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Microbicidal gentamicin-alginate hydrogels

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ABSTRACT

Sodium alginate (Alg) reacted with antibiotic gentamicin sulfate (GS) in an aqueous-phase condition mediated by carbodiimide chemistry, in the molar ratios Alg: GS of (1:0.5), (1:1) and (1:2). The Alg-GS conjugated derivatives were characterized by elemental analysis for nitrogen content, Fourier transform infrared spectroscopy in the attenuated total reflection mode (FTIR-ATR), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), thermogravimetric analyses (TGA) and water sorption measurements. XPS and FTIR-ATR analyses clearly indicated that GS molecules covalently attached to the backbone of the alginate chains by amide bond formation. The highest amount of GS bound to Alg (43.5 ± 0.4 wt%) and the highest swelling ratio (4962 ± 661%) were observed for the Alg-GS (1:2) sample. Bioluminescence assays with *Pseudomonas aeruginosa* PAO1/*lecA*:*lux* and colony forming counting of *Staphylococcus aureus* and *Escherichia coli* upon contact with all Alg-GS conjugates revealed microbicidal activity; however, Alg-GS (1:2) was the most efficient, due to the highest GS content.

1. Introduction

The increase of multidrug resistant pathogens is one of the top three threats to global public health listed by the World Health Organization (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/, accessed September 5, 2017). The most critical group of all includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and biomedical devices. They include *Acinetobacter*, *Pseudomonas* and various *Enterobacteriaceae*. They can cause severe and often deadly infections such as bloodstream infections and pneumonia (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/, accessed September 5, 2017). For this reason, there is increasing interest in the development of antimicrobial polymers for biomedical devices, food and food packing, textiles, health care products and water treatment systems. Antimicrobial polymers offer some advantages in comparison to low molecular weight quaternary ammonium compounds because they demonstrated to be more efficient, less toxic and long-term active (Jain et al., 2014).

Based on the polymer material type, the antimicrobial polymers can be divided into: (i) polymers with intrinsic antimicrobial activity; (ii) polymeric biocides, which are based on polymer backbones with biocide molecules attached; and (iii) biocide-releasing polymers, which consist of polymers loaded with biocide molecules (Santos et al., 2016; Siedenbiedel & Tiller, 2012). The polymers with cationic groups (such as QACs, quaternary phosphonium, guanidinium or tertiary sulfonium) on their polymeric backbones have intrinsic antimicrobial activity (Carmona-Ribeiro & de Melo Carrasco, 2013; Ganewatta & Tang, 2015). The polymeric biocides result from the chemical attachment of biocide molecules to the polymer chains. The biocide-releasing polymers are based on polymer matrices loaded with biocide molecules, which can be entrapped using different methods, or polymers containing biocides attached by cleavable linkages (Jämsä et al., 2013).

Polysaccharides represent an important class of materials because they are biodegradable, biocompatible, relatively inexpensive and available in a large scale, making them suitable candidates for several applications ranging from the biomedical to the food industry. Until now, chitosan (at pH lower than 6), polylysine and a variety of natural peptides are the only known biopolymers exhibiting intrinsic antimicrobial properties (Carmona-Ribeiro & de Melo Carrasco, 2014;

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Hernández-Montelongo et al., 2016; Rodríguez-Hernández, 2017), all other natural polysaccharides are not antimicrobial and require chemical modification or should be impregnated with antimicrobial agents. Recently, we reported about the outstanding antimicrobial properties of hydroxypropyl methylcellulose (HPMC)/xyloglucan (XG) blends loaded with gentamicin sulfate (GS), an aminoglycoside antibiotic with broad spectrum of antibacterial action and outstanding thermal stability (Kondaveeti, Damato, Carmona-Ribeiro, Sierakowski, & Petri, 2017). The amount of loaded GS was the highest for the 50:50 HPMC:XG blends, and the release followed a non-Fickian release mechanism with diffusional coefficient $n \sim 0.7$, regardless the blend composition (Kondaveeti et al., 2017). On the other hand, the release rate of GS from blends of chitosan and collagen could be adjusted by the chitosan content; the fastest release was observed for chitosan/collagen at the 75/25 ratio (Sionkowska, Kaczmarek, & Gadzala-Kopciuch, 2016). The physical incorporation of GS on crosslinked gellan gum, sodium alginate and collagen crosslinked with Ca^{2+} ions led to membranes that allowed controlled and localized delivery of GS for the treatment of post-operative bone infections (Cibor et al., 2017). Pullulan based superabsorbent hydrogels loaded with GS effectively suppressed bacterial proliferation to protect the wound from bacterial invasion (Li et al., 2011). The physical incorporation of GS to chitosan derivatives led to antimicrobial systems with superior performance in comparison to pure GS or pure chitosan derivatives (Yalinca, Yilmaz, Taneri, & Bullici, 2013). The delivery of GS from polysaccharides avoids problems faced by using synthetic polyesters (Sampath, Garvin, & Robinson, 1992) or poly(acrylic acid) (Nnamani, Kenechukwu, Dibua, Ogbonna, Monemeh, & Attama, 2013), such as contamination by the presence of residual monomers and catalysts, risks of toxicity and high costs.

Alginate (Alg) is an anionic polysaccharide found in the cell walls of brown algae, composed of β-(1–4) linked D-mannuronic acid (M) and α-(1–4) linked L-guluronic acid (G) blocks (Sabra & Deckwer, 2005). Alginate is a low cost, non-immunogenic, biocompatible, natural polymer free of catalysts, which has been successfully applied for the development of microbeads and films as delivery platforms of biocidal agents, such as silver nanoparticles (Ghasemzadeh & Ghanaat, 2014; Sharma, Sanpui, Chattopadhyay, & Ghosh, 2012), copper ions (Madzovska-Malagurski, Vukasinovic-Sekulic, Kostic, & Levic, 2016), enterocin (Marcos, Aymerich, Monfort, & Garriga, 2007), oregano or cinnamon essential oils (Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2006). Specifically for the delivery of GS, alginate was combined with hydroxyapatite (Sivakumar & Rao, 2003) or calcium phosphate bone cement (Chen, Chen, Shie, Huang, & Ding, 2011), crosslinked with aluminum, zinc and copper ions (Goh, Heng, Huang, Li, & Chan, 2008) or modified to alginate dialdehyde and crosslinked with casein (Bajpai, Shah, & Bajpai, 2017). In all these systems, GS molecules were physically attached to the polymeric matrix and were delivered after a given period. There is no report in the literature so far, to the best of our knowledge, which describes the chemical attachment of GS molecules to alginate chains mediated by carbodiimide chemistry, providing long-term polymeric biocides.

Carbodiimide chemistry mediated the attachment of alginate to galactosylated chitosan (Chung et al., 2002), GS molecules to chitosan (Liu, Ji, Lv, Qin, & Deng, 2017) or tobramycin molecules to collagen (Liu, Ren, Long, Wang, & Wang, 2014). We hypothesized that chemical modification of Alg with antibiotic GS by carbodiimide chemistry might be an easy strategy to create new antibacterial biomaterial, which are insoluble in water, and able to kill microbes by contact, without releasing biocides. This kind of antimicrobial material is of great importance to protect surfaces against bacteria for long periods; wound dressings and scaffolds for tissue engineering are potential applications. In order to test the hypothesis, we synthesized alginate-gentamicin (Alg-GS) conjugate films through covalently binding via carbodiimide chemistry using Alg and GS at different molar ratios. The characterization of Alg-GS derivatives comprised elemental analysis for nitrogen content, Fourier transform infrared spectroscopy in the attenuated total reflection mode (FTIR-ATR), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), thermogravimetric analyses (TGA) and water sorption measurements. Bioluminescence assays with *Pseudomonas aeruginosa* PAO1/*lecA*:*lux* and plating of *Staphylococcus aureus* and *Escherichia coli* with colony forming counting after contact with the Alg-GS conjugate hydrogels were performed to evaluate their microbicidal properties. After performing antimicrobial tests, the content of released Alg-GS conjugates to the medium was estimated by means of elemental analysis for nitrogen content.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt (Alg, Sigma 180947, mannuronate/guluronate ratio = 1.56, M_w from 120,000 g/mol to 190,000 g/mol), gentamicin sulfate (GS, Sigma G1914), 1-ethyl-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, Sigma 03450, 191.70 g/mol), 2-[*N*-morpholino]-ethanesulfonic acid (MES, Sigma M3671, 195.2 g/mol) buffer and *N*-hydroxysuccinimide (NHS, Sigma 130672, 115.09 g/mol) were purchased from Sigma Aldrich, Brazil. All reagents were of analytical grade and used as received. The chemical structures of sodium alginate and gentamicin sulfate are displayed in Fig. 1a and b, respectively.

2.2. Synthesis of alginate-gentamicin (Alg-GS) conjugates

Alginate-gentamicin (Alg-GS) conjugates were synthesized by carbodiimide chemistry, as schematically represented in Scheme 1. Firstly, 0.41–1.64 g (e.g. 0.5 mmol–2 mmol) of GS was added to a 1% (w/v) solution of Alg (20 mL solution, 1.00 mmol equivalent of alginate monomer) prepared in 0.10 mol/L MES buffer, pH 6.0. The mixture was stirred with magnetic bar at (23 ± 1) °C for 10 min to facilitate the complete dissolution of GS in the Alg solution. After that, 0.287 g (2.5 mmol) of NHS and 0.766 g (4 mmol) of EDC were added to the Alg solution (Chhatbar, Prasad, Chejara, & Siddhanta, 2012). Noteworthy, the molar ratio of NHS:EDC as 0.5:1.0 led to films with high solubility in water, which would not be adequate for topic applications. On the other hand, the molar ratio NHS:EDC as 2.5:4.0 was the one that led to insoluble Alg-GS films in water and, for this reason, this molar ration was kept constant for all reactions. Three different molar ratios Alg to GS were employed, namely, 1:0.5, 1:1 and 1:2. The reaction proceeded under magnetic stirring, at (23 ± 1) °C, during 12 h. After that, the resulting mixture was dialyzed (dialysis membrane cut off 14,000 MW, Viskase Corp. USA) against distilled water with changes until the conductivity of dialysis water reached 5 µS/cm. The dialyzed reaction mixtures were cast in polystyrene dishes (4.5 cm diameter) and allowed to dry in an oven at (45 ± 2) °C overnight in order to evaporate the

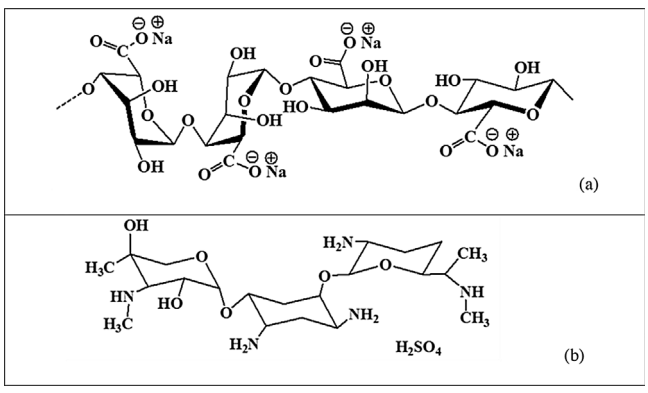

Fig. 1. Representation of the chemical structures of (a) sodium alginate (Alg) and (b) gentamicin sulfate (GS).