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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, vasoconstricion, 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 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% 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...
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 underlying 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% 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 saline treatment. The animals were allowed to survive for as long as 8 weeks as part of another experiment.

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 were created 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.
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. 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 200 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 autoclaved Milli-Q water (Millipore Corp., Billerica, MA), 207 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 213 method. The time of preparation did not affect the action of 214 the solution. We also tested some material that was prepared 215

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 using 3% and 11.0 ± 1.0 using 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.
Q15

Figs 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 (B), and 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. 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 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, and 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.

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 RAD16-I (NHS-1) 226self-assembling solution or with iced saline. In the groups Q13227treated with NHS-1 hemostasis was achieved in less than 10 228seconds in both hamsters and rats (Figure 2, A–D and Q14229Supplemental 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 235Q16 self-assembling peptide in rat cortex.”) Student’s t-test for 235 236two independent samples in both hamsters and rats showed 236 highly significant differences (P < .0001).

Hemostasis in a spinal cord injury

237Because 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.
251 Hemostasis in a high-pressure femoral artery wound

The femoral artery of 14 adult rats was surgically exposed, transacted, and then treated with 200 \( \mu \)L 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's \( t \)-test \( P < .0001 \)).

In the first liver experiment we made a sagittal cut in the 268 left lobe (\( n = 8 \)); upon treatment with 100 \( \mu \)L 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 using 1% self-assembling peptide treatment”). In one 272 set of controls (\( n = 3 \)) bleeding stopped 90 seconds after 273 cauterization of the wound; in the saline-treated control animals (\( n = 3 \)) bleeding continued for more than 5 minutes. Comparison of the cauterized and 276 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 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 saline-treated control animals (\( n = 4 \)) bleeding continued 283 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.

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In the third experiment using adult rats (308), we repeated the treatment and control side of the animal (309) for paired samples showed a significant difference between the treatment and control. We applied a solution of 1% NHS-1 was applied, and the controls (310) bled for less than 10 seconds. In the cauterized 311 experiment. We applied a solution of 1% NHS-1 to stop bleeding in less than 10 seconds. In the cautery 312 treated the area with 3% NHS-1 was applied, bleeding continued for more than 90 seconds (313). The controls, however, bled for more than 6 minutes. The dose response shows that treatment 314 concentration treatment cases complete hemostasis was 315 maintained after removing the excess assembled NHS-1 316 material (317). We found that the higher blood 317 pressure/flow rate transverse liver cut required a concentra- 318 tion of 2% NHS-1 or higher to bring about complete 319 hemostasis in less than 15 seconds. A significant difference 320 was found between the NHS-1–treated and control groups 321 using analysis of variance (ANOVA). When each treatment 322 group was compared to the control group those differences 323 were also significant; a Tukey test showed a 99% confidence 324 interval. There was no significant difference when the 325 various NHS-1 concentrations were compared, except for 326 the 1% NHS-1 solution treatment group.

In the third experiment using adult rats (308) we 327 punched 4-mm holes through the left lateral lobe and then 328 treated the area with 3% NHS-1, saline, or heat cautery to 329 bring about hemostasis (330). In the experimental 331 group (332) we applied a solution of 3% NHS-1 after 333 injury and hemostasis was achieved in about 10 seconds, 334 whereas the saline controls (335) required 3.5 minutes to 336 stop bleeding. In the heat cautery control group (337) 338 cessation of bleeding took more than 60 seconds, inclusive of 339 applying heat to cauterize the inside surface of the punch. We 340 allowed the NHS-1–treated animals to survive for as long as 6 341 months with no detrimental effect on the tissues. Using 342 ANOVA there was a significant difference between the 3% 343 NHS-1 treatment and the controls (P < .0001). In addition, 344 at 15 sec the Tukey test showed that each group was significantly 345 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 326 of 23 anesthetized adult nude mice for a total of 138 punches. 327 Three punches were treated with 1% NHS-1 solution and the 328 other three were left untreated, except for dabbing with cotton 329 every 15 seconds until bleeding stopped. Punched wounds 330 that bled for less than 10 seconds were excluded from the 331 experiment. We applied a solution of 1% NHS-1 10 seconds 332 after injury (n = 23), and hemostasis took less than 333 10 seconds; the controls (n = 23) continued to bleed for 334 more than 60 seconds (Figure 4, C). The bleeding times were 335 averaged for each side of the animal, and the Student’s t-test 336 for paired samples showed a significant difference between 337 the treatment and control side of the animal (P < .0001).

Comparison of three different materials

To learn more about the hemostatic properties and 338 mechanism of action of NHS-1 (RADA-16), we repeated both the sagittal and transverse liver experiments, comparing 341 them with two additional materials that are known to self-assemble and spontaneously form nanofibers: (1) RADA-12 342 (NHS-2), a sequence variation of NHS-1, and (2) EAK-16 343 (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 344 bleeding models [25-31].

Making a sagittal liver cut in adult rats (n = 9) we applied 345 100 μL of 2% NHS-2 solution to the wound, and bleeding stopped in less than 10 seconds. In the cautery group (n = 3) controls continued to bleed for more than 352 seconds (P < .0001). Upon repetition of the experiment 353 in adult rats (n = 8) using 100 μL of 2% TM-3, the material 354 assembled but did not achieve hemostasis; the animals 355 continued to bleed until the experiment was terminated after 356 more than 3 minutes.

The increased blood flow from the portal vein after 358 making a transverse liver cut allowed us to perform another 359 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 (360) were effective; however, the higher blood pressure and flow 361 rate after the transverse liver cut required a concentration of 362 2% or higher of NHS-1 to stop bleeding in less than 365 15 seconds.

TM-3 is a stiffer gel; 1% TM-3 is similar in stiffness to 363 3% NHS-1. We tried three different concentration levels 364 (1%, 2%, and 3%) and found that TM-3 was not effective at any concentration; the assembled material fractured and the 370 TM-3–treated animals continued to bleed regardless of the 371 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 hepatocytes and RBCs look 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 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 the NHS-1 and the neural tissue (Figure 5, D). We observed no RBCs and no evidence of platelet aggregation in the 394 assembled NHS-1. The RBCs that were present appeared 395
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 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 469 hemostasis will reduce radically the quantity of blood needed 470 during surgery of the future. As much as 50% of surgical time 471 can be spent packing wounds to reduce or control bleeding. The NHS solutions may represent a step change in 473 technology and could revolutionize bleeding control during 474 surgery and trauma; however, they still require clinical testing 475 before they can be used in humans.

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