

## **Nanotechnology and Regenerative Medicine**

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Mark Ratner, when he started this talk, this morning, this afternoon, he said, he asked, why nanotechnology. I'm going to start this afternoon by asking, why regenerative medicine? And regenerative medicine I believe is the greatest scientific challenge that we face today. It will remain that way for awhile, the century. It is a challenge that brings physical sciences, life sciences, engineering disciplines and, of course, clinical medicine together toward a common goal. It is really something that could push interdisciplinary science, which in my view is the most interesting type of science, to its limits. It is therefore, a great target for nanotechnology. Now, why is this important, well it is important, because people are living longer now, and so there are two problems, right? One is we live too long, so it's impractical to keep too many dysfunctional humans around, the economy would not be able to sustain that. Secondly, we live longer, and that also means that we demand a higher quality of life all the time. We don't want to be around in a highly unhappy dysfunctional state. So, humans really care about that, and this would be a great source of new businesses, and we will rejuvenate the economy by thinking about all the products that are going to be necessary to do regenerative medicine. It is, actually a good thing, instead of swallowing these weird organic molecules that we swallow everyday to correct all problems of dysfunction, perhaps we should think about regenerating tissues and organs, and reducing the number of molecules that we have to swallow in order to stay happy. So, therefore, we view this as a very important target, very important objective. Now, what are some of the targets that we could think about, and you might ask, you know, why is it or what is it going to take for us to be able to prevent or to reverse paralysis? Lots of people are confined to wheelchairs all their lives, so what is it really going to take? What type of science, what type of technologies are going to be necessary to do that? Or, perhaps to reverse or prevent blindness, return vision to human beings that have lost it, what is that really going to take? We cannot do it right now. People have heart attacks everyday, and after the heart attack there is a piece of your heart which becomes a dysfunctional scar, so you're not exactly the same individual you were before you had the heart attack. We all know that we want to cure Parkinson's disease and Alzheimer's disease, and all the other neurodegenerative diseases, all these are regenerative medicine problems. Strokes happen every day, we want to minimize the dysfunction from strokes, some of it is really devastating to many human beings. We have to cure diabetes in this century somehow, because it causes too many problems, and it would be great if people could have new cartilage and if, unless there are too many individuals here that are not 18 years old, or that are younger than 18, basically all of you are unable to re-grow cartilage, and tiny pieces of cartilage missing from your knee cause a lot of pain. And people are willing to pay lots of money to get rid of that pain. We need to be able to learn how to repair any bone fracture, it would be great if people could die with their own teeth, because nobody dies with their own teeth. People die with plastic, metal and ceramic in their mouths. So, there is lots of value in being able to do that. All

of these are regenerative targets, and if we want to tackle this problem, and we want to think about nanotechnology and where does it fit in, the first thing you will have to think about, is to think about the extracellular matrix, you saw this slide in Milan's talk, so this artist's rendition of an extracellular matrix is extremely, is a very important image for us to think of how nanotechnology can make a difference in regenerative medicine. Cells are hugged by all kinds of nanostructures, in particular, one-dimensional nanostructures, things that are very, very long and have, however, other dimensions that are very, very small. So just, in that magic regime of the, that Mark talked about in the 10-100 nanometer-scale, but they could be microns in length, and these nanostructures, they hug the cells, and these can send signals to them and they control these downstream events that make cells do what they do, proliferate or differentiate and build matrix and so on. So that's what we have to think about. And so, the question here is, can we reconstruct an extracellular matrix that is artificial, with nanostructures, because we know that extracellular matrices are indeed made up of nanostructures. It is at that scale that the cell receives the signals. And can we do that? So, what do we need? Well, we need basically, bioactive, organic, nanostructures, and they need to have sizes in the range or 1-100 nanometers. Ordinary chemistry doesn't do this, so you need a special kind of chemistry that connects to nanotechnology spirit, which Mark also talked about, self-assembly, and so we think the best synthesis of organic nanostructures in the nanotechnology range is to program molecules for self-assembly, and then create supramolecular structures. So, you make, maybe, a cylindrical object, like the one that is present in extracellular matrix, by thinking of a tiny molecule that then self-assembles with identical, or not so identical copies of the same molecule to build a supramolecular structure, a million or a billion molecules coming together into a nanostructure. So, we have lots of capabilities in this area today. I want to show you an example from my lab, which is very recent, in fact, even unpublished right now, which shows that we can make something that looks like a double helix, this is a SEM image of a double helix, which is basically made up of these special molecules we have designed and programmed to make twisted structures. They have been programmed to do that. This is not serendipity; they have been programmed to do it. And the pitch of this twisted structure we know now, very recently that we can basically dial it in through the chemistry. So, this is how, these are the kinds of capabilities that we will need to build an artificial extracellular matrix. So, we, a few years ago, started along this pathway by thinking about a molecule that could, or a set of molecules that could self-assemble into a cylindrical nanostructure, a nanofiber, with diameters in the order of just a few nanometers, again, in the magic nanotechnology range. And this was to emulate the architecture, not the composition, but the architecture of collagen fibrils, which are perhaps the most common components of extracellular matrix. The idea was, that when these molecules would self assemble into this nanostructure, then maybe we could put information right here on the surface, and remember nanostructures are surface rich objects, they are, that's their key property, lots of surface relative to their volume. And so, you could load a huge amount of information here in these nanostructures for cells to do things. In, and of course, because they, this happens by self-assembly, you can imagine taking a soup of molecules, and then integrate signals. So, artificial matrices for regeneration, what they should do, in my view, is they should manage early cell behavior, that's all they are

supposed to do, just a couple of weeks, three weeks. And in fact, what they should do, if you go down here and read, they should disappear when their function is complete, they should leave no trace. So, that's a design constraint, a chemical design constraint, and what we mean by managing early cell behavior, means they should somehow manage the proliferation of cells, the migration of cells, they should prevent apoptosis of cells. They need to, we need to have a feeding, effective feeding of cells, we need recruitment of cells, we need angiogenesis, without angiogenesis, of course, it's impossible to keep a mass of cells in an artificial matrix alive. And the "holy grail" of course, is that we need to somehow know how the artificial extracellular matrix loaded with nanostructures can mediate differentiation of the cells. And this matrix will provide then a set of integrated or perhaps even amplified signals. This is an artificial world. It's not biology, of course we have to follow biology to know what signals we need to provide, but we're going to do this in an abiotic way, and we're going to do it, in perhaps a way where we can integrate signals, sets of signals or amplified signals that biology doesn't particularly do. We also, want to program the delivery of the signals to the cells. This is very important, so you need to know, for example, if ten growth factors are needed for this event, and they need to come in according to new things we learn about systems biology, they need to come in, in a certain program, then we need to be able to, with chemistry, with nanotechnology deliver this program by design. So, this is really challenging to a chemist, challenging to material sciences, and so for that reason alone, also it is a great, great, great challenge. So, I will give you an example of how this works. We designed molecules, and this is the first molecule, Mark mentioned it in his lecture, this is a set of molecules, this happens to be the first one, but it now has turned into hundreds of molecules. They are called peptide amphiphiles, so we thought that what we should do is make a soap, that has a peptide, and of course, when we have an infinite number of permutations here to present signals and to give information. Peptides, amino acids are the language of biology, so you better design the nanostructures with peptides; otherwise they're not going to do very much. And then, there is a hydrophobic tail here, which can be that simple alkyl tail that you see here, but it could be more complex too. They're covalently linked right here, so this is a soap, its hydrophobic part, and it's more hydrophilic part. So, where is the challenge here? Remember, I said we want to make a cylindrical structure, with lots of surface area, so the challenge is that this molecule or the threaded molecules, regardless of what you, what amino acids you put here, let's say, in its terminal region. They need to make a cylinder, and that's not so simple, that is fundamental supramolecular chemistry. And so basically, we designed the system so that the molecules, these peptide amphiphiles will always be charged, always be soluble in water, because we are thinking about medicine, medicine you are going to need to inject something, perhaps it needs to be in water, so they need to be soluble and the idea is that once this charge is neutralized or is screened, then what we are interested in is in triggering the self-assembly of these molecules into this nanocylinder. And note, the peptide here, it's coming in to this nanostructure and it is perpendicular to the long axis of the fibers, so it is spatially in a disposition that is very convenient to signal the cell. If you were trying to do this with normal polymers, which are dominated by entropy, would be very difficult to control that spatial orientation, so you need an object that has a defined shape. The hydrophobic core, right here, is of course, composed of those hydrophobic tails and they go there,

because once the charge disappears, then the peptides are thermodynamically driven now to form hydrogen bonds, and the hydrophobic tails want to hide from the water, so everybody wants to form the cylinder, and if you put a soup of molecules of ten of them, they will want to form the cylinder as well. So, we have the nanofibers, they are very long, and they tend to bundle together, they are about six to eight nanometers in diameter and you look at the slide, and you think, well this looks like some kind of extracellular matrix, maybe some collagen fibrils aggregating together. So, you, one of the main properties of these molecules, that we've designed is that, you can have enormous diversity and structure as you see here in this gallery of peptide amphiphiles, but they all form the nanocylinders and so, there are some key sequences in there, which allow us to always gravitate to the cylinder, but then we can present, here we can put whatever chemistry is necessary to present to the cells, so for example, that peptide, that Milan was talking about, that consensus sequence, we could put it there and then display it on the surface of the nanostructure. And so, in the lab, what you see is the following, you have here a solution of the peptide amphiphile 1% by weight, this is in water, and so it's perfectly soluble. And I'm going to basically, eliminate the charge here, by putting some HCl vapor from the top, because the molecules are negatively charged, and so when that happens I'm going to trigger self-assembly and you see self-assembly is happening here. The nanofibers are forming and this solution becomes a gel. If you put ammonium hydroxide and reinstate the charge the gel becomes now a liquid again, and so it goes back and forth as many times as you want. So this is the spirit of self-assembly, right, you would design something, you now change something, in this case the charge and you trigger self-assembly, you make nanostructure, and the nanostructures together make a gel. Now, why do they make a gel microscopically? Well, they make a gel microscopically, because the very thin nanofibers that are forming they're bundling together, and they are making a network in the water until the water gels, and so the system is immobilized. Now think about what that means for medicine, you could have an injection, which is water based, you would inject it in the heart, or somewhere in the brain, and then what will happen is the nanofibers will form, and that solution will become a gel, so they won't escape. Whatever you put in there, the information you're putting in there stays there, and that's because of the nanofibers self-assembly. If you look in the microscope at the gel, it may look something like this. So, these are nanostructures, one dimensional, bundling together, forming a network and if you were to think about how to use this medicine, and I told you that you needed to manage the charge in order to trigger the self-assembly, well, one way to manage charge is not by putting HCl vapor, like protons, you know you wouldn't do that to humans very easily. Make them breathe HCl so that it self-assembles, no. What you would do is you would screen the charge, and the best way to screen the charge is to use our own natural electrolytes, so you see here on the slide, you started the same solution, you mix it with basically, cells and their media. This is just a suspension of cells and cell culture medium, you mix the two and the electrolytes in the cell culture medium trigger self-assembly in a matter of seconds, sometimes minutes, but often in seconds and you have a nanofiber network forming the gel, and the cells that used to be here, now are trapped in three dimensions by the nanostructures. This gel right here, is 99% by weight water, and only 1% by weight stuff, and the stuff is the nanostructures. The cells live there for weeks, you can basically feed them through the

water, provide nutrients to them, and they can proliferate, they can differentiate and do everything you want in the lab. Okay, so let me illustrate with the problems of spinal cord injury, how this could actually be used. Spinal cord injury, a devastating problem, one of the great targets of regenerative medicine, how to prevent or how to minimize spinal cord injury and paralysis? So, when you have spinal cord injury, as this histological slide suggests, lots of things happen, of course, axons get injured, some of them get severed, compressed and many things like that happen, but also a scar forms, and that scar is elaborated by the astrocytes, which are cells of the central nervous system, in fact. So, one of the things that we know now, and I will explain in a minute how we found this out, is that we can make nanofibers that actually suppress astrocytes, and so we wonder can this make a difference in the spinal cord injury problem. The glial scar basically blocks axons from reconnecting and then repairing the damage that has occurred, typically by compressive injury. Okay, so the idea, when as follows here is the design, so this is how a nanotechnologist works. Laminin, laminin-2, interesting protein, right, and this protein is known to interact with neurons. In laminin there is an epitope of five amino acids, IKVAV, which binds to alpha 6 beta 1 integrins, and it is known in the literature, that this interaction between the IKVAV epitope and the integrins, causes neurite extension, neurite sprouting. So, we designed, basically a nanofiber, which has this epitope on the terminal part of the peptide, so that IKVAV signal is displayed perpendicular to the axis of the fiber by these objects when they self assemble. One of the interesting things here, if you think about it, is when this fiber forms, it's packing at Van der Waals density, basically, these signals. Its nanostructure form, its nanostructure nature allows you to pack about  $10^{15}$  of these biological signals per square centimeter. If you make a gel of these nanofibers, they're roughly  $10^{15}$  signals per square centimeter of gel that can potentially impact the cells. Remember, this IKVAV epitope is known a neurite sprouting, or neurite guiding epitope is the one present in laminin. So, what we did in the first experiments is we, basically, encapsulated neuroprogenitor cells in a gel, that's what this bubble, this pink bubble is. We took them from the brains of embryonic mice and there were progenitor cells that had not yet differentiated into their final lineage, and they are surrounded by the fibers. We originally were interested in IKVAV, is because we said, well in spinal cord injury, maybe this will help with neurite extension, axon elongation, and this will help with repair. But, we were deeply surprised to find that those progenitor cells, in our nanofiber network, basically, start to become neurons after 24 hours or so, and for the first time in this kind of experiment, we observed, that astrocytes did not develop out of that population of neuroprogenitor cells. So, we were pretty excited about that, and of course, this connects to the glial scar because the glial scar, which blocks neurite outgrowth, after spinal cord injury, is elaborated by the astrocyte, and here we have a set of nanostructures that not only can elongate axons and neurites, but can also block the development of astrocytes from any progenitor cells that might be floating around the spinal cord. So, we have actually already tested this *in vivo*, this is a collaboration with my colleague Jack Kessler, in our neurology department, and we have a model, where one is a rat model, the other one is a mouse model, and basically, what we have found, and I will show you a slide of this in a minute, measuring the triple B score, which measures recovery from spinal cord injury, as a function of time, we find that those animals, that are receiving injections in the spinal cord from our soap solution, which

then forms nanofibers in the spinal cord, they seem to recover better from spinal cord injury, than the controls, and of course, this is very exciting, but I was first excited just to know that you could inject this liquid, and not kill the animals, because I was afraid of that, but so, the animals did not die. Secondly, also, we found that the injured animals, they survive socially always when the self assembly fluid is injected into their spinal cord, where as 15-30% of the injured, untreated animals, they die from the injury. So, there is a survival improvement from the injury itself, and then there is an accelerated recovery from the spinal cord injury. So, I'm going to show you what it means to have a triple B score of seven. So, you can understand the difference. This is an injured animal, and he is dragging his hind legs, he's paralyzed, so this would be a triple B score of seven. He's really paralyzed. Now, typical of the animals that get injected with our self assembling molecule, and forms these nanofibers in the spinal cord, we get easily into the range of 9, and 10, and 11 and so forth. Here is what 9 gives you, you see he is no longer dragging his hind legs; he's actually using his hind legs at a triple B score of 9. This is at least superficially speaking, one of the things that nanotechnology can do in this problem, somehow we are finding that the recovery is better, and it occurs, in fact, even protects animals from dying as a result of the injury, by just injecting a synthetic molecule that makes nanostructures in the spinal cord. We're not doing self therapies here. We're just doing nanotechnology, that's all we're doing. We have also a stroke model that is under development right now, also in collaboration with Jack Kessler and we've had a positive results from injecting the fluid in the brains of animals that have had a stroke, they recover faster and better from stroke, and in this slide what you see is an animal hanging from a rope, you measure the number of seconds the animal hangs to the rope as a way of scoring the recovery from stroke, if you stay longer you're better. This is, you know, pretty exciting, in both problems, the stroke model and the spinal cord injury we are, basically, looking at neuron repair, neuron regeneration, that's what we are looking at. It is being done through the same system. We have other models going on and one of them has to do with the use of these nanostructures to repair the skeleton, and so in that case we design a nanofiber, as you see here, that this nanofiber has biological epitopes of for example, RGV epitopes, to recruit cells, but also it has amino acids in there that can nucleate crystals of hydroxyapatite of the shape and the crystallographic orientation that you find in natural bone. This in fact, the combination of the epitopes here, and the crystals on top of the fibers, we believe can be a very powerful signal osteogenic signal to grow bone. So, if we make a defect, which is 5 mm long in an animal in the femur, and pack these nanostructures in mostly packing water here, then we find that after four weeks, this defect begins to be filled by bone, as shown by micro CT here. Whereas, the unfilled defect remains fairly empty. And in fact, this model of cutting out 5 mm of femur is supposed to be a non-healing defect. So, we have found that these nanofibers of the variety they grow a bone and recruit cells through cell adhesion epitopes are able to, within four weeks start growing bone in the defect. Well, the platform, this nanotechnology platform, can go many places from here, and so, just to give you some idea, of all the other ideas you can integrate to it, we are now thinking that this surface can be designed to adsorb proteins. So, this will definitely multiplex the bioactivity there, they can have, we can put specific proteins here, protein 1 and then put another protein on that surface and then signal through the epitopes here and then deliver in

special programs, these proteins. Now one of the ideas that a chemist would think about, is why not try to dial in the binding constant of the protein by changing the substrate? And that's in fact what we do, we also adsorb other molecules here, that are not proteins, but that can activate proteins as well. In the interior, which is hydrophobic, we can put some of those weird molecules that I talked about in the beginning, that are made by organic chemistry, to deliver drugs. So, for example, you could imagine putting drugs here, in this nanostructure, if endocytosed by a cell, you could first of all, target it through this protein, this may be an antibody for example. The fiber could be endocytosed, and in fact, we already know that these fibers are easily endocytosed and then the cargo can be released inside the cell, which carries therapeutic agents. So, we are already know also, that we can, basically, co-assemble molecules into these nanostructures, in this example you see that we have mixed two molecules, one short and one long, and we know from NMR, from two-dimensional NMR, we know that when these two molecules co-assemble this way, and are aligned this way, so what that means is that we can grow hairs on the nanostructures, we can grow extensions on the nanostructures this way, in this particular example, the hair, which is shown here, the part that sticks out, happens to be a peptide that binds to BMP2, a very important growth factor, for bone growth and also very important in neuron development. So this platform can be used to mix molecules and this way increase the bioactivity of the system. Using, in fact, and this is coming to the end of my talk, using in fact, all of these ideas we have very recently shown that we can engineer a system for angiogenesis. And angiogenesis of course, is a general target for whenever you want to do regenerative medicine you have to be concerned about angiogenesis. So, what you are looking at here, is this is a rat cornea assay, so I believe there is some connection to vision here, so I'm happy for that. We have the rat cornea assay, which is very common to test angiogenic potential. We make a tiny defect here in the center, and introduce our self-assembling nanostructures and what you see here is at 7-10 days later, after that has been placed in the cornea, we see a profuse development of blood vessels here, and we see just a few here, in this periphery, where we have, basically, introduced right there, the same stuff that goes here, except that the matrix, except that the material that is holding everything that's in the soup, in this case, is just taking collagen off the shelf, and then putting a bioactive molecule that you need for angiogenesis, like the growth factors, and here you put the same growth factors and activators of proteins, but you put them in a nano design network. And we design this network to the tee. We designed it to do everything, or at least some of the things that we know from basic biology that are needed to grow blood vessels. I think the picture speaks for itself, of the very pronounced difference in the ability of two materials to trigger the formation of blood vessels. In this case, the type of material that's there is the material that someone in nanotechnology would think about. In here, the material that's there, is the material that material scientists and other people have been thinking about for many years, as materials that could be used to perhaps grow tissues, or to substitute for tissues, in this case it happens to be ecology. So this, what's new here is that the nanostructures in there, and they are designed, and they are planned. At the nano scale to interact with proteins in a certain way, or with the activators of proteins in a certain way, that's what's new. As Mark says, that's what all the fuss is about. So, that's the fuss. Okay, this process that you just saw, we want to use it to regenerate

heart, that's the reason we are so interested in it. I mean it's important in general, but particularly in heart regeneration. And so, what we are thinking is that we're going to maybe through a catheter, we're going to carry this liquid, which has all the instructions to come together into nanostructures and then jump start the formation of blood vessels, exactly in the region of the infarct, where it is needed, and so that's why this is called liquid heart matrix, because you see it's going to go enter that way, we've already started these experiments, and we're very excited about them. Okay so, the vision is for regenerative medicine, the example I have shown you this afternoon is the use of liquids, you see, the use of liquids, which are mostly water, in fact, but they have some highly designed molecules, that somehow, when they go to tissues, they make the liquid become gel, but they make the liquid become a gel that interacts rationally with the biological environment, at the nano-scale, and that's what's different from things we did before. Thank you Richard for inviting me here today, and many thanks to all of you for your kind attention.