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Construction of a Chondrocyte Cell Sheet Using Temperature-Responsive Poly(*N*-isopropylacrylamide)-co-Acrylamide

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Abstract— **In this study, a novel temperature-responsive poly(N-isopropylacrylamide)-co-acrylamide was used to prepare a chondrocyte cell sheet. Chondrocytes were isolated from human articular cartilage and plated on the copolymer film grafted tissue culture plates. The cell attachment on the copolymer film was shown to be similar to that of the ungrafted surface. To harvest a cell sheet, the incubation temperature was reduced to 10°C for 30 minutes to allow the polymer chain to fully extend, changing the copolymer's phase from hydrophobicity to hydrophilicity. Additional incubation at 20°C for 60 minutes was necessary to activate the cellular metabolism required for cytoskeletal organization and cell detachment. A complete cell sheet recovery was achieved when a PVDF membrane was used as a cell sheet carrier. Unfortunately, the shrinkage of the cell sheet was observed. Nonetheless, the harvested cell sheet was shown to be viable and healthy.**

I. Introduction

Cell sheet engineering has received much attention in the recent years as a new approach of tissue engineering without the use of scaffold. Confluent cultured cells are harvested as an intact sheet using a temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PNIAM) and its derivatives [1]. Above the low critical solution temperature (LCST) of the polymer, the polymer becomes hydrophobic, allowing the cells to attach and grow. When the temperature was reduced to below its LCST, the polymer chains undergo rapid hydration and chain extension, making the polymer hydrophilic [2]. Due to different surface properties, the cell layer, together with the underlying extracellular matrix, is detached from the surface [3]. The extracellular matrix recovered from the culture surface allows for cell sheet stratification, making the construction of multi-layered cell sheets possible.

Temperature-responsive culture surfaces are now commercially available under the name "UpCell™" (Cell Seed, Inc., Tokyo, Japan). These special surfaces are fabricated by grafting PNIAM homopolymer onto tissue culture polystyrene dishes using electron beam (EB) irradiation. This cultureware is approximately 5 times more expensive than regular tissue culture products, making the construction of multi-layered cell sheets significantly expensive. In addition, the geometries of the cell sheets are constrained by the availability of the UpCell surfaces, limiting some applications in regenerative medicine. Therefore, a simple and inexpensive method to create temperature-responsive culture surfaces for customized applications is desired.

Recently, our research group has developed a novel method to graft PNIAM onto tissue culture surfaces using UV polymerization [4]. A UV light source is significantly cheaper than an electron beam instrument. However, UV irradiation was not as efficient as EB irradiation. To overcome this limitation, acrylamide (AM) monomer was added to NIAM monomer to help graft PNIAM onto polystyrene surfaces via radical polymerization using UV. AM possesses a high activity amide ($-NH_2$) group that would allow covalent bonding to other molecules even when polymerized at low irradiation intensity. According to our result, the copolymer film exhibited hydrophilicity-hydrophobicity transition at around 30 – 35°C, indicating that the addition of acrylamide did not interfere with the temperature-responsive property of PNIPAM. Mouse pre-osteoblast MC3T3-E1 cells were selected as a cell model in this study. The cells grew as successfully on the grafted surface as those on cultured on un-grafted surface. When the temperature was reduced to 10°C for 60 minutes, followed by 20°C for 60 minutes, the confluent cells could be detached as an intact sheet. The doubled and tripled layer cell sheets were shown to have tissue-like structure with relative abundant extracellular matrix proteins throughout the sheet.

Currently, partial thickness articular cartilage defects can be repaired using autologous chondrocyte implantation [5]. Although the clinical outcome is promising, several problems remain including low cell numbers, control of the location of the injected cells and incorporation to the host tissue. To overcome these problems, implantation of a chondrocyte cell sheet is suggested as an alternative.

In this study, human chondrocyte cell sheets were prepared using the temperature-responsive polymer, poly(*N*-isopropylacrylamide-co-acrylamide) (PNIAM-co-AM), grafted surface. An appropriate protocol to construct the cell sheet was determined. The cell viability of the harvest cell sheet was also evaluated for further clinical applications.

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II. Material and Methods

A. Preparation of PNIAM-co-AM grafted Tissue Culture Surface

Commercial 6 well plates were first exposed to UV (6W, 265 nm) for 30 minutes to activate the surface. Afterwards, a mixture of NIAM (1 M) and AM (1 M) monomers at 1:1 molar ratio, MBAM (20 mM) as a cross-linker, and KIO_4 (5 mM) as a photoinitator was added to the culture surface, incubated overnight at room temperature, and protected from light. The excess solution was removed from the plates before 1 hour UV exposure (6W, 265 nm). The PNIAM-co-AM grafted surfaces were rinsed with ethanol to remove unreacted monomer and dried in a vacuum chamber. For cell culture use, these surfaces were sterilized using acidic ethanol (70% EtOH, pH = 2) and washed twice with phosphate buffer saline (PBS). In all experiments, un-grafted commercial 6 well plates were used as a control.

B. Isolation and Culture of Human Chondrocytes

Human articular cartilage was obtained from a patient aged 42 years old, hospitalized in Siriraj Hospital, Bangkok, Thailand. A cartilage fragment was removed from a healthy region by arthroscopic biopsy and transported in an iced-cold sterile tube containing Hank's Balanced Salt Solution (HBSS). A sterile scalpel was used to cut the cartilage into small pieces. The cartilage suspension was washed twice with PBS and centrifuged to remove PBS. The chondrocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (1% penicillin-streptomycin and 1% Amphotericin B) at 37°C, in a 5% CO_2 humidified incubator until use. Cell morphology was observed using a bright-field inverted microscope (Sundrew MCXI600, Vienna, AUSTRIA).

C. Construction of a Chondrocyte Cell sheet

To construct a cell sheet, chondrocytes were plated on the control and PNIAM-co-AM grafted surfaces at a density of 2×10^6 cells/ml. The cells were cultured until confluence. The culture medium was removed from each well and a hydrophilically modified poly(vinylidenefluoride) (PVDF) membrane was placed over the confluent cell layer. An additional 100 µl of the fresh medium was added over the membrane to prevent dryness. To detach the cell sheet, the culture was incubated at 10°C for 30 minutes, followed by incubation at 20°C for 60 minutes. The membrane, together with the cell sheet, was peeled off using forceps and transferred to a new culture surface. The areas of the attached and detached cells were analyzed using ImageJ 1.44 software.

To release the membrane, the cell sheets, still attached to the membrane, were transferred to a Matrigel™ coated tissue culture dish and incubated incubated at 37°C for 1 - 2 hours to promote attachment to the new basal layer. Finally, the PVDF membrane was gently peeled off after adding fresh medium to the edge of culture dish, and the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

The viability of the recovered cell construct was examined using LIVE/DEAD® Assay Kit (L-3224, Invitrogen, U.S.A.).

III. Results And Discussion

A. Cell Attachment on the PNIAM-co-AM Grafted Surface

Primary human chondrocytes were cultured on both the ungrafted and the PNIAM-co-AM grafted surface at 37°C, as shown in Fig. 1. The cells grew and proliferated similarly on both culture surfaces, exhibiting uniform fibroblast-like morphology. The growth rates of the chondrocytes cultured on both surfaces were found to be similar, indicating that the copolymer film did not interfere with the metabolic activity of the cells.

B. Cell Sheet Detachment

The protocol to detach chondrocyte cell sheet was modified from the previous protocol developed for mouse pre-osteoblast cells, MC3T3-E1 [4]. Briefly, in the first step, the cells on the grafted surface was incubated at 10°C for 30 minutes to allow the polymer chain to be hydrated and fully extended. However, at this temperature, the metabolic activity of the cells was suppressed. As a result, the cells did not undergo cytoskeletal organization necessary for cell detachment. Thus, in the second step, the incubation temperature was increased to 20°C for 60 minutes to reactivate the cellular metabolic process.

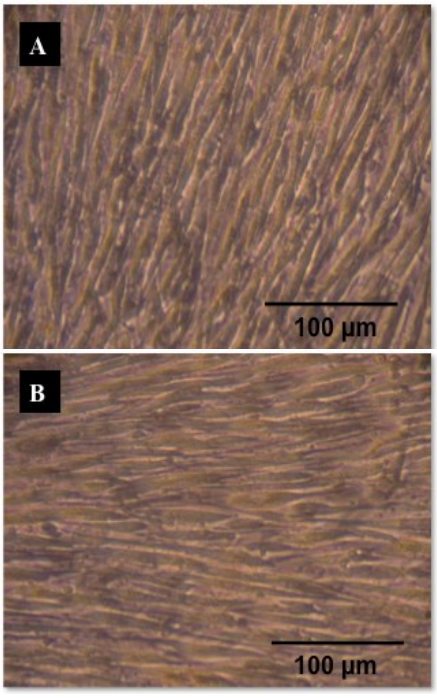


Figure 1. Human chondrocytes cultured on (A) the ungrafted and (B) PNIAM-co-AM grafted surface. Scale bar = 100 µm

In this study, the cells were allowed to reach 100% confluency to form strong tight junctions. Cells' tight junctions are important in the construction of a cell sheet to help pull adjacent cells away from the surface and enable the

cells to be connected into a layer without breakage. According to Fig. 2, after the low temperature treatment, the chondrocyte cell layer was slowly lifted off from the surface around the edge of the culture well toward the center of the sheet (Fig. 2b). Unlike the cells on the grafted surfaces, the cells on the control surface remained well attached regardless of temperature (Fig. 2a).

Table 1 shows the percentage of the cell detached area from the ungrafted and PNIAM-co-AM grafted surfaces. The cell detachment was performed with and without a PVDF membrane. Without the PVDF membrane, a cell layer was allowed to lift off from the surface primarily from the phase transition of the copolymer. Afterwards, a manual pipette was used to transfer the recovered cell sheet to another tissue culture dish for further investigation. As expected, the majority of the cells remained attached to the ungrafted surface. Only about 1% was detached. On the other hand, without the use of the PVDF membrane, approximately 70% of the cells were detached from the PNIAM-co-AM grafted surface after the temperature reduction. Most of the remaining cells on the grafted surface were observed near the edge of the well. It is possible that the density of the grafted PNIAM-co-AM film on each culture well might not be uniform. Lower density of the copolymer film was grafted around the edge of the well, resulting in the difficulty in the cell detachment. We hypothesized that the bottom of a tissue culture well plate may not be flat, leading to an uneven layer of the monomer solution and eventually different polymer thickness.

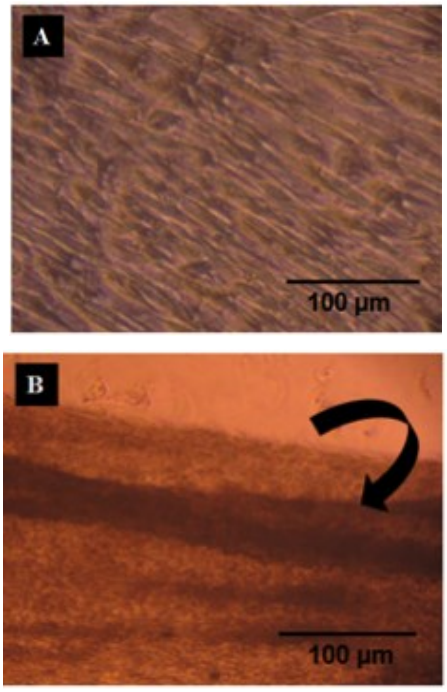
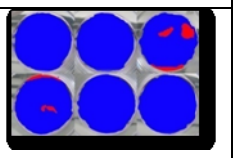
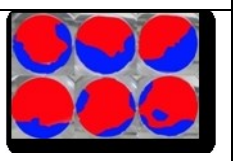
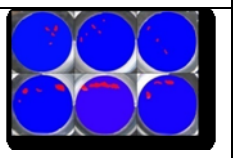
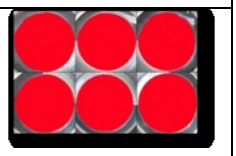


Figure 2. Images of the chondrocyte cells on (A) the ungrafted and (B) PNIAM-co-AM grafted surface after incubation at 10°C for 30 minutes, followed by additional incubation at 20°C for 60 minutes.

TABLE 1. THE AREA OF CELL DETACHMENT USING DIFFERENT CONDITIONS. NOTE THAT BLUE COLOR REPRESENTS THE AREA THAT THE CELLS REMAINED ATTACHED, WHILE THE RED COLOR REPRESENTS THE AREA THAT THE CELLS HAD ALREADY DETACHED.

Conditions		% of the detached area	Image analysis of the culture well
Without the PVDF membrane	Ungrafted surface	1 ± 3.31%	
	PNIAM-co-AM grafted surface	69 ± 11.45%	
With the PVDF membrane	Ungrafted surface	1 ± 1.04%	
	PNIAM-co-AM grafted surface	100	

When a PVDF membrane was used to harvest the cell sheet, a complete intact chondrocyte cell sheet (100% detachment) was achieved after the low temperature incubation. Thus, the PVDF membrane is considered necessary in harvesting a cell sheet.

After the PVDF membrane was removed, the chondrocyte cell sheet significantly shrank to approximately 30% of its original size (Figure 3). It has been reported that the cells in the cell sheet tend to contract due to the tension of cell-cell interaction and cell-matrix interaction [6]. Although the use of PVDF membrane has been shown to support the cell sheet structure, the shrinkage of cell sheet is still unavoidable.

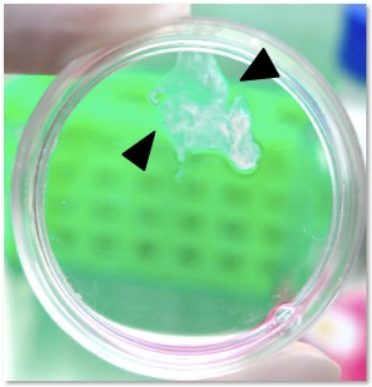


Figure 3. The harvested chondrocyte cell sheet after the PVDF membrane was removed.

Recently, a ring shaped membrane, in which the center of the membrane was cut out, was used to harvest cell sheets without shrinkage [7]. Another option would be to use

cell-adhesive and transparent gelatin hydrogel as a cell sheet carrier [8].

C. Viability of the Harvested Cell Sheet

The harvested cell sheet was placed on a Matrigel-coated surface and re-cultured for two days to let the cells completely adhere to the surface of the new culture dish. Because the cell sheet retained essential extracellular matrix on the basal side, the cell sheet could adhere to the new surface relatively more rapidly than single cells. The quality of the harvested cell sheet was examined using morphological observations and live/dead staining. Live cells were stained green, while red color indicated cell death. According to Fig. 4, the cell population was observed as green cells without any presence of red cells. The morphology of the chondrocytes in the cell sheet appeared fibroblast-like, similar to those in a conventional monolayer culture. In addition, the harvested cells started to spread and proliferate to the nearby area, confirming that the cells were still healthy and viable even after the low temperature treatment and cell manipulation process.

Although this study has demonstrated that PNIAM-co-AM grafted tissue culture plates could be successfully used to construct chondrocyte cell sheets, more thorough studies are required to assess the quality the cell sheet in depth. For example, the cellular properties of the chondrocytes should be investigated using the gene expression profile. Primary chondrocytes have been known to dedifferentiate upon monolayer cultivation, leading to the lower production of collagen and proteoglycan [9]. As a result, it is important to ensure that the chondrocyte cell sheets possess correct phenotype and genotype for the treatment of articular cartilage defects. In addition to the gene expression profile, the mechanical behavior of the chondrocyte cell sheet is also an important issue. The changes between the Young's modulus of a single chondrocyte cell sheet and that of the multilayered cell sheet would provide understanding on the chondrocyte-matrix interaction. This would be beneficial for the development of a tissue construct for cartilage repair.

IV. Conclusion

We successfully harvest a human chondrocyte cell sheet from temperature responsive PNIAM-co-AM grafted surfaces. The chondrocytes grew well and attached on the copolymer film, similar to those on the ungrafted surface. To harvest a cell sheet, the cells were allowed to grow to confluence. Afterwards the incubation temperature was reduced to 10°C for 30 minutes, followed by addition incubation at 20°C for 60 minutes. 100% cell detachment was achieved when a PVDF membrane was used. However, significant shrinkage of the cell sheet occurred. Nonetheless, the harvested chondrocyte cell sheet was shown to be healthy and viable.

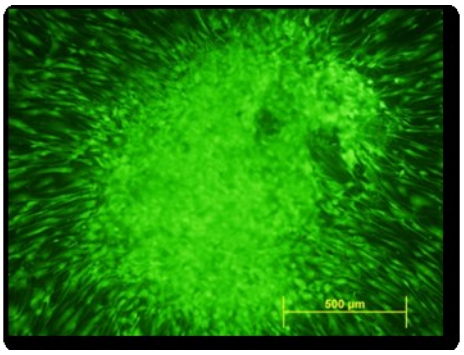


Figure 4. The chondrocyte cell sheet was stained with LIVE/DEAD stain, in which green color indicated viable cells, while red color indicated dead cells. Note that the scale bar represents 500 µm

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