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Highly versatile nanohydrogel platform based on riboflavin-polysaccharide derivatives useful in the development of intrinsically fluorescent and cytocompatible drug carriers

Chiara Di Meo*, Elita Montanari, Lucio Manzi, Claudio Villani, Tommasina Coviello, Pietro Matricardi

Department of Drug Chemistry and Technologies, "Sapienza" University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

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ABSTRACT

In this work we describe a new nanohydrogel platform, based on polysaccharides modified with the hydrophobic and fluorescent molecule riboflavin tetrabutyrate, which leads to innovative structures useful for drug delivery applications. Hyaluronic acid and pullulan were chosen as representative of anionic and neutral polysaccharides, respectively, and the bromohexyl derivative of riboflavin tetrabutyrate was chemically linked to these polymer chains. Because of such derivatization, polymer chains were able to self-assemble in aqueous environment thus forming nanohydrogels, with mean diameters of about 312 and 210 nm, for hyaluronan and pullulan, respectively. These new nanohydrogels showed low polydispersity index, and negative ζ-potential. Moreover, the nanohydrogels, which can be easily loaded with model drugs, showed long-term stability in water and physiological conditions and excellent cytocompatibility. All these properties allow to consider these intrinsically fluorescent nanohydrogels suitable for the formulation of innovative drug dosage forms.

1. Introduction

In recent years, hydrogel nanoparticles or nanohydrogels (NHs) have gained considerable attention as one of the most promising nanoparticulate systems capable to deliver therapeutic and/or diagnostic agents to specific tissues. The unique physicochemical properties of hydrogels, such as a soft consistency, high water content and low interfacial tension with water and biological fluids, together with their nano-sized dimensions, make NHs more similar to living tissues than many other classes of synthetic nanoparticulate systems (Qian, Fu, & Feng, 2013).

Among several types of polymeric NHs, those prepared from natural polymers, such as polysaccharides, offer several advantages because they are non-toxic, intrinsically hydrophilic, and generally show good biocompatibility and low immunogenicity, thus preventing inflammatory response. Among the various polysaccharide systems, pullulan (Akiyoshi et al., 1998), chitosan (Shutava & Lvov, 2006), alginate (Patel, Patel, Shah, & Modasiya, 2011), and hyaluronic acid (Montanari et al., 2013; Nakai et al., 2012; Choi et al., 2011) can be easily derivatized with hydrophobic moieties to form amphiphilic polymers that spontaneously self-assemble in aqueous media, thus forming NHs.

Polysaccharide self-assembling NHs, due to their amphiphilic nature, flexibility and versatility, can be used to deliver both hydrophobic and hydrophilic drugs as well as proteins. Akiyoshi extensively studied the ability of pullulan-cholesterol NHs to prevent irreversible aggregation of polypeptides and to assist protein refolding. It was demonstrated that these NHs are capable to form complexes with heat-denatured proteins and to release polypeptides in their refolded form upon dissociation of the NHs, showing an activity similar to that of molecular chaperones (Nomura, Ikeda, Yamaguchi, Aoyama, & Akiyoshi, 2003).

Among polysaccharides, hyaluronic acid (HA) seems to be the most interesting material for the development of biocompatible drug carriers; it is a bioactive polyanionic polysaccharide (the major glycosoaminoglycan constituent of extracellular matrices) capable to interact with several important cell receptors. There is an increasing interest to the use of such polysaccharide as a carrier for drug targeting to tumour cells characterized by the overexpression of the CD44 receptor. In this respect, several NHs carriers based on HA have been studied for targeting to solid tumours, e.g. HA-5β cholanic acid (Nakai et al., 2012), HA-ceramide (Cho et al., 2011),

* Corresponding author. Tel.: +39 06 49913961; fax: +39 06 49913133.
E-mail address: chiara.dimeo@uniroma1.it (C. Di Meo).

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HA-cholesterol (Montanari et al., 2013) and HA-glycyrrethinic acid (Zhang, Yao, Zhou, Wang, & Zhang, 2013). In the above-mentioned studies, anticancer drugs have been loaded within the NHs or have been covalently linked to the polymer chains, thus acting, at the same time, as the hydrophobic moiety responsible of the self-assembling properties.

Moreover, CD44 is over expressed also by inflammatory cells (Puré & Cuff, 2001), playing a major role in the clearance of apoptotic cells by macrophages (Teder et al., 2002).

Due to the importance to develop new systems that can be exploited in pharmaceutical and biomedical applications, we developed a new polymeric platform based on polysaccharides grafted with riboflavin tetrabutyrate, a hydrophobic derivative of riboflavin. Riboflavin (Rfv) is a vitamin of the B group (vitamin B2) consisting in a conjugated isoalloxazine ring (flavin) and a five-carbon carbohydrate (ribitol). It is widely distributed in nature, in plants and animals, as an essential constituent of all living cells. It is practically non-toxic and it is intrinsically fluorescent (Kotaki & Yagi, 1970). To demonstrate the general applicability of the strategy described here, we have chosen hyaluronic acid and pullulan, as representatives of anionic and neutral polysaccharides, respectively, and we obtained amphiphilic polymers by reaction of the polysaccharides with the Br-hexyl derivative of riboflavin tetrabutyrate. These modified polysaccharides, HA-Rfv and Pul-Rfv, spontaneously form fluorescent, stable and biocompatible NHs in water by macromolecular self-association.

In the present work we describe the synthesis of these amphiphilic polymers, the preparation of the NHs, their characterization in terms of dimension, stability, fluorescence and biocompatibility, along with the capability to be loaded with model drugs.

2. Experimental

2.1. Materials

Hyaluronan tetrabutylammonium salt (HATBA, $M_\eta = 2 \times 10^5$) was kindly provided by Fidia Advanced Biopolymers, Abano Terme (PD), Italy; pullulan (Pul, $M_\eta = 1.7 \times 10^5$) was by Hayascibara Co. Ltd., Japan; riboflavin tetrabutyrate (Rfv) was purchased from TCI Europe N.V., Belgium. Dimethyl sulfoxide (DMSO), *N*-methyl-2-pyrrolidone (NMP), 1,6-dibromohexane, 4-(dimethylamino) pyridine (DMAP), levofloxacin, piroxicam were Sigma products.

Other chemicals were reagent grade and were used without further purification.

2.2. Methods

2.2.1. Synthesis of 2′3′4′5′-tetrabutyril-3-(6-bromohexyl)riboflavin

To a 500 mg (0.78 mmol) solution of of 2′3′4′5′-tetrabutyrilriboflavin in 4.5 mL of dry *N*,*N*-dimethylformamide, anhydrous potassium carbonate (158 mg/1.14 mmol) was added in a single portion. The dark green suspension was stirred for 45 min under nitrogen atmosphere, then 0.470 mL of a solution of 1,6-dibromohexane (0.74 g, 3.0 mmol) in 2.5 mL of *N*,*N*-dimethylformamide was added drop wise and the mixture was stirred for 5 h. The reaction was monitored by TLC (SiO_2, dichloromethane:ethyl acetate 4:1) and ESI–MS. After neutralization with acetic acid, dichloromethane (10 mL) was added to the mixture and the organic phase was washed 5 times with an equal volume of water. The organic layer was separated, dried over sodium sulfate and evaporated to dryness. The product was purified by column chromatography (SiO_2, 40–63 μm) eluting first with hexane and then with dichloromethane/ethyl acetate 4/1. The title product was obtained as a yellow-orange solid (320 mg, 0.39 mmol, 50% isolated yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (s, 1*H*), 7.57 (s, 1*H*), 5.70 (br s, 1*H*), 5.47 (m, 2*H*), 4.91 (br s, 2*H*) 4.48 (d, *J* = 12.0 Hz, 1*H*), 4.23 (dd, *J* = 12.1, 6.1 Hz, 1*H*), 4.07 (t, *J* = 7.4 Hz, 2*H*), 3.40 (t, *J* = 6.7 Hz, 2*H*), 2.57 (s, 3*H*), 2.46 (s, 3*H*), 2.30 (t, *J* = 7.3 Hz, 2*H*), 2.07 (m, 1*H*), 1.86 (m, 3*H*), 1.74–1.60 (m, 10*H*), 1.66 (dd, *J* = 14.9, 7.4 Hz, 2*H*), 1.54–1.21 (m, 6*H*), 1.0 (q, *J* = 7.2 Hz, 6*H*), 0.94(t, *J* = 7.2 Hz, 3*H*), 0.63 (t, *J* = 7.2 Hz, 3*H*).

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.16, 173.12, 172.82, 172.79, 159.64, 154.80, 149.13, 147.29, 136.36, 135.72, 134.63, 132.89, 131.18, 115.42, 70.31, 69.01, 68.99, 61.73, 44.34, 41.78, 35.97, 35.83, 35.45, 33.84, 33.77, 32.65, 27.85, 27.52, 26.09, 21.29, 19.39, 18.38, 18.27, 18.23, 17.64, 13.59, 13.55, 13.54, 13.25.

FT-IR (KBr) cm^{-1} 2964, 2936, 2874, 1745, 1663, 1550, 1158.

ESI–MS (+) *m*/*z* 841, 843 $[M + Na]^+$.

2.2.2. HA-Rfv and Pul-Rfv synthesis

For the preparation of HA-Rfv, 1 mL of an Rfv-Br derivative solution in NMP (39.7 mg/mL) was added to 10 mL of an HATBA solution in NMP (10 mg/mL), in order to obtain a stoichiometric HATBA derivatization degree of 30% mol/mol (mol of Rfv per mol of HA repeating units).

The reaction was kept under magnetic stirring for 48 h at room temperature and then dialysed overnight against a NaCl 0.1 M solution, in order to exchange the free HA carboxylate groups from TBA^+ to Na^+ form. An exhaustive dialysis against distilled water was then performed (Visking tubing, cut-off: 12,000–14,000). The HA-Rfv sample was finally recovered as a yellow product by freeze-drying (yield ~90%).

Pul-Rfv derivative was obtained by dissolving 100 mg of Pul in 2 mL of anhydrous DMSO; 20 mg of DMAP were then added, followed by 40 mg of Rfv-Br dissolved in 0.5 mL of DMSO (1 mol of Rfv-Br per 4 mol of Pul repeating unit, i.e. stoichiometric derivatization degree of 25%). The reaction was kept for 48 h at room temperature under magnetic stirring and the product was then recovered by exhaustive dialysis against distilled water and freeze-dried (yield ~80%).

2.2.3. HA-Rfv and Pul-Rfv derivatization degree determination

For the evaluation of the derivatization degree, the modified polymers HA-Rfv and Pul-Rfv were solubilized in DMSO and analyzed by UV–vis spectrophotometry (Perkin-Elmer, double beam "Lambda 3A" model), using three linear calibration plots for riboflavin tetrabutyrate in DMSO: the calibration plots at λ = 445 nm and λ = 345 nm were obtained in the range 3.125–50 μg/mL, whereas the third calibration plot at λ = 272 nm was calculated in the range 1.125–18 μg/mL (*n* = 5, for each curve).

The derivatization degree of each polymer was evaluated as the average of the results obtained from the three calibration curves.

2.2.4. Preparation and characterization of sterile HA-Rfv and Pul-Rfv nanohydrogels

For the HA-Rfv NHs formation, in a typical preparation, 3 mg of the polymer was dispersed in 3 mL of distilled water (1 mg/mL) by magnetic stirring at room temperature; the suspension was then treated by the recently proposed autoclaving process (Montanari et al., 2014b). According to the proposed procedure, the samples were treated at 121 °C, 1.10 bar for 20 min, using a Juno Liarre autoclave (230 VAC, 50/60 Hz, 12 A, 2000 W). The process, that lasted for 20 min, led directly to sterile self-assembled NHs.

For the Pul-Rfv NHs formation, a different method was used: 3 mg of Pul-Rfv was dispersed in 3 mL of distilled water (1 mg/mL) by magnetic stirring at room temperature. The resulting suspension was then sonicated for 25 min, using an ultrasonic bath sonicator (Montanari et al., 2013) (Starsonic-35, Liarre). To prepare sterile samples for biological tests, Pul-Rfv was sterilized by filtration