Unlocking the secrets of mutable collagenous tissue

Himadri S. Gupta, Greg Szulgit, Jingyi Mo and Maurice R. Elphick (Queen Mary University of London and the University of Manchester, UK)

The mutable collagenous tissue (MCT) of echinoderms (sea cucumbers, starfish, sea urchins) is unique because of its ability to 'switch' mechanical states rapidly and reversibly – from stiff to soft and *vice versa*. This kind of tissue in humans, for example, in skin, tendons and ligaments, does not have this property. So what are the molecular-level secrets by which MCT achieves this transformative ability? New real-time ultrastructural investigations are beginning to shed light on this question. Synchrotron X-ray measurements of dynamic molecular conformational changes point to the key factor being the gel-like matrix between the collagen fibrils. These findings could have applications for developing treatments for collagen-based disorders.

Echinoderms (e.g. starfish, sea urchins, sea cucumbers) are weird. They have pentaradial symmetry, eat in funny ways and they can 'melt' their connective tissues under, presumably, neural control. Yet, as strange as echinoderms seem, they are more closely related to us than the vast majority of other invertebrates. Does this matter? Yes. Or, more carefully stated, it might, from a medical point of view.

The pentaradial symmetry of echinoderms won't feature in *The Lancet* anytime soon, but the ability to 'melt' their connective tissues is surprisingly pertinent. Our own connective tissues are made of collagen molecules that are very similar to those of echinoderms, but we can't alter the stiffness of our tissues on such short timescales. Furthermore, echinoderms can restiffen their tissues afterward. Researchers have, therefore, dubbed these 'mutable collagenous tissues', or MCTs. The key to the phenomenon seems to be the interaction of collagen *fibrils*, which are the intermediate structures created when collagen molecules are assembled into crystal-like arrays. Given that collagen is the most abundant protein in our bodies, you might expect that we would have characterized it at every level, but the fibrillar level is still rather mysterious. This is even more surprising when you consider the number of people with Ehlers–Danlos syndrome, a group of diseases in which connective tissues lose their structural integrity. If echinoderm collagenous tissues are mutable *because of* their fibrillar interactions, then learning more about this might shed some well-needed light on Ehlers–Danlos syndrome and on human tissues in general.

Teasing out an understanding of collagen fibrils

The problem with understanding mammalian collagenous fibrils is that we can't easily isolate them for study. In this sense, echinoderms offer a rare opportunity because, when their tissues 'melt', fibrils can be teased out without damaging them. This was first done in 1973 with starfish ligaments, showing them to differ from the commonly accepted fibrillar model. Further structural and biochemical studies in the 1990s, however, began to suggest that such fibrils are probably more similar to ours than was suspected and that, perhaps, it was the accepted model that was wrong.

Now, a fresh approach to imaging is offering further insights into fibrillar interactions. Small-angle X-ray diffraction (SAXD) is being used to examine the problem, in real-time, at the nanoscale. Thus far, the data has confirmed some ideas from the 1990s, but it is also refining them. As we improve our understanding of glycoproteins and other molecules responsible for intrafibrillar binding, we should be able to observe their effects at a higher resolution than ever before.

The collagenous tissue of echinoderms is an example of what materials scientists call a fibre composite. Stiff fibres of one material (in this case, collagen) are embedded in a more ductile matrix of less structured material, these are combined with the glycoproteins mentioned earlier, as well as other, as yet unknown,

Caption

proteins. Such fibre composites are common in engineering – from carbon fibre-reinforced tennis rackets to modern 'smart' textiles that can change their conductivity based on external environmental factors. They are also, interestingly, prevalent throughout biology – from the material making up plant cell walls to the elastic tendons in vertebrates. Such composites are well known for having structural properties that are inaccessible to the individual components on their own – and which is often the reason for making them. Viewing echinoderm tissues from this standpoint led materials engineers to wonder – how do the mysterious echinoderm collagen fibrils work collectively to change the properties of the whole tissue, and is the interaction between the fibrils important?

Getting on the right wavelength

The engineering challenge in studying echinoderm tissues as composites is one of size. The fibrils in echinoderms are really small – less than one-hundredth as thick as a human hair – and so a normal light microscope won't be able to tell us how they stretch or change as the whole tissue melts or restiffens. The solution lies in using a much more intense light source and a much smaller wavelength at the same scale as the size of the fibrils – i.e. X-rays rather than visible light. The wavelength of X-rays is about a thousand times shorter than visible light. At this scale, collagen fibrils appear striped in a dark/light pattern, due to the special way in which the crystal-like array of molecules is formed. When X-rays are incident on the fibril, they scatter differently from the dark and light regions, with constructive and destructive interference leading to a diffraction pattern. This phenomenon is called small-angle X-ray diffraction (SAXD), and is similar to the method used by macromolecular crystallographers in determining protein structure, but with the key difference that the periodic units aren't individual atoms or amino acid residues but rather regions of high and low density molecular packing (~100–1,000 times larger).

Having the peaks in the SAXD diffraction pattern linked to the collagen molecular packing is great, because that means when the fibrils deform, soften or stiffen, any small changes in the packing can be detected from the SAXD peak shifts. However, echinoderms change state very fast – within a matter of seconds. Standard lab X-ray set-ups available at universities are too slow to capture these changes, because they can take up to an hour to get enough signal intensity for a single SAXD pattern. The solution lies in using a much more brilliant source of X-rays. Such X-rays are available at central facilities called synchrotrons, where electrons are accelerated to near light speed and emit highly focused X-rays as a result. If the echinoderm tissue could be subjected to mechanical stimulation while a synchrotron X-ray beam was imaging the changes in the crystalline molecular arrays, we could obtain the first hints of what might be occurring at the level of fibrils.

Following this idea, our group developed a miniature mechanical tester especially designed to hold the soft collagenous body wall tissue of sea cucumbers and to fit into the small space of a synchrotron testing chamber. A stiff or soft mechanical state was induced by using an established method of raising the K^+ ion or Ca^{2+} ion concentrations, respectively, in the artificial seawater bathing the tissue. Although these treatments are non-physiological, they appear to trigger selective activation of molecular and cellular pathways that mediate the regulation of collagenous tissue 'mutability' naturally (see

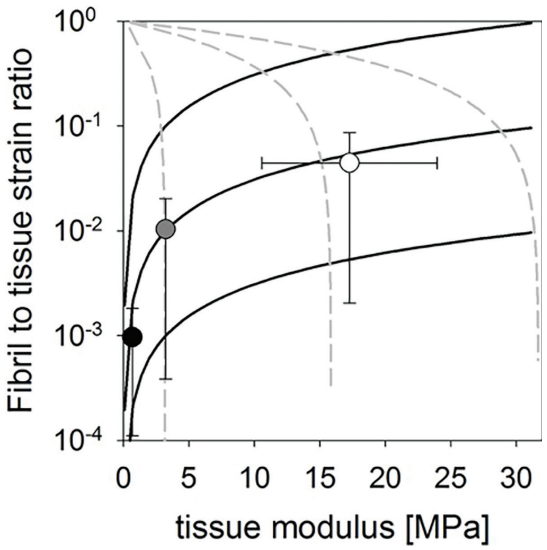


Figure 1: When sea cucumber dermis tissue is artificially stiffened or softened, the fibril strain shows a clear increase with the tissue modulus, which is the prediction of a fibre composite model where the mechanics of the interfibrillar matrix alters during a change of state (solid lines). An opposing behaviour (dashed lines) is predicted when the fibrils themselves change, and is not observed. Adapted from Mo, J. et al. (2016) PNAS.

below). Once the tissue was in one of the two different states (stiff or soft), an external mechanical strain was applied to see how the fibrils responded (via changes in the SAXD pattern), in comparison with untreated tissue.

The long and the short of collagenous tissue stiffening

We found that the fibrils in the stiffened and softened tissues behaved completely differently. In the softened case, it was as if the external stretching had no effect on the fibrils, which stayed exactly their original length and direction. In contrast, in stiffened MCT the fibrils showed a clear increase in length, up to a few per cent, as well as reorienting in line with the force much more rapidly. How could these results be explained? It was at this point that the lessons of fibre composites mentioned earlier became relevant. By treating the tissue as an assembly of the tapered collagen fibrils separated by the interfibrillar glycoprotein-rich matrix, it became possible to mathematically model both the overall mechanical response as well as the structural response of the individual fibrils. It was found that the change in the mechanical properties could be explained only if the glue-like interfibrillar matrix between the fibrils was changing state and becoming stiffer (Figure 1). Because the interfibrillar matrix acts as a transmitter of external forces between the adjacent fibrils – hence its description as a 'glue' – its overall flowability or viscosity has a huge effect on how much force is transmitted. When it is relatively stiff, quite small external strains will build up large shears in the matrix, which will then transmit these large forces to the fibrils which get stretched – exactly as observed.

Conversely, the hypothesis that the cause of echinoderm mutability lay in stiffening of individual fibrils was conclusively ruled out. Such a behaviour would result in overall tissue stiffening together with reduced fibril elongation (due to its higher stiffness). This is the exact opposite of the behaviour observed in the SAXD experiments, where increased fibril elongation was observed. Taken together, these results emphasize the importance of not treating the echinoderm collagen fibrils in isolation, but in a dynamic interaction with the surrounding glycoprotein-rich matrix. In essence, the stiffening or melting of echinoderms occurs not only because one individual phase (fibril or matrix) changes its mechanical state, but because the change in properties of the one (matrix) enables a greater stress take-up by the other (the 'passive' element of the collagen fibril). Indeed, calculations showed that because the fibril stiffness is always far larger than that of the matrix, the fibrils can take-up much larger stresses. So in a synergistic exploitation of complementary material properties, small changes in matrix properties translate into large stresses taken up by the fibrils.

Future directions

These experiments conclusively settle the question of whether echinoderm collagen fibrils, on their own, change their properties during mutability. They also show the importance of considering the fibrils *in situ* in their native environment in order to understand the mechanisms underpinning echinoderm mutability and functional changes. However, the experiments also lead naturally to the next fundamental question: *what* are the mechanisms by which the composition of the interfibrillar matrix is changed physiologically to achieve the soft and stiff mechanical states?

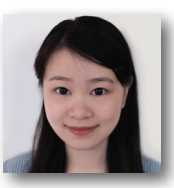
Analysis of the structure of MCT using electron microscopy has revealed the presence of presumptive effector cells, which are sometimes referred to as juxta-ligamental cells (JLCs), and the axonal processes of neurons. Thus, it is hypothesized that some nerve processes trigger activation of a subset of JLCs that release 'stiffening factors' and other nerve processes trigger activation of another subset of JLCs that release 'softening factors'. Testing this hypothesis will require detailed molecular characterization of the proteins that are expressed and released by JLCs and identification of neurotransmitters or neuromodulators secreted by the nerve processes. Some progress has been made in this regard, with identification of the proteins that cause stiffening (e.g. tensilin, NGIWYamide) or softening (e.g. softenin, holokinin) of sea cucumber body wall MCT. However, there is still much to learn, and by combining modern 'omics methods'

(transcriptomics, proteomics) with molecular and cellular anatomy and *in vitro* biomechanics combined with SAXS it may be possible to discover the mechanisms of MCT. And with these insights there lies ahead the exciting prospect of translating findings from the weird biology of echinoderms into the biomedical arena – perhaps to identify new therapies for the treatment of collagen-related disorders or to produce novel MCT-inspired 'smart' materials. ■

Himadri S. Gupta is a Senior Lecturer in Biomaterials at the School of Engineering & Materials Science at Queen Mary University of London. His research group investigates how the nanoscale structure–function relationships in hierarchical biological materials enable their biomechanical function, with experimental systems including echinoderm connective tissue, cartilage and arthropod cuticle. His group makes significant use of high brilliance synchrotron facilities, both in developing experimental probes and in ultrastructural modelling to reconstruct the architecture and dynamics of biomolecular aggregates. Email: h.gupta@qmul.ac.uk.

Greg Szulgit began studying the enigmatic connective tissues of echinoderms (MCTs) in the early 1990s, when the importance of the fibrillar structure was just becoming understood. Since then, he has broadened out into many areas, but has always retained a fondness for MCTs. He currently teaches full time at QMUL and is interested in too many things! Email: g.szulgit@qmul.ac.uk.

Maurice Elphick is Professor of Animal Physiology & Neuroscience in the School of Biological & Chemical Sciences at Queen Mary University of London. His research group is investigating the evolution and comparative physiology of neuropeptide signalling systems, focusing on echinoderms as experimental systems. He recently led a successful bid for the genome of the common European starfish Asterias rubens *to be sequenced as part of the Wellcome Trust Sanger Institute's 25 Genomes Project. Email: m.r.elphick@qmul.ac.uk.*



Jingyi Mo is currently a Research Associate at the University of Manchester, and based at the Rutherford Appleton Laboratory at Harwell, Oxford, UK. Her work centres on developing a multiscale understanding of materials using synchrotron X-ray techniques, including imaging and small-angle X-ray scattering. She recently completed her PhD in Biomedical Materials from Queen Mary University of London, with her thesis focused on the real-time understanding of nanoscale structure–function relationships in the echinoderm connective tissue.

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