Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture

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Properties of the organic matrix of bone[1] as well as its function in the microstructure[2] could be the key to the remarkable mechanical properties of bone[3]. Previously, it was found that on the molecular level, calcium-mediated sacrificial bonds increased stiffness and enhanced energy dissipation in bone constituent molecules[4,5]. Here we present evidence for how this sacrificial bond and hidden length mechanism contributes to the mechanical properties of the bone composite, by investigating the nanoscale arrangement of the bone constituents[6–8] and their interactions. We find evidence that bone consists of mineralized collagen fibrils and a non-fibrillar organic matrix[2], which acts as a 'glue' that holds the mineralized fibrils together. We believe that this glue may resist the separation of mineralized collagen fibrils. As in the case of the sacrificial bonds in single molecules[5], the effectiveness of this mechanism increases with the presence of Ca^{2+} ions.

It has become increasingly clear that, in addition to the influence of bone mineral density[9], the microscopic architecture[10] and microdamage[11,12], the nanoscopic arrangement of the bone constituents is vital for understanding the functioning[13] and mechanistic fracture criteria[14] of bone. Here we present data that shed light on nanoscale interactions between the mineralized collagen fibrils and a non-fibrous organic matrix present in the bone. These nanoscale interactions have profound implications for the behaviour of bone at the macroscopic scale.

Figure 1a shows scanning electron microscope (SEM) images of mineralized collagen fibrils on a fractured surface of human trabecular bone. Some fibrils are closely packed whereas others have spaces between them. These spaces are sometimes spanned by small filaments (see the arrows in Fig. 1b). The separation of mineralized collagen fibrils under loading is consistent with the findings of atomic force microscope (AFM) studies that crack formation and bone fracture occur between the mineralized collagen fibrils[6]. In the AFM image of Fig. 1c, such mineralized collagen fibrils can be seen, again with filaments spanning between the individual fibrils (arrows). Figure 1b and c indicates the presence of extrafibrillar organic material that was originally a thin layer between two fibrils (Fig. 1d) and is now stretched between the separated fibrils (Fig. 1e).

Do these filaments apply significant forces to resist the separation or shear of the mineralized collagen fibrils? The AFM can shed light on this question directly in a model system. We implemented a system, based on single-molecule force spectroscopy[15,16], composed of two pieces of bone in solution, one on the AFM cantilever and one as a sample. The pieces can be pressed together and then pulled apart (Fig. 2a). This process is repeated for several cycles. This system simulates the molecular interactions that occur during the separation of mineralized fibrils within bone. After the two bone pieces are put in contact, a force has to be applied to separate the pieces again, which indicates the existence of adhesion between the two bone pieces. The forces experienced during the separation were of the order of nanonewtons and persisted for distances of micrometres (Fig. 2b and d). The area under the curve of force versus distance (Fig. 2b) represents the energy required to separate the bone pieces. Most of the energy is dissipated, although some of this energy is stored elastically and recovered as the force is relaxed, if not all of the filaments have been broken (upper curve of Fig. 2b).

Energy dissipation at the single-molecule level has been previously shown[5,17,18] for biological materials and biological composites. An ion dependence of the molecular energy dissipation was found for bone and commercial bovine tendon collagen (which may also have included proteoglycans and other collagen-associated polymers); there was more energy dissipation when Ca^{2+} ions were present in the buffer in which the experiment was conducted[5]. Moreover, the energy dissipation increased if there was a delay of the order of 10 s between cycles of straining the molecules. Relative to this work[5], it has been pointed out that it is important to see whether this mechanism is involved in preventing the spread of microcracks[4].

Here we report that, for our experimental model that simulates the separation of mineralized fibrils, the energy dissipation is also greater if calcium ions are present in the buffer (Fig. 2c). For consistency, we used the same buffers as for the previously reported molecular pulling experiments[5] on bone ('calcium buffer': 40 mM $CaCl_2$, 110 mM NaCl, 10 mM HEPES, pH 7; 'sodium buffer': 150 mM NaCl, 10 mM HEPES, pH 7). The idea was to look for a perhaps subtle dependence on calcium ions by using one buffer that was much higher in calcium than physiological saline and one that was much lower. With Ca^{2+} ions present, the energy dissipation

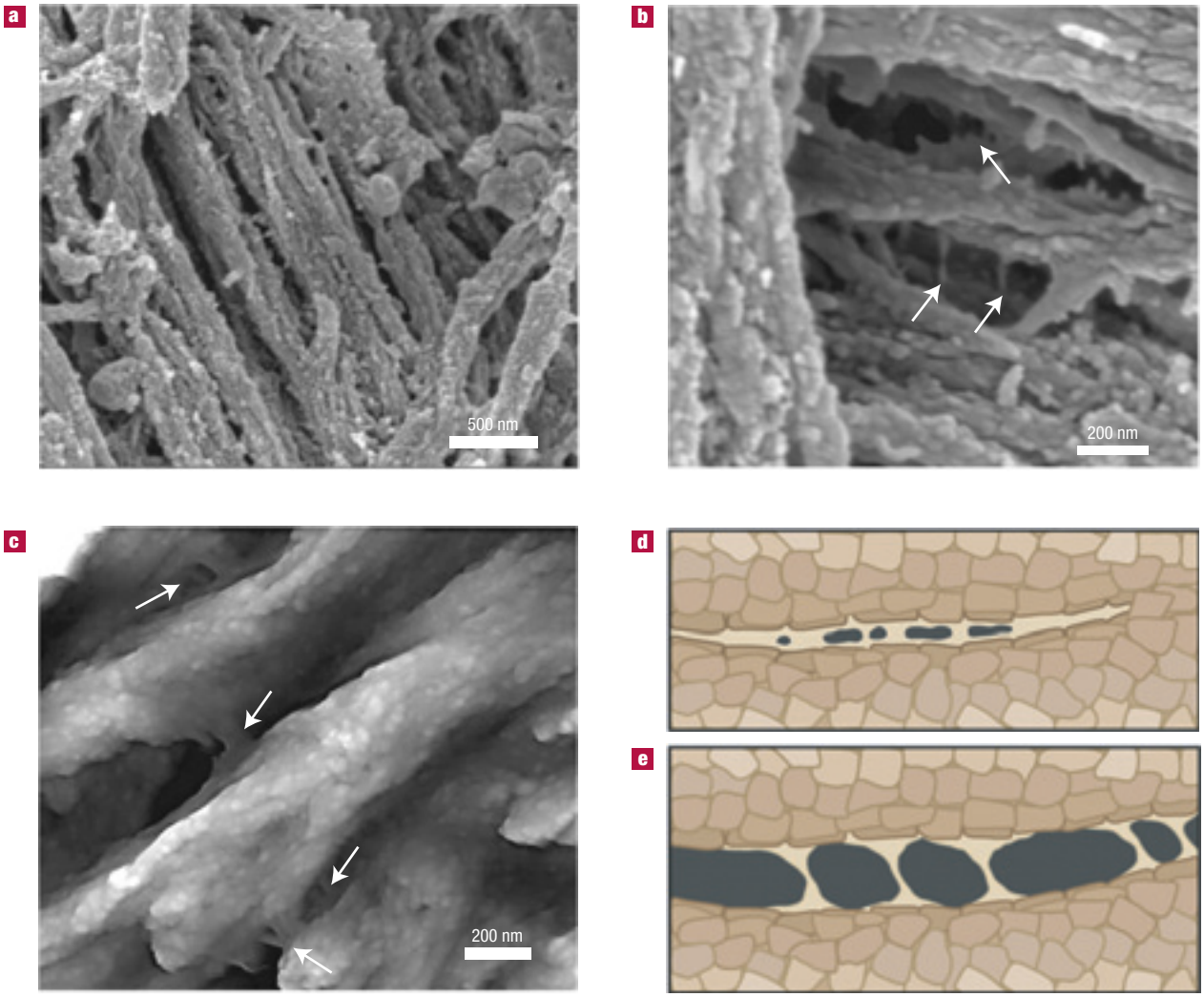

Figure 1 Mineralized collagen fibrils interconnected by a glue-like component. a, Fracture surface of human bone showing mineralized collagen fibrils. **b**, Individual collagen fibrils are held together with glue filaments (arrows), which might resist separation of the filaments. **c**, AFM image of a fractured surface also showing filaments (arrows) between neighbouring fibrils. **d**, Schematic showing how these filaments could resist the separation of fibrils. Initially, the mineralized collagen fibrils are glued together. **e**, When a force is applied, the glue resists the separation of the fibrils and filaments form between the mineralized fibrils.

increases significantly if there is a longer delay between cycles of separating the fibrils (Fig. 2c), whereas with only Na^+ ions present, there is almost no increase in energy dissipation with longer delays between cycles (1, 10, 30 s). We refer to this delay between cycles as the molecular restoring time to emphasize that within the time for which the bone pieces are left in contact, the connections between the mineralized collagen fibrils restore themselves partially so that for the next cycle of separation, one has to apply similarly high forces and dissipate a similar amount of energy to separate the bone pieces again. Additionally, the filaments adhere and stretch to a greater length in the presence of Ca ions. Figure 2d shows that the maximum length to which the filaments can be pulled before they finally break is larger if Ca ions are present. The average maximum pulling lengths were 2.7 ± 0.06 μm and 1.9 ± 0.09 μm for our experiments in Ca and Na buffers respectively, with an uncertainty of <0.001 in a Student's *t*-test.

On the basis of these measurements, we propose that the mineralized collagen fibrils that are the basic building blocks of the bone[19] are held together by a non-fibrillar organic matrix, which acts as a glue. When a force is applied on the bone, the glue resists the separation of the mineralized collagen fibrils, thereby counteracting the formation of cracks (see Fig. 1d and e). When the glue is stretched, energy is dissipated in the glue through rupturing of sacrificial bonds and the stretching of hidden length. Sacrificial bonds are additional, weak but reformable, bonds that break before the strong bonds that hold the structure together break. The energy that is needed to break the weak sacrificial bonds (and subsequently stretch the molecules) increases the total energy needed to fracture the material, thereby increasing the toughness of the material. After the force is relaxed, the glue bonds between the mineralized collagen fibrils can reform and the original strength of the bone is thereby at least partially restored. This mechanism contributes to the toughness of bone by increasing the amount of energy necessary for a crack to propagate[1], like for whole collagen fibrils that span cracks in bone on a larger size scale[14,20,21].

It is still unclear exactly what bonds and molecules are involved in this mechanism. We hypothesize that these include calcium-mediated sacrificial bonds (the opening of which reveals hidden length)[5,17]. These sacrificial bonds could be formed between, for example, Fig. 3b, (1) negative groups on one polymer molecule

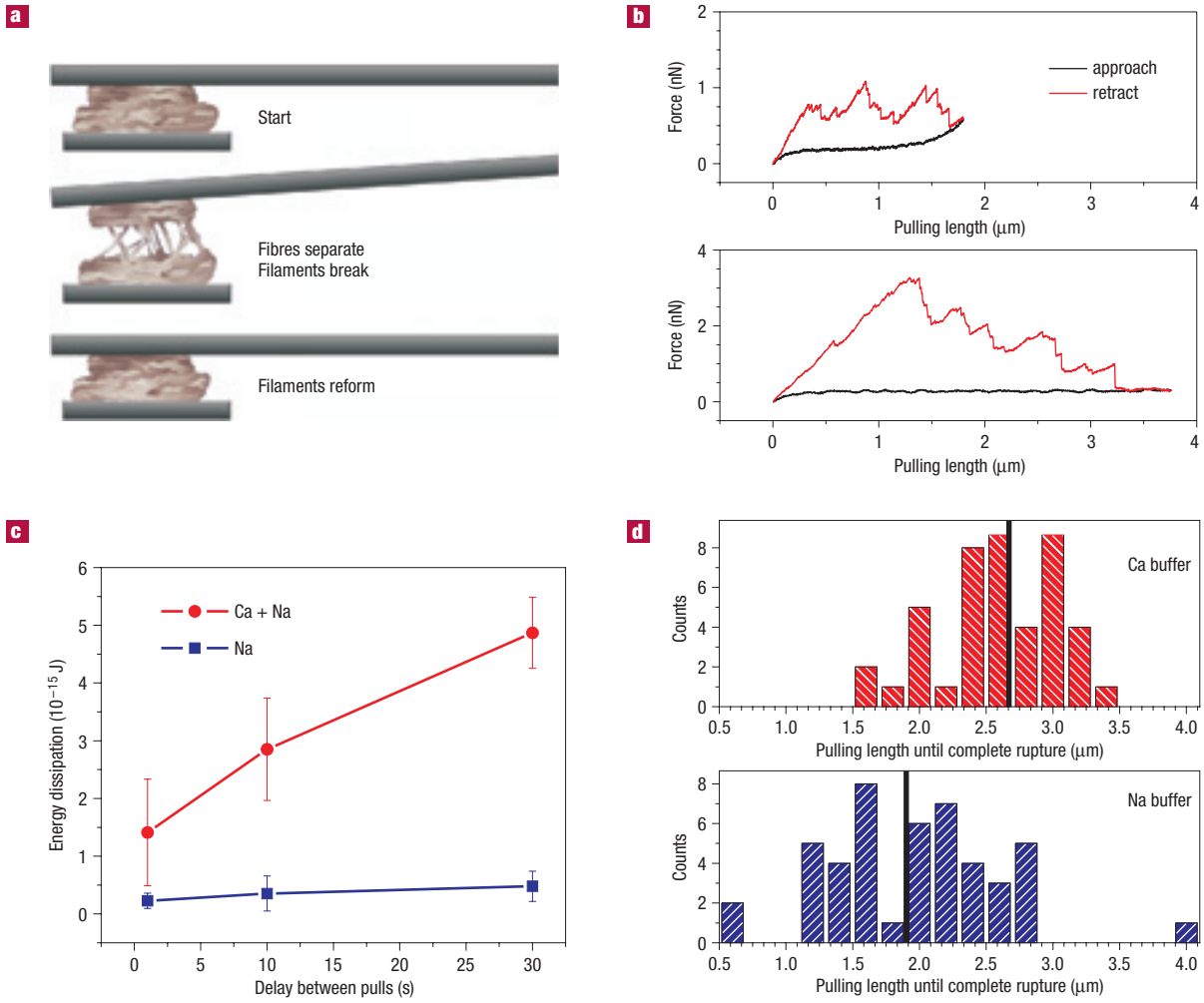

Figure 2 The AFM can measure the restoring forces between mineralized fibrils in the bone. a, A piece of bone glued to an AFM cantilever is pressed onto another piece of bone. As these are subsequently pulled apart, filaments exert forces that resist the separation of the mineralized fibrils. **b**, Representative pulling curves. Upper curve: not all filaments were broken; lower curve: all filaments were broken. The restorative forces are large relative to the forces involved in single polymer chains[15]. **c**, The total energy dissipation involved in separation of the bone fibrils is greater if calcium ions are present (data averaged over several hundred pulls). Red curve: with calcium and sodium ions in the buffer; blue curve: with sodium ions but without calcium ions in the buffer. The error bars are standard deviations. **d**, Filaments hold on for a longer pulling distance if Ca ions are present. The average lengths at which the filaments break completely (see the black lines) are 2.7 ± 0.06 μm and 1.9 ± 0.09 μm for Ca and Na buffer respectively, with a Student's- *t*-test uncertainty of > 0.001.

in the non-fibrillar organic matrix (for example, proteoglycan molecules[22], osteopontin[23] and other bone matrix proteins[24–26]), (2) multiple polymer molecules, (3) polymer molecules and the mineral plates of hydroxy-apatite[27], or (4) some combination of these. Some of these molecules and bonds may exist within the glue shown in Fig. 1b spanning the gap between mineralized collagen fibrils. They may also exist between collagen fibrils and mineral plates or within collagen fibrils. In addition to the contribution of sacrificial bonds and hidden length, a change in the surface energy of the mineralized fibrils due to the salt concentration could contribute to the adhesion between mineral particles at short distances, <10 nm. Essentially all of the energy dissipation that we measured in our AFM pulling experiments, however, comes from distances that are $\gg 10$ nm (see Fig. 2b–d).

The energy dissipation that we measure is of the order of 10^{-15} J (see Fig. 2), whereas the fracture toughness of bone (that is, the energy necessary for creating an additional crack surface) is of the order of 10^3 J m^{-2}. The ratio of these numbers indicates an effective contact area of the order of 1 nm^2. This effective contact area is much less than the geometric area of the samples, which is many μm^2, as would be expected as the rough fracture surfaces on the cantilever and bone on the sample disc do not mate. We only have a few areas on each in contact. We can see that, for example, only six real connections existed between the two bone pieces (number of major peaks) in the lower pull in Fig. 2. It is important, however, to be sure that in intact bone enough glue molecules could be present to be a major source of strength.

An order-of-magnitude calculation shows that less than 1% by weight of glue molecules could provide forces between mineralized collagen fibrils sufficient to give the known yield strength of compact bone in tension, <150 MPa. In short, if we assume that each glue molecule can provide 300 pN of force[5,17] and has a mass of the order of 100 kdalton, then to provide 150 MPa would require an area density of glue molecules of $<(150\ \text{MPa} \times 100\ \text{kdalton})/(300\ \text{pN}) = 9 \times 10^{-5}\ \text{kg m}^{-2}$. Braidotti's images of bone fractured in tension[2] show separated mineralized fibrils of length of the order of 5 μm on each fractured surface. If we make the conservative assumption that just the glue molecules

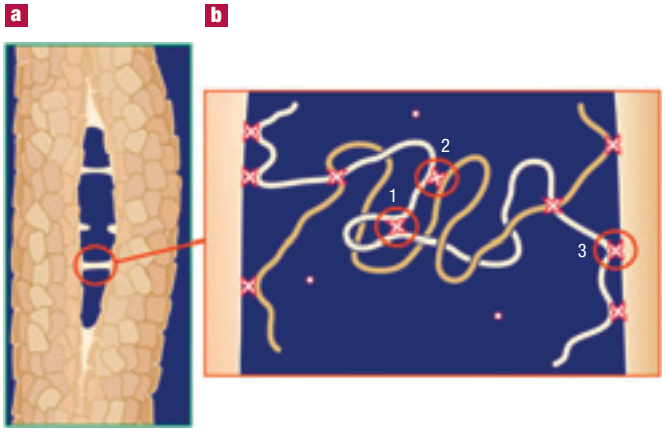

Figure 3 Possible kinds of sacrificial bonds involved in the glue between the mineralized collagen fibrils. a, Glue filaments could resist the separation of mineralized fibrils. **b**, The suspected, calcium-mediated sacrificial bonds in the bone could form between (1) two binding regions on one polymer, (2) two polymers or (3) a polymer and a mineral plate or a combination of these. For all cases the sacrificial bond might involve multiple weak bonds in parallel.

in this 5 μm region supplied all of the force that resisted the separation by sliding of fibrils, then the volume density of glue molecules would need to be $<(9\times10^{-6}\,\mathrm{g\,cm^{-2}})/(5\times10^{-4}\,\mathrm{cm}) = 0.018\,\mathrm{g\,cm^{-3}}$, which is less than 1% of the density of compact bone. As bone can be considered as a true nanocomposite, improvement of interfacial properties by this small fraction of the material can have a considerable impact on the whole composite due to the high surface-to-volume ratio of the constituents.

Many bone failure modes and energy dissipation mechanisms are based on delamination or slippage between mineralized fibrils. These modes and mechanisms would involve stretching the glue molecules that hold the mineralized fibrils together. In particular, during the formation of microcracks (and their deviation during propagation[28]) and crack bridging[20,29], work in stretching glue molecules with sacrificial bonds and hidden length would be required to separate the fibrils from the glue molecules with the sacrificial bonds. In addition to its role in these failure mechanisms, we believe that this molecular mechanism dissipates large amounts of energy even before or at the start of microcrack formation. In addition to the sacrificial bond and hidden length mechanism, other factors also play a role in resisting the separation or slippage of mineralized fibrils. There may also be a significant mechanical effect of the roughness of the mineralized fibrils, especially in tension along the fibrils; such a nanoscale friction mechanism has been proposed for nacre[30]. Clearly, the mineralized fibrils shown in Fig. 1 would be resistant to sliding relative to each other because of mineral plates on one mineralized fibril hitting mineral plates on another mineralized fibril. It is important to note, however, that without some glue-like material to hold the mineralized fibrils against each other during sliding, the effect of interlocking mineral particles would be small.

We believe proper functioning of the glue to be essential for the remarkable mechanical properties of bone. On the basis of its importance in natural materials, we propose that a glue that dissipates energy through the sacrificial bond and hidden length mechanism could improve stiffness and toughness of synthetic nanoscale composites as well.

MATERIALS AND METHODS

Trabecular bone samples were cut from fresh human or bovine vertebrae. They were frozen and cut on a bandsaw into cubes measuring about $4.5\times4.8\times4.0\,\mathrm{mm^3}$, where the shortest dimension was in the direction of the spinal column. The marrow was removed from the trabeculi using a pressurized stream of water. Samples were weighed and the exact dimensions were measured. Samples were kept wet throughout preparation and the experiments.

A buffer (40 mM CaCl, 110 mM NaCl, 10 mM HEPES, brought to pH 7.0 using 1.0 M NaOH) was used for studying the influence of calcium ions (in this paper referred to as Ca buffer) on the mechanical properties of bone. A control buffer (Na buffer: 150 mM NaCl, 10 mM HEPES, brought to pH 7.0 by adding small amounts of 1.0 M NaOH) was used as the control without added calcium.

SEM

The human bone samples were polished and afterwards compressed to partial fracture (under liquid) in a small, SEM-compatible vice. The amount of compression was monitored with a stereo microscope. After compression, residual salts were removed by rinsing the sample with Milli-Q water (the exposure time was insufficient for inducing demineralization artefacts). The samples were dried in a vacuum oven (10^{-3} torr, 30 °C) and coated with gold/palladium for SEM imaging with an FEI XL 40 Sirion SEM.

All images chosen for this paper are representative examples of features observed several times for different samples.

AFM

Bovine bone cubes were fractured in solution to create a fresh fracture surface. AFM images were taken in tapping mode on a Nanoscope IV system with PicoForce (Digital Instruments, Santa Barbara, California).

AFM FIBRIL SEPARATION EXPERIMENTS

Fragments scraped from trabecular bovine bone were glued to the tip of an AFM cantilever (Olympus bio-lever) using Devcon 2-Ton Epoxy. For the other half of the model system, a piece of trabecular bone was glued to a steel sample disc and, after drying, the sample was cleaved with a razor-blade to create a freshly cut surface. The bone pieces on the cantilever and on the sample disc were 'married' and separated as detailed in Fig. 2a. The pulling experiments were performed on a Nanoscope IV system with PicoForce (Veeco) under Ca or Na buffer. Paired experiments with Na and Ca buffer were performed at the same place on the same bone using a flow-through fluid system for the AFM, exchanging the solution between Na and Ca buffer and back several times. Several hundred pulls were collected and analysed. For the recovery of the energy dissipation (Fig. 2d), approximately 100 pulls were performed for the three intervals 1, 10, 30 s. The data points plotted in Fig. 2d are averages over these pulls.

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Competing financial interests

The authors declare that they have no competing financial interests.