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Do stress-whitening and optical clearing of collagenous tissue occur by the same mechanism?

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ABSTRACT

Bone is biphasic with an organic matrix and an inorganic mineral component. As we age bone's susceptibility to fracture increases. It has been shown that there is no change in mean mineralization with aging, but bone nevertheless becomes less tough. This aging effect is therefore likely related to the organic phase.

Under mechanical loading, immediately prior to failure, bone has been observed to visually become more opaque and has been termed stress-whitening. Stress-whitening is known to make materials tougher. The goal of this investigation was to investigate stress-whitening in the collagenous matrix of bone. Hydrogen bonds play a key role in collagen stability and we hypothesize that changes in hydrogen bonding will significantly affect matrix stiffness, toughness and stress whitening.

Demineralized bone specimens were loaded in tension and stress-whitening was monitored. The effect of hydrogen bonding on mechanical properties and stress-whitening process was probed by altering the Hansen's hydrogen bonding parameter (δ_h) of the immersing solution.

The Hansen's hydrogen bonding parameter of the immersing fluid affected the morphology, mechanical properties and stress whitening of specimens. Specimens were visually whiter in the absence of mechanical load in low δ_h solvents (the specimens solvent-whitened). Both the observed stress-whitening and solvent-whitening were reversible and repeatable processes.

The observed solvent-whitening that occurred without the presence of load was consistent with solvent-induced optical clearing (the opposite of whitening) in skin caused by collagen fibril swelling. Stress whitening and solvent whitening can be explained by a common mechanism, collagen fibril densification and thinning, leading to an increased distinction between the collagen fibrillar phase and immersing fluid, ultimately leading to more scattering. Bones may be at a greater risk for fracture as we age because solubility of the matrix changes, thus making the collagen less hydrated (and more brittle) even in the same solvent.

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1. Introduction

The risk of bone fracture increases with age, independent of its mineralization (Boivin and Meunier, 2002). Bone is a biphasic material composed of an organic matrix and an inorganic mineral component. The mineral phase is critical to bone stiffness and the organic phase is responsible for much of bone's toughness (J.D. Currey, 2003). There is little or no change in mean mineralization with aging (Boivin and Meunier, 2002), but the tissue nevertheless becomes weaker/less tough (R. Ritchie et al., 2009). Aged bone tissue has been shown to be stiffer and more brittle than tissue from younger subjects (Nagaraja et al., 2007). In the absence of significant changes in mineralization, this aging effect is likely related to the organic phase. This investigation was therefore focused upon the organic phase within bone.

1.1. Hydrogen bonding in bone

Hydrogen bonding plays a key role in the material properties of bone. Hydrogen bonds formed by glycine residues hold collagen's triple helix structure together (Wang and Puram, 2004). Collagen molecules will hydrogen bond with themselves (Kuznetsova et al., 1998) and the extent of hydrogen bonding within bone can be modified by submersion in solutions of different Hansen solubility parameters (HSP). The HSPs: hydrogen bonding parameter (δ_h), dispersion parameter (δ_d), and polar parameter (δ_p) characterize the solubility of a substance (the solute) in a solvent. HSPs have

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been extensively studied in polymer science (Hansen, 2007). Following the principle of "like dissolves like", solvents and solutes with sufficiently similar δ_h, δ_d, and δ_p, will interact and the solute will dissolve. Many solvents have measured and published HSP values; by mixing two (or more) solvents a solution with an intermediate value of the HSPs can be created and used to study the effect of HSPs on the properties of a substance such as bone matrix.

This investigation was focused on δ_h because of the importance of hydrogen bonding within collagenous matrices. The effect of dehydration has previously been studied in collagenous tissues by immersion of tissue matrix in solutions with lower δ_h than the in-vivo aqueous phase (Nalla et al., 2005). By varying the value of δ_h that specimens are immersed in, the hydrogen bonding between the solution and organic matrix can be controlled. When high δ_h solvents interact with the organic matrix the hydrogen bonds within the organic matrix are broken in favor of solvent-solute interaction. The hydration state of tissue has also been modified by air drying (Z.L. Shen et al., 2008). Bone matrix hydration decreases in-vivo as a result of increases in mineralization and cross-linking (Avery and Bailey, 2008). Increased mineralization and cross-linking have both been associated with bone that is less tough and therefore of greater fracture risk (J. Currey et al., 1996; Wang et al., 2002).

Reduced hydration has been shown to increase the stiffness and decrease the toughness of biological tissues. Exposing demineralized dentin to solvents with decreasing δ_h increased stiffness and decreased toughness (Pashley et al., 2003). Conversely, increased hydration of the collagen lattice increases the toughness of bone matrix and decreases the stability of the collagen molecule (Yan et al., 2008). Bembey found that bone tissue immersed in solvents with high δ_h had lower moduli (Bembey et al., 2006).

1.2. Stress-whitening in bone

Bone has been observed to visually whiten (stress-whiten) under load immediately prior to failure in a manner that is speculated to be similar to polymer crazing (J. D. Currey, 2006; Thurner et al., 2006). Polymer crazing is a known toughening mechanism that dissipates mechanical energy by micro-pore formation and molecular disentanglement (J. Lee, 2007). Stress-whitening within bone has been variously attributed to micro-cracks (Thurner et al., 2006) or to nano-pores formed between mineral crystals and collagen molecules (Sun et al., 2010).

In a previous study within our laboratory we found that stress-whitening occurred in demineralized bone specimens prior to failure, strongly suggesting that stress-whitening was a property of the organic matrix rather than of the mineral–collagen composite (Hardisty et al., 2012). Stress-whitening was explained as a densification (thinning) of the collagen fibrils that caused greater scattering of light.

1.3. Optical theory of bone

Light-scattering interactions in collagenous tissues (primarily skin and cornea) have been extensively studied (Maurice, 1957; Tuchin, 2000). Materials that are highly light-scattering appear turbid (cloudy or white) while materials that are less light-scattering appear more transparent. Scattering within collagenous tissues has previously been explained by modeling the collagen fibrils as scattering particles within a fluid media. In this optical theory collagenous tissues are described as biphasic; collagen fibrils and surrounding fluid. Scattering is quantified by the reduced scattering coefficient (μ'_s), the ratio of scattered photon flux to the incident flux normalized by the thickness of the material (a high μ'_s results in a whiter appearance, low values result in greater transparency). Mie scattering theory can be used to calculate μ'_s for a collagen matrix as (Liu et al., 1996; Rylander et al., 2006; Saidi et al., 1995):

$$\mu'_s = (\phi_{fibril}(1-\phi_{\text{fibril}}))\left(\left(\frac{n_{\text{fibril}}}{n_{\text{sol}}}-1\right)^{2.09}\right)\left(\frac{3.28\pi a^2\left(\frac{2\pi a n_{\text{sol}}}{\lambda}\right)^{0.37}}{V_{\text{fibril}}}\right) \quad (1)$$

Where, μ'_s is dependent upon the volume fraction of the collagen fibrils (ϕ_{fibril}) and the ratio of the refractive index of the collagen fibrils to that of the surrounding solution $\left(\frac{n_{\text{fibril}}}{n_{\text{sol}}}\right)$ (Liu et al., 1996; Rylander et al., 2006; Saidi et al., 1995). V_{fibril} is the volume of collagen fibrils, "a" is the radius of collagen fibrils and λ is the wavelength of incident light. n_{fibril} is a function of the molecular density(#/volume) within each collagen fibril and other molecular level parameters (Lalor, 1969).

Modeling collagen fibrils as scattering particles has been used to explain increases in skin clarity by air drying and glycerol immersion, both of which increase clarity by making skin more uniform ($\phi_{\text{fibril}} \to 1$)(Rylander et al., 2006; Saidi et al., 1995). Skin can be reversibly made clear by immersing it in solvents that interact with the collagenous matrix such as glycerol, dextrose, fructose, sucrose or sorbitol (Hirshburg et al., 2007; Rylander et al., 2006). Hirshburg linked collagen solubility to skin clarity, showing that good collagen solvents were the best clearing agents.

The present investigation was focused on understanding the interaction between solvent based whitening and stress whitening that we hypothesize are both manifestations of increased scattering caused by collagen fibrils. In our previous work we attributed the increase in scattering due to stress-whitening to a mechanically driven densification of the collagen fibrils within bone causing an increase in n_{fibril} and a decrease in ϕ_{fibril} both causing an increase in scattering (Hardisty et al., 2012).

The goal of this investigation was to study the mechanism causing stress-whitening in bone; of particular interest was the effect of hydrogen bonding on this process. We studied stress-whitening in demineralized bone matrix to isolate the role of the organic phase. We hypothesize that stress-whitening is caused by a non-damaging, reversible, and repeatable densification of the collagen fibrils within bone. Specifically we hypothesize that stress-whitening will be decreased when the demineralized bone is less solvated after immersion in solvents with lower δ_h. We hypothesize that solvent based whitening will occur in demineralized bone matrix when immersed in solvents of low δ_h (in which collagen is less soluble) without the presence of load. Further we hypothesize that mechanical properties and stress-whitening will be dependent upon δ_h because of the importance of hydrogen bonding within the organic matrix.

2. Methods

Cortical bone specimens were cut from the diaphysis of the third metacarpal bone (MC3) of necropsied thoroughbred race horses. Beams were cut ($2 \times 8 \times 30\ \text{mm}^3$) using an EXAKT 310 saw (EXAKT Technologies Inc., Oklahoma City, OK, USA) irrigated with deionized (DI) water.

The central 10 mm of the beams were demineralized using a buffered formic acid (22.5%) solution known to maintain the integrity of the collagen network (Yeni et al., 2002). The proximal and distal 10 mm ends of the beams were protected from demineralization by nail varnish (Coty, New York, USA) (Bowman et al., 1996) (Fig. 1). Demineralization was assessed by digital radiography (Eklin Mark III, Min

Fig. 1. 10 mm Gauge length demineralized specimen prior to mechanical loading. The specimens within this study were taken from disto-medial quadrant of the third metacarpal (cannon) bone of necropsied thoroughbred horses. Ends of specimen are coated with nail varnish to protect against demineralization.

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