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A novel method for producing electron transparent films of interfaces between cells and biomaterials

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Abstract Transmission electron microscopy (TEM) investigations of intact interfaces of cells and brittle biomaterials have proven difficult using common TEM preparation techniques. This paper describes a technique to fabricate thin sections for TEM investigation of intact interfaces between human monocytes and sintered hydroxylapatite by the use of focused ion beam (FIB) microscopy. The interfaces were examined using energy filtered TEM.

Introduction

The use of biomaterials to repair damaged structures is continuously growing with new indications and new materials. The success of a biomaterial is often determined at the zone of encounter between the material and the living tissue [1, 2]. Today, the interactions between biomaterials and cells are often studied in various in vitro systems. The cell response can be measured in many ways, e.g. cell viability and cell number. Cells can also be stabilised and imaged on the surface using scanning electron microscopy (SEM). However, high resolution imaging and analysis of interfaces usingTransmission electron microscopy (TEM) is very difficult. Partly because of the ultramicrotome technique's poor performance of cutting hard materials and its lack of precise selection of areas for TEM sample preparation [3]. It is of major importance for the scientific community to resolve the intact interface between cells and materials to be able to describe the adhesion and activation mechanisms in detail. Such descriptions would allow a better design of new biomaterials depending on the clinical use.

Focused ion beam (FIB) microscopy has been used within the materials science community to fabricate thin sections for TEM for some years [4, 5]. A FIB system scans a beam of positively charged gallium ions over the sample, similar to the electron beam in the SEM. More significantly, it is possible to increase the beam current of the primary ion beam and use the FIB as a fine-scale micro-machining tool. Although well known in the materials science community, the FIB technique to produce TEM samples of biological samples has not been fully explored. Recently the technique was used to produce TEM specimens of the interface between calcium aluminate ceramic and metallic implants and bone [6, 7]. This paper describes a method to fabricate thin sections of intact interfaces between human monocyte-like cells and brittle ceramic materials by using FIB.

Materials

The ceramic samples used in this study were prepared from commercially available hydroxylapatite (HA) powder. The

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powder was mixed with organic additives in a solvent and after mixing, drying and sieving, the powder was granulated. The granules were uniaxially pressed, and subsequently the resulting green bodies were sintered at 1,300 °C for 1 h in air to receive 99% dense ceramic discs. The surface roughness Ra ranged from 0.2 μm to 0.5 μm.

The human monocyte-like cell line, U-937 (DSMZ Deutsche Sammlung für Mikroorganismen und Zellkultur, Germany), was cultured in RPMI-1640 (Sigma, Germany) supplemented with 10 vol% fetal bovine serum (FBS, Sigma, Germany) and 1 vol% penicillin/streptomycin (Invitrogen, Germany) at 37 °C. Cells were grown as a suspension culture in 25 cm^2 culture flasks (Nunc, Denmark). After the cell culture became dense, cell suspension was centrifuged with 250*g* for 5 min. The supernatant was removed and the cell pellet was dissolved in fresh culture medium. Before cell seeding, the HA samples were cleaned by soaking in Extran (Merck, Germany) and SDS (sodium dodecyl sulphate, Sigma, Germany) solutions. Afterwards they were sterilised at 134 °C in an autoclave (Systec, Germany) and placed in a 24-well cell culture plate (Greiner, Germany). Thermanox® plastic coverslips (Nunc, Denmark) were used as standard materials.

U-937 cells were seeded at a concentration of 1,00,000 cells per mL. After 10 days the supernatant was discarded to remove the non-adherent cells. The adherent cells were cultured for 21 days. After 21 days the cultures were washed with PBS. Cells were fixed with a solution containing 3 vol% glutaraldehyde (Sigma, Germany) and 3 vol% paraformaldehyde (Sigma, Germany) in 0.2 M sodium cacodylate buffer and rinsed 3 times with PBS. For cell observation using scanning electron microscopy, all samples were dehydrated in a graded acetone series (10, 30, 50, 75, 90, 95, 98 and 100 vol%). Samples were maintained at 100 vol% acetone and were dried at critical-point. The samples were sputtered with gold before being further examined using FIB and TEM. The FIB used in this study was a dual-beam FEI Strata DB235 and the TEM images were taken in a FEI Tecnai F30 S-Twin TEM equipped with a Gatan Imaging Filter (GIF) and an acceleration voltage of 300 kV.

The method

Suitable samples/areas for microscopy were first examined using the non-destructive electron-beam in the FIB (Fig. 1a). TEM samples from the chosen areas were produced using FIB as follows. Initially, a thin layer of platinum (Pt) was deposited on top of the area to be thinned using the electron-beam, for protection of the topmost surface of the TEM sample from artefacts induced by the ions. A thicker layer was grown on top of this using the ion-beam for further protection and mechanical stability of the TEM sample. Trenches were then cut from both sides of the Pt-coating until a sheet with the area of interest was left (Fig. 1b). The sheet was then cut out with the ion-beam and subsequently lifted out and mounted on a TEM grid

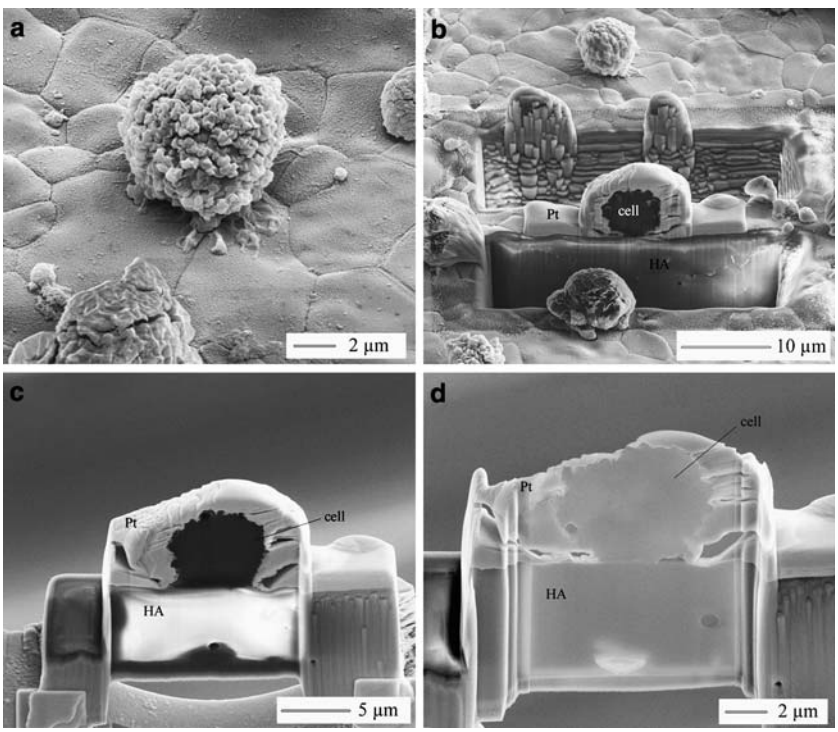

Fig. 1 SEM-images of the sample preparation process in the FIB showing: (**a**) localization of suitable specimen/area, (**b**) Pt-deposition and cutting of trenches, (**c**) attachment to TEM-grid and (**d**) finished TEM-sample

inside the FIB microscope using a fine needle made of tungsten (Fig. 1c). Finally, the sheet was milled to electron transparency in the FIB, normally 50–100 nm thick (Fig. 1d).

In order to obtain a smooth surface, free of interference from the different interfaces (i.e. Pt/cell/ceramic), the thinning of the sheet was performed in a stepwise manner. Initial rough polishing was performed using a high ion-beam current of about 3,000 pA and final polishing at the end was performed with an ion-beam current of about 50 pA. A uniform thickness of the sheet was achieved by slightly tilting it from the parallel orientation to the incident ion-beam. At 3,000–1,000 pA a tilt of 1° was used and at 300–100 pA 1.2°. At the final milling step of 50 pA the tilt was set to 0.5°. The overlap of two successive ion-beam scans was 50%. The thickness of the sheet can be monitored throughout the thinning-process by constantly grabbing electron-beam images. When the sheet becomes sufficiently thin the thickness of the Pt-layer is reduced quite rapidly and the thinning can be aborted.

A cell/HA-interface was briefly investigated in the TEM, operated in energy filtered (EF) mode for enhanced contrast in the images without staining [8]. Figure 2a shows a zero-loss image acquired using an electron energy loss of 0 ± 10 eV and the so called pre-carbon image in Fig. 2b was acquired using an electron energy loss of 250 ± 10 eV [9]. A 3 mm GIF entrance aperture slit and a 30 μm objective aperture was used during acquisition. Both images show a structurally modified HA surface layer of about 200 nm just beneath the cell interface. The HA grain size is much larger than the image and therefore only one HA grain is present in Fig. 2. Future studies will involve more detailed analysis of the interfaces, e.g. cell staining and elemental analysis, for deeper understanding of the adhesion mechanisms and cell/material interactions.

Summary and conclusions

The present technique of ion beam sectioning provided intact interfaces between synthetic hard materials and

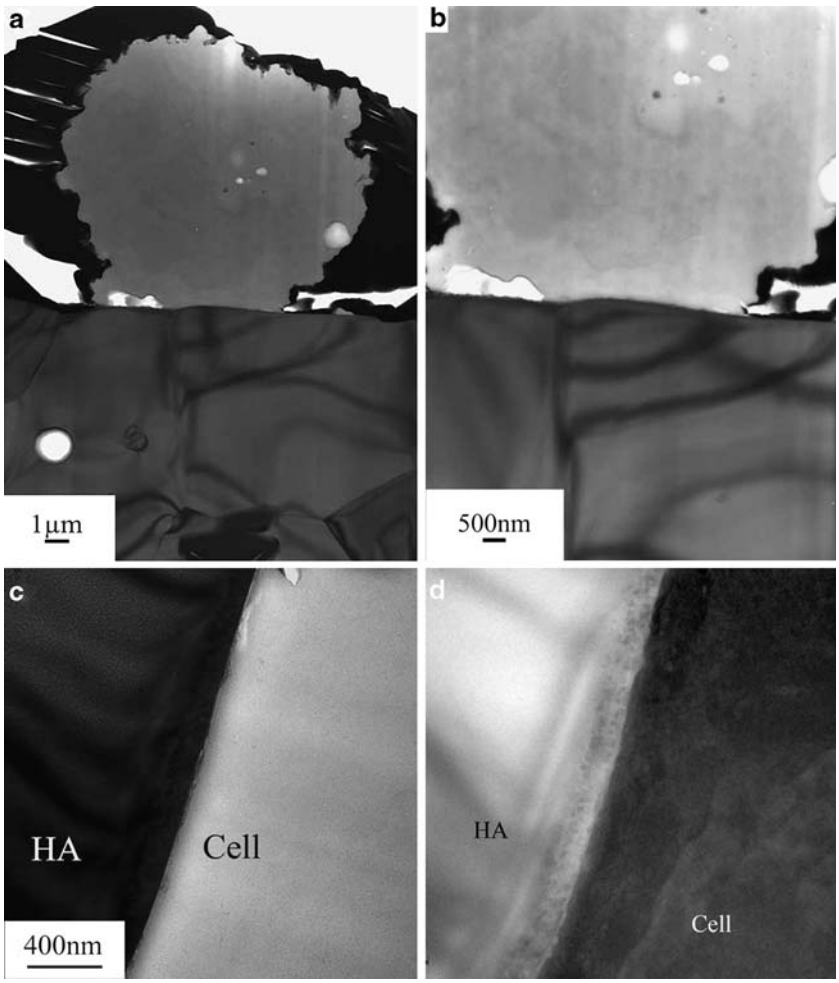

Fig. 2 (**a**) Overview image of the thin section incorporating cell and substrate, (**b**) high magnification of the interface, (**c**) zero-loss image of the HA-cell interface and (**d**) a pre-carbon image of the same area

preserved cells, allowing a solution to the previously regarded insurmountable problem of achieving ultra-thin sections. Despite large differences in sputtering rates and hardness among the materials the resulting thickness is uniform, due to the almost parallel scanning of the ion-beam in the thinning process. This in combination with the precise control of area for specimen preparation makes this technique very useful for studying cell/material interfaces. It is foreseen that the technique will provide the scientific community with a method to further understand the interface between tissue and biomaterial.

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