The structure-function relationships of a natural nanoscale photonic device in cuttlefish chromatophores

Progress towards elucidating the structure-function relationships of a natural nanoscale photonic device in cuttlefish chromatophores

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ABSTRACT

The adaptive coloration observed in cuttlefish *Sepia officinalis* skin is facilitated in part by properties of pigmented chromatophores that have not been previously reported. We found that chromatophore coloration is enabled by a tethering system that distributes layered pigment granules, comprised of fluorescent nanostructures, to optimize color intensity as the chromatophores are actuated. The design features gleaned from these studies provide intriguing insights into the development of artificial photonic systems useful for products ranging from conformable, high-definition color displays to optical fabrics capable of adapting their coloration within an ambient environment.

Keywords: Cephalopods, cuttlefish, chromatophores, pigment granules, optical organs, biophotonic, structure/function, luminescence

1. INTRODUCTION

Dermal coloration within cephalopods, such as the cuttlefish, *Sepia officinalis*, is regulated by its vertically coupled optical organelles consisting of (1) the leucophore, a scatterer having uniform reflectivity over the visible spectrum, (2) the iridophore, a reflector and (3) the chromatophore, a pigmented sac that functions as a tunable color filter.[1-5] The chromatophore contributes to active changes in dermal color across multiple spatial scales, beginning with the assembly of nanostructured pigment granules encapsulated within a pigment cell that give rise to its functionality.[6] While it is understood that chromatophores contribute to active coloration within the dermis, the role of its composition and the role of its intracellular granular architecture remain largely unknown.

The composition of the pigmented chromatophore granules have been previously linked to a class of redox active molecules comprised of substituted phenoxazinone derivatives known as ommochromes.[7] The only analytical report identifying the presence of ommochromes in cephalopod chromatophore pigment granules is by Van den Branden and Decleir from 1976.[8] They extracted three separate ommochrome pigments from cuttlefish skin based on solubility, which were predicted to correspond to the three chromatophore layers present. Characterization involved investigation of redox properties and halochrome formation, and collection of absorption spectra for the three extracts. Identification of yellow, red-brown, and violet ommochrome extracts was not reported.

In a recent report, purified brown pigment granules extracted from chromatophores in the dorsal mantle and ventral fin regions of the *S. officinalis* cuttlefish were observed to contained high refractive index proteins including isoforms of reflectin and crystallin.[6] Tandem mass spectrometry coupled with a series of systematic denaturation experiments in concentrated sodium hydroxide revealed that the degradation of the pigment granules correlated with a decrease in relative abundance of reflectin and crystallin, suggesting that these proteins are associated with the isolated chromatophores.[6] Optical properties of these granules were interrogated using micro-photoluminescence (μPL)

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spectroscopy, where an excitation sweep over the granules produced a local excitation maximum at 410 nm and emission maxima centered between 650-720 nm. Collectively, these observations suggest that *S. officinalis* chromatophore pigment granules more than just homogenous clusters of chromogenic pigments; they are uniquely assembled photonic nanostructures. In this report, we focus on further elucidating the role of reflectins in chromatophore pigment granules, while illustrating potential integration within flexible material substrates.

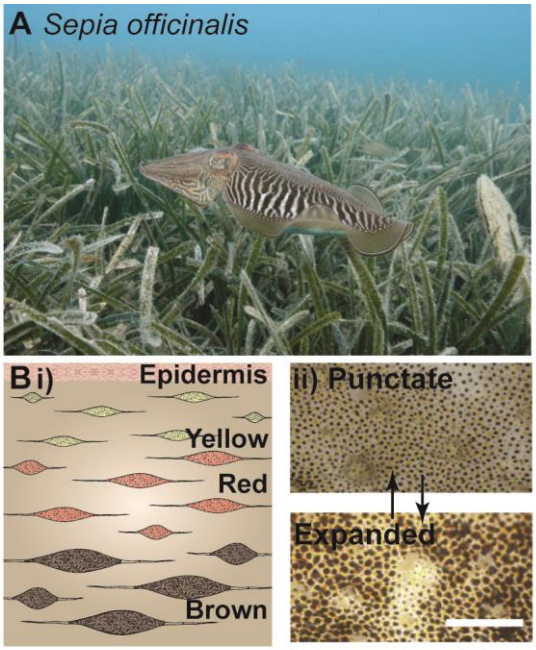

Figure 1. Dermal coloration in *S. officinalis*. (a) Adult cuttlefish *S. officinalis.* (b) Chromatophores are present as three colors, yellow over red over brown. i) Illustration of the layered chromatophores within the uppermost layer of the dermal tissue, (ii) when actuated in the dorsal mantle, chromatophores transition from a punctate to expanded state.

2. MATERIALS AND METHODS

2.1 Chromatophore pigment granule extraction, isolation, and purification

Adult cuttlefish, S. officinalis, (1+ year old) are raised and sacrificed at the Marine Resources Center (MBL, Woods Hole, USA). Prior to dissection, the whole animal is soaked in ethanol to minimize any microbial/protozoan contamination from its native habitat. Brown chromatophore pigment granules are isolated both from tissue biopsies and from single cells isolated using a laser capture micro-dissection (LCM) system (PALM Combi system, Carl Zeiss Microimaging) from the dorsal mantle or ventral fin. In the biopsy material, skin was removed from the dorsal mantle in 4 mm^2 pieces. Chromatophore tissue was manually dissected from the leucophores and iridophores and collected in homogenization buffer. The isolated tissue was then lysed via ultra-sonication and purified using a sucrose gradient for up to 60 min. Final pellets were resuspended in homogenization buffer and stored at 4ºC until use. Isolated chromatophore granules are purified using SDS-PAGE.

To mitigate the risk of contamination during our purification, we rinsed all tools in 70% ethanol and HPLC grade water prior to loading the gel. SDS-PAGE on the pigment pellet resulted in multiple protein bands spanning from 0-75 kD. Whole lanes from the gel are sectioned and analyzed using a fluorescent gel reader.

2.2 Microscopy and Image Analysis

Luminescing samples are imaged using a Zeiss LSM 510 laser scanning confocal microscope. Scanning electron microscopy images are collected using a field emission scanning electron microscope (FESEM; Carl Zeiss, Dresden,

Germany). The fiber samples are sputter coated using an Au target **(**Denton Vacuum, Moorestown, NJ**)** prior to imaging. All image analysis is done using ImageJ software (national Institutes of Health, Bethesda, MD).

2.3 Reflectance

Spectral reflectance measurements are obtained using a spectrometer (QE65000, Ocean Optics, Dunedin FL), attached with a 1000 micron diameter fiber optic cable to the c-mount of a stereo microscope (Zeiss Discovery V20). Illumination is provided by a high-power broadband Xenon light source (HPX2000, Ocean Optics, FL), allowing reflectance spectra to be collected from approximately 400 to 1000 nm (note that our microscope optics block UV wavebands).

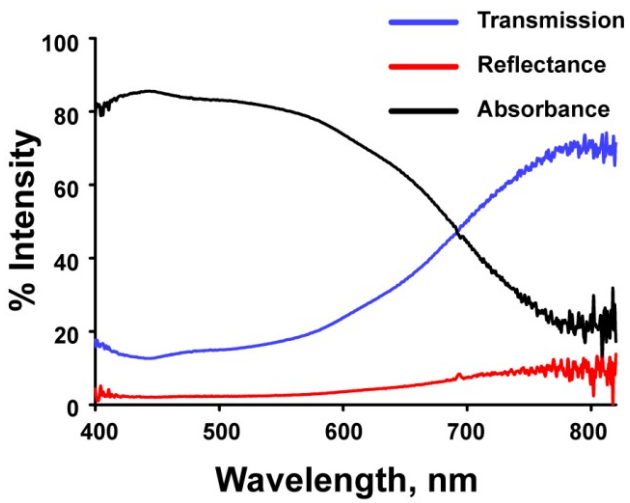

Figure 2. Absorbance, reflectance, and transmission of light in brown chromatophores. Data presented is averaged from n=10 chromatophores.

3. RESULTS AND DISCUSSION

Dermal coloration in *S. officinalis* (**figure 1a**) is accomplished through the coordination of the vertically layered chromatophore, iridophore, and leucophore optical organs in response to external environmental cues. Chromatophores populate the uppermost layer of the dermis and are present in three colors: yellow over red over brown (illustrated in **figure 1bi**). Depending on its actuated state (punctate or expanded, figure 1bii), all three or fewer of the chromatophore colors may interact with light to absorb or reflect light over a broad spectral range. Given that the millisecond actuation time of the chromatophores can result in a factor five times change in presented surface area (**figure 1bii)**, we asked how color modulation is achieved without loss of color fidelity or saturation. To test this, we used L-glutamate to keep chromatophores in expanded state to experimentally measure both reflectance and transmission (**figure 2**). The measured reflectance and transmittance of ten expanded brown chromatophores is then used to extrapolate absorbance, which is calculated assuming the sum of transmittance, reflectance, and absorbance is 100 (**figure 2**). These data suggest that brown chromatophores are strong absorbers of light (~80% from 400-650 nm) and that this strong absorbance may contribute to the richness in color in the cuttlefish during skin actuation.

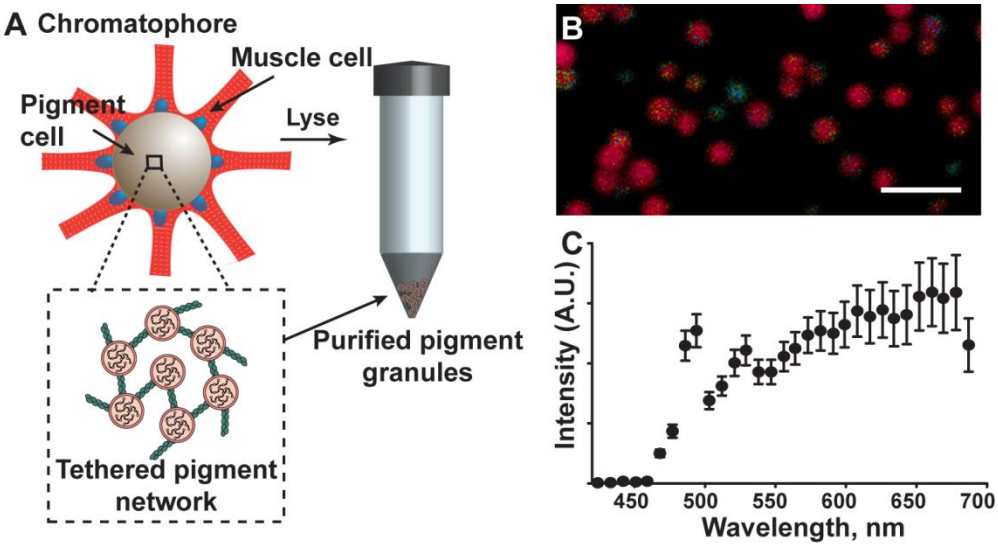

Figure 3. Chromatophore pigment granules as luminescing nanostructures. (a) Illustration of the tethered pigment granule network within the chromatophore. During analysis, the chromatophore cells are collected, lysed, and purified to collect granules for analysis. (b) Maximum projection laser scanning confocal image pseudo-colored to represent variations in emission wavelengths of the brown chromatophore pigment granules excited at 546 nm. Scale bar is 2µm. (c) Variations of luminescence intensity as a function of projected emission wavelength from image (b), n =24, error SEM.

Structural coloration is common to a wide range of animals,[9-11] where absorbance may be amplified by light scattering among and across adjacent nanostructures.[12] We reasoned that chromatophore pigment granules exhibit similar properties. Micro-photoluminescence (µPL) spectroscopy revealed pigment granule luminescence (ex 410, 505 nm/ em 650-720 nm).[6] This broad emission maximum suggests that emission intensity may vary as a function of focal plane. To further investigate these luminescing properties, laser scanning confocal microscopy is used to measure emission intensity of isolated pigment as function of focal plane (**figure 3**).First, nanostructured (~528 nm diameter) pigment granules are extracted from brown chromatophores, as depicted in **figure 3a**, and purified them using a series of centrifugation and washing cycles preceded by a sucrose gradient. Purified granules imaged using laser scanning confocal microscopy (**figure 3b**) displaying a broad emission profile when excited at 546 nm (**figure 3c**). These data suggest that brown chromatophore pigment granules behave as a non-uniform distribution of emitters, a property that may contribute to its broad emission adding to the complexity of the system. These properties demonstrate that brown

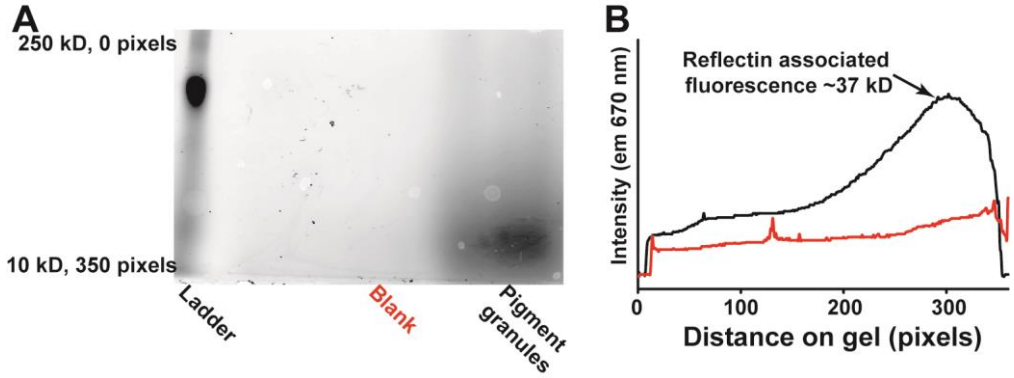

Figure 4. Fluorescence of SDS denatured and dialyzed pigment granules that are purified using gel electrophoresis. (a) Fluorescence image collected with 532 nm excitation and 670 nm emission. (b) Map of fluorescence intensity as a function of distance down the gel. Results show the strongest emission wavelength falls within ~35 kD range, suggesting reflectin protein is associated with the far red emission. Red is blank (loading buffer only) and black is the fluorescence signal from the denatured pigment granules.

chromatophore pigment granules are more than just homogenous aggregates of pigmented molecules; instead, they appear to be a heterogeneous ensemble of substructures.

In a previous report, an abundance of reflectin and crystallin proteins were observed within the isolated chromatophores.[6] Reflectins are best known as the high-refractive index structural protein in the iridophore layer[13] and had never previously been reported in the chromatophore. We asked whether the reflectins present in the chromatophore contribute to the far-red fluorescence of the pigment granules. To test this, purified brown pigment granules are denatured in sodium dodecyl sulfate (SDS) and purified using gel electrophoresis (**figure 4a**). Fluorescence associated with the unstained gel is measured using a Typhoon gel scanner (excitation 532 nm, emission 670 nm, **figure 4b**). Maximum fluorescence is observed in the molecular weight range (30-39 kD) of reflectin, suggesting that reflectin proteins are associated with the far-red emission profile observed in the pigment granules.

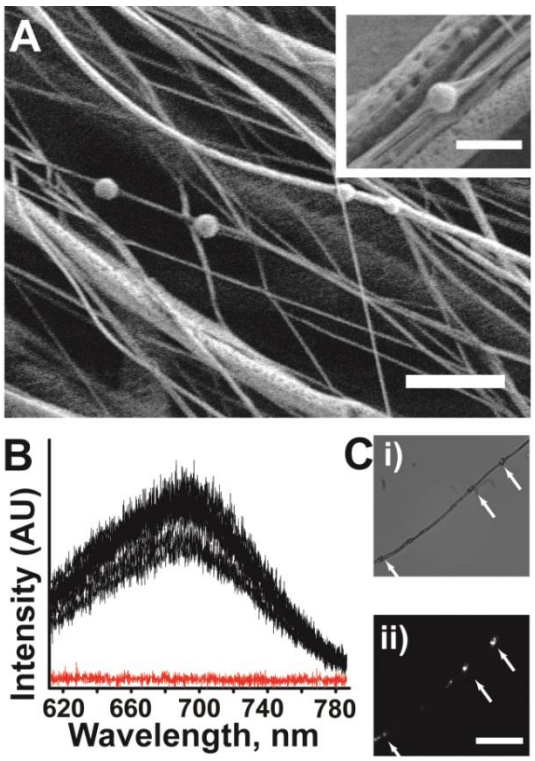

Figure 5. Manufacturing nanofibers using S. officinalis pigment granules. (A) SEM of protein composite fibers. Pigment granules (20 mg mL-1) embedded within PLA nanofibers as spun using the RJS. Scale bar is 5 µm. Scale bar of inset is 2 µm. (B) µ-PL was used to measure emission intensity (black) for n=8 pigment granules within the nanofibers excited at 410 nm. Red line represents emission spectrum for PLA fibers without pigment granules to show that PLA auto fluorescence does not contribute to luminescence. (C) Bright field (i) and fluorescence (ii) images of granules in a single fiber captured using confocal microscopy. Fibers are excited at 405 and 488 nm, and emission is collected using a 600 nm long pass filter to ensure only light emitted in the far red is collected. Scale bar is 50 µm.

These robust optical properties of *S. officinalis* chromatophores make them a compelling platform for bio-inspired design of new types of flexible displays. We asked whether pigment granules integrated into a flexible fiber matrix also retained its luminescing properties. Nanofibers were manufactured using the rotary jet spinning (RJS) technique.[14-16] RJS is a non-destructive nanofiber fabrication technique that uses centrifugal forces to extrude a composite polymer solution through a micron-sized orifice. Chromatophore pigment granules (20 mg) were suspended in 7.2 wt% poly-lactic acid in chloroform and were spun at 30,000 RPM to form fibers with an average diameter of 335 ± 220 nm. Samples were collected and imaged using scanning electron microscopy (**figure 4a**) and analyzed using micro-photoluminescence spectroscopy (**figure 4b**) and confocal microscopy (**figure 4c**). Whole granules were encapsulated within the nanofibers, exhibiting peak luminescence centered between 650-720 nm when excited at 410 nm. This data demonstrates that pigment granules are not only capable of withstanding the shear forces of solution extrusion during

RJS processing without loss in structure or optical function, but they also fluorescence with an emission maxima centered at 650-720 nm, similar to the 2D isotropic granules, depicted in figure 3.

4. CONCLUSIONS

Cephalopods, such as *S. officinalis*, have evolved the ability to alter the color patterning of their skin for the purpose of sensing, communication, defense, and reproduction. Adaptive coloration in these animals is facilitated by neurally-controlled, muscle-actuated pigment organs known as chromatophores that contain a tethering system of nanostructured pigment granules. Several synthetic approaches have been engineered to mimic the adaptive coloration of the cephalopod; however, existing strategies fail in imparting the dynamic range of visible color native to the cephalopod, which we reason is correlated with the limited understanding of the molecular composition of the chromatophore pigment granules.

Our data support previously reported observations suggesting that adaptive coloration in *S. officinalis* is regulated by the nanostructure and composition of the pigment granules and their ordered distribution within the cytoelastic sacculus. Such hierarchical structure makes possible the uniform, rapid transposition of color within the dermal tissue. An optical bonus within the chromatophore is the luminescence, which we predict may contribute to the richness in color of the expanded chromatophores. This report of luminescence in *S. officinalis* pigment granules also discourages the prevailing hypothesis that chromatophore granules are simply composed of ommochromes,[17] which has an emission maximum centered around 450-475 nm.[18] The differences in both the absorbance and emission shifts in pigments compared to the *S. officinalis* granules suggests that the addition of structural proteins, such as reflectins, can modify the optical properties of a pigment, red shifting both absorbance and emission. Understanding, mimicking, and modeling the molecular contributions to bulk coloration may provide insight into the development of next-generation flexible displays, dye-sensitized solar cells, or light-sensitive textiles.

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