Preparation, characterization, and silanization of 3D microporous PDMS structure with properly sized pores for endothelial cell culture

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Abstract

Nowadays, application of porous polydimethylsiloxane (PDMS) structure in biomedical is becoming widespread, and many methods have been established to create such structure. Although the pores created through these methods are mostly developed on the outer surface of PDMS membrane, this study offers a simple and cost-efficient technique for creating three-dimensional (3D) microporous PDMS structure with appropriate pore size for endothelial cell culture. In this study, combination of gas foaming and particulate leaching methods, with NaHCO₃ as effervescent salt and NaCl as progen are used to form a 3D PDMS sponge. The in situ chemical reaction between NaHCO₃ and HCl resulted in the formation of small pores and channels. Moreover, soaking the samples in HCl solution temporarily improved the hydrophilicity of PDMS, which then facilitated the penetration of water for further leaching of NaCl. The surface chemical modification process was performed by (3-aminopropyl)triethoxysilane to culture endothelial cells on porous PDMS matrix. The results are an indication of positive response of endothelial cells to the fabricated PDMS sponge. Because of simplicity and practicality of this method for preparing PDMS sponge with appropriate pore size and biological properties, the fabricated matrix can perfectly be applied to future studies in blood-contacting devices. © 2015 International Union of Biochemistry and Molecular Biology, Inc. Volume 0, Number 0, Pages 1–10, 2015

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1. Introduction

Although polymers suffer from thrombosis when exposed to blood cells, the application of polymers in blood-contacting devices is becoming widespread [1, 2]. Fortunately, covering the surface of polymer with an endothelial cell monolayer can mitigate thrombosis [3, 4]. It has been reported that porous polymer structure improves the interactions between cells, as it increases oxygen permeability and nutrient supply to deeper areas [5]. Moreover, microporous polymers can support the ingrowth of endothelial cells, whereas the sizes of the pores and porosity have significant effects on endothelial cells attachment, proliferation, and growth [6, 7]. As mentioned by Salem et al. [8], the formation of three-dimensional (3D) interconnected porous structures, with the pore size of less than 60 μm, is suitable for attachment and growth of endothelial cells.

Nowadays, polydimethylsiloxane (PDMS) has various applications in biomedical engineering because of its transparency, inertness, stability, flexibility, high oxygen permeability, and biocompatibility [9–11]. Recently, many approaches have been implemented to form microporous PDMS structure, including soft lithography [12], templating methods with polystyrene microspheres and anodized aluminum oxide [13], solvent evaporation-induced phase separation [14], CO₂ pulsed laser,
and applying salt particle on the outer surface of PDMS [15]. Because of the hydrophobic nature and high viscosity of PDMS prepolymer [16], fabrication of 3D porous PDMS is challenging, and the pores formed by mentioned techniques are mostly founded on the outer surface of PDMS membrane. In addition, the application of PDMS polymer in biology is limited by its hydrophobic nature, as reported in a previous study [16]. Several attempts have been made to improve the hydrophilicity of PDMS-based materials [17, 18]. Oxygen plasma, corona discharge, and UV/ozone are the most common surface modification methods to render a hydrophilic surface. However, the employed methods are of limited use in modifying 3D porous PDMS structure, which is because of the need for specialized instruments, incompatibility with complex features, rapid hydrophobic recovery, and the formation of surface cracks [18, 19]. Immobilizing different functional groups on the surface of material through surface-assembled monolayers (SAMs) based on organosilanes (silanization process) is a common technique to enhance the performance of polymer [20], and yet can be used to overcome the drawbacks caused by hydrophobicity of PDMS. In addition to the consideration of surface wettability, the types of functional groups in organosilanes influence cell attachment and growth [21]. According to a study performed by Jin et al. [22], functionalizing of hydrophobic polymers with amino groups (–NH₂) improves the hydrophilicity and enhances the endothelial cell attachment. Among the mostly studied amino terminated-organosilanes (SAMs), (3-aminopropyl)trimethoxysilane (APTES) is frequently used to prepare amino end groups on the surface of various materials [23–25]. It is hence that fabricating 3D porous structure with appropriate pore size along with improving the hydrophilicity and biological properties are the main challenges to forming an applicable structure for blood-contacting devices based on PDMS.

The purpose of this study is to develop a 3D porous PDMS structure with proper-sized pores that can be used for blood-contacting devices. First, it offers a new and simple technique based on particulate leaching–gas foaming methods, which uses NaCl as a progen for salt leaching and NaHCO₃ as an effervescent agent for gas foaming. To obtain desirable results, the size of salt particles and the salt-to-PDMS prepolymer ratio have been altered. Second, the whole parts of the fabricated 3D PDMS sponge have been modified by the salinization process to improve hydrophilicity and biological properties. Using APTES and anhydrous chloroform (as a solvent), the aminosilane layers have been formed throughout the PDMS sponge at room temperature. Finally, the interaction of human umbilical vein endothelial cells (HUVECs) with APTES-modified PDMS sponge has been evaluated.

2. Materials and Methods

2.1. Materials

For the objective of this study, Sylgard silicone elastomer base and Sylgard 184 silicone elastomer curing agent (Dow Corning Corporation, Germany), sodium chloride (NaCl; Merck, Germany), sodium bicarbonate (NaHCO₃; Merck, Germany), 30% hydrogen peroxide (Merck, Germany), 37% hydrochloric acid fuming (HCl) (Merck, Germany), 99% (3-aminopropyl)trimethoxysilane (APTES; Sigma, St. Louis, MO, USA; 440140), chloroform anhydrous ≥ 99% (Sigma, St. Louis, MO, USA), and 99.7% absolute ethanol (Hamoon Teb Markazi, Zarandieh, Iran) were purchased.

2.2. Preparing of porous PDMS sponge

The Sylgard 184 silicone elastomer includes base and curing agent, which were mixed carefully in a 10:1 ratio by weight in a beaker. After mixing, the precured samples were kept in vacuum desiccator for 30 Min. Later, different particle sizes of NaCl (65 wt%) and NaHCO₃ (35 wt%) were added to the precured liquid in 3:1, 5:1, and 7:1 ratios by weight. The code and composition of the porous PDMS specimens are summarized in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salt particle size (μm)</th>
<th>Salt-to-prepolymer ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>75–75 S1 S2 S3</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>75–150 S4 S5 S6</td>
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</tbody>
</table>

TABLE 1 The code and composition of different porous PDMS specimens

The silanization process was performed prior to cell culturing to improve the wettability of fabricated porous PDMS structure. As reported earlier, the presence of surface hydroxyl groups for bonding the organosilanes to the surfaces has been proven to be essential [23–26]; for this experiment, the porous PDMS structure was temporarily hydroxylated by soaking it in diluted acidic hydrogen peroxide solution (H₂O₂ [1 mL], HCl [1 mL], and water (5 mL)) for about 20 Min prior to bonding APTES to the surfaces. Next, the activated samples were washed carefully with deionized water and dried with N₂ gas. The silanization process was carried out immediately by soaking porous PDMS samples in 5% (v/v) APTES–chloroform solutions at room temperature for about 72 H under N₂ gas. Also, for the purpose of separating unreacted APTES, the PDMS sponges were washed with absolute ethanol and deionized water in an ultrasonic bath several times, and then dried by N₂ gas again. The sponges were then soaked in deionized water.
and kept in an incubator at 37 °C for about 48 H prior to surface characterization.

2.4. Characterization 3D porous PDMS structure
The internal pores morphology of porous PDMS samples before and after surface modification by APTES was observed by scanning electron microscopy (SEM; Essen Philips XL 30) using gold coating. The changes in the samples’ compositions were measured through energy-dispersive spectroscopy (EDS) in SEM. Using Image J software, the size of the pores, their distribution, the total area of the pores in each cross-section \(A_p\) , and the total area of each cross-section \(A_f\) were measured. The measurements were carried out from the five SEM images of each sample \((n = 5)\). Next, the porosity of the fabricated PDMS sponges was calculated using the following equation [27]:

\[
\text{Porosity} = \frac{A_p}{A_f}
\]

The surface structural changes in the porous PDMS structure before and after modification were examined through Fourier transform infrared spectroscopy in the attenuated total reflectance mode (FTIR-ATR; Equimo55 Bruker FTIR spectrometer). Moreover, based on the static sessile drop method, the changes in the water contact angle (WCA; OCA 20; Dataphysics Instruments) of porous PDMS samples before and after surface modification were measured. For each sample, the average of values obtained from three water droplets \((5 \mu L)\) is recorded.

2.5. Endothelial cell culture
HUVECs, from Pasteur Institute of Iran Cell Bank (NCBI), were maintained in Dulbecco’s modified Eagle medium (DMEM) with low glucose containing 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO₂ humidified incubator. Before cell seeding, disc-shaped samples were sterilized by autoclave at 120 °C for 20 Min, and then preincubated in cell culture medium for 24 H.

The direct 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Sigma) assay [28] was used to evaluate endothelial cell viability on the porous silanized PDMS structure after 3 and 7 days of culturing. One hundred microliters of culture medium containing \(3 \times 10^5\) cells/cm² was added to each sample and kept in an incubator \(5% \text{CO}_2\) at 37 °C for up to 7 days. For each of 3 and 7 days durations, the medium was replaced by 100 μL MTT solution \((0.5 \text{mg/mL})\) in PBS (Sigma) and then incubated at 37 °C. After 4 H of MTT incubation, for the purpose of dissolving purple formazan, 100 μL of isopropanol (Sigma) was added to each sample, mixed thoroughly, and then the samples were incubated at 37 °C for 15 Min. Next, using an ELISA reader (Stat Fax-2100; GMI, Inc., Miami, FL, USA), the absorbance of each well at 545 nm was measured. All the measurements were performed three times, and 24-well plates at the same condition were used as a control. Moreover, SEM using gold coating was used to examine the morphology of 48-H cultured endothelial cells \(3 \times 10^5\) cells/cm²). The cells were fixed with 4% \((\text{v/v})\) glutaraldehyde solution in PBS at 4 °C for 30 Min, dehydrated in graded alcohols \((10%, 30%, 50%, 70%, 80%, 85%, 90%, 95%, \text{and} 100%)\) each for 10 Min, and dried at room temperature.

2.6. Statistical analysis
All data were expressed as mean ± SD. A one-way analysis of variance was used to compare any significant differences. A \(P < 0.05\) was considered as the meaningful difference.

3. Results and Discussion
3.1. Pore size, pore size distribution, and porosity
Figures 1 and 2 show the SEM images of fabricated PDMS sponges and the corresponding normalized pore size distribution for each specimen. As mentioned earlier, this study uses two approaches of gas foaming and salt leaching to produce 3D porous PDMS structure. The experiment began by producing small pores and channels in the PDMS polymer by soaking the PDMS/salt blend in HCl (1 N) and expulsing the CO₂ gas from the mixture. In this process, the CO₂ gas was released because of \(\text{in situ}\) chemical reaction between NaHCO₃ and HCl, and soaking PDMS in HCl temporarily improved its hydrophilicity through hydrolysis of Si–O–Si bonds (as mentioned by Jong et al. [29]). As a result of this process, water could easily penetrate inside the PDMS structure through these channels and facilitate leaching of NaCl to the deeper regions. Leaching of NaCl salt from the cured PDMS resulted in the formation of large pores.

As mentioned earlier, previous studies confirmed that the microporous scaffolds with the pore size less than 60 μm are suitable for infiltration of the endothelial cells into the pores [8]. Data obtained from the distribution of pores in this study indicate that except for S1, in samples S2–S6 (shown in Figs. 1 and 2), more than 95% of the pores in the sponges are less than 60 μm in diameter and the numbers of large pores are insignificant. The SEM images along with data from normalized pore size distribution also indicate that increasing the salt to prepolymer ratio resulted in narrower pore size distribution (Figs. 1 and 2) and decrease in the average size of the pores (Fig. 3a). In the other words, higher salt to prepolymer ratio resulted in the development of more small pores. Furthermore, this suggests that when more salt added to the precured PDMS, the friction forces among salt particles increases, resulting in the smaller size of salt particles, which then leads to the formation of small pores after the leaching process. This illustrates that there is a correlation between the size of the salt particles and the size of the pores in the fabricated sponge.

As shown in Fig. 3a, increasing the size of NaCl particles from 75 μm (S1) to 150 μm (S4) for the same salt to prepolymer ratio, the average of pore size decreased from 39.76 ± 4.1 (S1) to 20.13 ± 1.3 (S4). This trend was observed for the other samples as well. Increasing friction between particles of different sizes during mixing process in prepolymer resulted in the breakage of particles, which decreased the average size of the pores.
Besides the average pore size and normalized pore size distribution, porosity is another key factor on designing porous implants [7]. Figure 3b shows that the porosity of the fabricated sponges in both group decreased by increasing the amount of added salt to precured polymer. The reduction in the amount of porosity by increasing the salts concentration is probably due to the increase in the stiffness of prepared mixture, which limits releasing of CO$_2$ and therefore channels formation.

Confirmed by earlier publications, 3D interconnected pores with the pore size less than 60 μm in biomaterials is required to facilitate endothelial cells penetration into the porous structure [8]. Therefore, in this experiment, among all the fabricated samples, the PDMS sponge with the average pore size of 20.136 ± 1.3 μm (95.8% of the pores are less than 60 μm in size) and 62.19 ± 3.96 μm, pore interconnectivity was selected for subsequent silanization and endothelial cell culturing.

3.2. IR spectroscopy
Figure 4 depicts the IR spectra of porous PDMS structure before and after treatment by APTES solution in the range of 900–1,700 cm$^{-1}$. The spectrum of untreated samples (Fig. 4a)
showed sharp peaks at 1,020 and 1,080 cm$^{-1}$, which were attributed to the Si–O–Si bands in the pristine PDMS structure [30, 31]. Furthermore, the peak located at 1,260 cm$^{-1}$ is originated from several C–H vibrations of methyl groups in the PDMS structure [32, 33]. Figure 4b presents the asymmetric stretching vibrations of siloxane linkage (Si–O–Si) at 1,012, 1,086, and 1,104 cm$^{-1}$ [34], and the vibration modes of NH$_2$ groups at 1,570 cm$^{-1}$ [34, 35] after the silanization process. The detection of the siloxane bonds after modification is presumably because of (i) the condensation reaction between APTES molecules and hydroxylated PDMS and/or (ii) the polymerization reaction between adjacent APTES molecules. The activation of PDMS structure after soaking in diluted acidic hydrogen peroxide solution leads to the formation of silanol (SiOH) groups throughout the PDMS sponges. Moreover, the hydrolysis of ethoxy groups in APTES molecules also created silanol (SiOH) groups. Subsequently, the condensation reaction between silanol groups in APTES with the PDMS silanol groups
resulted in the covalent bonding of APTES layer onto the PDMS surface via siloxane linkage (Si–O–Si) [26]. Therefore, the amino groups (–NH₂) in APTES molecules are oriented away from the PDMS surface. The schematic illustration of the possible reactions between APTES molecules and the PDMS surface is shown in Fig. 5.

As shown in the IR spectra of APTES-treated PDMS (Fig. 4b), a shoulder at 1,180 cm⁻¹ (related to the Si–O–C bond) [34] and the peaks centered at 1,263, 1,390, and 1,480 cm⁻¹ (assigned to the several C-H stretching modes) [31] reveal the presence of free ethoxy groups in the APTES-treated sponges, which are remnants of noncondensed APTES molecules in the formed aminosilane layers.

3.3. Pore morphology after silanization
Figure 6 illustrates SEM images of the cross-section and corresponding EDS spectra of porous PDMS structure before and after 72 H soaking in the APTES solution. As the SEM images depict, there is no evidence of cracks and wrinkles on the porous PDMS structure after the silanization process. Moreover, there is no obvious change in the pores morphology of fabricated porous PDMS after silanization (Fig. 6). However, the average of the pores’ wall size has increased from 7.175 ± 1.74 to 11.158 ± 2.78 μm after silanization, suggesting that aminosilane layers covered the entire fabricated porous PDMS. The appearance of nitrogen peaks in the corresponding EDS spectrum of APTES-modified sponge (Fig. 6) indicates that aminosilane layers formed throughout the PDMS sponge. However, the presence of carbon peak in the EDS spectrum of silanized surface suggests that some APTES molecules have retained their ethoxy groups. This result is in accordance with the results obtained from ATR-FTIR (Fig. 4).

3.4. Water contact angle
Figure 7 shows the WCA of porous PDMS structure before and after APTES treatment. The pristine PDMS structure has (126.2 ± 3)° water drop contact angle (Fig. 7a), which was close to the WCA of pristine PDMS in previous reports [16, 31]. As indicated in Fig. 7b, using APTES resulted in the reduction of WCA from (126.2 ± 3)° to (77 ± 2)°. Particularly, the hydrophobic nature of pristine PDMS sponge is because of the presence of methyl (CH₃) functional groups in its structure [10]. After silanization process with APTES, amine functional groups were formed on the surface of PDMS, which have tendency to form hydrogen bond with water molecules, and therefore improve hydrophilicity of PDMS surfaces.

3.5. Cell culture on APTES-modified surface
The endothelial cells (HUVEC) viability on the APTES-modified porous PDMS sponge is shown in Fig. 8. The figure shows the results of a direct MTT assay of the samples after 3 and 7 days of cell culture. The viabilities of cells exposed to the APTES-treated samples were approximately 10⁹ ± 4.3 at day 3 and 10⁶ ± 3.4 at day 7 of culturing. However, the cell viability of the APTES-treated porous PDMS was higher than the control group at each time point (P < 0.05). The results of cell viability indicate
that both fabrication method and silanization process did not show cytotoxic effect on HUVEC cells survival and growth. The high-resolution SEM images of HUVEC cultured after 48 h are shown in Fig. 9. The well-attached cells with small filopodia were observed in SEM images, which indicate the proper interaction and connection with the modified porous PDMS structure. Moreover, the cells have tendency to fusiform shape and were associated with the adjacent cell in some regions. This can prove the inductive role of hydrophilic and positively charged NH$_2$ groups of APTES for HUVEC cells attachment. It is generally believed that the cell–materials interaction on biomaterials depends on the surface characteristics, such as wettability and functionality [36–40]. Based on the previous findings, surface hydrophilicity and hydrophobicity strongly influence the cell–material interactions, in which the moderate hydrophilic surface is needed to improve cell attachment on the biomaterials surface [10]. In the current study, providing moderate hydrophilic surface ($\theta = 77^\circ \pm 2^\circ$) together with the
4. Conclusions

This study offers a simple and feasible method of preparing 3D porous PDMS sponge with proper-sized pores suitable for endothelial cells attachment and growth. Combination of gas foaming and particulate leaching methods, with NaHCO₃ as effervescent salt and NaCl as progen are used to form a 3D porous PDMS sponge. The in situ chemical reaction between NaHCO₃ and HCl resulted in the formation of small pores and channels. Moreover, soaking in HCl solution temporarily has improved the hydrophilicity of PDMS, which then facilitated the penetration of water for further leaching of NaCl. The size of the salt particles and the ratio of salt to PDMS prepolymer have been manipulated to alter the characteristics of pores in the porous PDMS structure. The SEM images and normalized pore size distribution confirm that by increasing the salt to prepolymer ratio, the average pore size decreases and the pore size distribution becomes narrower. The observation of this process shows that despite the increase in NaCl particle sizes, the average pore size of fabricated sponges has been reduced. A larger porosity (62.19 ± 3.96%) was obtained in the sample containing 65 wt% NaCl (150 μm) and 35%wt NaHCO₃ (75 μm) with the salt to prepolymer ratio 3:1. Prior to cell culturing, the sample was silanized with APTES-chloroform solution at room temperature. The obtained results after silanization revealed that APTES successfully bonds throughout the porous PDMS structure without changing the pore morphology. The sponge wettability as well as the endothelial cell viability was enhanced after bonding APTES throughout the fabricated porous PDMS. Furthermore, the well-attached endothelial cells inside the

presence of positively charged amino groups after silanization improved the endothelial cells interaction with porous PDMS surface. This supports the assumption of Zhu et al. [41], which indicates that aminolysis of polycaprolactone membrane enhances the endothelial cell attachments and growth.
The morphology of endothelial cells cultured on the PDMS sponge after 48 H of cell seeding: (a and b) magnification 1,000× and (c and d) magnification 2,000×.

pores of fabricated PDMS sponge were observed after 48 H of culturing.

5. References

Application of 3D Microporous PDMS Structure