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SUPRAMOLECULAR ENGINEERING STRATEGIES OF BIOMATERIALS

Professor Alvaro Mata

Professor in Biomedical Engineering & Biomaterials and Director of the Institute of Bioengineering, Queen Mary University of London

Thank you, Daniel, for that kind introduction and thank you to the organizers for giving me the opportunity to be part of your community. I was really excited to be here because I have not been before, and I really appreciate the focus this conference on quite fundamental wisdom such as 'standing on the shoulders of giants'. It is a very inspiring picture and I thought it was also appropriate if I look at some of the giants that we are trying to learn from.

Jean-Pierre Sauvage, Sir J. Fraser Stoddart and Bernard L. Feringa won the Nobel Prize in Chemistry in 2016 'For design and synthesis of molecular machines'. They are setting a path for the next generation of materials, showing how we move away from equilibrium materials to create and define dynamic structures that can provide energy, that will feed back, or that will be self-healing. I don't know if you are familiar with the salamander. You can cut off a limb and it grows another identical limb in an amazing process. So while we are starting to move into dynamic materials, there is still a long way to go.

I want to show you some of the steps that we are taking towards this goal; how we have been working from the bottom up using materials designed at the molecular scale using primarily proteins as well as peptides. You don't have to use the whole molecule, you can use small pieces that are known to elicit specific biologic responses and you can begin to build and organize them to create structure and function.

The first example, that I will discuss to introduce this concept is peptide amphiphiles (PAs), developed by Sam Stupp of Northwestern University. These are small molecules with four regions. One region is a carbon chain that is highly hydrophobic. Then you have a region that promotes beta-sheet interactions, where you take amino acids that promote beta sheet folding and you can introduce it into this molecule. Then you have a spacer and a bioactive sequence, such as

RGDS for cell adhesion or IKVAV for stimulating neuronal regeneration or differentiation. So you now have an amphiphilic molecule that is hydrophobic on one end, hydrophilic on the other, which you have basically designed like Lego out of building blocks.

You can dissolve these in water because they are charged, with the charge separating and stabilizing them. Then if you screen the charge the molecules will agglomerate into a structure with the hydrophobic ends inside hiding from the water outside, which will then form into a sheet structure due to the beta-sheet building block, before the sheets finally collapse into nanofibres with the hydrophilic end on the outside. This is very important if you are trying to create biomaterials to signal cells. With the library of peptides that is available, you can create a nanofibre structure with all the bioactivity on the surface of the fibre.

I am going to show you an example application for this material for bone repair. Bone has inorganic and organic components. You want to promote mineralization and you want to attract cells. As long as you have the same hydrophobic and beta-sheet segments, you can have assemble nanofibres with different bioactive ends. So we created a molecule that has an RGDS for cell adhesion, co-assembled with a molecule that has a phosphorylated serine, which promotes calcium phosphate binding and mineralization. These will rapidly self-assemble into a network of nanofibre so you can inject it as liquid and it will go from a liquid to a gel very quickly. This will be minimally invasive.

We tested this on bone defects in animals and showed that a month after injection that the nanofibre gel has bridged the gap and has formed significant amounts of new bone, with the bone healing in the normal way. You can create this material with molecular resolution so, for example, you can control the concentration of RGDS or the concentration of phosphoserine and you can tune the bioactivity. One of the big disadvantages of these materials still is that they are very weak.

Next, I want to tell you now about how we use molecular disorder to overcome this weakness. It has recently been discovered that the traditional paradigm of how proteins work, of having a defined structure that creates a particular function, is not entirely correct. There is an increasing literature showing proteins have more disorder than originally anticipated and this is an exciting new area, which is really opening new opportunities. As well as the conventional structures of alpha helixes and beta sheets, proteins also have a disordered, dynamic moving part, and there is more and more evidence that this disordered region plays a critical role in their functionality. We also have observed completely disordered proteins, which only acquires the specific order and functionality when it starts binding to other molecules.

So I would like to talk now about our work with a particular protein, an elastin-like protein (ELP) that we have combined with PAs to generate interesting

properties. ELPs are recombinant, which means genetically that they are made by bacteria, but are completely disordered in nature. They are called elastin-like because they are made by a repetition of a pentapeptide with an amino acid in one of the positions. Due to this sequence ELPs can change conformation from an extended open conformation below a particular temperature to a collapsed structure above it. In our work, using an ELP molecule developed by our collaborator Carlos Rodriguez Cabello in Valladolid, we use a segment of statherin, which is a protein forming in saliva, which binds calcium phosphate and helps maintain healthy enamel. This creates a long chain that has statherin segments that will bind calcium phosphate and that can be opened or closed. We used this to make little membranes, which look like a contact lens, with micron scale thickness, which is quite strong due to the elastin and is porous from the microfabrication techniques used. When we added stem cells on the membrane surface then they turned into osteoblasts, without any other signal, and at a faster rate than the standard control. We have used this membrane in-vitro on holes in rat craniums and saw that the fracture was beginning to heal after about a month.

When we put a similar but modified membrane in a calcium phosphate solution, you get hydroxyapatite nanocrystals that nucleate in the centre of the membrane and then spread when they reach the surface. This flat-ended morphology is typical of nano-crystals growing in an environment, where they are being directed by an organic matrix. The organic matrix is guides the growth and maintains this organization. These nanocrystals have a 10–20 nanometre coating of the organic matrix. After they nucleate, the organic matrix begins to template them and they grow towards the surface of the membrane because of the concentration gradient of the calcium phosphate from the outside to the inside. Once they reach the surface they begin to spread and they acquire a hierarchal, prismatic structure. The nanocrystals and prisms are about 50 nanometres and 5 micrometres in diameter, respectively. These prisms are made of high-density nanocrystals, with a crater in the centre of the structure, which over an 8-day period in solution will colonize the whole membrane and completely turn this organic matrix into a hierarchical hydroxyapatite membrane. If the membrane is less than 10 microns thick, the whole membrane becomes hydroxyapatite with 10 to 20 nanometre organic coating, so the organic matrix is still there.

We have been exploring using this to help repair enamel. Enamel is the outside of your teeth, which is extremely strong and resistant due to a well-defined hierarchical structure, with a highly-aligned prismatic structure of hydroxyapatite nanocrystals. Our structure has a similar organization and so we wanted to see if we can actually create this coating on teeth and this has now been demonstrated at the laboratory scale.

Now, I would like to discuss our work combining these ELPs with the PAs, which I discussed previously. The PAs are small molecules that self-assemble

into fibres while the ELPs are big molecules that can open and close. The PAs self-assemble when you screen the charges, by adding a molecule of opposite charge to neutralize and they collapse because of the hydrophobicity, which means that a solution of an ELP that is negative, and a solution of the PA that is positive, when combined will assemble and interface immediately into a membrane. If you inject a drop of PA solution in ELP then an ELP/PA membrane will form at the interface. This will start as a closed sphere but can open up into a tube.

The membrane forms a diffusion barrier which can continue to expand as molecules diffuse through it. So you go up from a spherical balloon or a sack to an open to an open tube. So similarly when you have a bubble of soap and you touch it with a wet surface, the bubble of soap opens up and seals the interface. So as long as you touch the right surface in the right environment, you will create openings in that structure that not only open but seal the air–water and water–plastic interfaces. The membrane always grows to about 20–30 microns in thickness, is limited by diffusion, and has multilayers made by PA nanofibres surrounded ELP, made by diffusion of the PA through the membrane. The mechanical properties can be controlled through the PA and ELP concentrations, where some membranes are more rigid while others are more flexible. The tube grows because you have molecules on both sides of the interface. Lower concentrations will limit the tube size. If there is sufficient concentration, the tube can be extended and the molecule will diffuse through and assemble at the interface. If we continuously inject one component on the inside and then we can grow them indefinitely.

We call this behaviour material morphogenesis because depending on where you touch it you can grow branching tubes, you can pick it up and you can stretch it. There is no cross-linking going on here; the strength comes only from the hierarchical assembly of two molecules. This material still weak but has more structural integrity than with PAs alone, and the surfaces can be colonized by cells.

I will finish by talking about the mechanism. Basically, you have an ELP and a PA site. As soon as you bring them together, you create a diffusion barrier, they co-assemble and you have this dense, thin layer that serves as a diffusion barrier for the PA to begin to diffuse, but the PAs are changing the conformation of the ELP which was being assembled above its collapse temperature, and now it is opening it up against its will. So, you are creating this internal stresses that will lead to instabilities in the membrane. Depending on the PA you can control the opening of the membrane. As the PAs diffuse through the membrane they begin to bind ELP molecules, which then begin to open up. The more PAs that bind, the more the ELP molecule opens up, which then opens up more areas for interaction with PAs, producing layers with different ELP–PA

concentrations and therefore different ELP opening. The less open structure will be more rigid.

To summarise, when you combine PAs and ELPs, you can change the conformation of them and assemble them in different ways. We are exploiting molecular disorder to create different orders, different structures and different properties.

I would like to finish by thanking all our lab members and our collaborators.

Transcription of Discussion

DISCUSSION CONTRIBUTIONS

Chair: Daniel Söderberg

FRC Chairman and KTH Royal Institute of Technology

Gil Garnier Monash University

Fascinating work. As you were speaking, I was thinking about the similarity and the complementarity of nanocellulose. Here with your proteins you have the primary, secondary and tertiary structure. We do have the same with cellular fibres, which is basically the nanocellulose fibres and crystals made of glucose units chained into cellular and soon on. However, what seems to be an issue for you is the strength; your composite is very weak, however, nanocellulose is very strong and could also gel. Have you considered complexing your protein with nanocellulose crystal to use glycoprotein, or basically to make a composite with cellulose crystals grafted with proteins?

Alvara Mata

Yes, we would love to do it. We haven't done, but I am sure we will be able to create different things. I don't know exactly what, but I can tell you we are now playing with other molecules other than the ELP, and they are also making different things. We are changing the formation in different ways and they are making different things, so we haven't, but I am sure there will be potentially.

Gil Garnier

Just to be specific, the dimension of your cylinder, and your rolls are exactly the same dimension as the nanocellulose crystal, so you can gain a lot of time to start with a nanocellulose crystal and functionalize its surface to assemble it with proteins.

Alvara Mata

Okay.

Discussion

Gil Garnier

So, very interesting.

Daniel Söderberg KTH Royal Institute of Technology

When you look at these growing disks, what is the timescale for growth?

Alvara Mata

So, the ones which were completely covering the membrane, it was about 8 days, but we start seeing them just visually after 4 or 5 hours. You can start seeing something starting to appear. It seems to be quite fast and then depending on the maintaining of the pH, we can about almost double the time, the speed, if you maintain the pH. It is days to completely mineralize the membrane.

Daniel Söderberg

Is there an effect of the temperature?

Alvara Mata

Yes, it is, but the problem we have with that is that the ELP's temperature sensitivity has a transition temperature, so we have not been able to control, or do systematic studies based on the temperature because if you do, you then completely change the confirmation of the ELP if you change the temperature. But what I can tell you is, we also know that depending on the concentration of the starting peptide, we can nucleate more points, and if you nucleate more points, you don't need so much time to be able to coat the membrane because now you have more volcanoes appearing and they don't need to grow as far to meet each other.

Daniel Söderberg

So, being a fibre network society, I think we are a part of here. When you talked about multilayers and you talked about fibres growing, what are the dimensions of these fibres and concentration of fibres.

Alvara Mata

The fibres of the PA are about 8 nm; single PA is about 4 nm in length, so a fibre will be 8 microns in length. We don't see any major difference when you have an ELP. Is that your question?

Daniel Söderberg

No, the question was when you form these, you call them gels or interfaces. What are the …

Alvara Mata

So, you mean when we start growing them?

Daniel Söderberg

Yes.

Alvara Mata

Yes, it depends on what you touch it with. If you touch it with a small tip, you will get a small tube. If you touch with a big one, you will get a bigger tube. But we have gone down to about 800 microns reproducibly at the moment and reproducibly from basically those videos they look nice, but you did not see the student very carefully pulling things, because we are doing it manually for now, but we think if we will be able to do it automatically, we will be able to go down in sizes and pull smaller tubes.

Daniel Söderberg

So, when you have this interface with fibres, what is the mechanics for controlling the strength of this? Is it a network?

Mata

The strength of the membrane is making a tube.

Daniel Söderberg

Is it the network?

Alvara Mata

It is a network. So, it is a tube that is made by multilayers and the multilayers are made by nanofibres and the nanofibres are made by PA and ELP. Now, the assembly which I did not have a lot of time to go through is basically PAs going

and diffusing, and when they diffuse they recruit ELP molecules which then turn into layers. Depending on the PA sequence, we think we can enhance the stiffness, but we have not quantified the stiffness yet. So we would just qualitatively see any changes, but it is still weak. In the video at least I wanted to show something to give you an idea. You can pick it up, you can stretch it a little bit, but you break it and I am very interested in starting to look for other molecules. Even if we don't do it, I think there is a lot of possibility of this concept of modulating conformation in this case using other molecules as core assembly systems.

Gil Garnier Monash University

Are the assembling protein systems pH and ionic strength dependent? Basically can you do in vivo growth, or you are limited to *in vitro*?

Alvara Mata

Yes. At the moment we can only do *in vitro*. It depends on pH, but we are using physiological conditions. At the moment, the growth of the tubes… we can only do it in water. If we have an ionic solution, it messes up the core assembly. The growth of the mineral, we have been able to do in a physiological condition in terms of calcium concentration, pH, temperature, but they are quite sensitive to that.

Hahn-Ning Chou Thepharak

Now this is a little bit unrelated to cellulose, but I was looking at your exciting presentation and it seems like there are a lot of benefits in medical application and you briefly mentioned stem cells. Now, I was wondering if there is synergistic effect and what is the medical community's reaction to your research.

Alvara Mata

Sorry, you mean synergistic effect of what?

Hahn-Ning Chou

With stem cell application, or the usage of the stem cells in maybe bone regeneration.

Alvara Mata

Yes. All of our lab is really focused on biomaterials for medical applications, the beauty of it is that the functionality of the stem cells signaling that we can provide can be engineered into the molecule and I basically just showed RGDS; so select cells adhering throughout, but the idea is that you can have other types of functionalities to stimulate differentiation, or perhaps differentiation in stem cells can also be based on stiffness and in the mineralization project we are trying to control the stiffness of the material so there are different ways in which you could stimulate stem cells. Does that answer your question?

Hahn-Ning Chou

Yes, I was just thinking, because a lot of the medical research is now trying to use the injection of stem cells to accomplish what you have talked about at least …

Alvara Mata

Yes.

Hahn-Ning Chou

… and I don't know if it works together, or you have to use it as an information activator, or something.

Alvara Mata

Yes, I guess we are using the material as a stimulator for stem cells, but not yet to incorporate it as part of the mechanism of assembly.

Hahn-Ning Chou

Yes, that was my question. Thank you.

Alvara Mata

Very good … very good idea.