

Biodegradable Poly(Butylene Succinate) Modified by Gas Plasmas and Their In vitro Functions as Bone Implants

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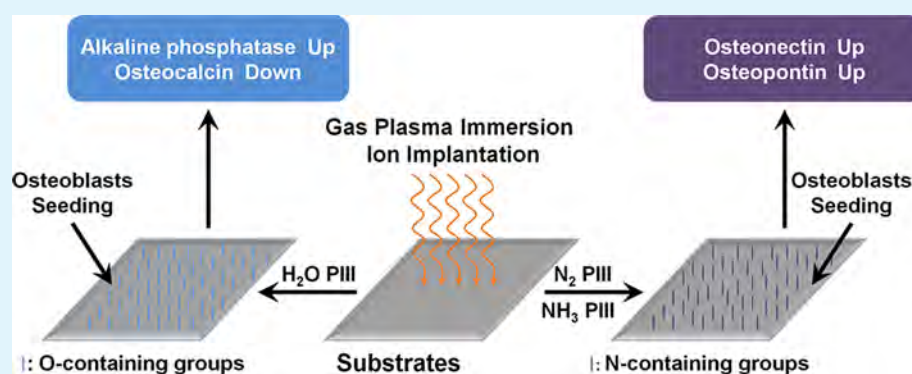
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S Supporting Information



ABSTRACT: Artificial implants are alternatives to autologous grafts in repairing severe bone damage and in many clinical applications, the artificial implant materials should be biodegradable in order to avoid chronic problems associated with biostable implants. In this study, a biodegradable biopolymer, poly(butylene succinate) (PBSu), is treated by N₂, NH₃ and H₂O plasmas and investigated as bone replacement materials in vitro to obtain a better understanding of the behavior of osteoblasts on the different plasma-treated materials. N₂, NH₃, and H₂O plasma immersion ion implantation (PIII) produces dominant C–N, C=N, and C–O surface functional groups, respectively rendering the materials with hydrophilic characteristics which favor osteoblast adhesion and early proliferation. In particular, N-containing groups, especially C=N, are more positive to osteogenic differentiation of the seeded osteoblasts than C–O. Among the 3 plasma treatments, NH₃ PIII is the most effective, yielding surface properties that are suitable for artificial bone implants.

KEYWORDS: poly(butylene succinate), plasma immersion ion implantation, surface chemistry, primary osteoblasts, cell adhesion and proliferation, osteogenic differentiation

1. INTRODUCTION

Bone damage is a serious medical problem worldwide as more than 2 million related therapies are carried out annually and the associated health cost is tens of billions of dollars.^{1,2} When bone loss is too severe to be regenerated by the routine repair mechanism in the human body, treatment with autologous grafts or artificial implants are considered. Compared to autologous grafts, artificial implants have advantages such as easy shaping, limitless supply, avoidance of donor-site morbidity, and so on.³ If the artificial implants are biodegradable, they even allow new tissues to take over the biological functions and potential chronic problems associated with biostable implants can be avoided.^{4–7} Poly(butylene succinate) (PBSu) is one of the biodegradable biopolymers (chemical structure shown in Figure S1 in the Supporting Information) with good processability, excellent mechanical properties, and harmless

degradation products (CO₂ and H₂O).^{8–10} Although it is promising as bone substitutes, its osteocompatibility is still insufficient. Hence, PBSu with better surface osteocompatibility is crucial to wider clinical acceptance.

Many techniques can be used to modify the surface of polymeric biomaterials, for instance, surface polymerization,¹¹ surface immobilization of biocompatible molecules,¹² and plasma-based methods. Among them, plasma immersion ion implantation (PIII) excels because of its simple operation, long-term effects, and non-line-of-sight characteristics, which bode well for the samples with a complex shape.^{13,14} By using different plasma sources, different chemical groups can be

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introduced into the specimen surface while the favorable bulk properties of the materials can be preserved. We have demonstrated that gas PIII is an effective method to achieve good biocompatibility and favorable osteogenic behavior on PBSu.¹⁵ In the work reported in this paper, PIII with three different plasma gases, N₂, NH₃, and H₂O, is conducted and the surface chemistry and effects on osteoblast functions are determined and discussed.

2. MATERIALS AND METHODS

2.1. Sample Preparation and Modification. The PBSu particles were purified twice by alternately dissolving and precipitating with dichloromethane and ethanol. The precipitated PBSu powders were molded to flakes (thickness of about 0.1 cm) by plastic injection molding and cut into uniform dimensions of 1 cm x 1 cm for subsequent experiments. The samples were put on a stainless-steel platen with a diameter of 10 cm and inserted into the plasma immersion ion implanter. N₂, NH₃, or H₂O was introduced into the chamber and PIII was conducted using conditions optimized in our previous study:¹⁵ bias voltage = -8 kV, voltage pulse width = 20 μ s, pulsing frequency = 30 Hz, gas flow = 20 sccm, radiofrequency power = 1000 W, and treatment time = 90 min. The PBSu samples after undergoing N₂ (nitrogen), NH₃ (ammonia), and H₂O (water) PIII are designated as N-PBSu, A-PBSu, and W-PBSu, respectively. The untreated PBSu termed U-PBSu is the control.

2.2. Surface Characterizations. The static contact angles were determined by the sessile drop method on a Rame'-'Hart instrument (USA) at ambient humidity and temperature to evaluate the surface hydrophilicity. Distilled water and glycerin were used as the media and the drop size was 10 μ L. Each data point represents the average and standard deviation of 10 measurements conducted on each specimen for statistical accountability and the statistical analysis was by the pair-sample t-test.

X-ray photoelectron spectroscopy (XPS) was conducted on the Physical Electronics PHI 5802 equipped with a monochromatic Al K α source to determine the surface chemical states of the 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.

The colorimetric method was employed to quantitatively determine the C=NH/C-NH₂ groups on the samples by using the Orange II dye (chemical structure shown in Figure S1 in the Supporting Information).^{16,17} The samples were initially immersed in the dye solution (15 mg mL⁻¹, adjusted to pH = 3 with 1 mol L⁻¹ HCl) at 37 °C for about 40 min and then rinsed thrice with an acidic solution (pH 3) to remove the unbound dye. After air drying, the stained samples were immersed in 1 mL of an alkaline solution (adjusted to pH = 12 with 1 mol L⁻¹ NaOH) separately and agitated gently to desorb the bound dye. Afterwards, the solution was adjusted to a pH of 3 and a final volume of 1.5 mL. The absorbance was measured spectrophotometrically at 484 nm. The amount of the surface C=NH/C-NH₂ groups is 1:1 proportional to dye adsorption and expressed in pmol mm⁻².

2.3. Cell Culture. Rat calvaria osteoblasts were acquired by sequential trypsin-collagenase digestion on calvaria of neonatal (<1 day old) Sprague-Dawley rats and then cultured in the Dulbecco's modified Eagle's medium (D-MEM, Invitrogen) supplemented with 10% newborn bovine serum (Hyclone). After an additional passage of the primary osteoblasts, they were seeded onto the samples at a density of 1.5 x 10⁴ cells per sample by using 24 well tissue culture plates as the holders. Before cell seeding, all the specimens were sterilized with 75% ethanol overnight and rinsed thrice with a sterile phosphate-buffered saline (PBS) solution. The cultures were incubated in a humidified atmosphere of 5% CO₂ at 37 °C and the medium was refreshed every 3 days.

2.3.1. Cell Viability Assay. A cell count kit-8 (CCK-8) was employed to quantitatively determine the viable osteoblasts on the samples after different culturing periods (6 h, 3 days, 6 days, and 12 days). At each time point, the samples with seeded cells were rinsed twice with sterile PBS solution and transferred to fresh 24-well tissue

culture plates. Subsequently, CCK-8 reagent was added to the wells with the samples and the performance was assessed on the basis of the manufacturer's instructions. For statistical accountability, four replicates of each sample at each time point were prepared and analyzed, and the statistical analysis was performed by one-way ANOVA.

2.3.2. Alkaline Phosphatase Activity Assay. After culturing for 3, 6, and 12 days, the alkaline phosphatase (ALP) activity of the seeded osteoblasts was evaluated using the ALP reagent. Succinctly speaking, the specimens were rinsed thrice with the PBS solution, followed by homogenization in an alkaline lysis buffer. After centrifugation, the homogenate was incubated with p-nitrophenyl phosphate (Sigma) at 37 °C to produce p-nitrophenol, and the reaction was terminated by adding NaOH solution 30 minutes later. The amount of p-nitrophenol produced was spectrophotometrically quantified by absorbance measurements at 405 nm and the ALP activity was expressed as the p-nitrophenol amount normalized to the total protein content. All the samples were prepared in quadruplicate for statistical accountability (performed by one-way ANOVA) and the unseeded disks with the same performance served as the blank control (the absorbance of blank control was subtracted from that of the samples).

2.3.3. Quantitative Real-Time Polymerase Chain Reaction (qPCR). The viable osteoblasts on the samples were determined for gene expressions after incubating for 3, 6, and 12 days. At each time point, the total RNA of the osteoblasts was isolated by using a TRIZOL reagent (Invitrogen). After a series of processes including addition of chloroform, shaking, and centrifuging, the RNA dispersed in the aqueous phase was recovered and precipitated by adding equal isopropanol. After washing with 75% ethanol treated with the RNase inhibitor diethyl pyrocarbonate (DEPC), the acquired RNA pellet was solubilized in sterile DEPC water for concentration determination and subsequent reverse transcription to cDNA by using a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa). Finally, qPCR was performed on the Bio-Rad iQ5 Real Time PCR detection system as follows: 40 cycles of PCR (95 °C for 10 s, and 60 °C for 20 s) following an 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. ALP, osteonectin (ON), osteopontin (OPN), and osteocalcin (OCN) were analyzed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the endogenous housekeeping gene for normalization. The primer sequences of the genes are shown in Table S1 in the Supporting Information and quantification of the gene expression was based on the comparative cycle-threshold (C_T) method (three replicates).

3. RESULTS AND DISCUSSION

Static contact angle measurement is a common used method to characterize biomaterials surfaces and provides information such as surface hydrophilicity which has a profound effect on cell adhesion and spreading. In this study, the water and glycerin static contact angles are measured and as shown in Figure 1, both sets of contact angles measured on the modified samples are much smaller than those on U-PBSu, implying much better hydrophilicity after the plasma treatment. The hydrophilicity of W-PBSu is a little better than that of N-PBSu and A-PBSu, but the difference among the three plasma-modified samples is negligible compared to that between U-PBSu and the treated samples. Hence, N₂, NH₃, and H₂O PIII can all endow PBSu with much better surface hydrophilicity.

PIII can change the surface chemistry of biopolymers and X-ray photoelectron spectroscopy (XPS) is conducted to characterize the surface chemical states before and after PIII. As shown in Figure 2 and Table 1, the C1s region of U-PBSu can be deconvoluted into 3 peaks corresponding to C*-H/C*-C (C₁ peak), C*-O (C₂ peak), and O=C*-O (C₃ peak). The peak areas are 67.3, 24.9, and 7.8%, respectively. After PIII, the surface C*-H/C*-C concentration decreases

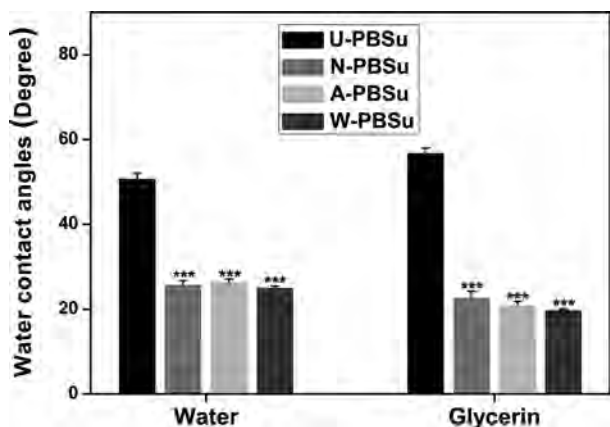


Figure 1. Water and glycerin contact angles measured on U-PBSu and the three sets of plasma-modified samples with the statistical significance indicated by ***, and *** referred to $p < 0.001$.

to a different extent whereas there are more C^*-O/C^*-N bonds (C^*-O and C^*-N are considered together in C_2 peak

Table 1. Percentages of Fitted Peaks Derived from the XPS Results Shown in Figure 2

	U-PBSu	N-PBSu	A-PBSu	W-PBSu
C1s peak proportions (%)				
C_1 peak	67.32	42.92	52.06	53.48
C_2 peak	24.86	34.81	34.82	29.07
C'_3 peak		13.92	7.57	8.23
C_3 peak	7.82	8.35	5.55	9.22
O1s peak proportions (%)				
O_1 peak	49.72	44.81	46.99	32.90
O_2 peak	50.28	55.19	53.01	67.10
N1s peak proportions (%)				
N_1 peak		42.10	61.93	
N_2 peak		57.90	38.07	

due to the binding energy overlap) and a new C'_3 peak (corresponding to $C^*=O$, $C^*=N$, $O-C^*-O$, and $N-C^*-O$) emerges. The area of the original C_3 peak (corresponding to $O=C^*-O$ and $N=C^*-O$) changes slightly after PIII. In the O1s spectra, the area percentages of O_1 peak ($C=O^*$) and O_2

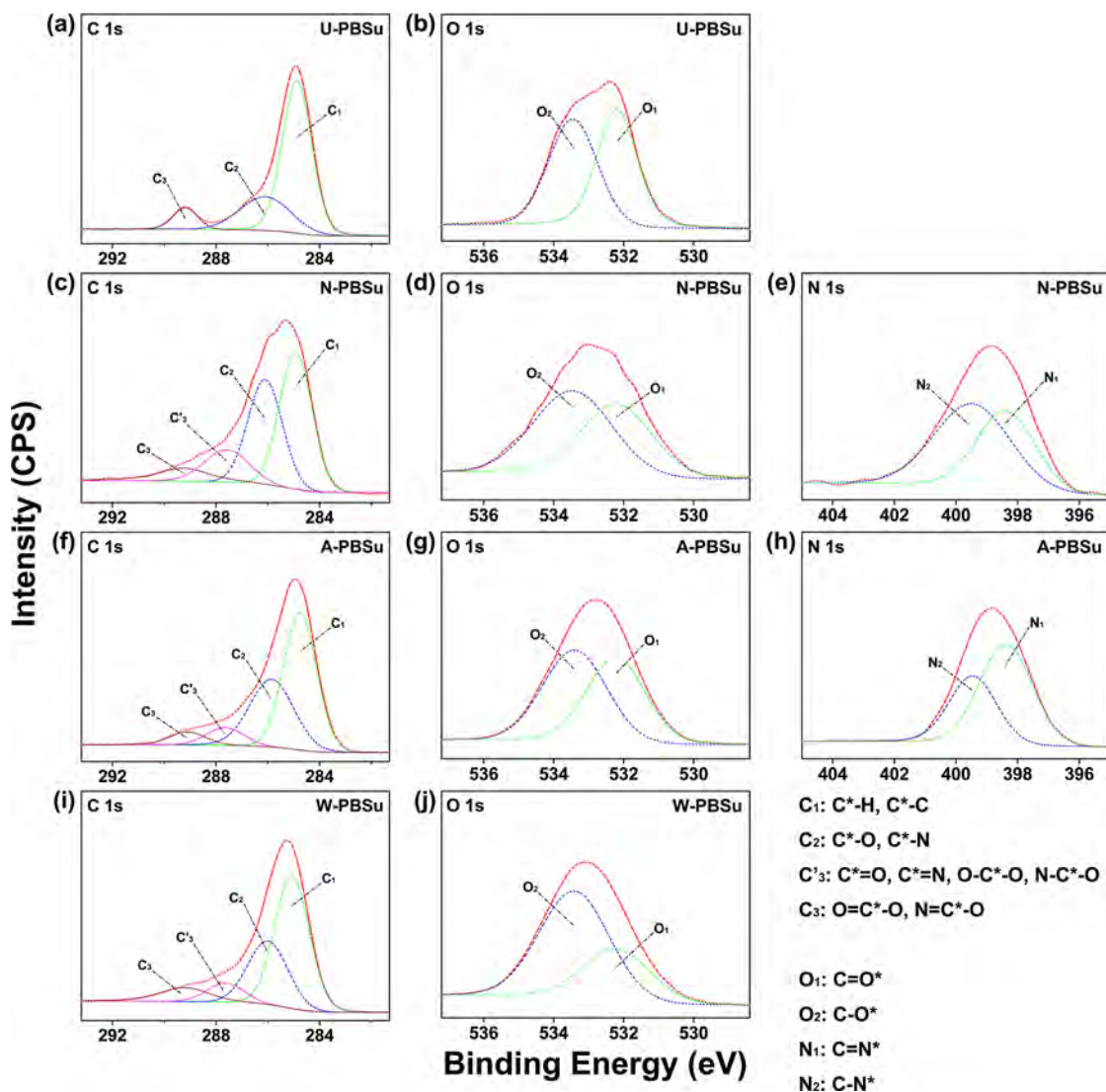


Figure 2. High-resolution XPS spectra acquired from the surface of (a, b) U-PBSu, (c-e) N-PBSu, (f-h) A-PBSu, and (i, j) W-PBSu. The N 1s regions of U-PBSu and W-PBSu are not shown because of low signals. Bottom right descriptions are referred to the attributions of fitted peaks.

peak (C–O*) on U-PBSu are 49.7% and 50.3%, respectively. After N₂ and NH₃ PIII, the peak area ratio of C–O* to C=O* is increased slightly, whereas the amount of surface C–O* bonds increases significantly after H₂O PIII. The N1s signal can be detected from N-PBSu and A-PBSu but not U-PBSu and W-PBSu. In particular, the N₁ peak (corresponding to C=N*) is preponderant on A-PBSu and N₂ peak (corresponding to C–N*) is dominant on N-PBSu. In general, XPS indicates that N-PBSu, A-PBSu, and W-PBSu have dominant surface C–N, C=N, and C–O functional groups, respectively. Different plasma gases yield different chemical groups on the samples.

Surface chemical groups are critical to the biofunctions of biomaterials. Curran, et al.¹⁸ have reported that amino groups on biomaterials have a positive effect on the osteogenesis of the cultured cells. Therefore, a colorimetric assay is performed by using Orange II dye to quantitatively determine the surface C=NH/C–NH₂ groups. This test which serves as a supplement to the semi-quantitative XPS measurements is based on the fact that C=NH/C–NH₂ groups are electro-positive under acidic conditions and they will adsorb electro-negative dyes in an acidic solution and desorb them under alkaline conditions. As shown in Figure 3, the amounts of C=

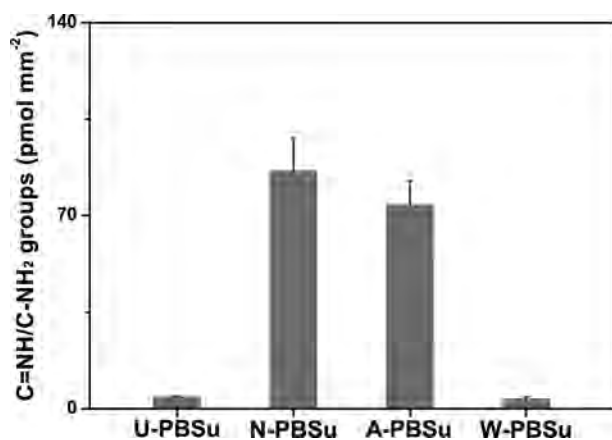


Figure 3. C=NH/C–NH₂ density on the samples assayed by Orange II dye (note the abundance of N-containing groups on N-PBSu and A-PBSu).

NH/C–NH₂ groups on N-PBSu and A-PBSu are significant as the surface C=NH/C–NH₂ density is both above 70 pmol mm⁻² (86.1 and 73.7 pmol mm⁻², respectively). On the other hand, the density of C=NH/C–NH₂ detected on U-PBSu and W-PBSu is below 5 pmol mm⁻² (4.2 and 3.5 pmol mm⁻², respectively). The colorimetric results are in line with the XPS data with both demonstrating that N-PBSu and A-PBSu have abundant N-containing surface functional groups. As aforementioned, XPS also indicates that the dominant N-containing groups on N-PBSu are C–N and C=N on A-PBSu. The different chemical states will probably affect the biofunctions of PBSu substrates differently, and this issue will be discussed in more detail later.

The 4 sets of samples are incubated with primary osteoblasts to assess the roles of the various plasma treatments in directing osteoblast behavior. Firstly, the time-dependent viabilities of the cultured osteoblasts are measured by a colorimetric assay CCK-8, and the results at 4 time points (6 h, 3 days, 6 days, and 12 days) are presented in Figure 4a–d. As shown in Figure 4a–c, up to 6 days, the modified samples, especially W-PBSu, are more positive in supporting primary osteoblast subsistence than

U-PBSu. However, when the culture is performed for 12 days (Figure 4d), the viable osteoblasts on U-PBSu are not statistically different from those on modified samples. The absorbance values measured from the various samples at this time point do not differ significantly. It is known that surface properties such as surface chemistry^{18,19} and morphology^{20,21} influence cell subsistence. In this study, the surface of U-PBSu is flat and there is no obvious change in the surface topography after PIII (confirmed by optical profilometry and the data are shown in Figure S2 in the Supporting Information). Hence, it can be inferred that the different cell behavior arises from the different surface chemistries. In other words, the new yielded C–N, C=N, and C–O bonds on the plasma-treated PBSu are positive to osteoblast adhesion and early proliferation.

Besides the osteoblast adhesion and proliferation, osteogenic differentiation of cultured osteoblasts is evaluated as it is crucial to the success of our samples as artificial implants. In particular, the alkaline phosphatase activity (Figure 5) and gene expressions related to osteogenic proteins (Figure 6a–d) are determined. ALP is a marker for early differentiation of osteoblasts. It regulates inorganic phosphate metabolism *via* hydrolyzing phosphate esters^{22,23} and functions as a plasma membrane transporter for inorganic phosphates.²⁴ As shown in Figures 5 and 6a, the trends of ALP activity and ALP gene expression are similar. Both of them are up-regulated when primary osteoblasts are cultured on W-PBSu after 6 and 12 days. The ALP gene expression on W-PBSu is even higher than those on N-PBSu and A-PBSu after culturing for 6 and 12 days. Osteonectin (ON) is an osteogenic protein which is actively involved in bone remodeling and ECM organization.²⁵ It is expressed in a very early stage of osteoblast differentiation and possesses the ability of binding to both type-I collagen and calcium.²⁶ As shown in Figure 6b, after culturing for 6 days, the ON gene expression of the osteoblasts on the modified samples is higher than that on U-PBSu, and then N-PBSu is the most positive sample with respect to ON gene expression. After additional 6 days of incubation, the ON gene on N-PBSu and A-PBSu is much more significant than that on U-PBSu and W-PBSu. Meanwhile, the ON level on A-PBSu is the highest among all. Osteopontin is a phosphoprotein of osteoblasts expressed during the active proliferation period^{27,28} and it serves as a bridge between osteoblasts and hydroxyapatite.²⁹ Figure 6c reveals that the OPN expression on N-PBSu and A-PBSu is higher than that on U-PBSu and W-PBSu after 6 days, and the OPN level is alone the highest on A-PBSu after 12 days. OCN is the protein that controls the nucleation and size of the hydroxyapatite crystals in the ECM of bone tissues and regulates bone crystal growth.³⁰ It is the most specific protein in osteoblast differentiation and only expressed by mature osteoblasts during the post-proliferative period.^{31–33} The osteoblasts cultured on all the samples are negative for OCN expression during the initial 3 days. After 6 days, the OCN gene of the osteoblasts is detectable and it is significantly up-regulated after 12 days. Interestingly, the OCN level detected on W-PBSu is much lower than that on the other 3 samples after 12 days of cell culture.

Summarizing the protein and gene expression results, samples N-PBSu and A-PBSu are positive to the differentiation of seeded osteoblasts as the ALP and OCN levels are comparable to those on U-PBSu and the ON and OPN expressions are even higher. Especially on A-PBSu, the ON and OPN expressions are the highest after 12 days. Since N-PBSu and A-PBSu have dominant surface C–N and C=N groups,

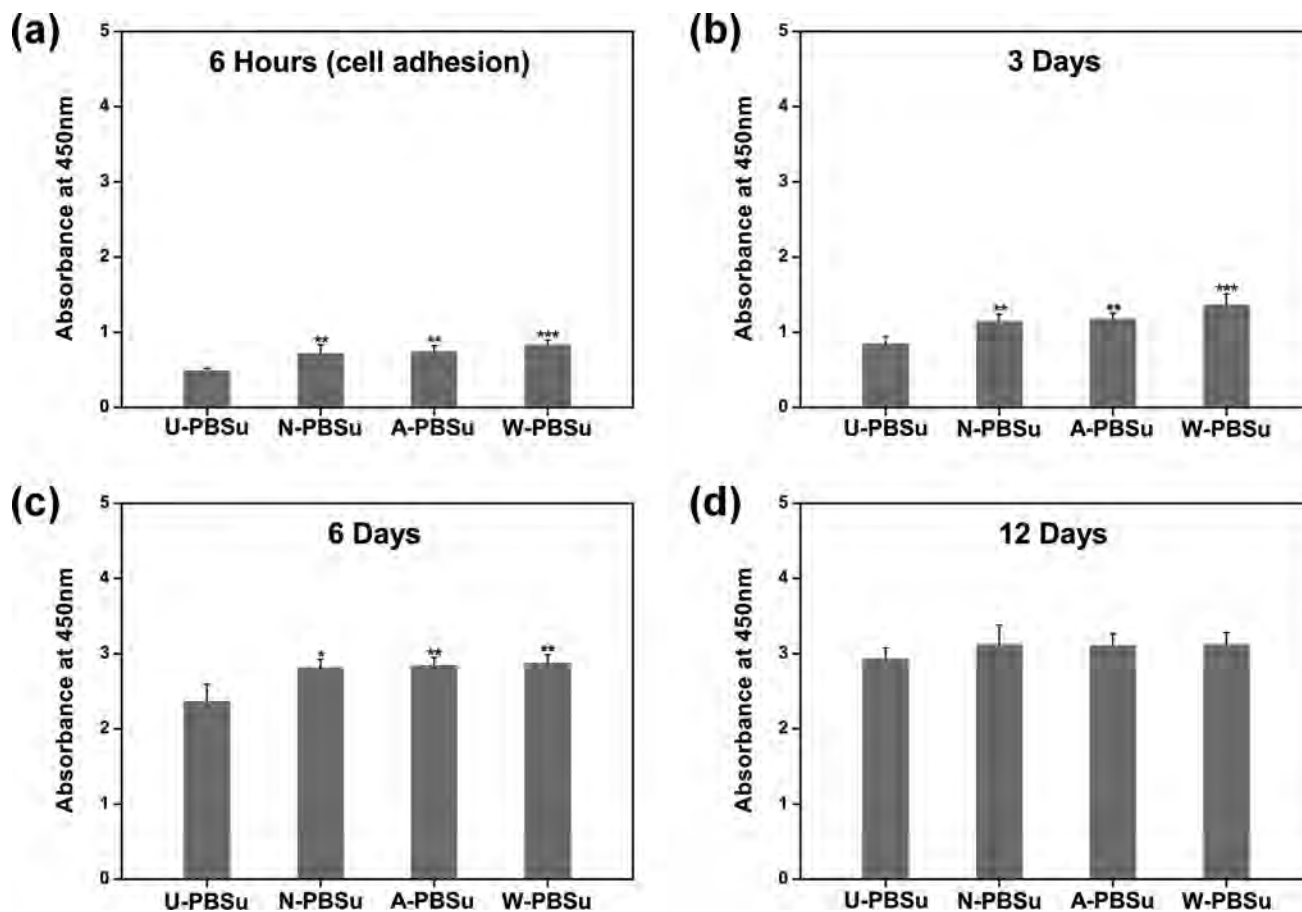


Figure 4. Viabilities of primary osteoblasts cultured on the samples for (a) 6 h, (b) 3 days, (c) 6 days, and (d) 12 days. Statistics evaluation is performed by one-way ANOVA compared to U-PBSu (indicated by *, ** and ***), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

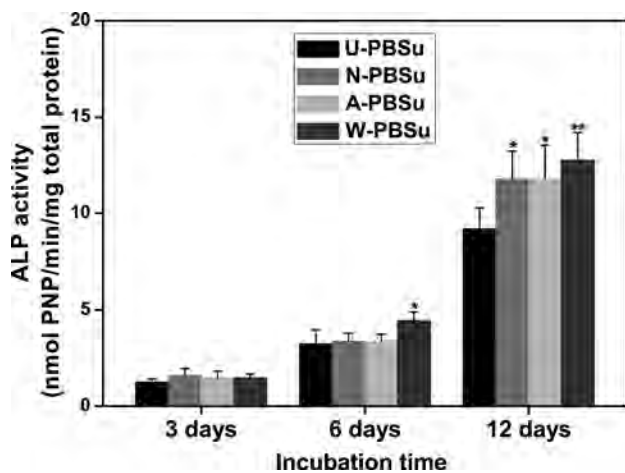


Figure 5. ALP activities of primary osteoblasts cultured on the samples for 3 days, 6 days, and 12 days. Statistics evaluation is performed by one-way ANOVA compared to U-PBSu (indicated by * and **), * $p < 0.05$, and ** $p < 0.01$.

respectively, the PCR results provide evidence that these N-containing groups are positive to the osteogenic differentiation of seeded osteoblasts. On the other hand, ALP is the highest on W-PBSu (dominant with surface C–O groups), but the ON and OPN levels are only comparable to those on U-PBSu. More importantly, the OCN expression on W-PBSu is much lower than that on the other 3 samples. We previously reported

that¹⁵ PBSu samples after O₂ PIII (dominant with C=O groups) were positive to ALP expression but negative to OCN expression and our new results are consistent. Our new data further demonstrate that A-PBSu is more positive than N-PBSu in supporting osteogenic differentiation of primary osteoblasts with respect to ON and OPN gene expressions. It is believed that the dominant surface C=N groups on A-PBSu are responsible for the enhanced osteogenic differentiation of osteoblasts on A-PBSu.

4. CONCLUSION

Nitrogen, ammonia, and water plasma immersion ion implantation (PIII) is conducted to modify biodegradable PBSu with different surface properties. Different chemical groups such as C–N, C=N, or C–O are produced after the PIII treatments although the surface morphology is not altered significantly. The osteoblast behavior on the three sets of samples is quite different. Osteoblast adhesion and early proliferation are promoted on all the plasma-modified samples but there is no difference in osteoblast late proliferation between the modified samples and U-PBSu. N-PBSu and A-PBSu are positive to osteogenic differentiation of the seeded osteoblasts with respect to up-regulating ON and OPN gene expressions. On the other hand, W-PBSu is positive to ALP levels but negative to OCN expression of the seeded osteoblasts. The observed difference in osteoblast differentiation is believed to stem from the different surface chemistry rendered by PIII of different gases. The N-containing

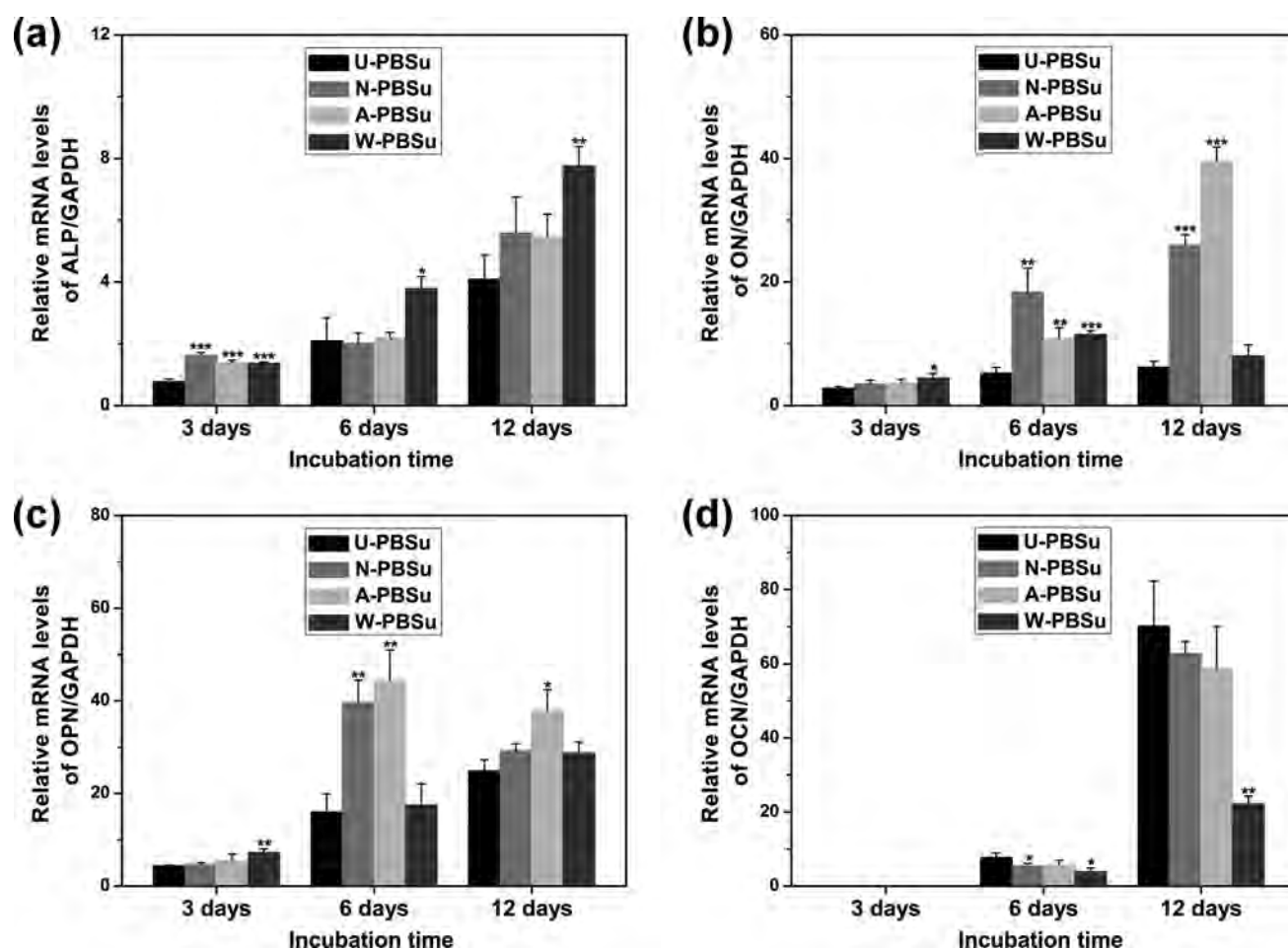


Figure 6. Osteogenic differentiation related gene expressions of primary osteoblasts cultured on the samples for 3, 6, and 12 days. Statistics evaluation is performed by one-way ANOVA compared to U-PBSu (indicated by *, ** and ***), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

groups, especially C=N, are more positive to osteogenic differentiation of the osteoblasts compared to C=O. Our results reveal that the PBSu treated by NH_3 PIII is the most promising bone replacement materials. Further work is being performed in our laboratory to understand more thoroughly how the surface properties affect the cell behavior.

■ ASSOCIATED CONTENT

Supporting Information

Sequences of the gene primers (Table S1); chemical structures of PBSu and Orange II dye (Figure S1); 3D images of various substrates obtained by optical profilometry (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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Supporting Information

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Table S1. Sequences of the gene primers used in the qPCR experiments.

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

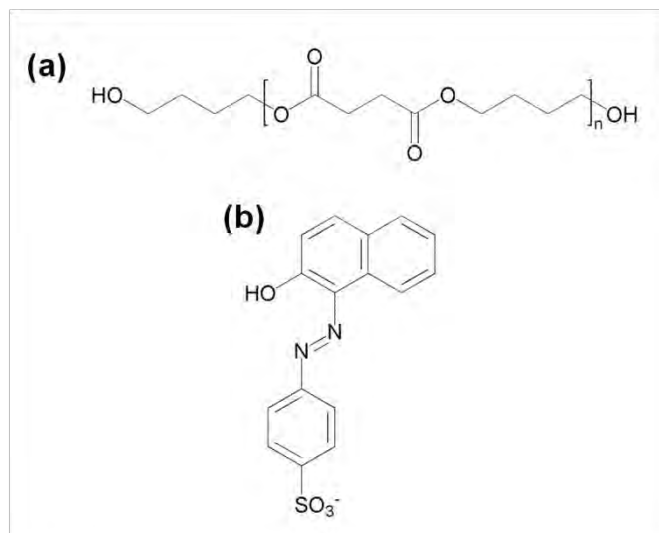


Figure S1. Chemical structures: (a) Biodegradable PBSu substrate and (b) Orange II dye used in the colorimetric method.

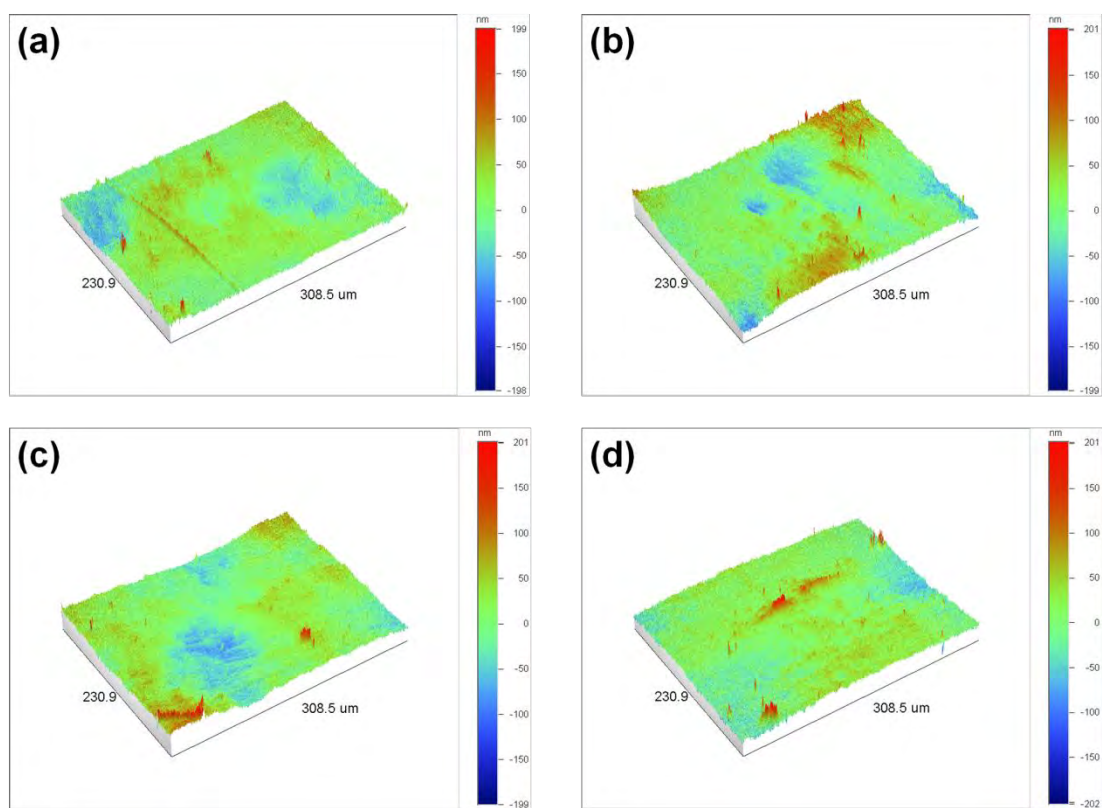


Figure S2. 3D images: (a) U-PBSu, (b) N-PBSu, (c) A-PBSu, and (d) W-PBSu determined by optical profilometry. An area of 230.9 μm x 308.5 μm is scanned and the surface roughness (R_a) values of U-PBSu, N-PBSu, A-PBSu and W-PBSu are 23.89 nm, 26.75 nm, 25.82 nm, and 22.39 nm, respectively.