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Osteoblast behavior on polytetrafluoroethylene modified by long pulse, high frequency oxygen plasma immersion ion implantation

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ABSTRACT

Polytetrafluoroethylene (PTFE) is a commonly used medical polymer due to its biological stability and other attractive properties such as high hardness and wear resistance. However, the low surface energy and lack of functional groups to interact with the cellular environment have severely limited its applications in bone or cartilage replacements. Plasma immersion ion implantation (PIII) is a proven effective surface modification technique. However, when conducted on polymeric substrates, conventional PIII experiments typically employ a low pulsing frequency and short pulse duration in order to avoid sample overheating, charging, and plasma sheath extension. In this paper, a long pulse, high frequency O_2 PIII process is described to modify PTFE substrates by implementing a shielded grid in the PIII equipment without these aforementioned adverse effects. X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and contact angle measurements are carried out to reveal the surface effects of PTFE after long pulse, high frequency O_2 PIII and the results are compared to those obtained from conventional short pulse, low frequency O_2 PIII, O_2 plasma immersion, and the untreated control samples. Our results show that less oxygen-containing, rougher, and more hydrophobic surfaces are produced on PTFE after long pulse, high frequency O_2 PIII compared to the other 2 treatments. Cell viability assay, ALP activity test, and real-time PCR analysis are also performed to investigate the osteoblast behavior. It is clear that all 3 surface modification techniques promote osteoblast adhesion and proliferation on the PTFE substrates. Improvements on the ALP, OPN, and ON expression of the seeded osteoblasts are also obvious. However, among these treatments, only long pulse, high frequency O_2 PIII can promote the OCN expression of osteoblasts when the incubation time is 12 days. Our data unequivocally disclose that the long pulse, high frequency O_2 PIII technique is better than the other two types of traditional plasma treatment in the development of PTFE for bone or cartilage repair.

1. Introduction

It is well known that a desirable biomaterial should co-exist with tissues in the human body without causing any unacceptable harm [1]. In particular, polytetrafluoroethylene (PTFE), which is one of the most commonly used polymers in medicine, is attractive for its high biological stability. It is non-toxic, leaves no residue, and is non-degradable *in vivo*. In biomedical applications, PTFE is usually expanded and serves as vascular prosthesis [2–4], membrane barriers for guided tissue regeneration (GTR) [5,6], and facial soft tissue augmentation materials [7,8]. PTFE is also a potential biomaterial in bone or cartilage replacement due to its high hardness and wear resistance. However, the high bio-inertness of PTFE hampers its applications as bone substitutes. In this case, improving the surface biocompatibility of PTFE is requisite.

Surface modification is a desirable approach to enhance materials performance while retaining their favorable bulk properties. Several methods such as coating of proteins, ultraviolet (UV) or vacuum ultraviolet (VUV) irradiation, ion implantation, and plasma treatment have been proposed for PTFE. Coating the inner surface with a thin layer of proteins (e.g. fibronectin) [9,10] is an effective method to enhance the autologous endothelialization of PTFE-based vascular grafts. As a result, the risk of thrombosis of small-caliber prosthesis is limited. However, pre-surface modification of the hydrophobic PTFE for proteins coating consists of multiple steps and is invariably time consuming. This lengthy process required to get ready for protein coating vascular graft is a negative factor in clinical treatment. Before UV surface irradiation [11,12], PTFE substrates should always be pre-coated with some reagents. Thus, it does not offer a time advantage either since extra time is need for pre-coating. Ion implantation,

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commonly beam-line ion implantation, is an attractive treatment for PTFE substrates on account of its flexibility, effectiveness, and environmental friendliness [13,14]. Both the morphological and chemical characteristics of the polymer surface can be easily changed by ion implantation. However, due to its light-of-sight constraint, it is difficult to treat irregular-shaped substrates uniformly with this method. Plasma treatment of PTFE is advantageous from the perspective of environmental factors, simple operation, and non-light-of-sight characteristics [15]. The general processing time ranges from 60 sec to several minutes, which is much less than required for protein coating and ultraviolet modification. Unfortunately, the effects of plasma treatments on polymeric substrates cannot be maintained for a long time because the treated region is relatively shallow and also due to the chain segment movement.

Plasma immersion ion implantation (PIII) is a convenient way to couple plasma modification and ion implantation [16,17]. In this process, the sample is immersed in a plasma and negative high voltage pulses are applied to the sample. When the sample is negatively biased, an ion sheath is established and ions are implanted into the sample. However, in order to avoid sample charging and overheating, conventional PIII can only be conducted on polymeric substrates with low pulsing frequency and short pulse duration. In our continuous efforts to refine PIII hardware and develop new experimental protocols, we have demonstrated that conventional PIII equipment can be ameliorated by adding a grounded conducting grid. Consequently, long pulse, high frequency O_2 PIII is now possible for PTFE without adverse effects encountered in conventional PIII [18]. In this study, we investigate and compare the rat calvaria osteoblast behavior on PTFE after long pulse, high frequency O_2 PIII, conventional short pulse, low frequency O_2 PIII, standard O_2 plasma treatment, and non-treatment. The surface chemistry, morphology, and wettability of these specimens are also evaluated and discussed together with the biological results.

2. Experimental details

2.1. Sample preparation

0.25 mm thick PTFE from Good Fellow were implanted by long pulse, high frequency oxygen PIII, conventional short pulse, low frequency oxygen PIII, and oxygen plasma immersion in the plasma laboratory of City University of Hong Kong [18,19]. The system consisted of a stainless steel plasma discharge chamber (ϕ600 mm × 300 mm) and a stainless steel plasma diffusion chamber (ϕ760 mm × 1030 mm). RF (radio frequency 13.56 MHz) power from 0 to 2 kW was coupled to the plasma discharge chamber.Negative high voltage pulses were applied to the metal sample stage through a ceramic high voltage feed-through underneath the plasma diffusion chamber.The sample stage was a steel cylindrical sample stage 55 mm tall and 160 mm in diameter. It was supported by a metal rod with a diameter of 10 mm. To conduct high frequency (500 Hz) and long pulse (200 μs) oxygen plasma immersion ion implantation, the high voltage sample stage and supporting voltage feed-though rod was shielded from the plasma by a metal cage made of aluminum. The metal cage consisted of a 2 mm thick cylindrical tube with a top cover. A 100 mm radius hole was opened at the center of the top cover. A mask with a square opening of 60 mm × 70 mm was covered by a stainless steel mesh with 120 meshes per 2.5 cm (1 inch) and 65.0 μm (0.0026 inch) wire diameter. The PTFE sample with lateral dimensions of 7 cm × 8 cm was positioned 1.0 cm away from the mask.

Oxygen gas was bled into the vacuum chamber at a flow rate of 6.0 sccm. The working pressure was 3.0×10^{-4} Torr. 1000 W radio frequency power was matched to the plasma discharge chamber to generate the oxygen plasma. The plasma density was estimated to be 3×10^9 cm^{-3} from a previous probe measurement [20]. −5 kV negative voltage pulses were applied to the sample stage during the PIII treatment. In long pulse, high frequency PIII, the pulse duration was 200 μs and frequency was 500 Hz (the treated PTFE sample labeled L-PTFE). In the conventional short pulse/low frequency PIII experiments, the pulse duration was 30 μs and frequency was 50 Hz (sample designated S-PTFE). In the plasma exposure treatment, no high voltage pulses were applied and the modified samples a denoted as P-PTFE. The total treatment time was 30 min. The untreated PTFE substrate denoted as U-PTFE serves as the control.

2.2. Surface characterization

X-ray photoelectron spectroscopy (XPS)was conducted on a Physical Electronics PHI 5802 equipped with a monochromatic Al K_α source to determine the surface chemical composition of the various samples.A constant pass energy of 11.75 eV was employed and all the data were collected at a take-off angle of 45° with a step size of 0.1 eV. To characterize the surface morphology of the various specimens, tapping mode AFM was performed with a force sensor using the NanoScope V MultiMode system (Veeco). All the measurements were performed under ambient conditions and the scanned area on each sample was 4.98 μm × 4.98 μm. Static contact angles were measured by the sessile drop method on a Ramé-Hart (USA) instrument at ambient humidity and temperature to determine the surface wettability of the pristine and modified PTFE. Distilled water was used as the medium and the drop size was 6 μl for L-PTFE and 2 μl for the others. Each data point represents the average and standard deviation of ten measurements conducted on different parts of each specimen for statistical accountability.

2.3. Cell culture

Rat calvaria osteoblasts were obtained by sequential trypsin-collagenase digestion on calvaria of neonatal (<1 day old) Sprague-Dawley rats and then cultured in a humidified atmosphere of 5% CO_2 in the Dulbecco's modified Eagle's medium (D-MEM, Invitrogen) supplemented with 10% newborn bovine serum (Hyclone). After being expanded for an additional passage, the osteoblasts were seeded onto the specimens at a density of 1.5×10^4 cells per sample by using 24-well tissue culture plates as the holders. Before cell culturing, all the substrates were sterilized with 75% alcohol overnight and then rinsed with sterile phosphate-buffered saline (PBS) thrice. The medium for cell culture was refreshed every 3 days.

2.3.1. Cell viability

A cell count kit-8 (CCK-8 Beyotime, China) was employed in the experiments to quantitatively identify the viable osteoblasts on the samples. After culturing for 6 h, 3 days, 6 days and 12 days, the specimens with seeded osteoblasts were rinsed twice with sterile PBS and transferred to fresh 24-well tissue culture plates. Subsequently, culture medium with 10% CCK-8 was added to these samples in a separate volume of 0.7 ml. After 4 h of incubation, the solution of

Table 1
Sequences of the primers.

Gene	Primers (F = forward, R = reverse)
ALP	F: 5′-AACGTGGCCAAGAACATCATCA-3′ R: 5′-TGTCCATCTCCAGCCGTGTC-′3′
OPN	F: 5′-AGACCATGCAGAGAGCGAG-3′ R: 5′-ACGTCTGCTTGTGTGCTGG-3′
ON	F: 5′-CTGCCACTTCTTTGCGACCA-3′ R: 5′-CTCCAGGCGCTTCTCGTTCTC-3′
OCN	F: 5′-GGTGCAGACCTAGCAGACACCA-3′ R: 5′-AGGTAGCGCCGGAGTCTATTCA-3′
GAPDH	F: 5′-GGCACAGTCAAGGCTGAGAATG-3′ R: 5′-ATGGTGGTGAAGACGCCAGTA-3′

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Table 2
Elemental ratios of O/C and F/C on various PTFE samples.

Elemental Percentage [%]	O/C	F/C
U-PTFE	1.4	183.2
L-PTFE	6.4	170.4
S-PTFE	12.6	127.1
P-PTFE	12.8	128.5

each sample was aspirated and the absorbance was measured spectrophotometrically at 450 nm. Four parallel replicates of each sample at each time point were prepared during this cell viability assay. The statistical analyses were performed by the one-way ANOVA analysis.

2.3.2. Alkaline phosphatase (ALP) activity

After culturing on various specimens for 3 days, 6 days, and 12 days, the alkaline phosphatase (ALP) activity of the seeded osteoblasts was determined. The seeded osteoblasts were first rinsed thrice with PBS and then homogenized in an alkaline lysis buffer. Afterwards, p-nitrophenyl phosphate was added to the resulting cell homogenate for co-incubation. After 30 min of culture, the reaction was stopped by NaOH and the amount of p-nitrophenol produced was quantified by absorbance measurements at 405 nm. The control (unseeded) disks were treated similarly and assayed as blank controls which were subtracted from the corresponding specimens. All the ALP activity measured was normalized to the total protein content at the end of the experiments. Each specimen at each time point was measured in quadruplicates for better statistics.

2.3.3. Quantitative real-time PCR

The osteogenic-associated genes were quantitatively analyzed by real-time PCR to investigate the influence of various modified specimens on the gene expression of the seeded osteoblasts. Initially, the total RNA of osteoblasts cultured on various substrates for 3, 6, and 12 days was isolated using a TRIZOL reagent (Invitrogen). After adding chloroform to the mixture, shaking, and centrifuging, the RNA was separated into an aqueous phase. The RNA was subsequently recovered and precipitated from the aqueous

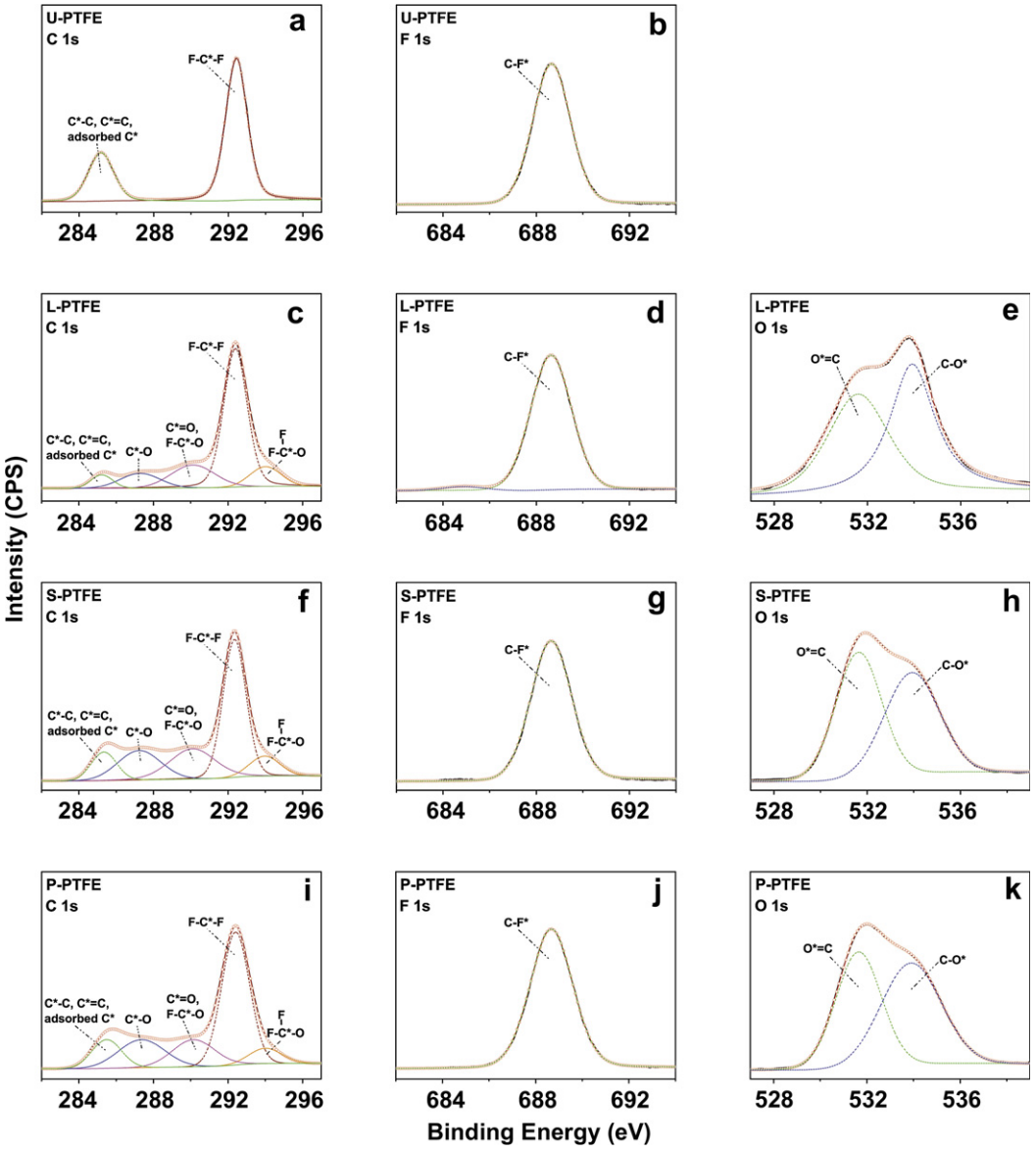

Fig. 1. XPS spectra acquired from the surface of: U-PTFE [(a) C1s and (b) F1s], L-PTFE [(c) C1s, (d) F1s and (e) O1s], S-PTFE [(f) C1s, (g) F1s and (h) O1s], and P-PTFE [(i) C1s, (j) F1s and (k) O1s]. The O1s regions of U-PTFE are not shown here as the O signals are very weak.

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phase by adding equivalent isopropanol. The acquired RNA pellet was washed with 75% ethanol treated with the RNase inhibitor diethyl pyrocarbonate (DEPC) and then solubilized in sterile DEPC water. After the concentration determination, a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa) was used to reverse transcribe the extracted RNA. Afterwards, real-time PCR (Bio-Rad iQ5 real time PCR detection system) was performed as follows: 40 cycles of PCR (95 °C for 10 s, and 60 °C for 20 s) after initial denaturation step of 30 s at 95 °C using a mixture of iQ5 SYBR Green I supermix, cDNA templates and each forward and reverse primers. Alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON) and osteocalcin (OCN) were evaluated when glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an endogenous housekeeping gene (the sequences of primers are shown in Table 1). Quantification of gene expression was based on the C_T (threshold cycle) value of each measurement and is presented as the average and standard deviation of three replicates.

3. Results

3.1. Characterization of samples

X-ray photoelectron spectroscopy (XPS) is employed to determine the surface chemical states. Table 2 presents the relative oxygen and fluorine contents relative to the carbon content. O can be detected on U-PTFE and the F/C ratio is less than 200%. It is believed to stem from little C and O_2 absorption on U-PTFE and disengagement of some C–F bonds. It can be observed that O is introduced to the PTFE samples after all the O_2 treatments and the F contents diminish accordingly. The O introduction and F reduction is less evident on L-PTFE compared to the two other treated specimens. No noticeable difference in the O/C and F/C ratios can be detected between S-PTFE and P-PTFE.

The high-resolution C1s, O1s, and F1s spectra are fitted and presented in Fig. 1. It is clear from the C1s spectra that only absorbed C/C–C/C=C and C–F bonds are detected on U-PTFE. After O_2 modification, C–O and C=O groups are formed on the PTFE substrates and the absorbed C/C–C/C=C and C–F are reduced simultaneously. In particular, formation of oxygen-containing groups on L-PTFE is less than that on S-PTFE and P-PTFE. However, reduction of absorbed C/C–C/C=C on the former is more prominent than that on the latter two. The spectra acquired from S-PTFE are quite similar to those from P-PTFE and it can thus be inferred that the surface chemical states of these 2 specimens are quite similar.

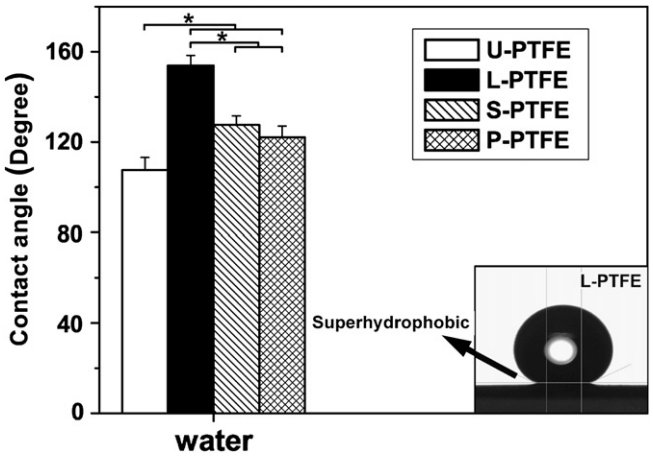

Fig. 3. Water contact angles measured on U-PTFE, L-PTFE, S-PTFE, and P-PTFE. The statistical significance indicated by *($p < 0.05$). The inset on the bottom right corner is the representative water droplet image (side view) on the top of L-PTFE.

The 3D AFM images of the different sample surfaces over a scanned range of 4.98 μm × 4.98 μm are shown in Fig. 2. These results are consistent with our SEM examination before [18]. U-PTFE displays a moderately rugged topography with some short buds with widths above 200 nm. After long pulse, high frequency O_2 PIII, there are many sharp rods with sizes of about hundreds of nanometers on PTFE substrates. Meanwhile, the topography change on the S-PTFE and P-PTFE are almost the same and only some smaller buds are detected on them. Apparently, the roughness of L-PTFE is much higher than that of the other 3 samples.

The measured contact angles using water as the media are summarized as a histogram in Fig. 3. It is clear that all the treatment processes increase the surface hydrophobicity of PTFE.L-PTFE is

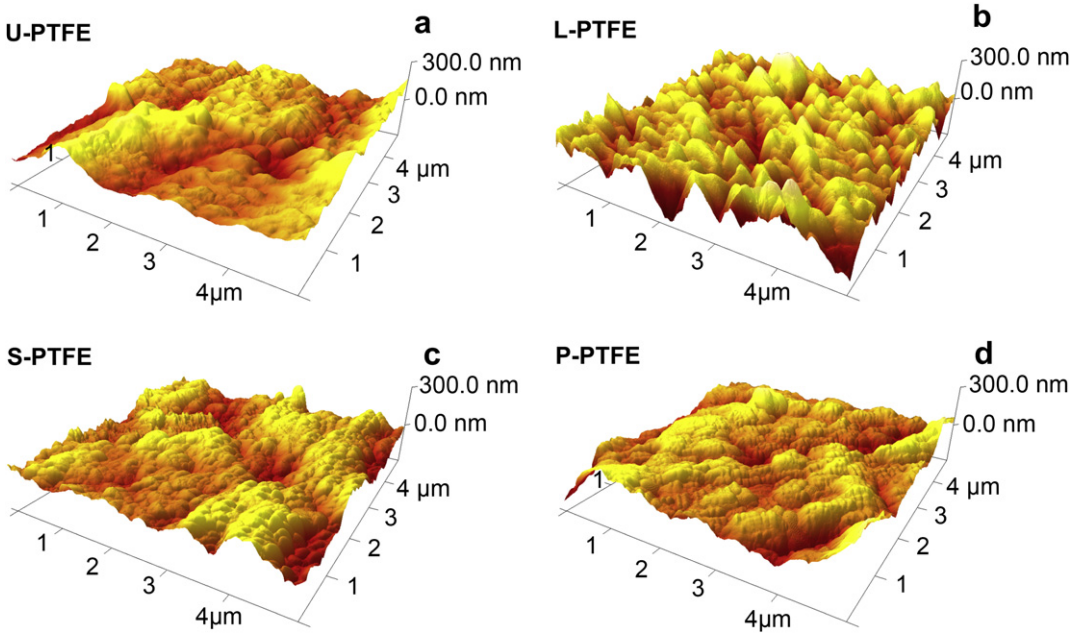

Fig. 2. 3D AFM images of (a) U-PTFE, (b) L-PTFE, (c) S-PTFE, and (d) P-PTFE.

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even superhydrophobic as revealed by a water contact angle of up to 153.9°. The water contact angles on PTFE also increase from 107.5° to 127.5° and 122.1° after short pulse, low frequency O_2 PIII as well as O_2 plasma immersion, respectively.

3.2. Adhesion and proliferation of osteoblasts

The time-dependent cell viability data of rat calvaria osteoblasts cultured on the various specimens are represented as a histogram in Fig. 4. It is obvious that the osteoblasts attachment and subsequent growth on the PTFE substrates are both improved after the 3 O_2 treatments. Furthermore, during the initial 6 days of incubation, the absorbance values which are proportional to viable osteoblasts are significantly lower on L-PTFE than on S-PTFE and P-PTFE. When the incubation time is up to 12 days, there is no difference in the data acquired from the 3 treated specimens.

3.3. ALP activity of osteoblasts

The ALP activities of the cultured rat calvaria osteoblasts are measured quantitatively to investigate osteoblast differentiation and the data are presented in Fig. 5. The results show that the ALP activities of the osteoblasts on the pristine and modified PTFE samples increase gradually with time during the assaying period. When the incubation time is 6 days or longer, the data obtained from the modified substrates are much higher than those on the untreated PTFE. Meanwhile, osteoblasts on S-PTFE exhibit the highest ALP activity among them.

3.4. Real-time PCR quantification of gene expression

Real-time PCR is utilized to quantify the mRNA expression of the rat osteoblasts relevant to the culture duration and types of substrates. The results are compared and presented as histograms in Fig. 6. It is clear that all the gene expression of the osteoblasts on various substrates increases gradually with culture time. When the osteoblasts are cultured on the various samples for 6 or more days, the gene expression is quite different. The trend of ALP gene expression is similar to that of the monitored ALP activity. After 6 days or longer incubation, all the O_2 treatments are found to promote ALP gene expression, and among the techniques, short pulse, low frequency O_2 PIII is the most effective. The OPN expression on U-PTFE is the highest during the first 6 days of incubation. However, when the incubation time is up to 12 days, all 3 treatments can dramatically promote the OPN expression of the seeded osteoblasts. The ON expression of osteoblasts on the modified substrates is always higher than that on the control and the difference in the ON expression between the untreated and treated PTFE becomes larger with time. No OCN expression of the seeded osteoblasts is detected during the initial 3 days of culture. When osteoblasts are cultured on the various substrates for 12 days, only long pulse, high frequency O_2 PIII induces significant up-regulation of OCN. Meanwhile, the OCN expression of osteoblasts on S-PTFE and P-PTFE is obviously lower than that on U-PTFE.

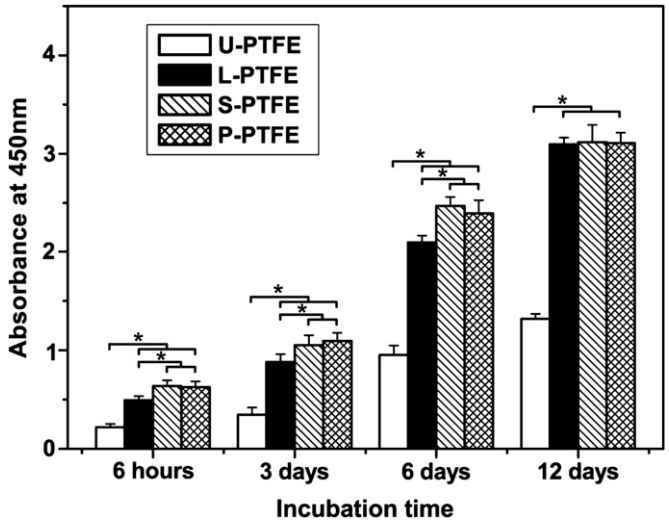

Fig. 4. The cell viability assay of rat calvaria osteoblasts cultured on U-PTFE, L-PTFE, S-PTFE, and P-PTFE for 6 h, 3 days, 6 days, and 12 days. The absorbance of the diluted Cell Counting Kit solution has been deducted from each data point and the statistical significance is indicated by $^*(p < 0.05)$.

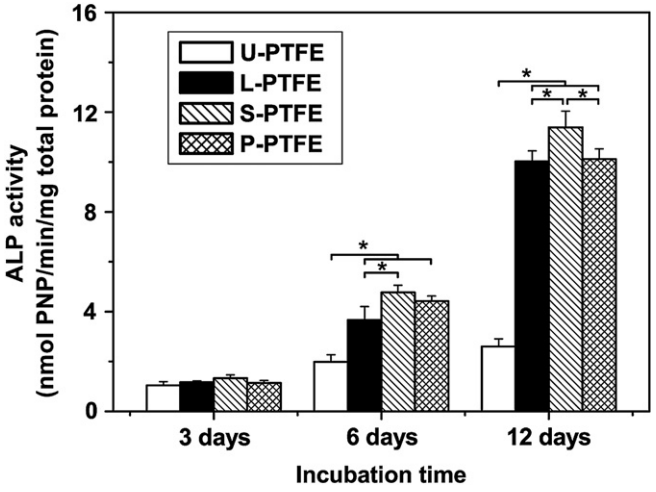

Fig. 5. ALP activity assay of rat calvaria osteoblasts cultured on U-PTFE, L-PTFE, S-PTFE, and P-PTFE for 3 days, 6 days, and 12 days. The statistical significance is indicated by $^*(p < 0.05)$.

4. Discussion

Primary rat calvaria osteoblasts are chosen in our experiments to evaluate the osteoblast response to the various treatments because some osteoblasts or osteoblast-like cell lines always exhibit abnormal phenotypes. The osteoblast adhesion, proliferation and differentiation on the various samples are evaluated and further analyzed according to the surface chemistry, surface topography and surface wettability. It can be observed from Table 2, Figs. 1 and 2 that both the surface chemistry and surface topography of PTFE are dramatically changed after the various treatements. Specifically, more surface oxygen groups are induced after short pulse, low frequency O_2 PIII and O_2 plasma immersion, whereas the surface roughness increase is more evident after long pulse, high frequency O_2 PIII. It is generally accepted that C–O and C═O groups are hydrophilic when they exist on polymeric substrates [21]. Feng and Jiang [22] have indicated that the surface roughness will increase the apparent contact angles when the true CA of this substrate is above 90°. Therefore, the superhydrophobicity of PTFE achieved after long pulse, high frequency O_2 PIII is the co-result of less oxygen groups and rougher surface morphology.

The cell viability assay indicates that all these treatments evidently improve osteoblast adhesion and subsequent proliferation on the PTFE substrates. In this respect, short pulse, low frequency O_2 PIII and O_2 plasma immersion are better than long pulse, high frequency O_2 PIII when the incubation time is 3 or 6 days. It has been demonstrated in our previous studies that the surface C–O and C═O groups positively affect osteoblasts adhesion and further proliferation [23,24]. More oxygen functional groups

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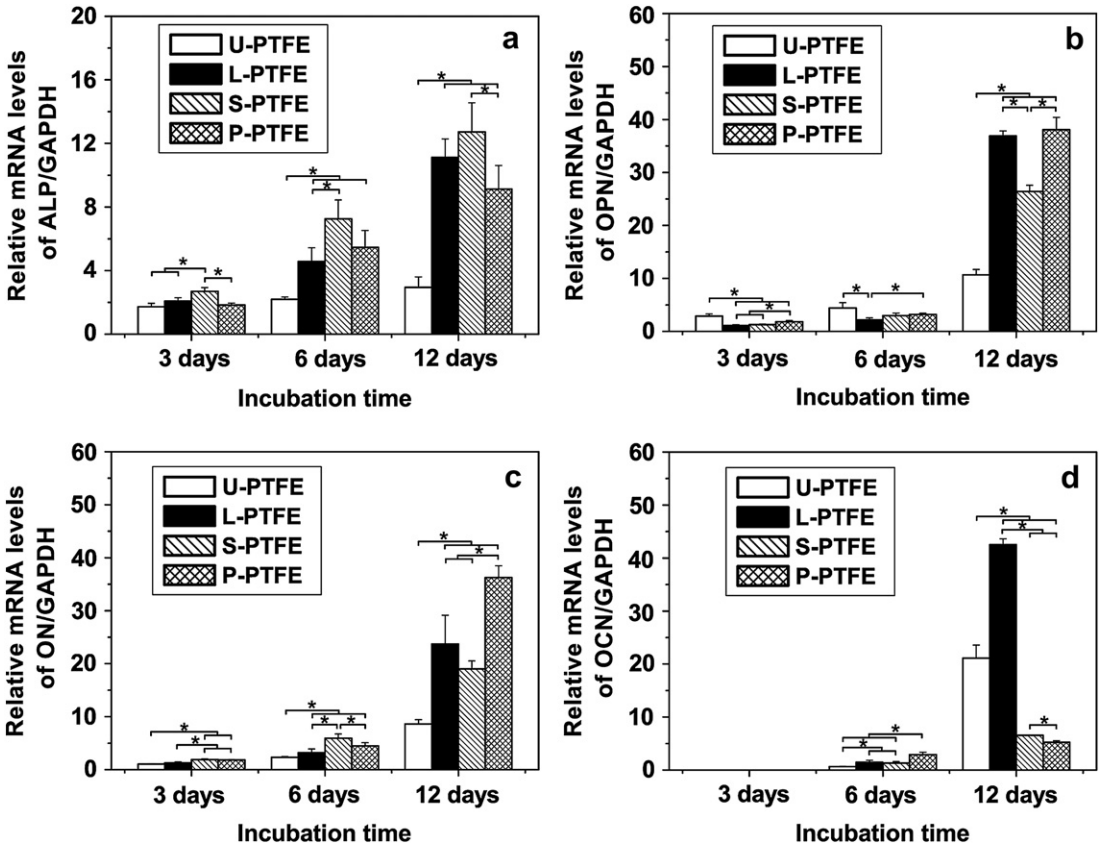

Fig. 6. Real-time PCR detection of osteogenic differentiation related gene expressions of rat calvaria osteoblasts cultured on U-PTFE, L-PTFE, S-PTFE, and P-PTFE for 3 days, 6 days and 12 days: (a) ALP, (b) OPN, (c) ON, and (d) OCN. The statistical significance is indicated by *($p < 0.05$).

lead to more osteoblasts on S-PTFE and P-PTFE compared to L-PTFE during the initial 6 days of incubation. However, if the incubation time is prolonged to 12 days, the quantities of living osteoblasts on these 3 modified substrates are very similar.

Osteoblast differentiation is determined by alkaline phosphatase activity measurements and quantification of gene expression related to osteogenic proteins. Among the osteogenic proteins, alkaline phosphatase is an enzyme regulating organic of inorganic phosphate metabolism via hydrolyzing phosphate esters [25,26]. It also functions as a plasma membrane transporter for inorganic phosphates [27] and is a marker indicating early differentiation of osteoblasts. Osteopontin is a phosohoprotein of osteoblasts expressed during the active proliferation period [28,29]. It serves as a bridge between the osteoblasts and hydroxyapatite through the RGD and polyaspartate sequences present [30]. It is also one of the earlier markers of osteoblast differentiation. Osteonectin is a non-specific osteogenic protein which is actively involved in bone remodeling and ECM organization [31,32]. It is expressed in a very early stage of osteoblast differentiation [33] and possesses the ability of binding to both type-I collagen and calcium [34,35]. Osteocalcin is a protein only synthesized by mature osteoblasts during the post-proliferative period [36–39]. It controls the nucleation and size of the hydroxyapatite crystals in the ECM of bone tissues then regulates bone crystal growth [40]. Furthermore, osteocalcin is the most specific osteogenic protein among the proteins mentioned above.

It is clear from Figs. 5 and 6 that all 3 treatments significantly improve the ALP activity and ALP gene expression of osteoblasts cultured after 6 or 12 days of incubation. Fig. 6 also indicates that the OPN and ON expressions are evidently developed by surface modification when the incubation time is 12 days. However, with regard to OCN, when osteoblasts are cultured on the various specimens for 12 days, only long pulse, high frequency O_2 PIII obviously promotes the gene expression of this most specific osteogenic protein.On the contrary, the OCN gene expression of the seeded osteoblasts are significantly down-regulated on the PTFE after short pulse, low frequency O_2 PIII and O_2 plasma immersion. It means that L-PTFE is better than the other 2 treated samples from the viewpoint of osteoblast differentiation. The L-PTFE surface with tiny and sharp rods is considered the key factor for the better osteoblast differentiation. Based on similar osteoblast proliferation and better osteoblast differentiation on L-PTFE, long pulse, high frequency O_2 PIII is determined to be superior to the other 2 treatments.

5. Conclusion

It is demonstrated in this study that long pulse, high frequency O_2 PIII, short pulse, low frequency O_2 PIII, and O_2 plasma immersion treatments all produce a oxygen-containing, roughened and hydrophobic surface on PTFE. The sample surface after long pulse, high frequency O_2 PIII is even superhydrophobic due to the formation of less oxygen groups and rougher surface morphology. Compared to the untreated PTFE, osteoblast adhesion and proliferation are much better on the modified samples. However, only long pulse, high frequency O_2 PIII up-regulate the OCN expression of the seeded osteoblasts among the 3 treatments. Consequently, this novel O_2 PIII treatment is better than conventional O_2 PIII and O_2 plasma immersion in the development of PTFE for bone or cartilage replacements.

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Appendix

Figures with essential color discrimination. Figs. 1 and 2 of this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.09.066.

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