JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 15 (2004) 355–359

The effect of silica nanoparticulate coatings on cellular response

B. G. COUSINS, P. J. DOHERTY*, R. L. WILLIAMS
Department of Clinical Engineering, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, UK
E-mail: b.g.cousins@liverpool.ac.uk; P.J.Doherty@liv.ac.uk

J. FINK, M. J. GARVEY
Department of Physics, University of Liverpool, Oliver Lodge Laboratory, Liverpool L69 7ZE, UK

The current techniques used to create patterned materials at the nanometer scale such as electron beam lithography are restricted to patterning small areas, which can be expensive and time consuming. A simple, cost-effective approach has been developed to create a reproducible surface topography to influence the cellular response. In this study, the cellular response of murine fibroblasts to 7, 14 and 21 nm colloidal silica particles were investigated over one, three and seven days and up to seven weeks. The surface topography and wettability of the surfaces were also studied. The results confirmed that silica particles create a nanoscale topography, which initiates a distinctive cellular response affecting the morphology, adhesion and proliferation of the fibroblasts. The effect was evident up to seven weeks with no adverse effects on cell viability.

Introduction

A variety of studies have established that the surface topography of the underlying substrate is an important physical property, which influences cell behaviour such as adhesion, proliferation and orientation of mammalian cells [1]. There is mounting evidence that a surface profile with nanometer scale dimensions (10^{-9} m) is of equal importance [2]. Murine macrophages (P388D1 cell line) have been shown to be highly sensitive to nanoscale topography and were guided and activated by features between 30 and 70 nm [3]. Nerve cells have also been shown to respond to 5 nm steps with increased cell adhesion, orientation and changes in cell behaviour [4, 5]. Recently, the effects of surface topography in terms of symmetry and regularity on the adhesion of fibroblasts and endothelial cells were investigated and the results showed that a regular topography reduced cell adhesion markedly [6].

The current techniques used to pattern materials include etching or ablation of the surface, which are restricted to patterning samples of relatively small areas and simple geometries. These methods are time consuming and costly hence the need for using alternative methods which are more economically viable. To overcome this, recent studies on the cellular behaviour have reported on physiochemical methods such as polymer blending, which endure phase separation upon drying to create nanoscale structures [7–9]. The approach we have developed involves the novel deposition of amorphous colloidal silica (SiO_2 – silicon dioxide) on to a wide range of materials [10]. Colloidal silicas are used in a diverse array of fields from pharmaceutical applications to coatings and thin films in optical and electronic components. The subunits of the aqueous silica sols are spherical, non-porous, and negatively charged in nature and are typically in the size range of 1–50 nm [11]. Silica is ubiquitous in biological systems and essential to some species such as diatoms and sponges that use it to make their rigid skeleton (frustules) [12]. Research in the 1970s proved that it was necessary for bone growth and is an essential nutrient for humans (total amount in the human body is approximately 1 g) [11]. All connective tissues in the body, along with the skin and hair contain silica.

It is clear that the topographical and physical properties of the surface have to be considered in order to provide understanding of the cellular behaviour at the nanometer scale. An ability to control the cell response at the biological–material interface is required to manufacture a new generation of biomaterials that are now in demand. This paper reports a simple procedure to modify the surface topography of materials and addresses the cellular response *in vitro* when colloidal silica is deposited on to glass substrates as a simple model surface.

*Author to whom all correspondence should be addressed.

Materials and methods

The substrates used for the deposition of silica particles were round (13 mm diameter) and square glass cover slips (22×40 mm diameter) purchased from BDH Ltd. and were used as received. Aqueous colloidal silica sols were purchased from W. R. Grace & Co. Three types of silica sols were selected for cell culture experiments with average particle diameters of 7, 14 and 21 nm (Ludox® SM-30, HS-40 and TM-50), respectively.

Deposition of colloidal silica

Glass cover slips were washed once with 100% methanol followed by three rinses with deionised H_2O and allowed to dry at room temperature. The cover slips were totally immersed in a 3.2 g/l aqueous dispersion of polycationic polymer, Zetag™ (Ciba Speciality Chemicals Ltd) for approximately 10 min. The substrates were removed from the polymer solution and rinsed with copious amounts of de-ionised H_2O for 3 min and allowed to dry at room temperature. The polymer-coated cover slips were immersed in 30% w/v colloidal silica sol for approximately 5 min to ensure monolayer attachment of the silica particles to the cationic polymer. The substrates were removed and again rinsed with copious amounts of deionised H_2O for 3 min and allowed to dry at room temperature. The silica-coated cover slips were then stored in a desiccator for approximately 24 h before use.

Surface analysis

Atomic force microscopy (AFM) analysis was performed using the Nanoscope IIIa from Digital Instruments Inc., USA. The substrates used for surface analysis were plain glass; Zetag™ and silica coated glass cover slips. All surfaces were imaged in air under standard conditions and analysed in tapping mode.

Contact angle measurements

In order to determine the surface properties of the materials contact angle measurements were conducted using the Wilhelmy method [14] and deionised H_2O as the solvent on square clean glass cover slips and each of the modified surfaces (cationic polymer, 7, 14 and 21 nm silica). Deionised H_2O was chosen as the solvent because of its polar nature and suitability for determining the hydrophilic and hydrophobic properties of surfaces. An automated dynamic contact angle surface-tensiometer was used (CDCA-100F, Camtel Ltd.) controlled by CDCA-100 software (Camtel Ltd.). The values reported for dynamic advancing and receding angles of glass and each of the modified surfaces are mean and standard deviations of 10 measurements ($n = 10$).

Cell culture experiments

L929 murine fibroblasts were purchased from the European Collection of Cell Cultures (ECACC) and grown as a monolayer culture in 50 cm^2 tissue culture polystyrene (TCPS) flasks. When confluent, the monolayer of cells were harvested by trypsinisation and subjected to serial dilution before seeding. All test materials were transferred in to sterile 24-well TCPS plates. Plain glass and Zetag™ coated cover slips were used as the control substrates. The control and silica modified glass substrates were seeded with 2 ml of L929 fibroblasts with cell concentrations of 1×10^5 cell/ml in MEM 199 (Gibco Ltd) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. For direct contact tests the substrates were transferred into 24-well TCPS plates and were incubated for a period of one, three and seven days. Three separate 24-well TCPS plates were set up to investigate the cellular response over a seven-week time period. One milliliter of the culture media was removed and replaced with 1 ml of fresh media on a weekly basis. After two weeks, three silica coated glass cover slips were carefully removed and placed into a sterile 12-well TCPS plate. The cover slips were then gently rinsed with 2 ml of phosphate buffered saline (Sigma). A plain glass microscope slide was transferred aseptically into a sterile 90 mm petri dish (Falcon) and seeded with the 2 ml of PBS supplemented with 8 ml of fresh media. All test substrates were incubated in 5% CO_2 at 37 °C and observed after 24-h periods using inverted phase-contrast microscopy.

Fixing and staining of the cells

All test substrates were dehydrated with 100% v/v methanol for 5 min, stained with 0.04% v/v methylene blue for approximately 1 min, rinsed with copious amounts of de-ionised H_2O and allowed to dry at room temperature. Optical micrographs were taken using the Axioplan 2 microscope (Carl Zeiss Ltd.).

Cell quantification

Quantitative analysis was carried out using the Ks400 light microscope (Carl Zeiss Ltd) from live images ($\times 250$ magnification). The percentage mean cell coverage was calculated using a pixelated computer programme (Carl Zeiss Ltd) to measure the total area taken up by the cells from control and modified glass substrates. The same threshold and illumination conditions were used throughout the analysis. Five replicate samples were analysed in 20 separate fields of view ($n = 100$). The standard deviation, mean cell count and student *t*-test were performed to compare the statistical significance between the control and test materials. The student *t*-tests were carried out using a two-tailed distribution and two-samples of equal variance with Microsoft Excel software. P values of < 0.01 were considered to be a significant difference between the control and test substrates.

Results

In Fig. 1, the topographic image of 21 nm silica showed an irregular, random close-packed array on the surface of the glass. This modification was representative of other particulate materials used in this study. Previous studies have shown that the surface coverage of the particles is uniform throughout the treated area with mean surface

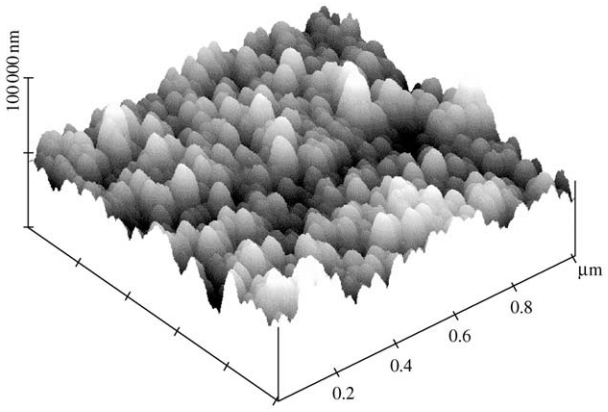

Figure 1 AFM topographical image (scan size $1 \times 1\,\mu m^2$) of 21 nm silica deposited on to glass substrates. The lighter areas are nanoparticles protruding from the surface and the darker features are the base substrate. The vertical scale range is 100 nm.

TABLE I Contact angles of modified glass surfaces using the Wilhelmy method and water as the solvent

Surface	Contact angle(°)	
	Advancing	Receding
Clean glass	27.8 ± 6.6	12.9 ± 7.2
Zetag™	37.2 ± 8.3	7.2 ± 7.9
7 nm silica	21.3 ± 4.1	15.5 ± 5.0
14 nm silica	30.6 ± 3.8	21.2 ± 3.7
21 nm silica	37.4 ± 11.6	21.3 ± 6.8

roughness measurements within the expected range for each particle size.

The results of dynamic contact angle measurements using water as the solvent for glass modified with Zetag™, 7, 14 and 21 nm silica are shown in Table I.

It can be seen that clean glass and cationic polymer are hydrophilic in nature with advancing contact angle values of 27.8 ± 6.6 and 37.2 ± 8.3, respectively. Contact angle measurements of silica generally increased with particle size. However, glass surfaces modified with 7 nm silica were found to be the most hydrophilic with advancing and receding angles of 21.3 ± 4.1 and 15.5 ± 5.0, respectively. Glass substrates modified with 14 and 21 nm silica had similar advancing angles to that seen with glass and Zetag™ coated substrates. The

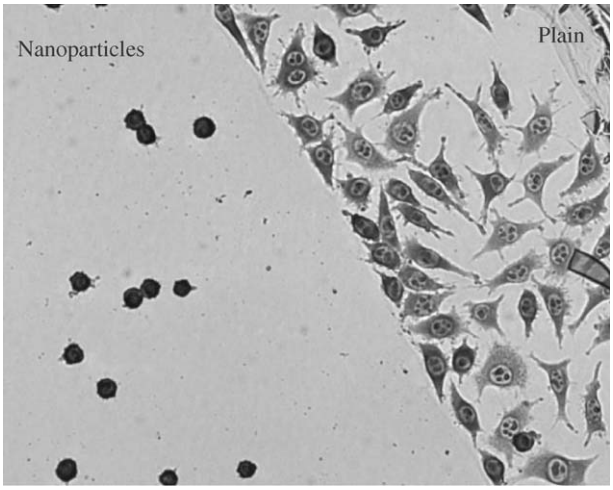

Figure 2 Optical image of the cellular response of L929 fibroblasts to 7 nm silica particles. The cells were placed in direct contact with the silica treated (left) and untreated (right) glass surface (× 200 magnification).

receding angles were low for all the samples although they increased slightly for the larger silica particles.

Fig. 2 shows the typical cellular response of L929 fibroblasts at the glass/silica interface. The silica interface can be clearly identified. When the fibroblasts were placed in direct contact with the silica surface the cells showed a rounded conformation, whereas, the cells on the untreated glass surface proliferated with spread morphology.

The cellular response to different-sized silica particles was analysed after one, three and seven days in cell culture. Generally, fewer cells were observed on the silica treated surfaces and hence a decrease in the number of spread and proliferated cells (Fig. 3). After three days a noticeable difference between the glass, Zetag™ and silica coated samples in terms of mean cell coverage and the morphology of the fibroblasts was apparent. At day seven, the cell coverage values for glass and polymer coated slides were confluent with values of 90% and 85%, respectively. There was more than a four-fold reduction in the number of cells adhered to the 7 nm silica particles (with a mean value of 19%).

The cellular response to 14 and 21 nm silica showed a two-fold reduction in the number of cells adhered to the materials. In Fig. 4, representative images of the data are

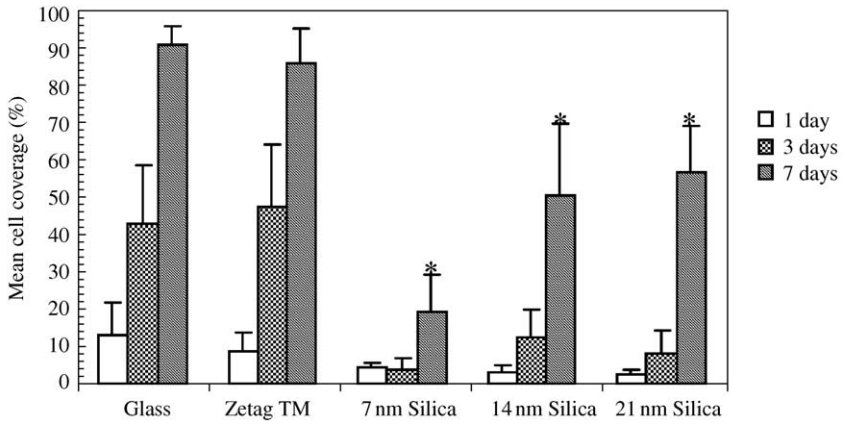

Figure 3 The mean percentage cell coverage of L929 fibroblasts on control and silica treated glass cover slips after one, three and seven days in cell culture. Five replicate samples were used ($n = 100$). * = Student's t-test where $P \ll 0.01$ when compared with the control samples.

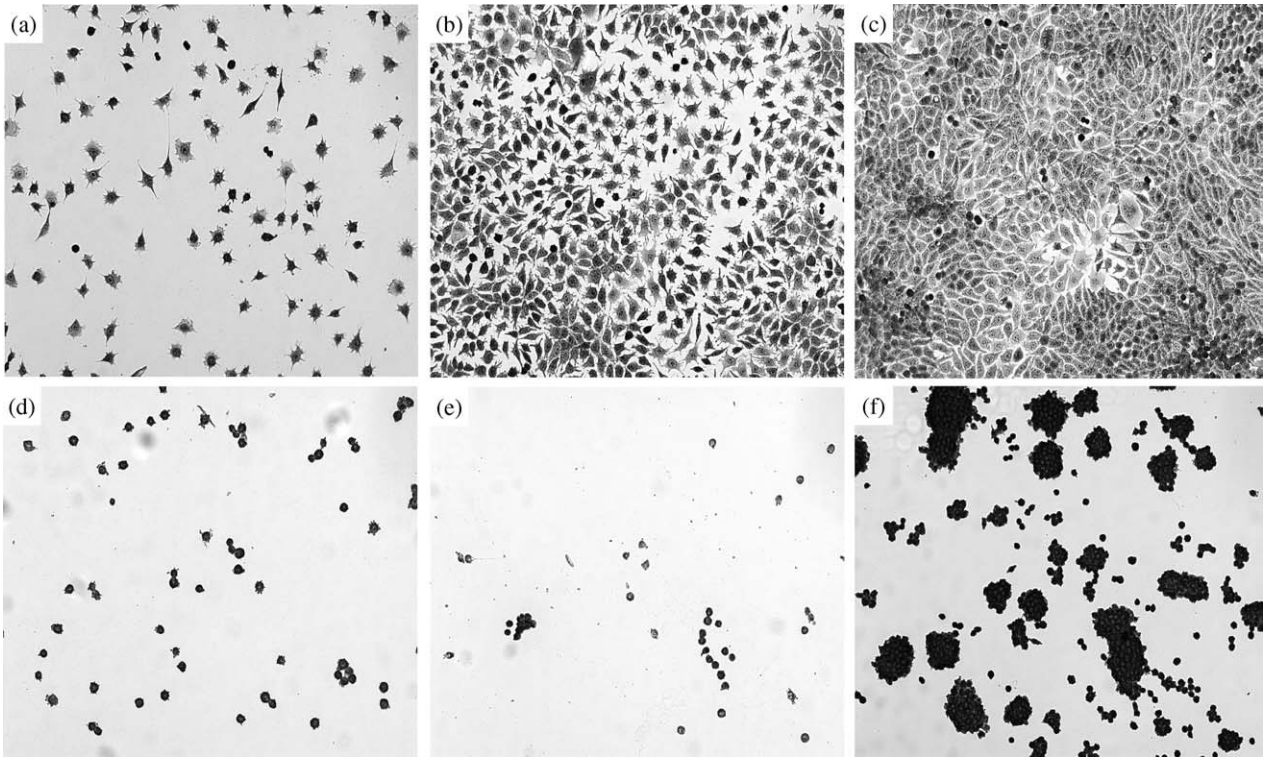

Figure 4 Optical images of L929 fibroblasts responding to plain glass after (a) one, (b) three and (c) seven days in tissue culture. In addition, the cell response to 7 nm silica particles is also shown after (d) one, (e) three and (f) seven days at × 50 magnification.

shown regarding the cell response to glass and 7 nm silica with time.

After 14 days, the fibroblasts on the silica treated surfaces showed the same rounded cellular response. Representative images of fibroblasts on 14 nm silica surfaces are shown in Fig. 5(a). The silica surface had a prolonged effect on the cellular behaviour of the fibroblasts. Fibroblasts on glass and Zetag™ coated surfaces remained confluent after this time period. The cellular response to silica treated surfaces can be seen in cell culture for up to seven weeks.

Furthermore, the fibroblasts were found to be weakly adhered to the silica treated substrates and could be removed with a gentle rinse with phosphate buffered saline. Subsequent reseeding of the cells in fresh media on glass substrates resulted in a change in the cellular behaviour back to the normal cell response of spread and proliferated cells. This change in cellular response when clusters of cells are removed and reseeded on to glass substrates is shown in Fig. 5(b).

Discussion

A novel approach to create nanotopographical features by the deposition of colloidal silica particles on to glass substrates can be achieved using a simple procedure, which leads to the ionic adsorption of negatively charged silica particles on to a thin film of cationic polymer. The particles condense and are highly cross-linked upon the removal of water [11]. Dynamic contact angle measurements were used to assess the wettability of the materials. Clean glass substrates have hydroxyl groups at the surface and were found to be hydrophilic as expected. Zetag™ coated substrates had a slightly higher advancing contact angle representing cationic functional groups in the form of polar amines along the hydrocarbon chain, which are hydrophilic in nature. The silica particles had hydrophilic surface properties as they have a high density of silanol groups (Si–OH) over a large surface area [12]. The difference in contact angles between the three silica surfaces could be due to the effect of surface roughness [14]. These measurements along with the AFM results

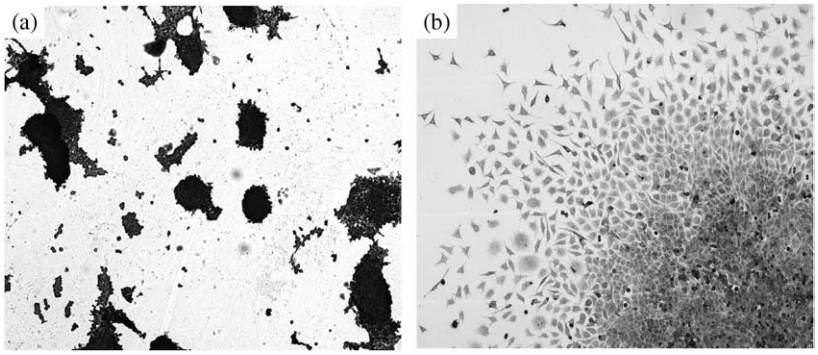

Figure 5 Optical images of L929 fibroblasts responding to 21 nm silica (a) after 14 days in tissue culture and (b) fibroblasts radiating away from the cell clusters on plain glass after being removed with PBS (× 50 magnification).

provide confidence that the surface properties of each substrate change with each modification step.

On control glass and Zetag™ coated substrates the cells spread and proliferated until confluent at day seven. It was demonstrated that the charged Zetag™ surface was more favourable for cell adhesion and proliferation after three days when compared with the glass control. However, cells placed in contact with colloidal silica led to a distinctive cellular response with rounded cell morphology. The results showed that the fibroblasts were responsive to the silica particles with low levels of cell attachment at day three. It was clearly evident that the silica particles prevented the spreading and proliferation of the cells. After seven days the fibroblasts formed clusters of cells rather than an even distribution on the surface of the substrate. It is known that cell adhesion to the substrate and spreading is necessary for cell cycle division [13]. If the cells are restricted in their spreading they will never enter the S phase of the cell cycle required for DNA synthesis [8, 13]. The cells on all silica treated surfaces were weakly adhered and could be removed with ease. Reseeding of the cell clusters on to control substrates changed the cellular response to spread and flattened cells. This suggested that the cells remained viable with no cytotoxic effect from the particles. All particulate surfaces were found to be as equally effective and that the particles influenced the cell response over a seven-week period. This effect of rounded, weakly adhered cells as a direct result of the nanoparticles may explain why the cells are undergoing reduced proliferation.

From the present study it is apparent that variations in cell adhesion and proliferation observed with the fibroblasts cannot be explained without mentioning the physiochemical properties of silica. The serum proteins will interact with such materials when placed in to the culture media. Previous studies have shown that increasing the serum concentration did not change the fibroblasts cellular response (results not shown). In addition, the surface chemistry and topography of the silica will govern the protein–material interaction creating a conditioned surface to which the cells respond. It could be hypothesised that the serum proteins adsorb differently to the nanostructures and this results in weakly adhered cells. As a consequence the focal adhesion sites of the cells may be limited to discrete localised regions in between the silica particles and adhere to the underlying glass substrate. This would influence the signal transduction of intergrin receptors responsible for normal cell adhesion and cytoskeletal arrangement. Another property of silica particles is that they undergo dissolution in aqueous environments to form monosilicic acid [11]. The dissolution rate of the reaction depends on a multitude of different factors including: silica surface area and radius of curvature, surface hydration (Si–OH), solvent pH, temperature and volume [11]. Generally, the smaller silica particles dissolve at a greater rate than larger ones [12]. Maybe the cells focal adhesion sites cannot be established because the surface is being continually remodelled and rearranged at the nanometer scale. The cells may also be responding to the concentration of dissolved silica in the culture media.

This nanoscale modification with silica has been tested with a range of mammalian cells from both primary and established cell lines and more recently, cells of prokaryotic origin and has proved to be equally effective as reported here [10]. This method is convenient, simple to perform, and adaptable to three-dimensional surfaces. All or part of the surface of the substrate can be coated. It can be applied to materials of clinical relevance from a range of organic substrates such as polymers or inorganic substrates such as glass, ceramics and metals using the same procedure. Future work will address the cell response in terms of gross morphology of different cell types. The cell response of bacterial adhesion is currently under investigation along with the physiochemical properties of the silica nanoparticles. Such nanoparticulate materials may be used for implantable devices or surfaces where cell attachment needs to be controlled.

Conclusions

It has been shown that silica particles initiate a distinct cellular response affecting the morphology, adhesion and proliferation of fibroblasts. Their overall response to silica changes the cellular behaviour over prolonged time periods with no detrimental effect on cell viability.

Acknowledgments

The authors gratefully acknowledge the financial support of Giltech Ltd, the EPSRC, and the Colloid Impact Faraday/DTI Scheme case award no. 01302879. We would also like to thank Dr. John Hunt for his help with image analysis.

References

1. R. G. FLEMMING, C. J. MURPHY, G. A. ABRAMS, S. L. GOODMAN and P. F. NEALEY, *Biomaterials* **20** (1999) 573.
2. A. S. G. CURTIS and C. WILKINSON, *Trends Biotechnol.* **19** (2001) 97.
3. B. WÓJCIAK-STOTHARD, A. S. G. CURTIS, W. MONAGHAN, K. MACDONALD and C. WILKINSON, *Exp. Cell. Res.* **223** (1996) 426.
4. A. M. RAJNICEK and C. D. MCCAIG, *J. Cell. Sci.* **110** (1997) 2905.
5. A. M. RAJNICEK and C. D. MCCAIG, *ibid.* **110** (1997) 2915.
6. A. S. G. CURTIS, B. CASEY, J. O. GALLAGHER, D. PASQUI, M. A. WOOD and C. WILKINSON, *Biophys. Chem.* **94** (2001) 275.
7. M. J. DALBY, S. J. YARWOOD, M. O. RIEHLE, H. J. H. JOHNSTONE, S. AFFROSSMAN and A. S. G. CURTIS, *Exp. Cell. Res.* **276** (2002) 1.
8. M. J. DALBY, S. CHILDS, M. O. RIEHLE, H. J. H. JOHNSTONE, S. AFFROSSMAN and A. S. G. CURTIS, *Biomaterials* **24** (2003) 927.
9. R. BARBUCCI, D. PASQUI, A. WIRSEN, S. AFFROSSMAN and A. S. G. CURTIS, *J. Mater. Sci. Mater. Med.* **14** (2003) 721.
10. M. J. GARVEY, R. L. WILLIAMS, P. J. DOHERTY, J. FINK and B. G. COUSINS, International Patent Application No. P 405296 WO (2002).
11. R. K. ILER, in ''The Chemistry of Silica'' (John Wiley & Sons, New York, 1979).
12. T. CORADIN and P. J. LOPEZ, *Chem. Bio. Chem.* **3** (2003) 1.
13. S. HUANG and D. E. INGBER, *Nat. Cell. Biol.* **1** (1999) E131.
14. J. D. ANDRADE, in ''Surface and Interfacial Aspects of Biomedical Polymers'' (Plenum Press, New York, 1985).

Received 4 October
and accepted 10 October 2003