WHAT DRIVES BONE GROWTH?

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The structure, property and functionality of bone have attracted considerable attention in the past and researchers from a number of disciplines have contributed to our understanding of bone. We now know that, in addition to providing a mechanical support to vertebrae, the complex architecture of bone also hosts cells, proteins and enzymes, which participate in the dynamic process of bone growth and resorption. We also know that at least part of this dynamic process is related to the mechanical environment that the bone is subjected to and surrounded by. Above all, bone serves a metabolic purpose by acting as a reservoir for on-demand supply of minerals, most notably Ca^{2+} ions.

Since the 1990s, biomimetic synthesis strategy has become an active area of research. The intention was to learn from nature's own way of creating different biological tissues and to use this teaching in the synthesis of biomaterials artificially. One important aspect of the research has been the endeavour towards biomimetic mineralisation. The impetus was based on the understanding that biomineralisation takes place by the process of precipitation of inorganic crystalline or amorphous materials into an organic matrix, the nature of which controls the form of the precipitate. Two important examples of such extra cellular biomineralisation are hydroxyapatite in bone and aragonite in coral.

In bone, according to Calvert [1], the collageneous matrix is associated with the nucleation and growth of the mineral, as well as soluble proteins, mucopolysaccharides, cells and vesicles. It is commonly believed that spaces within collagen fibrils provide the active nucleation sites for hydroxyapatite. On the other hand, coral forms by nucleation and growth from the outer surface of a cell into a sea-water medium, that possibly contains some mucopolysaccharides. The mineralisation here is apparently through nucleation at a site on a surface and results in crystals growing away from that surface.

However, one point that might have been missed out in this simplistic interpretation of biomineralisation is that the inorganic layer in mineralised tissue is highly organised and in turn participate in a hierarchical structure. There is ample evidence to suggest that the inorganic layer adopts one or two specific crystallographic orientation with respect to the organic surface. For example, in bone, the bimodal distribution of hydroxyapatite crystals exhibits orientation of the crystallographic *c*-axis along and perpendicular with the collagen fibril axis. The popular answer to this, as Mann [2] has identified, is that this is an epitaxial effect to the extent that the atomic spacings in the substrate induce the growth of mineral layer for which the atomic spacings almost match across the interface.

This concept however does not do a good job in explaining what actually works. For example, both hydroxyapatite and calcium carbonate can nucleate on surfaces that have been treated with anionic proteins from shells, surfaces with high roughness, surfaces that has been electrically polarised and surfaces that has permanent polarisation. So, in addition to epitaxy, stereochemistry and charge matching mechanisms need also to be considered as contributing factors [3,4]. In this article, we provide a critical review of literature to highlight the role of surface charge in the growth of bone with particular reference to biomineralisation. We discuss the phenomena of bone growth during the healing process in this regard, as bone healing constitutes the field where the phenomena of biomimetic mineralisation will find its immediate application.

Bone is a vascularised tissue consisting of cells and a mineralised extracellular matrix. Bone is deposited by bone forming cells *osteoblasts* and by *osteocytes*. Osteoblasts cease dividing when they transform into osteocytes. Bone is modelled, remodelled and/or removed by primarily *osteoclasts* and sometimes by osteocytes [5]. Type I collagen, composed of two α-I chains and one α-II chain is the major extracellular matrix component and osteocalcin, osteopontin and osteonectin are the major non-collageneous proteins. Bone matrix is highly permeable due to the canals, *canaliculi* that contain osteocyte processes. The canaliculi work as an interconnected transport system to connect osteocytes to other osteocytes, osteoblasts and osteogenic cells on the surface via gap junctions.

The first bone matrix deposited is unmineralised and known as *osteoids*, which is then impregnated with hydroxyapatite to form

bone, the *mineralised* tissue. Histological section of *Haversian* bone (FIGURE 1) reveals the hollow nature of bone, some of the holes containing nerves. The majority of holes represent tubules, *Haversian canals*, or canaliculi. There are also irregular canals, *lacuna* that represents sites of bone absorption and resorption.

Bone healing is essentially a cell-mediated phenomenon although factors that activates or stimulates such activity are expected to influence the healing process. Healing in a primary bone wound involves the initial formation of haematoma, followed by an inflammatory reaction and polymorphonuclear leucocytes (PMNs) infiltration [6]. Macrophages invade the clot and chemotactic agents attract marrow stormal cells that contain a small number of Mesenchymal stem cells (MSCs), which in a favourable environment can differentiate into a variety of cell types such as osteoblasts, chondrocytes and fibroblasts. In wound sites that are mechanically stable and vascularised, most of the cells will become osteoblasts, whereas in mechanically unstable and less vascularised sites cells form chondrocytes to form

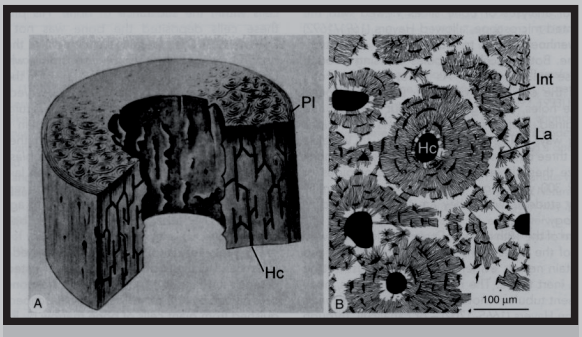

FIG.1. Three and two-dimensional views of Haversian bone [5]: (A) the lamellar structure of mammalian long bone. Haversian Canals (Hc) make up the bulk of the lamellar bone. Peripheral lamella (Pl) are found in periosteal surface; (B) histological section of a human femur showing Haversian canals (Hc) in cross-section, interstitial lamellae (Int) between more mature Haversian systems, and Lacunae (La) in which osteocytes would have been housed.

cartilage. If conditions are not optimal for bone or cartilage formation, cells may differentiate along a default pathway to become fibroblasts and non-union results.

When the ends of the wounded bone are in close approximation and the bone is mechanically stable, osteoblasts synthesise and *calcify* osteoids. Osteoblasts that are surrounded by calcified osteoids become osteocytes. This rapidly forming bone is termed *woven bone* because it lacks structural organisation. Woven bone after *remodelling*, is replaced by lamellar bone (FIGURE 1), including Haversian canals. This process takes varying lengths of time depending on the wound site and whether the bone is in mechanical function. Generally, bone healing and remodelling takes about 24 weeks and this may be longer in complicated or larger wounds.

If the wound is mechanically unstable, due to the chondroblastic activities that synthesise cartilage matrix, callous forms. The cartilage matrix undergoes endochondral differentiation, resulting in calcified cartilage, which is then remodelled by chondroclasts (osteoclasts that resorb calcified cartilage), leaving the newly formed underlying bone. The calcified cartilage thus serves as a natural scaffold for new bone formation and acts as an internal fixation device. As a result, wounds and defects that heal by callous formation are mechanically stiffer during healing than those heal by primary bone formation.

In the healing of a fractured bone four basic processes can be identified: the formation of a vascular haematoma; the formation of an early soft callous followed by a hard, mineralised callous; tissue transformation as the blood clot or cartilage of soft callous is replaced by bone and bone remodelling as the new bone adapts to local conditions of existence [5]. A number of factors play role in the process of bone healing and these factors can be judiciously applied to aide bone healing process. Bone growth factors such as TGF-β, PDGF, IGFs and FGFs are released by platelets aggregating at the wound site and by the injured bone itself [5,6]. For example, subperiosteal injection of TGF-β-1 or –2 has been found to enhance chondrogenesis and osteogenesis, the ratio of cartilage to bone varying with the dose administered.

The role of mechanical loading in helping bone repair is also known. For example, Rooij *et al.* [7] have found that cartilage has failed to form in a mechanically unloaded long bone fracture. In another interesting study, O'Driscoll and Salter [8] have found that *continuous passive motion* permits healing with hyaline cartilage in 80 percent rabbits in only 4 weeks, while *complete immobilisation* developed fibrous scar tissue and *normal motion* initiated repair in 20 percent of rabbits after 6 months. Also, under the constant passive motion, much more hexosamine, chondroitinin, keratin sulphates (markers of deposition of cartilaginous extracellular matrix) and type II collagen, were deposited. Moelcular studies reinforce and extend these findings. For example, IHH, Gli-3 and BMP-6 appear earlier in mobile tibial fractures than in immobile tibila fractures in mice [5].

The effect of mechanical loading at the microenvironment can by no means be taken as a verification of the so-called Wolff's law, which relates bone structure to bone function in terms of the forces and loads imposed on living active bone although mechanical loading can initiate bone formation in situations where no cartilage was present. Also, more recent studies provide evidence that the effects of pressure on bone growth can vary with the magnitude and type of mechanical loading. Stress beyond certain physiological threshold *inhibits* bone growth [5], while stress relaxation can stimulate growth. More importantly, clinical studies suggest that bone is adapted to *intermittent* loading: rapid transformation from quiescence to bone formation and increased synthetic activity can be recorded in osteocytes following a single brief period of bone healing.

How this mechanical loading is transducted in cellular activities put forward a perennial problem. One explanation can be sought from the polarisation response of bone when mechanically stressed, a property known as piezoelectricity. Fukada and Yasuda [9] demonstrated that application of a shearing stress along the long axis of bone caused a voltage to appear on bone surfaces parallel to the axis. Later on, in 1962, Bassett and Becker [10] independently found that bone produces electrical signal under the application of a mechanical stress [11]. Based on the Becker's previous works on electrical regeneration phenomenon, Bassett and Becker [10] conjectured that electrical potentials might be linked with the clinically observed adaptive response that occurs in children with healed malaligned fractures. Later, Shamos and Lavine [12] have reaffirmed that the observed bioelectric effect in bone is of a piezoelectric origin and they explained the importance of physiological functions of such electrical potentials in bone remodelling.

Piezoelectricity is fundamentally related to the crystal structure, order and polarisation as a result of mechanical stress. Piezoelectricity has been experimentally observed both in dry and wet state. The methods used include *inter alia* static, quasi static and low frequency dynamic methods using direct piezoelectric effect. Converse piezoelectric effect was also observed. Samples from different origins: human, bovine and horse have been tested. Most of these samples were obtained from femur, for the compactness of sample and convenience in handling. Piezoelectricity is represented by a third order tensor and known as piezoelectric strain coefficients popularly known in units of pC N^{-1}, which is a direct measure of generated charge (measured in Coulomb, C) as a function of applied force (measured in Newton, N). The value of piezoelectric polarisation can be directly found from the piezoelectric stress coefficient **e**, which is the product of the strain coefficient and the material's stiffness (a fourth-rank tensor) and measured by the amount of charge generated over a given area (C m^{-2}). Lang [13] proved that bone and tendon (predominantly made of collagen fibres with no apatite) both showed pyroeletricity, a subset of piezoelectric crystals. A pyroelectric crystal has at least one direction along which a spontaneous polarisation exists. As a result of this spontaneous polarisation surface charge exists in a pyroelectric crystal. In the case of bone, the direction of the spontaneous polarisation is along the bone fibre axis, in the case of tendon, it is the long fibrillar axis of collagen.

The effect of hydration, however, showed considerable effect on collagen as practically no piezoelectricity was found in tendon at 100% relative humidity. It has been suggested that the bound water in the material may change the symmetry to the point where no piezoelectricity can be observed [14, 15]. To the contrary, wet bone exhibited higher piezoelectric coefficients and some of the shear coefficients were over 50 times higher than those of dry bone [15].

The nature of electricity in bone is a steady-state potential measured as dc-potentials on the surface of living tissue and therefore relates to mechanically induced charge separation (i.e. piezoelectric polarisation) or to a concentration gradient between cations and anions at a surface giving rise to a surface potential called zeta (ζ-) potential [16]. Under an electric field across the bone/body fluid interface, electrokinetic phenomenon (streaming potential) results when one phase moves with respect to the other. Recent theories [17,18] suggest that, when considered in non-classical sense, both piezoelectric polarisation and electrokinetic potential can be responsible for the electromechanical response of bone.

As bone is pyroelectric, it has permanent dipoles. Atehe-

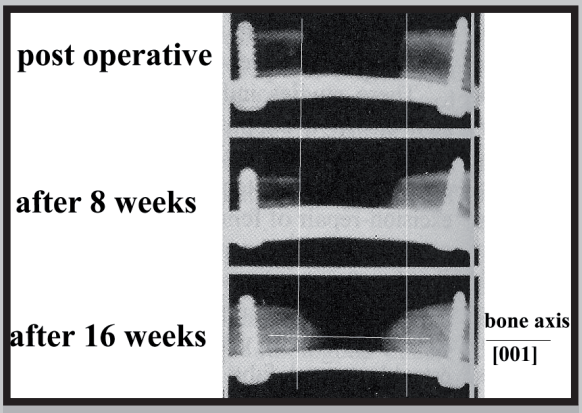

FIG.2. Directional natural healing in an empty bone defect, adapted from [21].

nstaedt [19] has demonstrated electric polarisation pattern in the lower extremity of an infant and explained the role of polarisation in ossification process. When a human reaches a certain age, radial polarisation disappears but longitudinal polarisation remains. So, if one creates an artificial radial polarisation vector in an area in the proximity of bone, then bone growth and regeneration can be induced locally in the radial direction [19,20].

One interesting phenomenon is revealed in radiographs shown in FIGURE 2c that shows the advancing plane in a naturally healing empty bone defect in the [001] direction, which happen to be the bone fibre axis along which bone pyroelectricity exist [21]. Despite evidence that bone morphogenesis and growth are polarised processes [5], little attention has been paid to analysing how polarity and axial symmetry are expressed during differentiation. Many authors have attempted a correlation between bone piezoelectricity and its physiological significance through the translation of mechanical stimuli into bioelectrical activity to which skeletal cells can respond. From the empirical evidence of artificial electrical osteogenesis induced, it can be surmised that enhanced chondrogenesis and acceleration of the growth of epiphyseal cartilage and bone occurs following direct application of an electrical field that the applied electric current in the μA range influence proliferation, differentiation and activity of skeletal cells. Analytical treatment of the correlation between a mechanical stimulus and concomitant bone growth is however rare as this empirical range of μA current does not tell much about the current or charge density that is available as a result of electrical stimulation. Based on piezoelectric polarisation in shear, Guzelsu calculated that, in wet bone, time required for deposition of an apatite layer having the same thickness (~ 10 μm), as that of an osteon, was about 12 days [20]. Guzelsu concluded that a bone, which is subjected to shear stress, adopts its functional shape in a relatively short time. In other words, the remodelling process is faster under shear stress than under normal stress condition with no stress gradient.

A Japanese group led by E. Fukada and I. Yasuda was first to demonstrate that surface charge from a bone graft could artificially induce bone growth [22-23]. They placed electrically polarised electret films (teflon) to induce callus formation in a rabbit and a rat. At first, the electret was thought to have permanent electrical polarisation *in vivo* as in air. However, the poled Teflon film was found to suffer from capacitive discharge to lose electrical surface charge completely by day 5. However, even this charge caused sufficient stimulation in undifferentiated mesenchymal cells to differentiate into bone forming cells and enhance bone growth. In later studies, Fukada used piezoelectric films of poly-γ-methyl-L-glutamate (PMLG) [24] and poly vinyledene fluoride (PVDF) [25] to stimulate bone growth. Two interesting findings were revealed in these studies. Firstly, the current produced by poled Teflon film and PMLG film was in the order of pA, which is of the same order of natural piezoelectricity of bone. Such a low level of current is six orders lower than the direct current that produces electrical osteogenesis. Secondly, it was found during the resorption study that after about eleven months, the newly formed bone near the Teflon film almost disappeared, but the bone near the PMLG film continued to grow. Fukada explained the observation considering the transient nature of electret polarisation in contrast to the perpetual nature of piezoelectric polarisation.

Electret polarisation of hydroxyapatite has shown enhanced apatite growth *in vitro* and accelerated osteobonding *in vitro* [26-30]. For a long time it has been observed that bone grows preferentially on the negatively polarised surface. Another Japanese group led by K. Yamshita in recent years has demonstrated that electret polarisation can be induced in hydroxyapatite, which can be used to selective growth of apatite on the negatively polarise surface. Yamashita and colleagues have found that electrical polarisation of piezoelectric ($CaTiO_3$, and $BaTiO_3$) and electret (hydroxyapatite) ceramic substrates can induce bone like apatite growth from a simulated body fluid. Most importantly, they found approximately 6 times higher growth of bone-like apatite on the negatively polarised surface of a hydroxyapatite ceramic polarised by a dc – electric field of 120 V mm^{-1} at 300 °C as compared to a control hydroxyapatite that has not been polarised. There was no growth of apatite on the positively polarised surface.

This finding along with the clinical results of better bone bonding and growth on the surface of polarised hydroxyapatite surface strengthens our earlier discussion on the role of polarisation in stimulating cellular activity and bone growth. Preliminary results from one of the author's (Tofail) own laboratory has confirmed the ability of hydroxyapatite to store electrical charge and exhibit polarisation. Whether the enhanced apatite growth due to the induced polarisation in hydroxyapatite is a bulk phenomenon or a local effect due to dipole orientation is currently under investigation.

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