New Biotechnology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

New Biotechnology

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

Full length Article

Hyaluronan-cholesterol nanohydrogels: Characterisation and effectiveness in carrying alginate lyase

Elita Montanari[a], Chiara Di Meo[a], Simona Sennato[b,c], Antonio Francioso[d], Anna Laura Marinelli[a], Francesca Ranzo[a,b,c], Serena Schippa[e], Tommasina Coviello[a], Federico Bordi[b,c], Pietro Matricardi[a,*]

[a] Department of Drug Chemistry and Technologies, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[b] Institute for Complex Systems, National Research Council, Via dei Taurini 19, 00185 Rome, Italy
[c] Physics Department, Sapienza University of Rome, P.le Aldo Moro 5, 00185, Rome, Italy
[d] Department of Biochemical Sciences, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[e] Department of Public Health and Infectious Diseases, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Nanohydrogel
Hyaluronan
Self-assembly
Protein delivery
Alginate lyase

ABSTRACT

Although in recent years several methods have been studied and developed to obtain different types of nanosized drug delivery systems, the set up of suitable procedures and materials remains highly expensive, their preparation is time consuming and often not feasible for a scale-up process. Furthermore, the sterilisation and storage of nanocarrier formulations represents a complicated but mandatory step for their effective use. In our previous work we assessed the use of an autoclaving process to achieve, in one simple step, sterile self-assembled hyaluronan-cholesterol (HA-CH) and hyaluronan-riboflavin (HA-Rfv) nanohydrogels (NHs). In the present work, the effect of the high temperature on HA-CH has been studied in detail. HA-CH suspensions were characterised in terms of size and polydispersity by Dynamic Light Scattering at different temperatures and conditions; the HA-CH chemical structure and its molecular weight were assessed via FT-IR and GPC analysis after the sterilising cycle in an autoclave at 121 °C for 20 min. The obtained NHs were then observed with TEM and AFM microscopy, in both dry and liquid conditions. The Young's modulus of the NHs was determined, evidencing the soft nature of these nanosystems; the critical aggregation concentration (c.a.c) of the nanosuspension was also assessed. Thereafter, alginate lyase (AL) was conjugated to NHs, with the aim of developing a useful system for therapies against bacterial infections producing alginate biofilms. The conjugation efficiency and the enzymatic activity of AL were determined after immobilisation. The AL-NHs system showed the ability to depolymerise alginate, offering an opportunity to be a useful nanosystem for the treatment of biofilm-associated infections.

Introduction

In recent years, self-assembled NHs, based on various polymer systems and prepared using different protocols, have been widely studied as carriers for drugs, therapeutic proteins and genetic material, showing a broad spectrum of effectiveness and drawbacks.

NHs are nanosized, three-dimensional networks able to absorb a high amount of water, showing swelling and de-swelling properties. NHs are usually soft, hydrophilic and biocompatible and represent a highly versatile nanosystem able to deliver a variety of bioactive molecules [1–3]. The porosity of the NH network provides a reservoir for loading molecular and macromolecular therapeutics, showing, in many cases, a protective action against degradation due to environmental conditions. In particular, self-assembled NHs have been recognised as promising carriers for macromolecular therapeutics [4], since their hydrophobic domains, surrounded by a hydrophilic outer shell, can serve as a reservoir for various hydrophobic and/or hydrophilic macromolecular drugs. Furthermore, Akiyoshi and collaborators have studied extensively the molecular chaperone-like activity of such self-assembled NHs based on pullulan [5].

Abbreviations: AL, alginate lyase; ALG, alginate; HA, hyaluronan; HA-CH, hyaluronan-cholesterol; HA-Rfv, hyaluronan-riboflavin; NHs, nanohydrogels; PDI, polydispersity index.

* Corresponding author.
E-mail addresses: pietro.matricardi@uniroma1.it, p.matricardi@libero.it (P. Matricardi).

http://dx.doi.org/10.1016/j.nbt.2016.08.004
1871-6784/

Hyaluronan (HA) is a well known polysaccharide, a linear and poly-anionic biopolymer composed of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine monomeric units linked together through β-1,4 and β-1,3 glycosidic bonds. In recent years, HA has received enormous attention as a biomaterial for the development of nanosystems. The major benefits of HA-based nanocarriers are a general biocompatibility, low-toxicity, biodegradability and ability to solubilise hydrophobic molecules and to actively target to cells where CD44 receptors are over-expressed [6]. Furthermore, HA demonstrates the natural ability to interact with several proteins in the body [7], enhancing and preserving their activity. The modification of HA chains (via ester or amide bonds) with small hydrophobic molecules, such as cholesterol, 5-β cholanic acid and tetrabutyril-riboflavin, leads to amphiphilic polymer chains which are able to self-assemble in aqueous media, thus forming NHs after suitable treatments. Nanoprecipitation, dialysis against water and sonication are the most frequently used methods to obtain self-assembled HA-based NHs. However, these treatments are not usually useful for scale-up intended for industrial applications.

In our previous work [1,8] we assessed the use of the autoclaving process to achieve, in one simple step, sterile self-assembled hyaluronan-cholesterol (HA-CH), hyaluronan-riboflavin (HA-Rfv) and gellan-cholesterol NHs. The resulting NHs were homogeneous in size and shape, with a low PDI, showing a good drug-loading capability, high stability in various storage conditions and, very importantly, sterility. Therefore, from the previously observed effect of the standard autoclave cycle in forming NHs and entrapping drugs during the same thermal cycle [1,8], the present work was aimed first to study the effect of temperature on HA-CH in achieving, in a fast and very reproducible step, sterile self-assembled hyaluronan-cholesterol NHs. The HA-CH NHs formed in this way were characterised *via* different techniques evidencing their spherical shape and soft nature; moreover, a correlation between the decrease in size and the polymer molecular weight (M_W) was observed.

These HA-CH NHs were also tested as useful carriers of the enzyme alginate lyase (AL). AL catalyses the degradation of alginate (ALG) by a β-elimination mechanism, forming 4-deoxy-5-keto-uronic acid and the unsaturated uronic acid moiety of the intermediate oligosaccharides [9,10]. ALG is the major component of the polymer matrix of biofilm and contributes to bacterial antibiotic resistance [11,12]. AL treatment in patients affected by biofilm-associated infections could represent an interesting approach, thanks to the ability of AL to reduce the viscosity of ALG. Hatch and collaborators demonstrated the ability of AL to promote the diffusion of aminoglycosides through the extracellular polysaccharide of *Pseudomonas aeruginosa* mucoid strains [13]. Therefore, with the aim of developing a potentially useful system for the treatment of biofilm-associated infections, AL was conjugated *via* non covalent interactions to HA-CH NHs and its enzymatic activity and ability to depolymerise alginate was observed following immobilisation into NHs.

Materials and methods

Chemicals

Hyaluronan tetrabutylammonium salt (HA^-TBA^+, M_w 150×10^3) was kindly provided by Fidia Advanced Biopolymers, Abano Terme (PD), Italy; riboflavin tetrabutyrate (Rfv) was purchased from TCI Europe N.V., Belgium. Alginate Lyase (AL) from *Flavobacterium* sp., sodium alginate (ALG) ($M_w = 1.2 \times 10^5$), periodic acid, sodium thiosulphate, 2-thiobarbituric acid (TBA), levofloxacin (LVF), dimethyl sulfoxide (DMSO), *N*-methyl-2-pyrrolidone. (NMP), 1,6-dibromohexane, 4-(Dimethylamino) pyridine (DMAP), were Sigma-Aldrich products.

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

Synthesis of hyaluronan-cholesterol derivative

The synthesis of HA-CH derivative was carried out as previously described [14]. Briefly, in the first step, cholesterol (CH) (500 mg, 1.3 mmol) was reacted with 4-bromo-butyric acid (648 mg, 3.9 mmol), EDC·HCl (744 mg, 3.9 mmol) and DMAP (79 mg, 0.65 mmol) in 10 mL of CH_2Cl_2 at room temperature, overnight [14] to form the CH-Br derivative.

HA^-TBA^+ (200 mg, 0.32 mmol) was dissolved in 10 mL of *N*-methyl-2-pyrrolidone (NMP) and the solution was mixed with the CH-Br derivative (34.3 mg) solubilised in 2 mL of NMP.

The reaction was mixed for 48 h at 38 °C. Exhaustive dialysis against distilled water (Visking tubing, cut-off: 12,000–14,000) was then carried out and HA-CH was finally recovered by freeze-drying. The resulting derivatisation degree was 18% (mol/mol), as determined by NMR [14].

NHs formation and characterisation

To produce NHs, HA-CH was dispersed in water (1.0 mg/mL) and kept under magnetic stirring overnight, at room temperature (RT). HA-CH suspension was then placed in an autoclave and a standard sterilising cycle performed (121 °C for 20 min).

Dynamic light scattering (DLS)

To study the effect of temperature on NHs formation, HA-CH (1.0 mg/mL) was dispersed in distilled water and kept under magnetic stirring overnight at RT. The suspension in a closed vial was then placed in an oven programmed with a temperature ramp from 25 °C to 121 °C. HA-CH suspension was removed at different temperature points (25, 80, 90, 100, 110 and 121 °C for 20 min). The hydrodynamic size and polydispersity (PDI) of the HA-CH suspensions were analysed by DLS measurements at room temperature, using a Submicron Particle Sizer Autodilute Model 370 (NanoZetaSizer, Malvern, UK).

In another experiment, size and PDI of HA-CH suspensions (1.0 mg/mL, in water) were also studied after thermal treatment in an autoclave (121 °C), with various time intervals lasting from 0 to 55 min. The DLS results were analysed using the cumulant method to derive the average hydrodynamic size and the corresponding PDI of the sample [15].

Fourier transform infrared spectroscopy (FTIR)

HA-CH suspension (1.0 mg/mL) was autoclaved (121 °C, 20 min) and then freeze-dried. FT-IR spectra of the solid HA-CH (before and after the thermal treatment) were performed using a Spectrum One FT-ATR spectrophotometer (PerkinElmer).

Gel permeation chromatography

HA^-TBA^+ aqueous solution (1.0 mg/mL) was treated by autoclaving (121 °C for 20 min) and freeze-dried. GPC analysis was carried out using a Varian HPLC system, equipped with a Varian Refractive index (356-LC) detector. Both untreated and thermally treated HA^-TBA^+ were dissolved in 0.01 M tetrabutylammonium bromide (TBA^+Br^-) and filtered through a 0.45 μm filter at a concentration of 1.5 mg/mL, before injection into a bank of three "TSK gel GMPWXL"- Tosoh Bioscience (13 μm, 300 × 7.8 mm) columns, at a flow rate of 1.0 mL/min. A calibration curve based on pullulan (range of M_W 11200–788000) in TBA^+Br^- (0.01 M) was used. GPC data were analysed using the Cirrus software.

HA-CH aqueous suspension (1.0 mg/mL) was treated by autoclaving (121 °C for 20 min) and then freeze-dried. GPC analysis

Please cite this article in press as: E. Montanari, et al., Hyaluronan-cholesterol nanohydrogels: Characterisation and effectiveness in carrying alginate lyase, New Biotechnol. (2016), http://dx.doi.org/10.1016/j.nbt.2016.08.004