Nanoscale Technologies: Nano-knitting, Healing Powers and Hemostasis

Rutledge Ellis-Behnke, PhD
Massachusetts Institute of Technology
Cambridge, MA

LEARNING OBJECTIVES

1. The difference between self-assembled and polymerized and the ramification in a wound.
2. How does the body react to non-assembled peptides and assembled peptide materials? Where do they go?
3. How these nano materials mimic how the body develops, stops bleeding and allows for healing.

CME QUESTIONS

1. Will self-assembled peptides cause an immune response?
2. How do self-assembled peptide materials differ from polymerized materials? Can self-assembled peptides also polymerize?
3. How does pH affect the local environment? What distance will the pH cause problems? Nanometer, micron, millimeter centimeter?

KEYWORDS

1. Blood
2. Eye
3. Sclera
4. Liver
5. Nanotechnology

INTRODUCTION

The intersection of nanotechnology and healthcare is rapidly taking center stage and the fruits of this marriage are on the visible horizon. While nano biomedicine has led to wildly futuristic promises, it has also presented real breakthroughs in drug research, development and formulation, as well as in the field of diagnostics. Two very different breakthroughs are explored: (1) the use of nanotechnology to repair the brain and show return of function; (2) the creation of a nano hemostatic agent that promptly stops bleeding, while also providing a clear visual operating field during surgery. Both developments represent significant nanobiomedical advances that hold great promise in treating human conditions in the foreseeable future.

There are several formidable barriers that must be overcome to achieve axonal regeneration after injury in the central nervous system (CNS), whether caused by a knife or a stroke. These obstacles are: (1) scar tissue formation after tissue injury; (2) gaps in nervous tissue formed during phagocytosis of dying cells after injury; (3) factors that inhibit axon growth in the mature mammalian CNS; and (4) failure of many adult neurons to initiate axonal extension.

We are now able to demonstrate the creation of a permissive environment for axonal regrowth using a synthetic biological nanomaterial that self assembles *in vivo*, with components that break down into beneficial building blocks and produce no adverse effects on the CNS. This discovery, by reducing or overcoming the first two obstacles and possibly more, allows for the reconnection of disconnected parts of the CNS after trauma[1].

SELF-ASSEMBLING PEPTIDE NANOFIBER SCAFFOLD (SAPNS)

The previously undiscovered treatment in this study used a designed self-assembling peptide that spontaneously forms nanofibers, creating a scaffold-like tissue-bridging structure that we found to provide a framework for partial reinnervation by axons with regenerative potential in young and adult animals. Because the peptide fibers are nanoscale, there is likely a direct interaction between the peptide scaffold, the extracellular matrix, and the neural tissue on both sides of the lesion. These structures create a scaffold that connects the two faces of the lesion, allowing movement of cells into the scaffold. The peptide scaffold in our experiments created a permissive environment for axonal growth while discouraging or preventing the scar formation that normally occurs at an early stage. This material appears to offer a treatment for ameliorating or bypassing tissue disruptions after neuronal damage.

SAPNSs are synthetic biological materials formed through the assembly of ionic self-complementary peptides and are designed by using alternating positive and negative L-amino acids that form highly hydrated scaffolds in the presence of physiological-concentration salts, i.e., saline, tissue culture media, physiological solutions, or human body fluids such as cerebrospinal fluid. The scaffold consists of β-sheet ionic peptide containing 50% charged residues. A number of additional self-assembling peptides have been designed, synthesized, and characterized for salt-facilitated matrix formation. The SAPNS consists of

interwoven nanofibers, and the individual fibers are 10 nm in diameter[1].

NANOFIBER DENSITY CORRELATES WITH PEPTIDE SOLUTION CONCENTRATION

This designed peptide nanofiber scaffold provides several benefits over currently available polymer biomaterials: (1) The peptide scaffold forms a network of nanofibers that are similar in scale to the native extracellular matrix and therefore provides an *in vivo* environment for cell growth, migration, and differentiation; (2) it can be broken down into natural L-amino acids and potentially used by the surrounding tissue, because the majority of the material is excreted in the urine; (3) it is synthetic and free of chemical and biological contaminants that typically are present in animal-derived biomaterials such as many collagens; and (4) it appears to be immunologically inert, thus avoiding the problem of neural tissue rejection[1].

All of these attributes make it very attractive for using the peptide nanofiber scaffold in both *in vitro* and *in vivo* studies. Our previous studies show that the SAPNS can support the attachment of a variety of mammalian primary cells in tissue culture. Additionally, one of the peptide scaffolds, arginine, alanine, aspartate, and alanine (RADA)16-I supports not only the growth of PC12 cells but also the formation of functional synapses *in vitro* using rat primary hippocampal neurons. Thus, RADA-I supports a wide range of neuronal growth and development using both *in vitro* and *in situ* cell culture systems.

A tissue gap caused by deep transection of the optic tract in the hamster midbrain and injection of saline can completely block reinnervation of the superior colliculus (SC) by the retina even at young ages [postnatal days (P) when the axons typically have more regenerative potential. Saline was used, because it is the standard irrigant for most neurosurgical procedures and is considered to be benign in the brain. Before the use of the SAPNS, we demonstrated substantial recovery of visual-orienting behavior in hamsters using a peripheral nerve optic tract bridge model. In this model, the optic tract was completely severed at the brachium of the SC, and the reconnection of the optic tract was accomplished with several surgically implanted segments of sciatic nerve taken from one of the animals' legs. However, the use of this model often results in leg disabilities in experimental animals[1].

In an attempt to facilitate optic tract regeneration with restoration of function, without additional clinical complications, we asked whether the SAPNS could create a permissive environment for regeneration in the damaged tissues as a substitute for sciatic nerve grafts. We examined both short- and long-term effects of injecting a peptide scaffold into the wound site in both young and adult animals using this model. Here we report that the SAPNS not only permitted significant axonal growth through the site of the treated lesion, partially restoring the optic tract, but also resulted in the return of functional vision in brachium transected experimental adult animals. We show that use of this biological nanofiber scaffold is an effective approach to facilitate the reconstruction of a continuous tissue substrate after CNS injury[1].

REGENERATED AXONS REVERSE BLINDNESS

We showed that the treatment with SAPNS solution enabled reconnection of brain tissue after acute injury that resulted in recovery of vision. The scaffold knitted together tissue in the mammalian CNS in both young and adult animals. Before the SAPNS, no treatment we tried created a permissive environment for axonal regeneration that allowed growth through the center of a lesion. These experiments show that, with a single SAPNS solution treatment at the site of injury, it is possible to overcome a major barrier to CNS regeneration in the optic tract of hamsters[1].

Our previous work using the optic tract transection peripheral nerve bridge model showed that a minimum level of 42% of normal local axonal innervation density in the target area is required for return of functional vision. In two adult animals of the present study, one at 30- and another at 45-day survival, there was evidence of axons in the SC, but the innervations density reached 20% of normal density and, in agreement with the earlier study, failed to restore functional vision. Because the SAPNS is highly hydrated, with 99% water content, it can fill an irregular void before assembly and then assemble to form the molecular nanofiber scaffold. This *in situ* self-assembly property may be critical, because most other materials do not conform to irregular voids created by injuries. This kind of intimate contact between nanofibers and the extracellular matrix may be critical to facilitate cell-scaffold interaction, thus encouraging brain injury healing[1].

We have shown that axons grew across the lesion site in 100% of the SAPNS-treated cases in both young and old animals, and that visually guided behavior was found in 75% of the adult animals receiving the SAPNS. The percentage would have been higher, except the vascular injury during surgery caused the SC to almost completely disappear in two adults. When the behavioral testing results for orienting are examined, there is a trend of improved responses as the testing progresses over time.

The SAPNS is nontoxic, and the degradation products, L-amino acids, could potentially be used by nearby cells for growth and repair. Coupled with the ability of the material to facilitate neural tissue reconstruction within the first 24 hours after injury, peptide scaffolds offer a promising alternative to autografts of peripheral nerve or other tissues for recovery from CNS injury. We also found, in each case where the peptide scaffold solution was injected in the brain, that there was no apparent inflammatory response as seen in the control cases, which resulted in considerable cell death and a tissue gap[1].

The SAPNS is nontoxic, and the degradation products, L-amino acids, could potentially be used by nearby cells

for growth and repair. Coupled with the ability of the material to facilitate neural tissue reconstruction within the first 24 hours after injury, peptide scaffolds offer a promising alternative to autografts of peripheral nerve or other tissues for recovery from CNS injury.

We also found, in each case where the peptide scaffold solution was injected in the brain, that there was no apparent inflammatory response as seen in the control cases, which resulted in considerable cell death and a tissue gap[1].

It is not clear exactly how the SAPNS promotes the closing of neural tissue gaps enabling axon regrowth. It is likely that it interacts at the nanoscale level with the extracellular matrix on both sides of the lesion. It is plausible that the SAPNS may promote cell migration into the lesion area, which creates a growth-permissive environment enabling axons to grow through the treated lesion site. It is also possible that it brings the two sides of the lesion together through a contractile process. By using the designed SAPNS solution, we have facilitated CNS healing and have overcome some of the barriers to CNS axon regeneration previously thought to be nearly impenetrable. This allowed functional recovery demonstrated by a return of lost vision[1].

Using nanobiomedical technology and molecular self-assembly to repair brain structures opens up a new field and a new source of hope for efficacious treatment of CNS trauma. This successful outcome gives us a glimpse of what reconstructive brain surgery may hold for the future.

USING NANOTECHNOLOGY TO STOP BLEEDING IN LESS THAN 15 SECONDS

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, 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. Chemical agents are those that change the clotting activity of the blood or act as vasoconstrictors, such as thromboxane A2, which causes vessels to contract thus reducing blood flow and promoting clotting. Thermal devices commonly involve cauterization using electrodes, lasers, or heat. There are also agents that react exothermically upon application that may create an effect similar to a standard two probe cautery device. Mechanical methods use pressure or ligature to slow the blood flow. A combination therapy might use both chemical and mechanical means to produce a hemostat that adsorbs fluid and swells, 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.

LIMITATIONS OF CURRENT HEMOSTATIC AGENTS

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, second, some liquid agents, such as cyanoacrylates, require a dry environment to be effective; 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; fourth, some agents have a short shelf-life and very specific handling requirements; and finally, many currently used hemostats are difficult to use in uncontrolled environments. 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. 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.

We 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[2].

RAPID HEMOSTASIS ACHIEVED IN MULTIPLE TISSUES

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. Nanohemostat-1 (NHS-1) and NHS-2 are synthetic, biodegradable 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. 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[2].

In our previous brain studies 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. 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[1].

The exact mechanism for the hemostasis reported here is not fully understood, but we know that the hemostasis is not explainable by clotting, or platelet aggregation, or 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.

The ability to speedily achieve hemostasis will radically reduce 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.

CME ANSWERS

1. It depends on the sequence. Some sequences are vasoactive and some are immunogenic.
2. Polymerized materials will form covalent bonds and can be very difficult to breakdown in the body. They can also form hard barriers that will not allow for migration of cells. Yes, some of them can polymerize and form very stable bonds.
3. pH can cause signalling of receptors and activation of the PAF system which can cause diffuse pain. The distance the pH acts is usually in the nanometer range; however, the effects can spread to centimetres. The change in pH can also cause materials to assemble differently.

REFERENCES

1. Ellis-Behnke, R. G., Liang, Y.-X. , You, S.-W. et al. Nano neuro knitting: peptide nanofiber scaffold for brain repair and axon regeneration with functional return of vision, Proc Nat Acad Sci U S. 103: 5054-5059 (2006).
2. Ellis-Behnke, R. G., Liang, Y.-X., Tay, D. K. C. et al. Nano hemostat solution: immediate hemostasis at the nanoscale. Nanomedicine: Nanotechnology, Biology, and Medicine. 2: 207-15 (2006).