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Endothelial cell adhesion on bioerodable polymers

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This paper presents the results of a preliminary screening of a new class of bioerodable polymers, partial esters of alternating copolymers of maleic anhydride and mono-methoxyoligoethyleneglycol vinyl ethers (PAM) for use in engineered vascular tissue. Different initial concentrations of PAM and human serum albumin (HSA) were spin-coated onto glass substrates and the surface properties of the resulting films and their relationship to endothelial cell adhesion was examined. An optimum PAM/HSA blend for use as the cell contact surface of a bioerodable scaffold was identified.

1. Introduction

With the recent emergence of tissue engineering as a potential solution to the problem of organ or tissue replacement, the possibility of erecting temporary scaffolds for tissue reconstruction is receiving great academic as well as public attention.

The applications of tissue engineering are vast, and range from disease control, wound repair, and organ replacement to plastic surgery and gene therapy.

One of the principal sectors of application is in cardiovascular prosthetic replacements, and in particular in arterial or venous substitution. In fact, one of the main goals of vascular prosthesis research is the development and realization of small diameter (less than about 6 mm) grafts lined with endothelial cells, which could prevent the onset of thrombosis. Up to now small diameter prosthesis have evaded success in implants because among other problems, the reduced diameter of the prosthetic vessel rapidly occludes blood flow owing to the formation of clots [1].

Several approaches have been used to render the biomaterials used in vascular prosthesis more suitable for endothelial cell adhesion. For example, the preparation of sufaces rich in RGD peptides, heparin and antibodies that recognize endothelial cell membrane receptors has been reported, [2–4].

In a recent and pioneering work, a group of Canadian researchers from the University of Laval reported the reconstruction of a completely biological tissue engineered blood vessel [5]. In this seminal work, smooth muscle cells were cultivated with ascorbic acid to produce a structure which could, after some time, be formed into a manageable tube. Subsequently, fibroblasts and endothelial cells were introduced to provide the adventitial and initimal layer, respectively. The entire process took about three months, mainly owing to the long length of time required to produce the smooth muscle cell tube. In some critical cases, shorter times would be more acceptable and this may be achieved by using a preformed bioerodable material as the structural base of the cylinder, rather than sheets of living cells.

In this paper we present the results of a study on a new class of bioerodable polymer, partial esters of alternating copolymers of maleic anhydride and mono-methoxy-oligoethyleneglycol vinyl ethers (PAM) [6]. These polymers have been used for controled release implants and show low levels of toxicity both *in vivo* and *in vitro*. When combined with albumin, the polymer shows enhanced biocompatibility and certain PAM/albumin blends exhibit hydrogel properties [7]. The purpose of this study was to investigate the feasibility of utilizing PAM in vascular prosthesis and for the reconstruction of small diameter tissue engineered vascular grafts. The polymer can be used as a temporary preformed bioerodable scaffold, upon which cells can be cultivated and which, compared with the Laval graft, may reduce the time required for vessel reconstruction. In our approach, we used PAM and its blends with human serum albumin (HSA) as a basic bioerodable structure with which to initiate tissue engineering studies. The results of a preliminary investigation on the surface structure of thin films of PAM obtained with different concentrations of HSA are reported. The relationship between surface properties and endothelial cell adhesion was also examined, and although several aspects still remain to be elucidated, the results show that PAM is an

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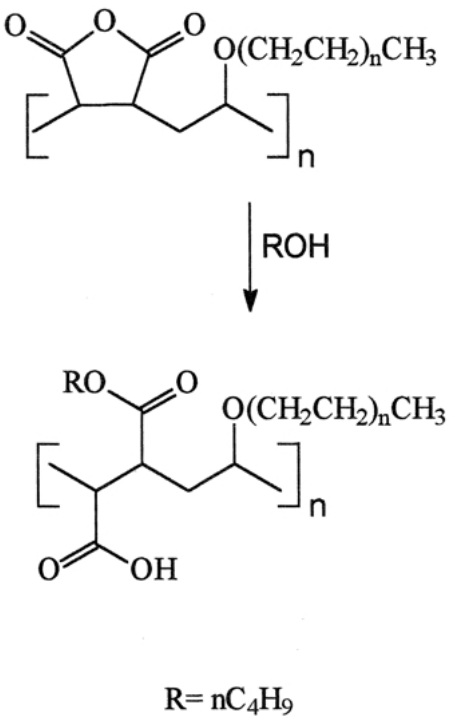
excellent substrate for endothelial cell adhesion and is suitable for use as a component of a bioerodable scaffold for tissue engineering.

2. Materials and methods

The structure of PAM is given in Scheme 1, and the polymer was prepared as described in [7]. Human serum albumin (HSA, 99% purity) was purchased from Sigma. All other substances were of reagent grade and milli-Q water was used in all aqueous solutions.

PAM is soluble in ethanol, and partially soluble in water. The solutions were prepared in different concentrations (1%, 5% and 10% w/v) in 1 : 4 ethanol : water. PAM and HSA blends were made up by adding aqueous HSA to PAM solutions in 1 : 4 ethanol : water. HSA was added to give the following final concentrations in the solution: 0.015%, 0.03%, 0.06%, 0.09%, 0.12%, 0.3%, 0.6%.

Films were deposited on 30 × 20 × 3 mm hydrophilic glass slides by spin-coating at a velocity of 2200 rpm. This velocity was found to be the optimum for obtaining smooth, transparent films. Subsequent to deposition, the samples were placed in tissue culture dishes (NUNC, Nalge) with wells corresponding to the dimensions of the slides and irradiated under u.v. light for several hours. This served not only to sterilize the samples, but also appeared to enhance cellular adhesion and rendered the samples less soluble in the culture medium owing to u.v. induced crosslinking between polymer chains.



Scheme 1 Structure of PAM (partial esters of alternating copolymers of maleic anhydride and mono-methoxyoligoethyleneglycol vinyl ethers).

2.1. HSA release

The amount of HSA released by PAM/HSA samples (spun from 10% PAM-0.3% HSA and 5% PAM-0.12% HSA solutions, respectively) was evaluated using the Pierce Micro BCA assay. In these experiments, films were spin-coated onto small slides which were able to fit directly into a spectrophotometer cuvette.

Slides were incubated for different lengths of time (from one to four days), and the protein content of the resulting solutions were assayed. A PAM (without HSA) sample was used to determine the background absorbance level since PAM itself appears to interfere with the assay.

2.2. Angle of contact measurements

The hydrophilic/hydrophobic nature of a surface appears to play an important role in cell surface interactions with wettable (hydrophilic) surfaces being more conductive to adhesion [8]. Uncoated glass slides as well as samples with PAM and PAM/HSA films were analyzed. Angle of contact measurements were made using the bubble-air method [9] in which a small bubble of air is introduced on a slide under water. The dimensions of the bubble, measured with a microscope with a graduated eyepiece, can be used to calculate the angle of contact between the air bubble and the sample. Using this technique, an angle of less than 90° is indicative of a hydrophilic surface whereas angles much larger than 90° are indicative of a non-wettable hydrophobic surface. Six points were measured per slide and averaged. The error on the value of contact angle is not constant and varies with the angle being measured. For the range of angles reported here, the error is about 5°.

2.3. Ellipsometry

Ellipsometry is a very sensitive optical technique for measuring the thickness and refractive index of thin films. An automated ellipsometer (Auto-EL II, Rudolph Instruments, USA) set at an incidence angle of 70° and with an incident beam of 633 nm was used. Each sample was measured in nine different points along the slide in order to construct a topological map of the substrate. Film thickness and refractive indices for each point were obtained by applying a minimization routine to the ellipsometry equations for a single layer non-absorbing isotropic film. The ellipsometry equations describe the relation between the ellipsometric parameters Δ and , and the optical constants (thickness and refractive index) of a film.

2.4. Surface potential

Whilst several of the mechanisms of cell-surface interactions remain unclear, it is well known that positively charged surfaces enhance cell adhesion with respect to neutral or negatively charged surfaces [10]. The surface potential measuring technique was used to examine the effect of surface potential or charge on cell adhesion. Surface potential measurements were made using a Kelvin vibrating plate (KSV Instruments, Sweden), with a nominal error of $\pm$ 10 mV. The Kelvin vibrating plate measures the surface potential between two conductors, the sample and probe respectively, placed about 1 mm apart. The difference in measured potential between a sample with no polymer

(the reference potential) and one which has the polymer deposited upon it is the potential difference due to the polymer, and depends on its thickness, dielectric constant and surface charge density. For these experiments the polymers and polymer–protein blends were spin-coated on aluminum plates, using the same conditions as for the glass slides.

2.5. Cell adhesion

Cell adhesion studies were performed using primary endothelial cells extracted from the umbilical cord using standard protocols [11]. Cells were cultured upto four passages in medium 199 containing 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml ampotherin B, 100 µg/ml porcine heparin and 50 µg/ml growth factor. Prior to seeding, the samples were equilibrated with serum free medium for about an hour. Endothelial cells were seeded onto the slides at a density of about 60 000 cells/cm^2. The samples were placed in a humidified incubator with 5% carbon dioxide. Gelatin and clean glass slides were used as controls.

Samples were observed using an optical microscope (Provis, Olympus, Japan) hourly for the first two hours, and then over a period of 1–3 days to evaluate the extent of endothelial cell adhesion and proliferation. The cells were identified as endothelial by their polygonal morphology. An estimate of the number of cells per unit area of the samples was obtained by counting cells in a Buerker chamber.

From some of the samples, the cell nuclei were also examined for signs of apoptosis by staining with propidium iodide [12].

2.6. Scanning force microscopy (SFM)

Samples with different concentrations of PAM were analyzed using scanning force microscopy to determine surface morphology and roughness. The SFM used was from Park Scientific Instruments (PSI, Sunnyvale, CA, USA) and equipped with a 100 µm scanner (with Scanmaster™, a closed loop position correction system) and a 5 µm scanner for higher resolution work. The scanners were calibrated in the x and y directions by using a 1 µm gold grating. For z-axis calibration, a VLSI (very large scale integration) standard step of 100 nm of height was used. Images in contact mode at constant deflection were taken using 300 µm-long, 0.6 µm-thick V-shaped cantilevers (supplied by PSI) which had a nominal spring constant $K = 0.01$ N/m, at the minimum possible interaction force. Images in non-contact and intermittent-contact mode were taken using 180 µm-long, 2 µm-thick Ultralevers (PSI) which had 90 KHz nominal resonance frequency.

Surface roughness analysis was performed using the standard instrument software after plane subtraction on each image and mean values were calculated on ten different images of the same size taken on each sample.

3. Results

3.1. Angle of contact and HSA release

The data presented in Fig. 1 are the angles of contact of PAM and PAM-HSA samples with respect to a clean hydrophilic slide (contact angle $19 \pm 4°$). PAM was found to be essentially hydrophilic with the angles of contact increasing slightly with increasing concentrations of PAM. The introduction of increasing concentrations of HSA appeared to increase the angle of contact significantly, rendering the films hydrophobic with respect to PAM.

The quantity of HSA released from both samples investigated was 0.85 ± 0.20 µg of HSA per mg of polymer per day, and did not change appreciably over the four days during which the measurements were made.

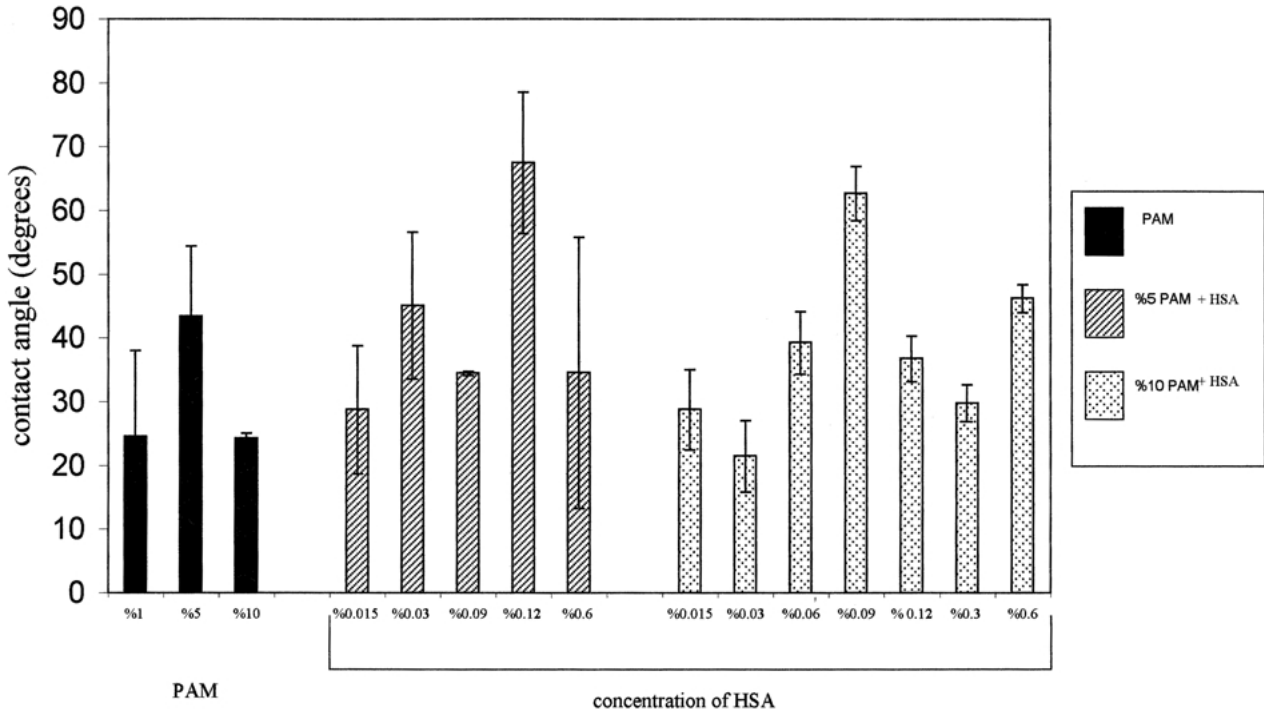


Figure 1 Angles of contact of PAM and PAM/HSA blends with respect to a clean hydrophilic glass slide.

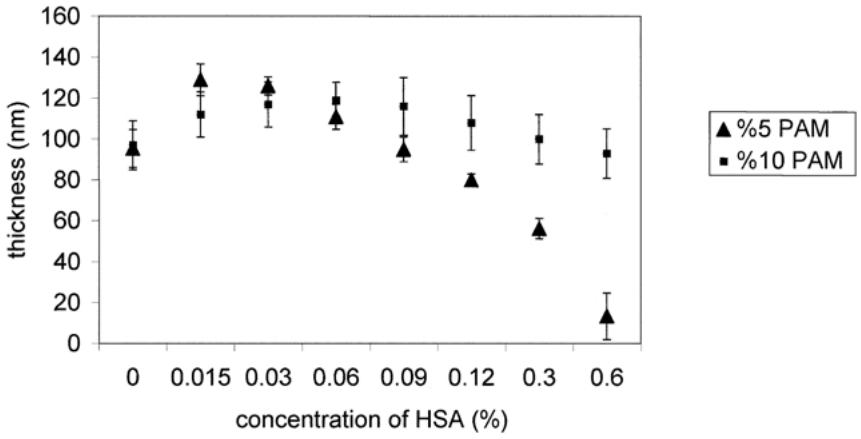


Figure 2 Thickness of PAM/HSA with increasing concentrations of HSA (■ 10%, ▲ 5%).

3.2. Ellipsometry

The refractive index for all samples remained more or less unchanged (1.471 ± 0.014), and for all three parent PAM concentrations used for spin-coating, the resulting PAM films were all 97 ± 10 nm thick. With the addition of HSA in the spinning solution, the thickness initially increased and then decreased with increasing amounts of protein (Fig. 2). This variation was particularly marked in samples prepared 5% PAM and HSA, and could be due to differences in the amount of water incorporated into the mixtures during the spinning process, or to phase separation of the blends at very high or low concentrations of HSA.

3.3. Surface potential

The surface potential of PAM was measured with and without HSA. With respect to an uncoated aluminum plate, films spun from 1% PAM and 10% PAM have positive surface potential values (about 100 mV), whereas those spun from 5% PAM have negative values of around -170 mV (Fig. 3). We also measured surface potentials of films spun from 3 and 6% PAM solutions, to ascertain that this behavior was not due to experimental error or other artifacts. As shown in Fig. 3, the surface potential shows a minimum at around a concentration of 5%. The reason for this variation in surface potential with PAM concentration is unclear, but it does tie in with the results on cell adhesion. In fact, cells did not adhere to films prepared from 5% PAM, which have a negative potential compared with the other two concentrations investigated.

In the curves shown in Fig. 4, the surface charge density σ (coulombs/m^2) of the PAM/HSA samples has been calculated using the following equation:

$$\sigma = \frac{2\varepsilon_o \varepsilon_f V}{d_f(\varepsilon_f - 1) + R} \tag{1}$$

where ε_o is the permittivity of free space (Farad/m), ε_f the relative permittivity of the polymer film, V (volts) the measured surface potential (difference between a coated and uncoated aluminum plate), d_f the film thickness (m), and R (m) the radius of the aluminum disk. This equation is derived from first principles using the approximation that R is much greater than both d_f and the distance between probe and sample. It should be noted that the values used for d_f and $\varepsilon_f(\varepsilon_f = n_f^2)$ were obtained from the ellipsometric measurements on spin-coated glass slides, and may not correspond exactly to those obtained by spin-coating on aluminum plates. All samples spun from 10% PAM and HSA show about the same surface charge density, independent of HSA concentration. The films spun from 5% PAM and HSA show a two-step variation in surface charge density, and the saturation values for 5% PAM and HSA blends are about five times higher than for 10% PAM and HSA blends.

3.4. Endothelial cell adhesion

An initial set of cell adhesion experiments was performed on PAM without the presence of HSA.

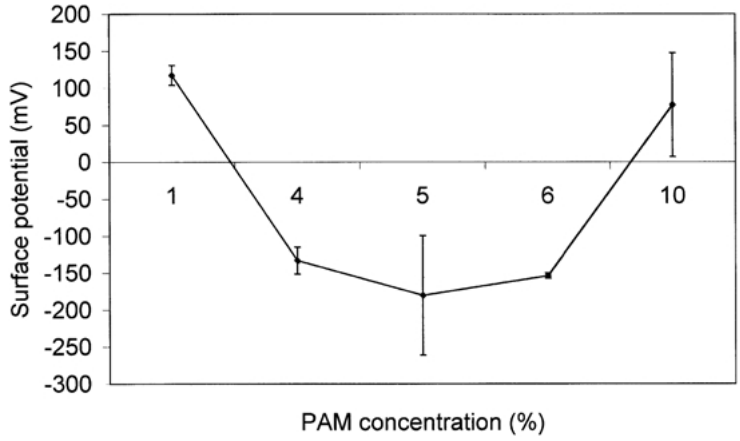


Figure 3 Surface potential of PAM films prepared from various parent concentrations.

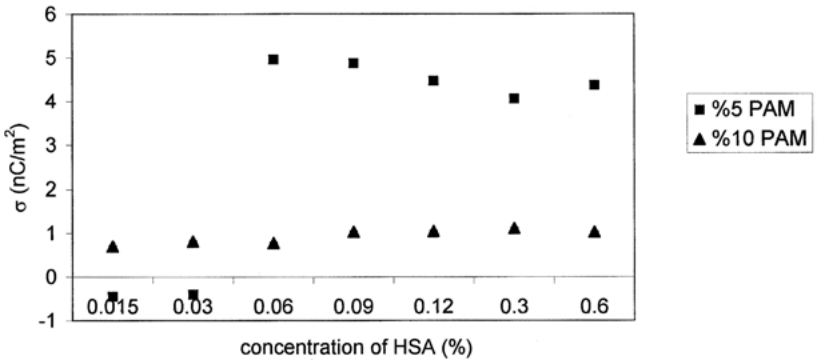


Figure 4 The surface charge density of PAM/HSA blends with increasing concentrations of HSA (▲ 10%, ■ 5%).

Films spin-coated from 1%, 5% and 10% PAM were seeded with endothelial cells and observed with an optical microscope over a period of about 2–3 days. The samples were monitored $\frac{1}{2}$, 1 and 2 h after seeding to monitor the initial process of cell adhesion, and to ensure that adhesion was substrate and not medium mediated. It was observed that while a fair number of cells adhered to films from 10% PAM, fewer cells adhered to 1% and less so to 5% PAM. Fig. 5 reports the cell densities 24 h after seeding, measured using a Beurker chamber.

1% PAM is a very low concentration and may result in structures that are mechanically and structurally too weak and thin to be useful in scaffold architecture. This concentration was thus not used for subsequent trails with HSA.

Following the initial experiments, 5 and 10% PAM were blended with different concentrations of HSA in an attempt to improve the cell adhesion properties of the polymer. It was observed that on several samples, particularly those prepared from 10% PAM, cell adhesion occurred within the first half hour.

Fig. 5 reports the variation in density of adhered cells after one day for different PAM and HSA blends. The addition of HSA enhances cell adhesion, but in what appears to be a fairly random manner, since the concentration of HSA does not correlate with cell density. The most promising was 0.3% HSA and 10% PAM, a blend which gave rise to cell adhesion and proliferation densities as well as morphologies similar to those of gelatin controls.

Samples with cell densities higher than about 40 000 cells/cm^2 were fixed and stained with propidium iodide. None of the samples showed any signs of apoptotic DNA.

3.5. SFM

The surface morphology of surfaces prepared from 1%, 5% and 10% PAM with and without HSA was evaluated using SFM and data on surface roughness using region analysis software was extracted.

Images of films spun from 1% PAM (Fig. 6(a)) show a uniform surface almost totally covered by small globular shape aggregates, with a surface RMS roughness of 3 nm. Images of films spun from 5% PAM (Fig. 6(b)) showed a much rougher surface (roughness up to 30 nm depending on the region scanned), with large randomly distributed aggregates. In the case of films spun from 10% PAM (Fig. 6(c)), images show an extremely smooth surface with a surface roughness of 0.8 nm, with small pit holes with diameters between 100 and 400 nm and depths from 1 to 10 nm.

In the case of samples containing HSA, a reduced selection of concentrations, on the basis of cell growth results, was examined: 0.09% and 0.3% HSA with 10% PAM and 0.12% and 0.03% HSA with 5% PAM. In all

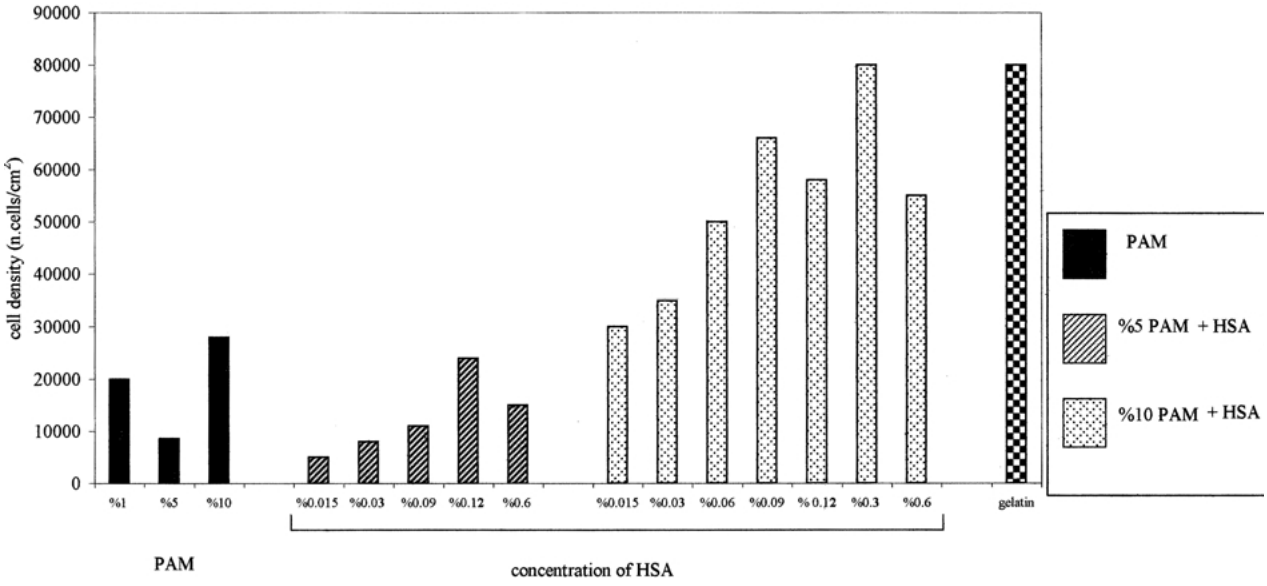


Figure 5 Cell densities on various samples after 24 h.

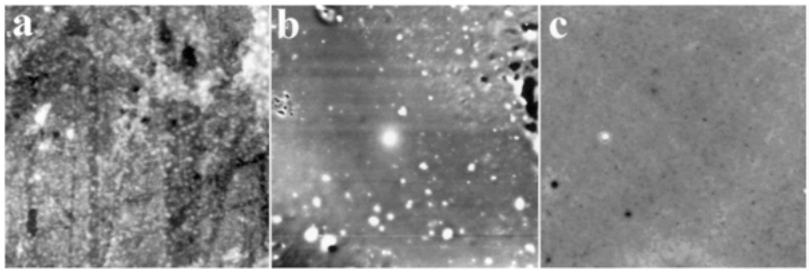


Figure 6 SFM images (10 μm × 10 μm) of spin-coated PAM films (a) 1% PAM, (b) 5% PAM and (c) 10% PAM.

images the surface was found to be made up of small spherical particles that have a size distribution that depends both on the PAM and HSA concentration (see Fig. 7). On samples prepared from 5% PAM, particles are larger and more uniform than on those prepared from 10% PAM.

Images of films spun from 5% PAM with 0.12% HSA (Fig. 7(a)) show a surface roughness of 12 nm with spherical particles having a diameter of 170 ± 16 nm, while the surface roughness of 5% PAM with 0.03% HSA (Fig. 7(b)) is about 19 nm. The spherical particles in Fig. 7(b) have a diameter of 200 ± 40 nm.

Images of films spun from 10% PAM with 0.09% HSA (Fig. 7(c)) show a surface roughness of 9 nm with spherical particles having a diameter of 80 ± 14 nm. 10% PAM-0.3% HSA films (Fig. 7(d)) have a similar surface roughness of 10 nm but with spherical particles with a random distribution of sizes ranging from 20 to 150 nm. In both samples prepared from solutions of 10% PAM and HSA, pinholes similar to those found in the case of 10% PAM without HSA were observed.

The characteristic of PAM to form spherical particles when blended with HSA has also been observed in PAM/HSA hydrogels. As reported in [13], PAM/HSA hydrogels prepared using a propriety technique result in regular monomodally distributed nanospheres that have been shown to be useful in controlled drug release applications.

4. Discussion

It is well known that there are very few empirical rules which allow one to predict the suitability of a particular surface for cell adhesion, and most of these rules appear to have several exceptions. For example, although there is clearly a difference in surface properties between films prepared from different concentrations of polymer and protein, there does not appear to be a particular cut-off value for physical parameters above or below which a minimum number of cells adhere. Furthermore, our best surface for cell adhesion, from 10% PAM and 0.3% HSA, appears not to have significantly different physical characteristics from other 10% PAM compositions investigated. Of note is 5% PAM which exhibits surface properties, in particular those of surface potential and surface morphology that do not appear to follow a

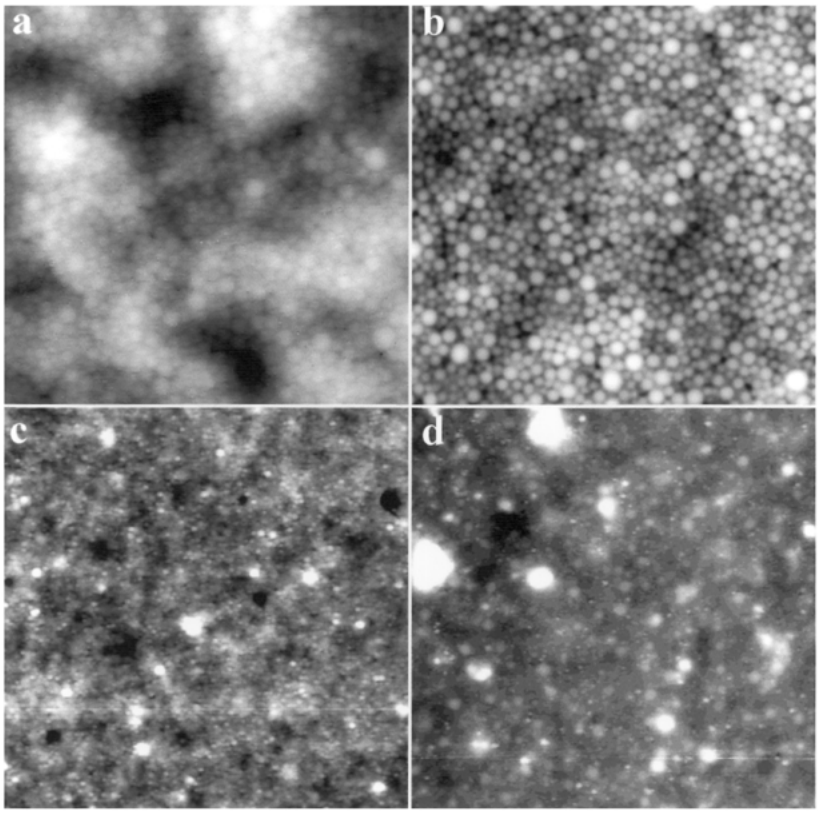


Figure 7 SFM images (5 μm × 5 μm) of spin-coated PAM HSA blends. (a) 5% PAM/ 0.12% HSA, (b) 5% PAM/ 0.03% HSA, (c) 10% PAM, 0.09% HSA, (d) 10% PAM, 0.3% HSA.

general trend, as would be expected from spin-coated films.

The presence of an HSA coating on polymers commonly used for tissue culture or on hydrophobic polymers generally inhibits cell adhesion [14]. We observed however that even in the first few hours of the adhesion process, the presence of small quantities of HSA in PAM noticeably enhances cell adhesion. Thus, while HSA release and exchange with other serum molecules probably plays an important role in the long-term adhesion process, the presence of HSA as an intermolecular binder for the PAM matrix [6] is essential for its biocompatibility and for initiating the adhesion process.

5. Conclusion

The surface properties of a novel bioerodable and highly biocompatible polymer, PAM, a copolymer of maleic anhydride and mono-methoxyoligoetheyleneglycol have been evaluated. Whilst we were unable to extract general rules to predict the extent of cell adhesion on the polymer and polymer/protein blends, it was shown that the polymer is able to support high densities of endothelial cells when blended with HSA. In particular, films spun from a blend of 10% PAM and 0.3% HSA were found to be optimum for supporting cell adhesion and growth.

This blend could be used as a component of the supporting scaffold for tissue engineered structures.

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