Radiation Physics and Chemistry 124 (2016) 124–129

Contents lists available at ScienceDirect

Radiation Physics and Chemistry

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

Hemostatic efficacy evaluation of radiation crosslinked carboxymethyl kappa-carrageenan and chitosan with varying degrees of substitution

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HIGHLIGHTS

- Multi-step carboxymethylation was done to modify κ-carrageenan (KC) and chitosan (Ch)
- Modified KC and Ch were successfully crosslinked by gamma radiation
- *In vitro* assays show carboxymethylation and crosslinking increased hemostatic ability
- Carboxymethyl KC and Ch hydrogels may be fabricated into hemostatic agents

ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form
30 January 2016
Accepted 2 February 2016
Available online 3 February 2016

Keywords:
Kappa-carrageenan
Chitosan
Carboxymethylation
Radiation crosslinking
Hemostatic efficacy

ABSTRACT

Carboxymethyl derivatives of kappa-carrageenan and chitosan, with varying degrees of substitution, were synthesized by multi-step reaction technique and evaluated for hemostatic efficacy through *in vitro* assays. FTIR analysis confirmed the presence of carboxymethyl group while ^{1}H NMR spectroscopy indicated degrees of substitution ranging from 1.15–1.58 and 0.45–0.51 for carboxymethyl-κ-carrageenan and carboxymethylchitosan, respectively. Derivatives formed into paste consistency (30% w/v) were successfully crosslinked by gamma irradiation at 30 kGy. The data obtained from whole blood clotting and platelet adhesion assays showed a significant increase in hemostatic capability of κ-carrageenan and chitosan as a consequence of carboxymethylation and crosslinking modifications. In addition, the level of efficacy was comparable to that of a chitosan-based commercial product. These results suggest the potential of κ-carrageenan and chitosan derivatives for development into hemostatic agents.

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1. Introduction

The hemostatic potential, or ability to control bleeding, of several natural and synthetic polymers has been previously studied for the development of radiation-crosslinked hydrogels as topical hemostat. Natural polymers that exhibited significant hemostatic property include κ-carrageenan and chitosan (Barba et al., 2016). However, these materials predominantly undergo degradation upon irradiation, (Abad et al., 2009) thus they can either be mixed with a crosslinking polymer like polyvinyl pyrolidone (Huang et al., 2007) or they can be chemically modified and utilized for synthesis of materials for biomedical applications (Luo et al., 2013; Leong et al., 2011; Yagi et al., 2010).

One of the most widely studied conversions of polysaccharides is carboxymethylation. This is due to its technical simplicity, low cost of chemicals used and non-toxicity of products (Silva et al., 2004). The pristine polysaccharide is activated with aqueous alkali hydroxide and converted with monochloroacetic acid or its sodium salt according to the Williamson ether synthesis. The resulting carboxymethyl derivative is usually a polyelectrolyte with various potential applications (Heinze and Koschella, 2005). Polysaccharides that were successfully modified through this reaction include cellulose (Aguir and M'Henni, 2005), starch (Kittipongpatana et al., 2006), xylan (Petzold et al., 2006) and many more.

Successful carboxymethylation of κ-carrageenan (KC) and chitosan (Ch) and their potential applications in the biomedical field have been reported in recent years. Carboxymethylchitosan (CMCh) has potential uses as controlled release drug delivery matrix (Guo and Gao, 2007), as hydrogel wound dressing and as injectable gel for *in-situ* bone tissue engineering (Mishra et al., 2011). Carboxymethylated κ-carrageenan (CMKC) was reported to

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http://dx.doi.org/10.1016/j.radphyschem.2016.02.003
0969-806X/© 2016 Published by Elsevier Ltd.

provide an efficient alternative approach for the oral delivery of hydrophilic macromolecules to the intestinal tract (Leong et al., 2011). The carboxymethyl groups conferred antibacterial activity and promoted anticoagulant activity onto KC. Moreover, CMKC modified with collagen peptide exhibited capacity to repair skin (Fan et al., 2016).

The degree of substitution has been shown to play a role in improving the properties of the parent polysaccharide. The water absorption and retention capacity (Fan et al., 2011), as well as the ionic conductivity (Mobarak et al., 2015) of kappa-carrageenan significantly increased with increase in DS. Recently, polysaccharide derivatives, such as carboxymethylcellulose and carboxymethylstarch, were observed to predominantly form crosslinks when irradiated in paste consistency (Wach et al., 2003; Nagasawa et al., 2004). Crosslinking of CMKC was also achieved when irradiated at high concentration (above 10 %w/v) and the crosslinking ability was influenced by DS (Yagi et al., 2010).

In this research work, the synthesis and characterization of CMKC and CMCh were described. The preparation of hydrogels from successfully carboxymethylated derivatives by gamma radiation, as well as evaluation of gel properties, were also presented. Lastly, the effect of these modifications on the hemostatic capability of KC and Ch were revealed through whole blood clotting, platelet adhesion and clotting time *in vitro* assays.

$$DS_{CMKC} = \sum x_i = \frac{A\left(H(s)\text{of the } CM \text{ carbon at position} O_i\right)}{A\left(H(s)\text{ofthe } CM \text{ carbon at position } O_i\right) + A\left(H(s)\text{of thenon}-CM \text{ carbon at position } O_i\right)} \quad (1)$$

$$DS_{CMCh} = \sum x_i = 0.5 \bullet \frac{\text{A (H(s)of the CM carbon at position} O_i \,\&N_i)}{\text{A}\left(H(s) \text{ of the CM carbon at position} O_i \,\& N_i\right) + \text{A(H(s)of the non-CM carbon at position} O_i \,\& N_i)} \quad (2)$$

2. Materials and methods

2.1. Materials

Refined κ-carrageenan (Benvisco LBP-2000, Lot No. N2515-1) was purchased from Shemberg Biotech Corporation (Cebu City, Philippines). Chitosan was obtained from VINAGAMMA (Ho Chi Minh, Vietnam) with a Mw 1.1 × 105 g/mol and ~84% degree of deacetylation. Sodium hydroxide, 2-propanol and ethanol were supplied by Alysons Chemical Enterprises, Inc. (Quezon City, Philippines). Monochloroacetic acid (HiMedia) was provided by Belman Laboratories (Manila, Philippines). Celox™ hemostatic granules (Medtrade Products Ltd., UK, Lot No. 094OA), was purchased in USA.

2.2. Multi-step carboxymethylation

Multi-step carboxymethylation of KC and Ch was carried out according to previously reported protocols (Tranquilan-Aranilla et al., 2012; Kusuma et al., 2015). The polysaccharide powder was suspended in 2-propanol-water (8:2) making up a 10% slurry. While stirring vigorously, an appropriate volume of 10 M NaOH was added drop-wise. Stirring was continued for another 30 min at 40 °C. After alkaline activation, a known weight of monochloroacetic acid was added in portions and the reaction was allowed to proceed for 3 h at 40 °C. The suspension was vacuum filtered and the collected product was resuspended in 2-propanol-water (8:2) for neutralization with acetic acid to pH 6.5. The derivative was extensively washed with 2-propanol-water (8:2) followed by 2-propanol and dried in the oven at 50 °C. Carboxymethylation was performed up to three conversion steps, using the same parameters, to obtain products with varying degrees of substitution. Products were designated as 1 s (one-step conversion), 2 s (two-step conversion) and 3 s (three-step conversion) for both KC and Ch.

2.3. Characterization of carboxymethyl derivatives

Prior to characterization, the derivatives were purified through precipitation and acidification. CMKC samples were suspended in isopropyl alcohol creating a 10% mixture and were stirred for an hour. On the other hand, CMCh samples were suspended in acidified ethanol creating a 5% mixture. The suspension was stirred for 1 h then filtered and extensively washed with pure ethanol. Samples were dried in the oven at 50 °C, dissolved in the appropriate solvent and precipitated.

2.3.1. Degree of substitution (DS) by ^1H NMR spectroscopy

The NMR analyses were done at the facility of the University of the Philippines-Diliman. The samples, consisting of the unmodified and carboxymethylated (CM) polysaccharides were prepared as specified from literature (Abreu and Campana-Filho, 2009; Leong et al., 2011). The samples were dissolved in recommended solvents to a concentration of 10 mg/ml. The solvent used for KC and CMKC was D_2O containing 20 mM Na_2HPO_4 while D_2O–HCl (100:1) was used for Ch and CMCh. All spectra were recorded at 25 °C on a Varian-500 NMR spectrometer operating at 500 MHz. Sixty-four scans were accumulated at a pulse angle of 90° with an interpose delay of 3.7 s and an acquisition time of 3.2 s. The DS was calculated using the following equations (Leong et al. 2011; Heinze et al., 2001):
where x_i is the partial DS, A is the peak area, O is the oxygen atom at position i and N is the nitrogen atom at position i. For KC and CMKC, O_i refers to positions C-2 and C-6 of the β-D-galactopyranose-4-sulfate unit (G) and position C-2 of the 3,6-anhydro-D-galactopyranose moiety (DA) while O_i refers to positions C-3 and C-6 and N_i to C-2 of the D-glucosamine for Ch and CMCh.

2.3.2. FT-IR analysis

All samples were mixed with potassium bromide (1:10) and pelletized into KBr disks. The spectra were obtained using a Nicolet 6700 FT-IR spectrometer in the range of 500–4000 cm^{-1}.

2.4. Preparation and crosslinking of hydrogels

Modified polymers were soaked for 1 h in deionized water to create a 3:7 mixture and further homogenized in a mixing/degassing machine (Thinky ARE-250). The resulting pastes were machine pressed at 1 bar for 1 min to form hydrogel sheets, then packed in 4 × 4" foil pouches and vacuum sealed. Irradiation was carried out by the Cobalt-60 facility of the Philippine Nuclear Research Institute-DOST, at ambient temperature with a dose rate of 4.8 kGy/h to deliver a dose of 30 kGy.

2.5. Gel fraction and swelling studies

Hydrogel samples were oven-dried at 50 °C and milled into granules. For measuring gel fraction, one-gram dried gels were enclosed in non-woven fabric pouches and immersed in deionized water for 48 h at ambient temperature. The pouches were then oven-dried at 50 °C and weighed. Gel fraction was calculated as follows:

$$\%\ \text{GF} = \frac{\text{wt}_{\text{dried gel after sol extraction}}}{\text{wt}_{\text{dried gel, initial}}} \times 100 \quad (3)$$

For swelling capacity, one-gram dried gels packed in non-woven fabric pouches were immersed in deionized water and pseudoextracellular fluid (PECF) as solvents, for a period of 72 h. Samples were retrieved, blotted with wipes and weighed at 24-h intervals while changing the solvent periodically. Degree of swelling (DSw) was calculated as follows:

$$\text{DSw (g absorbed water/gdried sample)} = \frac{\text{wt}_{\text{swollen gel}} - \text{wt}_{\text{dried gel}}}{\text{wt}_{\text{dried gel,initial}}} \quad (4)$$

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