Bone 110 (2018) 304–311

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Bone toughness at the molecular scale: A model for fracture toughness using crosslinked osteopontin on synthetic and biogenic mineral substrates

S. Cavelier [a], A.K. Dastjerdi [a], M.D. McKee [b,*], F. Barthelat [a,*]

[a] Department of Mechanical Engineering, McGill University, Montreal, Quebec, Canada
[b] Faculty of Dentistry, Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada

ARTICLE INFO

Article history:
Received 24 November 2017
Revised 20 February 2018
Accepted 22 February 2018
Available online 24 February 2018

Keywords:
Osteopontin
Collagen
Transglutaminase
Bone toughness
Mechanical testing

ABSTRACT

The most prominent structural components in bone are collagen and mineral. However, bone additionally contains a substantial amount of noncollagenous proteins (most notably of the SIBLING protein family), some of which may act as cohesive/adhesive "binders" for the composite hybrid collagen/mineral scaffolding, whether in the bulk phase of bone, or at its interfaces. One such noncollagenous protein – osteopontin (OPN) – appears to be critical to the deformability and fracture toughness of bone. In the present study, we used a reconstructed synthetic mineral-OPN-mineral interface, and a biogenic (natural tooth dentin) mineral/collagen-OPN-mineral/collagen interface, to measure the fracture toughness of OPN on mineralized substrates. We used this system to test the hypothesis that OPN crosslinking by the enzyme tissue transglutaminase 2 (TG2) that is found in bone enhances interfacial adhesion to increase the fracture toughness of bone. For this, we prepared double-cantilever beam substrates of synthetic pure hydroxyapatite mineral, and of narwhal dentin, and directly apposed them to one another under different intervening OPN/crosslinking conditions, and fracture toughness was tested using a miniaturized loading stage. The work-of-fracture of the OPN interface was measured for different OPN formulations (monomer *vs.* polymer), crosslinking states, and substrate composition. Noncrosslinked OPN provided negligible adhesion on pure hydroxyapatite, whereas OPN crosslinking (by the chemical crosslinker glutaraldehyde, and TG2 enzyme) provided strong interfacial adhesion for both hydroxyapatite and dentin using monomeric and polymeric OPN. Pre-coating of the substrate beams with monomeric OPN further improved the adhesive performance of the samples, likely by allowing effective binding of this nascent OPN form to mineral/matrix components, with this pre-attachment providing a protein layer for additional crosslinking between the substrates.

1. Introduction

Bone, as a hierarchically organized material, has a remarkably high combination of strength, stiffness and toughness, all of which are critical to the functions of the skeleton in support, protection and resistance to impacts. Bone is composed primarily of calcium phosphate-based mineral, collagen, noncollagenous proteins, small proteoglycans and water organized over the many levels of hierarchy [1–3]. By weight, the majority of protein found in bone is collagen type I which forms an extensive fibrillar network in the extracellular matrix. However, on a molar basis, noncollagenous proteins collectively are present in similar amounts as collagen. The noncollagenous proteins generally accumulate between the collagen fibrils in the so-called interfibrillar compartment [4,5] where it appears plausible that they may serve as a toughening "binder" within the bulk extracellular matrix [6]. Likewise, the prominent accumulation of osteopontin (OPN) at interfacial cement lines [7,8] arising from the reversal of bone resorption to bone formation during its remodeling cycle also points to a potential adhesive role. Indeed, many interfaces are present at the different levels of hierarchy in bones, and these are considered to be the initiators of powerful toughening mechanisms [4].

Intermolecular bonds within and between bone collagen fibrils, and also involving noncollagenous proteins, and collagen organization itself (*i.e.* between lamellae of the osteons), and the bonding also occurring across cement lines delimiting osteons (also called Haversian systems), can all generally be considered as "weak" interfaces which channel deformation [9] and deflect cracks [10] (Fig. 1). During the forces incurred

Abbreviations: dent, dentin; GTA, glutaraldehyde; HA, hydroxyapatite; LVDT, linear variable differential transformer; NC9, irreversible inhibitor of TG2; OPN, osteopontin; RDCB, rigid double cantilever beam; SIBLING, Small, Integrin-Binding Ligand N-linked Glycoprotein; TGs, transglutaminases; TG2, transglutaminase 2 (tissue transglutaminase).

* Corresponding authors.
E-mail addresses: marc.mckee@mcgill.ca (M.D. McKee), francois.barthelat@mcgill.ca (F. Barthelat).

https://doi.org/10.1016/j.bone.2018.02.022
8756-3282/

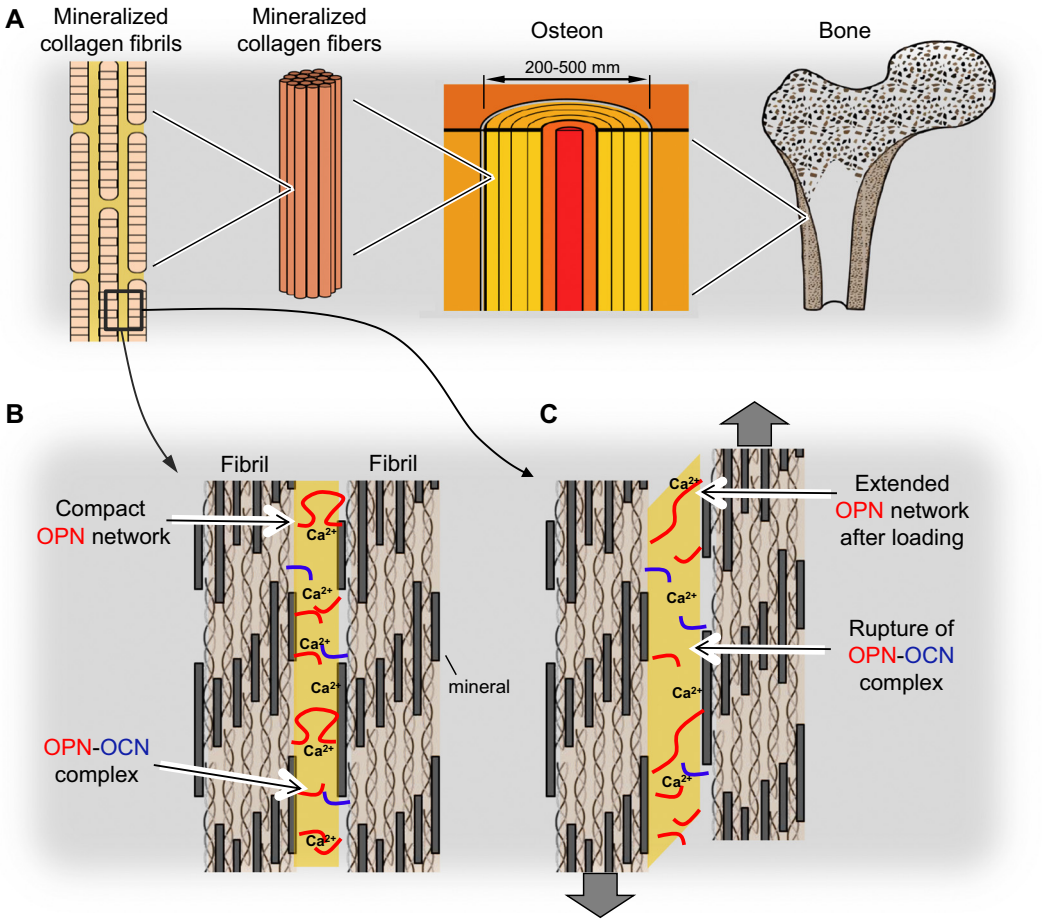

Fig. 1. (A) Four of the levels of hierarchy in bone. (B) Molecular interactions involving osteopontin (OPN, red) and osteocalcin (OCN, blue) between collagen type I fibrils. (C) Energy dissipation mechanisms involving OPN when two fibrils are subjected to shear load. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(Adapted from [4])

through skeletal function, cement lines/planes are a site of twisting and other deformations, where debonding, frictional pullout, crack bridging and crack deflection all may occur [10,11]. Previously, it has been speculated that OPN is responsible for matrix/mineral adhesion within cement lines in bone, and there may act as an interfacial adhesion promoter in mineralized tissues (between newly formed and older bone during remodeling) [8].

The weaker interfaces in bone which can deflect cracks (interfibrillar matrix and cement lines) are rich in noncollagenous proteins (and their networks) [4]. Prominent at these sites, OPN resides as a flexible, extended, intrinsically disordered phosphoprotein having a remarkably high negative charge that arises from an abundance of acidic amino acid residues (Asp and Glu) and the presence of many phosphorylated serine residues distributed along the full length of the protein [12]. While OPN is found in a wide variety of tissues and biological fluids from various species [13,14], its prominent abundance in bone extracellular matrix involves roles in regulating mineralization [15], providing cell adhesion ligands for integrin receptors [16], and generating mechanical performance enhancement, the latter observation supported by experiments showing that an absence of this protein (in OPN-deficient "knockout" mice) has a negative impact on the toughness of bone at the macroscale [17].

Cement lines/planes are not the only interfaces in bone in which OPN likely plays a mechanical role. In the molecular nanoenvironment of the interfibrillar compartment of the extracellular matrix, there are also interactions between the mineral crystallites and the abundant organic matrix molecules residing here, where specific deformation and toughening mechanisms would be expected to occur at the nanometer scale, in particular between collagen fibrils that in principle shear past one another under skeletal loading (Fig. 1A). Indeed, fracture-toughness tests on the weaker bones of knockout mice lacking OPN have shown the importance of this protein in toughening bone [17]. Relevant to a toughening mechanism for bone involving OPN is that this protein binds strongly both ionic and mineral lattice calcium atoms through its overall negative charge and specific acidic peptide motifs [7]. Moreover, OPN also binds to osteocalcin, another abundant noncollagenous bone protein [18]. With regard to the interaction between these two proteins, OPN forms a complex with osteocalcin as shown in Fig. 2B [4] that is capable of dissipating energy when two adjacent collagen fibrils are under shear load (Fig. 1C). The high negative charge of OPN allows for extensive binding to positively charged calcium ions to form sacrificial bonds (Fig. 1B) that break under shear load and allow energy-dissipating extension (without rupture) of the OPN molecule – such a toughening mechanism is depicted in Fig. 1C. Such sacrificial bonds can reform rapidly in the presence of calcium to allow nanoscale-level "healing" and repeated energy dissipation [4,6]. In principle, this particular process ends when the OPN molecule is fully stretched, but it can be repeated over multiple cycles of loading [6].

Many enzymes are known to modify components of extracellular matrices to modify their properties and/or to modulate resident cell behavior. For example, collagen is physiologically crosslinked through the action of the enzyme lysyl oxidase that catalyzes the formation of the lysine-derived aldehyde, allysine [19]. This process provides particularly bones, tendons and ligaments with high tensile strength [20]. The crosslinking of noncollagenous proteins such as OPN is predominantly conferred by the transglutaminase family of crosslinking enzymes,