Characterization of proteins and fibroblasts on thin inorganic films

CONSTANTINA S. GIANNOULIS, TEJAL A. DESAI*
Department of Bioengineering, University of Illinois at Chicago 60607, IL, USA
E-mail: Tdesai@uic.edu

The ability of biomaterial surfaces to regulate cell behavior requires control over surface chemistry and material microstructure. One of the goals in the development of silicon-based biomedical devices such as biosensors or drug delivery systems is improved biocompatibility which may be achieved through the deposition or adsorption of thin films. In this study, films of single crystal silicon, stoichiometric and low stress silicon nitride, doped and undoped polysilicon, as well as Arg-Gly-Asp (RGD) peptide adsorbed surfaces characterized in terms of protein adsorption or cellular adhesion for a period of four days. Protein adsorption studies using fibrinogen and albumin, two proteins implicated in cellular adhesion and surface activity, reveal that low stress silicon nitride surfaces have a 223% $\pm$ 2.50% greater protein adsorption compared to undoped polysilicon surfaces, followed by silicon nitride, unmodified silicon, and doped polysilicon surfaces, respectively. The thickness of the adsorbed albumin and fibrinogen layer on various thin films was measured by ellipsometry and compared to contact angle measurements. The greatest cellular adhesion was observed on undoped polysilicon, followed by unmodified (control) silicon, low stress silicon nitride, silicon nitride, and doped polysilicon surfaces. Cellular binding supports the differential protein adsorption found on modified and unmodified silicon surfaces. Understanding the biological response to thin films will allow us to design more appropriate interfaces for implantable diagnostic and therapeutic silicon-based microdevices.

1. Introduction

The application of micro-electromechanical or micro-mechanical systems to the biomedical arena (BioMEMS) has tremendous potential in terms of developing new diagnostic and therapeutic modalities. Micro- and nano-fabrication techniques are currently being used to develop implants that can record from, sense, stimulate, and deliver to biological systems. Micromachined neural prostheses, drug delivery micropumps/needles, and microfabricated immunoisolation biocapsules [1–3] have all been fabricated using precision-based silicon (PBS) technologies. However, as BioMEMS technology is rapidly being developed in the laboratory, the clinical use of implantable microdevices has been greatly limited due to the inability to effectively interface with the biological milieu, in a non-immunogenic and stable manner. Thus, there is a need to look at silicon-based interfaces that are compatible with both microfabrication processing and biological systems.

There has been little investigation into the biocompatibility of silicon thin films. As semiconductor materials, silicon and silicon-based materials have never been considered an optimal biomaterial, as compared to numerous other materials such as polymers, metals, and ceramics. Nonetheless, silicon has been shown in several studies to be non-toxic in cortical tissue, making it a potentially attractive biomaterial for use in biomedical microdevice fabrication [4]. Studies by L. T. Canham [5] have investigated silicon bioactivity with regards to *in vivo* bonding ability. It was found that while bulk silicon is relatively bioinert, hydrated microporous silicon coatings were both biocompatible and ''bioactive''. Results suggested that silicon and silicon containing films could and should be seriously considered for widespread *in vivo* investigations and applications as a biomaterial. Therefore, in light of the potential applications of silicon thin films, it is important to characterize these materials in terms of protein adsorption and cellular adhesion.

The nature of a cell-material or protein-material interaction is largely controlled by surface chemistry and microstructure. Thus, surface modification of biomaterials is the key to long term integrity and functionality of an implanted biomedical device. In particular, silicon biomedical microdevices may require surface modifications to improve their biocompatibility

*Author to whom correspondence should be addressed: Department of Bioengineering (MC 063), University of Illinois at Chicago, 851 South Morgan Street, Chicago IL 60607.

in terms of protein adsorption which can eventually lead to surface-induced thrombosis and/or excessive fibrosis [6, 7]. Moreover, cell adhesion and subsequent proliferation is dictated by initial protein adsorption. The activation of collagen specific receptors upon cell adhesion to a surface can lead to collagen deposition and fibrotic encapsulation of the implant [8]. At the same time, however, a surface that promotes favorable material-cell interactions is also desired.

Control over biomaterial surface chemistry and microstructure can result in a regulated cell response. For instance, the immobilization or coating of cell-adhesion peptides (i.e. RGD) to the surface of materials has been successful in enhancing adhesion of tissue-forming cells [9]. In general, materials can be surface modified by using physicochemical or biological methods. Physicochemical surface modifications fall into two categories: (1) chemically or physically altering the atoms, compounds or molecules in the existing surface (treatment, etching, chemical modification), or (2) overcoating the existing surface with a material having a different composition (coating, grafting, thin film deposition) [7].

In this study, thin films of silicon nitride, low stress silicon nitride, undoped polysilicon, doped polysilicon, or RGD adsorbed silicon were prepared. Peptides containing the RGD amino acid sequence have been found to promote adhesion of cells [10]. The biocompatibility of these surfaces was addressed by investigating their interactions with plasma proteins and cells. Albumin and fibrinogen were chosen as model proteins in this study, since they are implicated in cellular adhesion and surface activity. Fibroblasts were selected for the cell-surface interaction studies because of their anchorage-dependent properties and role in the fibrotic response [12].

2. Materials and methods

Human albumin and fibrinogen were purchased from Sigma, and stock solutions of 1 mg/ml in PBS were prepared. Single crystal silicon ($\langle 110 \rangle$ and $\langle 111 \rangle$ planes) and thin films of stoichiometric silicon nitride, low stress silicon nitride, undoped polysilicon, and doped polysilicon were prepared (Table I) as 8×12 mm slides. The slides were cleaned before use by immersion in a piranha solution (3 : 1 of v/v sulfuric acid : peroxide) for 10 min, followed by rinse in running deionized water until the water resistivity reached 12 mΩ. The UIC Microfabrication Applications Laboratory provided the piranha chemicals.

2.1. Albumin and fibrinogen adsorption

Prior to the adsorption of 1 mg/ml human albumin in PBS, silicon samples were rinsed with deionized water and blown dry using nitrogen. The individual samples were transferred into a 24-well tissue culture plate (14 mm across internal diameter). Next, 1000 µL of prepared albumin solution was added into each well. Adsorption proceeded for 1 h in an incubator at 37 °C. After adsorption was complete, the samples were washed three times with 1 mL of deionized water per well. The samples were dried with nitrogen and immediately transferred to an ellipsometer for measurement of the adsorbed protein thickness. The above procedures were repeated with a 1 mg/ml concentration of fibrinogen in PBS.

2.2. Ellipsometric measurement

Albumin and fibrinogen coatings were measured using an automatic Gaertner ellipsometer equipped with a 632.8 nm helium–neon laser. The thicknesses and refractive indices of control and protein films were determined using an ellipsometer computer program. Three ellipsometer measurements at different locations of each sample were taken and the average was calculated. Protein thickness measurements were carried out within 45 min after preparation of the coatings.

2.3. Contact angle measurements

Contact angles with water were measured in air by the sessile-drop method using a contact angle meter (Tantec, Inc.). Readings were made after the angles were observed to be stable with time. Contact angles stabilized in less than 90 min on all surfaces similar to data reported by Margel *et al*. [11]. Reported measurements are an average of three readings taken at different spots on each sample.

2.4. Fibroblast proliferation

Cell adhesion properties of the various surfaces were evaluated *in vitro* by cultivating a finite cell line of myocardial fibroblasts (provided by Dr Brenda Russell, UIC Department of Physiology and Biophysics) on these surfaces. This is an anchorage-dependent cell line harvested from Sprague-Dawley rats. Passage numbers 1–6 were employed in these experiments.

TABLE I Thin film processing parameters

Film type	Processing	Characteristic
Polysilicon	Chemical vapor deposition	High modulus, small (< 50 nm) grains
Doped polysilicon	Ion implantation	Boron concentration $> 7 \times 10^{19}$ cm^{-3}
Silicon nitride (stoichiometric)	Chemical vapor deposition (CVD)	High modulus (> 170 GPa), large tensile stress, silicon-rich nitride
Low stress silicon nitride	Low pressure chemical vapor deposition (LPCVD)	Low modulus (≈ 120 GPa), nitride rich surface

2.5. Culture conditions and cell number determination

Fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an incubator with 5% CO_2. The fibroblasts were removed from tissue culture flasks by incubating in 0.25% trypsin-EDTA for 5 min. Cell suspensions were counted with a hemacytometer, and then seeded on modified and unmodified silicon surfaces for cell growth observation.

2.6. Cell proliferation assay

As previously described by Zhang *et al*. [8], the measurement of change in number of attached cells on modified and unmodified silicon substrates was determined at one, two, and four days (28). Approximately 4×10^4 fibroblasts in 0.3 ml culture medium containing 10% FBS were pipetted into each well of 24-well tissue culture dishes containing the modified and unmodified silicon substrates. After designated incubation time, the sample substrates were transferred to empty culture plate wells and rinsed twice with 1 ml PBS to remove unattached cells. Substrates were then incubated with 0.2 ml of 0.05% trypsin-EDTA to remove attached cells. Next, trypsin was neutralized with 0.3 ml DMEM + 10% FBS. Cells detached from the substrates were counted twice with a hemacytometer. In addition, culture medium was replaced with fresh medium every 24 h.

3. Results

3.1. Protein adsorption

The thickness of clean, control surfaces, as well as thin films and adsorbed protein thickness is given in Table II. The thickness of the individual protein layers is determined by taking the difference of the thickness of the untreated surfaces and the thickness of the adsorbed surfaces. The thicknesses of the adsorbed albumin and fibrinogen layers on each thin film are shown in Fig. 1 and Table III. Both proteins displayed maximum adsorption on low stress silicon nitride surfaces. This may be due to amine groups located on the surface of these films which interact with the amine groups of proteins [13]. In contrast to this finding, stoichiometric nitride films exhibited much less adsorption of both proteins. Stoichiometric silicon nitride presents a greater ratio of silicon to nitrogen compared to low stress nitride. This suggests that proteins have greater affinity to amine groups versus silicon. The decrease in surface amine groups may result in less protein-film interaction, thus less adsorption. Furthermore, the greater stress state of silicon nitride versus low stress silicon nitride (170 GPa versus 120 Gpa) may also negatively influence adsorption.

The adsorbed thickness of fibrinogen is greater than that of albumin on all surfaces. The molecular sizes of fibrinogen and albumin are $60 \times 60 \times 450$ and $40 \times 40 \times 140$ Å, respectively [14]. The adsorbed thickness correlates reasonably well with relative protein molecular size. The least adsorption is obtained for both proteins on doped polysilicon, indicating that the presence of the high surface concentrations of boron found in the polysilicon may also inhibit protein adsorption. A water contact angle of 67° is measured on a clean, doped polysilicon surface (Table III). This surface has the greatest contact angle and is therefore, the least hydrophilic of all the films analyzed. The less hydrophilic nature of the film may contribute to the decrease in albumin and fibrinogen adsorption on doped polysilicon surfaces. This may be due to the immediate preference of the charged protein solution for a more hydrophilic surface. What is interesting to note is that, with the exception of low stress nitride films, there is an inverse relationship between contact angle and protein adsorption; the greater the contact angle, the smaller the adsorbed protein layer thickness (Fig. 2). Greater adsorption (i.e. larger thicknesses, > 98 Å) reveals the presence of protein multilayers, while less adsorption (thicknesses < 98 Å) is most probably due to the formation of a protein monolayer.

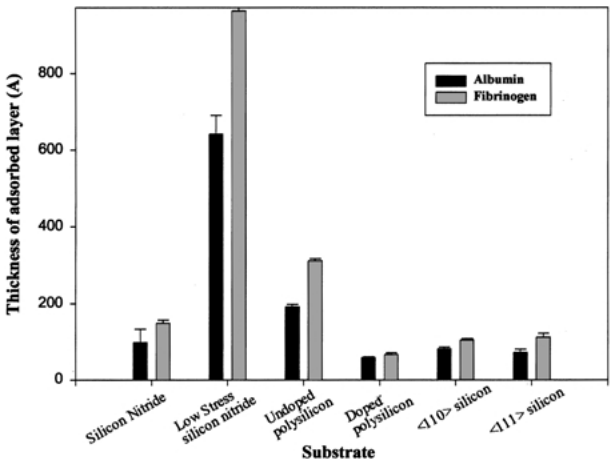

Figure 1 Thickness of adsorbed layer on different substrates. These values are computed by taking the difference between the thin film thickness and the thin film + adsorbed thickness at an incident angle of 70° given in Table I for each substrate. Reported are average values of three measurements taken at different locations of each sample.

3.2. Fibroblast proliferation

Fig. 3 shows the change in number of adherent fibroblasts on the various surfaces, determined after 1, 2, and 4 days, due to adherent cell proliferation, cell detachment, and/or cell death. Reported are average values of experiments performed four times with standard deviations. As seen in this graph, precoating silicon with a layer of adsorbed RGD peptide caused a significant increase in the number of adherent fibroblasts over time. This can be attributed to the receptor recognition sequence Arg-Gly-Asp (RGD) contained in many cell adhesion proteins, including fibronectin, vitronectin, and fibrinogen [15, 16]. Therefore, these RGD adsorbed surfaces produce adhesive substrates regardless of the underlying film. Cell viability was nearly 100% for all coatings, with a reproducible decrease in viability witnessed with doped polysilicon substrates (80–85% viability). The number of cells on all samples, except silicon nitride, increased with time and cell confluence was achieved within two days. Silicon nitride, on the other hand, had a reproducible

TABLE II Thickness of protein adsorbed vs. thickness of control thin films at two different incident angles. Each number is an average of three ellipsometric measurements conducted at three different locations of the sample

	Thin film thickness (Å)		Thin film + adsorbed albumin thickness (Å)		Thin film + adsorbed fibrinogen thickness (Å)	
	70°	50°	70°	50°	70°	50°
Silicon nitride	7673 ± 3.7	7745 ± 4.0	7771 ± 34.7	7773 ± 34.1	7822 ± 7.2	7991 ± 12.0
Low stress silicon nitride	8915 ± 4.4	9145 ± 8.9	9556 ± 50.0	9873 ± 2.0	9879 ± 7.2	9964 ± 6.1
Undoped polysilicon	98 ± 1.0	162 ± 2.6	289 ± 7.0	344 ± 10.4	410 ± 3.0	489 ± 4.0
Doped polysilicon	466 ± 0.0	494 ± 0.0	525 ± 1.7	523 ± 9.8	532 ± 5.0	540 ± 2.0
⟨110⟩ silicon	75 ± 1.7	97 ± 4	147 ± 4.1	178 ± 3.4	179 ± 3.6	182 ± 2.6
⟨111⟩ silicon	89 ± 1.7	96 ± 4	170 ± 8.2	175 ± 11.5	201 ± 9.5	219 ± 8.7

TABLE III Adsorbed protein thickness vs. contact angle reading

	Adsorbed albumin thickness (Å)	Adsorbed fibrinogen thickness (Å)	Contact angle[a] (degrees)
Si_3N_4	98.0 ± 34.9	149 ± 8.10	47
Low stress Si_3N_4	641 ± 50.2	964 ± 8.44	50
Undoped polysilicon	191 ± 7.07	312 ± 5.74	41
Doped polysilicon	59.0 ± 1.70	66.0 ± 5.00	67
⟨110⟩ silicon	72.0 ± 4.44	104 ± 3.98	53
⟨111⟩ silicon	81.0 ± 8.37	112 ± 9.65	52

[a]Contact angle is representative of non-adsorbed surfaces.

decrease in the number of adherent cells on day 2 of the experiment. The reason for this observation is not clear at this time. However, it may be due to unfavorable cell spreading on these surfaces which have high ratios of silicon to nitrogen. Initial cellular adhesion was equal and greatest on RGD adsorbed, undoped polysilicon, and ⟨110⟩ silicon. Least initial attachment was seen on doped polysilicon surfaces, similar to protein adsorption results.

The rates of change in cell number on ⟨111⟩ silicon, ⟨110⟩ silicon, undoped and doped polysilicon decreased with time. Furthermore, the rates of change of cell number for these substrates were similar despite significantly lower initial cellular adhesion on the doped polysilicon surfaces. This seems to indicate that doped polysilicon surfaces do not negatively affect cell functionality and differentiation, but rather inhibit initial cell attachment to the surface. Similar fibroblast behavior has been previously documented with polyethylene glycol (PEG) modified silicon [8]. The rates of change in cell number on low stress Si_3N_4 slightly increased with time, which is contrary to typical fibroblast behavior. This suggests that these surfaces may alter cell growth characteristics as time progresses. This may be caused by stable hydrogen and ionic bonds found at these cell-surface interfaces. The reflective microscope pictures in Fig. 4 reveal the morphology of the fibroblasts on three different surfaces. All pictures were taken on day 2 of the proliferation assay.

4. Conclusions

Thin films of single crystal silicon (⟨110⟩ and ⟨111⟩), stoichiometric and low stress silicon nitride, undoped and doped polysilicon and absorbed RGD on silicon were characterized in terms of protein adsorption and cellular adhesion. Low stress silicon nitride surfaces displayed

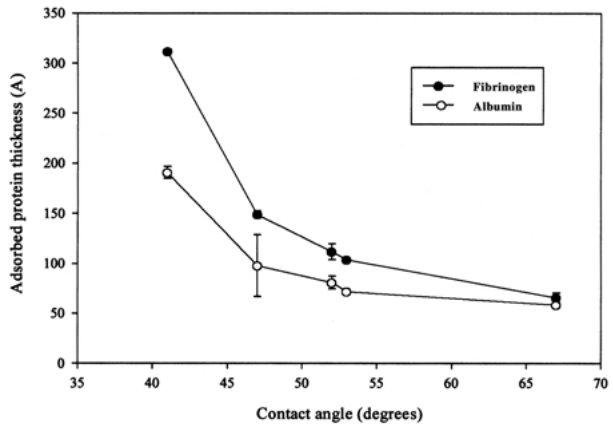

Figure 2 Adsorbed protein thickness versus contact angle reading of albumin and fibrinogen adsorbed silicon surfaces.

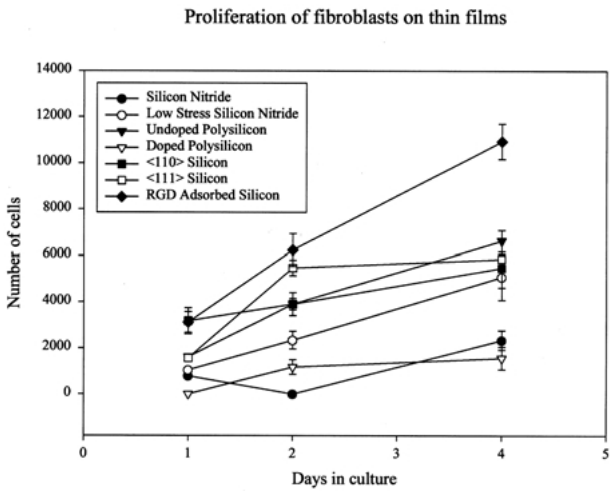

Figure 3 Proliferation of fibroblasts on thin films. Reported are average values of experiments performed 4 × with standard deviations.

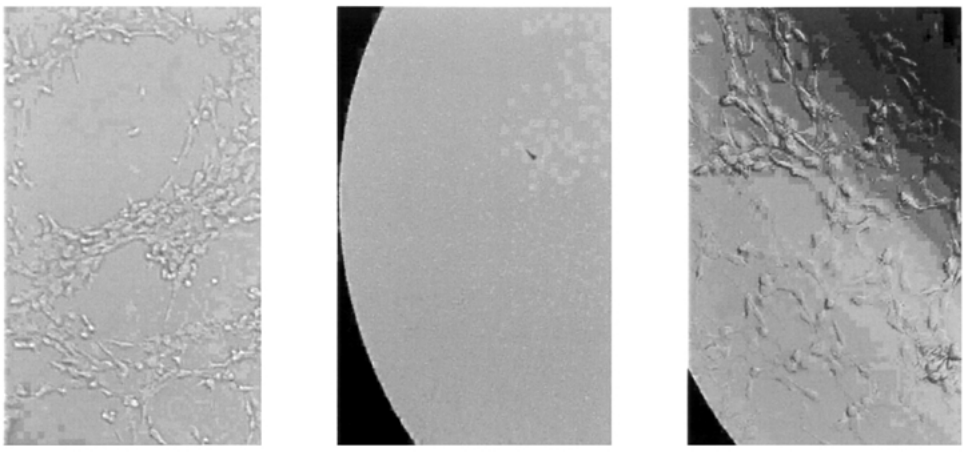

Figure 4 Morphology of fibroblasts on three different substrates. Using a 10 × objective, low stress nitride (a) and ⟨110⟩ silicon (c) surfaces display significantly greater fibroblast proliferation and attachment than doped polysilicon surfaces, which exhibit almost no cell growth (b).

the greatest adsorption of albumin and fibrinogen (223% ± 2.50% greater than undoped polysilicon surfaces), while doped polysilicon exhibited the least overall protein adsorption (over eleven times less than low stress silicon nitride). Although the surfaces are all relatively hydrophilic (contact angle $< 90°$), the results showed that more hydrophilic silicon-based surfaces result in greater albumin and fibrinogen adsorption, while less hydrophilic surfaces display less protein adsorption. An inverse relationship between protein adsorption and contact angle is seen for all surfaces except low stress silicon nitride, which had significantly greater adsorption than any other surface. The decreased protein adsorption on doped polysilicon may be explained by the presence of high surface concentrations of boron found in doped polysilicon [17]. Compared to the other substrates, boron doped surfaces also had the least cellular adhesion. This indicates that both proteins and cells are greatly inhibited from interacting with high boron concentrations.

RGD adsorbed silicon surfaces resulted in the greatest number of adherent cells (64.7% ± 4.80% greater than undoped polysilicon) on any given day during the four-day experiment. This is not surprising since recognition of the RGD peptide sequence is thought to occur by cell membrane proteins (integrins) resulting in specific receptor-ligand interactions at the cell-surface interface. The very small decrease in the rate of change in cell number on RGD adsorbed surfaces as time progressed suggests that RGD induces biochemical changes in the cell which affect its growth characteristics. Parallel to the protein adsorption results, least overall cell proliferation occurred on doped polysilicon surfaces.

In summary, protein adsorption and fibroblast proliferation was clearly sensitive to the differences in thin film chemistry and/or microstructure of the silicon substrates. It is clear that the overall biological process of cell adhesion and growth, as well as protein adsorption, are sensitive to the outermost functional groups of the modified silicon surfaces, although the exact mechanistic reasons for this distinction were not elucidated from these studies. As a consequence, well-defined surfaces with highly controled molecular and microstructural architecture have considerable potential in biomaterials investigations. Such information is important to the development of new silicon-based biomedical devices that may interact with cells and proteins in the body.

Acknowledgments

Funding for this project was provided by NSF ECS-9820829 and the Whitaker Foundation.

References

1. J. N. TUNER, W. SHAIN *et al.*, *Exp. Neurol.* **156** (1999) 33–49.
2. S. HENRY, D. V. MCALLISTER, M. G. ALLEN and M. R. PRAUSNITZ, *J. Pharm. Sci.* **88**(9) (1999) 948.
3. T. A. DESAI, W. H. CHU, J. K. TU, G. M. BEATTIE, A. HAYEK and M. FERRARI, *Biotechnol. and Bioeng.* **57** (1998) 118–120.
4. S. S. STENSAAS and L. J. STENSAAS, *Acta Neuropath.* **41** (1978) 145–155.
5. L. T. CANHAN, *Adv. Mater.* **7**(12), (1995) 1033–1037.
6. J. D. ANDRADE and V. HLADY, *Adv. Polym. Sci.* **79** (1986) 1–63.
7. B. D. RATNER and D. G. CASTNER, ''Surface Modification of Polymeric Biomaterials'' (Plenum Press, New York, 1997).
8. M. ZHANG, T. A. DESAI and M. FERRARI, *Biomaterials*, **19** (1998) 953–960.
9. S. P. MASSIA and J. A. HUBBELL, *Anal. Biochem.* **187** (1990) 292–301.
10. R. O. HYNES, *Cell*, **48**(4) (1987) 549–554.
11. S. MARGEL, E. A. VOGLER, L. FIRMENT, T. WATT, S. HAYNIE and D. Y. SOGAH, *J. Biomed. Mater. Res.* **27** (1993) 1463–1476.
12. B. D. RATNER, A. S. HOFFMAN, F. J. SCHOEN and J. E. LEMONS, ''Biomaterials Science: An Introduction to Materials in Medicine'' (Academic Press, San Diego, 1996).
13. D. J. HANSFORD, PhD. Dissertation, University of California at Berkeley (1999).
14. M. MALMSTEN and B. LASSEN, in ''Proteins at Interfaces II'', edited by T. A. Horbett and J. L. Brash (American Chemical Society, Washington DC, 1995) pp. 228–238.
15. C. F. MANDENIUS, S. WELIN, B. DANIELSSON, I. LUNDSTROM and K. MOSBACH, *Anal. Biochem.* **137** (1984) 106–114.

16. V. D. BHAT, B. KLITZMAN, K. KOGER, G. A. TRUSKEY and W. M. REICHERT, *J. Biomater. Sci. Polymer Edn.* **9**(11), (1998) 1117–1135.
17. R. C. JAEGER, ''Introduction to Microelectronic Fabrication'' **V** (Addison-Wesley, New York, 1998).
18. H. ELWING, *Biomaterials* **19** (1998) 397–406.
19. A. ULMAN, ''An Introduction to Ultrathin Organic Films'' (Academic Press, Boston, 1991).

Received 25 July 2000
and accepted 3 May 2001