Published on *Up Close* (https://upclose.unimelb.edu.au)

#272: Pore me another: Understanding how toxins target and overcome membranes

VOICEOVER Welcome to Up Close, the research talk show from the University of Melbourne, Australia.

SHANE HUNTINGTON
I?m Dr Shane Huntington. Thanks for joining us. Biological membranes are fundamental to many of the life processes of all organisms from the humble amoeba to complex animals like ourselves. In the microenvironment of a living cell, membranes help to control what comes in and what stays out. The proteins that sit on or reside in membranes play a vital role by relaying signals from one cell to another and by identifying and importing materials into the cell. In fact, the neurons in our brains wouldn't fire without the coordinated activity of membrane proteins.Membranes also figure in how toxins which have evolved to target both membranes and their proteins, are able to make the bridge. How do toxins, whether produced by humble bacteria or two-metre long Taipan snakes, do their destructive work on membranes. And how do we observe and model what's happening at the membrane level. Today on Up Close we are joined by two researchers who are attempting to answer these questions by combining laboratory studies with computational modelling. Our guests are Professor Frances Separovic, Professor of Chemistry and Head of the School of Chemistry at the University of Melbourne and Professor Terry Lybrand from the Departments of Chemistry and Pharmacology and the Centre of Structural Biology at Vanderbilt University. Welcome to Up Close, Frances and Terry.

TERRY LYBRAND
Thank you.

FRANCES SEPAROVIC
Thanks, Shane.

SHANE HUNTINGTON
Terry, I'm going to start with you. We're going to be talking a lot about membranes today so I thought a good point to start would be for you to define what we mean by

the term membrane in the biological context.

TERRY LYBRAND
I guess in the simplest sense you can say it's essentially the compartmentalisation. It's the barrier that separates what's outside the membrane from what's inside. It is a lot more than just a barrier. It has many functional roles. There have to be molecules transported across this from exterior to interior, a variety of molecules embedded in this membrane that perform a number of roles sensing what's on the outside, signalling from the outside to the inside, a whole host of activities like this.

SHANE HUNTINGTON
We're talking about a physical object here, aren?t we? There's a physical barrier. What's it made of?

TERRY LYBRAND
The primary constituents are lipids - fatty acid molecules - with head groups of various sorts, different chemical functionalities. There are a host of other species in these membranes, a wide array of proteins, other small organic molecules like cholesterol, so in fact it's quite a heterogenous and complex assembly of molecules.

SHANE HUNTINGTON
When we talk about these membranes in the body, are they many and varied or do we have just one type of membrane that serves all purposes?

TERRY LYBRAND
Generally there's variation, for example, from one type of cell to another. So you will see some variation when you're in different tissues or organs, also variations amongst different species. So the kinds of membranes you would find in microorganisms like bacteria often have different characteristics from those you would find in say, mammals such as humans. It has to do with what kinds of head groups are present, what kinds of fatty acid side-chains are in these lipid molecules and even what kinds of proteins and other components are there.

SHANE HUNTINGTON
Now, Frances, we talk about these proteins in the membranes. Is this the only thing that makes them different from one another and specific or do the lipids play a role as well?

FRANCES SEPAROVIC
The lipids are very important and I noticed Terry mention cholesterol. It's one of my favourite molecules because it tends to stabilise membranes and it's also involved in how proteins function but the other thing that I find fascinating is I work also with antimicrobial peptides and bacterial membranes are very different to our membranes and one of the things they don't have cholesterol, they have different charges, different lipids and you can really target things for different types of membranes.

SHANE HUNTINGTON

Most people would be thinking cholesterol, bad. You're saying this is a key and important feature of the way our membranes work?

FRANCES SEPAROVIC
It's bad in our arteries but it's good in our membranes.

SHANE HUNTINGTON
Terry, one of the first things we learn about biological membranes is that they are selectively permeable as in some things are allowed through, other things are not. How do different molecules get from one side of the membrane to the other and how do we determine which things can get through and which can't?

TERRY LYBRAND
Right, well there's several factors that control that. Molecules that are relatively non polar sometimes are actually able to absorb into the membrane and pass across, whereas something that's very polar like a water molecule typically is not going to pass through a membrane at all. In many cases there are actual transport systems here. They're either pores which are formed by peptides and proteins that allow smaller molecules to pass through. And in many systems we also see something called an active transport system. This will usually be a protein or a collection of proteins that bind a particular molecule on the outer surface and then actively transport if across the membrane. This generally requires energy to be expended. That's normally in the form of a molecule like ATP, which gets hydrolysed to provide the driving force.

SHANE HUNTINGTON
Now, can you speak a bit about this polar nature of some molecules? What exactly does the mean?

TERRY LYBRAND
So the pore forming nature, basically these molecules, often peptides, but they may be proteins, will assemble into an arrangement that in fact creates a small passage. You can think of it a bit like a tunnel through the membrane itself. Often times these pores that get formed will have some degree of selectivity, that is in many cases not just any small molecule could pass through there easily. They will select, for example for one type of ion versus another and it's very important in cells to maintain proper balance of certain ions in the interior versus the exterior. So pores of this sort sometimes are one of the ways that's managed or maintained.

SHANE HUNTINGTON
When we try and visualise these sorts of membranes I suppose some people would have an image of a very amorphous structure where things are just lying around anywhere. Are they very specifically structures to make sure that this transport role and so forth can be done effectively or are the proteins just sitting anywhere within the membrane itself?

TERRY LYBRAND

Yes, so they're frameworks, these cells and some of these membrane proteins actually anchor to the intracellular framework and that helps hold them in relative locations. So it's not amorphous but the answer's not simple and it's not fully understood, which is part of the reason a topic like this is interesting, at least for us, to talk about.

FRANCES SEPAROVIC
?and what we neglected to say was it's a lipid bilayer, so it's two lipid layers with hydrophilic head groups on the outside and hydrophobic chains in the inside.

SHANE HUNTINGTON
So water-loving and water hating.

FRANCES SEPAROVIC
Exactly.

TERRY LYBRAND
Yeah.

SHANE HUNTINGTON
Now, these structures must, I imagine, react very quickly because as some components try to get in there'll be others that shouldn't get in that have to be dealt with within very short time frames. How does that work, Terry? I mean are these membranes able to react so quickly that they can discriminate many things trying to beach their surfaces all at once?

TERRY LYBRAND
I think probably the simplest way to think about this and this is indeed gross oversimplification, but to a first approximation membranes are designed not to allow things to facilely cross either outside to inside or vice versa. In cases where you have this selective transport systems these transporters tend to be quite selective for the appropriate molecules. Now there are cases where that does get circumvented so there are some examples in drug delivery where medicinal chemists have made drug molecules that look much like the natural passenger for that transport system and then utilised that as a strategy to get the drug molecule inside particular cells. For example, some of the CNS active agents but as a general rule the system is designed not to permit routine and easy passage. And when there is some defect or aberration in the system that often leads to problems, some kind of pathology, either for the individual cell or for a collection of cells in the tissue.

FRANCES SEPAROVIC
I also find it fascinating how anaesthetics work and in the old days remember they used to give us ether? Ether goes actually into the membrane, dissolves in the lipid molecules, fluidises and disorders the membrane and then that affects how channels work and that is related to how anaesthetics work, or these particular types of anaesthetics work.

SHANE HUNTINGTON
It's lucky that that occurs. I'm Shane Huntington and you're listening to Up Close. Our guests today are chemists Frances Separovic and Terry Lybrand and we're talking about membranes. Now Terry, you and Frances here are collaborating to better understand a particularly nasty protein, or a toxin, called equinatoxin. Can you tell us a bit about this and why you're interested in it?

TERRY LYBRAND
Well, this is an example of one of these pore-forming proteins or peptides. It's a bit different than what we've discussed so far. This is a small protein or large peptide that's generated by certain marine organisms.

FRANCES SEPAROVIC
Sea anemone.

TERRY LYBRAND
Right, so it's actually secreted. It's, if you will, a sort of chemical weapon and this is a motif you see quite frequently in nature. Organisms will produce molecules like this that they can utilise either as a defence mechanism against invaders, be those invaders bacteria or larger species or in some cases these are used for actual attack. So many marine organisms use strategies like this to disable potential prey and prevent them from swimming away, for example.In particular an anemone since it can't exactly rush after its possible prey.

SHANE HUNTINGTON
They do move pretty fast, though. Anyone with a fish tank will know, some of them can move around pretty quickly but the interesting thing is these toxins are quite problematic for us. It's interesting that they have developed that capability against well, mammals, presumably actually not originally designed to affect us, but affect other sea borne creatures.

TERRY LYBRAND
Correct, and I think in many cases the reason these molecules which were clearly probably developed to deal with normal neighbours in that environment impact us is that there are similarities between a lot of the signalling molecules, a lot of the transport systems et cetera and us, compared to these other species. So there's a lot of related biochemistry here. And in a sense, you can say that it's just unfortunate that we use a lot of the same signalling molecules or transport systems and so we likewise are subject to the potentially severe consequences if we encounter compounds like these - equinatoxin.

FRANCES SEPAROVIC
This toxin actually binds to a specific lipid, which is called sphingomyelin and it's found in our red blood cells, for example.

SHANE HUNTINGTON
There you go. Unfortunately we're all related, aren?t we, if you go back far enough?

Now, Frances, you in particular, have got this particular toxin into your lab. What are you trying to find out about this toxin?

FRANCES SEPAROVIC
I actually want to work out how it works. That's the real thing that's interesting for me. I'd like to understand how this water-soluble protein gets into the membrane, which is hydrophobic.

SHANE HUNTINGTON
I assume for that you have to go after the structure of the protein. Is that correct?

FRANCES SEPAROVIC
Yeah, the structure is the way of understanding how it works.

SHANE HUNTINGTON
How do you do that?

FRANCES SEPAROVIC
My expertise actually comes from nuclear magnetic resonance spectroscopy. So there's two main ways of doing structures in biology at the atomic level. One is X-ray crystallography where you require crystal, and then there's NMR, or nuclear magnetic resonance, where you can study things either in solution or you can use a variation of that technique, solid state NMR, and look at things in membranes, and that's what I'm trying to do.

SHANE HUNTINGTON
Now, the crystal version is relatively simple from the sort of physics perspective. You look at orientations of the crystal, you shoot X-rays through it, you look at the diffraction patterns and you can back the information. Now, when you can't make a crystal, I can't sort of visualise how you go about pulling out the same information. It seems a lot more complicated.

FRANCES SEPAROVIC
I think of NMR as being a big magnet with lots of little magnets in it. And the little magnets are the nuclei and you can work out how far away they are from one another and at what angle they are to each other and from that you can get the structure.

SHANE HUNTINGTON
We're talking about proteins here, as you mentioned, are very large molecule, essentially. Are you able to pull out that complete structure or is there some ambiguity in what the NMR tells you? I assume that many parts of the protein are replicated, repeated. There's a lot of consistent atoms in that molecule. Are you able to completely look at the structure?

FRANCES SEPAROVIC
I wish. But we're lucky because equinatoxin can crystallise so we've got the crystal

structure, which had been done by another group. We're also lucky it's water-soluble and it tumbles and we can get the structure by solution NMR. So you can compare both the crystal and the solution NMR. But then when we put it with a membrane the structure changes but we can't get enough information because the lies are so broad and that's where people like Terry can help us because we can use molecular dynamics and molecular modelling to help understand the structure from sparse data.

SHANE HUNTINGTON
Now, Terry, this is where sooner or later when these things get this complex a computational biologist usually comes into the frame to help out.

TERRY LYBRAND
Yes.

SHANE HUNTINGTON
What sort of information can you put in? It sounds like you're filling in gaps that the instrumentation just can't pick up.

TERRY LYBRAND
Well, that's one of the things we try to do and when we're lucky we can provide some useful information. So we focus on a technique called molecular dynamics. The simplest way to think of this is we're solving Newton's equations of motion but we're solving them for collections of atoms. And so we're asking how the atoms move relative to each other in response to the forces that they experience either from each other or from other portions of the environment like the molecules and atoms in the membrane, for example. So we can generate the equivalent of an animation or a movie. We actually do these calculations frame by frame and you can imagine piecing these frames together and you see an animation that depicts how to protein or the system would move as a function of time. One of the things from that we can then compare to are the kinds of signals that Frances generates in her solid state NMR experiments. We ask do the motions and behaviours we see in these simulations seem compatible with the experimental data that are generated? Ad typically, in the modelling application you would try to make many comparisons and draw many correlations between the simulated results and the experimental data to see if the two are consistent. If they are, then you can in some cases cautiously assume that what you see in the simulation is reasonable depiction of the motions and that gives you some insight into function, which is what Frances was talking about. How in fact, do these systems really work? How does the equinatoxin, for example, actually bind to the membrane and begin this process of pore formation, which is the ultimately lethal event for the unfortunately cell that's the victim.

SHANE HUNTINGTON
You mentioned there the way in which the outputs of these models are compared with Frances's data to confirm their validity. What's the input to the model, though? What data is going in?

TERRY LYBRAND

So, historically these models have been calibrated or parameterised based on systems that are very well understood and very well studies experimentally. So we have to have a mechanism to describe forces between atoms, for example, if we're going to do this sort of atomic scale Newtonian mechanics. So we obtain that either from very high-level theoretical calculations using quantum mechanics on small reference molecules, molecule that would be representative of the components of something larger like a protein or you could also adjust the mathematical models that are used to describe the forces and the notions by comparison with very well characterised experimental systems.

FRANCES SEPAROVIC
We also got some results recently, which showed that when lipid bound to the equinatoxin, certain signals disappeared and by using Terry's techniques then we could see that these were actually the signals, which were proposed to be the binding site. So that's really reassuring. So by losing information we actually gained it.

SHANE HUNTINGTON
To some degree you take snap shots of these molecules with the NMR in time. And Terry, you're modelling these in a dynamic session. So are you able to - it sounds like you are but are you able to correlate a variety of points in time with these molecules and how they interact with the membranes with the data that Frances is pulling out at those points in time?

TERRY LYBRAND
So, that's our goal and we're not always able to do this in every single project but that's the goal and the way we generate our animations or our simulations in fact are with discrete time points. So it's not a continuous dynamics that classical physics often talks about. We do it in discrete time steps. Those discrete time steps then lead to a collection of different snapshots and it's that collection that we compare to data that Frances or others generate.And what that gives us that we don't generally get directly from most of the experimental techniques, is we can interrogate exactly where all the atoms are at any given point in time, how are they moving in relation to each other, which atoms seem to play more important roles in producing a function, in this case may be binding to the membrane and yes, then hopefully we can correlate those observations with the signals that Frances sees, including the signals that Frances observes, that disappear. That tells us some discrete bits of information and so think in a way of the simulation if nothing more, at a minimum we hope that it gives us a kind of atomistic view of what's happening and what the experimental data tell us is occurring.

FRANCES SEPAROVIC
The other thing is that I find Terry can save us time and money. So when you do biological NMR you tend to use hydrogen, which is cheap but carbon-13 and nitrogen-15 are the magnetic nuclei. And the cheapest way to do this is to enrich everything but when you're doing a large protein or solid state interactions with membranes you have too many signals. So what you want to do sometimes is specifically label, and that's expensive but Terry can tell us a good place to label. By

modelling you can work out the right place to do the labelling and you can test your theory how this interacts with membranes.

SHANE HUNTINGTON
So essentially, he gives you a path of a molecule that may be of particular interest and then you can then zoom in on that particular part and monitor what's going on as it interacts with the membrane with the NMR.

FRANCES SEPAROVIC
Exactly.

SHANE HUNTINGTON
Now, I want to drag people into the environments you'd work in for a moment. Terry, if I was in your office looking over your shoulder I'm sure I'd probably see email most of the time but on the days when you actually get to do some computational work, what do these models look like?

TERRY LYBRAND
So the first thing you would notice is that not only in my office but in my labs, quote, unquote, you'd not see anything with the exception of workstations with computer monitors in the desk, so in fact, that's my laboratory. It's purely computational. The other thing you'd notice immediately is that we'd be wearing these strange looking glasses, either wired directly to the monitors or with some sort of wireless transmission, looking at what appears to be quite blurring images on the screen because with large, complicated systems like these it's really important to get a good 3D visualisation of the molecules. So we use specialised hardware that gives us an effective 3D projection of these molecules from the screen. It's not unlike the 3D movie experience but the hardware we use is rather more expensive than the glasses they pass out at the theatre so that we get a richer 3D projection on our monitors. Again, that's crucial for complicated systems like this. You really want to be able to see the spatial relationships of all the atoms in your systema and also see how those spatial relationships fluctuate over time.So that would be the environment you'd see and on a really good day when I'm focussing just on research and not on answering all of those emails, you'd see us spending quite a lot of time in front of these monitors often with a group of people looking, perhaps with Frances, visiting and we're pointing out the latest simulation we've run and what we think it suggests and then discuss whether what we're seeing in the animation or the simulation seems to be consistent with and perhaps helps explain some data that Frances had generated. So that's very much the environment we live in and work in.

SHANE HUNTINGTON
And Frances, can you describe the NMR facility for us?

FRANCES SEPAROVIC
Yeah, the NMR lab at Bio21 has nine superconducting magnets and what these actually are is big steel cans which are actually like dewars, full of liquid nitrogen and liquid helium and the liquid helium keeps the superconducting wire very, very cold

and when it's cold it doesn?t have resistance and you can put a lot of electricity through and generate really large magnetic fields, typically half a million times that of the earth's magnetic field and that's what we use to get our signals from our nuclei.

SHANE HUNTINGTON
You're listening to Up Close. I'm Shane Huntington and today my guests are chemists Terry Lybrand and Frances Separovic. We're talking about membranes and toxins and how to examine their complex structures. Frances, one of the aspects of equinatoxin that you and Terry are looking at in particular brings us back to this point of membranes that we started the interview with. What, specifically, is of interest about this toxin and the membranes that it attacks?

FRANCES SEPAROVIC
It's actually very, very potent. One of the most lytic peptides that we think about is melittin from the bee.

SHANE HUNTINGTON
What is melittin?

FRANCES SEPAROVIC
It's bee venom and it's a small peptide in bee venom that punctures holes in membranes. The equinatoxin acts at a million times less concentration than that. It acts at micrograms per kilogram in a mouse, for example. So how does this actually first of all recognise the membrane, it doesn?t attack the sea anemone. It'll attack fish, it'll attack us. How does it work? What does it do? It's made up of a protein, which has beta sheets in it and then has a little end terminus at one end and looks very much like melittin, actually. So I thought it would act like melittin. What we actually did is we made the end terminus and we found it didn't put holes in membranes at all. So then we thought we'd better study the whole protein and work out how it works.

SHANE HUNTINGTON
When you say you made it, how did you go about making this artificially?

FRANCES SEPAROVIC
Oh, you do it by sulfated peptide synthesis and I must say I exaggerated: I don't specifically make it but our colleagues synthesise it.

SHANE HUNTINGTON
So obviously there was a pretty big difference between the version that was made in the laboratory and what we're finding in nature.

FRANCES SEPAROVIC
It's the same sequence. It's the same about 32 amino acids at one end but the whole protein's 179. So obviously this part, these 32 amino acids, which are proposed to fold away from the protein, insert into the membrane and form a pore like melittin, it doesn?t work like that.

SHANE HUNTINGTON
Do we have any idea of why this particular toxin doesn?t have an impact on the species that it is derived from?

FRANCES SEPAROVIC
It's believed it's because the specie doesn?t have sphingomyelin. So that's this particular phospholipid that you find in membranes in mammals and not sea anemones anyway.

SHANE HUNTINGTON
Not sea anemones. Terry, what is the sort of end goal of this work? Is it more understanding of how these particular things work or looking towards a way to combat people having their membranes damages by these particular toxins?

TERRY LYBRAND
Well, you could imagine it's both but at least at this stage it's understanding how it works. It really is desirable to understand how something works before you move to the next step, whether that's protecting unfortunate swimmers from exposure to this or potentially utilising the information you gain from that understanding to design something new and useful. These pore formers lyse cell membranes, they have different specificities for different kinds of membranes, as Frances said. So in some cases there are people who are interested in exploring the possibility of anti-bacterial strategies. If you find those pore formers that particularly like bacterial membranes but not mammalian membranes and as Frances said earlier, the bacterial membranes have a rather different chemical composition, so there are peptides out there that prefer binding to the bacterial membranes.

SHANE HUNTINGTON
Are we able to well, bring together both the potency, as Frances suggested, relative to things like bees, which we often think of as quite a nasty sting, with some of these other alternatives that you want, like the ability to affect bacterial membranes? I mean it sounds as though if you could somehow re-engineer these to bring these two benefits together you'd have something incredibly effective.

TERRY LYBRAND
Well, there's certainly people who are interested in that possibility. To my knowledge we're not terribly close to that right now, which is one reason that Frances and I are interested in really understanding the basics here. That's my philosophy. You really need to understand how these systems function before you probably are going to have much success in trying to design something very specific and useful.

FRANCES SEPAROVIC
This was actually brought to my attention by a colleague, Ray Norton, who at the time, who was at Weihai and this toxin actually acts through cardiac arrest as well. So he was actually interested more from the viewpoint of cardiac treatment.

SHANE HUNTINGTON

It's, I suppose, one of those areas where understanding the complexity of these proteins, they have so many different organisational components with them and pulling out that data is a particular challenge and I assume that the work, the techniques, would be applicable in particular to many other toxins and complex proteins that we would come across.

TERRY LYBRAND
Absolutely, and the modelling side, for example, one of Frances's students, Daniel Weber, has spent time with me at Vanderbilt and our first task in a project like this is to ensure that we have models that reasonably represent the behaviour of these various molecules involved. So some of the unique lipids that this toxin seems to like, in particular, were not lipids that we had studies previously. So the first phase of this work is a bit boring but it's that calibration step I told you about that the underlying mathematical model we use in the simulations has to reasonably reproduce the physics and the chemistry of the molecules we're studying. Now that we've developed what we think are some reasonable models for these particular lipids, those can certainly be used in other projects that also involved these kinds of lipids. So it is transferable and useful in a variety of other settings or contexts.

FRANCES SEPAROVIC
And I think what's special about equinatoxin is that we do have a lot of knowledge about it. We have the crystal structure, we have the solution NMR structure and now we can work on interacting with membranes and develop techniques so that we can do structures of membrane proteins in membranes that are actually still functioning.

SHANE HUNTINGTON
Well, it's fascinating work and I can imagine the amazing array, not just a fundamental understanding is coming out of this, but also potential ways to respond to toxins affecting us but also using some of these tricks of these toxins to actually help us, potentially, in the future. Frances and Terry, thank you very much for being our guests on Up Close today.

TERRY LYBRAND
Thank you.

FRANCES SEPAROVIC
Thanks, Shane.

SHANE HUNTINGTON
Professor Frances Separovic is Professor of Chemistry and Head of the School of Chemistry at the University of Melbourne and Professor Terry Lybrand is from the Departments of Chemistry and Pharmacology and the Centre for Structural Biology at Vanderbilt University. If you'd like more information on this episode, visit the Up Close website, where you'll also find a full transcript. Up Close is a production of the University of Melbourne, Australia. This episode was recorded on 22 October 2013. Producers were Eric Van Bemmel and Doctor Dyani Lewis. Audio engineering by Gavin Nebauer. Up Close is created by Eric Van Bemmel and Kelvin Param. I'm

Doctor Shane Huntington, until next time, good-bye.

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