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Hierarchical structure and mechanical properties of snake (*Naja atra*) and turtle (*Ocadia sinensis*) eggshells

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ABSTRACT

After hundreds of million years of evolution, natural armors have evolved in various organisms, and has manifested in diverse forms such as eggshells, abalone shells, alligator osteoderms, turtle shells, and fish scales. Eggshells serve as multifunctional shields for successful embryogenesis, such as protection, moisture control and thermal regulation. Unlike calcareous avian eggshells which are brittle and hard, reptilians have leathery eggshells that are tough and flexible. Reptilian eggshells can withstand collision damages when laid in holes and dropped onto each other, and reduce abrasion caused by buried sand. In this study, we investigate structure and mechanical properties of eggshells of Taiwan cobra snake (*Naja atra*) and Chinese striped-neck turtle (*Ocadia sinensis*). From Acid Fuchsin Orange G (AFOG) staining and ATR-FTIR examination, we found that both eggshells are mainly composed of keratin. The mechanical properties of demineralized snake and turtle eggshells were evaluated by tensile and fracture tests and show distinctly difference. Turtle eggshells are relatively stiff and rigid, while snake eggshells behave as elastomers, which are highly extensible and reversible. The exceptional deformability (110–230% tensile strain) and toughness of snake eggshells are contributed by the wavy and random arrangement of keratin fibers as well as collagen layers. Multi-scale toughening mechanisms of snake eggshells were observed and elucidated, including crack deflection and twisting, fibers reorientation, sliding and bridging, interlaminar shear effect, as well as the α–β phase transition of keratin. Inspirations from the structural and mechanical designs of reptilian eggshells may lead to the synthesis of tough, extensible, lightweight composites which could be further applied in the flexible devices, packaging and bio-medical fields.

Statement of significance

Amniotic eggshells serve as multifunctional shields for successful embryogenesis. The avian eggshells have been extensively studied while there are very few studies on reptilian eggshells and most of them focused on mineralization and embryotic development. For the first time, the hierarchical structure and mechanical properties of snake and turtle eggshells are comprehensively and comparatively studied. Both snake and turtle eggshells are multilayer, hierarchically-structured composites consisting mainly of keratin yet their mechanical behaviors are distinctly different. Turtle eggshells are stiff and rigid, while snake eggshells are highly extensible (>200%) and reversible due to multiple deformation stages, phase transition of keratin and various toughening mechanisms. We believe that this study will make positive scientific impact and interest the broad and multidisciplinary readership.

1. Introduction

Living organisms have developed optimum surviving strategies to adapt to different environments through hundreds of million years of evolution and natural armors have aroused increasing interests and been extensively investigated recently due to their superior mechanical performance yet considerably weak building blocks [1–5]. Most of natural armors are composites of organic proteins or polysaccharides and inorganic minerals self-assembled into hierarchical structures from molecular, nano-, micro-, meso-, to macro-scales [6,7]. The integrity of all hierarchical levels contributes to the bulk mechanical properties of biological materials synergistically by various toughening mechanisms, so even with the same building blocks, they show diverse and multifunctional performances [8,9].

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In addition to defense and structural support, to ensure the successful reproduction is a crucial task in nature. In 1866, Haeckel [10] categorized vertebrates based on their reproductive strategies: vertebrates that lay their eggs on lands such as reptiles, birds, and mammals were classified as amniotes. The amniotic egg was an important evolutionary development that allowed amniotes to fully adapt to terrestrial environment. On the other hand, anamniotics (fishes and amphibians), which typically lay their eggs in water, are restricted in aquatic and humid environments. The essential functions of amniotic eggshells are (1) to protect the eggs from damage and pathogens; (2) to control the exchange of gas and water; (3) to storage and provide minerals for embryonic development. Mikhailov et al. [11] comprehensively studied various fossil amniotic eggshells and classified them in detail by 5 basic types (testudoid, crocodyloid, dinosauroid, ornithoid, geckoid), 10 morphotypes (e.g. prismatic or spherulithic), 7 pore systems and 5 surface ornamentations. The porosity and water vapor conductance of amniotic eggshells are associated with nesting behavior and can be considered as an indication whether eggs are buried or left exposed [12,13]. For example, avian eggshells with lower porosity and water permeability can survive in drier conditions, while reptilian eggshells with high porosity are quite sensitive to humidity. Water loss is imperative for embryos, so the reptilian eggs are buried in sand to maintain their water content. Reptilian eggs can be divided into two groups based on their compositions: calcareous eggs and leathery eggs [14]. Unlike the hard, brittle, highly mineralized ($\sim$95 wt.%) avian eggshells, many reptiles lay leathery eggs which are tough and flexible with much lower mineral content ($\sim$20 wt.%), which can be considered as an evolutionary adaption in productivity. The flexible leathery eggshells can resist abrasion damages of buried debris more effectively than the hard ones, and avoid brittle fracture when being squeezed and tightly attached with other eggs. Moreover, the special surface texture on reptilian leathery eggshells is proposed to protect embryos from suffocation by preventing debris from blocking the pores [15,16].

Among amniotic eggshells, the avian eggshells are the ones that have been extensively studied in terms of composition, structure, biomineralization [17–20], and mechanical properties [21–30]. Across a wide variety of bird species, from the egg of hummingbird ($\sim$0.5 g) to that of ostrich ($\sim$2 kg), the mass of eggshells is approximately 10% of the total mass of the eggs. The avian eggshells are typically composed of an inner collagen-based membrane (type I, V and X collagen with relatively small amount of proteoglycans and glycoproteins), a bulk calcified shell consist of calcium carbonate in the calcite form, and an outer epithelium [18]. Motivated by the poultry industry [24], various mechanical tests were conducted on domestic hen (*Gallus domesticus*) eggshells, such as crushing [22,29], cracking, piercing, snapping [28,30], compression [26,31] and puncturing [32] tests. Tests were applied inwards and outwards, on the whole eggs and pieces of eggshells. Mechanical strength of eggshells has been correlated with calcium content, insecticides, incubation period, and shape index. Tyler et al. [33] found that the dominating factor of hen egg's strength is its thickness, and under the assumption of homogeneous and isotropic material properties, the eggshell's strength is applicable to the following relation based on the linear elastic shell theory [27]:

$$F = \sigma_f t^2 \tag{1}$$

where F is the applied force, t is the shell thickness, and σ_f is the ultimate tensile stress. This relation showed that for spherical shells, breaking force depends exclusively on shell thickness and the ultimate tensile strength of hen eggshells was estimated to be $\sim$53.6 MPa [23]. Evidences indicated that avian eggshells have evolved to sustain and adapt to varying loading conditions. For example, breaking strength of bird eggshells revealed strong dependence on their mother's weight [21,25]. Pritchard [25] and Ar et al. [21] considered the scaling effect and showed that in order for eggs to support the weight of their mother during incubation, the egg thickness (t) must scale with egg diameter (L) according to $t \sim L^{3/2}$. Their measurements of breaking force led to the following relation, $F = 1.7\ t^2$, and concluded that Eq. (1) provides an excellent estimate of breaking strength over a wide range of egg sizes and shapes.

There are limited studies on reptilian eggshells compared to avian eggshells. Tracy [34] and Vitt et al. [35] confirmed that humidity would influent the development of soft eggs but do not affect rigid eggs. Sexton et al. [36] studied and compared the amino acid distribution of eggshells of 24 species of lizards (7 families), 6 species of snakes (3 families) and 4 outgroups including avian eggshells and found that flexible eggshells contain significantly higher levels of proline compared to rigid eggshells. Flexible biopolymers are often abundant with hydro-proline, proline, and glycine [1], which are considered to contribute to the their flexibility. Most snakes, lizards, and tuataras lay soft eggs mainly composed of organic scaffolds with relatively small amount of minerals. Soloman and Baird [37] analyzed the eggshells of green–back turtles (*Chelonia mydas* L.) by XRD, SEM and TEM and found that the turtle eggshell consists mainly of crystalline calcium carbonate in the aragonite form (>95%) with a small amount of calcite (<5%). Sahoo et al. [38] studied the calcium metabolism in olive ridley turtle (*Lepidochelys olivacea*) eggs during embryonic development and discovered that the eggshell undergoes significant reduction in its calcium content from laying (21.1%) to hatching (9.4%). Calcite is the main mineral phase in snake eggshells and Pachard et al. [39] found that approximately 20% of the calcium required for the embryonic development in the oviparous snake (*Coluber constrictor*). The mineral content in reptilian eggshells are considerably low and varies at different stages of embryonic development. However, there is no comprehensive study on reptilian eggshell utilizing the Materials Science approaches that elucidates the structure–mechanical-function relationship. In this study, two representative types of reptilian eggshells from Taiwan cobra snake (*Naja atra*) (Fig. 1a and b) and Chinese striped-neck turtle (*Ocadia sinensis*) (Fig. 1c and d) are selected and investigated. Turtle eggshells are classified as calcareous eggs while snake eggshells are representative of leathery eggs among reptilian species. Moreover, the hatchling behaviors of snake and turtle are distinctly different. Newly hatchled snake utilize blade-like egg tooth to scratch and slice the eggshell (Fig. 1e) while turtle use drill-shaped turtle egg caruncle to penetrate and fracture the eggshell (Fig. 1f). This is the first comprehensive study on these reptilian eggshells in terms of compositional analysis, multi-scale structural characterization, mechanical properties and functions. Deformation and toughening mechanisms of turtle and snake eggshells are elucidated and the structure–property-function relationship is explicated. We hope this study can provide further understanding in reptilian eggshells, and offer inspirations for the synthesis of tough, extensible, lightweight composites which could be further applied in the flexible devices, functional textiles, novel armors, packaging and bio-medical fields.

2. Materials and methods

2.1. Sample preparation

Living eggs of Taiwan cobra eggshells (Fig. 1a and b) were obtained from the World Snake King Farm (Tainan, Taiwan). Living eggs of Chinese striped-neck turtle eggshells (Fig. 1c and d) were provided by the National Museum of Natural Science (Taichung, Taiwan). Because the hatchling times for both animals are