Carbohydrate Polymers 115 (2015) 502–509

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

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

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ARTICLE INFO

Article history:
Received 3 March 2014
Received in revised form 25 July 2014
Accepted 22 August 2014
Available online 16 September 2014

Keywords:
Nanohydrogel
Hyaluronan
Pullulan
Riboflavin
Self-assembling

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),

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http://dx.doi.org/10.1016/j.carbpol.2014.08.107
0144-8617/

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

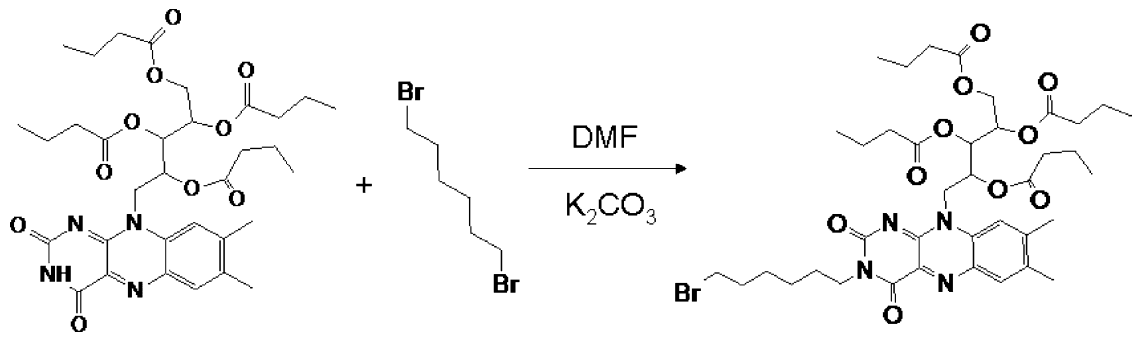
For the evaluation of the derivatization degree, the modified polymers HA-Rfv and Pul-Rfv were solubilized in DMSO and analyzed by UV–vis spectrophotometry (Perkin-Elmer, double beam "Lambda 3A" model), using three linear calibration plots for riboflavin tetrabutyrate in DMSO: the calibration plots at 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



Scheme 1. Synthesis of 2′3′4′5′-tetrabutyril-3-(6-bromohexyl)riboflavin.

through 0.22 μm pore-size filters, after NHs formation by bath sonication.

HA-Rfv and Pul-Rfv NHs size and polydispersity (PDI) were measured by Dynamic Light Scattering (DLS) using Submicron Particle Sizer Autodilute Model 370 (Nicomp).

ζ-Potential of the NHs in water was assessed with a Malvern NanoZetaSizer dynamic light scattering instrument (Malvern Instruments, Worcestershire, United Kingdom), equipped with a 5 mW HeNe (λ = 632.8 nm).

Stability studies of HA-Rfv and Pul-Rfv NH suspensions, prepared as above described, were carried out by checking the size and polidispersity of NHs in water at 4 °C for two months and 37 °C for one week. The stability of NH suspensions was also evaluated in physiological saline 0.9% w/v NaCl solution at 4 °C for two months and at 37 °C for 15 days in phosphate buffer 0.1 M, pH = 7.4.

2.2.5. Cryo-TEM analysis of HA-Rfv and Pul-Rfv NHs

Suspensions of HA-Rfv NHs (1 mg/mL, 4 mL) and of Pul-Rfv NHs (1 mg/mL, 4 mL) were prepared as described above and analyzed by cryogenic transmission electron microscopy (Cryo-TEM).

Cryo-TEM analyses were performed at the Institut Federatif de Recherche Biologie Integrative—IFR 83, Region Ile de France, Service de Microscopie Electronique; for the analysis, 4 μL of NH suspensions were laid onto a perforated carbon film mounted on a 200 mesh electron microscopy grid. Most of the drop was removed with blotting paper and the residual thin film, remaining within the holes, was vitrified by immersion in liquid ethane using a guillotine-like frame. The specimen was then transferred, using liquid nitrogen, to a cryospecimen holder and observed with a JEOL FEG-2010 electron microscope. Micrographs were recorded at 200 kV under low-dose conditions at a magnification of 40,000 on SO-163 Kodak films. Micrographs were digitized using a film scanner (Montanari et al., 2013; Sémiramoth et al., 2012).

2.2.6. Fluorescence measurements

Fluorescence emission spectra were recorded on a SPEX-Fluoromax spectrofluorimeter (Horiba Scientific) from λ = 400 to λ = 600 nm with excitation at λ = 345 nm using a 1 cm path length quartz cuvette. The excitation and emission slits were both set to 2.5 nm and scan speed was 120 nm/min. HA-Rfv and Pull-RFv NH samples were prepared as above described and diluted with distilled water up to 250 μg/mL. Fluorescence intensities were read at the maximum λ = 530 nm.

2.2.7. Cytocompatibility of HA-Rfv and Pul-Rfv NHs

Cytocompatibility of HA-Rfv and Pul-Rfv NHs was assessed according to the ISO 10993-5 protocol ("Biological evaluation of medical devices. Test for cytotoxicity: in vitro methods", Das et al., 2010). Briefly, for a qualitative analysis, 12 well plates containing 9 wells with a sub-confluent cell monolayer of mammal fibroblast ATCC BalbC 3T3 were treated with 50 μL of sterile sample suspensions (1 mg/mL, 0.5 mg/mL, 0.2 mg/mL and 0.1 mg/mL) deposed on a filter paper placed in the middle of the well (×3 wells), or with a negative control (filter paper alone × 3 wells) or a positive control (30 mm^2 of latex placed in the middle of the wells × 3 wells). The plates were then incubated at 37 ± 1 °C and 5% CO_2 atmosphere for 24 h, then observed using an inverted microscope to evaluate biological reactivity (cell degeneration and malformations); all experiments were carried out in triplicate.

For the quantitative evaluation of NHs cytocompatibility, cells were treated with 2 mL of Neutral Red Medium for 3 h, then the wells were washed with PBS and treated with 2 mL of Desorb Solution, stirring the plate for 10 min to homogenize the solution. The optic density (OD) was evaluated by spectrophotometric reading at 540 nm with a microplate reader (Bio-Tek) and the cell viability (%) vas calculated according to the following relationship:

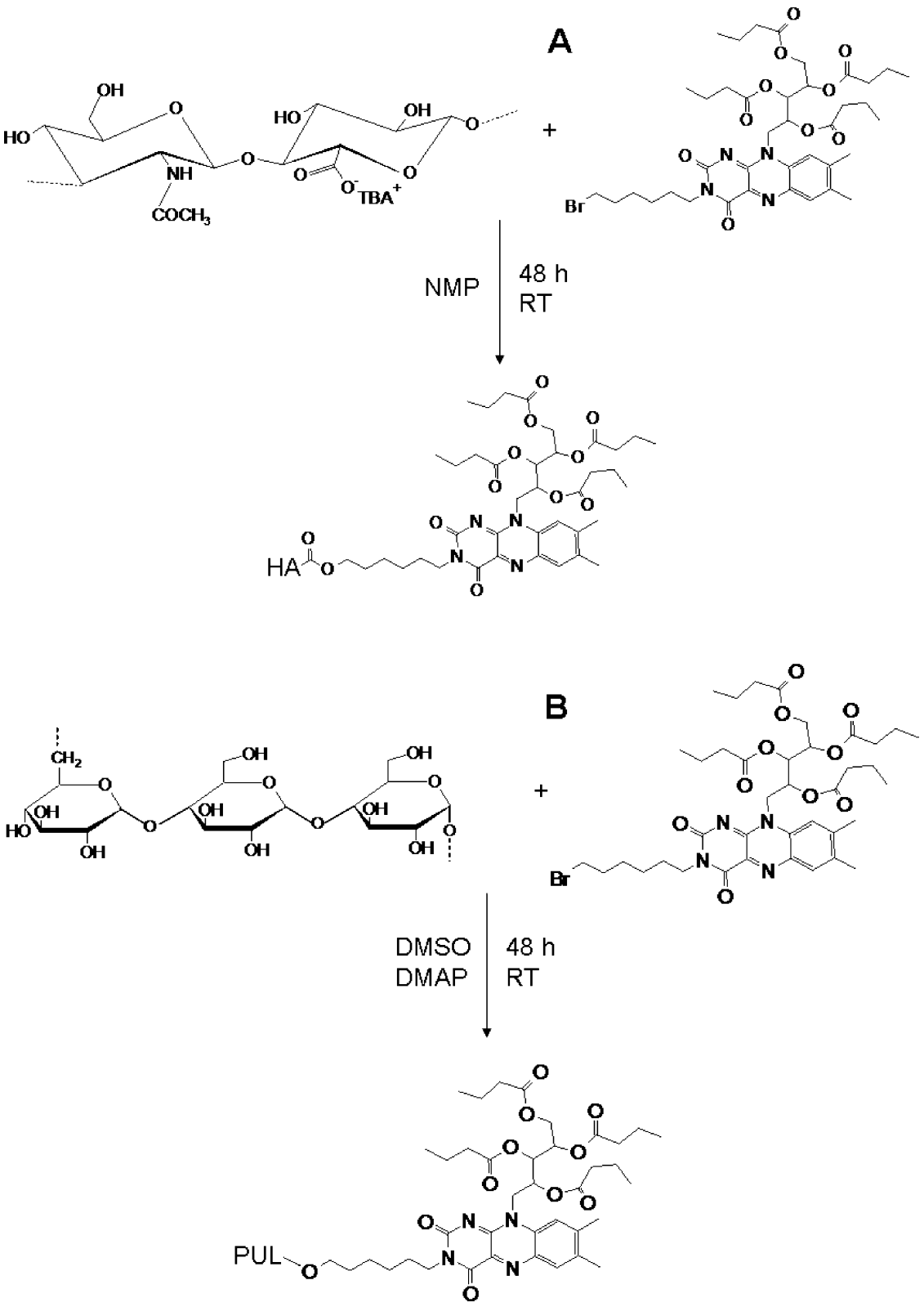
$$\%\,\text{cell viability} = \frac{OD_{test\_item} - OD_{blank}}{OD_{negative\_control} - OD_{blank}} \times 100$$

All experiments were carried out in triplicate.

2.2.8. Model drugs loading in HA-Rfv NHs

To test the ability of the new NHs to carry drugs, the antibiotic levofloxacin (LVF) was chosen as model hydrophilic molecule. HA-Rfv (6.0 mg) were dispersed in 4.0 mL of a LVF aqueous solution (0.5 mg/mL) and the sample was autoclaved (121 °C, 1.10 bar, 20 min), according to a new loading method recently proposed (Montanari et al., 2014a,b). LVF-loaded HA-Rfv NHs (LVF-HA-Rfv) were purified from the unloaded levofloxacin by dialysis against distilled water for 3 h. To evaluate the drug entrapment efficiency (EE%, w/w), LVF-HA-Rfv NHs were freeze-dried and dissolved in NMP (0.30 mg/mL). The LVF concentration was evaluated at λ = 302 nm with an UV–vis spectrophotometer, Perkin-Elmer, double beam "Lambda 3A", using a linear calibration plot for LVF in NMP obtained in the range 0.75–12.0 μg/mL ($n = 5$, $R^2 = 0.9994$). The EE% was determined as the ratio between the amount of the loaded LVF and the weight of dry polymer multiplied by 100.

As hydrophobic model drug, the non-steroidal anti-inflammatory drug piroxicam was used. In this case, 250 μg of the drug was dissolved in 250 μL of dichloromethane and the solvent was vacuum evaporated thus forming a film. The NHs aqueous suspension (1 mL, 2 mg/mL) was then added, vortexed and left overnight under magnetic stirring. The system was then autoclaved and the loaded NHs suspension was centrifuged (3000 rpm, 10 min, 4 °C) to precipitate non-loaded piroxicam which was re-dissolved in ethanol and analyzed by UV–vis spectrophotometry at λ = 356 nm; the amount of non-loaded piroxicam was evaluated using a linear calibration plot for piroxicam in ethanol obtained in the range 1.57–6.25 μg/mL ($n = 5$, $R^2 = 0.9998$). The EE% was determined as the ratio between the amount of the loaded piroxicam and the weight of dry polymer multiplied by 100.



Scheme 2. Synthesis of HA-Rfv (A) and Pul-Rfv (B) derivatives.

3. Results and discussions

The present work deals with the preparation of a new nanohydrogel platform based on self-assembling polysaccharide chains derivatized with Rfv. To overcome the difficulty originated from the poor solubility of Rfv, a tetrabutyril derivative of Rfv was used as starting molecule. The Br derivative was thus obtained by a simple chemical approach and the resulting Br-Rfv was directly linked to HA or Pul chains, in order to remarkably decrease water solubility of the polysaccharides. It is interesting to point out that the overall synthetic approach could be applied both to negatively charged and neutral polysaccharides. In the present work, the

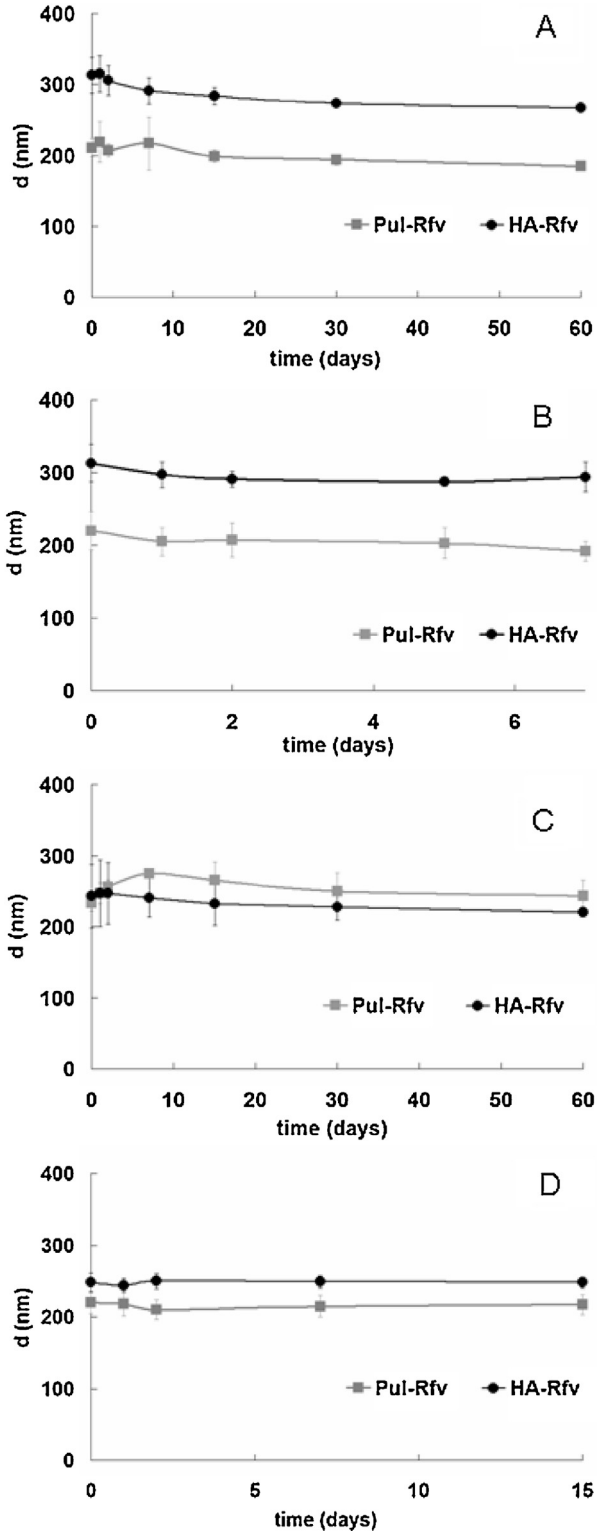


Fig. 1. Average dimensions of HA-Rfv and Pul-Rfv NHs as a function of time at 1 mg/mL (A) in water at 4 °C (HA-Rfv pH = 6.0, Pul-Rfv pH = 6.8), (B) in water at 37 °C (HA-Rfv pH = 6.0, Pul-Rfv pH = 6.8), (C) in NaCl 0.9% w/v at 4 °C, (HA-Rfv pH = 6.0, Pul-Rfv pH = 6.8) and (D) in phosphate buffered solution, 0.1 M, T = 37 °C, pH = 7.4.

negatively charged HA and the neutral Pul were chosen as model polymers.

The new polymeric compounds can be easily moulded in order to obtain cytocompatible and very stable nanohydrogel systems that can be exploited for biomedical and pharmaceutical applications, with a particular focus on drug delivery. The procedure here adopted to form nanohydrogels is based on the ability of polysaccharide chains, containing a hydrophobic moiety, to self-assemble in nanostructures in specific experimental conditions. The development of this platform and the cytocompatibility data are reported.

3.1. Synthesis of HA and pullulan derivatives

The synthesis of tetrabutyrilriboflavin derivative was carried out in DMF using potassium carbonate as a base to deprotonate the N3 nitrogen that was afterwards alkylated using an excess of 1,6-dibromohexane (Scheme 1). This step yielded the mono-NH alkylated derivative carrying a residual bromine at the terminal position of the hexyl chain, required for the subsequent polymer grafting. The reaction progress was easily monitored by ESI–MS that showed the exclusive presence of the mono-alkylated product after a reaction time of 5 h. The pure product was easily obtained by column chromatography.

The Br-derivative of tetrabutyrilriboflavin was able to react both with the carboxylic groups of HA, similarly to reactions previously carried out with paclitaxel (Leonelli et al., 2005), carboranes (Di Meo et al., 2007), and cholesterol (Montanari et al., 2013), as well as with the hydroxyl groups of Pul in the presence of DMAP as catalyst.

In the case of HA, the reactivity of the Br-Rfv derivatives towards the carboxylic groups present along the polymer chains was exploited to chemically link the hydrophobic moiety to the polysaccharide. This approach can generally be carried out with anionic polysaccharides. The reaction, which follows a general scheme already described in literature using different molecules (Leonelli et al., 2005; Di Meo et al., 2007; D'Arrigo et al., 2012; Montanari et al., 2013), was carried out by mixing the Rfv derivative and the polymer in the TBA form, leading to the formation of an ester bond. The reaction scheme is summarized in Scheme 2A.

In the case of Pul, and in general for neutral polysaccharides, an ether bond can be formed (according to the scheme depicted in Scheme 2B), by means of the activation of the polymer chains with DMAP and the following reaction with Br-tetrabutyrilriboflavin, leading to the formation of the more stable ether linkage.

Due to the natural fluorescence of riboflavin, it was possible to determine the HA and Pul derivatization degree by a calibration curve obtained by UV–vis spectrophotometry. The UV–vis absorption spectrum of riboflavin tetrabutyrate solubilized in DMSO (Drössler, Holzer, Penzkofer, & Hegemann, 2003) was evaluated in the range 200–600 nm where it exhibited maxima absorbances at 272, 345 and 445 nm. HA-Rfv and Pul-Rfv derivatives completely solubilized in DMSO showed the same maxima values. To assess the HA and Pul derivatization degree, the riboflavin concentration for a given polymer derivative solution in DMSO, was evaluated, exploiting the calibration plots carried out at the above mentioned peaks. The average derivatization degree for HA and Pul was 30% and 5% mol/mol, respectively. It is worth to note that the esterification reaction carried out in the case of HA was quantitative, leading to 3 derivatized repeating units out of 10, whereas the etherification reaction with pullulan/DMAP was less efficient: only the 20% of the used Br-tetrabutyrilriboflavin reacted and one pullulan repeating unit out of 20 was derivatized.

3.2. NH preparation, characterization and stability

NHs can be easily obtained from HA-Rfv and Pul-Rfv derivatives. Even though the sonication process was suitable for both systems, leading to NHs formation, for the HA-Rfv sample a new method to obtain NHs was tested, i.e. the autoclaving process, already used for the preparation of hyaluronic acid-cholesterol

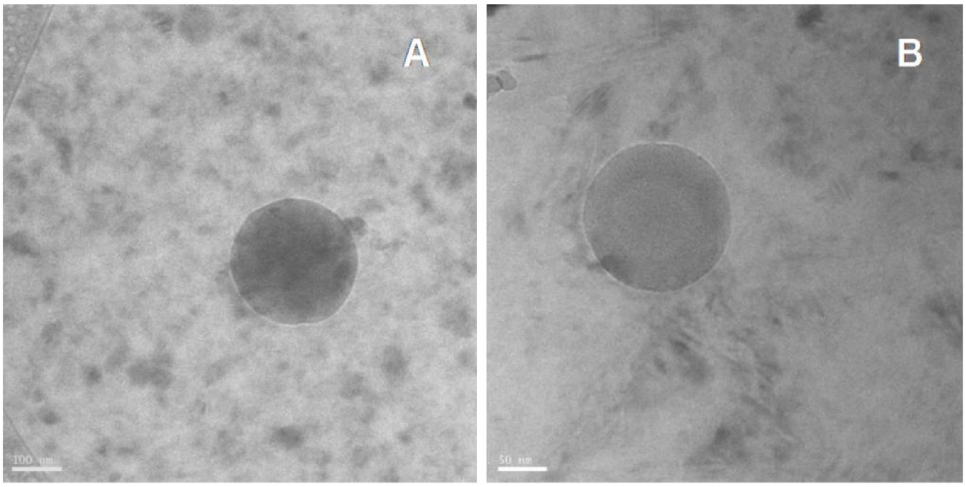


Fig. 2. Cryo-TEM micrographs of HA-Rfv NHs (A) and Pul-Rfv NHs (B).

and gellan-cholesterol (Montanari et al., 2014b) sterile NHs suspensions. In the present case, HA-Rfv suspended in water can be autoclaved to generate spontaneously NHs by self-assembling of the polymer chains. High pressure and high temperature conditions seem to be able to promote the interactions between hydrophobic moieties linked to the polymer chains (inter- and intramolecular interactions as well), thus forming sterile nanoparticulate suspensions. HA-Rfv NHs were actually prepared by treating the water suspension (1 mg/mL) of the polymer for 20 min at 121 °C, 1.10 bar. The obtained NHs showed a size of 312 ± 10 nm, a polydispersity index of 0.15 ± 0.05 and a ζ-potential of about −30 mV.

For the preparation of Pul-Rfv NHs a different procedure was adopted, as the autoclaving process was not efficient. Pul-Rfv were obtained in water at the concentration of 1 mg/mL by bath sonication for 25 min. The size of the obtained NHs was 210 ± 20 nm, with a PDI of 0.2 ± 0.05; the ζ-potential in water was −18 mV.

The stability of the HA-Rfv and Pul-Rfv NHs suspensions was investigated in water at 4 °C and, in this conditions, both NHs were stable for two months (Fig. 1A), whereas at 37 °C the suspensions were stable for one week (Fig. 1B). Stability studies carried out in physiological conditions showed that both NHs were stable for two months in NaCl 0.9% w/v at 4 °C and for at least 15 days in phosphate buffered solution 0.1 M, pH = 7.4 at T = 37 °C, as reported in Fig. 1C and D.

As expected, the mean dimensions of the HA-Rfv NHs were affected by the presence of the NaCl and a significant reduction was observed. This effect can be ascribed to the screening of the HA carboxylic groups negative charges by the Na^+ ions; the reduced repulsions allowed the polymer chains to be closer, thus reducing the NHs mean dimensions. For the same reason, the mean dimensions of the HA-Rfv NHs were lower in phosphate buffered solution 0.1 M, pH = 7.4.

It is important to note that HA-Rfv and Pul-Rfv NHs were stable in physiological conditions while in the case of NHs previously prepared with HA-cholesterol derivatives aggregation occurred in few hours (Montanari et al., 2013). This property represents a significant advantage of the new polysaccharide-Rfv NHs for their potential use in the field of pharmaceutics and biomedicine.

Cryo-TEM analyses (Fig. 2A and B) showed that HA-Rfv and Pul-Rfv NHs have a spherical shape with a diameter of about 250 nm and 150 nm for HA based and Pul based NHs, respectively. As expected, the mean size of the NHs obtained from Cryo-TEM micrographs was slightly smaller than that obtained from DLS analysis; such difference is to be related to the high hydrophilicity of the polysaccharides that increases the hydrodynamic radius of the nanospheres.

Fluorescence of HA-Rfv and Pul-Rfv NHs was measured in aqueous suspension at the concentration of 250 μg/mL, by exciting at λ = 345 nm. Fluorescence of riboflavin and riboflavin derivatives in solution is known to be strongly dependent on the solvent used (Kotaki & Yagi, 1970), due to particular interactions between the molecule and the medium that can cause fluorescence quenching. In particular, highly polar solvents induce a decrease in the fluorescence emission (Drössler et al., 2003).

The maximum emission of the NH suspensions in water was at λ = 530 nm, slightly red-shifted from the value of λ = 517 nm found for the Rfv-tetrabutyrate in DMSO (data not shown). The fluorescence intensity was higher for HA-Rfv NHs than for Pul-Rfv NHs, as shown in Fig. 3A and B, due to the lower derivatization degree

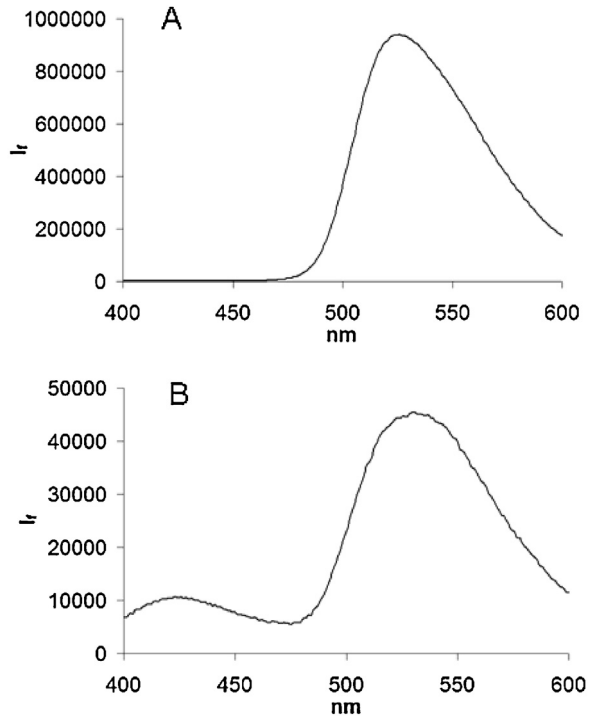


Fig. 3. Fluorescence emission spectra of HA-Rfv NHs (A) and Pul-Rfv NHs (B) 250 μg/mL in water at 25 °C; the excitation λ was 345 nm.

Table 1
Classification of cytocompatibility grades of a medical device according to ISO 10993-5 procedure.

Grade	Reactivity	Conditions of all cultures
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 1.0 cm
4	Severe	Zone extending farther than 1.0 cm beyond specimen

of the latter polymer, and probably to the sonication process. We suppose that sonication can improve the stacking of Rfv molecules, thus affecting, also in this case, the excitation/emission behaviour of the system. Despite these drawbacks, HA-Rfv NH and Pul-Rfv NH suspensions were fluorescent. This intrinsic characteristic represents a promising improvement in the detection of such systems in biological fluids or within cells when these innovative NHs are used in biomedical and pharmaceutical applications as drug delivery systems.

3.3. Cytocompatibility of HA-Rfv and Pul-Rfv NHs

According to the ISO 10993-5 standard procedure, the qualitative analysis of NHs cytocompatibility was evaluated by the direct observation of possible cell degenerations or malformations using an inverted microscope after 24 h of exposure to the samples. Obtained results were classified according to a scale ranging from 0 to 4 (Table 1).

According to this classification and referring to the positive control leading to a severe (grade 4) toxicity, both HA-Rfv and Pul-Rfv NH, in the form of undiluted suspensions and after the various dilutions appeared to be completely safe for cells (grade 0 of reactivity for all tested samples).

As reported in Fig. 4, quantitative analysis of NHs cytocompatibility, expressed as percentage cell viability, showed a complete safety of both NHs suspensions also in their undiluted form (1 mg/mL).

3.4. Model drug loading in HA-Rfv NHs

In order to test the drug carrier properties of the new NHs here described, HA-Rfv NHs were loaded with levofloxacin as water soluble model drug. The autoclaving process was exploited, allowing the NHs to be formed and loaded in one step (Montanari et al., 2014b). The amount of loaded LVF was determined by re-dissolving the freeze-dried sample in NMP, in order to completely dissolve the polymer and the loaded drug, and detecting LVF spectophotometrically. The drug encapsulation efficiency (i.e. % encapsulation, w/w) was 15.0 ± 2.0% w/w, a value higher than that previously obtained for the NHs based on HA-cholesterol derivatives (5.0 ± 0.5% w/w, Montanari et al., 2014a,b).

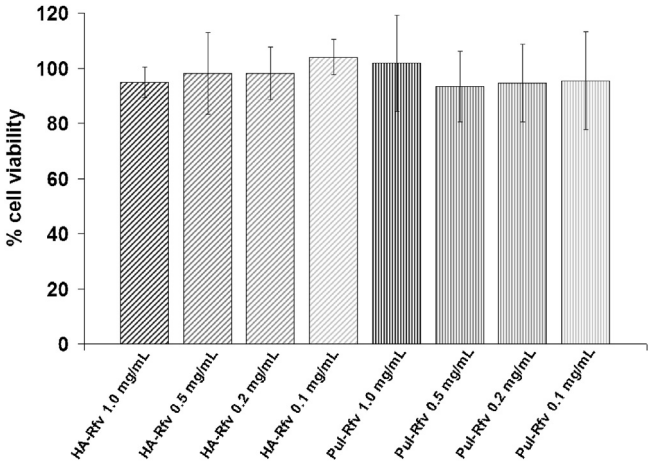


Fig. 4. Cytocompatibility at 24 h of HA-Rfv and Pul-Rfv NH suspensions at different concentrations on mammalian fibroblasts; percentage of cell vitality is referred to the 100% of the negative control. All experiments were carried out in triplicate.

Furthermore, NHs were shown to be able to load also hydrophobic drugs, acting as solubility enhancers. The anti-inflammatory piroxicam was loaded by the solvent-casting technique (D'Arrigo, Navarro, Di Meo, Matricardi, & Torchilin, 2014) followed by autoclave treatment. The purification of the system from unloaded drug was obtained by mild centrifugation and the free piroxicam was spectrophotometrically quantified (EE% = 11.0 ± 1.0% w/w).

4. Conclusions

New platforms capable to offer new opportunities and/or to solve old problematic issues is one of the main challenges that must be faced within the wide topic of drug delivery, in particular in the relatively young field of pharma-nanotechnology.

The new platform described here is based on the use of Rfv that is capable to induce self-assembling properties to the polysaccharide chains once chemically linked to such polymers. Since Rfv is insoluble and consequently difficult to handle, its tetrabutyril derivative was used as starting material. After a derivatization with a six carbon atom chain with a reactive Br atom at the end, Rfv was linked to two different polysaccharides: HA and Pul, two widely used biopolymers, negatively charged the former (due to the carboxylic groups on the glucuronic acid), neutral the latter.

The derivatization reaction, carried out on both polysaccharides, modified the polymer chains properties thus leading to self-assembling systems that are capable, following different preparation procedures – i.e. bath sonication, autoclave processing – to form nanohydrogels. Due to their biocompatibility, and stability in physiological conditions, these new nano-devices can be successfully exploited for biomedical and pharmaceutical applications. In this sense the new platform reported in the present paper can represent an interesting opportunity because of its versatility, fluorescence properties, stability in water and physiological conditions. Moreover, the simple procedures for the nanosystem formation, drug loading, sterilization, are very useful for an easy scale-up for industrial purposes.

Acknowledgements

Financial supports from University "Sapienza" (UNIV_2013), grant no. C26A13JTRW, are acknowledged.

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