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Uniformly size-controlled chondrocyte spheroid array and evaluation of its function

Tomomi Satomi[1,2], Hisatoshi Kobayashi[1], Junzo Tanaka[1], Yukio Nagasaki[3], Yoshinori Mitamura[2], Tetsuya Tateishi[1,2], Kazunori Kataoka[4], Hidenori Otsuka[1,2]*
[1]Biomaterials center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044,
[2] Division of Bioengineering and Bioinformatics, Graduate School of Information Science and Technology, Hokkaido University, [3]University of Tsukuba, [4]The University of Tokyo *corresponding author
Email: SATOMI.Tomomi@nims.go.jp
Present address: Tokyo University of Science, Department of applied chemistry, Faculty of Science, Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan

Abstract
Micro-patterning of cell is an important technique in medical field. In this study, protein repellent surface was constructed using poly (ethylene glycol) (PEG) having mercapto-group at the ω-end, subsequent plasma etching through metal mask exposure gold surfaces partly. PEG is a promising material to give protein repellent surface. Using different molecular weight of PEG, various PEG density surfaces were constructed on the gold surface, and effect of PEG density on micro-patterned cell attachment was investigated.
Keyword: poly(ethylene glycol), micro-patterning, cell array

INTRODUCTION

The spatial control of cell adhesion and growth is a critical issue in many areas of biotechnology, especially biosensing or tissue engineering using whole cells. Since cellular adhesion and spreading is regulated by protein adsorption, patterning of proteins responsible for cellular adhesion leads to spatially directed cellular adhesion. For example, tissue and cell-based biosensors (TBB and CBB) will facilitate clinical and pharmaceutical analysis of molecular targets, because living cells respond only to biologically active threats. In this study, micropatterned PEGylated substrates with two-dimensional arrays of plasma-etched circular domains (ϕ100-μm) were prepared by coating of mercapto-functionalized poly(ethylene glycol) (PEG) on Au surface, followed by plasma-etching through a metal mask pattern with circular holes. The PEGylated region on the patterned substrate works to repel proteins, and consequently, inhibits cell adhesion. Proteins are expected to adsorb from the serum-containing medium onto the plasma-etched circular domains, exposing the base gold surface. Then the micro-patterning of bovine articular hetero-spheroids underlaid with human umbilical endothelial cells (HUVEC) was achieved. In this study, micro-patterned PEGylated substrates with two-dimensional array of Au-circular domains of cell adhesion area were prepared on Au surface and evaluated its surface property. These arrayed spheroids are promising materials for TBB and CBB as well as tissue engineering technologies.

MATERIALS AND METHODS

Native gold surfaces are constructed by vacuum deposition on slide glass, using Cr as intermediate coat. PEG having mercapto group at its distal end (MeO-PEG-SH, *Mw*: 5k, 2k) was provided by NOF Corporation. Human umbilical endothelial cells (HUVEC) were purchased from Cambrex (Cambrex BioScience Walkersville, Inc. Walkersville, MD. USA). HUVECs were cultured in EBM-2 medium purchased from Cambrex. Chondrocytes were isolated under sterile conditions from the femoral-patellar groove of calf. Cartilage fragments were sharply curetted off the articular surface of the joint and digested using type 2 collagenase (SIGMA CHEMICAL, MO, USA). Fragments were digested for 12 hrs at 37°C with shaking. The resulting cell suspension was filtered using a sterilized filter (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). The filtrate was then centrifuged at 1200rpm for 5min at 4°C. Isolated chondrocytes were stored at passage 3 using Cell Banker® (Diatron, Tokyo, Japan) until use.

1. PEG immobilization study

Preparation of MeO-PEG-SH modified gold surface: Immobilization of PEG on the gold sensor chip surface was performed using surface plasmon resonance (SPR) instrument (Biacore X; Biacore AB, Uppsala, Sweden). Sodium phosphate buffer (PBS, pH7.4, 0.15M, containing 1M NaCl) of PEG solutions were injected at a flow rate of 10μL/min for 10 min at 37°C under running of PBS (pH7.4, 0.15M, containing 1M NaCl). PEG solutions with a different concentration were injected on a sensor chip, and then determined plateau region for PEG immobization. In order to increase (or change) the immobilized amount of PEG, the process of the PEG injection was repeated several times according to the previous paper[24] PEGylated surface prepared by

triple repetitive injections were denoted as PEG5k(3) surface. In a way similar to the above mentioned repeated process, a successive PEGylation with longer and shorter PEG was carried out. On the surface with the pre-constructed longer PEG brushes (PEG5k), a shorter PEG (PEG2k) as a filler was layerd by repetitive injection. PEG5k(1) surfaces with triple treatments with the filler PEG and PEG5k(2) with the four treatments with the filler PEG were denoted as PEG5k(1)/2k(3) and PEG5k(2)/2k(4) surfaces, respectively. Density of immobilized PEG chain was quantitatively estimated by quarts crystal microbalance (QCM) measurement. QCM measurements were performed using AT-cut gold spattered quartz crystal with a diameter of 5 mm and a resonant frequency of 9 MHz (SEIKIO EG&G, SEIKO Instruments Inc., Japan). The frequency of the QCMs was measured with a SEIKO EG&G Model 917 quartz analyser. The crystals were mounted in the cell by means of O-ring seals, with only one face in contact with the solution. The frequency was recorded after immersing the crystals into the PBS (pH7.4, 0.15M, containing 1M NaCl) at 37°C. After stabilization of baseline, PEG solutions (PEG5k, PEG2k) were injected to be a concentration of 0.01mg/mL, which is optimized above in the same repetitive manner studied in SPR measurement (PEG5k(3), PEG5k(1)/ 2k(3), PEG5k(2)/ 2k(4)).

2. Cell culture study

Construction of PEGylated surface: Glass substrates were subjected to a piranha etch (a boiling mixture of 50% (v/v) sulfuric acid and 50% (v/v) hydrogen peroxide) for 30 min and thoroughly rinsed in water. A thin layer of chromium (Cr, 1 nm) was then deposited on the cleaned glass plate and a gold film (20 nm) was subsequently deposited onto Cr at a deposition rate of 0.1 Å/s. PEGylated surfaces were prepared on gold deposited glass plate in the same manner as SPR measurements; PEG5k and PEG2k solutions were prepared to be 0.01mg/mL in PBS (pH 7.4, 0.15M, containing 1M NaCl) respectively. Then, PEG solutions with the appropriate conditions were retained on the gold film for 30 min to construct the PEGylated surfaces studied above (PEG5k(3), PEG5k(1)/2k(3), PEG5k(2)/2k(4)). Distilled water was flowed between each flow of PEG solution for the wash, retaining for 5 min.

Micropatterning of PEGylated surface: PEGylated surfaces thus constructed were then micropatterned by plasma etching (N_2+H_2) through a metal mask pattern Arrayed pattern is circular domain, 100μm diameter holes separated by 300μm (edge-to-edge distance) spacing. After construction of 2-well plastic chamber (Falcon BD) by silicon glue, all samples were sterilized by ethylene oxide gas.

Cell culture study: HUVECs were seeded onto the micropatterned PEGylated surface at the cell density of $1x10^6$cells/mL. They were cultured at 37°C in a humidified atmosphere with 5 % CO_2. Medium for cultivation was used EBM-2 (purchased from Cambrex). Medium was exchanged once in 2 day.

RESULT AND DISCUSSION

1. PEG immobilization study:

Surface property of PEG coating, which has most widely been used to minimize non-specific fouling of the device surface with bio-components, was studied in detail. At first, PBS solutions of PEG including 1M NaCl prepared in various concentrations were injected on gold surface using SPR instrument to optimize immobilized concentration on a sensor chip. The use of the buffer with high ionic strength caused an increase in the amount of immobilized PEG, due to the appreciably reduced solubility of PEG in the concentrated buffer solution.[1] Change of SPR angle in each concentration showed that immobilized PEG was increased with increase in the injected PEG concentration, showing saturated region over 0.01mg/mL. This result suggested that amount of possible immobilization is limited in constant area and optimized concentration to be enable to immobilize maximum amount in minimum concentration was 0.01mg/mL.

Then 3 types of PEG immobilization (PEG5k(3), PEG5k(1)/2k(3), PEG5k(2)/2k(4)) were performed in this concentration. It should be noted that SPR sensorgrams showed steep curve in PEG5k(1) of Figure 1-a, b, c and PEG2k(1) of Figure 1-b, c. This is a typical curve to adsorb molecules on a surface dominantly. Immobilization of long chain PEG5k for the first time increased largely in change of SPR angle, however, the shift decreased for the second injection of

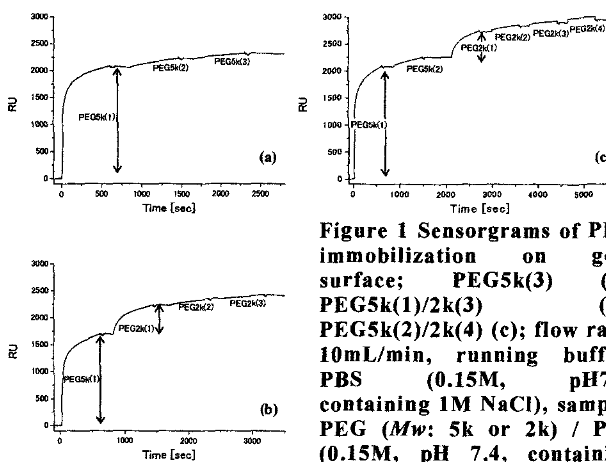

Figure 1 Sensorgrams of PEG immobilization on gold surface; PEG5k(3) (a), PEG5k(1)/2k(3) (b), PEG5k(2)/2k(4) (c); flow rate: 10mL/min, running buffer: PBS (0.15M, pH7.4, containing 1M NaCl), sample: PEG (*Mw*: 5k or 2k) / PBS (0.15M, pH 7.4, containing 1M NaCl) solution, sample injection: 100mL for each time.

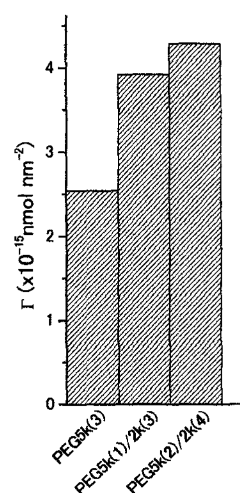

Figure 2 Comparison of 3 types of PEG immobilization measured by QCM: Γ ($x10^{-15}$nmol nm^{-2}) represent amount of immobilized PEG chains.

long chain PEG5k, further little change was seen for the third injection of long chain PEG (Figure 1a). On the other hand, immobilization of short chain PEG2k after long chain showed wide change again. It is considered that just long chain PEG immobilized surface have scarce density due to its exclusion volume effect although following short chain PEG immobilization filled the gap from these result. Then quantitative estimation of immobilized PEG was performed by QCM in the same manner as SPR (Figure 2). Mass conversion was calculated as $\Delta 1Hz = \Delta 1ng$, $15.9mm^2$ as an adsorbed area. Converted value to nmol/ nm^2 calculated from Δf was described as Γ in Figure 2. Final determination of immobilized PEGs estimated by QCM were 2.54, 3.92, 4.28 $x10^{-15}$ nmol per square nanometer on PEG5k(3), PEG5k(1)/ 2k(3), PEG5k(2)/ 2k(4) respectively. PEG5k and 2k mixed surface (PEG5k(1)/2k(3), PEG5k(2)/2k(4)) showed more PEG immobilization than only PEG5k immobilized surface (PEG5k(3)). As a result of QCM measurement, PEG5k(2)/2k(4) surface was determined to be the highest PEG chain density, and PEG5k(3) was the lowest PEG chain density, although PEG5k(1)/ 2k(3) and PEG5k(2)/ 2k(4) showed close value in mass conversion.

2. Cell culture study:

PEGylated surface to inhibit non-specific protein adsorption was constructed on gold surface, which is expected to work for cytophobic surface for the following cell patterning. Then PEG coated gold substrates were micropatterned by plasma etching (N_2+H_2) through a metal mask pattern with ϕ100μm circular holes separated by 100 μm distance in edge to edge, and then cell culture dish was set onto the surface thus constructed (2x2cm). Microscopic images when HUVEC was seeded were shown in Figure 3. In PEG5k(3) surface which suggested having lower PEG chain density in physicochemical studies, seeded HUVEC showed disorganized cellular attachment regardless of micro-patterning (Figure 3-a). On the other hand, PEG5k(1)/2k(3) surface (Figure 3-b) and PEG5k(2)/2k(4) surface (Figure 3-c) showed patterned cell attachment due to the suggested higher PEG chain density compared with the PEG5k(3) surface. Although overgrown cells beyond the pattern were still shown in PEG5k(1)/2k(3) surface. 1 week after cultivation of HUVECs was shown in Figure 3-d,e,f. Arrayed cellular attachment was observed only in PEG5k(2)/2k(4) surface contrary to the observation of sheet formation at intervals in PEG5k(1)/2k(3) surface and complete sheet formation in PEG5k(3) surface. It is clear that PEG chain density affect pattern-recognition in cell attachment. Cells were overgrew in weak protein-repellent surface, and when overgrown cells grew to close enough, overgrown cellular pattern attach each other, resulting in sheet formation. As mentioned above, pattern-recognition of cell attachment could not be seen in only long chain immobilized surface (PEG5k(3)) because of its lower inhibition effect of non-specific protein adsorption, in contrast, arrayed formation was observed by constructing long and short PEG chain mixed surface. Furthermore, PEG5k(2)/2k(4) surface have little non-specific cell attachment contrary to some non-specific cell attachment and bridging of each cellular pattern in PEG5k(1)/2k(3) surface. Considering from SPR result, cell-adhesive proteins are greatly repelled in PEG5k(2)/2k(4) surface to promote pattern-recognition of cell attachment. Some cell attachment between each cellular pattern, where suppose to be cytophobic region, was confirmed in PEG5k(3) and 5k(1)/2k(3) surface at 1 day co-culturing, and attached cells extended each other then bridging across each cellular pattern was occurred. Bridged cellular pattern grew everywhere in the surface, and then formed full-sheeted formation at last. The first cell attachment in cyotophobic region depends on the ability to repel protein of the surface, suggesting that was the reason why PEG5k(3) or PEG5k(1)/2k(3) surface resulted in full-sheeted cell attachment. Thus, cell culture study showed great agreement with surface properties, suggested PEG chain density played a critical role in micro-patterned cell attachment.

CONCLUSION

In conclusion, micro fabrication of cell adhesion controllable surface was achieved using different molecular weight of poly (ethylene glycol) for the cyto-phobic region. And micro cell patterning was well controlled by increasing its PEG chain density. This behaviour can be explained by evaluating PEG surface property such as protein inhibiting ability and surface contact angles in different PEG chain density. Although PEG gives higher inhibition effect of non-specific adsorption as its molecular weight increases, surface doesn't have higher density by immobilizing just long PEG chain no matter how many times it is immobilized because of its excluded volume effect. Here is our strategy, filling the gap with shorter PEG chain several times instead of immobilizing just long PEG chain. In terms of effect of PEG chain density on the surface property, different immobilizations of PEG surface were characterized by SPR which provided novel insight into a basis for the surface modification technology. Thus, we achieved improving PEG chain density higher of the surface, which having great ability to inhibit non-specific protein adsorption. Using the surface studied, micro-patterning was performed by plasma etching technique, eventually, ϕ=100μm circular domains that were edge to edge spaced in l=100μm

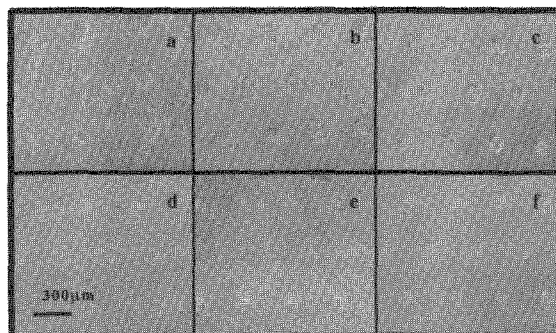

Figure3 Microscopic image of cell seeding study; 1day after culturing HUVEC onto micro-patterned PEG5k(3) surface (a), PEG5k(1)/ 2k(3) surface (b) and PEG5k(2)/ 2k(4) surface (c). 1week after culturing HUVEC onto micro-patterned PEG5k(3) surface (d), PEG5k(1)/ 2k(3) surface (e) and PEG5k(2)/ 2k(4) surface (f).

intervals were constructed. The surface property suggested that the micro-patterned surface thus constructed has great disparity between cyto-phobic and cyto-philic region from the surface property studies. Consequently, micro-cell-patterning was better performed in the surface having greater disparity between regions. And formed cellular pattern was maintained for more than 1 month. These cell culture studies showed good agreement with the surface property. It should be noted that precise surface property control in single-molecule order directly affected to micro-patterned cellular attachment, although large differences could not seen in surface property studies. Since micro-cell patterning is important technology for tissue/ cell based biosensors or tissue engineering, surface fabrication technique studied here is the promising technology in this field.

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