Tissue adhesive hemostatic microneedle arrays for rapid hemorrhage treatment

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A B S T R A C T
Blood loss by hemorrhaging wounds accounts for over one-third of ~5 million trauma fatalities worldwide every year. If not controlled in a timely manner, exsanguination can take lives within a few minutes. Developing new biomaterials that are easy to use by non-expert patients and promote rapid blood coagulation is an unmet medical need. Here, biocompatible, and biodegradable microneedle arrays (MNAs) based on gelatin methacryloyl (GelMA) biomaterial hybridized with silicate nanoplatelets (SNs) are developed for hemorrhage control. The SNs render the MNAs hemostatic, while the needle-shaped structure increases the contact area with blood, synergistically accelerating the clotting time from 11.5 min to 1.3 min in vitro. The engineered MNAs reduce bleeding by ~92% compared with the untreated injury group in a rat liver bleeding model. SN-containing MNAs outperform the hemostatic effect of needle-free patches and a commercial hemostat in vivo via combining micro- and nanoeengineered features. Furthermore, the tissue adhesive properties and mechanical interlocking support the suitability of MNAs for wound closure applications. These hemostatic MNAs may enable rapid hemorrhage control, particularly for patients in developing countries or remote areas with limited or no immediate access to hospitals.

1. Introduction

Uncontrolled, excessive bleeding caused by trauma [1-3], various diseases [4-7], and surgery is one of the current leading medical challenges. The injuries may be initiated externally, e.g., due to a blast or gunshot [8,9], or internally, as a result of visceral bleeding or aneurysm rupture [10,11]. Severe traumatic bleeding may result in hypothermia, hemorrhagic (hypovolemic) shock, organ failure, coma, and eventually death when more than 40% of blood is volumetrically drained [12]. Accordingly, immediate blockade and rapid cloting of blood may prevent the otherwise inevitable consequences of hemorrhage. To this end, numerous approaches have been implemented to manage hemorrhage using hemostatic biomaterials and devices [13-16]. Recently, biomaterials in the form of dry patches have received attention as a result of water absorption-mediated adhesion to wet tissues [17-19]. Surface microengineering of these patches through incorporating microneedle arrays (MNAs) has enabled better mechanical integration with soft tissues for a wide variety of applications, including wearable devices [20] and transdermal or intestinal drug delivery [21-24].

Hemostatic biomaterials can arrest bleeding by promoting blood coagulation [25]. They may activate blood coagulation cascade via the contact-activation of platelets and their aggregation, resulting in the release of pro-coagulation biomolecules (e.g., adenosine diphosphate, ADP) and ions (e.g., calcium) [26-28]. The most common hemostatic
bioactive materials are hydrogels, foams, powders, and tourniquets [13, 29]. Current hemostatic materials are mostly ineffective for individuals with clotting abnormalities [30]. Commercial hemostatic agents, such as fibrin-based sealants, suffer from weak mechanical and rheological properties, hindering their application under excessive blood flow [31]. Fibrin-based hemostats are not effective for coagulopathic patients [32]. Other biomaterials, including collagen and its derivatives (e.g., gelatin), have been used as material platforms to localize coagulation factors, induce thrombin secretion, and promote fibrin deposition [33]. Gelatin has frequently been used as a key biomaterial for developing hemostatic materials as a result of its biocompatibility and low cost [34,35]. Some of the commercially available gelatin-based products include Gelfoam® (gelatin sponge) [36], Surgifoam® powder, GFR Glue® (gelatin-n-resorcinol-formaldehyde) [37], GR-Dial® (gelatin-dialdehyde) [37], and FloSeal® (bovine gelatin granules and human thrombin) [38,39]. Unmodified gelatin may lose its stability in vivo as it can be diluted or washed away at the physiological temperature, increasing the risk of distant thrombosis. To address this issue, gelatin-based hemostats can be chemically modified to acquire mechanical strength and stability through various crosslinking mechanisms [40].

Gelatin has been modified with methacryloyl groups to yield tissue adhesive gelatin methacryloyl (GelMA) hydrogels [41–43], which can seal wounds and prevent air and/or body fluid leakage [44,45]. GelMA hydrogels are suitable for wound management due to their decent biodegradation and biocompatibility. In addition, they can be processed in the form of injectable sealants or dry patches [46]. Recently, gelatin-based shear-thinning biomaterials containing silicate nanoplatelets (SNs) have been developed for the minimally invasive control of internal bleeding. These SN-containing hydrogels may form a reversible network that breaks under shear forces and re-arranges upon the elimination of external shear [47–49]. The disc-shaped SNs (diameter ~30 nm, thickness ~1 nm) bear negative charges on their surfaces and positive charges on their edge. It has been hypothesized that the electrostatic charges lead to platelet aggregation and the activation of coagulation factors, which may eventually trigger the coagulation cascade [47]. While nanocomposite SN-gelatin hydrogels could accelerate blood coagulation and be injected using minimally invasive surgical devices, they lack sufficient mechanical resilience and tissue adhesion, which limit their application for wound management.

Here, we aim to engineer a biocompatible and biodegradable material platform with micro- and nanoscale features for hemorrhage control. Nanocomposite MNAs based on GelMA matrices incorporated with SNs are introduced to facilitate the rapid on-demand prevention of hemorrhage—biodegradable and biocompatible. In addition, they can be processed in the form of injectable sealants or dry patches [46]. Recently, gelatin-based shear-thinning biomaterials containing silicate nanoplatelets (SNs) have been developed for the minimally invasive control of internal bleeding. These SN-containing hydrogels may form a reversible network that breaks under shear forces and re-arranges upon the elimination of external shear [47–49]. The disc-shaped SNs (diameter ~30 nm, thickness ~1 nm) bear negative charges on their surfaces and positive charges on their edge. It has been hypothesized that the electrostatic charges lead to platelet aggregation and the activation of coagulation factors, which may eventually trigger the coagulation cascade [47]. While nanocomposite SN-gelatin hydrogels could accelerate blood coagulation and be injected using minimally invasive surgical devices, they lack sufficient mechanical resilience and tissue adhesion, which limit their application for wound management.

2. Materials and methods

2.1. Materials

Microneedle molds made up of polydimethylsiloxane (PDMS, 11 × 11 arrays) were purchased from Blueacre Technology (Co Louth, Ireland). Gelatin type A from porcine skin (300 bloom), Irgacure 2959 ultraviolet (UV) photoinitiator (PI) with the chemical name of 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropophenone, methacrylic anhydride (MA, 94%), fluorescein isothiocyanate (FITC), polyethylene glycol (PEG, Mn = 400), eosin Y, calcium chloride (99.99%), bovine serum albumin (BSA), triton X-100, Drabkin’s solution, pure human hemoglobin, glutaraldehyde solution (25% in water), dimethylsulfoxide-d6 (DMSO-d6), and sodium iodine X Selectophore™ were obtained from Sigma-Aldrich (MO, USA). Silicate nanoplatelets (SNs, Laponite® XLG-XR) were purchased from BYK Additives & Instruments (USA) and were used as obtained. Milli-Q integral system was used to produce type I ultrapure water (resistivity ~18.2 MΩ cm at 25 °C) was provided by Millipore (MA, USA). Spectra/Port™ 4 RC dialysis membrane tubing (12-14 kDa MWCO) was purchased from Spectrum (CA, USA). Biopsy punches (3 mm and 4 mm) were purchased from Integra Millex (NJ, USA). Fisherbrand™ superfrost® plus microscope slides (size: 25 × 75 × 1 mm), optimal cutting temperature (O.C.T.) compound, collagenase type II, ethanol 200 proof, paraformaldehyde 4% in PBS, Surgicel® absorbable hemostat by Ethicon, anti-CD68 mouse monoclonal antibody, PrestoBlue™ cell viability reagent, LIVE/DEAD™ viability/cytotoxicity kit, and PDMS (Krayden Dow Silgard 184 Silicone Elastomer Kit, 1.1 lb) used for the fabrication of tensile and needle-free molds were provided by Fisher Scientific (MA, USA). Evicel® fibrin sealant by Ethicon was bought from DOTmed (NY, USA). HemaToxilin, 10% neutral buffered formalin (NBF), and paraffin were provided by Leica Biosystems (IL, USA). Antigen Retrieval Buffer (100X Citrate Buffer pH 6.0), anti-CD3 rabbit monoclonal antibody, anti-mouse Alexa 488 conjugated secondary antibody, and anti-rabbit Alexa 555 conjugated secondary antibody were purchased from Abcam (MA, USA). VECTASHELD™ Vibrance™ antifade mounting medium with DAPI (4′,6-diamidino-2-phenylindole) was bought from Vector Laboratories (CA, USA). Standards USA carpenfo for veterinary (30 mg) was purchased from Fisher Bioreagents (PA, USA). Collagen sheets were bought from Weston (NC, USA). NIH/3T3 murine fibroblasts were procured by the American Type Culture Collection (ATCC, VA, USA). Fetal bovine serum (FBS) heat-inactivated, Dulbecco’s phosphate-buffered saline (DPBS, 1X), penicillin/streptomycin (P/S, 1X), and Trypan Blue solution (0.4%) were purchased from Gibco (NY, USA). Tissue culture flasks and tissue culture-treated polystyrene 96-well, 12-well and 24-well plates from Falcon (NC, USA) were bought from Corning (NY, USA) and used to culture cells or to perform in vitro blood clotting experiments.

2.2. Gelatin methacryloyl (GelMA) synthesis

GelMA was prepared according to the procedure mentioned in our previous publications [51,58,59]. In brief, porcine gelatin (10 g) was completely dissolved in DPBS (100 mL) at 50 °C on a magnetic stirrer with the speed of 240 rpm, followed by the dropwise addition of MA (final concentration of 8% v/v). The reaction continued at 50 °C for 2 h in dark, followed by the addition of an equal volume of DPBS to stop it. The solution was then purified via dialysis against DI water at 40 °C using dialysis tubing. After 7 days of dialysis, the solutions were deep-frozen at ~80 °C, freeze-dried (Free zone, 4.5 L benchtop freeze drier, Labconco, MO, USA), and stored at 4 °C until use.

2.3. Fabrication of hemostatic MNAs

Freeze-dried GelMA foam was dissolved in Milli-Q water at 37 °C for ~6 h to yield a solution with a final concentration of 20% w/v (i.e., an optimum concentration based on the tradeoff between viscosity before crosslinking and mechanical properties after crosslinking [60–62]), containing 0.5% w/v of the photoinitiator (Irgacure 2959). The solution was then mixed with varying concentrations of SN dispersion (final concentrations of 0, 0.5, 1, or 2% w/v) prepared in Milli-Q water using a speed mixer (FlackTek, Germany). The pre-gel mixture was introduced
into the microneedle mold via centrifugation (3000 rpm, 3 min at 37 ºC, Fisher Scientific, MA, USA), and crosslinked by UV irradiation (360 nm, Omnicure, Excellitas, CA, USA) at an intensity of 50 mW cm⁻² for 5 min. The MNAs were dried overnight at the ambient temperature and then removed from the molds.

2.4. Proton nuclear magnetic resonance (¹H NMR) spectroscopy

The samples were freeze dried and 20 mg of them was dissolved in 750 µL of DMSO-d₆. Bruker AV400 spectrometer at 400 MHz was used to record the spectra.

2.5. ζ-Potential measurements

All the samples were prepared in Milli-Q water at a concentration of 1 mg mL⁻¹ and added to folded capillary cells before the measurement. Panalytical Zetasizer Nano ZS (Malvern Instruments, U.K.) was used to measure the ζ-potential at the ambient temperature.

2.6. Scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS)

Shape, porosity, dimensions, and morphology of the dry needles were investigated using SEM (Supra 40 VP, Zeiss, Germany). MNAs were air-dried, adhered to specimen stubs with a double-sided carbon tape and a thin layer (22 Å) of iridium was sputter-coated on the surface of the samples using IBS/e, an ion beam sputter deposition and etching system (South Bay Technology, CA, USA). SEM images were obtained at an accelerating voltage of 12 kV. More than 15 needles were analyzed, and their sizes were measured using the ImageJ software (Version 1.52e, National Institute of Health, USA). For the EDS analysis, samples were coated with gold to render them electroconductive, and the elemental mapping was performed.

2.7. Mechanical characterisations

Mechanical properties of wet (hydrogels) and dry (MNAs) samples were examined using an Instron mechanical testing machine (model 5943, MA, USA) equipped with a 100 N load cell. Compression examinations were carried out on the crosslinked and dried MNAs containing varying concentrations of SNs (0, 0.5, 1, or 2% w/v) to evaluate their strength. The compression tests were performed at a 2 mm min⁻¹ displacement rate until the load reached 45 N, and the compression force versus displacement was recorded using the Bluehill software (version 3). For the tensile tests, hydrogel samples were prepared by adding 200 µL of a pre-gel mixture to a rectangular PDMS mold (25 mm × 5 mm × 2 mm). Subsequently, the samples were crosslinked via UV light exposure (intensity = 50 mW cm⁻², exposure time = 5 min), removed from the mold, rinsed with DPBS, and their dimensions were measured prior to testing. The tensile tests were executed at a constant strain rate of 2 mm min⁻¹ and continued until sample failure. Stress-strain curves were generated from the force-displacement data (force and displacement values were divided by the cross-sectional area and the initial length of samples, respectively). The maximum stress that the samples sustained before rupture (tensile strength) was also registered. All data were reported as a mean of 4 measurements ± standard deviation.

2.8. Tissue adhesion tests

Adhesive properties of wet (hydrogels) and dry (MNAs) samples were studied according to the ASTM F2392-04 standard protocol for in vitro burst pressure test with a slight modification [63]. Prior to the test, collagen sheets were cut into circular shapes (3 cm diameter) and soaked in DPBS for ~1 h. The excess DPBS was blotted with kimwipes and the center of collagen sheet was pierced (3 mm in diameter) with a biopsy punch. The collagen sheets were then clamped between two steel holders of a custom-made burst pressure device. For testing hydrogels, the desired GelMA mixture (20 µL) containing varying concentrations of SNs was pipetted onto the punctured hole and cured under the UV light (50 mW cm⁻²) for 5 min. For testing dry samples, the fabricated MNAs were directly placed onto the hole and pressed with thumb (pressure ~3–4 N cm⁻², 1 min) to seal it. Air was pumped into the sealed chamber at a constant rate of 20 mL min⁻¹, and a pressure sensor (Pasco Scientific, CA, USA) was used to register the pressure over time. The data were collected using the SPARKvue software (version 3.2.1.3, Pasco Scientific, CA, USA), the pressure was monitored until the samples failed, and the failure pressure was reported as the burst pressure. The data were reported for 5 measurements ± standard deviations.

2.9. Assessment of tissue penetration

To assess the ability of MNAs to penetrate the skin, we fabricated Trypan Blue-labeled MNAs by mixing Trypan Blue (0.4%) with the GelMA solution containing 2% (w/v) of SNs, followed by casting the solution in the MNA molds and crosslinking them using the UV light as explained in the fabrication section. The labeled MNAs were inserted into the porcine cadaver skin using thumb pressure for 1 min. Afterward, the MNAs were either removed to observe residual holes or stayed at the site for in situ imaging. After removing MNAs, the holes were stained with a Trypan Blue solution (0.4%) for 15 min, the excess dye was wiped with ethanol (70% v/v), and the skin was imaged. To observe the penetration of MNAs in the skin tissue, we fabricated fluorescently labeled MNAs by mixing FITC (0.05% w/v) and GelMA solutions similar to the procedure explained for the fabrication of Trypan Blue-labeled MNAs. FITC-labeled MNAs were inserted into the wet skin and left in the skin for ~2 h to allow dye diffusion into the holes. Thereafter, the MNAs were removed, the skin surface was cleaned, and imaged using the fluorescence microscope (Axio Observer 5, Zeiss, Germany). To characterize the penetration of MNAs in the porcine skin and determine MNA damage, the skin with the inserted MNAs was freshly frozen in the O.C.T. compound and sectioned in 20 µm slices using a cryostat (Leica, Germany). The cross-sectional images were captured using a brightfield microscope (Axio Imager A2, Zeiss, Germany).

2.10. Swelling tests

To determine time-dependent water uptake of MNAs, pre-gel mixtures (100 µL) containing different concentrations of SNs were pipetted in a PDMS mold (diameter = 8 mm, thickness = 1 mm) and crosslinked using the UV light (50 mW cm⁻², 5 min). The samples were maintained at 37 ºC to dry and form a thin film as a representative of MNA patch. The dry weight (m₀) of samples was measured, and the samples were incubated in DPBS at 37 ºC for 0, 2, 4, 8, 24, and 48 h. At each time point, the weight of wet samples (mₚ) was recorded after removing excess liquid with kimwipes. Replicates of 4 measurements were performed for each time point, and the swelling ratio was determined according to Equation (1):

\[
\text{Swelling ratio (\%)} = 100 \times \left( \frac{m_p - m_0}{m_0} \right) / m_d
\]

(1)

2.11. In vitro degradation tests

Samples were prepared similar to the swelling tests. The initial dry weight (m₀) of samples was recorded, and the dry samples were immersed in a freshly prepared solution of collagenase type II (2.5 U mL⁻¹ in DPBS) and incubated at 37 ºC. To ensure continuous degradation by enzymatic activity, the collagenase solution was refreshed twice a week. At pre-determined time points (0, 1, 7, 14, and 45 days), the samples were removed from the collagenase solution, rinsed with...
deionized water, thoroughly dried at 37 °C, and weighed to obtain the dry mass \(m_0\). The degradation rate was determined for 4 samples at each time point using Equation (2):

\[
\text{Degradation rate } (\%) = 100 \times \left( \frac{m_0 - m_n}{m_0} \right)
\]  

(2)

The MNA morphology undergoing degradation was also investigated via SEM imaging at different time intervals (day 1, 7, and 14).

2.12. In vitro toxicity assessments

NIH/3T3 murine fibroblast cells were seeded in 75 cm² tissue culture flasks containing high glucose DMEM, supplemented with 10% (v/v) of FBS and 1% (v/v) of P/S. The seeded cells were placed in a standard 5% CO₂ incubator (Forma incubators, ThermoFisher Scientific, US) at 37 °C and 95% of humidity. The DMEM was refreshed every other day, and the cells were passaged twice a week following a routine cell passaging protocol. The MNAs were sterilized using UV (254 nm) exposure for 1 h and placed in a 12-well plate. The cells at ~90% confluency were trypsinized using 0.5% trypsin-EDTA, counted using a hemocytometer, and placed in a 12-well plate. The cells at ~90% confluence were trypsinized using 0.5% trypsin-EDTA, counted using a hemocytometer, and resuspended in the medium at a density of 1 × 10⁵ cells in 1 mL. The cell suspension (200 μL) was then added carefully onto the MNAs in each well and allowed to adhere for ~2 h in the incubator. Thereafter, 2 mL of culture medium was gently dispensed in each well, and the samples were incubated at 37 °C for 24 h. To assess the metabolic activity of cells, PrestoBlue™ cell viability assay was performed on days 1, 3, and 7 following the manufacturer’s protocol. After 1.5 h incubation of cells with the medium containing the PrestoBlue™ reagent, fluorescence intensity was measured with a microplate reader (excitation = 530 nm, emission = 590 nm) and reported after subtracting the background signal (i.e., PrestoBlue™-containing cell-free media). The data were presented as 5 replicates ± standard deviation. After completing the PrestoBlue™ assay on day 7, the samples were thoroughly rinsed with DPBS and used for the live/dead assay. Live/dead staining solution was presented for 5 replicates with the medium containing the PrestoBlue™ reagent. The MNAs were sterilized using UV (254 nm) exposure for 1 h and placed in a 12-well plate. The cells at ~90% confluency were trypsinized using 0.5% trypsin-EDTA, counted using a hemocytometer, and resuspended in the medium at a density of 1 × 10⁵ cells in 1 mL. The cell suspension (200 μL) was then added carefully onto the MNAs in each well and allowed to adhere for ~2 h in the incubator. Thereafter, 2 mL of culture medium was gently dispensed in each well, and the samples were incubated at 37 °C for 24 h. To assess the metabolic activity of cells, PrestoBlue™ cell viability assay was performed on days 1, 3, and 7 following the manufacturer’s protocol. After 1.5 h incubation of cells with the medium containing the PrestoBlue™ reagent, fluorescence intensity was measured with a microplate reader (excitation = 530 nm, emission = 590 nm) and reported after subtracting the background signal (i.e., PrestoBlue™-containing cell-free media). The data were presented as 5 replicates ± standard deviation. After completing the PrestoBlue™ assay on day 7, the samples were thoroughly rinsed with DPBS and used for the live/dead assay. Live/dead staining solution was prepared by mixing ethidium homodimer-1 (20 μL) and calcein AM (5 μL) in DPBS (10 mL). The staining solution (1 mL) was added to each sample and incubated at 37 °C for 15 min in dark. The samples were then washed with DPBS, and a fluorescence microscope was used to capture images with the red channel (excitation/emission wavelengths of 528/567 nm) for ethidium homodimer-1 and the green channel (excitation/emission wavelengths of 494/515 nm) for calcein AM. The cell viability was then assessed by counting the number of live cells in ≥3 different fields using the ImageJ software (Version 1.52e) followed by normalizing the numbers with the total cells count [64].

2.13. Cell visualization

To visualize cell morphology and location after an extended culture on the MNAs, the culture medium was aspirated, and the cells were fixed with 2% (v/v) glutaraldehyde for ~30 min at the ambient temperature. Subsequently, the samples were washed with DPBS and dehydrated with a series of ethanol (50, 70, 90, 95, and 100% v/v) rinses for ~30 min each. The samples were then dried overnight at 37 °C, coated with iridium, and imaged by the SEM at an accelerating voltage of 14 kV. For imaging blood clot components, whole blood was applied to the surface of GelMA-SN (2% w/v) MNAs and incubated for 5 min to clot. The soluble left-over blood components were thoroughly washed with saline, and the samples were fixed with paraformaldehyde (4%) for 30 min. Thereafter, the samples were dehydrated with a series of ethanol rinses (30, 50, 70, and 100%, 15 min each), followed by drying at room temperature overnight before SEM imaging.

2.14. Assessment of hemolytic properties

We evaluated the concentration of released hemoglobin in plasma upon the exposure of blood to MNAs using a hemolysis assay following a standard protocol (ASTM E2524-08) [65]. In this assay, a colorimetric approach is used to quantify the oxidation of methemoglobin by cyanide (Drabkin’s reagent) at an absorbance wavelength of 540 nm. Briefly, a calibration curve was generated based on varying concentrations of human hemoglobin (0.025–1 mg/L in DPBS), which was used to quantitate the total hemoglobin in heparinized human whole blood (withdrawn from 3 healthy volunteer donors and purchased from ZenBio). All the blood samples used in the experiments were diluted with DPBS to a hemoglobin content of 10 ± 2 mg mL⁻¹ and analyzed in the lab following UCLA approved safety protocols. The desired MNAs were placed in a microcentrifuge tube, followed by adding 800 μL of DPBS and 100 μL of diluted blood. All samples were gently mixed, incubated for 3 h ± 30 min at 37 °C in a water bath, and centrifuged at 14,000 rpm for 15 min at the ambient temperature. The supernatant (100 μL) was then added to a 96-well plate, mixed with the Drabkin’s reagent (100 μL), and allowed to react at room temperature for 15 min in dark. The relative absorbance of samples in each well was assessed at 540 nm with a microplate reader (BioTek UV/vis Synergy 2, VT, USA) against the blank reagent, and was corrected with respect to the background absorbance (i.e., the absorbance of blood-free samples). Eventually, the calibration curve was used to evaluate the concentration of hemoglobin in each sample, taking the dilution factors into account. The hemolysis (%) was reported as the ratio of hemoglobin concentration in the samples to the total blood hemoglobin (i.e., ~10 mg mL⁻¹). Triton X-100 (1%) and PEG (4.4%) were used as positive and negative controls, respectively, for monitoring the assay performance. The experiments were repeated 3 times (n = 3) for each group.

2.15. In vitro blood clotting time

Fresh sodium citrate (3.8%)-treated whole human blood from healthy donors was purchased from ZenBio and analyzed with approved safety protocols in the lab. The blood was refrigerated not more than 48 h before use. In vitro clotting time was assessed for both wet (hydrogels) and dry (MNAs and needle-free) samples. To prepare wet samples, 200 μL of a pre-gel mixture was pipetted in a 24 well plate and crosslinked under UV light (50 mW cm⁻²) for 5 min. The dry samples were prepared either in the microneedle mold or in the needle-free square shape mold (15 × 15 × 1 mm) similar to the procedure explained for the fabrication of hemostatic MNAs. The dry samples were transferred to 24-well plates and used directly for the clotting test. Prior to the clotting test, calcium chloride (0.1 M) was added to the blood at a volumetric ratio of 9:1 and vortexed for 10 s to reverse anticoagulation. Thereafter, 200 μL of blood was pipetted onto each sample or in the empty wells as a control. At pre-determined time points, each well was repeatedly washed with a saline solution (0.9% w/v) until the solution became clear, indicating the removal of soluble blood components, leaving the clot in the well plate. The clotting time corresponding to the formation of a permanent clot on the surface of samples was recorded and compared with the clotting time of the control sample.

2.16. In vivo biocompatibility and hemostatic efficacy

All the animal experiments were carried out after the approval of our animal protocol by the UCLA Animal Research Committee (ARC# 2017-096-01). Animal handling procedures were performed following the “Guide for the Care of Laboratory Animals” [66]. Eight-week-old Sprague-Dawley male rats (n ≥ 6, weight of 250–300 g) were purchased from Charles River Laboratories (CA, USA) and acclimatized for 1 week before the experimentation. During the experimental periods, the rats were housed at pathogen-free conditions under a 12 h light/dark cycle at 25 °C and were fed with standard sterile laboratory pellets and purified water. All the survival surgeries were carried out under general anesthesia using isoflurane (1.5% in O₂), and analgesia was achieved by the subcutaneous injection of carprofen.

Subcutaneous implantation was conducted after shaving the dorsal
skin of rats, followed by sterilization with iodophor (0.2% w/v) and ethanol (70% v/v). A dorsal surgical incision of ~1.5 cm was created. Four small pockets were generated by blunt scissors, and a single MNA patch was implanted in each pocket. All the materials and MNAs implanted in the animals were sterilized under UV (wavelength of 254 nm) for 1 h. The rats were euthanized via CO₂ inhalation after 1, 2 and 4 weeks. The materials and MNAs along with their adjacent tissue were collected in NBF (10%) and used for hematoxylin and eosin (H&E) staining, followed by histological analyses.

For the hemostatic efficacy assessment, animals were divided into five experimental groups (n ≥ 6 per group), including injury (no treatment control), positive (+) control (commercial Surgicel™ hemostat dressing), GelMA MNAs, GelMA-SN MNAs (GelMA MNAs containing 2% w/v of SNs), and GelMA-SN flat (needle-free GelMA patch containing 2% w/v of SNs). Prior to the surgery, abdominal skin was shaved and wiped with a disinfectant. A moderate laparotomy was conducted, and the central lobe of liver was exposed. Excess blood was cleaned with surgical gauze, and the surrounding area was covered with Whatman filter paper for blood collection. A circular liver lesion (4 mm) was created using a biopsy punch. Immediately after the puncture, UV sterilized MNAs, flat patches, or Surgicel® were placed on the lesion and gently pressed for ~5 s. After ~20 s, the filter papers were removed and weighed to determine the amount of bleeding. We also used 8 animals as an untreated control (injury) group and left them without hemostat treatment to monitor the natural clotting process and the time required for the blood clotting. At the end of surgery, the abdomen region was closed using 4-0 absorbable sutures. Animals were monitored until recovered from anesthesia. Post operative pain was managed using carprofen cup and buprenorphine injection. After 7 and 14 days, animals were sacrificed by CO₂ inhalation. The site of lesion and degradation of materials were examined. Subsequently, the liver and vital organs (e.g., heart, liver, kidney, lungs, and spleen) were harvested in 10% formalin, reflecting the electrostatic attraction between the amine (-NH₂) groups in a broad spectrum of tissues while minimizing the tissue piercing pain [67].

2.17. Histology and immunofluorescence analyses

The collected tissue samples were fixed in NBF (10%), dehydrated in serial concentrations of ethanol (60%-100%), and embedded in paraffin. Tissue slides (4 μm thick section) were stained using the H&E staining kit. Microscopy images were collected using an inverted optical microscope (Amscope, USA).

For immunofluorescence analysis, citrate-buffered antigen retrieval buffer (1X) was used to liberate the antigen of sections (pressured cooker antigen retrieval for 30 min). Then the slides were permeabilized using PBST (PBS+0.3% Triton-X-100) and blocked with BSA (2% w/v) for 30 min at room temperature. Primary antibodies treated slides were incubated at 4 °C overnight. Anti-CD3 rabbit monoclonal antibody (1:100 dilution in PBST), and anti-CD68 mouse monoclonal antibody (1:100 dilution in PBST) were used. Anti-mouse Alexa 488 conjugated secondary antibody (1:1000 dilution in PBST) and anti-rabbit Alexa 555 conjugated secondary antibody (1:1000 dilution in PBST) were used as secondary antibodies. To visualize nuclei, sections were counterstained with an antifade mounting medium, containing DAPI, for 5 min. For the quantitative analysis, at least three 400x magnification fluorescence images of slides were acquired using an inverted fluorescence microscope (Zeiss Axio Observer). The specific fluorescence wavelength (green, red) images from the samples were counted using the Image-J software. The number of immunofluorescence-positive cells in each group was counted, and the relative intensity was obtained by comparing it with the 1-week timepoint of GelMA MNA group.

2.18. Statistical analyses

GraphPad Prism version 7.0 (La Jolla, USA) was used to perform statistical analyses. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison post test was conducted in all the tests, except the PrestoBlue™ metabolic activity test in which Two-way ANOVA was carried out to distinguish if there is any statistically significant difference between the independent groups of data sets. P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Fabrication of hemostatic microneedle arrays

The MNAs were fabricated using a commercial pre-made PDMS mold (nominal dimensions of mold are shown in Fig. S1), following the procedure shown in Fig. 1a. The molds were filled with GelMA-SN mixtures containing 20% w/v of GelMA and varying concentrations of hemostatic SNs via centrifugation, followed by photocrosslinking using UV light (intensity of 50 mW cm⁻², duration of 5 min). The crosslinked MNA hydrogels were dried at room temperature and peeled off from the molds. This yielded mechanically resilient and free-standing MNA patches, ready to be used without further preparation.

Chemical structure of GelMA was characterized using (¹H NMR). The methacryloyl hydrogen peaks at the chemical shifts of 5.2 and 5.6 ppm are shown in Fig. S2a. Based on the ¹H NMR results, the degree of substitution was ~69%. Physical interactions between GelMA and SNs were assessed via ξ-potential measurements (Fig. S2b). SNs have negative charges on the surface and positive charges on their edge, resulting in a ξ-potential of ~–11 mV in the medium. Mixing SNs with GelMA changed the ξ-potential to ~–2 mV for the GelMA-SNs mixture, reflecting the electrostatic attraction between the amine (-NH₂) groups of GelMA and the anionic groups of SNs. SEM coupled with EDS was used to analyze the surface morphology of dry MNAs and the distribution of SNs in GelMA (Fig. S3). SEM images show that dry MNAs are porous (Fig. S3a), with pore sizes of up to ~36 μm (Fig. S3b). The EDS spectrum shows silicon (Si, 2.02%) in the MNAs (Fig. S3c). The near uniform spatial distribution of Si originated from SNs was observed via the elemental mapping analysis (Fig. S3d).

Fig. 1b shows the facile application of MNAs to an injury, such as a bleeding wound. The MNAs are readily integrated with the tissue by applying gentle pressure, and the interlocking between the needles and tissue improves tissue adhesion. The MNAs are composed of an extra-cellular matrix (ECM)-mimicking biopolymer (GelMA), providing a cell-friendly environment to support wound closure and tissue regeneration. Fig. 1c is a snapshot of MNAs, which comprise ordered arrays of needles (11 × 11). Brightfield microscopy of MNAs showed a base diameter of ~300 μm and a pitch (center-to-center distance) of ~600 μm (Fig. 1d). The microneedles appeared sharp under SEM, showing a height of ~577 μm (Fig. 1e). This geometry supports the penetration of MNAs in a broad spectrum of tissues while minimizing the tissue piercing pain [67].

3.2. Mechanical characterizations

Mechanical strength of MNAs is a main design factor to ensure the successful penetration of needles into tissues without damaging the needle shape and compromising their function. Accordingly, the compression strength of MNAs was tested using a standard compression setup, comprising two stainless-steel plates applying force to MNAs, as shown in Fig. 2a. The MNAs (containing 121 needles) were subjected to a compressive force while registering the force and displacement. The needle failure force was identified by a local decrease in the force, shown with an arrow in Fig. 2b, indicating the deformation of needle tips (see the inset of Fig. 2a). The failure force for a single needle versus the SN concentration of MNAs is presented in Fig. 2c. While the incorporation of 0.5% and 1% w/v of SNs in GelMA did not significantly change the mechanical strength of MNAs, increasing the SN concentration to 2% w/v significantly improved the failure force to ~54 ± 4 mN per needle. This failure force was greater than the minimum force (~21 mN per
needle) reported in the literature to penetrate the stratum corneum layer of skin [68]. Therefore, the MNAs are mechanically resilient enough to penetrate soft tissues without breakage.

Open wounds can be subjected to transverse forces separating the wound edges away [69]. As a result, the attached MNAs can undergo tensile deformations. Therefore, measuring the tensile strength is important to ensure the localization of hydrated MNAs on tissues under tensile stress without breaking the MNA base. Fig. 2d presents the tensile...
characterization setup via which a hydrated MNA base (hydrogel) was attached to the instrument jaws using two metal grips, followed by stretching to identify the tensile stress required to break it. As shown in stress-strain curves (Fig. 2e), at varying concentrations of SNs, the MNAs had a linear elastic region followed by an abrupt failure at ~20–30% strain. The stress at which the wet MNA bases were broken (Fig. 2f) increased by increasing the SN concentration up to 2% w/v. The tensile strength of GelMA-SNs (2% w/v) was ~2.5-fold higher than the SN-free hydrogels. Such SN-mediated improvement in the tensile strength of wet MNAs is favorable for protecting them against stress during activities, such as walking, coughing, and running, which can introduce >20 kPa pressure to the injured (e.g., abdominal) organs [70].

3.3. MNA-assisted tissue adhesion and penetration

Tissue sealing capability of MNAs was tested using a standard burst pressure setup shown in Fig. 3a. The MNAs were gently pressed onto a perforated collagen sheet, and air was introduced into the chamber to detach the MNAs. The pressure versus time was registered, and the burst strength of MNAs was identified as an abrupt drop in the otherwise linearly increasing pressure. Fig. 3b presents the representative burst pressure profiles of MNAs versus their compositions, which shows that all compositions of MNAs are able to withstand minimum pressure of ~35 kPa. This pressure is much higher than the arterial pressure (~16 kPa) [71] and hepatic vein pressure (~1.5 kPa) [72], suggesting that the MNAs might be desirable for sealing the hemorrhaging wound in arteries and liver. We also compared the burst pressure of MNAs with their injectable hydrogel counterparts, crosslinked under the same conditions (Fig. 3c). At a given SN concentration (i.e., 2% w/v), the burst pressure of MNAs was ~43.9 ± 6.9 kPa, which was significantly higher than that of the injectable hydrogel sealant (22.1 ± 6.6 kPa) with a similar composition and was much higher than the Evicel® (7.6 ± 1.5 kPa), a commercial hemostatic hydrogel sealant. This improvement is attributed to the mechanical interlocking with the substrates due to microneedle penetration [57,73,74], as well as moisture absorption by the dry MNAs [17,18], facilitating interactions with the tissue surface via electrostatic forces and/or hydrogen bonding. It was further observed that the SN additives at the experimented concentrations did not have a significant effect on the adhesion capacity of wet hydrogels or MNAs. While injectable GelMA hydrogels have been used as promising platforms for tissue sealing [44,45], the MNAs provide a noticeably stronger attachment to tissues. This is important in developing tissue adhesive biomaterials and devices, wherein strong adhesion is required to ensure adequate residence time during hemorrhage.

We tested MNA ability to penetrate tissues, such as skin. For this purpose, MNAs were labeled with Trypan Blue (Fig. S4a) and pressed by thumb against fresh ex vivo porcine skin for 1 min (Fig. S4b). The MNAs could be manually removed after their application on the skin tissue. After removing the MNAs, the skin was stained with Trypan Blue, which left blue footprints on the skin tissue, suggesting tissue piercing (Fig. 3d). The cross-sectional images showed the tissue deformation by MNA penetration (Fig. S4c). The MNAs were also labeled using FITC (Fig. 3e) and pressed against the skin (Fig. 3f), which resulted in the puncture marks of MNAs on the tissue post penetration (Fig. S4d). The cross-sectional images of skin demonstrated successful penetration without a major physical damage to the MNAs, and the microneedles were able to interlock with the tissue (Fig. 3g).

3.4. Swelling and degradation properties

Swelling ratio of dried crosslinked GelMA at varying concentrations...
of SNs is shown in Fig. 4a. Up to 2% w/v of SNs did not significantly change the swelling behavior of GelMA, and all the dried nano-composites swelled about 3–4 times within 4 h and reached a plateau, similar to the SN-free GelMA. The degradation profiles of these nano-composite hydrogels are shown in Fig. 4b. Both GelMA biopolymer and SNs may degrade at physiological conditions and produce non-toxic byproducts [75]. Similar to the swelling ratio, the degradation profile was also not significantly affected by the SN content, and all the nano-composites degraded by ~20–30% within 45 days in DPBS containing 2.5 U mL⁻¹ of collagenase at 37 °C. The collagenase-mediated degradation of MNAs was monitored using SEM, as shown in Fig. 4c–e. After 1 day of degradation, the needles were stable and retained their sharpness and morphology (Fig. 4c). The height of needles was slightly reduced after 7 days of incubation in collagenase, although the tips remained sharp (Fig. 4d). After 14 days, the needles were partially degraded, and their tips became blunt (Fig. 4e). Although the degraded blunt needles may not effectively puncture tissues, they can still perform as pillars to support tissue adhesion. The enzymatic degradation of microneedles is favorable for ensuring the elimination of MNAs from the body once tissue remodeling and cell infiltration occur, and the healing process is completed. As an inorganic nanoparticle, the SN behavior is unlikely to change, as it does not undergo significant degradation in the period of study [76].

3.5. In vitro toxicity

Cytotoxicity of MNAs was tested via an in vitro two-dimensional (2D) cell culture. NIH/3T3 fibroblast cells were cultured on MNAs fabricated using GelMA and GelMA-SNs (2% w/v), and the cell viability and metabolic activity were monitored for 7 days. Live/dead assessment of the cells cultured on MNAs after 7 days indicated high cell viability (live cells stained in green, Fig. 5a). The cells successfully attached to the MNAs, regardless of the SN concentration, and remained viable within the experimental period. Fig. 5b shows the SEM images of adhered cells to the MNAs after 14 days. The cell viability, quantified by analyzing the fluorescence images showed ~95% of cells are viable on the MNAs across the SN content (Fig. 5c). The metabolic activity of cells cultured on the MNAs was assessed using the PrestoBlue™ reagent. Fig. 5d presents the metabolic activity in terms of fluorescence intensity for the cells cultured on MNAs made of GelMA and GelMA-SNs (2% w/v). Over time, the metabolic activity of cells increased as a result of cell proliferation. The metabolic activity on days 3 and 7 was ~6 and ~8 times higher than that of day 1, respectively. On a given day, the metabolic activity of cells on GelMA-SN MNAs was similar to that of SN-free MNAs, suggesting the cytocompatibility of SN-containing MNAs.

3.6. In vitro assessment of hemolysis and hemostatic properties

The effect of MNAs on red blood cells (RBCs) was investigated via a standard hemolysis assay in which the disruption of RBCs was calorimetrically quantified. Fig. 5a shows the mixture of diluted whole blood, incubated with various materials. When a positive control (PC, triton-X-100, 1% v/v) was used, RBCs were disrupted and released hemoglobin, resulting in the red color of solution, which remained persistent post centrifugation. The negative control (NC), i.e., PEG, did not have any significant effect on the RBCs, and the undamaged RBCs were separated at the bottom of tube via centrifugation. Incubating MNAs composed of GelMA and GelMA-SNs with the whole blood had a visually similar effect as the NC, i.e., no significant color change was observed, and the RBCs were successfully sedimented after centrifugation. Fig. 5b shows the hemolysis percentage induced by the contact of various materials with blood. The GelMA MNAs, including up to 2% w/v of SNs, resulted in ~5% hemolysis, which was within the permissible limit (ASTM F756-08) [77]. This was statistically nonsignificant (ns) compared with the NC and significantly lower than the PC. Accordingly, our results show that the MNAs do not disrupt RBCs.

To evaluate the hemostatic effect of GelMA and SNs, we performed a blood clotting assay on hydrated GelMA-SN (0–2% w/v) hydrogels. Fig. 5c shows the time-lapse of blood coagulation in the presence of hydrogels. While the untreated blood (control) formed a clot in ~12 min, the GelMA hydrogels did not significantly affect the coagulation process. Incorporating SNs to GelMA decreased the clotting time from ~6 min to ~5 min at 1% and 2% w/v of SNs, respectively. Fig. 5d shows the average clot formation time using various hydrogels. While GelMA hydrogel did not play a significant role in blood coagulation within 10.2 min, the SNs rendered the composite hydrogel hemostatic, reducing the clotting time by ~57% at 2% w/v of SNs. The MNA-mediated blood coagulation was assessed by measuring the coagulation time similarly. Fig. 5e shows the behavior of whole blood in contact with MNAs compared with the needle-free (flat) patches and a commercially available positive control, Surgicel®. In the absence of hemostatic agents, the early signs of blood coagulation were observed...
within ~12 min, whereas the SN-free GelMA MNAs reduced the coagulation time by at least half, and the clots were formed in the first 5 min. Eliminating the needles from the patch of GelMA increased the coagulation time to >8 min, which was close to the control (hemostat-free blood) sample. It is worth noting that the SN-free flat patches showed slightly shorter clotting time than the SN-free hydrogels, which could be attributed to the dry-state application of patches, allowing them to absorb more blood than the water-containing hydrogels. Increasing the SN content of MNAs to 2% w/v significantly reduced the clotting time to ~1 min, which was ~9-fold lower than the untreated blood control, ~6-fold lower than Surgicel®, and ~4-fold lower than the SN-free MNAs. The clotting time of needle-free GelMA-SN (2% w/v) flat patches was significantly higher (~5 min) than the MNA counterpart. The quantification of clotting time for the MNAs and needle-free patches is shown in Fig. 5f. The average blood clotting time in the absence of hemostatic agents was 11.5 ± 1.0 min, which decreased to 8.7 ± 1.5 min when needle-free GelMA patches were used. The clotting time further decreased to 5.4 ± 1.1 min for the GelMA patches with microneedle structures (i.e., GelMA MNAs). Incorporating the GelMA MNAs with SNs significantly decreased the clotting time to 1.3 ± 0.6 min, whereas the GelMA-SN (2% w/v) needle-free flat patches resulted in a similar clotting time as that of GelMA MNAs (5.4 min). Despite the hemostatic properties of SNs, lacking an extended contact area with blood hindered the hemostatic effect of SN-containing biomaterials, which was addressed via microengineering the tissue-biomaterial interface using microneedles. Our overall clotting time results highlighted the critical role of microneedles in inducing blood coagulation, possibly as a result of extended surface area and enhanced contact between the hemostatic material and blood. Accordingly, the best hemostatic performance was observed when the patch (i) was decorated with microneedles and (ii) doped with SNs. Note that at SN concentrations >2% w/v, the viscosity of GelMA-SN mixture was too high to penetrate the microneedle mold and form the MNAs, therefore, we did not investigate the effect of higher concentrations of SNs on blood coagulation. Moreover, 89% reduction in clotting time by GelMA-SN (2% w/v) MNAs exceeds the clotting time of the majority of commercially available solid-based hemostat agents (such as Surgicel®, dehydrated QuickClot®, and Instat™) which yield only ~20–60%
improvement in coagulation [78].

SEM imaging was performed to investigate the morphology and cellular composition of clots formed by GelMA-SN (2% w/v) MNAs. At the 5 min clotting time, a thick layer of hemostatic plug, rich in blood cells was formed on the entire surface of MNAs (Fig. 5g). A higher magnification of SEM micrograph showed fibrin filaments, indicated by arrows in Fig. 5h [79]. The activated platelets were agglomerated and trapped under the fibrin network. As shown in Fig. 5i, most of the clot components were activated platelets along with some RBCs. These findings suggest that the highly charged SNs can trigger blood coagulation cascade by attracting platelets and coagulation factors to the wound area, resulting in rapid hemostasis.

3.7. In vivo degradation and biocompatibility

Before evaluating the hemostatic efficacy of engineered MNAs in vivo, we assessed their degradation and degree of inflammation. For this purpose, GelMA MNAs and GelMA-SN (2% w/v) MNAs were implanted subcutaneously in the back of rats for 4 weeks (4 implants per animal, three animals, n = 3, per each time point/group). Mortality due to MNA implantation was not observed in any experimental group, and no significant weight loss or behavioral abnormalities were observed. In the gross analysis of subcutaneous tissue observed at each time point (1, 2, and 4 weeks), no adverse reactions such as redness or excessive vascular reaction were observed in both SN-free and SN-containing GelMA-based MNAs. As it can be seen from the results of H&E staining, both GelMA MNAs and GelMA-SN MNAs were degraded overtime for 4 weeks after implantation (Fig. 5g). The difference in the degradation rate of two MNA types was not significant. Both types of MNAs were degraded to about half their original size after 2 weeks, followed by further size reduction after 4 weeks. The overall in vivo degradation rate was much faster than the in vitro, possibly as a result of various enzymes in the body. At a higher magnification (400 ×), GelMA MNAs and GelMA-SN MNAs were encapsulated into a fibrous tissue, infiltrated with mild inflammatory cells at week 1. These fibrous capsules showed a general foreign body reaction pattern, becoming slightly thickened at week 2 and then transformed into loose connective tissue after 4 weeks. Interestingly, when GelMA MNAs and GelMA-SN MNAs were subcutaneously implanted, the infiltration of inflammatory cells observed at week 1 decreased gradually toward week 4 of implantation as the material disintegrated and the fibrous tissue was replaced with loose connective tissue.

We further analyzed T-cell and macrophage infiltration via immunofluorescence staining to assess the type of inflammatory cells infiltrating the fibrous capsule around MNAs (Fig. 5j). As a result, sporadic distributions of CD3⁺ T-cells and CD68⁺ macrophages were observed in both GelMA MNA and GelMA-SN MNA groups, but there was no significant difference between the groups. Similar to the H&E staining results, both T-cells and macrophages were mainly observed at week 1 and gradually decreased at week 4. In quantitative analysis, CD3⁺ T-cells and CD68⁺ macrophages did not show significant differences between the experimental groups. These results indicate that the GelMA MNAs and GelMA-SN MNAs are relatively safe to use and degrade in a time frame of approximately 4 weeks without exhibiting side effects, such as severe inflammation or tissue damage upon implantation. Subsequently, based on the promising biocompatibility results, we applied the hemostatic MNAs to an in vivo liver bleeding model and analyzed the hemostatic efficacy.

3.8. Bleeding management

Liver is a soft and fragile organ with ample blood supply. Because of these characteristics, it is difficult to treat liver hemorrhage using conventional methods, such as suturing or stapling [57]. Therefore, we selected rat liver bleeding model to investigate the potential of MNAs to stop bleeding in vivo. A standard liver bleeding model was prepared on the liver lobe using a 4 mm biopsy punch, followed by treatment using the hemostatic MNAs, as shown in Fig. 6a. The MNAs were able to easily penetrate the liver tissue, which has a lower Young’s modulus (0.6–2 kPa) [80] than skin. Immediately after creating the punctured wound on liver, engineered hemostatic MNAs, or a + control (Surgicel®, a commercially available hemostatic dressing) was applied to the injury site (n = 6 per group). To understand the effect of microneedles (contact area) on clotting, the blood clotting efficacy of GelMA-SN (2% w/v) needle-free flat patches was also evaluated and compared with the GelMA-SN (2% w/v) MNAs.

To evaluate the hemostatic efficacy of MNAs, we monitored the blood clotting time immediately after applying the engineered materials to the wound. All the animals in the treatment groups stopped bleeding in <20 s, whereas the untreated injury group stopped bleeding much slower, requiring >2 min. As shown in Fig. 6b, the amount of bleeding after treatment was analyzed using a filter paper placed around the liver. All the GelMA-based materials as well as the + control were able to stop bleeding efficiently compared with the untreated injured group. In particular, the animals treated with the + control and GelMA MNAs had similar bleeding, while flat patches and MNAs containing SN resulted in less bleeding. Nanoengineered MNAs, which were applied in a dry form to the bleeding area, absorbed blood quickly, became flexible and soft, and wrapped around the wound area to prevent bleeding. Both SN-containing flat patches and MNAs coagulated RBCs upon blood absorption, resulting in faster hemorrhage prevention. However, in case of flat GelMA-SN, the patches were easily dislodged from the applied area compared with the MNAs group. In the quantitative analyses (Fig. 6c), all the treatment groups yielded a substantial reduction in bleeding compared with the untreated injury group. The + control, GelMA MNAs, GelMA-SN flat patches, and GelMA-SN MNAs groups demonstrated 62.6 ± 20.1%, 59.5 ± 11.5%, 31.0 ± 5.1%, and 8.3 ± 2.0% bleeding, respectively, when compared with the untreated injury group. Notably, GelMA-SN MNAs had more pronounced hemostatic efficacy compared with the + control, whereas the bleeding percentage was not significantly different between GelMA MNAs and the + control. Interestingly, GelMA-SN MNAs had a significantly higher hemostatic efficacy than their flat patch counterparts. This observation indicates that the extended surface area promotes high-volume blood uptake and efficient interaction between the interlocked microneedles and lesion, leading to more platelet recruitment.

Next, we assessed the regeneration of punctured liver and the effects of biomaterials (+control, GelMA MNAs, GelMA-SN flat patches, and GelMA-SN MNAs) on other organs in a 2-week period compared with the control (injury with no treatment) group. As it can be seen in Fig. 6d, the injured group formed a scar tissue at the injured site and had a larger lesion after 2 weeks. In case of + control group, despite effective bleeding prevention, the hemostatic area was discolored to yellow. In addition, in more than 40% of implanted animals, the flat patches detached from the bleeding site and were unable to cover the wound area. The detached GelMA-SN flat patches did not impose adverse effects, such as inflammations or adhesion in the abdominal cavity. In contrast, GelMA MNAs and GelMA-SN MNAs were able to adhere effectively to the bleeding site, covering the hemorrhage site for up to 2 weeks.

In microscopic analyses (Fig. 6e), for a more detailed evaluation of the liver wound sites to which the experimental hemostatic materials were applied, all groups showed regenerative response/inflammatory response at week 1 and the response was relieved at week 2. The injury group showed inflammatory cell infiltration in the parenchyma of the liver, and the regenerative hepatocytes (hyperchromatic, nucleated) were arranged in the cord at 1-week post operation. In the + control group, the hemostatic material was attached to the liver capsule and decomposed, and the infiltration degree of inflammatory cells in the liver parenchyma during regeneration was higher than that of the other groups. GelMA MNAs, GelMA-SN flat patches, and GelMA-SN MNAs were all attached to the liver capsule and showed mild to moderate fibrotic reactions, but no significant inflammatory response was observed. In
(caption on next page)
other biologics to synergistically promote hemostasis and wound healing. Additionally, the hemostatic MNAs to the punctured liver wound with respect to the untreated injury control, the liver area, which was observed as discolored, changed to a fibrotic tissue. Accordingly, although the + control is able to quickly stop bleeding, it may not be conducive to regeneration in the parenchyma when it remains in the body. In all GelMA-based treatment groups (GelMA MNAs, GelMA-SN flat, and GelMA-SN MNAs) the boundary between the liver capsule and the material became unclear and gradually disappeared. The infiltration of fibrous tissue was observed at the interface between the liver and the patch, and the remaining GelMA-based material degraded over time. Compared with the + control group, the inflammatory response in the liver parenchyma of GelMA-based materials was low, and the regenerative response was still observed at 2-week post operation. Finally, the systemic toxicity following the application of hemostatic materials was evaluated (Fig. S8), showing no significant toxic reactions of vital organs including heart, lung, liver, kidney, and spleen in all groups after 2 weeks of implantation. Accordingly, hemostatic MNAs are localized in the liver bleeding wounds and do not cause significant systemic toxicity.

4. Conclusions and prospects
Excessive bleedings, particularly those resulting in trauma remain one of the main causes of death. Rapid prevention of bleeding after tissue rupture, e.g., post gunshot, is of utmost importance in controlling fatality. The standard practice for replacing sutures and staples for surgical applications has been based on hydrogels that undergo a phase transition from liquid to a crosslinked solid that adhere to tissue while providing hemostatic effects. However, these materials typically require excessive preparation and advanced processing, such as photo-/thermo-activation, which are not suitable for immediate use. We have developed hemostatic MNAs via the nanoeengineering of GelMA, a widely used cell-friendly biopolymer, using SNs which can significantly increase the contact surface with blood and adhere to the wound through an interlocking mechanism. We show that doping GelMA MNAs with only 2% w/v of SNs reduces the clotting time by 89% in vitro, which is otherwise impossible to achieve using hydrogels with identical compositions. We found ~92% reduction in bleeding after applying engineered GelMA-SN MNAs to the punctured liver wound with respect to the untreated injury group. Furthermore, the hemostatic MNAs could degrade in vivo in a time span (~4 weeks) that is compatible with the wound healing process without triggering major inflammation responses. The hemostatic MNAs may be left on the wound to prevent internal bleeding post-surgery without complications or need of removal as a result of decent bio/ hemocompatibility and biodegradability. Additionally, the hemostatic MNAs have the potential to be loaded with drugs, growth factors, or other biologics to synergistically promote hemostasis and wound healing. Future studies on the hemostatic effects of needle geometry and dimensions, and exploring other polymeric matrices for high pressure applications, such as cardiac bleeding in large animal models, are warranted to enable the clinical translation of hemostatic MNAs.

Author contributions
Reihaneh Haghniaz: contributed equally to this work, performed the in vitro and ex vivo experiments and helped with animal experiments, tissue sectioning, and immunofluorescence staining. Han-Jun Kim: contributed equally to this work, performed animal experiments and in vivo data analyses. Hossein Montazerian: contributed to mechanical testing, adhesion assessment, drawing schematics, and revising the paper. Avijit Baidya: contributed to the NMR data analyses and revising the paper. Maryam Tavafoghi: contributed to swelling assessments. Yi Chen: contributed to animal tissue sectioning. Yangzhi Zhu: contributed to revising the paper. Solmaz Karamikamkar: Contributed to revising the paper. Amir Sheikh: conceptualized the idea, supervised the research, and contributed to manuscript preparation and revision. Ali Khademhosseini: supervised the overall research, All the authors discussed the results and commented on the manuscript.

Ethical committee
The animal protocol is approved by the UCLA ethical committee, ARC # 2017-096-01.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data
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