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A self-assembling hydrophobically modified chitosan capable of reversible hemostatic action

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ABSTRACT

Blood loss at the site of a wound in mammals is curtailed by the rapid formation of a hemostatic plug, i.e., a self-assembled network of the protein, fibrin that locally transforms liquid blood into a gelled clot. Here, we report an amphiphilic biopolymer that exhibits a similar ability to rapidly gel blood; moreover, the self-assembly underlying the gelation readily allows for reversibility back into the liquid state via introduction of a sugar-based supramolecule. The biopolymer is a hydrophobically modified (hm) derivative of the polysaccharide, chitosan. When hm-chitosan is contacted with heparinized human blood, it rapidly transforms the liquid into an elastic gel. In contrast, the native chitosan (without hydrophobes) does not gel blood. Gelation occurs because the hydrophobes on hm-chitosan insert into the membranes of blood cells and thereby connect the cells into a sample-spanning network. Gelation is reversed by the addition of α-cyclodextrin, a supramolecule having an inner hydrophobic pocket: polymer hydrophobes unbind from blood cells and embed within the cyclodextrins, thereby disrupting the cell network. We believe that hm-chitosan has the potential to serve as an effective, yet low-cost hemostatic dressing for use by trauma centers and the military. Preliminary tests with small and large animal injury models show its increased efficacy at achieving hemostasis – e.g., a 90% reduction in bleeding time over controls for femoral vein transections in a rat model.

1. Introduction

The blood coagulation cascade is an exquisite example of a responsive self-assembly process in biology [1,2]. When a wound is formed, a cascade of self-assembly events occurs in blood at the site of the wound. The net outcome is the assembly of the globular protein, fibrinogen, catalyzed by a second protein, thrombin to yield chains of fibrin [3,4]. A network of insoluble fibrin chains forms the hemostatic "plug" or clot, which presents a physical barrier to the loss of blood from the wound [1,2]. The coagulation cascade is a delicately balanced series of events – if it was to occur too easily, blood clots may form in unwanted areas leading to strokes or other complications.

Scientists have long sought to harness the clotting power of fibrin to create hemostatic dressings or bandages [5–7]. Hemostatic dressings that can staunch the bleeding from serious wounds are a pressing need both in civilian trauma centers as well as for military personnel. Indeed, uncontrolled hemorrhage from severe injuries is a leading cause of death among young adults (e.g., accident victims) and it is also responsible for the majority of deaths on the battlefield [8–10]. Fibrin-based hemostatic sealants were first-developed in the 1940s and have proven to be quite effective [6,7]. For example, one form involves a dry powdered mixture of human fibrinogen and thrombin packed onto a solid bandage backing. When such a bandage is firmly pressed onto a bleeding injury, a strong fibrin seal quickly forms and bleeding is stopped [6]. However, fibrin bandages have limited practical applicability in trauma medicine because human fibrinogen and thrombin are highly expensive molecules that are scarce in supply [7].

A need thus exists for an inexpensive hemostatic agent based on widely available materials that could match the blood-clotting ability of fibrin. Although a variety of advanced hemostats have

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been brought to market [5,11–15], none have shown the efficacy of fibrin sealants [11,12]. Several products work simply by absorbing the blood at the site of the wound rather than by actively coagulating the blood [12,14]. Recently, a new approach has been put forward by Ellis-Behnke et al. [16], wherein the self-assembly of a synthetic peptide into a nano-fibrous network [17] is used to achieve hemostasis independent of the natural coagulation cascade. While this method is promising, the synthetic peptides employed are expensive and difficult to synthesize – therefore their practical viability is unclear. An additional factor to consider with hemostats such as the above is the risk of undesired gelation or clotting, i.e., embolization, in parts of the body that are peripheral to the site of injury [14,18]. To mitigate against such risks, it would be desirable to have the hemostat disassemble or "unclot" if and when desired; however, none of the hemostats described in the literature have been shown to have this ability.

In the present study, we explore the use of an amphiphilic biopolymer as a "reversible" hemostatic agent. The native (non-amphiphilic) biopolymer is chitosan, an inexpensive and widely available material with inherent anti-microbial properties [19]. We attach a small number of hydrophobic tails to the backbone of chitosan, thereby creating a hydrophobically modified or hm-chitosan (schematic in Fig. 1) [20,21]. Interestingly, native chitosan has seen use as a hemostatic material due to its cationic and anti-microbial nature [22–24], although its hemostatic efficacy in dealing with severe wounds has been questioned [11]. Our hypothesized mechanism of hm-chitosan's hemostatic action (Fig. 1) involves the anchoring of hydrophobes from the polymer into the hydrophobic interiors of blood cell membranes. Thereby, blood cells would become connected by biopolymer chains into a sample-spanning gel network, which could potentially halt the flow of blood. We probe this hypothesis both *in vitro* via rheological studies and *in vivo* using animal bleeding models. Furthermore, we also explore an important secondary objective: to reverse any hemostatic effect of this amphiphilic biopolymer, i.e., to "unclot" on demand. Our strategy here is to introduce an amphiphilic supramolecule, α-cyclodextrin (α-CD), which has been shown in the literature to sequester polymer hydrophobes within its hydrophobic pocket [25,26]. As such, we hypothesize that α-CD can be used to disassemble any hm-chitosan/blood gels that may form via hydrophobic interactions.

2. Materials and methods

2.1. Materials

Chitosan of medium molecular weight (190–310 K) and Brookfield viscosity of 286 cps was purchased from Sigma–Aldrich. The reported degree of deacetylation was about 80%. Chitosan was dissolved in 0.15 M of either L-lactic acid or acetic acid (both from Sigma–Aldrich) so as to mimic physiological ionic strength. The supramolecule α-cyclodextrin was purchased from TCI.

2.2. Synthesis of hm-chitosan

hm-chitosan was synthesized by attaching benzene-*n*-octadecyl tails to the chitosan backbone via reaction with 4-octadecylbenzaldehyde (purchased from TCI). The procedure is identical to that used in our earlier paper [21] and it also follows those described in the literature [20]. The degree of hydrophobic substitution follows the reaction stoichiometry and here it was fixed at 2.5 mol% of the available amine groups.

2.3. Preparation of hm-chitosan bandages

A solution of 0.5 wt% hm-chitosan was poured into trays of dimensions 10 cm (length) × 10 cm (width) × 4 cm (height). Trays were filled up to 2 cm in height with polymer solution. Samples were then frozen in a pilot-scale freeze drier at −40 °C, and then held under vacuum of 50 μbar for 5 days to remove all water. After drying, a Tegaderm® medical tape backing (from 3M Corp.) was placed on the back side of the dried solid for unhindered compression of the bandage during surgery.

2.4. Obtaining blood

5 human subjects volunteered to have 20 mL of blood drawn by a registered nurse at the UMD School of Medicine. Subjects were healthy adults ranging in age

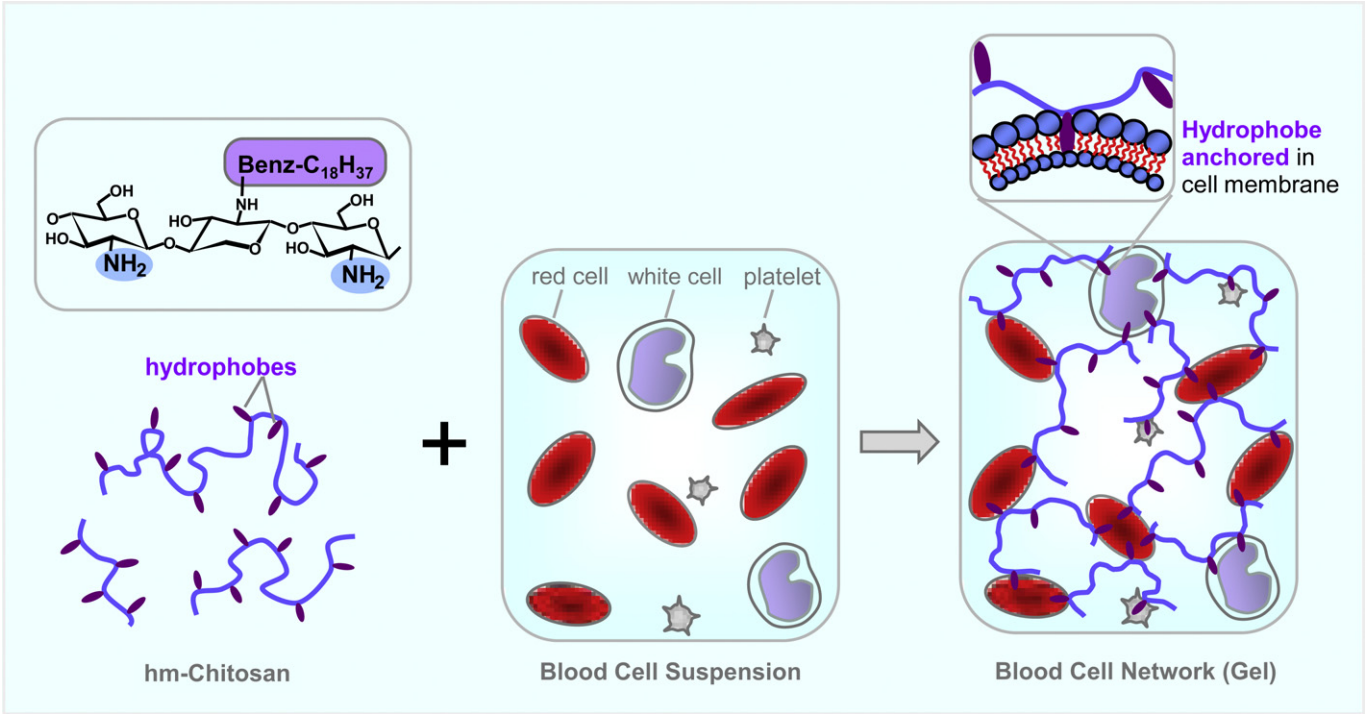

Fig. 1. Mechanism for gelation of blood by hm-chitosan. On the left the polymer is shown schematically with its hydrophilic backbone in blue and the grafted benzyloctadecyl hydrophobes in purple. When added to liquid blood, the components assemble into a three-dimensional network (gel), as shown on the right. This is driven by insertion of hydrophobes into blood cell membranes (as depicted in the top inset); thereby the polymer chains connect (bridge) the cells into a self-supporting network.

from 20 to 40 years (4 males, 1 female). 10 mL intervals of blood were drawn into Becton Dickinson Vacutainers® containing 143 USP units of sodium heparin. The protocol was approved by the Institutional Research Board (IRB) at UMD.

2.5. Rheological experiments

Steady and dynamic rheological experiments were performed on a Rheometrics AR2000 stress-controlled rheometer. A cone-and-plate geometry of 40 mm diameter and 4° cone angle was used and samples were run at the physiological temperature of 37 °C. Dynamic frequency spectra were obtained in the linear viscoelastic regime of the samples, as determined from dynamic strain sweep experiments.

2.6. Rat injury models

Surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UMD. 15 fasted male Long-Evans rats (250–275 g, from Harlan Laboratories) were anesthetized (60 mg/kg ketamine and 7.5 mg/kg xylazine given IP) and allowed to breathe air. Animals were maintained under pathogen-free conditions in 12 h diurnal cycles, with water and food *ad libitum*. Animal rooms were kept at 21 ± 3 °C with several changes of air per hour. All husbandry and animal procedures were in accordance with humane animal handling practices under the guidance of the Unit for Laboratory Animal Medicine at the UMD School of Medicine. At the end of each procedure, all animals were humanely sacrificed by ketamine administration.

Using a scalpel, the femoral vein was transected and allowed to bleed for 30 s, after a unilateral groin incision was made over the femoral canal. Exposure and isolation of at least 1 cm of the femoral vein was performed. 1 mL of test material was then dispensed onto 5 randomly selected animals via syringe (with a 22 gauge needle) after wiping away excess blood from the site of injury via cotton gauze. Bleeding time was measured via stopwatch, with the start time corresponding to the application of sample and the end time corresponding to visual observation of halted blood flow. Test materials studied were (1) saline buffer, (2) 0.5 wt% chitosan solution and (3) 0.5 wt% hm-chitosan solution.

2.7. Pig injury models

3 Yorkshire crossbred swine, age 2.5 months and weighing 39.6–42.8 kg, were used following screening by a veterinarian. Animals were allowed free access to water and to commercial food, which was withheld the night before the study. All animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The protocol was approved by the IACUC at UMD. The swine were anesthetized with 6 mg/kg of telazol and 0.01 mg/kg of glycopyrrolate that were given intramuscularly. They were intubated and placed on mechanical ventilation at a tidal volume of 12 mL/kg, a rate of 10 respirations per minute, and 100% oxygen. Anesthesia was maintained using isoflurane, and ventilatory parameters were maintained to attain an end-tidal CO_2 partial pressure of 40 mm Hg.

The protocol for injury creation and bandage application employed here is similar to that used in previous studies [14]. The animals underwent midline laparotomy, and a unilateral groin incision was made over their femoral canal. Exposure and isolation of at least 5 cm of the femoral artery was performed; band ligators were then applied to the proximal and distal ends of the exposed segment of the artery. Using surgical scissors, the femoral artery was transected (i.e., completely divided), the ligators were unfastened, and free bleeding was allowed for 15 s. Blood was collected by suction for this period and was designated as pretreatment total blood loss. Dry hm-chitosan bandages were cut to optimal sizes for wound approach and applied via 2 min of direct compression onto the injury site. Animals were observed under anesthesia for 180 min, at which point they were euthanized with pentobarbital IV 100–200 mg/kg.

3. Results and discussion

3.1. Gelling blood

First, we compare the effects of hm-chitosan and native chitosan on heparinized human whole blood. In each case, we adjusted the polymer concentration in the overall mixture to 0.25 wt%. Upon addition of hm-chitosan, liquid blood is instantly transformed into a self-supporting gel, as can be seen from the photograph in Fig. 2 where the sample holds its weight upon tube inversion [27]. In comparison, the mixture of chitosan and blood remains a freely flowing liquid, as seen from its corresponding photograph. These visual observations are also shown in real-time in Movies 1 and 2 (Supplementary information).

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.12.033.

The above differences in flow properties were quantified by rheological measurements. Fig. 2a shows data from dynamic rheology (oscillatory shear), which probes the linear viscoelastic response of the samples [28]. The data plotted are for the elastic (G') and viscous (G'') moduli as functions of the angular frequency ω. The hm-chitosan/blood sample shows a largely elastic response

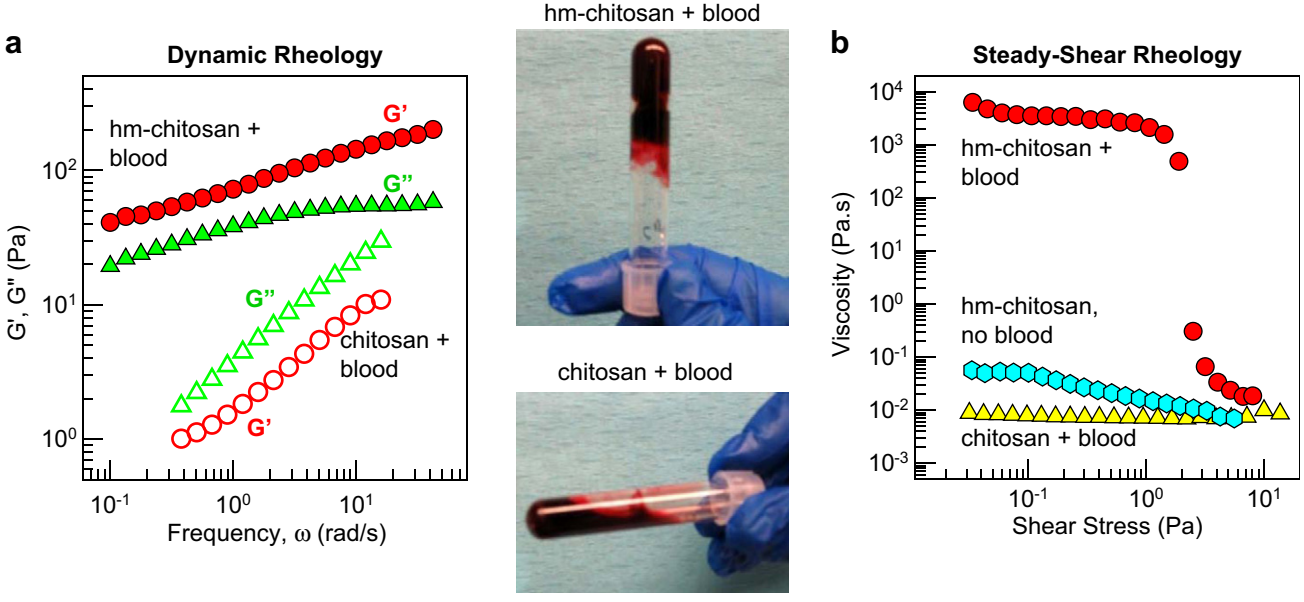

Fig. 2. Effect of 0.25 wt% hm-chitosan or chitosan on heparinized human blood. The photographs show that the hm-chitosan/blood mixture is a self-supporting gel that holds its weight in the inverted tube whereas the chitosan/blood mixture is a freely flowing liquid. In (a) dynamic rheological data for the elastic modulus G' and the viscous modulus G'' vs. frequency ω are shown for the two samples. The hm-chitosan sample (closed symbols) displays the rheology of a physical gel ($G' > G''$) whereas the chitosan sample (open symbols) responds like a viscous sol. In (b) steady-shear rheological data for the viscosity vs. shear stress are shown. The hm-chitosan/blood mixture (red circles) shows a significantly higher viscosity relative to both the chitosan/blood mixture (yellow triangles) as well as a 0.25 wt% solution of hm-chitosan with no blood (cyan hexagons).

typical of a physical gel: note that its G' exceeds G'' over the entire frequency range and moreover, both moduli are weakly dependent on frequency. The weak frequency dependence of the moduli is indicative of a sample-spanning network that is able to store the shear deformation [28]; relaxation of this network occurs very slowly over long time scales. Conversely, the chitosan/blood sample shows a viscous response: both its moduli decrease sharply with frequency and its G'' exceeds G' over the frequency range.

Data on the same samples via steady-shear rheology are shown in Fig. 2b, where the apparent viscosity is plotted as a function of shear stress. Here, we note that the chitosan/blood sample has a constant viscosity of about 0.01 Pa.s, which is about 4 times that of blood alone. A 0.25 wt% solution of hm-chitosan in water has a viscosity of about 0.07 Pa.s in the low-shear limit, indicating that the sample is slightly viscous but far from being a gel. In contrast, the sample of hm-chitosan/blood has a low-shear viscosity around 10,000 Pa.s, which is a *million-fold* higher than that of blood. Also, in this case, the steep drop in viscosity around a stress of 2 Pa is indicative of a *yield stress* [28], meaning that the sample hardly flows at stresses below this value. This accounts for the gel-like behavior seen in the photograph where the sample holds its weight and does not flow down in the inverted tube [27,28].

The data in Fig. 2 show that hm-chitosan gels blood while chitosan does not. Gelation occurs within a few seconds of mixing the polymer into blood, as can be seen from Movie 1 (Supplementary information). Thus gelation is rapid and the gel remains stable indefinitely. We also conducted gelation experiments with the components of whole blood: for this the blood plasma (containing the blood-clotting proteins) and the blood cells were separated by centrifugation. The blood cells were resuspended in an equal volume of Hank's Balanced Salt Solution and then mixed with 0.25 wt% hm-chitosan. This sample shows a gel-like response in dynamic rheology (Fig. 3, note $G' > G''$ over the frequency range) much like that observed earlier with whole blood. In contrast, the mixture of 0.25 wt% hm-chitosan with blood plasma shows a weakly viscous response in dynamic rheology: in this case, G'' is slightly higher than G' over the frequency range (Fig. 3). Photographs of the two samples are also shown in Fig. 3 and are consistent with the rheological results: the cell-containing sample holds its weight in the inverted tube whereas the plasma sample flows freely.

The results in Figs. 2 and 3 support the gelation mechanism depicted in Fig. 1. We have shown that gelation of blood requires the presence of hydrophobes on chitosan chains (Fig. 2); moreover, gelation takes place only in the presence of blood cells, indicating that it is the cells and not the plasma proteins that dictate gelation. In turn, the likely structure of the blood gels is as illustrated in Fig. 1, where some of the hydrophobes from hm-chitosan chains are shown embedded within the hydrophobic cores of cell membranes. The hydrophobes can be viewed as nanoscale analogs of hooks on a Velcro® pad – when hydrophobes encounter cells, they "hook on" to the cell membranes via hydrophobic interactions [21,29]. Individual polymer chains thereby connect (bridge) adjacent cells, and the net result is a sample-spanning three-dimensional network in which the cells act as the cross-link points or nodes. Such a polymer-bridged network structure has also been postulated to form in mixtures of amphiphilic polymers and lipid vesicles (which are nanocontainers enclosed by bilayer membranes) [21,29]. For example, in a study analogous to the present one, we found that gelation of vesicles could be induced by hm-chitosan (but not chitosan), and we postulated a similar network of vesicles bridged by hm-chitosan chains [21]. Similarly, gelation of both vesicles as well as cells has been demonstrated by Meier et al. using a polymer with a hydrophilic backbone and hydrophobic cholesteryl end-groups [29].

3.2. Reversal of blood gelling

As discussed above, gelation is driven by the hydrophobic affinity between the grafted tails on the polymer and the acyl tails of lipids in cell bilayers [21,29]. This also suggests a mechanism to reverse the gelation, which is via species that can preferentially bind to the polymer hydrophobes and thereby disengage the hydrophobes from the cells. We demonstrate such reversal using a sugar-based supramolecule called α-cyclodextrin (α-CD) [25,26]. This belongs to a family of barrel-shaped supramolecules where the exterior of the barrel is hydrophilic while its interior pocket is hydrophobic (Fig. 4). In particular, the pocket diameter of 0.57 nm in α-CD is perfect for sequestering single-tailed hydrophobes such as those on hm-chitosan whereas the pocket is too narrow to fit two-tailed lipids from cell membranes [26,30]. We should also point out that cyclodextrins are inexpensive, commercially available, and biocompatible – they are extensively used in pharmaceutical formulations [25,26].

Fig. 4 shows the result of adding 3 wt% of α-CD to a blood gel (much like that in Fig. 2) formed by adding 0.25 wt% hm-chitosan. From visual observations, it was clear that the α-CD was able to

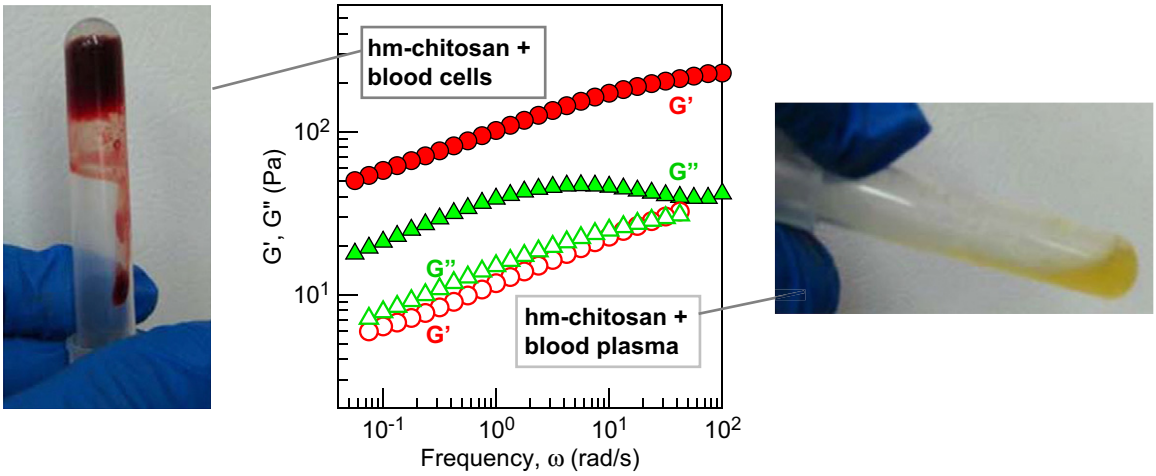

Fig. 3. Effect of 0.25 wt% hm-chitosan on separated blood cells and plasma. The photographs show that hm-chitosan gels blood cells whereas its mixture with plasma is a flowing viscous liquid. Dynamic rheology confirms the visual evidence, as the sample containing the blood cells (closed symbols) responds like a weak physical gel whereas the sample containing the plasma (open symbols) shows a viscous response.

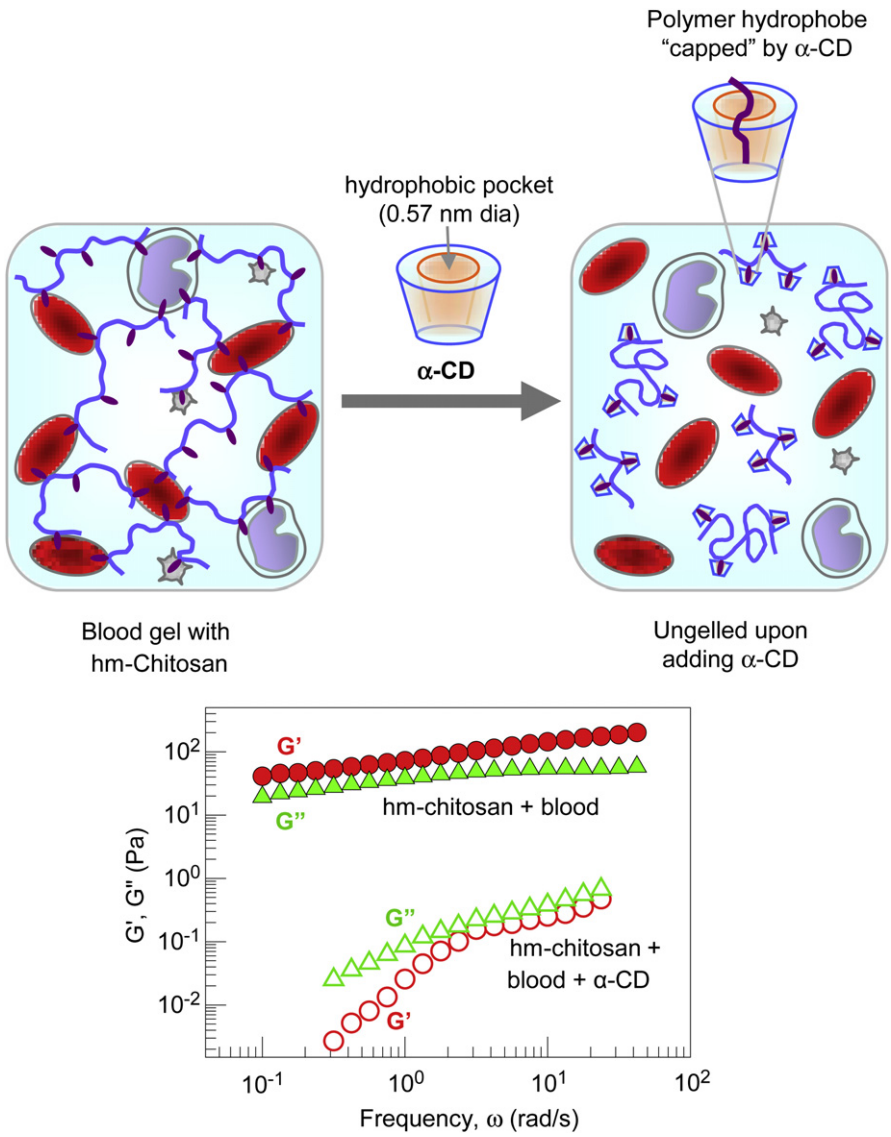

Fig. 4. Reversal of blood gelation by α-cyclodextrin (α-CD). The top panel illustrates the mechanism for this reversal. The α-CD molecule has a barrel shape with a hydrophobic pocket. When added to a hm-chitosan/blood gel, the polymer hydrophobes "unhook" from the cells and instead get buried within the hydrophobic pockets of α-CDs. The connections between the cells are thus eliminated and the gel is thereby liquefied. The bottom panel shows dynamic rheology data that confirm this effect. The initial blood gel based on 0.25 wt% hm-chitosan shows an elastic, gel-like response (closed symbols), whereas upon addition of 3 wt% α-CD, the response converts to that of a viscous liquid (open symbols).

instantly reverse the gelling and convert the sample into a thin, flowing liquid or "sol". Dynamic rheology confirms these observations: note from Fig. 4 that the response in the presence of α-CD is liquid-like with $G'' > G'$ over the frequency range. Fig. 4 also presents a schematic illustration of the action of α-CD: here, the hydrophobes on hm-chitosan chains are shown to be sequestered within the hydrophobic pockets of α-CD molecules [26,30]. In turn, the polymer chains no longer connect adjacent cells, allowing the cells to flow freely. Effectively, the strong affinity of α-CD for the hydrophobes causes these moieties to "unhook" from the cells and bind to the α-CDs instead. Note that the results with α-CD further demonstrate that free hydrophobes are required for gelation, as depicted in Fig. 1.

3.3. Animal injury models

The ability of hm-chitosan to gel blood is reminiscent of the natural clotting action of fibrin sealants. Therefore, it is pertinent to examine whether, like fibrin, hm-chitosan can also serve as a hemostatic sealant for bleeding injuries. To investigate this aspect, we conducted tests with animal injury models. First, we evaluated an injury in a small animal, and in this case, we tested a hm-chitosan solution as the hemostatic agent. Femoral vein injuries were created in Long-Evans adult rats ($n = 5$ per sample) via scalpel. We then applied a given test solution to the injury via a syringe with a 22 gauge needle and measured the time to hemostasis, i.e., for the bleeding to cease (Fig. 5A). First, 1 mL of a saline control was applied and in this case, hemostasis was achieved in 50 ± 4 s (hemostasis here results from the rat's own blood coagulation cascade). Next, we applied 1 mL of a 0.5 wt% native chitosan solution and it showed a similar time to hemostasis of 47 ± 3 s. Finally, 1 mL of a 0.5 wt% hm-chitosan solution was applied, and in this case, hemostasis was attained in 4.5 ± 0.6 s – this is a 90% reduction compared to the controls. The hm-chitosan was also able to control bleeding from a minor injury model in a larger animal (porcine femoral vein injury) in a comparable period of time (5.6 ± 0.7 s).

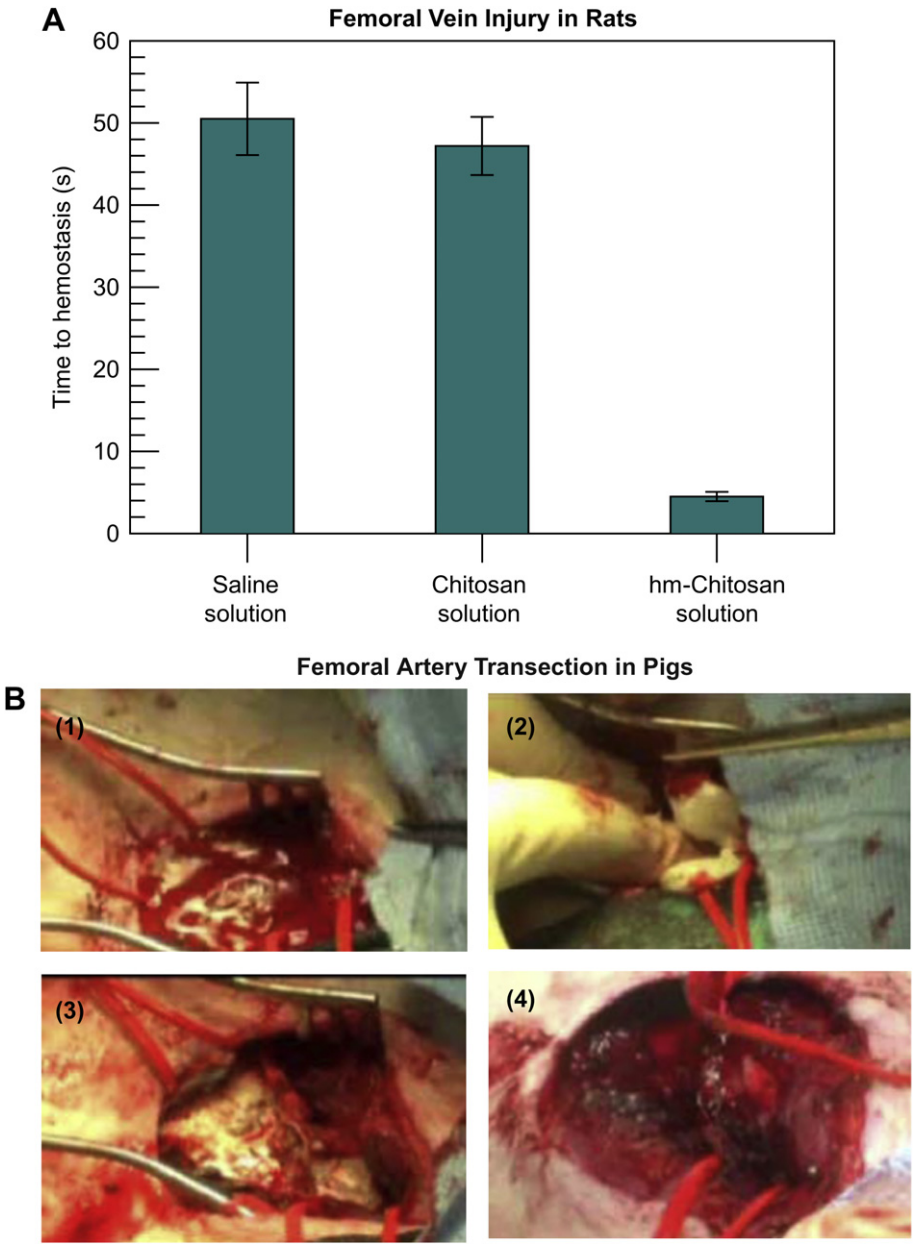

Fig. 5. Evaluation of hm-chitosan as a hemostatic agent in animal injury models. (A) Tests on a rat femoral vein injury model demonstrate a significantly lower time to hemostasis in the case of a 0.5 wt% hm-chitosan solution relative to both saline buffer and a 0.5 wt% native chitosan solution. (B) Use of a hm-chitosan bandage to treat a severe injury in a pig (femoral artery transection). The photographs are stills from Movie 3 (Supplementary information). First, (1) shows the rapid flow of blood upon creation of the injury in the femoral artery. Next, (2) shows the hm-chitosan bandage being applied to the wound by direct compression. After 2 min of compression, (3) shows the bandage being attached to the wound site and providing hemostasis. Lastly, (4) shows the wound site to be successfully clotted when the bandage is removed after 180 min.

Next, we evaluated a hm-chitosan bandage to treat high-pulsatile injuries in a large animal model. For this, we produced freeze-dried bandages of hm-chitosan (these are comparable to the fibrin bandages discussed in the Introduction). We chose to study a pig femoral artery injury model ($n = 3$), where the femoral artery is transected and allowed to bleed freely. This injury is fatal within 15 min if left untreated. Fig. 5B displays photographs from a representative experiment, and the same experiment is also depicted in Movie 3 (Supplementary information). The blood pools at the site of the injury upon creation (1), whereupon the hm-chitosan bandage is applied (2). The bandage is able to successfully halt bleeding after 2 min of compression (3). Finally, the arterial injury is shown to be successfully clotted when the bandage is removed 3 h after application (4). All pigs survived for the duration of the experiment, after which they were euthanized according to protocol.

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.12.033.

The above results show that the hm-chitosan bandage is highly effective at treating severe bleeding injuries in pigs. No secondary bleedings occurred during the studied timeframe, and no toxicity was observed. We speculate that the effectiveness of hm-chitosan as a hemostatic sealant occurs for the same reasons that the fibrin sealant is effective. That is, contact between the hm-chitosan and blood results in a gelled “seal” at the site of the wound, and this presumably acts as a barrier to blood loss through the wound. The local gel may also help to localize clotting factors from the

bloodstream in the vicinity of the wound, and thereby enhance the natural clotting action. Moreover, the gelling of the blood may ensure that the bandage itself does not get soaked with liquid. This means that the parts of the bandage in direct contact with tissue will remain adherent and not delaminate, thereby eliminating the possibility of secondary bleeding (the latter is known to occur with many existing hemostatic dressings[11,13]). The hydrophobes on the polymer may also possibly enhance the adherence of the solid bandage due to interactions with underlying tissue cells – again, in much the same way as Velcro® hooks, but at the nanoscale.

4. Conclusions

We have shown that the amphiphilic biopolymer, hm-chitosan, can act as an effective hemostatic agent. It has the ability to transform whole liquid blood into a gel, and it quickly stops bleeding from both minor and severe injuries in small and large animals. The gelling mechanism is predicated on the self-assembly of hm-chitosan chains in such a way that their hydrophobes anchor in blood cell membranes and thereby bridge the cells into a 3-dimensional network. The cells are thus active components (nodes or junction points) in the network rather than being physically trapped in a polymer mesh. The gelling ability of hm-chitosan is similar to that of fibrin-based sealants, but at a much lower cost and wider availability. An additional important aspect of hm-chitosan as a hemostatic agent is that its gelling effect on blood can be reversed by addition of the supramolecule α-CD. The reversal occurs because α-CD molecules sequester the hydrophobes and prevent them from attaching to blood cells. Our studies show that hm-chitosan could be an effective and safe hemostatic agent for treatment of both external and internal bleeding injuries.

Acknowledgments

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Appendix

Figures with essential colour discrimination. All figures in this article are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.033.

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