Exploring the behavior of concentrated collagen, approaches to tailor biomimetic materials.

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ABSTRACT

Dense ordered collagen matrices can be obtained by fine tuning the electrostatic interactions in highly concentrated solutions of type I collagen. Such biomimetic fibrillar materials have a high application potential in the field of tissue engineering, owing to their structural similarity with major biological tissues like bone, cornea, tendon and skin. We show here that an isotropic-to-cholesteric (I/N*) phase transition occurs at critical concentrations ranging from 50-60 mg/mL to 80-85 mg/mL depending on solvent composition. X-ray diffraction experiments revealed that the average distance between triple helices d_{ave} decreases linearly as a function of $\phi^{-1/2}$. Equilibrium concentrations and the order parameter of the nematic phase agree reasonably well with theoretical predictions for semiflexible macromolecules. In the same high concentration range, close to that of living tissues (40-300 mg/ml), we explored the structure of gels obtained by setting the pH close to physiological conditions.

Keywords: collagen, liquid crystal, fibrillogenesis, self-assembly, TEM, X-ray scattering.

1 RESULTS AND DISCUSSION

1.1 I/N* transition critical concentrations

Based on previous work in the group (1,2), we explored a wide collagen concentration domain in acidic solutions to locate as precisely as possible the transition from a disordered isotropic state to an ordered chiral nematic (N*) phase. The main conclusions of this work are reported in reference 3. We have demonstrated that the critical concentrations at which the isotropic-to-cholesteric (I/N*) phase transition occurs increase from 50-60 mg/mL to 80-85 mg/mL as the acetic acid concentration of the solvent increases from 5 mM to 500 mM. These values essentially correspond to the dilute boundary of the I/N* transition, C_i, *ie* the concentrations at which the ordered phase appears in an otherwise isotropic solution, thus producing a biphasic sample. The location of the threshold was determined by inserting solutions into capillary tubes and testing them for birefringence. Isotropic samples very close to the transition exhibited transitory flow birefringence that relaxed within minutes. We considered that samples were anisotropic, at higher concentrations, when the birefringence did not relax over months. The change in behavior occured in a rather narrow concentration range.

We then tested classical theories from statistical physics designed to predict the critical concentration of such transition in colloidal suspensions of elongated particles. The original theory developed by Onsager for rigid rods (4) yields largely underestimated values. Long-range electrostatic repulsions were explicitly included in the model as an effective diameter D_{eff} (5) for collagen molecules, instead of their structural diameter of about 1.5 nm. For collagen concentration series prepared in 500 mM acetic acid, Onsager's theory predicts a critical concentration of 2.95 mg/mL when the experimental data is in the 80-85 mg/ml range. In fact, Khokhlov and Semenov (6) proposed an adaptation of the aforementioned theory, that accounts for the marked flexibility of most biological macromolecules, characterized by the ratio $R=L/P$, where L is the contour length and P the persistence length. Values of the persistence length found in the literature vary from 57 (7) to 160 nm (8), to mention only data obtained in the hydrated state. Given the strong dependence of the model to flexibility, the predicted critical concentration obtained using Chen's simulations (9) largely vary with P from 111.8 to 15.4 mg/ml, essentially bracketing the experimental data.

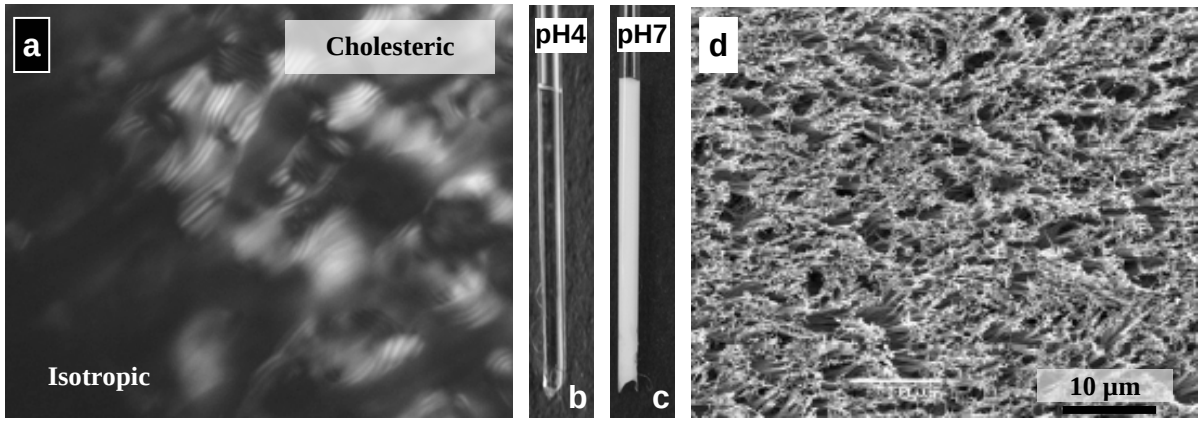


Figure 1: Formation of an ordered gel by fibrillogenesis of a concentrated collagen solution. (a) Biphasic sample viewed in polarized light microscopy. In a concentration gradient, the disordered isotropic (I) phase coexists with the birefringent cholesteric phase (N*). Concentrated collagen solutions (100 mg/mL) are transparent at acidic pH **(b)** and turn opaque when the pH is brought to neutral or basic pH **(c)**. The resulting gels, observed in SEM **(d)** are comprised of native-like fibrils with a long-range organization dependent on the initial collagen concentration (here 80 mg/mL) and the solution mechanical history (shear-aligned).

It is also possible to obtain birefringent samples by slowly evaporating the solvent directly in glass microchambers suitable for optical observations (2) and therefore explore them in polarized light with no external perturbation. In particular, by avoiding shearing, the anisotropic cholesteric phase exhibits intrinsic long range chirality visible as a typical fingerprint pattern with a helical pitch of a few microns. This chiral phase can be unwound into a uniaxial nematic phase, where molecules are aligned in the same direction, by applying moderate shear-stress. In a concentration gradient, which is established owing to the slow evaporation of acetic acid, the chiral nematic phase can be seen in coexistence with the isotropic one, separated by a well-defined interface (Figure 1a).

1.2 Local order in concentrated solutions

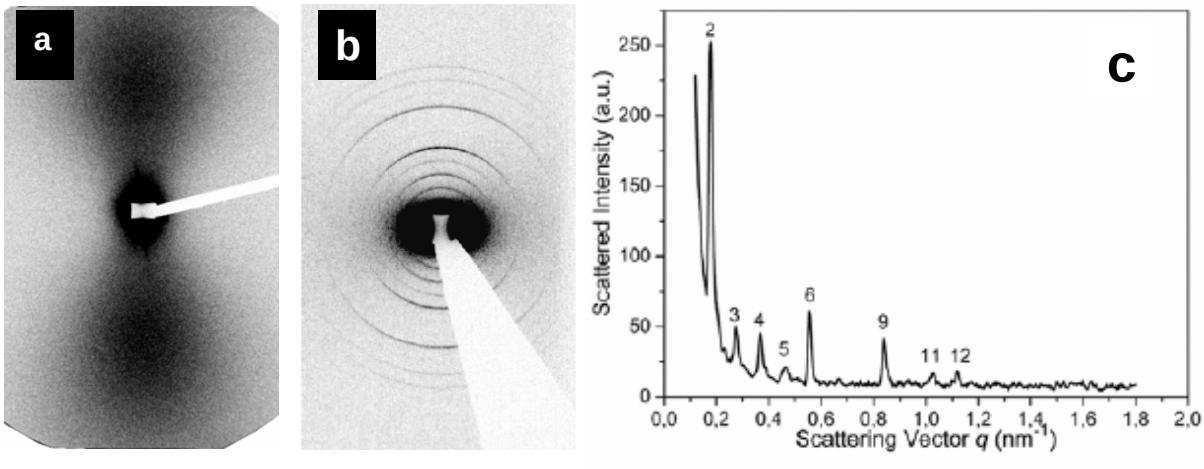
In concentrated solutions, interparticle scattering gives rise to a broad interference peak that we studied by small angle x-ray scattering. Strongly anisotropic diffraction patterns were obtained with highly concentrated collagen solutions submitted to very moderate shear (Figure 2a). The diffuse scattering intensity is higher in the vertical direction, indicating that the elongated collagen molecules are oriented horizontally along the flow direction. The average distance between triple helices d_{ave} can be determined from the position of the scattered intensity maximum. It was shown to decrease linearly as a function of $\phi^{-1/2}$ from 12.7 ±0.9 nm (22.5 mg/mL) to 5.0 ±0.6 nm (166.4 mg/mL). Taking a section through the 2D pattern at a fixed **q** modulus gives a profile of the intensity as a function of the azimuthal angle, the shape of which is directly related to the molecules' orientational distribution. Typically, for a 90 mg/mL collagen solution in 500 mM acetic acid, optically birefringent at rest but close to the phase boundary, the order parameter *S* is estimated to be 0.45 (±0.1). The predicted value (9) of *S* varies from 0.477 with the highest value of the persistence length (*P* = 160 nm) to 0.492 if the particles are considered more flexible (*P* = 57 nm).

Interestingly, we also found that fibrillar aggregates form in acidic solutions at collagen concentrations above 150 mg/mL. This is revealed by transmission electron microscopy by the presence of striated fibrils and by SAXS where harmonics of the 67-nm periodicity are visible in the diffraction patterns (10). Such evidences suggest a second phase transition, different from the Isotropic-Cholesteric phase transition, that is more like a N*/SmA transition (11).

1.3 Fibrillogenesis in concentrated solutions

In the same high collagen concentration range, close to that of living tissues (40-300 mg/ml), we explored (10) the structure of gels obtained by setting the pH close to physiological conditions, *ie* near neutrality.

In general, highly concentrated collagen solutions are transparent or translucent and turn opaque when brought to neutral pH (figure 1b,c). Fibrillogenesis yields strong elastic fibrillar gels for collagen concentrations higher than about 40 mg/ml. The SEM picture in figure 1d gives a general view of the internal structure of a gel prepared from an 80 mg/ml solution. It is mostly comprised of intertwined fibrils with a general orientation inherited from the long-range uniaxial organization of collagen triple helices induced by shear-alignement of the initial (acidic) solution.



Small-angle x-ray scattering of concentrated collagen. (a) Cholesteric collagen solution (91 mg/ml) in acetic acid recorded under moderate shear ($\dot{\gamma}$= 8 s^{-1}). **(b)** Fibrillar gel obtained with a 145 mg/mL collagen solution and **(c)** corresponding linear intensity profile. The peaks are labeled according to the order of the reflections arising from the staggered stacking of collagen molecules in fibrils.

The typical cross-striated patterns were visible in TEM in all conditions in the 6 to 12 pH range. Similarly, we have recorded the corresponding SAXS 67-nm diffraction peaks (figures 2b,c) in a wide range of pH [6-13] and ionic strength [24-794 mM].

The internal structure of fibrils does not seem to be influenced by the variations in physico-chemical conditions in the ranges given above, nor is it strongly affected by collagen concentration. However the latter parameter greatly influences the overall macroscopic structure of the resultant fibrillar gels, as well as the morphology and structure of the fibrils themselves. In the 40- to 80-mg/ml range, fibrils are small and thin (with a length/diameter aspect ratio of approximately 10–20), with little size polydispersity and no preferred orientation. Their width increases with concentration, from 40 to 80 nm at 40 mg/ml collagen to 80–120 nm at 80 mg/ml. At 100 mg/ml, there is a significant change in the fibril morphology with a much lower aspect ratio of about 4 to 5. Their size can reach up to 1.2 µm in width and 6 µm in length. The main axes of fibrils can have a preferred orientation imposed by the shearing of the initial solution. From 200 to 300 mg/ml, the fibrils are smaller (D=100 nm, L=1 µm) and are distributed more homogeneously. In the highest concentration range studied, above 300 mg/ml, a periodic arced pattern reminiscent of a helical structure (12) is sometimes visible, with a network of small interconnected fibrils.

2 CONCLUSIONS

We were able to assess the first-order nature of the isotropic/cholesteric phase transition in highly concentrated collagen solutions. Therefore, orientational ordering of soluble collagen in vitro occurs spontaneously without requiring any protein activity or cellular action.

Equilibrium concentrations and the order parameter of the nematic phase agree reasonably well with theoretical predictions for semiflexible macromolecules.

In concentrated collagen solutions, fibrillogenesis can be induced by the diffusion of fibril-forming buffers (FFBs). We show that it is possible to preserve the long-range organization of the initial acidic liquid-crystalline phase, either chiral or uniaxial, as done previously with ammonia vapor.

Biomimetic fibrillar matrixes exhibit the typical 67 nm X-ray diffraction signal. Negative staining of the samples reveals in TEM the succession of gap and overlap zones along the collagen fibrils present in the dense gels as in rat tail tendons. Native-like fibrils are formed and are stable over a pH range from pH 6.2 to 12.2, despite significant changes in surface electrical charge. In this same pH range, and from 24 to 794 mM ionic strength, fibrils exhibit typical striations observed by TEM, and the corresponding X-ray scattering signal is clearly visible with numerous harmonics.

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