Collagen Fibril Structure Is Affected by Collagen Concentration and Decorin

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Collagen fibrils were obtained in vitro by aggregation from acid-soluble type I collagen at different initial concentrations and with the addition of decorin core or intact decorin. All specimens were observed by scanning electron microscopy and atomic force microscopy. In line with the findings of other authors, lacking decorin, collagen fibrils undergo an extensive lateral association leading to the formation of a continuous three-dimensional network. The addition of intact decorin or decorin core was equally effective in preventing lateral fusion and restoring the normal fibril appearance. In addition, the fibril diameter was clearly dependent on the initial collagen concentration but not on the presence/absence of proteoglycans. An unusual fibril structure was observed as a result of a very low initial collagen concentration, leading to the formation of huge, irregular superfibrils apparently formed by the lateral coalescence of lesser fibrils, and with a distinctive coil-structured surface. Spots of incomplete fibrillogenesis were occasionally found, where all fibrils appeared made of individual, interwined subfibrils, confirming the presence of a hierarchical association mechanism.

Introduction

Much interest has focused on fibrillogenesis in recent years and, paradoxically, it is now better understood in vivo than in vitro. However, several apparently simple questions remain unanswered, including what limits the lateral accretion of collagen fibrils, which exhibit such different diameters among different tissues and, in some cases, within the same tissue.

It is known that collagen molecules aggregate in several different patterns depending on physicochemical conditions such as temperature, pH, ionic strength, and the presence of molecules such as glycosaminoglycans, nucleotides, or other small polyanions.[1,2] These usually exert an effect on the collagen superstructure without becoming part of the end product, thus making it even more difficult to understand the details of the intermolecular interactions.

Small leucine-rich proteoglycans (SLRPs) form a growing, heterogeneous family of proteoglycans associated with the fibril surface. They can bind several collagen types[3−5] and seem to fulfill different roles in different studies. A number of SLRPs can be expressed sequentially during the histogenesis of a given tissue,[5,6] and they can remain present simultaneously in adult tissues or be segregated in different locations (lumican, for instance, is mostly limited to the corneal stroma in the adult). Some SLRPs are known to bind avidly to the surface of collagen fibrils, where chondroitin sulfate/dermatan sulfate proteoglycans (CS/DS PGs), such as decorin and biglycan, bind at the d/e bands of the fibril binding pattern, and keratan sulfate proteoglycans (KS PGs), such as fibromodulin and lumican, bind at the a/c bands.[7,8] Fibromodulin and lumican were found to compete,[9] as were decorin and biglycan,[10] whereas decorin does not compete with fibromodulin or lumican.[9] The maximum binding force between decorin and collagen has been estimated at about 12.4×10^3 nN, a value exceeding the glycosaminoglycan chain's ultimate strength.[11]

One of the functions of decorin and collagen seems to be the control of lateral fibril growth, but SRLPs are likely to share this function with other processes since their absence in knockout organisms has relatively minor consequences[12,13] compared to other macromolecules of the extracellular matrix.

In a sense, decorin is the archetypal SLRP. It is the most abundant and widespread, it was the first to be deleted to obtain a knockout organism,[12] and its functional role is the subject of considerable interest.[14−18] Much attention has also been paid to decorin's role in fibrillogenesis, where it seems to influence the initial phases of the aggregation process in particular[19] in a different way from other SLRPs.[19,20] It it noteworthy that decorin is generally thought to slow up fibril formation,[20] but some studies report a decrease in diameter,[19−21] others an increase,[22] and yet other reports no effect at all.[23]

The present study allowed acid-soluble collagen to aggregate under controlled conditions of physiological pH and ionic strength, either alone or with the addition of decorin core or intact decorin. The fibrils obtained were then observed at the ultrastructure level by field-emission scanning electron microscopy (FESEM) or tapping-mode atomic force microscopy (TMAFM).

Experimental Procedures

Acid-soluble type I collagen (ASC I) was already available, purified from calf skin as previously described.[24] Briefly, delipidized dried calf skin was extracted with 0.15 M NaCl and 50 mM Tris-HCl, pH 7.4, containing protease inhibitors. The residue was extracted twice with 0.5 M acetic acid. The material dissolved by the acidic solvent was

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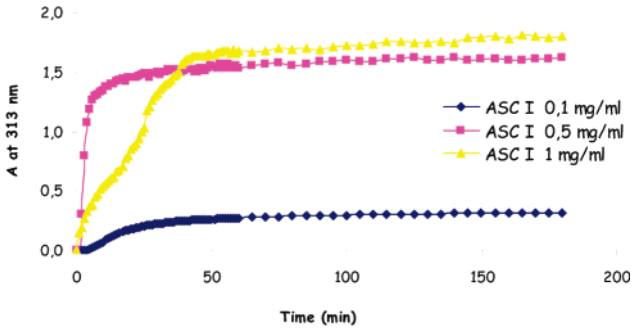

Figure 1. Turbidity curves of ASC I obtained at 313 nm and 32 °C in 10 mM phosphate and 130 mM NaCl at neutral pH and different concentrations (0.1, 0.5, and 1.0 mg/mL). The graph shows a remarkable change of kinetic curve for 0.1 mg/mL collagen concentration, while the kinetic curves obtained at 0.5 and 1 mg/mL collagen concentrations overlap.

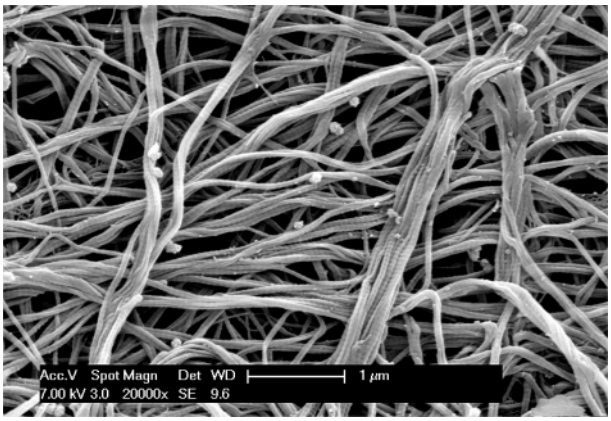

Figure 2. Scanning electron micrograph of collagen fibrils obtained at an initial concentration of 1 mg/mL. The fibrils form a branched network with frequent lateral fusions, while their D-banding is normal.

precipitated with NaCl (2 M final concentration), collected by centrifugation, redissolved in 0.5 M acetic acid, and subjected to a second NaCl precipitation. The final precipitate was dissolved in 0.1 M acetic acid, dialyzed exhaustively against the same solution, freeze-dried, and stored at −80 °C. The same batch was used for all the following experiments.

Decorin was purified from bovine tendon by extraction with 4 M guanidine hydrochloride, ultracentrifugation on a CsCl gradient, and ion-exchange chromatography as previously described.[25] Decorin was characterized by SDS−PAGE, N-terminal sequencing, and circular dichroic spectroscopy. The concentration of decorin solutions was determined by the Bradford method.

Collagen fibrils were obtained by incubation at 32 °C in 10 mM sodium phosphate and 130 mM NaCl, pH 7.4, at initial collagen concentrations of 1, 0.5, and 0.1 mg/mL. Fibril formation and aggregation kinetics were followed with a Beckman DU-62 spectrophotometer as the turbidimetric increase in absorbance at 313 nm immediately after mixing of the appropriate solutions.

Prior to fibrillation, some of the specimens were supplemented with decorin core or with intact decorin at a collagen:proteoglycan ratio of 5:1 by protein content. Most specimens were incubated for 2 h; some, for comparison, were incubated overnight.

The fibrils obtained were dehydrated in graded ethanol and hexamethyldisilazane. Some of the specimens were observed by tapping-mode atomic force microscopy (TMAFM) on a Digital Instruments Multi-Mode Nanoscope III/a SPM as previously described.[26] Other specimens, mounted on appropriate stubs with conductive adhesive, were gold-coated in an Emitech K225 apparatus and observed on a Philips XL30 field-emission scanning electron microscope (FESEM) FEG operated at 7 kV.

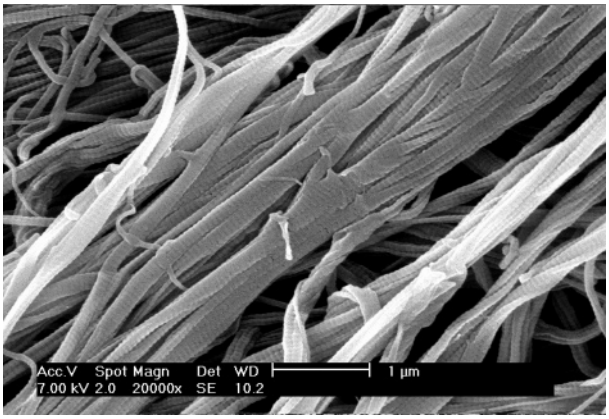

Figure 3. Scanning electron micrograph of collagen fibrils obtained at an initial concentration of 0.5 mg/mL, at the same magnification as in Figure 2. The fibril diameter is perceptibly larger, while lateral fusions and the network structure are unchanged.

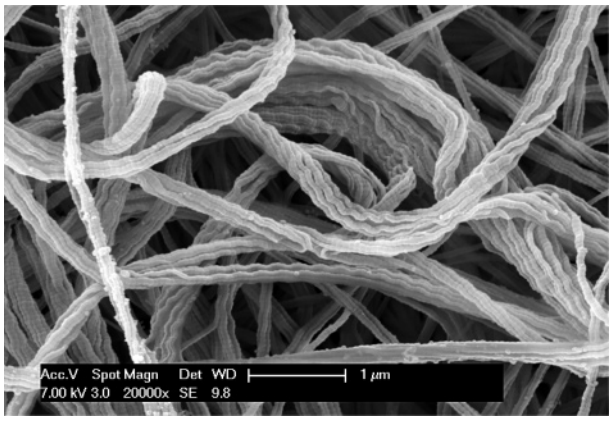

Figure 4. Collagen fibrils obtained at an initial concentration of 0.1 mg/mL appear as fluted superfibrils of large diameter, with a distinctive ripple along their course. Same magnification as Figures 2 and 3.

Table 1. Features of Collagen Aggregates from Different Initial Concentrations

collagen concn (mg/mL)	banding	diameter	assemblage	branching	notes
1.0	normal	+	3D network	++	
0.5	normal	++	3D network	+++	
0.1	normal	+++++	3D network	+++	fluted surface

Results and Discussion

A first stage of the research consisted of the characterization of collagen fibrils obtained in vitro from acid-soluble type I collagen from calf skin at initial concentrations of 1, 0.5, and 0.1 mg/mL. In all cases the fibrillation process produced a visible clot within 1 h, and longer times (up to overnight) caused no visible alteration or absorbance increase (Figure 1). The process always produced a thin, feltlike network of banded fibrils.

The size and shape of fibrils were not affected by the incubation time, while they varied widely depending on the initial concentration of collagen. At a collagen concentration of 1 mg/mL (Figure 2) the process yielded fibrils of small, variable size, with normal D-banding but partially fused laterally to form a continuous web.

A concentration of 0.5 mg/mL (Figure 3) yielded thicker but still irregular fibrils, interwoven into an extended three-

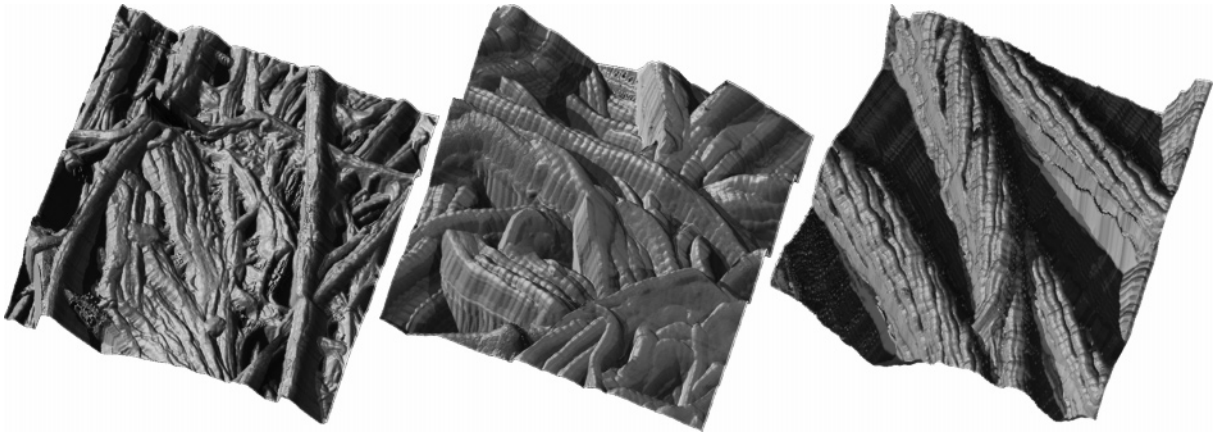

Figure 5. Atomic force micrographs of reconstituted fibrils obtained at initial concentrations of 1 mg/mL (left), 0.5 mg/mL (center), and 0.1 mg/mL (right). The fibril appearance is entirely consistent with the structure revealed by scanning electron microscopy. Each picture spans 3 × 3 μm.

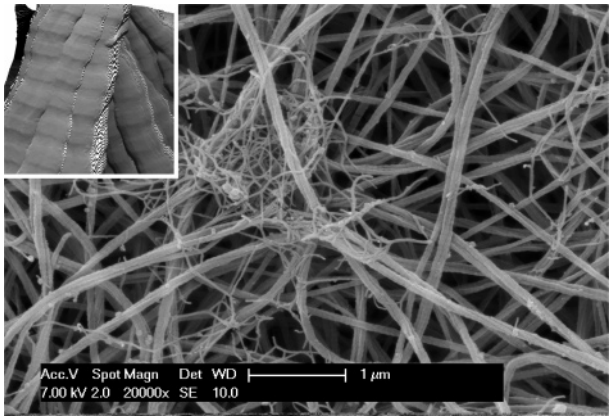

Figure 6. Scanning electron micrograph of collagen fibrils obtained in the presence of decorin core, at a molar ratio of 1:5 with collagen and an initial concentration of 0.5 mg/mL. Lateral fusion is no longer observable, and atomic force microscopy (inset) shows a normal gap/overlap sequence. The slender filaments among the fibrils correspond to subfibrils that did not complete the fibrillogenesis process.

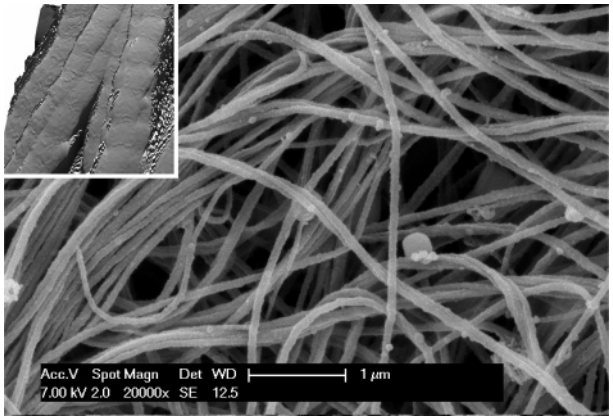

Figure 7. Collagen fibrils obtained in the presence of whole decorin, at a 1:5 collagen:proteoglycan ratio (by protein content) and an initial concentration of 0.5 mg/mL. Again, lateral fusion is not present and the fibrils remain separated along the whole field of view. Under the AFM (inset), the glycosaminoglycan side chains are easily visible across the collagen fibrils.

dimensional network. The lateral fusion of the fibrils was much more widespread than in the previous case, and the 3D network they formed was more evident. The D-banding disclosed no abnormalities, and except for the lateral fusion, the collagen fibrils showed no obvious differences from native fibrils.

At 0.1 mg/mL, however, the solution formed huge superfibrils (Figure 4) with an irregular, deeply grooved surface, appearing as bundles of smaller fibrils joined by extensive branchings and fusions. In addition to the fluted appearance caused by their irregular cross section, some superfibrils showed a distinctive longitudinal undulation, due to a supercoiling of their superficial subunits with a pitch on the order of 300 nm. A remarkable change was also evident in the kinetic curve (see Figure 1).

For all concentrations, AFM data were always entirely consistent with SEM micrographs (Figure 5), and both the axial D-banding and the lateral fusion were always readily appreciable under both the SEM and the AFM. These results are summarized in Table 1.

Due to the similarity of the results obtained at 1 and 0.5 mg/mL, we restricted the subsequent experimental phase to just two concentrations, 0.5 and 0.1 mg/mL.

The addition of decorin core to 0.5 mg/mL collagen completely inhibited the lateral fusion and restored a normal fibril appearance, while diameters were apparently not affected (Figure 6). Under the AFM, which has better spatial resolution,

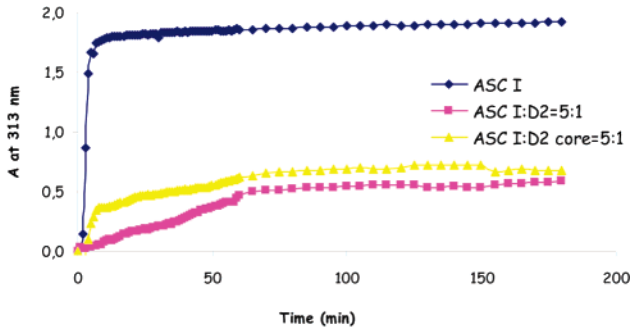

Figure 8. Turbidity curves of ASC I in the absence and in the presence of decorin (D2) or decorin core (D2 core) obtained at 313 nm and 32 °C in 10 mM phosphate and 130 mM NaCl at neutral pH. The initial concentration of collagen was 0.5 mg/mL, with a collagen:proteoglycan ratio of 5:1 by protein content. In the presence of decorin and decorin core, the kinetic curves are similar and show a delay in the fibrillogenesis process, with a considerable decrease of absorbance.

the fibrils showed a round cross-section and regular D-banding, with an evident gap/overlap alternation (Figure 6, inset).

The addition of intact decorin was equally effective in preventing fibril fusion (Figure 7), but in this case AFM clearly revealed the glycosaminoglycan side chains crossing the fibril surface (Figure 7, inset), where they followed a regular orthogonal arrangement not unlike that of native tissues.

Table 2. Features of Collagen Aggregates in the Presence of Decorin (Intact) or of Its Core Protein

composition	banding	diameter	assemblage	branching	notes
0.5 mg/mL ASC + DCN (core only)	normal	++	3D network	−	
0.5 mg/mL ASC + DCN	normal	++	3D network	−	side chains visible
0.1 mg/mL + DCN (core only)	normal	+++++	3D network	+++	fluted surface
0.1 mg/mL + DCN	normal	+++++	3D network	+++	fluted surface

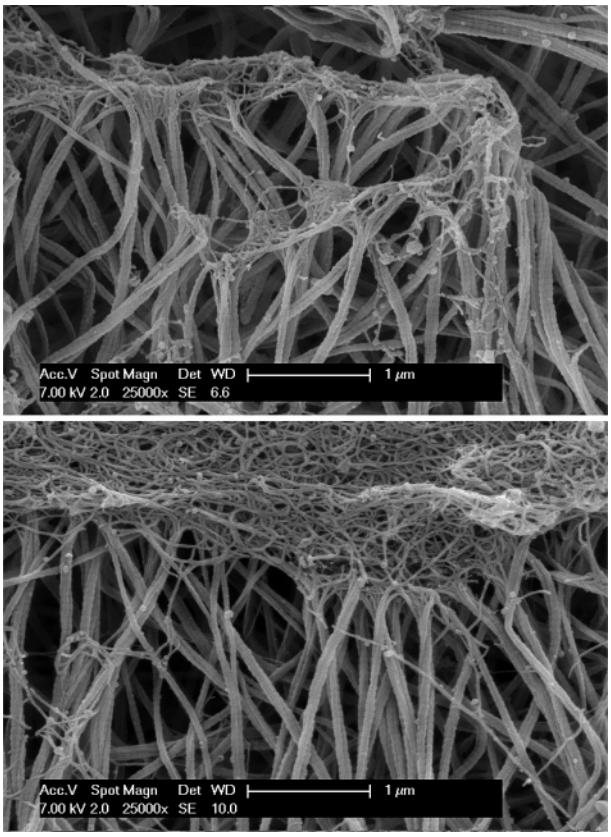

Figure 9. Incomplete fibrillogenesis is a common finding at all initial concentrations. In all cases, the fibrils appear made of intertwined, slowly spiralling subfibrils of a remarkably uniform thickness.

No obvious difference was found in the kinetic curves (Figure 8).

By contrast, both the decorin core and the intact proteoglycan were equally ineffective at lower collagen concentrations (not shown), which is noteworthy since in these larger aggregates the surface/volume ratio decreases so that even more decorin is available for the surface unit. These observations are summarized in Table 2.

The diameter of collagen fibrils precipitated in vitro varies widely in the literature, with sizes ranging from approximately 24 nm[15] to approximately 1.2 μm,[27] corresponding to a 50-fold increase. Under our experimental setup, decorin seems to affect the lateral association of fibrils much more than their diameter, which instead is more influenced by the initial concentration. This is explained by the simple fact that a higher collagen concentration translates into greater supersaturation, with the concomitant appearance of many nucleation centers. These centers compete with each other for aggregation of the remaining collagen molecules until the interfibrillar space quickly becomes depleted and fibril growth ceases, leaving many slender fibrils. On the contrary, an initial low concentration implies fewer nucleation centers and the eventual growth of fewer, larger fibrils.

The appearance of huge superfibrils at very low collagen concentration is therefore not surprising in itself. The superfibrils' cross section and, to a greater extent, the unprecedented supercoil shown on their surface are more perplexing. It is possible that their diameter is too large to allow for a complete rearrangement of molecules in the configuration having the lowest energy.

It is generally agreed that the addition of decorin to the collagen solution delays fibril formation,[20] while its influence on fibril thickness is more dubious.[15,22,23] Neither of these effects was evident in our specimens, but we did not carry out a quantitative morphometric analysis [a task for which a transmission electron microscope (TEM) is much better suited].

The effect of decorin on the lateral fusion of the fibrils is more obvious and is entirely consistent with the observation of irregular profiles and abnormal lateral coalescence of adjacent fibrils observed in vivo in Dcn −/− mice.[12,19] The addition of decorin (either the whole PG or the protein core alone) in our specimens always restored the normal fibril shape, with the notable exception of the superfibrils obtained at 0.1 mg/mL.

Branching fibrils have seldom been described.[19,28] However, parallel fibrils usually run with their D-period in phase, so that the parting of two adjoining fibrils in studies carried out on thin sections can easily be mistaken for a branching single fibril. The branching we observed in our SEM micrographs was unambiguous and its extent was, to our knowledge, unprecedented.

Finally, it was occasionally found that the fibrillogenesis process failed to achieve completion in isolated spots, and individual subfibrils and intermediate aggregates were plainly visible as slender filaments winding together to form fibrils (Figure 9; see also Figure 6). In all cases the fibrils were clearly the result of a lateral aggregation of elongated subunits of a uniform diameter of approximately 12−15 nm.

These fibrils must have a considerable fluidity, at least initially (at least at 1 and 0.5 mg/mL), because the intertwining of two or three subfibrils immediately gives rise to a single round fibril. This clearly requires the lateral rearrangement of molecules into a new, more favorable shape. It must also be noted that the subfibrils we observed were much thicker than the ≈4 nm that other authors report as the thickness of collagen layers,[29] while they are closer in value, when the different technical approaches are accounted for, to the subunits observed by tapping-mode AFM of fibrous-long-spacing (FLS) aggregates[30] or to the subfibrils observed in native tendon fibrils by fluid tapping-mode AFM.[26]

Conclusions

Our study found the size and shape of type I collagen fibrils formed in vitro barely affected by the incubation time, whereas

they varied widely depending on the initial concentration of collagen. Lacking decorin, lateral fusion of subfibrils (or maybe prefibrils) took place in our specimens to an unprecedented extent. In particular, at 0.1 mg/mL the solution formed huge superfibrils with extensive branchings and fusions. In addition, these fibrils often showed a distinctive supercoiling of their superficial subunits, with a pitch close to 300 nm.

It is noteworthy that the present study was carried out with inherently three-dimensional techniques such as SEM and AFM, whereas most previous research relied on TEM, that is, planar sections. Although this technique offers higher resolution and has been fundamental in the definition of the supramolecular structure of collagen, it has an inherent limit in the thinness of its sections (which is on the order of 60 nm). In these conditions the structure of the specimen above and below the section plane is unknown, and we cannot tell whether the irregular cross-section of fibrils observed by other authors in knockout animal models[12,19] and in pN-collagen[31] represents actual, individual fluted ("cauliflower") fibrils or if they portray smaller fibrils caught in the act of fusing.

Further research is presently underway to investigate the effect of other common SLRPs (fibromodulin, lumican, biglycan) and the timing of their addition to the fibril aggregation process.

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