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In vivo assessment of the osteointegrative potential of phosphatidylserine-based coatings

A. Merolli · M. Bosetti · L. Giannotta · A. W. Lloyd · S. P. Denyer · W. Rhys-Williams · W. G. Love · C. Gabbi · A. Cacchioli · P. Tranquilli Leali · M. Cannas · M. Santin

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Abstract The successful implantation of titanium-based implants for orthopaedic and dental applications is often hindered because of their mobility, which arises because of a lack of direct binding of the metal surface to the mineral phase of the surrounding bone. Ceramic coatings, although ensuring the integration of the implant within the tissue, are unstable and carry risks of delamination and of failure. Recently, a novel biomimetic approach has been developed where porous titanium implants are coated with calcium-binding phospholipids able to catalyse the nucleation of discrete apatite crystals after only 30 min incubation in simulated body fluids.

The present work assesses the osteointegrative potential of this new class of coatings in an *in vivo* rabbit model and compares its performance with those of bare porous titanium and hydroxyapatite-coated titanium.

The data obtained show that phosphatidylserine-based coatings, whilst resorbing, drive the growing bone into apposition with the metal surface. This is in contrast to the case of bare titanium.

A. Merolli (✉) · L. Giannotta
Universita' Cattolica, Clinica Ortopedica, Rome, Italy
e-mail: antoniomerolli@tiscali.it

M. Bosetti · M. Cannas
Universita' del Piemonte Orientale, Dipartimento Scienze Mediche, Novara, Italy

A. W. Lloyd · M. Santin
University of Brighton, School of Pharmacy & Biomolecular Sciences, Brighton, UK

S. P. Denyer
Welsh School of Pharmacy, Cardiff University, Cardiff, UK

W. Rhys-Williams · W. G. Love
Destiny Pharma Ltd, Brighton, UK

C. Gabbi · A. Cacchioli
Universita' di Parma, Dipartimento di Salute Animale, Parma, Italy

P. T. Leali
Universita' di Sassari, Clinica Ortopedica, Italy

1. Introduction

Growing bone is not always able to establish a strong adhesion with the surface of titanium-based implants commonly employed in orthopaedic and dental applications [1,2]. A few microns of non-mineralised tissue rich in collagen and proteoglycans usually forms at the interface impeding the direct contact of the bone with the implant surfaces [3]. Furthermore, in some circumstances a protracted inflammatory response can be triggered which leads to the formation of a relatively thick fibrotic capsule interposed between the bone and the implant [4]. Despite its thinness, the presence of non-mineralised tissue impairs the mechanical stability of the prosthesis leading to its mobilisation and failure of integration.

Hydroxyapatite (HA) is the coating of choice for orthopaedic and dental implants since it encourages osteointegration through a direct apposition of bone [5]. The high osteointegrative potential of this class of materials is due to its chemical nature which is very similar to that of the bone mineral phase thus providing an optimal substrate for the growth of the osteoblasts and for the adhesion of the bone mineral phase [5]. Nevertheless, drawbacks are associated with the use of this class of ceramic coatings: particularly,

crystalline HA (85%) renders the coating brittle under biomechanical stresses and prone to a slow degradation into particles [6], while low crystalline coatings seem to be prone to a relatively rapid degradation by hydrolysis [7]. As a consequence, the strong osteointegration of HA, combined with biomechanical stresses, may lead to the failure of the implant by coating delamination [8].

Alternative strategies have been suggested where the metallic surface is modified with bioactive molecules able either to encourage calcium phosphate crystal nucleation or to promote adhesion, spreading and proliferation of osteoblastic cells [9]. These so called biomimetic approaches are intended to achieve the integration of the implant by increasing the rate of deposition of new bone on to the biomaterial surface thus preventing the formation of an interposed non-mineralised tissue layer. One novel approach envisages the possibility of mimicking the mechanisms of bone regeneration mediated by the matrix vesicles [10]. Indeed, matrix vesicles rich in calcium-binding phospholipids are effective nucleation *loci* of new mineral phase in developmental bone and rapidly mineralising skeletal bone [11]. These vesicles are fragments of the cell membrane and possess calcium channels (Annexin V) able to facilitate the influx of calcium ions from the surrounding environment into the phosphate-rich interior. The calcium-binding properties of phospholipids such as phosphatidylserine (PS), as well as the saturating concentrations of calcium and phosphate in the vesicle interior, lead to the formation of crystals which eventually aggregate, disrupt the vesicle and merge with other nucleation centres [12,13]. These mechanisms of apatite crystal nucleation of the matrix vesicles were elucidated by *in vitro* studies which have mimicked the matrix vesicles through the preparation of liposomal systems made permeable to calcium through the use of synthetic ionophores [14,15]. More recently, phospholipid-based coatings for endo-osseous implants have been developed which are able to catalyse the formation of discrete apatite crystals after only 30-min incubation in simulated body fluids [10,16]. Such coatings are able to promote the rapid formation (within 24 h) of a consistent calcium phosphate mineral phase in simulated body fluids in vitro through their re-arrangement into 3D gels. The architecture of these gels depends on the of film hydration in the incubation medium as well as on the crosslinking action of the calcium ions [17]. Indeed, the calcium-binding anionic phospholipids are able to assume a porous structure in which a calcium phosphate-rich microenvironment forms and facilitates the formation of apatite crystals. The present study aims to compare the efficiency of this new class of coating *in vivo* with porous titanium and plasma sprayed HA coatings. Two formulations of phospholipid coatings were selected which were both based on the presence of the calcium-binding PS. This type of phospholipid was used alone or in combination with phosphatidylcholine (PC) and cholesterol (C) to obtain different 3-D re-arrangements of the coating upon hydration [17]. In particular, the study focused on the morphological analysis of the implant/bone interface by the use of high-magnification back-scattering scanning electron microscopy (BSEM).

2. Materials and methods

2.1. Implant preparation

Titanium rods of 10 mm × 3 mm size, plasma sprayed with porous titanium foam by a standardised industrial procedure, were kindly donated by SAMO SpA (Bologna, Italy) and used both as control materials and as metallic substrate for the HA-and phospholipids-based coatings. HA coatings were also applied by plasma spray by SAMO following the company's standard manufacturing procedure.

Phospholipid-based preparations were deposited onto the titanium rods by dip-coating into phospholipid solutions in chloroform. Two coatings with different concentrations of the calcium-binding PS were obtained from either a 62.5 mM PS solution (higher concentration of PS) or from a phosphatidyl choline: phosphatidyl serine: chloresterol (PC:PS:C) mixture in a 7:2:1 molar ratio (lower PS concentration). The coating was performed by rotating the titanium road into the phospholipid solution using a specifically designed apparatus (Fig. 1). The apparatus consisted of (i) a reservoir chamber equipped with a piston (Fig. 1, arrow 1) where the phospholipid solution was stored and (ii) a coating chamber (Fig. 1, arrow 2) where the titanium rod was coated under rotation by an electrically-driven motor (Fig. 1, arrow 3). After starting the rod rotation, the phospholipid solution was displaced from the reservoir chamber by lowering the piston and

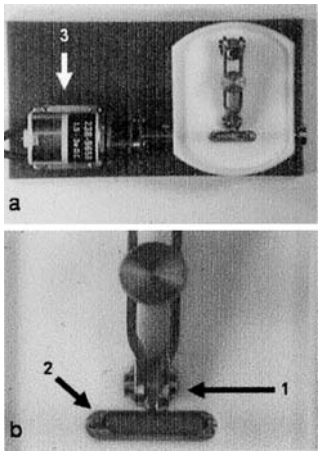


Fig. 1 The phospholipid coating apparatus consisting of a reservoir chamber with a piston (arrow 1) where the phospholipid solution was stored and a coating chamber (arrow 2) where the titanium rod was coated under rotation by an electrically-driven motor (arrow 3).

transferred into the coating chamber. The coating procedure lasted for 30 sec, followed by withdrawal of the phospholipid solution by the release of the piston and by a further rotation of the specimens for 15 sec in air to evenly distribute the excess of solution along the specimen surface. This standardised procedure provided the porous titanium rod with a uniform coating.

2.2. In vivo experiments

Young adult New Zealand White rabbits, weighing approximately 2.7 kg, were chosen as an animal model. The surgical implantation site selected was the distal femural canal (meta-epiphyseal region). Anaesthesia was achieved by intravenous injection of medetomidine (DOMITOR, Farmos Orion Corporation, Espoo, Finland) 0.3 mg/kg and ketamine chlorohydrate (KETAVET 100, Farmaceutici Gellini, Aprilia, Italy) 40 mg/kg. Antibiotic prophylaxis was provided by intravenous injection of enrofloxacin (BAYTRIL, Bayer AG, Leverkusen, Germany) 10 mg/kg/daily for 3 days. A hole (10 mm × 3 mm) was drilled from the intercondylar groove after access had been gained to the articular cavity of the knee by a lateral parapatellar approach. The rod was inserted along the main axis of the femur by press fitting. A total of 56 samples were implanted, divided into four groups, namely: group Ti (bare titanium foam), group HA (hydroxyapatite-coated titanium), group PS:PC:C (phosphatidyl-serine: phosphatidyl-choline: cholesterol-coated titanium), group PS (phosphatidylserine-coated titanium). The retrieval of 4, 4 and 6 samples took place at 4, 8 and 26 weeks, respectively. One scheduled 26 week group Ti sample retrieval failed due to the premature death of the animal unrelated to the presence of the implant.

2.3. Histological analysis

The retrieved femurs were fixed in formalin and embedded in poly(methyl methacrylate) (PMMA) resin after serial dehydration passages in ethanol following a standard procedure. These alcohol dehydration steps, although necessary to the preparation of the specimens, could not ensure the preservation of the ethanol-soluble phospholipid coatings. Resin blocks were ground, sputter-coated with palladium and analyzed by BSEM (Jeol JSM 6310, UK). Secondary scanning electron microscopy (SEM) and energy dispersive analysis (EDX) (Isis, Oxford Instruments Ltd, UK) were also used in some cases to facilitate a correct interpretation of the morphological analysis data.

BSEM images were analyzed by a semi-automated procedure (custom software) where a computer-generated radiating grid was centered and superimposed on the transverse section of the implanted cylinder (Fig. 2) and the sectors of bone-coating apposition were counted. There were 32 sectors in the grid and results were given as a fractional value of an index of trabecular bone apposition where 1.0 means 100% of apposition.

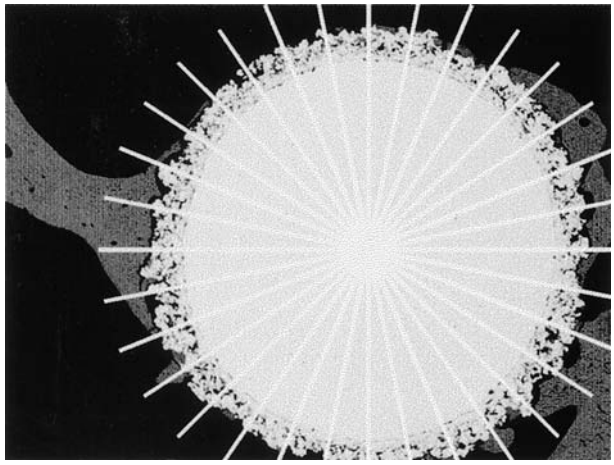


Fig. 2 A 15X BSEM image of a PS-coated rod analyzed by centering a computer-generated radiating grid on the transverse section of the implanted cylinder and counting the sectors of bone-coating apposition.

3. Results

3.1. Morphology in Ti group

After 4 weeks implantation, the control Ti shows bone trabeculae growing towards and around the implant (Fig. 3a). High magnification of the bone/implant interface, however, showed that the new bone did not establish a direct contact

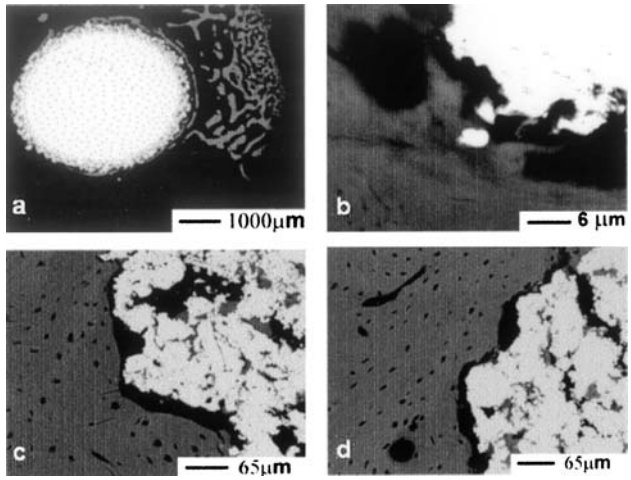


Fig. 3 After 4 weeks, implantation control Ti shows bone trabeculae growing towards and around the implant (a, 15X). High magnification showed that the new bone did not establish a direct contact with the material surface (b, 3000X). At 8 and 26 weeks the titanium foam allowed the penetration of a healthy and remodelling trabecular bone throughout its porosity, still showing areas of non-direct apposition (c and d, 250X).

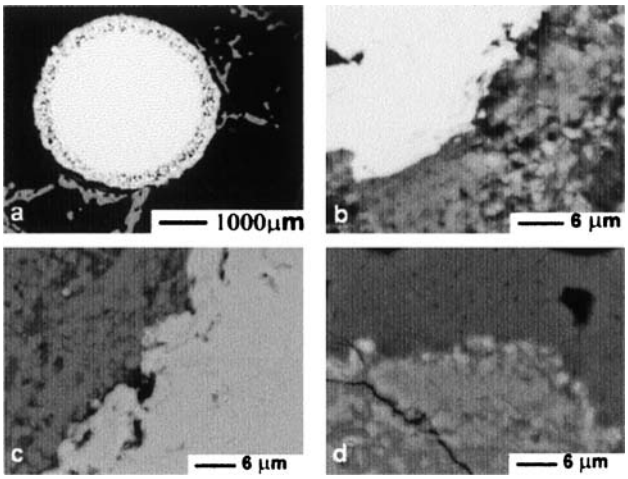


Fig. 4 New regenerating trabeculae pointing towards the HA surface after 4 weeks (a, 15X). High magnification showed the tight apposition of the bone to the ceramic material, but also areas of defects within its thickness (b, 3000X). The tight apposition of bone was preserved at 8 weeks (c, 3000X) and completed at 26 weeks, the newly-formed bone forming a continuous layer with the coating difficult to resolve even at very high magnification (d, 3000X).

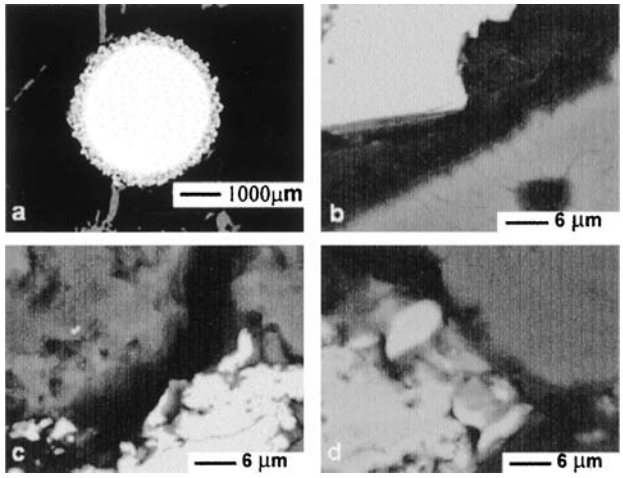


Fig. 5 New trabeculae were observed growing towards the implant coated with PC:PS:C after 4 weeks (a, 15X). BSEM high magnification of the interface at 4 and 8 weeks showed areas where newly-formed bone was separated by a thick electron dense layer (b and c, 3000X). After 26 weeks newly-formed trabecular bone encroached into the soft and carbon-rich layer to form large areas of direct contact with the implant (d, 3000X).

with the material surface (Fig. 3b). At 8 and 26 weeks the titanium foam allowed the penetration of a healthy and remodelling trabecular bone throughout its porosity, but still showed areas of incomplete apposition of tissue in several specimens when analysed at high magnification (Figs. 3c and d).

3.2. Morphology in HA group

New regenerating trabeculae pointing towards the HA surface were observed after 4 weeks (Fig. 4a). High magnification BSEM showed the tight apposition of the bone to the ceramic material, but also areas of defects within its thickness (Fig. 4b). The tight apposition of bone was preserved at 8 weeks (Fig. 4c) and completed at 26 weeks, the newly-formed bone presenting a continuous structure with the coating which was difficult to resolve even at very high magnification (Fig. 4d). After 26 weeks, coating fractures could be observed.

3.3. Morphology in PC:PS:C group

As for the other two groups, new trabeculae were observed growing towards the implant after 4 weeks (Fig. 5a). BSEM high magnification of the interface at 4 and 8 weeks showed areas where newly-formed bone was not directly apposed to the implant surface, but separated by a thick electron-dense layer (Figs. 5b and c). The combined SEM and EDX analysis showed the soft and carbon-rich nature of the interposed layer (data not shown). After 26 weeks newly-formed trabecular bone protruded into the soft and carbon-rich layer to form large areas of direct contact with the implant (Fig. 5d).

3.4. Morphology in PS group

The low magnification analysis after 4 weeks of implantation shows a bone formation with trabeculae growing towards the surface of the implant (Fig. 6a). The newly-formed bone was not adjacent to the implant surface when analysed at high magnification (Fig. 6b). After 8 weeks however the newly-formed trabecular bone in-growth had invaded almost completely the porous implant establishing a direct contact with its surface in most of the areas analysed (Fig. 6c). Tissue morphology at the implant surface was observed to be normal after 26 weeks when the invasion of the tissue was still readily visible and its apposition to the implant surface consolidated (Fig. 6d).

3.5. Image analysis

Results of the image analysis are summarized in Table 1. Legend names are: T for the Ti group; C for the PC:PS:C group; H for the HA group; S for the PS group.

Before reading the values, it is important to bear in mind that a sign of physiological bone remodelling, in *trabecular* bone, is a proper spacing between single trabeculae. Then, an index of trabecular bone apposition (surface of trabecular bone apposition/total surface of the implant) should be far from 1 (100% of bone apposition) to be considered physiological (while this is not the case in *cortical* bone). The

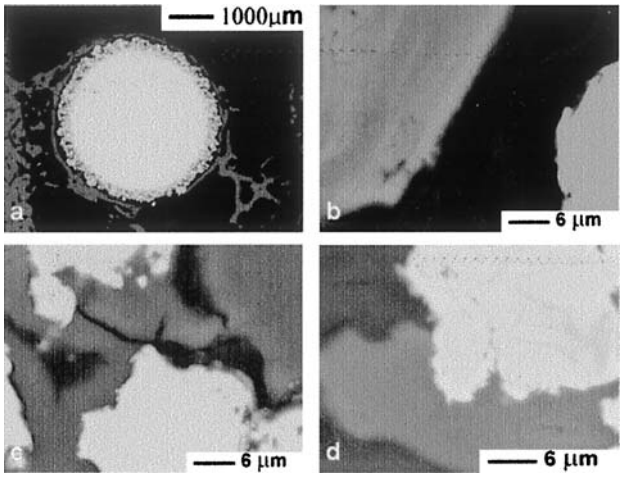
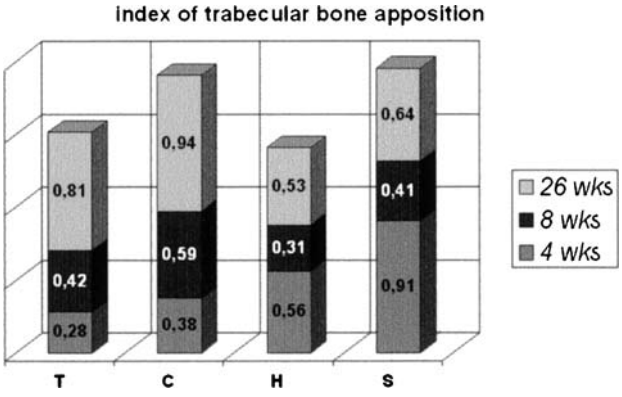


Fig. 6 Low magnification analysis of PS-coated rods after 4 weeks of implantation shows a bone formation (a, 15X). The newly-formed bone was shown not to be adjacent to the implant surface at high magnification (b, 3000X). After 8 weeks the newly-formed trabecular bone in-growth invaded almost completely the implant pores establishing a direct contact with its surface in most of the areas (c, 3000X). No significant difference in the tissue morphology at the implant surface was observed after 26 weeks where the invasion of the tissue was still clearly visible and its apposition to the implant surface consolidated (d, 3000X).

standard of reference is generally considered Hydroxyapatite coating.

After 4 weeks the highest apposition values are in the PS group, followed by the HA group; Ti group presenting the lowest values. After 8 weeks the task of bone integration should be considered accomplished and apposition values are comparable for PS, HA and Ti Groups but are higher in the PC:PS:C group. In the longer term, 26 weeks, apposition values are similar for PS and HA groups but higher in Ti and PC:PS:C groups.

Table 1 Results of the image analysis serialled at 4, 8 and 26 weeks. Legend names are: T for the Ti group; C for the PC:PS:C group; H for the HA group; S for the PS group. Before reading the values, it is important to bear in mind that a sign of physiological bone remodelling, in *trabecular* bone, is a proper spacing between single trabeculae. Then, an index of trabecular bone apposition should be far from 1 to be considered physiological (while this is not the case in *cortical* bone).



4. Discussion

The lack of a tight apposition of newly-formed bone onto the metal surface is a recognised limitation of the commercially-available titanium implants for orthopaedic and dental applications [3]. Although the manufacture of porous surfaces has improved the mechanical anchorage of the implant to the surrounding tissue, the formation of a thin non-mineralised tissue may still be one of the main causes of later implant mobilisation [3]. The high magnification analysis by BSEM undertaken in this current study both confirmed these literature findings for titanium and highlighted the advantages and disadvantages of the use of HA coatings. Indeed, BSEM images emphasised that while there is often a lack of bone contact with titanium it will nevertheless follow the contours of the implant without reaching its surface. Consistent with the literature, the analysis also demonstrated the high level of osteointegration with the HA coating, its heterogeneous nature and proneness to fracture [5]. Although these fractures were not necessarily generated *in vivo*, and instead could be possible artefacts arising during the sample preparation, it was peculiar to observe that they always took place within the HA coating and never at the bone/ceramic interface thus corroborating previous reports highlighting the risks of delamination of the HA coatings in clinical applications [8].

Both phospholipid-based coatings tested in this study showed no inhibitory action on bone apposition and growth and did not elicit any adverse fibrous reaction. Bone grew and matured around these implants. However, when the analysis of the bone/implant interfacial morphology was performed at high magnification at week 4, areas of bone with electron density typical of new tissue were found to grow very close to both phospholipid implants without reaching their surface. Unlike Ti, however, the bone surrounding the phospholipid-based coatings did not always follow the implant contours. Although BSEM, SEM and EDX could not identify the interposed layer as either embedding resin or a phospholipid layer, it can be speculated that after 4 weeks mature bone has already formed around the phospholipid coating. This hypothesis is supported by the different behaviour of the two phospholipid coatings at 8 weeks where the PC:PS:C coating was still separated from the bone by this interposed layer while, the PS coating showed large areas of direct contact of the newly-formed tissue with the metal surface. A different rate of resorption of the phospholipid layer would explain the gradual approach of the bone to the metal surface and its direct contact with the implant. In the case of the PC:PS:C coatings, the lower density of calcium-binding PS at the interface would promote a relatively slower in-growth

of bone into the titanium pores. Alternatively, the PS coating possessing more calcium-binding sites per unit area, would calcify more rapidly and consequently could have been resorbed/embedded in a shorter time.

Image analysis suggests that PS coating behaves in manner quite superimposable to HA coating (and, then, quite physiological), promoting even a wider bone apposition in the early stage (4 weeks). Ti coating drifting more toward a confinement of the implant operated by a rim of bone, a response which is well-known and which seems to be observed also in the PC:PS:C coating.

This data, together with in vitro information previously published showing the fast mineralization of the phospholipid coatings and their good substrate properties for osteoblast adhesion [18,19], would suggest that the calcium binding-phospholipids may gradually drive the in-growth of new bone tissue towards the implant surface allowing a final, intimate contact between the mineral phase and the metal.

5. Conclusions

The phospholipid coatings tested in this work emerge as a new type of biomimetic (matrix vesicle-like) surface treatment able to accelerate the osteointegration of titanium implants. Anionic phospholipid coating, uniquely rearranged into 3-D porous gels when immersed in calcium-containing aqueous media, may prevent the formation of non-mineralised tissue *in vivo* creating nucleation sites for the formation of calcium phosphate crystal seeds able to bridge the metal surface to the growing bone hydroxyapatite.

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