JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 119–123

The surface modification of polymers to modulate endothelial cell growth

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The effect of nitrogen ion implantation or ion-assisted film deposition on the growth of human endothelial cells *in vitro* on a substrate has been studied. It was shown that the largest change occurs when TiO_2 films were reactively sputtered onto the substrates, while the most passive surface was reactively sputtered SiO_2. SEM pictures of the cells adhering to the various treated surfaces show that the cells were healthy. Future studies on the effect of alloy films and their oxides and nitrides are proposed.

1. Introduction

The bioactivity of a material is closely related to its composition and structure [1]. However, its biological response may be changed by modifying its surface which may promote either an increase in cell interaction or a decrease. Both trends can have important applications; an increase in cell growth of human endothelial cells on the inside surfaces of vascular graft material would provide a non-thrombogenic lining, while a decrease in cell growth on the walls of catheters, for example, would limit blockage and retention of potential pathogens. Unfortunately, commonly used materials for vascular grafts, such as expanded polytetrafluoroethylene (ePTFE) or woven polyester (Dacron) do not readily allow seeding of human endothelial cells [2, 3]. Kirkpatrick *et al.* [4] have recently reviewed methods for modifying such surfaces by:

(a) absorption of bioactive molecules from applied solutions;
(b) formation of reactive groups at the polymer surface; or
(c) covalent coupling of bioactive molecules.

Earlier reports [5–7] described surface modification using inorganic coatings for improved wear and bonding but have not addressed the biomedical aspects of the work. Such types of inorganic coating can provide a well-bonded coating without resorting to high temperature or chemical processes which would weaken, and even destroy, the bulk polymer material.

A relatively simple solution to the problem of wear in medical implants has been described by Dearnaley [5] who showed that nitrogen ion implantation of Ti reduced the wear of the Ti ball in a hip-joint by a factor of over 500. Legg *et al.* [6] described methods for creating a biocompatible layer by using a 1.5 keV Ar^+ ion beam to bombard a Ti6A14V substrate onto which is sputtered hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$). However, the additional Ar^+ ion energy was not sufficient to produce crystalline films and an improved enhanced plasma method was developed by Legg *et al.* [7] and shown to produce well-bonded polycrystalline coatings.

As previous work does not assess the biomedical performance of surface-modified inorganic layers, it is the purpose of the present study to evaluate this aspect of the coatings. Polymeric materials have been treated by sputter-depositing films with and without ion bombardment and the subsequent growth rates of human endothelial cells evaluated. This is a preliminary study which is intended to demonstrate the possibilities of using surface modification to alter cell adhesion. Flat samples only were used for the evaluations and only when optimium surface layers have been identified will the problems of coating complex geometrical surfaces be addressed. Experimental methods and preliminary results for such samples are described and discussed.

2. Experimental procedures

Substrates were prepared by cutting polymer materials into pieces of size 1 cm × 1 cm. The following materials were used: Tissue culture polystyrene (Bibby Sterilin Co., Stone, England), Bacteriological polystyrene (Greiner, Nurtingen, FRG) and PETP (poly ethylene terephthalate) (ICI Rotterdam, The Netherlands). Samples were cleaned in an ultrasonic bath according to the following sequence:

(i) 10 min in 10% quadraline (industrial detergent containing 2–5% NH3 from Quadraline Chemical Products., Ltd., Derby, England);
(ii) 5 min in deionized water, this step repeated three times;
(iii) 10 min in absolute ethylene (analar).

Samples were then transferred in a multi-well container for the various stages of the treatment and analysis.

The apparatus used for modification of the samples is shown schematically in Fig. 1. The two ion sources were of broad-beam Kaufman-type. The vertical source provided a 7.5 cm diameter beam of 0–10 mA 1 keV Ar^+ ions which sputtered the target material T onto the samples mounted at S. The second source provided a 7 cm diameter beam of 0–30 $\mu A/cm^2$ Ar^+, O_2^+, or, N_2^+ ions at energies from 0–500 eV. The vacuum chamber was pumped to a pressure below 5×10^{-5} Pa but the pressure rose to 3×10^{-2} Pa during deposition owing to the introduction of gases into the ion sources. A quartz crystal thickness monitor was mounted near the sample to determine arrival rates of sputtered atoms and a Faraday cup was used to measure the ion current bombarding the sample. A thermocouple on the substrate holder showed that the temperature rose only slightly during deposition to a maximum of 40 °C. In all experiments using sputtering the targets were first sputter-cleaned for about 10 min (with substrates shielded) to remove impurities and oxides.

The samples were treated in three different ways:

Method (i): simple nitrogen ion implantation using the horizontal ion source.
Method (ii): simple reactive sputtering using argon ions from the vertical ion source and a high partial pressure of oxygen in the chamber.
Method (iii): a combination of reactive sputtering and nitrogen ion bombardment of the growing film (using both sources)

Following the surface treatment, which normally provided a batch of four identical samples, samples were transferred to the multi-well container for cell-proliferation studies. Before commencing this latter stage, however, samples were sterilized using either ethylenoxide, or, gamma irradiation. Human umbilical vein endothelial cells (HUVEC) were cultured in a mixture of Ham's F-12 and Iscove's modified Dulbecco's medium in a ratio of 1:1, supplemented with penicillin-streptomycin and L-glutamine (Gibco, Eggenstein, FRG) and, in addition, 20% pooled human serum. HUVEC were detached from the culture vessels with a solution consisting of collagenase (Worthington, supplied by Biozol, Eching, FRG) and EDTA (Sigma, Deisenhofen, FRG) in HEPES buffer (Sigma) supplemented with bovine serum albumin (Sigma). Further details of the culture of human endothelial cells are given in [8]. The HUVEC were then used for seeding at a density of 10 000 cells/cm^2. At the time points indicated in the figures, the number of adhering cells was determined by detaching the cells with a trypsin/EDTA (Sigma) solution and counting them with a Coulter Counter (Coulter Electronics, Krefeld, FRG). In all experiments control surfaces, on which endothelial cells grow well, were included. These controls were new tissue culture plates (tissue culture polystyrene; from Becton and Dickinson, Heidelberg, FRG) and Thermanox discs (from Nunc, Wiesbaden, FRG).

Electron microscopic studies of the cells were performed in a Philips SEM using an accelerating voltage of 20 kV. The cells were fixed in 1.5% glutaraldehyde and dehydrated in graded alcohols before a final drying in air. They were then sputter-coated with gold.

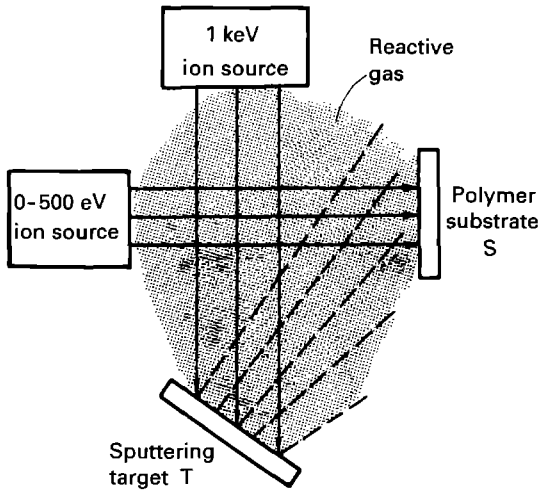

Figure 1 Schematic diagram of the dual ion beam surface treatment system.

The composition of the thin films was determined using Rutherford backscattering spectroscopy (RBS). This gives quantitative information on the amount of each element present in the coating (e.g. Si,O) but does not indicate whether compounds (such as SiO_2) have formed.

3. Results and discussion

The data for various treatments of tissue culture polystyrene (TCP) substrates, obtained from an average from four samples, are presented in Fig. 2. The lowest cell proliferation rates occur for both untreated (but cleaned) TCP and 60 nm thick SiO_2-coated samples, whereas the highest rates occur for uncleaned and untreated TCP. This result implies that the cleaning process itself has a marked effect. Surface treatment by method (i), where implantation was by 10^{16} ions/cm^2 of 150 eV nitrogen, produces a similar result to that for standard Thermanox substrates. The best of the coated samples are those treated by method (ii) which have a 60 nm thick TiO_2 coating but these do not promote adhesion as well as the untreated and uncleaned TC polystyrene samples.

Further experiments were performed to see if there was an optimum thickness in the TiO_2 layer, or, an optimum nitrogen implant dose. The results of this study are presented in Fig. 3 and show no change with thickness of TiO_2 between 20 nm and 120 nm. There is, however, an increase in cell density with nitrogen implant dose which, from other ion implantation work [9], would be expected to have reached saturation above about 5×10^{16} ions/cm^2 owing to reliberation of previously implanted nitrogen at the higher doses.

The same surface treatments were applied to bacteriological polystyrene substrates and again, as shown in Fig. 4, the presence of TiO_2, with or without treatment by nitrogen ion bombardment (methods (ii) or (iii)), increased the cell proliferation rates.

Finally, because it was difficult to deduce whether the SiO_2 films were having an effect at all (because the

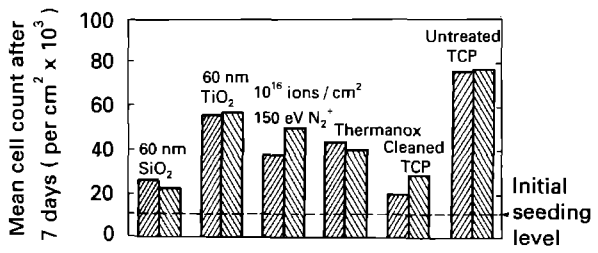

Figure 2 Cell density after 7 days incubation on treated TCP samples sterilized by gamma irradiation (■) or treatment in ethylene oxide (■).

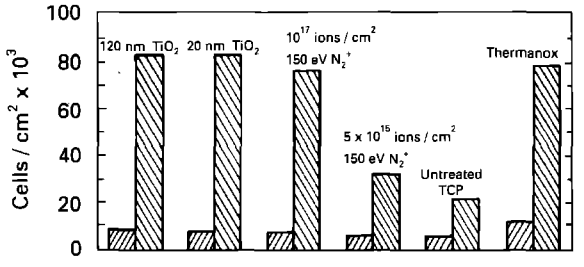

Figure 3 Cell density at day 1 (■) and after 7 days (■) incubation on treated TCP samples.

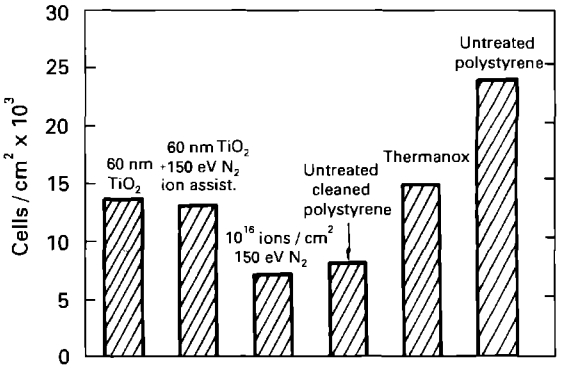

Figure 4 Cell density after 10 days incubation on treated bacteriological polystyrene samples.

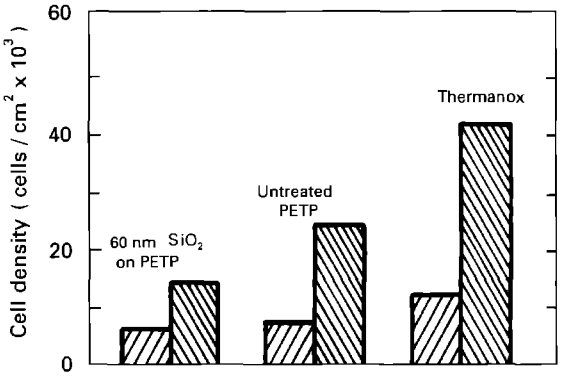

Figure 5 Cell density at day 1 and after 7 days incubation on treated PETP samples.

cell growth was the same as on untreated samples), a few experiments were performed with SiO_2 films on PETP substrates on which endothelial cells normally proliferate moderately. Fig. 5 shows that, for the few samples tested, SiO_2 appears to reduce the rate of cell growth even below the limited rate normally found on PETP.

The RBS data for 60 nm SiO_2 and TiO_2 reactively sputtered films, shown in Figs 6 and 7, respectively, indicate that the films are stoichiometric and, apart from the small argon peak to the higher energy side of the silicon edge, contain no significant levels of impurity. Other work using the same rig has shown that the argon content of such films is below 2% [10].

Scanning electron micrographs of endothelial cells adhering to the various surfaces were examined for cell spreading and coverage of the surface and the results are shown in Fig. 8. Extended flattening of cells adherent to a material indicates good interaction between cell and surface. Moreover, this means that the surface is not toxic to the cells.

As reported above, cell counting showed that a relatively large number of endothelial cells adhered to the TCP surface coated with 60 nm of TiO_2, both with and without nitrogen ion-assistance during film growth. Scanning micrographs of cells adhering to these surfaces reflected these results (Fig. 8a and b). At a relatively low magnification a surface fully covered

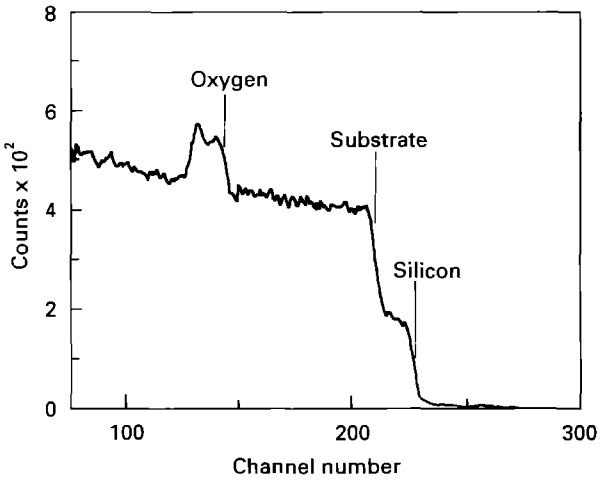

Figure 6 RBS spectra for 60 nm thick SiO_2 film on bacteriological polystyrene samples.

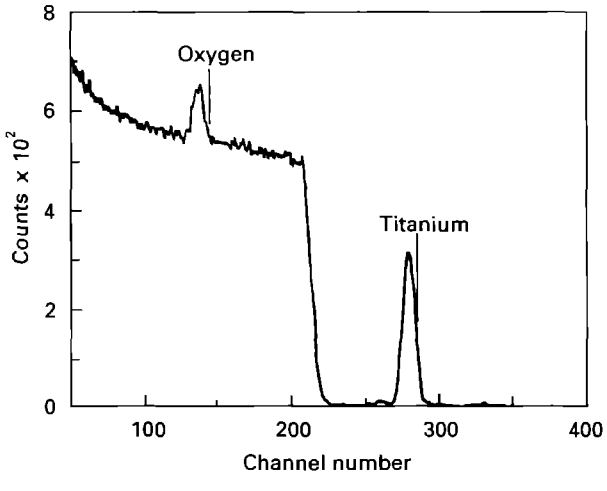

Figure 7 RBS spectra for 60 nm thick TiO_2 film on bacteriological polystyrene samples.

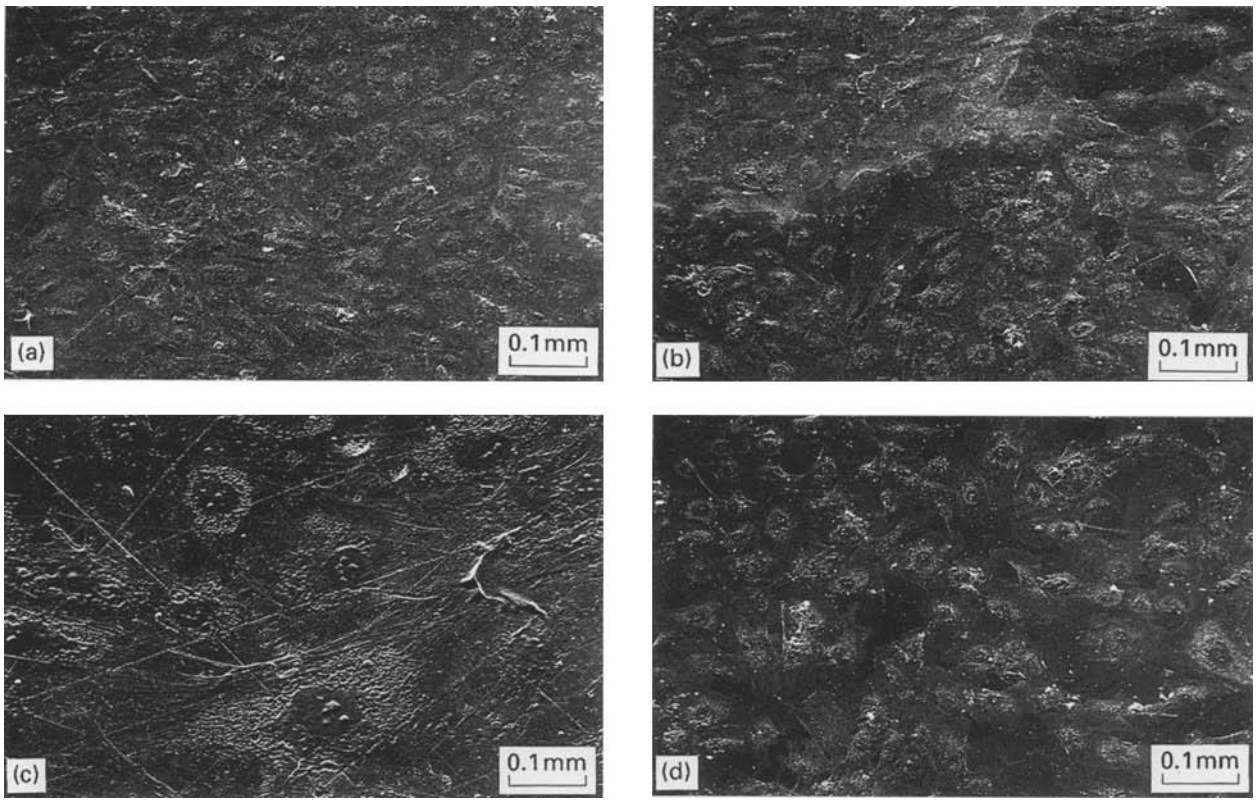

Figure 8 Scanning electron micrographs of endothelial cells adhering to various surfaces: (a) TCP coated with 60 nm of TiO_2; (b) TCP coated with 60 nm of TiO_2 using 150 eV N_2 ion assistance; (c) Enlarged view of 8(b); (d) Thermanox

with endothelial cells could be observed and the cells were well spread on these surfaces, as can be seen from the magnified view of the ion-assisted TiO_2 shown in Fig. 8c. The cells have flattened so well that surface irregularities could be seen through the cells (Fig. 8c). This means that the adherent endothelial cells are healthy and there is good interaction between the cells and the surface. A similar coverage was seen on the control material Thermanox (Fig. 8d), to which large numbers of cells also adhered. Endothelial cells adhered less well to the TCP surfaces coated with SiO_2. In this case SEM micrographs showed a less-populated surface although the cells still had a well-spread morphology. This indicates that the minor adhesion to these surfaces is not simply a result of cytotoxic influences of the material and probably is caused by lower interaction between cells and this surface.

4. Conclusions

The results show that the cell proliferation rate on a substrate can be changed by surface coating or nitrogen ion implantation. The largest increase occurs when TiO_2 films are reactively sputtered onto the substrates, while the most passive surface studied here is provided by reactively sputtered SiO_2. SEM pictures support the cell-count data and show that cells are well adhered to TiO_2. Cells are fewer in number but are well spread on SiO_2 indicating that the reduced number is not a result of cytotoxic effects. Even moderate reduction in cell adhesion could have important applications in catheters, where reduced blockage and retention of blood cells would extend their lifetime and reduced adhesion of pathogens would lessen the need for antibiotics. Future studies will concentrate on the production of alloy films and their oxides and nitrides. Physical properties of such films prepared by ion-assisted deposition in the apparatus described here have already been reported [10–12].

Acknowledgements

The authors are grateful to Dr S. D. Pringle and Dr S. P. Kaye for carrying out the deposition work, to Mrs D. Ruchatz and Mrs G. Dinger for performing the cell proliferation studies, to Mr M. Bovi for preparing the SEM specimens and making the micrographs and to the British Council and DAAD for partial financial support of this programme.

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Received 20 February
and accepted 11 May 1995