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Nano hemostat solution: immediate hemostasis at the nanoscale

Rutledge G. Ellis-Behnke, PhD,[a,b,c,*] Yu-Xiang Liang, PhD,[b,c] David K.C. Tay, PhD,[b,c] Phillis W.F. Kau, BSc,[b,c] Gerald E. Schneider, PhD,[a] Shuguang Zhang, PhD,[d] Wutian Wu, MD, PhD,[b,c] Kwok-Fai So, PhD[b,c,e,*]

[a]*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA*
[b]*Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China*
[c]*State Key Laboratory for Brain and Cognitive Science, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China*
[d]*Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA*
[e]*Research Centre of Heart, Brain, Hormone and Healthy Aging, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China*

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Abstract Hemostasis is a major problem in surgical procedures and after major trauma. There are few effective methods to stop bleeding without causing secondary damage. We used a self-assembling peptide that establishes a nanofiber barrier to achieve complete hemostasis immediately when applied directly to a wound in the brain, spinal cord, femoral artery, liver, or skin of mammals. This novel therapy stops bleeding without the use of pressure, cauterization, vasoconstriction, coagulation, or cross-linked adhesives. The self-assembling solution is nontoxic and nonimmunogenic, and the breakdown products are amino acids, which are tissue building blocks that can be used to repair the site of injury. Here we report the first use of nanotechnology to achieve complete hemostasis in less than 15 seconds, which could fundamentally change how much blood is needed during surgery of the future.

Key words: Hemostasis; Surgery; Trauma; Nanotechnology; Self-assembling peptide

Through the ages doctors have found ways to achieve hemostasis, beginning with the simple act of applying pressure, then cauterization, ligation, and clinically induced vasoconstriction [1-10], but nanotechnology brings new possibilities for changes in medical technology. Here we present a novel method to stop bleeding using materials that self-assemble at the nanoscale when applied to a wound. This method results in the formation of a nanofiber barrier that stops bleeding in any wet ionic environment in the body; furthermore, the material is broken down into natural l-amino acids that can be used by the surrounding tissue for repair.

Currently there are three basic categories of hemostatic agents or procedures: chemical, thermal, and mechanical [1,3,6,8,10-15]. Chemical agents are those that change the clotting activity of the blood or act as vasoconstrictors, such as thromboxane A2 [16], which causes vessels to contract thus reducing blood flow and promoting clotting [7,16,17]. Thermal devices commonly involve cauterization using electrodes, lasers [8,14], or heat. There are also agents that react exothermically upon application that may create an effect similar to a standard two probe cautery device [1,14]. Mechanical methods use pressure or ligature to slow the blood flow [3]. A combination therapy might use both chemical and mechanical means to produce a hemostat that adsorbs fluid and swells [18], producing

The authors declare a competing financial interest: S.Z. is a co-founder and board member of 3D Matrix, the provider of one of the materials used.

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* Corresponding authors. R.G. Ellis-Behnke, Department of Brain and Cognitive Sciences, 46-6007, Massachusetts Institute of Technology, Cambridge, MA 02139, USA; K.-F. So, Department of Anatomy, University of Hong Kong Faculty of Medicine, Hong Kong, SAR, China.
E-mail addresses: rutledg@mit.edu (R.G. Ellis-Behnke), hrmaskf@hkucc.hku.hk (K.F. So).

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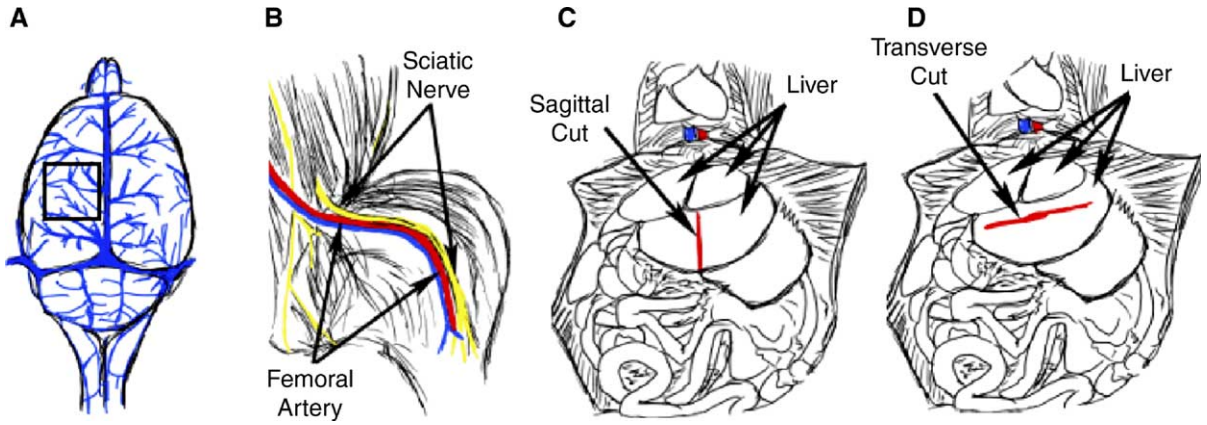

Fig 1. Schematics of surgical procedures. Rostral is up and caudal is down in all figures. **A**, Dorsal view of the rat brain. The blue lines depict the blood vessels superficial to the cortex. The boxed area corresponds to location of the lesion and treatment. **B**, Drawing of ventral view of the lower limb of a rat with the femoral artery in red and sciatic nerve in yellow. **C** and **D**, Drawings of a ventral view of rat with abdomen open. Overlying structures have been removed exposing the liver. The lobe was transected with a cut (depicted in red) in both sagittal (**C**) and transverse (**D**) directions.

pressure to slow the blood flow and allow clotting, or it may involve the introduction of fibrinogen, thrombin, and calcium to produce fibrin glue, which acts as an artificial clot [1,2,5,6,8,10,14,19].

There are five major issues related to the limitations and applicability of many of these hemostatic agents. First, some of the materials are solid, such as powder formulations, and are not able to flow into the area of injury to bring about their hemostatic effects [1,10,14]; second, some liquid agents, such as cyanoacrylates, require a dry environment to be effective [8]; third, some materials can create an immune response resulting in the death of adjacent cells, placing additional stress on the body that can prolong or prevent healing [8,10,14,15,20]; fourth, some agents have a short shelf-life and very specific handling requirements [6,10,14,16,17]; and finally, many currently used hemostats are difficult to use in uncontrolled environments [1,7,8,10,14]. Moreover, if a therapy uses swelling as part of its hemostatic action, then extra care must be taken to ensure that the local blood supply is not reduced or stopped, which could cause additional tissue damage or even death. This is particularly crucial when using expanding foams [19]. Many hemostatic agents must be prepared just before use because of their short shelf-life. Surgical instruments, such as cauterization devices, clamps and clips, must be used by a skilled individual in a controlled environment [2,5,8-10,16,20].

Our discovery, observed during a neurosurgical procedure, introduces a new way to stop bleeding using a self-assembling peptide that establishes a nanofiber barrier and incorporates it into the surrounding tissue to form an extracellular matrix (ECM). Surmising that nanotechnology might be useful in our central nervous system regeneration studies, we injected the material into wound sites in the brain of hamsters to determine whether it would facilitate neuronal regeneration [21]. To our surprise, it also stopped bleeding.

We then wanted to know if the rapid hemostasis that we had observed in our nerve regeneration experiments was tissue specific or would also work in other tissues. The seven experiments we designed and performed demonstrate that in less than 15 seconds complete hemostasis can be achieved after (1) a transection of a blood vessel leading to the superior sagittal sinus in both hamsters and rats, (2) a spinal cord cut, (3) a femoral artery cut, (4) a sagittal transection of the left lateral liver lobe, (5) a transverse transection of the left lateral liver lobe including a cut in a primary branch of the portal vein, (6) a 4-mm liver punch biopsy, and (7) multiple 4-mm skin punch biopsies on nude mice.

Materials and methods

Adult Syrian hamsters were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and adult rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg). The experimental procedures adhered strictly to the protocol approved by the Department of Health and endorsed by the Committee on the Use of Laboratory Animals for Teaching and Research of the University of Hong Kong and the Massachusetts Institute of Technology Committee on Animal Care.

Cortical vessel cut experiment

The animals were fitted in a head holder. The left lateral part of the cortex was exposed, and each animal received a transection of a blood vessel leading to the superior sagittal sinus (Figure 1, *A*). With the aid of a sterile glass micropipette, 20 μL of 1% wt/vol NHS-1 solution (see below under "Preparation of the self-assembling solutions") was applied to the site of injury, or iced saline in the control cases. The animals were allowed to survive for as long as 6 months.

Spinal cord injury experiment

Under an operating microscope, the second thoracic spinal cord segment (T2) was identified before performing a dorsal

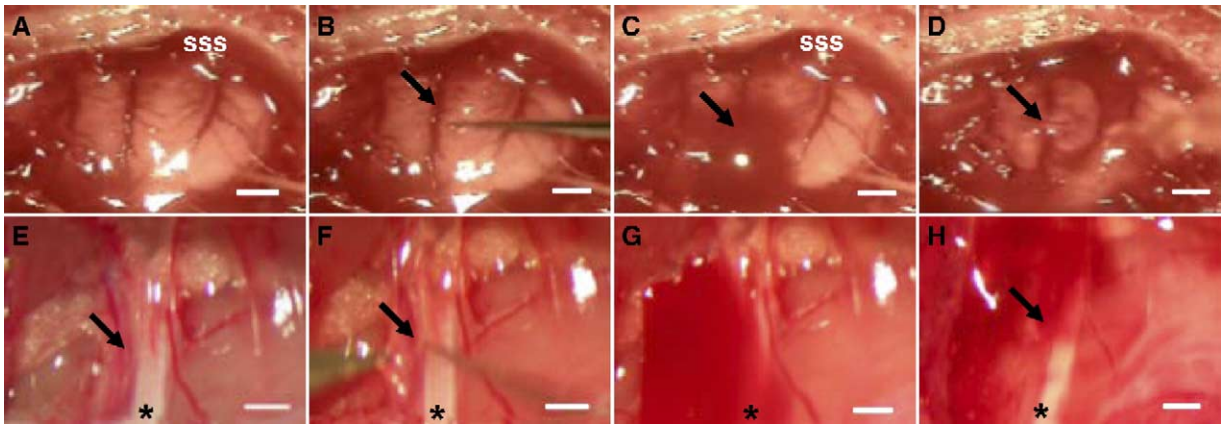

Fig 2. Complete hemostasis in brain and femoral artery. The pictures are time-lapse images at each stage of the experiment for brain (**A**-**D**) and femoral artery (**E**-**H**). **A**-**D**, Adult rat cortex hemostasis. Part of the overlying skull has been removed in an adult rat, and one of the veins of the superior sagittal sinus is transected and treated with 1% self-assembling NHS-1. **A**, The brain and veins of the superior sagittal sinus (SSS) are exposed. **B**, Cutting of the vein (arrow). **C**, Bleeding of the ruptured vein (arrow). **D**, The same area 5 seconds after application of the self-assembling NHS-1 to the location of the cut (arrow) as seen under the clear NHS-1. **E**-**H**, Rat femoral artery hemostasis. Exposure of the neurovascular bundle in the thigh showing the sciatic nerve (*) in each panel. **E**, Femoral artery and vein exposed. **F**, Cutting of the artery (arrow). **G**, Bleeding, masking the artery completely and sciatic nerve partially. **H**, The same area 5 seconds after application of the self-assembling peptide to the cut (arrow). Note that there is complete hemostasis in the area formed by NHS-1 (covering the entire picture) as it self-assembles in the presence of blood and plasma, revealing the underlying structures. Complete hemostasis was achieved in 10.6 ± 4.1 seconds, significantly different from 367.5 ± 37.7 seconds in controls irrigated with saline ($P < 0.0001$). Scale bars represent 1 mm.

laminectomy in anesthetized adult rats [22,23]. After opening the dura mater, we performed a right hemisection using a ceramic knife. Immediately after the cord hemisection 20 μL of a 1% wt/vol solution of NHS-1 was applied to the area of the cut for bleeding control. The controls received a saline treatment. The animals were allowed to survive for as long as 8 weeks as part of another experiment.

Femoral artery cut experiment

Rats were placed on their backs, and the hind limb was extended to expose the medial aspect of the thigh (Figure 1, *B*). The skin was removed, and the overlying muscles were cut to expose the femoral artery and sciatic nerve. The femoral artery was cut to produce a high-pressure bleeder (Figure 2, *F*). With a 27-gauge needle, 200 μL of 1% wt/vol NHS-1 solution was applied over the site of injury. In two cases we applied the dry powder of NHS-1 to the injury site, which also was effective. (Data are not shown and were not included in the analysis.) Controls were treated with a combination of saline and pressure with a gauge. All animals were killed 4 hours after the experiment.

Liver wound experiments

Rats were anesthetized and placed on their back, and the abdomen was opened exposing the liver (Figure 1, *C*). The left lobe of the liver was cut using a scalpel in the rostral-to-caudal direction, separating the two halves of the lobe (Figure 3, *B*) in the sagittal cut. With a 27-gauge needle, 100 μL of 1% or 2% wt/vol NHS-1, NHS-2, or TM-3 solution was applied to the site of injury (Figure 3, *B*). Livers of the controls were treated with saline or cauterized. Cauterization was performed using a thermal cautery device and was applied to the entire surface of the injury. In another group of 28 adult rats the same procedure was followed for the liver, which was cut transversely (Figure 3, *D*). With a 27-gauge needle, 400 μL of 1%, 2%, 3%, or 4% wt/vol NHS-1 or TM-3 solution was applied to the site of injury (Figure 3, *H*).

In another group of anesthetized adult rats the liver was exposed, and a 4-mm punch biopsy done from the ventral aspect through the liver to the dorsal surface of the left liver lobe. The resulting core was removed from the liver, after which one of three treatments was applied. For the treatment group 200 μL of 3% NHS-1 solution was applied to the site of injury, whereas in the controls either saline was applied or cauterization of the exposed liver surface was carried out. The superficial material was then wiped clear of the injury site. The abdominal incision was closed, and the animals were allowed to survive for as long as 8 weeks.

Skin punch experiment

In anesthetized adult nude mice using aseptic precautions, a 4-mm punch was used to create three wounds on each side of the back of the animal. On one side of the animal the wounds created were treated with 1% wt/vol NHS-1 solution, and the wounds on the opposite side were left untreated to provide a control. The punch biopsies were made through the full thickness of the skin. If the wound did not bleed for 10 seconds the punch would be excluded from the data analyzed. All procedures were videotaped, and the analysis consisted of reviewing the tapes. The animals were allowed to survive for as long as 2 months. If animals involved in any of the above experiments appeared to experience any discomfort they were euthanized.

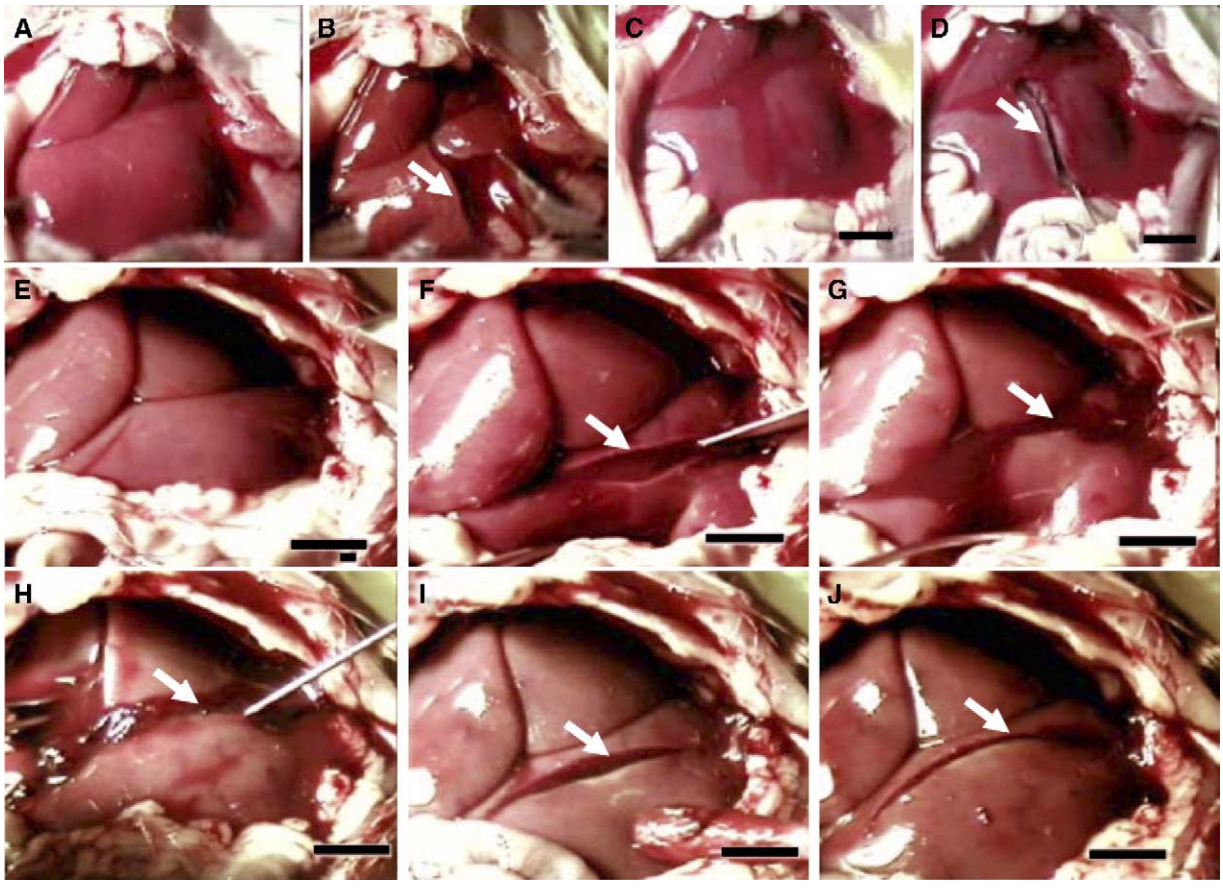

Fig 3. Rat liver hemostasis. This series of pictures is of an adult rat wherein the skin covering the intraperitoneal cavity was excised, exposing the liver. **A** - **D**, Sagittal cut. **A**, The left lateral lobe received a sagittal cut completely transecting a portion of the liver lobe. **B**, The liver is separated (arrow). Note the profuse bleeding. **C**, The two halves are allowed to come back together, and the bleeding continues (arrow). **D**, The 1% NHS-1 solution was applied, and the extent of the incision was visible under the transparent assembled NHS-1 (arrow). Complete hemostasis was achieved in 8.6 ± 1.7 seconds, statistically significant when compared to 90.0 ± 5.0 seconds when cauterization was applied, or 301.6 ± 33.2 seconds if irrigated with saline. **E** - **J**, Transverse cut. This series of pictures is of a transverse cut to the left lateral lobe in an adult rat. **E**, The exposed intact liver. **F**, Applying a transverse cut in the lobe (arrow). **G**, Profuse bleeding produced when a major branch of the portal vein is cut (arrows). **H**, Treatment with self-assembling NHS-1. Note the complete cessation of bleeding (in 10.3 ± 0.5 seconds using 2% concentration; 10.0 ± 1.0 seconds and 11.0 ± 1.0 using 3% and 4%, respectively) seen under the clear assembled NHS-1 (arrow). **I**, 2 minutes after treatment and after the superficial self-assembling NHS-1 has been removed (arrows) to show the extent of cut. **J**, Bleeding ceased 15 seconds after treatment, and hemostasis was maintained. Scale bars represent 1 mm.

Transmission electron microscopy sample preparation

In the brain and liver of anesthetized adult rats a 1% or 2% NHS-1 solution was injected immediately after making a cut, and the treatment site was sampled. Samples were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde wt/vol in 0.1 M phosphate buffer (PB) for 4 hours. The samples were washed in 0.1 M PB three times for 10 minutes each at 4°C and embedded in 2% agar (wt/vol); blocks were postfixed in 4°C 1% (wt/vol) osmium tetroxide for 2 hours and then washed in buffer three times for 10 minutes each at 4°C. The sample blocks were dehydrated in ethanol, infiltrated, and embedded in pure epon with Lynx EM tissue processor. Ultrathin 70-nm sections were cut (Reichert-Jung ultra cut) and collected on no. 200 mesh grids. Sections and grids were stained with uranyl acetate and lead citrate and examined under a Philip EM208S transmission electron microscope.

Preparation of the self-assembling solutions

The NHS-1 solution was prepared using RADA16-I synthetic dry powder (obtained from the Massachusetts Institute of Technology Center for Cancer Research Biopolymers Laboratory, Cambridge, MA; the Zhang laboratory, and 3-DMatrix, Cambridge, MA) dissolved in an Eppendorf tube. The 1% wt/vol NHS-1 solution was prepared by dissolving 10 mg of RADA16-I powder in 1 mL of autoclaved Milli-Q water (Millipore Corp., Billerica, MA), sonicated for as long as 5 minutes, and filtered. This was repeated with 20 mg/mL, 30 mg/mL, and 40 mg/mL to produce 2%, 3%, and 4% wt/vol concentrations. NHS-2 and TM-3 dry powders (made by the Massachusetts Institute of Technology Center for Cancer Research Biopolymers Laboratory, Cambridge, MA) were prepared using the same method. The time of preparation did not affect the action of the solution. We also tested some material that was prepared

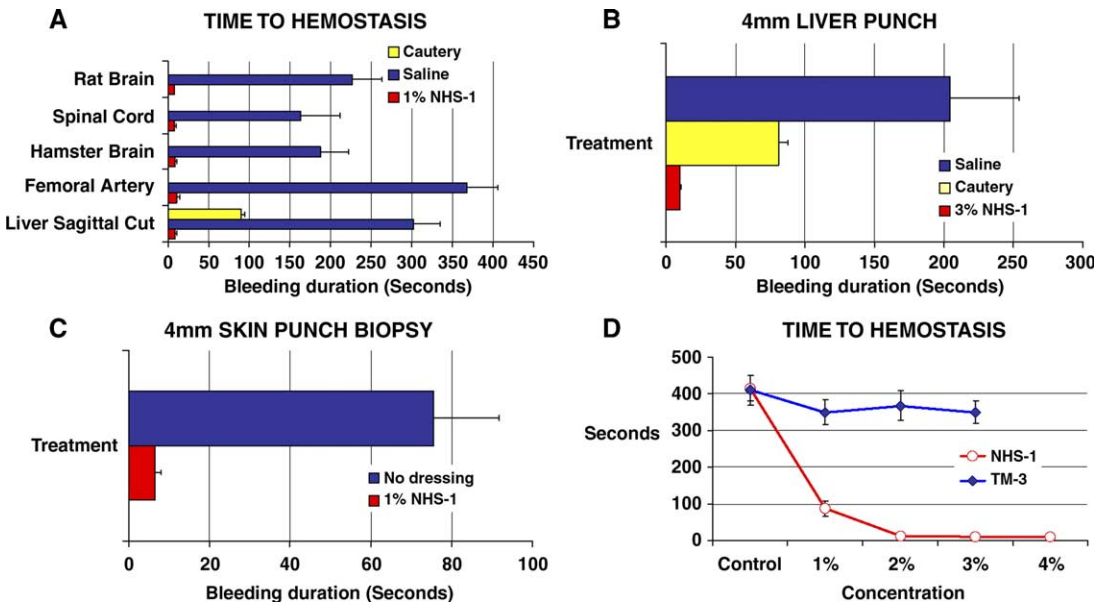

Fig 4. Time required to achieve hemostasis. Graphs illustrate bleeding durations in cases treated with 1% NHS-1 self-assembling solution compared with those cautery- and saline-treated controls for brain, femoral artery, and liver cuts (**A**), liver punches in rats (**B**), and nude mice skin punches (**C**). Each bar shows the mean time in seconds for NHS-1–treated cases (in red), saline controls (in blue), and cautery controls (in yellow). **A**, In the rat brain cut, durations were measured from the start of application of self-assembling NHS-1 to the completion of hemostasis after transection of the veins leading to the superior sagittal sinus in the brain of adult rats. Complete hemostasis was achieved in 8.4 ± 2.1 seconds. In the saline controls bleeding continued until 227.0 ± 36.6 seconds. In the hamster brain cut, complete hemostasis was achieved in 9.0 ± 1.8 seconds. In the saline controls bleeding continued until 187.6 ± 34.7 seconds. In the femoral artery cut, complete hemostasis was achieved in 10.5 ± 4.1 seconds. In the saline controls bleeding continued until 367.5 ± 37.7 seconds. In the liver sagittal cut, complete hemostasis was achieved in 8.6 ± 1.7 seconds. In the cautery control (yellow), bleeding continued until 90.0 ± 5.0 seconds, and the saline controls bled for 301.6 ± 33.2 seconds. **B,** Liver 4-mm punch biopsy. A 4-mm core was removed from the left liver lobe, and the hole was treated with NHS-1, heat cautery, or saline. Treatment with 3% NHS-1 brought about complete hemostasis in 9.7 ± 1.2 seconds. In the cautery controls (yellow) bleeding continued for 81.2 ± 6.7 seconds, and the saline controls bled for 204.3 ± 49.6 seconds. **C,** Skin 4-mm punch biopsy. A 4-mm punch biopsy was made on the backs of nude mice. The biopsy extended through the dermis, and the core was removed. Care was taken not to disrupt the underlying muscle. The three wounds on one side were treated with 1% NHS-1, and complete hemostasis was achieved in 6.4 ± 1.5 seconds. On the opposite side of the animal the wounds were not treated. Bleeding continued until normal clotting occurred at 75.5 ± 16.3 seconds. **D**, In rats, concentration response curves of NHS-1 and TM-3. The left lateral liver lobe received a transverse cut severing a portion of the liver lobe and branch of the portal vein. A higher concentration of NHS-1 (open circles) is more effective in higher pressure and volume hemorrhages. NHS-1 at concentrations of 4%, 3%, and 2% were effective in achieving hemostasis in 11.0 ± 1.0 seconds, 10.0 ± 1.0 seconds, and 10.3 ± 0.5 seconds, respectively. The 1% NHS-1 solution required 86.6 ± 20.8 seconds at the area of the most severe bleeding. TM-3 (diamonds) was not effective at any concentration; in the saline controls bleeding continued until 377.5 ± 85.0 seconds, and one animal died. Time (seconds) is shown on the x-axis, concentration on the y-axis.

(obtained from the Zhang laboratory) and stored in solution at room temperature, for 3 years before use, and it performed as well as the newly mixed material.

Results

Hemostasis in a brain injury

We began our experiments in the brain, removing the overlying skull and performing a complete transection of a branch of the superior sagittal sinus in the brain of rats (n = 15) and hamsters (n = 15) (Figure 1, *A*). The areas were treated with 20 μL of a 1% wt/vol solution of RADA16-I (NHS-1) self-assembling solution or with iced saline. In the groups treated with NHS-1 hemostasis was achieved in less than 10 seconds in both hamsters and rats (Figure 2, *A-D* and Supplemental Video 1, "Hemostasis in rat cortex with self-assembling peptide treatment" which can be found in the online version of this article). Control group hamsters (n = 5) and rats (n = 5) irrigated with saline bled for more than 3 minutes (Figure 4, *A*). A truncated iced-saline control and subsequent treatment with NHS-1 is shown in Supplemental Video 2 ("Saline control and treatment with self-assembling peptide in rat cortex.") Student's t-test for two independent samples in both hamsters and rats showed highly significant differences ($P < .0001$).

Hemostasis in a spinal cord injury

Because blood has been shown to be toxic in neural tissue [24] we wanted to know if the spinal environment was different from the brain. By quickly bringing bleeding under control secondary damage caused by surgery can be reduced. After laminectomy and removal of the dura, the spinal cord was hemisected at T2, from the dorsal to ventral aspect, and treated (n = 5) with 20 μl of 1% wt/vol NHS-1. Hemostasis was achieved in just over 10 seconds. In the saline controls (n = 5) bleeding continued for as long as 5 minutes. Comparison of the treated group and the saline controls shows a significant difference using the Tukey test with a 99% confidence interval.

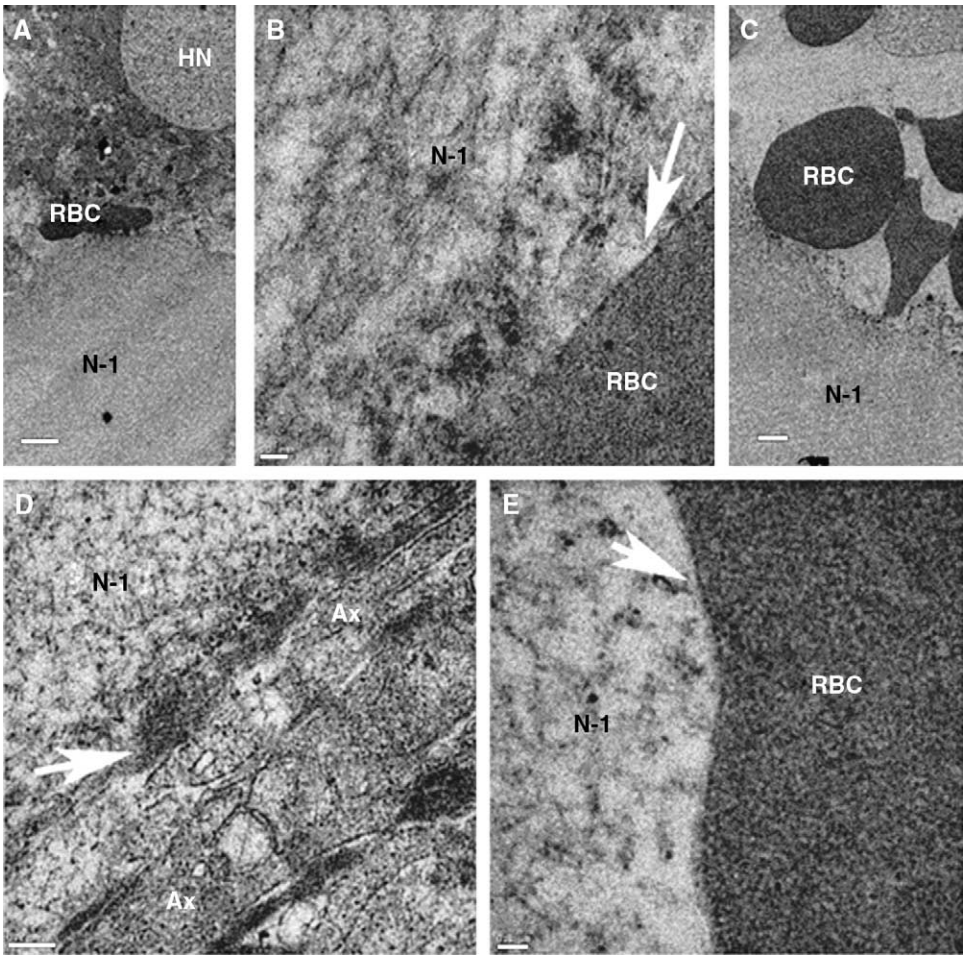

Fig 5. Electron micrographs. This series of TEM images shows the interactions of NHS-1 with liver, cortex, and red blood cells. **A**, The left lateral lobe was treated with NHS-1, and the tissue was taken shortly after treatment. Note the hepatocyte and its nucleus (HN). There is a red blood cell (RBC) between the assembled NHS-1 (N-1) fields. Scale bar represents 2 μm. **B**, A closer look at the interface of the RBC and the material. Scale bar represents 50 nm. **C**, In the liver the RBC do not appear to mix with the NHS-1. Scale bar represents 1 μm. **D**, Application of 1% NHS-1 solution to a cut in the cortex. Note the close interface with the axons (Ax). Scale bar represents 0.2 μm. **E**, In another part of the brain the interface between the RBC and the NHS-1 appears to be similar to that in the liver. Scale bar represents 0.1 μm.

Hemostasis in a high-pressure femoral artery wound

The femoral artery of 14 adult rats was surgically exposed, transected, and then treated with 200 μL of a 1% wt/vol solution of NHS-1 or iced saline and packing (Figure 2, *E-H*). In the treated rats (n = 10) about 10 seconds elapsed before hemostasis occurred (Figure 4, *A*). The controls (n = 4) continued to bleed for more than 6 minutes. The difference in times to achieve complete hemostasis was highly significant (Student's t-test $P < .0001$).

Hemostasis in highly vascularized liver wounds

Using a group of 76 rats, we performed three different liver cuts: (1) a sagittal (rostrocaudal) cut (Figure 3, *A and B*) to test NHS-1 in an irregular-shaped laceration wound, (2) a transverse (lateral-medial) cut involving the transection of a major branch of the hepatic portal vein to intensify bleeding (Figure 3, *E-J*), and (3) 4-mm punches through the liver lobe to observe the material in uniform wounds.

In the first liver experiment we made a sagittal cut in the left lobe (n = 8); upon treatment with 100 μL of 1% wt/vol NHS-1 solution bleeding ceased in less than 10 seconds (Figure 3, *A-D* and Supplemental Video 3, "Sagittal cut of left liver lobe using 1% wt/vol self-assembling peptide treatment"). In one set of controls (n = 3) bleeding stopped 90 seconds (Figure 4, *A*) after cauterization of the wound; in the saline-treated control animals (n = 3) bleeding continued for more than 5 minutes. Comparison of the cauterized and the saline-treated controls shows a significant difference using the Tukey test with a 99% confidence interval.

In the second experiment we severed a major branch of the portal vein while making a transverse cut in the left lobe to test NHS-1 in a environment with a high flow rate. Four concentrations of NHS-1 were tested (n = 12) along with (n = 4) control animals. We applied 400 μL of 4% wt/vol concentration NHS-1, and bleeding stopped in 11 seconds (Figure 3, *E-J* and Supplemental Video 4, "Transverse cut of left liver lobe using 4% self-assembling peptide treatment").

We duplicated the test successfully with 400 μL of both 3% wt/vol and 2% NHS-1 solution; bleeding ceased in 10 and 10.3 seconds, respectively (Figure 4, *D*). When 400 μL of 1% wt/vol NHS-1 was applied, bleeding continued for more than 60 seconds (Figure 4, *D*). The controls, however, bled for more than 6 minutes. The dose response shows that treatment results using 3% and 4% NHS-1 are nearly the same as with the 2% concentration. Furthermore, in the 2%, 3%, and 4% concentration treatment cases complete hemostasis was maintained after removing the excess assembled NHS-1 material (Figure 3, *I* and *J*). We found that the higher blood pressure/flow rate transverse liver cut required a concentration of 2% wt/vol NHS-1 or higher to bring about complete hemostasis in less than 15 seconds. A significant difference was found between the NHS-1–treated and control groups using analysis of variance (ANOVA). When each treatment group was compared to the control group those differences were also significant; a Tukey test showed a 99% confidence interval. There was no significant difference when the various NHS-1 concentrations were compared, except for the 1% NHS-1 solution treatment group.

In the third experiment using adult rats ($n = 45$) we punched 4-mm holes through the left lateral lobe and then treated the area with 3% wt/vol NHS-1, saline, or heat cautery to bring about hemostasis (Figure 4, *B*). In the experimental group ($n = 15$) we applied a solution of 3% NHS-1 after injury and hemostasis was achieved in about 10 seconds, whereas the saline controls ($n = 15$) required 3.5 minutes to stop bleeding. In the heat cautery control group ($n = 15$) cessation of bleeding took more than 60 seconds, inclusive of applying heat to cauterize the inside surface of the punch. We allowed the NHS-1–treated animals to survive for as long as 6 months with no detrimental effect on the tissues. Using ANOVA there was a significant difference between the 3% NHS-1 treatment and the controls ($P < .0001$). In addition, the Tukey test showed that each group was significantly different from the other with a 99% confidence interval.

Hemostasis in skin punch biopsies

Six 4-mm punch biopsies were made on the backs of each of 23 anesthetized adult nude mice for a total of 138 punches. Three punches were treated with 1% wt/vol NHS-1 solution and the other three were left untreated, except for dabbing with cotton every 15 seconds until bleeding stopped. Punched wounds that bled for less than 10 seconds were excluded from the experiment. We applied a solution of 1% wt/vol NHS-1 10 seconds after injury ($n = 23$), and hemostasis took less than 10 seconds; the controls ($n = 23$) continued to bleed for more than 60 seconds (Figure 4, *C*). The bleeding times were averaged for each side of the animal, and the Student's *t*-test for paired samples showed a significant difference between the treatment and control side of the animal ($P < 0.0001$).

Comparison of three different materials

To learn more about the hemostatic properties and mechanism of action of NHS-1 (RADA-16), we repeated both the sagittal and transverse liver experiments, comparing them with two additional materials that are known to self-assemble and spontaneously form nanofibers: (1) RADA-12 (NHS-2), a sequence variation of NHS-1, and (2) EAK-16 (TM-3), a different sequence in the same family of self-assembling peptides used to determine if the material's length and stiffness altered its hemostatic effectiveness in bleeding models [25-31].

Making a sagittal liver cut in adult rats ($n = 9$) we applied 100 μL of 2% wt/vol NHS-2 solution to the wound, and bleeding stopped in less than 10 seconds. In the cautery controls ($n = 3$) bleeding continued for more than 90 seconds ($P < 0.0001$). Upon repetition of the experiment in adult rats ($n = 8$) using 100 μL of 2% wt/vol TM-3, the material assembled but did not achieve hemostasis; the animals continued to bleed until the experiment was terminated after more than 3 minutes.

The increased blood flow from the portal vein after making a transverse liver cut allowed us to perform another dose response experiment in which we compared various concentrations of NHS-1 (1% to 4%) and TM-3 (1% to 3%) wt/vol with controls (Figure 4, *D*). All concentrations of NHS-1 were effective; however, the higher blood pressure and flow rate after the transverse liver cut required a concentration of 2% or higher of NHS-1 to stop bleeding in less than 15 seconds.

TM-3 is a stiffer gel; 1% wt/vol TM-3 is similar in stiffness to 3% NHS-1. We tried three different concentration levels (1%, 2%, and 3%) and found that TM-3 was not effective at any concentration; the assembled material fractured and the TM-3–treated animals continued to bleed regardless of the concentration used. There was actually no significant difference between TM-3 and the controls (Figure 4, *D*) in achieving hemostasis.

Interface of NHS-1 and tissues

Still looking for mechanism clues as well as further understanding of the relationship of the NHS-1 blood/tissue interface in both the brain and liver, we also examined the treated tissues using transmission electron microscopy (TEM), interested in learning how the red blood cells (RBCs), platelets, tissue, and the ECM interact with the material.

We applied 1% wt/vol NHS-1 to a liver wound and immediately harvested the tissue. In the electron micrograph the hepatocyte and RBC looks to be intact with the assembled NHS-1 at the interface (Figure 5, *A*). When applied shortly after injury, the material appeared to stop the movement of blood from the vessels without detrimental effects to the liver's RBCs; there was also no evidence of lysing (Figure 5, *B*). Furthermore, there was no evidence of platelet aggregation [32] at the blood/NHS-1 interface (Figure 5, *C*) when samples were taken at various time points after treatment.

In the brain we found a very tight interaction between NHS-1 and the neural tissue (Figure 5, *D*). We observed no RBCs and no evidence of platelet aggregation in the assembled NHS-1. The RBCs that were present appeared

intact at the edges of the assembled NHS-1 with no evidence of lysing (Figure 5, *E*). Furthermore, no evidence of thrombi was observed in the brain, lung, or liver of the animals treated with NHS-1 and NHS-2.

Discussion

Our study demonstrates that hemostasis can be achieved in less than 15 seconds in multiple tissues as well as a variety of different wounds. This is the first time that nanotechnology has been used to stop bleeding in a surgical setting for animal models and seems to demonstrate a new class of hemostatic agent that does not rely on heat, pressure, platelet activation, adhesion, or desiccation to stop bleeding. NHS-1 and NHS-2 are synthetic, biodegradable [10,19] and do not contain any blood products, collagens, or biological contaminants that may be present in human- or animal-derived hemostatic agents such as fibrin glue [1,8,10,14,20]. They can be applied directly onto, or into, a wound without the concern that the material may expand, thus reducing the risk of secondary tissue damage as well as the problems caused by constricted blood flow. In our previous brain studies [21] we looked for evidence of the production of prion-like substances or fibril tangles in animals that had the material implanted in their brain for as long as 6 months but found none. Furthermore, the breakdown products of NHS-1 are amino acids, which can be used by the body as tissue building blocks for the repair of the injury [21]. Independent third-party testing of the material found no pyrogenicity, which has been found with some other hemostatic agents, and no systemic coagulation or other safety issues in animals [33].

The exact mechanism for the hemostasis reported here is not fully understood, but we have uncovered several clues. First, we know that the hemostasis is not explainable by clotting. Blood clots are produced after injury, but do not begin to form until 1 to 2 minutes have elapsed, depending upon the status and coagulation history of the patient [6,12,34].

Second, the electron micrographs show no evidence of platelet aggregation at the interface of the material and wound site. That arginine inhibits platelet aggregation suggests that the arginine in NHS-1 plays a role in this effect [4,35-37]; this seems to be consistent with our data. The NHS-1 and NHS-2 solutions appear to self-assemble into a barrier, stemming the flow of blood and facilitating the movement of adjacent cells to repair the injured site [21].

Third, in our experiments the NHS-1 and NHS-2 solutions easily filled in and conformed to the irregular shapes of the wounds before assembling, as shown in the electron micrographs. We believe this tight contact is crucial to the hemostatic action because of the size of the self-assembling peptide units. The micrographs also showed that the material does not cause the RBCs to lyse, apparently protecting them from normal degradation when exposed to the air.

Fourth, we do not believe that the hemostasis can be explained by gelation kinetics. One would think that a stiffer gel would be more effective for higher pressure bleeders; however, we found the opposite to be true. TM-3, which is from the same family of peptides as NHS-1 and NHS-2, and is the stiffest of the three self-assembling peptides tested, did not arrest bleeding; it fractured at the tissue interface and within the resultant gel. We surmise that TM-3 may have fractured because of (1) the pulsations of the liver and (2) the inability of the material to flex with the tissue as blood pumped through the organ. This is similar to the fracturing of an artery when grown in a laminar flow environment and then transplanted to a pulsed environment. The cells line up along the direction of flow, unlike the natural helical coil [38-41] seen in a pulsed environment, which allows for expansion and contraction, without splitting, as blood moves though the artery. Conversely, NHS-1 and NHS-2 were able to flex with the tissue.

Finally, NHS-2, the most pliable of the three materials, seemed to perform identically to NHS-1, probably as a result of their similar structure and modulus.

With this discovery the ability to speedily achieve hemostasis will reduce radically the quantity of blood needed during surgery of the future. As much as 50% of surgical time can be spent packing wounds to reduce or control bleeding. The NHS solutions may represent a step change in technology and could revolutionize bleeding control during surgery and trauma; however, they still require clinical testing before they can be used in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nano.2006.08.001.

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