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POLYSACCHARIDE-BASED CROSSLINKED GEL MATERIALS AND THEIR PROPERTIES

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Abstract

The article presents research on obtaining gel-based delivery systems from hyaluronic acid and low molecular weight chitosan in H₂O and DMF environments, utilizing activators CIMPI/HOBt. It was determined that the obtained gel-based delivery systems form saturation curves at the highest percentage in aqueous solution. Additionally, studies on the acute toxicity of the gel-based delivery systems revealed that these samples belong to class VI-non-toxic compounds, and it was found during the experiments that the hemostatic properties of the obtained gel-based delivery systems are 3.0 times higher compared to the control group.

Keywords: Hyaluronic acid, chitosan, low molecular weight chitosan, hydrogel, swelling degree, hemostatic pharmacotoxicology

Introduction

Depending on their location and functions in the body, hyaluronic acid can exist in various molecular weights (5–20000 kDa) (Chistyakov et al., 2019; Xue et al., 2020). High molecular weight, low molecular weight, and (Gao et al., 2019) forms of hyaluronic acid participate in various biochemical and physiological processes as signaling molecules. In addition to its signaling function, high molecular weight hyaluronic acid also plays a role in the structure of cells

and tissues. Hyaluronic acid is particularly important for obtaining therapeutic delivery systems in various forms, such as conjugates with biologically active compounds, nanomaterials, and hydrogel materials (Bencherif et al., 2008; Guo et al., 2021; Nikjoo et al., 2021). Gel-based delivery systems derived from hyaluronic acid can be prepared by conjugating with biomolecules, including chito-oligosaccharides, which have the ability to enhance anti-cancer immunity by activating macrophages in the body through

polysaccharide chains (Chokradjaroen et al., 2018; Lee et al., 2002). In our recent research, an efficient and glycosidic bond-specific method for preparing functional oligosaccharides has been developed (Toole et al., 2008). Preliminary research results on applying these findings to other biopolymers indicate that this method can be used to obtain low molecular weight chitosans (LMW-CS) with any desired degree of polymerization. However, it remains crucial to deeply analyze the optimal reaction conditions for producing LMW-CS's and the changes that may occur in the polysaccharide chain during the reaction. Recently, there has been an increasing interest in developing biomaterials with the ability to form injectable hydrogels, therapeutic implants, and drug delivery systems (Drury & Mooney, 2003; Hoffman, 2002). Traditional hydrogel materials do not allow for direct administration into the body, requiring surgical procedures. To address these shortcomings, it is crucial to develop injectable hydrogel matrices based on biocompatible, biodegradable polysaccharides that have minimal adverse effects on the body. These types of hydrogel matrices can be administered into the body using a syringe. Therefore, obtaining hydrogel materials based on hyaluronic acid and LMW-CS that can be directly injected into the body and investigating the optimal reaction conditions is one of the urgent tasks.

The purpose of this scientific work is to prepare hydrogel samples through covalent bonding of hyaluronic acid and LMW-CS under the influence of activating reagents, as well as to study their physicochemical properties and biological activity.

Research Objectives:

- to synthesize covalently bonded hydrogel samples based on LMW-CS using sodium salt of high molecular weight hyaluronic acid at various molar ratios under the influence of activating reagents;
- to purify the obtained hydrogel samples from additives. to study the physicochemical properties of prepared hydrogel samples;
- to study the swelling ratio, pharmacotoxicology, and hemostatic activity of the hydrogel samples.

Materials and methods:

Obtaining Hydrogels Based on hyaluronic acid and LMW-CS

A 40.0 mg/ml concentration solution of hyaluronic acid (Mw = 1480 kDa, DP = 3691, DPI = 4.11) was prepared. After hyaluronic acid formed a clear viscous solution in the solvent, a 15.0 mg/ml concentration solution of LMW-CS (Mw = 12.2 kDa, DP = 75.3, DPI = 1.25) was added at a ratio of 0.5 mol/mol of hyaluronic acid unit (HAU) and stirred for 30–60 minutes. Subsequently, 2-chloro-1-methylpyridinium iodide (ClMPI)/1-hydroxybenzotriazole (HOBt) was added to the reaction mixture at a ratio of 0.6/1.0 mol/mol of HAU and the reactions continued until hydrogel samples were formed. The resulting hyaluronan/chitosan-based (H-LMW-CS) hydrogel matrices were purified by dialysis for 48 hours in a phosphate buffer solution (pH = 7.4) and for 24 hours in deionized water to remove additives.

Study of the Structures of Samples

The IR spectra of the samples were obtained using the Thermo Nicolet AVATAR 370 FTIR spectrometer by the KBr pellet method or directly using the Shimadzu IRTracer-100 spectrometer in the absorption range of 400–4000 cm⁻¹.

Determining the Swelling Degree of H-LMW-CS

The swelling ratio of the prepared hydrogel samples was observed in three types of solutions: deionized water, phosphate buffer solution (pH = 7.4), and 0.9% NaCl solution. The initial weights of the hydrogels were 15 ± 3 g. To determine the swelling ratios of each gel system, 400 ml of the above solutions were placed in 500 ml beakers, and the prepared gel samples were immersed in the solutions. At specific time intervals, the gel samples were removed from the solutions, excess water was blotted with filter paper, and they were immediately weighed. This process was repeated three times, and the average value was adjusted for measuring the swelling ratio (%). The swelling degree was calculated using the following equation.

$$A = \frac{W_0 - W_1}{W_1} \cdot 100\% \quad (2.1)$$

In the formula: W_0 is the mass of the swollen gel in water, and W_1 is the initial mass of the gel.

Determining the Pharmacotoxicological Properties of H-LMW-CS

The acute toxicity of H-LMW-CS was studied in male and female mice (weight 20 ± 2.0 g, $n = 6$) according to the Litchfield and Wilcoxon method. In the experiments, H-LMW-CS gel samples were administered subcutaneously via syringe at doses of 5000–10000 mg/kg. Changes in the overall condition, tremors, and mortality were recorded hourly on the first day of the experiment after the gel samples were administered to the mice. In the subsequent phase, changes in the functional status of the animals were observed over a period of 14 days under experimental conditions, including indicators such as general condition, activity, behavior, respiration, changes in the skin, body weight variation, survival, convulsions, and mortality. All mice in the experiment were kept under uniform conditions and fed a common diet. At the end of the experiment, the average lethal dose (LD50) and toxicity class were determined.

Hemostatic Properties of H-LMW-CS

The hemostatic properties of H-LMW-CS samples were studied in male white rats weighing 180 ± 20 g, which were anesthetized by administering 1% sodium thiopental (50 mg/kg). The experiment involved opening

the abdominal cavity of the anesthetized rats using a surgical instrument (scalpel). For hepatic hemostasis, an incision was made longitudinally along the midline of the abdominal cavity, and a wide incision was created in the thoracic cavity using special clamps. The anterior part of the liver was exposed and delineated with a special gauze or filter paper. The liver was resected with a scalpel. The segment cut in the vertical projection resembled a circle or an ellipse, maintaining a constant size. A wound with soft edges and uniform curvature was created, with an approximate area of 1–1.5 cm in depth and about 0.3 cm in diameter. Free bleeding was allowed for 20 seconds, after which 0.3 ml of H-LMW-CS was immediately applied to the defect. At the end of the experiment, the hemostatic properties of the sample were analyzed.

Results and discussion

This study explored the possibilities of obtaining gel-based delivery systems from hyaluronic acid and LMW-CS. Additionally, during the research, the effects of reaction conditions on the molecular sizes of the obtained products were examined to determine optimal reaction conditions, and gel systems necessary for investigating biological activity were prepared. The research on obtaining H-LMW-CS gel samples was conducted in H_2O and DMF environments with the activators ClMPI/HOBt.

Figure 1. Reaction for obtaining hydrogel under the influence of activating reagents based on sodium hyaluronate and low molecular weight chitosan



The studies indicated that to obtain gel samples with suitable molecular sizes, a ratio of 0.5 mol/mol of chitosan to HAU, along with 0.6 and 1.0 mol/mol of ClMPI and HOBt respectively, and a hyaluronic acid concentration of 40 mg/ml at 20 °C were optimal. The reaction for the formation of hydrogel samples proceeded through the formation of direct amide bonds ($-\text{CO}-\text{N}(\text{H}, \text{R})-$) between the carboxyl groups in hyaluronic acid and the amino groups in LMW-CS. The reaction scheme for the gel-based delivery system involving high molecular weight hyaluron-

ic acid and LMW-CS with ClMPI/HOBt is shown in Figure 1.

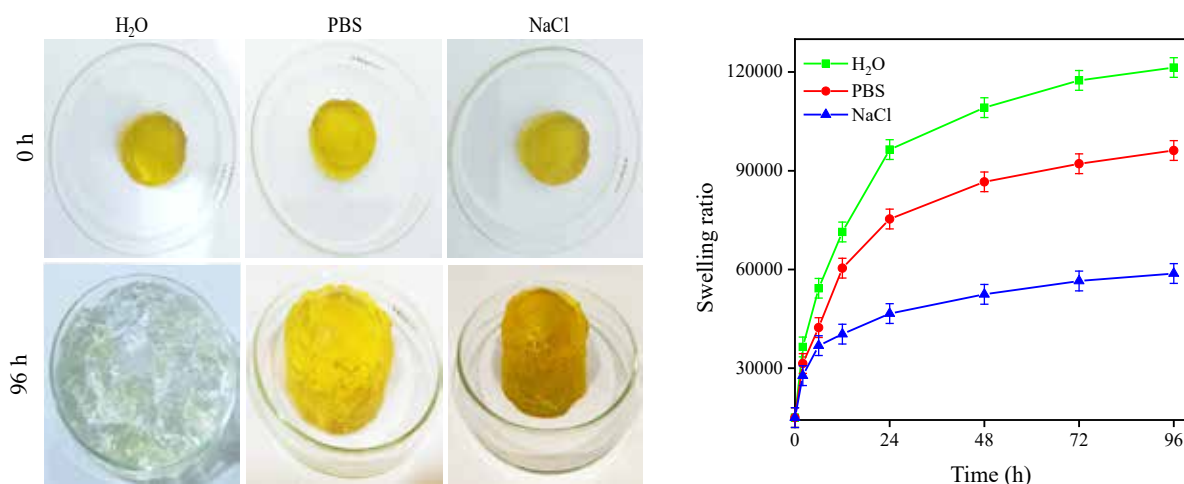
The FTIR spectra of the obtained gel samples based on high molecular weight hyaluronic acid and LMW-CS were analyzed, revealing characteristic signals corresponding to the O–H, N–H, C6–H, amide II, C–O–C, and C–C bonds in hyaluronic acid and LMW-CS samples. These signals were observed in the spectral regions of 3200–3600, 2900, 1556, 1060, and 600–894 cm^{-1} , respectively.

The swelling ratio of H-LMW-CS was determined in three different solutions: distilled

water, phosphate buffer solution (pH=7.4), and 0.9% NaCl solution. The swelling of gel materials in these solutions is of great importance, as even a small change in the swelling value leads to changes in the free volumes of water molecules passing through the polysaccharide matrix. Thus, this significantly affects the swelling properties of the gel materials. In the study, the equilibrium swell-

ing behavior of H-LMW-CS was examined at 4–8 °C over a period of 0–96 hours. The swelling ratios of H-LMW-CS in H₂O, PBS, and NaCl solutions are presented in Figure 2(a). The results of the swelling study (Figure 2(b)) showed that the swelling ratios reached a maximum of 120±3% in the aqueous solution, indicating the gel materials' insolubility and their high water retention properties.

Figure 2. Swelling degree of H-LMW-CS. In the figures: (a) Swelling state of H-LMW-CS in H₂O, PBS, and NaCl solutions; (b) Swelling dynamics of H-LMW-CS over different time intervals



According to the swelling dynamics of H-LMW-CS, the gel materials exhibited high water absorption during the initial 0–24 hours, with maximum water absorption observed at 96 hours. In PBS (pH = 7.4), H-LMW-CS showed an initial maximum swelling coefficient of $15 \pm 3 - 75 \pm 3\%$ over 0–24 hours, reaching $96 \pm 3\%$ saturation after 96 hours. This indicates that the swelling characteristics of H-LMW-CS depend on the solution environment and nature. In experiments, the presence of a small amount of 0.9% NaCl in the dialysis solution was observed to slightly reduce the swelling degree of the gel materials. It was found that increasing the concentration of the saline solution significantly affects the swelling characteristics of the gel materials. In 0.9% NaCl solution, H-LMW-CS initially exhibited a swelling of $15 \pm 3 - 46 \pm 3\%$ over 0–24 hours, with minor changes in the following hours and demonstrating a saturation of $58 \pm 3\%$ after 96 hours.

The studies investigated the overall effect and acute toxicity of H-LMW-CS on animal organisms. The experiments were conduct-

ed on male, non-purebred white laboratory mice with a body weight of 20 ± 2.0 g. All pharmacological tests were carried out on healthy, sexually mature mice that were kept under quarantine for 10–14 days. H-LMW-CS was administered subcutaneously to the mice at doses of 5000, 7000, 9000, and 10000 mg/kg. The control group received an equal volume of physiological solution.

During the first day of the experiments in laboratory conditions, the general condition of the animals in both the experimental and control groups was monitored hourly, along with any signs of tremors and mortality as indicators of their functional status. Over the next two weeks, daily assessments were made of the general condition, activity, coat condition, skin status, respiratory rate and depth, urination, body weight changes, and other parameters of all groups of animals under vivarium conditions. All experimental animals were maintained on a uniform feeding regimen, with unrestricted access to water and food.

At the end of the experiment, the average lethal dose (LD_{50}) and toxicity class of the tested samples were determined. No acute toxicity effects were observed in the aforementioned parameters at any of the doses of H-LMW-CS. Throughout the entire experiment, no deaths among the animals were recorded at these doses. Based on the results, it was determined that the average

lethal dose (LD_{50}) for the preparation when administered subcutaneously is greater than 10000 mg/kg. The results are presented in the following Table 1. The findings indicate that the acute toxicity properties of H-LMW-CS classify these samples in the VI – non-toxic compounds category, with an average lethal dose (LD_{50}) greater than 10000 mg/kg.

Table 1. Acute toxicity indicators of H-LMW-CS material when administered subcutaneously to mice in a single dose

Samples	Animal Type / Administration Method	Dose (mg/kg)	Number of Dead/Surviving Animals	$LD_{50} - m + m$ (mg/kg)
H-LMW-CS	Mouse / Subcutaneous	5000	5/0	>10000 mg/kg
		7000	5/0	
		9000	5/0	
		10000	5/0	
Control		0.5	5/0	

The hemostatic properties of H-LMW-CS were studied. Groups treated with medical gauze were used as controls. In the experiments, bleeding time (in seconds) and blood loss were recorded immediately. The results showed that when medical gauze was applied to the control group, the bleeding time from the liver's parenchyma was 125 ± 5 seconds, while in the groups treated with H-LMW-CS, bleeding was stopped within intervals of 40 ± 5 seconds. It was determined that the hemostatic properties of H-LMW-CS were 3.0 times higher compared to the control group.

Conclusion

The gels were obtained based on hyaluronic acid ($M_w = 1480$ kDa, $DP = 3691$, $DPI = 4.11$) and LMW-CS ($M_w = 12.2$ kDa, $DP = 75.3$, $DPI = 1.25$). The study of the degree of saturation of the obtained samples showed that they formed saturation curves with a maximum of $120 \pm 3\%$ in an aqueous solution. The results of the study of the acute toxicity characteristics of H-LMW-CS indicated that these samples belong to the class of VI-non-toxic compounds, and when administered subcutaneously, the average lethal dose (LD_{50}) was found to be > 10000 mg/kg.

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