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UV-ozone modification of plasma-polymerised acetonitrile films for enhanced cell attachment

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Abstract

Plasma polymerisation is of great interest for modifying the surface properties of biomedical devices in order to control, for example, protein adsorption and cell attachment. In this paper we present results for plasma-polymerised acetonitrile deposited onto silicon or polystyrene substrates. The chemistry of films deposited under a range of experimental conditions was studied by X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR). XPS provided evidence that the elemental composition of the films varied with rf power to flow rate parameter (W/F) with films produced at higher W/F being deficient in nitrogen. FTIR revealed that the plasma deposited film contained a wide range of nitrogen functional groups including amine, imine and nitrile. Oxidation of the films by exposure to radiation from a low pressure mercury vapour lamp in an air ambient increased the surface oxygen levels from 3 to 17 at.% after 300 s exposure. XPS also revealed that the oxidation process proceeded via the formation of carbonyl groups at short exposure times (<60 s) while longer treatment times (>60 s) resulted in an increase in the concentration of carboxyl groups. To assess their potential to support cell growth, polystyrene culture dishes coated with plasma deposited films and UV-ozone oxidised films were seeded with 1BR.3.N human fibroblast cells and incubated for up to 72 h. Un-oxidised plasma-polymerised acetonitrile films were found to give comparable cell attachment densities as tissue culture polystyrene. The greatest cell attachment density was found with plasma polymer films which had been UV-ozone treated for the longest time (300 s). Enhanced attachment to this surface was attributed to the high level of carboxylic groups found on this substrate.
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Keywords: Plasma polymer; Surface modification; Cell attachment; Acetonitrile; UV-ozone

1. Introduction

The production of coatings with well-defined surface chemistries for cellular attachment has received considerable attention in recent years. Previous studies have shown that certain functional groups are greatly beneficial to cellular attachment either directly or by influencing the adsorption of attachment proteins. The hydrophilicity of the surface is believed to influence cell attachment through the adsorption of adhesion proteins such as fibronectin and vitronectin. However, the presence of specific functional groups is known to enhance cell attachment. Although this is a complex process which is not presently fully understood, it is clear that substrate chemistry influences culture serum and cellular protein adsorption via polar and electrostatic interactions. It has been suggested that protonated amino groups introduce a localised positive charge to the surface which at the physiological pH of 7.4 may lead to enhanced cell attachment since most proteins and cell membranes carry a net negative charge [1]. The presence of amino functional groups has been shown to be beneficial for the attachment of keratinocytes [2] and neuronal cells [3]. France et al. have shown that the level of cell attachment increases with the surface concentration of nitrogen-containing functional groups [2]. Electrostatic interactions are obviously not the only driver for cell attachment as numerous studies have demonstrated improved cell attachment to surfaces with predominantly negative charged oxygen-containing groups.

Several methods have been used to incorporate chemical functionalities into the surface in order to support cell growth: these include self-assembled monolayers (SAMs) [4,5], UV-ozone treatments [6,7] and plasma polymerisation [2,3].

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SAMs are usually deposited by the coating of gold-coated surfaces with alkanethiols to create a monolayer containing very pure chemically functionalities. Although SAMs are ideal for use as model surfaces to probe fundamental biological interactions their more general application to biomaterials is limited to substrates of simple geometry by the requirement for gold coatings and their relative complexity of production coupled with high cost is likely to restrict their technological application.

Plasma polymerisation has many benefits over the alternative surface modification techniques. Uniform films can be deposited onto almost any surface in a completely dry process leaving no trapped solvent molecules or other residual contaminants. Although considerable fragmentation of the monomer occurs during plasma polymerisation it is possible to retain much of the original chemical functionalities by the use of a pulsed plasma [8] or by using reduced powers [9]. The functional group concentration of the film can be controlled by plasma co-polymerisation whereby the functionalised monomer is diluted with a hydrocarbon monomer [2,10]. In addition, secondary heterogeneous functionalisation offers additional control of the final surface chemistry of the film.

Nitrogen-containing monomers are often used for the production of plasma polymers. Acrylonitrile is generally known to be one of the best monomers for plasma polymerisation and consequently has been extensively studied [11–15]. In contrast, *Acetonitrile* is in some respects an unconventional monomer for plasma polymerisation because the nitrile group is the only unsaturated site available for polymerisation. However, the nitrile group is readily reduced by hydrogen, which is a major constituent in plasmas of organic vapours, to form both primary and secondary amines. Films deposited from acetonitrile are known to have a composition low in nitrile but high in imine and amine groups [14].

The principal objective of this study was to produce coatings, which possess a variety of oxygen- and nitrogen-containing functional groups and to compare their cell attachment properties with those of established culture substrates such as tissue culture polystyrene. Plasma polymerisation of acetonitrile will be used to produce films with high levels of nitrogen functionalities. A post-deposition UV-ozone oxidation treatment has then been used to modify the surfaces to convert existing functional groups, e.g. amine to amide, and also introduce additional oxygen-containing species such as carboxylic acid. A comparison has then been made of the suitability of the variously treated surfaces for cell attachment. Although the films studied here are not model substrates, in terms of having only one functional group, the range of nitrogen functionalities and carbon–oxygen species produced by this two stage process provide a surface which is conducive to the attachment of cells. In this study, we describe the optimisation of both the coating process and the subsequent UV-ozone modification.

2. Experimental

2.1. Plasma polymerisation

Polymer deposition was carried out in a home-built plasma chamber consisting of a 10 cm diameter, 50 cm long Pyrex cylinder. The rf power at 13.56 MHz was coupled to the reactor via the matching network using an externally wound copper wire (four turns over a spacing of 8 cm). The chamber was pumped via a liquid nitrogen cooled trap by a two-stage rotary vane pump to a base pressure of approximately 1×10^{-3} mbar. The acetonitrile monomer (99%, Sigma–Aldrich) was degassed through several freeze-pump-thaw cycles. The monomer flow rate was controlled with a fine needle valve located between the glass reservoir and the reactor. The monomer flow rate was measured by closing off the valve to the pump and recording the initial pressure increase in the reactor. The substrates were placed in the glow region and all depositions were carried out under dynamic pumping conditions i.e. the valve to the pump remained open throughout the 5 min deposition.

Two sets of samples were prepared for this paper. The first, to study the influence of rf power and flow rate on film composition were prepared at a constant rf power of 40 W with flow rates varying from 1 to 40 cm^3 (STP) min^{-1}. These flow rates corresponded to a chamber pressure in the range 10–100 mBar. The second group of samples, used for UVO irradiation and cell culture, were prepared with constant plasma conditions—40 W rf power and a flow rate of 8 cm^3 (STP) min^{-1}.

The films produced by this method were found to be conformal, uniform and very smooth. The RMS surface roughness obtained from atomic force microscope (AFM) studies was found to be in the range of 1–2 nm. This is much less than that expected to influence cellular attachment and is similar to that found on commercially available tissue culture polystyrene dishes. AFM analysis of plasma polymer films also provided a value for thickness which for a 5 min deposition time was approximately 300 nm.

2.2. UV-ozone treatment

A number of the films were prepared with constant plasma conditions—40 W rf power and 8 cm^3 (STP) min^{-1}—for surface modifcation in a Jelight UV-ozone reactor (model number 42–220). This reactor contains a high-intensity, low-pressure mercury vapour grid lamp which emits strongly at 184.9 and 253.7 nm wavelengths [16]. These wavelengths are known to excite oxygen to generate ozone and also to photosensitise polymer surfaces [17]. Before use, the lamp was preheated for 1 h and all samples were treated for the appropriate time period of between 15 s and 5 min at a constant distance of 3 cm from the source.

2.3. Substrate preparation

The substrates used for XPS and FTIR spectroscopy were ⟨1 0 0⟩ orientated silicon wafers, which were ultrasonically cleaned with acetone and rinsed in ethanol before being dried with a nitrogen jet. For cell culture, films were deposited on untreated polystyrene dishes. Untreated polystyrene dishes and tissue culture polystyrene dishes were left uncoated for a comparison with the plasma polymer coated and UV-ozone treated samples.

2.4. Analysis of films

XPS analyses were performed in a Kratos Axis HSi spectrometer equipped with a 5-channel detector and a monochromated Al Kα X-ray source with an energy of 1486.6 eV operating at ∼150 W. All spectra were obtained in fixed analyser transmission mode with pass energies of 80 and 20 eV being used for the survey and high-resolution scans, respectively. Elemental compositions were calculated from peak areas obtained from the survey spectra using the appropriate sensitivity factors after subtraction of a linear background. A low energy electron flood gun was used to compensate for surface charging on the insulating films. The carbon 1 s signal from each film was corrected for surface charging to the hydrocarbon signal at 285.0 eV. The peak was deconvolved into four Guassian components: hydrocarbon (C–C, C–H) at 285.0 eV; carbon–nitrogen (C–N, C=N and C≡N) or alcohol/ether (C–O) at a shift of +1.5 eV; amide or carbonyl (CNO or C=O) at a shift of +3.0 eV and carboxylic acid or ester (COOH/R) at a shift of +4.5 eV.

FTIR spectroscopy was found to be a very appropriate technique for the study of the various functional groups present in the plasma polymerised films before and after UV-ozone treatment. Transmission FTIR measurements were performed on a Perkin-Elmer Spectrum GX FTIR system with a resolution of 8 cm^{-1} averaging over 25 scans. All FTIR measurements were performed within 1 h of deposition and UV-ozone treatment.

2.5. Cell attachment

1BR.3.N Human skin fibroblast, (transformed), cells were grown in 75 cm^2 canted neck TCPS flasks (Iwaki Glass, Japan) and incubated at 37 °C under a 5% CO_2 atmosphere. The culture media used was EMEM (Labtech International, Ringmer, UK).

All cells were cultured in media containing 25 mM HEPES, 10% (v/v) Foetal Calf Serum (Labtech International, Ringmer, UK), 1 unit/ml penicillin (Labtech International, Ringmer, UK), 0.1 mg/ml streptomycin solution (Labtech International, Ringmer, UK), 0.1 mg/ml non-essential amino acids (Labtech International, Ringmer, UK) and 2 mM L-glutamine (Lancaster Synthesis, UK).

Prior to harvesting, cells were rinsed in phosphate buffered saline (PBS) and harvested with 1 ml of Trypsin–EDTA solution (Labtech International, Ringmer, UK) for a maximum of 5 min at 37 °C with 5% CO_2. The trypsin was deactivated by the addition of 20 ml culture media. After centrifugation for 3 min at 340 × g, the pelleted cells were gently re-suspended in fresh serum containing media. For the cell attachment profile and patterning experiments, 1 ml of this cell suspension, equivalent to a cell density of 5000, was added to each treated dish and untreated control dish. The dishes were left undisturbed at 37 °C in a 5% CO_2 atmosphere between observations. After inoculation the cells were observed at 10× magnification (optical microscope (Leica HTML, Germany)) at selected time intervals (24, 48 and 72 h) to determine their attachment profile and counted using an overlaid grid with dimensions calibrated using a graticule observed at the same magnification.

3. Results and discussion

Plasma polymerisation of acetonitrile resulted in a brown film being deposited on the walls of the reactor and substrates. XPS analyses of the films deposited on silicon wafer substrates showed that the films contained carbon and nitrogen with typically less than 3 at.% oxygen. The presence of this oxygen is most likely a result of quenching the trapped free radicals in air when the films are removed from the reactor [18]. This oxygen may also have originated from reaction between the depositing film and water desorbed from the reactor walls during polymerisation.

The relationship between the atomic concentrations of nitrogen and carbon (N/C) and the W/F parameter (ratio of rf power-to-monomer flow rate) is shown in Fig. 1. Films prepared with lower W/F were found to contain a higher nitrogen concentration compared to those prepared at a higher W/F. The decrease in N/C ratio with increasing W/F found in this study is believed to result from increased fragmentation

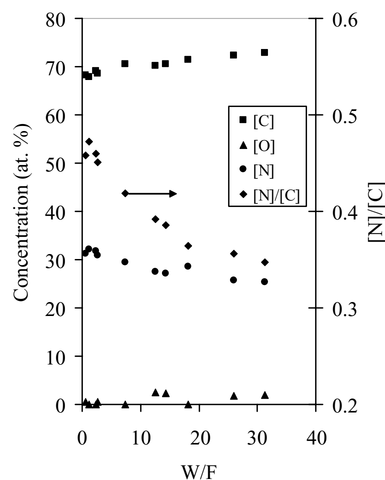

Fig. 1. Nitrogen, oxygen and carbon atomic concentrations and nitrogen-to-carbon ratio of plasma-polymerised acetonitrile films as a function of rf power to flow rate parameter (W/F).

of the monomer in the plasma which results in a loss of nitrogen in the form of low molecular weight volatile species. Cyano-containing monomers such as acrylonitrile are known to produce hydrogen cyanide (HCN) during plasma polymerisation which may be removed from the reaction chamber without being incorporated into the growing film [15]. Increased rf power to flow rate parameter results in a greater degree of monomer dissociation increasing the gas phase concentration of hydrogen molecules. Such an occurrence would account for the observed reduction in nitrogen content with increasing plasma power.

Lefohn et al. found no dependence of film composition on plasma power for acetonitrile plasmas [14]. However, it should be noted that this statement appears to be based on FTIR spectra recorded from films produced at only three powers (2, 14 and 50 W) and although assumed to be constant, there is no mention of monomer flow rate. A previous investigation by Munro et al. on acrylonitrile plasma polymers using XPS found the N/C ratio decreased from 0.27 to 0.21 when rf power was increased from 1 to 35 W but was found increase to 0.32 when the power was further increased to 55 W [11]. This was attributed to changes in the gas-phase composition in the plasma chamber and a change in the sticking coefficient with the accompanying increase in substrate temperature. However, it must be remembered when making comparisons of this type that XPS is much more surface sensitive than FTIR with the respective sampled depths being approximately 10 nm and 1 μm.

The composition of plasma polymer films, prepared with rf power of 40 W and 8 cm^3 (STP) min^{-1} flow rate, were studied by XPS after exposure to UV-ozone treatment. Fig. 2 shows the change in film composition with UV-ozone treatment time. The atomic concentration of oxygen increases sharply with UV-ozone treatment to a saturation level at about 16 at.% after ∼60 s. The nitrogen content of the films does not vary significantly with UV-ozone treatment from an original concentration of ∼28 at.%.

Further information on the chemical modifications resulting from the UV-ozone treatment can be obtained by analysing the carbon 1 s peak envelope. Examples of spectra obtained from a film deposited with an rf power of 40 W and flow rate of approximately 8 cm^3 (STP) min^{-1} and similar films UV-ozone treated for 45 and 300 s are shown in Fig. 3. The envelopes from the as-deposited and UV-ozone treated films were fitted with four peaks: C−C/C−H; C−OR/C−OH/CN; C=O/CON and COOH/COOR at 285.0, 286.5, 288.0 and 289.5 eV respectively. A summary of the results from the peak fits is shown in Table 1. Analysis of the peak fit information reveals that the as-deposited film contained only 1% carboxyl or ester groups with 4% of the C 1 s signal arising from carbonyl or amide groups. For the un-modified film the peak at 286.5 eV (amine, nitrile, imine, ether or alcohol) accounted for 43% of the total envelope area. As the oxygen content of the un-modified film was only 2 at.% it is clear that the bulk of this peak can be assigned to nitrogen functionalities.

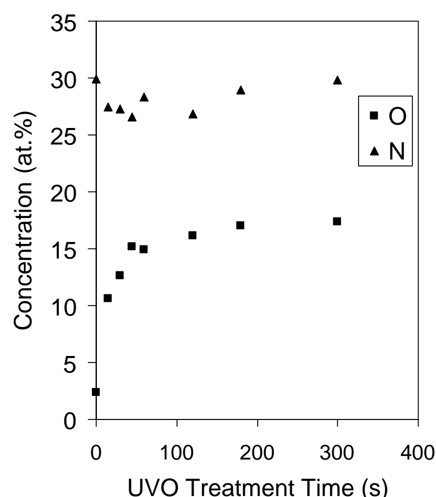

Fig. 2. Variation in oxygen and nitrogen atomic concentrations with UV-ozone treatment time. From films prepared with constant rf power (40 W) and flow rate (8 cm^3 (STP) min^{-1}).

Evaluation of the carbon 1 s spectra following UV-ozone treatment show that for short treatment times, i.e. <60 s, the oxidation process proceeds via the formation of carbonyl groups with a modest increase in the concentration of carboxyl groups from 1 to 6 at.%. The concentration of carbonyl/amide groups reaches saturation at ∼20 at.% after 60 s but the concentration of carboxyl groups continued to

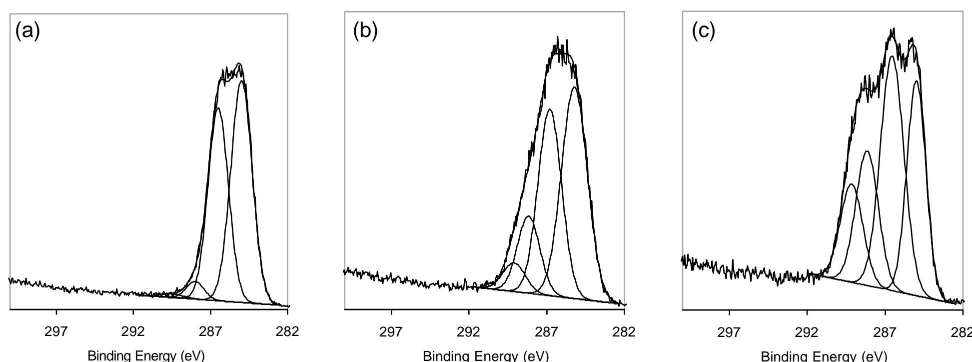

Fig. 3. C 1 s XPS spectra for (a) as-deposited film, (b) UV-ozone treated for 45 s and (c) film treated for 300 s.

Table 1
Components of carbon 1 s XPS peak as a function of UV-ozone treatment time

UVO treatment time (s)	Percentage of C 1 s peak			
	Component 1	Component 2	Component 3	Component 4
0	52.4	43.3	3.8	0.5
15	42.2	42.3	13.7	1.8
30	40.3	42.0	15.5	2.2
45	44.8	35.9	14.1	5.2
60	41.9	31.7	20.9	5.5
120	37.8	37.9	20.3	4.0
180	37.5	33.1	22.5	6.9
300	27.7	37.3	20.1	14.9
Assignation	C–C, C–H	C–N, C=N, C≡N, C–O	C=O, CONH	COOH, O–C=O

increase, becoming 15% of the total carbon 1 s peak after 300 s UV-ozone treatment. As the total oxygen concentration (as shown in Fig. 2) saturates after 60 s it is clear that conversion of existing oxygen–carbon groups (e.g. alcohol or carbonyl to carboxylic acid) is occurring.

Three regions of interest from the FTIR spectra from these plasma polymers are shown in Fig. 4. A broad absorbance is seen between 1400 and 1700 cm^{-1} (Fig. 4a). The peak at 1620 cm^{-1} is generally believed to result from imine (C=N) stretching [14,19]. In the region 2000 to 2300 cm^{-1} there are three bands of interest: the conjugated imine stretch at 2138 cm^{-1}; conjugated nitrile at 2187 cm^{-1} and nitrile stretch at 2245 cm^{-1} (Fig. 4b) [13,14]. Bands resulting from N–H in amines or amides at ∼3340 cm^{-1} with N–H stretching in imine groups resulting in the shoulder found at ∼3600 cm^{-1} (Fig. 4c). Hydrocarbon stretching (CH_2 and CH_3) accounts for bands at ∼2950 cm^{-1} [19].

UV-ozone treatment resulted in several changes to the FTIR spectra obtained from the films. After a treatment time of 45 s a number of new absorbance bands appeared in the carbonyl region of the spectrum (Fig. 4a). The peaks appearing between 1573 and 1521 cm^{-1} are characteristic of N–H bending in secondary amides. Carbonyl stretching in secondary amides also accounts for the appearance of bands between 1634 and 1666 cm^{-1}. Bands at 1730, 1712, 1696 cm^{-1} can be attributed to carbonyl absorptions in carboxylic acid groups. The presence of ester groups would be expected to result in a group of peaks at higher wavenumber values of between 1730 and 1750 cm^{-1} [19]. The absence of absorbances in this region allows us to conclude that the XPS peak at 289.5 eV originates predominantly from carboxylic acid and not ester groups. The appearance of these bands was accompanied by a disappearance of the band at 2138 cm^{-1} which was attributed to conjugated imine C=C–C=N–H. Obviously, the use of plasma polymers and UV-ozone treatments results in a wide range of nitrogen and oxygen-containing functional groups being incorporated in the film.

The suitability of the plasma polymer thin films for cell attachment was assessed before and after UV-ozone treatment by seeding coated polystyrene Petri dishes with human skin cells (1BR.3.N). The attachment profiles were measured at 24 h intervals up to a total of 72 h. As-deposited plasma polymer films were compared with UV-ozone treated films, untreated polystyrene and tissue culture polystyrene. Fig. 5 shows the mean number of fibroblast cells per mm^2 attached to each dish after 24, 48 and 72 h. Cells grown on untreated polystyrene were found to have a rounded morphology characteristic of weak attachment. In contrast cells grown on tissue culture polystyrene (TCPS), and both as-deposited and UV-ozone treated plasma-polymerised acetonitrile films were found to be evenly distributed and exhibited

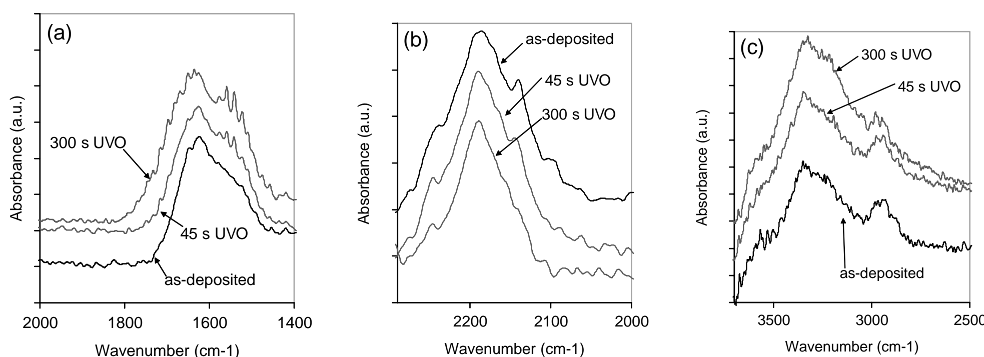

Fig. 4. Variation of FTIR spectrum with UVO treatment time.

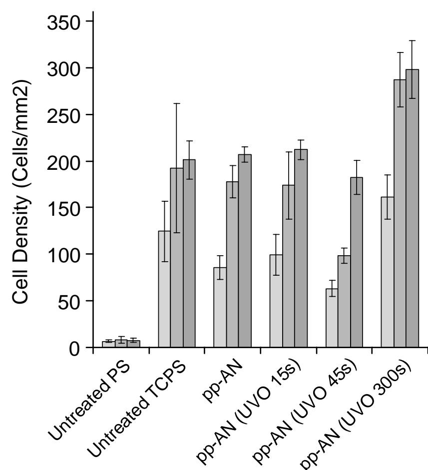

Fig. 5. Cell attachment to polystyrene, TCPS, plasma-polymerised acetonitrile and UV-ozone treated plasma-polymerised acetonitrile after 24, 48 and 72 h.

a flat morphology—characteristic of well-attached cells. Fig. 6 shows examples of human fibroblasts attached to (a) untreated polystyrene, (b) TCPS, (c) as-deposited plasma-polymerised acetonitrile and (d) plasma-polymerised acetonitrile after 300 s UV-ozone treatment.

The level of attachment of 1BR.3.N cells to as-deposited plasma-polymerised acetonitrile was comparable to TCPS. Since the as-deposited film contains <3 at.% surface oxygen the majority of cell attachment must be attributed to nitrogen-containing groups. For the film UV-ozone treated for 15 s, despite an increase in carbonyl groups, the level of cell attachment is comparable to the unmodified film. UV-ozone treatment for 45 s results in an increase in the concentration of carboxylic acid groups but the number of cells attached after 24 and 48 h was found to be lower than TCPS and the untreated polymer film. The film treated for 300 s showed the highest level of cell attachment. This film was shown by high-resolution XPS to contain the highest level of carboxylic acid groups. Brandley et al. have previously shown that carboxylic acid derivitised surfaces supported vigorous long term growth of fibroblast cells [20].

Owing to the complex chemistry of films produced by this route it was impossible to positively identify all of the functional groups present on the surface. However, a number of researchers have found that surfaces containing multiple chemical functionalities promote cell adhesion. Tidwell et al. have observed increased cell attachment on tissue culture polystyrene as compared to homogeneous self-assembled monolayers which strengthens the argument that a chemically complex surface is more attractive for cell growth [21]. Films produced by this two stage process produced films with multiple oxygen and nitrogen containing groups which have been shown to give enhanced attachment of fibroblast cells as compared to TCPS.

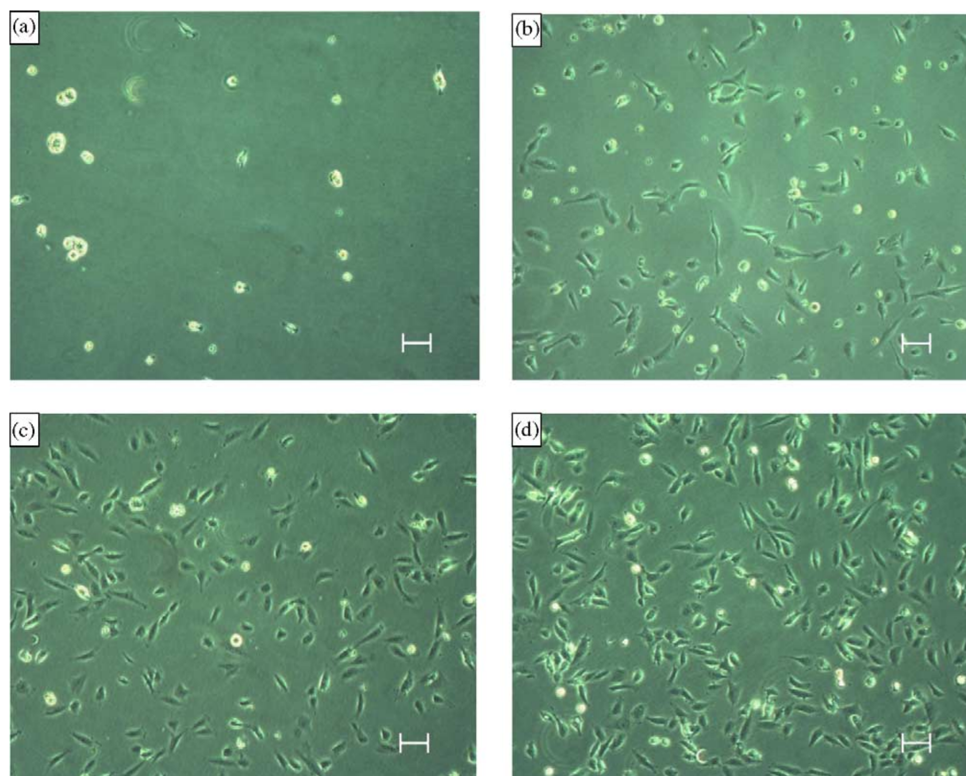

Fig. 6. Cell attachment after 72 h to (a) untreated polystyrene, (b) TCPS, (c) plasma-polymerised acetonitrile and (d) plasma-polymerised acetonitrile UV-ozone treated for 300 s. Scale bar = 100 μm.

4. Conclusion

Plasma polymerisation was used to produce films containing high levels of nitrogen functionalities. These films were then oxidised in a UV-ozone reactor to provide films with a range of surface chemistries. XPS and FTIR spectroscopy were used to provide information on the various oxygen and nitrogen groups present in the films. The as-deposited films were found to consist of mostly carbon and nitrogen with typically less than 3 at.% oxygen. Films produced at higher power densities were found to have a lower nitrogen content which was attributed to increased levels of fragmentation of the monomer in the plasma resulting in a loss of nitrogen in the form of volatile species.

UV-ozone treatment of the plasma-polymerised acetonitrile was found to increase the oxygen content of the films with saturation levels of oxygen at around 17 at.% occurring after around 60 s. High resolution XPS spectra indicate that UV-ozone treatment results in an increase in C=O groups immediately after treatment begins. FTIR spectroscopy supports the findings of the XPS with the appearance of a number of new bands in the carbonyl region of the spectrum after UV-ozone treatment. At longer treatment times further oxidation occurs with the formation of carboxylic acid groups. As-deposited plasma-polymerised acetonitrile were found to give comparable attachment rates to commercial tissue culture polystyrene. The 300 s UV-ozone treated film exhibited the optimum surface in terms of cell attachment density which was attributed to the high surface concentration of carboxylic acid groups.

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