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Characterization of a water-soluble chitosan derivative and its potential for submucosal injection in endoscopic techniques

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ABSTRACT

To examine the potential of chitosan-based agents for submucosal injection in endoscopic techniques, a chitosan derivative was prepared with lactose moieties linked to the amino groups of its glucosamine units (CH-LA). After dissolving CH-LA in neutral pH solutions, including physiological saline (CH-LA-S), its response to different concentrations of anionic glycosaminoglycans and proteins in the surrounding environment was examined. The CH-LA-S form changed in the presence of sulfated glycosaminoglycans (heparin, chondroitin sulfate, and mucin) and protein (fibrinogen). High concentrations of sulfated substrates in the solution caused the formation of larger structures. In contrast, in the presence of hyaluronan, 30 mg/mL CH-LA-S did not form any large structures. Submucosal injection of 30 mg/mL CH-LA-S into extracted swine stomachs showed a strong lifting effect of the gastric mucosa. These results indicate the potential utility of CH-LA-S as a submucosal injection for endoscopic techniques such as endoscopic submucosal dissection and mucosal resection of tumors.

1. Introduction

The primary surgical treatments for early gastric cancer that has not spread to the lymph nodes are endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) (Hirasaki et al., 2004; Ohkuwa et al., 2001; Uedo, Takeuchi, & Ishihara, 2012). The success of these endoscopic techniques relies on suitable submucosal injections beneath the tumor, which provide a sufficiently high submucosal fluid cushion to facilitate perfect and clean dissection and resection of the tumor. Various types of submucosal injection agents have been developed and employed in EMRs and ESDs, such as normal physiological saline, hypertonic saline, dextrose, glycerol, and hyaluronic acid, with distinct advantages and disadvantages (Asge Technology Committee et al., 2008). Specifically, although normal physiological saline, hypertonic saline, dextrose, and glycerol are easy to inject, low in cost, and readily available, physiological saline tends to dissipate quickly during surgery, and the other three agents tend to cause tissue damage and local inflammation at the sites of injection. Moreover, although hyaluronic acid is the longest-lasting cushion among available agents, it is expensive and is not available in most endoscopy units, and has also been shown to stimulate the growth of residual tumor cells.

Chitosan is obtained by the alkaline deacetylation of chitin, which is considered to be the second most abundant natural biopolymer and is positively charged owing to the generation of free amino groups by deacetylation. Accordingly, chitosan has several advantageous biological properties, including hemostatic activity, platelet activation ability, biodegradability, antibacterial activity, antidiabetic activity, and antitumor effects (Hattori & Ishihara, 2015; Hattori & Ishihara, 2017; Karadeniz, & Kim, 2014; Lin, Lin, & Chen, 2009; Ratajska et al., 2003; Rinaudo, 2006; Tan et al., 2014; Yang et al., 2008). Chitosan is commonly used in biomedical materials for wound dressings, artificial skin, hemostasis, and adhesion, and as a raw material for cosmetic and antibacterial agents. However, chitosan is only soluble in acidic solvents such as diluted hydrochloric and acetic acids, and is extremely viscous, making it difficult to handle in wound dressings and biological adhesion treatments. In addition, because thick chitosan

Abbreviations: AB, Alcian blue; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; PAS, periodic acid-Schiff; PCA, photocrosslinkable chitosan; UV, ultraviolet.

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solutions do not retain at a physiological pH, chitosan is ineffective when combined with therapeutic physiologically active reagents, which has thus far limited its applications in healthcare and medicine.

Modification of polysaccharides is an attractive strategy for the development of novel materials with beneficial properties, with potential to be used in a wider range of applications. Hence, to utilize the advantages and compensate for the disadvantages of chitosan, various chitosan derivatives have been reported to modify water solubility over a broad pH range to improve advanced functionalities (Desbrières, Martinez, & Rinaudo, 1996; Mourya & Inamdar, 2008), such as maltosyl- (Lopes-da-Silva, 2012), N-alkylated (Yang, Chou, & Li, 2002), and carbohydrate-branched (Morimoto et al., 2001) chitosan derivatives. Furthermore, N-alkylation at the C-2 positon could effectively enhance the solubility of chitosan using different types of disaccharides, including lactose, maltose, and cellobiose (Yang et al., 2002).

We also prepared a photocrosslinkable chitosan (PCH) derivative (Ishihara, Hattori, & Nakamura, 2015; Ono et al., 2000) containing lactose moieties and a photoreactive azide group (*p*-azidobenzoic acid). PCH exhibited high aqueous solubility at neutral pH, which was demonstrated by the formation of insoluble hydrogels after cross-linking induced with ultraviolet (UV) irradiation. PCH has been used as a drug delivery material for topical cancer treatments (Ishihara et al., 2006; Obara et al., 2005; Ta, Dass, & Dunstan, 2008). Almost all of the PCH in the medium was retained in the submucosa for more than 4 weeks during the formation of granulation tissue with *in vivo* vascularization and neutrophil infiltration; moreover, PCH in the medium was completely degraded and replaced with tissue matrix within 8 weeks (Ishizuka et al., 2009; Kumano et al., 2012). Furthermore, given that PCH shows good tissue adhesion and hemostasis, it has potential as a new submucosal injection with applications in the development of hemostasis and tissue adherent biomaterials (Hattori, Amano, Nogami, Takase, & Ishihara, 2010; Hattori et al., 2013; Horio et al., 2010; Ishihara et al., 2001). However, the hydrogelation of PCH remains a hindrance to its wide application, requiring a photoreactive azide group as cross-linker and an expensive UV irradiation device with a potential negative influence to the normal tissues. Therefore, further modification of chitosan is required before it can be a suitable agent for submucosal injection in endoscopy techniques.

Various negatively charged polysaccharides (Prydz, 2015; Souza-Fernandes, Pelosi, & Rocco, 2006), proteins (Huttner, 1988; Liu & Lipmann, 1985), and lipids (Iwamori, Suzuki, Ito, Iwamori, & Hanaoka, 2005; Vos, Lopes-Cardozo, & Gadella, 1994) are present in human and animal tissues. Among these, glycosaminoglycans with anionic sulfate groups and carboxylic groups such as heparin, heparan sulfate, chondroitin sulfate, and hyaluronic acid are present in various organizations and tissues (Prydz, 2015; Souza-Fernandes et al., 2006). Mucin, with an anionic nature, is particularly abundant on the gastric mucosa (Owen, 1996). We hypothesized that positively charged chitosan may form a hydrogel following electrostatic interactions with negatively charged polysaccharides in the connective tissues, thus avoiding the need for introducing chitosan azide groups and applying hazardous UV irradiation. Thus, clarification of the interaction of a chitosan derivative, involving lactose moieties linked to the amino groups of glucosamine units without photoreactive azide groups, with anionic glycosaminoglycans and proteins would greatly contribute to the development of a novel submucosal injection for endoscopic techniques.

Herein, we developed and characterized a water-soluble chitosan derivative, and examined its interactions with anionic polysaccharides and proteins to determine its potential use as a submucosal injection agent for endoscopic techniques.

2. Materials and methods

2.1. Synthesis of lactose-linked chitosan (CH-LA) (Fig. 1)

CH-LA was prepared as previously reported (Ono et al., 2000). In brief, 125 g of chitosan with a degree of deacetylation of 80% and a molecular weight (Mw) of approximately 1,000,000 (Yaizu Suisankagaku Industry Co., Shizuoka, Japan) was mixed into 3 L of a 50 mM aqueous solution of tetramethylethylenediamine (Wako Pure Chemical Industries, Osaka, Japan), and then dissolved in 56.25 mL of concentrated hydrochloric acid (36%). Subsequently, 32.5 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Wako Pure Chemical Industries) and 0.25 g of lactobionic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were added, and the mixture was stirred for 24 h at room temperature. The solution was then ultra-filtered to remove unreacted substances with Mws below 10,000, and a powder of CH-LA was obtained by freeze-drying.

Through a conventional phenolsulfuric acid colorimetric assay for carbohydrates, it was estimated that 2% of the amino groups in the chitosan molecule were linked via lactobionic acid (Ono et al., 2000). CH-LA was dissolved in physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) (CH-LA-S) (40, 30, 20, 10, and 5 mg/mL) for further experiments.

2.2. Viscosity of CH-LA-S

The viscosity of CH-LA-S (40, 30, 20, 10, and 5 mg/mL) was measured with a series of tuning fork vibro viscometers, SV-10 (A&D Company, Limited, Tokyo, Japan). After CH-LA-S was warmed to 40 °C, the viscosity was measured at 5-s intervals until it reached a temperature of 35 °C. Distilled water was used as the zero calibration.

2.3. Measurement of turbidity

Sodium heparin (5000 IU, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), sodium chondroitin sulfate B (Mw: 11,000–25,000; Seikagaku Co., Tokyo, Japan), sodium hyaluronate (Mitsubishi-Kagaku Foods Co., Tokyo, Japan), and mucin from the porcine stomach (Sigma-Aldrich Co., LLC., St. Louis, MO, USA) were dissolved in physiological saline. Subsequently, 0.1-mL aliquots of 30 mg/mL CH-LA-S were added to 1-mL solutions (1000, 500, 250, 125, 62.5, and 0 IU/mL for sodium heparin, and 5, 2, 1, 0.5, and 0.2 mg/mL for sodium chondroitin sulfate B, sodium hyaluronate, and mucin), and after shaking for 30 s, the turbidity was measured at 660 nm using a spectrophotometer (Jasco Co., Tokyo, Japan). To observe the colorless and transparent CH-LA-S in video and still images, 0.5 mL of CH-LA-S was stained with 12.5 μL of a 0.05% toluidine blue solution (Wako Pure Chemical Industries).

2.4. Gel formation of CH-LA-S to fibrinogen

Fibrinogen from bovine plasma (Sigma-Aldrich Co., LLC) and bovine serum albumin (Wako Pure Chemical Industries) was dissolved in physiological saline, and 0.5 mL of the solution of each concentration (10, 5, 1, 0.5, and 0 mg/mL) was then mixed with 0.5 mL of fetal bovine serum (FBS) (Funakoshi, Tokyo, Japan) in a microtube. After 0.1-mL aliquots of 30 mg/mL CH-LA-S were added to the mixture solution, it was shaken for 30 s and incubated for 10, 60, and 180 min at room temperature. After the microtube was gently turned upside down five times, the formed gel was picked up with a needle and removed, and the volume of the remaining solution was measured with a syringe. The obtained value was subtracted from 1.1 (representing the total volume of the mixture