

Preface

Professor J.E.Scott was awarded the Barbara Robert Medal and gave the eponymous lecture entitled "*Interactions between glycosaminoglycans in the extracellular matrix and the shape module concept*" earlier this year. The Barbara Robert Medal is awarded annually by the Societe Francaise du Tissu Conjonctif. This was the 25th occasion on which it was given, in Rennes at their Annual General Meeting. The French Society is slightly older than the British society.

The following article is based partly on the Barabara Robert Lecture and partly on the plenary lecture Professor Scott gave to HYALURONAN 2000, entitled "*Biological Properties of hyaluronan are controlled by tertiary structures*".

Introduction

Why do we study connective tissues? Because they are there, is the short answer. You have to study something, otherwise you don't get your degree, C.V., salary, tenured position, is the defensive and practical answer. How about the pleasure of making discoveries in a universally

important field, in which there is still a chance of driving your own bandwagon?

When the legs on proteoglycan centipedes have all been counted, gene sequences decoded and microtitre plates filled to overflowing with antigens, there remain the questions of structure-function, which attract and repel scientists in unequal measure, because boundaries have to be crossed and new fields studied without the benefit of organised curricula and timetables. Departments with a rich culture are hard work and thin on the ground. It is easier to conform to national guidelines and easier to get an interview if you stay in line.

Chemical morphology is still a new subject, although my Chair was recognised by Manchester University 25 years ago. It is a clear concept, unlike the hyped redundancy 'Structural Biology'. There is no biology without structure. Chemical morphology is the chemistry of shape and the shape of chemicals. The former without the latter does not exist. What better field in which to establish the fundamentals of the subject than that of connective tissues?

STRUCTURE and FUNCTION in EXTRACELLULAR MATRICES DEPEND on INTERACTIONS BETWEEN ANIONIC GLYCOSAMINOGLYCANS

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Proteoglycan:collagen fibril interactions

Connective tissues are about shape. Their extracellular matrices (ECMs) were defined as 'networks of fibres and soluble polymers which evolved to take the stresses of movement and the maintenance of shape' [1]. Pulling stresses are taken by the fibrils and compressive forces are opposed by the expansile water-soluble carbohydrate-rich polyanions (mainly proteoglycans (PGs))[1]; a neat division of labour. PGs should have fixed relationships with

the fibrils to stop them moving randomly under compressive stress, like beans in a bean bag, thus losing the essential element of a permanent shape [1]. This scenario was widely accepted but there were questions. What keeps collagen fibrils in the regular alignment found in tendons, skin etc.and most obviously in corneal stroma, when there are no visible regular connections between fibrils? This article reviews evidence that the anionic glycosaminoglycans (AGAGs) chondroitin, keratan and dermochondan

sulphates and hyaluronan, can form supramolecular organisations which take tensile stresses as well as compressions. They assist in orienting collagen fibrils, helping to maintain shape, as part of '**shape modules**'. Specific PG-collagen relationships not only tie down the PGs, they tie down the collagen fibrils as well.

Specific binding of PGs to collagen fibrils was demonstrated by electron microscopy, using reagents (Cupromeronic blue etc) that showed water-soluble PGs in something like their solution configuration [2]. PG AGAGs were seen to bridge between collagen fibrils in beautifully regular orthogonal arrays (Fig.1a). They 'decorate' the fibrils, hence the romantic name 'decoran' - which should apply to the entire group of collagen-associated PGs but which,

due to the multiplication of trivial names, is limited to dermochondan (DS) PGs. The AGAGs are carried on small leucine-rich PGs that can associate at four specific binding sites per fibril D period (Fig 1b). Keratan sulphate (KS) PGs were found attached to collagen fibrils only in the corneal stroma of the eye, whereas (DS) PGs associate with collagen fibrils in every soft (non-mineralised) ECM. Patterns of occupancy of these sites differed between tissues and species and with age. Biochemical and ultrastructural studies demonstrated a non-covalent link between the protein part of the PG and specific amino acid sequences along the collagen fibrils [3].

The electron histochemical reagent Cupromeronic blue shows the AGAG part of the bridge, the length of which is measurable in electron micrographs [4]. AGAG chains in the corneal stroma exactly spanned the interfibrillar spaces, suggesting that AGAGs play a part in determining the distance between fibrils, which is important in establishing the shape of the ECM. Accurate and constant interfibrillar spacings between corneal collagen fibrils are vital in maintaining corneal transparency [4]. The combination of **collagen fibril_ PG protein-AGAG-PG protein_ collagen fibril** constitutes a module which is repeated every

60nm (i.e. per D period) along the collagen fibrils, hence it was termed a '**shape module**' (Fig.2).

Crucially, cultures of cells from a human foetus that did not express the protein core (decoran) of decoran were unable to form shape modules and ordered collagen fibrils, although DS chains were produced in normal amounts [5]. The ECMs in the decoranless proband cell cultures were totally disorganised, unlike normal cell cultures from donors of all ages - and even cultures produced by cells from patients with different types of osteogenesis imperfecta. These results proved the fundamental importance of decoran PG in organising ECMs.

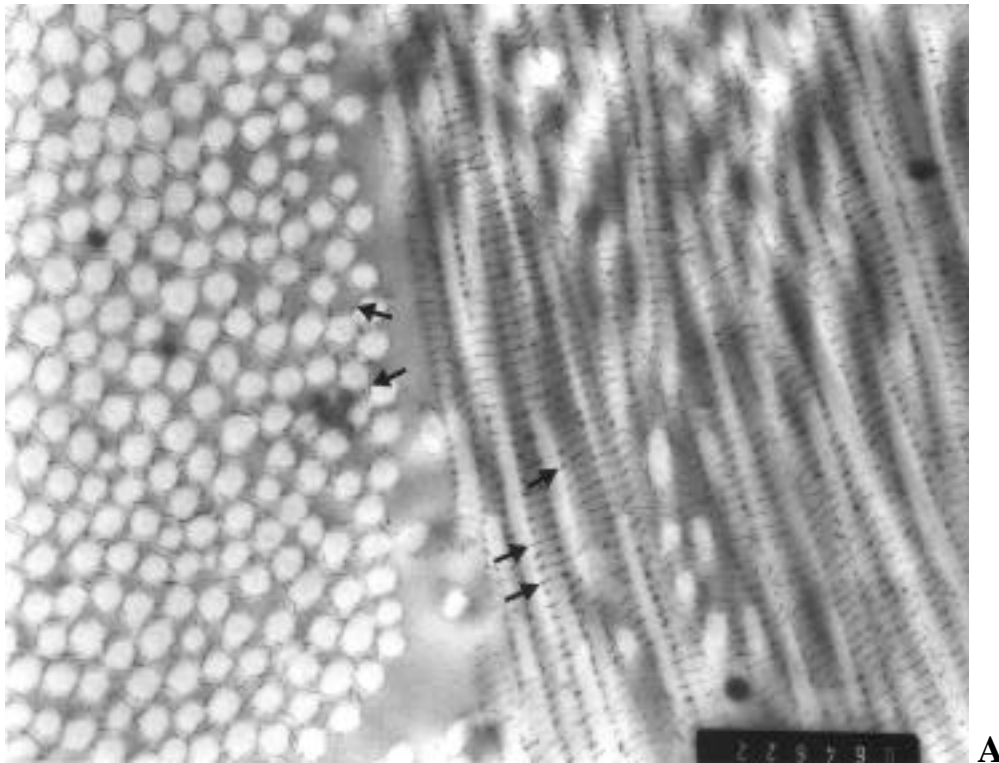
The number of binding sites on collagen fibril surfaces is equivalent to the number of available decoran molecules, i.e each fibril surface binding site is occupied by a decoran molecule [6]. The number of fibril surface binding sites and hence decoran molecules depends on the diameter of the fibril (~30 per D period for a diameter of 50nm) but the number of AGAG bridges is much less, so each bridge must contain more than one AGAG chain. Stereological studies suggested that more than two AGAG chains were present in each bridge, implying that AGAG chains collaborate together in the shape module [4].

The Shape Module and AGAG-AGAG interactions

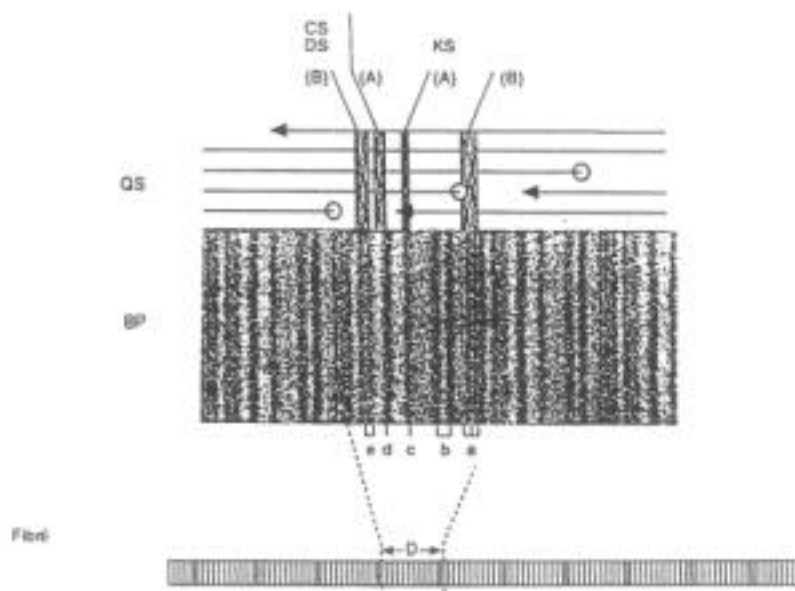
Two interactions underpin the shape module; 1) PG protein-collagen and 2) AGAG-AGAG. There is evidence from tissues [6,7] and *in vitro* experiments that the protein core of the PG interacts with the fibril. Protein-protein interactions are the lifeblood of biochemistry but AGAG-AGAG interactions are a new theme. They seem unlikely, since the participants are highly charged, which should result in mutual repulsion rather than aggregation. In the 1970s L-A Fransson's group produced evidence for self-aggregation of DS and heparan sulphates in solution, but the mechanism was unknown and aggregate structures were equally mysterious. This article

will emphasise the resemblance to DNA aggregation, in which attractive forces counter mutual repulsion. The key structure in the shape modules, which keep the collagen fibrils oriented, in register and probably correctly

spaced, is the AGAG bridge. The total contribution to the bridges must be shared between PGs based on the participating collagen fibrils. Two possibilities arise; - either the AGAG chains in a bridge are wholly



A

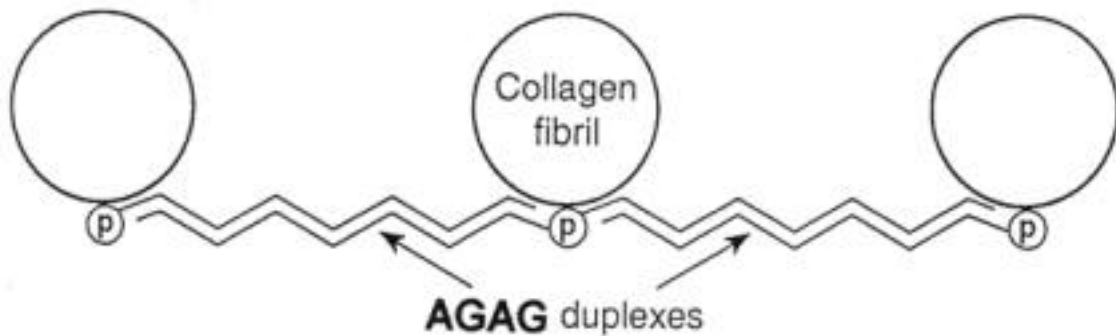


B

Figure 1 (A) Electron micrograph of rabbit skin stained with Cupromeronic blue to show AGAGs which are attached to small leucine-rich proteins. The dark filaments (arrowed) are AGAGs. The collagen fibrils show white against the electron-dense plastic embedment. Left; the fibrils are in cross section (~100 nm diameter) and Right; in longitudinal section. AGAGs are in regular orthogonal interfibrillar arrays at separations of ~60nm (right), frequently spanning space between fibrils.

(B) Map of binding sites against fibril a-e banding pattern produced by staining with uranyl. The a-e banding pattern (BP), repeated 4.4 times per collagen molecule (i.e. the D period) arises from differential uptake of uranyl by bands of aminoacids of differing polarity, providing a guide to the position of the PG binding site against the primary structure of collagen in the fibril. The PG binding regions are shown as filled bands in the quarter-staggered (QS) array at the top.

PGs carrying DS AGAG chains are specific to the d- or e-bands. KS PGs are found at the a- or c-bands, so far only in the corneal stroma of the eye. The AGAGs in the the d band are distinguishable chemically from those in the e band, as are the KS AGAGs (A) and (B) in the c-bands from the a-band. Relative occupancies a/c and c/d vary with species (Taken from Ref. 7).



The Shape Module

Figure 2. Generalised scheme of shape module (interfibrillar bridges) regularly occurring every ~60nm along the fibrils. PG protein cores associate specifically but non-covalently to binding sites on the fibril (Fig.1). Collagen fibrils (in cross section) are linked by antiparallel duplexes or higher aggregates of AGAGs of the CS/DS or KS families, depending on the tissue. This scheme shows the central PG with two AGAG chains, but probably the structure involving PGs (such as decoran) carrying only one AGAG chain, is repeated in a staggered array above and below the plane of the diagram.

parallel, or they are antiparallel in varying proportions. The evidence, from modelling and observation, suggests that the latter predominates. A crucial observation by electron microscopy of rotary-shadowed decoran complexes showed beyond doubt that aggregation was *spontaneous* between the AGAG chains in **antiparallel** arrays [7,8]. 'Antiparallel' is the key concept in understanding the structure and properties of the AGAG bridge. Two PG proteins based on adjoining collagen fibrils are linked by a bridge of two or more antiparallel AGAG chains (Fig.2)

Although electrostatic repulsion works at comparatively long ranges, attractive forces (van der Waals, dipole-dipole etc) critically depend on close approach of the participants. Thus, the shapes of the aggregating molecules must be highly complementary for these forces to be important.

AGAG molecular shape was for long a confused topic. Classical methods of polymer study in aqueous solution produced pictures based on averaged behaviours of huge numbers

of molecules. Interpretations were based on treatments which did not involve chemical specificity. Conversely, X-ray fibre diffraction provided valuable insights - but also a range of possible structures that were difficult to relate to solution properties. Three techniques give the necessary resolution and chemical specificity. NMR resolved the position of atoms within AGAG molecules in aqueous solution, allowing secondary structures to be identified; electron microscopy of rotary-shadowed polymers showed supramolecular organisational possibilities and molecular modelling helped to bridge the gap between the two. If modelling with a secondary structure determined by NMR led to a tertiary structure compatible with electron micrographs, a basis for understanding biological function was available [6-8].

Historically, the first phase concentrated on the question, *do AGAG molecules take up preferred configurations in aqueous solution?* The consensus had suggested that random coils were all that was present. However, a specific chemical reaction, glycol cleavage of the uronate residues by periodate, indicated that

hyaluronan (HA) and the chondroitin sulphates (CS) had a polymer shape, - a secondary structure. This structure fitted X-ray diffraction data of Prof. Ted Atkins and a well-defined highly hydrogen-bonded two-fold helix was developed from the twin approaches [9] (Fig. 3C). Detailed NMR studies with my colleague Dr. Frank Heatley of the Manchester University Chemistry Dept. over almost a decade proved the existence of the two-fold helix in water, dimethyl sulphoxide and in mixed solvents (Fig. 3A). There was some controversy about how much of this structure existed at any one moment, but unequivocally the twofold helix exists in a variety of conditions, and its geometry emerges easily from the primary structures of the entire group of ECM AGAGs (HA, CS, DS and KS) [10].

With hindsight we were, to an extent, off-target. To use the DNA analogy, if attention had concentrated on single-stranded polynucleotides it would not have paid the enormous dividends that the investigation of the double helix provided. Similarly, the work on fragmented and uncomplexed AGAG molecules gave only ghostly hints of the dramatic functional properties of supramolecular organisations - but it provided the foundation for the elucidation of tertiary structures.

Subsequent work on supramolecular AGAG structures tended to concentrate on HA, since it has the simplest chemistry of the ECM AGAG family, with no sulphate groups, epimerised residues etc. The lessons learnt from HA studies nevertheless can be extended to the other ECM AGAGs, since they are also 1e-4e, 1e-3e linear polymers of C1 chair-shaped pyranose sugars. An early unexpected dividend from the two-fold helix was the realisation that there was an extended hydrophobic patch, spread over three sugar units, present on both sides of the polymer, which is tapelike in shape [11]. HA and the other AGAGs are thus amphiphilic, which was a new slant on their properties, previously thought of as totally hydrophilic. Interactions with phospholipids were demonstrated by Peter Ghosh and Yvonne Pasquali-Ronchetti, and it is relevant that HA is

biosynthesised in lipid cell membranes, as shown by Peter Prehm. Each side of the tape is identical with the other, but they run in opposite senses, i.e. antiparallel. I called these molecules 'ambidexterans', from ambidexterous, able to use both hands equivalently.

Can AGAG molecules interact with themselves, via hydrophobic bonding, based on the hydrophobic patch? Modelling of the secondary structure derived from NMR studies showed that intimate overlap of two HA molecules could be achieved only if they were antiparallel. The gentle curves in the HA twofold helices, present both in plan and elevation, were then complementary (Fig. 3B). Very significantly, in this duplex the acetamido NH was in a position to H-bond perfectly with carboxylate on the partnering molecule [12]. Two forms of bonding could then operate, H-bonding and hydrophobic bonding, which might outweigh electrostatic repulsion between the two AGAG molecules. The resemblance to the DNA double helix story is very close, in general terms.

Proof of the existence of the H-bond approximates to complete proof that the structures of the aggregates are as modelled. It is crucial that the acetamido group is fixed in space by the H-bond (Fig. 3B). Because it is not free to rotate the ¹³C carbonyl NMR signal is greatly broadened [13]. Small HA oligosaccharides do not form a tertiary structure and their acetamido groups are free to rotate, giving a sharp NMR carbonyl signal. The capacity of the carboxylate to accept a H-bond is removed by methyl ester formation and the acetamido group is then free to rotate [13]. The tertiary structure thus demonstrated is reversible on heating and cooling and on raising and lowering the pH between 7.0 and 12.5 (Scott and Heatley, in press).

The beautiful tertiary structures are exactly analogous to the β -sheets found in proteins. The AGAGs share the properties of both proteins and polysaccharides in this respect, with no other glycans able to adopt β -sheets being known to me.

It is now clear that the tertiary structure hides and sequesters active sites that have important biological properties, in the fields of angiogenesis, complement inhibition etc. HA is a molecule that has different biological activities depending on its molecular weight and/or tertiary structure, in which it may also be unique.

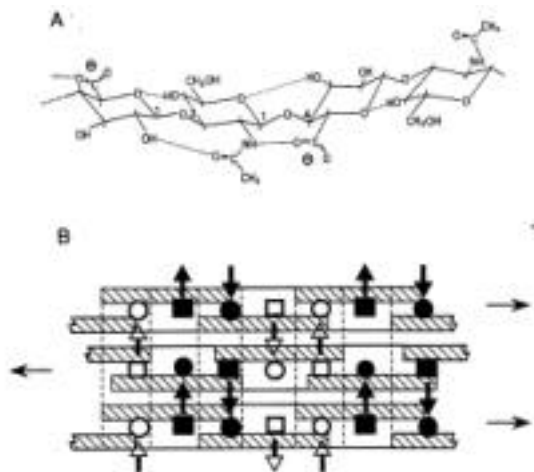


Figure 3. Primary, secondary and tertiary structures taken up by HA in aqueous environments.

(A) A tetrasaccharide fragment of hyaluronan, showing H-bonds (dotted lines) in a twofold helix. H-bonds between acetamido NH and carboxylate seen in dimethyl sulphoxide solution are largely replaced in aqueous solution by a water bridge.

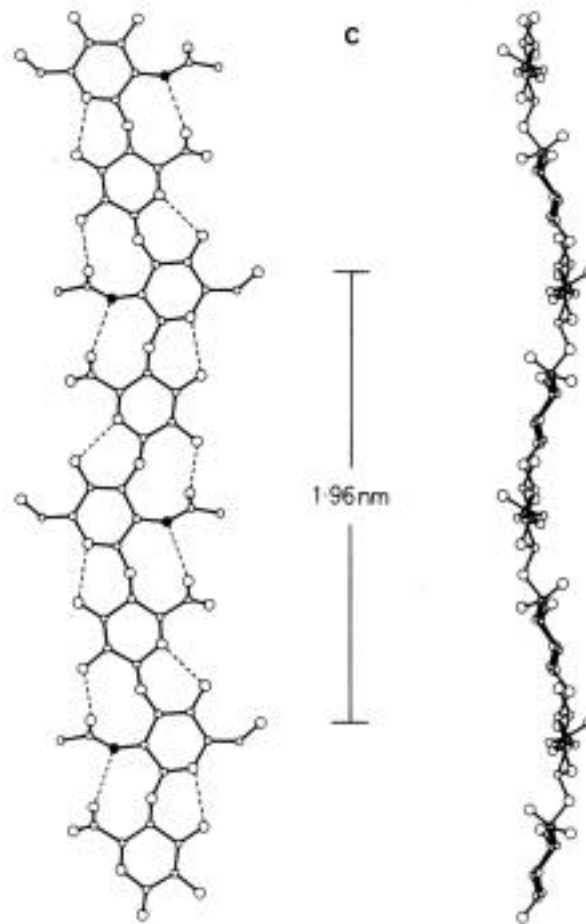
(B) Three HA chains in a tertiary structure based on NMR and X-ray findings, i.e. twofold helices with gentle curves in the polymer backbone in two planes at right angles [9,13] (see (C) below). Vertical dotted lines delineate sugar units and the arrows at the right and left sides indicate the reducing terminal direction. Hydrophobic patches (hatched) on alternate sides of the polymer [12] stretch across 3 sugar units.

Only in antiparallel orientation do the gentle curves in the backbones of the participating molecules complement each other so that interactions are optimal. The acetamido (ζ and γ) and carboxylate groups (ζ and γ) are then positioned so that H-bonds form, as indicated by arrows pointing from donor to acceptor groups. Open symbols refer to groups on the proximal side of the tape-like molecule and the closed symbols are on the distal side.

The potentials of the other ECM AGAGs are very similar to those of HA [10]. These 1e-4e,1e-3e linked polymers prefer to take up twofold helical shapes in solution with similar hydrophobic patches of similar size and location [9]. They interact with themselves and with

Hydrophobic patches (hatched) on neighbouring molecules are contiguous and hydrophobic bonding between them can occur.

This structure is formally equivalent to that of the β -sheet in proteins, in which pairs of H-bonds are disposed in alternate directions (up and down) between antiparallel polypeptide chains (Taken from Ref 13).



(C) plan and elevation of the 2-fold helix showing gentle curves in planes at right angles which must be complementary to each other in the parallel array shown above (taken from Ref.9).

Cooperative interactions enable large numbers of HA molecules to aggregate specifically, opposed by electrostatic repulsion. This situation is analogous to that of the DNA double helix, in which repulsive forces are countered by hydrophobic and H-bonding, DNA helices are without the ambidexteran property that permits extensive lateral aggregation. Similar self-aggregates of CS, DS or KS are proposed to be part of the shape modules in ECMs.

other AGAGs, probably by the same pairing of hydrophobic and hydrogen bonding. *Thus the properties of the shape module bridges are deducible from the solution behaviour of HA.*

Protein-protein interactions have long been recognised as important in building up the

collagenous matrix. Glycan-glycan interactions are a newly recognised way of constructing supramolecular structures of great beauty and functional significance in the ECM, - a paradigm whose time has come.

Abbreviations. AGAG; anionic glycosaminoglycan, CS, chondroitin sulphate, DS; dermochondan (formerly dermatan)

sulphate, ECM; extracellular matrix, HA; hyaluronan, KS; keratan sulphate, PG; proteoglycan.

Keywords secondary structure, tertiary structure, two-fold helix, β -sheet, collagen fibril, decoran, shape module, cornea, Cupromeronic blue

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