FEATURE ARTICLE

Light Manipulation by Guanine Crystals in Organisms: Biogenic Scatterers, Mirrors, Multilayer Reflectors and Photonic Crystals

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Guanine crystals are widely used in nature to manipulate light. The first part of this feature article explores how organisms are able to construct an extraordinary array of optical "devices" including diffuse scatterers, broadband and narrowband reflectors, tunable photonic crystals, and image-forming mirrors by varying the size, morphology, and arrangement of guanine crystals. The second part presents an overview of some of the properties of crystalline guanine to explain why this material is ideally suited for such optical applications. The high reflectivity of many natural optical systems ultimately derives from the fact that guanine crystals have an extremely high refractive index—a product of its anisotropic crystal structure comprised of densely stacked H-bonded layers. In order to optimize their reflectivity, many organisms exert exquisite control over the crystal morphology, forming plate-like single crystals in which the high refractive index face is preferentially expressed. Guanine-based optics are used in a wide range of biological functions such as in camouflage, display, and vision, and exhibit a degree of versatility, tunability, and complexity that is difficult to incorporate into artificial devices using conventional engineering approaches. These biological systems could inspire the next generation of advanced optical materials.

1. Introduction

Guanine crystals are responsible for some of the most amazing colors in animals, and are also often integral components of their visual systems. The focus of this feature article is to address the questions of how guanine crystals are used by animals to manipulate light and why crystals of guanine in particular are so well-suited to such optical applications. Answers to these questions may well provide ideas for the development of new synthetic optical devices.

Guanine, an essential component of DNA and RNA, was first identified in guano in 1845 (cited in Rao et al.),[1] and in 1861, guanine crystals were identified in the scales and air bladders of fish.[2] In 1882, guanine crystals were found in reptiles and arthropods.[3] and by 1893, it was well established that guanine crystals are responsible for the iridescence of fish skin and that the crystals are located inside specialized cells called iridocytes.[4] Since then, many studies have reported the presence of guanine crystals in animals[5–7] and also in a few phyla outside the animal kingdom.[8] **Figure 1** shows the taxonomic distribution of guanine crystals in the animal kingdom. Surprisingly, only three phyla out of the 33 phyla reported by Margulis and Schwartz[9] produce guanine crystals, and within these three phyla, guanine crystals are found in only some taxonomic classes, but not in all. This "localized" production of guanine crystals is consistent with a convergent evolutionary scenario, namely that guanine crystal production evolved independently many times. This is reasonable, as all organisms essentially have the same metabolic degradation pathways for the nucleate bases, guanine and adenine. We can thus assume that the independent evolution of guanine crystals resulted from manipulations of this metabolism in order to accumulate large amounts of guanine in a crystalline form. Rao already pointed out in 1917 that guanine is one of the end products of nucleic acid degradation metabolism in certain organisms, and that this metabolite is utilized by some animals for functional purposes.[1] Guanase is the enzyme that converts guanine into xanthine. Low levels of guanase activity[10,11] or its artificial inhibition induce the spontaneous formation of guanine crystals in mammals that do not normally produce them.[12] In organisms where guanine is the actual end product of the metabolism, guanine crystals are sometimes secreted as a waste product and only in the case of this secretory function does an entire taxonomic class produce guanine crystals (spiders and amphibians).[10,13]

Only a handful of functional organic crystals have been found in nature,[14] and of these, crystalline guanine is one of the most widespread. Guanine crystals have a variety of different functions in organisms,[15] including manipulation of light to produce structural colors,[5] mirror components of visual systems,[16] protection against extreme heat,[17] and prevention of gas diffusion.[18] The use of guanine for light manipulation is probably due to the fact that guanine crystals have one of the highest known refractive indices (n = 1.83) for any

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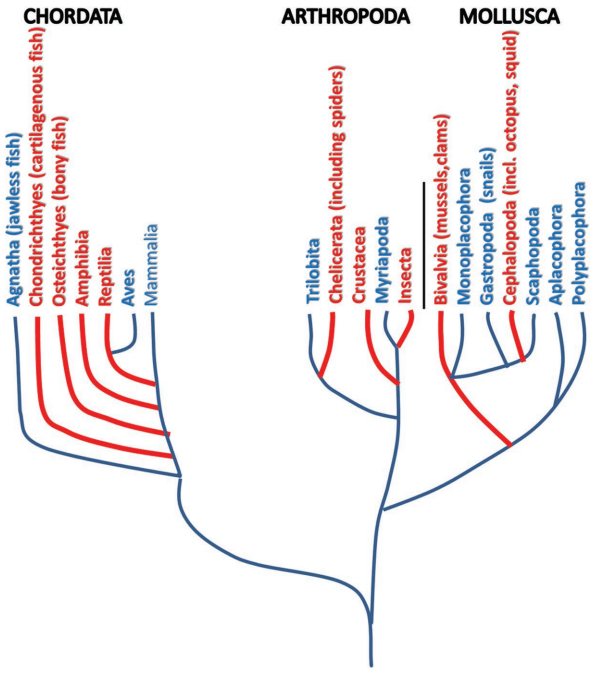

Figure 1. Taxonomic distribution of guanine crystals in the animal kingdom. Within the 3 phyla producing guanine crystals, the taxonomic classes where they are produced are marked in red. Note that this is a partial phylogenetic tree, including only the 3 phyla where guanine nanocrystals are found.

natural biological material.[19] This high refractive index occurs along one crystallographic axis, corresponding to the direction of the stacking of guanine molecules in the crystal structure, whereas the refractive indices along the orthogonal directions are estimated to be much lower, namely around $n = 1.45$.[20] Thus, the interaction with light will depend critically on the direction in which the light impinges on the crystal and on the crystal shape and size. Here, we first explore some of the ways in which guanine crystals are used by animals to manipulate light and then rationalize why crystals of guanine are suited to perform such functions.

2. How do Animals Use Guanine to Manipulate Light?

Guanine crystals are used in many biological optical systems to perform an astonishing variety of functions,[7] from the production of white color in certain spiders to the metallic silvery reflectance of certain fish, to the brilliant iridescent colors of planktonic crustaceans and fish, and for vision in complex mirrored eyes.[16,21,22] All the optical functions described in this article stem from light reflection. The white color observed in certain organisms results from the diffuse reflection of light (i.e., light scattering), in which all wavelengths of visible light undergo multiple scattering events, producing a white coloration. On the other hand, silver reflectance, iridescence, and structural colors are produced by specular (i.e., directional) reflection either of all wavelengths (in the case of silver reflectance) or of specific wavelengths of light (in the case of structural colors). Light reflection may have some advantages over absorption. For example it is energetically efficient, avoids heating due to absorption, is more conducive to tunability, can facilitate control over polarization and directionality, and often produces enhanced brightness.[23,24]

Here, we describe how the vastly different functions of guanine-based optical systems are achieved by modifications of just three components: crystal morphology, crystal size, and the ultrastructural arrangement of the guanine crystals. By reference to specific examples, including examples from our own work, we describe how these parameters vary in diffuse scatterers, broadband and narrowband reflectors, tunable photonic crystals, and image-forming mirrors in nature. We relate these observations to the underlying physical mechanism/principles to rationalize the observed optical properties.

2.1. Light Scatterers

The striking matte-white appearance of certain spiders (**Figure 2**a) (such as *Enoplogatha ovata*, *Nephila komaci*,

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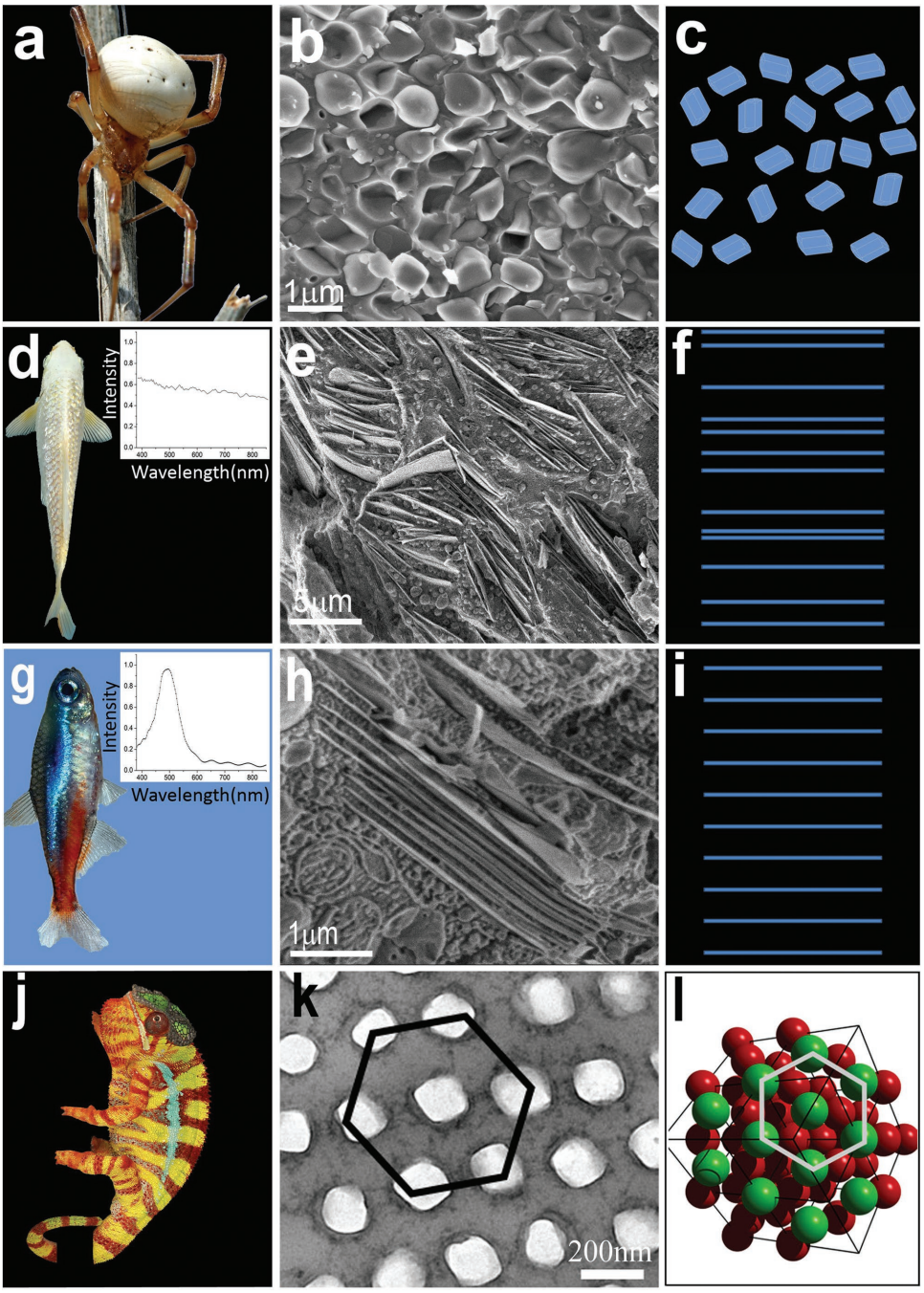

Figure 2. Examples of guanine crystal-based optical systems with different functionality, and their corresponding architecture. a–c) The white widow spider (*Latrodectus pallidus)*: The integument of the spider (a) is white due to coherent scattering from a thick dense layer of blocky guanine crystals ≈1 μm in size (b), located beneath the integument. Impinging light is scattered multiple times from neighboring randomly distributed crystals (c) before emerging. d–f) The Japanese Koi fish (*Cyprinus carpio*). The silvery reflection of the fish (d) arises from disordered multilayer reflectors located in the epithelial layer underneath the scales of the fish (e). The thin (≈20 nm) plate-like guanine crystals are not all parallel to each other, but there is local order within each crystal stack. f) The cytoplasm spacings are widely distributed. Constructive interference of different wavelengths reflected from individual stacks results in diffuse broadband reflection, appearing silvery to the human eye (d, inset). g–i) The neon tetra fish (*Paracheirodon innesi*): The brilliant blue color of the lateral stripe of the neon tetra (g) arises from ordered, well aligned multilayer reflectors (h,i) with narrow distribution of thicknesses of both the thin guanine crystals (≈22 nm) and the thicker cytoplasm spacings (≈155 nm). Constructive interference of selected wavelengths reflected from individual stacks results in narrow band reflection centered at 500 nm (g, inset). j–l) The panther chameleon (*Furcifer pardalis*): The observed skin color of the panther chameleon (j) originates from a combination of both pigments and guanine-based 3D photonic crystals. Light is reflected from small ≈130 nm guanine crystallites (k) arranged in face-centered cubic geometry (l). The panther chameleon can change its color by changing the lattice spacing of the crystallites. a,d,g,j) Optical images of the animals. b,e,h) Cryo-SEM, and k) TEM images showing the guanine crystals in the tissues. c,f,i,l) Schematic representation of the tissue architectures. Figure (a) was supplied by Guy Haimovitch, and Figures (j–l) were reproduced with permission from Prof. Milinkovitch.[25]

Latrodectus pallidus, and *Argiope lobata*) is produced by light-scattering from block-shaped prismatic guanine crystals about one micrometer in size (Figure 2b).[22,26] Their white color results from multiple scattering events from a layer tens of micrometers thick, which is full of randomly oriented guanine crystals (Figure 2c). In some spiders, the white color is used to provide camouflage against a white background (for example flowers) in order to both avoid predators and deceive prey.[27] In other spiders (such as *T. grallator)*, layers of guanine crystals reside specifically underneath pigmented areas of the hypodermis,[28] acting as a white background to the opaque pigmented patterns.

Guanine-based scatterers are also found in the eyes of certain fish such as the elephant nose fish (*Gnathonemus petersii)*.[29] These unusual eyes possess grouped retina, where many cones are included inside a cup containing a constriction at its base. The walls of the cup are lined by a highly ordered multilayer reflector constructed from 4 rows of plate-shaped guanine crystals, which concentrate light (by a factor of <5) onto the cone cells at the base of the cup. The tissue beneath the cup wall contains a layer of disordered ≈1 μm sized prismatic-shaped guanine crystals together with light-absorbing melanin granules in the vicinity of the rod cells. These crystals scatter the incoming light, increasing light absorption by the pigments and reducing the amount of light impinging on the rods. This remarkably complex optical design enables the simultaneous activation of both rod and cone cells. The cone cells would normally be "blind" in dim light conditions but are activated by the increased illumination provided by the mirror. On the other hand, the rod cells, which would normally be saturated when the cones are active, receive a reduced level of illumination as a result of scattering. This unusual matching of the rod and cone sensitivity provides the fish with an enhanced response to colored stimuli in the dim light conditions associated with the murky water environment in which it lives.

The whiteness of such systems arises from diffuse scattering in which light of all wavelengths is reflected multiple times in all directions that sum up until they emerge as white light. For this reason, white color generally requires a relatively thick layer of randomly positioned high refractive-index scattering centers.[23] In general, larger particles scatter more light, regardless of their refractive index. However, as the particles get larger, due to interference they scatter more and more light in the direction of the propagation of incident light (forward scattering). The propensity to forward scatter light is lower for high refractive index materials, and therefore the use of high refractive index materials such as guanine crystals enables organisms to simultaneously attain high scattering efficiency (using large particles) while ensuring that light is scattered in all directions.[23,30]

2.2. Multilayer Reflectors

Probably the best known use of guanine crystals in nature is in silvery reflectance of fish scales and skin. The silvery color results from the reflection of light from stacks of irregularly spaced thin guanine crystal plates that are deposited in specialized cells (iridophores) in the epithelial layer of the fish (Figures 2d and e).[31,32] The crystal plates operate as multilayer reflectors (Figure 2f). The same phenomenon is responsible for the silvery appearance of the cuticles of certain spiders.[22,26] When uniform bright colors are produced, such as in certain fish scales (Figure 2g and i), the spacing between guanine crystals is more regular.[33]

Biological as well as artificial multilayer reflectors, also defined as one-dimensional photonic crystals, are composed of periodically stacked, alternating layers of high and low refractive index materials. When light is reflected from a multilayer consisting of different materials A and B with refractive indices n_A and n_B and thicknesses d_A and d_B, the condition for constructive interference is

$$2\left(n_A d_A \cos\theta_A + n_B d_B \cos\theta_B\right) = m\lambda \quad (1)$$

where θ_A, θ_B are the incidence angles of the light, m is an integer, and λ is the wavelength of the reflected light. This equation shows that the light reflected from the multilayer reflector varies with the observation angle: as the angle of observation increases, the reflected color shifts towards shorter wavelengths (an angle of 90° defines the direction perpendicular to the orientation of each layer of the stack). The most efficient reflector of this type is produced when both the high and low refractive index layers have an optical thickness $n\ d$ equal to a quarter-wavelength of light. This arrangement produces the highest reflectance for the smallest number of layers. For many years, the quarter wavelength arrangement was considered to be the predominant type of reflector found in nature.[31] However, recent studies have demonstrated that this might not be the case.[34–36] For guanine-based biological reflectors, the high refractive index material is guanine, with n = 1.83,[7] and the low refractive index material is cytoplasm, with n = 1.34.[37] The high value of Δn also enhances the reflector efficiency. The degree of order within the system dictates the broadness of the reflectance band, such that high degree of order will promote a relatively narrow reflectance band and high degree of disorder will promote a broad reflectance band.

2.2.1. Broadband Reflectors

The silvery metallic reflectance observed in many varieties of fish provides them with camouflage over a wide range of angles.[38] The guanine reflectors of fish scales make the animal essentially invisible when viewed from almost any direction against the background of light penetrating the sea water, because light entering the sea rapidly loses any preferred direction due to scattering. Since the ambient light conditions inside the oceans are dominated by blue wavelengths, these reflectors are required to efficiently reflect blue light across a large range of incidence angles to provide effective camouflage.[39]

One well-studied system is the Koi fish (*Cyprinus carpio*) and its different varieties (Figures 2D-F).[35] The Koi fish scale tissue ultrastructure (Figures 2d-f) shows that the crystals are not all parallel to each other, and the cytoplasm spacings in each crystal stack are variable within a wide range (60–300 nm).

The optical thickness of the alternating layers strongly deviates from the ideal quarter wavelength reflector. This disordered arrangement results in broadband reflection in which most or all wavelengths of light are simultaneously reflected. Structures appearing pure silver or gold to the human eye can be achieved by at least three different arrangements of a multilayer stack: (i) a composite of ordered multilayer stacks each tuned to a specific wavelength,[40] (ii) a 'chirped stack' (a stack with systematically changing thicknesses),[41] and (iii) a 'chaotic' stack (a stack with large variations in the spacings of both crystal and cytoplasm layers around a mean value).[42] In the cases that we have examined, using techniques that preserve biological tissues in close to physiological conditions, the solution adopted by the Koi fish are closer to the chaotic arrangement but are not completely chaotic. The cytoplasm spacings have some consistency within stacks and, more important, the crystal thickness is constant at ≈22 nm.[35] The same narrow size distribution of the guanine crystal thickness was reported in other systems such as the eye of the spider *Drassodes cupreus*[34] and the silvery integument of the spider *Tetragnatha montana*.[26] The partial order results in more uniform reflectance across the whole visible range.[35] In fact, totally chaotic reflectors produce a blueish metallic reflectance,[23] whereas the silver reflectance of the fish scales is better reproduced using locally ordered multilayer stacks.

The Koi fish were also the subject of a study aimed at understanding how biology modulates the amount of reflectivity observed in different organisms.[35] A comparative study was performed on two variants of Koi fish: one 'common' Koi fish and a variant (*Gin rin*) that exhibits enhanced reflectivity. The study showed that the higher reflectance observed in the *Gin rin* originated from (a) a higher density of iridophore cells in the skin, (b) a thicker layer of iridophore layers, (c) more crystal stacks inside each iridophore, and (d) the crystal stacks are better aligned with the scale surface.[35] Furthermore, the presence of multiple crystal stacks inside a single iridophore cell leads to the interdigitation of crystals from neighboring crystal stacks, and consequently to a much wider distribution of spacings between the crystals.[35] A recent study found that this inherited trait of higher reflectance in the Koi fish is due to a dominant mutation of a single gene.[43] An interesting variant has been reported in which the platey guanine crystals within a stack have alternating orientations of their optic axes to produce a non-polarizing broadband reflector.[44] It is difficult to understand how the iridophore cells could actually produce such an arrangement.

2.2.2. Narrowband Reflectors

In contrast to broadband silver reflection, several species of colored fish, such as the blue damselfish (*Chrysiptera cyanea*),[45] the Siamese fighting fish (*Betta splendens*),[46] and the neon tetra fish (*Paracheirodon innesi*) (Figure 2g)[31,33,47] reflect light of specific wavelengths, resulting in defined colors. This narrowband colored reflectivity is obtained when the variability in the thicknesses of the crystals and the cytoplasm layers is low. This is exemplified in the bright blue lateral stripe of the neon tetra fish, where the thicknesses of both the guanine crystal plates (≈22 nm) and the cytoplasm spacings are uniform (≈155 nm).[36] While the thicknesses of the guanine crystals are comparable to those observed in the broadband reflectors of silvery fish, the variability in the thickness of the cytoplasm layers is much lower. Simulations of the reflectance based on matrix transfer calculations[48] reproduce well the measured reflectance spectrum, namely a blue-green peak at ≈500 nm (Figure 2g, inset).[36] This is the color observed in the lateral stripe of the neon tetra when the fish swims in bright light. However, when the fish swims into a dark location, the color of the stripe changes within 15 minutes from blue-green to indigo. The function of the brightly colored stripes is hypothesized to provide a mirror image decoy and in this way to confuse predators,[49] whereas the darker color may reduce the contrast against the dark background. Interestingly, the color change persists even in animals soon after death, indicating that the phenomenon is not exclusively under neurological control.

2.2.3. Tunable Reflectors

In addition to the neon tetra, tunable-color multilayer reflectors have been reported in the blue damselfish (*Chrysiptera cyanea*),[45] the Siamese fighting fish (*Betta splendens*),[46] the common surgeonfish fish (*Paracanthurus hepatus*),[50] the dark sleeper (*Odontobutis obscura*),[51] and the domino damsel (*Dascillus trimaculatus*).[50] There are also some examples of tunable reflectors in other animals such as the panther chameleon[25] (Figure 2j) and the *Sapphirinidae* copepods (**Figure 3**a,d).[52] One of the major challenges here (particularly from the viewpoint of bio-inspired materials science) is to understand the mechanisms by which these color changes occur.

In order to tune the color of a given reflector, a change in the optical thickness of at least one of the components is required. In principle this could be obtained by changing the refractive index of one of the layers or changing the thickness of one of the layers. The former effect could be conceivably obtained by secreting or absorbing optically active compounds into the layer, whereas the latter effect could be achieved through layer expansion or compression induced by changes, for example, in osmotic pressure or by mechanical forces. An array of different techniques, including in situ microspot X-ray diffraction was used to show that the change in color of the lateral stripe of the neon tetra fish is achieved by tilting of the guanine crystal/cytoplasm stacks,[36] confirming the proposed "venetian blind" model.[47] The tilting results in a change in the crystal spacings. This does not, however, exclude the possibility that changes in osmotic pressure cause the tilting of the stacks.

The male sapphirinid copepods are small planktonic crustaceans that exhibit brilliant iridescent colors (Figure 3). The copepods possess multilayer reflectors underneath their chitin cuticle. These reflectors are composed of regularly alternating layers of exquisitely formed and perfectly tiled thin hexagonal-shaped guanine crystals[53] and cytoplasm to produce spectacular structural colors.[54–56] Correlative cryo-SEM and reflectance measurements showed that the males have the remarkable ability to reversibly change their reflectance spectrum in response to changes in the light conditions.[52]

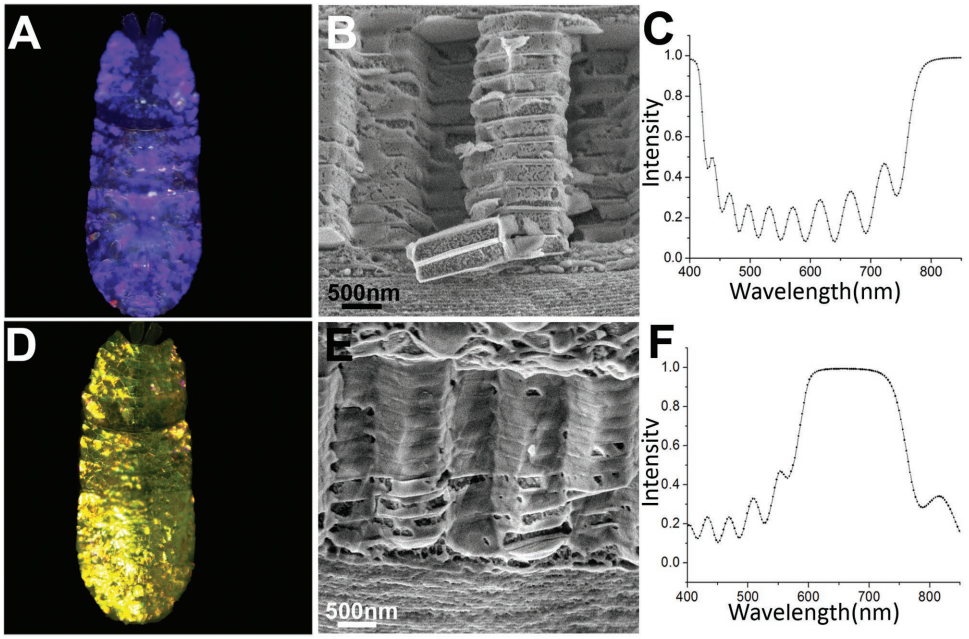

Figure 3. The reflectance and structural properties associated to color change in the male sapphirinid copepod. a-c) Dark-adapted *S. metallina*. d-f) Light-adapted *S. metallina*. (a,d): light microscopy images of the specimens. (b, e): cryo-SEM images of cuticle cross sections, showing the stacks of parallel hexagonal guanine crystals seen edge-on to the page. (c,f): simulated reflectance spectrum. The simulated reflectance was calculated based on the cytoplasm and the crystal thicknesses measured from the cryo-SEM images (b) and (e). The thickness of the crystals is similar in both the light and the dark adapted (≈70 nm) states, whereas the thickness of the cytoplasm spacings changes dramatically, shrinking from ≈190 nm in the dark adapted states to ≈135 nm in the light adapted state. The shrinkage and expansion of the cytoplasm spacings causes the observed shift in the reflected light.

This color change is achieved by varying the thickness of the cytoplasm layers between the guanine crystals (Figures 3b,e). In the two species examined, exposure to light and the subsequent shrinking of the cytoplasm thicknesses cause the reflectance peak to 'blue shift'. In the magenta dark adapted *Sapphirina metallina* (Figure 3a), where the first order reflection peak is at ≈800 nm and the second order reflection peak is at ≈420 nm (Figure 3c), light promotes a blue shift of both peaks. The first order peak shifts to yellow and the second order peak shifts into the UV, resulting in yellow reflectance (Figure 3d, f). In the deep blue dark adapted *Copilia mirabilis*, only the first order reflection peak is in the visible range (≈450 nm). In this case the blue shift 'pushes' the reflection peak into the UV, making the animal transparent and thus virtually invisible to potential predators that do not possess photosensitivity in the UV region. These changes were found to be both intensity and wavelength dependent.[52] The capability to tune their color provides the males with the ability to efficiently reflect light for intra-species communication in the deep sea, while remaining camouflaged when close to the surface.[52,55,57] Both the neon tetra and the sapphirinid copepods are examples of light-induced tunable photonic crystals. This capability has, to the best of our knowledge, not yet been achieved even in the most advanced artificial functional materials.

2.3. 3D Photonic Crystals

The only known example to date of a guanine-based 3D photonic crystal is in the panther chameleon (Figure 2j).[25] The panther chameleon has bright coloration that changes within minutes during male contests or courtship. Two different types of iridophores were identified: superficial iridophores (S-iridophores) and deep-lying iridophores (D-iridophores). The D-iridophores contain disorganized brick-shaped guanine crystals (≈150–600nm), which appear to function as a broadband reflector, ranging across the visible and into the infrared. The functions of the D-iridophores may include heat protection, because of the extension into the infrared. The S-iridophores contain smaller crystallites (≈130 nm) arranged in face-centered cubic geometry, which are also assumed to be guanine crystals (Figure 2k). The periodicity along all 3 spatial dimensions results in constructive interference in any direction of impinging light, producing a 3D photonic crystal. In the 3D photonic crystals generally found in nature, the geometries are highly conserved,[58] and most of the structures described have a face-centered cubic geometry,[58] as in the case of the panther chameleon.[25] The change in color is attributed to changes in the lattice spacing of the crystallites in the S-iridophores.

2.4. Mirrored Eyes

Guanine crystals are commonly used as mirrors in animal eyes.[59] These mirrors are used in a variety of operations from image formation (vision) to light collection and in light-doubling tapeta, (reflective layers used to enhance light sensitivity in eyes).[60] In general, mirrors in eyes serve to increase the amount of light available for vision and are

typically found in nocturnal animals or in organisms living in low-light habitats.

2.4.1. Image Formation

Although rare, certain organisms utilize mirrors comprising stacks of guanine crystals to reflect light onto a focal point on the retina to produce an image. In principle, image formation requires an extremely high degree of ultrastructural organization since light must not only be reflected but also focused. Clearly the mirror(s) in such eyes must be oriented such that light rays entering the eye from many angles can be precisely directed onto a single spot on the retina. Indeed, it is observed that organisms displaying image-forming mirrored eyes exert unprecedented control over the hierarchical organization of crystals. Not only are the individual guanine crystals arranged in multilayer stacks to form a mirror, but the stacks themselves are arranged into a geometry that enables the focusing ability of the eye.

A spectacular example is found in the *Pecten* scallop (Mollusca, Bivalvia), which has a remarkable visual system with dozens to hundreds (1 mm) of iridescent eyes residing along the mantle tissue edges (**Figures 4**a,b).[61–63] Architecturally the eye resembles the so-called 'fisheye camera' comprising a single chamber and a lens. However, after ascertaining that the lens was in fact made of a jelly-like low refractive index substance, Land[61] discovered that an image is formed when light is reflected off a concave mirror lying at the back of the eye (the argentea) onto a focal point on the retina (Figure 4d). The photoreceptor cells of the distal retina give "off" responses when illumination is reduced and are stimulated when a dark object crosses the retina in the reflected image. This causes the scallop to shut abruptly. The main function of the eye is thought to be in predator detection, although more recent behavioral studies suggest that scallops may also use their vision for feeding.[64] The main drawback of this optical arrangement is that unfocussed light must first pass through the retina before being reflected and imaged on the photoreceptors, resulting in significant degradation of the image contrast. However, due to the extremely high light-gathering power of the eye, what the organism loses in contrast it gains in light sensitivity—the main concern for an animal residing in relatively low light conditions.

The brilliantly reflecting argentea of the scallop eye is constructed from stacks of 30–40 square guanine crystal plates with a thickness of around 80 nm and a presumable cytoplasm thickness of around 100 nm (Figure 4c).[62,63] The system is very close to an ideal quarter wavelength stack, giving a peak in the reflectance at 490 nm. The reflector appears to be ideally suited both to efficiently accept the blue/green light available in the scallops habitat while also providing optimal reflectivity in the part of the spectrum in which the photoreceptive pigments are maximally sensitive (490–540 nm).[65]

One problem with concave mirrors is that they suffer from spherical aberration where rays reflected at a distance from the axis are over-focused (Figure 4). The scallop has arrived at an ingenious solution to this problem. The geometry of the outer surface of its lens ensures that there is more focusing power closer to the lens axis than at the periphery, which perhaps corrects for the over-focusing of the concave mirror.[66]

There are only two known examples of vertebrates that use mirrors for image formation: the extremely unusual deep sea fish, the so-called spookfish barreleye *Dolichopteryx*

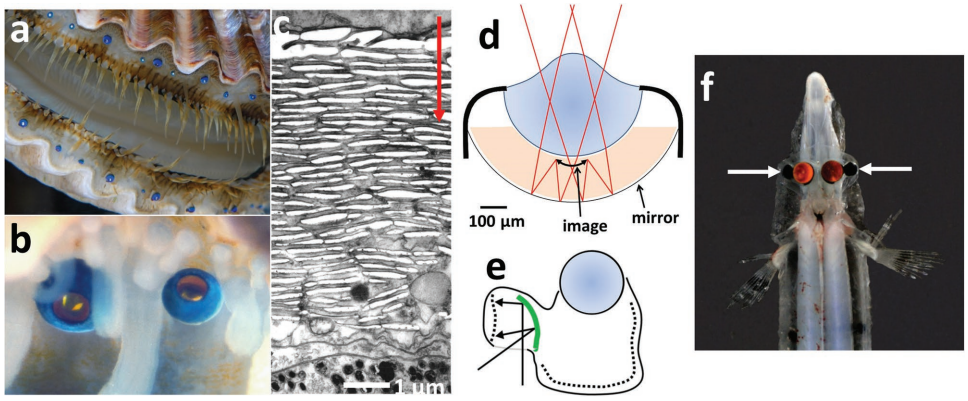

Figure 4. a) Photograph of the eyes of the scallop residing along the mantle tissue of the scallop *Argopecten irradians* (we are obliged to Prof. Sonke Johnson for supplying this photograph). b) Close-up photograph of two eyes the Pecten scallop (photo provided by Prof. Dan Eric Nilsson). c) Transmission electron micrograph of a complete vertical section (cut at right angles to the surface) though the argentea of the eye of the scallop *Pecten maximus*. (TEM image reproduced with permission.[62] Copyright 1966, The Company of Biologists.) The crystals were dissolved during preparation of the tissue (staining with lead citrate). The electron transparent holes mark the positions of the guanine crystals and clearly show a multilayer of 30–40 layers. The red arrow shows the direction that light will impinge on the argentea. d) Schematic of the eye of the pecten scallop, which forms an image by reflection off a concave mirror. The argentea forms an image on the distal section of the retinal cells. The dome-shaped lens probably functions to correct spherical aberration of the mirror. e) Schematic of a transverse section through the visual of the deep sea spook fish *D. longipes*, showing the positions of the retinae (dotted line) in the both the main and auxiliary eyes and the mirror (green) which receives light through a transparent window facing ventrally. The secondary eye has a downward pointing field of view of 48°. Adapted with permission.[67] Copyright 2009, Elsevier. f) Photograph showing the double eyes of the deep sea spookfish *D. longipes*. Photograph taken from above showing the main eyes pointing upwards and the auxillary eyes pointing sidewards marked by white arrows (photo provided by Dr. Tamara Frank).

longipes[67] and *Rhynchohyalus natalensis*.[68] Both these fish contain a double eye, with a normal tubular eye projected upwards towards the surface and a laterally facing lensless basic eye, called an ocular diverticulum, allowing the deep sea fish to simultaneously maximize their sensitivity to brighter downwelling light and to detect the presence of bioluminescent objects beneath them (Figures 4e and f). The diverticulum contains a ventrally pointing transparent window (allowing light to be accepted from downwards directions), and its medial part is lined by a concave parabolic mirror comprising a multilayer stack of (presumably) guanine crystals. Unlike in the *Pecten* scallop, which possesses 'front-silvered' mirror eyes, in *D. longipes* the guanine crystals are tilted with respect to the substrate and the tilt angle progressively changes along the parabolic mirror forming a Fresnel-type reflector plate. Computer modelling demonstrated that this set up is able to produce a well-focused image avoiding spherical aberration.[67] The presence of the ventrally facing transparent window also ensures that reflection takes place before the light reaches the retina, allowing the fish to circumvent the problem of the scallops by first passing unfocused light through the retina before it returns again following reflection.

2.4.2. Light Collection

Some invertebrates utilize guanine crystals in their mirrored eyes for light collection. Examples are the cardium cockle (Mollusca, Bivalvia);[69] the small 'nauplius' eyes of the ostracod *Notodromas* containing three cups, each with a small number of photoreceptors;[70] the enormous (1 cm) mirrored eyes of the ostracod *Gigantocypris* (Crustacea);[60] and in the deep sea amphipod *Scypholanceola* (Crustacea).[71] However, the purpose of these less sophisticated eyes seems to be for extremely rudimentary imaging or phototaxis or to concentrate as much light as possible, rather than to produce a well-focused image.

2.4.3. Light-Doubling Tapeta

A large number of vertebrates and invertebrates possess a tapetum lucidum ('silvery carpet')—mirror lying behind the retina. Tapeta composed of stacks of guanine crystals have been reported in many different phyla including sharks,[72] spiders,[34,73] moths, crabs and crocodiles.[74] The function of the tapetum lucidum is to reflect focused light back to the retina that has been missed on the first pass, increasing the light sensitivity of the eye. This results in the observation of spectacular eye shine.

3. Why Do Animals Use Guanine to Manipulate Light?

In the following sections we will examine the chemical and physical properties of anhydrous guanine crystals that make it especially attractive as a component in reflection-based optical systems.

3.1. Guanine Crystal Structure and Properties

Guanine (2-amino-6-hydroxypurine, **Figure 5**a), with the molecular formula $C_5N_5H_5O$, belongs to the purine family of

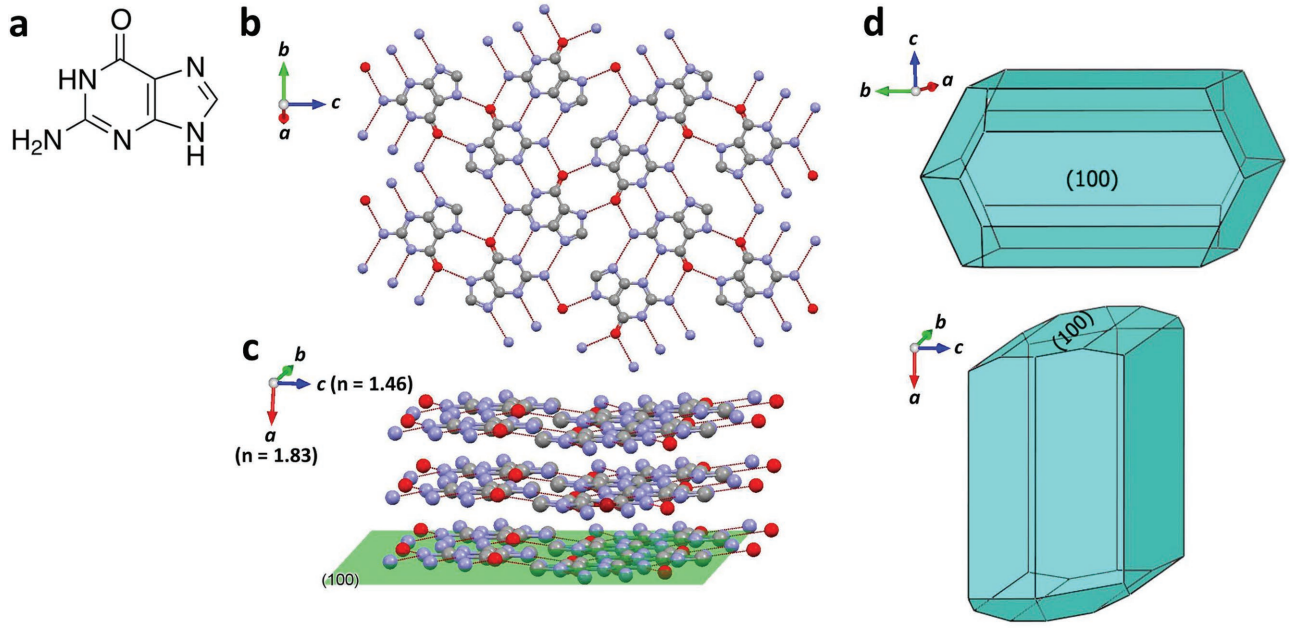

Figure 5. Guanine molecular and crystal structure. a) Guanine molecular formula. b) The crystal structure of the β polymorph of anhydrous guanine in the (100) plane, showing the dense hydrogen-bonded layer. c) The same structure projected in a direction almost perpendicular to the (100) plane, showing the stacking of the guanine molecules in the hydrogen-bonded layers along the short $a = 3.59$ Å axis. The position of (100) plane and the direction of the a axis, where the high refractive index $n = 1.83$ is measured, are indicated. d) The crystal theoretical morphology, calculated from the crystal structure, viewed along down a axis (above) and down the b axis (below), as in the structure views in (b) and (c), respectively.

molecules, heterocyclic aromatic compounds characterized by fused pyrimidine and imidazole rings. Guanine is a planar molecule, sandwiched by clouds of delocalized electrons. Each molecule can form eight hydrogen bonds in the plane of the molecule, creating extended molecular networks. The molecule has a mixed hydrophobic/hydrophilic nature and a strong tendency to form molecular aggregates held together by hydrogen bonds and stacking interactions between the aromatic rings. For this reason, guanine in its neutral form is essentially insoluble in water and is only sparingly soluble in many other common solvents.[75]

Three crystal structures have been determined to date for guanine in its neutral form: the monohydrate and the α and β anhydrous structures.[76] The structure of the α polymorph was determined in synthetic guanine crystals only a decade ago, whereas the very recent elucidation of the structure of the β polymorph, characteristic of the biogenic crystals, was the subject of such a suspense story that it was referred to as a 'guanigma'.[76] The two structures of anhydrous guanine are very similar and have similar calculated lattice energies. Both are predictably characterized by extended hydrogen-bonded layers in the (100) crystallographic plane, stacked along the very short 3.55 Å (α) or 3.59 Å (β) *a* axis (Figures 5b,c). The stable H-bonded layer motif is the same in both α and β structures, but they differ in the stacking of the layers, which are offset along the *c*-axis and the *b*-axis in the α and in the β forms, respectively.

Ultimately, the ability of guanine crystals to act as an effective reflective material in biology derives from the fact that crystalline guanine has an extremely high refractive index along the molecular stacking direction.[32] From ellipsometry measurements on assembled guanine thin layers on silicon, Hinrichs et al.[20] measured the refractive index as $n = 1.76$ in the molecular stacking direction and $n = 1.45$ in the perpendicular direction. On the other hand, measurements of the refractive index of guanine crystals in fish scales by Huxley[19] using an interference microscope gave a refractive index of $n = 1.83$. Our own unpublished measurements confirmed a refractive index value of $n > 1.83$.

This high refractive index together with the difference in refractive indices along different crystallographic directions (i.e., the birefringence) derives from the anisotropic crystal structure of guanine and particularly depends on the efficiency of electronic transitions parallel to and perpendicular to the H-bonded layers. The high refractive index in the molecular stacking direction along the *a* crystal axis (Figure 5c) is due to the high polarizability of the electrons in this direction. The polarizability determines the relative permittivity (ε_r), a complex quantity reflecting the ease with which an electron cloud in a dielectric is distorted by an applied field, and $n = \sqrt{\in_r}$.

The relative permittivity in different crystallographic directions is a function of all the electronic transitions polarized in those directions. In a crystal of guanine the electron band dispersion along the stacking direction is dominant over the dispersion in the H-bonded plane because of the efficient overlap of the π orbitals, relative to the H-bonded plane. The exact electronic transitions responsible for the high polarizability in this direction are still a matter of debate, partly due to the difficulty in accurately measuring reflectance spectra along different crystal faces on small guanine crystals. However, the interaction of the electric field vector and $n \rightarrow \pi^*$ transitions polarized perpendicular to the stacking plane is thought to be a dominant factor. We note that a value of $n = 1.83$ is extremely high not only for organic materials but is also relative to common inorganic materials.

3.2. Biogenic Guanine Morphologies

The flexibility exercised by organisms in the control of guanine crystal morphology is the fundamental reason for the impressive variety of guanine crystal-based biological scatterers and reflectors. How organisms achieve this level of control is still far from being understood.

The theoretical morphology of anhydrous guanine is determined by the strong aromatic stacking interactions in the direction of the *a* axis and not quite as strong interactions within the hydrogen-bonded layers. As a result of the balance between these interactions, the predicted growth form of guanine is prisms elongated in the direction of the *a* crystallographic axis (Figure 5d).

The crystal morphology of the light scattering layer in white spiders (**Figure 6**a), as well as in other organisms using guanine crystals for light scattering functions, is similar to the theoretical prismatic growth form.[26] It is also similar to the morphology of synthetic crystals grown from aqueous solutions[77] (**Figure 7**a). The crystals are prisms, with an irregular hexagonal cross-section and typical sizes of 1–2 μm. The size, slightly larger than the wavelengths of visible light, is very well suited to light scattering. White spiders thus do not appear to have evolved modifications to the intrinsic guanine crystal morphology.

In contrast to the white spiders, silver-colored fish, silver spiders, certain crustaceans, frogs, and all organisms using guanine multilayer reflectors, produce thin crystals with plate-shaped morphology, where the large plate faces are parallel to the (100) plane. This morphology is specifically adapted to optimize the reflective properties of the individual crystals and, eventually, the cumulative reflective properties of the biogenic photonic crystals. The shortest dimension, the direction in which the light impinges on the thin crystal plates, is in the direction of the *a* axis, where the refractive index is highest.[20,76]

The guanine crystals in the fish scales are elongated hexagonal (100) plates, >10 μm long, a few μm wide, and only 20–25 nm thick[26] (Figures 6b,c). The optical thickness of the high refractive index crystal component is $4\ n\ d \approx 40$ nm—far from a perfect quarterwavelength reflector for visible light. It appears that the abundance of the crystals over the extended area of the fish scales made further evolution toward optimization of the reflecting apparatus unnecessary.

Achieving the thin plate morphology requires extensive biological intervention to control the intrinsic tendency of the crystals to grow as prisms in an aqueous environment. Major challenges are to determine how the thin plate morphology is achieved in biology, and how it is stabilized at such a low thickness value.

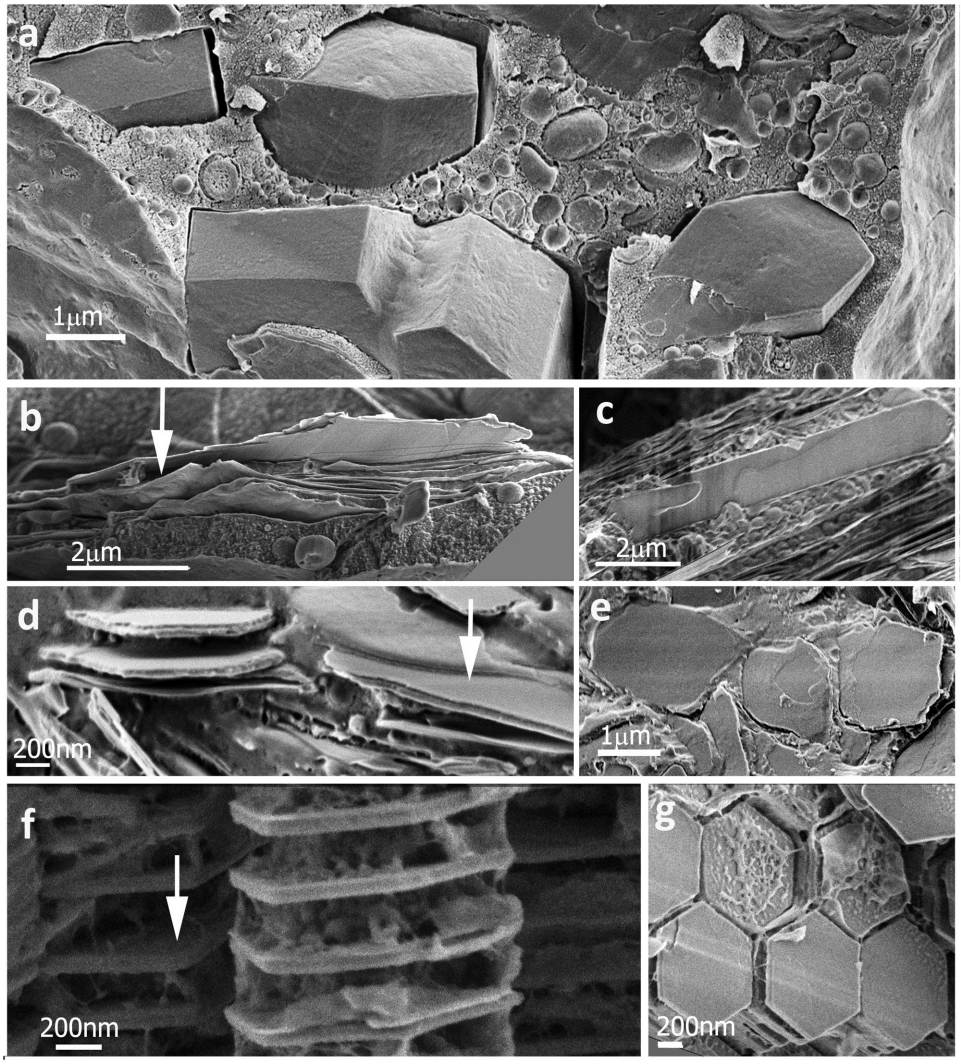

Figure 6. Cryo-scanning electron micrographs of biogenic guanine crystals imaged in the intact tissues after freeze fracture. In (b), (d), (f), the thin plate-shaped crystals were imaged edge-on, whereas in (c), (e), (g) they were imaged on the plate face. The arrows in (b), (d), and (f) indicate the direction of impinging light, where the refractive index $n = 1.83$. a) Anhydrous guanine prismatic crystals under the epithelial layer of the white spider *Latrodectus pallidus*. b,c) Anhydrous guanine crystal plates from the gin-rin Koi fish scales, 20–25 nm thick. d,e) Crystals from under the epithelial layer of the silver spider *Tetragnatha extensa*. Each high refractive index unit is 70 nm thick and is composed of an amorphous guanine layer 30 nm thick, sandwiched between two ≈20 nm thick anhydrous guanine crystal plates. f,g) Anhydrous guanine crystal plates from the cuticle of the copepod *Sapphirina metallina*. Each crystal plate is a perfect hexagon, achieved through multiple twinning perpendicular to the (100) crystallographic plane (labeled in Figure 5).

This conundrum becomes even more baffling when considering the crystal morphology in the silver spiders.[26] The morphology in the plane of the plates is irregular. The crystal layer is ≈70 nm thick, achieving maximum reflectance for $\lambda \sim 500$ nm well within the visible range. Surprisingly, the ≈70 nm thickness is achieved by combining two ≈20 nm thick crystals enclosing a 30 nm thick layer of amorphous guanine (Figures 6d,e). It thus appears that the 20–25 nm thickness may be "hard-wired" in biology, as it is preserved in fish and spiders, even though it is far from ideal in terms of reflection. On the other hand, sapphirinid copepods produce stacks containing ≈70 nm thick perfectly hexagonal guanine crystals (Figures 6f,g). This crystal thickness results in an almost ideal quarter wavelength reflector, where the cytoplasm layer thickness modulates the reflector wavelength.[56] It will be interesting to determine the crystal morphology in the panther chameleon 3D photonic crystals that, based on the published TEM images taken in two projections, appear to be prismatic.

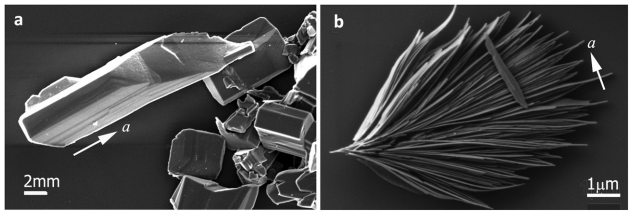

Figure 7. Synthetic crystals of anhydrous guanine. a) Anhydrous guanine crystals obtained in aqueous solutions. b) Anhydrous guanine crystals obtained from DMSO. The direction of the *a* axes is indicated by an arrow for one of the crystals both in (a) and (b).

3.3. Control of Guanine Crystal Morphology and Crystal Formation

The fact that guanine is practically insoluble in water at neutral pH presumably represents a physiological advantage because the crystals are not likely to easily dissolve if exposed to sudden fluctuations in the concentration of the cellular environment, or even of the extracellular matrix environment. On the other hand, the very low solubility must complicate the transport and concentration of the guanine molecules needed to build a crystal. The principle of compartmentalization of each crystal within an individual membrane is common to all the light manipulation guanine-based systems that have been analyzed so far (**Figure 8**). It is conceivable, but not demonstrated that, because the solubility of guanine substantially increases at acidic or basic pH, local control of the pH in the compartment may facilitate transport and concentration at the site of crystallization.[77]

A hint of what may well be an additional common crystal building strategy was obtained from the Koi fish scales, where crystals form from an amorphous guanine precursor phase enclosed in spherical vesicles (Figure 8a).[78] Similar vesicles containing apparently solid organic material were observed in silver and white spiders.[26] We note that crystal formation through transient amorphous precursor phases is a widespread strategy evolved by organisms of different phyla for building skeletal materials from close-to-insoluble compounds.[79]

Crystal morphology can conceivably be controlled at several levels: large (100) plates might be induced to form by direct templating on the (100) crystal plane over a large surface. In this case the templating surface, presumably membrane attached, must be hydrophobic and must form specific interactions with the aromatic side of the rings exposed on the (100) plate face. This is not impossible, but is hard to conceive.

The chemistry of the micro-environment in which the crystals form may also be involved in controlling the crystal morphology, either through specific intermolecular interactions or through the establishment of local chemical and physical properties, such as dielectric constants and chemical potentials, conducive to preferred development of hydrophobic surfaces. Consistent with this general idea, it is relevant that synthetic guanine forms from DMSO as thin (100) plates (Figure 7b). DMSO is a polar aprotic solvent that dissolves compounds with mixed hydrophobic and hydrophilic character and with low solubility in other solvents, such as guanine.

An alternative possibility is that control of crystal morphology is achieved through additives that specifically adsorb on the (100) face, inhibiting crystal growth in the *a* crystallographic direction. A small amount of xanthine and hypoxanthine might in principle be sufficient to inhibit guanine crystal growth. Both xanthine, hypoxanthine and uric acid have been reported to be present in some biogenic crystals.[80] All three compounds are closely related to guanine in their molecular structure and in the metabolic pathway, making it conceivable that they may be available at the site of crystallization to control crystal morphology.

4. Concluding Remarks

Perhaps the strongest theme that arises from surveying this field is the versatility of guanine-based optical systems in nature. The white scattering of certain spiders, the silvery reflectance of fish and other spiders, the brilliant and diverse colors of copepod crustaceans and tropical fish and the diverse mirrors of animal eyes are all produced by manipulation of a single material—crystalline guanine. This astonishing diversity stems from nature's ability to exquisitely control the morphology and arrangement of these highly reflective guanine crystals.

From an evolutionary perspective it seems that nature has converged on the 'guanine solution' several times independently. Although we can explain why crystalline guanine is well suited for such optical functions, we are not able to explain why there are so few other organic bio-crystals in natural optical systems. Why are other purines such as adenine, uric acid, hypoxanthine, and xanthine not found more widely in natural optical systems? In order to obtain deeper insights into this subject, a better understanding of the crystal structures and resulting optical properties of purines and other related molecules is required.

One striking feature is that across a range of classes in 3 different phyla the thin (usually around 20 nm) plate-like crystal

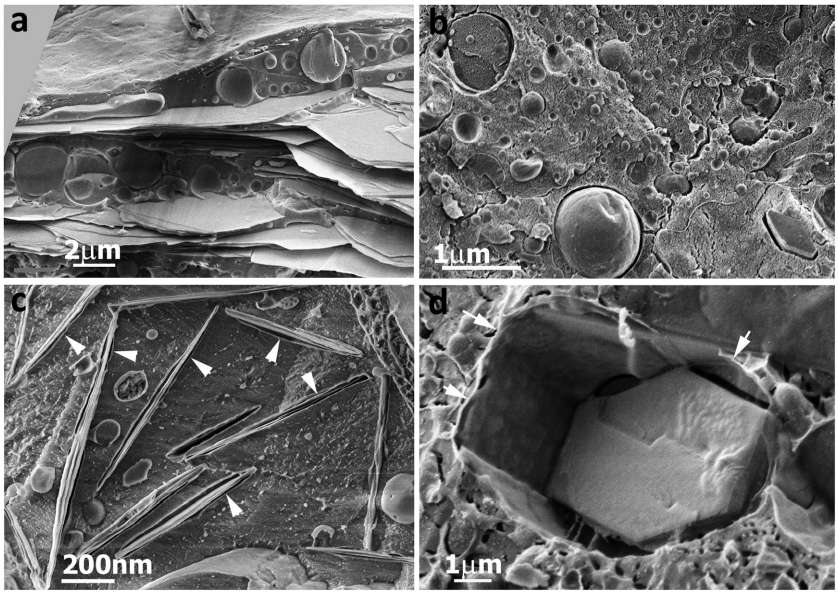

Figure 8. Guanine is deposited and guanine crystals are formed inside membrane-delimited compartments. Cryo-SEM images: (a), (c) Sections through the iridophore layers under gin-rin Koi fish scales: a) the membrane-delimited vesicles interspersed between crystal layers contain solid material that was proven to be amorphous guanine;[78] c) each thin crystal plate is enclosed within a membrane, called the 'crystal chamber'. White arrows indicate the thin membranes. b,d) Section under the epithelial layer of the white spider *Latrodectus pallidus*: b) membrane-delimited vesicles are abundant, interspersed with crystals; d) a crystal enclosed within a membrane. White arrows indicate the thin membrane.

morphology of guanine has been strongly conserved, despite it being far from ideal in terms of its optical thickness. Often it is the control of the cytoplasm thickness and not crystal morphology that is critical for determining the optical function of a material. Is there a fundamental biological or chemical reason why nature has consistently found this plate-like crystal solution?

Although the physics of these systems is well understood, many fundamental questions on the chemistry and biology remain unanswered. In many ways, the field of organic biomineralization is in its infancy and many of the questions that have been asked in conventional inorganic biomineralization still remain to be posed (let alone answered!). How do organisms control the morphology of guanine crystals? Are amorphous guanine precursor phases common in nature? Given the extreme insolubility of guanine in water, how is guanine transported through aqueous media to the site of crystallization without nucleating? In tunable optical systems, what cellular machinery is used to vary the spacings between crystal and cytoplasm?

The body of literature existing on biological light manipulation using guanine crystals shows just how fascinating and diverse these systems are. We have little doubt that there are still many natural systems involving the manipulation of light by guanine crystals yet to be discovered. Some of these will no doubt reveal more "neat" solutions to problems that may be applied to synthetic systems. Furthermore, a basic understanding of the open questions outlined above will further facilitate better exploitation of guanine in artificial systems.

Apart from the use of guanine crystals in the cosmetic industry (where it is used to produce the pearlescence effect),[81] we know of no other direct artificial application of guanine crystals. However, of greater importance than using guanine crystals directly is applying the underlying principles exhibited by guanine-based optical systems in nature. Of particular interest to materials science may be taking inspiration from nature for the manufacture of tunable photonic crystals that could be used in a range of applications such as sensors, optical fibers, photovoltaics, Bragg mirrors, and displays.[82,83]

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