JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 120–124

# Scanning electron microscopy of the undersurface of cell monolayers grown on metallic implants

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When cells, cultured on a plastic disc, are fixed, dehydrated and embedded in acrylic resin their undersurfaces can be studied by scanning electron microscopy after the disc has been separated and removed from the resin, using a sharp knife, and the resin etched away using glow discharge before sputter coating the now exposed cell undersurface with gold. This method does not work for metallic discs, which do not separate cleanly, leaving the cells in the resin attached to the metal. Rapid cooling of the discs on an aluminium block cooled with nitrogen slush was found to be a successful method, leaving no resin on the metal and without any observable morphological damage to the cells in the resin block. This then allowed direct adhesion studies with the SEM, on the cells' undersurfaces. Focal adhesion processes and stress fibres were observed, which were related to the cell's adhesion and shape.

## 1. Introduction

Both scanning and transmission electron microscopy (SEM and TEM) have been used to study cell adhesion to substrates. By employing special embedding techniques sections of cell adhesions to glass, taken perpendicular to the substrate surface, have been studied using TEM [1, 2], and the undersurface of cells grown on plastic with SEM [3].

In order to study the ultrastructural effects of introducing implant metals to living cells it is also necessary to investigate the nature of attachments made by the cells to such metallic interfaces by microscopy. Observing cells attached to the metal surface using scanning electron microscopy (SEM), after fixation and critical point drying, provides useful information regarding any changes in general cellular morphology which result from confronting such materials, as opposed to other surfaces upon which cells are usually grown. An adhesive film "stripping" technique [4], which removes the cell's uppermembrane and most of the cytoplasm thus exposing the intracellular components remaining attached to the disc, can also be used to investigate the cytoskeletal ultrastructure associated with attachment regions in cell monolayers.

For direct TEM and SEM of the contact interfaces a new approach is required. SEM of the undersurface of cells, grown on plastic culture discs, is possible by utilizing the resin embedding–glow discharge etching approach [3]. This entails the chemical fixation of the cells, followed by dehydration and embedding in a suitable resin. Once the culture surface material has been removed it is possible either to submit the resin surface to glow discharge etching or to re-embed the material in resin, to allow for ultrathin sectioning for TEM. However, the bonding between the embedding resin and the metallic implant disc is very strong, making it virtually impossible to separate the embedded cells from the metal using the physical force method employed with plastic disc embedded material. For microscopic study of the contact areas of the cells the metal must be removed.

One approach, already established for light microscopy work [6], is to utilize electrolytic dissolution to remove the metal. Another is to make use of the fact that metal and resin will contract at differing rates when cooled rapidly, thereby ensuring separation of the two materials. The cells, being embedded and held in the resin, are then completely removed from the metal. This paper describes the merits of applying such techniques to the study of the adhesion of fibroblastic monolayer cultures grown on steel and titanium metal implant discs.

## 2. Materials and methods

### 2.1. Cell culturing

Balb/c-3T3 cells were maintained according to the method of Elvin and Evans [5]. An inoculum of 20 000 cells per well in 1 ml of Dulbecco's minimal essential medium and 10% foetal calf serum, without antibiotics was cultured on 13 mm implant quality steel (ISO5832/1) or titanium (ISO5832/2) discs, or on plastic "Thermanox" discs in tissue culture wells.


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## 2.2. Fixation

The following fixation protocol was carried out at 20 °C. The culture medium was removed and the cells were rinsed in 0.1 M PIPES (Piperazine-NN′-bis-2-ethane sulphonic acid) buffer at pH.7.4. The cells were fixed in 2.5% glutaraldehyde in 0.1 M PIPES at pH 7.4 for 5 min and were then rinsed three times for 2 min each in 0.1 M PIPES, pH 7.4. Postfixation was in 0.5% osmium tetroxide in 0.1 M PIPES, pH 6.8 for 5 min. The cells were then rinsed three times for 2 min each in double distilled water and stained with 2% aqueous uranyl acetate for 15 min (Fig. 1a).

## 2.3. Dehydration and embedding

The fixed cell culture was taken through an ethanol series – 50%, 70%, 96% and 100% for 5 min each followed by LR white resin for 1 h to allow complete infiltration of the resin into the cells. The discs were removed from the wells and placed cell side uppermost in 15 mm wells in a silicon mould, fresh resin was poured onto them and cured thermally at 65 °C for 12–16 h (Fig. 1b). After polymerization the resin blocks were easily removed from their moulds and the blocks were cleaned of excess resin under the disc.

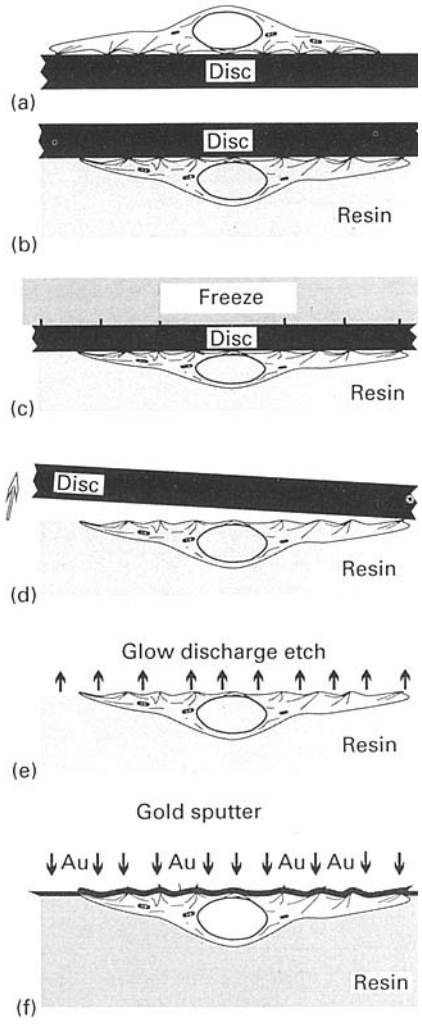


*Figure 1* Diagramatic representation of the technique employed to view the undersurface of cells cultured on metallic implant discs: (a) culture cells on the test disc; (b) fix, dehydrate and embed cells in resin; (c) rapid cooling of test disc with aluminium block cooled in melting nitrogen; (d) easy removal of detached disc with knife; (e) glow discharge etch for optimal duration; (f) sputter coat, if necessary with a thin layer of suitable metal.

## 2.4. Electrolytic dissolution of the metal disc

This technique used electrochemical dissolution, which was a slight modification of the method described by Brown and Simpson [6], to corrode away the metallic discs. An anodic potential of 5 V was applied to the disc by means of an insulated crocodile clip which allowed a current of 0.8 A initially to flow from the disc to the steel cathode, the current decreasing as the metal corroded. The cathode was a steel cylindrical ring through which the jet emerged, the flow of water removing dissoluted ions from the near vicinity of the corroding disc. LR White resin was used, since it has properties of good ultrathin sectioning, retaining biological activity and is stable under the electron beam. Spurr epoxy resin was also used because of its property to low shrinkage during polymerization and section stability under the electron beam [7]. Various concentrations of acetic acid and hydrochloric acid were used for various durations up to 6 h to attempt to remove remaining metal particles on the resin block.

## 2.5. Removal by cooling

The difficulty of removing the metal cleanly led to the development of the second technique involving the rapid cooling of the metal disc to detach it from the resin. The block, metal facing downwards, was immersed in nitrogen slush at − 210 °C, made by subjecting liquid nitrogen to a low vacuum. Nitrogen slush was used because when the discs were immersed in liquid nitrogen, the nitrogen would boil by the Leidenfrost effect and the interface became metal–gas–liquid instead of metal–liquid. This retards the cooling rate due to the low thermal conductivity of the gas. High plunge velocity results in improved cooling rates, but if the resin block is plunged deeper than the metal into the cryogen then the resin itself shatters. This method was improved on by cooling an aluminium block in a bath of nitrogen slush. The combined resin block and metal disc was placed in contact with the cold aluminium block, with the metal disc touching the aluminium. The rapid contraction of the metal, due to rapid heat transfer to the aluminium block, caused it to detach from the resin because the metal contracted much faster than the resin (Fig. 1c). Once loosened in this way the discs were then easily removed from the resin with a sharp knife inserted between the resin and the metal disc, leaving the resin block containing the cell monolayer intact (Fig. 1d).

## 2.6. Etching

The resin blocks containing the cells were then etched in the vacuum chamber of an Edwards 12E6 high vacuum coating unit using glow discharge to expose the undersurface of the cells [3] for various durations

from 5 min up to 80 min (Fig. 1e). The cells were then sputter coated with 10–20 nm of gold, using argon gas in a Polaron E5000 diode sputter coater (Fig. 1f) and then studied with a Jeol 840 SEM operated at various accelerating voltages and in both secondary and back-scattered electron emission detection modes of operation.

## 3. Results and discussion

### 3.1. Electrolytic dissolution

The duration of the electrolytic dissolution for a 1 mm by 13 mm steel disc was between 6 and 12 h. This depended upon the distance of the jet from the disc, the closer the jet the higher the rate of dissolution and the flow rate of the saline, the higher the flow rate the lower the duration of dissolution. Fig. 2 shows the resin blocks after dissolution of the metal block with a large amount of metal remaining at the contact point with the anode. A scattering of fine pieces of metal is found on the surface of the resin, which have not corroded due to other areas corroding away and breaking the continuity of electrical contact. The only cellular material that was observed was after dissolution of polished implant steel from Spurr resin blocks followed by 4 h of immersion in 1 M hydrochloric acid and then for 1 h in TAAB resin solvent, but the cells appeared totally distorted after such treatment.

This method of removing the metal followed by sectioning was adequate for light microscopical examination of the interface between the implant and the tissue, but did not remove enough metal to allow glow discharge etching of the resin from the cell surface. A fine layer of metal always remained attached to the resin, after dissolution, which prevented any further preparation to enable visualization of the cells' undersurfaces. The metal particles interfere with good ultrathin sectioning for TEM or prevent glow discharge etching for SEM. The fine metal layer could be removed, but only by using strong acid attack for prolonged periods which resulted in damaged cells. Damage to the cells probably occurred both as a result of heating and acid attack. Reducing the applied voltage would result in lower heat production at the implant disc surface because of the lower current flow, increasing the duration of dissolution. The lower temperature would prevent heated particles of metal from melting the resin. However, if the metal disc was not smooth then there would always be some small areas of metal particles remaining in the resin. This is because eventually, during dissolution, the electrical continuity would corrode away. This method therefore proved inadequate for the study of various metal discs with various types of surfaces.

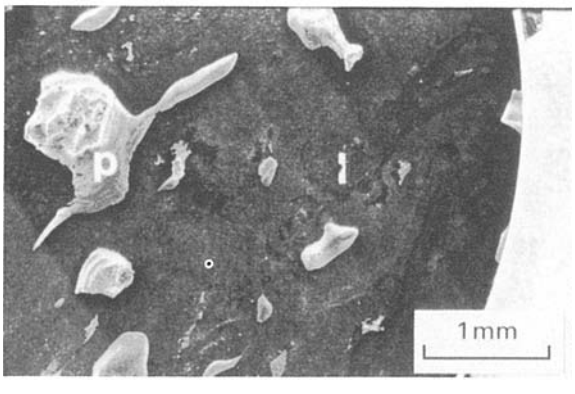


*Figure 2* Low magnification view of resin block containing a cell monolayer after 12 h electrolytic dissolution. Large particles (p) and a very fine layer (l) of metal still remains.

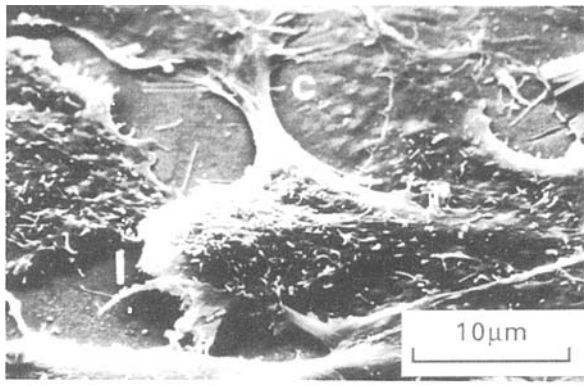


*Figure 3* Fibroblast culture on implant steel displaying varying degrees of cell–substrate contact. Close cell–substrate contract (c) and low cell–substrate contact (l) can be observed.

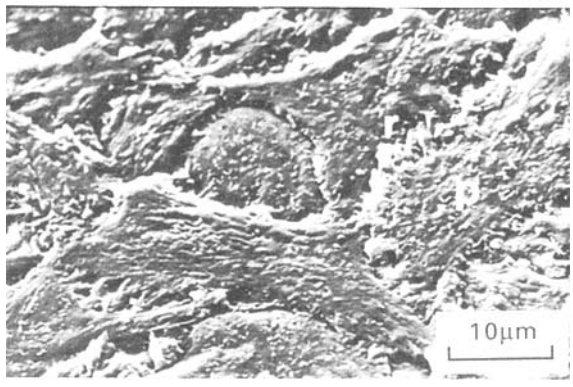


*Figure 4* Undersurface of fibroblasts embedded in resin cultured on Thermanox plastic discs, after removal of the disc and 20 minutes glow discharge etching. Ruffling of the lamellipodia edges (r) and numerous processes (p) can be observed.

### 3.2. Removal by rapid cooling

The depth of immersion of the resin block and metal into the cryogen could not easily be controlled when plunge-cooling, and some of the cryogen would still boil off causing a gas–metal interface, thereby slowing the cooling rate. Cooling of the resin also caused it to fracture resulting in damage to the cells within (Fig. 5). Impact cooling of the metal discs on an aluminium block immersed in nitrogen slush eliminated the problem of depth of immersion into the cryogen and the resulting fracturing of the resin (Fig. 6).

Normal SEM imaging of the uppersurface of a Balb/c-3T3 cell grown on a steel implant disc shows that a large proportion of the cell does not contact the

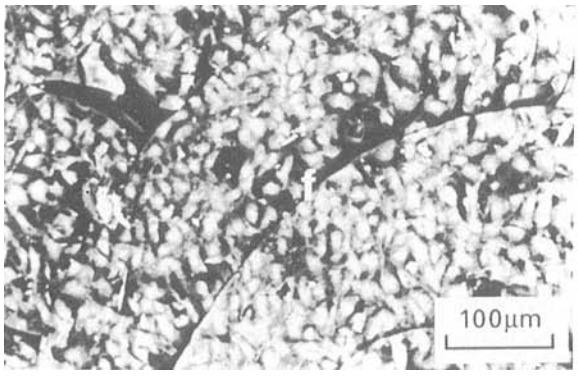


*Figure 5* Backscattered electron image of the resin block containing cells that were cultured on implant titanium after removal of the disc by immersion into liquid nitrogen causing fracturing of the resin (f) and damage to the cells.

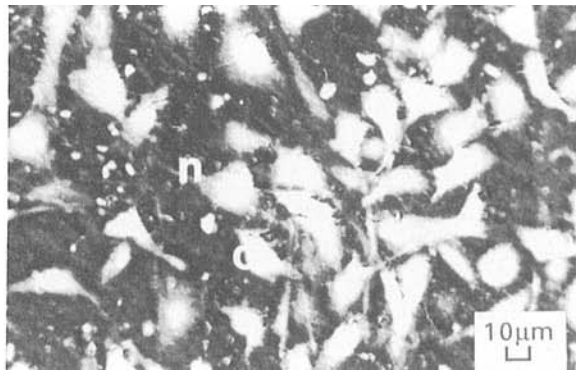


*Figure 7* The same area of cultured cells as in Fig. 6. in backscattered electron detection mode. The non-contacting area (n) of the cells covered with resin is revealed by the deeper penetrating power of electrons accelerated by 20 kV. The contact area of the cells (c) is also observed, as well as the overall shapes of the cells.

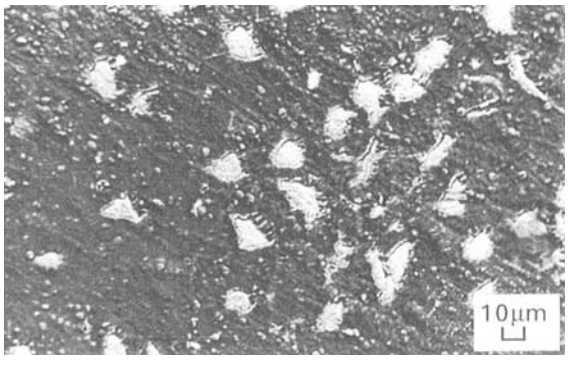


*Figure 6* Impact cooling removal of the titanium implant disc leaving intact cells within the resin. The accelerating voltage of 10 kV in backscattered electron detection mode allows visualization of only the undersurface areas of the cells that were in contact with the implant. 20 minutes glow discharge etching.

metal surface (Fig. 3). This area of non-contact is filled with resin during the process of embedding. This occludes the non-contacting area of the cell's undersurface from detection using ordinary secondary electron emission in the SEM, and also with backscattered electron emission at the normal operating accelerating voltages (up to 10 kV) used in biology (Fig. 6). The area can, however, be observed using backscattered electron detection at higher accelerating voltage, such as 20 kV (Fig. 7).

Cell adhesion is essential for a wide range of biological processes both in the developing and adult organism. Evidence has been provided that the cytoskeleton plays a central role in the adhesion process [8], probably mediated by the redistribution of cell surface molecules. This interpretation is based on the analysis of F-actin organization in fibroblasts after using drugs that inhibited the adhesion process. The cytoskeleton was also found to be disrupted by the drugs.

As has been shown in a recent review [9], the actin cortex has been identified as the principal cytoskeletal component that appears to be attached, via plasmalemmal receptors, to the extra cellular matrix (ECM). Living C3T3 cells display a complex array of fibrous structures which can be shown by staining with fluorescently labelled anti-actin antibody to contain actin. Transmission electron microscopy shows that these structures are submembranous bundles of microfilaments located mainly in those regions of cells which attach to substrates. Similar patterns of "tension striae" were originally described back in 1924 [10]. Since then these fibres have been described in BHK21 cells [11, 12] and embryonic rat cells [13], demonstrating the relationship between the fibres seen in living cells and the bundles of microfilaments observed by electron microscopy. The array of fibres is detectable in both the living and fixed cell, although the fibres are more pronounced in the fixed cells.

In cell culture many cell types develop specialized adhesions to extracellular matrix (ECM) components, that have been adsorbed to the underlying substratum and provide a model for studying cell interactions with the ECM. Focal adhesions, first identified in electron microscopic studies of cultured fibroblasts [14], are characteristic of stationary or slow moving cells as the tight adhesion they provide impedes cell migration. The intracellular part of the focal adhesions provide attachment points for actin microfilament bundles. These are the stress fibres which are thought to generate tension on the focal adhesions [15–17]. It is considered that in the majority of cultured cells focal adhesions and their associated stress fibres are artifactual, resulting from their strong adhesion to the rigid planar surface. Such conditions would not normally be found within soft tissues. A strong adhesion is also likely to form between cells and implant materials, as they also have a rigid planar surface. Therefore their study in culture is relevant not only from this point of view but also as a useful model of cytoskeleton–ECM interactions.

The undersurfaces of cells grown on "Thermanox" plastic discs (Fig. 4) show a large number of focal adhesion processes and also the lamellipodia between cells are ruffled at their edges. Many of the cells grown on the plastic show numerous, and prominent, sub-

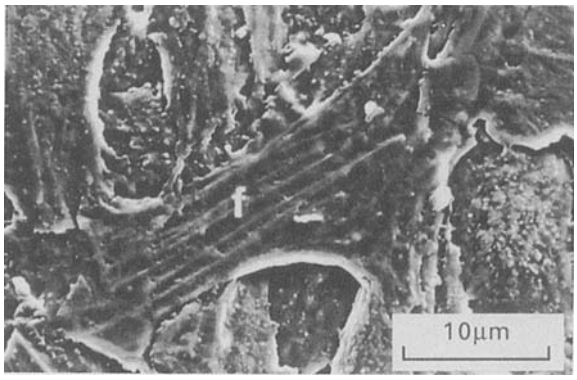


*Figure 8* Undersurface of a fibroblast cultured on Thermanox plastic, displaying numerous longitudinal stress fibres (f) close to the adhesive surface of the cell. 20 minutes glow discharge etching.

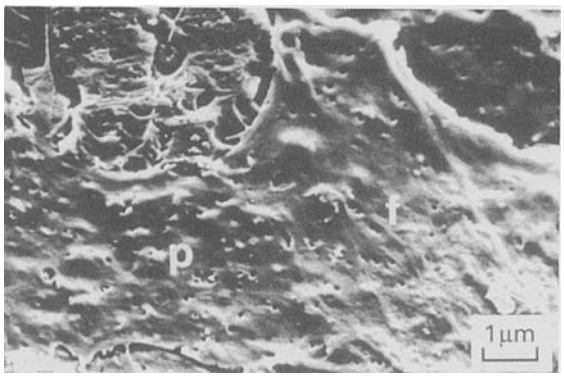


*Figure 9* Numerous focal adhesion processes (p) on the undersurface of a fibroblast cultured on implant steel which was impact freeze removed. Some evidence of "stress fibres" (f) can also be seen. 20 minutes glow discharge etching.

membranous "stress fibres" on the undersurface that was in contact with the substrate (Fig. 8). "Stress fibres" are also seen in cells grown on both steel and titanium discs (Fig. 9), but are not as well developed as those found in cells grown on plastic surfaces. These stress fibres are likely to be composed of actin microfilaments, that are active in, among other things, the control of cell shape. Confirmation of this will need to be carried out by applying immunocytochemical techniques to these cells utilizing antibodies specific for cytoskeletal components.

The fibres run parallel for long distances along the cell and their distribution is in agreement with the finding [18] that such structures in living cells are displaced towards the adhesive side of the cell. What appear to be numerous focal adhesion processes can also be seen on the surface, again immunolabeling for integrins should confirm this. Focal adhesion processes may also be numerous on the undersurface of cells grown on steel (Fig. 9).

## 4. Conclusions

The method of employing rapid cold block cooling to remove implant metal discs from embedded cell monolayers in resin is shown to be a clean and fast technique which does not cause damage to the embedded material. Preliminary comparative results indicate significant morphological differences between cells cultured on different surfaces.

## Acknowledgements

The authors gratefully acknowledge receipt of AO/ASIF Foundation Research grant (No. 93-G14) and thank P. C. Lloyd, D. Williams and R. Peter for technical assistance.

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*Received 8 December 1993*
*and accepted 7 April 1994*