Carbohydrate Polymers 142 (2016) 213–221

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Assembly of HE800 exopolysaccharide produced by a deep-sea hydrothermal bacterium into microgels for protein delivery applications

Agata Zykwinska[a,*], Mélanie Marquis[b], Corinne Sinquin[a], Stéphane Cuenot[c], Sylvia Colliec-Jouault[a]

[a] Ifremer, Laboratoire Ecosystèmes Microbiens et Molécules Marines pour les Biotechnologies, 44311 Nantes, France
[b] INRA, UR1268 Biopolymères Interactions Assemblages, F-44300 Nantes, France
[c] Institut des Matériaux Jean Rouxel (IMN), Université de Nantes-CNRS, 44322 Nantes, France

ARTICLE INFO

Article history:
Received 21 October 2015
Received in revised form 14 January 2016
Accepted 25 January 2016
Available online 29 January 2016

Keywords:
Marine exopolysaccharide
Microgel particles
Microfibers
Microfluidic
Microencapsulation
Protein release

ABSTRACT

Assembly of biopolymers into microgels is an elegant strategy for bioencapsulation with various potential biomedical applications. Such biocompatible and biodegradable microassemblies are developed not only to protect the encapsulated molecule but also to ensure its sustained local delivery. The present study describes the fabrication of microassemblies from a marine HE800 exopolysaccharide (EPS), which displays a glycosaminoglycan (GAG)-like structure and biological properties. HE800 EPS was assembled, through physical cross-linking with divalent ions, into microgel particles and microfibers using microfluidics. The microparticle morphology was highly affected by the polysaccharide concentration and its molecular weight. A model protein, namely Bovine Serum Albumin (BSA) was subsequently encapsulated within HE800 microparticles in one-step process using microfluidics. The protein release was tuned by the microparticle morphology with a lower protein amount released from the most homogeneous structures. Our findings demonstrate the high potential of HE800 EPS based microassemblies as innovative protein microcarriers for further biomedical applications.

1. Introduction

Hydrogels are biomaterials widely explored for their broad range of applications in medicine and pharmaceutics. These highly hydrophilic three-dimensional polymer networks present some similarities to the extracellular matrix of connective tissue and are therefore frequently used as scaffolds to engineer new tissues in combination with cells and biological signaling molecules (e.g. proteins) (Lee & Mooney, 2001). Because these biological molecules crucial for cellular responses are extremely fragile, new strategies for their protection and delivery have been developed based on hydrogels structured at micrometer scale (DeFail, Chu, Izzo, & Marra, 2006; Park, Na, Woo, Yang, & Park, 2009; Bian et al., 2011; Ansboro et al., 2014). Indeed, microencapsulation of bioactive species allows not only to protect them from external degradation conditions (e.g. chemical, physical or enzymatic), but also offers the possibility for their sustained local delivery, which considerably enhances their bioavailability and efficiency. Hydrogels engineered for biological applications need to fulfill several requirements to ensure their therapeutic efficacy. Because biocompatibility and biodegrability are the most critical parameters, natural polymers such as polysaccharides appear as ideal candidates for the development of smart delivery microgel-based systems. Among all polysaccharides, alginate (Bian et al., 2011; Silva, Ribeiro, Ferreira, & Veiga, 2006; Jay & Saltzman, 2009) and chitosan (Bugamelli, Raggi, Orienti, & Zecchi, 1998; Niu, Feng, Wang, Guo, & Zheng, 2009; Koppolu et al., 2014) microgels (e.g. microparticles, microcapsules) have been used widely for controlled delivery of proteins. Due to its polyanionic nature, alginate gels easily through ionic cross-linking in the presence of divalent cations. Mild gelation conditions and the use of non-toxic reactants are extremely important when encapsulating proteins. However, alginate is biologically inert and chemical modifications are required to enhance its bioactivity (Genes, Rowley, Mooney, & Bonassar, 2004; Freeman, Kedem, & Cohen, 2008). On the contrary to alginate, chitosan presents some structural similarities with glycosaminoglycans (GAGs), natural constituents of the extracellular matrix, which participate in many biological processes through specific

* Corresponding author. Tel.: +33 240374065.
E-mail address: Agata.Zykwinska@ifremer.fr (A. Zykwinska).

http://dx.doi.org/10.1016/j.carbpol.2016.01.056
0144-8617/

interactions with growth factors, receptors and adhesion proteins (Casu & Lindahl, 2001). It can then be thought that the analogous structure may also have related bioactivities. However, GAGs are all anionic polysaccharides, on the contrary to chitosan, which is a cationic polyelectrolyte. Therefore, chitosan is often used in association with negatively charged GAGs and other polyanions to form ionic complexes (Suh & Matthew, 2000). Polysaccharides from GAG family, such as hyaluronic acid (HA) (Ansboro et al., 2014) and chondroitin sulfate (Lim et al., 2011) were also explored as microcarriers for signaling agent delivery because of their natural presence and their crucial functions within the extracellular matrix of connective tissue. However, in order to induce gel formation, chemical modifications of these polysaccharides are required. The toxicity of cross-linking molecules as well as non-degradable cross-linking setting must therefore be considered and may become limiting factors in biological applications. Thus, the research of new molecules that can easily be structured into microgels for bioactive compounds delivery and which are endowed with biological activities (e.g. similar to GAGs) is still encouraged.

HE800 exopolysaccharide (EPS) is an innovative polysaccharide secreted by deep-sea hydrothermal bacteria *Vibrio diabolicus*, which displays interesting biological activities resulting from its unique structure (Raguénès, Christen, Guezennec, Pignet, & Barbier, 1997; Rougeaux, Kervarec, Pichon, & Guezennec, 1999; Zanchetta, Lagarde, & Guezennec, 2003; Senni et al., 2013). HE800 EPS is a linear non-sulfated polysaccharide of a tetrasaccharidic repeating unit composed of *N*-acetyl-glucosamine (GlcNAc), two glucuronic acids (GlcA) and *N*-acetyl-galactosamine (GalNAc) covalently linked in the following sequence: →3)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 4)-β-D-Glc*p*A-(1 → 4)-α-D-GalpNAc-(1→ (Rougeaux et al., 1999). This unusual structure presents some similarities to the HA structure, which contains alternating GlcNAc and GlcA residues linked by β-(1 → 4) and β-(1 → 3) bonds (Atkins & Sheehan, 1971). Besides its HA-like structure, native HE800 EPS, either in dry or in soluble state, was demonstrated to possess GAG-like properties since it enhanced *in vivo* bone regeneration (Zanchetta et al., 2003) and stimulated collagen structuring as well as extracellular matrix settle by fibroblasts in reconstructed dermis (Senni et al., 2013). Both structural and functional features of HE800 EPS could therefore be explored to elaborate new microcarriers for biological molecule delivery that can further be used in tissue engineering applications.

When developing microgel-based delivery systems, it is important to properly control the particle size and size distribution, which both highly influence release kinetics. Microgels are usually produced by emulsification methods and different techniques are applied, e.g. dripping, jetting, sonication (Fundueanu, Nastruzzi, Carpov, Desbrieres, & Rinaudo, 1999). However, none of these techniques allows to obtain microgels with a narrow size distribution and permits to encapsulate the totality of the bioactive species. Moreover, the use of high energy and temperature may lead to alteration of the polysaccharide structure and to degradation of the biological molecules to be encapsulated within the microgels. Taking into account all these parameters, microfluidic technology, manipulating multiphase laminar flows to produce homogeneous structures, appeared as a versatile method for generating micrometer-sized droplets with controllable size and functionality (Zhang, Tumarkin, Sullan, Walker, & Kumacheva, 2007; Marquis, Renard, & Cathala, 2012; Marquis, Davy, Fang, & Renard, 2014). Another incontestable advantage of microfluidics is the possibility to produce microstructures and, at the same time, to encapsulate the totality of the bioactive molecules, in one-step process. This process conducts to a homogeneous distribution of these molecules within microgel (Xu et al., 2009). Although highly advantageous, the use of microfluidic technology to produce microgels for bioactive compound encapsulation has not completely been explored yet as few studies were only reported (Xu et al., 2009; Chen et al., 2013).

In the present work, HE800 EPS, innovative polysaccharide from marine origin displaying both GAG-like structure and biological properties has been used for the first time to generate microgels that could further be used as carriers for bioactive molecule delivery, especially in regenerative medicine field, e.g. to reconstruct bone or cartilage tissues. Indeed, when embedded into hydrogel scaffold, these composite carriers are expected to support cell morphogenesis and proliferation through a sustained delivery of encapsulated compounds. HE800 EPS was physically structured in the presence of divalent cations into microparticles and microfibers using microfluidics. In order to optimize the experimental microfluidic parameters, numerical simulations were developed as predictive tools. The influence of the polysaccharide concentration and its molecular weight on the microparticle morphology was assessed. Furthermore, the use of these microgels as vehicles for sustained delivery of a model protein, namely Bovine Serum Albumin (BSA), was evaluated and the protein release was studied.

2. Materials and methods

2.1. Production of the native HE800 EPS and HE800 derivatives, and their characterization

HE800 EPS is naturally produced under controlled conditions by fermentation of a non-pathogenic marine bacteria, *V. diabolicus*, HE800 strain (CNCM: I-1629), isolated in a deep sea hydrothermal vent in the East Pacific Rise from a polychaete annelid *Alvinella pompejana*. HE800 EPS was produced as previously described (Raguénès et al., 1997; Rougeaux et al., 1999) using a 2 L fermenter containing 1 L of marine 2216 broth medium supplemented with glucose, at atmospheric pressure, at 25 °C and pH 7.2. HE800 derivatives (HE800 DRs) were obtained by a free-radical depolymerization process (Senni et al., 2008). Briefly, native EPS was dissolved in water at 7 wt% and 2 g of $(CH_3COO)_2Cu$ were added. The resulting mixture was then heated at 60 °C and the pH was set at 7.5. A diluted H_2O_2 solution was continuously added (1 mL/min) to the EPS solution under controlled pH conditions using a pHstat (Hache and Lange). The polysaccharide chains were stabilized by an overnight room temperature reduction reaction with $NaBH_4$. Excess of $NaBH_4$ was quenched with CH_3COOH (10 M). Copper cations were chelated on Chelex® 20 resin (sodium form). The resulting solution of HE800 derivatives in the form of sodium salts was ultrafiltrated before being freeze-dried.

Sugar composition of the native HE800 EPS and HE800 DRs was determined by gas chromatography analysis of trimethylsilyl derivatives after acidic methanolysis (Kamerling, Gerwing, Vliegenthart, & Clamp, 1975). The weight-average molecular weight (M_w) was determined by High-Performance Size Exclusion Chromatography (HPSEC) coupled with a multi-angle light scattering detector (MALS, Dawn Heleos-II™, Wyatt technology) and a differential refractive index (RI) detector (Hitachi L2490). HPSEC system was composed of an HPLC system Prominence Shimadzu™, a PL aquagel-OH mixed (Varian) guard column, and a PL aquagel-OH mixed (Varian) separation column.

2.2. HE800 DR sample preparation for AFM imaging

HE800 DR (300,000 g/mol) was solubilized overnight at 0.1 wt% in MilliQ water. The solution was then diluted at 5 μg/mL in water. $CuCl_2$ solution at 0.5 wt% was prepared in MilliQ water and was mixed (1:1, v/v) with an aqueous HE800 DR solution at 0.1 wt%. The mixture was diluted at 5 μg/mL. 10 μL of each diluted solution