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Development of carboxymethyl cellulose nonwoven sheet as a novel hemostatic agent

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Carboxymethyl cellulose (CMC) is a plant-derived material that has high biocompatibility and water solubility. We developed a CMC nonwoven sheet as a hemostatic agent by carboxymethylating a continuous filament cellulose nonwoven sheet. The CMC nonwoven sheet was able to absorb water and dissolve in it. The rates of absorption and dissolution depended on the degree of carboxymethylation. After dissolving in blood, CMC accelerated clot development (possibly owing to the incorporation of CMC into fibrin fibers) and increased the viscosity of the blood, both of which would contribute to the improved blood clotting of an injured surface. *In vivo* experiments using a rat tail cutting method showed that a CMC nonwoven sheet shortened the bleeding time of the tail when applied to the cut surface. The hemostatic effect of the CMC nonwoven sheet was almost at the same level as a commercial hemostatic bandage. These results suggest that a CMC nonwoven sheet could be used as a novel sheet-type hemostatic agent.

[**Key words:** Carboxymethyl cellulose; Nonwoven sheet; Hemostasis; Blood clotting; Bandage; Biomaterial]

Topical hemostatic agents have been widely used in surgical procedures. Various forms of hemostatic agents, including powders, gels, and sheets, are now commercially available, and the choice of form depends on the intended use (1). For example, powders and gels are suitable to cover irregular surfaces, though their effective use is limited to relatively minor bleeding because they are easily forced out of the wound by severe bleeding. On the other hand, as sheet-type hemostatic agents are easy to handle and able to be used with pressure application, they have been used in wide variety of wounds, including severe bleeding cases. Many kinds of materials, such as collagen (2,3), oxidized cellulose (4), and chitosan (5,6), have been utilized as hemostatic sheets to date. It has been reported that these hemostatic sheets improve blood clotting, which is induced by the interaction between the sheet material and the blood components (e.g., platelet activation induced by contact with collagen (7)). However, there are also some problems with the currently available sheet-type hemostatic agents (8). For example, risk of infectious disease cannot be avoided when using animal-derived materials such as collagen (9,10). Low pH-induced inflammation can also occur when oxidized cellulose is applied to a bleeding site (11,12). Therefore, development of a novel hemostatic sheet is still required in clinical practice (13–15).

Carboxymethyl cellulose (CMC) is a cellulose derivative in which some of the hydroxyl groups in the cellulose backbone are modified into carboxymethyl groups. CMC is known as a plant-derived biocompatible material and has good water solubility resulting from carboxymethylation (16). Owing to these characteristics, CMC has been widely used in the food and healthcare industries (e.g., as a thickener in foods and a lubricant in eye-drops). Its potential use in biomedical area, such as in wound healing, regenerative medicine and drug delivery, is also currently being investigated (17,18). Some groups have reported that CMC dissolved in blood can promote blood clotting (19–21). For example, Aoshima et al recently showed that dissolved CMC worked as a bridge for fibrin polymerization, leading to thick fibrin fiber formation and thus improving blood clotting (19). Activation of platelet coagulation by dissolved CMC was also reported by Wang et al. (20).

Their works inspired us to develop a CMC nonwoven sheet as a hemostatic agent. In previous studies, we achieved large-scale fabrication of a continuous filament cellulose nonwoven sheet that has low lint and excellent water absorption property (22). It is expected that by carboxymethylating this sheet, we can fabricate a novel hemostatic product that combines the characteristics of CMC with those of the nonwoven sheet; high hemostatic activity and biocompatibility (i.e., low risk of infectious disease or inflammation) can be derived from the CMC, whereas good water absorption (i.e., blood absorption) and easy handling are expected from the structure of the nonwoven sheet. CMC nonwoven sheets with

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various degrees of carboxymethylation were fabricated in this study. The hemostatic activity of these sheets was examined *in vitro* and *in vivo*.

MATERIALS AND METHODS

Sodium hydroxide, acetic acid, and sodium chloroacetate were purchased from Kishida Chemical (Osaka, Japan). Ethanol, calcium chloride ($CaCl_2$), and sodium citrate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Normal saline was purchased from Otsuka Pharmaceutical (Tokyo, Japan). Standard human plasma (Coagtrol N) was purchased from Sysmex (Hyogo, Japan) and human fresh frozen plasma (FFP) was supplied by the Japanese Red Cross Society (Tokyo, Japan).

Animals The animal experiments were performed at the Animal Research Section, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo. The Animal Care Committee of The University of Tokyo approved all procedures in this study before it began. Male Sprague–Dawley rats (9–11 weeks old) were used in this study. All the rats were acclimated to the laboratory environment for at least 1 week before the experiment. The animals were kept in metal cages and maintained under controlled conditions of temperature (21–24 °C), humidity (40–60%) and light (12-h light–dark cycles).

Fabrication of CMC nonwoven sheets A nonwoven sheet of cellulose was fabricated from cotton linter as described in our previous report (22). In brief, prepurified cotton linter was dissolved in cuprammonium solution. The solution was then ejected from the spinning nozzles on a net conveyor, leading to the formation of a cellulose nonwoven sheet. After that, the sheet was washed to remove the solvent, dried up, and then collected. A 10 × 75 cm piece of the obtained cellulose nonwoven sheet was immersed in 172 mL of sodium hydroxide solution (3.3 M in 50 vol% ethanol aqueous solution) for 30 min. The sheet was then immersed in the mixture of sodium chloroacetate and sodium hydroxide solution (0.35 M sodium chloroacetate and 0.33 M sodium hydroxide in 300 mL of 80 vol% ethanol aqueous solution) and stirred at 50 °C for carboxymethylation. The reaction was allowed to proceed for 2–6 h, depending on the intended degree of substitution (DS). The reaction was quenched by adjusting pH to about 6.0–8.0 using acetic acid. The obtained CMC nonwoven sheet was washed twice with 80 vol% ethanol and dried at ambient temperature. Carboxymethylation of the sheet was confirmed by an attenuated total reflectance–infrared (ATR-IR) spectrometer (4200ST, JASCO, Tokyo, Japan). Degree of modification was determined by titration analysis, according to a previous report (23).

Water absorption and dissolution property of CMC nonwoven sheets The rate of water absorption was evaluated by dropping 60 μL of normal saline on a 20 × 20 mm CMC nonwoven sheet. We measured the time required for the drop to become flat on the sheet, and used it as an indicator of water absorption rate. The dissolution property of the CMC sheets was evaluated by measuring the weight change of CMC sheets in normal saline. CMC sheets (10 × 10 mm) were incubated with normal saline at 37 °C. The weights of the CMC sheets were measured by electric balance at given time intervals to monitor their dissolution kinetics. The obtained weights of the CMC sheets were normalized by those of CMC sheets after 2 min incubation, in which water absorption was supposed to have reached equilibrium but dissolution had not yet begun, and then plotted against time.

Effect of CMC nonwoven sheets on clotting process of blood Clotting time was measured using a semi-automatic coagulation analyzer (KC10, Tcoag Ireland Ltd., Bray, Ireland). A CMC nonwoven sheet dissolved in 100 μL of normal saline (1.0 mg/mL) was added to 100 μL of human FFP and incubated at 37 °C for 3 min. In a control experiment, 100 μL of normal saline without CMC was used. The clotting time was then measured immediately after the addition of 100 μL of $CaCl_2$ solution (12.5, 25, 50, or 70 mM). The effect of the CMC nonwoven sheet on the clotting process was also examined using a Sonoclot coagulation and platelet function analyzer (Sienco Inc., Boulder, CO, USA), which enabled us to monitor the mechanical change of blood samples during coagulation (24,25). The Sonoclot analyzer detected mechanical changes in the blood samples with an oscillating probe, converted them to a clot signal using its built-in program, and recorded it with time. A CMC nonwoven sheet (DS = 0.26, 0.40, or 0.53) dissolved in 120 μL of normal saline (1.0 mg/mL) was mixed with standard human plasma and warmed at 37 °C. In the control experiment, 120 μL of normal saline without CMC was used. A 100 μL volume of $CaCl_2$ solution (25 mM) was then added and the mechanical change was measured by the Sonoclot analyzer.

Effect of CMC nonwoven sheets on blood viscosity Blood viscosity after contact with the CMC nonwoven sheets was examined using a cone-type viscometer (TVE-22, Toki Sangyo, Tokyo, Japan). Whole blood was taken from rats and mixed with 3.8% sodium citrate. CMC sheets (15 × 15 mm) were immersed in 1.2 mL of the rat whole blood for 2 min and then removed. The viscosity of the blood was then measured by the viscometer at a shear rate of 40 s^{-1}.

Hemostatic activity of CMC nonwoven sheets *in vivo* The hemostatic activity of the CMC nonwoven sheets was examined using a rat tail cutting method (26–28). The experiments were carried out by more than one investigator (S. O., T. N., or K. M.) to avoid bias. CMC nonwoven sheets were sterilized with ethylene oxide gas prior to the experiment. Heparin (300 U/kg) was administered intraperitoneally using a syringe with a 24-gauge needle (Terumo, Tokyo, Japan). Anesthesia was not used in this experiment to avoid any possibility of it affecting bleeding. After 15 min, the rat was placed in a plastic cylinder with an opening, from which the tail of the rat emerged. The tail was then transected 4 mm from the tip using a surgical blade. Immediately after the tail cutting, a CMC nonwoven sheet was applied to the cut surface and the bleeding time was measured. The CMC sheet was temporarily removed at a given time interval to observe the bleeding status. Hemostasis was judged by applying new gauze to the cut surface to determine if blood was attached to the gauze. The same experiment was conducted using HemCon bandage (HemCon Medical Technologies Inc., Portland, OR, USA), which is a commercial chitosan-based hemostatic bandage, for comparison.

RESULTS

Properties of the fabricated CMC nonwoven sheet A CMC nonwoven sheet was fabricated by carboxymethylating the continuous filament cellulose nonwoven sheet that we had developed previously (22). The morphology of the nonwoven sheet did not change after carboxymethylation (Fig. 1A). Thickness and fiber diameter of the nonwoven sheet also did not change so much after carboxymethylation; from 0.44 mm to 0.48 mm in thickness and 11.0–11.1 μm in fiber diameter. On the other hand, density of the nonwoven sheet was increased from 0.19 to 0.31 g/m^3 after carboxymethylation, which would reflect changes in chemical structure of the nonwoven sheet. The ATR-IR spectra of the nonwoven sheets carboxymethylated for different reaction times are shown in Fig. 1B. The stretching vibration of –CH (2892 cm^{-1}) and the bending vibration of –OH (3348 cm^{-1}) were observed in the spectrum of the original cellulose nonwoven sheet. After the reaction, a new peak around 1591 cm^{-1}, which corresponds to the carbonyl stretching vibration, emerged in the ATR-IR spectra. The relative intensity of the new peak around 1591 cm^{-1} to that around 2892 cm^{-1} increased with increasing reaction time. These results confirmed that the continuous filament cellulose nonwoven sheets had been successfully carboxymethylated. We estimated the degree of carboxymethylation by titration analysis (23). The DS of the CMC nonwoven sheet reacted for 2, 3, and 6 h was 0.26, 0.40, and 0.53, respectively. There was a linear relationship between the ratio of peak at 1591 cm^{-1} (C=O) to that at 2892 cm^{-1} (C=H) in the ATR-IR spectra and DS value of CMC determined by the titration (Fig. S1), which further confirmed the consistency of our results. We used these CMC nonwoven sheets with different DS values in the following experiments.

Water absorption and dissolution property of CMC nonwoven sheets with different DS The effect of the DS on the water absorption rate of the CMC nonwoven sheet is shown in Fig. 2A. Absorption time was prolonged with increasing DS of the CMC, meaning that an increase in the DS decreased the rate of water absorption. In contrast, an increase in the DS enhanced the dissolution property of the CMC nonwoven sheet. Fig. 2B shows the dissolution behavior of the CMC nonwoven sheets with different DS values in normal saline. The weight of the CMC sheets decreased with time during the incubation, indicating that the CMC sheets were gradually dissolved in normal saline over time. The dissolution rate of the CMC sheets was accelerated by increasing the DS values; while approximately 80% of the CMC remained after 8 h incubation in the case of a DS of 0.26, almost all the CMC was dissolved within 4 h with DS values of 0.40 and 0.53.

Interaction of CMC nonwoven sheets with blood examined *in vitro* After absorbing blood and dissolving in it, the CMC nonwoven sheet is expected to interact with blood components and promote clotting. We measured the clotting time of human plasma containing 1.0 mg/mL of dissolved CMC nonwoven sheet.