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Sucker Rings from the Humboldt Squid *Dosidicus gigas*: The Role of Nanotubule Architecture on the Mechanical Properties

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ABSTRACT

The suckers that line the arms and tentacles of squid are equipped with rigid toothed ring-like elements that increase the gripping power during prey capture and handling. The sucker rings of the Humboldt squid *Dosidicus gigas*, are fully proteinaceous and contain nanotubules with diameters ranging from 100 to 250 nm. It has been shown previously that the ensuing porosity is a prime determinant of the local elastic modulus [A. Miserez *et al.*, *Adv. Mater.* **21**, 401 (2009)]. Here additional nanoindentation data are presented together with structural analyses. The nanomechanical data support our model that the measured modulus is determined by the local porosity. The dry moduli reach ca. 8 GPa and are reduced about two-fold in the hydrated state. This surprisingly small reduction is discussed in relation to possible chemistries responsible for assembly of these structures.

INTRODUCTION

The cephalopods, which include squid and octopi, grasp onto objects through the action of muscularized suckers regularly placed along their tentacles and arms. Despite these similarities in prey capture strategies, the design of squid and octopi suckers differ significantly from each other: While the suckers of octopi rely exclusively on the suction power generated by muscular contraction and the conforming nature of the flexible outer margin [1], squid suckers are mounted on flexible stalks and are equipped with additional rigid toothed ring-like elements [2] as illustrated in Figure 1A and 1B. We recently showed that the sucker rings of the up to 2 m long red devil or Humboldt squid, *Dosidicus gigas*, contain parallel tubular elements with diameters in the 100-250 nm range [3]. In addition to this unusual microstructure, the sucker rings were shown to be proteinaceous and devoid of chitin. They were also found to be non-crystalline and contain, in addition to their organic constituents, only sulfur and chlorine (as determined by the detection limits of energy dispersive spectroscopy) [3]. Here we review our previous results and provide new data on the local mechanical properties as probed by nanoindentation and speculate on the underlying chemistry responsible for these observations.

EXPERIMENT

The sucker rings used in this study were isolated from Humboldt squid collected from Hueneme Canyon (N 34.06, W 119.16) during May to July, 2007 at 110-183 m depths [3]. The

sucker rings were isolated from 10 individuals and the residual salt was removed by soaking them overnight in deionized water. Scanning electron microscopy (SEM) was performed using either an FEI field emission NovaSEM 600, an XL30 (FEI, Eindhoven, the Netherlands), a CamScan MaXim 2040 EnVac SEM (CamScan, Waterbeach, Cambridgeshire, UK), or a Tescan Vega TS5130-MM (Tescan, Brno Czech Republic).

Spatial mapping of mechanical properties was assessed by nanoindentation, using a Triboindenter nanomechanical testing system (Hysitron, Minneapolis, USA), equipped with a 30 mN full load force transducer. An elongated cube-corner tip allowing testing under both dry and hydrated conditions and a custom made fluid cell were employed as described elsewhere [4]. The area function of the tip was carefully calibrated prior to testing by performing indentation on fused quartz and PMMA standards. Previous investigations [3] indicated that variations in mechanical properties across the sucker ring teeth are related to gradients in nanotubule volume fraction and size, and that the measured values of mechanical properties depend on the orientation of microstructural features relative the loading axis during indentation. In order to further access this relationship, individual teeth were mounted in epoxy resin with their longitudinal axis parallel to the face of the embedding mold (Ted Pella, Redding, CA) with subsequent flat surfaces prepared on a Leica ultramicrotome (Leica Mikrosysteme GmbH, Wetzlar, Germany). From our previous structural characterization, it is assumed that the tubules were oriented parallel to the cutting plane after microtoming. An electron micrograph showing the region of the tooth and the section plane that was investigated is shown in Figure 2A and the assumed indentation axis/tubule orientation angle is presented in Figure 2B.

RESULTS AND DISCUSSION

The sucker rings (Figure 1A) can be extracted from the suckers with forceps and a photograph of one such ring is shown in Figure 1B. Only rings from the tentacles will be discussed in the present paper. The rings are elliptical in shape and contain a series of triangular teeth, Figure 1A, that can penetrate the skin of a prey item during capture and handling. When fractured, it is revealed that the sucker rings are porous and are composed of very high aspect ratio nanotubules, Figures 1E-H [3]. Figure 1C shows a fracture surface made perpendicular to the plane of the basal ring. The orientation of the constituent nanotubules can be obtained from such images and a sketch of their general arrangement is shown in Figure 1D [3]. Within the teeth, the tubules run parallel to the tooth long axis. The tubules are locally parallel and densely packed with a tubule diameter and spacing that varies with location (Figures 1E and 1F). Indeed, the tubule diameter is smallest near the tooth exterior surface and then progressively increases toward the tooth core. The innermost region of the circular ring base is solid and lacks any detectable porosity (Figure 1D) [3]. Measurements of tubule diameter in an individual tooth showed that they are close to 100 nm near the tooth surface and increase to a plateau of ca. 250 nm at a distance corresponding to ca. 40% of the radial distance into the tooth interior [3]. Surprisingly, there is no symmetry or periodicity in the arrangement of the tubules, Figure 1H. The increased size of the tubules results in an increased porosity and the pore volume fraction increases from close to zero near the surface to ca. 20% in the core region of the teeth [3]. Figure 1G shows a fractured surface of a tooth, revealing useful information regarding its failure mode. The highly regular nature of the fractured surface suggests that the wall structure is uniform on the 10-100 nm length scale.

Figure 1. Sucker ring morphology and architecture. (A) Contained within squid suckers are the sucker rings, which often exhibit formidable teeth (in this case, from the genus *Loligo*). (B) Photograph of an isolated sucker ring from *D. gigas* lying on a piece of ‘mm-paper’ where each square is $1 \times 1 \text{ mm}^2$. Note the brownish green color of the basal ring. (C) Scanning electron micrograph of a fracture surface made perpendicular to the plane of the basal ring. (D) Diagram illustrating the approximate tubule orientation throughout the ring section. (E) The tubules are highly aligned and densely packed. (E) The tubules terminate near the exterior of the sucker ring, seen at the right of this image of a tooth longitudinal section. (G) In some places, the tube walls spontaneously fracture revealing their interior architecture. (H) Tubules in a tooth displayed end-on; note the lack of symmetry or periodicity in tubule packing.

Mechanical properties

Maps of local elastic moduli determined by nanoindentation are presented in Figure 2 for both wet (Figure 2C) and dry conditions (Figure 2D). Overall, previous trends [3] are confirmed: the modulus decreases from the periphery to core under both dry and wet conditions. Using a simple Ashby-Gibson analysis, we have previously suggested [3] that the relative decrease of elastic modulus (E_p/E_e) from the periphery to the core measured by nanoindentation depended on the local porosity and the orientation of the tubules relative to the loading axis, where E_p and E_e are the moduli in the interior and periphery, respectively. Here, it can reasonably be assumed that the tubules are oriented roughly parallel to the surface on the microtomed plane of Figure 2A. In this situation, the dominant loading mode is bending of the tubule walls and the modulus ratio (E_p/E_e) is expected to decrease in cubic dependence of the local porosity content V_p :

$$\frac{E_p}{E_e} \approx \left(\frac{C_1 - V_p}{C_1 - V_e} \right)^m \quad (1)$$

with exponent $m=3$, C_1 being a constant close to unity, and V_e being the porosity content on the periphery. Quantitative measurements of nanotubule volume fraction by image analysis indicated that the maximal porosity content in the core of each tooth is ca. 20% [3]. The associated reduction in elastic modulus relative to that of the periphery is predicted by Eq. 1 to be 0.54. This is in excellent agreement with the measured decrease in moduli derived from the map in Figure 2D ($V_e/V_p \sim 0.52$) as illustrated graphically in Figure 3, which shows a plot of (E_p/E_e) vs. V_p . The agreement confirms that (1) the mechanical properties are governed predominantly by the architectural organization of the nanotubules and not by biochemical gradients, and (2) that fine changes in structural features can be captured by nanoindentation.

The absolute values of elastic modulus are quite remarkable considering the fully proteinaceous nature of the sucker rings. A dry modulus of about 8 GPa is in the range of highly cross-linked polymers. Intriguingly, the softening in the periphery in the hydrated state is only a factor of 2 with moduli of 3.5-4 GPa. This raises important questions regarding the chemistry responsible for such interactions. One hypothesis could be that electrostatic interactions play a role, possibly in the form of positively charged histidine bridged by chloride, even though this would place special demands on the environment during synthesis since histidine has a pKa of ~ 6.5 and thus is uncharged at the pH 8 of sea water.

Figure 2. Mapping of elastic modulus by nanoindentation. (A) Scanning electron micrograph of an individual sucker ring showing the plane of sectioning used for the indentation measurements. (B) SEM image of a sucker rings after freeze-fracture with indication of the expected loading axis deduced from the structural information in Figure 1. (C) and (D) Maps of elastic modulus in hydrated (C) and dry conditions (D); both maps were measured using the same elongated cube-corner tip and under identical loading conditions.

Figure 3. Relative modulus vs. porosity content, V_p . The black curve corresponds to a simple Ashby-Gibson analysis of loading perpendicular to the tubule direction (Eq. 1). The measured range of moduli inferred from Figure 2 is indicated on the y-axis and is in good agreement with the values inferred from the analysis.

Synthetic polyelectrolyte multilayers have also been shown to obtain similar large dry moduli. Using poly(allylamine hydrochloride) (PAH) and an azobenzene-containing carboxylate functionalized polyelectrolyte, Mermut et al. obtained polyelectrolyte multilayers with a modulus of 6.5 GPa when assembling the films at a pH where both components were highly charged [5]. Pavor et al. used PAH and poly(acrylic acid) (PAA) to obtain moduli on the order of 10 GPa in some cases even up to 12 GPa when both components were fully charged [6]. However, when submerged in water, the films swelled, leading to a reduction in modulus by two orders of magnitude, e.g. from 10.9 GPa in the dry state to 0.07 GPa in pH 7 water [6]. In the *D. gigas* sucker rings, the modulus is reduced by far less than an order of magnitude, suggesting that bonding interactions other than just electrostatics are at play. We have found that the rings can be completely dissolved in concentrated formic acid, indicating that they cannot be heavily covalently cross-linked or at least that the cross links will have to be highly acid labile. In our previous work, we determined the average amino acid composition of the sucker rings. They are rich in glycine (37% of all amino acids), tyrosine (14%), histidine (13%) and in hydrophobic amino acids other than tyrosine (25.5% total for leucine, alanine, valine, phenylalanine, isoleucine and methionine) [3]. We speculate that the large quantity of hydrophobic amino acids may hinder the modulus-reducing capabilities of water by reducing access. In this way, the electrostatic stiffening process discussed above could conceivably be of importance in the sucker rings even though they do not ‘loosen up’ under water. It should be stressed here that the chemical state of Cl in the sucker rings is currently unknown. It could be present as chloride and act as counterion or it could be covalently attached to the proteins, with tyrosine chlorination being a likely candidate [7].

The observation that the rings are soluble in formic acid is surprising also in view of the brownish green color of the base (Figure 1A), which is typically associated with protein tanning, i.e. covalent cross linking, which is expected to render them insoluble [7,8,9,10]. The ease of dissolution in formic acid suggests that tanning in the traditional sense is unlikely and that other factors are responsible for the coloration. We are currently undertaking experiments to establish the water content of the sucker rings, to probe the nature of Cl and to shed more light on the proteins that make up these intriguing structures.

CONCLUSIONS

The porous protein-based design of the sucker rings of *D. gigas* is, to the best of our knowledge, unprecedented in the animal kingdom. The use of porosity to control the mechanical properties could serve as a useful design in tissue engineering applications [11]. In the present paper, we have used additional nanoindentation measurements to lend support to our model of this structure-property relationship. The lack of chitin and metals is surprising considering the high modulus of these structures. Several questions remain as to understand the fundamental biochemistry of the sucker rings, the role of chlorine and how these influence their mechanical performance. Once these questions are addressed, the biochemical principles learned from this model structure could teach us valuable lessons in making robust, environmentally friendly, and biodegradable polymers.

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