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Biomimetic hydroxyapatite coating on glass coverslips for the assay of osteoclast activity in vitro

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Abstract The osteoclast (OC) is the cell type responsible for the resorption of bone. The activity of this cell is important in the aetiology of a large number of skeletal pathologies, and also for the biocompatibility and osseointegration of orthopaedic implant materials. OC mediated acid hydrolysis of calcium phosphate from the bone matrix offers a prime means of studying the biology and activity of this cell type. We have developed a method of coating glass coverslips with a hydroxyapatite (HA)-like mineral, using a biomimetic approach. Hydroxylation followed by formation of a self assembled monolayer (SAM) using the surfactant triethoxysilylpropyl succinic anhydride (TESPSA), allowed biomimetic deposition of HA-like mineral from a simulated body fluid (SBF). The biocompatibility of the TESPSA SAM-HA coated glass coverslips was tested by culturing human mature OC present in samples of giant cell tumour of bone (GCT). Parameters of OC activity were assayed, including F-actin ring formation, release of calcium and formation of osteoclastic resorption pits, confirming that OC were able to attach to and resorb the coated surface. This approach for the preparation of HA coatings on glass coverslips could have wide applicability for the study of osteoclast behaviour in vitro.

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1 Introduction

The osteoclast (OC) is the principal cell responsible for the resorption of bone during bone remodelling [1]. Bone remodelling is critical for skeletal development and integrity, as well as for fracture repair, tooth eruption and calcium and phosphate homeostasis [2]. Many skeletal diseases involving low bone density and mass, such as osteoporosis, rickets, rheumatoid arthritis, periodontal disease, Paget's disease and cancer-related bone loss, are due, at least in part, to excess osteoclastic activity, which leads to an imbalance in bone remodelling that favours resorption [1, 3]. Excessive osteoclast activity also plays a role in peri-prosthetic (aseptic) loosening, an important problem in orthopaedics. Peri-prosthetic loosening primarily arises from osteoclastic resorption of surrounding bone. The major cause of this excess osteoclastic differentiation and activity appears to be the cellular response to wear particles of the prosthetic material(s), including metal, ceramic and polyethylene (PE) components [4–7]. Thus, the biology of the OC and measuring its activity is of considerable interest to biomedical research. Furthermore, there is great demand for model culture systems with which to study bone cell activity, and a need to study the interactions of bone cells with potential biomaterials.

Whilst the enzymic marker, tartrate resistant acid phosphatase (TRAP) is a common histochemical means for identifying OC-like cells, the single most indicative measure of the activity of OC is their ability to resorb

mineralised substrates. OC resorb bone by dissolving the hydroxyapatite (HA)-like calcium phosphate mineral by secreting acid and breaking down the principally collagenous matrix [8]. In vitro cell culture assays based on measuring this principle generally utilise natural bone substrates, including cortical bone, usually either human or bovine, or dentine such as sperm whale tooth or elephant tusk. However, the use of natural substrates for this type of assay presents several disadvantages. Natural bone, while readily resorbed by OC in vitro, has a complex surface topography, often making it difficult to identify and quantify resorptive activity. While the surface topography of dentine or tusk is more uniform, these are often difficult to obtain. Osteoclastic resorption on natural substrates is also often irregular because these surfaces exhibit a degree of variability [9]. Additionally, their opacity makes imaging of OC on their surface difficult. Hence, specialist and expensive methods such as scanning electron microscopy are often required to visualise the degree of osteoclastic activity.

More recently, artificial substrates have become available that consist of culture wells coated with thin layers (approximately 0.6 μm) of HA (e.g. Osteologic^{TM} slides). These have the advantage of commercial availability and give higher reproducibility between replicate samples. Imaging of the OC is also easier as due to their relatively thin coatings and superior light transmission properties. However, the HA coating of such artificial substrates is usually produced by a commercial physical or chemical means, such as plasma spray or sol–gel techniques. A problem with these methods is that they do not always produce a high crystallinity in the HA coating, an attribute considered essential for achieving optimal bioactivity [10].

Whilst this problem may be overcome by thermal treatment of the HA coating [10], this precludes integration into coatings of proteins (e.g. collagen) and growth factors that can improve the bioactive characteristics of the HA surface. An alternative method for producing the thin HA required on these artificial substrates could be to exploit a biomimetic approach, reproducing the biological deposition of HA. During natural bone mineralisation, HA crystals in bone are formed when heterogeneous nucleation overwhelms homogeneous nucleation. Furthermore, bone mineral is normally assembled through the orderly deposition of apatite minerals within a type I collagenous matrix [11].

To replicate this natural process a biomimetic approach usually employs macromolecules—or self-assembled monolayers (SAM)—as templates on a surface to initiate and control crystal formation. A SAM molecule comprises a functional top group, an anchor end and an intervening alkyl chain. Once attached to a surface, they are highly ordered and oriented. A wide range of groups can be incorporated in both the alkyl chain and at the chain terminal [12]. The surface template formed by the SAM therefore allows direct synthesis of crystals by providing the surface with the requisite functionality for heterogeneous nucleation [13–15]. In the case of HA formation, the surface templated material is immersed in a simulated body fluid (SBF) or in a HA depositing solution, such as a calcium phosphate solution. Thus, the biomimetic approach creates the same nanoscopic and hierarchical structures present in natural bone through biological principles and the process of self-assembly [16]. Additionally, the entire process can be conducted under conditions similar to normal body temperatures, allowing the integration into coatings of proteins and growth factors whilst retaining their bioactive characteristics.

The use of biomimetic methods has already been successfully demonstrated for producing HA coating on materials such as Ti and glass for use with orthopaedic implants [13, 17]. Furthermore, it has been observed that negatively charged SAMs have better HA nucleating ability compared to positively charged SAMs [18]. We recently reported the relative performance of different SAMs for HA coatings on Ti [19]. A SAM containing a –COOH functional group produced a highly crystalline HA coating with crystalline characteristics most like bone [19]. Thus, the negatively charged self-assembled monolayer (SAM) is critical for successful biomimetic deposition of HA.

In this paper, we report on the quantification of OC resorptive activity and visualisation of OC morphology on thin layer HA coatings produced on glass coverslips by a biomimetic method using a negatively charged –COOH SAM.

2 Materials and methods

2.1 Preparation of HA coated glass coverslips

The preparation of the –COOH SAM and the HA coating on glass coverslips followed the synthesis procedure of Liu et al. [15], with modifications. All glassware was first washed with 1% v/v Extran^{TM} once, rinsed with tap water, subsequently with reverse osmosis water (RO water), and finally with milli-Q water (the tissue culture (TC) wash). The plastic boxes used for immersing sample holders in SBF were also subjected to a TC wash prior to their use and dried inside a biohazard class II hood under sterile conditions. Glass coverslips were of 13 mm diameter (Menzel GmbH & Co, Braunschweig, Germany) which fit in standard 24 well (15 mm well size) tissue culture plates. The –COOH SAM was synthesized on the glass coverslips by vertically immersing them for 20 h in a solution containing anhydrous toluene (Sigma Chemical Co., St. Louis, MO,

Table 1 Composition of simulated body fluid (SBF) in comparison to plasma. Adapted from Kokubu et al. [20]

Ion	Blood plasma (mM)		SBF (mM)
	Total	Dissociated	
Na^+	142.0	142.0	142.0
K^+	5.0	5.0	5.0
Mg^{2+}	1.5	1.0	1.5
Ca^{2+}	2.5	1.3	2.5
Cl^-	103.0	103.0	147.8
HCO_3^-	27.0	27.0	4.2
HPO_4^{2-}	1.0	1.0	1.0
SO_4^{2-}	0.5	0.5	0.5

USA) and 10% v/v Triethoxysilylpropyl succinic anhydride TESPSA (Gelest Inc., Morrisville, PA, USA), as described by Toworfe et al. [14]. The coverslips were then rinsed in toluene (Sigma) and subsequently in milli-Q water and sonicated in sterile milli-Q water for 1 h, in order to remove excess surfactant molecules on the surface of the coverslips.

The composition of the SBF solution, identical to that employed by Kokubo & Kawashita [20], is shown in Table 1. SBF was first sterilised by filtration through 0.2 μm filter (VacuoCap® 60, Gelman Laboratories, Melbourne, Australia). The TESPSA SAM coated coverslips were loaded onto 316 stainless steel holders and immersed in the sterile boxes filled with SBF at 37°C for 18 days. The coverslips were then removed, rinsed with RO water and dried inside a biohazard class II hood under sterile conditions.

2.2 Analysis of SAM layer & HA coating

Successful application of the SAM layer on the glass coverslips was confirmed by water contact angle measurement and FTIR analysis. The static contact angle of water on the SAM-coated glass coverslips was measured at 25°C using a proprietary contact angle goniometer (Ian Wark Research Institute, Adelaide, Australia) by placing a 10 μl drop of distilled water on the wafer surface. The droplet shape was imaged with a video camera and contact angle calculated by proprietary software (Ian Wark Research Institute, Adelaide, Australia).

FTIR spectra of the SAM-coated glass coverslips were collected in attenuated total reflection (ATR) mode with a Nicolet Magna-IR 750 spectrometer (Thermo Electron Corporation, Massachusetts, USA) between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}. Background measurements were performed with KBr powder (Sigma).

The presence and uniformity of HA layer on the SAM-coated glass coverslips (following immersion in the SBF) was verified by direct observation of surface topography by scanning electron microscopy (SEM, XL20, Philips) with the working distance adjusted to 33 ± 0.1 mm for each sample.

2.3 Osteoclast culture experiments

In order to test human OC resorptive activity, we utilised the active mature OC found in giant cell tumours of bone (GCT), the activity of which is regulated in a similar way to non-tumour derived OC [21–23]. GCT cells were obtained and processed from a patient as previously described [21]. GCT cells were seeded onto glass coverslips in 24-well tissue culture microplates (8×10^5 cells/well), in αMEM medium (SAFC Biosciences, Lenexa, KA, USA) containing 10% v/v foetal calf serum (FCS) (MultiSer™, Thermo, Melbourne, Australia), penicillin, gentamycin and L-glutamine. As a positive control, cells were also plated onto sterilised sections (4 × 4 × 0.1 mm) of sperm whale dentine (SA Whale Centre, Victor Harbour, SA, Australia).

2.4 F-Actin ring formation

To examine the ability of OC to form an F-actin ring, a prerequisite for osteoclastic resorption of a mineralized substrate, GCT OC were cultured for 24 h on the various substrates. Culture media were aspirated and the cells rinsed three times with PBS and then fixed with paraformaldehyde (4% in PBS) for 20 min. Subsequently, the cells were washed again with PBS and were further permeabilised with Triton X-100 (0.1% in PBS) for 5 min on ice. Following an additional washing step as described above, the cells were blocked with 5% v/v normal goat serum (IMVS Animal Services, Gilles Plains, SA) for 1 h at ambient temperature. Cells were then stained with phalloidin-TRITC (25 μg/ml; Sigma) diluted in 1.5% w/v bovine serum albumin (BSA) in PBS for 1 h in the dark at room temperature. Following a further washing step as described above, the cells were stained with the nuclear stain, 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 1 mg/ml in methanol, Roche Diagnostics, Castle Hill, NSW, Australia) for 5 min in the dark at room temperature. The cells were washed again and fixed in 1% w/v paraformaldehyde, 1% v/v formalin in PBS for 10 min. The cells were washed once more, and the coated coverslips were mounted inverted onto 30 mm coverslips using ProLong® Gold Antifade reagent (Molecular Probes™, Invitrogen). The coverslips were finally sealed with nail polish and the cells examined for F-actin ring formation by dual laser confocal microscopy (Nikon D-Eclipse C1).

2.5 Colorimetric assay for Ca^{2+} concentration

As a rapid and quantitative readout of OC activity, a technique used routinely to measure osteoblast in vitro mineralization [24] was adapted to measure the calcium release from the HA substrates by OC into the culture medium. After growing GCT cells on coated the coverslips for 2 days, media were collected and stored at 4°C until they were assayed. Soluble calcium levels were determined using a colorimetric assay, as per manufacturer's instructions (TRACE Laboratories, Melbourne, VIC, Australia). Calcium standards ($CaCl_2$) were diluted to create a standard curve. The UV absorbance at 570 nm was measured using a MR7000 microplate reader (Dynatech Laboratories, Guernsey, Channel Islands).

2.6 SEM analysis for osteoclastic resorption

To assess OC activity, samples were examined by SEM. For this, coverslips or dentine were dehydrated in pure ethanol and mounted onto stubs and coated with carbon-gold, as we have previously described [22]. Samples were analysed on a Philips XL-20 scanning electron microscope.

3 Results and discussion

3.1 Analysis of the SAM layer and HA coating

The water-contact angle of the SAM-coated glass coverslips was 32.8°, consistent with that reported for COOH-functionalized SAMs [14]. The FTIR spectra (not shown) for the SAM-coated coverslips revealed a clear peak at 1710 cm^{-1} indicating the presence of C=O bonds in saturated carboxyl groups. SEM analysis verified that a uniform HA layer was successfully formed on the glass coverslips (Fig. 1).

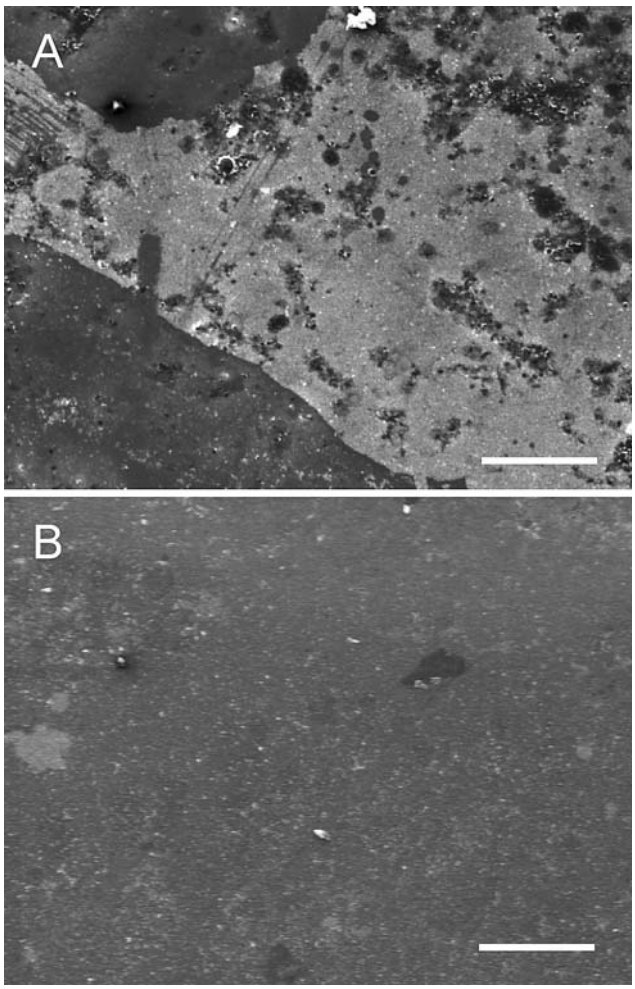

Fig. 1 SEM analysis of **a** unmodified glass surfaces and **b** HA coated glass surface. Original magnification = 50×

3.2 Osteoclast activity

Figure 2 shows the F-actin ring formation observed by confocal microscopy of GCT OC cultured for 24 h on untreated glass, HA coated glass and whale dentine. The formation of the F-actin ring is an essential prerequisite for proper osteoclastic resorption of a mineralised substrate, allowing a tight junction to form between the bone surface and basal membrane of the OC. This junction is termed the sealing zone, and is characterised by several types of adhesion interactions, including that of $\alpha v\beta 3$ integrin adhesion to bone matrix proteins that contain specific arginine-glycine-asparagine (RGD) sequences [25]. Evidence suggests that a true sealing zone forms only when an OC is in contact with HA crystals [26]. Protons and proteases are secreted into the sealing zone to resorb the mineralised collagen matrix [1, 2, 27]. In the absence of a proteinaceous substratum, the F-actin ring appears as a condensed region of actin at the periphery of the OC plasma membrane [27]. As shown in Fig. 2, after 24 h of culture, mature OC present in samples of GCT formed clear F-actin rings at their periphery when cultured on HA-coated glass. In contrast, OC did not form visible F-actin rings when cultured on non-SAM coated glass. As expected, classical F-actin rings were evident when cells were cultured on dentine. These observations suggest that TESPSA SAM-HA coatings provide a suitable mineralised substrate for OC resorption activity. Culture supernatants from OC plated onto the coverslips was performed using a colorimetric assay for calcium. Figure 3 gives the calcium concentration in supernatant for HA coated glass coverslips, with or without OC after 2.5 days of incubation. The analysis revealed a significant release of

calcium by the OC compared to the no cell control (Fig. 3), demonstrating that OC functionality is intact on mineralised glass surfaces, and that the colorimetric detection of calcium is a viable method for measuring OC activity.

The visual hallmark of osteoclastic resorption is the formation of resorption lacunae or pits, which in 2-dimensional images appear as circular clearings, or as trails if formed by motile OC [8]. OC were cultured on the HA coated coverslips for 3 days before the surfaces were investigated using SEM. Figure 4 presents the SEM images of OC resorption of HA after 3 days of culture on whale dentine and the HA coated coverslips. On HA coated coverslips without SAM, true OC activity was difficult to distinguish. However, on both dentine and on TESPSA SAM-HA coated coverslips, characteristic resorption pits and trails were evident. A clear difference in resorption pit size was observed between those on dentine and those on TESPSA SAM-HA coated glass. Resorption pits on dentine measured approximately 15 μm in diameter, whereas those on TESPSA SAM-HA substrate were up to three times this size. This size difference is consistent with the larger F-actin rings observed on TESPSA SAM-HA, as the

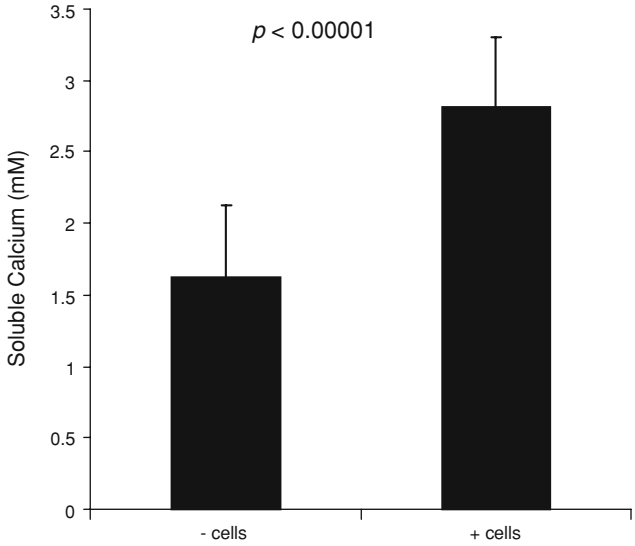

Fig. 3 Calcium concentration in supernatant after incubation of HA coated glass coverslips, with of without GCT-OC for 2.5 days. Ca^{2+} concentration was determined using a colorimetric assay, as described in Sect. 2. Data shown are means of 6 replicates±standard deviation. Significance (indicated) was determined using Student's *t*-test (2-tailed, unpaired)

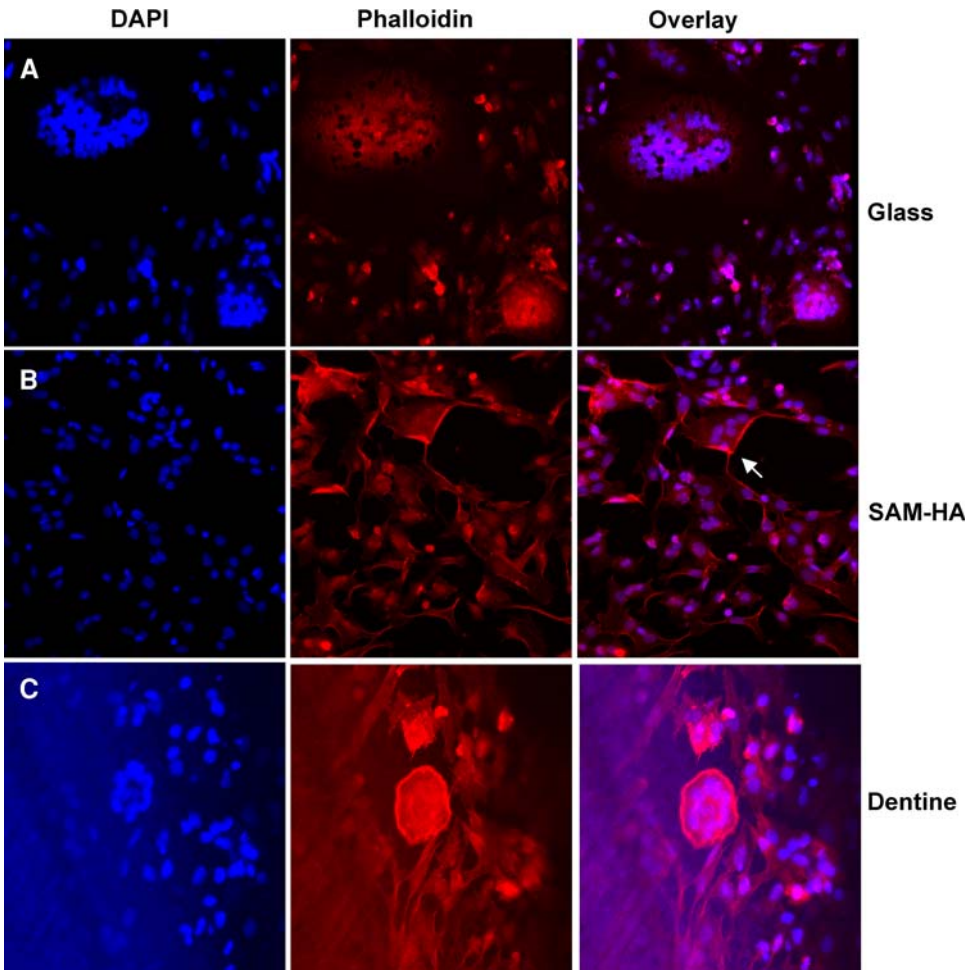

Fig. 2 F-actin ring formation by GCT OC, cultured for 24 h on **a** glass **b** HA coated glass, **c** whale dentine. Shown are representative fields generated by confocal microscopy, of nuclei stained with DAPI, phalloidin-TRITC stained actin, and the merged image for each field. Images were taken using a 20× objective

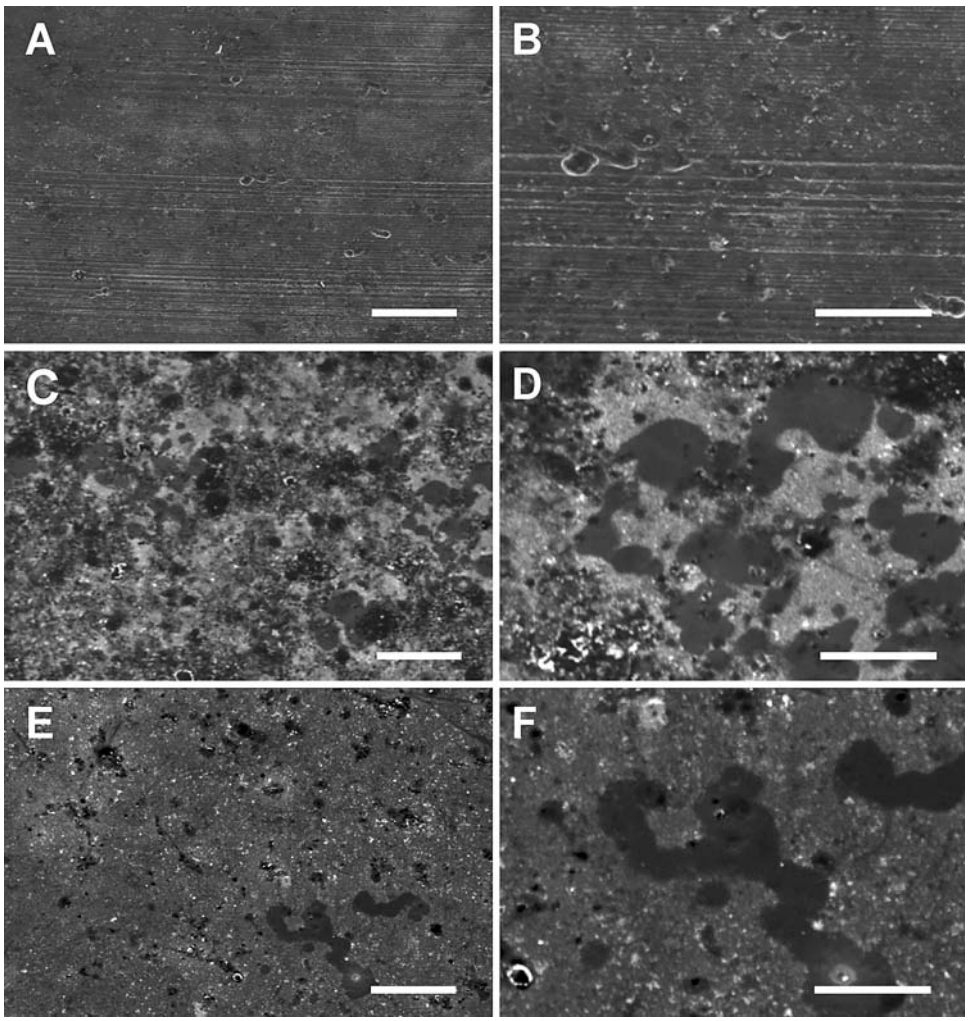

Fig. 4 SEM images of OC resorption of HA after 3 days of culture on **a** whale dentine (200× magnification) and **b** at 400× magnification; **c** HA coated without SAM coverslip (200× magnification) and **d** at 400× magnification; **e** TESPSA SAM-HA coated coverslip (200× magnification) and **f** at 400× magnification. White bars in **a**, **c** & **e** represent 200 μm, and in **b**, **d** and **f**, 100 μm

F-actin ring defines the size of the sealing zone, which borders the resorption space itself. The larger size is likely partially due to the thinner resorbable layer of HA on the SAM coated coverslips compared with that on dentine, the slices of which were approximately 0.1 mm thick. Another likely contributing factor for this observation is the absence of protein incorporated into the biomimetically deposited HA, and hence the absence of $\alpha v\beta 3$ integrin adhesion to bone matrix proteins containing RGD sequences [25]. Therefore, the OC is presumably less strongly anchored to the surface and so can migrate and spread more freely, giving rise to longer resorption trails. Together, the results from this study indicate that OC attach to and efficiently resorb biomimetically deposited mineral. Since matrix proteins such as type I collagen, fibronectin and laminin deposit spontaneously onto glass surfaces, it should be possible to incorporate protein matrices of choice into TESPSA SAM-HA surfaces to allow the study of OC activity on customised substrates.

4 Conclusions

In summary, this study describes a relatively simple and inexpensive method for generating HA coatings through a biomimetic route on TESPSA SAM coated coverslips for the assay of OC function. The use of coated glass, as developed in this study, will allow a more extensive study of OC activity and the interaction of these cells with their substrate, by virtue of its excellent light transmission properties. In addition, our results imply that biomimetically produced HA coatings on orthopaedic implants, for the purpose of repair of bone defects for example, have the potential to provide direct interaction with OC and may therefore promote osseointegration through bone remodelling.

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