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Possible transient liquid crystal phase during the laying out of connective tissues: α-chitin and collagen as models

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Abstract

Morphogenesis of extracellular matrices can be considered from different perspectives. One is that of ontogenesis, i.e., an organism's development, which is mostly concerned with the spatiotemporal regulation of genes, cell differentiation and migration. Complementary to this purely biological point of view, a physico-chemical approach can help in understanding complex mechanisms by highlighting specific events that do not require direct cellular control. Because of a structural similarity between some biological systems and liquid crystals, it was supposed that similar mechanisms could be involved. In this respect, it is important to determine the intrinsic self-assembly properties driving the ordering of biological macromolecules. Here we review *in vitro* studies of the condensed state of major biological macromolecules from extracellular matrices and related theories describing a mesophase transition in suspensions of rodlike particles. Dilute suspensions of collagen or chitin are isotropic, i.e., the macromolecules can take on any orientation in the fluid. Beyond a critical concentration, an ordered nematic phase appears with a higher volume fraction. The two-phase coexistence can be seen between crossed polarizers since the nematic phase is strongly birefringent and appears bright, whereas the isotropic phase remains dark. A widespread property of these structural macromolecular scaffolds is their chirality. Although the origin of chirality in colloidal suspensions is still a subject of debate, the helical nature of the cholesteric phase can be quantified. Small angle x-ray scattering performed on shear-aligned samples can help demonstrate the cholesteric nature of the anisotropic phase, inferred from optical observations. Liquid-like positional local order is revealed by the presence of broad interference peaks at low angle. The azimuthal profiles of these patterns are fitted to determine the value of the nematic order parameter at the transition. A few physico-chemistry experiments can assess the nature of the transition, and in turn, applying theoretical models can prove useful in predicting and controlling the structure of assemblies of biological macromolecules.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Skeletal materials found in living organisms offer a variety of complex and subtle architectures with specific properties that inspire material scientists in physics and chemistry [1]. An essential characteristic of biological materials is their hierarchical organization from the nanometre to the millimetre scale and higher. Mollusc shells, crustacean carapaces, fish scales, and compact bones provide remarkable examples. During the course of evolution, a limited number of structural macromolecules have been selected, sometimes adopting the same principles of organization, despite their biochemical diversity. We chose to study the self-assembly mechanisms of the two most abundant in the animal world: a protein, collagen, and a polysaccharide, chitin. Cellulose, present in plant cell walls, is another polysaccharide chemically close to chitin that exhibits a very similar hierarchical structure. In each case, the macromolecules are synthesized by specialized cells as the result of gene expression controlled by complex regulatory and signalling pathways. Although the ordered organic matrix laid out in the extracellular space is comprised of many other macromolecules, the fibrous components (collagen, chitin) predominate and support the long-range tridimensional organization. In some cases, a mineral phase of either carbonate (calcite) or phosphate (apatite) calcium salts is deposited in the spaces left free within the fibrillar network [2]. Bones and crustacean carapaces are periodically regenerated throughout the animal's lifetime. Both biological tissues are dynamic materials: arthropods have moulting (ecdysis) cycles, where shedding of the old 'skin' requires its enzymatic resorption and replacement by a new larger exoskeleton. Likewise, bone can be remodelled by the complementary (or competitive) action of osteoclasts (degradation) and osteoblasts (synthesis). The picture of static hard tissues built up once and for all is therefore rather naïve. In this respect, it is important to fully comprehend the mechanisms that not only yield such a complex structure, but also allow its permanent renewal. One long-lasting belief is that the highly ordered three-dimensional organization is the result of a genetic determinism through which tissue-specific cells—osteoblasts in bone and epidermal cells in carapace, for instance—not only synthesize collagen and chitin but also position and organize it by means of their cytoskeleton. There are numerous lines of evidence today supporting another approach based on the intrinsic properties of biological macromolecules to self-assemble and spontaneously form complex ordered networks without the intervention of cells. Initially, a strong structural similarity was demonstrated some 30 years ago between the supramolecular arrangement of biomacromolecules in connective tissues, and the ordering of small molecules with thermotropic liquid-crystal (LC) properties. Following this, it was shown that many biological macromolecules with a strong shape anisotropy (length $\gg$ diameter) can self-assemble *in vitro* in the absence of cells and produce the same kind of supramolecular arrangements as those found in living tissues. For instance, extracted collagen and chitin form lyotropic cholesteric mesophases with fluidities characteristic of liquid crystals that mature biological tissues like bone and carapaces are lacking. However, the forces at play are quite different between thermotropic LCs and lyotropic mesophases. The system undergoes a transition from a disordered to an ordered state due to an increase in volume fraction in lyotropics, and to a decrease in temperature in thermotropics. The isotropic/mesophase transition in lyotropic systems is mainly due to excluded volume repulsive forces [3, 4], whereas in thermotropic liquid crystal the transition is mostly driven by attractive van der Waals interactions [5]. In both cases, the transition is first order, although weakly so, and corresponds to a discontinuity in the order parameter.

The aims of this review are (i) to recall the structural analogy between some important biological tissues and liquid crystals, (ii) to summarize the main features of Onsager's theoretical treatment of the isotropic–nematic transition, and finally (iii) to compare this model

with recent experimental data of *in vitro* reassembly of biological macromolecules. The experimental systems we investigate, chitin colloidal dispersions and type I collagen solutions, are of particular relevance since they constitute the skeleton of most animal species.

2. Biological liquid crystals and analogues

2.1. Structural basis for an analogy with cholesteric LCs

Numerous biological tissues, networks and actuators exhibit highly ordered and anisotropic tridimensional organizations. Some of them are true liquid crystals *in vivo* in physiological conditions, with functions associated with the fluidity and anisotropy of the mesophase. Cell membranes composed of phospholipid bilayers are by far the most widely known example of a biological LC. Stacks of bilayers are a typical lyotropic lamellar phase that corresponds to the smectic structure initially described by Friedel [6]. Plasma and nuclear membranes are limited to one and two periods, respectively. In contrast, myelin of neural cells, the thylakoid membrane of chloroplasts and its equivalent in small marine phytoplanktonic organisms are made of several concentric stacked bilayers. A similar onion-like multilamellar structure can be produced *in vitro* by shearing a lamellar phase of surfactants [7]. A second example of a biological macromolecule that exhibits liquid crystallinity *in vivo* was suggested by Livolant's work on dinoflagellate chromosomes [8, 9]. The helical organization of DNA in these exceptionally large chromosomes free from histones was revealed by characteristic arced patterns in transmission electron microscopy TEM [9, 10]. Mucilage from quince seeds has also been studied as a possible example of a fluid ordered state *in vivo* [11–14]. When dry quince seeds are imbibed with water, a fluid substance is released that exhibits optical birefringence when observed between crossed polarizers.

Here again, images obtained by TEM [13–15] of thin sections of seeds display an arced pattern, revealing the helical organization of cellulose fibres (microcrystals), as is observed in other plant cell walls [16]. The authors suggest that cellulose microcrystals inside the seeds are helically arranged and are able to flow upon wetting. The actin–myosin network in muscle cells is often cited as an example of biological LC. The periodical structure of sarcomeres lined up along the fibre direction bears a strong resemblance to smectic geometry and transverse sections through the A band in insect flight muscles sometimes exhibit a double hexagonal array of interdigitating myosin and actin filaments [17, 18]. The two molecules have the ability to slide with respect to each other during muscle contraction. However, this movement, due to a conformational change of the myosin heads, requires energy produced by the hydrolysis of ATP. Therefore, the myosin/actin system hardly qualifies as a liquid crystal, although it remains a beautiful example of a sophisticated self-organized biological actuator.

Let us now turn our attention to connective tissues whose structure bears a striking resemblance to that of LCs [19, 20], but that exhibit no or very little fluidity in their functional state. Compact bones [21, 22], fish scales [23, 24] and cornea [25–27], for instance, are highly ordered collagenous matrices. To a lesser extent, skin, tendon and ligaments also have anisotropic structural features, suggesting an ordered fluid state at some point in their morphogenesis. The exoskeleton of arthropods, i.e., the carapace of crustaceans, insects, myriapods and arachnids, is comprised of chitin fibres organized in a helical fashion. Crab cuticles, for instance, when observed as thin sections in TEM, show characteristic series of nested arcs. It was shown by Bouligand [18, 28] and Neville [29, 30] that these patterns reveal the chiral arrangement of chitin microcrystals throughout the carapace. This was the starting point of a *school of thought* whose purpose was, and still is, to determine whether self-assembly is the leading mechanism in laying out ordered extracellular matrices based on the analogy with

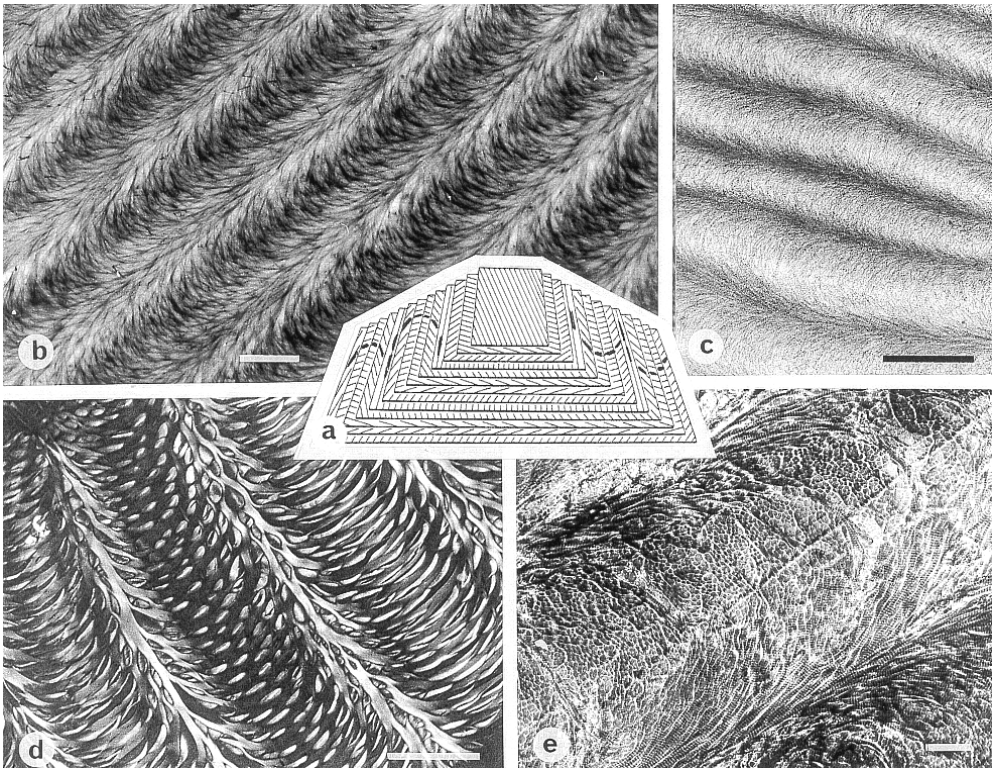

Figure 1. Examples of twisted plywoods in biological fibrous systems. (a) Progressive rotation of fibrillar direction in series of equidistant planes. The fibrous orientations draw series of nested arcs on the oblique side of the model. (b) Chitin–protein organic matrix of a decalcified crab cuticle showing superimposed series of nested arcs in TEM, bar = 2 μm. (c) Plant cell walls from the skin of a pear where cellulose microcrystals exhibit the same pattern in TEM, bar = 0.5 μm (courtesy of D Reis). (d) Collagen network observed in optical polarized light microscopy showing arced patterns in the dermal scutes of fishes, bar = 40 μm (courtesy of L Besseau). (e) Decalcified human compact bone osteon observed in TEM, showing the helical organization of cross-striated collagen fibrils, bar = 1 μm. Reprinted from [19] with permission from Elsevier.

cholesteric LCs. Following the lead of this pioneering work, several authors described similar helical organizations in other tissues with functions similar to those of cellulose in plant cell walls [13–16] and type I collagen in compact bones [21]. It should be noted that chitin and cellulose are both linear polysaccharides that form long and thin microcrystals, whereas type I collagen is a large protein made of three polypeptide chains folded into an elongated triple helix. Incidentally, the same was shown to be true for DNA, a double helix of polynucleotides. In spite of very different chemical structures, these three biological macromolecules adopt the same long-range tridimensional organization. As proposed in Bouligand's model, schematically represented in the centre of figure 1, the helical geometry arises from a continuous rotation of the direction of molecules that are otherwise fairly well aligned with respect to each other at a local scale. It is important to point out that in most cases the planes drawn in the model are virtual and have no physical meaning, the rotation of chitin microcrystals or collagen molecules being continuous along the helical axis. In some cases, however, it is possible to identify layers of some thickness and to determine a finite angle of rotation between adjacent layers. The distance that separates equivalent orientations of the molecules after 360° rotation is the helical

pitch. It usually ranges from hundreds of nanometres to several microns in biological tissues. The resulting periodicity roughly spans the spectral region from visible light to mid- and even far infrared. Some insects and plants exhibit interference colours as a consequence of light interaction with a periodic structure [31–35]. When the helical pitch has values larger than the wavelength of visible light, the same phenomenon should apply to infrared radiation. It was thus suggested that the helical organization of insect cuticles may act as a cooling system, in a way similar to that described for some butterfly species where photonic-crystal-type structures present in the scales is suggested to play a role in thermal regulation [36]. Modulations in the chirality of the tissues have also been described ([37] and P 172 in [30]). Sections taken through the tibia of a desert locust excised at night shows stacks of helical (cholesteric) layers and when excised during the day, uniaxial (nematic) arrangements are seen [30]. The source of this difference is not known but it has been shown to be related to control by circadian clocks located in the epidermis, independent of the nervous and endocrine systems. It should be noted that, apart from exhibiting a helical organization throughout the carapace, chitin rods were shown to be arranged locally on a quasi-hexagonal lattice.

2.2. Biosynthesis, a possible transient fluid state

Cells build macromolecular compounds through biochemical processes inside cellular compartments often in close association with membranes, and usually at their surface (transmembrane synthases). The complete biochemical pathway can be complex and often involves multiple enzymatic activities, as in the case of chitin biosynthesis [38, 39]. The biosynthesis of type I collagen, which is a heterotrimer composed of two α_1 chains and one α_2 chain, follows the classical scheme of RNA translation on membrane-bound ribosomes. Individual polypeptide chains with carboxy- and amino-terminal propeptides are released into the lumen of the endoplasmic reticulum (ER). The non-helical propeptides initiate the trimerization of the individual chains and folding into a triple helix. Finally, propeptides are responsible for the solubility of collagen at physiological pH. Collagen can be processed through the ER, modified by hydroxylations and glycosylations, and finally secreted by Golgi secretory vesicles. Once the collagen molecules (triple helices) are in the extracellular space, the propeptides are chopped off by specific proteases and the resulting tropocollagen, from now on referred to as 'collagen', precipitates into fibrils. The structure and assembly mechanisms of collagen fibrils are out of the scope of this review and an abundant literature is devoted to these aspects [40–43]. Two crucial steps that still need to be clarified are how the three polypeptide chains are assembled and secreted by the cell. One important question to address is whether the procollagen in the secretory vesicles is packed [44] at such a high volume fraction that it forms a cholesteric liquid crystal, thus imprinting chirality on the solid tissue formed outside the cell. Woodhead-Galloway, Knight and co-workers made major contributions to the identification of liquid crystal phases of collagen *in vivo*, especially with their work on shark fins [45] and dogfish egg cases [46, 47]. In the latter study, a series of molecular assembly states were identified by TEM in secretory droplets. As in the case of DNA from dinoflagellate chromosomes, patterns observed in TEM reveal the organization of collagen molecules somewhat indirectly after chemical fixation and ultrathin sectioning. Likewise, it was suggested that spider silk proteins are stored within intracellular secretory vesicles in a mesomorphic state [48], and in particular a hexagonal columnar phase [49]. Going back to vertebrate collagen, if a liquid crystalline state should occur before excretion, i.e., in secretory vesicles, it would be in the form of procollagen. *In vitro* reassembly of procollagen extracted from chick embryo tendons [50] yielded a birefringent fluid phase with a 'precholesteric' texture very similar to that of birefringent shear bands [51]. No cholesteric phase was obtained,

but the study was limited by the small quantities of product available due to the painstaking processes of production and purification. More work would be needed to study the self-assembly properties of procollagen at physiological pH. This would require large amounts of the molecule, which might be possible by expressing collagen in recombinant systems [52, 53].

3. *In vitro* reassembly, entropy-driven processes

Confronted with the question of whether biological macromolecules have the intrinsic ability to form mesophases, several groups designed suitable purification protocols to test self-assembly properties *in vitro*. It must be noted that the extracted molecules have often been matured *in vivo* and therefore physicochemical studies *in vitro* are usually conducted in conditions slightly different from physiological ones. Cellulose [54] and chitin [55, 56] microcrystals and collagen triple helices [57–59] all gave birefringent fingerprint textures typical of a cholesteric phase when brought to high enough concentrations. Crucial intracellular molecules like DNA [60, 61] and components of the cytoskeleton, like F-actin filaments [62–64] and microtubules [65], were also shown to form mesophases *in vitro*. In the following part, we recall the main statistical physics models describing the isotropic–nematic transition in dispersions of rodlike particles and how their main features are tested experimentally.

3.1. Models for self-assembly of elongated particles

Every elongated macromolecule or filament, stably dispersed in a solvent, is likely to produce anisotropic assemblies when brought to high enough concentrations. This property of lyotropic liquid crystals was theoretically addressed in a seminal paper by Lars Onsager in 1949 [3], later to be reviewed and extended [66–70]. At the centre of this model is the idea that elongated particles with a high aspect ratio align spontaneously above a critical volume fraction. Beyond this threshold, the isotropic phase becomes unstable and the system undergoes an isotropic/nematic first-order transition towards a much more ordered state (figure 2). The lowest free energy is reached when the entropy is largest. The excess Helmholtz free energy ΔF (equation (1)), thermodynamically describing the dispersion of rigid rods, contains two competing entropic terms, in the second virial coefficient approximation:

$$\frac{\Delta F}{NkT} = \int \ln(4\pi f)\, \mathrm{d}\Omega - \frac{\rho}{2} \int f_1 f_2 \beta_m \, \mathrm{d}\Omega_1 \, \mathrm{d}\Omega_2. \tag{1}$$

The orientational distribution function $f(\Omega)$ is related to the probability of finding a rod pointing in a given direction within a small solid angle $\mathrm{d}\Omega$. Of the two competing entropic terms the first one corresponds to the entropy of mixing and favours random orientation of the rods; it is lower in the nematic phase. The second term represents the excluded volume entropy and increases as the average angle between neighbouring particles decreases. Since it is subtracted from ΔF, tighter packing in the nematic decreases the total free energy. Onsager's theory considers ideal infinitely long rods and is accurate for $L/D > 100$.

More recently, another expression of the free energy of spherocylinder suspensions was proposed by Lee [71], in order to describe the transition for particles of low aspect ratio. The original theory [3] was also implemented to include a twisting force arising from electrostatic effects [72], and extended to account for semiflexibility [73, 74, 83], three-body interactions, and polydispersity [70, 75, 76]. Predicting the coexistence concentration was done principally in two ways. One way consists of using a trial function for the orientational distribution and minimizing the free energy with adjustable parameters. Onsager's trial function is almost Gaussian in the nematic phase and yields coexistence concentrations $c_\mathrm{i} = 3.340$ and $c_\mathrm{a} =$

4.486 in the isotropic and anisotropic phases, respectively. The parameters 'c' used here are dimensionless and are related to the number density, i.e., the number of particles (N) per unit volume (V), $c' = N/V$, through $c = bc'$, where $b = (\pi/4)L^2D$ is half the excluded volume between two rods in the isotropic phase. The other approach is the direct minimization of equation (1), and the analysis of Kayser and Reveché gives $c_i = 3.291$ and $c_a = 4.223$. Very similar values were found by Lekkerkerker *et al* [76]: $c_i = 3.290$ and $c_a = 4.191$. Finally, the scaled particle theory, yet another statistical mechanics treatment of the same problem, was proposed by Cotter [77] and Lasher [78], in which virial coefficients of order higher than two are approximated. This theory was also extended to account for some flexibility of the particles [79] and proved to give good predictions for coexistence concentrations, except at low ionic strength [80, 81]. The sharp discontinuity in density is a unique feature of liquid crystals composed of hard rods suspended in solvent. The situation is very different from that of thermotropic liquid crystal, where attractive interactions, mostly dipole–dipole, predominate. In fact, Maier and Saupe found a very small difference in density between isotropic and nematic phases for a thermotropic LC [5]. In the case of dispersed rigid rods, infinite repulsion prevents the particles from overlapping in the hard rod limit.

Another connected characteristic feature of the first-order I/N transition is a jump in the value of the order parameter that describes orientational order as follows (p 37 in [82]):

$$S = \langle P_2(\cos\theta)\rangle = \int f(\theta)\left(\tfrac{3}{2}\cos^2\theta - \tfrac{1}{2}\right)\mathrm{d}\Omega. \tag{2}$$

Here, theta (θ) is the angle between two rods and P_2 is the Legendre polynomial of order two. If the orientation is totally random as in the isotropic phase, $S = 0$. For perfectly aligned rods, it would be equal to unity, with all the particles pointing exactly along the director **n**. Different values have been proposed for the nematic phase coexisting with the isotropic phase in the case of long rigid rods. Typically, $S = 0.848$ [3], 0.792 [76] or 0.784 [78], depending on the theoretical treatment. The value of S at the transition decreases when the particles exhibit some flexibility [73, 70]. For instance, the bacteriophage fd has a ratio of the persistence length to the contour length p/L of about 2.5 ($L = 880$ nm and $p = 2200$ nm), and S is predicted to be 0.55 [73, 83], which is confirmed to some extent by experiments [80, 81]. The particles we study are dispersed in aqueous solvents and bear charges on their surface that stabilize the suspensions. This must also be the case in living tissues, which are immersed in aqueous biological fluids. The counter-ions that compensate the surface charges form a repulsive coat around the rods so that their bare diameter D is increased by a layer of thickness δ. It has been demonstrated that the models are still valid and can be applied to charged particles, considering an effective diameter $D_{\mathrm{eff}} = D_{\mathrm{bare}} + \delta$. In aqueous solvents, the thickness is related to the Debye length κ^{-1}, which essentially depends on the ionic strength. Coexistence conditions can be expressed as volume fractions: $\phi_i = 3.3L/D_{\mathrm{eff}}$ and $\phi_a = 4.2L/D_{\mathrm{eff}}$. Since D_{eff} decreases as the ionic strength increases, adding salt to a suspension of rodlike particles should increase the isotropic–nematic coexistence concentrations. The transition described in the models is first order and the two phases should coexist in a domain whose concentration range should vary with ionic strength, but with a constant width. In contrast, the theory proposed by Lee for spherocylinders is sensitive to the aspect ratio of the particles. For $L/D < 100$, not only are the predicted concentrations lower than in Onsager's model, but the width of the coexistence domain is predicted to narrow as L/D decreases, down to a critical value where the phase separation does not occur anymore. The order parameter of the nematic phase at the transition also varies and goes from 0.783 at high aspect ratio ($\sim$100) down to 0.604 for $L/D = 2$.

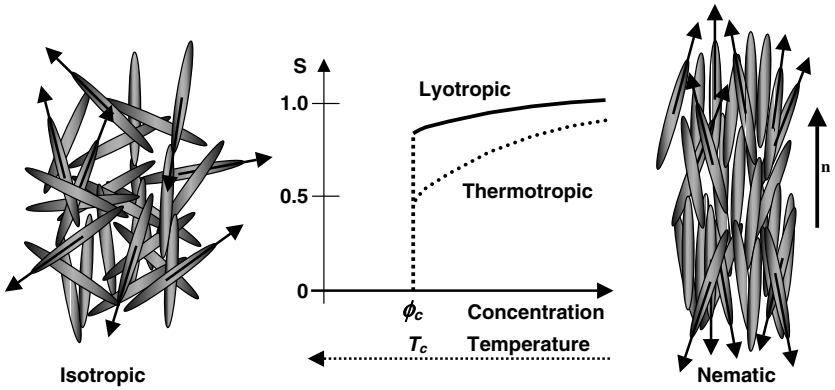

Figure 2. Schematic diagram of an assembly of rods undergoing an isotropic/nematic phase transition. The orientation of rods in the isotropic phase is random, whereas it follows an average direction (director **n**) in the nematic phase. The order parameter S jumps from 0 in the isotropic phase to about 0.5 in thermotropic nematics below a critical temperature T_C (dotted line) and almost 0.8 for rigid rods forming a lyotropic nematic phase beyond a critical concentration ϕ_C (solid line).

3.2. Testing the models

We study liquid crystallinity of biological macromolecules *in vitro* because controlling or even only monitoring the physico-chemical parameters *in vivo* is extremely difficult. Extraction conditions must preserve the structural integrity of the macromolecules, which is necessary for *in vitro* self-assembly studies. The collagen we use comes from rat tail tendons, and with several purification steps solubilization in 0.5 M acetic acid is simply achieved. It was shown that mild ultrasound treatment could promote the formation of collagen mesophases [84], probably due to an increased mobility of shorter fragments, the triple helical structure of the protein being preserved. Chitin from crustacean shells, the most frequent industrial source, is subjected to much harsher treatments, in particular boiling in concentrated HCl for one hour. Wide-angle x-ray diffraction is used to confirm that extracted chitin rods still exhibit a typical α-chitin crystal structure [85], and titration methods are used to estimate the charge density, which depends on the extraction conditions [86, 87]. Whether or not a given system can be described by Onsager's model can be tested by performing a few rather simple experiments. The most sensitive parameter is the orientational distribution function, although it is usually not very easy to evaluate. Let us suppose that we know our system exhibits optical birefringence at high volume fractions, suggesting a lyotropic phase transition from a disordered to an ordered state. One of the main features of the model is that the transition is first order and there should be a concentration range where the two phases coexists. The most practical way to proceed is to prepare a series of samples with increasing concentrations and pick up the last isotropic sample and the first anisotropic one. Because the density of the nematic phase is higher than that of the isotropic phase, anisotropic droplets form and sediment to the bottom of the vial, where they coalesce (figure 3(a)) and form a continuous phase that appears bright between crossed polarizers (figure 3(b)). When the phase separation process is complete, a sharp interface separates the two coexisting phases (figure 3(c)). The bright nematic phase exhibits the characteristic fingerprint pattern, revealing the chiral nature of the cholesteric phase (figures 3(b), 4(a), (c)). Collagen solutions in 0.5 M acetic acid exhibit strong birefringence for concentrations above about 100 mg ml^{-1} [57–59].

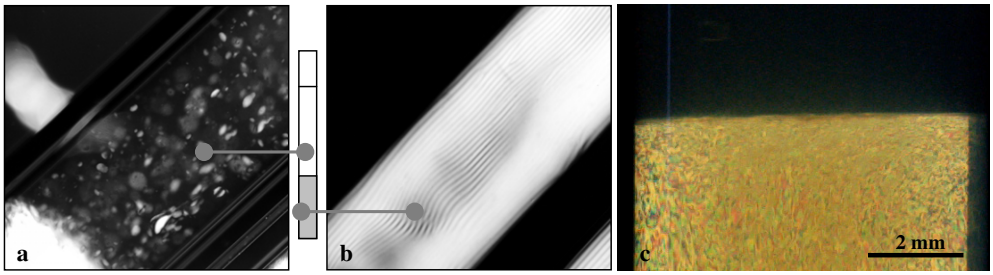

Figure 3. Polarized light microscopy of two characteristic regions in a capillary tube filled with biphasic chitin suspension during the phase separation process. (a) is taken above the interface and shows sedimenting droplets; (b) corresponds to a homogeneously oriented cholesteric phase with the chiral axis perpendicular to the lateral sides of the tube. (c) Close-up of the I/N interface in a wide tube viewed between crossed polarizers.

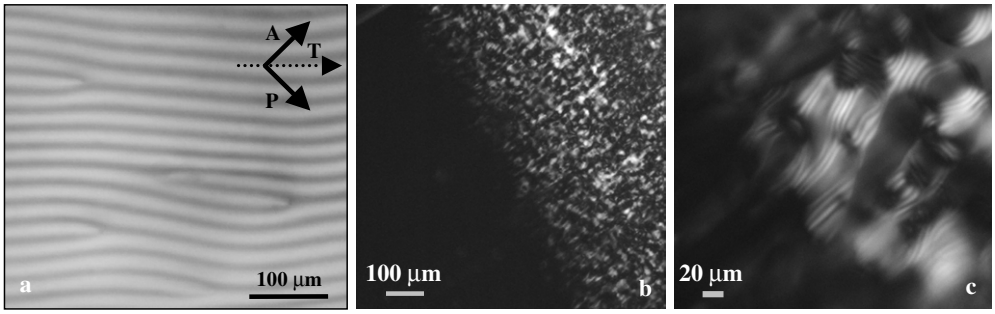

Figure 4. (a) Cholesteric phase in a chitin suspension (2.05 w%). (b) I/N interface observed in polarized light in a capillary tube. Type I collagen solution at 5 mg ml^{-1} in 0.5 M acetic acid was left to evaporate slowly until birefringence was observed near the air/solution interface. (c) The same region of the sample at a higher magnification showing the typical fingerprint pattern.

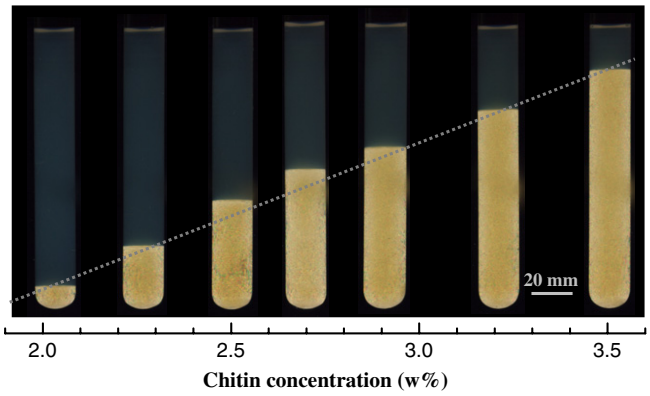

Figure 5. Series of suspensions with increasing total chitin concentrations (2.05–3.50%) observed in polarized light. The upper isotropic phase appears dark, whereas the nematic phase at the bottom is very bright. As the total concentration of chitin is increased, the proportion of the nematic phase in the biphasic samples increases.

At such volume fractions, the solutions are strongly visco-elastic and behave like a weak gel. When such solutions, with concentrations presumably in the coexistence domain, are introduced into a capillary tube, shearing induced by the flow produces a homogeneously birefringent sample that does not phase-separate over long periods of time. However, coexistence can be observed by placing a dilute collagen solution in a capillary tube and letting the solvent evaporate slowly at one end, that is left open. Birefringence appears near the air/solution interface and slowly develops inside the solution. A sharp interface is visible between the completely isotropic dilute solution and the birefringent phase (figure 4(b)). A more elaborate set-up composed of small glass chambers, into which a dilute collagen solution is continuously injected at a very low flow rate, allowed a thorough investigation of the textures produced over the whole concentration range [88]. This strongly suggests that the I/N transition in collagen solutions is also first order, although the observations are made in a concentration gradient and thus not strictly at equilibrium. The measurement of the isotropic and nematic phase volume fractions at coexistence can be done in different ways. Collagen titration requires complete hydrolysis and is thus destructive but requires little material. Chitin concentrations, on the other hand, are determined by weighing a small volume of the suspension before and after drying. Assuming that one knows the density of the particles and their dimensions, the concentration in weight percentage can be converted into the volume fraction. The major drawback of this technique is that it consumes large amounts of sample. Large quantities of suspension were prepared for one composition of the suspending medium and placed into large tubes that were left standing for several days until the phase separation was complete (figure 5). The volume fraction was measured in each phase for the entire concentration series. This revealed that, given the low ionic strength of the supporting medium, the contribution of the particle surface charges to the total ionic strength was not negligible.

Small angle x-ray diffraction is a powerful tool to probe the positional and orientational correlations between particles. In the chitin nematic phase, the rods are tens of nanometres apart with only local liquid-like order. The structure factor represents interparticle interferences and, when the interactions are repulsive, the scattered intensity presents a maximum at a wavevector q (nm^{-1}) inversely proportional to the average distance between the rods ($q = d_{\mathrm{ave}}/2\pi$). Due to a relatively low electronic contrast with the solvent, the diffraction signal from chitin suspensions is rather weak, and even more so for collagen, which is a highly hydrated protein. In both cases, we used shearing in capillaries or in a cylindrical shear-cell to produce nematic monodomains. Strong magnetic fields are often applied to homogeneously align the nematic phase, but chitin [56], cellulose [89], and collagen [57] particles align perpendicular to the magnetic field and the entire texture rotates instead of unwinding. SAXS patterns of nematic monodomains also contain information on the orientational distribution function (ODF) of the particles about the average direction represented by the director. The azimuthal (angular) spreading of the interference diffraction peak is a function of the ODF. It is thus possible to estimate the order parameter by fitting angular profiles of the diffracted intensity [80, 90]. For the nematic phase at coexistence, we found the expected value of 0.8, which confirmed that chitin suspensions are lyotropic nematic liquid crystals essentially described by Onsager's model. The situation with collagen is probably complicated by the large flexibility of the particles. Collagen has a persistence length estimated around 160 nm [91–93] and a contour length of 300 nm, which gives a flexibility $\alpha = L/p$ of about 1.9. Chen [83] predicts the order parameter to be around 0.45 with such a flexible chain, much less than for rigid rods and chitin microcrystals, in particular. Preliminary experimental results on shear aligned collagen solutions suggest that the order parameter is indeed less than 0.5. In the temperature range possible with aqueous samples, from 0 to 100 °C, we observed that samples of chitin suspensions at coexistence were perfectly stable. Sealed capillaries with different proportions

of isotropic and nematic phases were kept for several weeks at different temperatures and no variation was seen. This simple experiment confirms that the system is essentially athermal.

4. Conclusions

Liquid crystallinity surely plays an important role *in vivo* during the laying out of major biological tissues. Theories describing the thermodynamics of dispersed rigid rods suitably describe the behaviour of polysaccharide microcrystals like chitin and cellulose *in vitro*. And since the 3D long-range organization of concentrated suspensions mimics that of the corresponding biological tissues, it seems reasonable to suggest that the formation of plant cell walls and arthropod cuticles largely relies on self-organization mechanisms. Similarly, collagen solutions form cholesteric phases and exhibit most of the features characteristic of the I/N transition theoretical models. The chiral fluid phase can be stabilized by an increase in pH and the resulting dense fibrillar gels still exhibit the helical symmetry observed *in vivo* in compact bones [88, 94, 95]. An abundant theoretical literature and numerous lines of experimental evidence support the theory that macromolecules are produced by specialized cells, excreted into the extracellular space in a dense state, and then escape cellular control to self-organize into highly ordered biological tissues. Neither chitin microcrystals nor tropocollagen are the original macromolecules as produced by cells, before they form a solid tissue. Chitin chains crystallize into long rigid rods as soon as they are released at the outer cell membrane. As the acetylated polysaccharide is insoluble in water, its charged derivative chitosan was studied in the condensed state and shown to form solid crystals [96] and mesophases [97], but never produced regular cholesteric phases. Likewise, procollagen concentrated solutions are strongly birefringent and exhibit patterns suggesting a nematic phase with chiral perturbation, but did not produce a typical fingerprint pattern [51]. Harvesting the macromolecules directly after their release by cultured cells [50, 98, 99] or recombinant systems [52, 53], maintaining them in an intact and dispersed state, and testing their self-organization properties in small confined set-ups could be a way to tackle the early stages of physiological assembly processes. Confinement between the cell membrane and the existing matrix is indeed likely to play a role in restraining the biological molecules in a given space, allowing concentrations to reach the critical threshold for phase separation. In addition, the presence of an existing organized interface could act as a template and orientate the regular growth of a homogeneous matrix. Finally, besides the major components giving the overall 3D structure, less abundant molecules undoubtedly play a role in fine tuning local associations, supramolecular organization and function. It will be necessary to identify precisely their effects and the molecular interactions involved.

The extensive work carried out on both biological structures and *in vitro* systems has provided us with elegant tools to produce ordered materials with a structural control on multiple scales. Biological macromolecules are widely available from renewable sources, can be extracted in mild conditions, and are generally highly biocompatible. A number of applications have already been considered in a wide range of technological and biomedical domains, taking advantage of their liquid crystal properties. The alternation of hydrophilic and hydrophobic layers in lamellar phases of phospholipids, for instance, can be used to solubilize drugs, immobilize enzymes [100], and precipitate inorganic phases [101–103], etc. Cellulose or chitin suspensions can be simply evaporated to prepare films with an adjustable helical pitch, reflecting circularly polarized light and producing interference colours [104], or associated with a mineral phase to form ordered hybrid or mesoporous materials [105, 106]. Collagen solutions were also successfully used to prepare ordered hybrids with silica [107], although the most obvious application for dense ordered collagen matrices with a biomimetic structure is in tissue engineering in association with stem or specialized cells [108] for skin or bone replacement, for

instance. Apart from further fundamental research dealing with *in vivo* biological mechanisms of self-assembly, more effort must now be put into providing materials scientists with robust processes to tailor-make new ordered biomimetic materials.

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