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Fabrication of Injectable and Superelastic Nanofiber Rectangle Matrices (“Peanuts”) and Their Potential Applications in Hemostasis

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Abstract

Uncontrolled hemorrhage, which typically involves the torso and/or limb junctional zones, remains a great challenge in the prehospital setting. Here, we for the first time report an injectable and superelastic nanofiber rectangle matrix (“peanut”) fabricated by a combination of electrospinning, gas foaming, hydrogel coating and crosslinking techniques. The compressed nanofiber peanut is capable of re-expanding to its original shape in atmosphere, water and blood within ten seconds. Such nanofiber peanuts exhibit greater capacity of water/blood absorption compared to current commercial products and high efficacy in whole blood clotting assay, in particular for thrombin-immobilized samples. These nanofiber peanuts are capable of being packed into a syringe for injection. Further *in vivo* tests indicated the effectiveness of nanofiber peanuts for hemostasis in a porcine liver injury model. This new class of nanofiber-based materials may hold great promise for hemostatic applications.

Graphic Abstract

This study reports the fabrication of injectable and superelastic nanofiber peanuts and their potential applications for hemostasis.

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Conflict of Interest

The authors declare no conflict of interest.

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Keywords

Electrospinning; nanofiber rectangle matrix; injectable; superelastic; hemostasis

1. Introduction

In modern warfare, nearly 1 in 4 fatal battlefield injuries are potentially survivable; 90% of these potentially survivable injuries involve noncompressible (i.e., not amenable to manual/physical compression) truncal or junctional (limb-trunk intersection) hemorrhage [1,2]. In order to improve the outcomes from these potentially survivable injuries, therapy that prevents exsanguination must be applied in the prehospital setting (e.g., on the battlefield), i.e., prior to the casualty reaching a surgeon [1,3,4]. Of note, most battlefield-injured patients will now survive if they reach a field hospital/forward surgical unit [5]. Unfortunately, in modern warfare the time required for extrication of a wounded warfighter from the battlefield with transport to a hospital can extend to 6 h or longer [4]. Under these conditions, effective therapies are needed to stabilize patients with noncompressible truncal hemorrhage long enough to get them to the hospital alive; implementation of such therapies should improve outcomes from potentially survivable traumatic injury [1,2,6,7]. Current field treatment for noncompressible torso hemorrhage includes fluid and blood product resuscitation, and rapid evacuation to a forward surgical facility [8,9].

Development of novel treatments for noncompressible hemorrhage remains a research priority for the United States Army [10]. The only well-published device intended for direct treatment of noncompressible torso hemorrhage is the expansile polyurethane foam, but this device is still in the preclinical stage [11–13]. This foam is not biodegradable. It also generates dangerously high intraabdominal pressures (~100 mm Hg) during injection [11]. Recently, an aortic occlusion catheter was introduced which has been intended for field stabilization of severe hemorrhage [14,15]. This device decreases hemorrhage by reducing perfusion to the torso, pelvis and lower extremities, but is not a direct treatment for hemorrhage. Field experience with this device is very early, so its utility in the pre-hospital setting is not known. Nevertheless, there currently is no direct field treatment for noncompressible torso hemorrhage that is available for clinical use. In this report we describe an expansile, shape-recoverable, nanofiber cylindrical matrix (“peanut”) which has the potential to be formulated into an injectable therapy to help address problem of uncontrollable hemorrhage in the field.

Previous studies have proposed the use of electrospun nanofibers in hemostatic applications. However, traditional electrospinning typically produces uncontrolled and densely packed fibers, resulting in compact two-dimensional (2D) nanofiber mats/membranes which are limited in their applications [16]. Due to limitations imposed by this 2D structure, most previous studies utilizing electrospun nanofibers for hemostatic applications focused on utilizing a bandage without incorporation of hemostatic agents for the treatment of compressible hemorrhage (i.e., bleeding which can be controlled by manual/physical compression, in contradistinction to noncompressible hemorrhage) [17,18]. Of note, there are numerous bandages already approved for field treatment of compressible hemorrhage, a clinical scenario not considered to be in dire need of improved therapy [19].

A recent study proposed direct electrospinning of medical glue fibers onto the wound to control hemorrhage [20]. Such an approach would not be practical for use in noncompressible torso hemorrhage. In addition, this approach is time consuming and necessitates the use of electrospinning setup. Another study attempted to fabricate sonicated chitosan nanofiber mats with enlarged porosity for use as hemostatic materials [21]. The thickness obtained for sonicated nanofiber mats was only about 3-fold of the original thickness. Use of electrospun nanofibers in the management of noncompressible torso hemorrhage has not been reported in the literature, likely because of the difficulty in making nanofiber membranes injectable and re-expandable. Here, we sought to develop injectable, expansile, shape-recoverable nanofiber peanuts which might be useful in the treatment of noncompressible torso or marginally compressible junctional hemorrhage. Fig. 1a shows the schematic illustrating the fabrication of nanofiber peanuts and pellets. Briefly, nanofiber mats were electrospun from polycaprolactone (PCL; an FDA-approved polymer), then expanded using our established gas-foaming technique, and next performed gelatin coating and crosslinking followed by immobilization of clotting factors [16, 22, 23]. The expanded nanofiber peanuts then were compressed into pellets and packed with fine nanofiber strips made of water-soluble polymers (e.g., polyvinylpyrrolidone (PVP)) [24–26]. It was predicted that the nanofiber pellets injected through a syringe or catheter would re-expand upon contact with blood and exhibit a tamponade effect to activate hemostasis in a closed cavity (such as within the abdomen, uterus, or junctional wounds) (Fig. 1b).

2. Materials and Methods

2.1. Materials

PCL (Mw= 80 kDa), gelatin and sodium borohydride were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM) and N, N-dimethylformamide (DMF) were purchased from BDH Chemicals (Dawsonville, GA). Human whole blood and platelet rich plasma were purchased from Research Blood Components, LLC (Boston, MA). Thrombin and PierceTM BCA Protein Assay Kits were purchased from Fisher (Hampton, NH, USA). PEI was purchased from Polymer Science Inc. (Monticello, IN, USA). Thrombin ELISA kit, CD41 primary antibody, goat anti-rat secondary antibody, and goat anti mouse secondary antibody were purchased from Abcam (Cambridge, MA, USA). CD62p primary antibody was ordered from Invitrogen (Carlsbad, CA, USA).

2.2. Fabrication of PCL Nanofiber Peanuts

Electrospun PCL nanofiber mats were fabricated following our previous studies [16,22,45,46]. Two grams PCL beads were dissolved in 20 ml DCM and DMF mixed solvent at a ratio of 4:1 (v/v). After PCL solution was transparent, 50 ml PCL solution was pumped at a flow rate of 0.7 ml/h using a syringe pump while a potential of 18 kV was applied between the spinneret (22 Gauge needle) and a grounded collector. Around 1 mm thick aligned PCL nanofiber mat was collected by a high-speed rotating drum. To avoid deformation on the edges, PCL nanofiber mats were first cut into small rectangular pieces (1 cm × 1 cm × 1 mm) in liquid nitrogen. The expansion process was followed by our previous studies [16, 22]. Briefly, the small rectangular pieces were subsequently immersed in 1 M NaBH₄ solution, which was gently shaken for 24 h. After expansion, the PCL nanofiber peanuts were transferred into distilled water and exposed to a vacuum (~200 Pa) for 10 s, repeated this process for 3 times. Finally, distilled water was removed, and the PCL nanofiber peanuts were exposed to a vacuum until it froze then freeze dried. The optimization of the ratio of the PCL peanuts and the gelatin was determined by the mechanical property and water absorption. The mechanism for the expansion was briefly described in SI [16].

2.3. Fabrication of Gelatin-coated PCL Nanofiber Peanuts without and with Thrombin Immobilization

After the PCL nanofiber peanuts were fabricated, they were immersed in 0.1%, 0.5%, and 1% (w/v) gelatin solutions, and exposed to a vacuum (~200 Pa) for 10 s. Then, the gelatin solutions were discarded. The 0.1%, 0.5%, and 1% (w/v) gelatin-coated PCL nanofiber peanuts were exposed to a vacuum until it froze then freeze dried. Finally, the freeze-dried PCL nanofiber peanuts were cross-linked by 25% glutaraldehyde vapor for 6 h. The immobilization of thrombin was performed by a layer-by-layer technique. PCL membranes (1cm×1cm) and PCL nanofiber peanuts (1cm×1cm×1cm) were immersed in PEI solution (2 mg/mL in 0.01 M PBS) for 20 min and then washed with PBS and freeze dried. Subsequently, the PCL membrane and PCL nanofiber peanut were alternately immersed in thrombin (100 IU/mL in 0.01 M PBS) and tannic acid (2 mg/mL in 0.01 M PBS) each for 5 min, totally for 10 layers (Thrombin 5 layers and tannic acid 5 layers). Finally, these samples were freeze dried and stored at -20 °C for further use. The amount of thrombin immobilized on PCL membranes and PCL nanofiber peanuts was quantified by deducting the final concentration from the original concentration of thrombin solutions. The concentrations of thrombin solutions were measured by thrombin ELISA kit.

2.4. Characterization of Morphology

The morphologies of PCL nanofiber peanuts and gelatin-coated PCL nanofiber peanuts were recorded by a digital camera. The structures of cross sections (X-Y, Y-Z, X-Z planes) of PCL nanofiber mats before and after expansion and 0.1%, 0.5%, and 1% (w/v) gelatin-coated PCL nanofiber peanuts were characterized by scanning electron microscopy (SEM, FEI, Quanta 200, Oregon, USA).

2.5. Water Absorption Ratio Analysis

The gelatin (0.1%, 0.5%, and 1%)-coated PCL nanofiber peanuts, Gelfoam[®], Gauze, Surgicel[®] were weighted (W_d), immersed in distilled water and exposed to a vacuum (200 Pa) for 1 min, and then the gelatin-coated PCL nanofiber peanuts, Gelfoam[®], Gauze, and Surgicel[®] were taken out and the wet weights were determined (W_w). The water absorption ratio of each product (n=5) was calculated according to Equation (1). Water absorption ratio (%) = $(W_w - W_d)/W_d * 100\%$ (1)

2.6. Whole Blood Clotting Assay

The whole blood clotting assay was performed based on previous studies. The gelatin-coated PCL nanofiber peanuts, gelatin-coated, thrombin-immobilized PCL nanofiber peanuts, Gelfoam[®], Gauze, and Surgicel[®] were placed in a 15 ml-centrifuge tube and pre-warmed at 37 °C for 1 h. Then, 100 μ l human whole blood was dispensed onto these materials and 10 μ l of 0.2 M CaCl₂ solution was added to start coagulation. After that these tubes were incubated at 37 °C and shaken for 10 min. Finally, 12.5 ml distilled water was added and the absorbance (n=5) was measured at 540 nm.

2.7. Platelet Adhesion Assay

The gelatin-coated PCL nanofiber peanuts, thrombin-immobilized PCL nanofiber peanuts, Gelfoam[®], Gauze, and Surgicel[®] were placed in a 24-well culture plate, and then 2 ml human platelet rich plasma was added to the surface of these materials, and incubated at 37 °C for 1 h. Then, the platelet rich plasma was removed and washed with PBS for 5 times to removed non-adherent platelets. Finally, all samples were fixed in 4% paraformaldehyde and dehydrated in a series of graded alcohol solutions for SEM observation. For CD41 and CD62p immunostaining, platelets adhered 0.5% gelatin-coated PCL nanofiber peanuts, thrombin-immobilized, 0.5% gelatin-coated PCL nanofiber peanuts, Gelfoam[®], Gauze, and Surgicel[®] were treated with 0.5% Triton-X100 for 15 min at room temperature and then rinsed with PBS for 3 times. Subsequently, samples were blocked with 5% BSA for 30 min and then incubated with CD41 (1:300) and CD62p (1:250) overnight at 4 °C. The samples were then rinsed with 0.25% PBST for 3 times and incubated with goat anti-rat (1:200) and goat anti-mouse (1:200) for 1 h at room temperature. Finally, samples were rinsed with 0.25% PBST for 3 times. The samples (n=5) were then imaged by laser scanning confocal microscopy.

2.8. Blood Clotting Time Test

Duo to inaccessibility to fresh human blood, we used fresh swine blood for the blood clotting time test. About 12 mg Surgicel[®], Gauze, Gelform[®], gelatin-coated PCL nanofiber peanuts, thrombin-immobilized PCL nanofiber peanuts were weighted and placed into 15 mL centrifuge tube respectively, then 2 mL fresh swine blood was added to each tube. Every 15 s, the tubes were tilted to examine the flow ability of the blood. The time was recorded when the blood clotting was formed (n = 3).

2.9. Blood Proteins Absorption Assay

The human serum was obtained by centrifugation of human blood at 250 *g* at 37 °C for 15 min. About 12 mg Surgicel®, Gauze, Gelform®, gelatin-coated PCL nanofiber peanuts, thrombin-immobilized PCL nanofiber peanuts were weighted and immersed into 1 mL human serum of 15 mL centrifuge tubes, then incubated at 37 °C for 10 min. Subsequently, all samples were placed into 24-well culture plate after removing residual serum, followed by adding one mL protein extraction solution to each well for 10 min and centrifuging at 1500 rpm/min for 5 min. The supernatant of each sample containing human blood proteins was collected. Finally, the protein concentration was quantified using commercial Pierce™ BCA Protein Assay Kit (n = 3).

2.10. Hemostatic Test In Vivo

The details of liver injury model can refer to our previous study. A midline laparotomy was performed. The liver was brought up into the incision, and our standard liver injury clamp (5 cm diameter X-tines) was applied with the tines over the dome of the liver, within 2 cm of and directly anterior to the vena cava, ensuring shredding of multiple hepatic veins. The 0.5% gelatin coated PCL nanofiber peanuts (about 500 mL) then were stuffed immediately into the injury; the result appeared hemostatic from early on. Laparotomy pads were then placed over dome of liver, covering the injury site. One pad was placed and under liver. The abdominal incision was then closed with towel clips. Time from injury to completion of incisional closure was 8 min. After 60 min, the subject was intentionally exsanguinated from euthanasia. The blood loss (suction+clot+lap pads) was quantified. Totally two swine were used for the *in vivo* test.

2.11. Histology

After the swine was sacrificed, the liver wound and surrounding normal liver tissue (1 cm) were carefully excised, then fixed in 4% Paraformaldehyde (PFA). Samples were dehydrated in a graded ethanol series (70–100%) and embedded in paraffin. Five-micrometer sections were prepared. According to the standard procedures, paraffin sections were stained with either Hematoxylin & Eosin (H&E).

2.12. Statistical Analysis

The data were presented as the mean ± S.D, and the statistical analysis was performed using SPSS 13.0 software. Differences among different groups were assessed using one-way ANOVA followed by post hoc tests. The values of $p < 0.05$ were considered statistically significant. The values of $p < 0.01$ were considered statistically very significant. The significance levels were set at $*p < 0.05$ and $**p < 0.01$.

3. Results

3.1. Fabrication and Characterization of Injectable and Superelastic Nanofiber Peanuts

Fig. 2a shows a schematic illustrating the 3D structure of expanded PCL nanofiber peanuts. Fig. 2b and 2c show PCL nanofiber mats before (≈ 1 mm thick) and after expansion. The thickness of expanded PCL nanofiber peanuts was mainly in the range of 5–6 cm (58.33%),

and the expanded PCL nanofiber peanuts with thickness over 4 cm accounted for 88.89% (Fig. 2d). Also, the nanofiber peanuts exhibited a highly porous structure (Fig. 2e). In addition, the width reduced from 10 ± 0.89 mm before expansion to 8 ± 0.53 mm after expansion. The volume of peanuts increased from 100 ± 12 mm³ to 3456 ± 563 mm³. The average gap distances between layers increased from 84 ± 13 μ m to 859 ± 37 μ m. And the porosity increased from (83.6 $\pm$ 2.42)% to (99.2 $\pm$ 0.26)% (Fig. S1). To enhance the mechanical property and render the superelastic property, we coated the nanofiber peanuts with gelatin followed by crosslinking. Fig. 2f shows the morphology of nanofiber peanuts without and with gelatin coating. Gelatin coating can significantly enhance the compressive modulus along different planes (Fig. S2 and S3). Intriguingly, such gelatin-coated nanofiber peanuts were re-expandable after compression (Fig. 2g). Cyclic compression tests indicate that gelatin-coated nanofiber peanuts were able to fully re-expand to their original shape in the atmosphere after compression for 5 times (Fig. 2h and Video S1). Following our previous study, we also fabricated PVP nanofiber mats and cut into fine strips which were rolled or twisted into threads [24–26]. Gelatin-coated nanofiber peanuts which had been compressed into pellets and packed with PVP nanofiber fine threads rapidly re-expanded to their original shape when immersed in water or blood (Video S2 and S3). Fig. S4 shows a series of photographs of compressed nanofiber pellets after putting in water at different times, suggesting that compressed nanofiber pellets can re-expand to the original shape in less than 10 s upon contact with water. Similarly, the compressed nanofiber pellets were able to re-expand to the original shape in blood within 10 s (Fig. 3). In order to make a device for injecting nanofiber peanuts to the intradominal or junctional wounds, the nanofiber peanuts were compressed to form pellets and packed in a delivery device. Fig. 4 shows the process of packing and injection device of compressed nanofiber peanuts. After the packed nanofiber peanuts were loaded in a syringe and then injected into water, the packed peanuts can re-expand to their original shapes within 10 s. Such injectable and shape recoverable properties of nanofiber peanuts suggest great potential for use in noncompressible torso and marginally compressible junctional hemorrhage.

3.2. Water Absorption

To test the hemostatic efficacy of nanofiber “peanuts”, we firstly examined their water absorption rate and capability compared to commercially available hemostatic materials for compressible hemorrhage including Gauze, Gelfoam[®] and Surgicel[®] although they are not used in noncompressible and marginally compressible junction hemorrhage. Gauze and Surgicel[®] were composed of cotton fibers and cellulose fibers, and Gelfoam[®] exhibited a typical porous structure (Fig. S5). 0.5% gelatin-coated PCL nanofiber peanuts, Gauze, and Surgicel[®] quickly sank into water, while Gelfoam[®] stayed on the surface of water (Fig. 5a). Moreover, the water uptake rate of 0.5% gelatin-coated PCL nanofiber “peanuts”, Gauze and Surgicel[®] were obviously faster than that of Gelfoam[®] (Fig. 5a). Gauze and Surgicel[®] reached the maximum water absorption after immersion for 2 s, (807 $\pm$ 34)% and (459 $\pm$ 53)%, respectively. The water absorption of 0.5% gelatin-coated PCL nanofiber peanuts increased rapidly from (2561 $\pm$ 315)% at 2 s to (4326 $\pm$ 279)% at 10 s, and achieved the swelling equilibrium at 10 s. By comparison, the water uptake rate of Gelfoam[®] was much slower, which gradually increased from (101 $\pm$ 15)% at 60 s to (661 $\pm$ 51)% at 360 s (Fig. 5b). The 0.5% gelatin-coated nanofiber peanuts can absorb more than 70-fold of its weight

of water (Fig. 5c). Comparing to three commercial hemostatic materials (e.g., Gauze, Gelfoam[®], and Surgicel[®]), gelatin-coated nanofiber peanuts showed the highest capacity of water absorption (Fig. 5c). It is worth mentioning that the gelatin content only occupied around 10.54% of the total weight of 0.5% gelatin-coated nanofiber peanuts (Fig. 5d).

3.3 In Vitro Hemostatic Efficacy

We then tested the capability of blood absorption for nanofiber peanuts. Fig. 6a shows the photographs of different hemostatic materials before and after blood absorption. The color of blood could remain the same after absorption for Gauze, Gelfoam[®], and 0.5% gelatin-coated nanofiber peanuts. However, the blood absorbed on the Surgicel[®] gave rise to black color, which could be due to the production of an acidic medium that converted hemoglobin to acid hematin [27]. Similarly, nanofiber peanuts showed the highest blood absorption among the tested hemostatic materials (Fig. 6b).

To further enhance hemostatic efficacy of 0.5% gelatin-coated PCL nanofiber peanuts, we immobilized thrombin, a clinically used clotting factor, to nanofiber peanuts using a layer-by-layer technique based on previous studies [28]. Thrombin-immobilized, 0.5% gelatin-coated PCL nanofiber peanuts contained (6.47 ± 0.59) ng thrombin per mg of nanofiber peanuts. To characterize the hemostatic efficacy *in vitro*, we performed the whole blood clotting assay for nanofiber peanuts with and without thrombin immobilization and other commercially available hemostatic materials [21]. Nanofiber peanuts with thrombin immobilization and Surgicel[®] displayed the best and comparable whole blood clotting efficacy and hemoglobin binding efficiency among the tested materials (Fig. 6c and 6d). However, pH value of diluted blood solution after Surgicel[®] treatment was around 2.83, which confirmed the acidic effect. In contrast, the whole blood clotting efficacy and hemoglobin binding efficiency of 0.5% gelatin-coated PCL nanofiber peanuts without immobilization of thrombin were close to those of Surgicel[®] and thrombin immobilized peanuts (Fig. 6c and 6d). Importantly, the pH value of diluted blood solution after the treatment of nanofiber peanuts without and with thrombin immobilization was around 7.1 which is very close to the physiological pH value.

Moreover, the blood clotting times of Surgicel[®] and Gauze were about 100 and 110 s close to the blood clotting time of whole blood without any treatment (110 s). The blood clotting time of Gelfoam[®] was reduced (80 s) compared with Surgicel[®] and Gauze. By comparison, the blood formed clotting much faster in 0.5% gelatin-coated nanofiber peanuts (55 s), and thrombin-immobilized, 0.5% gelatin coated PCL nanofiber peanuts (20 s) compared to the other three commercial products (Fig. 6E). Furthermore, the proteins absorbed by Surgicel[®] and Gauze were 0.59 ± 0.12 g and 0.60 ± 0.09 g per g materials. The proteins absorbed by Gelfoam[®] increased to 0.87 ± 0.16 g per g materials. Interestingly, the protein absorption of 0.5% gelatin-coated nanofiber peanuts (1.54 ± 0.32) significantly increased, in particular for thrombin-immobilized samples (5.93 ± 0.59) (Fig. 6F).

3.4. Interaction of Platelets and Hemostatic Materials

To understand the hemostatic mechanism of nanofiber peanuts, we also cultured human platelet rich plasma with the tested hemostatic materials. Interestingly, it is seen that more

platelets were adhered on 0.5% gelatin-coated PCL nanofiber peanuts without thrombin immobilization than on Surgicel[®], commercial Gauze, and Gelfoam[®] (Fig. 7a). Thrombin-immobilized, 0.5% gelatin-coated PCL nanofiber peanuts showed the highest number of platelet adhesion among the tested samples. We further confirmed the platelet adhesion on the nanofibers by culturing platelets on the 2D nanofiber membranes with and without thrombin immobilization (Fig. S6). Similarly, more platelets were adhered on the thrombin-immobilized, 0.5% gelatin-coated PCL nanofiber membranes compared to 0.5% gelatin-coated PCL nanofiber membranes. In addition, some platelets adhered on the nanofibers were polarized indicating their activation. We also performed immunostaining of adhered platelets on the hemostatic materials (Fig. 7b). On the nanofiber peanut samples, adhered platelets expressed CD41 (glycoprotein IIb of the GPIIb/IIIa complex), a marker for surface bound platelets. The activation of adhered platelets on the nanofiber peanuts was confirmed by immunostaining of CD62p, a marker for P-selectin. The activated platelets co-expressed CD41 and CD62p. A significantly higher number of activated platelets were observed on the thrombin immobilized, 0.5% gelatin-coated PCL nanofiber peanuts than other samples, which was in line with the SEM observation. In contrast, no positive staining including CD41 and CD62p was observed on Surgicel[®], Gauze, and Gelfoam[®]. Interestingly, a layer of hydrogel was found on the surface of thrombin immobilized, 0.5% gelatin-coated PCL nanofiber peanuts, which may be due to the absorption of plasma proteins and crosslinking of absorbed fibrinogen into fibrin catalyzed by immobilized thrombin (Fig. S7).

3.5. Hemostatic Efficacy In Vivo

In order to provide some preliminary indication (proof-of-principle) of hemostatic efficacy *in vivo*, 0.5% gelatin-coated PCL nanofiber peanuts were examined in a porcine model of compressible hemorrhage (stellate liver injury), as shown in Fig. 8a. This grade V liver injury model was similar to that previously described, except that bimanual compression was used during the treatment phase; i.e., the peanuts were compressed into the injury site, with one hand applying pressure anteriorly while the other hand applied counter-pressure behind the organ [29]. Our intent with these pilot procedures was to determine whether the nanofiber peanuts would promote hemostasis in a standard hemorrhagic wound. We specifically were not intending this to be a test of treatment efficacy for noncompressible hemorrhage. The manually-placed peanuts conformed to the irregular-shaped cavitory wound in the liver dome, allowing tamponade and clotting to ensue. Two subjects that were injured and treated in this manner easily survived for 60 min, with final mean artery pressure (MAP, mm Hg) in the 90's, blood loss ~300 ml, and no ongoing hemorrhage. The nanofiber peanuts were intact/in place at the injury site, and were adherent to the wound (Fig. 8a). In comparison, no-treatment historical controls (i.e., weight- and age-matched pigs with the identical injury, but no treatment) (n=6) recorded final MAP and blood loss of 65 ± 24 mm Hg and 1140 ± 684 ml, respectively. The retrieved nanofiber peanuts subsequently were imaged by SEM. We found that the inside surface and outside surface of retrieved nanofiber peanuts were covered by absorbed proteins. The platelet adhesion was also identified by the black arrows in Fig. 8b. We further performed hematoxylin and eosin (H & E) staining of injured liver tissue (Fig. 8c). Blood clotting was evidently noticed around the wound edge.

3.6 Working Mechanism of Superelastic Property

As shown in Fig. 2 and Videos S1–S3, the 0.5% gelatin-coated PCL nanofiber peanuts after compression can re-expand to their original shapes not only in water but also in atmosphere. The mechanism of shape-recovery after compression (superelastic property) may be different from water induced shape-memorable materials [30, 31]. The working mechanism of superelastic property was presented in Fig. 9. The superelastic property (shape-recovery after compression) is most likely attributed to the structure of expanded nanofiber peanuts that have hierarchical lamellar/layer architectures composed of many micro/milli-scale arches serving as elastic units and the cross-linked gelatin coating further strengthening the existing arches (Fig. 2 and Fig. 9). As shown in Fig. 9, PCL nanofiber peanuts with cross-linked gelatin coating exhibit higher stress at the same compressive strain compared to non-coated ones (Fig. S3). When the stress is applied, the deformation of peanuts is mainly due to the bending or compress of the arches/pores. During compression, because of the densification of the pores, the pore walls start to touch each other and become the major loading-bearing portion. The cross-linked gelatin coatings “lock” the fibers together at crossing point, nodes, and contacting surfaces and provide strong fatigue resistance [32]. Therefore, 0.5% gelatin coated nanofiber peanuts could completely recover the shape and maintain the structural integrity in each compress-release cycle under 70% strain (Fig. 2). Based on the similar principle, previous studies demonstrated the superelastic property of aerogels prepared by bidirectional freezing and/or graphene coating [33].

4. Discussion

Hemorrhage is the leading cause of mortality in battlefield trauma and the second leading cause of death in civilian trauma [34,35]. Although therapies have been developed for compressible hemorrhage, non-compressible torso hemorrhage that cannot be controlled by external compression is a leading cause of potentially preventable trauma mortality [36]. Currently, there is no active intervention for non-compressible hemorrhage available to civilian or military medics and physicians. Research of non-compressible hemorrhage control methods may offer solutions that could save lives. The injectable and superelastic nanofiber peanuts we developed in this study could potentially be used for management of these unmet needs.

Electrospun nanofibers have been demonstrated for use in many biomedical applications including drug delivery, tissue engineering, and hemostasis [20,21,37–39]. However, previous studies were mainly limited to the development of nanofiber bandages for compressible hemorrhage due to the difficulty in fabrication of injectable and shape-memorable electrospun nanofiber materials [18]. In this work, we for the first time reported the injectable and shape-memorable nanofiber peanuts using a combination of electrospinning, gas-foaming, gelatin coating and crosslinking techniques.

The fabricated nanofiber peanuts can be compressed into pellets and packed with fine strips made of hydrophilic polymers. Such compressed nanofiber peanuts can re-expand to their original shapes within seconds in air, water and blood. We further demonstrate a syringe device which could be used to load and inject compressed nanofiber peanuts to the cavity (e.g., junctional wounds, abdomen, and uterus) for hemostasis. Comparing to the current

commercially available hemostatic materials, nanofiber peanuts show much faster rate and higher capacity of water absorption. In addition, 0.5% gelatin-coated nanofiber peanuts show better efficacy in whole blood clotting assay than commercial Gauze and Gelfoam[®]. Thrombin-immobilized, 0.5% gelatin-coated nanofiber peanuts show comparable efficacy as Surgicel[®] in whole blood clotting assay, which is superior to other testing samples. Moreover, the 0.5% gelatin-coated PCL nanofiber peanuts exhibit a shorter blood clotting time and higher blood protein absorption capability compared to the other 3 commercial products. In particular, for thrombin-immobilized, 0.5% gelatin-coated nanofiber peanuts, the blood clotting time is further dramatically reduced and the protein absorption is further significantly increased compared to 0.5% gelatin-coated nanofiber peanuts (Fig. 6). It is worth mentioning that Surgicel[®] treatment cause very acidic environment, while the pH of blood treated with nanofiber peanuts is close to the physiological pH. In addition, the blood clotting time and protein absorption capability for Surgicel[®] is longer than that of nanofiber peanuts, meanwhile the protein absorption capability for Surgicel[®] is lower compared to that of nanofiber peanuts.

Platelets play an important role during primary hemostasis as they are activated and form platelet plug [40]. The interaction of platelets and hemostatic materials was examined by culturing human platelet rich plasma with different testing materials. 0.5% gelatin-coated nanofiber peanuts showed much better platelet adhesion comparing to commercial Gauze, Gelfoam[®] and Surgicel[®]. Intriguingly, some activated platelets were also observed on the 0.5% gelatin-coated nanofiber peanuts. Thrombin a clinically used hemostatic agent acts as catalyst for the conversion of fibrinogen to fibrin during the secondary hemostasis, which has been immobilized to the surface of 0.5% gelatin coated nanofiber peanuts using a layer-by-layer technology based on previous studies [28]. Thrombin-immobilized, 0.5% gelatin-coated nanofiber peanuts showed the best platelet adhesion among tested samples. A higher number of activated platelets were observed on thrombin-immobilized, 0.5% gelatin-coated thrombin-immobilized, 0.5% gelatin-coated nanofiber peanuts. The CD41 and CD62p positive staining also proved the platelet adhesion and activation on the nanofiber peanuts with and without thrombin immobilization. The similar phenomena were also observed on 2D nanofiber membranes (Fig. S5). The promoting effects on platelet adhesion and activation of electrospun nanofibers, in particular, thrombin immobilized nanofibers, which may partially contribute to their hemostatic effect other than the strong blood absorption capability. It is also possible to incorporate other clotting factors (e.g., fibrinogen, Von Willebrand factors, factor XIII) to the injectable and shape-memorable nanofiber peanuts for enhancing the hemostatic efficacy using different surface immobilization techniques [41,42].

5. Conclusions

In summary, we have developed a novel means to generate injectable and shape re-expandable nanofiber peanuts for hemostasis. Such nanofiber peanuts provide the following unique features: *i*) biodegradability, *ii*) superelastic and re-expandable, *iii*) a high expansion ratio, *iv*) a nanofibrous structure, and *v*) ease of incorporation of hemostatic agents. These novel features distinguish our nanofiber peanuts from those currently available and suggest that this approach is expected to overcome problems associated with other means of non-degradability and safety issues. It is expected that the compressed nanofiber pellets could be

delivered through a cannula or syringe (8 mm in diameter) to the injured site, and then re-expand to their original shapes in seconds upon contact with blood. The nanofiber peanuts materials made of PCL and gelatin are FDA approved for other clinical uses, making these peanuts readily translatable to clinics [23,43]. In addition, thrombin is in clinical use [44].

Although we tested the hemostatic efficacy of nanofiber peanuts in porcine liver injury model, further studies should be carried out to identify the role of injectable and shape memorable nanofiber peanuts for hemostasis. For example, examining whether nanofiber peanuts are effective for noncompressible hemorrhage and marginally compressed junctional hemorrhage in the relevant animal models [45,46]. Another intriguing study would be investigating the incorporation of other clotting factors including factor XIII and fibrinogen to 0.5% gelatin-coated PCL nanofiber peanuts through surface immobilization for further enhancing their hemostatic efficacy. We envision that the reported nanofiber peanuts may be useful as scaffolds for 3D tissue construction and tissue regeneration. We also envision that the superelastic nanofiber peanuts could be used as injectable carriers for delivery of cells and tissues to injury sites for repair/regeneration through minimally invasive surgery [47,48]. Moreover, we will devote to further increasing the water and blood absorption capability of PCL nanofiber peanuts. The absorbed water could be divided by two parts, namely free water and bound water (or structural water). On one hand, the expanded nanofiber peanuts have high porosities, which could hold high amount of free water. On the other hand, the gelatin-coated nanofiber peanuts are mainly composed of PCL and gelatin. The surface of PCL nanofibers after plasma treatment contains –OH and C=O groups, while gelatin contains N-H, C=O, C-O, -OH groups. Therefore, it is possible to form hydrogen bonding with water based on these functional groups. In our future work, we will attempt to increase the hydrogen bonding between PCL nanofiber peanuts and water to further enhance the water absorption capability. For example, we may use oxygen or nitrogen containing plasma to treat the PCL nanofiber peanuts, or graft more hydrophilic functional groups to achieve higher water absorption through hydrogen bonding [31].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic showing the fabrication and the use of injectable and shape memorable nanofiber peanuts. (A) Illustration of the process of fabrication of injectable and shape memorable nanofiber peanuts. (B) Illustration of the use of injectable and shape memorable nanofiber peanuts. Left: nanofiber pellets delivery through a cannula or syringe. Right: The expansion of nanofiber peanuts upon contact with blood, the blood plasma absorption, and the tamponade effect control bleeding.

Figure 2.

Fabrication and cauterization of superelastic nanofiber peanuts. (A) The schematic shows the 3D structure of expanded PCL nanofiber peanuts. The red arrow indicates the direction of fiber alignment. (B) Photographs showing the morphology of PCL nanofiber mats before and after expansion. (C) Photograph showing a pile of PCL nanofiber peanuts. (D) The thickness distribution of PCL nanofiber peanuts. (E) The cross-section structures (Y-Z, X-Z, X-Y planes) of PCL nanofiber mats before and after expansion. (F) Morphological characterization of PCL nanofiber peanuts and gelatin-coated PCL nanofiber peanuts. Top row: photographs of PCL nanofiber peanuts before and after gelatin coating. Bottom row: SEM images showing the cross-sectional morphologies of PCL nanofiber peanuts before and after gelatin coating. (G) A series of photographs suggesting the compressed nanofiber peanuts can re-expand. (H) Cyclic compressive test of 0.5% gelatin-coated PCL nanofiber peanuts (5 cycles). In order to accommodate the testing equipment, the nanofiber peanuts cut in 2 cm thick were used for this test. Insets: the corresponding photographs of nanofiber peanuts at different stages during the compressive test.

Figure 3. Photographs showing compressed nanofiber pellets after putting into blood for different times. (A) $t = 0$ s. (B) $t = 1$ s. (C) $t = 9$ s. (D) $t = 17$ s.

Figure 4. Photographs illustrating the packing and injection of nanofiber peanuts. (A) Expanded nanofiber peanuts. (B) PVP nanofiber threads made by rolling the fine strips. (C) Packed nanofiber peanuts. (D) Packed nanofiber peanuts loaded in the syringe. (E) Inject packed nanofiber peanuts into water for different times.

Figure 5.

The water uptake of hemostatic materials. (A) Photographs showing 0.5% gelatin-coated PCL nanofiber peanuts, commercial Gauze, Gelfoam®, and Surgicel® after putting in water. (B) The rate of water uptake of 0.5% gelatin-coated PCL nanofiber peanuts, commercial Gauze, Gelfoam®, and Surgicel®. (C) The amount of water absorbed of PCL nanofiber peanuts and 0.1%, 0.5% and 1% gelatin-coated PCL nanofiber peanuts. *p<0.05, **p<0.01. (D) Gelatin content in 0.1%, 0.5% and 1% gelatin-coated PCL nanofiber peanuts.

Figure 6.

In vitro hemostatic efficacy test. (A) Photographs showing the hemostatic materials before and after blood absorption. (B) The blood absorption of 0.5% gelatin-coated PCL nanofiber peanuts, commercial Gauze, Gelfoam®, and Surgicel®. ** $p < 0.01$. (C) The whole blood clotting assay of Surgicel®, Gauze, Gelfoam®, 0.5% gelatin coated PCL nanofiber peanuts, and thrombin-immobilized, 0.5% gelatin coated PCL nanofiber peanuts and the pH value of each group after whole blood clotting assay. (D) The hemoglobin binding efficiency of Surgicel®, Gauze, Gelfoam®, 0.5% gelatin coated PCL nanofiber peanuts, and thrombin-immobilized, 0.5% gelatin coated PCL nanofiber peanuts. (E) The blood clotting time of Surgicel®, Gauze, Gelfoam®, 0.5% gelatin coated PCL nanofiber peanuts, and thrombin-immobilized, 0.5% gelatin coated PCL nanofiber peanuts. (F) The blood protein absorption of Surgicel®, Gauze, Gelfoam®, 0.5% gelatin coated PCL nanofiber peanuts, and thrombin-immobilized, 0.5% gelatin coated PCL nanofiber peanuts. * $p < 0.05$, ** $p < 0.01$.

Figure 7.

The platelet adhesion assay of Surgicel®, commercial Gauze, Gelfoam®, 0.5% gelatin-coated PCL nanofiber peanuts, and thrombin-immobilized, 0.5% gelatin-coated PCL nanofiber peanuts. (A) SEM images showing platelet adhesion and activation. (B) Immunostaining of adhered platelets with CD41 (a marker for surface bound platelets) and CD62P (a marker for activated platelets).

Figure 8.

Hemostatic efficacy test in vivo. (A) *In vivo* test procedure. (I) A pile of 0.5% gelatin-coated nanofiber peanuts. (II) Exposure of injury site (Grade V hepatic dome laceration) 1 h after treatment with nanofiber peanuts. Cotton lap pads have been removed, showing 0.5% gelatin-coated PCL nanofiber peanuts still packed into the injury defect. (III) Close-up of injury site. Forceps is beginning to extract the peanuts. (IV) Nanofiber peanuts after removal from wound. (B) SEM images showing the protein absorption and platelet adhesion on the outer surface and inner surface of 0.5% gelatin-coated PCL nanofiber peanuts after removal from wound. Insets: the corresponding highly magnified images. Black arrows indicate platelet adhesion on the nanofiber peanuts. (C) H&E staining of the wound. Green dots indicate the wound edge.

Figure 9.

The schematic illustrating the working mechanism of superelastic property after compression. (A) PCL nanofiber peanuts. The PCL nanofiber peanuts failed to recover the shape completely after compression under 70% compressive strain. (B) 0.5% gelatin-coated PCL nanofiber peanuts. The 0.5% gelatin-coated PCL nanofiber peanuts were able to fully recover after compression under 70% compressive strain.