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

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A B S T R A C T

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 opposed 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 to mineral/matrix components, with this pre-attachment providing a protein layer for additional crosslinking between the substrates.

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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
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 nano-environment 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,
mainly tissue transglutaminase 2 (TG2) and Factor XIIIA. As shown in Fig. 2A, OPN includes glutamine and lysine residues in its primary structure – two amino acids that can be physiologically crosslinked by TG2 and Factor XIIIA, by forming an isopeptide bond [21,22] (Fig. 2A,B). Kaartinen et al. [21] described that TG2 crosslinks two other substrates in addition to OPN – bone sialoprotein, and fetuin-A (αHS-glycoprotein). Importantly, it has been previously demonstrated that TG2 increases the binding properties of OPN to collagen [23], and the number of TG2-crosslinked OPN complexes correlates with an increase in mechanical strain [24]. Finally, in cartilage lesions, application of TG2 provided an adhesive performance superior to that of fibrin [25].

Glutaraldehyde (GTA) is a well-known, extensively used industrial crosslinker that has also been used for decades to preserve biological structure by “fixation” of samples primarily for microscopy and other protein immobilization/stabilization purposes [26]. GTA crosslinks proteins with a higher efficiency than TG2 [27] (Fig. 2D), and it can be used as a reference to establish the degree of crosslinking of OPN in terms of its relative adhesive performance.

As a basis for our hypothesis that OPN provides adhesive/cohesive properties to bone, here we build upon our previous studies that demonstrated i) OPN accumulation at interfaces such as cement lines/planes where new bone is bonded onto older bone during remodeling of the skeleton [8], or after bonding to existing bone after cutting/drilling [7,28], and ii) in the bulk of bone extracellular matrix, where it binds to mineral and collagen fibrils and accumulates in the interfibrillar compartment [29]. Given this, we analyzed whether the cohesive/adhesive binding properties of OPN are physiologically increased by the covalent crosslinks generated by the enzyme TG2 (and potentially other TGs like Factor XIIIA) to enhance the fracture toughness of bone.

2. Materials and methods

2.1. Proteins, enzymes and reagents

Purified, well-characterized bovine milk-derived osteopontin (OPN) kindly provided Dr. Esben Sørensen and Arla Foods was used in this study (University of Aarhus, Denmark, and Arla Foods, Viby, Denmark) [14,30,31]. Briefly, the extraction of OPN consisted of a two-step protocol where the proteose peptone was obtained from milk, from which OPN was extracted. Centrifugation, heating and cooling, and pH adjustment separated caseins and denatured whey proteins from solution. Trichloroacetic acid-precipitated proteins, including OPN, were purified using urea and Sephadex G-75 gel chromatography.

We used two crosslinking agents (one physiologic, an enzyme found in bone, and the other a small-molecule chemical crosslinker) to model a toughness mechanism for OPN interactions in bone. The first crosslinker that we used was the enzyme tissue transglutaminase 2 (TG2) which is abundant in bone, as extracted and purified from guinea pig liver (Sigma Aldrich Cat# T5398). As a control, to specifically inhibit TG2 activity, an irreversible inhibitor of TG2 known as NC9 was used; this small molecule occupies a surface site on TG2 with the direct result of forcing the enzyme into an open conformation that inhibits its activity [32]. NC9 inhibitor was used in this study in combination with TG2 to verify that the increase in toughness was indeed correlated with the enzymatic action of TG2. We also examined the effect of the small-molecule, chemical fixative glutaraldehyde (GTA, Electron Microscopy Sciences, Cat#16210) on OPN toughness, since GTA crosslinks collagens and noncollagenous proteins with a higher efficiency than does the physiologic enzyme TG2 [27]. Calcium chloride, Tris-HCl buffer and all other reagents were from Sigma unless otherwise specified.

2.2. Sample preparation

In this study we used a well-controlled, interface-substrate mechanical testing system (see Fig. 3A) to measure the adhesion produced by OPN when administered between apposing substrate synthetic hydroxyapatite and biogenic tooth dentin slabs/beams. With a thin layer of aqueous OPN solution sandwiched between two hydroxyapatite beams pressed into contact, a miniaturized mechanical testing device was used to fracture the interface and measure the energy consumed in the process (toughness). The different OPN preparations (see below and Table 1) were used on two types of flat substrates: synthetic hydroxyapatite and biogenic tooth dentin. We used a commercially available hydroxyapatite (Himed Old Bethpage, NY, USA) for the synthetic mineral model. For the biogenic substrate, cortical bovine bone was at first considered and tried, but the inherent porosity (e.g. vascular and remodeling canals) and heterogeneities of bone structure made it difficult to produce consistent, comparable beams. Tooth dentin is avascular and relatively homogeneous, and is a well-accepted alternative for in vitro bone cell-matrix/mineral interaction studies, as it shares a
homogeneity, low porosity and large dimensions necessary for us to prepare cantilever substrate beams for our testing apparatus. Another advantage for selecting dentin was that the polished dentin and synthetic hydroxyapatite surfaces had a similar surface roughness (data not shown).

Pairs of rectangular beams (dimensions 8 × 2 × 1 mm) were cut from both mineral substrates using a diamond saw (Accutom-5, Struers, Denmark). The beams, which served as substrates for the OPN adhesive formulations, were then attached onto custom-made steel fixtures using epoxy (Fig. 3A). The steel fixtures were longer than the substrates, and included pinholes which were used to transmit opening forces to the sample (Fig. 3A) in a configuration similar to a rigid double-cantilever beam (RDCB) [34–36]. The open faces of the substrates were then polished using metallographic methods on a polishing wheel down to a particle size of 0.05 μm. The two pairs of RDCB fixtures were stored in deionized water at room temperature until the preparation of the adhesive and mechanical testing, all of which occurred within 4 h (including the incubation time for the OPN solutions, see below).

To prepare these samples for incubation with OPN solutions, the RDCB mineral beams were first washed with acetone, and then with ethanol. They were then extensively rinsed with deionized water and dried under air flow at room temperature. The basic protein incubation procedure consisted of placing 2.5 μL of 100 g/L (2.96 mM) OPN solution onto each of the two RDCB mineral substrates (corresponding to 250 μg of OPN for each substrate, for a combined total of 500 μg of OPN in the assembled interface). Differently treated substrate beams (for details of formulations and crosslinking see below) were then apposed and loaded with a 60 g weight, and together transferred into a sealed box that contained a small reservoir of water to maintain 100% humidity, and the sealed box was then placed into an incubator at 37 °C (physiologic temperature) for 2 h for proper crosslinking of OPN [37]. The box was then removed from the incubator and the samples were immediately tested using a miniaturized mechanical testing machine as detailed in Section 2.3 below.

As indicated in Table 1, there were multiple variations and additions for crosslinking based on the basic procedural plan described above.

A total of nine combinations of OPN-based adhesives and substrates were considered in this study. The preparation of the samples was based on a “reference” protocol which was used to create the sample group labeled (Ref). The reference protocol was a basic protocol where noncrosslinked OPN was deposited on the substrates.

For samples in the group labeled (TG), we crosslinked the OPN into a polymerized network with the enzyme tissue transglutaminase (TG2). Transglutaminases are calcium-dependent enzymes, and require calcium-binding to catalyze a crosslinking reaction in OPN [38]. Crosslinked OPN was prepared by mixing 7.5 μL of 100 g/L (2.96 mM) of OPN solution, 5.25 μL of TG2 solution (2 mU/μL), and 2.25 μL Reaction Buffer (64 mM Tris-HCl, pH 8, 20 mM CaCl2, and 6.4 mM dithiothreitol) for a total volume of 15 μL. Tris-HCl was used to maintain pH at 8 during the chemical reaction between TG2 and OPN, and the addition of dithiothreitol (DTT) solution was used as a reducing agent to keep the active sites (thiol groups) of the enzyme open for chemical reaction [38]. Each mineral beam received 5 μL of the mixture before assembling them in apposition. These samples were then incubated for 2 h at 37 °C.

Samples for the pre-coated group TG-C followed the same procedure, but the substrates were pre-coated with pure OPN. For that purpose, 5 μL of 100 g/L (2.96 mM) of the OPN solution was applied to the surface of each of the substrates, which were then left at room temperature for 2 h for drying before the adhesive was applied.

Samples from the group TG-C-NC9 were prepared using the same procedure as for TG-C, but a solution of 2.5 μL of a 6 mM NC9 solution was additionally added. The total volume of the mixture was then 17.5 μL, and each substrate received 5.83 μL of the mixture before assembling the beams together and incubation. The total quantity of OPN used was also 500 μg for these samples.

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Table 1
Summary of the formulations tested in this work.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adhesive</th>
<th>Substrate</th>
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<tr>
<td>(Ref)</td>
<td>Pure OPN</td>
<td>Bare HA surface</td>
</tr>
<tr>
<td>(TG)</td>
<td>OPN crosslinked with TG2</td>
<td>Bare HA surface</td>
</tr>
<tr>
<td>(TG-C)</td>
<td>OPN crosslinked with TG2</td>
<td>OPN-coated HA surface</td>
</tr>
<tr>
<td>(TG-C-NC9)</td>
<td>OPN crosslinked with TG2 and NC9</td>
<td>OPN-coated HA surface</td>
</tr>
<tr>
<td>(GTA)</td>
<td>OPN crosslinked with GTA</td>
<td>Bare HA surface</td>
</tr>
<tr>
<td>(GTA-C)</td>
<td>OPN crosslinked with GTA</td>
<td>OPN-coated HA surface</td>
</tr>
<tr>
<td>(Ref-dent)</td>
<td>Pure OPN</td>
<td>Bare dentin surface</td>
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<tr>
<td>(GTA-dent)</td>
<td>OPN crosslinked with GTA</td>
<td>Bare dentin surface</td>
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<tr>
<td>(GTA-C-dent)</td>
<td>OPN crosslinked with GTA</td>
<td>OPN-coated dentin surface</td>
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GTA is a universal protein crosslinker that we also used in this study; as an aliphatic dialdehyde, it crosslinks proteins rapidly, effectively and irreversibly [26]. Glutaraldehyde reacts with many nucleophiles, of which amine groups in proteins contribute the most to the crosslinking since they are in the greatest abundance. Sulphydryl groups from cysteine and imidazole side chains of histidine also participate in the crosslinking reaction within and between proteins. To obtain these samples we used the same basic procedure, but with 7.5 μL of a 5% GTA solution added to 7.5 μL of the OPN solution concentrated at 100 g/L (2.96 mM). Each of the beams received 5 μL of this mixture before apposition and incubation. The quantity of OPN between the two substrates was the same for the monomeric OPN samples and the GTA-crosslinked OPN samples (250 μg on each substrate, for a total of 500 μg). This protocol was used on bare hydroxyapatite beams (group GTA) and OPN pre-coated hydroxyapatite beams (group GTA-C).

Finally, we prepared three groups where we used natural narwhal dentin in place of the synthetic hydroxyapatite as the cantilever beams (Table 1). We tested pure OPN monomer on dentin (group Ref-dent), OPN crosslinked with GTA on dentin (group GTA-dent), and OPN crosslinked with GTA on OPN-coated dentin (group GTA-C-dent).

2.3. Mechanical testing protocol

A custom-made fixture was supported by two pins, with each of the pins sliding into the pinholes of the RDCB sample beams (Fig. 3B). This setup was designed for the pins to transmit the opening force $F$ onto the sample. The force was recorded with a 5 lb capacity load cell, while the opening was measured with a linear variable differential transformer (LVDT). During the test, the opening was slowly increased at a rate of 3 μm/s, which opened the beams and progressively separated the OPN interface until complete failure. The test was stopped once the force had dropped to values below 0.5 N. A typical force-opening curve is shown in Fig. 3C. From this data, we computed the work-of-fracture of the OPN interface, obtained by dividing the energy under the force-opening curve (unit of Joule) by the initial area of the bond (unit of m²). The work-of-fracture (in J/m²) provided a measure of the toughness of the OPN-based adhesives on hydroxyapatite and dentin, corresponding to the amount of energy required to separate a unit surface of that adhesive.

3. Results and discussion

Fig. 4 shows the force-opening curves for the first six groups of adhesives (OPN on synthetic hydroxyapatite beams). In our first experiments, pure monomeric OPN solutions (Ref group) on the bare hydroxyapatite beams showed no evidence of adhesion, which underscored the necessity of crosslinking the protein to generate adhesion, as predicted by our hypothesis [23]. This is likely explained by the rapid engagement of the majority of the mineral-reactive side groups of OPN (carboxylates from Asp and Glu, and phosphates from P-Ser) [39] on one hydroxyapatite beam or the other prior to the weighted apposition step of the procedure. In all other subsequent groups/experiments, OPN was crosslinked into a polymer network, producing force-opening curves which increased essentially monotonically up to a maximum force, followed by a progressive decrease of force up to a maximum opening of about 200 μm at failure. The progressive failure of some samples suggests toughening mechanisms such as crack bridging by intact proteinaceous ligaments. Our experimental approach to measuring adhesion is at a much larger scale and using different substrates than that used by Fantner et al. [6], who elegantly assessed pulling forces on OPN at the single-molecule scale using atomic force microscopy.

Fig. 4. Force-opening curves for the six tests performed on hydroxyapatite beams.
As expected, crosslinking of OPN by the addition of TG2 produced a tougher adhesive. A pre-coating of OPN onto the hydroxyapatite beam further increased the toughness of the interface, to values slightly above 10 J/m². Pre-coating hydroxyapatite beams with OPN provided an initial surface composed of both hydroxyapatite and OPN, a combination that appeared to be more favorable to subsequent OPN binding. It is likely that the secondarily added OPN molecules in solution developed linkages with OPN molecules from the pre-coat that in turn were already strongly bound (as monomer) to the hydroxyapatite mineral of the beam, a two-step process which resulted in a higher overall toughness. Indeed, monomeric OPN binds strongly to HA [40,41], and our previous work demonstrated that OPN molecules interact together homotypically with each other, and that the strength of OPN-OPN bonds is large enough to influence self-assembly and adhesion in mineralized tissue [39].

Our results also show that the TG2 inhibitor NC9 inhibited TG2 crosslinking, so that samples from the group TG-C-NC9 exhibited negligible adhesion, similar to that observed for pure monomeric OPN. As expected, the strong chemical crosslinker GTA increased the adhesive performance of OPN significantly more than the enzymatic crosslinking by TG2; toughness was 33% higher by crosslinking OPN with GTA compared to crosslinking with TG2 (noncoated substrates), and 124% higher using OPN-coated substrates. The highest toughness we measured was about 25 J/m².

As shown in Table 1, we also used dentin as substrate to measure the toughness of three OPN-based adhesives: pure OPN (Ref-dent group), OPN crosslinked with GTA (GTA-dent group) and OPN crosslinked with GTA on OPN-coated dentin (GTA-C-dent group). Representative force-opening curves for these three groups are shown in Fig. 5. Interestingly, pure monomeric OPN did in this case provide adhesion on dentin, a result that contrasted with the lack of adhesion for pure monomeric OPN on hydroxyapatite. Besides slight differences in the biogenic dentin compared to the synthetic pure hydroxyapatite, it is possible that the presence of organic dentin components such as collagen and/or other noncollagenous proteins provided additional binding of the applied OPN even though they were not crosslinked, an additional binding which was sufficient to detect a small, measurable adhesion between the substrate beams. The GTA-dent and GTA-C-dent groups produced toughness values which were significantly higher than the Ref-dent group.

A summary of all the results is shown in Fig. 6. To provide relative context using the same mechanical device and setup, we compared the toughness values we obtained for OPN to that of a well-known standard adhesive – “office tape” on smooth glass substrate beams [35]. In seven tests out of nine, the adhesive performance of OPN was significantly greater than that of tape on glass. OPN adhesion was 3 times (Ref-dent group) to 17 times (GTA-C-dent group) superior to tape adhesion.

Overall, the results show that crosslinking of OPN into a polymer network after application between two mineral slabs increases toughness when assessed by mechanical testing. Moreover, as expected, chemical treatment with GTA is a more efficient crosslinker than enzymatic treatment with TG2 enzyme, but both treatments indeed showed remarkable toughness gains compared to using monomeric OPN alone (without crosslinking). Pre-coating the mineral substrate beams with OPN significantly improved adhesion as toughness increased by 20–70% on the hydroxyapatite substrate, and by 240% on dentin substrate. The GTA-dent group did not exhibit significant changes in toughness when compared to the same test on hydroxyapatite (GTA, p < 0.05). Of particular note, the overall adhesive performance of OPN on the organic-inorganic composite dentin was superior to OPN on hydroxyapatite mineral alone; the Ref-dent group exhibited a toughness of 6.3 J/m² while the Ref group exhibited no toughness, and the GTA-C-dent group was 62% higher than the GTA-C group. Superior adhesion using crosslinked OPN and biogenic dentin beams/slabs as compared to the hydroxyapatite beams likely results from the mineral phase of dentin having a preferred orientation relative to the synthetic sintered hydroxyapatite, and from the fact that TG2 crosslinking of OPN enhances binding to collagen (as present in the dentin sample) [23].

TG2 found in bone crosslinks proteins (including OPN) predominantly by forming γ glutamyl ε lysine isopeptide bonds between the polypeptide chains of proteins, as shown schematically in Fig. 2A, B [12]. This inter- and intramolecular crosslinking between glutamine and lysine leads to changes in protein conformation from the open flexible monomeric state, to the heavily crosslinked polymeric state [42] (Fig. 2B). Fig. 2C shows the chemical reaction leading to a γ glutamyl ε lysine bond. Sorensen [12] demonstrated that this type of bond was predominant in OPN crosslinked by TG2 and occurred between Gln42 and Lys4, and Gln193 and Lys154, Lys157 or Lys231. In addition, Gln248 [43], Gln34 and Gln36 [14] are also three other TG2-reactive glutamines. In the case of Gln34 and Gln36, the sequence alignments containing these glutamines are conserved in all known OPN, thus suggesting a functional importance at these particular sites [14]. The very precise TG2-mediated crosslinking sites differ from the essentially indiscriminate, nonphysiologic crosslinking reaction of proteins with GTA where polymerization products mainly occur as a result of extensive crosslinking between many functional groups of amino acids such as amine, thiol, phenol, and imidazole groups. Using GTA, many other amino acids (lysine, tyrosine, phenylalanine, tryptophan, histidine, proline, serine and others) of the OPN amino acid sequence are expected to react with the aldehydes, forming many other linkages for adhesion as depicted in Fig. 2D [44]. The higher number of linkages in OPN molecules crosslinked nonphysiologically by the chemical GTA leads to higher changes in the conformation and adhesive function of OPN.

In summary, here we provide evidence that polymer networks of OPN established by the crosslinking action of the enzyme TG2 may play an important functional adhesive/cohesive role in bone. In addition to being dispersed throughout the bone extracellular matrix, OPN is also enriched in the skeleton at the interface where new bone is deposited onto older bone in cement lines (actually planes in three dimensions) as part of the bone remodeling cycle. At both these locations, adhesion afforded by crosslinking would seem to be advantageous across different length scales – a feature particularly important given the highly hierarchical nature of bone structure. At the molecular scale, bonding and crosslinking interactions of OPN between and with collagen fibrils and/or mineral crystallites likely participate in dissipating energy under mechanical strain, as do sacrificial bonds in the extracellular matrix. The present study has revealed that the monomeric form of OPN, when applied onto a pure hydroxyapatite substrate, cannot develop bonds sufficiently strong enough to be adhesive; in this case, the presence of TG2 appears to be a necessary requirement for an adhesive function. However, monomeric OPN binding to dentin does provide some adhesion, likely attributable to interactions of OPN with the organic components.
of this tissue, and/or from slight variations in the biogenic mineral phase. Most importantly, the crosslinking of OPN occurring as a result of addition of either GTA or TG2 substantially improves the adhesive performance of OPN on both substrates (hydroxapatite and dentin). By comparing the performances of TG2- and GTA-crosslinked OPN, it appears that the extent of crosslinking of the protein is an important factor for mechanical performance. The results also confirm that blocking TG2 enzymatic activity by adding the inhibitor NC9 effectively cancels the adhesive performance of OPN. Pre-coating of the substrates with OPN further improves the adhesive performances of the samples, likely by allowing monomeric OPN to initially bind to the mineral and matrix components, with this initial attachment providing a protein layer for additional crosslinking between the substrates. The samples and loading we used are highly controlled in terms of geometry and scale, which provided the first estimate of the “engineering” fracture toughness of OPN. While this configuration is highly idealized, the toughness reported here reflects adhesive properties and contributions of monomer and polymer (crosslinked) OPN, and mechanisms of adhesion of OPN on mineral. In this sense, our results bring new insight into the adhesive behavior of OPN in the context of toughening mechanisms that might occur in bone across different length scales.

Acknowledgments

This work was supported by a Discovery Accelerator Supplement from the Natural Sciences and Engineering Research Council of Canada (NSERC) [grant numbers RGPAS 429451-2012] to F.B. and by a Discovery Grants from NSERC [grant numbers RGPIN-2016-05031] to M.D.M. S.C. and A.K.D were partially supported by a McGill Engineering Doctoral Award. The authors thank Dr. Esben Sorensen (Aarhus University) and Aria Foods (Denmark) for the purified osteopontin protein, Dr. Mari Kaartinen and Ms. Betty Hoac of McGill University for suggestions and help with the transglutaminase crosslinking of osteopontin, Dr. Jeffrey Keillor (University of Ottawa) for providing the NC9 inhibitor, and Dr. Rene Harrison (University of Toronto) for supplying the narwhal dentin. M.D.M. is a member of the FRQ-S Network for Oral and Bone Health Research.

Conflict of interest

The authors have no conflicts of interest to report.

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