Section 1

CHAPTER 1

The Mutable Collagenous Tissue of Echinoderms: From Biology to Biomedical Applications

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1.1 Introduction

Collagenous tissue in all extant classes of the phylum Echinodermata (starfish, sea-urchins and their close relations) has the capacity to drastically alter its mechanical properties within a timescale of seconds under the direct control of the nervous system. Such mutable collagenous tissue (MCT) has not been found in any other animal phyla and therefore is likely to be an echinoderm synapomorphy – a derived trait shared by all members of the phylum including their most recent common ancestor. This is corroborated by indirect evidence for the presence of MCT in Middle Cambrian stylophorans and other Palaeozoic echinoderms.[1,2] Although MCT is exclusive to echinoderms, some specific features underpinning its mechanical adaptability – particularly the absence of

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permanently stable crosslinks between its constituent collagen fibrils – may be ancestral and have permitted the emergence of comparable phenomena in other phyla. There are, for example, remarkable parallels between MCT and the collagenous mesohyl of demosponges whose tensile properties are under non-neural physiological control.[3]

MCT demonstrates a micro-architectural diversity comparable to that of vertebrate fibrous connective tissue, occurring as three-dimensional fibre networks in dermal layers, parallel-fibred ligaments interconnecting skeletal components and crossed-fibre helical arrays in the walls of tubular organs. These anatomical structures perform the same functions as their vertebrate counterparts, *i.e.*, they resist, transmit and dissipate mechanical forces. Their variable tensility, however, adds another dimension to their functional repertoire, which is of widespread importance to echinoderm biology and may have contributed to the evolutionary success of the phylum.

Many echinoderms maintain the whole body or its appendages in a rigid posture for prolonged periods of time for the purpose of defence or food collection. Such postural fixation depends on passive MCT stiffening rather than active muscle contraction, resulting in considerable energy saving.[4,5] The irreversible destabilisation of MCT is, on the other hand, the basis of all investigated echinoderm autotomy (defensive self-detachment) mechanisms[6] and of the processes that effect fission (asexual reproduction by division of the whole body) in brittlestars, starfish and sea-cucumbers.[7–9] Possibly related is the liquefaction ('autolysis' or 'melting') of the whole dermis exhibited by some sea-cucumbers in adverse conditions, a pathological phenomenon of great commercial importance that is the subject of intensive ongoing investigation.[10–12] Another surprising attribute of MCT, though one that has so far been demonstrated only in certain featherstar and sea-lily ligaments, is the capacity to actively generate tensile force.[13] This may actuate quite complex behaviour.[14] However, as the mechanism of active contractility is unknown, its relationship to variable tensility remains uncertain, and it will not be addressed further in this chapter.

In the 15 years since the last comprehensive review on MCT was published[15] there has been a significant expansion of information on its basic biology and, in step with the increasing attention being paid to 'marine collagens' in general,[16–19] MCT has attracted interest as a source of constituents for the construction of novel materials with potential biomedical applications[20–25] and for the food and cosmetics industries,[26,27] and as a source of inspiration for the design of entirely artificial materials with adaptable and controllable mechanical properties.[28–34] Although during this period several informative articles have reviewed specific aspects of MCT biology,[2,3,35–37] a survey of the whole field is overdue. In this chapter we first provide an overview of MCT organisation and functioning, focusing on recent advances in knowledge, and we then detail research that highlights its potential biomedical applications.

1.2 Biology of MCT

1.2.1 Organisation

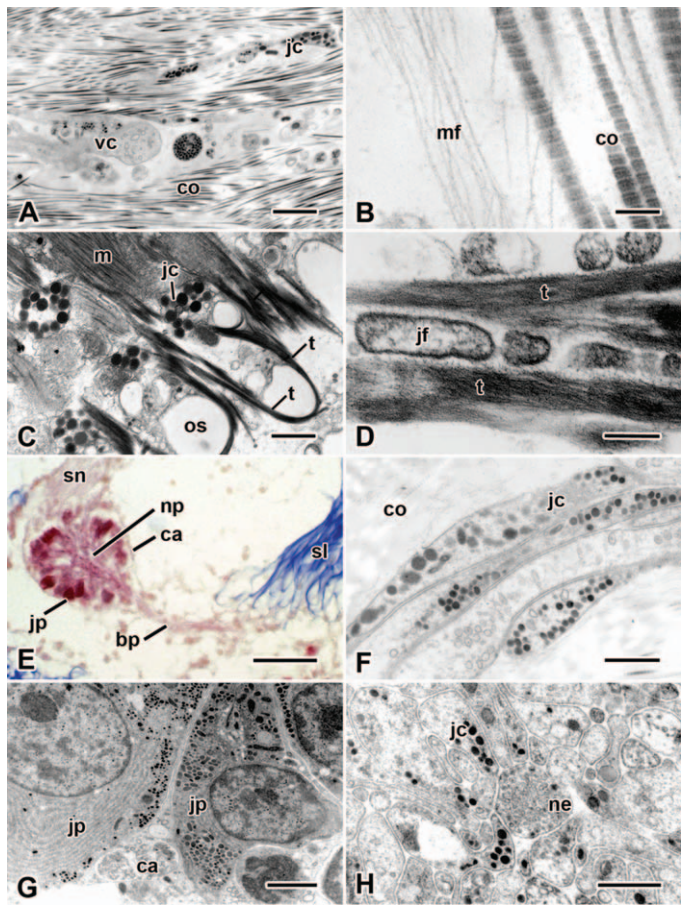
1.2.1.1 Extracellular Components

All echinoderm mutable collagenous structures consist predominantly of extracellular materials associated with a relatively small volume fraction of cellular components. With one exception, the extracellular materials, as observed by transmission electron microscopy, comprise mainly transversely banded collagen fibrils usually aggregated into bundles (fibres) and accompanied by loose arrangements of beaded microfibrils; specific staining methods also reveal the presence of interfibrillar proteoglycans (Figure 1.1A and B). The exception is the tendon tissue of the intervertebral muscles at the autotomy planes of brittlestar arms, which is an extension of the basement membrane of the muscle cells (Figure 1.1C and D).

The banded collagen fibrils of MCT, like those of vertebrate connective tissue, are parallel arrays of trimeric collagen molecules with a regular stagger between adjacent molecules ranging from 40 to 80 nm, a much wider variability than the 65–67 nm reported for vertebrate fibrils.[38,39] The collagen molecules of most investigated echinoderm fibrils comprise two fibrillar α chains (1α and 2α) which form $(1\alpha)_2 2\alpha$ heterotrimers; although fibrils from sea-cucumber dermis were previously thought to be $(1\alpha)_3$ homotrimers, recent proteomic evidence suggests they are heterotypic.[40–42] A small proportion of collagen molecules in sea-urchin fibrils, including those of MCT, contain a third fibrillar chain (5α) and have a $(1\alpha)_2 5\alpha$ stoichiometry.[40] The 5α chain is unusual in that its N-propeptide is not removed prior to fibril assembly, as occurs in the echinoderm 2α chain and in all vertebrate fibrillar procollagens,[43] and is located at the surface of the fibrils. The 5α N-propeptide is also notable because it contains 11 SURF ('sea-urchin fibrillar') modules, which are also present in the 2α N-propeptide and in fibrosurfin – an interfibrillar protein of unknown function. Since cleaved 2α *N*-propeptides have been immunolocalised to the periphery of fibril bundles in a sea-urchin mutable ligament,[44] it has been suggested that these SURF-containing molecules play a role in MCT variable tensility.[40]

Surveys of the *Strongylocentrotus purpuratus* genome and transcriptome analysis of a sea-cucumber body wall have revealed no unusual features of the extracellular matrix (ECM) that could be linked to MCT variable tensility. For example, echinoderms have up to four fibrillar collagen genes of the vertebrate I/II/III type and two of the V type, all of which encode molecules occurring in varying combinations in the banded fibrils of vertebrates.[9,45,46] These investigations also demonstrated the presence of fibrillin genes and their transcripts, complementing biochemical and immunological evidence that the beaded microfibrils that are ubiquitous in MCT and non-mutable echinoderm ligaments[38,47,48] consist at least partly of fibrillin-like proteins. These microfibrils may facilitate slippage between adjacent fibril bundles during MCT deformation and contribute to passive elastic recoil after the removal of external forces.[15]

Other interfibrillar components must include molecules that are responsible for the cohesion between adjacent collagen fibrils and therefore have a major influence on the mechanical properties of MCT. Proteoglycans, which consist of a protein core and glycosaminoglycan (GAG) sidechains, are present both within and on the surface of the collagen fibrils.[15] There is biochemical evidence that surface proteoglycans act as binding sites for other molecules that form interfibrillar crossbridges (see Section 1.2.3), but electron histochemistry suggests they may also be components of such crossbridges. Staining with the cationic dyes cuprolinic blue or cupromeronic blue, which label GAG sidechains, reveals both punctate/globular precipitates on the surface of fibrils and linear structures that extend between adjacent fibrils and are attached to specific sites within each fibril *D*-period.[38,49,50] In featherstar ligaments and sea-cucumber dermis the GAG components of these surface proteoglycans have been identified as chondroitin sulphate.[49,50] Fibrillar MCT contains several other molecules that contribute to interfibrillar cohesion but whose extracellular disposition is unknown; these are discussed in Section 1.2.3.



Brittlestar tendons are continuations of the basement membrane (or, more accurately, the basal lamina) of muscle cells (Figure 1.1C and D).[48,51,52] Those at arm autotomy planes undergo destabilisation at autotomy, which allows the arm muscles to separate 'cleanly' from the skeleton. The autotomy tendons are indistinguishable from the basal lamina of the muscles and epithelia in terms of their histochemical properties and granular/microfilamentous ultrastructure.[51] Although the specific molecular composition of brittlestar basal laminae has not been investigated, genomic and transcriptomic analyses have indicated that echinoderms possess the complete basic ''basement membrane ECM toolkit'' common to protostomes and deuterostomes, including genes encoding collagen types IV and XV/XVIII, laminin subunits and the proteoglycan perlecan.[9,45,46,53]

1.2.1.2 Cellular Components

In terms of its composition and organisation, the extracellular compartment of MCT generally resembles that of the collagenous tissue of non-echinoderms, including vertebrates. By way of contrast, the cellular assemblage associated with MCT is unique. The most abundant and characteristic cellular components are neurosecretory-like cell processes, and sometimes cell bodies, containing large (diameter $\geq$ *ca.* 100 nm) dense core vesicles (normally referred to as 'granules'). These were first described in a brittlestar ligament and named 'juxtaligamental cells' (JLCs) because in brittlestars the perikarya are always located outside, but usually closely adjacent to, collagenous structures (Figure 1.1E).[54] In almost all investigated mutable collagenous structures there are two populations of granule-containing processes distinguished by the size, and sometimes the shape and electron density, of their granules: one type ('type 1' herein) generally has spherical granules of diameter 100–200 nm, and

Figure 1.1 Organisation of mutable collagenous tissue: spine ligament and autotomy tendons of the brittlestar *Ophiocomina nigra*. (A) Spine ligament: general view. Scalebar: 2 μm. (B) Spine ligament: edge of fibril bundle. Scalebar: 0.2 μm. (C) Autotomy tendons of aboral intervertebral muscle: general view of intact tendons. Scalebar: 1 μm. (D) Autotomy tendons of aboral intervertebral muscle: fixed after autotomy. Scalebar: 0.2 μm. (E–H) Juxtaligamental cell components of spine ligament. (E) Relationship between node and ligament. Scalebar: 20 μm. (F) Juxtaligamental cell processes in spine ligament. Scalebar: 1 μm. (G) Edge of node, showing juxtaligamental cells and capsular epithelium. Scalebar: 2 μm. (H) Centre of node, showing neuropil-like region. Scalebar: 1 μm. bp, bundle of juxtaligamental cell processes; ca, capsular epithelium; co, collagen fibrils; jc, juxtaligamental cell process; jp, juxtaligamental cell perikaryon; jf, fragmented juxtaligamental cell process; m, aboral intervertebral muscle; mf, microfibrils; ne, neural cell process; np, neuropil-like region; os, decalcified skeletal ossicle; sl, spine ligament; sn, spine nerve; t, tendon; vc, heterogeneous vacuole-containing cell.
(A, B and E–H) Adapted from ref. 48, https://doi.org/10.1371/journal.pone.0167533, under the terms of the CC BY 4.0 license, https://creativecommons.org/licenses/by/4.0/ and (C, D) Adapted from ref. 51 with permission and from Springer Nature, Copyright 1987.

the other ('type 2') generally ellipsoidal granules of maximal diameter 200–1000 nm (Figure 1.1F).[48,55–58] Two examples of MCT with a single cell type containing two types of granules have been described.[38,59]

An expanding body of evidence indicates that cell types 1 and 2 are neurons. In brittlestars both are unipolar cells with pyriform perikarya most of which are located in aggregations, known as juxtaligamental nodes, that have a central, neuropil-like region penetrated by the axons of hyponeural motoneurons, with typical chemical synapses occurring between axonal and juxtaligamental processes (Figure 1.1E, G and H).[48,52,55,60,61] The presence of an outer capsule of neuroglia-like cells with centrally directed partitions that compartmentalise the juxtaligamental perikarya (Figure 1.1G) gives the nodes a very ganglion-like appearance and suggests they could be integrating centres that coordinate changes in the tensile properties of MCT with the activities of other effector systems.[48] Immunohistochemistry employing antibodies against neuronal markers (such as synaptotagmin, calbindin-D32k and acetylated tubulin)[3,61–64] and a neurotransmitter (L-glutamate)[65] has provided abundant evidence for the presence of neural elements in MCT, although at the light microscope level it has not been possible to relate immunopositive cell types to those distinguishable at the ultrastructural level with certainty. At least some of the type 1 processes may be peptidergic axons, since their granules are within the size range of the neuropeptide-containing granules of other phyla (*ca.* 50–300 nm).[66–69]

It is highly likely that JLCs are the effectors that directly modulate the tensile properties of MCT, since their processes terminate in MCT, have no possible cellular targets and link the ECM to the motor nervous system, and since they are absent from non-mutable echinoderm collagenous structures.[15] However, evidence supporting this supposition remains circumstantial. Putative effector molecules occur in sea-cucumber JLCs: the stiffening proteins tensilin and stiparin have been immunolocalised to the granules of type 2 cells in the dermis[70] (also Keene and Trotter, unpubl.), and tensilin has been detected by immunohistochemistry and *in situ* hybridisation in type 2 cells of Cuvierian tubule connective tissue.[58] In a minority of mutable collagenous structures alterations of tensile state are accompanied by changes in JLC ultrastructure. Such changes have been seen mainly in structures undergoing irreversible destabilisation or stiffening and usually include evidence that granules or their contents are released into the extracellular compartment.[38,55,58,59] It has been observed consistently that granule-containing processes tend to occur in heterogeneous clusters in which the two types of process are in close apposition, implying a functional relationship.[48,55–57] Whilst this might represent a reciprocal effector system, with one cell type destabilising the ECM and the other type stabilising it, the peptidergic neuron-like features of type 1 JLCs suggest that they are 'conventional' neurons that regulate the effector activity of type 2 JLCs. It is now recognised, however, that the granules of vertebrate neurosecretory neurons, including peptide-secreting types, handle a diversity of molecules that are involved in a wide range of biological processes, including ECM degradation.[71–73] Each of the two cell types in MCT could

therefore have both effector and regulatory functions. An entirely different role for JLCs is intimated by the observation that the regeneration of the visceral mass in a featherstar involves the transdifferentiation of apparent JLCs (identifiable by the presence of two granule types) into enterocytes.[74] The occurrence of 'central' juxtaligamental-like cells in the radial nerve cord of a brittlestar[61] is a further indication that these cells may have a range of functions that varies between different echinoderm classes.

Other cells present in MCT include heterogeneous vacuole-containing cells (Figure 1.1A), which may have a phagocytic function, and, in a few starfish and sea-urchin structures, myocytes.[15,75] Most echinoderm collagenous tissue, including MCT, appears to lack cells recognisable as fibroblasts.[48,57–59,76] One exception to this is a mutable sea-urchin ligament in which collagen-synthesising cells have been identified on the basis of their ultrastructure and labelling with an antibody against prolyl 4-hydroxylase, an enzyme involved in collagen biosynthesis.[3] It is possible that the more usual absence of fibroblasts is indicative of echinoderm ECM components having a very low turnover rate. However, echinoderms have indeterminate growth[77] and therefore must possess as yet unidentified populations of cells with the capacity to maintain the continuous expansion of connective tissue structures.

1.2.2 Biomechanics

1.2.2.1 Protocols

A range of different biomechanical test protocols has been used to assess MCT mechanical properties. These include quasi-static (strain-rates $\sim 10^{-4}$–$10^{-2}\ s^{-1}$) tensile tests to failure, from which tangent modulus, failure strain and failure stress are evaluated. Tangent modulus usually increases with strain, so several reports use only maximum (and/or initial) values. A second type of test frequently used is dynamic mechanical analysis (DMA), which refers to cyclic loading to fixed strain-amplitudes below yield and provides information on elastic and viscous behaviour by reporting storage (elastic) and loss (viscous) moduli. Not all work on MCT using this type of test reports the full range of DMA parameters. Concerning time-dependent or viscoelastic effects, transient tests like stress-relaxation (reduction of stress when the tissue is held at a fixed strain) and creep (elongation of the tissue under a fixed stress) are frequently carried out. Stress-relaxation tests on MCT – often under different states of mechanical mutability – report the relaxation modulus (time-varying stress divided by strain), as well as the timescale of relaxation (denoted as τ) and equilibrium modulus (when the stress no longer decreases). Multiple timescales (*e.g.*, short ~seconds and long ~hours) may be obtained, depending on the model used as well as system/stimulation tested.

There are several technical challenges involved in mechanical testing of MCT-containing tissues, some of which are inherent in biomechanics of soft tissues. These include obtaining regular shaped sections with as homogeneous a microstructure as possible – this is to ensure that the reported elastic modulus and other parameters correspond to MCT itself rather than

a mixture of other tissue components. Obtaining such samples is a challenge for the complex shapes of certain MCT-containing structures, like the compass depressor ligament in sea-urchins or the ossicle-connective tissue-muscle network in the body wall of starfish. A second challenge is to avoid inducing mechanical stiffness *via* the process of dissection of the tissue itself, which can be ameliorated in some cases by allowing the tissue to relax following dissection. Related to both the above is the choice of whether to test whole structures (which enables more physiological conditions) or to test individual parts of a structure (which allows more standardised mechanical tests to be conducted and parameters to be extracted). Lastly, the stress is obtained by dividing by cross-sectional area, which can be difficult to estimate for multi-component structures or complex shapes.

1.2.2.2 Comparison of MCT Properties with Those of Other Tissues

Table 1.1 provides elastic moduli of MCT-containing tissues in the normal (unstimulated) state. The moduli of sea-cucumber dermis in its normal state have been reported to be 0.01–5 MPa. Possible reasons for this wide variation may be the nonlinear nature of the stress–strain curve, with much lower gradients at 10% tissue strain compared to between 20–30%, along with variation in the maximum strain applied across different studies and biological inter-individual variability. It should be noted that for the values in Table 1.1, in some cases where explicit values for moduli are not provided, they have been estimated approximately from the published stress–strain curves. Starfish body wall – both isolated samples as well as whole arm – has generally been reported to be stiffer than sea-cucumber dermis (Table 1.1). Values range from ∼8 MPa for whole arm[78] to ∼28 MPa for the isolated dermis,[57] with much larger values of ∼200–350 MPa reported for isolated

Table 1.1 Stiffness (elastic modulus) of MCT-containing structures.[a]

Class	Species	Structure	Elastic modulus (MPa)	Reference
Asteroidea (starfish)	*Linckia laevigata*	Whole arm[b]	8.0	78
	Linckia laevigata	Body wall[c]	28.0	57
	Echinaster spinulosus	Body wall[d]	250–350	79
Echinoidea (sea-urchins)	*Paracentrotus lividus*	CDL	16.0	80
	Paracentrotus lividus	CDL	20.0	81
	Paracentrotus lividus	Tube foot stem	200–400	82
Holothuroidea (sea-cucumbers)	*Thyonella gemmata*	Dermis	0.13	83
	Stichopus chloronotus	Dermis	0.024	84
	Actinopyga mauritiana	Dermis	1.0	85
	Cucumaria frondosa	Dermis	0.07–0.08	86
	Holothuria leucospilota	Dermis	3.0	87

[a]CDL, compass depressor ligament.
[b]Bending tests.
[c]Uniaxial tests (longitudinal direction).
[d]Uniaxial tests (longitudinal, transverse and bias directions).

samples from another species.[79] The compass depressor ligament in the sea-urchin *Paracentrotus lividus* has dynamic mechanical moduli of ~16 MPa.[80] However, the tube feet in this species have much higher moduli of 200–400 MPa in artificial sea-water (ASW).[82] Comparing these values as a whole with those of vertebrate unmineralised collagenous tissues, it is observed that moduli of MCT-containing tissues (~0.1–400 MPa) are much lower than that of vertebrate tendon (1–2 GPa[88]) and closer to that of articular cartilage (1–10 MPa[89]). The maximum (failure) strains of MCT-containing tissues like sea-cucumber dermis (40–50%)[87] or starfish body wall (~25–30%)[57] are between those for dense, aligned collagenous tissues like tendons (~10–20%) and highly extensible tissues like skin (>100%).[88] It is likely that both intrinsic differences in ECM composition (material differences) and fibre organisation and orientation (architectural differences) contribute to these differences in failure strain, but the details are not yet fully clear.

1.2.2.3 Response to Stimulating Agents

Induction of mechanical changes can be through either mechanical (*e.g.*, rubbing the tissue), chemical (changing the ionic composition of the bathing media) or biochemical means (adding specific proteins or peptides believed to alter the fibrillar-level mechanics). Mechanical stimulation can lead to stiffening, with Motokawa and Wainwright[78] observing an increase in stiffness of starfish (*Linckia laevigata*) whole arm from 8 MPa to 20.5 MPa. In intact *L. laevigata* dermis, an increase in (uniaxial stretch to failure) modulus from 27.5 to 40.7 MPa was observed on mechanical stimulation.[57] The use of ASW with different ionic compositions (enriched or depleted in K^+, Ca^{2+} *etc.*) has been historically one of the first ways to induce mechanical changes in MCT in the laboratory. These include potassium-enriched ASW (KASW) or calcium-chelated ASW (CaFASW). Motokawa[84] found, in the sea cucumber *Stichopus chloronotus*, a five-fold increase in modulus from 0.024 MPa to 0.128 MPa on treating with KASW. KASW treatment of *L. laevigata* dermis also increased its stiffness at 10% strain by a factor of six.[57] Mo *et al.*[87] found four-fold increases in stiffness with KASW treatment of the dermis of the sea-cucumber *Holothuria leucospilota* and 80% lower modulus in CaFASW compared with normal ASW. Greenberg and Eylers[83] found that both NaCl and $CaCl_2$ solutions, relative to deionised water, caused a reduction in relaxation modulus in stress-relaxation tests. Initial hypotheses (reviewed by Wilkie[90]) suggested that calcium and other ions could be causing formation of intermolecular bonds (and consequent stiffening) in the ECM directly. However, subsequent work[91,92] suggested that the effect of calcium was *via* Ca^{2+}-dependent cellular processes, since cell-lysed tissues did not show such mutability.

1.2.2.4 Viscosity and Bending Creep Parameters

To estimate the viscous flow in the ECM matrix, a frequently used measure of MCT tissue mechanics is the creep test, whether the creep is due to an

external load or to the time-dependent flexion of a horizontal tissue section (cantilever-geometry) under its own weight.[92] This measure also has the advantage that it can be used for whole structures without the mechanical stimulation caused by dissection of strips of tissue. In the case of gravity-bending, if the creep is assumed as arising solely due to the viscosity η of the tissue, an approximate estimate of η can be made from the time t to flex (drop) a given distance, *via* the Newtonian relation $\sigma = \eta(d\varepsilon/dt)$.[92] Taking the stress to be a constant (which is linked to the tissue weight) as well as the strain ε (as the distance is fixed) and approximating the strain-rate $(d\varepsilon/dt) \sim \varepsilon/t$, it is seen that $\eta \propto t$ when the weight or mass of samples is comparable. This method was used to estimate the softening, flow and stiffening associated with Ca^{2+} concentration with and without cell-lysing agents,[92] as well as the effect of proteins like tensilin.[93] While obtaining absolute values of viscosity from the load-deflection curves is not easy, relative changes of viscosity can be calculated. For example, changes of ten-fold or more in viscosity were estimated in sea-cucumber (*Cucumaria frondosa*) dermis treated with agents that block calcium channels or chelate calcium.[92] However, without a structural viscoelastic model, such measurements are not easy to directly link to material-parameters of MCT.

1.2.2.5 Modelling and Role of Ultrastructure

Despite the considerable amount of experimental mechanical data on the variable tensility of MCT-containing tissues, the basic biophysical/biochemical processes are not completely understood (see Section 1.2.3). A limited amount of information exists on the mechanical properties of the individual constituents. Eppell *et al.*[94] used small-scale micromechanical testing on individual echinoderm collagen fibrils, obtaining stiffnesses in the order of ~0.5 GPa. Thurmond and Trotter[95] characterised the mechanics of the microfibrillar network that surrounds the collagen fibril bundles by extracting and purifying it with guanidine and bacterial collagenase. The microfibrillar network behaved as a highly extensible elastomer (~300% elongation) whose elastic modulus can be estimated as ~4 kPa from the stress–strain data reported. On the basis of dynamic mechanical tests Motokawa and Tsuchi[85] developed a model according to which sea-cucumber dermis can adopt three different tensile states: compliant, standard and stiff, with stiffness increasing in that order. The standard state is the non-stimulated basal condition, but is not transitional between the other two, since they show qualitatively different features: compliant dermis can undergo stress softening; the stress–strain curves of stiff dermis lack a prominent toe region; and standard dermis shows neither of these features. These qualitative differences suggest that different mechanisms are involved in compliant ← → standard and standard ← → stiff shifts.

Direct visualisation of alterations in MCT structure during mechanical alterations is difficult, due to the experimental challenge of characterising the ECM ultrastructure *in situ*. Mo *et al.*[87] used synchrotron small-angle X-ray

scattering (SAXS) with tensile testing on sea-cucumber dermis to quantify fibrillar dynamics in response to mechanical mutability induced by ASW of different ionic compositions. The results showed that stiffening and destiffening of MCT can be explained in terms of a fibre-composite model at the nanoscale, where the collagen fibrils are separated by an interfibrillar matrix whose stiffness changes dramatically on going between stiff and standard or between standard and compliant states. The reason for these changes in interfibrillar matrix may be due to interfibrillar diffusion of tensilin or other factors discussed in Section 1.2.3. In the shear-lag model proposed by Mo *et al.*,[87] the increased interfibrillar matrix stiffening also leads to an increased stress transferred to the collagen fibrillar network, which becomes effectively more interconnected as a result, whilst on destiffening there is very little stress borne by the fibrils. Whatever the biochemical details of the mechanism, the combination of fibrillar-level and tissue-level stress and strain measurements indicated that the collagen fibrils themselves do not alter in mechanical properties. More recently, Goh and Holmes[81] modelled the mechanics of MCT deformation and failure using shear-lag models of the fibrils embedded in the interfibrillar matrix, highlighting the importance of factors such as aspect ratio and linking the structure of MCT to that of other collagenous tissues. Further research, combining biophysical, biochemical and proteomic studies is clearly necessary to elucidate these mechanisms in detail.

1.2.3 Mechanisms of Tensile Change

MCT shows four patterns of tensile change: (1) only irreversible destabilisation (as occurs during autotomy), (2) reversible stiffening and destiffening, as well as irreversible destabilisation, (3) only reversible stiffening and destiffening and (4) only irreversible stiffening.[58] These varying capacities are made possible by an important feature of MCT that distinguishes it from the collagenous tissues of vertebrates: interfibrillar crosslinking in the latter is permanently stable, whereas in MCT interfibrillar crosslinking is labile and under physiological control. This is illustrated by the contrast between the extractability of echinoderm collagen fibrils, which can be isolated by mild chemical and mechanical methods,[96,97] and the inextractability of collagen fibrils from normal adult vertebrate collagenous tissues.[98–100]

In recent years most ideas on the possible molecular mechanisms underpinning the physiological control of MCT variable tensility have been derived from investigations of sea-cucumber dermis and have focused on chemical factors that can be isolated from the dermis and that influence its mechanical behaviour *in vitro* (Figure 1.2). The best characterised are the tensilins, a group of molecules with a high degree of sequence identity to TIMP (tissue inhibitor of metalloproteinase) proteins. Tensilins can be isolated only after the dermis is subjected to treatments that cause cell lysis, indicating that they are stored intracellularly. They cause calcium-independent aggregation of isolated collagen fibrils and stiffen samples of whole dermis *in vitro*. Their binding to fibrils is probably mediated by surface GAGs, but their specific contribution to

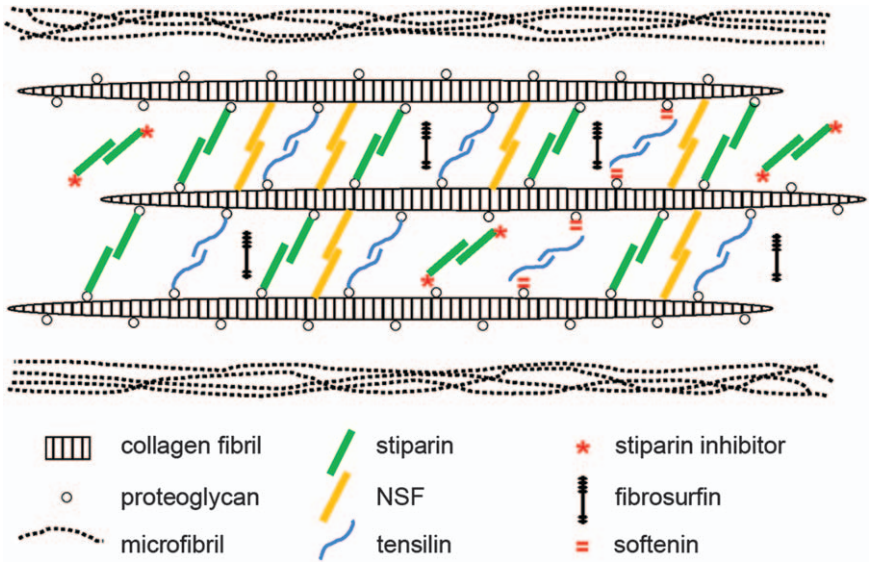


Figure 1.2 Model of MCT molecular organisation based primarily on sea-cucumber dermis and including some of the factors known to influence the mechanical properties of that tissue. For simplicity, the model assumes that stiparin, novel stiffening factor (NSF) and tensilin form dimers that act as interfibrillar crossbridges. Fibrosurfin has been detected in sea-urchin MCT but its function and microstructural disposition are unknown. Adapted from ref. 15 with permission from Springer Nature, Copyright 2005.

interfibrillar linkage is not known.[70,101] Despite their TIMP-like structure, it is unlikely that this involves inhibition of metalloproteinase activity, since the peptide sequences of all known tensilins differ from those of mammalian and sea-urchin TIMPs in the N-terminal domain, which is critical for their inhibitory activity (Figure 1.3). Sea-cucumber tensilins have a limited stiffening effect: Tamori *et al.*[101] found that the tensilin from *Holothuria leucospilota* applied *in vitro* could convert the dermis from (using the terminology of the 'three state model': see Section 1.2.2.5) the compliant to the standard state but not from the standard to the stiff state. The phylum-wide significance of tensilins is also not clear, since a sea-urchin tensilin has no consistent effect on the mutable compass depressor ligament of the sea-urchin *Paracentrotus lividus*.[102] A second stiffening protein – 'novel stiffening factor' (NSF) – converts sea-cucumber dermis from the standard to the stiff state but has no effect on compliant dermis and does not aggregate isolated collagen fibrils.[103] The differential stiffening effects of tensilin and NSF support the view that separate molecular mechanisms are responsible for the compliant → standard and standard → stiff transitions (see Section 1.2.2.5). Another protein – stiparin – causes calcium-independent aggregation of collagen fibrils, but has no effect on whole dermis. It is extractable by prolonged immersion of the dermis in sea-water alone and is the most abundant soluble glycoprotein in the dermis of

Human TIMP-1			C	T			C	V	P	P	H	P	Q	T	A	F	C
Human TIMP-2			C	S			C	S	P	V	H	P	Q	Q	A	F	C
Human TIMP-3			C	T			C	S	P	S	H	P	Q	D	A	F	C
Human TIMP-4			C	S			C	A	P	A	H	P	Q	Q	H	I	C
Strongylocentrotus purpuratus	TIMP-1		C	T			C	L	P	K	H	P	Q	Q	H	Y	C
Strongylocentrotus purpuratus	TIMP-3		C	S			C	M	P	A	H	Y	Q	T	L	F	C
Strongylocentrotus purpuratus	TIMP-4		C	M			C	Q	P	S	H	P	Q	Q	Q	Y	C
Strongylocentrotus purpuratus	TLP		C	S	H	T	C	L	S	L	H	P	Q	Q	R	F	C
Apostichopus japonicus	tensilin		C	G	A		C	D	T	K	H	P	Q	Q	H	Y	C
Cucumaria frondosa	tensilin	Q	C	A	G		C	S	V	K	H	P	Q	H	H	F	C
Holothuria forskali	tensilin		C		G	Q	C	V	S	D	H	P	Q	Q	H	Y	C
Holothuria leucospilota	tensilin			W	G	Q	H	S	T	N	H	P	Q				

Figure 1.3 Partial N-terminal domain amino acid sequences of human and *Strongylocentrotus purpuratus* TIMPs and of some echinoderm tensilins, including sea-urchin tensilin-like protein (TLP). Identical amino acids are highlighted.
(Sources of data: ref. 48 and 58).

Cucumaria frondosa. Stiparin is thought to be a constitutive component of the ECM that maintains a basal level of interfibrillar cohesion, while allowing slippage between adjacent collagen fibrils.[70]

Destiffening molecules have also been extracted from the sea-cucumber dermis. 'Stiparin inhibitor' is a 62 kDa sulphated glycoprotein that binds stiparin and inhibits its fibril aggregating activity. 'Plasticiser' is a small (<15 kDa) protein that has a direct destiffening effect on the dermal ECM and, like tensilin, is released only after cytolytic treatments.[70] Softenin is a *ca.* 20 kDa destiffening protein that is extractable without cell lysis. As it disaggregates tensilin-aggregated collagen fibrils *in vitro* and reversibly destiffens cell-dead dermis in the standard state, it may compete for tensilin binding sites on the collagen fibrils.[104] Because of the reversibility and short time course of its effect, softenin is unlikely to be an enzyme. However, enzymes have been suggested as possible MCT destiffeners, particularly matrix metalloproteinases (MMPs), which are extensively involved in the ECM remodelling that accompanies echinoderm development and regeneration.[105–107] The synthetic MMP inhibitor galardin was found to stiffen sea-urchin compass depressor ligaments in all three mechanical states, though its effect was much lower on stiff ligaments than on compliant and standard ligaments. Ribeiro *et al.*[80] speculated that ligament stiffness is adjusted through crosslink degradation by constitutive MMP activity regulated by the cellular release of an endogenous MMP inhibitor. MMPs have also been implicated in the phenomenon of sea-cucumber dermal liquefaction. Although the activity of several endogenous proteinases increases in liquefying dermis,[10,11] liquefaction is blocked by MMP inhibitors and only MMP activity achieves the complete disaggregation of collagen fibres into smaller fibril bundles and fibrils (possibly *via* the degradation of interfibrillar proteoglycans) that characterise the process.[11,12]

Echinoderms possess a surprising number of MMPs. For example, the *S. purpuratus* genome includes 26 genes encoding MMPs, whereas there are 24 human MMPs.[9,108] More unexpected is the huge assemblage of echinoderm TIMPs: a phylum-wide transcriptomic survey identified 405 TIMP genes, with up to 45 genes per species, in contrast to the four human TIMP genes.[109] Whilst structural features indicate that not all of these TIMPs could function as MMP inhibitors,[9] it is still obvious that MMP-dependent mechanisms must be very important for echinoderm biology. This may be partly due to the extensive regenerative capacities of echinoderms but could also be related to the wide functional diversity of echinoderm collagenous tissues, including their mechanical adaptability.

The mechanical behaviour of MCT preparations is affected *in vitro* by several endogenous peptides, which are discussed fully in Section 1.2.4. Most of their effects are likely to be cell-mediated. However, it has been suggested that two peptides – holokinin-1 and holokinin-2, whose heptapeptide sequence is present in the C-terminal domain of 5α collagen, destiffen the dermis by disrupting putative interfibrillar linkages involving 5α collagen.[110] It is therefore intriguing that 5α collagen is one of only two proteins found to be downregulated at all stages of progression of the sea-cucumber disease known as 'skin ulceration syndrome'.[111] Zhao *et al.*[111] argued that, if 5α is a target protein of the causative pathogen, its downregulation could be a protective response. However, if 5α collagen is involved in interfibrillar crosslinking, its downregulation could also be a factor in the pathogenesis of the full-thickness dermal lesions that develop in this disease and in which collagen fibres are disorganised.[112]

More evidence that separate stiffening mechanisms are responsible for the compliant → standard and standard → stiff transitions has been obtained from comparisons of the water content and distribution in MCTs in different mechanical states. On the basis of measurements of volume and mass, Tamori *et al.*[113] concluded that water moves out of sea-cucumber dermis during the standard → stiff shift but not during the compliant → standard shift. Similarly, confocal Raman spectroscopy has shown that water moves from the interior to the surface of sea-urchin compass depressor ligaments during the standard → stiff but not the compliant → standard shift.[114] Furthermore, in both these MCTs the standard → stiff transition alone is accompanied by a significant increase in the collagen fibril packing density.[38,115] The transmission electron microscope observations of Tamori *et al.*[115] also revealed that in both transitions in sea-cucumber dermis there is a significant increase in the number of crossbridges connecting adjacent collagen fibrils, leading the authors to propose that dermal stiffening is achieved by three mechanisms: increased crossbridge formation in both transitions, tensilin-dependent stiffening of collagen fibrils (through subfibril fusion) in the compliant → standard transition and, in the standard → stiff transition, strengthening of interfibrillar cohesive forces through (as yet unidentified) bond formation that increases fibril packing density and causes water exudation.

At present the applicability of the above model to MCT stiffening across the phylum is not clear, due to the paucity of information on the MCTs of

other echinoderm classes. Nor can its relevance to destiffening be evaluated: is this achieved simply through the reversal of the stiffening mechanisms, or are qualitatively different mechanisms, *e.g.*, enzymatic degradation, employed? Are different mechanisms involved in reversible and irreversible destiffening? The model certainly does not apply to the non-fibrillar basal lamina-derived tendons of brittlestar intervertebral muscles, which undergo irreversible destabilisation at arm autotomy and which, like basal laminae throughout the Metazoa, must be assumed to consist of meshworks of type IV-like collagen, laminin glycoproteins and proteoglycans.[116] Although the microstructure of the autotomy tendons thus differs fundamentally from that of fibrillar MCTs, the parsimonious inference is that the molecular mechanisms responsible for their variable tensility have not evolved independently and that they share common features.

1.2.4 Neural Control

MCT functions as a mechano-effector whose passive tensile properties are adjusted under neural control and in coordination with the activity of muscles.[117] Insights into the mechanisms by which the nervous system controls the mechanical state of MCT have been obtained using a variety of experimental approaches. As discussed above, detailed analysis of the microstructure of MCT in brittlestars has revealed innervation from branches of the hyponeural nerves, which contain the axons of motoneurons.[48,52,55,60,61] However, it is not known if there are populations of hyponeural motoneurons that specifically innervate MCT or if hyponeural motoneurons exert dual control of muscles and MCT in echinoderms.[118] *In vitro* pharmacological studies have revealed that acetylcholine (ACh) causes contraction of muscle preparations in echinoderms, indicating that it acts as a general excitatory neuromuscular transmitter in this phylum. Furthermore, there is evidence that the effects of ACh on echinoderm muscle are mediated by muscarinic and/or nicotinic receptors.[119–125] Analysis of the effects of ACh on the body wall dermis of the sea-cucumber *Holothuria leucospilota* revealed that ACh and nicotinic receptor agonists cause an initial increase in viscosity followed by a later decrease in viscosity, whereas muscarinic agonists cause a decrease in viscosity.[126] These findings suggest that both stiffening and destiffening of sea-cucumber dermal MCT are controlled by cholinergic motoneurons. Different patterns of response have been reported in other echinoderm classes. Sea-urchin spine ligaments are stiffened by both nicotinic and muscarinic agonists, the latter having a slower effect, as in vertebrate cholinergic systems,[127] whereas both types of cholinomimetic destiffen the cirral ligaments of a sea-lily, the muscarinic effect again being slower.[128] This interclass diversity of cholinergic responses is perhaps a further indication of the basal origin of the mutability phenomenon.

In addition to ACh, the effects of other 'classical' neurotransmitters/hormones on MCT have been investigated. The catecholamines adrenaline, noradrenaline and dopamine produce a biphasic (destiffening then stiffening)

response in sea-urchin spine ligaments; β-phenylethylamine has the same biphasic effect, but its derivatives, tyramine and octopamine cause only destiffening.[127] The presence of glutamate-like immunoreactivity has been revealed in neuron-like cells in the arms of the featherstar *Antedon mediterranea*. *In vitro* pharmacological tests on isolated arm pieces revealed that glutamate causes irreversible destiffening of MCT-containing ligaments at skeletal joints (syzygies) in the arms of *A. mediterranea*, indicating that glutamate signalling may mediate neural control of autotomy in featherstars.[65] Although there is evidence that serotonin acts as a modulator of neuromuscular transmission in echinoderms[129] and gamma-amino butyric acid (GABA) has a variety of effects on echinoderm muscle preparations,[119–121,123] there appear to be no reports of either neurotransmitter affecting MCT stiffness. Opportunities to gain further insights into the molecular and cellular mechanisms by which neurotransmitters such as glutamate exert effects on MCT in *A. mediterranea* and in other echinoderms are afforded by the availability of transcriptome/genome sequence data.[45,130–133] Thus, candidate neurotransmitter receptors could be identified, pharmacologically characterised and localised in MCT.

Neuropeptides are an evolutionarily ancient and diverse class of neuronal signalling molecules that regulate a huge variety of physiological processes in animals.[134,135] The first neuropeptides to be identified in echinoderms were SALMFamides, which act as muscle relaxants.[136,137] Subsequently, an extensive chemical analysis of extracts of the sea-cucumber *Apostichopus japonicus* revealed the identity of other myoactive neuropeptides, which cause muscle relaxation or contraction or have modulatory effects on neuromuscular preparations.[138,139] Several of these myoactive peptides also affect the stiffness of sea-cucumber dermal MCT. Thus, the amidated pentapeptide NGIWYamide causes stiffening, heptapeptides known as holokinins (PLGYMFR and an oxidised derivative) cause destiffening and the cyclic peptide stichopin (DRQGWPACYDSKGNYKC, with a disulphide bridge between the cysteine residues) suppresses the stiffening effect of ACh on the dermis.[140] Furthermore, the expression patterns of NGIWYamide and stichopin in *A. japonicus* have been investigated using immunohistochemical methods. NGIWYamide-immunoreactivity was detected in hyponeural nerves and in nerve fibres located within the dermis, consistent with the stiffening effect of NGIWYamide on the dermis.[141] Recently, a candidate receptor for NGIWYamide was identified in *A. japonicus* in parallel with pharmacological characterisation of the receptor for NGFFFamide, an orthologue of NGIWYamide in the sea-urchin *Strongylocentrotus purpuratus*.[142] Therefore, pharmacological characterisation of the candidate NGIWYamide receptor and localisation of its expression in *A. japonicus* could provide insights into the molecular and cellular mechanisms by which NGIWYamide causes stiffening of sea-cucumber dermis.

Localisation of stichopin in *A. japonicus* revealed immunoreactivity in nerve fibres in the dermis, consistent with its effects on the mechanical properties of the dermis.[62] Stichopin-like immunoreactivity was also detected in oval-shaped cells without processes, which are located in the dermis and in other connective tissues, and it was suggested that these cells may release stichopin

as a hormone.[62] The sequence of the precursor protein that stichopin is derived from has also been determined by analysis of transcriptome sequence data, revealing that it comprises an N-terminal signal peptide, as would be expected for the precursor of a secreted neuropeptide or hormone, followed by the stichopin peptide sequence.[110] Furthermore, analysis of genome sequence data has revealed that the coding sequence for the signal peptide of the stichopin precursor is interrupted by an intron.[143] With the availability of the transcript/gene sequence for the stichopin precursor, it would now be possible to investigate its expression in *A. japonicus* using mRNA *in situ* hybridisation methods, facilitating comparison with the immunohistochemical data reported previously.[62]

Analysis of *A. japonicus* transcriptome sequence data has also provided insights into the molecular properties of holokinins, revealing that they are not neuropeptides, as originally supposed,[140] but are fragments of the C-terminal region of a 5α type collagen.[110] As highlighted above (Section 1.2.3), this was an interesting finding because it provides a basis for explanations of how holokinins could influence the mechanical properties of MCT. However, the physiological relevance of the effect of holokinins on sea-cucumber dermis is unclear because holokinins could be a product of collagen degradation generated when body wall extracts are prepared. Nevertheless, it would be interesting to investigate the mechanisms by which holokinins cause softening of the sea-cucumber dermis.

Transcriptome/genome sequencing has recently facilitated identification of a large number of candidate precursors of neuropeptides in several echinoderm species.[144–149] Furthermore, using the starfish *Asterias rubens* as a model experimental system, there are extensive ongoing efforts to investigate the physiological roles of neuropeptides identified by analysis of neural transcriptome sequence data in this species.[146] Having determined the structures of novel neuropeptides using mass spectrometry, neuropeptide expression in *A. rubens* has been examined using mRNA *in situ* hybridisation and immunohistochemical methods. Expression of several neuropeptides was detected in the cell bodies of hyponeural neurons, indicating potential roles in motoneuronal signalling. For example, these include pedal peptide/orcokinin (PP/OK)-type, gonadotropin-releasing hormone (GnRH)-type and calcitonin-type neuropeptides.[150–153] Consistent with hyponeural expression, use of immunohistochemistry revealed that PP/OK-type and calcitonin-type neuropeptides are present in motor nerves and in the innervation of muscles between the skeletal ossicles. The effects of these neuropeptides on interossicular muscles are not known, but *in vitro* pharmacological tests have revealed that they cause relaxation of other muscular preparations.[150–152] It is perhaps significant that PP/OK-type or calcitonin-type neuropeptides were not detected in the innervation of collagenous tissue in the body wall of *A. rubens*. Therefore, hyponeural motoneurons expressing PP/OK-type or calcitonin-type neuropeptides may be uniquely associated with innervation of interossicular muscles. Conversely, there may be neuropeptides that are uniquely associated with

hyponeural innervation of body wall collagenous tissue in *A. rubens*, but as yet such neuropeptides remain to be identified. If neuropeptides present in the innervation of body wall collagenous tissue of *A. rubens* (or other echinoderms) can be identified, then this would provide a basis for investigation of their effects on MCT, which may facilitate further advances in our understanding of the molecular and cellular mechanisms by which the nervous system regulates the mechanical properties of MCT in echinoderms.

1.3 Biomedical Applications of MCT

Since Trotter *et al.*[70] first introduced the notion of MCT-inspired synthetic systems, interest in the potential biomedical applications of MCT has been steadily growing. This has involved two approaches: exploiting MCT as a concept generator (the biomimetic approach) and using extracted MCT components for deployment in synthetic biomedical materials (the biotechnological approach).[154]

1.3.1 Biomimetic Approach

This approach imitates natural models without using any of their actual constituents. The biomimetic approach can be adopted for *biology-inspired developments* (biology-push processes) or *technology-derived developments* (technology-pull processes).[154] Although both of these are types of biomimetic development in which technical solutions are inspired by living organisms, they differ in the nature of their starting point: this is a *biological question* in the case of the former and a *technical problem* in the latter case.

1.3.1.1 Biology-inspired Developments: Biology-push Processes

Here the starting point for knowledge transfer is an inspiring idea derived from the structural features and/or operating principles of a biological model, which leads to a biomimetic development that benefits clinical practice or healthcare.[154] With regard to MCT as a concept generator, the most 'popular' in a range of potential echinoderm models has been the sea-cucumber dermis.

Trotter *et al.*[70] first proposed, designed and tested a synthetic analogue of sea-cucumber dermis that was composed of collagen fibrils in an artificial elastomeric matrix and included reversible interfibrillar crosslinks formed by photo- or electro-sensitive reagents. Although in its first version this model was a hybrid, since it was inspired by MCT mechanical properties as a concept generator but incorporated natural fibrils extracted from sea-cucumber dermis, the final version was completely biomimetic and employed a fully synthetic fibrous composite with dynamically controlled stiffness. The bottom-up, biology-push strategy of Trotter *et al.*[70] was a source of inspiration for a series of applied top-down projects (technology-pull processes) leading to successful technology-derived developments (see below).

Xia[31] described sea-cucumber dermis as ''an intriguing biological model for the biomimetic design of artificial polymer nanocomposites that exhibit similar architecture and chemo-mechanical behavior''. He suggested that, to mimic the striking stiffening/destiffening performance of MCT, new artificial adaptive materials could be developed by employing a framework of nanofibres immersed in a soft matrix, with reversible bonds between the nanofibres and stress transfer being controlled by modifying interactions between nanofibres and/or between nanofibres and the matrix polymer *via* external stimuli. Mo *et al.*[87] also highlighted how the adaptive mechanical properties of MCT could provide new practical perspectives for the treatment of connective tissue pathologies, such as the weakening of tendons or ligaments following surgery or immobilisation, and for the design of a new generation of mechanically tunable implants.

1.3.1.2 Technology-derived Developments: Technology-pull Processes

In this case the starting point for knowledge transfer is a technical problem whose solution requires an appropriate technical product developed through an engineer-driven process.[154] Biological models are a source of inspiration, but the related biomimetic technical products do not necessarily appear to be morphologically similar to them and frequently do not display the same functions.

The pioneering example relevant to MCT was developed by Capadona *et al.*,[28] who assembled the first mechanically adaptive material inspired by sea-cucumber dermis, their aim being to exploit dynamic mechanical materials for specific neuro-medical applications requiring adaptive substrates, *viz.* implanted intracortical microelectrodes. The importance of adaptability or stimulus-responsiveness is critical for implanted materials, since a common cause of implant failure *in vivo* is the inability of engineered materials to adapt to their biological environment. The model of Capadona *et al.* was both structurally and functionally biomimetic, since it reproduced the natural composite-material structure by employing tunicate (sea-squirt)-derived cellulose nanofibres (*t*-CNCs) immersed in a polymeric matrix (EO-EPI or PVAc), and it also displayed adaptive chemoresponsive mechanical behaviour. In this model, mechanical changes (stiffening/destiffening) occurred in response to chemical stimuli through (1) inter-nanofibre crosslinks (hydrogen bonds) that could be switched on or off and (2) competitive nanocomposite–water interactions related to water uptake.[155] Exposure to water caused competitive hydrogen bond formation between water and CNCs with a consequent stiffness reduction. It should be noted that, although all versions of this model were intrinsically biomimetic,[28,156] they also utilised a biotechnological approach (see below) since they employed a bio-derived product as a structural material, *i.e.*, cellulose nanofibres extracted from tunicates or cotton. Advances in the field of intracortical implantation have included the development of mechanically-compliant microelectrodes (again employing *t*-CNC nanocomposites) that are more compatible with the tissue mechanical needs,

reduce long-term neurodegenerative and neuroinflammatory responses and preserve both neuronal and glial integrity.[157]

Stimulus-responsive nanocomposite systems with predictable and programmable behaviour ('smart' or 'intelligent' materials) have recently been exploited in a range of different biomedical applications employing minimally invasive medical devices. In particular, the concept of 'self-shaping materials' has been developed.[29] A widely adopted application of the sea-cucumber dermis model, inspired by its stiffening, destiffening and autolytic capabilities, has been utilised for the development of hydrogels – three dimensional crosslinked polymeric networks swollen with water. Such hydrogels have attracted widespread attention as functional biomimetic systems that can respond to external and internal physical or chemical stimuli (temperature, electric fields, chemical compounds, pH) (reviewed by Lim *et al.*[158]). Specific attention has been given to their employment as injectable biomaterials, tunable surfaces for cell sheet engineering, sensors and actuators (see for example Gao *et al.*[30]). *In vitro* cytotoxicity tests and preliminary subcutaneous implantation have indicated that supramolecular polymer hydrogels can be biocompatible and autolytic *in vivo*, with the potential to be used as temporary devices for intestinal drug delivery or for injectable filling to assist the suturing of small vessels.

In their review of bioinspired polymer systems with stimuli-responsive mechanical properties, Montero de Espinosa *et al.*[159] discussed examples of sea-cucumber dermis-inspired materials that are able to reversibly alter their stiffness, shape, porosity, density or hardness upon remote stimulation. It is interesting that the switching principles underpinning sea-cucumber dermis mutability are also exploitable in the design of shape-memory polymer/CNC nanocomposites capable of adopting one or more temporary shapes while remembering their original shape.[29,155] Starting from initial applications where water movement was employed to regulate hydrogen-bonding interactions in the nanocomposite framework, more recent studies have involved models that respond to other, more specific physico-chemical environmental stimuli, such as temperature or pH changes, UV irradiation and light.[32,159,160]

There has been recent interest in light-induced molecular switches that modify the shape and stiffness of soft materials. Lancia *et al.*[34] showed that incorporating artificial molecular switches into anisotropic soft matter could facilitate the development of mechano-responsive materials that are able to combine fast and complex deformation with mechanical adaptability such as is seen in soft tissues such as skeletal muscles and MCT. Such mechano-adaptive materials, which have the capacity to actively tune their rigidity, could certainly make a significant contribution to the top-down approach needed in the development of human-friendly and soft robotics.

1.3.2 Biotechnological Approach: Native or Hybrid Biomaterials

This second, fundamentally different, approach involves the extraction from natural models of one or more tissues or tissue components (*e.g.*, specific

molecules), which are then redeployed, in either an unmodified or modified form, in human products and devices. In this case biological systems are used only indirectly as concept generators, the target products being biotechnological applications that utilise biological components or their derivatives.[154]

The main applications of echinoderm MCT in terms of biomaterials employed in biomedicine and applied research are currently represented by (1) the employment of a single MCT-component, namely collagen, to produce 'simple' but highly versatile biomaterials and (2) the use of decellularised MCT explants, to obtain more complex matrices/biomaterials. In both cases, the final purpose is to develop bioscaffolds for tissue engineering or cell culture studies.

1.3.2.1 Employment of Extracted Collagen Fibrils

Collagen is the main structural protein of human tissues. Recent technological advances have facilitated the design of new collagen-based materials and structures (*e.g.*, dressings for wound healing, organoid stroma) for use in regenerative medicine where native connective tissue needs to be replaced with biomaterials, *e.g.*, for the repair and tissue engineering of hard tissues, cartilage, skin and nervous system. Freedman and Mooney[161] reviewed several biomaterial-based strategies for improving the healing of these tissues and highlighted guidance documents and standards that should be considered for translating new biomaterials into clinical practice.

In this context the material sciences are currently paying much attention to biocomposites based on collagen isolated from marine invertebrates, as is evidenced for example by patent registrations.[162,163] 'Marine collagens' are being increasingly used as alternatives to mammalian collagens for biomedical applications.[17,19,164,165] Echinoderm connective tissue, particularly MCT, is a particularly amenable source of marine collagen, due to the ease with which intact and native (*i.e.*, with GAGs still attached) collagen fibrils can be extracted from it (see Section 1.2.3 above). Sea-urchin collagen has been evaluated as a potential low-cost alternative for the production of biofilms or bioscaffolds.[20–23] This has involved different approaches, including the development of a specific protocol for collagen fibril extraction and matrix preparation, and a detailed evaluation of the structure, mechanical properties and *in vitro* biocompatibility of the constructed 'matrices'. Since sea-urchin collagen extracted from ligaments and peristomial membrane is similar to mammalian type I collagen in terms of chain composition, immunoreactivity and ultrastructure[15] (see Section 1.2.1.1 above), it is a potential alternative to the latter for biomedical applications. Accordingly, different types of sea-urchin collagen membranes (thin films, three-dimensional porous scaffolds) potentially suitable for tissue regeneration have been developed.[22,23] The matrices produced so far have proven to be mechanically more resistant than commercially available membranes normally used in tissue regeneration. They also possess the appropriate properties for specific tissue engineering applications, such as skin

regeneration, and can be optimally designed for use as dermal stitches for surgical purposes or skin tape for the treatment of lacerations and burns.[166] It has also been shown that sea-urchin collagen matrices may be successfully seeded with different mammalian cell types, indicating their potential as clinical tools for mammalian tissue regeneration.[22,23] Sea-urchin collagen has the further advantage of being salvageable from a by-product of the food industry that would otherwise be discarded as waste, *i.e.*, the remains of sea-urchins from which the edible gonads have been removed. It is therefore a low-cost, eco-friendly and sustainable resource that fits well with the modern concept of the 'circular economy' (Sugni *et al.*, work in progress).

1.3.2.2 Employment of Decellularised Tissue Explants

A range of bioscaffolds prepared by the decellularisation of various mammalian or human tissues is now commercially available and widely applied in surgical tissue engineering.[167–169] As far as echinoderm MCT is concerned, this application area has been explored in pilot studies. In particular, decellularised MCT from sea-urchin peristomial membrane has been used to produce scaffolds for sea-urchin cell cultures.[170] These substrates are very complex, native-mimicking types of MCT-derived bioscaffolds and are designed to retain most of the components (collagen, GAGs *etc.*) of natural MCT, apart from the cells. At present, these scaffolds have been used to facilitate the development of invertebrate (echinoderm) cell culture, a research field that is still largely unexplored. However, it is possible they could eventually have a role in human tissue engineering, which would require them to be treated appropriately to make them safe for use in biomedical applications. As such, recent reviews[25,81] have identified the technical challenges and problems arising from the employment of decellularised echinoderm tissues. For example, before being acceptable as components of marketed products, echinoderm connective tissues would need to be subjected to physical/mechanical and biochemical treatments (decellularisation, hydration/dehydration, sterilisation, lyophilisation *etc.*) that may affect both the structural and mechanical integrity of the bioscaffold and therefore need to be accurately optimised and controlled step by step. Meyer[169] has also commented on the expected requirements of purity and quality control of such materials.

1.4 Conclusions

Although much new information on the biology of MCT has been acquired in recent years, serious gaps in knowledge remain. Key to understanding the mechanics of MCT, the mechanisms underpinning its variable tensility, and its potential for biomedical exploitation, is the full characterisation of the molecular components responsible for interfibrillar cohesion and stress transfer. Whilst possible constitutive (*e.g.*, stiparin) and regulatory (*e.g.*, tensilin) factors have been identified, their exact roles in interfibrillar crosslinking remain unknown. This is not surprising, given that the

components transmitting interfibrillar shear forces in mammalian collagenous tissue have yet to be determined.[171]

Regarding the mechanisms of tensile change, the three-state model of Motokawa and Tsuchi[85] has proved to be a productive paradigm that accommodates the effects of a number of endogenous, and possibly regulatory, molecules and has given rise to the specific stiffening hypothesis of Tamori *et al.*[115] It is notable that the three-state model was derived from the mechanical behaviour of sea-cucumber dermis and that most of the endogenous factors that influence MCT properties have been isolated from this tissue alone. The wider relevance of the three-state model is not known. That sea-cucumbers possess many more TIMP genes than the other four echinoderm classes[109] could indicate that aspects of their MCT are not typical and emphasise the need for a wider range of models to be explored. Starfish body wall,[79] sea-urchin compass depressor ligaments[102] and brittlestar oral arm plate ligaments[172] are all easily accessible and amenable to *in vitro* experimentation.

In linking the motor nervous system to the ECM, JLCs have no known parallels outside the Echinodermata. There has been a proliferation of micro-anatomical information on the neural supply to MCT and on the expression of neurotransmitters and, especially, neuropeptides, which supports the view that, being the only possible target of such innervation, JLCs are the cellular effectors of tensile change. There remains, however, a lack of data pinpointing their exact contribution to the effector mechanism. This could be rectified through the transcriptomic and/or metabolomic profiling of the cells,[173,174] with the ultimate aim of defining the juxtaligamental secretome. This is feasible, because certain juxtaligamental nodes in brittlestar arms[54] can be accessed and removed surgically, offering scope for their constituent JLCs to be isolated and analysed.

The reversible destiffening of MCT that occurs in coordination with muscle activity[117] and the irreversible destabilisation associated with autotomy[6] are adaptive phenomena. Their relationship to the non-adaptive liquefaction of sea-cucumber dermis is far from clear. Investigation of the latter has focused exclusively on enzymatic mechanisms,[10–12] yet liquefaction must succeed a sequence of events that starts with sensory input and is likely to involve motor pathways, in which case adaptive MCT mechanisms may be invoked in at least the earlier aetiological stages. It might therefore be worth exploring the possible role of putative effector molecules that have been isolated from sea-cucumber dermis (see Section 1.2.3); this is another area that might be enlightened by comparative ''-omics'' approaches.[9,109] The need to investigate the possible contribution of dysfunctioning MCT mechanisms also applies to other pathologies such as sea-cucumber skin ulceration syndrome, which, like dermal liquefaction, has serious commercial implications,[111,112] and sea-star wasting syndrome, which has caused significant ecological disturbance.[175] Starfish affected by the latter demonstrate evidence of calcium homoeostasis derangement, which could influence MCT physiology and explain the body wall softening seen in this condition.[175]

The exploitation of MCT as a fundamental marine-derived biological material for biomedical applications is a realistic prospect as new technologies employing mechanically adaptive biomaterials become more prevalent. Like many natural processes, MCT mechanisms are intrinsically very complex, their multi-scale character making it particularly difficult to reproduce them in optimised artificial models. Therefore, although basic knowledge of MCT principles is already available, before any realistically employable devices can be developed for biomedicine and other applied fields, initial goals should be (1) to acquire a deeper understanding of structure–function relationships at all hierarchical levels of a range of biological models[159] and (2) to develop an interdisciplinary materials science approach.

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