The Molecular Self-Healing Mechanism of Bone – Localization and Mechanical Properties of Noncollagenous Proteins

4+1 Thurner, P.J.; 2 Zappone, B.; 3 Lam, S.; 4 Adams, J.; 5 Weaver J C; Fantner G E; 1 Morse D E; 1 Hansma, P K
4+1 University of Southampton, Southampton, United Kingdom, 2 University of California, Santa Barbara, CA, 3 University of Calabria, Italy

Introduction
Noncollagenous proteins in bone have previously been reported to exhibit a molecular self-healing mechanism. Using atomic force microscopy (AFM) in force spectroscopy mode, thin layers of highly negatively charged proteins such as osteopontin (OPN) and bone sialoprotein (BSP) have been found to be able to repeatedly dissipate large amounts of energy in the presence of Ca2+ ions [1]. Similar effects have previously been reported for freshly fractured surfaces of trabecular bone and been hypothesized to be due to noncollagenous proteins or proteoglycans [2]. However, it is not clear to date whether proteins such as OPN are truly making up the failing interface in bone and their macromechanical impact is unknown. In the presented study we present further data on the nanomechanical and micromechanical properties of thin layers of purified OPN. In addition, we present evidence from immunohistochemical investigations showing that fracture surfaces in trabecular human bone are indeed densely covered with a layer of noncollagenous protein.

Methods
Using an AFM and a Surface Force Apparatus (SFA) we have measured the surface coverage, adhesion and mechanical properties of layers of OPN adsorbed on mica. For SFA OPN was adsorbed from different solutions and one SFA sample was subsequently also measured using AFM. The other OPN films for AFM experiments were adsorbed by letting a droplet of OPN, dissolved in purified water, dry on a freshly cleaved mica surface. AFM and SFA experiments were carried out in different buffer solutions either containing only monovalent ions, or containing also Ca2+ ions. For immunohistochemistry, freshly cleaved trabecular bone was subjected to labeling for phosphoserine, using antiphosphoserine from rabbit (Product Number AB1603, Chemicon International Inc., Temecula, CA) in TBS, 1% BSA, 0.05% Tween 20 (concentrations of 1:5, 1:50 and 1:500) for 3 h, secondary antibody – goat anti-rabbit with 20 nm Au colloids (Product Number EM.GAR20, BBInternational Ltd., Cardiff, UK) – in TBS, 1% BSA, 1% goat serum, 0.05% Tween 20 (concentration of 1:50) for 3 h, and a final incubation with silver enhancement for 18 min. Negative controls for each staining step were carried out, samples blocked with appropriate agents and washed after each step. Labeling for OPN and BSP was done in a similar fashion using antibodies donated by Dr. Larry W. Fisher. Labeled surfaces were then subjected to carbon coating and imaged with a scanning electron microscope (SEM) in secondary and backscattered electron mode. Images from the latter mode were subjected to a custom particle detection algorithm, which was used to determine significant differences between labeled and negative control samples using a student’s T-test.

Results
AFM normal force measurements showed large adhesion and energy dissipation upon retraction of the tip, which were due to the breaking of the many OPN-OPN and OPN-mica bonds formed during tip-sample contact. The dissipated energy increased in the presence of Ca2+ ions due to the formation of additional OPN-OPN and OPN-mica salt bridges between negative charges. The forces measured by SFA between two macroscopic mica surfaces were mainly repulsive and became hysteretic only in the presence of Ca2+. adsorbed layers underwent an irreversible compaction during compression due to the formation of long-lived calcium salt bridges. This provides an energy storage mechanism, which is complementary to energy dissipation and may be equally relevant to bone recovery after yield. Strong adhesion of adsorbed OPN layers was only detected when the protein was adsorbed with the two mica-surfaces in contact (cp. Figure 1A), similarly as previously reported for an adhesive protein found in the foot of the mussel mytilus californianus [3]. Immunohistochemistry showed that fractured surfaces in trabecular bone are indeed densely coated with noncollagenous proteins (cp. Figure 1 Band C), comparison of retrieved SEM images showed large significant signals for Phosphoserine, OPN and BSP. In summary, our study shows that noncollagenous proteins such as OPN and BSP exhibit adhesive, cohesive and energy storage mechanisms, which might be important for bone mechanics and fracture resistance, especially in light of the fact that the amount of noncollagenous proteins bone significantly decreases with age and disease [4].

Figure 1: A) SFA force distance curves for OPN layers adsorbed with mica surfaces in contact showing large adhesion. B) SEM image of fractured surface of trabecular bone labeled for phosphoserine. C) Negative control.