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## Hydrophobically-modified chitosan foam: description and hemostatic efficacy

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### ABSTRACT

**Background:** Trauma represents a significant public health burden, and hemorrhage alone is responsible for 40% of deaths within the first 24 h after injury. Noncompressible hemorrhage accounts for the majority of hemorrhage-related deaths. Thus, materials which can arrest bleeding rapidly are necessary for improved clinical outcomes. This preliminary study evaluated several self-expanding hydrophobically modified chitosan (HM-CS) foams to determine their efficacy on a noncompressible severe liver injury under resuscitation.

**Methods:** Six HM-CS foam formulations (HM-CS1, HM-CS2, HM-CS3, HM-CS4, HM-CS5, and HM-CS6) of different graft types and densities were synthesized, characterized, and packaged into spray canisters using dimethyl ether as the propellant. Expansion profiles of the foams were evaluated in bench testing. Foams were then evaluated *in vitro*, interaction with blood cells was determined via microscopy, and cytotoxicity was assessed via live–dead cell assay on MCF7 breast cancer cells. For *in vivo* evaluation, rats underwent a  $14 \pm 3\%$  hepatectomy. The animals were treated with either: (1) an HM-CS foam formulation, (2) CS foam, and (3) no treatment (NT). All animals were resuscitated with lactated Ringer solution. Survival, total blood loss, mean arterial pressures (MAP), and resuscitation volume were recorded for 60 min. **Results:** Microscopy showed blood cells immobilizing into colonies within tight groups of adjacent foam bubbles. HM-CS foam did not display any toxic effects *in vitro* on MCF7 cells over a 72 h period studied. Application of HM-CS foam after hepatectomy decreased total blood loss ( $29.3 \pm 7.8$  mL/kg in HM-CS5 group versus  $90.9 \pm 20.3$  mL/kg in the control group;  $P < 0.001$ ) and improved survival from 0% in controls to 100% in the HM-CS5 group ( $P < 0.001$ ).

**Conclusions:** In this model of severe liver injury, spraying HM-CS foams directly on the injured liver surface decreased blood loss and increased survival. HM-CS formulations with the highest levels of hydrophobic modification (HM-CS4 and HM-CS5) resulted in the lowest total blood loss and highest survival rates. This pilot study suggests HM-CS foam may be useful as a hemostatic adjunct or solitary hemostatic intervention.

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## 1. Introduction

On the battlefield, traumatic hemorrhage remains the leading cause of preventable death, accounting for 50% of all deaths before the injured patient reaches a treatment facility [1–7]. Noncompressible, or intracavitary, hemorrhage accounts for approximately 80% of all hemorrhage-related deaths [8]. Although several advanced hemostatic products have been deployed for military use, most notably Combat Gauze, Hemcon Bandage, none are designed to treat intracavitary bleeding [9–13]. Currently, no effective solutions for noncompressible hemorrhage exist outside of the surgical intervention [8,14].

A number of biomaterials have been evaluated as potential treatments of noncompressible hemorrhage. The largest effort has come from the United States Army in their evaluation of sprayable fibrin foams for use in truncal bleeding [15,16]. However, results with the fibrin sprays have been equivocal [8] and fibrin use in the field is limited due to its cost, storage requirements, and preparation before application. Other products, such as thrombin based hemostatic agents, lyophilized platelets, conjugated red blood cells, and fibrinogen-coated albumin microparticles showed limited success or practicality [8,17].

Chitosan (CS) is a highly abundant, low-cost polysaccharide, which has been used commercially as a solid, compressible, and hemostatic agent since 2003 [19,20]. It has not, however, been used as a flowable or sprayable agent. Furthermore, its efficacy in solid format for compressible hemorrhage models has been questioned [21–23]. In the previous work, we have shown that the modification of CS with hydrophobic grafts enhances its hemostatic capabilities, particularly in its sprayable format [24,25].

In this study, we perform an initial screening of the efficacy of a new hydrophobically modified chitosan (HM-CS) foam for use in treatment of noncompressible hemorrhage. On dispensing the material from the canister, unlike native CS formulations, the HM-CS self-expands into a foam which fills cavities rapidly. We hypothesized that the HM-CS foam would reduce blood loss and improve survival in the absence of direct pressure in a rat model of severe hepatic hemorrhage.

## 2. Materials and methods

### 2.1. Test materials

Six sets of HM-CSs were synthesized according to previous methods [24]. The variants of HM-CS synthesized are shown in Table 1, and the chemical structures of the corresponding C-12 and C-18 CS variants are displayed in Figure 1. Through this set of HM-CS biopolymers, we aimed to gain important insight on the effect hydrophobic grafting density and hydrophobic length on hemostatic ability. Foams were created as follows: HM-CS (1.0 wt%) was dissolved in 0.2 M acetic acid (Sigma–Aldrich, St Louis, MO). The resulting solution was co-injected into a pressure-resistant handheld, lightweight aluminum canister along with dimethyl ether (DME) as the propellant material. The canister is shaken for 10 s to mix well

**Table 1 – List of HM-CS variants synthesized.**

| Polymer variant | Graft type | Grafting density (theoretical % mol) |
|-----------------|------------|--------------------------------------|
| CS              | None       | 0                                    |
| HM-CS1          | C-12       | 1                                    |
| HM-CS2          | C-18       | 1                                    |
| HM-CS3          | C-12       | 2.5                                  |
| HM-CS4          | C-18       | 2.5                                  |
| HM-CS5          | C-12       | 5                                    |
| HM-CS6          | C-18       | 5                                    |

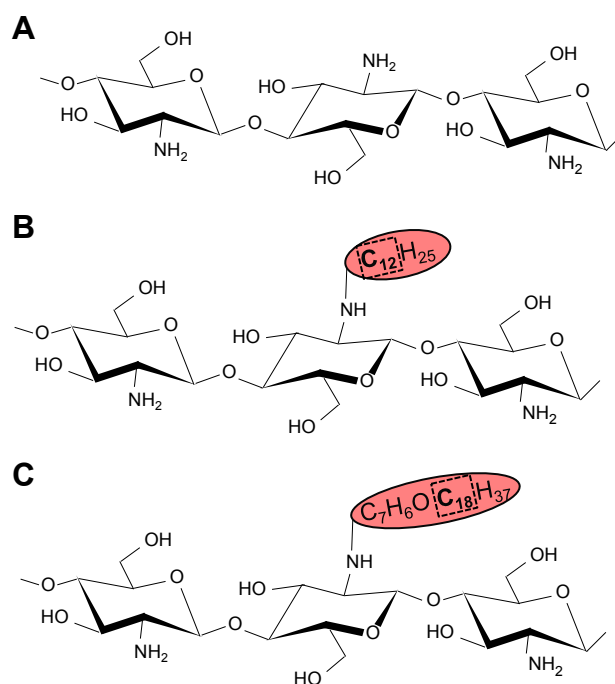
and the contents was sprayed onto the injured tissue at a constant pressure. This apparatus was optimized for smooth dispensing of the foam onto tissue. The canisters were then crimped and filled with DME at a ratio of 30:70 (v/v) (DME):(polymer concentrate).

### 2.2. Optical microscopy

Bovine heparinized blood was mixed with sample HM-CS4 and pressed down a glass slide by a cover slip. A Zeiss (Jena, Germany) Axiovert 135 TV inverted microscope equipped with the Motic Image Plus imaging system was used to visualize blood cells on the glass slide with a  $\times 10$  objective lens.

### 2.3. Cell culture

MCF7 human breast cancer cells (American Type Culture Collection, Manassas, VA) were used for testing of



**Fig. 1 – Structures of CS and HM-CS. In (A), the structure of unmodified CS is shown. In (B), the structure of a HM-CS with a C-12 graft is shown. Finally, in (C), the structure of an HM-CS with a C-18 graft is shown. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)**

cytocompatibility of the HM-CS foams. These cells are sensitive to outside polymer matrix molecules, and as such, act as a common platform for testing for initial safety of new biomaterials. For culture of MCF7 cells, high-glucose Dulbecco's Modified Eagle media supplemented with 5  $\mu\text{L}/\text{mL}$  of penicillin or streptomycin and 10% fetal bovine serum was used. MCF7s were cultured separately in T75 flasks in a 37°C incubator with 5%  $\text{CO}_2$ . Cells were subcultured every 5–7 d by trypsinization with 0.25% and/or 0.02% trypsin and/or EDTA.

#### 2.4. Live–dead cell assay

A solution containing 4  $\mu\text{M}$  of live (calcein-AM; Life Technologies, Grand Island, NY) and dead (ethidium homodimer; Life Technologies, Grand Island, NY) assay reagent was prepared in phosphate buffered saline. To stain the cells, 10  $\mu\text{L}$  of this solution was added to the culture media, incubated at room temperature for 15 min, and then imaged on a confocal microscope (Leica SP5 X; Leica Microsystems, Buffalo Grove, IL). For imaging calcein-AM, the excitation was done at 495 nm and emitted light was recorded using a 505–554 nm band-pass filter. For imaging ethidium homodimer, the excitation was done at 556 nm and imaging was done with a 568–700 nm band-pass filter. Images were captured 72 h after addition to the MCF7 cells, the test samples which were prepared by extraction of 0.5 wt% CS and HM-CS5 foam samples in complete DMEM for 24 at 37°C.

#### 2.5. Animals

Thirty-five male Sprague–Dawley rats (278–346 g) were housed inside a climate controlled facility. Food and water was available *ad libitum*. All animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care and Use Committee of the Massachusetts General Hospital, Boston, MA. All animals received care in strict compliance with the National Research Council's Guide for Care and Use of All Laboratory Animals.

#### 2.6. Liver injury model

A modified liver injury model was adopted from Matsouka et al. [26], and Holcomb et al. [15], of reproducible, nonheparinized severe liver injury model of noncompressible hemorrhage. Anesthesia was induced with pentobarbital sodium (60 mg/kg intraperitoneally) administered through a 25-gauge needle. Femoral arterial and femoral venous catheters were placed via cervical cutdown. The internal venous line (25 gauge) was connected to a pump for resuscitation. A rectal temperature probe was placed and connected to a digital thermometer. The pre-injury temperature was maintained at 37°C with a warming blanket and heat lamp. Mean arterial pressure (MAP) and systolic and diastolic blood pressures as well as heart rate were recorded at 10-s intervals throughout the study period using a continuous data collection system connected to the femoral arterial line.

A midline laparotomy was performed. Using a small plastic ruler, the capsule of the median lobe was scored in three spots (lateral, medial, and in the midline), 1 cm from the suprahepatic vena cava, with a handheld cautery. The portion of

median lobe distal to cautery marks was excised with scissors. No manipulation of the rapidly bleeding liver occurred. Immediately after completion of the liver injury, animals were resuscitated with warm (40°C) lactated Ringer solution at the infusion rate of 1.0 mL/min. The abdominal cavity was left open and animals were allowed to bleed for 60 s before applying the foam and no blood was removed from the peritoneal cavity or cut liver surface during this bleeding period. Single gauze 2  $\times$  2's was used as necessary to collect blood if it overflowed the peritoneal cavity and weighed for quantification of blood loss. After 60 s, HM-CS or CS concentrates in aluminum canisters with DME as the propellant were sprayed into the open-abdominal cavity. The canister was held 6 inches from the cut surface of the liver, and the actuator was pressed for 6 s to dispense the foam.

Animals were monitored for 60 min or until death, whichever came first. Death before 60 min was defined as a respiratory rate of 0 and no arterial waveform. After 60 min, the animals that were still alive were euthanized with sodium pentobarbital.

The dose (grams) of foam applied was recorded as the difference in weight of the foam components and container immediately before and just after application. At the end of the 60 min study period, shed blood in the abdominal cavity was quantified. The total blood loss was calculated as the difference between blood-soaked sponges minus the weight of pre-weighed dry 2  $\times$  2's for each animal. Total resuscitation fluid used and time to death was recorded. Each liver was removed and the remaining median lobe was dissected from the liver and each section individually weighed.

#### 2.7. Procedure and statistics

Animals were assigned to treatments according to a random number table. Treatment groups were designated as following: (1) HM-CS foam, (2) CS foam, and (3) no treatment control group (NT). The CS foam was a placebo foam and differed from the HM-CS in that hydrophobes are grafted along the backbone of the CS. The CS does have known hemostatic qualities, although it is not rapidly effective in a liquid or flowable format. The investigators were blinded to treatment for groups 1 and 2.

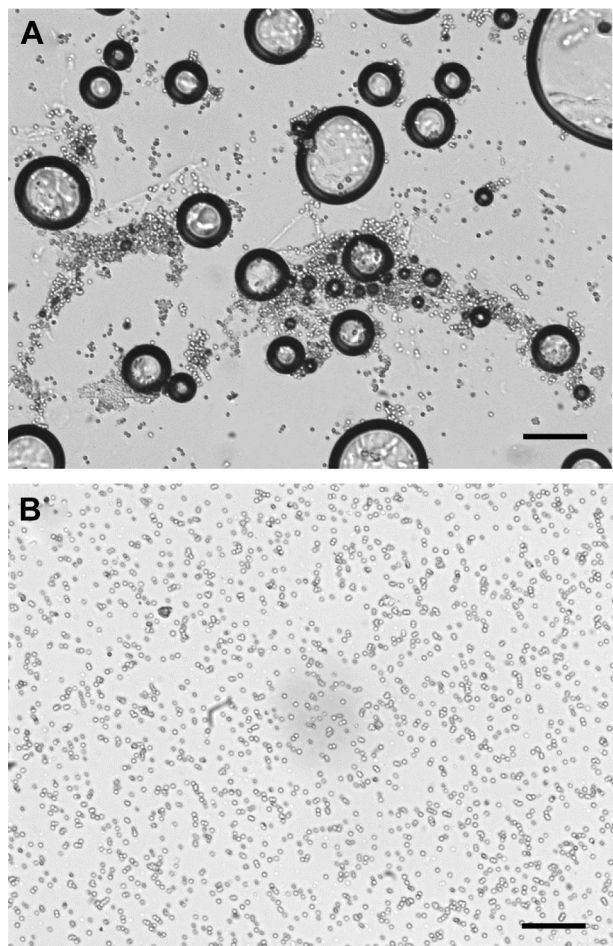
The weight of the excised median lobe divided by the pre-injury total body weight of the rat was used as a measure of the reproducibility of the injury. Blood loss was corrected for body weight (mL/kg). All measures are presented as mean  $\pm$  standard deviation. For measures with differences between group means, direct comparisons of the test sample groups with the CS and NT groups were performed using the Tukey–Kramer multiple comparisons test. Statistical significance was assigned at a >95% confidence level ( $P < 0.05$ ).

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### 3. Results

#### 3.1. Sample exclusions

HM-CS6 was observed to be too viscous to be sprayed from the aerosol canister, and as such, it was excluded from the study.



**Fig. 2 – Photomicrograph of blood mixed with HM-CS foam. (A) Stabilized foam bubbles in a sample of HM-CS5 mixed with heparinized bovine blood are shown between a glass slide and cover slip. Blood cells are observed to immobilize and aggregate between neighboring foam bubbles. In areas where no bubbles are present, blood cells move uninhibited with the convective flow of plasma. (B) For unmodified CS samples sprayed with propellant, no bubbles are observed. Furthermore, blood cells do not aggregate significantly, and they move uninhibited with the convective flow of plasma. Scale bar is 100  $\mu\text{m}$ .**

All other HM-CS foam samples were sprayable and thus included in the study for evaluation.

### 3.2. Microscopy

Figure 2 displays a photomicrograph of bovine heparinized blood mixed with sample HM-CS5 magnified by  $\times 100$ . This image was representative of other HM-CS samples mixed with whole blood. Blood cells were seen to become trapped between foam bubbles stabilized by the HM-CS. Once the blood cells were trapped, they were observed to become immobile. In contrast, unmodified CS or CS, does not form bubbles, and the blood cells are shown to be mobile under the cover slip. This experiment suggests *in vitro* that HM-CS would be more effective at halting the flow of blood, and

potentially assisting in accelerating clotting by gathering blood cells into tight colonies.

### 3.3. Live–dead cell assay

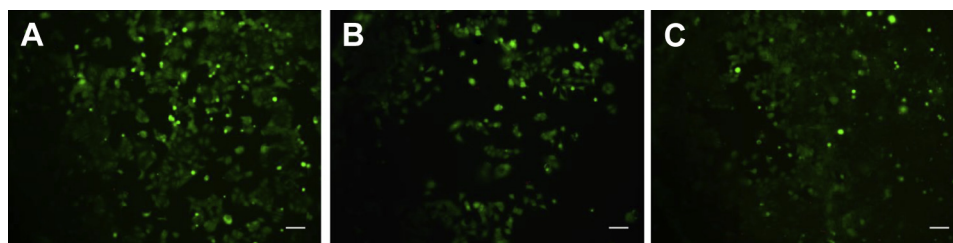
Figure 3 shows the results of the live–dead cell assay of an HM-CS sample relative to controls. This assay was carried out to gain a preliminary assessment of the cytotoxicity of the HM-CS foam formulations. HM-CS5 was used as the representative-modified biopolymer test sample. The foam was soaked in complete DMEM at a weight ratio of 0.5% overnight at 27°C. Calcein-AM was used as the live stain and ethidium homodimer as the dead stain. After exposure of cells to these stains, confocal microscopy was used to monitor the resulting fluorescence. A green fluorescence due to calcein is indicative of live cells whereas red fluorescence due to intercalation of ethidium is indicative of dead cells. In Figure 3A, the live–dead overlay for the cell media control at 72 h is shown; as expected, most of the cells absorb the live stain, but do not absorb the dead stain. In Figure 3B, the live–dead overlay for a CS control foam (0.5 wt%) is shown; qualitatively, the image resembles that of the cell control media, with slight inhibition of cell growth. Finally, in Figure 3C the live–dead overlay for an HM-CS5 foam (0.5 wt%) is shown. Like the cell control media and CS foam, the vast majority of cells treated with HM-CS5 foam extract absorbed the live-stain only. This is an initial indication that HM-CS foams, even at high grafting densities like HM-CS5, are benign biomaterials.

### 3.4. In vivo injury model development

Rats were used as the test subjects for each of the HM-CS formulations ( $n = 5$  per test group), with the addition of an unmodified CS control and NT control. There was no statistical difference among categories of numbers of animals tested, amount of foam applied, liver excision weight, and resuscitation volume. To create the injury and hemorrhage, a section of the medial hepatic lobe was resected, representing  $14 \pm 3\%$  of initial liver weight was excised with surgical scissors, and no difference in liver resection percentage in studied groups. This injury was lethal within the timeframe studied; the average survival time was  $17.2 \pm 0.8$  min. Figure 4A shows a photograph of a rat subject, which had HM-CS foam applied to the resected liver; a post-mortality image of the medial lobe of the liver next to its excised pieces is shown in Figure 4B. The foam was applied easily and quickly into the body cavity on canister actuation. In our model, a clear association between total blood loss and lethality was established. We measured blood loss through the weight change in gauze used to absorb the blood in the peritoneal cavity at the end of the experiment.

### 3.5. Survival

An important aspect of this work was to determine if application of the CS foam improves survival following liver trauma. Table 2 displays the baseline characteristics of these animals and Table 3 displays the summary results of the study. The cumulative study data show that there was a clear trend demonstrating decreased blood loss and increased survival following application of HM-CS foam ( $P < 0.001$  versus NT,  $P < 0.05$  versus CS). Total blood loss and survival

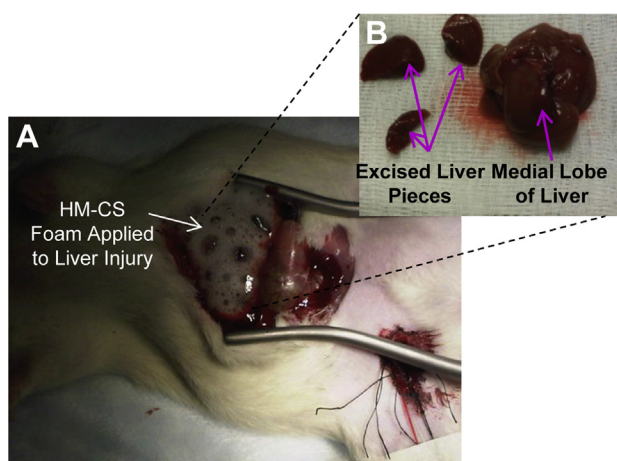


**Fig. 3** – Live–dead cell assay of HM-CS foam. In (A), the live-stained and dead-stained cells for a cell media control are shown. In (B), the live-stained and dead-stained cells for a CS control foam (0.5 wt%) are shown. In (C), the live-stained and dead-stained cells for an HM-CS5 foam (0.5 wt%) are shown, respectively. All three samples showed that the vast majority of cells were alive at 72 h. Scale bar is 100  $\mu$ m. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

times varied among samples; each HM-CS versus NT was statistically significant ( $P < 0.01$ ). Both the NT and CS controls showed a 0% survival rate, with 0 out of 5 animals surviving the 60 min observation period. In stark contrast, survival rates improve for all injuries addressed with HM-CS4 and HM-CS5, which achieved 100% survival in the 60 min timeframe studied. Figure 5 displays a Kaplan–Meier survival analysis of the animal test subjects in the groups for NT, CS, and HM-CS5. The HM-CS5 (dot-dashed line) maintained hemostasis and supported survival of animals for the full 60 min of the observation testing. In contrast, the CS (dashed line) and NT (black dotted line) controls resulted in deaths among all test subjects within 30 min. The statistical difference for NT versus HM-CS5 and CS versus HM-CS5 curves were both very stark ( $P < 0.001$ ).

#### 4. Discussion

Previous studies have shown that HM-CS samples in flowable or bandage format possess advantages over unmodified CS for



**Fig. 4** – Photograph of application of foam to rat liver injury. In (A), a photograph of HM-CS foam filling the peritoneal cavity after application to the injured site is shown. (B) shows a post-mortem image of the excised pieces of liver juxtaposed to the remaining medial lobe of the liver. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

treatment of bleeding [24,25]. However, in this study, a particularly novel format of the material, a sprayable foam, is applied to a specific type of soft tissue bleeding injury without any compression. The sprayable foam format shows a visually distinct contrast between HM-CS and CS, as the HM-CS foam expands significantly in volume on exit from the aluminum canister, whereas CS sprays do not expand at all on dispensing. This physical expansion aspect likely had an effect on the ability of the samples to address the pulsating bleed coming from the liver without the aid of manual compression. Thus, when the results of previous hemostatic studies on the HM-CS molecule are placed within the context of the outcome of the present study showing the physical capabilities of HM-CS in the sprayable foam format, we find appreciable promise for the use of this material in noncompressible applications. Note that no additional foaming agents were added to these formulations, only the dissolved biopolymer and the propellant were added into the spray canisters. As such, HM-CS is able to act as its own foaming vehicle, which is an advantage to the safety profile of this application. Many traditional foaming agents are cytotoxic surfactants.

In further expansion beyond earlier HM-CS studies, the observation of these samples mixed with blood under the microscope is a step forward with respect to characterizing the material for use in biomedical applications. Blood cells were observed to cluster around the bubbles formed by the

**Table 2** – Baseline parameters and animal characteristics.

| Variable                 | Mean $\pm$ SD    |
|--------------------------|------------------|
| Body weight (g)          | 303.2 $\pm$ 18.4 |
| Hematocrit (%)           | 31.8 $\pm$ 4.1   |
| pH                       | 7.44 $\pm$ 0.02  |
| Lactate (mM)             | 1.15 $\pm$ 0.42  |
| pCO <sub>2</sub> (mm Hg) | 30.9 $\pm$ 4.9   |
| pO <sub>2</sub> (mm Hg)  | 98.7 $\pm$ 16.0  |
| Heart rate (bpm)         | 247.9 $\pm$ 57.2 |
| Preinjury MAP (mm Hg)    | 105.2 $\pm$ 14.3 |

Data expressed as mean  $\pm$  standard deviation.

pCO<sub>2</sub> = partial pressure of blood carbon dioxide; pO<sub>2</sub> = partial pressure of blood oxygen; SD = standard deviation.

**Table 3 – Outcomes for treatment of severe liver hemorrhage with different hemostatic foams in rats.**

| Foam type | Number of animals | Amount of gr3foam applied (g) | Excision weight/liver weight ( $\times 100\%$ ) | Resuscitation volume (mL) | Total blood loss (mL/kg)      | Survival time (min)           |
|-----------|-------------------|-------------------------------|---|---------------------------|-------------------------------|-------------------------------|
| NT        | 5                 | NS                            | 15 $\pm$ 4                                      | 28.5 $\pm$ 5.0            | 90.9 $\pm$ 20.3               | 17.2 $\pm$ 0.84               |
| CS        | 5                 | 6.2 $\pm$ 1.8                 | 14 $\pm$ 4                                      | 27.2 $\pm$ 5.2            | 75.1 $\pm$ 14.4 <sup>*</sup>  | 21.6 $\pm$ 4.8 <sup>†</sup>   |
| HM-CS1    | 5                 | 7.6 $\pm$ 1.2                 | 13 $\pm$ 4                                      | 28.9 $\pm$ 14.9           | 45.0 $\pm$ 12.3 <sup>‡</sup>  | 34.6 $\pm$ 17.6 <sup>§</sup>  |
| HM-CS2    | 5                 | 6.7 $\pm$ 1.6                 | 15 $\pm$ 3                                      | 26.0 $\pm$ 14.0           | 48.9 $\pm$ 11.4 <sup>  </sup> | 36.0 $\pm$ 22.1 <sup>¶</sup>  |
| HM-CS3    | 5                 | 6.2 $\pm$ 0.9                 | 14 $\pm$ 4                                      | 29.7 $\pm$ 10.2           | 39.8 $\pm$ 12.4 <sup>#</sup>  | 44.2 $\pm$ 14.8 <sup>**</sup> |
| HM-CS4    | 5                 | 6.3 $\pm$ 0.9                 | 15 $\pm$ 3                                      | 30.5 $\pm$ 8.7            | 31.4 $\pm$ 7.2 <sup>††</sup>  | 60 <sup>‡‡</sup>              |
| HM-CS5    | 5                 | 6.6 $\pm$ 1.1                 | 15 $\pm$ 2                                      | 26.5 $\pm$ 19.8           | 29.3 $\pm$ 7.8 <sup>§§</sup>  | 60 <sup>   </sup>             |

Data expressed as mean  $\pm$  standard deviation.

NS = not significant.

P values were calculated by Tukey–Kramer multiple comparisons test.

<sup>\*</sup> vs. NT, NS.

<sup>†</sup> vs. NT, NS.

<sup>‡</sup> vs. NT,  $P < 0.001$ ; versus CS,  $P < 0.05$ .

<sup>§</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.05$ .

<sup>||</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.05$ .

<sup>¶</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.05$ .

<sup>#</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.01$ .

<sup>\*\*</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.05$ .

<sup>††</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.001$ .

<sup>‡‡</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.001$ .

<sup>§§</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.001$ .

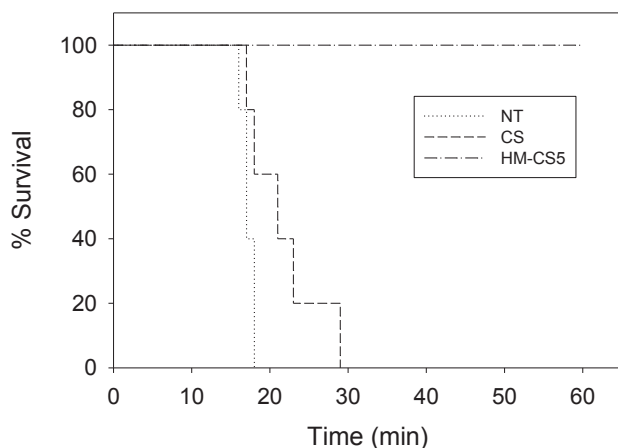
<sup>|||</sup> vs. NT,  $P < 0.001$ ; vs. CS,  $P < 0.001$ .

HM-CS foam samples. Although the hypothesis presented in earlier work regarding the passive insertion of hydrophobes into cell bilayers [24], and resultant 3-D networking of cells, may provide some insight into the current results, further studies are required to understand the actual interaction between HM-CS and blood cells. This result may suggest that the blood cells become physically trapped in the vicinity of the thick HM-CS interfaces, which act to stabilize the surface of the foam bubbles. In addition to some further insight around the mechanism of action, the initial safety profiling is a distinguishing feature of this work. In general, the study of

biocompatibility of amphiphilic biopolymers is surprisingly nascent, and previous hemostatic studies on HM-CS did not include any element of safety evaluation. With the results presented in Figure 3, we have observed an encouraging initial indication that the hydrophobic modification of polymers can be executed in ways which are benign to biological cells. Note that others have observed similar results when testing the biocompatibility of HM-CS [27].

Several materials have now been tested for use in noncompressible hemorrhage models. The subject of a series of several noncompressible studies was a fibrin sealant foam, which delivered a mixture of fibrinogen and thrombin to the site of an animal liver resection [15,16]. More recently, a polyurethane-based injectable material has been evaluated in a closed-abdomen *in vivo* model [18]. Results were encouraging, with 73% survival relative to only 8% under fluid resuscitation only over a 3-h observation period. The novel system, designed for internal application only, requires safety testing before field use. Additionally, an expanding cellulosic mini-sponge coated with CS, applied via syringe injection, has shown promise for treatment of intracavitary bleeding [28]. The material significantly improved survival times relative to Combat Gauze, which is the current standard of care for US Military field dressings, in a swine injury model, which involved transection of the subclavian artery, followed by dressing application through a 4.5-cm aperture.

In past studies on HM-CS, only single variants of hydrophobic length or grafting density were studied. Here, we synthesized a series of HM-CS formulations of varying hydrophobic grafting lengths and densities to determine their effect on foaming capability and hemostatic efficacy. We observed that most hydrophobic samples (HM-CS4 and HM-CS5) displayed the highest efficacy on the animal model. However, a more highly grafted sample HM-CS6 could not be



**Fig. 5 – Kaplan–Meier analysis of HM-CS foam on lethal liver injury. The HM-CS5 (dot-dashed line) maintained hemostasis and supported survival of animals for the full 60 min of observation testing. The HM-CS5, CS (dashed line), and NT (black dotted line) controls resulted in < 30 min deaths among all test subjects.**

used in the study because it was too viscous to spray from the canister. Early studies have shown that the higher the grafting density and the bigger the size of the grafts of the HM-CS sample, the higher the viscosity [29]. Therefore, an optimum efficacy material may be achieved by maximizing the amount of hydrophobic grafts along the backbone with a graft size which still allows for practical flowability of the material such that it can be sprayed efficiently.

The HM-CS foam canisters present a dual use for both external and internal, open-abdominal, and applications. The foams could also be potentially introduced into a body cavity via laparotomy tube actuator in closed abdominal settings.

It is important here to discuss the limitations of the present study. First, the sample sizes per study group ( $n = 5$ ) are considered small. Ideally, such a study would have a minimum of eight subjects per test group for robust statistical variance. Second, treating hemorrhage from a rodent solid organ has little fidelity with respect to a comparable injury in a human. Resection of a proportionally sized section of a human liver would be an extremely difficult bleeding injury to treat. Finally, a 1 h survival time is a significant limitation as the pre-hospital period for patients in the field often last several hours. Despite these clear limitations, this study was a valuable initial screening of the efficacy of several HM-CS foams for treatment of bleeding in soft tissues without manual compression. We will use the findings from this study to design new HM-CS foams, which may translate to similar bleeds in large animal models with respect to efficacy.

## 5. Conclusions

This pilot study suggests HM-CS foam may be useful as a hemostatic adjunct or solitary hemostatic intervention. HM-CS foams immobilize blood cells into clusters between adjacent bubbles in the foams, whereas CS controls do not immobilize blood cells. The HM-CS foam also showed no significant toxicity to cells relative to CS foams and cell media controls. The HM-CS concentrates were able to be packaged and sprayed as expanding foams from standard lightweight aluminum canisters, which were pressurized with bioinert DME propellant. In this model of severe liver injury, spraying HM-CS foams directly on the injured liver surface significantly decreased blood loss and increased survival. HM-CS formulations with the highest levels of hydrophobic modification (HM-CS4 and HM-CS5) resulted in the lowest total blood loss and highest survival rates. Further efficacy studies on large animals during longer time-intervals and tissue compatibility studies must be completed to determine the suitability of HM-CS foams for human use.

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## Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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