Rat calvaria osteoblast behavior and antibacterial properties of O_2 and N_2 plasma-implanted biodegradable poly(butylene succinate)

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ABSTRACT

Poly(butylene succinate), a novel biodegradable aliphatic polyester with excellent processability and mechanical properties, was modified by O_2 or N_2 plasma immersion ion implantation (PIII). X-ray photoelectron spectroscopy and contact angle measurements were carried out to reveal the surface characteristics of the treated and control specimens. The in vitro effects of the materials on seeded osteoblasts were detected by cell viability assay, alkaline phosphatase activity test, and real-time polymerase chain reaction analysis. Plate counting was performed to investigate the antibacterial properties. Our results show that both PIII treatments significantly improve the hydrophilicity of PBSu, and C=O and nitrogen groups (C=NH and C—NH_2) can be detected on the PBSu after O_2 and N_2 PIII, respectively. The modified samples exhibit similar compatibility to osteoblasts, which is better than that of the control, but O_2 PIII and N_2 PIII produce different effects according to the osteogenic gene expressions of seeded osteoblasts on the materials. Moreover, the N_2 plasma-modified PBSu exhibits anti-infection effects against *Staphylococcus aureus* and *Escherichia coli* but no such effects can be achieved after O_2 PIII.

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1. Introduction

Tissue engineering is usually considered when the damaged tissues of patients are too severe to be regenerated by the routine repair mechanism of the body. Among the various types of materials used in tissue engineering, biodegradable polymers are superior to others for their easy shaping and avoidance of chronic problems associated with the permanent presence of biostable implants [1–5]. A variety of synthetic biodegradable polymers has been investigated for bone tissue engineering. A suitable biodegradable material for bone regeneration should have high compressive strength and allow cell attachment, proliferation, extracellular matrix (ECM) deposition, ultimately supporting in vivo bone regeneration with a suitable degradation rate. In particular, poly(butylene succinate) (PBSu), a biodegradable aliphatic polyester (the chemical structure of which is shown in Fig. 1), is a promising material for bone and cartilage repair. It provides good processability, supe-

rior mechanical properties, harmless degradation products (CO_2 and H_2O), and an adjustable degradation rate [6–9]—hence fulfilling the requirements demanded by bone tissue engineering. It was found in our previous studies that osteoblasts can grow on PBSu [10]. However, similar to most other biopolymers, PBSu is prone to bacterial infection and insufficient osteocompatibility after implantation into the body. Hence, in order for the materials to be used as a bone substitute, both the anti-infection activity and the biocompatibility of PBSu must be improved.

Surface modification is a desirable approach to develop materials while retaining the favorable bulk properties [11,12]. Among the various possible surface treatments, plasma immersion ion implantation (PIII) excels due to its simple operation and its non-light-of-sight characteristics, and hence shows much promise for biomedical implants with complex geometric shapes [13–16]. The technique is quite versatile in that different chemical groups can be introduced onto the specimens by performing PIII using different gases. In the work reported here, PBSu is modified by O_2 or N_2 PIII, and the resultant changes in the surface chemistry and hydrophilicity are investigated. The effects of the treatments on the bacteria and on osteoblast behavior are also studied and discussed.

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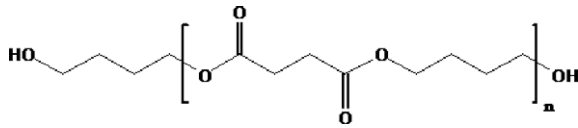E-mail address: paul.chu@cityu.edu.hk (P.K. Chu).

Fig. 1. Chemical structure of biodegradable poly(butylene succinate).

2. Materials and methods

2.1. Sample preparation and modification

The PBSu samples were acquired from HKH National Engineering Research Center of Plastics Co., Ltd., and purified twice by alternately dissolving and precipitating with dichloromethane and ethanol. The precipitated PBSu powders were molded to flake shapes by Plastics Injection Molding and cut to uniform dimensions of 2 cm × 2 cm × 0.1 cm for antibacterial assay and 1 cm × 1 cm × 0.1 cm for cell culture. The samples were laid on stainless-steel substrates with a diameter of 10 cm and inserted into the plasma immersion ion implanter. Various gas plasma treatments were performed under optimal instrument conditions based on our trial and previous experiments [10,16]: bias voltage = −8 kV, voltage pulse width = 20 μs, pulsing frequency = 30 Hz, gas flow = 20 sccm, radiofrequency power = 1000 W, and treatment time = 90 min.

2.2. Surface characterization

To determine the surface hydrophilicity of the modified substrates, static contact angles using distilled water or glycerin as the medium were performed by the sessile drop method on a Ramé-Hart (USA) instrument at ambient humidity and temperature. The drop size was 10 μl. Each data point represents the average and standard deviation of five measurements conducted on different parts of each specimen for better statistics. Statistical analyses were performed by Student's *t*-test.

To investigate the surface chemical composition of the modified samples, X-ray photoelectron spectroscopy (XPS) was conducted on a Physical Electronics PHI 5802 equipped with a monochromatic Al Kα source. The step size and the constant pass energy of XPS was 0.1 and 11.75 eV, respectively. All the data were collected at a take-off angle of 45° and curve fitting was performed using the Scienta ESCA300 data system software [17].

2.3. Cell culture

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

2.4. Cell viability

A cell count kit-8 (CCK-8 Beyotime, China) was employed in this experiment to quantitatively evaluate the cell viability. After culturing for 6 h, 3, 6 and 12 days, the osteoblast-seeded samples were rinsed twice with sterile PBS and transferred to fresh 24 well tissue culture plates. The samples were subsequently incubated with 0.7 ml culture medium containing 10% CCK-8 for 4 h. The solution of 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 for statistical accountability. Statistical analyses were performed by one-way ANOVA analysis.

2.5. Alkaline phosphatase (ALP) activity

The ALP activity of the osteoblasts seeded on various substrates was measured spectrophotometrically after incubation for 3, 6 and 12 days. The cell-seeded substrates were rinsed three times with PBS, followed by homogenization in an alkaline lysis buffer. After centrifugation, the resulting cell homogenate was incubated with *p*-nitrophenyl phosphate (Sigma) at 37 °C for 30 min. NaOH was added to stop the reaction and the amount of *p*-nitrophenol produced was quantified by absorbance measurements at 405 nm. The control (unseeded) discs were treated similarly and assayed as blank controls, which were then subtracted from the corresponding samples. The ALP activity was normalized to the total protein content at the end of the experiments. All experiments were performed in quadruplicate.

2.6. Quantitative real-time polymerase chain reaction

To investigate the influence of various modified substrates on the gene expression of seeded osteoblasts, the osteogenic-associated genes were analyzed using real-time polymerase chain reaction (PCR). The total RNA from osteoblasts grown on various substrates for 3, 6 and 12 days was isolated using a TRIZOL reagent (Invitrogen). By adding chloroform to the mixture, shaking and centrifugating, the RNA was separated into an aqueous phase, which was subsequently recovered and precipitated by 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 concentration determination, the extracted RNA was reverse transcribed by using PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa). Real-time PCR (Bio-Rad iQ5 real-time PCR detection system) was performed by the following method: 40 cycles of PCR (95 °C for 10 s, and 60 °C for 20 s) after initial an denaturation step of 30 s at 95 °C using a mixture of iQ5 SYBR Green I supermix and each of the forward and reverse primers and cDNA templates. The markers for osteoblast differentiation—osteocalcin (OCN), alkaline phosphatase (ALP), osteopontin (OPN) and osteonectin (ON)—were evaluated. 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 for each sample calculated as the average of three replicate measurements. The relative expression levels for each gene of interest were normalized to that of GAPDH.

Table 1
Sequences of the primers.

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

2.7. Antibacterial assays

The antibacterial properties of the modified samples were determined by the method of plate-counting [18,19] utilizing *Staphylococcus aureus* ATCC6538 (Gram-positive) and *Escherichia coli* (Gram-negative) as the test bacteria. All the samples were first sterilized with 75% ethanol overnight and air dried. A 0.1 ml solution of bacteria ($2–5 \times 10^5$ CFU ml^{-1}) was separately added onto the surface and then the surface was covered by a polyethylene film (1.6×1.6 cm). The bacteria on modified and control PBSu were incubated in a humidified incubator at 37 °C for 24 h. Afterwards, they were thoroughly washed with the sterile PBS solution that contained 0.1% Tween 80 with a pH of 7.2–7.4. For active bacteria counting, PBS eluents with various volumes (1, 0.1 or 0.01 ml) were added onto the different dishes containing liquid agar and incubated under similar conditions for 24 h. The subsequent macroscopic bacterial colonies were counted and the antibacterial effect was quantitatively determined using the following relationship:

$$R\ (\%) = ((B - C)/B) \times 100,$$

where R is the antibacterial effect (%), B is the mean number of bacteria on the control samples (CFU sample^{-1}), and C is the mean number of bacteria on the modified samples (CFU sample^{-1}).

3. Results

3.1. Characterization of O_2 and N_2 PIII PBSu

The measured contact angles using water and glycerin as the media are summarized as a histogram in Fig. 2. It is clear that both PIII processes can significantly improve the surface hydrophilicity of PBSu, but no significant statistical difference is found between the O_2 PIII and N_2 PIII groups. XPS was employed to determine their surface chemical states. Table 2 shows the quantitative surface atomic composition and indicates that N_2 PIII can introduce large nitrogen contents into the substrates; the increase in the total oxygen contents after O_2 PIII PBSu is significant as well. The high-resolution C1s, O1s and N1s spectra are fitted and presented in Fig. 3. It can be observed that some new nitrogen groups (C=NH and C—NH$_2$) are formed on substrates after N_2 PIII. The increased C_3 and O_1 components in the O_2 PIII samples demonstrate that C=O is the dominant functional group after O_2 PIII.

Table 2
Elemental ratios of O/C and N/C in the samples.

Elemental percentage [%]	O/C	N/C
Control	57.1	1.3
O_2 PIII	88.2	0.8
N_2 PIII	62.9	33.9

3.2. Adhesion and proliferation of osteoblasts

The time-dependent cell viability data of rat osteoblasts cultured on the various substrates are displayed as a histogram in Fig. 4. It is evident that both PIII treatments can improve attachment of osteoblasts and their subsequent growth on PBSu. In particular, during the initial 3 days of incubation, the absorbance values corresponding to the cell numbers on O_2 PIII are slightly higher than those on the N_2 PIII sample. The absorbance values of the treated samples are both remarkably higher than those of the control. When the incubation time is 6 days, the absorbance values of these two types of treated substrates are almost the same and much higher than those on untreated PBSu. When the incubation time is extended to 12 days, the absorbance values of the treated PBSu samples are about the same and still slightly higher than those determined from the control, but the differences among them are not significant.

3.3. ALP activity of osteoblasts

In order to investigate the differentiation ability of osteoblasts cultured on the various samples, the ALP activity of those cells was examined. The quantitative data in Fig. 5 illustrate that the ALP activity of cells cultured on all the substrates increases gradually with time during the assay period. After 6 days of incubation, the ALP activity of the osteoblasts on the O_2 PIII PBSu is much higher than that on the other two substrates. When the incubation time is extended to 12 days, the ALP activities on both the O_2 PIII PBSu and on N_2 PIII PBSu are much higher than that on the control. The ALP activity detected on the O_2 PIII PBSu is then a little higher than that on the N_2 PIII PBSu.

3.4. Real-time PCR quantification of gene expression

Variations of the mRNA expressions as a function of the culture duration and types of substrates are quantified using real-time PCR. The results are compared and illustrated as histograms in Fig. 6. The osteoblasts cultured on the O_2 and N_2 PIII PBSu show different gene expressions compared to those found on untreated ones. After 3 days of culture, both O_2 and N_2 PIII can induce up-regulation of ALP, and O_2 PIII can even promote ON expression. At the same time, no significant difference is found on OPN and no OCN expression is detected. After 6 days of incubation, the ON, ALP expressions on O_2 PIII PBSu and the OPN, ON expressions on N_2 PIII PBSu are dramatically higher than those on the control. Meanwhile, both treatments suppress the OCN expressions, and the down-regulation of OCN on O_2 PIII PBSu is more significant than that on the N_2 PIII samples. Interestingly, after culturing for 6 more days, only the ALP is still the highest on the O_2 PIII substrate, whereas the OCN and OPN expressions on them are both lower than those on the control, and the ON expression on the O_2 PIII PBSu is equal to that on the untreated one. At the same time point, the OPN and ON expressions on the N_2 PIII samples are remarkably higher than those on the control, and there is no significant difference in the OCN and ALP expressions between the N_2 PIII and control PBSu.
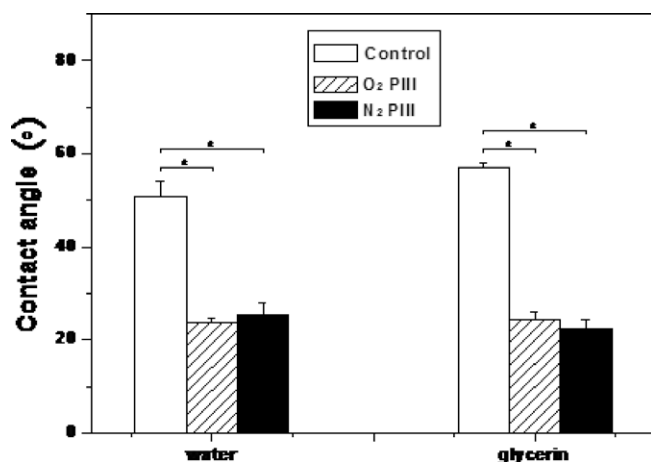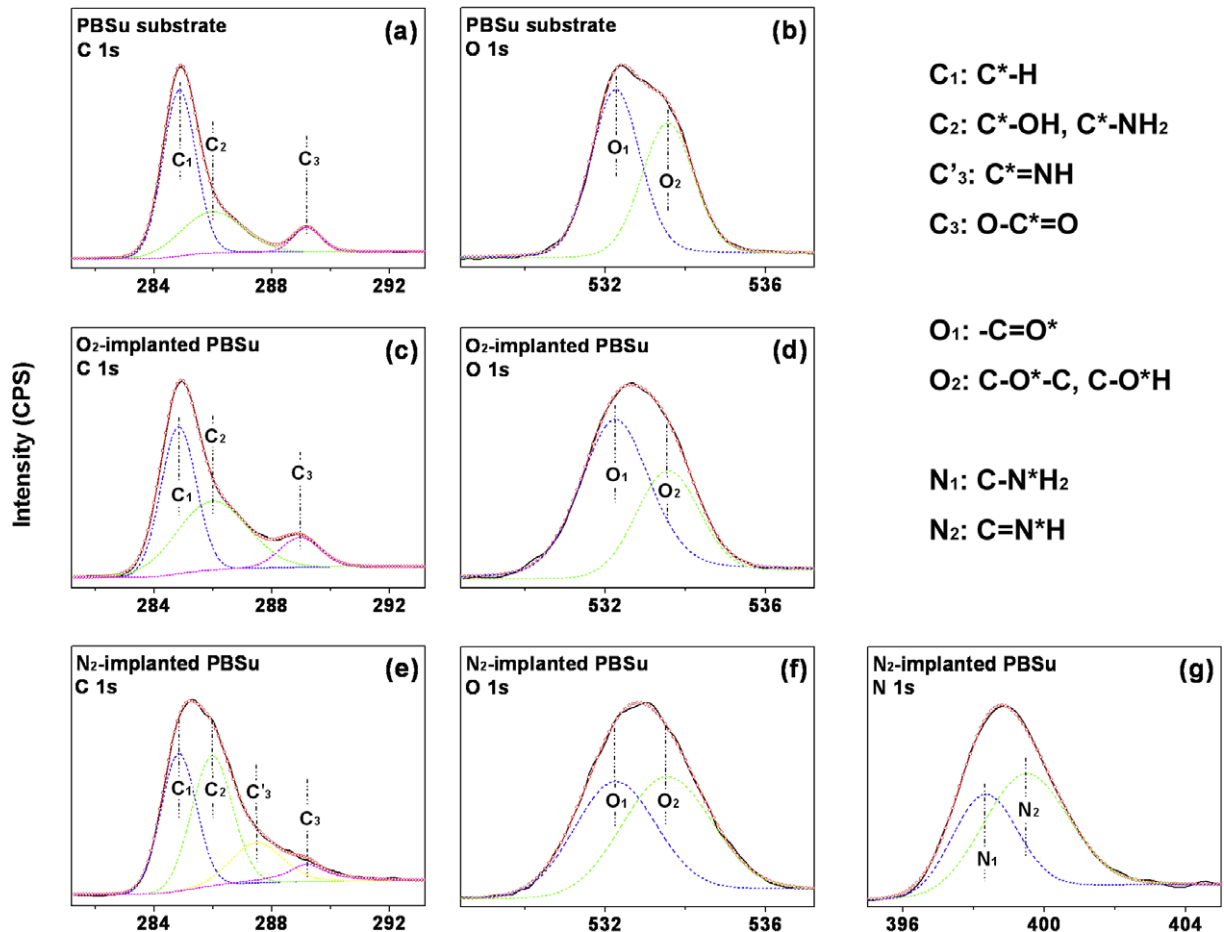

Fig. 2. Water and glycerin contact angles measured on the control PBSu, O_2 PIII PBSu and N_2 PIII PBSu. *Statistically significant at $P < 0.05$.

C_1: C*-H

C_2: C*-OH, C*-NH_2

C'_3: C*=NH

C_3: O-C*=O

O_1: -C=O*

O_2: C-O*-C, C-O*H

N_1: C-N*H_2

N_2: C=N*H

Fig. 3. XPS spectra acquired from the surface of control PBSu ((a) C1s and (b) O1s), O_2 PIII PBSu ((c) C1s and (d) O1s), and N_2 PIII PBSu ((e) C1s, (f) O1s and (g) N1s). The N1s regions of the control PBSu and O_2 PIII PBSu are not shown here as the N signals are very weak.
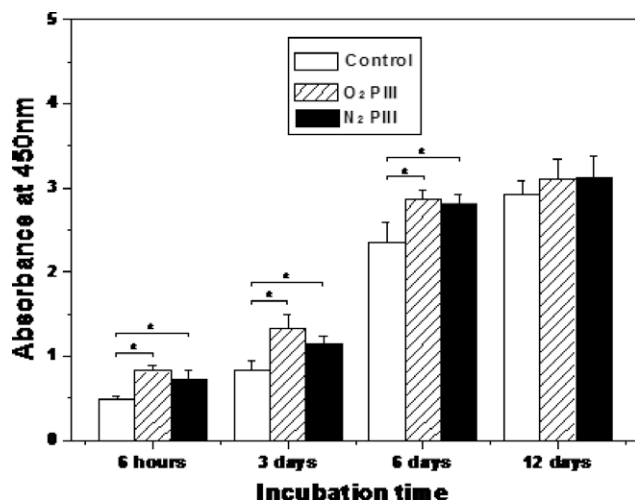

Fig. 4. The cell viability assay of rat calvaria osteoblasts cultured on the control PBSu, O_2 PIII PBSu and N_2 PIII PBSu for 6 h, 3, 6 and 12 days. The absorbance of the diluted cell counting kit solution has been deducted from each data point. *Statistically significant at $P < 0.05$.
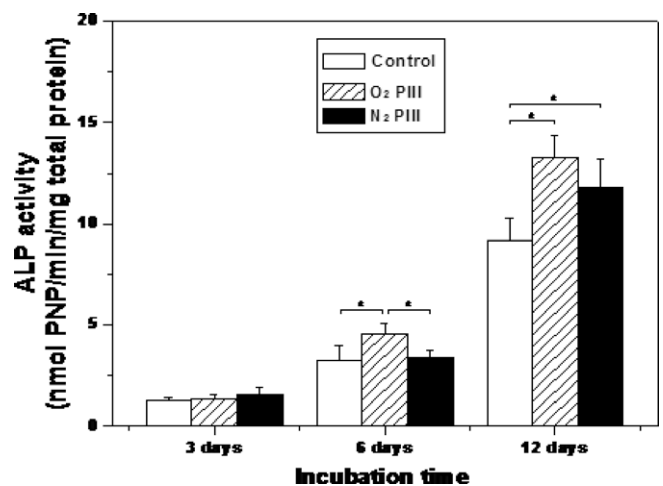

Fig. 5. ALP activity assay of rat calvaria osteoblasts cultured on the control PBSu, O_2 PIII PBSu and N_2 PIII PBSu for 3, 6 and 12 days. *Statistically significant at $P < 0.05$.

3.5. Antibacterial properties

To evaluate the antibacterial properties of PBSu after O_2 and N_2 PIII, the plate counting method is used to determine the active bacteria amounts after culturing on the various substrates for 24 h and the data are presented in Fig. 7. The amounts of active bacteria on the N_2 PIII PBSu are much less than those on the control, but there is no statistical difference between the O_2 PIII PBSu and control. The calculated antibacterial effects of N_2 PIII PBSu against *S. aureus* and *E. coli* are 91.41% and 90.34%, respectively.
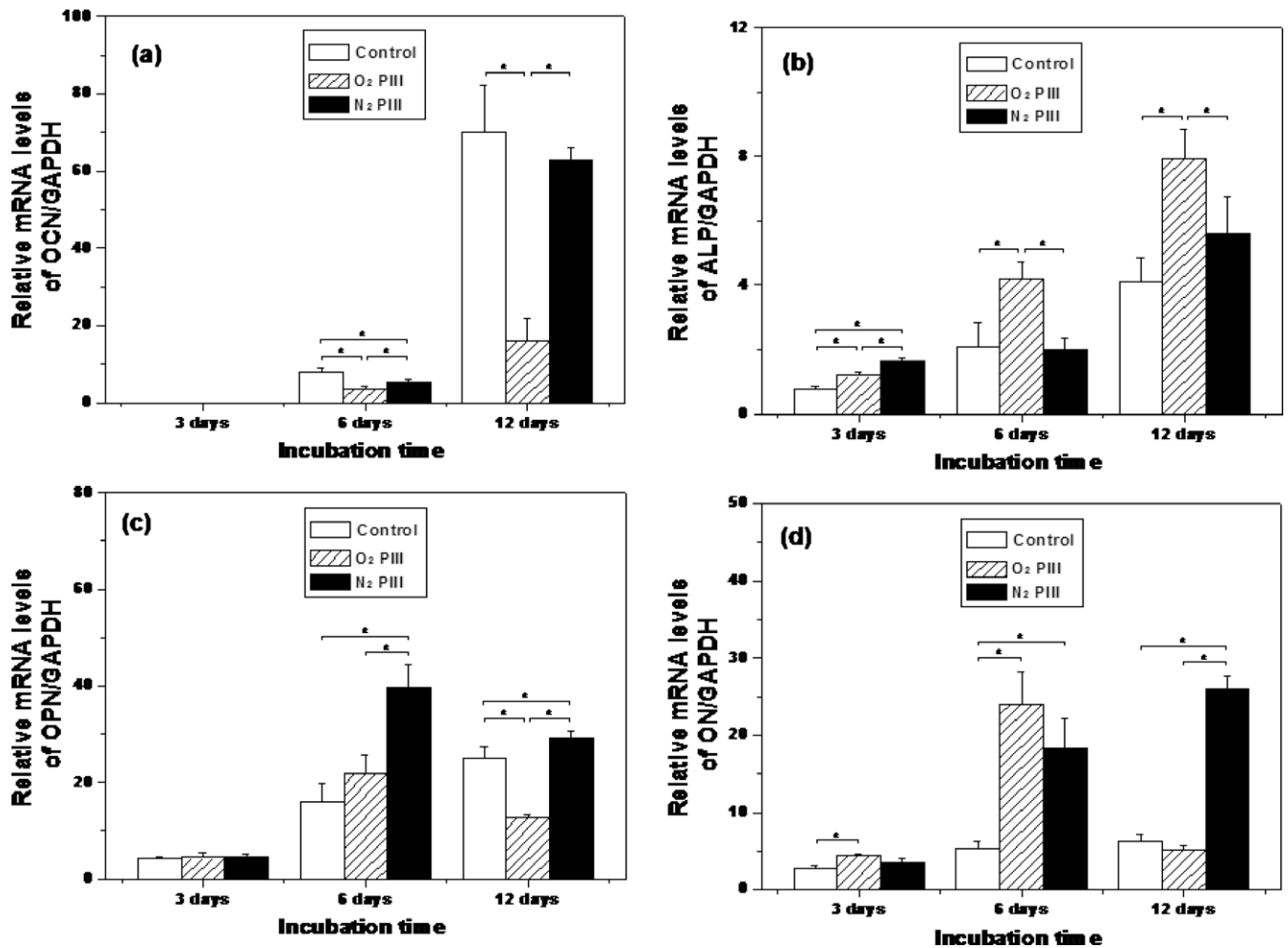
Fig. 6. Real-time PCR detection of osteogenic differentiation-related gene expressions of rat calvaria osteoblasts cultured on the control PBSu, O_2 PIII PBSu and N_2 PIII PBSu for 3, 6 and 12 days: (a) OCN, (b) ALP, (c) OPN and (d) ON. *Statistically significant at $P < 0.05$.
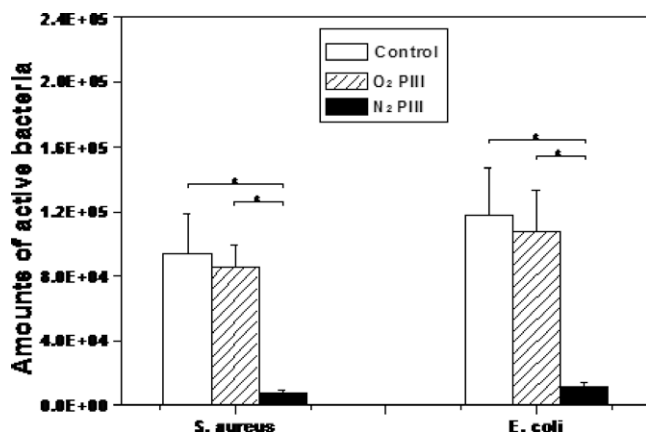

Fig. 7. Amounts of active *S. aureus* and *E. coli* cultured on the control PBSu, O_2 PIII PBSu and N_2 PIII PBSu for 1 day. *Statistically significant at $P < 0.05$.

4. Discussion

Owing to the difficulty in obtaining human primary osteoblasts and abnormal phenotypes of some osteoblast or osteoblast-like cell lines, rat calvaria osteoblasts are chosen in our experiments to evaluate osteoblast response to the materials. The cell viability, alkaline phosphatase activity and gene expressions are determined and analyzed in terms of the surface chemistry and hydrophilicity of the specimens. The antibacterial properties are also evaluated and discussed in the context of surface characteristics.

The cell adhesion and spreading not only indicate the cell–material interaction, but also influence the subsequent cell performance of the implants. Following initial adhesion of osteoblasts on the materials, a sequential process comprising proliferation, ECM deposition, differentiation and maturation takes place [20]. Thus, the attachment and subsequent growth of osteoblasts on the various specimens are first evaluated. The cell viability measurements reveal that both O_2 and N_2 PIII can promote osteoblast attachment and growth on the PBSu substrates, but no significant difference can be observed from these two treatment protocols. In addition, the osteoblast growth is fast during the initial 6 days of incubation but slows down considerably thereafter. This observation is consistent with that by Stein and Lian [21] who indicated that the proliferation genes were down-regulated by the functionally coupled relationship between osteoblast proliferation and differentiation at the initiation of differentiation. On account of the similar hydrophilicities and different surface chemical states of PBSu after these two kinds of treatments, it can be inferred that the induced nitrogen groups (C=NH and C—NH$_2$) impose similar positive effects on osteoblast attachment and growth compared to the C=O groups.

Osteoblast gene expression is also one of the key issues determining the success of bone regeneration in a tissue engineering approach. Among the non-collagen genes that are active in the osteoblast differentiation process, OCN is the most specific protein in osteoblast dif-

ferentiation and mineralization [22]. It regulates bone growth [23] and is only expressed by mature osteoblasts during the initial stages of mineralization [24,25]. ALP is a marker indicating early differentiation of osteoblasts. It is shown to regulate organic of inorganic phosphate metabolism via hydrolyzation of phosphate esters [26,27] and function as a plasma membrane transporter for inorganic phosphates [28]. OPN is a phosphoprotein of osteoblasts which serves as a bridge between the cells and hydroxyapatite. It is expressed during the active proliferation period of osteoblasts [29,30]. Although ON is not an osteoblast-specific protein, it is actively involved in bone remodeling and ECM organization [31,32], and it is expressed in a very early stage of osteoblast differentiation.

Fig. 6 shows that the highest ALP is detected on the O_2 PIII PBSu from 6 to 12 days post-seeding. The gene expression of ALP is observed to follow a trend similar to the ALP activity detected. However, when the incubation time is 12 days, opposite effects on the OCN, OPN and ON gene expressions are observed from the N_2 PIII and O_2 PIII samples. In comparison with the control, considerable OCN levels and much higher expressions of OPN and ON are detected on the N_2 PIII substrates. The OCN and OPN are significantly down-regulated after O_2 PIII, and the ON expression is maintained at the same level. Only the ALP expression on the O_2 PIII PBSu is higher than that on the N_2 PIII PBSu, whereas the OCN, OPN and ON expressions on the former sample are all evidently lower than those on the latter sample. It can be inferred that the surface C=NH and C—NH_2 groups after N_2 PIII and the C=O groups induced by O_2 PIII have different effects on the gene expressions of the seeded osteoblasts. The N_2 PIII substrates are superior to the O_2 PIII PBSu ones according to differentiation of the seeded osteoblasts.

The antibacterial assay data reveal that N_2 PIII can improve the antibacterial property of PBSu surface when O_2 PIII does not render this effect. It means that in addition to promoting osteoblast proliferation, differentiation and mineralization, the surface C=NH and C—NH_2 groups after N_2 PIII can protect the PBSu substrates from bacterial infection while the C=O formed is adiaphorous.

5. Conclusion

Rat calvaria osteoblasts attach and proliferate better on both the O_2 and N_2 PIII modified substrates. N_2 PIII and O_2 PIII produce different effects according to differentiation of the seeded osteoblasts, and in this respect, N_2 PIII is better than O_2 PIII. N_2 PIII can even protect the PBSu samples from bacterial infection when O_2 PIII appears to be ineffective. The observed differences are believed to arise from the different surface chemical states on these two types of modified surfaces. Our results reveal that N_2 PIII is better than O_2 PIII from the perspective of osteoblast culture and anti-infection properties. The N_2 PIII PBSu samples are potential implant materials for bone regeneration in tissue engineering applications.

Acknowledgments

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figure 3 is difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2009.07.026.

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