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Investigation into the formation and mechanical properties of a bioactive material based on collagen and calcium phosphate

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A biomaterial composite was formed by the room-temperature precipitation of calcium phosphate (in the form of brushite) on to collagen. It was found that the addition of 1 mM O-phosphoserine (Ser P) causes the morphology of the brushite crystals to change from large plates to small needles. An increase in the surface coverage and weight fraction of brushite incorporated into the collagen was observed. The mechanical properties of this composite were tested in the wet state. The ultimate tensile strength (UTS) was 45 MPa compared with 34 MPa for the wet collagen. Osteoblastic differentiation was promoted on the surface of the material and new bone formed.

1. Introduction

A material that does not produce any adverse biological side effects when placed in the body is termed a biomaterial, and as such is classified as either bioinert or bioactive. Osteogenic bioactive materials stimulate bone regrowth, and are generally composed of natural materials. Implants of porous hydroxyapatite (HA) [1], collagen combined with gelatin [2] and HA immersed in a collagen gel [3] have all shown excellent bone regenerative capacity, but unfortunately have been unable to provide the mechanical properties for use as implants in stressed regions. The mechanical properties of many different types of animal bone were tested by Currey [4], giving the Young's modulus and UTS values as between 2 and 50 GPa and between 40 and 200 MPa, respectively. The mechanical properties of bioinert materials, such as Ti alloys and stainless steels, have been shown to be far superior to those of natural bone. Stress shielding resulting in bone resorption is one of the main reasons why these mechanical properties are not conducive to implant longevity. In addition, they are not recognized by the immune system, so no interaction with the living tissue occurs.

There is therefore a need for a biomaterial with mechanical properties closely matched to those of bone, with bioactive capacity, and which will stimulate bone regrowth. In this paper we present the results of a study into the formation, mechanical properties and bioactivity of a composite formed from calcium phosphate and collagen which goes some way to meeting this need. This is part of an on-going research project [5, 6]. Some preliminary results have already been published [7, 8].

The thermodynamic factors governing the nucleation and growth of calcium phosphate phases on both HA seed crystals and reconstituted collagen have been studied extensively by Nancollas and co-workers [9, 10]. The involvement of kinetic factors has been investigated by Boistelle and Lopez-Valero [11]. It is known that bone-specific proteins may influence the *in vivo* bone formation process. Indeed, many experimenters have investigated the effect of proteins on mineralization *in vitro*. Glimcher and Endo [12] studied the effect of incorporating the protein Ser P with collagen in the solid state before mineralization, and found that the lag-time occurring before mineral precipitation decreased upon adding the protein. Termine *et al.* [13] found osteonectin to enhance the mineralization process and suggested that it formed a "link" between collagen and mineral, whereas the experiments of Doi *et al.* [14] deemed the same protein to inhibit mineralization. Similar arguments have taken place over osteocalcin and osteopontin. Overall, the results of the effects of proteins on mineralization are inconclusive, due to conflicting information. However, it will be shown that the general approach of using a protein, or other organic molecule, to aid in mineral precipitation on to collagen is potentially powerful.

2. Experimental procedure

Type 1 collagen in the form of decalcified bone was obtained from rabbit femur by ethylenediaminetetraacetic acid (EDTA) extraction. The femurs were decalcified over 2–4 days, depending on the size of the original bones, with stirring in 10% EDTA. The ratio

0957–4530

of Ca^{2+}: EDTA was 1:10 (w/w), assuming the calcium content of the bone was 25%. The ratio was achieved by adjusting the volume of the EDTA solution. Before the remineralization experiments the decalcified femurs were stored in 0.9% NaCl and 0.01% thiomersal at 4 °C.

The growth studies entailed immersing the decalcified femur in a 50 mM Ca^{2+} ion HA solution at pH 5.5. This calcifying solution was made by dissolving the appropriate amount of HA powder in hydrochloric acid at pH 2 and then making up to the required pH with 0.5 M KOH. Small samples of decalcified bone (10 mm × 10 mm × 1 mm) were left in 40 ml of this solution for 5 days before examination in a Hitachi scanning electron microscope (SEM). A larger piece (5 mm × 20 mm × 1 mm), for use as a tensile test specimen, was mineralized in two separate identical solutions each for 10 days, consecutively, before being dried in a desiccator overnight. Ser P is a bone-specific phosphoprotein found at initial mineralization sites in bone. To determine any effect that this may have, the two growth experiments were repeated using the same mineralizing solution with additions of 1 and 10 mM Ser P. To determine the weight percentage mineral contained in the tensile specimens, a preweighed sample was heated to 800 °C in a furnace in a platinum crucible. The remaining ash was weighed.

The tensile tests were performed on an Instron 1195 at a crosshead speed of 0.5 mm min^{-1}. The samples were tested in the wet condition, having been immersed overnight in their respective mineralizing solutions. Also, as a control, a tensile test specimen of unmineralized decalcified bone was prepared and tested wet. The Young's modulus and UTS were determined from the load–displacement data. The original cross-section of the dry samples, after mineralization, was measured. A slight swelling after rewetting occurred, but this was ignored in the subsequent calculations.

The bioactivity of this material was assessed by coculturing with human-derived bone cells and examining the effects histologically. The cells were obtained by outgrowth from normal human trabecular bone using the method described in [15]. The test material was cut into fragments 6 mm × 3 mm and sterilized using ethylene oxide for 30 min. Single fragments were then added to the wells of a 24-well multiwell culture plate with 1 ml culture medium. The culture medium used was Dulbecco's modified Eagles minimum essential medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 20 mM N-(2-hydroxyethyl)-1-piperazine – N′-2-ethanesulphonic acid (Hepes), 100 μM L-ascorbic acid 2-phosphate, 100 units ml^{-1} benzyl penicillin and 100 μg ml^{-1} streptomycin. The cells were added at a density of 2.4×10^4 cells $well^{-1}$ and incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The medium was changed every 2 days. After 32 days the material fragments were removed and fixed in 95% methanol at 4 °C and embedded in methacrylate. Sections were cut for light microscopy and stained with toluidine blue and Von Kossa stains, or with toluidine blue alone.

3. Results and discussion

Plate-like crystals approximately 100 μm × 30 μm in area were deposited on the surface of the collagen in the solution without Ser P (Fig. 1a). Speciation calculations using the modified Debye–Huckle equation were performed. In agreement with other workers [9, 10], the precipitation of brushite was verified. This was confirmed using a Cameca microprobe to measure the Ca/P ratio of the crystals. Upon addition of Ser P the morphology of the crystals changed to give tight efflorescences of much smaller needle-like crystals (Fig. 1b), and surface coverage of mineral increased by a factor of 3 as the number of nucleation sites increased. However, when 10 mM Ser P was added the surface cover of mineral decreased drastically to only 5%, yet the tight efflorescent morphology remained. The phase of the crystals did not change upon addition of the protein. The calcium ion concentration in solution was found to be reduced upon addition of Ser P. These results indicate that the presence of Ser P was influencing the mineralization process.

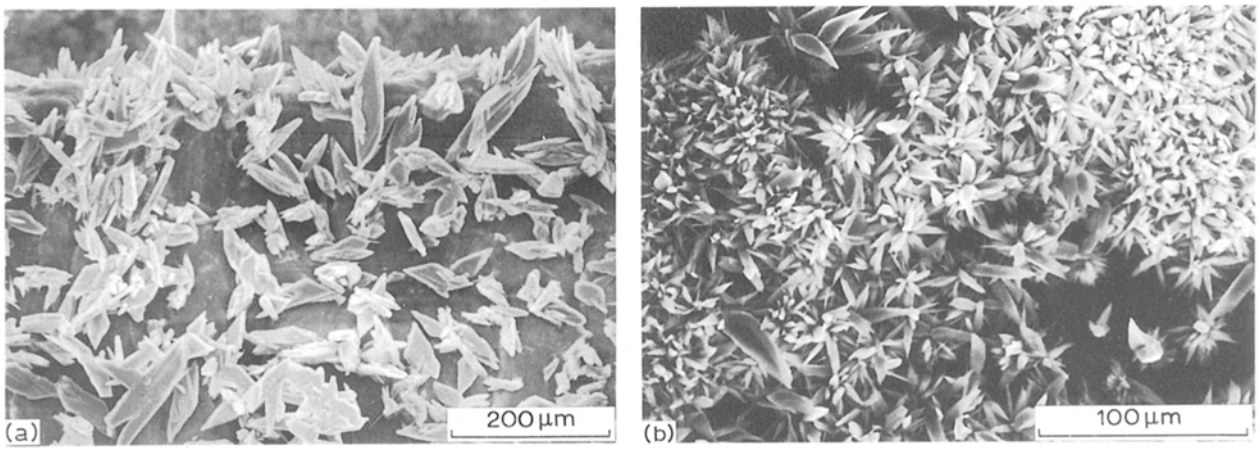


Figure 1 SEM micrographs showing (a) plate-like crystals of dicalcium phosphate dihydrate grown on decalcified bone and (b) the smaller needle-like efflorescences of dicalcium phosphate dihydrate grown on decalcified bone with the addition of 1 mM Ser P.

Since addition of Ser P reduces the calcium ion concentration, it must be complexing with the Ca^{2+} ions in solution. This reduction in ion concentration will then affect the precipitation rates, by lowering the supersaturation. If an excess of Ser P is present (as in the 10 mM experiment), more-complete complexing of the Ca^{2+} will occur and fewer free Ca^{2+} ions will be available for mineral growth. In addition, to increase the amount of surface coverage the Ser P probably also forms a bridge between the collagen and the mineral, making use of the charged groups that exist on the amino-acid molecule in solution [16]. The actual cause of the morphology change is not yet known, and further work is in progress to clarify this.

The stress–strain curves for the various samples tested are shown in Fig. 2. As mineralization proceeded the modulus increased from 344.8 MPa for the collagen alone to 513.2 and 698.4 MPa for decalcified bone mineralized without Ser P (containing 9.1 wt % mineral after mineralization) and with OPS (containing 13.1 wt % mineral after mineralization) additions, respectively. This represents an overall modulus increase of 100%. The UTS also increased by approximately 40% from 34.66 to 44.87 MPa upon mineralization, and then increased further to 45.83 MPa upon addition of Ser P to the mineralizing solution. It can be seen that the strain to failure decreases as the strength increases. The fracture surfaces of one unmineralized and one remineralized tensile specimen are shown in Fig. 3a and b, respectively. The osteons are visible in the unmineralized case, but after mineralization the mineral can be seen in the interior of the sample, obscuring the osteons and demonstrating that this growth technique produces mineral throughout the cross-section of the sample (regions arrowed A in Fig. 3b), and not just on the surface.

Fig. 2 shows that the strain to failure of the collagen after mineralization is lower than that before remineralization. This means that the crystalline content of the material must be affecting the energy-absorption mechanisms in operation. The general shape of the stress–strain curves is typical of a soft and tough elastomer, exhibiting rubber-like elasticity, although at relatively low elongations due to the complexity of the collagen molecule. The increase in modulus is due to the incorporation of the mineral phase and, as such, a simple composite law of mixtures may be applied (see, for example [17]). Further growth experiments are now in progress; in particular, varying the volume fraction of mineral phase to show which model of elasticity will give the closest agreement. The strengthening may be attributed to a variety of mechanisms, such as the extra energy requirement of breaking the collagen–mineral bonds to allow collagen fibre pull-out or extension. This behaviour is typical of a "filled" polymeric material for which addition of filler generally causes the Young's modulus to increase and the strain to failure to decrease (see, for example [18]). The Young's modulus of the decalcified rabbit femur is lower than that generally obtained with collagen: a value of 1–2 GPa would have been expected. However, our collagen sample was highly porous (as bone generally is), and this accounts for our lower values. In addition, the stress–strain behaviour of organic polymeric materials is dependent on the strain rate. Previous work has shown the increase in Young's modulus with strain rate of the same type of collagen (see, for example [5, 6]). Thus, the comparatively low values were not unexpected.

In Fig. 4a the Von Kossa stain, specific for phosphate, demonstrates a phosphate gradient with higher

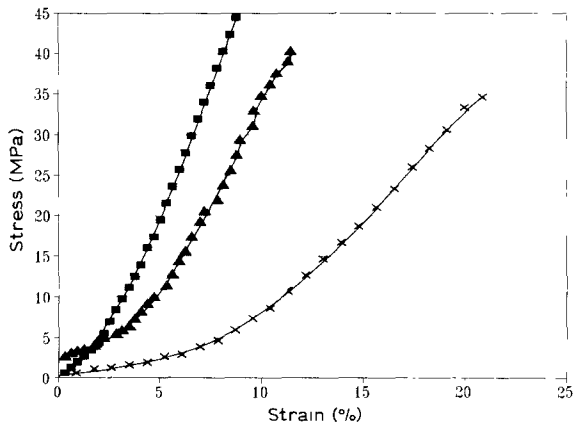
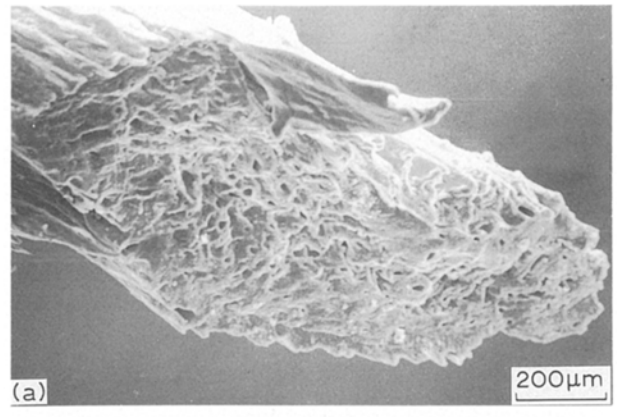


Figure 2 Stress–strain curves of the prepared tensile specimens (crosshead speed 1 mm min^{-1}): (X) for collagen, (▲) for collagen with calcium phosphate and (■) for collagen with calcium phosphate and Ser P.



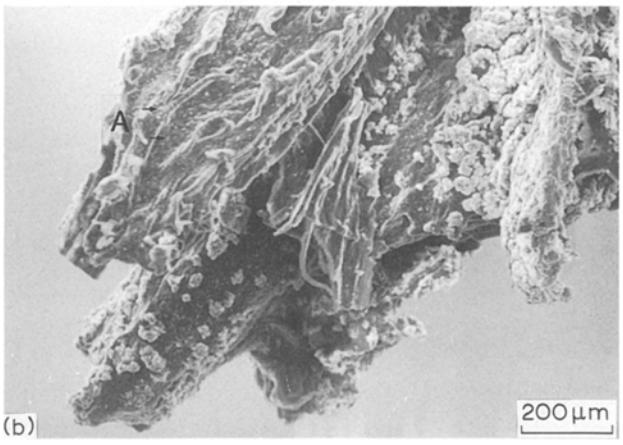


Figure 3 SEM micrographs showing the fracture surface of the decalcified bone. (a) without remineralization (note the visibility of the osteons) and (b) remineralized using Ser P. Note that the osteons are no longer visible and that mineral can be seen on internal surfaces as small crystals (A).

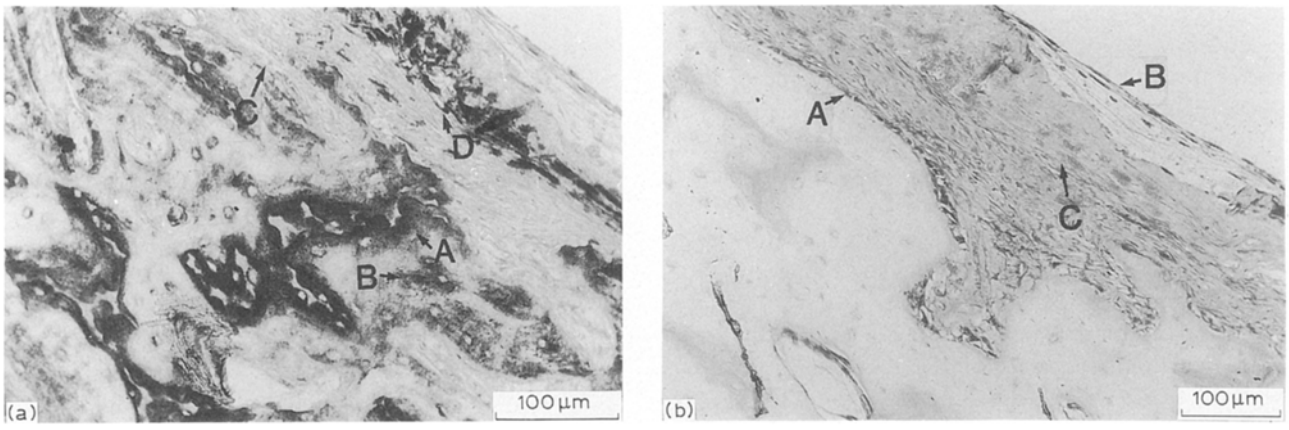


Figure 4 (a) (stained with Von Kossa and Toluidine blue). The Von Kossa stain (dark) shows that there is a concentration gradient of phosphate in the fragment which is highest at the surface (A), although there is also staining within fragment B. The cells have adhered to fragment C and there is mineralization of the matrix produced by the cells (D). (b) (stained with Toluidine blue). The cells on the surface of the fragment have a similar appearance to active osteoblasts found on the bone surface *in vivo* (A). The cells at the periphery have formed a loose connective tissue and have a fibroblastic appearance (B). In between the matrix is more dense and has an appearance similar to osteoid (C).

amounts of phosphate close to the surface (marked A) of the fragment, but also penetrating well into the fragment in some areas (for example B). The bone-derived cells have adhered to the fragment (marked C) and produced a substantial amount of matrix that had mineralized in some areas (marked D). Fig. 4b shows that the cells adjacent to the fragment surface (marked A) had taken on a plump cuboidal appearance that is typical of active osteoblasts found on bone surfaces *in vivo*. At the periphery the cells were fibroblastic in nature and had formed a loose connective tissue (region B). Between these two areas there was a dense matrix which had the appearance of newly formed osteoid (region C). This differentiation pattern had a spatial relationship to the fragment, indicating that this material had promoted osteoblastic differentiation. Thus, this material had caused new bone to form, and in this respect seemed to behave like young bone. Similar coculturing experiments using only collagen and only mature bone did not produce new bone.

3. Conclusions

A composite of collagen and brushite was formed at room temperature by precipitating the mineral from solution. In this work we used Ser P to enhance the number of nucleation sites, reduce the calcium phosphate crystal size and increase the final weight fraction of mineral precipitated. From this preliminary study it has been postulated that the Ser P acts as an interlayer between the mineral and the collagen to promote nucleation. Further work on this aspect is currently in progress. This use of an organic interlayer may have general applications. In this particular case it has been shown to lead to an increase in the wet UTS to > 45 MPa with a Young's modulus of 0.76 GPa. The collagen samples under the same experimental conditions had values of 34 MPa and 0.34 GPa, respectively. The osteogenic bioactivity of this novel composite has also been demonstrated in this work. Thus, we have shown that it is now possible to create a bioactive material with mechanical properties approaching those of bone.

Acknowledgements

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