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Dark-field transmission electron microscopy of cortical bone reveals details of extrafibrillar crystals

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ABSTRACT

In a previous study we showed that most of the mineral in bone is present in the form of "mineral structures", 5–6 nm-thick, elongated plates which surround and are oriented parallel to collagen fibrils. Using dark-field transmission electron microscopy, we viewed mineral structures in ion-milled sections of cortical human bone cut parallel to the collagen fibrils. Within the mineral structures we observe single crystals of apatite averaging 5.8 ± 2.7 nm in width and 28 ± 19 nm in length, their long axes oriented parallel to the fibril axis. Some appear to be composite, co-aligned crystals as thin as 2 nm. From their similarity to TEM images of crystals liberated from deproteinated bone we infer that we are viewing sections through platy crystals of apatite that are assembled together to form the mineral structures.

1. Introduction

It is well known that bone is a composite material made up of approximately equal volume amounts of mineral usually described as hydroxyapatite (HA) and protein mainly consisting of collagen. The collagen occurs exclusively in the form of fibrils. These are bundles of triple helices of collagen molecules, cross-linked together in a three-dimensional structure in which the positions of the collagen molecules are staggered with respect to one another by a characteristic repeat distance (*D*) of approximately 67 nm. This structure was originally described by Hodge and Petruska (1963) and recently refined by Orgel et al. (2006). Besides collagen, bone contains a few percent of other proteins (non-collagenous proteins), sugar-based molecules (glycans) and other organic molecules.

The topological and geometrical relationships between the mineral and collagen fibrils have been a matter of great interest. In the Hodge-Petruska model the collagen triple helices are arranged so that there is a gap approximately 40 nm long between the end of one helix and the next collinear helix. The staggered arrangement of these helices in the fibril structure results in these gaps being aligned across the width of the fibril, creating so-called gap-zones, adjacent to which are zones across the width of the fibril in which no gaps are encountered, called overlap zones. In the literature on the ultrastructure of bone or on the mineralization of fibrils *in vitro* most authors state that the majority of the mineral in bone resides in gap zones (Weiner et al., 1991; Siperko and Landis, 2001; McEwen et al., 1992; Arsenault, 1989; Hamed et al., 2012). According to some authors, mineral also occurs outside the fibrils, in the interfibrillar space (Landis et al., 1996). As well, some authors suggest that mineral extends into the overlap zone (Su et al., 2003).

By contrast, Lees and colleagues (Bonar et al., 1985; Lees and Prostak, 1988; Lees, 1987) showed transmission electron microscopic (TEM) images which appeared to display large amounts of mineral surrounding fibrils in cross sections of bone cut normal to the fibrils. From geometric relationships of these components Lees (1987) calculated that 70–80% of the mineral must be situated outside of the fibrils. Other researchers came to similar conclusions based on indirect evidence such as the dynamic properties of bone (Pidaparti et al., 1996). Nevertheless, little attention is given to extrafibrillar mineralization in recent literature, and *in vitro*

Abbreviations: HA, hydroxyapatite; TEM, transmission electron microscopy; BF, bright field; DF, dark field; MS, mineral structure (defined in text); MSs, plural of MS; SAED, selected area electron diffraction; XRD, X-ray diffraction; SAXS, small angle X-ray scattering.

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experiments have appeared showing that HA can be induced to grow inside collagen fibrils (Nudelman et al., 2010; Olszta et al., 2007).

Using TEM images of ion-milled sections of human and other bones, we have found evidence (McNally et al., 2012, 2013) which supports and extends the model of Lees and colleagues. We showed that most of the mineral in bone is not in the gap zones within fibrils but instead lies outside the fibrils in the form of elongated plates, about 5 nm thick, 60 nm wide and several hundreds of nm long. Cross-sections of these plates are seen in sections cut perpendicular to the fibril axes (approximately perpendicular to the axis of long bones) as shown in Fig. 1a. The mineral-rich plates are organized around the periphery of the fibrils, somewhat like staves around a barrel; typically four to five such plates are stacked between any two adjacent fibrils. Where a section has been fortuitously oriented precisely perpendicular to the mineral plates, we are able to visualize a ~1 nm gap between adjacent plates (Fig. 1b). Images very similar to Fig. 1a have been obtained by us in bones of mouse, elephant, and cow, by Jantou et al. (2009) in elephant dentine, and by Cressey and Cressey (2003) and Reznikov et al. (2014, Fig. 10A) in human bone.

In sections cut parallel to the fibril axes we see the same mineral plates edge on, arranged in lanes spaced about 50 nm apart (Fig. 2). From images like Fig. 1a we infer that lying between these lanes are other plates, their long axes parallel to the ones visible here, but with their flat surfaces lying close to the plane of the section. In this orientation they are too thin to be revealed in the TEM images. The mineral-rich plates viewed edgewise display much higher electron contrast than the gap zones, suggesting that most of the mineral is located in these plates. Note also that it is possible, by tilting the stage of the electron microscope, to see a complete transition from images like Fig. 1 to views that look like Fig. 2; this is illustrated in Fig. 7 of McNally et al. (2012) and also in the Supplementary Material of McNally et al. (2013).

We coined the term "mineral structures" to denote these extrafibrillar, mineral rich plates. Other workers have recognized the presence of extrafibrillar mineral, but none prior to McNally et al. (2012) recognized that it was organized into well-defined structures; it is for this reason that we invented the new term. Jantou et al. (2009), using focused ion beam milling of dentine, obtained images similar to ours, but did not recognize the existence of these structures.

Images such as these do not appear in older TEM studies of bone using microtome-cut sections. For example Rubin et al. (2003), in a detailed description of the relation between mineral and collagen, show a TEM image of a section of bone (Rubin et al., 2003: Fig. 2A) that is strikingly similar in appearance to those presented here and in McNally et al. (2012). Similarly, Lees et al. (1994; Fig. 1A) show a section of turkey tendon in which mineral is wrapped around a circular fibril section. However the use of a microtome in the preparation of both of these sections has resulted in the shattering of the mineral structures into fragments. This is presumably why Rubin et al., Lees et al., and other previous researchers failed to identify mineral structures in bone. By contrast, the mineral in gap zones inside the fibrils tends not to be damaged by a microtome, which led earlier researchers to identify this as the main locus of mineral in bone.

It should be noted, however, that some mineral does occurs in the gap zones (Weiner et al., 1991; Siperko and Landis, 2001), imparting electron contrast to them and allowing us to visualize them in relation to the adjacent overlap zone (Figs. 2 and 3). In sections oriented so that the boundary between the gap and overlap zones are normal to the plane of the section, we can see that this boundary is sharply defined with no indication of mineral extending from gap- to overlap-zone (Fig. 3a). Using energy-dispersive X-ray spectroscopy (EDXS) we have previously shown (McNally et al., 2012) that the Ca concentration (and thus mineral content) of the gap zone is much less than in the mineral structures. We

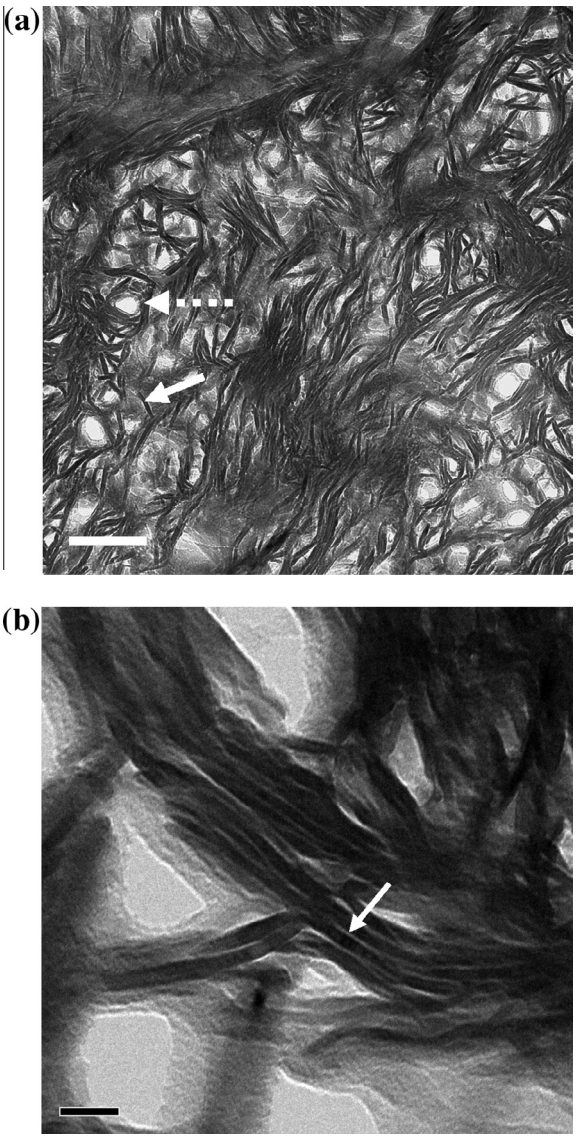

Fig.1. (a) Bright-field image of section of a sample of human femoral cortical bone cut normal to the femoral axis. Solid arrow: single mineral structure; dashed arrow: hole marking site of collagen fibril; star: stack of mineral structures. Scale bar = 100 nm; (b) bright-field image of section of cortical bone cut normal to the femoral axis, showing ~1 nm gap between two adjacent mineral structures (white arrow). Scale bar = 20 nm.

estimated that 80% of the Ca in bone is in the mineral structures, while about 20% resides in the gap zones. Fig. 4 shows a hypothetical model for the structure of bone, showing the spatial relationships between the mineral structures and collagen fibrils.

The present paper is concerned with observations made on dark-field (DF) images of ion milled sections of bone. DF images exploit the principle that it is possible to construct TEM images of a wholly or partly crystalline sample by using electrons diffracted from a specific set of lattice planes of the crystals. In this way we obtain an image which is dark, except at points which map the location of crystals appropriately oriented so as to scatter electrons from a specific lattice plane. In principle, the only illuminated areas in a DF image of a sample containing crystals of apatite[1] should be areas

[1] Throughout this paper we refer to mineral in bone as "apatite" rather than hydroxyapatite. While both X-ray diffraction and selected area electron diffraction (SAED) studies confirm that the mineral can be classified as an apatite, numerous studies have failed to find significant amounts of hydroxyl ions in the structure using both FTIR and NMR methods (Rey et al., 1989, 1995; Pasteris et al., 2004).