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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′