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Molecular Crowding of Collagen: A Pathway to Produce Highly-Organized Collagenous Structures

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Abstract

Collagen in vertebrate animals is often arranged in alternating lamellae or in bundles of aligned fibrils which are designed to withstand *in vivo* mechanical loads. The formation of these organized structures is thought to result from a complex, large-area integration of individual cell motion and locally-controlled synthesis of fibrillar arrays via cell-surface fibripositors (direct matrix printing). The difficulty of reproducing such a process *in vitro* has prevented tissue engineers from constructing clinically useful load-bearing connective tissue directly from collagen. However, we and others have taken the view that long-range organizational information is potentially encoded into the structure of the collagen molecule itself, allowing the control of fibril organization to extend far from cell (or bounding) surfaces. We here demonstrate a simple, fast, cell-free method capable of producing highly-organized, anisotropic collagen fibrillar lamellae *de novo* which persist over relatively long-distances (tens to hundreds of microns). Our approach to nanoscale organizational control takes advantage of the intrinsic physiochemical properties of collagen molecules by inducing collagen association through molecular crowding and geometric confinement. To mimic biological tissues which comprise planar, aligned collagen lamellae (e.g. cornea, lamellar bone or annulus fibrosus), type I collagen was confined to a thin, planar geometry, concentrated through molecular crowding and polymerized. The resulting fibrillar lamellae show a striking resemblance to native load-bearing lamellae in that the fibrils are small,

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generally aligned in the plane of the confining space and change direction *en masse* throughout the thickness of the construct. The process of organizational control is consistent with embryonic development where the bounded planar cell sheets produced by fibroblasts suggest a similar confinement/concentration strategy. Such a simple approach to nanoscale organizational control of structure not only makes *de novo* tissue engineering a possibility, but also suggests a clearer pathway to organization for fibroblasts than direct matrix printing.

1. Introduction

The facility with which large scale structural organization is generated by Nature has captivated scientists for centuries. This fascination has deepened with the realization that macroscale organization is the result of highly-controlled hierarchical self-assembly of nanometer-scale subunits. Bimolecular self-assembled structures are found both intracellularly (e.g. actin [1] and microtubules [2]) and extracellularly (e.g. collagen [1, 3–4] and nacre [5]) and play central roles in all phases of vertebrate life from embryogenesis to the mature adult organism (e.g. morphogenesis [6–8], wound healing [9–10], mechanosensing [11–12], and cell locomotion [13–15]). The processes which produce these structures are mainly characterized by the rapid self-assembly of molecules into organized filaments (or fibrils) followed by the controlled disassembly of the fibrils into their constituent molecules (this is particularly true for intracellular components). For more permanent molecular assemblies, which are often extracellular, stabilization of the assembled fibrillar networks is accomplished with covalent cross-linking [7] and/or calcification (e.g. collagen).

In recent years scientists have begun to tease apart precisely how nature guides molecular self-assembly to build and maintain tissue in an energy efficient fashion. In addition to the obvious basic scientific importance, understanding and replicating the processes which control molecular self-assembly *in vitro* has a potentially significant translational/biotechnological impact. Gaining control over molecular self-assembly could usher in a new era of bioinspired structural engineering which would permit the industrial fabrication of sophisticated and complex structures not possible before.

A majority of the experimental and theoretical studies on the assembly/disassembly of biomolecules have been focused on the cytoskeletal filaments [16–17]. The results of these investigations have revealed that the volume exclusion and confinement provided by the crowded cell environment play a critical role in self-association of the molecules into the organized bundles [18–20]. In this context the macromolecular crowding refers to effects attributed to volume excluded by one soluble macromolecule to another, and macromolecular confinement refers to effects attributed to volume excluded by a fixed (or confining) boundary to a soluble macromolecule [18].

We propose that a similar crowding/confining mechanism is involved in the development of the highly organized collagenous structures of the extracellular matrices (ECM) in vertebrates. The proposal is based on the careful examination of the physiological events leading to the development of such organized structures during embryogenesis (i.e. in cornea, annulus fibrosus, and bone, please see the discussion section).

The goal of this investigation is to attempt to mimic a potential mechanism used by Nature to produce highly-organized collagenous arrays in the extracellular matrix (ECM). *In vivo*, collagen is produced in the form of soluble procollagen which comprises a right-handed helical region (i.e. collagen monomer, 300 nm in length and 1.5 nm in diameter) flanked by two non-triple-helical C- and N- terminus. Due to their non-helical structures, the propeptides occupy a large space around each collagen molecule. Therefore, it has been

suggested that the function of the propeptides is to inhibit premature collagen self-assembly [21]. Following enzymatic cleavage of the propeptides from the tropocollagen, spontaneous self-assembly results in the production of alternating lamellae or bundles of highly-organized fibrils well-suited to carry substantial *in vivo* mechanical loads (i.e. interwoven bundles of skin, parallel bundles in tendon and ligaments [22], orthogonal lamellae in cornea [23] and ± 60 to $\pm 45^\circ$ alternating lamellae in annulus fibrosus [24–25]). The production of highly-organized collagenous arrays during development is thought to be either a derivative of direct cell-mediated collagen fibril deposition [26–31] or a derivative of physical factors which exert control over fibrillogenesis via liquid crystal phasing of the collagen [32–33]. We believe that collagen fibril formation is more likely driven by the latter physicochemical effect and that the molecular crowding plays a significant role in driving the formation of organized structure (perhaps benefitting by some guidance via cell processes or shape).

Collagen is of interest to bioengineers for a number of reasons: i) It is the predominant structural material in vertebrate animals and its organizational modulation produces structures with an extreme range of mechanical properties (dentin, bone, cartilage, ligament, tendon, skin, cornea, intervertebral disk etc.) ii) In vertebrates, collagen-based, load-bearing tissues exhibit 3 orders of magnitude in structural scalability (for example female *Paedocypris micromegethes* of length approx. $0.008\mu\text{m}$ and the blue whale *Balaenoptera musculus* of length approx. $30\mu\text{m}$) iii) Collagen is the principal embryonic template molecule used to produce the initial organized musculoskeletal anlagen, iv) Collagen-related diseases such as osteoarthritis and intervertebral disk degeneration significantly impact quality of life and have an enormous economic impact [33] and v) Recent data suggest that collagen fibrils are stabilized by mechanical strain and along with collagen's complementary ECM molecules may comprise the basis of a smart, load-adaptive structural system on which all vertebrate animals are built [34–39].

In this investigation, we examine the effect of the molecular crowding and confinement on the self-assembly and organization of the collagen fibrils. The crowding was achieved by concentrating pure collagen monomer in planar confined geometries (self-crowding or unimolecular crowding) or in open, but crowded solutions comprising a high-concentration of a strong biomolecular co-nonsolvent (polyethylene glycol or hyaluronic acid).

2. Materials and methods

2.1. Molecular Crowding and Confinement

2.1.1. Unimolecular Crowding—Unimolecular crowding of collagen was produced by concentrating type I collagen molecules (PURECOL[®], Inamed, Freemont, CA) to densities found in the load-bearing tissues and beyond (100–400 mg/mL). Two ranges of collagen concentration were targeted in these experiments. Concentrated collagen (i.e. crowded collagen) was produced by dialyzing 3 mg/mL acidic solution of type I collagen monomers against polyethylene glycol (PEG, 20 kMWCO, Sigma-Aldrich, St. Louis, MO) at 4°C. For the first range of the concentration, the dialyzing procedure proceeds until the concentration of the collagen molecules reached the range of 175 ± 25 mg/mL (LC range). At this point, the concentrated solution was removed, neutralized, confined between two coverslips, and left at 37 °C, 100% relative humidity to start collagen fibrillogenesis. For the second range of the concentration (375 ± 25 mg/mL, HC collagen) the dialyzing was further proceeded by injecting the LC solution into a dialysis cassette and dispensing the cassette in the PEG solution. The collagen solution was then neutralized (by titrating the PEG solution and permitting the system to equilibrate) and transferred into a 37 °C incubator. The collagenous constructs were then carefully removed for further ultra-structural assessment.

2.1.2. Bimolecular Crowding—The bimolecular crowding was achieved by mixing low concentration of collagen molecules (3 mg/mL) with graded solutions of polyethylene glycol (PEG, 20k MWCO) or hyaluronic acid (HA; 10%, 20% and 40%). Fibrillogenesis was induced by neutralization and warming under physiological conditions *in situ*. Hyaluronic acid was chosen because it is present during the development of collagenous rudiments [40–42].

2.2. Differential Interference Contrast (DIC) microscopy

The long range organization of the collagen fibrils was investigated using DIC [43]. The collagenous constructs were transferred between two coverslips and placed on the stage of an inverted microscope (TE2000E; Nikon). The alignment of collagen fibrils was studied using series of in-plane and Z-stacks.

2.3. Small Angle X-ray Diffraction (SAX)

Measurements were taken at Beamline X6B at the National Synchrotron Light Source, Brookhaven National Laboratory, at wavelength $1.54 \text{ \AA}^{-1}$ and spot size $0.3 \times 0.3 \text{ mm}$, in transmission with the beam normal to the collagen sheets. Samples were sandwiched between a 40mm #1 glass cover slip for rigidity, and a 3.6 μm thick mylar sheet for x-ray transmission on the downstream side. Silver Behenate powder was included in the sample sandwich alongside the collagen, and measured separately by translating the sample transverse to the beam, for calibration of the distance from sample to area detector (Princeton Instruments SCX CCD). Acquisition times ranged from 90 to 360 seconds.

2.4. Transmission Electron Microscopy (TEM)

Constructs were fixed overnight in modified Karnovsky fixative (2.5% Glutaraldehyde, 2.5% formaldehyde, 0.1M cacodylate buffer, pH 7.2), washed with 0.1M buffer, post fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, and dehydrated in a graded series of ethanol. The samples were infiltrated and embedded in a mixture of Spurr's resin and Quetol according to Ellis [44]. 60–80nm cross sections and *en face* sections were cut on an Ultracut E microtome (Reichert, Depew, NY) using a diamond knife. Thin sections were stained with 5% uranyl acetate and Reynolds lead citrate. The sections were viewed with a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) and images were digitally captured on an AMT XR-41B CCD camera system (Advanced Microscopy Techniques Inc., Danvers, MA).

2.5. Scanning Electron Microscopy (SEM)

To prepare for SEM, samples were fixed and dehydrated similar to the methods described for TEM. Following the dehydration, constructs were critically point dried using a Samdri Pvt 3 (Tousimis Research Corp., Rockville, MD). The specimens were coated with platinum-palladium (80–20%) by thermal evaporation in a Denton DV 502 vacuum evaporator (Denton Vacuum Inc., Cherry Hill, NJ) and examined with a Hitachi S4800 SEM.

3. Results

The unimolecular crowding of the collagen molecules resulted in transparent collagenous constructs (Supplementary Fig. 1). DIC images of unimolecular crowded LC (Fig. 1A) and HC (Fig. 1B) collagen fibrils shows that the fibrillar matrix was uniformly aligned over long distances in the x-y plane (100s of microns). In the plane of the confined constructs, TEM generally confirmed the DIC images by demonstrating large areas of complete fibril alignment parallel to the confining surfaces (Figs. 1C and D). In the lower range of

concentrations the fibrils would sometimes adopt a wavy pattern which suggests the production of open space as monomers are incorporated into the aggregating fibrils (Fig. 1C). At higher concentration the collagen fibrils are more tightly packed and highly-aligned parallel to the confining surface (Fig. 1D). Close inspection of the collagen fibrils at high magnification in longitudinal section shows them to be thin (~20 nm), dense and highly-aligned (data not shown). DIC images also revealed bimolecular crowding of the collagen monomers in the presence of PEG (Fig. 2) and HA (Supplementary Fig. 2) results in highly-aligned arrays of collagen fibrils. Bimolecular crowding of collagen molecules resulted in the packing of the fibrils into aligned bundles and a very limited number of individual collagen fibrils in the open space.

The small-angle X-ray diffraction method (SAXS) was used to investigate the organization of the collagen fibrils over large areas as well as the molecular packing of the monomers within the fibrils. SAXS patterns of the constructs show several orders of the ~ 65 nm periodicity (Fig. 1E and F) which corresponds to the D-banding patterns of the native collagen fibrils (see Supplementary Fig. 3). These peaks show orientational alignment that varies from about 30 to 90 degrees azimuthally, as expected for aligned fiber bundles with a distribution of domains in the 300 micron beam spot. The wings of diffuse scattered intensity normal to the fiber axis indicate that neighboring dense bundles are of order 10 nm apart.

DIC, z-scan optical imaging revealed that the *de novo* constructs possessed multiple lamellae (up to seven layers; Fig. 3C and D; also see Supplementary Movie). The organization of collagen fibrils within the lamellae and the change of the orientation of the fibrils in the adjacent lamellae by large angles (Fig. 3A) are suggestive of similar lamellar organizations in native tissues. In the normal human cornea, collagen lamellae are approximately 2 microns thick and the angle between successive lamellae is generally 90° [45]. In annulus fibrosus, collagen lamella are 100 to 400 microns thick and change direction by 45 to 65 degrees [46]. Low magnification cross-sectional sTEM and SEM micrographs of confined collagen constructs corroborated the results of the DIC imaging by revealing alternating arrays of aligned fibrils in spontaneously-formed *de novo* lamellae (Fig. 3B and Fig. 3E–H). Low magnification TEMs of the constructs bear a striking resemblance to TEM images of the normal human cornea with its arrays of alternating fibrils packed into lamellae (Fig. 3E and F). Similar to the DIC and TEM images, cross-sectional SEM also confirms the high fibril density, high-degree of fibril alignment and lamellar structure in crowded collagen solution (Fig. 3G and H).

The higher magnification sTEM and SEM image of the collagenous constructs (Fig. 4) reveals generally small diameter, polydisperse and laterally fused fibrils (Fig. 4D). Interestingly, the fibrils have an appearance similar to collagen fibrils produced by a proteoglycan (lumican) knock-out mouse [47]. Proteoglycans and their associated glycosaminoglycans have been shown to be directly involved in the control of fibril diameter and spacing. Therefore, in our system we do not expect to see uniform diameter or circular fibrils without the aid of auxiliary ECM molecules such as proteoglycans, glycosaminoglycans or other types of collagens [47–49]. This raises the possibility of using these collagenous matrices to test the effect of knocking-in matrix controlling molecules.

4. Discussion

In this investigation, we have demonstrated that the molecular crowding results in the production of highly organized arrays of collagen fibrils. In addition, fibrils are organized in the multi-layer structures similar to the structure of the collagen fibrils in the ECM of the

native tissues. These results not only have a significant basic scientific impact but also create a new avenue for the tissue engineering of load-bearing tissues.

Onsager was first to predict that crowding of molecules with anisotropic geometries results in the generation of organized arrays [50]. Since then a significant body of theoretical and experimental investigations revealed that crowding of biomolecules and synthetic molecules induces organization and self-assembly. It has also been shown that cells use a crowding mechanism to guide the self-assembly of the monomeric G-actin into filamentous F-actin and tubulin into microtubules [20]. The self-assembly and organization of these two intracellular molecules is essential in cell locomotion, mechanosensation, and mechanotransduction. If biological systems have been developed to take advantage of this simple mechanism to control the organization of the cytoskeleton, there is a reasonable possibility that a similar mechanism is used to control the organization of the ECM. The data presented in this study strongly support the notion that, by providing a crowding condition, cells may take advantage of the physiochemical properties of individual collagen molecules to build macroscale organization. In crowded conditions, high-aspect ratio molecules spontaneously self-organize in the direction of the molecules' long axis. The spontaneous ordering of the molecules is entropy-driven because under the packing constraints, once the molecules are aligned along a common axis the translational entropy that the molecules gain greatly overcomes the rotational entropy that is lost [50–52]. As a result, in the crowded condition the anisotropic state (i.e. orientationally ordered) is more stable than the isotropic state. Using the same analogy, at similar volume fractions a lower number of particles with higher aspect ratios is more entropically advantageous. Therefore, in a system comprising molecules with self-aggregating properties, similar to actin and collagen, the molecules will spontaneously organize into linear aggregates to reduce the free energy of the system. The theory also predicts that self-assembly in the crowded conditions would result in the polydisperse diameter fibrils. Our TEM micrographs indeed revealed that collagen fibrils formed under the crowded conditions have polydisperse and irregular shapes.

It has been shown that collagen exhibits cholesteric liquid crystalline phase behavior [53]. However the structures generated in previous studies are not similar to the lamellar and uniaxial fibrillar organization found in most load-bearing tissues. In addition, translation of these structures into highly-organized fibrillar arrays which persist over large length scales has been unsuccessful [54].

It should be noted that the concentrations at which we observe self-organizing behavior of collagen molecules under the unimolecular crowding condition is generally higher than that found under physiological conditions. We point out, however, that *in vivo*, the ECM is crowded with many other macromolecules which could induce collagen self-assembly and organization (similar to our crowded experimental conditions). For example, it is well-documented that HA is present during development of the corneal stroma [42]. HA is a high molecular weight, negatively-charged moiety with high osmotic pressure proteoglycans. We postulate that the HA likely plays a critical role in collagen organization during development by absorbing a high volume of available water molecules, crowding the ECM and subsequently driving collagen molecules into organization. Consistent with this postulate, the HA molecules are degraded rapidly after the deposition of the organized collagen matrix [42].

To better understand the processes which govern the formation of organized collagenous structures *in vivo* and to assess the relevance of this study to the *in vivo* condition we have also re-examined connective tissue morphogenesis. Tissue morphogenesis is the stage during which the rapid tissue development occurs. In vertebrates, the prospective cornea

forms in a thin confined space between the lens and epithelium [23]. Hyaluronic acid is the main macromolecule present in the space prior to the appearance of fibroblasts and collagen fibrils [42]. During development of the avian corneal stroma, a highly anisotropic primary collagenous template matrix is deposited by a confluent layer of epithelial cells through a continuous basement membrane [42, 55] during which, the epithelium is thought to be immobilized by intercellular tight junctional complexes. In addition to collagen, there are significant quantities of other molecules present, particularly hydrophilic proteoglycans and hyaluronate [56–58]. Because of the tight junctional complexes, there is likely little cell motion or cell-mediated control of the orientation of the fibrils. In addition, the development of anisotropy in the deposited stromal rudiment only proceeds after the endothelium becomes confluent, suggesting that confinement between the epithelium and endothelium is a mediator of organization [55]. Similarly, during the development of the annulus fibrosus in chick, collagen fibrils appear within the spaces confined between confluent layers of organized cells [1].

These observations suggest that immobilized, confluent cell layers synthesize a high concentration of procollagen into the space filled with hyaluronic acid and confined between the confluent cell layers. Indeed the rate of monomer production has been estimated to be $1-3 \times 10^6$ monomers per hour per cell during chick corneal development (from day 11 to 16 of development) which suggests that a single cell could produce $\sim 10-20$ cubic microns per hour of collagen [59]. Such a prolific rate of collagen production in a confined space should result in a highly crowded condition for the collagen molecules in a relatively short time.

Fibroblasts in the bounding confluent layers may provide external guidance cues (i.e. surface charge, protein expression, topological changes, etc.) while embedded fibroblasts could control local directionality of the monomers via internal guidance cues (e.g. elongated cell shape and filipodial extensions). To test this hypothesis, the organization of the collagen fibrils in the vicinity of a high-aspect-ratio object was investigated. Glass microcylinders (6 microns diameter and 20 microns length) were introduced to the high concentration solution of collagen molecules prior to fibrillogenesis. The representative results of this study (Supplementary Fig. 4A) showed that, at high concentrations, the direction of collagen fibrils can be locally influenced by the presence of a high-aspect-ratio object. It is thus possible that the combination of geometric confinement and internal guidance cues can be used to fully control the orientation of collagen fibrils and the thickness of the lamellae precipitated from dense solutions of collagen monomers. Due to their self-organizing capacity at crowded conditions, collagen molecules could possibly be used to organize other molecules. To explore this possibility, we performed a preliminary experiment to use molecularly-crowded collagen molecules to organize single-walled nanotubes (SWNT; Supplementary Fig. 4B). This is a potentially highly valuable method of secondary organizational control which could have significant applications in the nano- and biotechnology industry.

There are compelling observations which suggest that the formation of highly-organized collagenous structures *in vivo* is primarily a consequence of intrinsic collagen physicochemistry which is merely guided (as opposed to fully-controlled) by resident fibroblastic cells [60–61]. In this investigation we found that collagen is a highly cooperative molecule which exhibits a propensity to form natural load-bearing structures in the form of alternating lamellar sheets of fibrils. It is difficult to imagine that biology would not take advantage of this intrinsic property of collagen when producing the complex load-bearing structures necessary to successfully negotiate a world replete with mechanical challenges. The idea that the triple helix in atelo-collagen is encoded with long-range structural information which can be leveraged merely by tight confinement and concentration is both simplifying and empowering. It is simplifying because it changes

conceptually the idea that fibroblasts need to actively stitch together complex, highly-organized 2-D+ lamellar structures like the cornea. Instead, they would merely need to confine and concentrate collagen monomer while providing low-energy directional cues (which could be active cell attachments to the matrix, filipodia or the spindle shape, high aspect ratio cell body). The concept of cooperative collagen molecules is empowering because engineers may now be able to envision new ways to produce organized collagenous tissues and scaffoldings which do not entail the use of millions of intelligent, mobile, ~20 micron diameter, collagen extrusion machines (i.e. cells) operating independently.

5. Conclusions

In this investigation we demonstrate that uni- or bimolecular crowding can force collagen monomers to self-organize and assemble into compact aligned arrays of fibrils. If we combine molecular crowding with planar confinement the result is the formation of organized fibrillar matrices which show a striking resemblance to native load-bearing collagenous lamellae such as those found in the cornea and annulus fibrosus. The facility with which highly-organized collagenous matrices can be produced using this method should permit bottom-up approaches for the generation of *de novo* tissues as well as for cell-based *in vitro* applications (such as providing organized templates for cells). We further show that crowded collagen can be induced to either “follow” the contour of a large high-aspect ratio guidance structure or cause the organization of small high-aspect ratio objects (SWNTs). The ability to readily control the organization of collagen molecules by unimolecular or bimolecular crowding, confinement and geometrical guidance should enable us to fabricate highly-organized and potentially complex bio-inspired structures for a variety of applications including tissue engineering and nanotechnology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. DIC and TEM micrographs of *de novo* collagenous matrix alignment

A and B, DIC images of typical matrix alignment formed in the unimolecular crowded collagen solution in low concentration (**A**) and high concentration (**B**). **C and D**, En face sTEMs of fibril organization produced from LC (**A**) and HC (**B**) concentration collagen monomers. Over large distances fibrils were completely in alignment. **E and F**, Small angle x-ray diffraction pattern of low concentration (LC, (**E**)) and high concentration (HC, (**F**)) unimolecular crowded collagen fibrils. SAX pattern of constructs shows harmonics of the ~65 nm periodicity (arrows), which corresponds to the D-banding patterns of native collagen fibrils. The butterfly-shaped bands of diffuse scattering in the orthogonal direction are indicative of the ~10 nm length scales for fibrils grouped into bundles. Bars are 20 microns in **A** and **B**, and 500nm in **C** and **D**.

Figure 2. DIC micrographs of bimolecular crowded collagen fibrils

Collagen molecules were crowded in the using PEG: 0% PEG (**A**, control), 25% PEG (**B**), 50% PEG (**C**), and 75% PEG (**D**). Bimolecular crowding of collagen molecules resulted in the packing of the fibrils into aligned bundles and very limited number of individual collagen fibrils. Bar =20 microns.

Figure 3. DIC, TEM and SEM micrographs of the lamellar structure of the *de novo* collagenous matrix constructs

A, Artistic representation of the lamellar structure of the collagenous matrices found *in vivo* and produced *de novo* in this investigation. **B**, Optical thick section of unimolecular crowded collagen. Five morphologically distinct layers (at low magnification light microscopy) are clearly visible in this 100 micron thick cross-section (white arrows indicate individual layers). **C** and **D**, DIC images extracted from z-scans demonstrating the change in direction of the matrix in the LC construct. The matrix alignment angle changes 30° over a depth of 30 microns. **E** and **F**, Lamellar structures spontaneously formed in *de novo* construct comprises aligned collagen fibrillar arrays (black arrows indicate width of a lamella, white arrow indicates direction of fibril alignment in a lamellar plate. **G** and **H**, SEM of fractured construct clearly demonstrates the multi-layer structure of the collagenous constructs where the organization of the collagen fibrils spontaneously changes across the thickness of the sample. Bars are 50 microns in **B**, 20 microns in **C** and **D**, 5 microns in **E**, microns in **F**, 500 microns in **G**, and 2 microns in **H**.

Figure 4. SEM and sTEM micrographs of collagen in high-concentration *de novo* constructs in cross-section

A, SEM of fractured cross-section of construct where a clear change of direction in highly-aligned collagen fibrils in lamellae may be discerned. **B**, Fibrils which are cut in cross-section are so densely packed that the construct appears to be “solid”. **C**, Low magnification sTEM of large uniform area in cross-section confirming the high-density and uniformity of the fibrillar array. **D**, High-magnification sTEM of collagen in cross-section demonstrating the presence of individual fibrils. In general, fibrils are small and have a polydisperse diameter distribution. The micrograph shows numerous fused and irregular fibrils. Such fibrillar fusion and irregularity can also be found in lumican knockout mouse corneas [1]. Bars are 10 microns in **A**, 2 microns in **B**, 1 microns in **C**, and 200nm in **D**.