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In vitro cell culture, platelet adhesion tests and *in vivo* implant tests of plasma-polymerized *para*-xylene films

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ABSTRACT

Plasma-polymerized *para*-xylene (PPX) was developed in a previous study by adjusting the process parameters: pulse frequency of the power supply (ω_p) and *para*-xylene monomer flow rate (f_p). All the obtained PPX films exhibit an amorphous structure and present hydrophobicity (water contact angle ranging from 98.5° to 121.1°), higher film growth rate and good fibroblast cell proliferation. In this study, *in vitro* tests (fibroblast cell compatibility and platelet adhesion) and an *in vivo* animal study were performed by using PPX deposited industrial-grade silicone sheets (IGS) and compared with medical-grade silicone ones (MS), which were commonly manufactured into catheters or drainage tubes in clinical use.

The results reveal that PPX deposited at high ω_p or high f_p, in comparison with MS, exhibit better cell proliferation and clearly shows less cell adhesion regardless of ω_p and f_p. PPX also exhibit a comparatively lower level of platelet adhesion than MS. In the animal study, PPX-coated IGS result in similar local tissue responses at 3, 7 and 28 days (short-term) and 84 days (long-term) after subcutaneous implantation the abdominal wall of rodents compared with respective responses to MS. These results suggest that PPX-coated industrial-grade silicone is one alternative to high cost medical-grade silicone.

1. Introduction

Implanted biomaterials are directly in contact with host tissues or fluids when they are applied clinically. So, good biocompatibility is always the ultimate goal of developing a medical device, which necessitates proper functionality, non-toxicity, bioinertness, non-carcinogenicity, non-allergenicity and sufficient durability for a reasonable lifespan [1–4]. Surface modifications of existing biomaterials to improve biocompatibility have got much attention in the literature over the past few decades, and it is well known that the advantage of using the plasma surface modification process is its ability to modify the surface properties of materials without changing the bulk physical properties [1,4,5]. The surface characteristics of biomaterials, such as surface topography, surface chemistry and surface charge, strongly correlate with the cell-material interactions [6–8]. Among these, surface wettability is a key physicochemical property that can affect cell behaviors, including cell adhesion, cell spreading and cell proliferation [2,4,9]. *In vitro*, some studies have reported that enhancing surface hydrophilicity of polymers increases cell spreading and adhesion [5,10,11]. In contrast, some authors have shown that hydrophobic surfaces promote increased cell adhesion and proliferation [12,13]. Moreover, other studies have demonstrated that different types of cells may specifically adhere to or proliferate on some surfaces within a particular range of water contact angle [14,15]. These controversies exist because the balance between hydrophilicity and hydrophobicity on the surface influences the biocompatibility of biomaterials [2,15].

For blood-contacting medical devices, thrombus formation is still the main barrier of development and clinical application. The formation of thrombi on biomaterials is known to be related to a complex hemagglutination reaction, which involves blood plasma coagulation and platelet-mediated reactions [4,16]. Even though studies have clearly shown that hydrophobic surfaces significantly reduce activation of blood plasma coagulation and lower level of platelet adhesion [16–18], implanting a test specimen into a laboratory animal is ultimately the most effective method to observe the potential effects of a biomaterial on the living tissue [19].

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Silicones, or polydimethylsiloxane (PDMS), are very competent synthetic polymeric biomaterials because they possess some advantages, such as excellent mechanical resistance, intrinsic stability, non-irritation and non-toxicity. They are broadly used in medical applications, such as catheters, drainage tubes, membrane oxygenators, prostheses, implants and excipients of transdermal drug delivery systems [9,20,21]. However, medical-grade silicone is more expensive than industrial-grade silicone, and failure of PDMS-based medical devices may eventually occur due to blood clotting or obstruction which have been tried to dissolve in some studies by plasma polymerization or photo-grafting [22–24]. We established a pulsed-dc plasma polymerization system by adjusting the process parameters, *para*-xylene monomer flow rate (f_p) and pulse frequency of the power supply (ω_p), to prepare plasma-polymerized *para*-xylene (PPX) films. The deposited PPX films exhibit hydrophobic surface characteristics with good mechanical properties and non-cytotoxicity in fibroblast culture test [25,26]. In the present study, *in vitro* tests (fibroblast cell compatibility and platelet adhesion) and *in vivo* animal study were performed to compare differences between PPX-coated industrial-grade silicone sheets and medical-grade silicone sheets in order to determine the potential of PPX-coated industrial-grade silicone to take the place of PDMS in biomedical applications.

2. Materials and methods

PPX films deposited in a pulsed-dc plasma polymerization system has been prescribed in our previous work [25,26] and briefly summarized as follows. The substrate table was connected to a pulsed-dc power supply and placed in an electrically grounded vacuum chamber capable of pumping down to an ultimate vacuum degree about 1 mTorr (0.133 Pa) and the pressure in the chamber to a working pressure of 37.2 ± 10.6 Pa. The heated *para*-xylene monomer (starting material) was carried into the deposition chamber using argon with a flow rate of 10, 20, 30, 40 and 50 sccm, which subsequently determined *para*-xylene monomer flow rate f_p as 1.15, 2.30, 3.45, 4.60 and 5.75 sccm, respectively. Pulse frequency of the power supply (ω_p) was set at 25, 50, 75 and 100 kHz, respectively. Then the deposited PPX films were obtained at near room temperature for 60 min. Other power supply settings were fixed at a duty cycle ($T_{on}/T_{on} + T_{off}$) of 70%, and the applied voltages at T_{on} and T_{off} were −350 V and +52 V, respectively. The industrial-grade silicone sheets (IGS) substrates (manufactured by Yowsong Enterprise Co., Ltd., Taichung, Taiwan) were cut into squares measuring $20\,mm \times 20\,mm \times 1\,mm$ and $10\,mm \times 10\,mm \times 1\,mm$, respectively, for different tests. After coating, they were denoted as the specimens of the experimental group. Medical-grade silicone sheets (MS) (PERTHESE®, manufactured by LPI, Bornel, France and already in clinical use) were cut into the same sizes as aforementioned and were denoted as the specimens of the control group.

Cell compatibility tests, including cell adhesion and proliferation, were performed under the guidance of Biocompatibility Safety Assessment of Medical Devices, ISO-10993-5 [27]. A cell line, ATCC CRL-4001 fibroblast, was purchased from American Type Culture Collection. Cells were cultured in culture flasks (BD Falcon, USA) at 37 °C in a 5% CO_2 humidified incubator. An initial concentration of 2×10^4 cells/mL was seeded into each culture well (1 mL/well) (NUNC, Taiwan), where the specimens of the experimental and control group had been placed. After incubation for 48 h, the specimens for cell adhesion test were washed to remove non-adherent cells. Following rinsing, fixation, dehydration, and ambient drying, the specimens were then freeze-dried, coated with a thin layer of Au–Pd alloy and observed by using a scanning electron microscope (SEM, Hitachi S-3000). For the cell proliferation test, cell counting was performed after incubation for 3 days.

For the platelet adhesion test, fresh platelet-rich plasma (PRP) was obtained from healthy male volunteers. All subjects enrolled in this research have responded to an Informed Consent which has been approved by the Institutional Committee on Human Research of Taichung Veterans General Hospital (followed by Declaration of Helsinki and ICH-GCP guidelines), and that this protocol has been found acceptable by them. The specimens of both groups were equilibrated with phosphate buffered saline (PBS, pH 7.40) first and incubated with PRP at 37 °C for 60 min. Then the specimens were washed three times with PBS to remove non-adherent platelets, followed by soaking in 2.5% glutaraldehyde for 30 min to fix the adhered platelets. Following rinsing, fixation, dehydration, and ambient drying, the specimens were then freeze-dried and Au–Pd alloy was deposited prior to observation by SEM.

In the *in vivo* animal study, this protocol has been approved by the Institutional Animal Care and Use Committee (IACUC) of Taichung Veterans General Hospital, and NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev 1985) have been observed. The specimens were implanted in the subcutaneous layer of SD (Sprague-Dawley) male rodents (Initial body weight 251–275 g) to evaluate the local effects after implantation under the guidance of ISO-10993-6 [19]. The specimens in the experimental group and the control group all measured $10\,mm \times 10\,mm \times 1\,mm$ in size and were sterilized before the surgery. After the rodents were anesthetized and the skin was prepared, one experimental specimen was implanted in the subcutaneous layer of the lower abdominal wall on one side and one control specimen was implanted on the opposite side. Another group of rodents was prepared for a blank test involving the same surgery without implants. Local tissue response was assessed after 3, 7, 28, and 84 days and followed by histopathological examinations. At the microscopic level, the nature and extent of cellular reaction to implants were evaluated and scored. Degree of lesions, including inflammatory area and cellular responses (infiltration of polymorphonuclear leukocytes, lymphocytes, eosinophils, plasma cells, macrophages and giant cells; degree of degeneration, necrosis, fibrosis and edema; material residue), was graded from one to five for each subcategory depending on severity: 1 = minimal (<1%); 2 = slight (1–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); 5 = severe/high (76–100%) [28]. A mean histoscore was determined by averaging these twelve specific scores. A higher histoscore means more severe inflammatory reactions. The differences among the experimental group, the control group and the blank test were measured by Student's *t*-test.

3. Results and discussion

3.1. Results of cell compatibility tests

In the cell adhesion study, it could be observed that the ATCC CRL-4001 fibroblasts cultured on PPX-coated IGS, regardless of the ω_p and f_p, all showed less cell adhesion. In one example shown in Fig. 1(a), fibroblasts cultured on the PPX film deposited specimens at $\omega_p = 25$ kHz and $f_p = 1.15$ sccm, where the specimen possesses a relatively lower water contact angle compared with that of all the PPX deposited ones, and exhibits sparse cell adhesion. In Fig. 1(b), SEM observation revealed that the fibroblast cells on MS spread somewhat more actively than those on PPX-coated IGS. This is consistent with the results found in many studies that fibroblasts tend to adhere on more hydrophilic surfaces than hydrophobic surfaces [1,7,12,14]. According to the results of water contact angle measurement in our previous work (shown in Fig. 2a), it is known that all the deposited PPX films exhibited hydrophobicity, while MS (water contact angle $98.3 \pm 2.8°$) exhibited less hydrophobicity which subsequently stimulated cell spreading with observable