The effect of surface microtopography of poly(dimethylsiloxane) on protein adsorption, platelet and cell adhesion

Hong Chen\textsuperscript{a,b,*}, Wei Song\textsuperscript{a,*}, Feng Zhou\textsuperscript{a,b}, Zhongkui Wu\textsuperscript{b}, He Huang\textsuperscript{b}, Junhu Zhang\textsuperscript{c}, Quan Lin\textsuperscript{c}, Bai Yang\textsuperscript{c}

\textsuperscript{a} State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, Wuhan 430070, PR China
\textsuperscript{b} School of Materials Science and Engineering, Wuhan University of Technology, Wuhan 430070, PR China
\textsuperscript{c} State Key Laboratory of Supramolecular Structure and Materials, Jilin University, Changchun 130012, PR China

\textbf{Abstract}

Chemical homogeneous poly(dimethylsiloxane) (PDMS) surface with dot-like protrusion pattern was used to investigate the individual effect of surface microtopography on protein adsorption and subsequent biological responses. Fibrinogen (Fg) and fibronectin (Fn) were chosen as model proteins due to their effect on platelet and cell adhesion, respectively. Fg labeled with \textsuperscript{125}I and fluorescein isothiocyanate (FITC) was used to study its adsorption on flat and patterned surfaces. Patterned surface has a 46\% increase in the adsorption of Fg when compared with flat surface. However, the surface area of the patterned surface was only 8\% larger than that of the flat surface. Therefore, the increase in the surface area was not the only factor responsible for the increase in protein adsorption. Clear fluorescent pattern was visualized on patterned surface, indicating that adsorbed Fg regularly distributed and adsorbed most on the flanks and valleys of the protrusions. Such distribution and local enrichment of Fg presumably caused the specific location of platelets adhered from platelet-rich plasma (PRP) and flowing whole blood (FWB) on patterned surface. Furthermore, the different combination of surface topography and pre-adsorbed Fn could influence the adhesion of L929 cells. The flat surface with pre-adsorbed Fn was the optimum substrate while the virgin patterned surface was the poor substrate in terms of L929 cells spread.

\textcopyright 2009 Elsevier B.V. All rights reserved.

1. Introduction

Protein adsorption, the first response after biomaterials contacting the biological environment, plays a crucial role in mediating the subsequent reactions such as platelet adhesion and in determining the final biocompatibility of biomaterials [1]. Besides, the designs of biosensor [2], medical diagnosis device and drug delivery vehicle should also take protein adsorption into account. Therefore, it is vital to investigate and understand the interaction between protein and material’s surface.

During the past several decades, many efforts have been focused on controlling protein adsorption to biomaterials by chemical modification [3–7], whereas fewer reports have been involved with the simplex effect of surface topography on protein adsorption. In fact, protein adsorption depends not only on the surface chemistry but also on the surface topography. Due to the complexity of the surface properties, it is difficult to separate the effect of surface topography from that of surface chemistry. Very recently, coarse surfaces with distinct roughness [8], silica particles with various diameters [9,10] and titanium surfaces with particular patterns [11] were used as model substrates to investigate the impact of surface topography on protein adsorption. These studies indicated that chemical homogeneous surfaces with nanotopographical features including roughness, curvature and geometrical figures, could affect protein adsorption behavior [12].

However, large numbers of polymeric biomaterials have microscale surface topographies or patterns. These surface features are either unconsciously introduced during material processing or intentionally fabricated for biomedical application such as scaffolds used in tissue engineering. Plenty of researches are focused on controlling protein adsorption to biomaterials by chemical modification [3–7], whereas fewer reports have been involved with the simplex effect of surface topography on protein adsorption. In fact, protein adsorption depends not only on the surface chemistry but also on the surface topography. Due to the complexity of the surface properties, it is difficult to separate the effect of surface topography from that of surface chemistry. Very recently, coarse surfaces with distinct roughness [8], silica particles with various diameters [9,10] and titanium surfaces with particular patterns [11] were used as model substrates to investigate the impact of surface topography on protein adsorption. These studies indicated that chemical homogeneous surfaces with nanotopographical features including roughness, curvature and geometrical figures, could affect protein adsorption behavior [12].

However, large numbers of polymeric biomaterials have microscale surface topographies or patterns. These surface features are either unconsciously introduced during material processing or intentionally fabricated for biomedical application such as scaffolds used in tissue engineering. Plenty of researches are focused on controlling protein adsorption to biomaterials by chemical modification [3–7], whereas fewer reports have been involved with the simplex effect of surface topography on protein adsorption. In fact, protein adsorption depends not only on the surface chemistry but also on the surface topography. Due to the complexity of the surface properties, it is difficult to separate the effect of surface topography from that of surface chemistry. Very recently, coarse surfaces with distinct roughness [8], silica particles with various diameters [9,10] and titanium surfaces with particular patterns [11] were used as model substrates to investigate the impact of surface topography on protein adsorption. These studies indicated that chemical homogeneous surfaces with nanotopographical features including roughness, curvature and geometrical figures, could affect protein adsorption behavior [12].

However, large numbers of polymeric biomaterials have microscale surface topographies or patterns. These surface features are either unconsciously introduced during material processing or intentionally fabricated for biomedical application such as scaffolds used in tissue engineering. Plenty of researches are focused on controlling protein adsorption to biomaterials by chemical modification [3–7], whereas fewer reports have been involved with the simplex effect of surface topography on protein adsorption. In fact, protein adsorption depends not only on the surface chemistry but also on the surface topography. Due to the complexity of the surface properties, it is difficult to separate the effect of surface topography from that of surface chemistry. Very recently, coarse surfaces with distinct roughness [8], silica particles with various diameters [9,10] and titanium surfaces with particular patterns [11] were used as model substrates to investigate the impact of surface topography on protein adsorption. These studies indicated that chemical homogeneous surfaces with nanotopographical features including roughness, curvature and geometrical figures, could affect protein adsorption behavior [12].

© 2009 Elsevier B.V. All rights reserved.

0927-7765/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfb.2009.02.018
complicated adsorption process, and the fourth dimension-time [17], surface microtopography might influence cell behaviors by virtue of its initial effects on previous adsorbed protein. Thus, it may be an effective way to clarify this intricate relation by tailoring specific micropattern bearing homogeneous chemical composition on biomaterials surfaces.

Based on the above hypothesis, we proposed a simple and straightforward method to investigate the individual effect of surface microtopography on protein adsorption and subsequent biological response. Microtopography on poly(dimethylsiloxane) (PDMS), a widely used biomaterial, was fabricated as the method used in soft lithography technique [18]. Instead of being used as a stamp to transfer spheres [19–21], chemical and biological molecules [22], PDMS with specific microtopography but homogeneous chemical composition was directly utilized as substrate. Fibrinogen (Fg) and fibronectin (Fn) were used as model proteins due to their pivotal effects on platelet and cell behaviors. Fg adsorption and subsequent platelet adhesion from platelet-rich plasma (PRP) and flowing whole blood (FWB) to patterned PDMS surface were studied. In addition, the effect of patterned PDMS surface on adsorbed Fn was indirectly evaluated in terms of the morphology of adherent L929 cells.

2. Materials and methods

2.1. Materials and reagents

PDMS from the Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) was used to prepare substrates. Fibrinogen (Plasminogen-Depleted, Human Plasma) was obtained from Cal-BioChem (CAS No. 9001-32-5). Fibronectin (From human plasma) was purchased from Sigma (Product No. F2006). Na125I was bought from BioChem (CAS No. 9001-32-5). Fibronectin (From human plasma) (Plasminogen-Depleted, Human Plasma) was obtained from Cal-Ing, Midland, MI) was used to prepare substrates. Fibrinogen (Fg) and fibronectin (Fn) were used as model proteins as a stamp to transfer spheres [19–21], chemical and biological [18]. Instead of being used in soft lithography technique [18].

2.4. Fibrinogen adsorption

Fg was labeled with 125I by using the ICl method [23]. For studies of Fg adsorption from buffer, labeled Fg was mixed with unlabeled Fg (1:19, labeled:unlabeled) at a total concentration of 1.0 mg/ml with TBS buffer (50 mM, pH 7.4). In all cases, surfaces were equilibrated in TBS for 10 h prior to the adsorption experiments. The patterned surface was incubated with 125I-labeled Fg solution for 2 h at room temperature, rinsed three times, 10 min each with TBS. Small amount of TBS left on surfaces was removed with filter paper, and surfaces were transferred to clean tubes for radioactivity determination by gamma counter (PerkinElmer, Wallac 1480 Wizard). In all cases, radioactivity was converted to adsorbed protein amount.

To label Fg with FITC, 100 mg FITC was dissolved in 10 ml of anhydrous DMF immediately before use. 100 μl FITC solution was added to Fg solution and stirred at room temperature for 60 min. 0.1 ml hydroxyl ammonium chloride solution was added and stirred for 10 min at room temperature to remove any unreacted FITC. The protein was then purified using a 10 mm × 300 mm column with Sephadex G-50 equilibrated in 0.1 M sodium bicarbonate buffer (pH 8.5). The obtained FITC-labeled Fg solution was adjusted to 1.0 mg/ml for adsorption experiment.

2.5. Platelets adhesion

Venous blood from a healthy human volunteers was collected with a vacuum syringe and mixed with acid citrate dextrose (ACD) anticoagulant (Blood: ACD = 9:1, v/v). The blood was centrifuged at 200 × g for 10 min at 25 °C and PRP was withdrawn with a pipette and placed in clean vials. The harvested PRP was centrifuged at 1500 × g for 10 min at 25 °C. The platelet pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4) to obtain platelet solution (PS). 100 μl PRP or PS was placed on flat and patterned PDMS surfaces and allowed to stand for 1 h at 37 °C. The samples were then washed with PBS to remove any non-adherent platelets and fixed with 2.5% glutaraldehyde in saline at 25 °C for 1 h. The samples were then dehydrated with ethanol (0, 30, 50, 70, 90, 100%) and dried naturally before SEM ( scanning electron microscope, JSM-5610LV) observation.

Platelet adhesion from FWB was conducted in a cone-and-plate device [24]. Fresh blood was collected from healthy aspirin-free donors and anticoagulated with ACD (Blood: ACD = 6:1, v/v). PRP, heparinized platelet-poor plasma (PPP) and a suspension of red cells were prepared from the aforementioned blood sample. The washed platelets were resuspended in heparinized PPP containing 0.03 μl/ml apyrase. The obtained whole blood contained platelets with a concentration of 250,000/μl. Finally, washed red cells were added to give a hematocrit of 40%. Surfaces were equilibrated in Tyrodes buffer overnight. They were removed from the buffer immediately prior to the experiment and assembled in the cone-and-plate apparatus. 1 ml whole blood was then added to the wells. The cones were lowered until the cone tips touched the surfaces and rotated at 200 rpm (fluid shear rate of 300 s-1) to give flow conditions typical of those in the arterial circulation. After 15 min, the test surfaces were removed and rinsed with Tyrodes buffer containing 0.01 M EDTA. For SEM test, the surfaces with adherent platelets were immersed in 0.2% solution of glutaraldehyde in Tyrodes buffer for 30 min at room temperature and overnight at 4 °C for fixation. They were then dehydrated through graded ethanol, dried in a CO2...
critical point dryer, mounted on aluminum specimen stubs, coated with gold, and examined using SEM (JSM-840, JEOL).

2.6. Fibronectin adsorption

Flat and patterned PDMS surfaces were sterilized in 70% ethanol for 1 h, rinsed three times with Milli-Q water and air dried in clean bench. All the surfaces were transferred into a 96-well tissue culture plate (Costar, Corning Incorporated). 200 μl 0.025 mg/ml Fn was added into each well, and then incubated in 37°C for 1 h. After protein adsorption, 200 μl PBS was used to rinse each surface for three times. The surfaces with pre-adsorbed Fn were immediately used in following cell experiment.

2.7. Cell culture

L929 cells were cultured in RPMI medium 1640 (Gibco) with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin and incubated at 37°C with 98% humidity in air containing 5% CO2. Cells were harvested by trypsinization and trypsinblau staining test showed that cell viability was above 95% after trypsinization. L929 cells were seeded onto flat and patterned PDMS surfaces with or without pre-adsorbed Fn. The cell density was 20,000 cells/cm2 on each surface. For optical microscope observation, cell culture plate was removed from the incubator (Galaxy S, RSBiotech, UK) and micrographs were captured by using inverted optical microscope (XDS-1B, Chongqing Optical & Electrical Instrument Co, China) equipped with a CMOS camera (GDS310, Guang Di Optical & Electrical Instrument Co, China). For cell counting, nine measurements were obtained from three samples treated identically. A 25×magnification and a field size of 0.002 cm² were used to count the cells adhered on the surfaces.

2.8. Statistics

Data are expressed as mean±standard deviation. Statistical analysis was performed using OriginPro 7.5 software. Statistical comparisons were made by one-way analysis of variance (ANOVA) (Tukey test). P values less than 0.05 were considered to be significant.

3. Results and discussion

3.1. Surface characterization

PDMS is an excellent material for biomedical applications [25] and widely used as stamp to form chemical or biological heterogeneous patterns on many substrates [22]. Herein, patterned PDMS surface with dot-like protrusions was prepared as shown in Fig. 1 and its dimensional parameters were summarized in Table 1. Spontaneously, the chemical composition of resulting surface was identical. Obviously, when the patterned surface directly contacts protein solution, the individual effect of surface topography on protein adsorption can be examined. Moreover, because the diameter

| Table 1 |
| Dimensional parameters of dot-like protrusion. |
|---------------------------------|-----------------|-----------------|
|                                | Horizontal analysis | Diagonal analysis |
| Diameter of protrusion’s top    | 4.12 ± 0.41 μm    | 4.09 ± 0.46 μm  |
| Diameter of protrusion’s bottom | 12.13 ± 0.18 μm  | 8.73 ± 0.31 μm  |
| Interval of two protrusions     | 7.52 ± 0.09 μm    | 3.85 ± 0.12 μm  |
| Height of protrusion            | 1.27 ± 0.08 μm    | 0.72 ± 0.06 μm  |

| Table 2 |
| The difference in nanometer-scale roughness between flat surface and top of protrusion. Size: 1 μm × 1 μm. |
|---------------------------------|-----------------|-----------------|
|                                | \( R_a \) (nm)  | \( R_q \) (nm)  |
| Flat surface                   | 5.88 ± 1.67     | 7.09 ± 4.01     |
| Top of protrusion              | 9.05 ± 3.50     | 10.98 ± 4.07    |
of the protrusion’s top is about 4 μm, 1 μm × 1 μm area in the middle part of the top was chosen to analyze the nanometer-scale roughness and it was compared with the parallel region on flat surface. The result is shown in Table 2. The difference in nanometer-scale roughness between protrusion’s top and flat surface is not significant.

3.2. Fibrinogen adsorption

Fg is a key protein involved in blood clotting process, thus, the quantity of adsorbed Fg on surfaces was always used to evaluate the blood compatibility of biomaterials. In present study, it is used as a model protein to study protein adsorption on patterned surface. As a precise technique to quantify the amount of adsorbed protein on surfaces, the radiolabeling method is employed in this experiment [23]. The adsorption results obtained from 1.0 mg/ml Fg solution on flat surface and patterned surface were summarized in Fig. 2. Although the surface area increased only 8% after introducing the dot-like protrusions, the amount of adsorbed Fg was much higher on the patterned surface (0.386 μg) than the flat surface (0.265 μg), an increase of 46% was found. The increase of adsorbed Fg was not proportional to the increase of surface area.

In addition, by exposing both surfaces to FITC-labeled Fg (1.0 mg/ml) for 2 h, a clear fluorescent pattern was visualized on patterned surface whereas a random fluorescent signal was observed on flat surface as shown in Fig. 3. Such result suggests that surface topography significantly affect the distribution of adsorbed Fg. Moreover, fluorescent images show that very considerable amount of Fg has been adsorbed on the interspaces between protrusions, which partially explains the disproportionate relationship between Fg quantity and surface area after microtopography being introduced on PDMS surface.

Han et al. [26] and Cai et al. [27] reported that surfaces displaying nanometer-scale roughness did not have significant effect on the amount of adsorbed proteins. In our case, there was no significant difference in nanometer-scale roughness between protrusion’s top and flat surface. Therefore, our results indicate that surface microtopography rather than nanometer-scale roughness is able to influence protein adsorption. Although microtopography may appear indistinctive to an individual protein, protein adsorption is a dynamic and interactive process which involves a great deal of lively proteins other than the individual protein. Consequently, the effect of surface microtopography on protein adsorption might derive from the vast number and dynamic characteristic of protein as well as various surface energy of introduced micropattern.

3.3. Platelet adhesion

Among plasma proteins, Fg plays an important role in mediating platelet adhesion because of its binding to the platelet Glycoprotein IIIb/IIIa receptor [28]. As both distribution and amount of adsorbed Fg were influenced by surface topography, subsequent platelet adhesion from PRP and FWB was investigated in vitro.

Fig. 4 exhibited SEM micrographs of adhered platelets from PRP on flat and patterned surfaces. The morphologies of adhered platelets were similar on flat and patterned surfaces. They were round with some pseudopodia extension. However, the distribution of adhered platelets resembled that of adsorbed Fg, that is, on patterned surface most platelets adhered on the flanks or valleys of protrusions, whereas on flat surface platelets adhered rather dispersedly. To confirm whether the surface topography or proteins adsorbed from PRP caused the location of adhered platelets, patterned surface was incubated in pure platelet solution without plasma proteins. As a result, platelets adhered from such solution were very sparse and random on patterned surface (result not shown). These results indicate that patterned surface indirectly influenced the location of platelets via affecting protein adsorption.

Although PRP is a widely used method to evaluate blood contacting materials, it has several inherent disadvantages such as the absence of red cell and static incubation condition. To overcome these disadvantages and simulate blood circulation, FWB was adopted and thus platelet adhesion was studied in a more lifelike system. As shown in Fig. 5, the morphology of adhered platelets was different from that in PRP condition. Both on flat
and patterned surfaces, adhered platelets were generally in activated state with somewhat aggregation. Hyaloplasm can be clearly visualized between platelets and some platelets reached their fully spread state appearing as a characteristic “fried egg”. The discrepant morphologies of adhered platelets in PRP and FWB indicate that different test conditions should be carefully compared when evaluating the blood contacting biomaterials. Furthermore, the quantity of adhered platelets was higher on patterned surface than on flat one and similar to PRP condition, many platelets adhered from FWB were on the flanks or valleys of protrusions (see Fig. 5 (D) dot-dashed profile).

The mechanism of the correlation between adsorbed proteins and specific location of adhered platelets is complicated partially because there are hundreds of plasma proteins in PRP and FWB. However, based on our Fg adsorption result, it was plausible that among these plasma proteins, adsorbed Fg is of great importance in platelet adhesion. Massa et al. [29] also reported that the distribution of Fg correlates to the platelet adhesion pattern on fluorinated surface-modified polyetherurethane. Additionally, the conformation of adsorbed Fg adopted on surface topography should be responsible for platelet adhesion [28,30]. In our case, Fg adsorbed on the flanks of protrusions may have favorable conformation for the interaction between ligands (Arg–Gly–Asp sequence) in Fg and receptors (Glycoprotein IIb/IIIa) on platelet membrane [31,32]. Besides, on the flanks and valleys, protection from fluid stress and local enrichment of Fg (see Fig. 3) could also have synergic effect on the location of platelets.

3.4. Cell’s morphology

Fn was chosen as another model protein in order to further investigate the effect of surface microtopography on protein adsorption. As a component of the extracellular matrix, Fn has been frequently immobilized onto a variety of surfaces in order to induce cell adhesion and spreading [33]. Herein, the morphology of adherent L929 cells was used to indirectly study adsorbed Fn on flat and patterned PDMS surface.

As shown in Fig. 6, there are round and spread cells on all the surfaces no matter with or without pre-adsorbed Fn. Cells proliferated very well and cell density increased significantly as culture time prolonged (see Fig. 7). The percentage of spread cells, however, was different on surfaces with or without pre-adsorbed Fn (see Fig. 8). It was clearly shown that the percentage of spread cells consistently increased on surfaces with pre-adsorbed Fn. In contrast, at 72 h the percentage of spread cells decreased on surfaces without pre-adsorbed Fn. Among all the surfaces, virgin pattern surface was the worst substrate whereas Flat + Fn surface was the best substrate for L929 cell spreading. Such results indicate that both topography and pre-adsorbed Fn could influence the morphology of adhered cells.
Due to the protrusion's array on patterned surface, the formation of focal contacts and the maturation of these newly formed contacts [34] might be negatively affected, resulting in the inhibition of cell spreading process. On the other hand, Fn is well known to induce cell attachment and spreading through its cell binding site and related synergy sites. Thereby, the percentage of spread cells significantly increased on Pattern + Fn surface though this percentage was still lower than that on the Flat + Fn surface, which is easy to understand when considering the adverse effect of virgin pattern surface on cell spreading. However, the conformation and orientation of adsorbed Fn on material's surface which determine

![Fig. 6](image-url)  
*Fig. 6. The representative optical micrographs of cells on flat and patterned PDMS surfaces (A–C) flat surface; (D–F) patterned surface; (G–I) flat surface with pre-adsorbed fibronectin; (J–L) patterned surface with pre-adsorbed fibronectin. Scale bar: 50 μm.

![Fig. 7](image-url)  
*Fig. 7. Cell density on different surfaces as culture time prolonged. *P < 0.05.

![Fig. 8](image-url)  
*Fig. 8. The percentage of spread cells on different surfaces as culture time prolonged. *P < 0.05.
the exposure of cell-binding domain, such as Arg–Gly–Asp (RGD) sequence, should not be overlooked either. The adsorbed Fn alters the substrate to which cells will adhere and the active or correct conformation of adsorbed Fn is of great importance for cell adhesion [35,36]. Herein, compared with flat surface, the protrusion pattern probably caused the change of some adsorbed Fn’s conformation, thus, the cell-binding domain could not be properly exposed or the orientation of these domains could not be identified by adhered cells [37]. In addition, the different states of adsorbed Fn on flat and patterned surfaces (conformation, amount, orientation, etc.) might have mutual effects on the adsorption of serum proteins in culture medium [38], ECM proteins secreted by cells [39] and subsequent cell behaviors. Our future work will focus on the investigation of aforementioned conceivable factors in serum-free culture.

4. Conclusions

In conclusion, chemical homogenous micropatterned PDMS surface was prepared by a universal and simple method. The amount and distribution of adsorbed Fg were influenced by such microtopography, suggesting that microtopography could also affect protein adsorption and should not be neglected. The location of platelets adhered from PRP and FWB on patterned surface followed the similar pattern of adsorbed Fg. In addition, the surface topography and pre-adsorbed Fn influenced the morphology of adhered L929 cell. These results illustrate the correlation between protein adsorption and surface topography, and demonstrate that protein adsorption as well as subsequent biological responses could be regulated by introducing particular topography onto biomaterials surfaces.

Acknowledgments

The authors thank John Brash’s group in McMaster University for help with flowing whole blood experiment. This work was financially supported by the National Natural Science Foundation (20534040, 90606013, 20634030), the Ministry of Education (107080), and the Ministry of Science and Technology of China (2008CB617510).

References