Hierarchical Nanoporous Layer (HNL) glass characteristics and an overview of cell behavior

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1. Introduction

Adhesion at the interface between the artificial material and the living tissue is important when replacing tissues with artificial objects due to accidents, diseases, or other reasons. To get a beneficial tissue response, it is essential to study the interactions between living tissues and non-living materials to ensure it does not harm the patient. Developing bioactive materials based on glass for medical purposes is considered. In recent years Hierarchical Nanoporous Layer (HNL) glass was developed. The alkaline etching treatment of glass easily forms the Hierarchical Nanoporous Layer, and the etching conditions can control its thickness [1]. However, the mechanism of Hierarchical Nanoporous Layer (HNL) formation on glass is still unelucidated. Hierarchical Nanoporous Layer (HNL) glass has nanoscale pores on its surface and exhibits long-life superhydrophilic [1]. Furthermore, since it is superhydrophilic, it has, antifogging, antifouling, and low reflectivity functionalities [1]. Given that the Hierarchical Nanoporous Layer (HNL) glass is superhydrophilicity, superhydrophilicity characteristics may help find new cell viability control perspectives. The wettability - hydrophilicity- of the material plays a significant role in cell adhesion and proliferation. The first step to take when biocompatibility evaluation is, wettability measurement. When cells adhere to a material's surface or biomaterial implant into an organism, the proteins will adsorb to its surface, allowing cell adhesion. Adhere cells will release active compounds allowing cell proliferation. From that, Protein adsorption is affected by wettability, causing cell adhesion. However, the roughness and Surface Charge is another vital parameter influencing cell adhesion. The surface's physicochemical properties are deeply involved in the control of cell adhesion. This study provides a general view of Hierarchical Nanoporous Layer (HNL) glass characteristics and an overview of adhering cells' behavior on its surface. Due to that, application in the medical field will be determined.

2. Materials and method

2.1 Materials

2.1.1 Hierarchical Nanoporous Layer (HNL) glass

Hierarchical Nanoporous Layer (HNL) glass characterize by superhydrophilicity and optical anti-reflection property [1]. Borosilicate glass (TEMPAX Float®; Schott Jenaer Glas GmbH, Jena, Germany) was used as the substrate. The etching time was 18h. Hierarchical Nanoporous Layer (HNL) was formed on the glass according to the reference [2][3]; pristine glass was heated in a sodium-bicarbonate aqueous solution of 0.5 mol dm^{-3} at 110 ◦C for 18 h. Then, the samples were rinsed with deionized water and dried. By using a scanning electron microscope (Hitachi High-Technologies, SU-8230, Tokyo, Japan) Hierarchical Nanoporous Layer (HNL) structure was confirmed. The size of the test pieces used in all the experiments was 10x10 mm. Except in the cell culture experiment, the size was 20x20mm.

2.1.2 Borosilicate glass

Borosilicate glass is untreated glass and was used as a control group. The size of the test pieces used in all the experiments was 10x10 mm. Except in the cell viability experiment, the size was 20x20mm.

2.2 Method

2.2.1 Hierarchical Nanoporous Layer (HNL) glass surface analysis

Scanning Electron Microscopy (SEM, SU-8230, Hitachi High-Technologies Corporation) was used to determine the surface's microstructure. Furthermore, the surface's roughness was measured using White Interference microscopes (BW-S507, Nikon Instech Co., Ltd.). X-Ray Photoelectron Spectroscopy (XPS, SSX-100, SURFACE SCIENCE INSTRUMENTS Company) was used to find the atomic concentration of the surface. The contact angle of the surface was measured with a contact angle meter (DM-301, manufactured by Kyowa Interface Science Co., Ltd.) to search for hydrophilicity. Ultrapure water was dropped on the surface of the test pieces, and the contact angle was calculated by the A half-angle Method. Furthermore, to get more information about the surface charge Zeta potential (Zeta potential measurement system ELSZ-2000Z, Otsuka Electronics Co., Ltd.,) was measured.

2.2.2 Cell culture

The study was carried out using the MC3T3-E1 cells. MC3T3-E1 cells are from mouse calvaria and were purchased from Cell Bank (Tsukuba, Japan), Institute of Science and Technology. For the medium, MEM Alpha (containing Ribonucleosides, Deoxyribonucleosides, Phenol Red, and L-glutamine, manufactured by Thermo Fisher) containing 10% of FBS (fetal bovine serum) was used. The test pieces were used after being ultrasonically cleaned with ethanol, then UV sterilized for 24 h. MC3T3 cells were seeded on each test piece, and cell adhesion and proliferation were evaluated. First, the test pieces were placed on a 6-well plate bottom surface, and MC3T3 cells of 1.0 × 104 cells suspended in the medium were seeded and cultured in an incubator (37 ° C, CO2 5%). The medium used is MEM Alpha (containing Ribonucleosides, Deoxyribonucleosides, Phenol Red, and L-glutamine, manufactured by Thermo Fisher), including 10% of FBS (fetal bovine serum). After 6,24,48,72h from the start of the culture, the cells were washed with PBS (phosphate-buffered saline), and adhered cells were collected from the test pieces using a hemocytometer. For cell viability a Trypan Blue solution and hemocytometer were used to count the number of live and dead cells at 3,24,48,72h. Cell viability was calculated by the ratio of total live/total cells (live and dead). To observe the morphology of cell adhesion at 3,24,48,72h on the surface of the test piece with a scanning electron microscope (SEM), the test piece was immersed in the treatment solutions shown in Table 1 to Fixation, dehydration, and dry the cells. To observe the adherent cell's nucleus and cytoskeleton under a microscope the fluorescent staining method was used. First, the cells were fixation on the surface by paraformaldehyde with 0.5% Triton X-100 solution. Then, stained by adding acridine orange staining solution (AAT Bioquest) as the primary antibody. Furthermore, add Acti-stainTM670phalloidin staining solution (Cytoskeleton, INC.) as a secondary antibody. Staining cells were observed at 3,24,48,72h through a confocal laser scanning microscope (FLUOVIEW FV1000-D, Olympus Co., Ltd.).

Table 1 Preparation process for Scanning Electron Microscope

Order	Process	Liquid
1	Washing	0.1M phosphate buffer
2	Primary fixation	Phosphate buffer containing 2.5% glutaraldehyde (room temperature, 3 hours)
3	Washing	0.1M phosphate buffer
4	Secondary fixation	2% osmic acid aqueous solution (4°C, 2 hours)
5	Dehydration	Ethanol (50%→70%→90%→100% 10 minutes each)
6	Drying	t-Butyl alcohol freeze-drying method

3. Result

3.1 Hierarchical Nanoporous Layer (HNL) glass surface analysis

3.1.1 Microstructure and Topographical

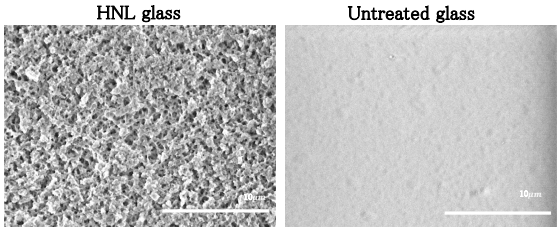

Figure 1 Microstructure of the surface.

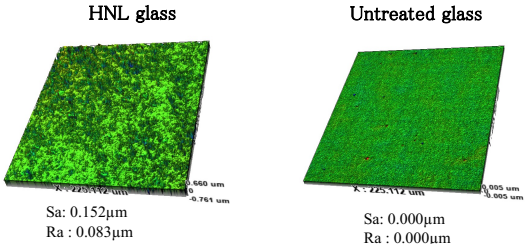

Figure 2 Topographical of the surface

Figure 1 shows the Microstructure of the surface. The Microstructure of Hierarchical Nanoporous Layer (HNL) glass is formed with different pore sizes at the surface. In Borosilicate glass (untreated glass), the surface is flat. Figure 2 shows the topographical and roughness of the surface. The Hierarchical Nanoporous Layer in Hierarchical Nanoporous Layer (HNL) glass is formed through alkaline etching treatment and has nanoscale details, causing roughness compared to Borosilicate glass (untreated glass).

3.1.2 Hydrophilicity

For the Hydrophilicity, on Hierarchical Nanoporous Layer (HNL) glass, the average contact angle measurement is 4.5 degree, which means it is a superhydrophilic surface. On Borosilicate glass (untreated glass), the average contact angle measurement is 57.0 degree, which means it is a hydrophilic surface.

3.1.3 Atomic concentration

Table 2 Atomic concentration of the surface

HNL glass	
Element	Atom%
Al 2p	8%
Si 2p	17%
C 1s	19%
Ca 2p	1%
O 1s	52%
Na 1s	2%
total	100%

Untreated glass	
Element	Atom%
Si 2p	24%
C 1s	16%
Ca 2p	1%
Sn 3d	2%
O 1s	57%
total	100%

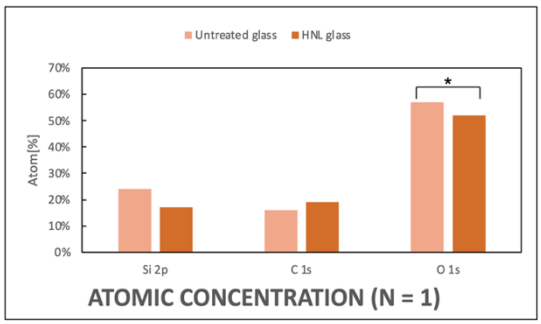

Figure 3 Atomic concentration of the surface

Figure 3 shows the atomic concentration of the surface. Oxgang(O1s) and Silicon (Si2p) content in Hierarchical Nanoporous Layer (HNL) glass are a little bit less than in Borosilicate glass (untreated glass). Nevertheless, carbon(C1s) content in Hierarchical Nanoporous Layer (HNL) glass is higher than in Borosilicate glass (untreated glass). Although the differences are slight, so the components are almost similar.

3.1.4 Zeta potential

For zeta potential, as shown in table 3 Hierarchical Nanoporous Layer (HNL) glass has more negative charge compared to Borosilicate glass (untreated glass).

Table 3 Zeta potential of the surface (n=3)

	HNL glass	Untreated glass
zeta potential (mV)	-1.61	-1.07

3.1 Cell Viability, Adhesion, and Proliferation on Hierarchical Nanoporous Layer (HNL) glass

3.1.1 Cell proliferation

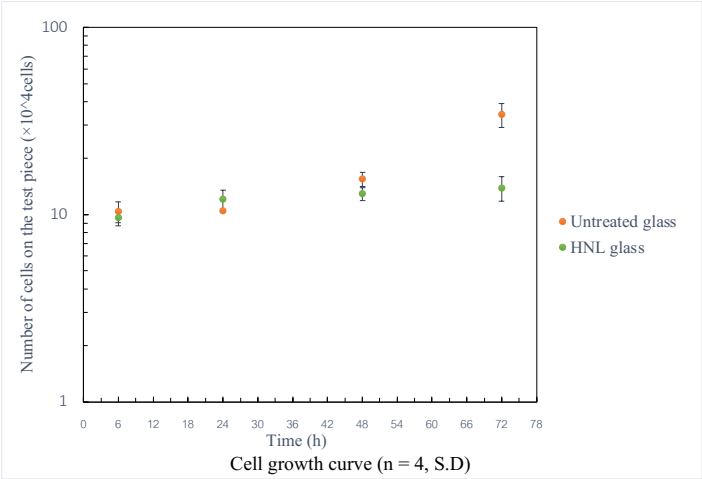

Figure 4 Cell proliferation curve

Figure 4 shows the cell proliferation curve on the Hierarchical Nanoporous Layer (HNL) glass surface. It is shown that the cell number on Hierarchical Nanoporous Layer (HNL) glass is lower than on Borosilicate glass (Untreated glass). On the Hierarchical Nanoporous Layer (HNL) glass surface, cell proliferation between 6h and 24h is increased like Borosilicate glass (Untreated glass), but at 48,72h, the number of increasing was slight.

3.1.2 Microscopic observation of the cells

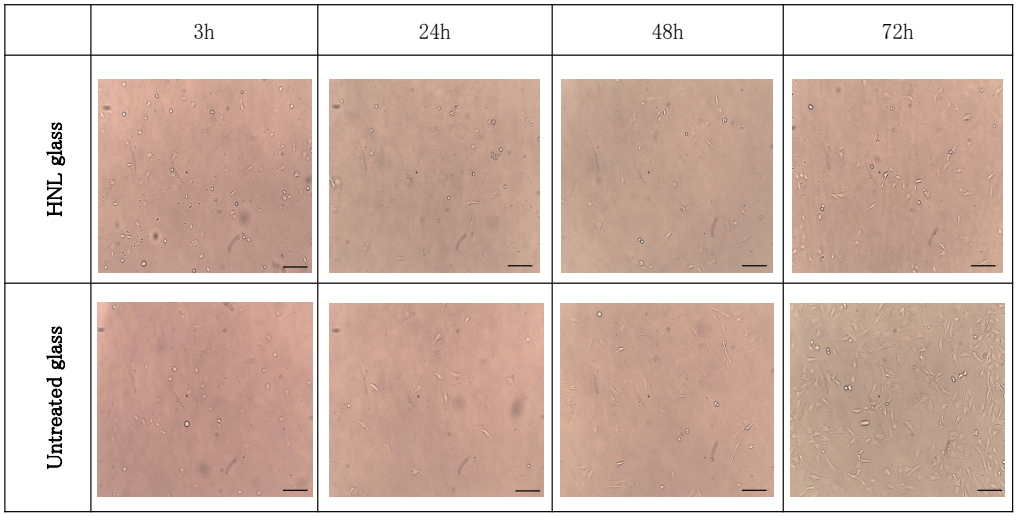

Figure 5 Microscopic observation of the cells at different times (x10, scale bar: 100 um)

The cell status on the Borosilicate glass (untreated glass) and Hierarchical Nanoporous Layer (HNL) glass test piece are shown in Figure 5. The number of cells on the Hierarchical Nanoporous Layer (HNL) glass test piece did not change over time significantly.

3.2.3 Cell viability

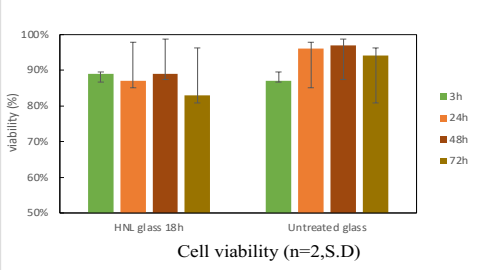

Figure 6 Cell viability on the surface

Figure 6 shows the cell viability on Hierarchical Nanoporous Layer (HNL) glass at different times. At 3h, cell viability is almost the same in Hierarchical Nanoporous Layer (HNL) glass and On Borosilicate glass (untreated glass). However, after 24h, cell viability on Hierarchical Nanoporous Layer (HNL) glass becomes lower than on Borosilicate glass (untreated glass).

3.2.4 Images of a stained adherent cells

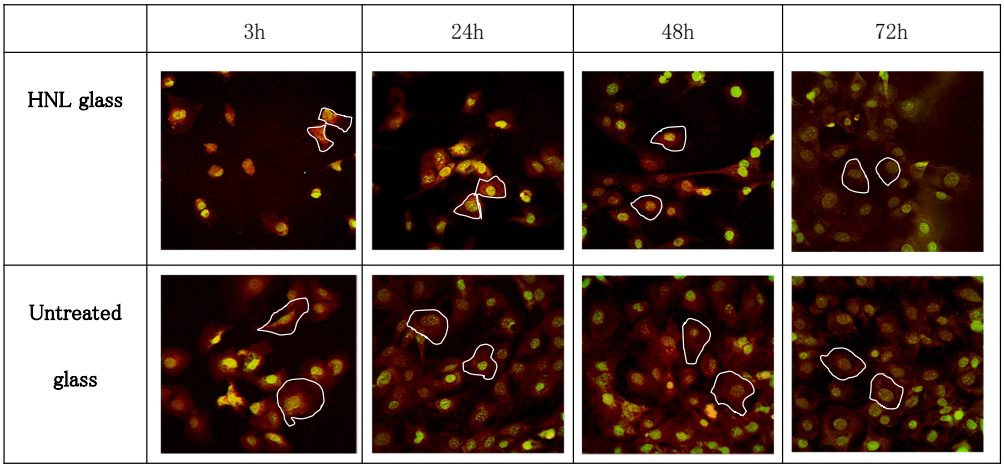

Figure 7 Staining adherent cell image

Figure 7 shows the Staining adherent cell image where the nucleus is green, and the cytoskeleton is red. The cells on Hierarchical Nanoporous Layer (HNL) glass have slower Filopodia growth than on Borosilicate glass (untreated glass).

3.2.5 Morphology of adhered cells

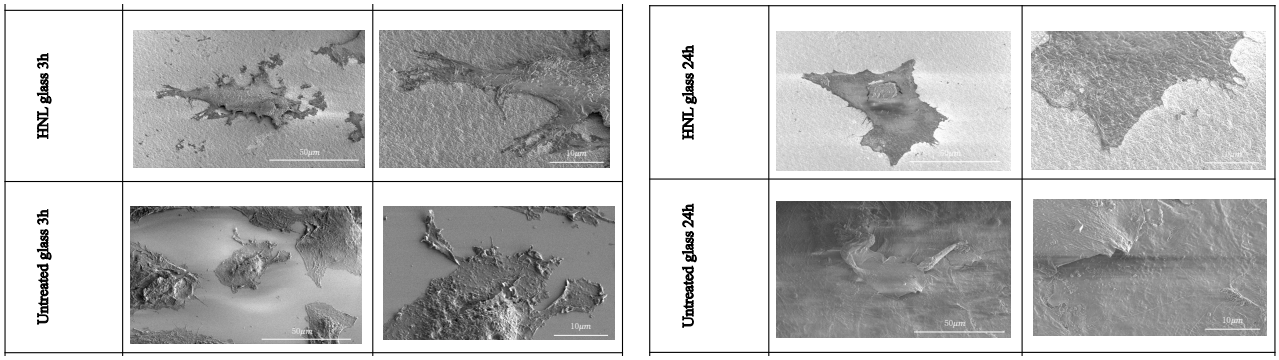

Figure 8 The morphology of adhered cells

As shown in figure 8, At 3h, The cells on Hierarchical Nanoporous Layer (HNL) glass are attached to the surface strongly from the sides, but the nucleus appears loosely attached –there is a space between the cell and the surface -. On Borosilicate glass (untreated glass), The cells attached strongly to the surface.

4 Discussion

4.1 Effects of Surface Properties on Cellular Behaviors

Wettability is one of the properties that influence protein adsorption and thus influence cell behavior. Based on the experiment, Hierarchical Nanoporous Layer (HNL) glass water contact angle is about 4.5 ◦, and it is superhydrophilic. When it comes to superhydrophilic surfaces, seem unsuitable surfaces for cell adhesion based on the experiment's result. One study shows that the reason for decreased cell adhesion on superhydrophilic surfaces is the low absorption of protein [4,5]. On superhydrophilic surfaces, the adsorption of water molecules is higher leading to a larger repulsive force on the proteins and then lower protein adsorption [4,5]. when the repulsive force on the proteins is large, the cells will not be able to bind to surface chemical groups [4,5]. In other words, the cell adhesion rate tends to decrease when superhydrophilic degree is too high. The roughness of the material plays a significant role in cell adhesion and proliferation. Based on the results of the experiment, Hierarchical Nanoporous Layer (HNL) glass is rough compared to Borosilicate glass (untreated glass) due to it having nanoscale details. Furthermore, it was founded that nanoscale materials have a large surface area [6]. Increasing the surface area allows more cells to come into contact [6]. Should note that each characteristic of the rough or smooth surface, hydrophilic or hydrophobic, stimulates different kinds of cell responses [7]. Rough surfaces are preferred by osteoblast cells, and smooth surfaces are preferred by fibroblast cells and epithelial cells [7]. In addition, the surface charge also plays an important role in influencing the responses of cells. Based on the electrophoretic measurement, mammalian cells have a negative charge [8]. When culturing cells on the negative charge surface, electrostatic repulsion will minimize cell adhesion [9]. On the contrary, the positive surface charge will enhance cell proliferation and adhesion. In addition to surface charge, surface potential affects cell adhesion, density, and morphology. one study shows that increasing surface (zeta) potential enhances cell adhesion because more positive surface potential means less electrostatic repulsion with negatively charged cell membranes [10]. Due to better cell adhesion, cell density increased with increasing surface potential [10]. Also, it was found that when surface potential increased, the cell morphology of NIH3T3 cells changed to a flat and spreading shape instead of a bipolar shape [10]. Hierarchical Nanoporous Layer (HNL) glass has more negative charge compared to Borosilicate glass (untreated glass) so electrostatic forces might play a role in protein adsorption and cell adhesion.

4.2 Cell proliferation rate on Hierarchical Nanoporous Layer (HNL) glass

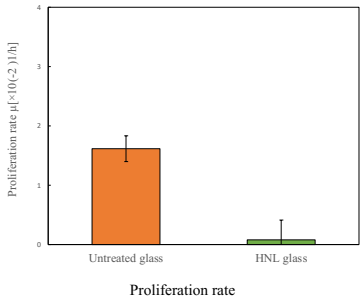

Figure 9 Proliferation rate

Figure 9 shows the Proliferation rate of Hierarchical Nanoporous Layer (HNL) glass compared to Borosilicate glass (Untreated glass). The number of cells at 48h and 72h after culturing on the test piece was set as $N0$ and N, respectively.

$$\mu = 2.303 \times \frac{\ln \frac{N}{N_0}}{t \ln 2} \log 2$$

Using the above formula [11], the specific growth rate μ and then the proliferation rate was founded. It can be considered that the cell proliferation rate on Hierarchical Nanoporous Layer (HNL) glass is lower than on Borosilicate glass (Untreated glass).

4.3 Cell adhesion mechanism

A general view of cell adhesion's mechanism should help to consider cells' behavior on Hierarchical Nanoporous Layer (HNL) glass.

4.3.1 First-stage adhesion and second-stage adhesion [12]

Cells adhere to the surface materials in the early stage through two levels. One is first-stage adhesion, and the other is second-stage adhesion. first-stage adhesion is the initial adhesion stage controlled by the physicochemical interaction between cells and materials. At this stage, the adhesive force is weak, and the cells are easily detached. Therefore, it is possible to control cells' adhesiveness by controlling the surface's wettability. In general, the higher the hydrophilicity of the material surface, the smaller the interface energy between the material and water, and the larger the surface free energy, which weakens the adhesive force of cells. Second-stage adhesion is a more advanced stage of adhesion. Second-stage adhesion is accompanied by intracellular energy metabolism. Also, changes in intracellular metabolism, reorganization of the cytoskeleton, and proliferation occur.

4.4 Cells' behavior on Hierarchical Nanoporous Layer (HNL) glass

The superhydrophilic surface of the Hierarchical Nanoporous Layer (HNL) glass significantly affected the cells' behavior. The reason is that the water contact angle on Hierarchical Nanoporous Layer is about 5 ◦, which means that interfacial energy between the material and water is tiny—causing surface free energy to become more prominent, which weakens the adhesive force of cells. Based on the experiment of culturing Hierarchical Nanoporous Layer (HNL) glass, a tiny increased number of cells on Hierarchical Nanoporous Layer (HNL) glass over time. That means the cells did not proliferate compared to untreated glass because of protein adsorption inhibition on the Hierarchical Nanoporous Layer (HNL) glass. Accordingly, the second-stage adhesion was inhibited.

Conclusion

In this study, Hierarchical Nanoporous Layer (HNL) glass characteristics were observed. Also, adhering cells' behavior on Hierarchical Nanoporous Layer (HNL) glass was determined. Based on the experiment, Hierarchical Nanoporous Layer (HNL) glass is formed with different pore sizes at the surface. The nanoscale detail causes roughness. It is a Superhydrophilic surface with a 4.5-degree contact angle. The atomic concentration is almost the same as Borosilicate glass (untreated glass). Hierarchical Nanoporous Layer (HNL) glass has more negative charge compared to Borosilicate glass (untreated glass). The cells adhere to the surface of Hierarchical Nanoporous Layer (HNL) glass but spread slowly. Because Hierarchical Nanoporous Layer (HNL) glass is Superhydrophilic, lowering protein adsorption, the cell proliferation rate was lower. Also, since Hierarchical Nanoporous Layer (HNL) glass has more negative charge compared to Borosilicate glass (untreated glass) so electrostatic forces might play a role in protein adsorption and cell adhesion. Cell behavior such as adhesion is not affected by just wettability. The other surface's physicochemical properties are deeply involved in the control of cell adhesion.

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