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Review

Extracellular matrix, supramolecular organisation and shape

JOHN E. SCOTT

Chemical Morphology, Biological Sciences, Manchester University, Manchester, UK

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ABSTRACT

Connective tissue function is defined as the formation and maintenance of shape, without which centralised physiologies (circulatory, digestive or nervous) could not have evolved. Two elements, inextensible (collagenous) fibrils and compression-resistant interfibrillar soluble polymers (proteoglycans), cope with all usual stresses. Relationships between the two are highly specific, as demonstrated by electron histochemistry based on Cupromeronic blue and critical electrolyte concentration (CEC) methodologies. Recent ideas on (1) the protofibrillar or modular structure of collagen fibrils, (2) the nature of specific binding sites for proteoglycans on fibrils, and (3) fundamental similarities in secondary and tertiary structures of the glycosaminoglycans (hyaluronan, chondroitin, keratan and dermatan sulphates) are described. They have greatly illuminated the study of extracellular matrix structure and function in normal, pathological (osteogenesis imperfecta) and ageing tissues. The small proteoglycans are proposed to be tissue organisers, orienting and ordering the collagen fibrils—thus shaping the tissue at a molecular and ultimately macro level. These interfibrillar structures are based on their bifunctional character, the protein parts binding to collagen fibrils at specific sites and the glycosaminoglycans duplexing and aggregating to hold the proteins and hence the collagen fibrils at defined distances from each other, rather like yardsticks. Examples of the way these functions work in specific tissues are drawn from the cornea and vitreous humour of the eye and developing tendon.

Key words: Anionic glycosaminoglycan; collagen; dermochondran (dermatan) sulphate; hyaluronan; keratan sulphate; proteoglycan; cornea; vitreous humour.

INTRODUCTION

There are limits to what one cell can do, working alone. Division of labour extended cellular potential almost without limit, encouraging vast increases in genetic resources, giving rise to specialised and central functions. Organisms thus endowed could easily expand into new environments. However, digestive, circulatory and nervous systems depend totally on fixed spatial relationships determined by the framework in which they function. The achievement of permanent, reproducible shapes by multicelled organisms was thus of supreme importance. Shape is maintained by connective tissues. The evolution of single cells into multicelled organisms and thence into higher animals was therefore also the evolution of connective tissue (Scott, 1975).

The development of a nervous system to control internal workings, and of a sensorium which registered positions and contacts at the periphery was facilitated by a permanent shape and conversely, impossible without it. Particularly significant in the evolution of intelligence was the cognitive, externalised role of the sensorium. The evolution of intelligence and shape must often have been mutually interdependent. The ability to recognise shapes is often a matter of life and death. When a wolf recognises a sheep, and vice versa, there is an interplay of intelligence with shape—as determined by the extracellular matrix (ECM) of the connective tissues.

The ECM exemplifies the concept, as do membranes, chromosomes and the contractile machinery, of a successful design that has been used in myriads of organisms with modifications that were small com-

Correspondence to Professor John E. Scott, Chemical Morphology, Biological Sciences, Chemistry Building, Manchester University, Manchester M20 9PL, UK.

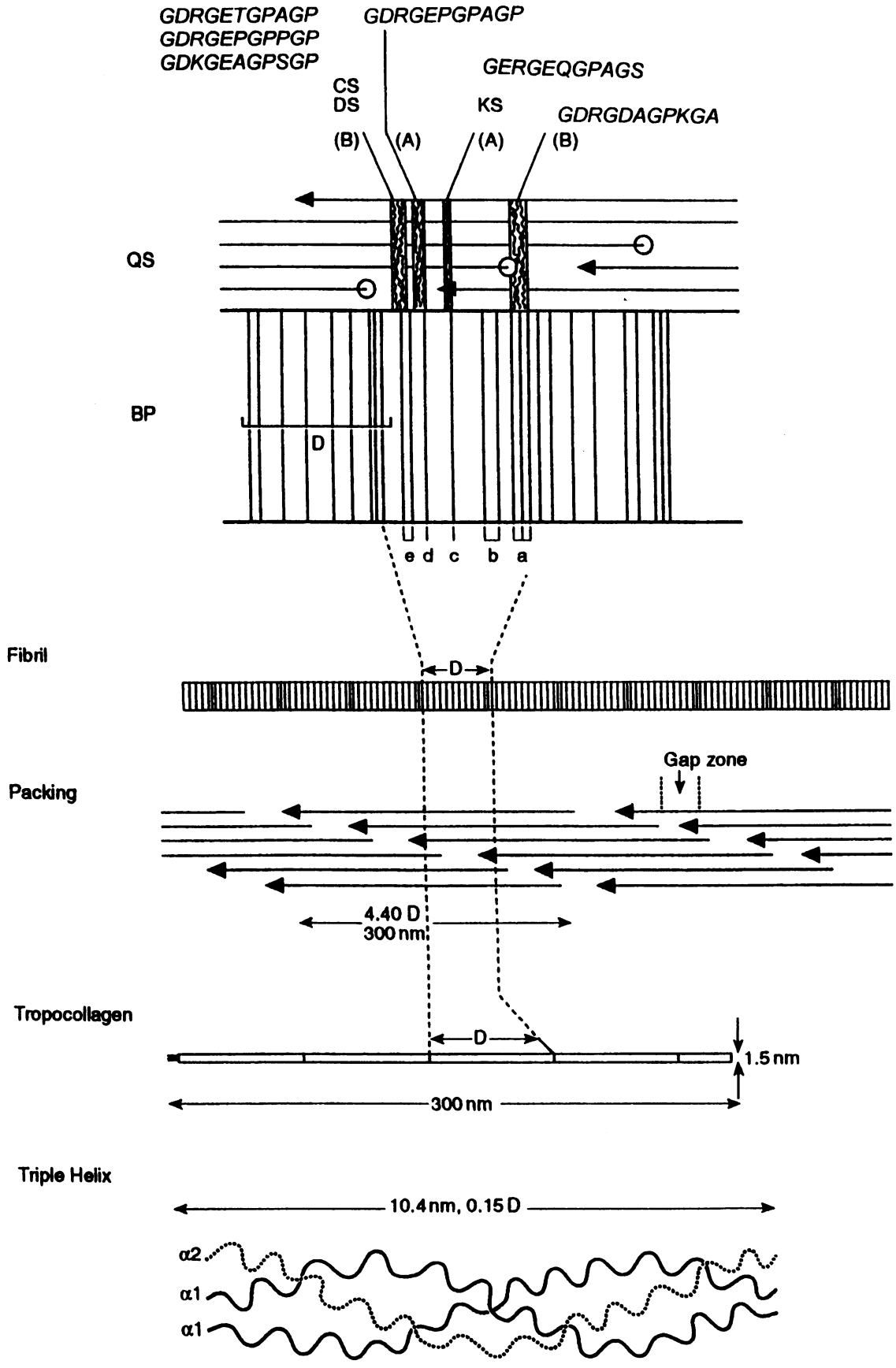

Fig. 1. For legend see facing page.

pared with the great diversity of finished products. This was achieved with a few fundamental molecules, which, when in place, require minimum maintenance.

Blueprint for an ECM

Microscopists have long known that ECM consisted of fibres, mostly of collagen, with a basophilic interfibrillar jelly, the 'amorphous ground substance', that showed no obvious structure. These facts were developed into a 'core concept' of connective tissue, valid in all species at all ages; i.e. connective tissues are systems of insoluble fibrils and soluble polymers that evolved to take the stresses of movement and the maintenance of shape (Scott, 1975).

Collagen fibrils (Fig. 1) are inextensible; they resist and transmit tensile stresses, *defining the maximum size* of the tissue. The production of controlled shapes depends on getting collagen fibrils of the right size into the right place—and keeping them there.

The water-soluble polymers are polysaccharide-rich proteoglycans (PGs, Fig. 2) and/or hyaluronan. They swell the aqueous spaces between fibrils, taking compressive stresses, limiting tissue collapse under pressure. Using two functional elements the ECM copes with the pulls and pushes that flesh is heir to. Water-soluble molecules diffuse through the aqueous PG channels to and from cells.

Replacement of the hydraulic fluid (water, in the PG space) in these shock absorbers with a solid mineral phase produced bone, inter alia. This, the last word in permanent shapes coupled with high load bearing, was achieved at a cost in terms of regulatory mechanisms which keep the mineral under control, neither allowing it to disappear into solution nor grow beyond allotted bounds. In practice bones combine both types of ECM, with shock absorbers (the cartilages) providing cushions between mineralised units.

COLLAGEN FIBRILS ARE OPTIMALLY DESIGNED TO TAKE TENSILE STRESS

Our concern is with fibril-forming collagens (primarily types I, II and III). A group of 'fibril-associated collagens with interrupted triple helices' (FACIT) are involved in structural fibrils, but they do not appear to form independent fibrils. One of them (type IX) is also a PG (see below) by virtue of an attached glycan chain.

In the fibril, collagen molecules are aligned in parallel, but staggered, overlapping neighbours ahead and behind with three quarters of their length (Fig. 1). Collagen molecules attract and reinforce each other optimally in this configuration, which has no weak points at which transverse rupture could occur under tensile stress. The polypeptides in a collagen fibre are oriented along the lines of stress that it is meant to withstand, carrying the principle of stress-aligned polypeptides through to every level of tissue organisation.

The regular modular fibril contains orthogonal bands of polar amino acids that take up heavy metals in a repeated 'bar-code' pattern visible in electron microscopy (Fig. 1). Patterns vary between collagen types and sometimes drastically between species (e.g. Erlinger et al. 1993). In principle, it is possible to work back from the staining pattern to amino acid sequences in the collagen molecules (see Fig. 1).

Why don't collagen fibrils thicken indefinitely?

Collagen molecules aggregate spontaneously to fibrils under physiological conditions. It is vital that this does not continue indefinitely in vivo. Indeed, fibril diameters in tissues are regulated and often characteristic of the individual ECM. Thick fibrils are thought to be intrinsically stronger than thin fibrils. 'Young' fibrils are cylindrical and often as much as 20 times thinner than in old tissues (Parry & Craig, 1984), where cross-sections may have very irregular

Fig. 1. The collagen fibril hierarchy. Fibril-forming collagen types I, II and III contain 3 left-handed helical polypeptides called α chains, each with glycine in every third position. Type I collagen contains 2 α_1 chains and 1 α_2 chain. The triple super helix has a right-handed twist. Problems of classification (i.e. collagen or not) arise when the triple helix is not a major part of the molecule, nor even specific to the ECM (as in the case of the complement component, C1q). In the ordered quarter-staggered ('packing', QS) array bands of polar amino acids lined up across the fibril take up heavy metal stains in a bar-code pattern ('fibril') labelled *a–e*, which is asymmetric (BP), indicating in which direction the collagen molecules are pointing. The pattern repeats every D-period, of which there are 4.4 within the length of a collagen molecule. If 5 quarter-staggered molecules are rolled into a cylinder, a 'pentafibril' results, in which all molecules interact optimally with 2 neighbours, ahead and behind. Cupromeronic blue staining showing PG filaments at the surface of the fibril demonstrated 4 PG binding sites, in the *a*, *c*, *d* and *e* bands (top). The 4 PGs are different, giving rise to the one PG: one binding site hypothesis. In corneas of large animals all 4 are occupied by dermochondan sulphate (DS) or keratan sulphate (KS). In small animals (mouse, etc.) there is little or no keratan sulphate and the *a* and *c* bands carry no PGs (Scott, 1988). Each binding site contains at least one copy of an 11-amino acid homology (GDR, etc.) which has been found only in fibrillar collagens, in the PG-associated regions (top) (Scott & Glanville, 1993). In old human costal cartilage PG filaments are present inside and oriented along the axis of very thick collagen fibrils. They are regularly spaced and appear to be associated with protofibrils (Scott, 1990).

outlines—possibly because the thick fibrils arise from fusion of smaller fibrils (Scott & Parry, 1992). Fibril thickening is one of the most obvious and easily measured age-related phenomena. Nevertheless, it does not take place in all tissues, e.g. corneal stroma.

The thinnest viable collagen fibril cannot be smaller than the so-called pentafibril (Fig. 1), in which 5 collagen molecules form an annulus of ~ 4 nm diameter, quarter staggered with respect to their nearest neighbours, thus optimising intermolecular attractions. However, the thinnest fibrils observed in tissues are ~ 10 nm thick. Moreover, the much thicker (> 25 nm) fibrils that are the norm in most tissues appear to be aggregates—which are readily and reversibly dissociated—of ~ 10 nm thick 'protofibrils'. As a corollary, there are no covalent cross links *between* protofibrils, but only inside them. A protofibril is a gigantic covalent structure, a super building block. Modular architecture may allow economical remodelling of collagen fibrils via recycling of the protofibrils, avoiding the energy-expensive route of breakdown into peptides and amino acids followed by resynthesis (Scott, 1990).

What determines the ~ 10 nm diameter of protofibrils? Probably the geometry of the collagen molecule limits the extent to which it can accommodate to an increasing radius of fibril cylinder. In the pentafibril the angle between lines-of-centre joining 3 adjacent molecules is 108°, while it approaches 180° at the periphery of a very large fibril. The amino acids interacting between neighbouring molecules cannot be the same in both situations. This has repercussions on the formation of intermolecular covalent cross-links, which require good fits between molecules. It would also affect the energies with which free collagen molecules bind to the exteriors of growing fibrils. Given the choice between binding sites on different fibrils, they would go to those offering the best fit.

Fibrils are polarised, since the component molecules point in the same direction. Gieseking (1962) recognised that half the fibril polarities (as shown by heavy metal staining) were antiparallel to those of the other half. This is the picture in all normal (and fibrotic) vertebrate tissues so far examined. It could be a cell-mediated phenomenon or the product of random chance (Scott & Parry, 1992). The two groups of fibrils therefore cannot fuse or exchange protofibrils, thus limiting the extent of fibril diameter growth.

Echinoderm (sea cucumbers, featherstars, etc.) collagen fibrils disaggregate under neuronal control. In less than a second after an appropriate stimulus echinoderm 'mutable connective tissue' loses its strength, and collagen fibrils fray into protofibrils. This drastic self-destruct process might have survival value in allowing predators to get away with easily removable bits, rather than the whole animal. In the echinoderm *Antedon bifida* most fibril polarities were parallel (Erlinger et al. 1993). Perhaps this facilitates rapid reaggregation of the greater proportion of protofibrils, after neuronally stimulated disaggregation.

PGs probably play a role in limiting fibril lateral growth (Scott & Parry, 1992). Tissues with high PG contents tend to have thin collagen fibrils. This is partly due to hindrance to fibril fusion caused by the interfibrillar jelly. As ECM matures, the amount of perifibrillar jelly diminishes and, for example, in old tendons there is insufficient to prevent fibrils from growing large and knobbly by fusion. Conversely, when new connective tissue is laid down in mature tissues, as in fibrotic liver (Scott et al. 1994), the new collagen fibrils are thin and associated with considerable amounts of new PGs.

SOLUBLE POLYMERS—VERSATILE POLYANIONS

The soluble polymers are highly negatively charged, containing 50–100% by weight of polysaccharides known as anionic glycosaminoglycans (AGAGs, Fig. 2). Their charges repel each other, their associated counterions (Na^+ etc.) induce osmotic swelling and the AGAGs are stiff chains. Consequently they occupy much space, and exert strong swelling pressure at relatively low concentrations within the fibrillar matrix, an example of economy in ECM design.

PGs are proteins with attached AGAG chains. The proteins are gene products and the AGAGs are post-translational modifications. Trivial names have been given to whole PGs, but where a trivial name is used in this article, it applies only to the protein. Many proteins, including at least one collagen (type IX), carry AGAG chains. Certain amino acid sequences act as signals for the growth of AGAG chains on the protein, but the cause-effect relationship is not 100%.

The greater part of ECM AGAG is associated with a few types of PG, simply described as small, large and very large. Their proteins have different primary structures and shapes (Fig. 3). The small proteins are globular, with 1 or 2 AGAG chains from the chondroitin, keratan or dermochondan groups. The PGs often look like tadpoles on electron microscopy. The large and very large PGs have at least 1 globular and 1 linear polypeptide domain with 5–10, and up to 100 AGAG chains, respectively.

Very large PG(s) are responsible for the elastic response of articular cartilage to loading, like the stuffing in a cushion. There is so much of it in cartilages that, when stained, it completely overshadows everything else, in contrast to skin, cornea, etc., where the PGs are 5–50 fold less prevalent. The design of the large PGs suggests that they are also space-filling molecules, with a pressure-resistant function. The small PGs are found in a variety of specific relationships with other biopolymers.

Tying down the proteoglycans

There was an important question. Were the PGs free to move among collagen fibrils like fish in a net? Would the application of pressure then displace them and lead to permanent deformation of stressed tissue (Scott, 1975)? The problem was that PGs, unlike collagen fibrils, are invisible, dissolved in water. They had to be stained in a way that would preserve solution structures, if there were any. An electron-dense cationic dye (Cupromeronic blue) was designed and synthesised to do this, using 'critical electrolyte concentration' methodology. Some semblance of the shapes, sizes and orientations of the tissue polyanions were retained in the dye-substrate complexes, allowing supramolecular organisations involving PGs and collagen fibrils to be examined by electron microscopy (Scott, 1992*c*).

It was then seen that PGs in tendons (Scott & Orford, 1981), skin, etc., were associated regularly along collagen fibrils at 60–65 nm intervals (Figs 2, 3*a*). There was order in the 'amorphous ground substance'. Cupromeronic blue staining for AGAGs was found very frequently at the 'd' band and less frequently at the 'e' band of the collagen fibril (Fig. 2). This was so in skin, the sclera of the eye and all soft (as opposed to mineralised) ECMs containing type I collagen fibres, from many animal species. The AGAGs were usually dermochondan sulphate, and immunohistochemistry confirmed that the PGs contained decoron proteins. Binding to the fibrils is probably via the protein (Scott, 1988) (see below).

The corneal stroma presented a similar picture, with additional PG binding sites at the 'a' and 'c' bands, associated with keratan sulphates. Cornea is the only tissue with PGs at these loci. The hypothesis of one PG:1 binding site was proposed, and a map embodying this concept was drawn up (Scott, 1988) (Fig. 2). Relative frequencies of occupancies of these sites varies with the tissue and the species.

Regular orthogonal PG:collagen fibril-surface association was observed in quite remote animals, the echinoderms. Their PGs contain chondroitin sulphate-based AGAGs which differ in important details from those found in vertebrates, and their fibrils show a very different D period staining pattern. Nevertheless, this demonstrates the great evolutionary antiquity and hence functional importance of PG:collagen fibril interactions (Erlinger et al. 1993).

A tissue which contains much of the type I collagen in the body, but little or no proteodermochondan sulphate (PDS), is bone (Scott, 1988). Correspondingly, no orthogonal PGs were observed at the gap zones. Since PDS in nonmineralised tissue occurs at or near a site of calcification of the collagen fibril, it was speculated that in its presence mineralisation would not occur. This would be an important function, since we might otherwise solidify. Calcification of collagen fibrils is a serious threat in some pathologies, especially in ageing animals.

Intra-fibrillar AGAGs are seen in some tissues, located parallel rather than orthogonal to the fibril axis. They are probably anchored at the gap zone at the surface of protofibrils, with about 1 AGAG per collagen molecule length ($\sim$ 300 nm) of protofibril (Scott, 1993*a*). They are seen in thick fibrils, particularly in cartilages and intervertebral discs, that are rich in type II collagen (Scott, 1990). They may belong to type IX collagen molecules, present at the surface of protofibrils. Covalent crosslinks between type IX and type II collagens have been found in cartilage.

The presence of similar intrafibrillar structures in echinoderms attests again to the importance of these interactions throughout evolution. The AGAGs inside the fibrils were different from those on the fibril surface (Erlinger et al. 1993).

What do the PG proteins do?

It is highly likely that the specific association of small PGs with collagen fibrils is mediated through the protein. The primary and secondary structures of the small PG proteins (e.g. decoron) strongly resemble each other. It seemed likely that their binding sites would also be similar and indeed homologous sets of 11-amino acid sequences were found only in the a, c, d and e bands, where small PGs associate, and only in the fibril-forming types I, II and III collagens (Scott & Glanville, 1993). The sequences (Fig. 1) were highly conserved in the 4 species (man, cow, mouse and chick) so far investigated, as were also the PG binding patterns in these species.

The sequences were not affected in any case of osteogenesis imperfecta in which a mutated glycine residue in collagen was identified, which by now

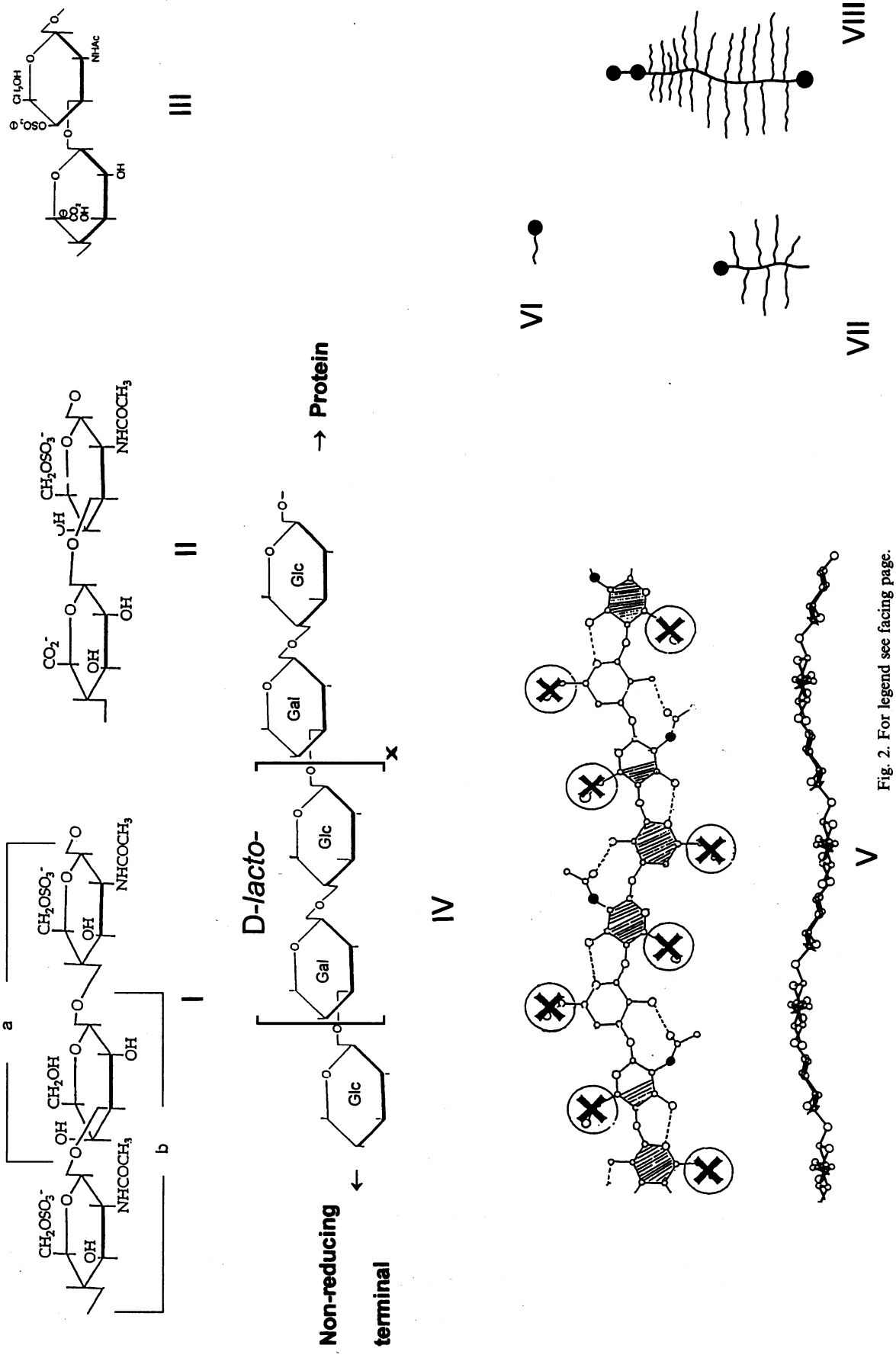

Fig. 2. For legend see facing page.

include nearly 80 of the possible total of 334 (R. Tenni & J. E. Scott, unpublished). Arguing from the premise that the more important an interaction is in vivo, the less likely it is that mutations affecting it could carry through to a viable animal, the absence (up till now) of mutations affecting these sequences in living offspring is evidence for their vital significance.

Even in dermatosparactic cow hides, which are so weakened by inability to form normal collagen fibrils that the skin tears under its own weight, the small PG PDS was found at the d band wherever there was enough organised collagen to produce a recognisable D periodicity (Scott et al. 1989). The ECM which accumulates in abnormal amounts in fibrotic liver showed normal PDS-binding patterns (Scott et al. 1994). In the very old and the very young, in mechanically stressed and in unstressed tissue, PDS was found at the d band. It is not yet proven that PDS associates similarly with types II and III collagen fibrils, but it is likely.

PG : COLLAGEN INTERACTIONS AND ECM SHAPES

More than any other organ, the eye demonstrates the vital roles played by ECM in providing the right shapes and conditions for it to function.

Corneal stroma is an elegant ECM, of constant morphology and morphometry. The diameters of the collagen fibrils and the interfibrillar gaps must not vary significantly or the tissue is opaque and the eye is blind. Relationships between PGs and collagen fibrils are consistent and quantitative.

The simplest and most widespread shape in stained corneal AGAG structures was a fibril-to-fibril tie or bridge stretching across as many as 4 fibrils and the intervening spaces (Fig. 3). Their lengths and thicknesses implied that they contained several AGAG molecules.

Dermochondan sulphate molecules isolated from corneal stroma were as long as the gap spanned by AGAG bridges between 2 fibrils was wide. Moreover, the molecular lengths were all similar. One end of the AGAG is very probably anchored to a fibril via the PG protein. Contact between the distal tips of the anchored AGAG chains and neighbouring fibrils is unlikely to be avid and permanent. However, an antiparallel duplex in which 2 AGAG chains support each other, with each linked noncovalently via their proteins to neighbouring collagen fibrils fits the data (Scott, 1992*a*, *c*). The postulated dumbbell-like structures (Fig. 3) form spontaneously in solution. PDS is thus a bifunctional cross-linking molecule, with the PG protein binding to collagen, and AGAG aggregating with AGAG (see below).

In this scenario the AGAG chains are yardsticks which maintain collagen fibrils at separations determined by the AGAG chain lengths. Formerly it was a mystery as to how the collagen fibrils in the corneal stroma maintain a regular arrangement, since

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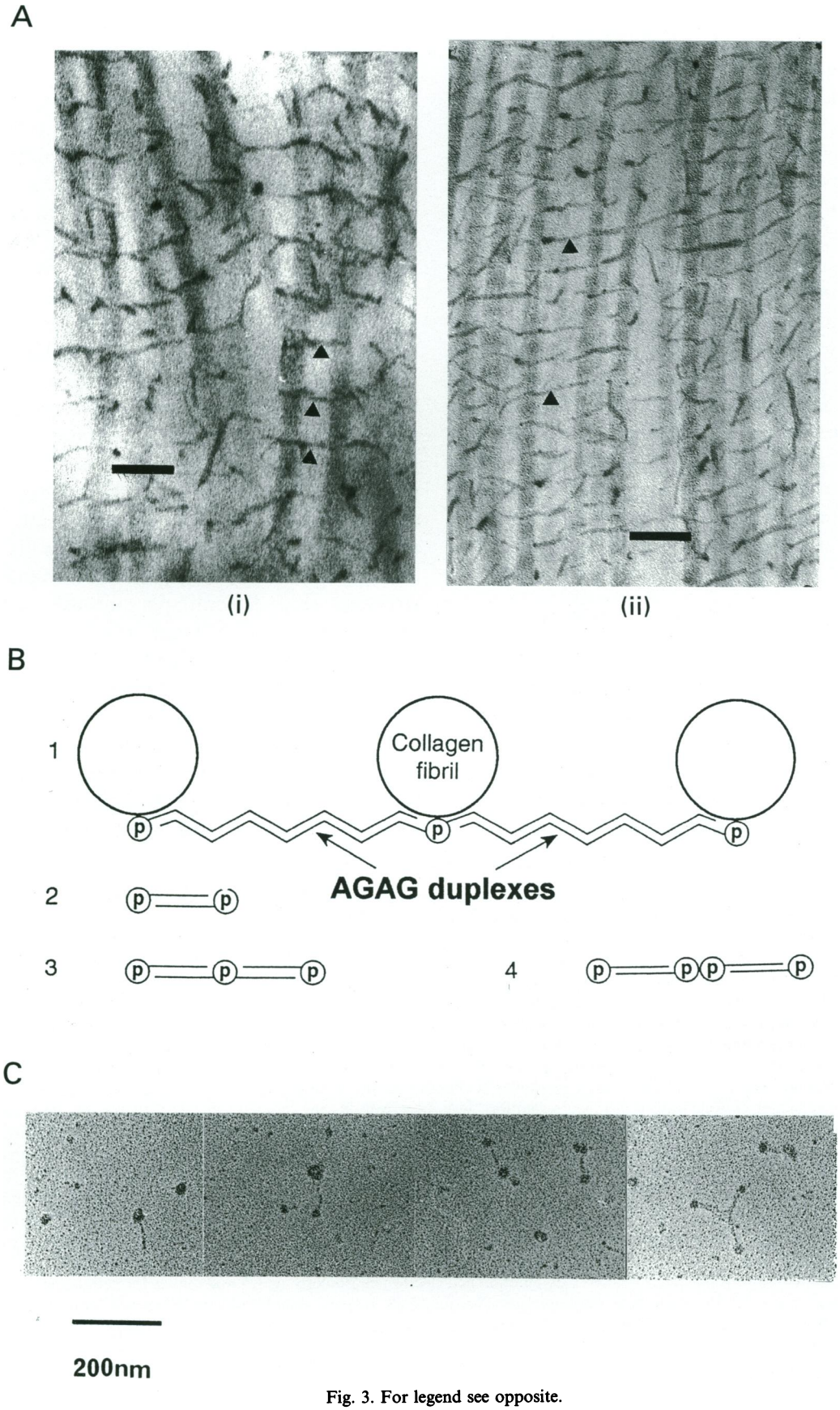
Fig. 2. Soluble polymers. *Carbohydrate polyanions* (*AGAGs*). (*a*) *Primary structures.* ECM PG-associated AGAGs are based on the same polymer backbone, polylactose (IV), which carries acetamido (CH_3CONH-) and sulphate ester (-OSO_3^-) substituents. I*a* (or I*b*), II and III are representative disaccharides from the keratan, chondroitin and dermochondan (formerly dermatan sulphate; Scott, 1993*b*) series, respectively. None of the sugars are oxidised in the keratans, the glucose residues are oxidised to D-glucuronates in the chondroitins and some of the carboxylate groups of the D-glucuronates (on the left in II) are epimerised to L-iduronates (on the left in III) in the dermochondans. I*a*, II and III are written with the hexosamine residues to the right, according to convention going back to the 1950s. It would now be more logical to write them all with the glucose-based ring to the right as in I*b*, but for the fact that current analytical methods based on enzyme digestions measure disaccharides II and III and their analogues. The hyaluronan backbone is the same as that of the chondroitins, except that both rings are glucose-based. (*b*) *Secondary structures.* The shapes that matter are those in solution. NMR showed that geometries around the glycosidic bonds were very similar in chondroitin and keratan sulphates, implying that the identical backbones took up very similar shapes, twofold helices, in aqueous solution. V shows a twofold helix characteristic of ECM AGAGs. The cross-hatched parts are hydrophobic patches. The structure is a keratan, with sites usually carrying a negative charge (sulphate esters) marked X. The anionic sites are identically placed in chondroitin-6-sulphate, half being carboxylate and the others sulphate esters. The dotted lines are probable hydrogen bonds. Dermochondan sulphate adopts a very similar twofold helix in water (Heatley & Scott, unpublished). The AGAG twofold helix has 2 waves, seen in plan in V upper, and in side view in V lower. (*c*) *Tertiary structures* (*aggregation*). The more highly charged the AGAGs, the greater their mutual repulsion and the lower the stability of their aggregates. The positions of anionic sulphate ester groups determine whether duplexes can form (Scott, 1992). In chondroitin-4-sulphate they are present along the central axis of the polymer, giving a highly concentrated polyanionic field. In chondroitin-6-sulphate, sulphates are at the periphery of the twofold helix (V); the polyanionic field is less intense and mutual repulsion is weaker. Computation and electron microscopy agreed that duplex formation was possible in this case but not in that of chondroitin 4 sulphate. *Proteoglycans* The small PGs (VI) are globular proteins (decoron, lumicon, etc.) with 1 or 2 AGAG chains. They associate specifically with collagen fibrils at the gap zone (see Fig. 1). The very large PGs (VIII, based on the protein core, aggrecon) concentrate as much charge in as small a volume as possible. So much of the molecule is present in so many side arms that cleavage of even a complete AGAG chain (e.g. by free radical damage) represents a small change in the properties of the whole molecule. Hyaluronan, on the other hand, suffers disproportionately more damage from most cleavages of its single chain. Keratan and chondroitin sulphates are present together in the very large PG. The possibility (and some in vitro evidence) exists that these AGAGs could interact in multivalent aggregates to stabilise and organise the interfibrillar jelly in the cartilage shock-absorber. The large PGs (VII) share some of the features of VI and VIII, resembling the latter in shape and the former in possessing dermochondan chains, which have not been found in VIII.



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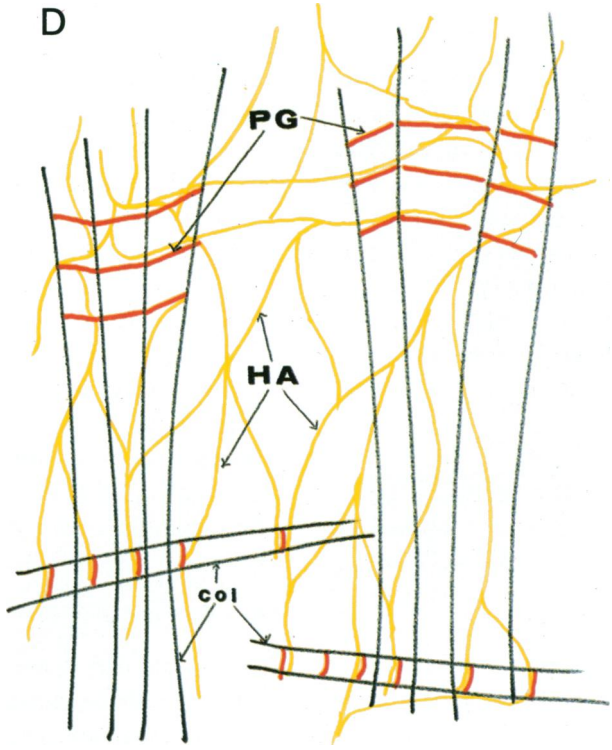

Fig. 3. Cornea, developing tendon and vitreous humour. The cornea is a slice of water (80% of the wet weight) stabilised by fibrils and PGs. The vitreous is ~ 97% water, stabilised similarly. Vitreous and cornea use analogous ultrastructural motifs, based on specific PG:collagen interactions. These motifs are also clearly visible in developing connective tissues, e.g. young tendons. Collagen fibrils in corneal stroma are of roughly constant diameter (~ 26 nm, in plastic sections for electron microscopy) separated by a constant distance (~ 66 nm, centre to centre). PG filaments (arrowed) stained by Cupromeronic blue bridge 2 or more collagen fibrils (A1, bar, 60 nm). This (bovine) tissue was digested with keratanase, removing keratan sulphate to demonstrate dermochondan sulphate more clearly. The lengths and thicknesses of many of the filaments suggest that they contain more than one PG molecule. Similar structures dominate in developing (7 day postpartum) rat tail tendons (A2, undigested; bar, 100 nm). Both tissues were lightly stained with UO_2^{++} to show a faint banding pattern on the collagen fibrils. The PG proteins (p) are attached noncovalently to collagen fibrils. The extended length of dermochondan chains isolated from cornea corresponds to the fibril centre–centre distance, and all are of about the same length. The PG filament bridges are tangential to the fibrils (structure B). The bridges, of antiparallel duplexed AGAGs, accommodate the latter without overlaps. Their average length then determines the average separation of the collagen fibrils. This organises the fibrils with respect to each other, and helps determine the shape of the ECM. Proteodermatan sulphates (PDSs) spontaneously form the dumbell shaped structures postulated in B2, as shown by electron microscopy (C). Other arrangements of PDSs that could give extended bridges looking like the filaments in A are shown in C3 and C4. So far only dermochondan and keratan sulphates have been implicated in such structures. Both can self-aggregate in solution. Aggregates involving both together may be present in corneal stroma (Scott, 1994). The vitreous contains very thin (~ 10 nm) collagen fibrils in sprays, quite widely separated, as in D. They are bridged by AGAGs, probably chondroitin-6-sulphate. To keep the sprays in order, so that some are at right angles to others and they do not collapse together, hyaluronan is suggested to form a quarternary system (D, taken from Scott, 1992*b*). Hyaluronan-AGAG interactions occur in vitro, and computation suggests that they are likely (Scott, 1992*a*). The dramatic consequences of breakdown of this invisible system are seen with age. Slow deterioration occurs from water-clear infant samples until, in old vitreous, 'floaters' are formed which adversely affect vision (Sebag, 1987).

each fibril is surrounded by 'space'. Consistent with the yardstick hypothesis, the lengths of dermochondan sulphate chains vary in different tissues, being shorter in the sclera of the eye or skin, where collagen fibrils are packed more closely than in corneal stroma (Scott, 1992*c*). PDS is proposed as an organiser of ECM shape, orienting collagen fibrils with respect to each other.

Even more transparent than cornea is the vitreous humour of the eye, which is water stabilised by small amounts of very thin type II collagen fibrils and AGAGs, the least possible amount of nonaqueous material in the light path from the front of the eye to the retina. It was thought to be a relatively random structure, but recent work with Cupromeronic blue showed that at least some of the fibrils were cross-linked via AGAG bridges in parallel bundles similar to those in corneas, developing tendons, etc. The vitreous, in this respect, looks like very dilute cornea (Scott, 1992*b*). It is speculated that hyaluronan, which is present in some large mammalian eyes in high (up to 1% w/v) concentrations, interacts with the other AGAGs (mainly chondroitin sulphate) as shown in vitro and by computer modelling, to give even more

extensive supramolecular complexes (see below). In later life the AGAG components of this jelly-like material are less well organised, possibly due to free radical damage. The collagen fibrils then stick together, interfering with the passage of light, giving rise to vision problems called 'floaters' that particularly affect myopic people (Sebag, 1987). This dramatic change illustrates the importance of supramolecular organisation in maintaining ECM function. The capacity to prevent collagen fibrils from fusing together is one that fails with age in many ECMs.

AGAGS AND THEIR SHAPES

Supramolecular organisation is the key to biological function, and the key to ECM supramolecular organisation is the shapes of the AGAGs, which determine their ability to form tertiary structures. ECM AGAGs, mainly elucidated by Karl Meyer's group, comprise the chondroitin, keratan and dermochondan (formerly dermatan) sulphates and hyaluronan. They are chains of disaccharides (Fig. 2). There are 16 possible chondroitin sulphate disaccharides (Scott, 1993*a*), of which about 10 have already been found in tissues, and 32 each from keratan and dermochondan sulphates. There are astronomical numbers of possible polymers in each group, e.g. 10^{45} possible keratan sulphate molecules of 30 disaccharides (a common size in tissues)!

The demonstration of domains within keratan sulphate chains by Greiling's group, block copolymers in chondroitin sulphates by Fransson's group, and the development of specific endolyases by Suzuki's group (see Scott 1993*a* for reviews) has forced a reassessment of AGAGs in tissues. Disaccharide *sequences* are now considered significant and inevitably they will be written in single letter codes, as are amino acids and nucleotides in proteins and nucleic acids. The immense variability in AGAG structures is a potential goldmine of molecular recognition phenomena.

A major reorientation came with the realisation that chondroitins and keratans were not fundamentally dissimilar, as had been thought for 40 years, but were based on an identical polymer backbone, polylactose (Scott, 1992*c*). This strange oversight was due to the convention of writing disaccharide structures (Fig. 2) with hexosamine residues to the right, aligning galactosamine with glucosamine, rather than galactose with galactosamine. This 'frame-shift' is significant because identical backbones imply very similar repertoires of polymer shapes. All ECM AGAGs have very similar shapes in solution based on a twofold helix and similar potentials for tertiary (supramolecular) structure formation (Scott, 1992*a*). The substituents (acetamido, sulphate) may stabilise or forbid some shapes by attractive or repulsive forces.

Models of twofold helical AGAGs reveal another remarkable similarity. They all have large hydrophobic patches, stretching over 3 sugar rings, consisting of 7–9 CH units—equivalent to a fatty acid. This raises possibilities of hydrophobic bonding to other molecules with hydrophobic structures, including lipids, proteins—and themselves (Scott, 1992*a*).

These properties could explain why hyaluronan increases the fluidity of red blood cell membranes, enhances the transport of lipids from the intestine, and binds inflammatory lipid-like molecules in arthritic joints (see Scott, 1992*a*, for review).

How do the AGAGs get together?

The twofold helices are flat tapelike chains, each face of the backbone polymer being identical with the other, although antiparallel. The hydrophobic patches are present on alternate sides along the molecule (Fig. 2). This pattern was termed 'ambidexteran' (from ambidextrous), since both 'hands' of the molecule can be used equally.

Duplex and higher aggregate formation was demonstrated by electron microscopy. The gap between the atomic dimensions of NMR and the supramolecular scale of electron microscopy was bridged using computer simulations by molecular dynamics in a 'water' environment. The emerging scenario is that AGAG aggregation is driven by hydrophobic and hydrogen bonding, opposed by electrostatic repulsion (Scott, 1992*a*). This is precisely analogous to DNA double helix formation.

Moreover, AGAGs of different structures can form mixed aggregates. The similarly shaped secondary structures shared by all ECM AGAGs offer many opportunities of this kind. Less highly charged AGAGs, e.g. hyaluronan, keratan and chondroitin are intrinsically more promiscuous in their liaisons, enabling them to interlink between structures containing more highly charged AGAGs, as proposed in the vitreous of the eye (Scott, 1992*b*) (see Fig. 3).

Hyaluronan

Hyaluronan forms a typical twofold helix in solution with an extensive hydrophobic patch, as do the other ECM AGAGs (Scott, 1989). It was implicated in supramolecular organisation in cartilages over 2 decades ago, as a molecular thread along which

aggrecon PGs were strung in very large complexes, comparable in size to a cell. This interaction involves the PG protein and hyaluronan but, in the light of recent findings on AGAG-AGAG aggregation, there may be additional intermolecular liaisons.

Hyaluronan is much larger than the other AGAGs. Because it is also less highly charged, it forms infinite meshworks in very dilute solution, consisting of thin re-entrant 3-dimensional networks, changing into much thicker strands and finally into sheets at physiological concentrations (e.g. > 1 mg/ml, as in synovial fluid). These changing morphologies are based on easily reversible, ordered interactions, produced by growth on both faces of hyaluronan ambidexteran molecules. Low molecular mass hyaluronan forms aggregates which do not always overlap at low dilutions, appearing as independent islands (Scott et al. 1991).

Many cells possess receptors that anchor hyaluronan meshworks as pericellular halos, thus preventing close approach of other cells and macromolecules. High molecular mass hyaluronan was found to protect fibroblasts against free radicals generated by enzymes that probably could not get close to the cells. Low molecular mass hyaluronan was less effective (Presti & Scott, 1994). Conversely, these aggregates may hide physiological properties that could be important. Chang et al. (1985) demonstrated that heat 'denatured' hyaluronan was far more active in inhibiting complement activation of red blood cell lysis than unheated hyaluronan. The effect of heating was reversible.

Conclusion

The concept of 'amorphous ground substance', with its implied randomness, has had its day. There is real order and organising potential among the water-soluble AGAG components of ECM. It seems highly probable that these properties help define the shapes of macroscopic ECMs, and hence of that of the organism.

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