Published under licence in IOP Conference Series: by IOP Publishing Ltd.
Inst. Phys. Conf. Ser. ***179*** *(2004) pp 87-90*
Electron Microscopy & Analysis Conference, EMAG 2003, Oxford

Scanning Electron Microscopy of Biomaterials

K.J. McKinlay,[1] C.A. Scotchford,[1,2] D.M. Grant,[1] J.M. Oliver,[3] J.R. King[3] and P.D. Brown[1]

[1]School of Mechanical, Materials, Manufacturing Engineering and Management; [2]School of Biomedical Sciences; and [3]School of Mathematical Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK.

Abstract
A comparison of conventional high vacuum scanning electron microscopy (HVSEM), environmental SEM (ESEM) and confocal laser scanning microscopy (CLSM) in the assessment of cell-material interactions is made. The processing of cells cultured for conventional HVSEM leads to the loss of morphological features that are retained when using ESEM. The use of ESEM in conjunction with CLSM of the labeled cytoskeleton gives an indication of changes to the cell morphology as a consequence of incubation time and substrate surface features.

1. Introduction

The investigation of biological systems on the molecular scale using high vacuum SEM (HVSEM) requires several rigorous processing steps to dehydrate the cells and prevent the sample from charging when exposed to the imaging electron beam [1,2]. Such processing steps can lead to the destruction of finer features and the introduction of artefacts on the cell membrane. In addition, dehydration implements alcohols such as ethanol and acetone and as more polymeric materials are being studied for implant materials, such processing can lead to the degradation of the material. With the advent of environmental SEM (ESEM) [3,4], these dehydration steps can be avoided allowing the morphology of cells to be assessed at high resolution in a state closer to their natural morphology *in vitro*. A comparison of the HVSEM and ESEM techniques in the imaging of cell morphology is discussed here. The advantages of using ESEM to image cell morphology in conjunction with confocal laser scanning microscopy (CLSM) are emphasised. The application of ESEM and CLSM to the assessment of cell response to implant material surface features is also described.

2. Methodology

Primary human osteoblasts (HOBs), derived from femoral head trabecular bone, were seeded at an initial cell density of $2x10^4$ cells/cm^2 onto a variety of substrates sterilised by exposure to ultra-violet radiation. Substrates were incubated in full culture medium

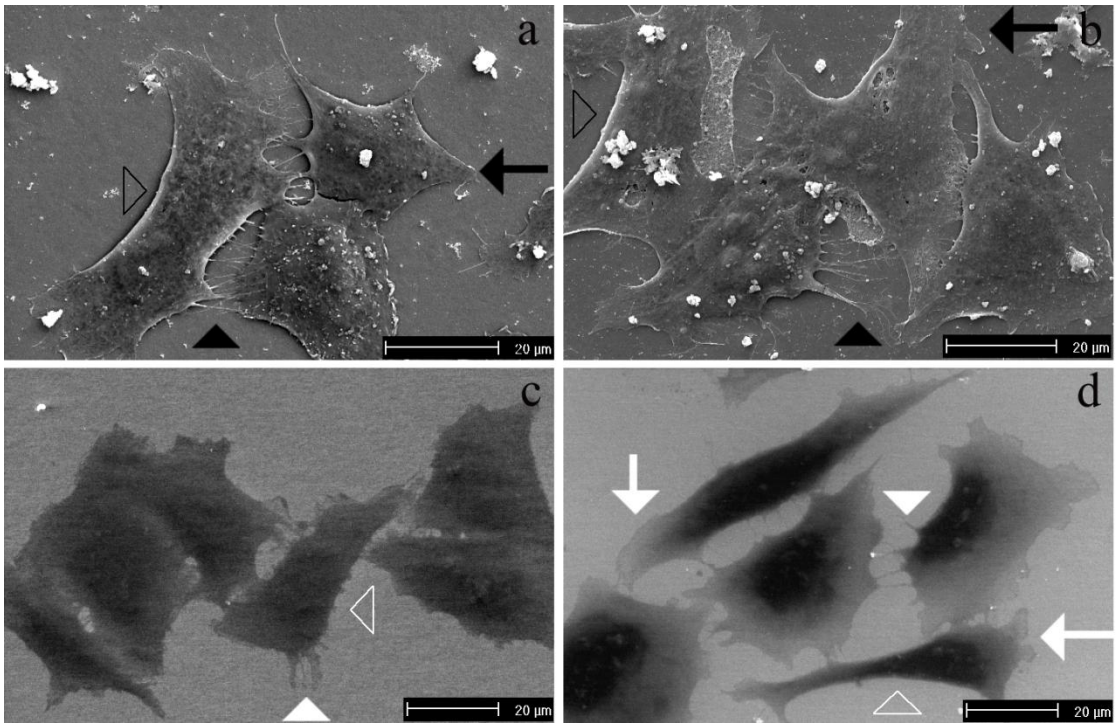

Figure 1: a,b) HVSEM of HOBs cultured on a titania surface and dehydrated; c,d) the equivalent samples imaged using ESEM. Samples were fixed after a,c) 90 minutes and b,d) 6 hours (micron markers = 20µm).

at 37°C and 5% CO_2 for times of 90 minutes and 6 hours before processing for microscopy.

Secondary electron imaging (SEI) using an FEI XL30 field emission gun (FEG)-ESEM was performed in high vacuum and wet (i.e. water vapour imaging gas) modes. A 30 µm incident beam aperture with a spot size of 6 nm and an accelerating voltage of 10 kV were typically used. Secondary electron (SE) and gaseous secondary electron (GSED) detectors were employed to image the cells.

After the selected culture period, samples for ESEM analysis were fixed with 3% glutaraldehyde in 0.1M sodium cacodylate (NaCaco) buffer. Prior to imaging, samples were washed with deionised water to remove residual buffer and prevent interference by salt crystal precipitation. Samples were placed on a copper stub, which was in turn placed in good thermal contact with a Peltier effect cooling stage. The sample chamber was pumped down to a pressure of 6 Torr (above the pressure required for imaging) before flooding with water vapour several times to 10 Torr to ensure saturation of the sample environment with water. The pressure was decreased slowly to the imaging pressure to prevent the sample temperature dropping below 4°C, which would otherwise result in the formation of ice crystals and the subsequent destruction of the cells.

After the selected culture period, samples for HVSEM analysis were fixed with 3% glutaraldehyde in 0.1M NaCaco buffer and post fixed with 1% osmium tetraoxide in Millonig's buffer. Dehydration was implemented using a typical route by submersion in ethanol solutions of increasing concentration before submersion in hexamethyl disilazane (HMDS). The majority of HMDS was removed and the remaining solvent was left to evaporate overnight in air. Samples were mounted on SEM stubs, gold coated and visualised using the FEG-ESEM operated in high vacuum mode.

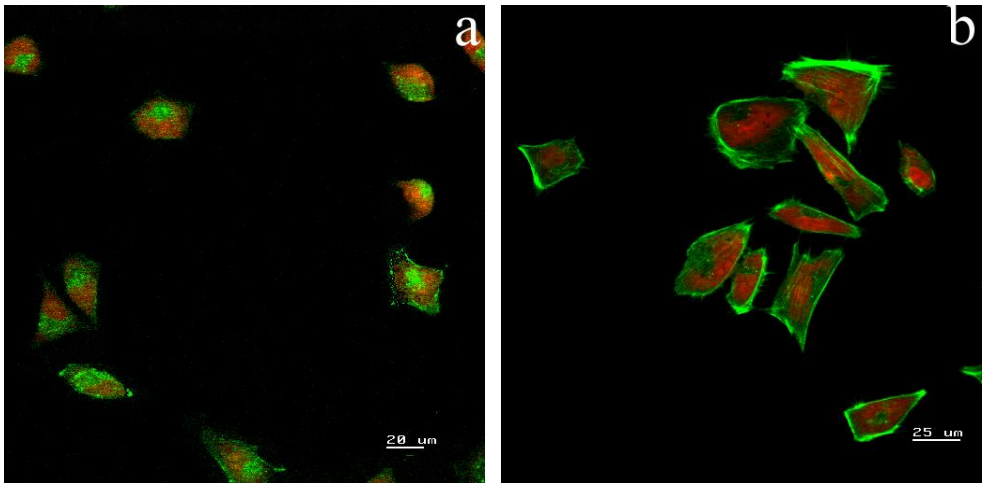

Figure 2: CLSM of HOBs cultured on a titania surface with actin network labeled after a) 90 minutes and b) 6 hours incubation (micron markers = 20 and 25μm, respectively).

After the selected culture period, samples for CLSM were fixed with 4% paraformaldehyde at 4°C and stained with FITC-conjugated phalloidin (10μg/ml,Sigma, Dorset, UK) to label the actin and a 0.5% aqueous solution of propidium iodide (Sigma, Dorset, UK) to label the nucleus of each cell. Samples were then mounted and the cell actin networks were imaged using a Leica TCS 4D confocal laser scanning microscope.

3 Results and Discussion

A comparison of the morphology of HOBs cultured on a smooth titania surface and imaged using HVSEM and ESEM is shown in Figure 1. Both HVSEM and ESEM show that increased incubation time, from 90 minutes to 6 hours, leads to an increase in cell spread as would be expected. However some differences in the detail are evident. In HVSEM, the cell spreading is indicated after 6 hours by broad thin lamellipodia (Figs. 1a,b; black arrows) which show cracking and perforation compared to those cells incubated for just 90 minutes. However, there is very little difference in the morphology of the cells when the two time points are compared with HVSEM. At both time points there is evidence of fine contact processes (Figs. 1a,b; black arrowheads) which have snapped due to the processing are and well defined cell edges (Figs. 1a,b; black-lined arrowheads).

If the images obtained with ESEM are considered, a greater difference between the cells at the two time points is observed. Once again the HOBs are clearly spread to a greater extent after 6 hours and fine contact processes are evident (Figs. 1c,d; white arrowheads). However, the cells are now less precise in their attachment after 90 minutes compared to 6 hours, whereas there was no obvious difference in the shape of the fine processes with incubation time in the dehydrated specimens. The extension of pseudopodia and elongation of the cells after 6 hours incubation is also more obviously seen in the ESEM images (Figs. 1c,d; white arrows). There is also a clearer difference in the edges of the cells (Figs. 1c,d; white-lined arrowheads). After 6 hours the edges of the cells are in general smoother and more clearly defined. The ruffled edges seen in the ESEM image of HOBs incubated for 90 mins are clearly lost during the dehydration processing for HVSEM. This is probably due to membrane shrinkage and the fact that the less well defined processes are not strongly attached. The increased definition of the cell edge seen in the ESEM images after 6 hours incubation could indicate an increased order of the substructure [5].

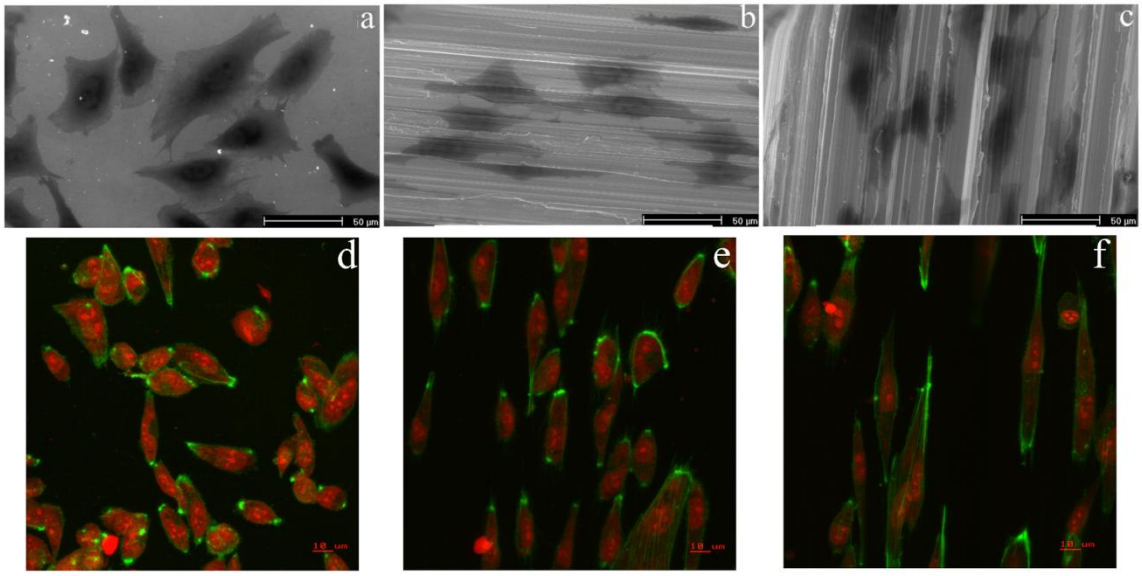

Figure 3: ESEM (a-c) and CLSM (d-f) of HOBs culture for 6 hours on stainless steel surfaces (a,d) polished; (b,e) roughened with P80 SiC and (c,f) roughened with P40 SiC paper.

Surface morphology only provides an indication of changes in the structural organisation of a cell, while labeling and visualisation of the distribution of the cytoskeletal components involved is still required to confirm the state of organisation.

Figure 2 shows equivalent CLSM images of the actin distribution of HOBs cultured for 90 minutes and 6 hours on titania surfaces. The increased definition of the fibrous network and the short fibres extending from the cell body seen in the 6 hour culture but not the 90 minute culture, consistent with the change in cell morphology observed using ESEM, but is not evident in the HVSEM images.

Imaging of cell morphology on roughened surfaces using optical microscopy techniques can prove difficult due to the short focal length, ESEM overcomes this limitation. Figures 3a-c are examples of ESEM images obtained from HOBs cultured on stainless steel surfaces with increasingly roughened surfaces. The overall alignment of the cells with the surface texture is evident, however, the small cell features observed on smooth surfaces are difficult to distinguish from the substrate texture and as such the filopodia and morphology of the cell outline cannot easily be determined. Therefore, CLSM of the cytoskeletal organisation is used to complement the ESEM observations.

The main advantage of using ESEM to image cell cultures grown on biomaterial surfaces lies in the fact that the processing of the samples prior to observation does not destroy features that are indicative of the cell morphology. The processing for HVSEM imaging is found to destroy fine features especially at the early time points.

References

1. Bozzola J J and Russell L D 1998 *Electron Microscopy: Principles and techniques for biologists,* (Massachusetts: Jones and Bartlett)
2. Hayat M 2000 *Principles and techniques of electron microscopy: biological applications* (Cambridge: Cambridge University)
3. Danilatos G D 1993 Micr. Res. Tech., 25 354-361.
4. Uwins P J R 1994 Mat. For. 18 51-75.
5. Bray D 2001 *Cell movements: from molecules to motility* (New York: Garland)