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Nano hemostat solution: immediate hemostasis at the nanoscale

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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 in less than 15 seconds 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 ligation 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 51 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 cautery 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% 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...
opening the dura mater, we performed a right hemisection using a ceramic knife. Immediately after the cord hemisection, dorsolateral laminectomy in anesthetized adult rats [22,23]. After exposing the liver surface, we performed a right hemisection transversely (Figure 3, D). With a 27-gauge needle, 400 μL of 1%, 2%, 3%, or 4% 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 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% NHS-1 or TM-3 solution was applied to the site of injury (Figure 3, H).

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% 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.

Rats were anesthetized and placed on their backs, 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% 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. Cautery 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% NHS-1 or TM-3 solution was applied to the site of injury (Figure 3, H).
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 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; blocks were postfixed in 4°C 1% osmium tetroxide for 2 hours and then washed in buffer three times for 10 minutes each at 192°C. The sample blocks were dehydrated in ethanol, infiltrated, and embedded in pure epon with Lynx EM 194 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.

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 cautery 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 had ceased 15 minutes after NHS-1 treatment. Scale bars represent 1 mm.

Preparation of the self-assembling solutions

The NHS-1 solution was prepared using RADA16-I 200 synthetic dry powder (obtained from the Massachusetts Institute of Technology Center for Cancer Research 202 Biopolymers Laboratory, Cambridge, MA; the Zhang 203 laboratory, and 3-DMatrix, Cambridge, MA) dissolved in 204 an Eppendorf tube. The 1% NHS-1 solution was prepared by dissolving 10 mg of RADA16-I powder in 1 mL of 205 autoclaved Milli-Q water (Millipore Corp., Billerica, MA), sonicated for as long as 5 minutes, and filtered. This was 208 repeated with 20 mg/mL, 30 mg/mL, and 40 mg/mL to 209 produce 2%, 3%, and 4% concentrations. NHS-2 and TM-3 210 dry powders (made by the Massachusetts Institute of 211 Technology Center for Cancer Research Biopolymers Laboratory, Cambridge, MA) were prepared using the same 212 method. The time of preparation did not affect the action of 213 the solution. We also tested some material that was prepared 214
216(obtained from the Zhang laboratory) and stored in solution 217at room temperature, for 3 years before use, and it 218performed as well as the newly mixed material.

219Results

220Hemostasis in a brain injury

221We began our experiments in the brain, removing the 222overlying skull and performing a complete transection of a 223branch of the superior sagittal sinus in the brain of rats ($n = 22415$) and hamsters ($n = 15$) (Figure 1, A). The areas were 225treated with 20 μL of a 1% solution of RADA16-I (NHS-1) 226self-assembling solution or with iced saline. In the groups 227Q13227 treated with NHS-1 hemostasis was achieved in less than 10 228seconds in both hamsters and rats (Figure 2, A–D and 229Q14229 Supplemental Video 1, “Hemostasis in rat cortex with self- 230assembling peptide treatment”). Control group hamsters 231($n = 5$) and rats ($n = 5$) irrigated with saline bled for more 232than 3 minutes (Figure 4, A). A truncated iced-saline control 233and subsequent treatment with NHS-1 is shown in 233 234Supplemental Video 2 (“Saline control and treatment with 235Q14229 self-assembling peptide in rat cortex.”) Student’s $t$-test for 236two independent samples in both hamsters and rats showed 237highly significant differences ($P < .0001$).

**Hemostasis in a spinal cord injury**

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Because blood has been shown to be toxic in neural tissue 239[24] we wanted to know if the spinal environment was 240different from the brain. By quickly bringing bleeding under 241control secondary damage caused by surgery can be reduced. 242After laminectomy and removal of the dura, the spinal cord 243was hemisected at T2, from the dorsal to ventral aspect, 244and treated ($n = 5$) with 20 μL of 1% NHS-1. Hemostasis 245was achieved in just over 10 seconds. In the saline controls 246($n = 5$) bleeding continued for as long as 5 minutes. 247Comparison of the treated group and the saline controls 248shows a significant difference using the Tukey test with a 24999% confidence interval.
Hemostasis in a high-pressure femoral artery wound

The femoral artery of 14 adult rats was surgically exposed, transacted, and then treated with 200 lL of a 1% 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 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 268 left lobe (n = 8); upon treatment with 100 lL of 1% NHS-1 269 solution bleeding ceased in less than 10 seconds (Figure 3, 270 A-D and Supplemental Video 3, “Sagittal cut of left liver lobe 271 using 1% self-assembling peptide treatment”). In one 272 set of controls (n = 3) bleeding stopped 90 seconds 273 (Figure 4, A) after cauterization of the wound; in the 274 saline-treated control animals (n = 3) bleeding continued 275 for more than 5 minutes. Comparison of the cauterized and 276 the saline-treated controls shows a significant difference 277 using the Tukey test with a 99% confidence interval.

In the second experiment we severed a major branch of the 279 portal vein while making a transverse cut in the left lobe to 280 test NHS-1 in an environment with a high flow rate. Four 281 concentrations of NHS-1 were tested (n = 12) along with 282 (n = 4) control animals. We applied 400 lL of 4% NHS-1 283 concentration NHS-1, and bleeding stopped in 11 seconds 284 (Figure 3, E-J and Supplemental Video 4, “Transverse cut of 285 left liver lobe using 4% self-assembling peptide treatment”). 286
Six 4-mm punch biopsies were made on the backs of each animal and hemostasis was achieved in about 10 seconds (Figure 4, D). The controls, however, bled for more than 6 minutes. The dose response shows that treatment with 3% NHS-1 and 4% NHS-1 is nearly the same as with 2% NHS-1. Furthermore, in the 2%, 3%, and 4% NHS-1 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% NHS-1 or higher to bring about complete hemostasis in less than 15 seconds. A significant difference between the NHS-1–treated and control groups was found 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% NHS-1. We applied 1% NHS-1 to a liver wound and immediately assembled peptides used to determine if the material appeared to stop the movement of blood from the liver interface (Figure 5, A). We observed no evidence of lysing (Figure 5, B). We applied 100 µL of 2% TM-3, the material assembled but did not achieve hemostasis; the animals continued to bleed until the experiment was terminated after 3 minutes. Making a sagittal liver cut in adult rats (n = 9) we applied 100 µL of 2% NHS-2 solution to the wound, and bled for more than 10 seconds. In the controls the difference between TM-3 and the controls (n = 15) we found that 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. The controls, however, bled for more than 6 minutes. The dose response experiment in which we compared various concentrations of NHS-1 (1% to 4%) and TM-3 (1% to 3%) with controls (Figure 4, D). All concentrations of NHS-1 are effective; however, the higher blood pressure and flow rate after the transverse liver cut required a concentration of 3% NHS-1. We tried three different concentration levels of NHS-1 (1% to 4%) and TM-3 (1% to 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% NHS-1 to a liver wound and immediately harvested the tissue. In the electron micrograph the hepato-383 cyte and RBC looks to be intact with the assembled NHS-1 at the blood/NHS-1 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 harvested at various time points after treatment.

In the brain we found a very tight interaction between the NHS-1 and the neural tissue (Figure 5, D). We observed no RBCs and no evidence of platelet aggregation in the area.
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 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 460 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 through 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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References

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