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Cell attachment and biocompatibility of polytetrafluoroethylene (PTFE) treated with glow-discharge plasma of mixed ammonia and oxygen

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Abstract—The plasma generated from a gas mixture of NH_3 plus O_2 ($NH_3 + O_2$) has been used to impart unique chemical and biological characteristics to polytetrafluoroethylene (PTFE). PTFE treated with $NH_3 + O_2$ plasma was physiochemically distinct from surfaces treated with plasma of either NH_3 or O_2 alone, as determined by electron spectroscopy for chemical analysis (ESCA). The contact angle analysis revealed that the PTFE surfaces became less hydrophobic after plasma treatments. ESCA results indicate the presence of oxygen-containing groups and nitrogen-containing groups at the plasma-treated surfaces. PTFE treated with $NH_3 + O_2$ plasma resisted the attachment of platelets and leukocytes in a manner similar to untreated PTFE; however, the attachment of bovine aorta endothelial cells was substantially increased. Once attached, these cells grew to confluency. The increased endothelial cell attachment was higher than that observed following plasma treatment with each gas used separately, which could be attributed to the considerable amount of $CF(OR)_2$-CF_2 formed on the $NH_3 + O_2$ plasma-treated PTFE surface. At 14 days after subcutaneous implantation in rats, the PTFE wafers treated with $NH_3 + O_2$ plasma demonstrated less encapsulation and lower levels of inflammatory cells compared to controls. Collectively, the results suggest that $NH_3 + O_2$ plasma treatment imparts a unique character to PTFE and could be useful in certain *in vivo* applications.

Key words: Glow-discharge plasma; ammonia; oxygen; cell attachment; polytetrafluoroethylene (PTFE).

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INTRODUCTION

In recent years, plasma surface modifications have been used more and more in the field of biomedical materials research [1]. Those biomaterials include metals such as stainless steel, nitinol, titanium, and polymers such as polypropylene, polyurethane, nylon and polytetrafluoroethylene (PTFE). The unique advantage of plasma surface modifications is that the surface properties and biocompatibility can be enhanced selectively while the bulk properties of the materials remain unchanged. Furthermore, a wide range of surface modification can be realized with different glow-discharge plasmas by changing the working gas, discharge power, pressure, mass flow rate and the like.

Because of the advantages of chemical stability, physical robustness and biological inertness, expanded PTFE has been extensively used as a biomedical material for applications such as blood filter membranes and vascular grafts. Optimal interaction of endothelial cells with graft surface is necessary for cell attachment, spreading and proliferation. A number of strategies have been used to increase the attachment of endothelial cells to PTFE, especially as applied to small-bore vascular grafts [2–7]. Many of these strategies involve coating the graft with adhesion proteins such as fibronectin, although glow-discharge plasmas have also been used [8, 9]. Treatment of materials with oxygen plasma has been reported to increase endothelial cell attachment [8]. Surfaces having incorporated nitrogen were more effective than those of oxygen-containing functional groups in promoting endothelial cell attachment [10]. Similarly, amide and amine-containing plasmas have been reported to increase the attachment of endothelial cells [4, 9]. In the present study, O_2, NH_3 and the gas mixture of $NH_3 + O_2$ plasmas were used to modify PTFE surface for improving its biocompatibility and cell adhesion. The surface hydrophilicity of plasma-treated PTFE was studied by measuring the contact angle. The elemental composition at PTFE surfaces was analyzed with Electron Spectroscopy for Chemical Analysis (ESCA). The cell culture tests include endothelial cell attachment, protein absorption and subcutaneous implantation of PTFE in adult rats.

MATERIALS AND METHODS

Plasma treatment

Sheets of PTFE were obtained commercially from McMaster-Carr Supply Company (New Brunswick, NJ, USA) and cut into wafers with a total surface area of approximately 1 cm^2. Prior to use, the wafers were cleaned with acetone for 10 min; and then dried in air for 5 min.

Clean wafers were exposed for 45 s to gas plasmas composed of oxygen, ammonia, or mixtures of oxygen plus ammonia. O_2 gas with purity of 99.9%, industry grade, was purchased from Air Products and Chemicals (Allentown, PA, USA). NH_3 (99.999%, electronic grade) was also purchased from Air Products and Chemicals. The plasmas were generated at 110 W under a pressure of

50 mtorr and using a total mass flow rate of 50 standard cubic centimeter per minute (sccm) for oxygen (O_2), ammonia (NH_3) and oxygen plus ammonia gas mixture ($NH_3 + O_2$). For $NH_3 + O_2$ gas mixture plasma, the mass flow rate ratio of NH_3 to O_2 was set at 2 : 3 to see how oxygen and nitrogen, activated in the plasma atmosphere, incorporate into the PTFE surface and consequently affect cell adhesion to the surface. A detailed description of the radio-frequency (RF) glow-discharge plasma deposition system has been reported elsewhere [11, 12]. Following plasma treatment, all the wafers were stored in sealed plastic vials in an attempt to alleviate the reaction of air to plasma-treated surfaces and prevent dust from falling onto wafer surfaces.

Electron spectroscopy for chemical analysis (ESCA)

PTFE samples, including those with no plasma treatment and those that were plasma treated were analyzed using ESCA. ESCA was performed with a VG Scientific 220i-XL instrument equipped with a monochromatic Al K_α X-ray (1486.7 eV) source at the Chemistry Department, University of Utah. Typical operating conditions for the X-ray source were 400-μm nominal X-ray spot size (FWHM) operating at 15 kV, 8.9 mA setting and 100 W for both survey and high-resolution spectra. The take-off angle was fixed at 90°. A 2-μm-thick aluminum window was used to isolate the X-ray chamber from the sample analysis chamber in order to prevent high-energy electrons from impinging on the sample. Survey spectra, from 0 to 1200 eV binding energy, were collected at 100-eV pass energy with an energy resolution of approx. 1.0 eV, a dwell time of 100 ms and a total of one scan averaged. High-resolution spectra were collected at a pass energy of 20 eV, energy resolution of 0.1 eV, dwell time of 100 ms and a total of seven scans averaged in the respective binding energy ranges for F1s, C1s, O1s and N1s. For those insulating PTFE samples, a low-energy electron flood gun was used to compensate for sample charging. The nominal settings for this gun were 6 eV, 0.09 mA emission current resulting from 3.0 A filament current. Peak areas were fitted using VG Eclipse software. Quantification was performed from the peak area using atomic sensitivity factors specified for the spectrometer (F1s, 4.43; C1s, 1.00; O1s, 2.93; N1s, 1.8). The operating pressure of the spectrometer was typically in the 10^{-9} mbar range with a system base pressure of 2×10^{-10} mbar.

Contact angle measurement

The static water contact angle was determined at room temperature (approx. 21°C). The contact angle formed between a sessile drop and the PTFE wafer surface is directly related to the forces at the liquid–solid interface, indicating the hydrophilic or hydrophobic characteristics of the surface. The measurement was performed with a NRL 100-00 Contact Angle Goniometer (Ramé-Hart, Mountain Lakes, NJ, USA) using distilled and de-ionized water for the drops. For each condition, PTFE wafers in triplicate were measured to obtain the average value and standard

deviation. The samples were stored in sealed plastic vials after being removed from the plasma reactor. Control samples (without plasma treatment) were stored in a similar manner.

Endothelial cell attachment

The wafers were placed in wells of 24-well tissue-culture plates. Aliquots of 10^4 bovine aorta endothelial (BAE) cells (BioWhitaker Clonetics; Walkersville, MD, USA) were seeded into the wells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics and incubated for various times as indicated in the results section. After the incubation period, the medium was gently aspirated, the cultures rinsed once in phosphate-buffered saline (PBS, pH 7.4) and the specimens fixed in 10% buffered formalin. After fixation, the cells were stained in 70% ethanol containing 0.1% eosin Y. After rinsing to remove unbound stain, the specimens were examined by epifluorescence and digitally photographed. Tissue-culture medium, serum, antibiotics and eosin Y were purchased from Sigma Aldrich (St. Louis, MO, USA).

Human platelets and peripheral leukocytes

Human blood was obtained from normal donors who had not received aspirin or anti-inflammatory medication within one week and collected into heparinized tubes. Thereafter, all manipulations were performed in plastic ware.

Leukocytes were isolated by centrifugation over Ficoll-Hypaque® (Amersham Pharmacia, Piscataway, NJ, USA), rinsed once in a completed medium composed of DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin and resuspended in complete medium to a concentration of 10^6 cells/ml. Aliquots of cells in 0.5 ml were added to wells of low-attachment tissue-culture cluster plates (Fisher Scientific, Pittsburgh, PA) containing PTFE wafers. The wafers were used following glow-discharge plasma treatment or used without treatment. After two hours, the wafers were gently rinsed in PBS (pH 7.4) and the cells fixed by immersing the specimens in buffered formalin. In all experiments, samples were in at least triplicate and the experiment was repeated twice.

Platelets were used as platelet-rich-plasma (PRP). Blood was collected by venipuncture into heparinized tubes and thereafter processed with plastic ware. The PRP was obtained by centrifuging the tube at $150 \times g$ for 15 min at room temperature. The supernatant (the PRP) was pipetted into a clean plastic tube and used immediately to prepare working platelet solutions. PRP was diluted 1 : 3 with complete medium before use and contained approximately 1×10^5 platelets/μl. The medium containing the PRP was added in 0.5-ml aliquots to wells of low-attachment tissue-culture cluster plates containing treated or non-treated PTFE wafers. After two hours the wafers were gently rinsed in PBS (pH 7.4) and the cells fixed by immersing the specimens in buffered formalin.

All specimens were stained with eosin Y in 70% ethanol, rinsed and viewed by epifluorescence microscopy. Platelets were viewed at a magnification of 400× and recorded as digitized computer images. Leukocytes were viewed at 200× and similarly recorded. Regions of the images were evaluated to determine the number of bound cells. In all experiments, samples were in at least triplicate and the experiment was repeated twice.

Adsorption of plasma proteins

Human plasma was obtained from normal human donors or purchased as a lyophilized preparation (Sigma Aldrich). The plasma was diluted 1 : 5 in PBS and filtered through a 0.2-μm filter. Stainless steel wafers coated at 37°C with fibrinogen (100 mg/ml), fibronectin (10 mg/ml) or vitronectin (10 mg/ml) were used as positive controls. Wafers coated with 1% bovine serum albumin were used as negative controls. Experimental samples were immersed in the human plasma solution and the specimens incubated for 30 min. After extensive rinsing in PBS all wafers were immersed in PBS containing 1% BSA and a 1 : 500 dilution of either goat anti-human fibrinogen IgG, sheep anti-human vitronectin IgG (Accurate Chemical, Westbury, NY, USA) or rabbit anti-human fibronectin IgG (Sigma Aldrich). The specimens were incubated for 1 h and rinsed. After rinsing, the specimens were transferred to fresh tubes. Aliquots of PBS/BSA containing 1 : 250 dilutions of the appropriate species specific HRPO-conjugated polyclonal IgG were added and the specimens incubated for 1 h. After rinsing, a chromogenic solution containing 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfinic acid) (ABTS; Pierce Biotechnology, Rockford, IL, USA) was added and the color subsequently read at 410 nm in a spectrophotometer.

Implant

The animal studies were done under an approved animal protocol. PTFE wafers with and without plasma treatments were implanted subcutaneously in adult rats and 4 animals were used for each material. After pentobarbital (i.p.) anesthesia, an incision was made laterally across the side of the animal and the fascia loosened with a hemostat. The wafer was placed distal from the incision site and the surgical wound closed with stainless steel surgical clips. The animals were allowed to recover and then returned to the animal colony. Each animal received two implants randomized as to type. After 7 or 14 days, the animals were killed and the implanted area was surgically excised. The tissue was fixed in formalin, imbedded in paraffin, sectioned and stained with hemotoxylin and eosin.

RESULTS

Surface characterization

Figure 1 presents the contact angle data for untreated PTFE and for PTFE treated with glow-discharge plasmas of O_2, NH_3, or $NH_3 + O_2$. All the samples treated

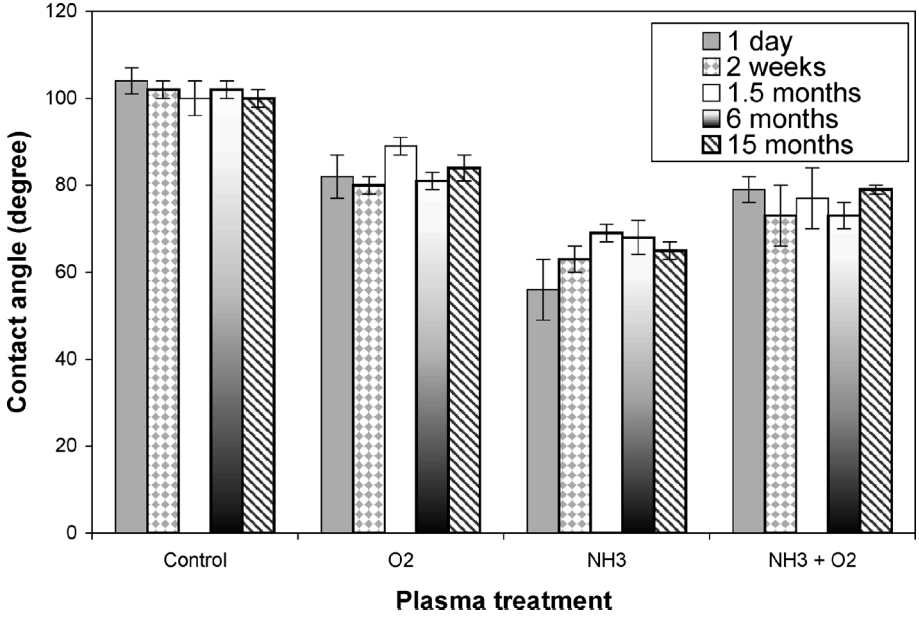


Figure 1. Contact angle data at 1 day, 2 weeks, 1.5 months, 6 months and 15 months of PTFE samples after plasma treatments. (a) Control (none), (b) O_2 plasma treated, (c) NH_3 plasma treated and (d) $NH_3 + O_2$ plasma treated. Error bars represent means±standard deviation for $n = 3$.

by O_2, NH_3 or $NH_3 + O_2$ plasma exhibited a decrease in contact angle relative to controls indicating a substantial increase in surface hydrophilicity. NH_3 plasma treatment resulted in the most pronounced decrease in contact angle (highest hydrophilicity). To establish if the surface properties resulting from plasma treatment of PTFE were stable, contact angle measurements were performed at different time intervals after sample preparation. Taking into account the error bars shown in Fig. 1, the contact angle of PTFE samples treated with O_2 plasma or $NH_3 + O_2$ plasma did not appear to change within 15 months after plasma treatment, whereas the NH_3 plasma-treated PTFE tended to increase the contact angle in the first 1.5 months after its exposure to air.

The ESCA survey scan spectra of PTFE samples are shown in Fig. 2. The non-control PTFE samples were used for ESCA at 4 months after the plasma treatments. In each case, including control, only 1 specimen was analyzed. For all 4 types of PTFE samples, there were three common peaks F1s, C1s and O1s. For the latter two samples, which were treated with NH_3 and $NH_3 + O_2$ plasma respectively, one more peak (N1s) was detected. The elemental composition on the PTFE samples surface detected using ESCA survey scans was calculated and listed in Table 1. Nitrogen was detected on the PTFE specimen only following treatment with NH_3 plasma or $NH_3 + O_2$ plasma. Surface oxygen was detected on all specimens tested. The small amount of oxygen present on the untreated PTFE was probably due to the absorption of oxygen from air and has been observed by others [13].

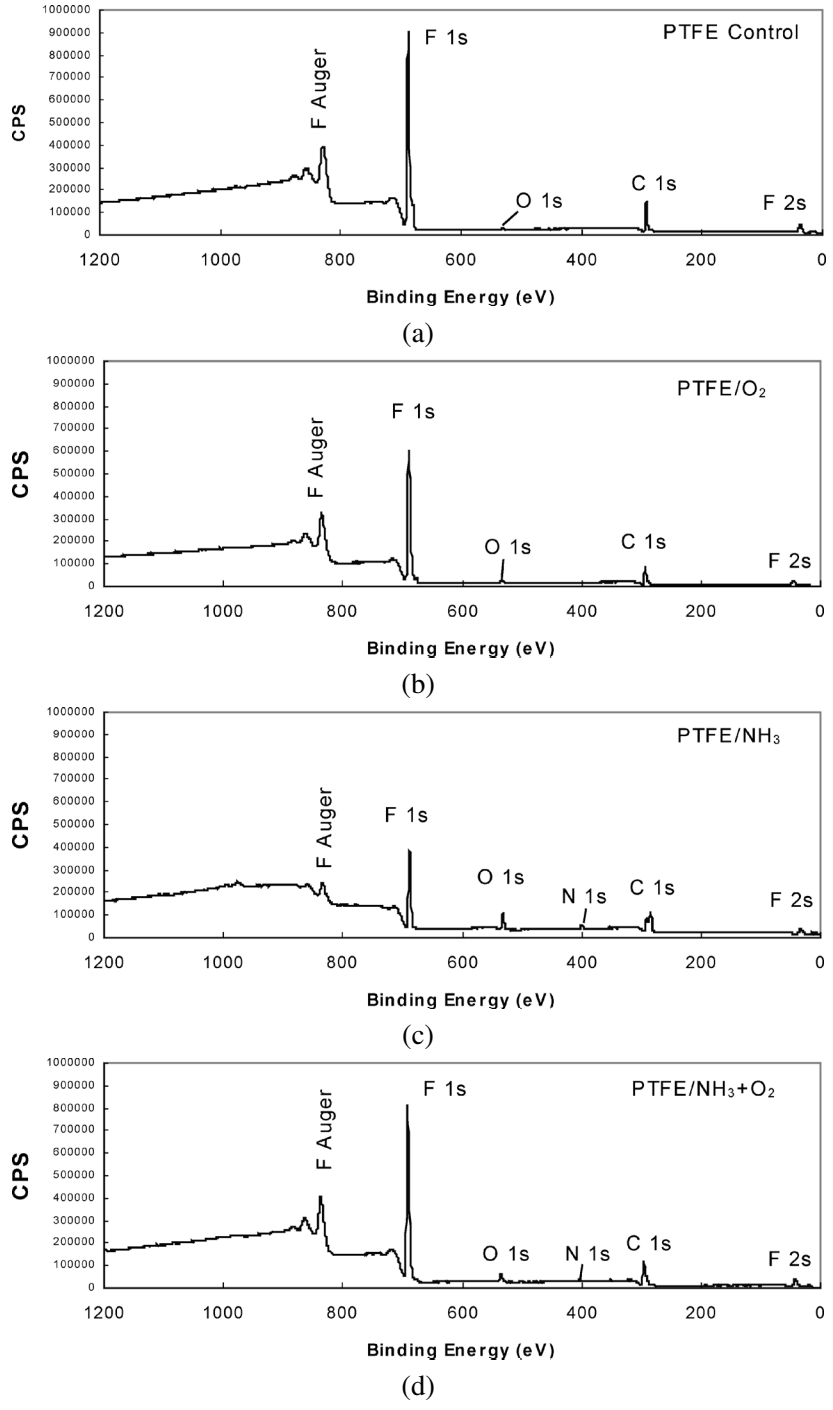


Figure 2. ESCA survey spectra of the PTFE samples at 4 months after plasma treatments. (a) Control (no plasma treatment), (b) O_2 plasma treated, (c) NH_3 plasma treated and (d) $NH_3 + O_2$ plasma treated.

Table 1.
Elemental composition at the surface of PTFE treated with various plasmas as determined ESCA

Plasma process	Fluorine	Oxygen	Carbon	Nitrogen
None (control)	66.2	0.5	33.3	0
O_2	64.6	1.4	34.0	0
NH_3	30.2	8.7	56.8	4.3
$NH_3 + O_2$	55.2	4.4	39.4	1.0

Fluorine, oxygen, carbon and nitrogen were determined from F1s, O1s, C1s and N1s peaks. Data are reported in atomic percent.

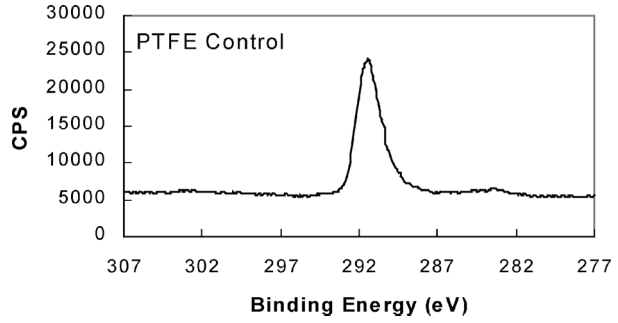
Table 2.
Atomic percent ratios of fluorine to carbon (F/C) and oxygen to carbon (O/C) with reference to decreasing contact angle values (1.5 months) at PTFE surfaces

Plasma process	Ratio of atomic percents		Contact angle (°)
	F/C	O/C	(mean±SD, $n = 3$)
None (control)	1.99	0.02	100 ± 4
O_2	1.90	0.04	89 ± 2
NH_3	0.53	0.15	69 ± 2
$NH_3 + O_2$	1.40	0.11	77 ± 7

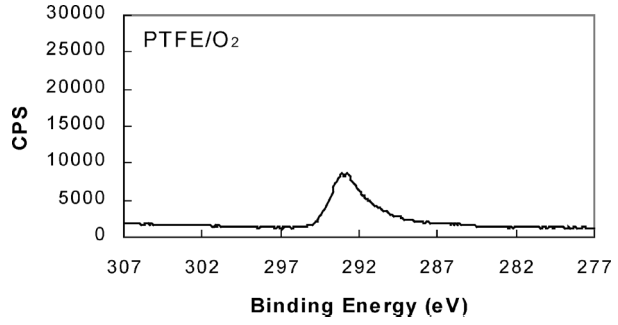
SD, standard deviation.

All plasma treatments reduced the atomic number ratio of fluorine to carbon (F/C) (Table 2). The reduction was from 1.99 (untreated PTFE) to 1.90, 0.53 and 1.40 by the treatment of O_2, NH_3 and $NH_3 + O_2$ plasma, respectively. The decrease in F/C ratio indicated that the plasma treatments caused defluorination of the PTFE surface. In contrast, the O/C ratio increased from 0.02 (untreated PTFE) to 0.04, 0.15 and 0.11 by the treatment of O_2, NH_3 and $NH_3 + O_2$ plasma, respectively. The highest defluorination occurring on the ammonia plasma-treated surfaces as represented by the lowest ratio of F/C indicated that the plasma treatment led to a formation of many carbon free radicals, which then resulted in stronger oxidation than the other two plasma treatments. Actually, the elemental composition of oxygen at the NH_3 plasma-treated PTFE surface was the highest among the 4 types of PTFE samples, implying a significant post-oxidation happening on the NH_3 plasma-treated surface and causing the contact angle to increase with time.

Figure 3 illustrates the high-resolution C1s peaks of the PTFE samples. The NH_3 plasma-treated PTFE sample had two C1s binding energy peaks, whereas the other three only had one peak. The distribution of functional groups, as percentages of total carbon, is shown in Table 3. The carbon atom, with asterisk, was the objective carbon used for the binding energy assignment. For untreated PTFE, the C1s peak was resolved into two component peaks: a major peak at 291.8 eV ($C^*F_2{-}CF_2$) and a minor peak at 289.6 eV ($C^*OO{-}CF_2$). After O_2 plasma treatment, there was a slight percentage decrease in the 291.8 eV subpeak, a marked increase in the



(a)



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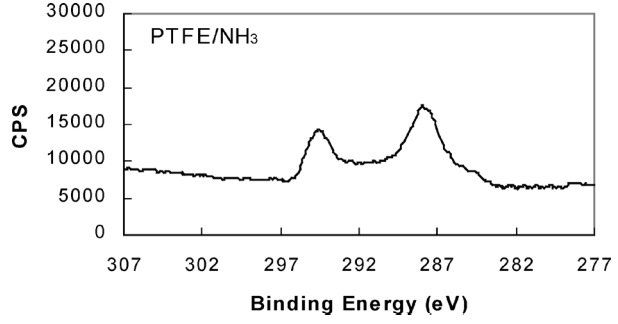

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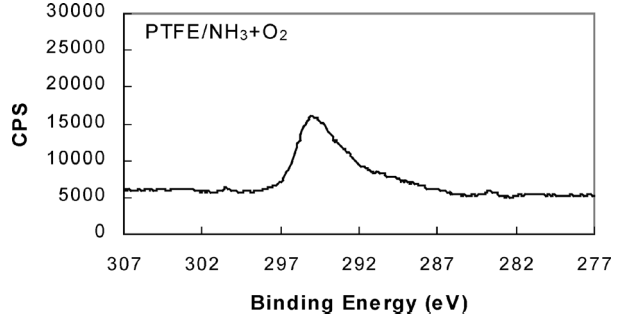

(d)

Figure 3. The high-resolution C1s peaks of the PTFE samples. (a) Control (no plasma treatment), (b) O_2 plasma treated, (c) NH_3 plasma treated and (d) $NH_3 + O_2$ plasma treated.

Table 3.
Percentage of carbon functional groups from C1s peaks as determined from high-resolution ESCA

Plasma process	285.2 eV	287.6 eV	289.6 eV	291.8 eV	294.3 eV
	C^*H_2-CHF, $-C^*NH_2$	C^*HF-CH_2, $C^*=O$	$C^*H(OR)-CHF$, C^*OO-CF_2	$C^*F_2-CF_2$	$C^*F_3-CF_2$, $C^*F(OR)_2-CF_2$
None (control)	—	—	13.5%	86.5%	—
O_2	—	6.9%	20.7%	72.4%	—
NH_3	9.6%	40.5%	12.6%	10.9%	26.4%
$NH_3 + O_2$	—	6.0%	13.7%	24.8%	55.5%

The reference carbon is illustrated with an asterisk (*). The functional groups are co-listed by the peak eV.

289.6 eV subpeak and the appearance of a new component at 287.6 eV ($C^*=O$). The profile of the PTFE sample treated with NH_3 plasma was more complex and contained component peaks at 294.3 eV ($C^*F_3-CF_2$ and $C^*F(OR)_2-CF_2$), 287.6 eV (C^*HF-CH_2) and 285.2 eV (C^*H_2-CHF and $-C^*NH_2$), where R stands for alkyl. The 285.2 eV peak was present only on the NH_3 plasma-treated PTFE because of the formation of amine groups on the surface and hydrogen incorporation with carbon. The 291.8 eV ($C^*F_2-CF_2$) peak, indicative of the major components of PTFE, was sharply reduced due to defluorination. A substantial increase at peak of 287.6 eV was attributed to oxidation of carbon free radicals upon exposure to air.

For the $NH_3 + O_2$ plasma-treated PTFE sample, a higher component peak than in the NH_3 plasma-treated sample was observed at 294.3 eV, indicating more $C^*F_3-CF_2$ and $C^*F(OR)_2-CF_2$ groups were formed during the $NH_3 + O_2$ plasma treatment. It is likely that $C^*F(OR)_2-CF_2$ was preferentially formed rather than $C^*F_3-CF_2$, as $NH_3 + O_2$ at a molar ratio of 2 : 3 was used. This suggests a single bond of the oxygen to the carbon ($-C-O-$) in contradistinction to the double-bonded oxygen ($-C=O$) of the ammonia-treated specimens (see Table 3).

Adsorption of blood proteins

Blood plasma was used to monitor if proteins associated with cell attachment could be adsorbed onto the surfaces of PTFE with and without plasma treatments (Table 4). The plasma treatments were performed 1 month before the protein adsorption test. Relative to untreated specimens, PTFE treated with $NH_3 + O_2$ plasma absorbed significantly higher amounts of fibrinogen and less vitronectin, whereas fibronectin adsorption was unaffected. Ammonia plasmas increased the relative amount of fibrinogen and fibronectin, and decreased vitronectin levels. Oxygen plasmas increased fibronectin levels, whereas fibrinogen levels were unaffected and vitronectin levels decreased. Thus, each type of plasma had a distinct and different effect on adsorption profiles.

Table 4.
Absorbed blood plasma-proteins on PTFE with and without glow-discharge plasma treatment

Plasma process	Absorbance at 405 nm		
	Fibrinogen	Fibronectin	Vitronectin
None (control)	0.36 ± 0.09	0.19 ± 0.11	0.46 ± 0.09
O_2	0.35 ± 0.14	0.36 ± 0.05	0.10 ± 0.00
NH_3	0.50 ± 0.15	0.37 ± 0.18	0.18 ± 0.02
$NH_3 + O_2$	0.83 ± 0.16	0.19 ± 0.01	0.07 ± 0.05

Data are means±SD, $n = 4$.

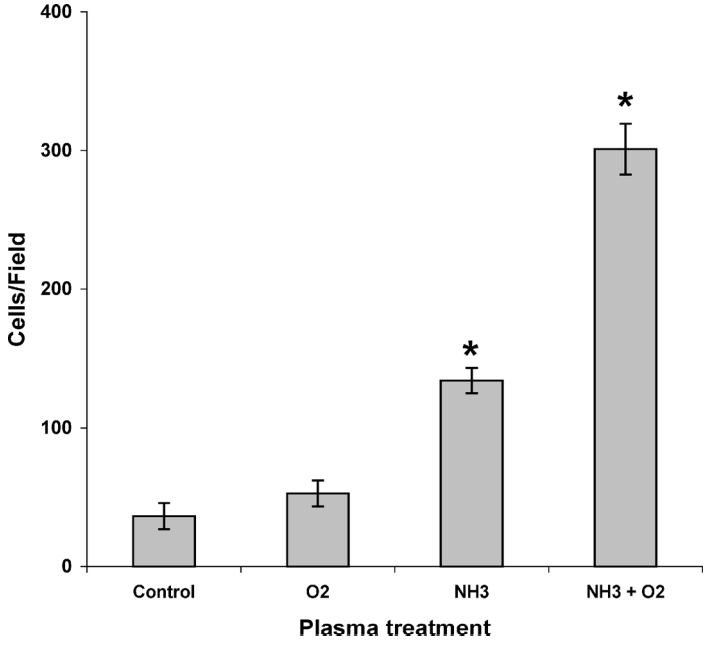

Figure 4. Attachment of bovine aorta endothelial cells to PTFE wafers at 2 h post-seeding as determined by microscopic examination (fluorescence, 100× magnification) after staining with eosin Y. The PTFE wafers were at 1 week after plasma treatments. Data are presented as the average±SD for $n = 3$. Asterisks indicate $P < 0.05$ relative to untreated controls (none).

Cell attachment and growth

The effect of treating PTFE surfaces with mixed gas plasmas was evaluated with endothelial cells. Three endpoints were monitored relative to untreated PTFE: cell attachment, cell spreading and growth to confluency. The plasma-treated samples were used at 1 month after plasma treatment.

Cell attachment was monitored 2 h after addition of the cells (Fig. 4). At this time point, few endothelial cells had attached to untreated PTFE (controls) or to PTFE treated with oxygen. PTFE treated with ammonia plasma resulted in an approx. 3-fold increase in cell attachment. PTFE treated with $NH_3 + O_2$ plasma had nearly a

10-fold increase in cell attachment at 2 h. Cell spreading and growth were monitored at 24 h and 4 days, respectively, after addition of cells. Cells that did attach were largely spread onto the surface by 24 h, regardless of treatment, although the density of cells was clearly highest for PTFE treated with $NH_3 + O_2$ plasma (Fig. 5). By 4 days, cells on PTFE treated with $NH_3 + O_2$ plasma were confluent. Confluency was not achieved by cells grown for 4 days on PTFE with no treatment or PTFE treated with either oxygen plasma or ammonia plasma.

The attachment of leukocytes and platelets was also evaluated on PTFE specimens treated with $NH_3 + O_2$ plasma at 2 days after the plasma treatment and compared to untreated PTFE and found to be essentially the same (data not shown).

Subcutaneous implant

PTFE wafers implanted in rats for 1 week developed a capsule surrounding the implant (Fig. 6). Those plasma-treated PTFE wafers had been in storage for 1 month before they were implanted into rats. Leukocytes, indicative of inflammation, were found juxtaposing the implant surface. The tissue surrounding PTFE treated with oxygen plasma had a moderately thick capsule and was widely infiltrated with leukocytes, especially near the interface with the implant. With NH_3 plasma treatment, the interface between the implant and tissue contained extensive thrombus (clot material) with inter-dispersed connective tissue. On the other hand, PTFE treated with NH_3+O_2 plasma had little or no evidence of thrombus formation, little or no inflammation and a thin capsule. A similar morphology was found at two weeks of implant (Fig. 7).

DISCUSSION

The contact angle was lower for PTFE following all plasma treatments, as compared to untreated controls. The change in contact angle clearly suggested a significant change in the surface chemistry following plasma treatment. The contact angles of specimens treated with $NH_3 + O_2$ plasmas did not change over time suggesting that the chemical change introduced by plasma treatment was stable for at least 15 months.

Nitrogen was detected on the PTFE specimen only following treatment with NH_3 plasma or $NH_3 + O_2$ plasma. It is not unexpected that PTFE treated with NH_3 plasma had a higher nitrogen content compared to PTFE treated with O_2 or NH_3/O_2 mixtures. The small amount of oxygen present on the untreated PTFE was probably due to the absorption of oxygen from air. The oxygen found on PTFE treated with NH_3 plasmas was thought to arise from a different set of reactions occurring upon exposure to room air. The incorporation of nitrogen and oxygen on expanded PTFE vascular grafts treated with ammonia plasma was also reported in the literature [14].

The NH_3 plasma was expected to produce carbon free radicals and it is these carbon free radicals that ultimately could react with atmospheric oxygen. In NH_3

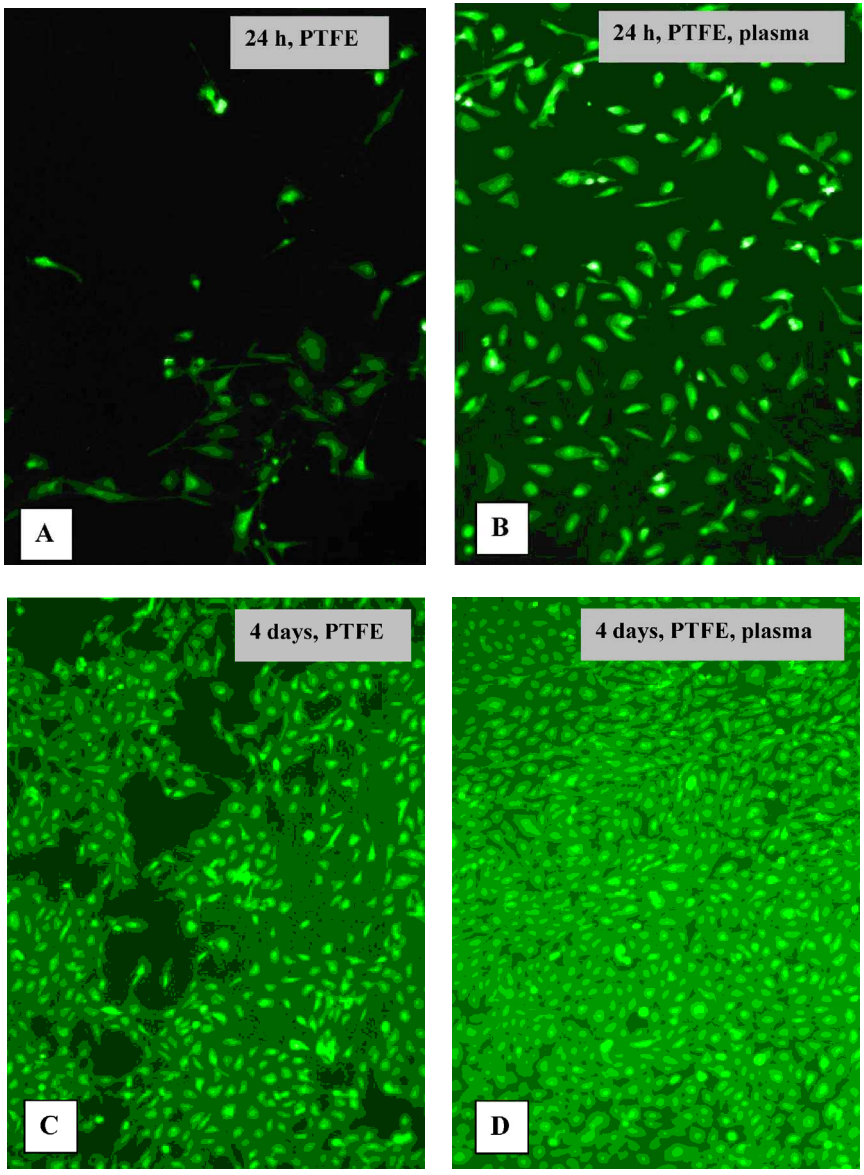

Figure 5. Enhanced attachment and growth of endothelial cells on PTFE with and without treatment with $NH_3 + O_2$ plasma treatment. The PTFE wafers were at 1 week after plasma treatments. Top panels: fluorescence micrographs of bovine aorta endothelial cells on PTFE at 24 h post-seeding (A, control; B, plasma treated). Bottom panels: fluorescence micrographs of bovine aorta endothelial cells on PTFE at 4 days post-seeding (C, control; D, plasma treated). This figure is published in colour on http://www.ingenta.com

glow-discharge plasmas (and also those composed of $NH_3 + O_2$), NH_2, NH, H, excited NH_3 and NH_4^+, NH_3^+, H^+ would be expected to be formed by the collision

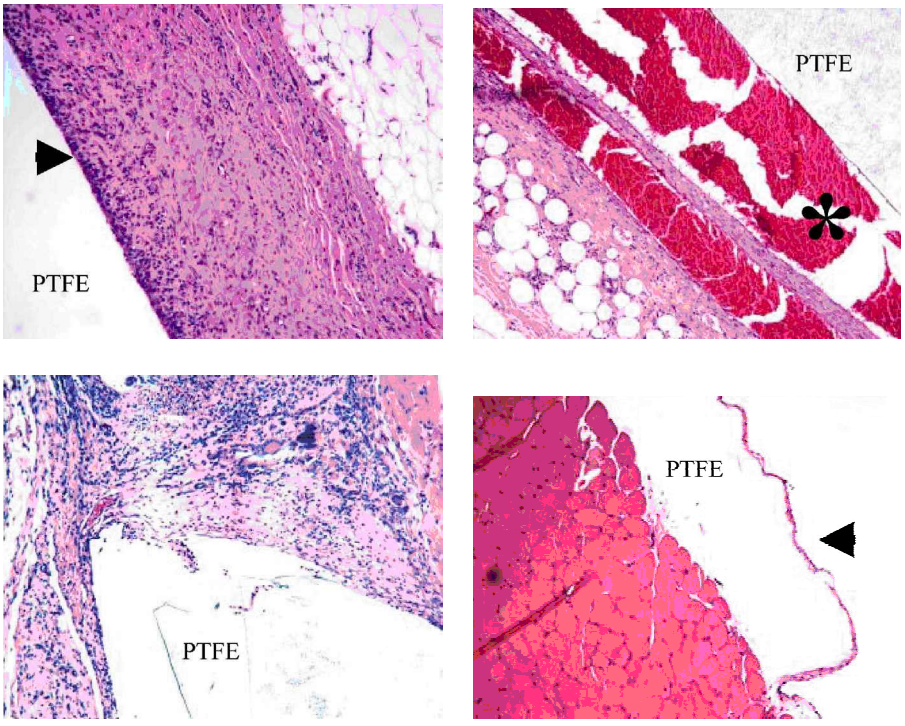

Figure 6. Micrographs of tissue capsules surrounding PTFE without treatment and with various plasma treatments. PTFE wafers were implanted subcutaneously into adult rats at 1 month after plasma treatments and collected after 7 days. Without treatment (upper left), the surrounding tissue capsule was moderately thick with pronounced inflammatory cells at the tissue capsule interface (arrow). With NH_3 plasma treatment (upper right), the interface between the implant and tissue contained extensive thrombus (*) with interdispersed connective tissue material. With O_2 plasma treatment (lower left), the tissue evidenced inflammatory cells. With $NH_3 + O_2$ treatment, only a thin capsule (arrowhead) was found with no substantial inflammation or clotting. Original magnification 100×, hemotoxylin/eosin stain. This figure is also published in colour on http://www.ingenta.com

of NH_3 molecules with energetic electrons produced in the discharge [15, 16]. The hydrogen ions, with light mass and high kinetic energy, then bombard the PTFE sample surface resulting in the generation of carbon free radicals and defluorination of the surface. This would be similar to defluorination reactions caused by H_2 plasma [13] on PTFE surfaces. Upon exposure to atmospheric oxygen, the carbon free radicals would react to form carbonyl and carboxyl groups. Other researchers found that N_2 plasma-treated PTFE surface also had oxygen-containing group, even though no oxygen-containing gas was used for plasma treatment [17].

Within an oxygen plasma there would be no hydrogens to displace the fluorines of PTFE and the amount of defluorination should be less, as was seen from the ESCA data. Nonetheless, within an oxygen plasma O, O_2^+, O_2^-, metastable-excited O_2 and free electrons are expected to be present [18, 19], thus it is not unexpected that at

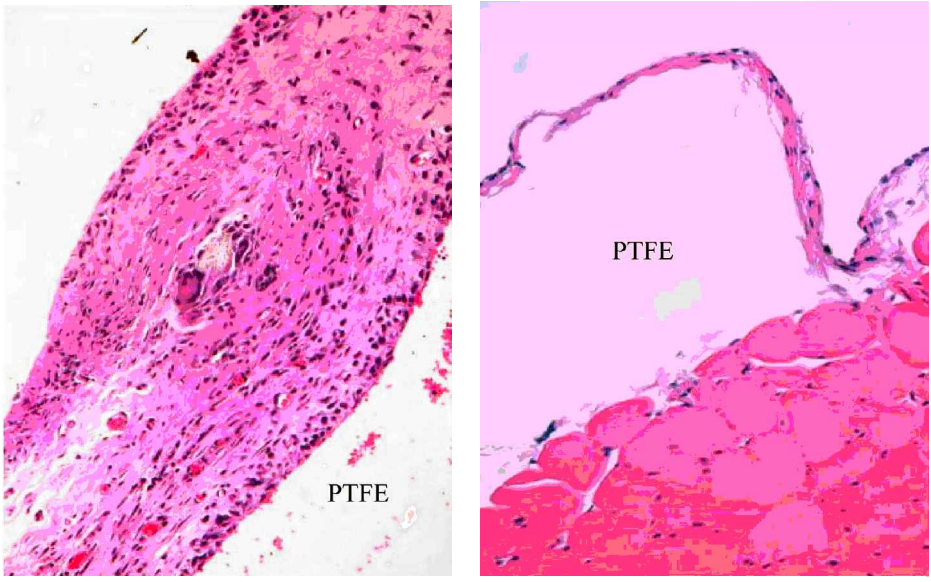

Figure 7. Micrographs of tissue capsules surrounding PTFE without treatment (left) and with $NH_3 + O_2$ treatment (right). PTFE wafers were implanted subcutaneously into adult rats at 1 month after plasma treatments and collected after 14 days. Without treatment (left), the capsule is moderately thick with pronounced connective tissue and marked vascularization. With $NH_3 + O_2$ treatment (right), the capsule is thin with little connective tissue. Original magnification 200×, hemotoxylin/ eosin stain. This figure is published in colour on http://www.ingenta.com

least some of these would react with the PTFE and generate carbonyl and carboxyl groups.

In a mixed plasma environment of $NH_3 + O_2$, reactive species formed from both molecules would be present and include excited NH_3, NH_4^+, NH_3^+, H^+, NH_2, NH, $H^\bullet$, O_2^+, O_2^-, $O^\bullet$, etc. In such a plasma, NH_3 (and its ionic species) would be expected to undergo oxidation. The oxidation of ammonia should occur in the plasma phase as well as on the solid phase, thereby leading to surface bound oxygen-containing groups. The high-resolution ESCA data support this hypothesis in that, following treatment with $NH_3 + O_2$ plasma, the amount of $CF(OR)_2{-}CF_2$ was substantially increased compared to all other plasma treatments. Furthermore, no $-C-NH_2$ was detected. This was a clear contradistinction to results with NH_3 plasmas where $-C-NH_2$ was detected in substantial amounts.

In comparing the F/C ratio data and the contact angle data, a clear correlation was found; the lower the F/C ratio, the lower the contact angle and, therefore, the higher the hydrophilicity. A converse correlation was found comparing the O/C ratio to the contract angle data; the higher the O/C ratio, the lower the contact angle, indicative of higher hydrophilicity. Hydrophilicity has been suggested to be a key determinant of cell attachment [20, 21]. Hydrophilicity is known to influence the adsorption of blood proteins and through these proteins to regulate a variety of cell behaviors such as cellular attachment.

We examined the pattern of adsorption of three blood proteins known to mediate cell attachment. Vitronectin, fibronectin and fibrinogen belong to a group of structurally and functionally homologous adhesive proteins found in blood that are essential in the pro-coagulant phase of the hemostatic system where they interact with platelets and the vessel wall [22, 23]. These molecules have structural motifs that are responsible for the attachment of cells including platelets and nucleated cells like endothelial cells and smooth muscle cells. In our studies, the overall pattern of adhesion-protein adsorption was altered relative to untreated PTFE by every plasma treatment. The pattern of the adsorbed proteins compared to untreated controls was unique for each plasma and may well reflect the chemical changes introduced by the respective plasma, e.g. amine, carbonyl and carboxyl groups. The pattern following $NH_3 + O_2$ plasma treatment included a higher adsorption of fibrinogen, a decrease in vitronectin and no change in the adsorption of fibronectin, relative to untreated PTFE and may contribute to the enhancement of endothelial cell attachment. On the other hand, it was noted that the $NH_3 + O_2$ plasma treatment did not result in a change in platelet or leukocyte attachment relative to controls.

The higher amount of fibrinogen adsorption on $NH_3 + O_2$ plasma-treated specimens is noteworthy. Fibrinogen is a complex molecule with multiple cell binding domains including those of the integrin type and those of the non-integrin type [24]. Leukocytes, endothelial cells and platelets each have different integrin and non-integrin receptors, yet all can attach to fibrinogen. Adsorbed fibrinogen can undergo post-adsorptive conformational changes leading to modulation of cell binding domains [25]. It is possible that such types of conformational changes in adsorbed fibrinogen, if occurring following treatment of PTFE with $NH_3 + O_2$ plasma, could play a role in the differential binding of these cell types. It should also be noted that while it has generally been accepted that biomaterials adsorbing the least amount of the fibrinogen will support the least platelet adhesion, recent studies indicate that fluid shear, residence time of the adsorbed protein, nature of the co-adsorbed proteins and surface chemistry of the material all play important roles in influencing platelet-surface interactions and that they act in a complex manner to influence platelet interaction [26, 27].

The amount of endothelial cell attachment observed following treatment of PTFE with $NH_3 + O_2$ plasma was substantially higher than that seen following treatment with oxygen or ammonia plasma alone. Oxygen plasma treatment has been reported to increase the amount of fibronectin bound and to subsequently increase the extent of endothelial attachment [8]. Similarly, ammonia has been used to increase the attachment of endothelial cells to PTFE [4]. Our study confirms both those observations and indicates that the $NH_3 + O_2$ plasma treatment results in greater endothelial cell attachment. The enhanced endothelial cell attachment resulting from the $NH_3 + O_2$ plasma is unique to the separate effect of oxygen or ammonia plasma acting alone. As for the effect of elemental composition at sample surface, it was reported in the literature that oxygen content was correlated with the endothelial cell growth [28] and surface carboxyl groups were important

to cellular adherence [29, 30]. The nitrogen-rich functional groups were also speculated to contribute to endothelial cell attachment [9]. In the present study, the results demonstrated that higher percentage of carboxyl carbons contained in the $C^*F(OR)_2-CF_2$, characterized by a high-resolution ESCA scan of C1s, was well correlated to the cellular affinity.

The contact angle data presented herein suggest that treatment of PTFE with $NH_3 + O_2$ plasma results in stable surface modification. This is also supported by attachment study with endothelial cells on PTFE at 15 months after treatment wherein specimens treated with $NH_3 + O_2$ plasma retained nearly a 7-fold increase in cell attachment at 2 h after addition of cells as compared to controls, which is only a slight decrease from the 8-fold increase at 1 week after plasma treatment (see Fig. 4).

That endothelial cells attached and grew to confluency on PTFE treated with $NH_3 + O_2$ plasma suggests a potential clinical application on ePTFE vascular grafts. These grafts do not support effective endothelialization and, as a consequence, are at chronic risk of developing thrombosis especially in small-bore grafts. To overcome this problem, seeding of endothelial cells on the luminal surface of small-diameter vascular grafts has been explored and is a promising method to avoid occlusion of these prostheses [31].

PTFE implants without plasma treatment had modestly thick capsules and modest inflammation, particularly at the tissue/implant interface. Implants of PTFE treated with NH_3 plasma was marked by extensive thrombosis; PTFE treated with O_2 plasma had marked inflammation; and PTFE treated with $NH_3 + O_2$ plasma had largely benign responses like little inflammation and negligible thrombosis.

PTFE and extended PTFE (ePTFE) have a long history in clinical use [32]. The *in vivo* response to PTFE has also been documented in several different animals and implants [3, 33, 34]. The tissue response to ePTFE vascular grafts [34], for example, is marked by albuminal inflammation at the graft/interface along with neovascularization of tissue along the surface of the graft next to the tissue. This is thought to be similar to the response to the PTFE implants of our study. The studies with $NH_3 + O_2$ presented here suggest that this treatment may pacify the inflammatory reactions and limit fibrous tissue build-up as a reaction to PTFE implants. Our ongoing studies indicate that a similar enhancement of endothelial cell attachment, as found for PTFE in this study, can be expected on ePTFE vascular graft material. These results, in light of the current study, suggest that follow-on studies with plasma-treated ePTFE in a vascular setting with histomorphometric analysis may be warranted.

CONCLUSIONS

Surface modification of polytetrafluoroethylene (PTFE) was performed using NH_3, O_2 and $NH_3 + O_2$ plasma without altering its bulk properties. All the plasma-treated PTFE surfaces exhibited an decrease in contact angle, as compared to

untreated controls. The contact angle of PTFE treated with O_2 and $NH_3 + O_2$ plasma seems to be stable over the period of 15 months, while that of NH_3 plasma-treated one had a tendency to increase within the first 1 month and a half after the plasma surface modification. The improvement of surface hydrophilicity was considered to be conducive to cell attachment to the PTFE surface. ESCA result indicates the presence of oxygen-containing groups such as C═O, COO—CF_2 and $CF(OR)_2$—CF_2, as well as nitrogen-containing groups at the surface of plasma-treated PTFE surfaces. PTFE treated with $NH_3 + O_2$ plasma resisted the attachment of platelets and leukocytes in a manner similar to untreated PTFE. However, the attachment of bovine aorta endothelial cells was substantially increased both at 1 week and 15 months after plasma treatment, which could be attributed to the considerable amount of $CF(OR)_2$—CF_2 formed on the $NH_3 + O_2$ plasma-treated PTFE surface. At 14 days after subcutaneous implantation in rats, the PTFE wafers treated with $NH_3 + O_2$ plasma revealed a reduced amount of encapsulation and reduced levels of inflammatory cells compared to controls. These results suggest that $NH_3 + O_2$ plasma surface modification produces a unique character to PTFE and may be useful for surface property improvement of vascular grafts made of expanded PTFE.

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REFERENCES

1. P. K. Chu, J. Y. Chen, L. P. Wang and N. Huang, *Mater. Sci. Eng. R.* **36**, 143 (2002).
2. V. D. Bhat, B. Klitzman, K. Koger, G. A. Truskey and W. M. Reichert, *J. Biomater. Sci. Polymer Edn* **9**, 1117 (1998).
3. R. Sbarbati, D. Giannessi, M. C. Cenni, G. Lazzerini, F. Verni and R. De Caterina, *Int. J. Artif. Organs* **14**, 491 (1991).
4. R. Sipehia, *Biomater. Artif. Cells Artif. Organs* **18**, 437 (1990).
5. K. S. Tweden, H. Harasaki, M. Jones, J. M. Blevitt, W. S. Craig, M. Pierschbacher and M. N. Helmus, *J. Heart Valve Dis.* **4** (Suppl. 1), S90 (1995).
6. R. Vohra, G. J. Thomson, H. M. Carr, H. Sharma, M. Welch and M. G. Walker, *Eur. J. Vasc. Surg.* **5**, 93 (1991).
7. S. P. Massia and J. A. Hubbell, *J. Biomed. Mater. Res.* **25**, 223 (1991).
8. D. L. Mooradian, P. Trescony, K. Keeney and L. T. Furcht, *J. Surg. Res.* **53**, 74 (1992).
9. D. Y. Tseng and E. R. Edelman, *J. Biomed. Mater. Res.* **42**, 188 (1998).
10. J. G. Steele, G. Johnson, C. McFarland, B. A. Dalton, T. R. Gengenbach, R. C. Chatelier, R. A. Underwood and H. J. Griesser, *J. Biomater. Sci. Polymer Edn* **6**, 511 (1994).
11. M. Chen, P. O. Zamora, L. Peña, P. Som and S. Osaki, *J. Biomed. Mater. Res.* (in press).
12. M. Chen, S. Osaki, P. O. Zamora and M. Potekhin, *J. Appl. Polym. Sci.* **89**, 1875 (2003).
13. N. Inagaki (Ed.), *Plasma Surface Modification and Plasma Polymerization*. Technical Publishing Company, Lancaster, PA (1996).
14. R. Sipehia, M. Liszkowski and A. Liu, *J. Cardiovasc. Surg.* **42**, 537 (2001).

15. S. Pringle, V. Joss and C. Jones, *Surf. Interface Anal.* **24**, 821 (1996).
16. P. McCurdy, C. Butoi, K. William and E. Fisher, *J. Phys. Chem. B* **103**, 6919 (1999).
17. A. Dekker, K. Reitsma, T. Beugeling, A. Bantjes, J. Feijen and W. G. van Aken, *Biomaterials* **12**, 130 (1991).
18. B. Tissington, G. Pollard and I. M. Ward, *Composite Sci. Technol.* **44**, 185 (1992).
19. L. Lianos, D. Parrat, T. Q. Hoc and T. M. Duc, *J. Vac. Sci. Technol. A* **12**, 2491 (1994).
20. P. B. van Wachem, A. H. Hogt, T. Beugeling, J. Feyen, A. Bantjies, J. P. Detmers and W. G. van Aken, *Biomaterials* **8**, 323 (1987).
21. J. H. Lee and H. B. Lee, *J. Biomater. Sci. Polymer Edn* **5**, 467 (1993).
22. K. T. Preissner and D. Seiffert, *Thromb. Res.* **89**, 1 (1998).
23. G. C. Troy, *Vet. Clin. North Am. Small Anim. Pract.* **18**, 5 (1988).
24. S. Herrick, O. Blanc-Brude, A. Gray and G. Laurent, *Int. J. Biochem. Cell Biol.* **31**, 741 (1999).
25. J. Grunkemeier, C. Wan and T. Horbett, *J. Biomater. Sci. Polymer Edn* **8**, 189 (1996).
26. V. Balasubramanian, N. K. Grusin, R. W. Bucher, V. T. Turitto and S. M. Slack, *J. Biomed. Mater. Res.* **44**, 253 (1999).
27. V. Balasubramanian and S. M. Slack, *J. Biomater. Sci. Polymer Edn* **13**, 543 (2002).
28. S. I. Ertel, B. D. Ratner and T. A. Horbett, *J. Biomed. Mater. Res.* **24**, 1637 (1990).
29. E. A. Voger and R. W. Bussian, *J. Biomed. Mater. Res.* **21**, 1197 (1987).
30. W. S. Ramsey, W. Hertl, E. D. Nowlan and N. J. Binkowski, *In Vitro* **20**, 802 (1984).
31. P. M. Consigny, *J. Long Term Eff. Med. Implants* **10**, 79 (2000).
32. J. J. Bauer, M. T. Harris, I. Kreel and I. M. Gelernt, *Mt. Sinai J. Med.* **66**, 20 (1999).
33. J. M. Bellon, J. Bujan, L. A. Contreras, A. Hernando and F. Jurado, *J. Biomed. Mater. Res.* **31**, 1 (1996).
34. D. C. Yee, S. K. Williams, D. L. Salzmann, G. D. Pond, V. Patula, S. S. Berman and D. J. Roach, *J. Vasc. Interv. Radiol.* **9**, 609 (1998).