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FORMATION OF ULTRACRACKS IN METHACRYLATE-EMBEDDED UNDECALCIFIED BONE SAMPLES BY EXPOSURE TO AQUEOUS SOLUTIONS

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Abstract

Back-scattered electron (BSE) imaging allows the visualization and evaluation of mineralized bone structures down to the micrometer range. To produce undecalcified bone sections with adequate structural and surface integrity, bone specimens are usually resin-embedded, followed by cutting, grinding, and polishing procedures. In samples prepared this way, so-called "ultracracks" were detected as black clefts in the lamellar bone matrix by BSE-imaging at magnifications ranging from 1000x to 3000x. By charging phenomena in the secondary electron (SE) mode of the scanning electron microscope (SEM), these clefts can be proven to be open cracks in the sample surface, and thus, as being created after embedding. These "ultracracks" seem to be a swelling effect of the bone matrix when it is exposed to water on the sample surface, followed by shrinking during drying. They did not occur, when water-free preparation techniques, like micromilling, were used and all water contact with the sample surface was avoided. This observation using the BSE-technique in SEM, and the simple method of discrimination between cracks existing before embedding and cracks newly generated during or after embedding, seem important for ultrastructural investigations of mineralized bone tissue, particularly for the evaluation of microcracks after loading or for the study of bone-implant interfaces.

Key Words: Artifactual ultracracks, surface cracks, microcracks, bone, methacrylate-embedding, mineralized collagen fibrils, backscattered electron imaging, secondary electron imaging, water-free surface preparation technique, micromilling.

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Introduction

Microtome sections as well as ground and polished sections of resin-embedded undecalcified bone samples are the prerequisites for the light microscopical evaluation of structures, cellular activities and the mineral distribution in bone (Schenk *et al.*, 1984; Plenk, 1986). The same preparation techniques are usually applied to bone samples to be examined by the back scattered electron (BSE)-imaging method in scanning electron microscopy (SEM) (Boyde and Jones, 1983; Reid and Boyde, 1987). During our own BSE-investigations of collagen fibril-mineral crystal relationships (Roschger *et al.*, 1993a), formations of multiple cracks within the lamellar zones of bone matrix was detected in conventionally prepared samples at higher magnifications (1000x to 3000x); apparently, these cracks were artifacts. Since these "ultracracks" were not seen after surface preparation by water-free micromilling, the potential effect of water on the sample surface was investigated. In this report, we discuss the methods of production of these cracks, their distinction from cracks pre-existing to embedding, and their avoidance.

Material and Methods

For a representative experiment to demonstrate the potential effect of water on the integrity of a sample surface, a bone biopsy from a patient suffering from idiopathic femoral head necrosis (male, age 42 years) was fixed for 72 hours in Burkhardt's solution, dehydrated in a graded series of ethanol, and routinely embedded in methylmethacrylate (Plenk, 1986). From the block, a 10 mm thick section with two parallel surfaces was cut by a water-cooled diamond saw (Isomet®, Buehler Ltd., Lake Bluff, IL, USA), and made exactly plane parallel on a surface grinding machine (Stephan-Werke, Hameln, Germany) using sand-paper (type: P150, silicon carbide, TRIM-ITE paper, 3M Comp., St. Paul, MN, USA). Both sides of the section were consecutively ground, avoiding any further water contact. The final surface preparation was performed water-free by diamond micromilling (Ultramiller®, Jung-Leica, Vienna, Austria).

After carbon coating (SCD 004 Balzers, Liechten-stein) of the micromilled surface, BSE-images of the microstructure of trabecular bone were taken on a digital SEM (DSM 962, Zeiss, Germany) operated at 15 kV and equipped with a four quadrant semiconductor BSE-detector. Then the thin carbon layer was carefully removed by polishing with a diamond paste (0.25 μm DIAPLAST, Winter, Hamburg, Germany) on a cotton wool bud. The surface was cleaned by cotton-wool buds dipped in petroleum ether (boiling range 50°C-70°C, Merck, Darmstadt, Germany). The same area of the sample was then covered with distilled water for 5 to 10 minutes, wiped, air dried, and carbon coated again. The identical area was then BSE-imaged for a second time.

Other areas were first inspected by BSE after the usual aqueous grinding and polishing procedures. Parallel to the BSE-mode, the fields were viewed by the secondary electron emission (SE) mode of SEM. Thereafter, at least 10 μm of the surface was removed by water-free micromilling and the new surface was examined again after carbon coating.

Results and Discussion

On the bone surface prepared by water-free micromilling, differently arranged bundles of mineralized collagen fibrils became visible in the trabeculae by BSE-imaging in SEM at magnifications ranging from 1000x to 3000x (Fig. 1a). Depending on the section plane and the type of bone, the bundles formed a characteristic pattern, each lamellar zone having its own orientation of bundles. Such a structurally intact BSE-image of the bone matrix could only be obtained when the surface was treated without water contact. However, exposing the identical surface area to distilled water for a few minutes resulted in multiple black clefts between the collagen fibril bundles, disturbing the normal bone matrix structure in the BSE-image (Fig. 1b). The possibility that this artifact to the bone structure can occur after any transient water contact, particularly during normal cutting and grinding techniques that use water for cooling and rinsing, was considered.

Bone surfaces of all specimens (n = 102), which had water contact, showed multiple cracks separating collagen bundles within the lamellae (Fig. 2a). If the same bone surfaces were viewed in the SE-mode in SEM (Fig. 2b), bright, shining, fuzzy structures, corresponding to the black clefts in Figure 2a, could be discerned. Such electrical charging and edge effects are typical for surface defects or cracks which have not been properly carbon coated. Since cracks existing before embedding would be filled by resin, these cracks must have been produced after embedding and can, therefore, be considered as artifacts. These cracks are restricted to the areas of sectioned bone matrix and do not extend into the surrounding areas of bone marrow penetrated by embedding medium. If such a bone surface with cracks after aqueous preparation was micromilled for at least 10 μm, the artifactual cracks disappeared (Fig. 3). However, no

Figure 1. Corresponding BSE-images of the same trabecular bone surface, prepared first without water contact **(a)**, and then treated with distilled water **(b)**. Compare the matrix structure before (a) and after water treatment (b). OC: osteocyte lacuna with canaliculi (white arrows); black arrows: examples of "ultracracks".

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systematic analysis of crack depth has yet been performed and therefore, milling away about 15 μm from the surface, as was done for Figure 3, will not guarantee the removal of all these cracks in all cases.

To distinguish them from microcracks, we call this new category of cracks "ultracracks", since this ultrastructural damage of bone matrix is only visible in SEM images. The microcracks are normally observed during light microscopical examination of fatigue damaged bone (Forwood and Parker, 1989; Schaffler et al., 1989; Mori and Burr, 1993). "Ultracracks" could be a swelling artifact, probably caused by the penetration of water into the dehydrated, but not resin-infiltrated, bone matrix, which was exposed by the surface preparation. To what extent can even the low viscosity methylmethacrylate infiltrate such a bone matrix after dehydration is an open question. Any rehydration of the organic bone matrix, however, will result in a swelling which is then followed by shrinking (when the surface is dried) accompanied by crack formation.

It is noteworthy that "ultracracks" are not distributed and oriented randomly within the bone matrix, but show a dependency on the arrangement of collagen. In Figure 1b, they run parallel to the collagen fibrils; while in Figure 2a, the multiple cracks predominate in the wider lamellae running perpendicular to the trabecular surface, and thereby, separate collagen fibril bundles. They can stop at and join the adjacent lamellae, or they can extend over several lamellae. The pattern of these ultracracks is strikingly similar to a model of mechanical, stress-induced ultra-damage of composite materials, proposed by Reifsnider (1990). As Reifsnider explained, stresses exceeding critical levels will cause continuation of crack formation over structural boundaries. This is also visible in some "ultracracks" of the bone sample in Figure 2a.

These "ultracracks" should be distinguished by their dimension from other larger cracks, often to be seen by light and electron microscopy in methacrylate-embedded samples, which are the result of stresses during or after resin polymerization. Both forms of crack, however, appear post-embedding as "open" cracks and seem to indicate zones prone to fatigue damage.

Since SEM and the BSE-mode will apparently replace and supplement light microscopy for the evaluations of bone structure and mineral distribution (Reid and Boyde, 1987; Grynpas and Holmyard, 1988; Skedros et al., 1993; Roschger et al., 1993b), the first description of this new category of artifactual "ultracracks", and the simple method of distinguishing them from cracks existing before embedding, should be of interest.
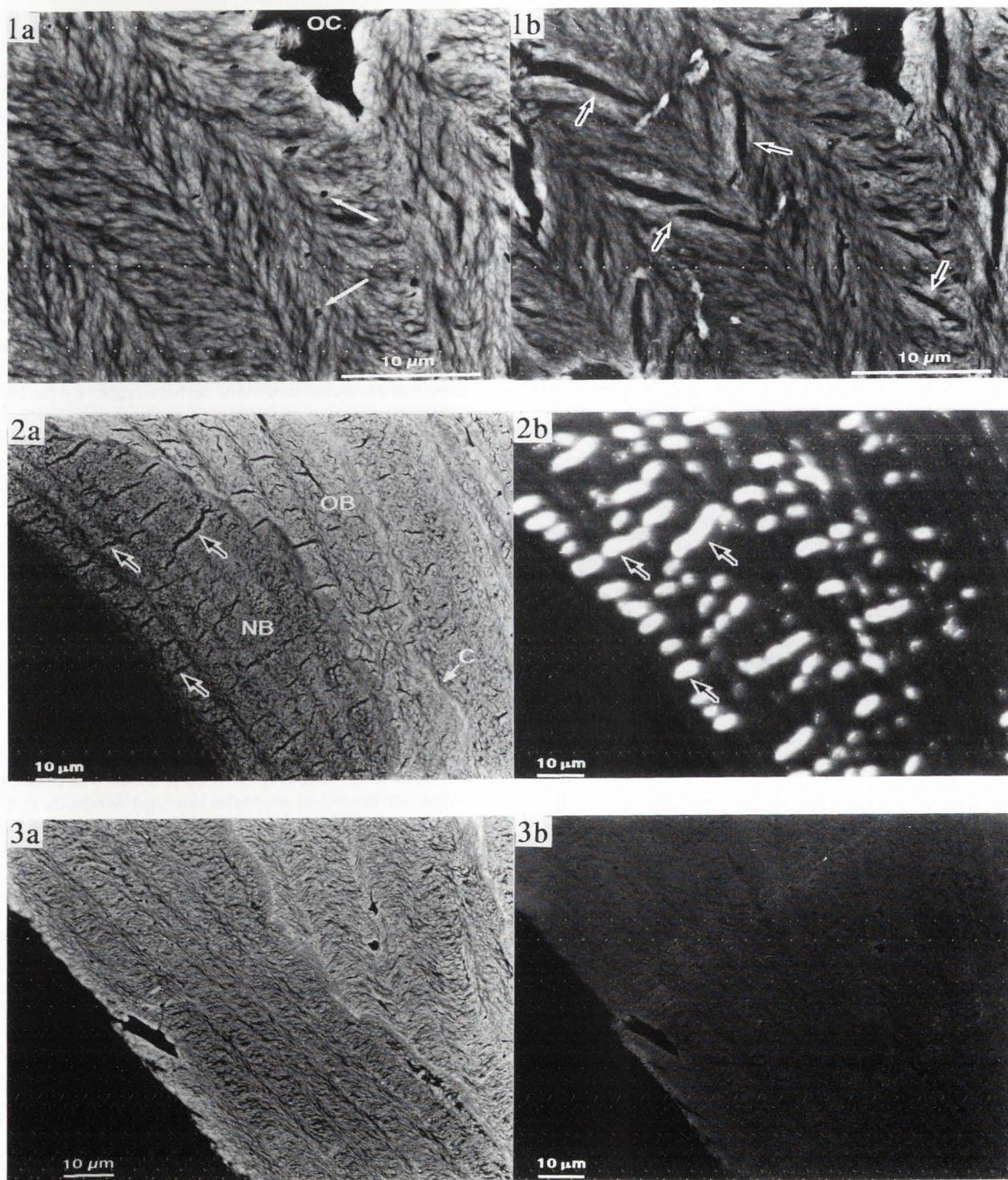
Figure 2. BSE (a) and the corresponding SE (b) images of a surface preparation of trabecular bone with water contact. Within the lamellar structure of the bone matrix, areas with different degree of mineralization (different BSE grey-levels) can be seen. OB: old bone; NB: newly formed bone; C: cementing lines. The black "ultracracks" (arrows) in (a) appear as shining structures (arrows) in (b).

Figure 3. BSE (a) and corresponding SE (b) images of a surface preparation of trabecular bone with water contact, but consecutively about 15 μm were removed by water-free micromilling. No "ultracracks" are visible in (a) and (b).

Furthermore, the recommendation to avoid any water contact, during sample surface preparation procedures, seems important for the investigation of the microstructure of the bone matrix; in particular for the detection of fatigue micro-damage in bone, or the analysis of bone/implant interfaces.

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Discussion with Reviewers

Reviewer I: The microcrack theory originally offered by Frost (1960) is to date an experimentally "questionable theory" and requires more scientific clarity. This paper, if published, would only confuse the issue. If these authors are to prove their thesis, they must first prove, using SEM techniques, that limited or no microcracks were present initially. After this is accomplished, quantitative methods should be used to prove the number of microcracks had increased statistically. Finally, the microcrack theory of Frost would best be proven using cortical bone from healthy human or animal cortical bone. One might argue that cracks seen in diseased tissue are a result of the pathology and make the tissue move sensitive to processing artifacts.

Authors: The aim of this report was not to investigate or to discuss the "microcrack theory". Our intention was: (1) to report our observations of artifactual formation of multiple "ultracracks" in the surface of methacrylate embedded bone samples apparently caused, by transient water contact; (2) to present a method to identify these "ultracracks" and other surface-cracks by the combined examination of the same bone area with BSE- and SE-imaging. In our opinion, however, these two observations are of particular importance if fatigue micro-damage in bone is investigated in SEM using BSE-imaging methods.

Reviewer I: There is further confusion when a careful study of Figures 1a and 1b is made. The osteocyte lacunae in Figure 1a can be superimposed over Figure 1b. So can the three canaliculi (two of which are distinguished by white arrows in Figure 1a). It is curious as to how this could have been accomplished. Was the same field heated under the electron beam to create these artifacts? This is disturbing and confusing.

Authors: As is correctly pointed out, Figures 1a and 1b can be superimposed over each other, as was the aim of the experiment, because the identical area was examined twice, as described in **Materials and Methods** and in **Results and Discussion**. In order to find the identical specimen area after the water treatment for the second BSE-imaging session, a series of BSE-images with descending magnifications was made at the first BSE-image session. The generation of these artifacts by electron beam damage could be excluded, because: (1) no visible alterations could be detected in sample appearance during each BSE-imaging session; and (2) water treated areas only once scanned by the electron beam showed the same artifacts (see for example, Fig. 2).

Reviewer I: Figures 2a and 2b are an excellent example of additional confusion. If the sample changes on secondary (Fig. 2b) emission, why does not it change on BSE-images of bone known to charge if proper conductive coatings are not applied. Why secondary charging and not BSE? The physics must be explained.

Authors: During the impact of the primary electron beam on the sample, secondary electrons (SE, electrons of low energy) are emitted from the very outermost surface layer, whereas backscattered electrons (BSE, electrons with high energy) are emitted from somewhat deeper surface layers. Because of the low energy of the SE, the SE are very sensitive to already weak local

electric fields, causing emission phenomena by charging, as seen in Figure 2b, whereas the high energy BSE are not yet visibly influenced. Of course, if the charging becomes severe, the primary beam as well as the BSE can be influenced giving rise to charging artifacts; however, these are different in appearance (Goldstein *et al.*, 1992).

Reviewer I: The investigators also need to establish how they can distinguish canaliculi from microcracks. I have never observed canaliculi that is as small as those depicted in Figure 1a.

Authors: In fact, as long as only morphological criteria are applied, the accuracy by which canaliculi can be identified in the BSE-image depends on the plane in which the canaliculi are sectioned. Perpendicular cross-sections of canaliculi show characteristic circular black spots within the white-grey mass of mineralized collagen matrix (Boyde, 1972). These cross-sections have a large variety in diameter. Cracks cannot have such circular forms. In longitudinal sections, the appearance of canaliculi might sometimes interfere with potential microcracks.

D.B. Burr: No method is presented to distinguish real from artifactual cracks. Only a method of preparation to prevent the proliferation of cracks is presented. It has not been demonstrated that water-free preparation of specimens prevents all artifactual cracks, only that using water in preparation creates additional cracks. Please comment.

Authors: Indeed we are not able to directly identify in SEM crack-like structures as "real" microcracks caused by fatigue damage. Nevertheless, we can distinguish, by this method, cracks which are clefts in the sample surface, from potential "real" cracks. Naturally, artifactual cracks, resulting from preparation procedures before embedding, cannot be distinguished from the latter, as well as dark crack-like structures in the bone matrix representing intact bone (e.g., longitudinal sections of canaliculi). Also, in water-free preparation, tension-cracks, caused by shrinking processes during resin polymerization or by mechanical stress after embedding etc., can exist. In general, these type of cracks are not restricted to the bone matrix area, but instead, extend into intertrabecular space. These cracks are of larger dimensions than the described "ultracracks".

P.O. Gerrits: "Ultracracks" may have contained highly hydrophilic structures, which were relatively resin-free after embedment with the hydrophobic methyl methacrylate. It is likely that these poorly embedded structures bulge out of the tissue and are affected during grinding with water. Do the authors have any idea which tissue structure/components remain detectable in petroleum ether treated specimens?

C.A. Scotchford: It is suggested that incomplete infiltration with methylmethacrylate is a contributing factor to microcrack formation. Do the authors feel that that improved infiltration would reduce the occurrence of such artifacts? Leaching of mineral from bone and calcified cartilage exposed by aqueous media and the use of non-aqueous preparation techniques to prevent this is well documented. Have the authors considered the possibility of partial dissolution of mineral from areas of embedded bone mineral as a contributory factor to the observed surface cracks?

Authors: We think that the water-induced formation of surface cracks in the methacrylate embedded bone samples is not primarily a question of poor local infiltration, but instead, is generally a question of rehydration of dehydrated organic structures. Aqueous media may directly contact and rehydrate these structures, when they are exposed on the sample surface during grinding. They may also come into contact with water by penetration of water through some distances of the polymeric resin structure (otherwise a surface-staining of embedded ground sections or staining of semi-thin sections would not be possible). No loss of tissue structure/components after cleaning of the sample surface with petroleum ether was observed by SEM and/or by light microscopy. We do not think that leaching of mineral from the sample surface during water contact is a essential contributory factor for crack formation because we did not observe a visible demineralization effect in the BSE-image.

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