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The chiral structure of porous chitin within the wing-scales of Callophrys rubi

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ABSTRACT

The structure of the porous three-dimensional reticulated pattern in the wing scales of the butterfly Callophrys rubi (the Green Hairstreak) is explored in detail, via scanning and transmission electron microscopy. A full 3D tomographic reconstruction of a section of this material reveals that the predominantly chitin material is assembled in the wing scale to form a structure whose geometry bears a remarkable correspondence to the **srs** net, well-known in solid state chemistry and soft materials science. The porous solid is bounded to an excellent approximation by a parallel surface to the Gyroid, a three-periodic minimal surface with cubic crystallographic symmetry $I4_132$, as foreshadowed by Stavenga and Michielson. The scale of the structure is commensurate with the wavelength of visible light, with an edge of the conventional cubic unit cell of the parallel-Gyroid of approximately 310 nm. The genesis of this structure is discussed, and we suggest it affords a remarkable example of templating of a chiral material via soft matter, analogous to the formation of mesoporous silica via surfactant assemblies in solution. In the butterfly, the templating is achieved by the lipid-protein membranes within the smooth endoplasmic reticulum (while it remains in the chrysalis), that likely form cubic membranes, folded according to the form of the Gyroid. The subsequent formation of the chiral hard chitin framework is suggested to be driven by the gradual polymerisation of the chitin precursors, whose inherent chiral assembly in solution (during growth) promotes the formation of a single enantiomer.

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1. Introduction

The basis of structural colour is the interaction of light with a periodic structure in one, two or three dimensions whose periodicity is of a comparable size to the wavelength. These structures are known as *photonic crystals*, and can result in strong reflection for a range of wavelengths. The reflections are due to constructive interference, which creates a photonic band gap – a range of wavelengths that cannot propagate through the crystal. The central wavelength and the width of the photonic band gap both depend on the direction of propagation through the crystal. Complete photonic bandgaps imply that there is a range of wavelengths that cannot propagate through the crystal from any direction. In principle this property allows the structure to appear the same colour when viewed from any angle – however, a requirement of such a bandgap is that the microstructure has a refractive index contrast exceeding two (Joannopoulos et al., 2008).

The technological applications of photonic crystals however go far beyond colouration. The exquisite control they offer over the emission and transmission of light has led them to be used for such diverse applications as optical security devices (van Renesse, 1997), solar cells (Bermel et al., 2007), low threshold lasers (Akahane et al., 2003; Gong et al., 2010) and for displays (Ha et al., 2008) and as an enabling technology for photonic chips (Joannopoulos et al., 2008; Ha et al., 2008). The fact that such structures also exist in nature (such as in the wing-scales of certain butterfly species) is not only intriguing, but also they offer important fabrication and design insights. Three-dimensional photonic crystals are challenging to make, and the refractive index contrast in organic materials is much smaller than is possible for inorganic systems, making it impossible to achieve complete photonic bandgaps. The vast database of natural optical microstructures found in biology has emerged through many generations of evolutionary optimisation and the microstructures offer ingenious implementation of polarisation effects, compound structures and randomisation to achieve optical functionality, despite the limited contrast



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in refractive indices available to biological materials. Such solutions may be particularly important for low cost polymer-based photonic devices, where the index contrast is also restricted.

Butterflies exhibit a variety of optical microstructures (Ghiradella, 1984; Ingram and Parker, 2008), and three-dimensional photonic crystals have now been identified in the wingscales of several butterfly species, including the papilionids Parides sesostris and Teinopalpus imperialis and the lycaenids Mitoura gryneus, Mitoura siva, Callophrys dunetorum and Callophrys rubi. Photonic activity is induced by polymerised chitin material (with lesser fractions of unidentified biomolecular species) that is structured at optical wavelengths. However the size of these structures, their complex topology and natural variation within a single wingscale and between distinct specimens has made conclusive structural assignation difficult, if not uncertain. The earliest proposed structure for *C. rubi* was a simple cubic array of polymeric chitin spheres (Morris, 1975), while later studies suggested face-centred cubic packings (Ghiradella and Radigan, 2005) and most recently, a three-dimensional connected network related to the Gyroid structure (Michielsen and Stavenga, 2008; Michielsen et al., 2010; Saranathan et al., 2010). Face-centred cubic structures have been proposed for a number of species (Vukusic and Sambles, 2003; Prum et al., 2006; Kertész et al., 2006), while a triclinic structure has been proposed for T. imperialis (Argyros et al., 2002). To date, structural studies have relied on indirect methods, from analysis of earlier electron micrographs of two-dimensional sections (Michielsen and Stavenga, 2008; Michielsen et al., 2010) to small-angle scattering X-ray (Saranathan et al., 2010).

Here we give the first direct three-dimensional structural data for the organised chitin network found in wing-scales of *C. rubi*. The excellent resolution of the data allows us to quantitatively compare the structure to the Gyroid, resolving definitively any doubts regarding the occurrence of this intriguing structure in the wing-scales of *C. rubi*. We have performed electron tomography on a single sample of the wing scales of *C. rubi*. The conclusion, based on skeletonisation of the chitin phase and on explicit comparison of the imaged interface to a mathematical model surface, is that the spatial structure of the investigated probe is commensurate with the channel structure to one side of the Gyroid surface, i.e. a single Gyroid structure based on the **srs** net (O'Keeffe et al., 2008), with cubic symmetry group $I4_132$, and lattice parameter $a = (311 \pm 5)$ nm. The structure is illustrated in Fig. 1.

2. Structure determination from 3D electron tomography

Dried specimens of C. rubi were purchased from a commercial insect supplier (www.insectcompany.com). Small samples of the green wing areas were prepared for TEM tomography data collection in the standard manner (Ghiradella, 1985). Wing pieces of approximately 1 cm² were treated with a primary fixative (2.5% glutaraldehyde in 0.1 M phosphate butter at pH 7.2), rinsed with 0.1 M buffer and then treated with a secondary fixative (1% Osmium tetraoxide in 0.1 M phosphate buffer). They were then dehydrated in a graded series of ethanol (first 50% ethanol in water, then 70%, 90%, 99%, and finally 100% ethanol), infiltrated in Spurr's Resin under mild vacuum (approx. 400 torr) and left to polymerise at 60 °C. The embedded blocks were sectioned using an ultramicrotome and the sections picked up on copper grids and stained with uranyl acetate. A slice of thickness 500 nm was chosen, as specimens of this thickness had been successfully imaged by TEM previously (Argyros et al., 2002). Colloidal gold particles (diameter 10 nm) were embedded in the sample on both sides of the sections to act as fiducial markers for the tomographic reconstruction. Dual-axis tilt series were obtained on a Tecnai TF30 300 kV Transmission Electron Microscope, with a Gatan 650 tilt-rotate holder,



Fig. 1. Spatial structure of the chitin phase of *C rubi*. The right fraction of the threedimensional body represents a subset of the tomographic data, segmented to yield 50% volume fraction, suitably rotated. The left side represents a solid body bounded by Schoen's triply-periodic *Gyroid* minimal surface. Also shown is a single **srs** network tracing the centres of the void phase (orange) and projections onto the planes perpendicular to one of the threefold rotation and threefold screw axes (bottom) and one of the fourfold rotation and fourfold screw axes (top).

using the SerialEM program for automated data collection. Tilt images were collected in 1° increments for the range $[-60^\circ, 60^\circ]$, at a magnification of 20,000×. Separate samples were prepared for SEM and the TEM (Figs. 4,5) by mounting wing scales on copper grids then exposing the scales to a 3–5 kV Ar ion beam for 1–10 minutes. The scanning electron samples were milled in a beam set at 15° to the exposed flat scale sample, which was glued to the grid then imaged in a Hitachi 4300S/N FESEM operating at 15 keV. Transmission electron micrographs samples were suspended within grid gaps and milled from both sides for a similar time. Those samples were observed in a Philips CM300, operated at 300 kV.

Tomograms were generated from the data and combined using the software package IMOD (Kremer et al., 1996). The resulting density dataset has voxel size $dx = (1.28 \pm 0.08)$ nm and overall size of approximately $2100 \times 1900 \times 400$ nm³. The largest rectangular subset representing an ordered structure without grain boundaries has size $850 \times 1280 \times 400$ nm³; this subset is referred to as subset "L". The analyses below are carried out on this subset "L" and also on a smaller subset, called "S", of approximate size $640 \times 640 \times 400$ nm³.

The grey-scale density of the electron-tomography dataset was smoothed with a Gaussian kernel of width $\sigma = 6$ nm. It was segmented by the *converging active contour* method, CAC (Sheppard et al., 2004), followed by removal of small isolated clusters such that the chitin and the void phase form a pair of single-connected components. (The CAC method uses a combination of watershed

and active contour methods. Initial low and high phase seed regions were chosen; these regions were then evolved according to a speed function dependent on gradient and intensity.)

The sample porosity cannot be precisely determined from the 3D density dataset: the obtained volume fraction depends on the choice of segmentation parameters. We note however that this uncertainty does not weaken our structural analysis below, since both the quality of the segmentation and the variations of the interface position from the corresponding parallel surface to the Gyroid minimal surface are similar for all intermediate parameter values of the segmentation parameter. Therefore, a precise estimate for the volume fraction ϕ of the chitin phase cannot be extracted. A rough estimate, determined by the minimal and maximal volume fraction beyond which the segmentation does not yield an ordered connected phase, is $15\% < \phi < 70\%$.

A triangulation of the interface between the chitin and the void phase, as given by the segmented data, is obtained by the Marching Cubes algorithm (Lorensen and Cline, 1987) followed by mesh decimation. We then estimated a best fit of the resulting interface to a "parallel-Gyroid" surface as follows. The Gyroid minimal surface, discovered by Alan Schoen (Schoen, 1970), bisects space into a pair of labyrinths; filling one labyrinth with chitin results in a pattern with 50% porosity. A one-parameter (x say) family of parallel-Gyroid surfaces form by displacing the Gyroid interface an equal distance x at all points along the surface normal of each point. The volume fraction of the parallel-Gyroid formed by a displacement of x is determined by the Steiner formulae $\phi(x) = 1/2 + A_0 x/a^3 (1 - 2\pi \chi x^2/3)$ with $A_0 = 3.09$ and $\chi = -8$ for cubic lattice parameter *a* if r/a < 0.19, (Schröder et al., 2003; Schröder-Turk et al., 2007). We chose the parallel-Gyroid interface within that family whose porosity best matched the selected porosity. The parameters of a transformation (consisting of three rotations, a translation and a rescaling of the coordinates by a factor) were determined that minimised the average square distance $(1/N)\sum_{i=1}^{N} [d(p_i)]^2$ between the *N* vertices **p**_i of the interface triangulation to the nearest point $\mathbf{f}(\mathbf{p}_i)$ on the model parallel–Gyroid interface, with the usual distance function, $d(\mathbf{p}_i) = |\mathbf{p}_i - \mathbf{f}(\mathbf{p}_i)|$. The optimal orientation, or rather an approximation thereof, is determined by a sequence of Monte-Carlo-like small random moves (translations, rescaling, rotation of the whole dataset) combined with a visual choice of a suitable starting configuration.

Following this rigid-body transformation of the tomographic data representing the chitin interface, it was visually evident that the structure resembled closely the corresponding Gyroid interface, for all analysed segmentation parameters that gave reasonable porosities ϕ , see Fig. 1. Further, a representation of the underlying net of the chitin phase by its thinned centred skeleton (its medial axis computed by distance ordered homotopic thinning) is in good agreement with the **srs** net that lines each channel of the Gyroid or its parallel relatives, see Fig. 2.

The width of the distribution of distances $d(p_i)$ to the corresponding parallel Gyroid interface, shown in Fig. 3, affords a quantitative measure for the fidelity of the match between the porous chitin matrix and the Gyroid geometry. Perfect congruence would imply an infinitely sharp distribution; the fact that the distribution is approximately given by a Gaussian distribution, with maximal likelihood for the points to be on the corresponding parallel-Gyroid interface and rapid decay of this probability away from this interface, offers firm quantitative evidence that the porous chitin structure is given by the Gyroid $I4_132$ structure. The fact that the deviations from the Gyroid structure are Gaussian is commensurate with these deviations being caused by noise or other smallscale deviations. The agreement is similarly good for all porosities analysed. While analysis on the smaller subset "S" yields the closest match, the match of the larger subset "L" remains very convincing.



Fig. 2. Voxelised Medial axis of the chitin phase (blue) superposed on the single **srs** net of symmetry $I4_132$. (a) View along a fourfold direction. (b) View along a threefold axis (animated versions of these images are provided in the supplementary material). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This structural stability, regardless of porosity is striking. Though we provide detailed analysis of only a small region of the wing-scale of a single specimen, the analysis allows us to conclude that the structure based on a parallel–Gyroid interface is formed in *C. rubi*. Lower magnification views of a single wing-scale (of a different specimen) show that there are significant variations in porosity in different regions of the scale, varying approximately over the range of values explored in our quantitative analysis, see Fig. 4.

In addition, it is clear that these crystalline domains are rather small, extending only over a few unit cells in any direction. Often, the angles between neighbouring domains are small, and distinct structures within the grain boundaries are not seen; the chitin



Fig. 3. Distribution of distances from the chitin interface in the optimally oriented tomographic dataset to the nearest point on the corresponding Gyroid surface. The lattice parameter, also determined by the Monte Carlo technique described in the text, is $a = (311 \pm 5)$ nm. The largest possible deviation from the minimal Gyroid interface (applicable to $\phi = 50\%$) is given by the maximal domain size 0.23 *a* of the Gyroid minimal surface (Schröder et al., 2003).



Fig. 4. Scanning electron micrographs of different regions within a single wingscale of *C. rubi*: (left) $10,000 \times$ magnification with a 5 µm scale bar; (right) $25,000 \times$ magnification with a 2 µm scale bar. Note the differences in porosity and the presence of smaller domains, separated by small-angle grain boundaries (the SEM instrument used was a Hitachi 4300S/N FESEM).

network appears to traverse distinct "grains" uninterrupted, though significant misorientations between neighbouring domains have been reported in other studies (Michielsen and Stavenga, 2008). Typical domain sizes in a high-porosity region of a single wing-scale analysed to date are between 5 and 10 μ m; each

domain gives slightly different optical activity, homogenising somewhat the optical activity of the scale (Fig. 5).

3. Chirality

A remarkable feature of the µm-scale chitin framework is its chirality. The **srs** net that describes the channel array of the structure is inherently handed. A recent study has suggested that both enantiomers are to be found in wing scales of the related lycaenid *C. dumetorum* (Saranathan et al., 2010). This suggestion was based on the observation of both right- and left-handed helices in micrographs. However, their suggestion is inconclusive in our view, given the presence of both left- and right-handed helices in a single enantiomer of **srs** (parallel to the <100> and <111> axes of the cubic lattice with slightly different radii and pitches equal to the lattice parameter and $\sqrt{3}/2$ times the lattice parameter, respectively). The possibility of a single enantiomer of chitin within individual wing-scales, within a single butterfly or indeed within the species can therefore not be excluded on current evidence; further studies are essential to resolve this intriguing question.

4. Closing

The finding that the spatial structure of *C. rubi* wing scales is a chiral photonic crystal is significant for a variety of reasons and this ultrastructural chirality is in principle independent of the molecular-scale chirality of chitin. First, the material is optically active, that is, it rotates the polarisation of incoming light. This is particularly interesting in the context of photonic crystals, as it has been argued that such structures could result in polarisation bandgaps: frequency bands in which one polarisation state can be transmitted through the crystal, while the other is reflected (Poladian et al., 2009). Detailed modelling of the photonic features of this chitin structure confirm the presence of partial band gaps for circularly polarised light (Saba et al., accepted for publication).



Fig. 5. Transmission electron micrograph of a small region within a single wing-scale of *C. rubi* (2 µm scale bar). This wing-scale has been thinned by ion milling and the viewing direction here is perpendicular to the plane of the scale. A number of distinct domains are imaged, each with its chitin framework pattern distinctly misoriented with respect to its neighbours. Domain boundaries have been marked with white lines to aid visualisation. Inset: The variation of optical activity within the wing-scale, likely corresponding to domains like those imaged by TEM, can be seen in this optical micrograph (reflected light) of a single scale lying flat. The scale bar is 10 μ m.

Even more intriguing are the implications for metamaterials. Recent intense interest in this field has been driven by the theoretical possibility of designing structures to produce a range of extraordinary effects, including negative refractive index materials, electromagnetic cloaking and manipulation of the near-field (such as superlensing and hyperlensing). Conventional approaches to this problem involve designing structures to manipulate the magnetic and electrical response of the material (specifically the permittivity μ and permeability ϵ) to induce negative refractive index. However, the refractive index of highly chiral materials can be negative though both μ and ϵ remain positive (Pendry, 2004; Zhou et al., 2009; Zhang and Cui, 2007). Combined with the availability of chemical templating mechanisms to convert chitin structures into e.g. rutile (TiO₂) structures (Weatherspoon et al., 2008) butterfly scales may provide an intermediate method for generation of chiral inorganic photonic crystals with lattice size of approximately 300 nm while no suitable synthetic self-assembly mechanism for this length scale is available.

The formation mechanism of this extraordinarily complex chitin framework in vivo is of interest on many fronts. Biological studies suggest that the chitin slowly polymerises in the larval stage of the butterfly, guided by mutual folding of the smooth endoplasmic reticulum (SER) and plasma lipid-protein membranes to give a Gyroid-like pattern (Ghiradella, 1994, 2008; Saranathan et al., 2010). This "cubic membrane" folding geometry has been detected in many membrane organelles, across virtually all kingdoms of life (Landh, 1995; Almsherqi et al., 2006; Hyde et al., 1997). The same structure, realised at a much smaller length-scale, is well-known in hard atomic and molecular as well as soft molecular materials (Hyde et al., 2008), including lyotropic liquid crystals of lipids (and lipid-protein mixtures) in water (Larsson, 1989), where its presence is a signature of molecular self-assembly into a 2D layer subject to bending energy (Helfrich and Rennschuh, 1990; Hyde, 1990), with a strongly preferred local membrane (Gaussian) curvature and a preference for uniform channel sizes (Hyde et al., 1997; Schröder-Turk et al., 2006). Much coarser patterns, but still cubic, have been observed in butterfly larvae (Ghiradella, 1994). It is therefore reasonable to conclude that the chitin network emerges via self-assembly of the SER membranes, which then template the harder chitin matrix, qualitatively similar to the synthetic route to formation of mesoporous materials.

Despite the clear links between this biological material and condensed materials formed by self-assembly in vitro, aspects of the genesis of this self-assembled structure remain poorly understood. In particular, no clear explanation has been given for the stability of such a highly swollen Gyroid pattern, whose lattice parameter is two orders of magnitude greater than typical dimensions in lipid-water or lipid-protein-water mesophases (Larsson, 1989). Electron micrographs indicate that the SER membrane – possibly accompanied by the plasma membrane – folds as a coherent stack of multiple bilayers, in contrast to the usual single bilayer characteristic of cubic bicontinuous mesophases (Ghiradella, 1994); Saranathan et al. (2010) suggest that single SER and plasma membrane bilayers condense and fold in concert into the Gyroid morphology. The enhanced membrane rigidity associated with a stack of two bilayers compared with a single bilayer goes some way towards explaining the crystallinity of this massively swollen structure, as follows. Since the crystalline Gyroid geometry results from minimisation of membrane bending energy, the enhanced modulus of bending rigidity associated with the double bilayer may explain the enhanced swelling of the crystalline pattern without melting.

A second aspect of this structure requires further investigation. The chitin framework contains a single, chiral **srs** net, in distinction to the pair of (enantiomeric) **srs** nets that line Gyroid channels. Straightforward templating of chitin within the pair of aqueous channels formed by a multilayer stack folded onto the Gyroid, or within the space between parallel membranes, should result in a pair of interwoven (left- and right-handed) frameworks or an achiral sheet. What gives rise to this symmetry-breaking, resulting in a chiral structure? We do not yet know, and any explanation must await more careful studies of the specific chirality, if any, of the chitin matrix. It is tempting to suggest that the chitin oligomeric precursors themselves preferentially grow within a single aqueous channel. Chitin oligomers are themselves chiral, chiral aggregation has been seen in a number of biological chitin materials (Giraud-Guille et al., 2004) and chitin suspensions in water are known to spontaneously form (chiral) cholesteric mesophases (Revol and Marchessault, 1993). A similar mechanism has been employed in the lab to prepare single chiral srs mesoporous networks. In that case, chiral (sucrose) precursors were absorbed and then polymerised within MCM-48 (an amorphous mesoporous silica with the Gyroid structure): the carbonaceous materials that remained after removal of the silica was itself chiral, with symmetry 14132, indicative of a single srs network (Ryoo et al., 1999). A similar mechanism at work in C. rubi would likely imply just a single enantiomer of chitin in the wing-scales, a scenario whose likelihood is in our view at present unclear. This scenario is, though, not essential. A second example of a chiral srs mesoporous network, again templated from MCM-48, but made of platinum, shows that a chiral species is not needed to form srs (Terasaki et al., 2002). We note in this case, however, that either enantiomer of this Pt network is equally likely to form, though the origin of the single network remains unclear. This example shows, however, that the formation of a single srs need not imply a single enantiomer.

Clearly, full understanding of the genesis of this remarkable chitin material must await further studies. However, the clear similarities to synthetic self-assembled meso-scale materials suggest concrete future directions to explore in order to achieve an ultimate goal of *in vitro* self-assembly of these pattern for further photonics research.

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