Determination of efficacy of novel modified chitosan sponge dressing in a lethal arterial injury model in swine

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BACKGROUND: Chitosan is a functional biopolymer that has been widely used as a hemostat. Recently, its efficacy has been questioned due to clinical failures as a result of poor adhesiveness. The purpose of this study was to compare, in a severe groin injury model in swine, the hemostatic properties of an unmodified standard chitosan sponge with standard gauze dressing and a novel hydrophobically modified (hm) chitosan sponge. Previous studies have demonstrated that hm-chitosan provides greatly enhanced cellular adhesion and hemostatic effect via noncovalent insertion of hydrophobic pendant groups into cell membranes.

METHODS: Twenty-four Yorkshire swine were randomized to receive hm-chitosan (n = 8), unmodified chitosan (n = 8), or standard Accu-Sorb gauze dressing (n = 8) for hemostatic control. A complex groin injury involving arterial puncture (4.4-mm punch) of the femoral artery was made after splenectomy. After 30 seconds of uncontrolled hemorrhage, the randomized dressing was applied and compression was held for 3 minutes. Fluid resuscitation was initiated to achieve and maintain the baseline mean arterial pressure and the wound was inspected for bleeding. Failure of hemostasis was defined as pooling of blood outside the wound. Animals were then monitored for 180 minutes and surviving animals were killed.

RESULTS: Blood loss before treatment was similar between groups ($p < 0.1$). Compared with the hm-chitosan sponge group, which had no failures, the unmodified chitosan sponge group and the standard gauze group each had eight failures over the 180-minute observation period. For the unmodified chitosan sponge failures, six of which provided initial hemostasis, secondary rebleeding was observed 44 minutes ± 28 minutes after application. Standard gauze provided no initial hemostasis after the 3-minute compression interval.

CONCLUSIONS: Hm-chitosan is superior to unmodified chitosan sponges ($p < 0.001$) or standard gauze for controlling bleeding from a lethal arterial injury. The hm-chitosan technology may provide an advantage over native chitosan-based dressings for control of active hemorrhage. (*J Trauma.* 2012;72: 899–907. Copyright © 2012 by Lippincott Williams & Wilkins)

KEY WORDS: Hemostatic dressing; modified chitosan dressing; hemostasis; trauma; hm-chitosan.

Despite substantial advances in hemostatic technologies over the past two decades,[1–3] uncontrolled hemorrhage still remains the leading cause of death among combat casualties and the second leading cause of death in civilian trauma patients (first among patients aged 1–44 years).[4–6] Hence, innovative strategies for treatment of acute wounds continue to be paramount to the mission of trauma medicine and critical care. Recently, advanced hemostat design has gravitated toward a parlaying of novel active raw materials into improved outcomes in hemorrhage control. Such materials, e.g., clays or zeolites, are able to clot blood very rapidly relative to standard field dressings (i.e., cotton gauze), which, surprisingly, had been held as the standard dressing in military medicine for nearly 2000 years.[7] The key catalyst toward displacement of gauze field dressings as state-of-the-art came in 1993 when US Special Forces at the Mogadishu battle in Somalia suffered excess mortality from extremity wound hemorrhage. Post-mortem analysis showed that this result was due to highly inadequate field hemostasis.[8]

Just before the start of Operation Iraqi Freedom in 2003, the Department of Defense (DoD) quickly authorized the deployment and use of three Food and Drug Administration-approved hemostatic agents. This "first generation" of advanced hemostatic products included the zeolite Quickclot Powder, chitosanic HemCon Bandage, and the American Red Cross fibrin dressing. These hemostats had shown significantly improved efficacy over gauze in controlled animal models,[9–11] and they would eventually save lives on the battlefield which gauze could not.[12] However, as similar

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nonlinear, or "urban," combat settings persisted throughout conflicts in Iraq and Afghanistan in the 2000s, greater improvements in hemostats remained in demand by the DoD. Injured soldiers often remained under fire in urban settings for several hours before being transported to a combat support hospital.[13] Casualty analysis demonstrated that hemorrhage remained a leading cause of preventable deaths[13]; survival in these cases may have been improved by better early control of bleeding via highly effective field hemostats.

This continued need further spawned the development of a "second generation" of high-efficacy products such as Woundstat (clay mineral powder), Celox (chitosan powder), and Quickclot Combat Gauze (kaolin-incorporating gauze) which entered into the military arena in the latter half of the 2000s. Indeed, the second-generation products represented a higher level of efficacy over the first generation.[2,14,15] Unfortunately, however, neither generation was without concerning side effects or practicality issues. The Quickclot technologies suffered from severe heat generation[16,17] and/or difficulty of resection (leaching into tissue),[18] Woundstat suffered from dangerous peripheral clotting,[2,18] and the fibrin sealant dressing was limited in supply, difficult to store, and highly expensive.[10,19]

Together, the first- and second-generation biomaterials for treatment of severe bleeds fall into one of the three categories: (1) proteins (fibrinogen/thrombin), (2) aluminosilicates (kaolin and zeolite), and (3) polysaccharides (cellulose, dextran, and chitosan). Protein-based technologies are generally very effective; however, their cost and storage difficulties make them continually unattractive for military field use. Aluminosilicates are also very effective, yet low cost, making them more attractive than proteins for field use. Unfortunately, aluminosilicates generally have unfavorable toxicity profiles,[20] and they have been shown to cause permanent tissue damage[15] and undesired peripheral clotting.[18] Polysaccharides, however, are the only group to provide low cost and low toxicity jointly. Indeed, cellulose has been used since ancient times as a wound care material,[21] and its toxicity profile is well understood.[22] Unfortunately, its hemostatic effectiveness is very limited. Dextran-based products, e.g., TraumaDex, have shown improved efficacy over cellulose,[16] although the improvement has been incremental and the availability of dextran is relatively low. Nonetheless, chitosan, the second most abundant biopolymer in nature next to cellulose, has shown significantly more promise in hemostatic efficacy[11,23,24] than either cellulose or dextran, and it has also been shown to be biologically nontoxic.[25] An added advantage is its substantial antimicrobial capability,[26] further strengthening chitosan's position as an ideal raw material for constructing wound care devices.

Chitosan has been studied as a hemostatic agent since the 1980s.[27–29] Since that time, a considerable amount of research on its use as a wound care material has been conducted.[30,31] Coincidentally, it still remains one of the least understood hemostats out of any of those noted above with regard to its actual mechanism of action. This lack of certainty is related to the observation that not all forms of chitosan or its precursor, chitin, are equally effective. The effectiveness of chitin/chitosan may vary from batch to batch, which may reflect sourcing issues as well as manufacturing difficulties. Jewelewicz et al.[32] found that the first Food and Drug Administration-approved chitin bandage, the RDH ("Rapid Deployment Hemostat") Bandage (Marine Polymer Technologies, Danvers, MA) was extremely effective, while Pusateri et al.[33] found that it was completely ineffective in the same animal model involving a liver injury. Commercial chitosan bandages, most notably the Hemcon Bandage, which were later and more advanced relative to chitin bandages, have been similarly equivocal with regard to their efficacy.[2,16,19] The physical form of chitosan along with its molecular characteristics of molecular weight, degree of deacetylation, and degree of ionization have been attributed to its hemostatic action and efficacy.[31] However, because of the batch-to-batch molecular variation inherent to the natural sources of chitin and to the postprocessing of chitin to chitosan, these characteristics of chitosan are difficult to control.[34] Hence, the inconsistency in hemostatic efficacy of chitosan reported in the literature is unsurprising.

Herein, we describe a modification to chitosan that enhances its performance as a hemostatic material. Hence, we aim to optimize an existing material for advanced hemorrhage control by means of biomolecular engineering. We attach a small number of hydrophobic tails to the backbone of chitosan, thereby creating a hydrophobically modified (hm) chitosan. In previous work, we have shown that hm-chitosan in dilute solution format is able to clot heparinized blood very rapidly relative to unmodified chitosan controls.[35] The proposed mechanism of action was a three-dimensional cross-linking of blood cells via anchoring of the hydrophobes along the chitosan backbone into the fatty membranes of adjacent cells. Because of the amphiphilicity of the hm-chitosan molecules, they are able to quickly self-assemble into gel-forming networks in the presence of cells. In that same study, a limited porcine injury trial ($n = 3$) was performed by applying a freeze-dried form of the hm-chitosan to an arterial transection model. Herein, we expand on that result by conducting a much large-scale study on a more stringent, femoral artery puncture, injury model with standard gauze dressing and unmodified chitosan dressing acting as controls.

The proposed mechanism of action of the hm-chitosan dressing (Fig. 1) is that upon compression of the sponge onto bleeding tissues, the fatty grafts anchor themselves noncovalently into stationary soft tissue cells, thereby increasing the tissue adhesion and the holding strength of the bandage. The grafting of hydrophobes onto the chitosan backbone is expected to create an independent set of "bonding opportunities" with the tissue upon compression of the chitosan dressing onto the injury. The results of this study suggest that the addition of the hydrophobes to chitosan may overcome the molecular variance issues that plague native chitosan.

MATERIALS AND METHODS

Materials

Chitosan of medium molecular weight (190–310 K) and Brookfield viscosity of 286 cps was purchased from

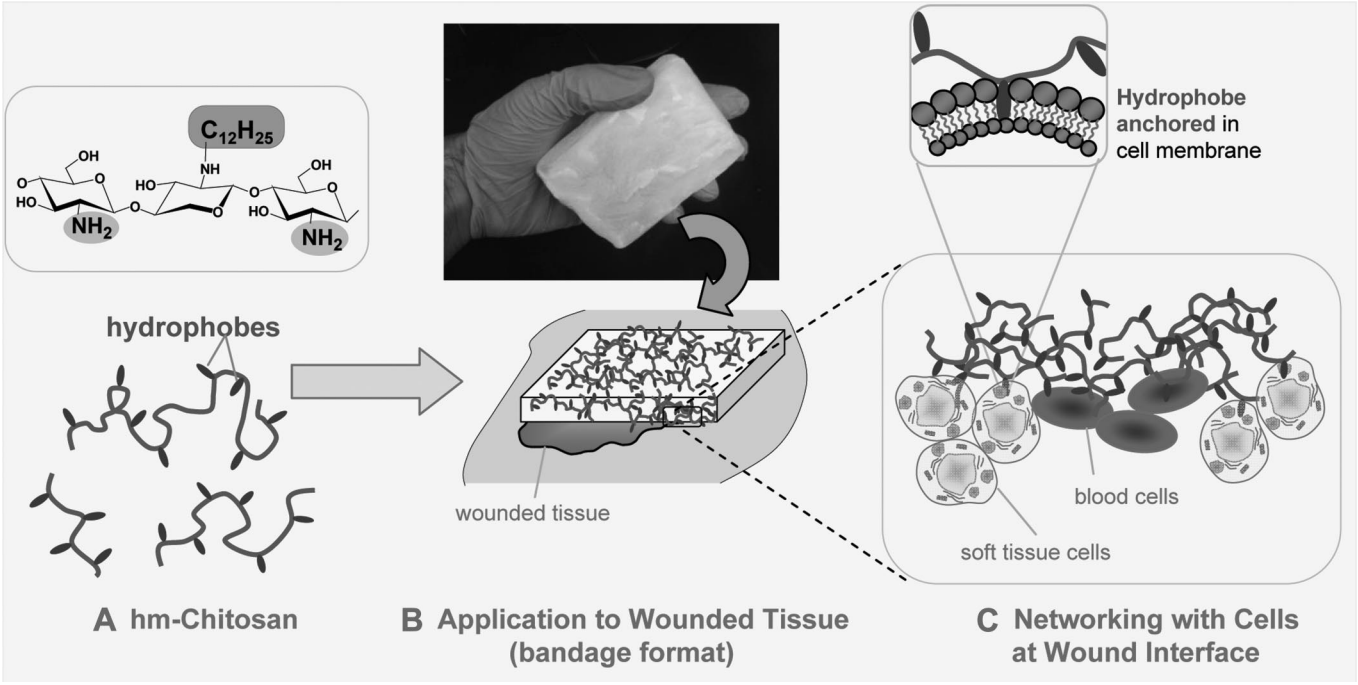

Figure 1. Mechanism for effective hemostasis of hm-chitosan. *A*, The polymer is shown schematically with its hydrophilic backbone in blue and the grafted dodecyl hydrophobes in purple. *B*, A bandage composed of freeze-dried hm-chitosan below which a schematic of its compression onto wounded tissue is demonstrated. *C*, Illustration of the expected mechanism of bandage-tissue networking. This interaction is driven by insertion of hydrophobes into soft tissue and blood cell membranes (as depicted in the top inset) upon compression on the injury. As such, a large, robust set of "bonding opportunities" with the wounded tissue are created via the addition of hydrophobes to the chitosan backbone.

Sigma-Aldrich. The reported degree of deacetylation was about 80%. Chitosan was dissolved in 0.2 mol/L of acetic acid (Sigma-Aldrich).

Synthesis of hm-Chitosan

hm-chitosan was synthesized by attaching *n*-dodecyl tails to the chitosan backbone via reaction with dodecyl aldehyde (purchased from Sigma). The procedure is identical to that used in our earlier study.[35] The degree of hydrophobic substitution follows the reaction stoichiometry. Here, several levels of modification were prepared (1, 2.5, 3.5, and 6 mol%) so as to screen for optimal tissue adhesion properties. The hm-chitosan with the most adhesiveness would be selected as the raw material for test bandage preparation.

Preparation of Chitosan and hm-Chitosan Bandages

A solution of 1.0 wt% chitosan or hm-chitosan was poured into trays of dimensions 10 cm (length) × 10 cm (width) × 4 cm (height). Trays were filled up to 1.27 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 3 mol/L) was placed on the back side of the dried solid for unhindered compression of the bandage during surgery. The dressings were physically and visually identical to each other.

Tissue Adhesion Studies

Dressing test pieces (punched 1 cm × 1 cm) were each compressed at the center of individual strips of bovine muscle tissue. Dressings were held at 20 kPa compression for a set hold time of 3 minutes. At the conclusion of the hold time, the tissue was fixed taut on two ends with vice grips and the dressing on the bottom side. Hanging weights (0.01 N) were then attached sequentially to the test pieces. The adhesion strength (kPa) was determined by the dressing detachment weight divided by the contact surface area. Testing was be performed with eight test pieces in the unmodified chitosan test group and eight test pieces in the modified chitosan test group.

Surgical Preparation and Instrumentation

Female Yorkshire pigs, weighing 39.2 kg ± 2.3 kg were obtained from the Thomas D. Morris Institute of Surgical Research (Reisterstown, MD). 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 Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. The swine were anesthetized with 6 mg/kg of telazol and 0.01 mg/kg of glycopyrrolate that were given intramuscularly. They were incubated and placed

on mechanical ventilation at a tidal volume 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.

Preoperative blood samples were collected during the quarantine period. Complete blood counts, coagulation profiles (prothrombin time, activated partial thromboplastin time, and fibrinogen), and serum chemistries were measured to ensure complete health status of the animals. Pigs were fasted for 24 hours for the surgical procedure but had free access to water. On the day of the surgery, a venous blood sample was collected to verify normal complete blood count values.

Surgical Procedures

All the surgical procedures were performed using standard aseptic methods. The ear vein was cannulated with a Teflon catheter (21 gauge), and lactated Ringer's (LR) solution was administered (5 mL/kg/h) throughout the operation to compensate for fluid evaporation. The left femoral artery and vein were cannulated for arterial blood sample collection and intravenous drug and fluid infusion. The arterial line was also connected to a telemetry device that permitted monitoring and recording of blood pressure, heart rate, electrocardiogram, and body temperature of the animals without restricting movement. The animals were randomized to chitosan, hm-chitosan, or gauze dressings. The surgeons were blinded to the type of dressing being utilized for each animal.

Next, a midline laparotomy was made followed by a splenectomy to minimize hematologic changes that may occur from autotransfusion by pig's contractile spleen. The blood loss from splenectomy was replaced by infusing LR at three times the weight of the removed organ.

To create a severe hemorrhage in the groin area, approximately 5 cm of the right femoral artery was dissected free from surrounding tissues and the overlying abductor muscle was removed. Injury to the adjacent femoral vein and nerve was avoided. The vessel was then bathed with a few milliliters of 2% lidocaine to relax vasospasm and dilate the artery to its normal size. A 10-minute stabilization period was allowed (no manipulation), and baseline data including mean arterial pressure (MAP) and body temperature were recorded. A stable MAP of 60 mm Hg or higher was required before proceeding with the rest of the experiment. The artery was clamped proximally and distally, and a 4.4-mm diameter arteriotomy was made on the anterior surface of the vessel using a vascular punch. The clamps were then released and free bleeding was allowed for 30 seconds. The shed blood was collected by suction, weighed, and recorded as pretreatment blood loss. While the femoral artery was bleeding, a dressing was applied to the injury site and manually compressed against the wound with sufficient pressure to occlude arterial blood flow. After a 3-minute compression, hemostasis was visually checked; if hemostasis was not achieved or if rebleeding occurred within the next 10 minutes, the dressing was removed and replaced with a new dressing of the same type, with an additional 3-minute compression. The second dressing was applied under the same conditions as the first one. If hemostasis again was not achieved, a third dressing was applied in the same manner as the second. After failure of a third dressing, hemorrhage was allowed without any additional intervention until the animal exsanguinated. This event was recorded as a failure of the dressing to achieve initial hemostasis; time of death was recorded once MAP and end-tidal P_{CO_2} fell below 20 mm Hg and 15 mm Hg, respectively. The blood and blood clots were collected from the peritoneal cavity and weighed, and the volume of posttreatment blood loss was determined. If hemostasis was achieved and was stable for 10 minutes, the animal was resuscitated intravenously with warm LR solution (three times the volume of pretreatment blood loss at 100 mL/min) to its baseline, preinjury MAP. The abdominal incision was then closed in layers.

Animal survival was defined as the presence of a heart rate at the end of 180 minutes. Any surviving animals at the end of the study period were killed with pentobarbital IV 100 mg/kg to 200 mg/kg.

Data Analysis

Data are expressed as mean ± SD and analyzed by analysis of variance (paired *t* test), Fisher's exact test, and log-rank test for statistical comparisons. *p* values were adjusted according to false discovery rate method for bigroup comparison. The data with high variance were log-transformed for analysis of variance. Statistical significance was assigned at a greater than 95% confidence level ($p < 0.05$).

RESULTS

Results of the tissue adhesion studies are shown in Figure 2. Our results indicate that the adhesiveness of the bandages increase linearly with increase in the level of hydrophobic modification according to the following equation:

$$\eta = 11.92h + \eta_0 \quad (1)$$

where η is the stress to remove the film bandage, h is the mol % of available amines along the chitosan backbone which were hydrophobically modified, and η_0 is the force to remove an unmodified chitosan film (the measured value being 31.77 kPa in this case). The 6 mol% modified chitosan was selected as the raw material for test bandages to be used in vivo as it displayed the highest adhesion strength at 101.3 kPa ± 10.3 kPa.

Body weight, temperature, hematologic measurements, and preinjury MAP are listed in Table 1. All the parameters were similar among treatment groups. The overall results are summarized in Table 2. Application of three consecutive gauze dressings with 2-minute compression times did not produce hemostasis. All animals treated with the gauze bled to death shortly after reestablishment of blood flow. The animals did not receive fluid resuscitation because gauze application did not produce initial hemostasis.

The efficacy of the chitosan dressing was tested in eight animals, and hemostasis was achieved in six. These results show greater efficacy of chitosan dressing to stop arterial bleeding compared with gauze treatment ($p = 0.02$). A total of 12 dressings from a single freeze-dry lot were tested, of

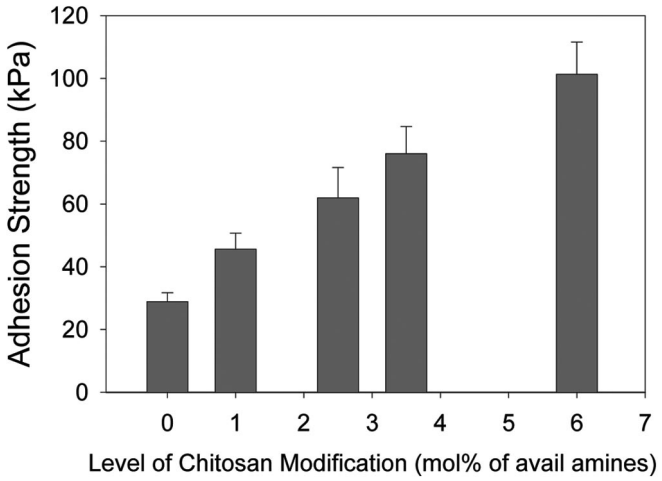

Figure 2. Tissue adhesion strength of hydrophobically modified chitosans. Chitosans with varying degrees of hydrophobic grafts were synthesized and subsequently made into freeze-dried bandages. The bandages were then evaluated for tissue adhesion via uniaxial load testing. Our results indicate that the adhesiveness of the bandages increase linearly with increase in the level of hydrophobic modification according to the equation $\varsigma = 11.92h + \varsigma_o$, where ς is the adhesion strength the bandage, h is the mol% of available amines along the chitosan backbone which were hydrophobically modified, and ς_o is the force to remove an unmodified chitosan film (the measured value 31.77 kPa in this case). Only hm-Chitosan of 6 mol% grafting density was selected for in vivo studies.

TABLE 1. Baseline Parameters and Animal Characteristics

Variable	
Body weight (kg)	39.2 ± 2.3
Body temperature (°C)	37.18 ± 0.47
Hematocrit (%)	30.7 ± 1.2
Hemoglobin (g/dL)	11.06 ± 0.55
Platelets (1000/μL)	308 ± 49
PT (s)	9.6 ± 0.4
aPTT (s)	15.8 ± 1.09
Fibrinogen (mg/dL)	221 ± 53
pH	7.45 ± 0.02
Preinjury MAP (mm Hg)	67.8 ± 7.7

Data expressed as mean ± SD.
aPTT, activated partial thromboplastin time; PT, prothrombin time.

which 6 (50%) were successful and stopped hemorrhage. Hence, hemostasis from the chitosan dressing was limited. The dressings failed at 48, 52, 53, 58, 64, and 73 minutes after application. Nonetheless, overall survival time was significantly longer in the chitosan-treated animals ($p = 0.0001$) than in the controls.

The hm-chitosan dressings produced hemostasis in all eight animals tested ($p = 0.002$ vs. gauze). A total of 10 dressings were used in this group, of which 8 were effective and stopped arterial hemorrhage.

Figure 3 shows the total blood loss (pretreatment + posttreatment) for each test group. The data show that the hm-chitosan dressings significantly reduce the amount of blood loss experienced for the animals; an average of 15.09 mL/kg of blood was loss in the hm-chitosan group relative to 44.62 mL/kg and 42.16 mL/kg for the chitosan and gauze groups, respectively.

Figure 4 shows typical mean arterial pressure profiles for pigs treated with the three tested hemostats. The hm-chitosan dressing produced initial hemostasis in all eight animals tested. A total of 10 dressings were used in this group, of which eight were effective and stopped hemorrhage. There was a trend for a higher percentage of hm-chitosan dressings than chitosan dressings to be efficacious (80% vs. 50%, $p = 0.16$). In Figure 4, the hm-chitosan dressing remained in place and prevented secondary bleeding despite a substantial rise in blood pressure during the 3-hour period tested. Hemostasis was sustained in all animals treated with hm-chitosan dressing for the 3-hour duration of the experiment. Hemostasis duration (Table 2) and survival time were significantly longer for the pigs treated with hm-chitosan dressing than the other two groups ($p < 0.0001$).

A Kaplan-Meier analysis of the survival data are shown in Figure 5. The trends from each control group show a progression from short survival times (gauze), improved survival times (chitosan), and long survival times (hm-chitosan) over a 180-minute observation period. All gauze-treated pigs expired within 7 minutes posttreatment of the third dressing. Pigs treated with the chitosan bandages expired at 15, 22, 37, 55, 67, 73, 78, and 93 minutes. In contrast, all pigs treated with the hm-chitosan bandages survive the 180-minute interval.

DISCUSSION

The tissue adhesion tests revealed that there is a linear relationship between tissue adhesion and the level of hydrophobic modification of the chitosan and suggests that the driving force behind adhesion is a summation of noncovalent bonding with the tissue. Because the strength each bond between hydrophobe and cell membrane would be roughly of equal value (<1 kcal/mol), the addition of new each new hydrophobe-membrane bond would cause a linearly proportional increase to the total tissue adhesion strength. Chitosans of hydrophobic modification levels greater than 6% were not tested, although the results suggest that a future study should be conducted which aims to identify the upper limit in hydrophobicity. As more hydrophobes are added to the chitosan, it becomes more difficult to dissolve in aqueous media and also becomes much more viscous, both of which would limit the processing capabilities for new bandage production. Here, we did not reach that upper limit and simply chose the most hydrophobic chitosan as it met our criteria of highest tissue adhesion strength.

Beyond the prelude of tissue adhesion studies, the bulk of this work compared the efficacy of two hemostatic dressings based on the biopolymer chitosan. One dressing was a medium molecular weight chitosan in freeze-dried 4″ × 4″ bandage format. The other was the same chitosan but modi-

TABLE 2. Outcomes for Treatment of a Severe Arterial Hemorrhage With Different Hemostatic Dressings in Swine

Dressing Type	Number of Animals	Pretreatment Blood Loss (mL/kg)	% Initial Hemostasis Achieved*	Number of Dressings Used	Duration of Hemostasis (h)	Posttreatment Blood Loss (mL/kg)	Survival Time (h)
Gauze (Kerlix)†	8	6.9 ± 1.7	0 (0/8)	24	0	35.2 ± 5.0	0.05 ± 0.03
Chitosan	8	7.6 ± 2.1	75 (6/8)‡	12	0.73 ± 0.47§	37.1 ± 6.0‖	0.92 ± 0.47¶
hm-Chitosan	8	8.2 ± 1.8	100 (8/8)#	10	3**	6.9 ± 6.0††	3‡‡

Data expressed as mean ± SD.
* Initial hemostasis was considered to occur after bleeding was stopped for at least 3 min after compression.
† Gauze testing was stopped after three unsuccessful experiments.
‡ vs. gauze, $p = 0.0035$ (Fisher's exact test).
§ vs. gauze, $p = 0.0027$ (log-rank test).
‖ vs. gauze, NS (paired t test).
¶ vs. gauze, $p = 0.000105$ (log-rank test).
# vs. gauze, $p < 0.0001$; vs. chitosan, not significant (NS) (Fisher's exact test).
** vs. gauze, $p = 1.62 \times 10^{-6}$, vs. chitosan $p = 1.85 \times 10^{-8}$ (log-rank test).
†† vs. gauze, $p = 0.0001$; vs. chitosan, $p < 0.0001$ (paired t test).
‡‡ vs. gauze, $p = 4.56 \times 10^{-8}$, vs. chitosan $p = 4.56 \times 10^{-8}$ (log-rank test).

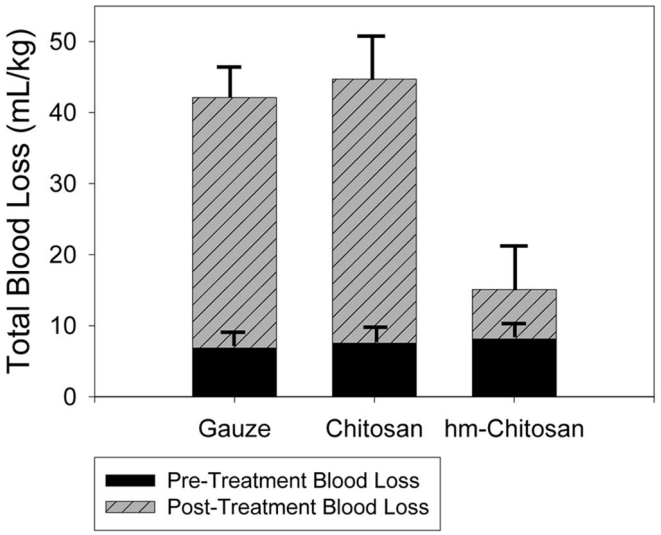

Figure 3. Total blood loss in porcine injury models. Average values of pretreatment and posttreatment blood losses of pigs treated with three hemostatic agents were summed to give total blood loss from femoral artery injury. The posttreatment blood loss was significantly less in hm-Chitosan group (n = 8) as compared with unmodified chitosan (n = 8) and gauze controls (n = 8).

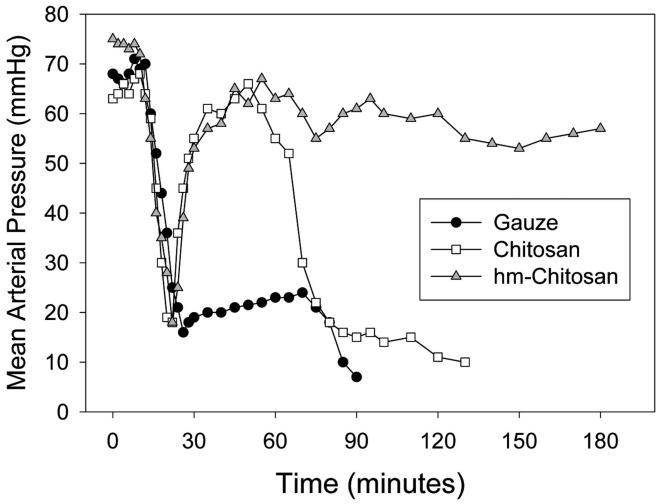

Figure 4. Profile of femoral artery mean arterial pressure (MAP) of typical animals receiving standard gauze (closed cirles), chitosan dressing (open squares), or hm-chitosan dressing (open triangles). MAP remained at near-normal levels posttreatment with hm-chitosan dressing for the 180-minute observation period. In contrast, unmodified chitosan dressings generally experienced rebleeds within 100 minutes, and gauze controls were ineffective at resuscitating MAP postinjury.

fied with a limited number (6 mol% of available amines) of C-12 alkyl tags along its backbone, rendering the biopolymer amphiphilic in nature. The hm-chitosan was processed in the same freeze-dried 4″ × 4″ bandage format. 4″ × 4″ gauze dressing served as the control. Hm-chitosan dressings produced initial hemostasis in all eight animals tested ($p = 0.002$ vs. gauze). In contrast, only six of eight dressings in the chitosan group and none of eight dressings in the gauze group provided initial hemostasis. The secondary bleedings observed in the chitosan group occurred at 48, 52, 53, 58, 64, and 73 minutes after application and correlate well with similar comparative studies.[2,19,36] Relative to other chitosan-based dressings, the hm-chitosan dressing showed promising results, particularly with regard to survival (100%), over the 180-minute observation period.

Contrary to the results from the chitosan arm of our study, there have been a number of studies where chitosan bandages have been shown to work very effectively. Hemcon bandages have been shown to be very effective in treating hemorrhage and improving the survival of pigs subjected to both femoral artery punctures[23] and grade V liver injuries.[11] Furthermore, a field report of 64 unique hemorrhage cases from Operation Iraqi Freedom and Operation Enduring Freedom showed that Hemcon dressings were 97% effective in staunching bleeding or improving hemostasis.[24] An additional field study was carried out a level III US combat support hospital in Iraq in 2006. In all 44 studied cases studied, the Hemcon bandages were described by the attending surgeon as successful in achieving hemostasis.[12] The high

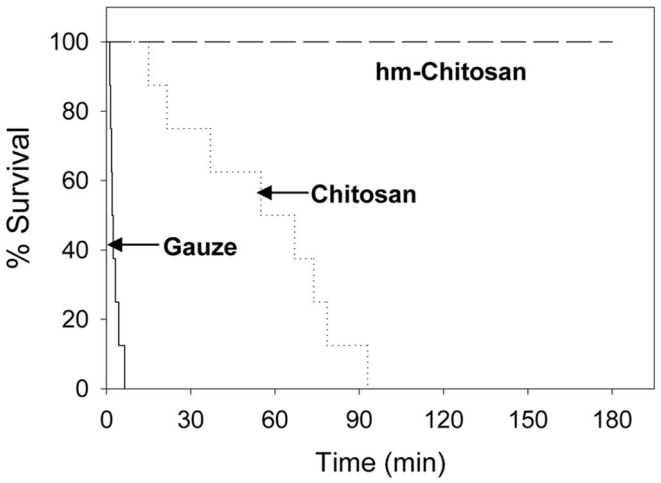

Figure 5. Kaplan-Meier analysis of survival data. The hm-chitosan dressing (dashed line) maintained hemostasis and supported survival of animals for the full 180 minutes of observation testing ($p < 0.0001$, log-rank test). The unmodified chitosan dressing was also found to be more effective than standard gauze ($p < 0.001$, log-rank test).

level of success in the field may suggest that the animal model used here and in similar studies may not be relevant to most battle injuries. However, even within the same injury models, chitosan bandages have shown considerable variation,[14,16] highlighting significant room for improvement either in sourcing, molecular engineering, manufacturing, or all three.

Tissue adherence has been cited in literature as an important variable for the achievement of hemostasis and the duration of hemostasis for chitosan bandages.[1,37] In an earlier study from our group, we showed that a chitosan patch, whose surface was preexposed to a saturated water vapor, has the capability to stop a severe liver bleed in a hypothermic, coagulopathic grade V liver injury model in swine.[38] The success of this "modified chitosan patch" on such a stringent injury model was also attributed to increased tissue adhesion (adhesion data not shown). In the current study, increased tissue adhesion is achieved through a different modification to chitosan, albeit with qualitatively similar success. Such results further bolster the importance of tissue adhesiveness of chitosan bandages with respect to their hemostatic efficacy.

In reviewing the ideal characteristics for a hemostatic dressing for prehospital use, as stated by Pusateri et al.,[37] lyophilized chitosan itself is an ideal starting point for a hemostatic dressing. It is durable, low cost, antimicrobial, safe, ready-for-use and easy to apply. The results of this study suggest that the hydrophobic modification to chitosan may enhance efficacy relative to a regular chitosan dressing such that hemostasis is consistently achieved for the entire prehospital period. The hydrophobic modification to chitosan is low cost and ostensibly safe, as such the hm-chitosan dressings should continue to fulfill the remaining criteria.

Recently, an additional dressing criterion has been requested: it should be easily removable.[39] This likely was inspired by the difficulty in removing powder-based products such as WoundStat, Celox, and Quickclot. Difficulty in resection of the hemostat makes the job of the trauma surgeon extremely cumbersome upon receiving a patient from the field. Also, if the material is not bioresorbable, as is the case with WoundStat and Quickclot, this creates a significant risk for the patient postsurgery; removal of all residual material for any given case is doubtful. Fortunately, the hm-chitosan bandages were able to be removed from the tissue without much difficulty after the procedure (data not shown). This observation is consistent with the noncovalent nature of the hydrophobic interactions with the tissue. That is, each individual interaction is very weak; the sum of all these interactions results in a substantial adhesion that is versatile enough to remove. Hence, we can easily remove these "Velcro-like" bandages[35] on demand without any residual material left behind.

There were two hm-chitosan bandages which failed to provide initial hemostasis, and although it is difficult to make any conclusions as to why this occurred, we will offer possible explanations. As with any "compression-required" hemostat, there is an unavoidable element of user error involved in these evaluations. The injury sites in some cases can be difficult to cover on a first pass attempt with a bandage either due to difficulty in visualization of the injury site or interference of interposing tissues. Such issues have been noted by other authors.[23,24] In the two cases where the hm-chitosan did not provide initial hemostasis, we believe that the puncture simply was not covered completely by the bandage on the first compression. In contrast, the follow-up attempts, with suction of the pooled blood preceding it, were generally easier circumstances to visualize the site of puncture and to address it with compression. In addition, as with any chitosan-based technology, there will be batch-to-batch variation in the chitosan molecular weight, and it is possible that this variation might affect the hm-chitosan bandages as well, although to a lesser degree than the native chitosan bandages.

In future work, we would like to study the survival of animals for a significantly longer period of time (up to 96 hours). Although we have identified the first 3 hours after injury as a critical interval for chitosan-based hemostats, it is clearly important to understand the failure risk for the hm-chitosan bandages over a more substantial timeframe. As mentioned previously, nonlinear combat settings can be extremely unpredictable and rescue operations are often difficult to complete within 3 hours. In addition to long-term survival studies, it would be useful to understand how the hm-chitosan bandage performs under coagulopathic conditions by applying it to recently developed animal injury models which simulate coagulopathy.[38,40] Such models are relevant as trauma victims who experience voluminous blood loss often develop coagulopathy during resuscitation, making hemostasis significantly more difficult to achieve. Finally, we plan to study the tissue reactivity and the biocompatibility of the hm-chitosan relative to the native chitosan. While neither animal death nor any noticeable inflammation was observed in the hm-chitosan group, this aspect of the material is crucial

to its potential use as a hemostatic device. Histologic sections of the injured vessel and surrounding tissue should be collected, stained, and evaluated for unwanted blockage of the artery and undesirable inflammation. It is worth noting that a study using an *N*-palmitoyl (C_{16} acyl) chitosan as an in situ forming hydrogel was found to be nontoxic in vitro in culture with mouse fibroblast cells.[41] In rats, the hydrogel was found to elicit an initial macrophage response which decreased over a 6-week period.[41] Although biocompatibility studies on these C-12 alkyl chitosan must be performed before human use, these prior toxicity studies using similar compounds project biological benignity.

In summary, a highly tissue-adhesive hm-chitosan bandage of 6 mol% C-12 alkyl tails was identified. This material was used to make bandages to be used in vivo on a large animal bleeding model. Hm-chitosan is superior to unmodified chitosan sponges ($p < 0.001$) or standard gauze for controlling bleeding from a lethal arterial injury. The dressings were tested for their ability to repair a 4.4-mm femoral artery injury in a lethal hemorrhage model in pigs. Their ability to stop the initial hemorrhage and subsequently maintain hemostasis up to 3 hours was tested and compared with gauze dressing. Both test dressings were able to stop the initial femoral artery bleeding and achieve hemostasis that could not be controlled by standard gauze treatment, with a success rate of 75% for the chitosan bandage and 100% for the hm-chitosan dressing. However, the hemostasis produced by the chitosan dressing was unstable and the dressings tended failed on an average of 44 minutes (range, 0–73 minutes), whereas the hm-chitosan dressing produced secure hemostasis for 3 hours. As such, the hm-chitosan dressings promise improved outcomes relative to native chitosan-based dressings for control of active hemorrhage.

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DISCLOSURE

The authors declare no conflicts of interest.

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