Application of epifluorescence scanning for monitoring the efficacy of protein removal by RF gas–plasma decontamination

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Abstract. The development of methods for measuring the efficiency of gas–plasma decontamination has lagged far behind application. An approach to measuring the efficiency of protein removal from solid surfaces using fluorescein-labelled bovine serum albumin and epifluorescence scanning (EFSCAN) is described. A method for fluorescently labelling proteins, which are adsorbed and denatured on metal surfaces, has been developed. Both approaches have been used to evaluate the efficiency of radio frequency (RF) gas–plasma decontamination protocols. Examples with ‘real’ surgical instruments demonstrate that an argon–oxygen RF gas–plasma treatment can routinely reduce the protein load by about three orders of magnitude beyond that achieved by current decontamination methods.
1. Introduction

A variety of gas–plasma techniques are capable of destroying both small organic molecules and larger biomolecular structures adhering to, or adsorbed on, a variety of surfaces without causing significant damage to the surfaces themselves [1]–[4]. This has led to a large number of decontamination applications in the medical and electronics industries. However, it would be true to say that, while the technique is capable of very significant reduction of the surface load of organic contamination, the development of methods to quantify the efficacy of particular treatments lags far behind the development of the plasma processes themselves.

The interest in radiofrequency (RF) gas–plasmas in our laboratory lies not in their application for medical sterilization but rather for the removal of protein contamination from surgical instruments [5]–[7]. This has particular relevance to the destruction and removal of infective proteins associated with the transmission of Creutzfeldt–Jacob disease (CJD) [6]. However, the diverse shapes and the different nature of the materials used in the manufacture of surgical instruments together with the efficiency of modern decontamination methods make measuring protein contamination on their surfaces a difficult analytical problem. The cleaning protocols used in surgical instrument reprocessing by sterile service departments (SSDs) in hospitals involve washing procedures that typically reduce the surface residual tissue contamination, of even very heavily contaminated instruments, to levels which cannot be detected by the naked eye. Typically this represents a level of residual protein contamination (averaged over an instrument surface) of about 10 µg cm⁻² with localized deposits, which rarely exceed a loading of 1 µg mm⁻² [8, 9]. Scanning electron microscopy (SEM), especially when linked to energy dispersive x-ray (EDX) spectroscopic analysis, has proven an invaluable tool for the location and analysis of tissue residues on conventionally cleaned instruments [5, 7, 8, 10]. However, SEM analysis is limited in scope: it is non-quantitative, restricted to elemental analysis, applicable only to small objects and is very time consuming and labour intensive.

RF gas–plasma treatment can reduce the amount of residual contamination on instruments to a level where no organic residues can be detected by SEM [7] and this has led us to examine other more sensitive types of analysis. While a range of very sensitive spectroscopic techniques can, in principle, be employed for direct detection of deposits on surfaces at the molecular level, many of these, like surface plasmon resonance (SPR) [11]–[13], surface-enhanced Raman spectroscopy (SERS) [14]–[17] and attenuated total reflection Fourier transform infrared
(ATR-FTIR) spectroscopy [18], are limited to specially prepared solid matrices and have more relevance to the development of sensor technology for quantification of materials in solution than for adsorbed species in decontamination applications. Immunological assays using antibodies are routinely capable of measuring concentrations of specific proteins down to about 1 pg ml\(^{-1}\) in solution. However, antibody techniques applied to measurement of proteins on surfaces have proven much less sensitive; specific detection of bovine albumin and bovine \(\gamma\)-globulin adsorbed on metal tokens can be achieved but the limit of detection appears to be about 1 \(\mu g\) mm\(^{-2}\) [19]. Two factors militate against the use of antibody detection methods for quantification of adsorbed proteins before and after gas–plasma treatment: (a) the method is intrinsically insensitive even with undenatured proteins and (b) even minor chemical modifications to the side chains of residues of the protein caused by plasma treatment can result in loss of antibody binding, even if the bulk of the protein remains intact.

The requirement for a quantitative routine method for analysis of protein molecules bound to surfaces, suitable for measurement of sub-nanogram mm\(^{-2}\) levels, which could be employed as a routine method for contamination quantification both before and after gas–plasma decontamination procedures, has led us to develop a more general approach. This involves \textit{in situ} covalent derivatization of the amino and thiol groups of surface-bound protein molecules with fluorescein-based fluorophores and development of a surface scanning fluorimeter capable of measuring the resultant fluorescence of labelled proteins bound to the surfaces.

2. Materials and methods

\textbf{Surgical instruments:} the instruments used in this work were supplied by the SSD at the Royal Infirmary of Edinburgh. Prior to our studies the instruments were stringently cleaned by conventional hospital procedures, fully compliant with Quality Management System EN ISO 9002. While the instruments were judged suitable for reuse for surgical procedures they were permanently withdrawn from service for this study.

\textbf{Low pressure RF gas–plasma treatments} were carried out using a Plasma-Etch BT1 (Plasma Etch, Carson City, NV, USA). A schematic of the instrument is shown in figure 1. The SSD-cleaned instruments were soaked in distilled water for 20 min and subjected to the plasma procedure while still wet [7, 20]. The electrodes of the instrument consist of perforated aluminium plates \((300 \times 300 \times 3\) mm\) fitted with cooling coils through which a glycol–water mixture was pumped to hold their temperature at 20 °C. In the decontamination experiments described, an \(\text{Ar} : \text{O}_2\) \((1 : 1)\) mixture (at 0.250 Torr) was subjected to RF excitation \((13.5\) MHz) at a power density of \(>6\) mW cm\(^{-3}\) for 1 h. The rise in the temperature of treated discs or instruments observed was \(<2\) °C over 1 h under these conditions.

\textbf{SEM} was conducted using a Philips XL30CP instrument, operating at 12 kV, providing a resolution better than 5 nm. Backscatter electron imaging enabled regions with a mean atomic number difference greater than 0.1 to be resolved. Energy dispersive x-ray (EDX) spectroscopic analysis was carried out in the SEM using a PGT Spirit X-ray analyser, capable of detecting elements of atomic number greater than 6. The imaged areas \((5\mu m^2)\) were subjected to elemental analysis to a depth of about 3 \(\mu m\) \((V = 6 \times 10^{-17}\) m\(^3\)) using an x-ray fluorimeter capable of detecting elements comprising 1% mass of the sample volume. No standardization could be applied to the EDX spectra, and thus the intensities of the peaks only indicate the relative proportions of elements present in the sample volume.

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Confocal fluorescence microscopy of fluorescently labelled protein on surfaces was carried out using a Bio-Rad Radiance 2100MP confocal microscope. A ×4 objective was used and fluorescence was excited using the 488 nm line of an argon ion laser.

Epifluorescence scanning (EFSCAN) was carried out using a computer controlled optical-fibre-based system incorporating an optical head with an epifluorescence geometry, which we have described previously [21]. Excitation of the sample was achieved by a 468 nm, fibre-coupled light emitting diode (LED) (Ocean Optics). The fluorescence, detected collinearly with the excitation, was coupled into a fused silica fibre bundle for transmission to a photomultiplier tube (PMT). The sample was mounted on a computer-controlled motorized x–y translation stage (Ocean Optics) which allowed successive measurement of the amplitude of fluorescence signals from an 0.5 mm² area of the surface under study. The control software program was written in-house in LabView (National Instruments, Austin, TX, USA).

Standard samples: calibration of the instrument was carried out using bovine serum albumin (BSA) covalently labelled with fluorescein (at the thiol group of Cys-34), which was prepared by reaction of the protein with fluorescein isothiocyanate (FITC, Pierce Biotechnology, IL, USA) in carbonate buffer at pH 9. Analysis of UV-VIS spectra of the product showed that it contained a single fluorescein per protein molecule. Known amounts of an aqueous solution of the labelled protein (20 µM) were applied to stainless steel discs (5 mm diameter, 316 stainless steel) and these were dried overnight at room temperature before measurement.
Figure 2. (a) Three-dimensional representation of EFSCAN data showing the spatial distribution of fluorescence intensity for a calibration series of five stainless steel tokens with fluorescein-labelled BSA loadings from 2 µg to 0.2 ng. (b) The relationship between total emission intensity (integrated over the area of the protein deposit) and the amount of fluorescein-labelled BSA deposited, for this set of stainless steel tokens.

**In situ protein labelling:** to assess labelling efficacy, known femtomolar amounts of BSA were deposited from aqueous solution onto steel discs (5 mm diameter, 316 stainless steel), which were held at 80 °C to fix the protein to the disc and evaporate the water. Using this technique the protein is adsorbed nonspecifically on the metal surfaces. The protein-coated discs were then soaked in a solution of FITC (3 mM in dimethylsulfoxide) for 15 min with light orbital shaking. The discs were then rinsed with water to remove excess reagent and allowed to dry at room temperature overnight in foil covered Petri dishes. Covalent labelling of residual tissue deposits on surgical instruments was carried out using the same labelling procedure.

3. Results and discussion

3.1. Measuring the efficiency of RF gas–plasma decontamination

The general approach we have developed involves covalent derivatization of nucleophilic protein functionalities, such as amino and thiol groups, with a fluorophore and subsequently determining the amount of bound fluorophore by EFSCAN of the surface. To determine the response of the scanning instrument to labelled protein on metal surfaces, we prepared serial dilutions of BSA singly labelled with fluorescein in solution and deposited this on stainless steel discs. The protein was then ‘fixed’ by heating the discs at 80 °C. This heating step evaporates the water and denatures the protein. Figure 2(a) shows EFSCAN data for a series of five discs with labelled BSA loadings of between 2 µg and 0.2 ng and figure 2(b) a calibration curve, which may be used to relate the observed fluorescence intensity to the amount of labelled BSA present in an unknown deposit. Our current practical limit for detection of singly labelled BSA is about 5 pg in an area of 0.5 mm² and the dynamic range of detection is greater than 10⁴.
Figure 3. EFSCAN illustrating the progressive removal of fluorescein-labelled BSA from 5 mm diameter stainless steel discs using an Ar : O$_2$ RF gas–plasma (1 : 1 Ar : O$_2$, 0.25 Torr). Each disc was loaded with 1 µg of labelled protein. Five time points (0, 5, 10, 20 and 40 min) are shown.

Figure 4. SEM backscatter images of fluorescein-labelled BSA on a 5 mm diameter stainless steel disc before (left-hand image) and after (right-hand image) RF gas–plasma treatment (1 : 1 Ar : O$_2$, 0.25 Torr, 1 h). 2 µg of labelled protein was heat-fixed to the disc before plasma treatment.

We have used this method to measure the efficiency of Ar : O$_2$ RF gas–plasmas in removing labelled protein from stainless steel surfaces. The scanning method is of special value in optimizing the conditions and exposure time required for decontamination. Figure 3 shows EFSCAN ‘snapshots’ of contaminated discs (1 µg labelled protein/disc) examined after different durations of gas–plasma treatment. After 40 min the level of protein contamination is below the detection level of EFSCAN. Figure 4 shows SEM images of a contaminated stainless steel disc (1 µg labelled protein/disc) before and after plasma treatment for 1 h. Although the SEM technique is not quantitative, the absence of detectable protein after treatment is consistent with EFSCAN data.

To assess the extent to which irreversible photobleaching of the fluorophore under plasma conditions might reduce the fluorescence intensity, fluorescein-labelled BSA samples (0.1 and
1 μg/disc, in triplicate) were sealed in fused silica tubes through which UV light can pass. Examination of these samples after plasma treatment for 1 h showed about 5% decrease in fluorescence in comparison to untreated controls. This is very small compared to the reduction achieved by plasma treatment of unshielded samples (see figure 3). This is consistent with the evidence for protein removal obtained by SEM (see figure 4) and indicates that the large reduction in fluorescence observed under low pressure gas–plasma treatment conditions is due to destruction of the protein. We would not expect that UV irradiation alone would result in significant protein destruction, since it is likely that the main mechanism of protein removal under Ar : O₂ RF gas–plasma conditions involves a combination of oxidation reactions and fragmentations induced by ionic and atomic bombardment of the protein [22, 23].

3.2. In situ labelling of proteins on surfaces

In real-life applications, such as surgical instrument decontamination, labelling of denatured protein must be carried out in situ, that is, with the protein already bound to the surface; this is more challenging. It is extremely unlikely that proteins bound to surgical instruments will be in their native conformations. In normal practice, medical instruments undergo successive heat treatments at 40–60 °C in the washers, and steam sterilization at 132–137 °C. This denatures any residual proteins and increases their adhesion to the surfaces. Formation of protein aggregates or specific binding interactions with the metal surface would be expected to reduce the accessibility of reactive groups on a protein, potentially limiting the derivatization level. In order to quantify the level of in situ labelling possible with conventional fluorescent probes, it was necessary to produce standard samples which would mimic the condition of protein deposits expected on real surgical instruments. To this end test samples were prepared by depositing known amounts of BSA onto stainless steel discs held at 80 °C. Without this heat-fixing, losses of 50–80% of the initial deposit were observed during the subsequent labelling process; however, in the heat-fixed samples, this loss was reduced to 10–20%.

A variety of conditions and reagents were evaluated for labelling of the denatured protein on the surface and the most successful combination proved to be immersion in 3 mM FITC in dimethylsulfoxide solution for 15 min at room temperature, with light orbital shaking, followed by rinsing with water to remove the excess reagent. While this labelled the BSA, the fluorescence intensities of the protein deposits were, reproducibly, about 30% of the values obtained with pre-labelled proteins.

Confocal fluorescence microscopy was used to investigate the penetration of the FITC reagent and the uniformity of protein labelling. In order to visualize the depth of the protein deposit the BSA was initially mixed with rhodamine 101 (R101), to give non-covalent staining of the protein, and heat-fixed onto stainless steel discs. The protein was then covalently labelled in situ with FITC dissolved in various solvents. Simultaneous excitation of both fluorophores was possible with the 488 nm line of an argon ion laser, and the spectral separation of their emission allowed simultaneous measurement of their fluorescence in two separate detection channels.

Figure 5 shows confocal fluorescence images of a 12 μm section, at a distance of about 100 μm above the metal surface, for a 100 μg protein deposit containing R101, before and after in situ labelling with FITC. The images show a section of a thick ring of protein at the boundary of the protein deposit and a thinner central region. The thick outer ring and marbled pattern in the central region are characteristic of macroscopic heat-fixed protein samples deposited from solution. The similarity of the R101 and FITC images shows that FITC does effectively label the
protein deposit. Rhodamine 101 is dispersed throughout and thus its emission can be detected throughout the entire depth of the deposit. The FITC fluorescence can be observed within the thick layer, but its intensity here is less than that of R101, suggesting that the labelling reagent is not able to penetrate fully.

Increases in labelling times did not result in significant improvements in labelling, but these studies did show that the most extensive and uniform labelling was achieved when dimethylsulfoxide was used as the solvent. Labelling from acetone, dimethylformamide and methanol was poor, suggesting that an important factor in \textit{in situ} labelling efficiency may be the penetration of the protein deposit by the labelling solution.

The major reason for the inefficiency of the labelling reaction thus appears to be the lack of accessibility to the reactive sites of the denatured protein on the metal surface. Perhaps this is not surprising since a similar effect occurs with insoluble denatured proteins aggregates in aqueous suspensions. This is exacerbated in the case of thick deposits such as that illustrated in figure 5. However, for practical decontamination monitoring purposes, quantification of the initial contaminant load may not be of major importance. The important criterion in decontamination is not the amount of material at the start of the process but rather the amount remaining afterwards.

3.3. Visualization of protein contamination on surgical instruments

The labelling method described above has been applied to locate and evaluate protein contamination on reprocessed surgical instruments that have undergone hospital cleaning and decontamination methods and are classified as acceptable for surgical use. In preliminary experiments, we used SEM and confocal fluorescence microscopy to refine and evaluate the labelling and washing procedures. Figure 6 shows a SEM image of the tips of a pair of Darik surgical clamps before labelling and an image of the same area obtained by confocal fluorescence microscopy after labelling. That the residues identified in the SEM image are predominately protein is supported both by the elemental composition identified by EDX sampling and by reaction with the fluorescent reagent\textsuperscript{6}.

\textsuperscript{6} Although tissue samples are very heterogeneous in composition the material adhering to the metal surfaces of surgical instruments after washing is, in our experience, almost exclusively proteinaceous.

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Figure 6. Reprocessed Darik clamps (a) a backscatter SEM image showing distinct areas of residual contamination, (b) the EDX spectrum of the main deposit, which shows signals for carbon, nitrogen, oxygen and sulphur characteristic of the presence of protein and signals due to inorganic salts and silicon and (c) the confocal image of the fluorescently labelled instrument indicates that the labelling corresponds to the areas of residual contamination identified in the SEM image.

Figure 7. Images and EDX spectrum of reprocessed titanium ophthalmic micro forceps (a) shows the area of the instrument that is shown in the SEM images, (b) shows a backscatter SEM image of distinct areas of residual contamination, (c) the EDX spectrum of the red circled area in (b) which shows the presence of both organic and inorganic residues indicative of both proteins and salts, (d) shows the backscatter SEM image of the decontaminated surfaces after RF gas–plasma treatment (1 : 1 Ar : O\textsubscript{2}, 0.25 Torr, 1 h) and (e) the EDX spectra surface of the red circled area in (d). EDX analysis (not shown) of the area in the white square indicated only traces of salt residues.

We have demonstrated previously that RF gas–plasma treatments using Ar : O\textsubscript{2} mixtures can reduce tissue contamination on surgical instruments to below the levels detectable by SEM [7]. Figure 7 shows a typical example of this type of experiment on a reprocessed pair of titanium ophthalmic micro forceps. Heavy contamination, indicated by the dark regions, is
Figure 8. EFSCAN monitoring of RF gas–plasma decontamination of a pair of titanium ophthalmic micro forceps after labelling with FITC: (a) indicates the section of the forceps shown in the scanned images, (b) shows the scan obtained before gas–plasma treatment and (c) shows that after treatment (1 : 1 Ar : O₂, 0.25 Torr, 1 h). Note the difference in the intensity scales.

seen on the forceps tips and this is removed by the gas–plasma treatment. Assessing removal of contamination using this approach is effective, if non-quantitative, but only small areas of an instrument, approximately 200 × 500 µm, can be examined at one time. It is not a method that is suitable for either routine monitoring or for large instruments, which cannot be accommodated in the microscope.

A comparative experiment on a pair of ophthalmic micro forceps, of similar provenance, using the EFSCAN system is shown in figure 8. Distinct areas of residual protein contamination on the forceps were mapped by EFSCAN after labelling the proteins with FITC. Integration of the EFSCAN data indicates a total fluorescence of 13.078 units, which correlates to a minimum observed residual protein loading in the region of 10 pmoles—or about 1.8 µg if the average protein molecular weight is taken at 60 000 amu and the labelling efficiency is 30%. This should be compared to the level of fluorescence measured after RF gas–plasma decontamination, which shows a total fluorescence of 0.030 unit. This indicates removal of more than 99.8% of the labelled protein.

Figure 9 shows the results of a similar procedure carried out with a pair of stainless steel curved artery forceps. Various areas of residual contamination, particularly on the tips and the box joint of the instrument, were visualized by EFSCAN after FITC labelling, and the level of

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7 While we have not attempted further quantification in this study, in earlier work we carried out SEM examinations and measured the protein contamination (using acid striping and chemical analysis) on a large number of instruments [8]. The values obtained here by EFSCAN are of the same order of magnitude.
residual contamination equates to 30.19 units of fluorescence. This was reduced to 0.017 unit by a single RF gas–plasma treatment. Again, the difference in the integrated fluorescence intensity scale on the two EFSCAN plots should be noted. More than 99.5% of the labelled protein is removed by the RF gas–plasma treatment.

4. Conclusions

Measurement of the concentrations of biological molecules adsorbed on surfaces presents a difficult analytical problem. In the surgical instrument decontamination field, current solution methods of decontamination typically reduce the burden of tissue contamination to below the level of visual detection. This is around 0.5 µg tissue mm⁻²—and most of the residual contamination consists of adsorbed proteins.

In our studies, we have adopted an integrated approach, which involves covalent labelling of proteins with a fluorescent reagent to enable detection, and development of an epifluorescence scanner that is capable of mapping the fluorescence of labelled protein on surfaces. Using pre-labelled BSA this technique has enabled us to measure the efficiency and rates removal of the protein from various solid substrates using RF gas–plasma. Ar : O₂ gas–plasma treatment for 1 h typically reduces the level of labelled protein contamination below the detection limit of the scanning method.
A method for the derivatization of proteins adsorbed and denatured on metal surfaces has been developed. The labelling efficiency here falls to about 30% of the values typically obtained with solution phase labelling. While a small amount of ‘heat fixed protein’ is removed by the solvents employed in derivatization, the main reason for the lowered labelling efficiency is that not all the reactive functional groups of protein molecules contained in the surface-adherent denatured aggregates are exposed to the reagent. Nonetheless, labelling of proteins in pre-existing tissue deposits on the surfaces of ‘cleaned’ surgical instruments enables us to determine a minimum level of surface contamination and this has proven to be a useful tool in evaluating experimental decontamination protocols. Examples with ‘real’ surgical instruments show that single RF gas–plasma treatments using Ar : O\textsubscript{2} can routinely reduce the ‘apparent protein load’ by two to three orders of magnitude below the level normally achieved using current decontamination methods.

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References

[1] Aronsson B O, Lausmaa J and Kasemo B 1997 J. Biomed. Mater. Res. 35 49
[2] Herrmann F H W, Henins I, Park J and Selwyn G S 1999 Phys. Plasmas 6 2285
[3] Rossi F, Kylian O and Haswa M 2006 Plasma Process. Polym. 3 431
[4] Moreau M, Orange N and Feuilloley M G J 2008 Biotechnol. Adv. 26 610
[5] Whittaker A G, Graham E M, Baxter R L, Jones A C, Richardson P R, Meek G, Campbell G A, Aitken A and Baxter H C 2004 J. Hosp. Infect. 56 37
[6] Baxter H C, Campbell G A, Whittaker A G, Aitken A, Simpson A H, Casey M, Bountiff L, Gibbard L and Baxter R L 2008 J. Gen. Virol. 86 2393
[7] Baxter H C, Campbell G A, Richardson P R, Jones A C, Whittle I R, Casey M, Whittaker A G and Baxter R L 2006 IEEE Trans. Plasma Sci. 34 1337
[8] Baxter R L, Baxter H C, Campbell G A, Grant K, Jones A C, Richardson P and Whittaker A G 2006 J. Hosp. Infect. 63 439
[9] Lipscomb I P, Sihota A K, Botham M, Harris K L and Keevil C W 2006 J. Hosp. Infect. 62 141
[10] Vandervoort K Microscopy as an analysis tool for studying plasma applications Biological and Environmental Applications of Gas Discharge Plasma ed G Brellés-Mariño (New York: Nova Science Publishers) at press
[11] Westphal P and Bommann A 2002 Sensors Actuators B 884 278
[12] Calander N 2006 Curr. Anal. Chem. 2 203
[13] Phillips K S and Cheng Q 2007 Anal. Bioanal. Chem. 387 1831
[14] Haynes C L, Yonzon C R, Zhang X and Van Duyne R P 2005 J. Spectrosc. 36 471
[15] Li T, Guo L and Wang Z 2008 Biosens. Bioelectron. 23 1125
[16] Scholes F H, Bendavid A, Glenn F L, Critchley M, Davis T J and Sexton B A 2008 J. Raman Spectrosc. 39 673
[17] Porter M D, Lipert R J, Siperko L M, Wang G and Narayanan R 2008 Chem. Soc. Rev. 37 1001

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[18] Rigler P, Ulrich W-P, Hoffmann P, Mayer M and Vogel H 2003 Chem. Phys. Chem. 4 268
[19] Merritt K, Edwards C R and Brown S A 1988 J. Biomed. Mater. Res. 22 99
[20] Baxter R L and Baxter H C 2006 PTC Int Appln. WO 2006079801
[21] Richardson P R, Jones A C, Baxter R L, Baxter H C, Whittaker A G and Campbell G A 2004 Proc. SPIE 5502 291
[22] Opretzka J, Benedikt J, Awakowicz P, Wonderlich J and von Keudell A 2007 J. Phys. D: Appl. Phys. 40 2826
[23] Kylián O, Benedikt J, Sirghi L, Reuter R, Rauscher H, von Keudell A and Rossi F 2009 Plasma Process. Polym. 6 255