JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **17** (2006) 43–48

The mechanical properties of calcium phospate ceramics modified by collagen coating and populated by osteoblasts

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Bioceramics containing hydroxyapatite (HA), tricalcium phosphate (TCP) or composites which combine the best properties of both materials are among the principal candidates for bone replacement grafts. In this study we have investigated the mechanical strength of HA, TCP and composites of the two in the ratios 75;25 (H75), 50:50 (H50) and 25:75 (H25). The strength of each material was investigated in the presence and absence of collagen coating, and the influence of osteoblast culture for up to 28 days on strength was determined. TCP, H25 and H75 were significantly weakened by collagen coating, the strengths of the other materials were either not affected (HA) or increased (H50). Culture with osteoblasts significantly increased the strength of uncoated HA and H50, but this effect was not observed when the materials were coated with collagen. Our results indicate that ceramic composition affects the interactions between collagen coating, culture with osteoblasts and mechanical strength of the material. Although collagen coating has been found to increase the proliferation of osteoblasts into these ceramic materials, it may be necessary to stabilise and optimise the coating process to minimise effects on mechanical strength.

Introduction

Calcium phosphate based bioceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) are the principal candidates for bone replacement implant materials, although low mechanical strength limits their range of applications. HA is a stronger material than TCP and, in general, blends of the two materials have intermediate mechanical properties [1]. They are considerably stiffer than natural bone and lack its viscoelastic properties [2]. Although there have been some studies of the mechanical properties of calcium phosphate ceramics following implantation into living bone [3, 4], the effect of infiltration of cells into the materials *in vitro* on their mechanical properties has not been investigated.

The fact that collagen comprises 90% of the extracellular matrix of bone suggests that it occupies a pivotal role in the interaction of osteoblasts and their environment. Indeed, we have recently found that collagen coating increases the biocompatibility of HA and TCP [5], increasing the rate of osteoblast infiltration *in vitro*. Collagen coating may therefore increase the rate of osseointegration of calcium phosphate ceramic implants *in vivo*, but before advocating this it would be desirable to assess the effect of collagen coating on the mechanical strength of the ceramics. In this study we have cultured osteoblasts on composite ceramics composed of HA and TCP blends coated and uncoated with collagen and determined the effects on their mechanical properties over a period of 28 days.

Methods

Preparation of the materials for culture experiments

The ceramic materials used in the experimental work were HA, TCP and composite materials of the two in the following proportions: 75:25 (H75), 50:50 (H50), 25:75 (H25). These were produced and generously supplied by Hi-Por Ceramics (Sheffield, UK). The materials were supplied in the form of discs of their commercial product

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Synhapor™. Discs were 12 mm in diameter by 5 mm in height, to the following specifications: mean pore size: 200 μm; pore size range: 50–500 μm; porosity: 82–86%; density: 0.44–0.56 g/cm^3. The materials were sterilised by autoclaving. Discs were coated with a preparation of type-I collagen extracted from rat tail tendons according to the method of Elsdale and Bard [6]. A modified version of the pressure/flow method developed previously [5] was used for coating the discs. Heat-shrinkable 10 mm polyolefin tubing (Maplin, F-100, YR17T) was cut into lengths of approximately 4 cm and one end stretched slightly, in order to accommodate the disc, before being sterilised by soaking in ethanol for 30 s to 1 min and air-drying in a laminar flow hood overnight. A sterile disc was then inserted into the end of the tubing and the tubing shrunk around the disc by the application of heat from a hot-air gun. The disc-tubing assembly was then placed in a sterile, capped 15 ml conical-bottomed polypropylene tube and microwaved at 800 W for 5 min to ensure sterility. The disc-tubing assembly was attached to a sterile 1 ml syringe through an adaptor and a 3-way valve, the third outlet of which was connected to a 2 μm air-filter. 2 ml of sterile type-I collagen solution (4.68 mg/ml in 1% acetic acid) was pumped repeatedly up and down through the disc for a period of 2 min. Excess collagen solution was expelled using air drawn into the assembly through the filter. The disc-tubing assembly was then placed back in the 15 ml tube for centrifugation at 3000 rpm for 1 minute to ensure that excess collagen solution was completely removed from the disc-interior. The disc was recovered from the tubing and dried overnight in the flow hood.

Measurement of the retention of the collagen coating

In order to investigate the retention of the collagen coating, discs were incubated in 1 ml of phosphate buffered saline (PBS, pH 7.4) for 1 week at 37°C. Solution was then recovered from the discs by centrifugation and stored for subsequent analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In order to assess how much collagen adsorbed onto the discs, the coating was stripped off using a solution of 1% SDS (w/v in dH_2O). Coated discs were incubated overnight at room temperature in 1 ml of SDS solution, which was then pumped through the disc-tubing assembly for 5 min. Recovery was maximised by centrifugation of the disc. This procedure was repeated for a total of 5 washes. The recovered solution was stored at −20°C for subsequent analysis by SDS-PAGE.

SDS-PAGE was carried out using a Bio-Rad Mini-Protean II™ protein mini-gel apparatus, according to the manufacturer's instructions. Following electrophoretic separation, proteins were visualised by staining with Coomassie Brilliant Blue R-250. Destained gels were scanned using a Bio-Rad GS 690 Imaging Densitometer and analysed using ScionImage image analysis software.

Culturing the FFC rat osteoblasts on the ceramics

Immortalised rat osteoblasts (FFC cells) were used in these experiments. These cells were derived by T-antigen transfection of primary neonatal rat calvarial osteoblasts and were propagated as described previously [7]. Sterile discs were placed in the wells of sterile 24-well tissue culture plates, pre-wetted with 100 μl of complete Dulbecco's Minimum Essential Medium (DMEM) containing 10% foetal calf serum (Sigma Chemical Co) and incubated at 37°C for 30 min −1 h to allow the medium to fully penetrate the disc. Cells (2.26×10^4), suspended in a volume of 200 μl of DMEM, were seeded onto the surface of the discs and incubated for at least 10 min at 37°C to allow them to settle onto the surface and begin attaching. A further 1 ml of pre-warmed DMEM was then added, covering the discs to a depth of several mm. The plate was then incubated at 37°C in an atmosphere of 5% CO_2 in air. For the first week of incubation, the medium was aspirated and replaced with an equal volume of fresh, pre-warmed DMEM every second day. From the 8th day onwards, the medium was replaced daily.

Confocal laser scanning microscopy (CLSM)

Following 3 washes with PBS (pH 6.75), samples were stained with ethidium bromide (EtBr) (to visualise dead cells) and 5-carboxyfluorescein diacetate (CFDA) as previously described [8]. The samples were viewed in PBS pH 6.75, using a Leitz Aristoplan microscope, coupled to a Leica model 1A confocal laser scanning microscope, with a 25X, 0.75 numerical aperture water immersion lens. In order to determine the depth of cellular penetration into the materials, discs were preserved by fixing in 4% formalin at 4°C in the dark. Crude sectioning was carried out by bisecting the discs using a razor blade positioned across the middle of the flat surface and driving it through with a sharp blow from a hammer. Discs could then be up-ended and examined in cross-section by CLSM, following re-staining with EtBr.

Mechanical testing

Discs were tested to destruction by compression using an Instron model 4500 universal testing machine with a 10 kN load cell. The testing was controlled by computer, running Instron Series IX software (version 8.24.00). The machine crosshead speed was set to 1.0 mm/min and data were collected at a frequency of 10 Hz.

Statistical analysis

Student's t-tests were used to assess whether differences observed between groups were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. The confidence level was set at 95% for all tests, unless otherwise stated.

Results

Collagen coating of calcium phosphate ceramics represents a promising means of improving their osseointegration properties [9]. In addition to the effects on biocompatibility, any alteration in the mechanical properties of these materials must also be considered. To reveal the effects of collagen coating and culture with osteoblasts on the mechanical strength of the materials used in this study, discs were tested to destruction by compression. FFC cells were seeded onto collagen-coated and uncoated discs of HA, H75, H50, H25 and TCP and cultured for periods of 7, 14, 21 and 28 days. Specimens of each material at each time point, along with cell-free controls, were tested to determine the maximum load supported by discs prior to fracture.

The retention of the collagen coating on the various materials is another important consideration in the development of implants and this parameter was also examined.

Retention of the collagen coating

The retention of the collagen coating was examined by incubating coated discs in PBS for 1 week at 37°C, recovering the solution from within the discs by centrifugation and analysing the samples by SDS-PAGE. The amounts of collagen recovered, expressed as collagen eluted per gram of material, are shown in Fig. 1. These results show that the coating is most stable on HA and least stable on H50, with intermediate levels of stability on H25, H75 and TCP.

CLSM images of cells growing on material surfaces

Cells were visualised on the material surfaces by CLSM at each stage of culture, following staining with CFDA and EtBr. The images in Fig. 2(A) and (B) show cells growing on TCP (coated and uncoated) at 28 days of culture, demonstrating that the cells have effectively covered the ceramic surface by this stage. Fig. 2(C) shows a cross-section of the cell layer on the surface of coated HA following 21 days of culture. The main body of cells form a layer penetrating approximately 100 μm into the disc surface, although there are also scattered groups of cells at greater depths (not shown).

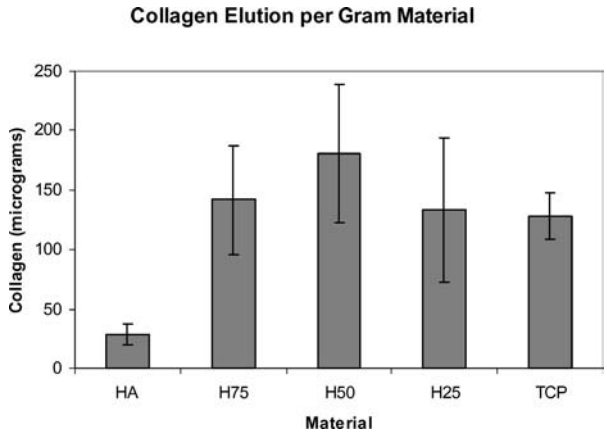

Figure 1 Retention of the collagen coating. Collagen eluted from the various materials expressed as micrograms of collagen released per gram of ceramic. Each experiment was repeated in triplicate and the results given are the mean values ±SD.

Mechanical strength of the materials

The results of the mechanical testing are shown in Fig. 3. The results show that the mechanical strength of these materials is affected by collagen coating and culture with osteoblasts and that the effects are highly dependent on blend composition.

HA: The coating process itself does not appear to affect the strength of the HA discs as there is no difference between coated and uncoated samples in the cell-free controls (see Fig. 3(a)). It appears, however, that osteoblasts do affect the material's strength, with a significant increase in the strength of uncoated cultured material over cell free day 0 controls. The strength of coated materials does not appear to increase upon culture with osteoblasts and shows a weak downward trend.

H75: The coating process weakens this material (see Fig. 3(b)). The strength of both coated and uncoated samples appears to increase following culture with osteoblasts, though the differences from cell free day 0 control values are not statistically significant.

H50: Collagen coating increases the strength of H50 (see Fig. 3(c)). As was the case with HA, the strength of uncoated material is also increased, relative to cell free day 0 controls, by culture with osteoblasts (to the point where it is similar to the coated material), while the strength of coated material does not seem to be affected.

H25: Collagen coating weakens H25 (see Fig. 3(d)). This material is unique amongst those tested in this study in that its strength was reduced by cell-free incubation (compare c0 and c28) and that culture with osteoblasts initially reduced the strength of uncoated specimens. This, however, appears to recover during the culture period, so that by day 28 the strength is not different from the cell free day 28 control. The

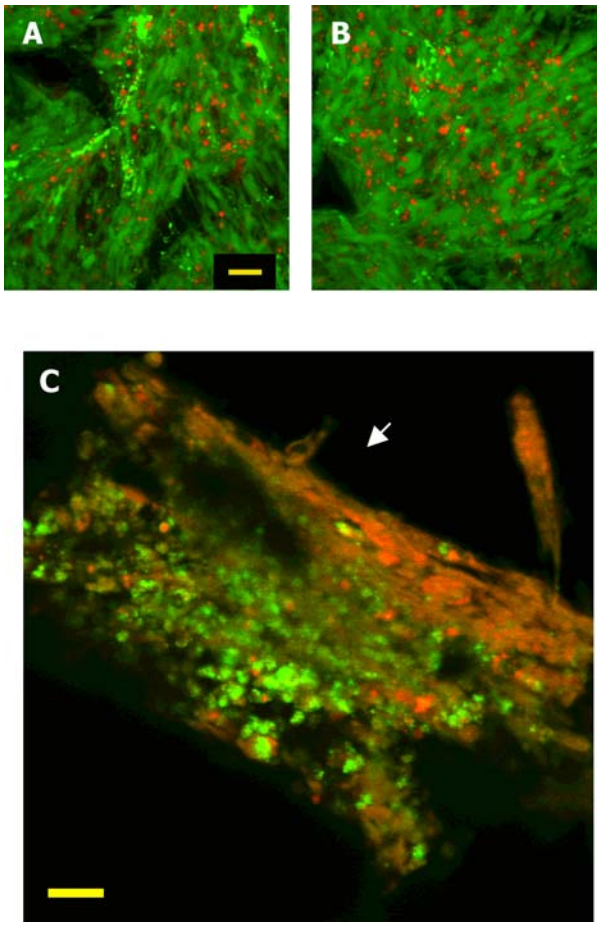

Figure 2 FFC osteoblasts growing on the material surface. (A, B). Uncoated (A) and coated (B) TCP discs following 28 days of culure. Live cells are stained green by CFDA and dead cells are stained red by EtBr. Voltage = 378/438 (red channel/green channel). (C). Coated HA following 21 days of culture. The cells shown above have previously been stained with CFDA/EtBr, followed by fixation and storage in 4% formalin. The disc was then split perpendicular to the original flat surface, restained with EtBr, and re-examined by confocal microscopy. This view therefore shows cells growing down into the disc in cross-section. The top of the disc is indicated by a white arrow. Voltage = 582/486. Scale bars (A, C) = 20 μm.

strength of coated material does not appear to be affected by osteoblast culture, and at day 28 the sample with osteoblasts is not significantly different from the cell free controls at the same time point.

TCP: The strength of the TCP discs is reduced by the coating process (see Fig. 3(e)), but there do not seem to be any significant strength changes engendered by culture with osteoblasts.

Discussion

We have previously demonstrated that collagen coating increases the rate of cell growth on calcium phosphate based ceramics [1–3]. CLSM images of the cells growing on coated and uncoated TCP showed that the surface of the discs was completely covered with cells by day

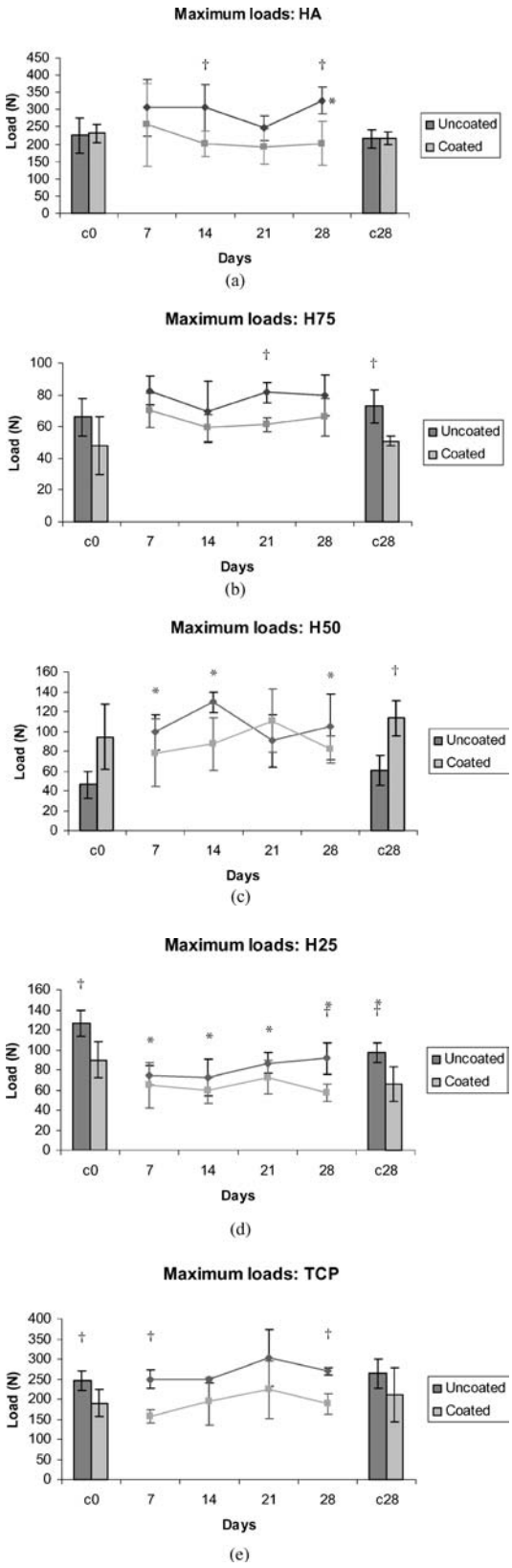

Figure 3 Maximum loads borne by discs following culture with osteoblasts. The values for coated and uncoated specimens are shown for each time point, along with the day o and day 28 cell-free controls, co and c28 (bars at either end of the x-axis). †Significant difference between coated and uncoated. *Significant difference from cell free day o controls (co). Confidence level = 95%.

28 (Fig. 2). Examination of HA discs after 21 days culture demonstrated that the cell growth on the surface of the disc was 80–100 μm thick. Furthermore, there were scattered groups of cells throughout the disc interior, showing that enough oxygen and nutrients were able to diffuse through the porous structure of the ceramic to support cell growth albeit on a limited scale. *In vivo* the process of vascularisation would further support cellular ingrowth into the implant by providing active transport of nutrients.

Collagen coating may prove to be a useful method of improving osseointegration *in vivo*, and the retention of the collagen coating on the ceramic is thus of paramount importance. The stability of the coating following incubation in PBS for 1 week at 37°C ranges from 95% for HA to approximately 80% for H50. The factors affecting coating stability on the ceramic surface are likely to involve a complex interaction between solubility, zeta potential, crystal structure and surface topology [11]. It may prove possible to increase stability through collagen crosslinking. However, before advocating collagen coating of ceramic implants as a means to enhance osseointegration *in vivo* it is essential to determine the effect of the collagen coating on material strength.

In the present investigation the effects of collagen coating and osteoblast growth on the materials were determined. Coated and uncoated discs were tested to destruction by compression following culture with FFC osteoblasts, along with day 0 and day 28 cell-free controls, in order to distinguish the effects, if any, caused by coating, cell growth and incubation in tissue culture medium. To our knowledge, the literature does not contain any studies dealing with the mechanical properties of calcium phosphate ceramics following *in vitro* culture with osteoblasts. The interactions between collagen coating, osteoblast culture and mechanical strength observed in this study were complex and depended on the composition of the material. Significant differences were seen between both coated and uncoated materials and between specimens cultured with osteoblasts and cell-free controls, indicating that both processes affect the strength of the materials.

For HA, the cell-free controls show that neither the coating process itself nor the 28-day incubation in DMEM affect the strength of the material. Rodriguez-Lorenzo and coworkers also noted that the mechanical strength of HA was maintained following incubation for 21 days in simulated body fluid [12]. The growth of osteoblasts does, however, result in an increase in strength of the uncoated material though, intriguingly, this is not the case on the coated material. This difference between coated and uncoated specimens is surprising, as we have previously reported that osteoblasts proliferate more quickly on the coated material during the first 7–14 days of culture [9]. This might be expected to enhance the increase in strength engendered by osteoblast activity and indeed, collagen-coated tissue culture plastic has been reported to support higher levels of mineralisation [13]. One possible explanation is that lower pH levels, caused by the increased cell numbers on coated specimens (it was observed when changing the medium during the culture period that wells containing coated discs acidified more rapidly), caused some weakening of the material, as acidic conditions have been reported to do [14].

In the case of H75, collagen coating does weaken the material, with significant differences between coated and uncoated specimens on the cell-free controls at day 28 and on cultured specimens at day 21. Neither the coated nor the uncoated specimens, however, show significant differences between cultured and control samples. As was seen for HA, the strength of cell-free control samples was not affected by incubation in DMEM.

H50 is somewhat unique among the materials tested in this study, in that its strength was actually increased by collagen coating. It does, however, share the feature with HA that the strength of uncoated samples is increased, relative to controls, by culture with osteoblasts, while coated materials are not affected. In common with HA and H75, the strength of the controls did not vary across the culture period.

H25 is the only material examined in this study that was actually degraded by culture in DMEM, with cell-free controls showing a significant reduction in strength by day 28. The uncoated material is also weakened by culture with osteoblasts, although this appears to stabilise so that the cultured specimens, though significantly weaker than the day 0 controls, are not significantly different from the day 28 controls.

The pattern for TCP replicates that seen for H75. The material was weakened by the coating process, but neither incubation in tissue culture medium nor culture with osteoblasts affected the strength of the samples.

The effects of the various parameters examined in this study on material strength are summarised in Table I. Our results indicate that ceramic composition affects the interactions between collagen coating, culture with osteoblasts and strength of the material. Culture with osteoblasts significantly increases the strength of uncoated HA and H50, but this effect is not observed when the materials are coated with collagen. Although collagen

TABLE I Summary of significant effects on mechanical strength caused by collagen coating (coating), incubation in DMEM (soaking) and culture with osteoblasts (osteoblasts). ↑ Indicates increased strength. ↓ Indicates decreased strength. – Indicates no change in strength. u = uncoated. c = coated.

	HA		H75		H50		H25		TCP	
Coating	–		↓		↑		↓		↓	
Soaking	–	–	–	–	–	–	↓	–	–	–
Osteoblasts	↑	–	–	–	↑	–	↓	–	–	–
	u	c	u	c	u	c	u	c	u	c

coating has been shown to increase the proliferation of osteoblasts on the materials, it may be necessary to stabilise and optimise the coating process to achieve biocompatibility and mechanical strength.

Acknowledgments

JCB was in receipt of an EPSRC studentship. The assistance of David Smith with the Instron testing is acknowledged.

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Received 11 June 2004
and accepted 25 May 2005