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# Formation of hydrogels based on chitosan/alginate for the delivery of lysozyme and their antibacterial activity

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ABSTRACT

Novel hydrogels based on chitosan/sodium alginate (CS-ALG) were prepared to deliver and protect lysozyme while eliminating food-borne microorganisms. These hydrogels were characterized according to the zeta potential, optical microscopy, scanning electron microscopy (SEM), UV–visible spectroscopy (UV–vis), fourier transform infrared (FT-IR), and small-angle X-ray scattering (SAXS). The results demonstrated that the resultant hydrogels were negatively charged and spherical in shape. In addition, the maximum swelling ratio was 45.66 ± 7.62 for CS-ALG hydrogels loaded with lysozyme. The relative activity of the released lysozyme was 87.72 ± 3.96%, indicating that CS-ALG hydrogels are promising matrices for enzyme loading and adsorption. Furthermore, a 100% bacterial clearance rate of CS/ALG loaded with lysozyme was observed to correspond to the superposition effect stimulated by CS and lysozyme, which improved the antibacterial activity against *E. coli* and *S. aureus* compared to CS/ALG, suggesting its potential use in the food industry as well as other applications.



## 1. Introduction

Hydrogels have been demonstrated to be important tools in drug-delivery systems for controlled and sustained release due to excellent mechanical properties and similarity to soft biological tissue (Appel, Forster, Rowland, & Scherman, 2014). Recently, discussion of hydrogels based on various biomaterials has been on the rise (Douglas et al., 2014; Dragan, Cocarta, & Gierszewska, 2016; Facin, Moret, Baretta, Belfiore, & Paulino, 2015; Ma, Zhang, Xie, Liu, & Xie, 2011; Tu et al., 2017). Natural polymers, in particular polysaccharides such as chitosan (CS), pectin, sodium alginate and starch, are well-developed as the matrix to fabricate hydrogels to meet different demands (Gupta, Tummalapalli, Deopura, & Alam, 2014; Li et al., 2014; Ventura & Biancopeled, 2015; Zhang et al., 2014).

Chitosan (CS), obtained by the deacetylation of chitin, is a popular polysaccharide biomaterial composed of β-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose with a macro pKa value varying from 6.3 to 6.5 (Meng et al., 2010). CS has aroused widespread concern regarding biocompatibility, degradability, non-mammalian origin, and antibacterial properties (Kong, Chen, Xing, & Park, 2010). The amino group at the C-2 position below the pKa (pH 6.3) of the glucosamine residue contributes to antimicrobial activity (Tian et al., 2016). As CS is rarely used alone due to limited flexibility, various methods such as cross-linking, grafting and blending have been developed to broaden applications. Acting as a cationic polymer to synthesize hydrogels is one of those developed methods (Hoffman, 2012; Meng et al., 2010).

Sodium alginate (ALG), a non-toxic biodegradable anionic natural polymer, is composed of different proportions of β-D-mannuronic acid (M block) and α-L-guluronic acid (G block) units. Previously, studies about the formation of CS/ALG hydrogels or micro-nano particles have been primarily conducted using $Ca^{2+}$, $Zn^{2+}$ or $Cu^{2+}$ as cross-linkers, as ALG is able to form three-dimensional networks with these multivalent metal cations (Treenate & Monvisade, 2017). The most recognized mechanism of $Ca^{2+}$ serving as the cross-linker for ALG through the G-blocks was named the egg-box model, where specific Ca-mediated interactions involve two polymer chains (Sikorski, Mo, Skjåkbræk, & Stokke, 2007). Alternatively, chitosan/sodium alginate (CS/ALG) composites can be assembled by adjusting the pH value. Polyelectrolytes with opposing charges may gel or precipitate when mixed, which depends on the concentration, ionic strength, and pH of the solution. And the products of such ion-crosslinked systems are called as complex coacervates or polyion complexes (Hoffman, 2012). ALG is sensitive to pH: ALG can shrink in acidic pH with neg-

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ative charges, but it swells when in neutral or alkaline systems (Pawar & Edgar, 2012). Compared to acidic conditions, the negative charge on the alginate molecules is higher under neutral conditions, resulting from the partial protonation of the carboxylic acid groups ($-COO^- \rightarrow COOH$, $pKa \approx 3.5$) on the mannuronic and guluronic acid groups (Lee & Mooney, 2012). A study by Rassu et al., 2016 reported that the formation of a hydrogel based on ALG and CS can be achieved by adjusting the pH of the mixing solution without adding calcium cations. In another study, Meng et al., 2010 prepared CS/alginate composite film by adjusting the pH to 5.0.

Meanwhile, lysozyme is a monomeric protein that can be used in pharmacology and the food industry, but the relatively narrow antimicrobial spectrum, instability and easy inactivation make the practical application of free lysozyme quite limited (Wu et al., 2017). Considering these reasons, hydrogels based on CS and ALG were prepared in this study by adjusting the pH. The effect of lysozyme addition on the properties of composite hydrogels was assayed by the zeta potential, optical microscopy, and scanning electron microscopy (SEM). Moreover, the chemical structure of CS/ALG and CS/ALG loaded with lysozyme are characterized by UV–visible spectroscopy (UV–vis), fourier transform infrared (FT-IR), and small-angle X-ray scattering (SAXS) measurements. In addition, the antibacterial actions of the hydrogels against *E. coli* and *S. aureus* are also determined. The morphologies of *S. aureus* treated by hydrogels are observed by atomic force microscopy (AFM). This study aims to provide detailed information about the synthesis of CS/ALG composites and to provide protection for lysozyme from the surrounding environment to prolong antibacterial activities and generate a new natural, non-toxic, and affordable material to meet the demand for preservation in food industries or other fields.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate with molecular weight (*Mw*) and mannuronate/guluronate ratio (M/G) of approximately 140,000 Da and 1:1, respectively, and lysozyme (from eggs, 5000 U/mg) were obtained from Aladdin Industrial Co. (Shanghai, China); chitosan (CS, degree of acetylation 80–95%) and hydrochloric acid (guarantee reagent, GR) were purchased from Sinopharm Chemical Reagent Co., Ltd.; *Escherichia coli* O157:H7 (ATCC25922) (*E. coli*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*), nutrient broth (NB) and nutrient agar (NA) were purchased from Qingdao Hope Bio-Technology Co., Ltd. *Micrococcus lysodeikticus* cells were obtained from Nanjing Jiancheng Biology , China.

### 2.2. Hydrogel preparation

Hydrogel preparation was performed according to previous woks by Rassu et al. (2016) with slight modifications. Sodium alginate solution (2.50%, w/v) was made dissolving alginate powder in water under magnetic stirring at room temperature. Separately, chitosan (2.75%, w/v) was dissolved in hydrochloric acid solution of pH 0.7, under magnetic stirring. Then, 1.25 mL of chitosan solution was added to 3.75 mL alginate solution (pH 7.0) and stirred vigorously to obtain the final pH of 3.0. The prepared hydrogel was labelled as CS/ALG-0.

Loaded hydrogels were prepared by dissolving lysozyme (1, 5, 10 mg) in 1.25 mL chitosan solution before mixing polymer solution, and the resultants were labelled as CS/ALG-1, CS/ALG-2, CS/ALG-3, respectively. Therefore, the content of lysozyme presented in CS/ALG-0, CS/ALG-1, CS/ALG-2, CS/ALG-3 was 0, 0.2, 1, 2 mg/mL, respectively. After preparation, the CS/ALG with or without lysozyme were stored at 4 °C in solution.

### 2.3. Morphology

The hydrogels were observed with an optical microscope (MODEL UB200i, Chongqing Aopu Photoelectric Technology Co., Ltd). A droplet of the hydrogel was placed on a microscope slide and was gently covered with a cover slip.

The morphology of the hydrogels was characterized by scanning electron microscopy (SEM, SU-8010, Japan). The resultant hydrogels were freeze-dried with a freeze dryer (Labconco, Beijing Light Ace HK Limited, China) for 36 h under −80 °C before further analysis. A thin layer of gold was covered on the sample surface before the observation.

### 2.4. Zeta potential measurement

The zeta potential (ζ-potential) of hydrogels was determined using a zeta potential analyser (Zetasizer Nano ZS90, UK). The mean ζ-potential (ZP) values (±SD (standard deviation) were obtained from the instrument. Samples were diluted with 10 mM phosphate buffer (at the same pH as the sample) prior to analysis.

### 2.5. Swelling ratio measurement

The swelling ability of hydrogels was measured according to the method of Xu, Kang, and Neoh (2006), with slight modifications. The samples were freeze-dried and the weight ($W_d$) of samples was measured (initial weight was 100 mg). Then samples were added to distilled water. At appropriate intervals, the excess water on the sample surfaces was wiped off with moist filter papers, and the swollen weight ($W_w$) of the hydrogels was determined. The swelling ratio (q) of the hydrogels was calculated from the Eq. (1):

$$q = \frac{W_W - W_d}{W_d} \quad (1)$$

### 2.6. UV–vis absorption measurement

UV absorption spectra were measured from 250 nm to 800 nm with a UV–vis spectrophotometer (UV-2550, Japan) in a 1 cm quartz cuvette at 25 °C. Five milligrams of lyophilized samples were diluted in 10 mL of deionized water before assay.

### 2.7. Fourier transform infrared (FT-IR) measurements

Fourier transform infrared (FT-IR) spectroscopy of the lyophilized samples was conducted according to the KBr pellet method on a Nicolet Avatar 370 (Thermo Scientific, USA) to confirm information about chemical bonds or functional groups. The spectra was collected from 4000 to 400 $cm^{-1}$ at room temperature at 4 $cm^{-1}$ resolution.

### 2.8. Small-angle X-ray scattering (SAXS) measurement

Diffraction patterns of samples exposed to small-angle X-ray scattering (SAXS) were obtained using a SAXS system diffractometer (Xenocs-3D SAXS, France). Samples were scanned with 600 s of exposure time at room temperature.

### 2.9. Enzymatic activity of lysozyme

The enzymatic activity of lysozyme released from CS-ALG was measured according to the method of Higashi, Tajima, Motoyama, and Arima (2012) with slight modifications. A 0.1 mL