THE CONCENTRATION-INDEPENDENCE CELLULAR EFFECTS OF FIBRONECTIN ADSORBED ON MATERIAL SURFACES WITH DIFFERENT HYDROPHOBICITIES

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SUMMARY

In tissue engineering, coating biomaterial’s surface with extracellular matrix (ECM) proteins can promote many biological activities, including cellular responses, wound healing, and rejection reduction. Several interactions occur upon binding proteins onto the surfaces, leading to changes in the protein structural conformation, directly affecting the cell-host interactions. Therefore, this study investigates the impacts of surfaces’ wettability on protein conformation. In order to get the insights, organosilicate (OGS) was utilized to modify the tissue culture plate, resulting in surfaces with different wettability, followed by fibronectin (FN) immobilization. Then, the surfaces were used to study the fibrinogen interaction, cell attachment, and spreading. The results showed that OGS-modified surfaces produced four different wettability, ranging from super-hydrophilic (OGS150), hydrophilic (OGS100), hydrophobic (OGS60), to super-hydrophobic (OGS5). Each surface possessed particular nature, resulting in the variation of FN molecules’ structural change. The amount of FN adsorbed on the OGS-coated surfaces was shown not to be perfectly proportional to the results of fibrinogen interaction, cell attachment, and spreading. The super-hydrophobic surfaces (OGS5) were highest in the amount of immobilized FN and the efficiency in subsequent experiments among the OGS-coated surfaces group. Notably, the hydrophobic surface adsorbed the lowest amount of FN but achieved remarkable results in the following experiments. Thus, this study holds a promising potential in producing biocompatible materials in tissue engineering.

Keywords: cell activities, fibronectin conformation, hydrophobicity, protein interaction, wettability

INTRODUCTION

Fibronectin (FN) is an essential extracellular matrix (ECM) protein in vertebrates, which is well-known for playing a vital role in different stages of wound healing (Lenselink, 2015). For instance, FN assembly is the initial step to constituting an ECM network.
In other words, FN unfolds and exposes many hidden binding sites after the binding with cell integrins. The aggregate then recruits many ECM components, including collagen, proteoglycans, laminin, and cells, which jointly develop into mature and stable ECM (Schwarzbauer, Sechler, 1999).

Interestingly, many studies on the FN assembly process in the cell-free system demonstrated that FN fibrillogenesis could also occur via chemical (Mosher, Johnson, 1983) and mechanical (Ejim et al., 1993; Nguyen et al., 2013) approaches. Generally, these approaches provided many impressive methods to produce FN fibrils with variation in sizes and morphologies, promising ECM biological functions outcomes.

On the basis of the phenomenon, many studies were conducted for medical purposes, notably in cell-biomaterials interactions, which can be utilized to manipulate cellular processes, including guidance, proliferation, and differentiation (Ruoslantii, 1996). When investigating the influence of material characteristics such as topography and wettability on protein adsorption and cell behavior, each research had advancements in different biological activities (Papenburg et al., 2010). In this research, we are specifically interested in approaching the topic through studies regarding the nature of FN and its interaction with biomaterials.

Cells usually bind to the protein layers rather than attach directly to the surface of materials since the formation of protein always occurs first when materials contact the biological environment (Adam Hacking, Khademhosseini, 2013). Despite cell adhesion and spreading can occur in the absence of serum proteins, this is an inappropriate environment for cell activities, which might lead to cell impairment or death (Wilson et al., 2005). However, coating the material surface with ECM molecules, such as FN, could enhance the biocompatibility upon the implantation into biological environments (Weidt et al., 2019). Many studies have been conducted to improve the biocompatibility of pre-coated biomaterial surfaces with FN, regarding cell response, wound healing, and tissue regeneration (Papenburg et al., 2010; Parisi et al., 2020).

After binding to the substratum surface, FN molecules would undergo the conformational change, exposing more binding sites. The process is influenced by, but not limited to, intramolecular bonds (hydrophobic interactions, ionic bonds, and charge transfer) (Schmidt et al., 2009), chemical composition, physical properties, surface topography (Weidt et al., 2019), and electrostatic force (Matsui et al., 2015). Depending on the conditions, FN molecules will have different performances regarding structural changes, resulting in different biological activities outcomes. For example, some researchers have studied the impact of water wettability on FN's biological responses at the cell-material interfaces. Their study showed that hydrophobic polystyrene (PS) surfaces exhibited a better performance in FN adsorption and cell adhesion compared to super-hydrophobic polystyrene (SP-HS) (Ballester-Beltrán et al., 2011).

In this study, we aimed to investigate the impact of surface attributes, including wettability and topography, on FN adsorption, which in turn affect protein interaction, cell attachment, and cell spreading. The organosilicate compounds were used as the coating solution to generate surfaces with different wettability and topography. Accordingly, we aimed to examine how FN's conformation changes can influence the subsequent bioactivity of protein (fibrinogen) and cells (fibroblast) after interacting with different surface wettability. As a result, this study can provide a good premise for manufacturing or modifying biomaterials with higher biocompatibility in tissue engineering.

MATERIALS AND METHODS

Materials

24-well plates were purchased from SPL Life
Fibronectin was extracted from human plasma, which was obtained from Blood Transfusion Hematology Hospital. All other reagents were purchased from major suppliers.

**Surface modification**

**Organosilicate coatings**

Polystyrene-tissue culture plate substrates were modified using organosilicate (OGS) compounds as described elsewhere (Park et al., 2019). Briefly, tetraethyl orthosilicate (TEOS) was used as a basic precursor to stabilize the OGS compounds. The TEOS was then used alone by itself or mixed with either silicon dioxide (SiO$_2$) particles or a combination of SiO$_2$ particles and 1H,1H,2H,2H-perfluorodecyltriethoxysilane (PFAS) to form the final compounds for coating process, respectively represented as super-hydrophilic, hydrophilic, or super-hydrophobic surfaces. The contact angle of each coated surface was denoted as a group label, which was described in the table below.

<table>
<thead>
<tr>
<th>OGS</th>
<th>Contact angle</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>OGS5</td>
<td>5°</td>
<td>TEOS/SiO$_2$</td>
</tr>
<tr>
<td>OGS60</td>
<td>60°</td>
<td>TEOS</td>
</tr>
<tr>
<td>OGS100</td>
<td>100°</td>
<td>TEOS/PFAS</td>
</tr>
<tr>
<td>OGS150</td>
<td>150°</td>
<td>TEOS/SiO$_2$/PFAS</td>
</tr>
</tbody>
</table>

**FN immobilization**

After the OGS-modification, surfaces were incubated in 50 µg/ml of extracted human plasma FN for 3 hours at room temperature. Next, the surfaces were rinsed with PBS buffer to remove unbound FN. Fresh surfaces (non-OGS-modified surfaces) were used as control.

**Modified surface characterization**

**Wettability and surface morphology of OGS-modified surfaces**

The water-contacted angle (WCA) of all OGS-modified surfaces was measured to analyze the wettability of the coating layer. The Nikon Eclipse Ti-S inverted microscope was used to observe the morphology of the coated surfaces.

**FN-loaded efficiency**

SDS 1% was used to re-dissolve immobilized FN, to examine the efficiency of FN immobilization. The extracted solutions were then centrifuged for 10 minutes at 1000 rpm and room temperature, followed by optical density (OD) measurement at the wavelength of 280 nm. The amount of absorbed FN was calculated based on the equation:

$$y = \frac{x}{1.28}$$

where $x$ is OD, $y$ is FN concentration (mg/ml) and 1.28 is the extinction coefficient (1%) of FN (Rocco et al., 1983).

**Protein binding of immobilized FN**

All the modified surfaces were incubated in BSA 1% for 1 hour at 37°C to block the FN-uncovered areas. Next, fibrinogen conjugated alexa flour 488 (Thermo Fisher Scientific) at 10 µg/ml was added to all the surfaces in the dark condition, at 37°C for 1 hour. The surfaces were then washed with PBS pH 7.4 buffer and the fluorescence intensity was recorded using the multimode microplate reader (VarioskanTM LUX, Thermo Fisher Scientific).

**Effect of immobilized FN on cell activity**

To study the impact of immobilized FN, cell attachment and cell spreading assay were performed. The mouse L929 fibroblasts, purchased from the Cell Bank at ATCC, were cultured at a density of 10^4 cells/ml, then was added to all surfaces and incubated at 37°C for 1 hour.
Cell attachment

After 1 h of incubation, all surfaces were rinsed twice with PBS buffer (pH 7.4). All the surfaces were incubated in MTT 10% v/v followed by DMSO addition as described in previous studies (Chang et al., 2016; Ponsonnet et al., 2002). The OD at 570 nm was recorded. In another set of experiments, all rinsed surfaces were submerged in formaldehyde 4% for 20 minutes prior to DAPI staining at room temperature in the dark condition for 5 minutes. The cell distribution was observed using fluorescence microscopy (Nikon Eclipse Ti-S, Japan).

Cell spreading on adsorbed FN

In a parallel set of experiments, after 1 hour of being cultured, the cell spreading was studied based on the cell morphology using phase-contrast microscopy (Nikon Eclipse Ti-S, Japan). Three random areas were used to quantify the number of spreading cells based on their morphology using ImageJ.

\[
\text{Spreading Ratio(\%)} = \frac{\text{No. of extended cells}}{\text{total No. of attached cells}} \times 100
\]

Statistics

All the experiments were repeated at least 3 times and the number of repeats was noted as n. Results are shown as average ± standard deviation. Optical density and FN concentration were analyzed by one-way ANOVA with Tukey post hoc test, while cell attachment and cell spreading ratio were analyzed by two-way ANOVA with * p < 0.05, ** p < 0.001, *** p < 0.0001.

RESULTS AND DISCUSSION

Characterization of modified surfaces

The TCP surfaces were modified with OGS and FN. All the surfaces were then characterized by the wettability, surface morphology, and amount of immobilized FN. Figure 1A showed the contact angle value of all coated surfaces, which were 5° - super-hydrophilic (OGS5), 60° - hydrophilic (OGS60), 100° - hydrophobic (OGS100), 150° - super-hydrophobic (OGS150). Notably, the hydrophilic and hydrophobic coating surfaces were identically smooth, while the two remaining samples were fragmental. The area of each piece in OGS5 was more significant than in OGS150, as well as the distances between each piece (478.7 µm vs. 146.1 µm, p = 0.0001, n=80) (Table 2).

Table 2. Distances from OGS5 and OGS150 coated area to other coated pieces (µm).

<table>
<thead>
<tr>
<th>Types of surfaces</th>
<th>OGS5</th>
<th>OGS150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average distances (µm)</td>
<td>478.7</td>
<td>146.1</td>
</tr>
<tr>
<td>S.D</td>
<td>308.7</td>
<td>79.62</td>
</tr>
<tr>
<td>Min</td>
<td>67.22</td>
<td>37.81</td>
</tr>
<tr>
<td>Max</td>
<td>1857</td>
<td>415.9</td>
</tr>
</tbody>
</table>

Figure 1B demonstrated the amount of FN adsorbed on five types of surfaces, including the control and four OGS-coated surfaces. The unmodified surfaces obtained the highest FN concentration (40.3125 µg/ml) after the immobilization. It might be due to the material of the tissue culture plate made of polystyrene (PS), which is used widely in the experiment of protein adsorption and cell culture (Lerman et al., 2018). Among four OGS-modified surfaces, the super-hydrophobic surface had the highest adsorbed protein (32.0833 µg/ml), followed by the super-hydrophilic surface (27.1354 µg/ml), the hydrophilic surface (18.5938 µg/ml), and the
hydrophobic surface (about 9μg/ml). Several studies delineated that super-hydrophobic surfaces are unsuitable substrates for protein binding (Ballester-Beltrán et al., 2011; Falde et al., 2016). However, various factors can compensate for the efficiency of protein adsorption on hydrophobic surfaces, such as wettability, roughness, chemical compositions, and types of protein (Schmidt et al., 2009; Weidt et al., 2019). Thus, it might explain the contrasting results in this experiment. Indeed, the two surfaces that adsorbed the most FN concentration were OGS5 and OGS150-coated surfaces, which have heterogeneous and rough layers (Figure 1A).

![Figure 1. TCP surface characteristics after coating. (A): Representative images showing the morphology of TCP treated surfaces after coating OGS compounds using phase-contrast microscopy. Inset: Wettability property of OGS-treated dependent surfaces. Scale bars are 200 µm. (B): The amount of the extracted FN after immobilizing onto OGS-modified surfaces by measuring the optical density of solution at 280 nm using a spectrophotometer (n=3). (C): The quantified concentration of coated FN onto OGS-modified surfaces using OD280 nm to divide to the extinction coefficient of FN (1.28) (n=3). All data were presented as mean ± standard deviations. * p < 0.05, ** p < 0.001, *** p < 0.0001.](image-url)
**FN-coated surfaces enhance protein affinity**

FN binds to other fibrinogen molecules via the fibrinogen binding regions located at the C-terminal of FNI1-5 and C-terminal of FNI10-12 (Makogonenko et al., 2002). To study the protein binding activity of immobilized FN on the substrates, substrates were incubated with fibrinogen conjugated Alexa flour 488. Figure 2 shows the binding affinity of fibrinogen to FN-modified surfaces. The OGS150-coated surfaces had the highest adsorbed FN and the most remarkable fibrinogen interaction (0.04442) in comparison to other samples. On this super-hydrophobic surface, the topography was rough and heterogeneous. Hence, it might provide more areas for trapping and unfolding FN, which impact the affinity between FN and fibrinogen.

Combining with Figure 1C, we obtained many intriguing results. The amount of FN on the OGS5-coated surface was 3-fold higher than the amount on the OGS100-coated surface. However, the fibrinogen interaction of the FN layer on the OGS100 surfaces was four times more dynamic than on the OGS5 surfaces. In addition, although the control surfaces adsorbed the most considerable amount of protein, the structural changes of the FN molecules did not support the fibrinogen interaction. It was approximately two-thirds of the fibrinogen interaction value on the OGS100 sample. Thus, the OGS100-coated surface provided an optimal condition for FN to change its conformation and enhanced fibrinogen binding efficiency compared with other OGS-coated ones. Further studies on conformational changes of FN adsorbed on these material surfaces should be conducted to get more insights into molecular mechanisms governing FN-fibrinogen interaction.

**Figure 2.** FN concentration proportion to fibrinogen interaction. Data were shown as mean ± standard deviations. * Showing significant differences (p < 0.001) between OGS100 with other groups, # showing significant differences (p < 0.001) with other groups, ns nonsignificant difference.

**Cell adhesion and spreading on adsorbed FN**

Different coated polymers interact with FN resulting in different cell behaviors. Hence cell adhesion and cell spreading become effective parameters to get more insight into the phenomenon. In this experiment, FN immobilization layers were demonstrated to significantly improve cell adhesion on all surfaces (Figure 3C). Besides, the number of adhered cells was considerably independent of the concentration of adsorbed FN. There was no significant difference between the three samples having the highest cell attachment, namely the control (1.197), OGS60- (1.204), and OGS150-modified surfaces (1.212) (p = 0.99097).
Among the examined samples, the FN layer on OGS60 demonstrated its better suitability in structural change for cell adhesion. The amount of protein on this sample was two times lower than on the control surfaces and 1.7 times lower than on the OGS150 samples. Similarly, FN molecules on the OGS100 surfaces had a higher performance in cell attachment than those on the OGS5 surfaces despite the two samples having the same effect on cell attachment which were 0.919667 and 0.86233, respectively (p = 0.5533).

Figure 3. Cell activity on modified surfaces with or without FN immobilization. (A) Results of DAPI staining and (B) phase-contrast microscope of the adherent fibroblast cells on the four OGS-modified surfaces and control surface in the presence or absence of adsorbed FN; (C) and (D) Comparison the cell attachment and spreading ratio between coated and uncoated FN surfaces of five groups of modified surfaces. * Showing significant differences with other substrates within groups (p<0.05) All the data was presented as mean ± standard deviations (n=3).
In the absence of FN immobilization, the super-hydrophobic surface obtained the highest cell attachment efficiency (0.354), followed by the super-hydrophilic samples (0.187). Two samples, including hydrophilic and hydrophobic surfaces, showed similar results in cell adhesion, which were 0.121 and 0.125, respectively. Meanwhile, the control sample achieved the lowest efficiency among all groups of surfaces, which was only 0.082. In this study, the hydrophilic (OGS60) and the control surfaces achieved significantly lower cell attachments efficiency than the super-hydrophobic surface. The cell behaviors might vary depending on the difference in topography and roughness (Nguyen et al., 2016) or types of cells (Wilson et al., 2005). Thus, the OGS150-coated layer, which was heterogeneous and included both smooth and rough coating fragments, might reveal a suitable place for trapping cells. As a result, it might lead to the unexpected higher affinity of super-hydrophobic surface to fibroblasts.

Cell nucleus DAPI staining was performed to further investigate the adhesion of fibroblasts onto the coated polymer surface, as shown in Figure 3A. Our results showed that fibroblasts distributed equally over the surfaces in the presence of FN (Figures 3A and 3B). It suggests that FN adsorbed on OGS-modified surfaces has a remarkable impact on cellular biological response.

Surface hydrophobicity was shown to affect the conformation of adsorbed proteins. We hypothesized that adsorbed FN achieved different conformations on different OGS surfaces. The cell spreading ratio was negligible on uncoated FN surfaces, while OGS60-treated samples had the highest ratio, which was about 10.321% (Figure 3C-D). There was no statistical difference in the spreading ratio between OGS100 and OGS150 samples. The ratios on OGS5-modified surfaces were lower than on OGS60-modified surfaces (5.56%) (p = 0.0153) but higher than on OGS100- (2.83%) and OGS150-modified surfaces (3.3%) (p = 0.033832). In the presence of coated FN on the surface, the cells spread identically on the substrates, without regard to the differences in FN adsorption onto the OGS-modified surface (as shown in Figure 1C). It can be concluded that there is no proportional relationship between adsorbed FN concentration and the ratio of cell spreading.

In this study, our data pointed out that the structure of adsorbed FN in specific, or proteins in general, is more influential than the concentration in the biological activity. Further study on cell responses to the adsorbed protein on OGS-modified surfaces should be conducted with different cell types to define the influences of FN conformational change on cell guidance and differentiation. Accordingly, we can develop novel biomaterials focusing on particular types of tissue engineering applications.

CONCLUSION

In this study, we have investigated the impacts of material wettability and topography on the adsorption of FN. They subsequently influenced cell attachment, spreading, and the ability to interact with fibrinogen. Particularly, the OGS150-modified surfaces had the most remarkable performance in FN adsorption, fibrinogen interaction, and cell attachment, spreading in all experiments. FN adsorbed to OGS60-modified surfaces demonstrated a medium affinity to fibrinogen. However, it possessed a high cell adhesion rate, similar to control and OGS150-modified wells. Despite the higher affinity of FN to fibrinogen, the OGS100-modified surface exhibited a lower affinity to fibroblasts compared to OGS60, which might be due to the lower amount of adsorbed FN on the substrate.

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REFERENCES


