Microscopy at the life sciences / physical sciences interface

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Abstract. Procedures for investigating biomedical implant surfaces and biological cell / surface interactions in plan-view and cross-sectional geometries are described. Ultramicrotomy, FIBSEM and cryogenic-FIBSEM techniques, as developed for the controlled cross-sectioning of human osteoblasts on Ti substrates for TEM investigation, are compared.

1. Introduction

The four basic design considerations for load bearing biomedical implants are biocompatibility, corrosion resistance, tribological performance and the matching of mechanical properties. Such design considerations have led to many exciting advances in ceramic, polymeric or composite-based biomaterials in recent years, although it is recognised that the vast majority of biomedical implants are still metallic-based, covering a wide range of sites and time dependencies in the body. In particular, Ti-based alloys are made interesting due to their beneficial osteo-conductive properties, with the development of native passivating oxide or hydroxide surfaces that continue to grow within the body, helping to provide protection against metal ion release [1]. Such alloys may be used within orthopaedic devices such as hip and knee joints and bone fixation plates, along with heart valves, maxillofacial implants and dental implants, and even though there is a huge industry dedicated to the application of biomedical implants, better understanding of how living organisms interact with foreign material is still required, in order to progress the development of biomaterials with appropriately designed surfaces.

The process of introducing a surface-modified, foreign material into the body often seeks to promote controlled cell differentiation and proliferation, with the aim of stimulating rapid tissue repair. However, it is recognised that body tissue combines many functions, e.g. the partitioning of biochemical reactions, the transmission of mechanical loads and neurological signals, and the provision of energy sources, in addition to incorporating mechanisms for functional repair. The cellular level, in particular, introduces complicated associations of proteins, nucleic acids, polysaccharides and lipids that adopt 3D conformations according to the local chemical environment. In this context, the initial stage of bio-integration is concerned with the sequential process of protein layer adsorption, biomolecule absorption, cell interaction and tissue integration. However, close

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The challenge is to tailor the implant with appropriate levels of bioactivity (or bioinertness) depending on the intended application.

By way of example, research on the controlled modification of Ti-based alloy surfaces has shown clear differences in the biological cell response to surface chemistry [2]. The surface modification process of shot peening (grit blasting), presently favoured by the bioengineering industry, can lead to the amorphisation of surfaces, in turn offering better corrosion resistance behaviour [3]. Further, simple heat treatments in air can be effective in reducing metal ion release, e.g. reducing platelet activation or the oxidative stress of endothelial cells [4]. Other approaches, such as highly polished surfaces, can have advantages in terms of sterilisation but may lead to other problems such as higher initial corrosion behaviour. Adding hard ceramic layers to prevent metal ion release is also an option.

In this context, there is need for an appropriate combination of complementary techniques to characterise (i) the interaction of biological cells with implant materials (in planar-view geometry), in terms of cell morphology, motility and viability (complementing genetic marker and cell assay-based investigations); (ii) the surface modified implant material in terms of morphology, crystal structure and chemistry in advance of introduction to the biological environment and in vitro (or in vivo) cell trials; and ideally (iii) the interfaces between biological cells and biomedical implant materials (in cross-sectional geometry), on the molecular scale. Here, we comment briefly on these various approaches to inform the design and development of biomaterials and biomedical coatings.

2. Overview of characterisation techniques
2.1 Planar view investigation of cells on surfaces

The combined techniques of environmental scanning electron microscopy (ESEM), high-vacuum SEM and confocal laser scanning microscopy (CLSM) provide valuable information on cell morphology and the internal actin distribution. ESEM investigation, in particular, when performed in environmental (wet) mode, allows the morphology of hydrated cells (Figure 1a) to be assessed in relation to the local chemistry of a material surface without the extraneous effects of the dehydration process (Figure 1b). The operation of the ESEM naturally creates a conducting pathway in the vicinity of an insulating material under examination due to exploitation of the ‘poor vacuum’ ambient and this obviates any concerns about charging effects during sample observation and analysis [5]. For the purpose of CLSM, fixed cells may be labelled using antibodies against vinculin to visualize focal adhesions, and FITC tagged phalloidin to visualize F-actin filaments, thereby giving an indication of the organisation of the cytoskeleton (Figure 1c).
Conversely, reflectance differential interference contrast (DIC) microscopy may be used for time lapse microscopy of cell motility on opaque surfaces (Figure 2), recognising that migration is essential for the colonisation of biomaterial scaffolds in tissue engineering and of orthopaedic implants. From images of viable cells crawling on a biomaterial surface, two types of sustained motion are generally observed: i) a steady gliding movement where the various crawling processes take place simultaneously and ii) a two-step movement where a slow lamellipod extension occurs, followed by adhesion and then translocation, detachment and retraction, in accordance with the cell crawling models of Abercrombe [6] and Alt and Dembo [7].

These combined planar-view techniques are ideally suited to the investigation of cell-surface associations, given that the process of cell migration over surfaces and through tissue is fundamental to many biological phenomena. Indeed, this approach has been used to inform the development of a mathematical model of cell crawling activity [8].

2.2 Characterisation of biomaterial surfaces and surface modified implants

Ideally, the biomedical implant surface should be fully characterised in terms of morphology, crystal structure and chemistry, in advance of cell seeding and in vitro cell trials. Hence, it is recognised that SEM, atomic force microscopy (AFM) and profilometry techniques are valuable for providing insight into surface morphology and roughness, whilst reflection high energy electron diffraction (RHEED) and X-ray photoelectron spectroscopy (XPS) are ideal for the assessment of the surface crystal structure and chemistry, respectively, complementing X-ray diffractometry (XRD) and energy dispersive X-ray (EDX) analysis in the SEM that provide for more ‘bulk’ information on the sample structure and chemistry. Given that the surface structure of the biomedical implant is very often graded, e.g. as a consequence of the surface modification procedures applied during processing, the technique of cross-sectional transmission electron microscopy (TEM) potentially provides valuable insight into the nature of the surface microstructure being presented to the biological environment. For example, Figure 3 shows the TiO₂ rutile scale developed at the surface of a mechanically polished...
Figure 5: (a,b) Bright field TEM images of a HOb/Ti-foil sample prepared by ultramicrotomy containing a cell (c), Ti foil (Ti) and resin (r). The cell ultrastructure (nucleus (N), mitochondria (M) and rough endoplasmic reticulum (RER)) is rendered visible by staining with osmium tetroxide and uranyl acetate.

Ti sample following annealing at 900°C. The limitation, as ever, is the time consuming nature of the conventional sample preparation procedures of sequential mechanical polishing and low angle Ar ion milling, for cross-sectional TEM investigation. Accordingly, attention is drawn to the little used technique of RHEED that can be used to characterise rapidly the near surface crystal structure of a biomaterial. The RHEED technique is sensitive to the top few nanometres and hence is found to be very useful for the rapid assessment of surface modified titanium, allowing for a ‘process-map’ for thermal oxidation to be constructed, in advance of more detailed TEM investigation. For example, the RHEED pattern of Figure 4 confirms the development of very fine grained TiO$_2$ rutile phase at the surface of a Ti sample annealed in air for 10 h at 600°C.

2.3 Cross-sectional TEM investigation of biological cells on metallic implants

Extending this investigation process to the appraisal of the biological cell / metallic implant interface constitutes a significant technological challenge, given the very different mechanical properties of the material either side of the interface. For the purpose of cross-sectional TEM investigation, the challenge is to retain representative material from the interfacial region, free of artefacts, for the purpose of detailed analytical investigation. In this context, the techniques of ultramicrotomy, and conventional and cryogenic focused ion beam scanning electron microscopy (FIBSEM) have been developed for the controlled cross-sectioning of model system implant metallic and ceramic templates, exposed in-vitro to human osteoblasts (HObs) [9].

Cells cultured on Ti-foil substrates were fixed, dehydrated, stained and embedded in Araldite-Epon resin for the purpose of sectioning using a Reichert Jung Ultracut microtome. Conversely, two different sample geometries were employed for the sectioning of cell/Ti disc or foil samples using an FEI Quanta 200 3D FIBSEM, featuring a Quorum Technologies PP2000T cryo-transfer unit, an Omniprobe micromanipulator and an Oxford Instruments INCA energy dispersive X-ray (EDX) analysis system. Conventional FIBSEM site-specific ‘lift-out’ procedures were applied to fixed, dehydrated, stained and embedded cell/Ti samples, whilst ‘H-bar’ procedures were used for the cryogenic FIBSEM preparation and investigation of fixed, stained and hydrated cell/Ti-foil ‘edge-on’ samples. The details of the experimental procedures used are given by Edwards et al [9]. In particular, the use of a cryogenic-transfer system allows hydrated biological specimens to be rapidly frozen in slush liquid nitrogen, thereby stabilising the specimen for subsequent imaging and milling at low temperature [10]. Electron transparent specimens were examined using an FEI Tecnai 12 Biotwin TEM (100 and 120 kV) and a Jeol 2100F field emission gun TEM (100 and 200 kV) for which a Gatan liquid nitrogen cooled sample holder was employed for the support of both FIBSEM and cryo-FIBSEM sectioned specimens.

Figures 5a,b present bright field TEM images of an ultramicrotomed HOb on a Ti-foil substrate, viewed in cross-section. The cell ultrastructure is clearly visible, with high contrast, and the cell morphology has been well maintained. Mitochondria (M), rough endoplasmic reticulum (RER) and cell nucleus (N), may be discerned within the cell.
Figure 6: Secondary electron images of the two site-specific FIBSEM lift-out geometries adopted. (a) A ‘top-down’ HOb/Ti lift-out section (cultured for 5 days) containing cell (c), Ti and W; and (b) a ‘side-on’ HOb/Ti lift-out section (cultured for 21 days) containing cell (c), resin (r), Ti and W, both attached to TEM support grids.

Figure 6a shows a thinned area of a HOb attached to a Ti substrate during the preparation of a ‘top down’ lift-out section, whilst Figure 6b shows a HOb/Ti specimen attached to a TEM support grid following ‘side-on’ lift-out. The ‘top down’ geometry favours the lift-out of a single cell, whilst the ‘side-on’ lift-out approach produces a section containing resin as well as cells, favouring the preparation of specimens containing multilayered cells.

Figure 7: Bright field TEM image of embedded multilayered HObs (cultured for 21 days) on a Ti-foil prepared through FIBSEM ‘side-on’ lift-out, showing cells (c), collagenous extra-cellular matrix (ECM), resin (r), Ti and artefact W at a point of delamination between the cell and Ti.

Figure 8: HAADF image of a HOb/Ti electron transparent section produced through cryo-FIBSEM milling showing the morphology of the cell (c) with respect to the Ti-foil, limited detail within the cell and milling artefacts across the specimen and illustrating the build up of ice crystals formed during transfer of the specimen from FIBSEM to TEM (I).

Figure 7 presents a bright field TEM image of a ‘side-on’ lift-out section containing HObs cultured for 21 days on a Ti-foil. In this instance, interpretable contrast attributable to the cell ultrastructure and collagenous extra cellular matrix (ECM) can be discerned, and the region of the cell/Ti interface has been preserved and although the cell appears to exhibit intermittent contact with the Ti substrate.

Figure 8 presents an HAADF image a HOb/Ti-foil specimen (cultured for 21 days) produced through cryo-FIBSEM milling in the H-bar configuration. A pronounced curtaining effect was found to be more strongly associated with hydrated specimens prepared through using cryo-FIBSEM procedures as compared with conventional FIBSEM techniques applied to dehydrated specimens, being attributed to the non-uniformity of the gas injection system (GIS) deposited W coating at cryogenic temperatures. By reducing the GIS temperature and carrying out metal deposition in the absence of the ion beam [10], superior coatings with reduced porosity were achieved.
By way of general overview, it was found that conventional ultramicrotomy was capable of producing cross-sections with a large field of view and high contrast, with the sectioning of cell/Ti-foil specimens embedded in resin, but significant problems, e.g. with cell detachment, using this technique were found to restrict its general applicability. Conventional FIBSEM ‘top-down’ and ‘edge-on’ lift-out procedures were found to be effective for the preparation of uniform sections of fixed and dehydrated cell/Ti specimens, but the control of cell staining was found to be an issue. Cryogenic-FIBSEM procedures used with an ‘H-bar’ sample geometry enabled hydrated cell/Ti-foil specimens to be sectioned, but issues remain over ion beam induced artefacts. This was due in part to practical problems associated with the ion beam deposition of protective W coatings under cryogenic conditions, leading to the ‘curtaining’ effect. Further, refined procedures for the complete inhibition of ice formation on such specimens during cryo-transfer are also required.

3. Summary
Procedures for the characterisation of biological cells on biomedical implant materials have been described. In particular, ultramicrotomy, FIBSEM and cryogenic FIBSEM techniques, as developed for the controlled cross-sectioning of human osteoblasts on titanium substrates for TEM investigation, have been compared. Each method was found to be viable, enabling the initial response of biological cells on hard metallic implants to be investigated, but technical challenges still remain for the refinement of these techniques. It is considered that the fundamental understanding gained through the TEM assessment of such sectioned biological cell / engineering alloy specimens will have a significant effect on the design and development of future biomaterials and biocompatible coatings.

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