Enhanced cytocompatibility and reduced genotoxicity of polydimethylsiloxane modified by plasma immersion ion implantation

Liping Tong\textsuperscript{a,1}, Wenhua Zhou\textsuperscript{a,1}, Yuetao Zhao\textsuperscript{a}, Xuefeng Yu\textsuperscript{a}, Huaiyu Wang\textsuperscript{a,\ast}, Paul K. Chu\textsuperscript{b,\ast}

\textsuperscript{a} Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, PR China
\textsuperscript{b} Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

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\textbf{A B S T R A C T}

Polydimethylsiloxane (PDMS) is a common industrial polymer with advantages such as ease of fabrication, tunable hardness, and other desirable properties, but the basic (-OSi(CH\textsubscript{3})\textsubscript{2}-)\textsubscript{n} structure in PDMS is inherently hydrophobic thereby hampering application to biomedical engineering. In this study, plasma immersion ion implantation (PIII) is conducted on PDMS to improve the biological properties. PIII forms wrinkled “herringbone” patterns and abundant O-containing functional groups on PDMS to alter the surface hydrophilicity. The biocompatibility of the modified PDMS is assessed with Chinese hamster ovarian cells and compared to that of the untreated PDMS. Our results reveal that the PDMS samples after undergoing PIII have better cytocompatibility and lower genotoxicity. PIII which is a non-line-of-sight technique extends the application of PDMS to the biomedical field.

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1. Introduction

Polydimethylsiloxane (PDMS), one of the high-performance polymers in many types of applications, has attracted increasing interest from materials scientists and engineers due to its unique physical and chemical properties [1]. PDMS which is flexible, thermo-tolerant, resistant to oxidation, and tunable in hardness is very promising in microelectronics encapsulation [2,3]. Owing to the ease of fabrication and texturing, PDMS is even regarded as the cornerstone of soft lithography thus playing an important role in the development of microfluidics techniques based on soft lithographic patterning on the microscale and nanoscale [4,5]. In the biomedical field, PDMS is particularly suitable as contact lenses and implants [6,7] on account of its transparency, high gas permeability, and long-term durability in aqueous solutions. Nevertheless, PDMS with the basic structure of (-OSi(CH\textsubscript{3})\textsubscript{2}-)\textsubscript{n} has many methyl groups (-CH\textsubscript{3}) and the surface free energy of PDMS is quite small (22–25 mJ/m\textsuperscript{2}) [8]. The inherent hydrophobicity and concomitant biocompatibility inadequacy have hitherto restricted the application of PDMS in biomedical engineering.

The hydrophilicity and biocompatibility of PDMS can be enhanced by surface techniques [9–12] such as plasma treatment, silanization, and plasma-enhanced chemical vapor deposition, but the activation tends to be temporary because the modified layer on the surface is typically quite thin and the surface polymer chains of PDMS can migrate into the bulk in a relatively short time. In this respect, energetic ion beam treatment may be more useful, and plasma immersion ion implantation (PIII) a non-line-of-sight plasma- and ion-beam-based surface treatment technique, is particularly suitable for biomedical devices with an irregular shape [13–18]. By using different plasma gases, PIII can create different chemical groups on the surface of different types of samples including PDMS to enhance the biological performances.

In this work, O\textsubscript{2} PIII is conducted on PDMS in the presence of a grounded conducting grid to reduce surface charging (Fig. S1, Supporting information) [19–21] and improve the effectiveness of the surface treatment. The surface properties and cellular response are studied systematically and our results reveal that PIII is a viable technique to improve the cytocompatibility and reduce the genotoxicity of PDMS.
2. Materials and methods

2.1. Sample preparation

The PDMS samples were prepared by a commercial polydimethylsiloxane kit (Sylgard-184, Dow Corning) according to the manufacturer’s instructions. In brief, the PDMS base was mixed with the cross-linking agent at a ratio of 10:1 and out-gassed in a vacuum chamber. Afterwards, the solution was spin-coated on a clean petri dish (Model WS-400 BZ-6NPP/LITE) at 300 rpm for 2 min and baked at 50 °C for 10 h. The solidified PDMS disk had a diameter of 100 mm and thickness of 1.5 mm.

O2 plasma exposure and O2 PIII were conducted on the PDMS samples. In the O2 plasma exposure experiments, the PDMS disk was cut in half, cleaned by alcohol and distilled water, and exposed to an O2 plasma for 1 h. The O2 PIII apparatus consisted of a stainless steel plasma discharge chamber (600 mm × 300 mm) and stainless steel plasma diffusion chamber (670 mm × 1030 mm) [Fig. S1, supporting information]. Negative high voltage pulses were applied to the metal sample stage underneath the plasma diffusion chamber and radio frequency (RF) power from 0 to 2 kW was coupled to the plasma discharge chamber. To reduce charging during O2 PIII, the high voltage sample stage was shielded from the plasma by a metal cage consisting of a 2 mm thick cylindrical tube and a top cover with a 200 mm diameter hole. The hole was covered by a stainless steel mesh (15 meshes per inch and 0.5 mm wire diameter). The PDMS sample was placed on the metal sample stage 50 mm away from the top mask. The typical instrumental conditions in O2 PIII were: sample bias voltage = -10 kW, voltage pulse width = 200 μs, pulse frequency = 500 Hz, gas flow = 20 sccm, radio frequency power = 1 kW, and treatment time = 1 h. The samples were designated as PDMS (untreated), P-PDMS (plasma exposure), and PIII-PDMS (PIII treatment).

2.2. Surface characterization

The surface morphology was examined by optical microscopy, profilometry (VVKO NT9100, Veeco) and scanning electron microscopy (SEM, JEOL JS-820). The samples were dried and sputter-coated with gold prior to SEM examination. The static contact angles were measured on a Ramé-Hart (USA) instrument by the sessile drop method using distilled water as the medium (10 μl per drop). To evaluate the change in the surface hydrophobicity with time, the water contact angles were measured every 12 h and each data point represents the average of seven measurements conducted on different parts of each specimen to improve the statistics. The surface chemical states were determined by X-ray photoelectron spectroscopy (XPS) on a Physical Electronics PHI 5802 equipped with an amonochromatic AlKα source using a constant pass energy of 11.75 eV, take-off angle of 45°, and step size of 0.1 eV.

2.3. Cell culture

The Chinese hamster ovarian (CHO) cells were obtained from ATCC and cultured in a basic medium (Dulbecco’s Modified Eagle Medium:F-12 =1:1, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a humidified atmosphere of 5% CO2 at 37 °C. Before cell seeding, the PDMS samples were exposed to ambient air for about 5 days, cut into 10 mm × 10 mm pieces, sterilized with 75% alcohol, rinsed twice with sterile phosphate-buffered saline (PBS), and soaked in a complete cell culture medium overnight.

2.3.1. Cell adhesion and morphology

The CHO cells were seeded at a density of 2 × 10⁴ cells per sample on 24-well tissue culture plates. After incubation for 6 h, the attached cells were rinsed twice with PBS and trypsinized before the attached cells were counted by a hemocytometer. After incubation for 2 days, the samples were rinsed twice with PBS and fixed by 2% paraformaldehyde (Sinopharm Chemical Regent) to determine the cell morphology. The cells were stained for the cytoskeleton protein filamentous actin (F-actin) by phallolidin (fluoresceinisothiocyanate labeled, Sigma-Aldrich) and counterstained for nuclei by Hoechst33342 (Sigma-Aldrich). The samples were mounted on slides and images were acquired on a fluorescence microscope (Axio Observer Z1, Carl Zeiss).

2.3.2. Assays of cell viability and genotoxicity

The CHO cells were cultured at a density of 2 × 10⁴ cells per sample to assess the cytotoxicity and genotoxicity on the various specimens. After incubation for 2 days, the cell viability was separately determined by the MTT (Beyotime) assay and Live/Dead assay (Calcein-AM/PI Double Stain Kit, Yeasen) according to the manufacturer’s instruction. In the MTT assay, the samples were rinsed twice with PBS and incubated with the MTT solution for 4 h at 37 °C to form formazan which was then dissolved by dimethylsulfoxide. The optical density was determined spectrophoto metrically at 570 nm. In the Live/Dead assay, the CHO cells were trypsinized, rinsed, resuspended in the buffer solution with the same concentration, and co-stained with Calcein-AM and PI for 15 min at 37 °C. Afterwards, the cells in the suspension were quantitatively determined on a microplate reader and dripped onto slides to acquire the fluorescent images.

The corresponding genotoxicity was evaluated by the cytokines block microneuron (MN) technique [22]. Briefly, the CHO cells were trypsinized and inoculated on the tissue culture dishes using the complete culture medium with 2.5 μg/ml cytochalasin B (Sigma-Aldrich). After incubation for another 12 h, the cells on the dishes were rinsed by PBS, fixed in methanol/acetic acid (v/v = 9:1), and air-dried for further staining with 10 mg/ml acridine orange (Lifetechnology). The MN in the binucleated cells were scored and classified by fluorescence microscopy according to standard criteria [23]. In the genotoxicity assay, at least 1000 binucleated cells were scored on each sample to determine the MN ratio in the binucleated cells.

2.3.3. Assays of reactive oxygen species (ROS)

2 × 10⁴ CHO cells were cultured for 2 days using the completed culture medium added with 0.1% dimethylsulfoxide (DMSO, a scavenger of ROS). The samples were evaluated by the MTT assay and MN test as described above. To determine the level of cellular ROS, 1 × 10⁴ CHO cells suspended in 1 ml complete culture medium were cultured on each specimen for 2 days. The medium in each well was added with 20 μM H2DCFDA (oxidative stress indicator, Invitrogen) and incubated for 30 min. The samples after H2DCFDA incubation were washed twice with cold Hank’s solution containing 1% serum, added with 0.02% digitonin (Sigma, pH = 4.2), and cultured for 20 min at 37 °C. Afterwards, the mixture was centrifuged at 3000 rpm for 5 min and the supernatant was collected in an ice-bath and analyzed on a microplate fluorescent reader (excitation 486 nm/emission 520 nm). The CHO cells treated with 8.8 mM H2O2 served as the positive control, whereas the negative control referred to the cells cultured on the petri dishes. The relative ROS level of each group was normalized to the cell number with the data acquired from the PDMS being set as 1 for easy comparison.

2.3.4. Determination of superoxide anion (O2−)

1 ml of the culture medium containing 2.0 × 10⁴ cells were added to each specimen and incubated for 1 day. Afterwards, the
samples were rinsed twice with Hanks' solution and stained by hydroethidine (HE, an indicator of $O_2^-$. Invitrogen). The relative level of $O_2^-$ was determined on a fluorescence microplate assay (485 nm excitation and 520 nm emission) [24]. The corresponding results were normalized to the cell number and the data obtained from PDMS was set as 1 for easy comparison.

In the visual assay, the samples after HE staining were counterstained for nuclei by Hoechst33342 and mounted on slides. The images were acquired on a fluorescence microscope (Axio Observer Z1, Carl Zeiss).

2.4. Statistic analysis

The in vitro assays were performed in triplicate and each value was expressed as mean ± standard deviation. Each in vitro experiment was repeated three times and there presentative data were presented. The Student’s t-test was utilized to determine the level of significance and $p < 0.05$ was considered significant.

3. Results and discussion

Optical microscopy, profilometry and SEM are employed to examine the surface morphology of PDMS, P-PDMS and PIII-PDMS and the corresponding results are depicted in Fig. 1. In contrast to the flat morphology observed from PDMS and P-PDMS, wrinkled “herringbone” patterns with lateral feature several micrometers in size are observed from the surface of PIII-PDMS. This unique topographical feature is probably formed by the compressive strain originating from the mismatched thermal expansion of the top and underneath layers during O$_2$ PIII [25]. Similar wrinkled patterns with different sizes (Fig. S2, supporting information) can also be observed after the PDMS samples undergo Ar, N$_2$, and NH$_3$ PIII under similar conditions.

It is well recognized that the surface hydrophilicity of biomaterials is crucial to their biological functions. In this study, the time-dependent hydrophilicity is determined by the static sessile drop method and the water contact angles are plotted versus time in Fig. 2A. PDMS is inherently hydrophobic with the water contact angle consistently being about 110°. In contrast, the freshly prepared P-PDMS and PIII-PDMS are hydrophilic showing initial water contact angles of 14.8° and 11.5°, respectively. However, the hydrophilicity on P-PDMS is temporary and the water contact angle recovers to the same level as PDMS in 84 h. PIII-PDMS shows time-dependent hydrophilicity similar to P-PDMS with the water contact angles increasing from 11.5° to 68.2° in the initial 48 h. In comparison, the water contact angle on PIII-PDMS is 79.8° after 120 h and it is quite different from those on PDMS (110°) and P-PDMS (109.8°). Our data show that O$_2$ PIII is more effective than simple plasma exposure from the perspective of long-term hydrophilic effects.

The surface chemical states of PDMS, P-PDMS, and PIII-PDMS are determined by X-ray photoelectron spectroscopy (XPS) as shown in Figs. 2B–D. It is obvious from the survey XPS spectra in Fig. 2B and corresponding atomic percentages in Fig. 2C that O$_2$ plasma exposure and O$_2$ PIII are both effective in introducing O-containing groups onto the PDMS surface. The fitted high-resolution XPS spectra in Fig. 2D indicate that Si-CH$_3$ on PDMS is partially replaced by
Si-OH/Si=O after O2 plasma exposure or O2 PIII. In the Si2p spectra of PIII-PDMS, a new peak with a larger binding energy emerges as a result of the drastic chemical conversion from (CH3)3Si(OH)3 to (-O)2Si(OH)2 or (-O)2Si=O. The O-containing functional groups are more abundant on PIII-PDMS than PDMS.

The samples are cultured with CHO cells to assess the cell behavior. Fig. 3A reveals that PIII-PDMS and P-PDMS are much better than PDMS in terms of initial cell adhesion. After incubation for 2 days, the CHO cells are stained for cytoskeleton to determine the morphology. As shown in Fig. 3B, the CHO cells on PDMS exhibit a spherical morphology with lack of F-actin organization. In contrast to PDMS, the CHO cells spread more on P-PDMS and the elongated cells on PIII-PDMS are comparable to those on the culture plate abundant with F-actin in the skeleton.

To evaluate the cytotoxicity of the samples, a Live/Dead assay is implemented. Fig. 4A shows that the pristine PDMS has higher cytotoxicity than P-PDMS and PIII-PDMS. Quantitative analysis indicates that the relative Dead/Live cells ratios decrease from 1 ± 0.037 on PDMS to 0.653 ± 0.026 on P-PDMS and 0.425 ± 0.033 on PIII-PDMS (Fig. 4B). The MTT assay is further performed and Fig. 4C shows that the viability of the CHO cells after 2 days follows the following order: PIII-PDMS > P-PDMS > PDMS. The data are normalized to the results determined from the culture plate and the value of PIII-PDMS is as large as 105.53% ± 4.30%. At the same time point, all 3 types of PDMS samples are subjected to the MN test to determine possible chromosome damages. As a typical indicator of genotoxicity, MN is caused by the loss of the entire or a portion of the chromosomes from the daughter nucleus at mitosis, which exists separately from the main nucleus of a cell[26,27]. Fig. 4D shows that the MN ratio in the binucleated cells on PDMS is 23.25% ± 0.55%, whereas it is about 20.02% ± 1.43% on P-PDMS. The value on PIII-PDMS is only 16.02% ± 0.53%, which is as small as that of the culture plate (16.76% ± 0.52%) in terms of genotoxicity.

The PDMS samples after Ar, N2, and NH3 PIII are compared to PIII-PDMS (O2 PIII treatment) from the perspective of cell adhesion, morphology, viability, as well as genotoxicity. Figs. S3 and S4 in the supporting information demonstrate that all the PIII samples support the bio-functions of CHO cells.

Many factors such as chemicals, radiation, or oxidative stress can induce MN in mammalian cells. As one of the possible factors inducing MN, intracellular ROS is evaluated by involving DMSO (a scavenger of ROS) after CHO cells have been cultured for 2 days. The MTT result in Fig. 5A shows that addition of 0.1% DMSO to the culture medium does not compromise the viability of the
CHO cells. In contrast, the DMSO treatment decreases the MN frequency from 22.79% ± 1.23% to 15.82% ± 0.92% on PDMS and from 20.06% ± 0.52% to 16.49% ± 0.80% on P-PDMS (Fig. 5B), indicating that cells on PDMS and P-PDMS exist with intracellular ROS. On the other hand, the MN ratios determined on PIII-PDMS and culture plate show the same level (from 14.86% ± 2.5% to 14.78% ± 2.1%, and from 15.95% ± 0.6% to 14.95% ± 1.24%) after addition of 0.1% DMSO. Hence, the cells cultured on PIII-PDMS and culture plate do not have much intracellular ROS contrary to PDMS and P-PDMS.

In addition to the assays involving DMSO, the intracellular level of ROS is determined quantitatively by the H$_2$DCFDA (an oxidative stress probe) assay. Fig. 6A indicates that the intracellular ROS level follows the order of PDMS > P-PDMS > PIII-PDMS after incubation for 2 days. The intracellular ROS determined on PIII-PDMS and the other PDMS samples after Ar, N$_2$ and NH$_3$ PIII (Fig. S5, Supporting information) can even reach that of the culture plate which serves as the negative control in the H$_2$DCFDA assay.

As a member of the intracellular ROS family, the intracellular superoxide anion (O$_2^-$) is further evaluated by the assay of hydroethidine (HE) staining. HE is the sodium borohydride-reduced derivative of ethidium bromide (EB) which can cross cell membranes easily and be oxidized to EB exhibiting red fluorescence so that intracellular O$_2^-$ can be probed. The red fluorescence intensity is directly proportional to the concentration of intracellular O$_2^-$. Fig. 6B and C show that the intracellular O$_2^-$ on PIII-PDMS is much lower than those on PDMS and P-PDMS, and even as low as that on the cell culture plate. The intracellular O$_2^-$ assay results are consistent with those of MN (Figs. 4 B and 5 B) and intracellular ROS accumulation (Fig. 6A) suggesting that PIII-PDMS has much lower genotoxicity than PDMS and P-PDMS.
4. Discussion

PIII is a versatile surface modification technique for different types of biomaterials. Since polymeric materials are typically not electrically conducting, a low voltage, small pulsing frequency, and short pulse duration are usually implemented to alleviate sample charging and overheating in addition to the use of a grounded conducting grid [19–21]. In fact, in the presence of the grounded conducting grid, PIII can be performed on insulating polymeric substrates with higher pulsing frequency and longer pulse duration without adverse effects. Based on our trial experiments, the optimal conditions for the PDMS experiments are 500 Hz (pulsing frequency) and 200 μs (pulse duration). Under the optimal PIII conditions, active functional groups and “herringbone” topographical features are formed on the PDMS surface to tailor the surface hydrophilicity and consequently biological properties.

The PDMS specimens after PIII are compared to the untreated PDMS and those after plasma exposure (P-PDMS) and PIII indeed leads to better cell adhesion, morphology, and viability. In fact, the PIII samples are as good as the culture plate with regard to low MN formation and intracellular ROS. The excellent cellular properties originate from the chemical and topographical alteration introduced by PIII. Integrin plays a pivotal role in these processes as both the active functional groups and micro-patterned topographies are favorable to the binding between integrin and the extracellular environment.
matrix (ECM) [28–32]. The cellular behavior can be benefitted by modulating integrin clustering on the substrate.

5. Conclusion

Plasma immersion ion implantation is employed to modify the surface properties of PDMS. Different with simple plasma exposure, O2 PIII not only produces wrinkled “herringbone” patterns on the PDMS surface, but also improves the surface hydrophilicity. The PDMS samples after PIII better cytocompatibility and lower genotoxicity than both the untreated and plasma-exposed sample and the performance is even comparable to that observed from the culture plate. PIII is demonstrated to be a viable and effective way to improve the surface properties of PDMS in biomedical applications.

Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2016.08.057.

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Liping Tong\textsuperscript{a,†}, Wenhua Zhou\textsuperscript{a,†}, Yuetao Zhao\textsuperscript{a}, Xue-Feng Yu\textsuperscript{a},
Huaiyu Wang\textsuperscript{a,*}, Paul K. Chu\textsuperscript{b,*}

\textsuperscript{a} Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, P.R. China

\textsuperscript{b} Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

* Corresponding authors, E-mail addresses: hy.wang1@siat.ac.cn (H.Y. Wang); paul.chu@cityu.edu.hk (P.K. Chu)

† These authors contributed equally to this work.
Figure S1. Schematic diagram of the plasma immersion ion implantation (PIII) apparatus with the grounded conducting grid.
Figure S2. Optical photographs of the PDMS samples modified by Ar, O\textsubscript{2}, N\textsubscript{2}, and NH\textsubscript{3} PIII (scale bar is 40 μm).
Figure S3. (A) Adhesion of CHO cells on the different PIII samples after 6 h. (B) Morphology of CHO cells on the different PIII samples after 2 days with the green fluorescence referred to cytoskeleton and blue fluorescence referred to the nuclei.
Figure S4. (A) Viability and (B) Micronuclei formation of CHO cells on the different PIII samples after 2 days.
Figure S5. (A) Intracellular ROS accumulation of CHO cells on the various PIII samples after 2 days. The CHO cells cultured on the culture plate serves as the negative control and those treated with 8.8 mM H$_2$O$_2$ serve as the positive control. The student’s t test is utilized to determine the level of significance and ** stands for $p < 0.05$. 