH