Video Article

Preparation of Thermoresponsive Nanostructured Surfaces for Tissue Engineering

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URL: https://www.jove.com/video/53465
DOI: doi:10.3791/53465

Keywords: Bioengineering, Issue 109, Langmuir film, Langmuir-Schaefer method, reversible addition-fragmentation transfer radical polymerization, nanostructured surface, thermoresponsive surface, cell adhesion, cell detachment, cell sheet

Date Published: 3/1/2016

Citation: Sakuma, M., Kumashiro, Y., Nakayama, M., Tanaka, N., Haraguchi, Y., Umemura, K., Shimizu, T., Yamato, M., Okano, T. Preparation of Thermoresponsive Nanostructured Surfaces for Tissue Engineering. *J. Vis. Exp.* (109), e53465, doi:10.3791/53465 (2016).

Abstract

Thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-immobilized surfaces for controlling cell adhesion and detachment were fabricated by the Langmuir-Schaefer method. Amphiphilic block copolymers composed of polystyrene and PIPAAm (St-IPAAms) were synthesized by reversible addition-fragmentation chain transfer (RAFT) radical polymerization. A chloroform solution of St-IPAAm molecules was gently dropped into a Langmuir-trough apparatus, and both barriers of the apparatus were moved horizontally to compress the film to regulate its density. Then, the St-IPAAm Langmuir film was horizontally transferred onto a hydrophobically modified glass substrate by a surface-fixed device. Atomic force microscopy images clearly revealed nanoscale sea-island structures on the surface. The strength, rate, and quality of cell adhesion and detachment on the prepared surface were modulated by changes in temperature across the lower critical solution temperature range of PIPAAm molecules. In addition, a two-dimensional cell structure (cell sheet) was successfully recovered on the optimized surfaces. These unique PIPAAm surfaces may be useful for controlling the strength of cell adhesion and detachment.

Video Link

The video component of this article can be found at https://www.jove.com/video/53465/

Introduction

Nanostructured surfaces have recently attracted substantial attention due to their various potential applications, including patterning, cell culture, cleaning, and surface switching. For example, superhydrophobic surfaces inspired by the nanostructure of the lotus leaf and other responsive surfaces are capable of reacting to external stimuli[1-4].

The Langmuir film is one of the most widely studied polymer coatings. A Langmuir film is formed by dropping amphiphilic molecules onto an air-water interface[5-8]. The film can then be transferred onto a solid surface by physical or chemical adsorption, and the molecular conformation on a solid surface can be controlled using vertical and horizontal transfer methods[9-12]. The density of the Langmuir film can be precisely regulated by compressing the air-water interface. Recently, this method has also proven effective for fabricating nanoscaled sea-island structures by utilizing amphiphilic block copolymers. The nanostructures are assumed to consist of a core of hydrophobic segments and a shell of hydrophilic segments[13-17]. In addition, the number of nanostructures on a surface is regulated by controlling the area per molecule (A_m) of the block copolymer at the interface.

We have focused on an original, unique scaffold-free tissue engineering approach, cell sheet engineering, using a temperature-responsive culture surface. The developed technology has been applied to regenerative therapies for various organs[18]. A temperature-responsive culture surface was fabricated by grafting poly(*N*-isopropylacrylamide) (PIPAAm), a temperature-responsive molecule, onto a surface[19-27]. PIPAAm and its copolymers exhibit a lower critical solution temperature (LCST) in aqueous media at temperatures near 32 °C. The culture surface also exhibited a temperature-responsive alternation between hydrophobicity and hydrophilicity. At 37 °C, the PIPAAm-grafted surface became hydrophobic, and cells readily attached and proliferated on the surface as well as on conventional tissue culture polystyrene. When the temperature was lowered to 20 °C, the surface became hydrophilic, and cells spontaneously detached from the surface. Therefore, cultured confluent cells on the surface could be harvested as an intact sheet by changing the temperature. These cell adhesion and detachment properties were also displayed by a surface fabricated by Langmuir film coating for laboratory demonstration[26, 27]. A Langmuir film of block copolymers composed of polystyrene (P(St)) and PIPAAm (St-IPAAm) was fabricated. The Langmuir film with a specific A_m could be horizontally transferred to a hydrophobically modified glass substrate. In addition, cell adhesion on and detachment from the prepared surface in response to temperature were evaluated.

Here, we describe protocols for the fabrication of a nanostructured Langmuir film composed of thermo-responsive amphiphilic block copolymers on a glass substrate. Our method may provide an effective fabrication technique for organic nanofilms in various fields of surface science and may facilitate more effective control of cell adhesion on and spontaneous detachment from a surface.

Protocol

1. Synthesis of Polystyrene-*block*-poly(*N*-isopropylacrylamide) by Two-step Reversible Addition-fragmentation Chain Transfer (RAFT) Radical Polymerization

1. Dissolve styrene (153.6 mmol), 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT; 0.2 mmol), and 4,4'-Azobis(4-cyanovaleric acid) (ACVA; 0.04 mmol) in 40 ml of 1,4-dioxane. Freeze the solution in liquid nitrogen under vacuum for 15-20 min to remove the reactive species and gradually thaw at RT. Make sure that the solution is completely thawed and repeat this freeze-pump-thaw degassing cycle three times.
2. Obtain the polystyrene (PSt) (Mw: 13,500) as a macro RAFT agent by polymerization at 70 °C for 15 hr in an oil bath.
3. Precipitate PSt macro RAFT agent with 800 ml of ether and dry *in vacuo*.
4. Dissolve IPAAm monomer (4.32 mmol), PSt macro RAFT agent (0.022 mmol), and ACVA (0.004 mmol) in 4 ml of 1,4-dioxane.
5. Remove the oxygen in the solution by the freeze-pump-thaw degassing cycles as mentioned in step 1.1.
6. Perform a polymerization at 70 °C for 15 hr in an oil bath after degassing. Obtain synthesized St-IPAAm molecule (Mw: 32,800) in the same manner as the PSt macro RAFT agent.

2. Preparation of Silanized Hydrophobic Modified Glass Substrates

1. Wash glass substrates (24 mm x 50 mm) with an excess of acetone and ethanol and sonicate for 5 min to remove surface contaminants.
2. Dry the substrates in an oven at 65 °C for 30 min. Then use oxygen plasma (400 W, 3 min) to activate the surfaces of the substrates at RT.
3. Immerse the substrates in toluene containing 1% hexyltrimethoxysilane overnight at RT to silanize the substrate.
4. Wash the silanized substrates in toluene and immerse in acetone for 30 min to remove unreacted agents.
5. Anneal substrates for 2 hr at 110 °C to thoroughly immobilize the surface.
6. Cut the silanized substrates by a glass cutter to 25 mm x 24 mm to fit the cell culture dishes (dish size: φ35 mm).

3. Preparation of Langmuir Films and Film-transferred Surface

1. Place the Langmuir film instrument in a cabinet to prevent the accumulation of dust.
2. Wash the Langmuir trough (size: 580 mm x 145 mm) and barriers with distilled water and ethanol to remove contaminants.
3. Dry the trough and barriers by wiping with a lintless towel. Then fill the trough with approximately 110 ml of distilled water, and set the barriers on both sides of the trough. Note that distilled water should be added without spilling in the following steps from 3.5 to 3.13.
4. Heat a platinum Wilhelmy plate (perimeter: 39.24 mm) for monitoring the surface tension with a gas burner until the plate turns red and then wash with distilled water to remove contaminants. Suspend the Wilhelmy plate on a wire attached to the surface-pressure-measurement instrument.
5. Zero the surface-pressure-measurement instrument according to manufacturer's protocol. Compress the air-water interface on the trough by the barriers on the both sides of the trough until the interface reaches approximately 50 cm^2 without any drops of polymer.
6. Aspirate small contaminants until the surface pressure is nearly 0 mN/m.
7. Reposition the barriers on both sides, and add distilled water to compensate for the decrease of distilled water from step 3.6.
8. Dissolve 5 mg of the synthesized St-IPAAm molecule in 5 ml of a development solution of chloroform.
   Note: Dichloromethane or toluene can also be used as the solvent.
9. Gently drop 27 µl of St-IPAAm dissolved in chloroform onto the trough using a microsyringe or micropipette.
10. After waiting for 5 min to allow complete evaporation of chloroform, move both barriers horizontally to compress the St-IPAAm molecule at the interface. Maintain compression rate of the barriers at 0.5 mm/sec until the target area of 50 cm^2 is reached.
    Note: A rapid compression rate causes defects in the Langmuir film.
11. Measure the surface pressure (π)-A_m isotherms with the platinum Wilhelmy plate attached to the surface-pressure-measurement instrument during compression according to manufacturer's protocol.
12. After reaching the target area size, maintain the surface for 5 min to allow the St-IPAAm molecules to relax; the molecules do not reach equilibrium immediately after compression.
13. Transfer the Langmuir film to a hydrophobically modified glass substrate using a transfer apparatus for 5 min to robustly adsorb the film. Fix the hydrophobic glass substrate in parallel on the device. Connect the device to an alignment stage and move perpendicularly.
14. Lift the substrate horizontally with the transfer apparatus and dry for 1 day in a desiccator.

4. Culturing Cells and Optimizing Cell Adhesion and Detachment on the Langmuir Film Transferred Surface

1. To prepare cell suspensions, culture bovine carotid artery endothelial cells (BAECs) to one third confluence at 37 °C in 5% CO_2 and 95% air on tissue culture polystyrene (TCPS) with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin.
2. After confluence is reached, treat BAECs with 3 ml of 0.25% trypsin-EDTA for 3 min at 37 °C in 5% CO_2 and 95% air.
3. Deactivate the trypsin-EDTA by adding 10 ml of the DMEM containing 10% FBS, and collect the cell suspension to a 50 ml conical tube.
4. Centrifuge at 120 x g for 5 min, and aspirate the supernatant. Re-suspend the cells with 10 ml of the DMEM.

5. Place the St-IPAAm surfaces under ultraviolet light on a clean bench for sterilizing for 5 min.
6. Seed the recovered cells on the St-IPAAm surfaces at a concentration of 1.0 x 10^4 cells/cm^2 counted by a disposable hemocytometer and observe the cells on the surfaces by a microscope equipped with an incubator at 37 °C with 5% CO_2 and 95% air.
   Note: Sterilize the St-IPAAm surfaces by ultraviolet light equipped to a clean bench.
7. Record time-lapse images of adherent BAECs for approximately 24.5 hr at 37 °C by a phase-contrast microscope with 10X magnification. After BAEC adhesion, record detachment of the BAECs from the St-IPAAm surface at 20 °C for approximately 3.5 hr.

5. Cell Sheet Fabrication on the Langmuir Film-transferred Surfaces

1. Culture BAECs used in the same manner described in Section 4.
2. Seed a total of 1.0 x 10^5 cells/cm^2 on St-IPAAm surfaces and incubate for 3 days at 37 °C in 5% CO_2. Confluent BAECs spontaneously detached at 20 °C.

Representative Results

Block copolymers composed of polystyrene and poly(*N*-isopropylacrylamide) (St-IPAAms) with specific molecular weights were synthesized by RAFT radical polymerization. ECT was prepared as a chain-transfer agent as described in Moad *et al.*[28]. Two St-IPAAm molecules of different PIPAAm chain lengths were synthesized, and the obtained block polymers were characterized by ^1H nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC). The molecular weights of St-IPAAms were 32,800 and 67,900, with a narrow molecular weight distribution (1.31 and 1.50). The monomer conversions of polystyrene macro RAFT agent and PIPAAm were found to be 17.4% and more than 85.0%. The synthesized St-IPAAms were named St-IPAAm170 and St-IPAAm480, respectively.

Langmuir films with various areas per molecule (A_m) were fabricated at an air-water interface by dropping St-IPAAm molecules dissolved in chloroform solution (**Figure 1A**). After dropping St-IPAAm molecules, the surface pressure of the air-water interface was fixed at 0 mN/m and continuously increased by closing the interface during compression with the barriers (**Figure 1B**). Any defects in Langmuir films formed on an air-water interface could be inferred from the π-A_m isotherm. The prepared St-IPAAm Langmuir film was transferred onto a hydrophobically modified glass substrate (St-IPAAm surface) (**Figure 1C**). The film-transferred surface was evaluated by atomic force microscopy (AFM). **Figure 2** shows AFM topographic images (1 x 1 µm) of the St-IPAAm170 and St-IPAAm480 surfaces. Nanostructures were observed on the St-IPAAm surfaces, while such structures were only rarely observed on the bare hydrophobic substrate. The size and shape of the nanostructures were strongly dependent on A_m and the composition of St-IPAAm. AFM topographic images confirmed that the Langmuir films could be homogeneously transferred onto the hydrophobic modified glass substrate and that the surface morphologies could be controlled by the molecular composition and A_m.

The stability of the St-IPAAm Langmuir film was evaluated by an attenuated total reflection Fourier-transform infrared spectroscope (ATR/FT-IR). The amount of PIPAAms on the hydrophobic glass substrate could be estimated by a calibration line obtained from peak intensity ratio of 1,000 cm^{-1} derived from glass (Si-O) and 1,650 cm^{-1} derived from PIPAAm (C=O). The calibration line was calculated from a series of known amounts of PIPAAm cast on the hydrophobic glass. The amounts of PIPAAm of St-IPAAm170 (10 nm^2/molecule) and St-IPAAm480 (40 nm^2/molecule) on the substrate were found to be 0.87 µg/cm^2 and 0.63 µg/cm^2, respectively. The amount of PIPAAm on the surface after washing with distilled water was almost the same as those on the surfaces without washing. These results indicated that the fabricated St-IPAAm surfaces were stable in a water condition.

We next examined the adhesion and detachment of bovine carotid artery endothelial cells (BAECs) on the St-IPAAm surfaces. Time-lapse photography of adherent and detaching BAECs on the St-IPAAm480 surface at 40 nm^2/molecule is shown in the Animated **Figure 1**. Adherent BAECs at 37 °C were rapidly detached from both St-IPAAm170 and St-IPAAm480 after decreasing the temperature to 20 °C. The number of adherent cells on St-IPAAm170 and St-IPAAm480 at 37 °C was 0.6 x 10^4 cells/cm^2 and 0.9 x 10^4 cells/cm^2, respectively, indicating that the number of adherent cells was modulated by the composition of the St-IPAAm surface. Cell sheets could also be recovered by detaching confluent cultured BAECs from these St-IPAAm surfaces. The recovered cell sheet was visualized as a two-dimensional cell structure (**Figure 3**). Cells reached confluence after three days in culture on St-IPAAm170 and St-IPAAm480 surfaces at 37 °C, and the cell sheet was rapidly recovered after reducing the temperature from 37 °C to 20 °C. The effect of molecular weight and A_m of St-IPAAms on cell sheet recovery is summarized in **Table 1**. The viability of cell sheets was evaluated by trypan blue staining after detachment of BAECs sheet. Cells treated with trypsin-EDTA on the glass substrate at 37 ºC were used as a control. Dead cell ratios on St-IPAAm170, St-IPAAm480 and the hydrophobic glass substrate as a control were calculated to be approximately 11.0%, 7.7%, and 11.7%, respectively. These results indicated that cell viability was hardly affected by temperature reduction.

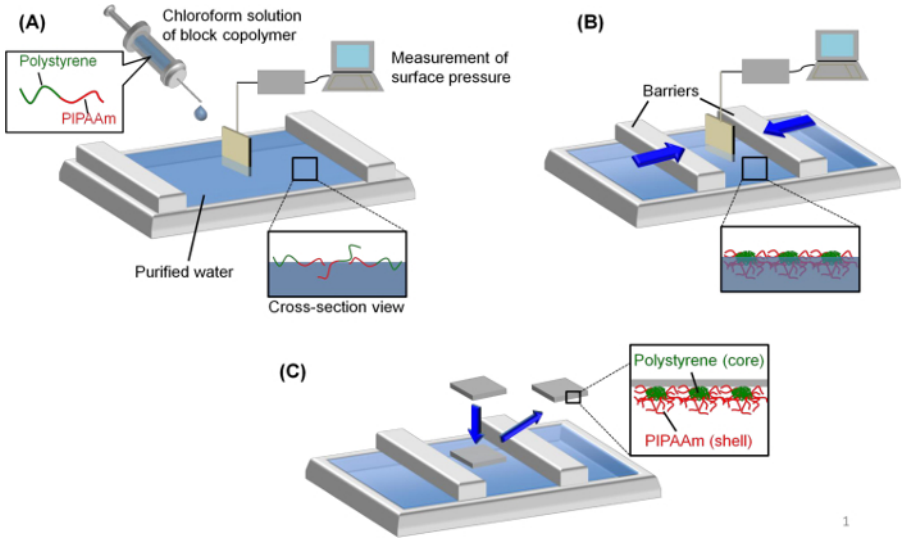


Figure 1: Preparation of a thermoresponsive Langmuir film-transferred surface. (**A**) The polystyrene-*block*-poly(*N*-isopropylacrylamide) (St-IPAAm) chloroform solution was gently dropped onto an air-water interface. The surface pressure was measured during compression to detect any defects in the Langmuir film. (**B**) Two barriers were used to compress the St-IPAAm molecules on the interface until a target area of 50 cm^2 was reached. (**C**) After compression, a hydrophobically modified cover glass substrate was horizontally placed on the interface with an alignment stage for 5 min. The substrate was lifted horizontally and dried for 1 day. This figure has been slightly modified from the publication by Sakuma *et al.*[27]

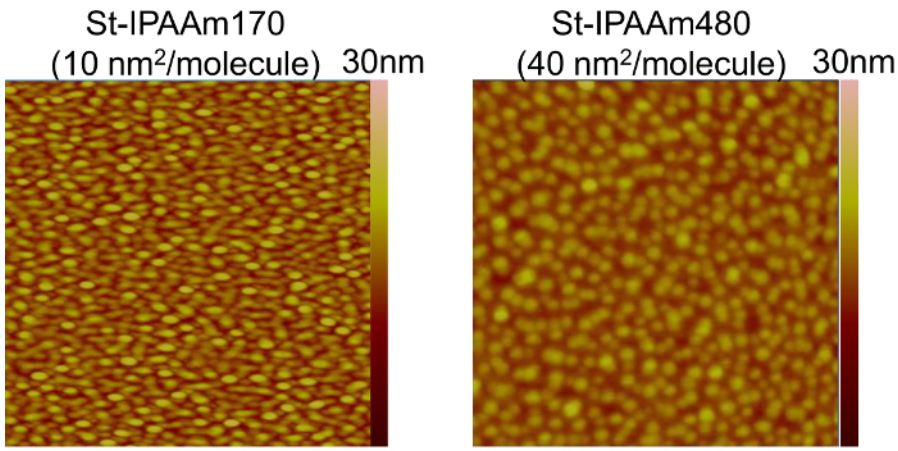


Figure 2: Atomic force microscopy (AFM) topographic images (1 x 1 µm) of Langmuir film-transferred surfaces (St-IPAAm surface). Left panel: Nanostructured surfaces of St-IPAAm170 with an A_m of 10 nm^2/molecule. Right panel: Nanostructured surfaces of St-IPAAm480 with an A_m of 40 nm^2/molecule. The AFM images were obtained in tapping mode using a phosphate-doped silicon cantilever with a spring constant of 3 N/m and resonant frequency of 70-90 kHz.

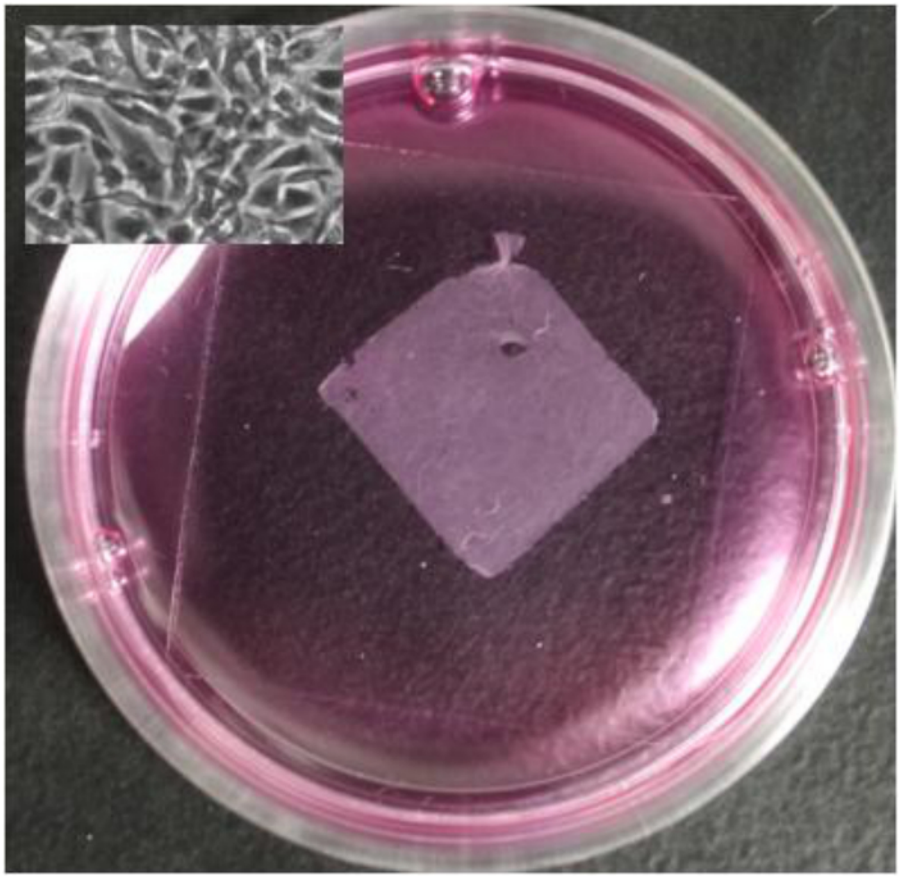
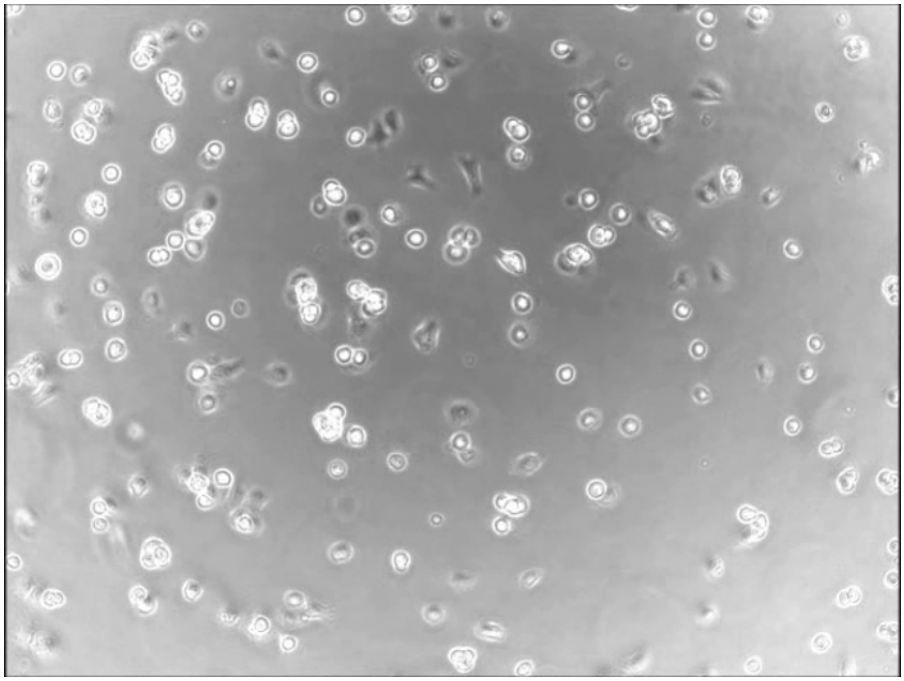


Figure 3: Macroscopic image of a recovered cell sheet on a Langmuir film-transferred surface with St-IPAAm480 and an A_m of 40 nm^2/molecule. Bovine carotid artery endothelial cells (BAECs) were seeded on St-IPAAm480 surfaces and incubated for 3 days at 37 °C. After reaching confluence, BAECs spontaneously detached at 20 °C, and a cell sheet was recovered after approximately 30 min. Inset image shows the confluently cultured BAECs on the surface observed by a phase contrast microscope (10X).



Animated Figure 1: (Right click to download). Time-lapse photographs of BAECs adhesion and detachment on a Langmuir film-transferred surface with St-IPAAm480 and an Am of 40 nm^2/molecule. The images were collected at 2-min intervals and the collected pictures were provided as a movie at 960x speed. The scale bar in the movie is 50 μm.

St-IPAAm170			St-IPAAm480		
3 [nm^2/molecule]	10 [nm^2/molecule]	40 [nm^2/molecule]	3 [nm^2/molecule]	10 [nm^2/molecule]	40 [nm^2/molecule]
Weak adhesion	Good	Hardly recovered	Weak adhesion	Weak adhesion	Good

Table 1: Bovine carotid artery endothelial cell (BAEC) sheet recovery of a Langmuir film-transferred surface with various densities and PIPAAm chain lengths. "Good" represents intact cell sheet recovery. "Hardly recovered" means that the cells could be confluently cultured and the cultured cells showed little or no detachment from the surface even after a reduction in temperature. "Weak adhesion" means that cells were not cultured to confluence at 37 °C for 3 days.

Discussion

A temperature-responsive surface was fabricated by the Langmuir-Schaefer method, and the surface properties for cell adhesion/detachment and cell sheet recovery were optimized. When using this method for the fabrication of surfaces, several steps are critical. The molecular composition of the St-IPAAm molecules has a great effect on the surface structure and the stability of the surface, and by extension, on cell adhesion and detachment. In particular, the St-IPAAm molecules should have a narrow molecular weight distribution. In our method, two St-IPAAm molecules with different PIPAAm chain lengths were synthesized by RAFT polymerization, allowing control of the molecular weight and molecular weight distribution.

Steps should be taken to prevent contamination of the air-water interface during the preparation of the St-IPAAm surface to avoid defects in the nanostructures. Before dropping polymer molecules onto the interface, contaminants should be aspirated until the surface pressure reaches approximately 0 mN/m. Contamination tends to accumulate around the edges of the trough and Wilhelmy plate. Because the Wilhelmy plate had some contamination on its surface, it was annealed. When a paper Wilhelmy plate is used, the aspiration step at the interface should be repeated at least twice. The π-A_m isotherm curve also depends on the presence of contamination at the air-water interface. We recommend that the curve of the isotherms be obtained more than once before the fabrication of the film. Because contamination of the hydrophobic modified glass substrate can also occur, the substrate should be blown with fresh air or nitrogen gas to remove contamination.

To consistently fabricate Langmuir film-transferred surfaces (St-IPAAm surface), the hydrophobic segment of a polymer is important because the hydrophobic interaction between the Langmuir film and hydrophobic modified glass is the driving force of the reaction. In this study, because the block copolymer was composed of polystyrene, which is strongly hydrophobic, and hydrophilic PIPAAm at room temperature, the transferred film was stably adhered to the substrate even under water or cell culture conditions. This indicates that hydrophobic segments play an important role in fabricating a robust St-IPAAm surface.

To control cell adhesion and detachment on a temperature-responsive surface, both the molecular composition and the precise control of density are important. In this Langmuir-Schaefer method, the area per molecule (A_m) of polymer synthesized by a given amount of dropped molecules and the target area of an air-water interface can be controlled by compression. The A_m of dropping St-IPAAm molecules can in theory be calculated by the following equation:

$$A_m = \frac{AM_w}{cN_AV}$$

where A is the area of the interface, M_w is the molecular weight of St-IPAAms, c is the concentration of the chloroform solution, N_A is Avogadro's number, and V is the volume of the polymer solution dropped. Nanoscale sea-island structures are observed on St-IPAAm surfaces after fabrication because amphiphilic polymers form self-organizing structures on an air-water interface, as described in previous studies[13-17]. The size and amount of nanoscale structures was controlled by A_m and the molecular weight of St-IPAAms.

Cell adhesion and detachment were evaluated on PIPAAm-coated surfaces fabricated by various methods, including electron beam irradiation, surface-initiated RAFT polymerization and spin coating[19-27]. Because density, molecular weight, and PIPAAm structure affect cell adhesion and detachment, precise techniques for fabricating PIPAAm-coated surface are important. In this Langmuir-Schaefer method, density, molecular weight and surface nanostructures could be controlled as described above. Cell adhesion and detachment on the St-IPAAm surfaces were drastically affected by various A_m and St-IPAAm molecular composition, and some conditions of St-IPAAm surfaces for cell adhesion and detachment could be further optimized. These results indicate that this method can control the interaction between the St-IPAAm surface and cells.

For reproducibly recovering intact cell sheet, the design of an amphiphilic polymer is most important. When only hydrophilic polymers are fabricated on a surface by a Langmuir-Schaefer method, coated polymers are easily washed out by distilled water or culture medium. This result indicated that the weak hydrophobic polymer should not be used for reproducibly recovering cell sheet in this method because the polymer modified-surface was unstable in a water condition. In our method, the hydrophilic segment in an amphiphilic polymer enables robust fabrication on a surface without washing out by an aqueous solution. In addition, since the Langmuir film was parallelly transferred to a glass substrate in this study, nano-scaled sea-island structures were observed on the surface. Although the structure of Langmuir film on the basal side is well known to be different from that on the apical side, the structure was fixed by a parallel transference.

Cell therapy and regenerative medicine have been focused on the possibility of healing patients who are unresponsive to other treatments. Our laboratory has developed an original, unique scaffold-free tissue engineering approach using cell sheet engineering fabricated on a temperature-responsive culture surface with potential applications in tissue engineering. Several clinical trials with cell sheets are already underway and have demonstrated successful outcomes for several types of tissues[29-31]. Although BAECs were used as the cell source to fabricate cell sheets, other cell sources, including stem cells, can be used to fabricate sheets on a St-IPAAm surface by optimizing the combinations of A_m

and the molecular composition of St-IPAAm molecules. This non-conventional technology will contribute not only to the easy production of nanostructured surfaces but also to fundamental cell culture strategies for use in regenerative medicine.

Disclosures

All authors contributed equally to writing the manuscript and have approved the final version. The authors declare that they have no competing financial interests.

Acknowledgements

This study was financially supported by the Creation of Innovation Centers for Advanced Interdisciplinary Research Program's Project for Developing Innovation Systems "Cell Sheet Tissue Engineering Center (CSTEC)" of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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