THE TEMPERATURE RESPONSIVE POLY (N-ISOPROPYLACRYLAMIDE-CO-ACRYLAMIDE) GRAFTED CULTURE SURFACE FOR CELL SHEET ENGINEERING

MISS SOPITA WONG-IN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOLOGICAL ENGINEERING)
FACULTY OF ENGINEERING
KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI
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Thesis Title	Temperature Responsive Poly (*N*-isopropylacrylamide-co-acrylamide) Grafted Culture Surface for Cell Sheet Engineering
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Abstract

A novel temperature-responsive tissue culture dish is prepared by using ultraviolet irradiation to graft poly *N*-isopropylacrylamide-co-acrylamide (PNIAM-co-AM) onto commercial tissue culture polystyrene surface (TCPS). These surfaces are able to promote cell adhesion/detachment by controllable hydrophobicity of the surface. The physical properties of this temperature-responsive surface were investigated by dynamic contact angle measurement, Atomic Force Microscopy (AFM) and Fourier Transform Infrared Spectroscopy (FTIR). The *in vitro* toxicity of polymer grafted surface was evaluated by using MTT assay. The PNIAM-co-AM was found to be non-toxic. The cell attachment/detachment on the graft copolymer film was investigated by using the mouse fibroblast cell line (L929), mouse pre-osteoblast cell line (MC3T3-E1), and human chondrocyte primary cells as cell models. Our results demonstrated that L929, MC3T3-E1, and chondrocyte cells could adhere and grow on the grafted surface similar to those on the ungrafted TCPS under normal condition at 37°C. When temperature was reduced below 32°C, the confluent L929, MC3T3-E1 cells, and chondrocyte cells could be detached as single cells and L929, MC3T3-E1 sheet without any enzymatic treatment. The harvested cells could be grown on a new tissue culture dish to promote reattachment of the cells. The cell sheet harvested using the low temperature treatment which is preserved the ECM and adhesive factors, allowing the sheet to adhere to another culture surface or other cell sheets as a double-layered sheet. This cell sheet was in a good condition as indicated by LIVE/DEAD stain.

Keywords: temperature-responsive polymer / UV grafting / L929 cells / MC3T3-E1 cells / human chondrocyte cell / cell attachment / cell detachment / single layer cell sheet /double layer cell sheet

หัวข้อวิทยานิพนธ์	การสังเคราะห์พื้นผิวที่ตอบสนองต่ออุณหภูมิด้วยโคพอลิเมอร์ชนิด Poly (*N*-isopropylacrylamide-co-acrylamide) เพื่อนำไปใช้ในการสร้างเซลล์ชีท
หน่วยกิต	12
ผู้เขียน	นางสาวโศภิตา วงศ์อินทร์
อาจารย์ที่ปรึกษา	ผศ.ดร.ขวัญชนก พสุวัต
หลักสูตร	วิทยาศาสตรมหาบัณฑิต
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คณะ	วิศวกรรมศาสตร์
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บทคัดย่อ

ภาชนะเลี้ยงเซลล์แบบใหม่ที่สามารถตอบสนองต่ออุณหภูมิได้ ถูกสร้างขึ้นโดยการกราฟต์โคพอลิเมอร์ชนิดพอลิเอ็นไอโซโพรพิลอะคริลาไมด์โคอะคริลาไมด์ (poly (*N*-isopropylacrylamide-co-acrylamide) (PNIAM-co-AM)) ลงบนภาชนะเลี้ยงเซลล์แบบพอลิสไตรีน (TCPS) โดยภาชนะเลี้ยงเซลล์แบบใหม่นี้มีคุณสมบัติเหมาะสมในการเกาะและการหลุดออกของเซลล์เมื่อมีการควบคุมความชอบน้ำและไม่ชอบน้ำของพื้นผิวชนิดนี้ได้ การตรวจสอบคุณสมบัติทางกายภาพของภาชนะเลี้ยงเซลล์ที่สามารถตอบสนองต่ออุณหภูมินี้ทำได้โดยใช้การวิเคราะห์ค่ามุมสัมผัส การวิเ คราะห์พื้นผิวด้วยแรงอะตอม และการตรวจสอบเอกลักษณ์เฉพาะ โดยเปรียบเทียบระหว่างพื้นผิวการเลี้ยงเซลล์ปกติแบบพอลิสไตรีนและพื้นผิวที่ถูกกราฟต์ด้วยโคพอลิเมอร์ วิเคราะห์ความเป็นพิษของพื้นผิวที่ถูกกราฟต์ก่อนนำมาใช้ศึกษากับเซลล์โดยการทดสอบความมีชีวิตของเซลล์ด้วยวิธี MTT assay ซึ่งผลการทดสอบพบว่าพื้นผิวไม่เป็นพิษสามารถนำมาใช้ในการเลี้ยงเซลล์ได้ การศึกษาการเกาะ (cell attachment) และการหลุดออก (cell detachment) ของเซลล์ ได้ทดสอบกับเซลล์ไลน์สองชนิด คือ mouse fibroblast cell line (L929) และ pre-osteoblast cell line (MC3T3-E1) รวมทั้งได้มีการทดสอบกับเซลล์ปฐมภูมิหนึ่งชนิด คือเซลล์กระดูกอ่อนของมนุษย์ (human chondrocyte cell) จากการศึกษาพบว่า เซลล์ทั้งสองชนิดสามารถเกาะแผ่และเจริญเติบโตบนพื้นผิวที่ถูกกราฟต์ด้วยโคพอลิเมอร์ที่อุณหภูมิ 37 องศาเซลเซียส เช่นเดียวกับภาชนะเลี้ยงเซลล์ปกติที่ไม่ถูกกราฟต์ เมื่อลดอุณหภูมิลง ต่ำกว่า 32 องศาเซลเซียส เซลล์สามารถหลุดออกจากพื้นผิวที่ถูกกราฟต์เป็นเซลล์เดี่ยว (single cells) และเซลล์ชีท (cell sheet)ได้ โดยไม่ต้องอาศัยเอนไซม์ ซึ่งเซลล์ชีทที่หลุดออกมาสามา รถสร้างเป็นสองชั้นโดยการวางเซลล์ชีทแผ่นแรกลงบนเซลล์ชีทแผ่นอื่นโดยการใช้เมมเบรน เซลล์ ที่หลุดออกเป็นเซลล์ชีท (cell sheet) สามารถนำไปถ่ายเลี้ยงบนพื้นผิวที่เคลือบด้วยโปรตีนใหม่ได้ โดยที่เซลล์ยังคงมีชีวิตรอดเมื่อทำการทดสอบด้วยการย้อมสีเซลล์

คำสำคัญ: พอลิเมอร์ที่ ตอบสนองต่ออุณหภูมิ / การกราฟต์ด้วยรังสียูวี / เซลล์ L929 / เซลล์ MC3T3-E1 / เซลล์กระดูก อ่อน / การเกาะของเซลล์ / การหลุดของเซลล์ / เซลล์ชีทแบบหนึ่งชั้น / เซลล์ชีทแบบสองชั้น

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PNIPAM	=	Poly(*N*-isopropylacrylamide)
NIPAM	=	*N*-isopropylacrylamide
AM	=	Acrylamide
PNIAM-co-AM	=	Poly(*N*-isopropyl acrylamide-co-acrylamide)
MBAAm	=	N,N'-Methylenebisacrylamide
KIO4	=	potassium periodate
LCST	=	Lower critical Solution Temperature
TCPS	=	Tissue Culture Polystyrene
ATRP	=	Atom Transfer Radical Polymerization
XPS	=	X-ray Photoelectron Spectroscopy
ToF-SIMS	=	Time of- Flight Secondary-Ion Mass Spectroscopy
FT-IR	=	Fourier transform Infrared Spectroscopy
AFM	=	Atomic Force Microscopy
SEM	=	Scanning Electron Microscopy
DMSO	=	Dimethylsulfoxide
PVDF	=	Poly(vinylidene difluoride)
EDTA	=	Ethylene Diamene tetraacetic Acid
PBS	=	Phosphate Buffered Saline
DMEM	=	Dulbecco's Modified Eagle's Medium
Alpha-MEM	=	Alpha-Minimal Essential Medium
Pt/St	=	Penicillin-Streptomycin
FBS	=	Fetal Bovine Serum
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDL	=	Periodontal Ligament cell
Cells/ml	=	Cells per milliliter
ECM	=	Extra Cellular Matrix
g	=	molar (mole per liter)
m	=	meter
mg	=	milligram
ml	=	milliliter
µl	=	microliter

CHAPTER 1 INTRODUCTION

1.1 Motivation

Tissue engineering is a technology that applies the principles of engineering and life sciences to develop biological substitutes that can restore, maintain, or improve tissue function [1]. This technology uses biodegradable polymer scaffolds for cell seeding to direct the cell growth into the three dimensional tissues as target tissue shapes. Various tissues include: cartilage, bone, blood vessel, and urinary bladder have been reconstructed. Although this technology has been applied to many tissues, there are still some limitations. The cells harvested by trypsinization lose cell surface proteins, which are important for the interactions between the cell and its environment. For large constructs, cells in the center of the constructs will die due to insufficient oxygen, nutrients and removal of metabolic waste [2]. Thus, this method is not suitable for complex tissue such as cardiac muscle, liver and kidney. To overcome these problems, anew tissue engineering technique has been developed without using biodegradable polymer scaffolds. This method is called "cell sheet engineering". This technology uses temperature-responsive culture dish to detach cells in the form of cell sheet [3]. Temperature-responsive culture plates are prepared by surface modification with poly(*N*-isopropylacrylamide)(PNIPAM) and its derivatives, which enables reversible cell adhesion to and detachment from the dish by controlling hydrophobicity of the surface. Previous studies have been shown that various types of cells cultured on the temperature-responsive culture at 37°C attach, spread and proliferate similarly to those on un-grafted tissue culture polystyrene (TCPS) dishes [4]. When the temperature is reduced below the lower critical solution temperature (LCST~32°C), this polymer changes to hydrophilic phase. Subsequently cells are detached from the temperature-responsive surface due to rapid hydration and swelling of the grafted PNIPAM surface without enzyme (Figure 1.1).

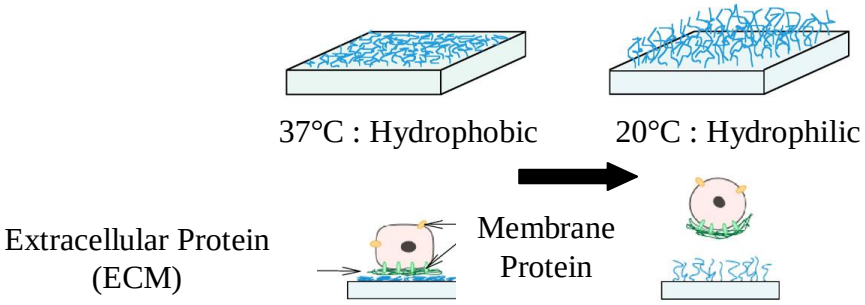


Figure 1.1 Cells attach onto temperature-responsive surfaces at 37°C. By lowering the temperature below 32°C, the surfaces become non-adhesive to cells [5].

By lowering the temperature, the confluent cells are harvested as single, continuous cell sheets with intact cell-cell junctions and deposited extracellular matrix (ECM) protein. In contrast, cell harvesting by enzymatic digestion results in the disruption of both adhesive proteins and membrane receptors (Figure 1.2) [6]. The harvested cell sheets have been used for various tissue reconstructions, including ocular surfaces, periodontal ligaments, cardiac patches, and bladder augmentation [7].

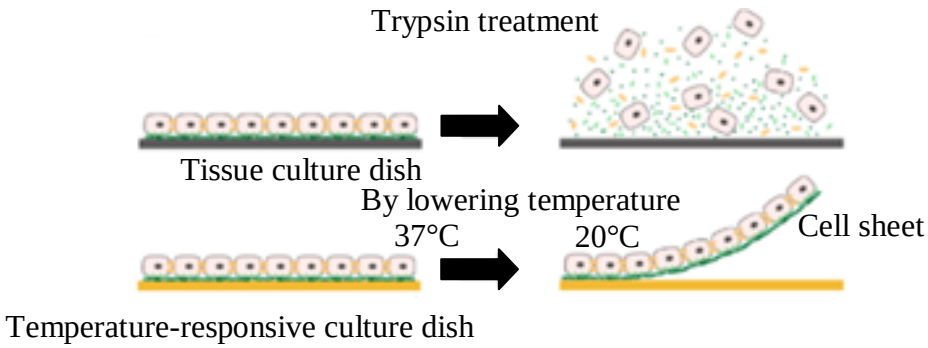


Figure 1.2 By lowering temperature, cell sheet preserved cell junction and deposited protein [5].

There are several methods to create the temperature-responsive culture dish for cell culture, such as, UV irradiation, Electron Beam (EB) irradiation, Plasma polymerization, 'Grafting to' approach, 'Grafting from' approach, and surface-initiated living radical polymerization (ATRP) [3]. Recently, various tissue regeneration without a scaffold has been studied using a commercial-available temperature-responsive culture dish (UpCell™CellSeed Inc., Tokyo, Japan), in which PNIAM is grafted by Electron Beam (EB) irradiation [8]. The fabrication process is expensive due to the requirement of specialized equipment and well-trained personnel. In addition, the culture surface is only limited to the size available in the market, making it difficult to create a larger tissue construct. Therefore, a more economical technique to graft PNIAM onto tissue-culture surface is desired.

In this work, the surface modification of tissue culture polystyrene (TCPS) dish with poly(*N*-isopropyl acrylamide-co-acrylamide)(PNIAM-co-AM) using UV irradiation was investigated. UV irradiation was performed using an in-house equipment. The method is easy and inexpensive. The physical properties of the grafted surface were characterized and subsequently used to culture and detach various cell types, including cell lines and primary cells. The cell sheets harvested from PNIAM-co-AM grafted surface were used to construct multi-layer tissue for future tissue engineering applications.

1.2 Objectives

1. To synthesize temperature-responsive polymer using UV irradiation. The physical and biological properties of the grafted surface were characterized.
2. To evaluate the ability of the grafted surface to attach and detach various cell types as single cells and cell sheets.
3. To harvest the cells as intact sheets by simple temperature changes, without proteolytic enzymes and held together by normal cell junctions and extracellular matrix (ECM). The cell sheets were re-grown on different culture surfaces.
4. To compare the properties of the cell sheet detached from PNIAM-co-AM grafted culture surface synthesized by UV irradiation with that of the commercial PNIAM-co-AM grafted culture surface synthesized by electron beam irradiation.

1.3 Scope of study

1. The temperature responsive surface was made of PNIAM-co-AM and synthesized using UV irradiation method.
2. The types of cells used in the study included L929, MC3T3-E1 and human chondrocyte cells.

3. The commercial PNIAM-co-AM tissue culture dishes were UpCell^{TM}, prepared by electron beam irradiation.

1.4 Expected output

1. Confirmation that the grafted surface has temperature sensitive properties and can be used in cell-sheet engineering.
2. A protocol to harvest mono-layered and double-layered sheets from the thermo-responsive polymer, PNIAM-co-AM, grafted surface for further cell sheet applications.

CHAPER 2 LITERATURE REVIEWS

2.1 Cell Sheet Engineering

Recently, a new tissue engineering technique involving the cell sheet was reported to be successful for some tissues [9]. Many published papers have been progressing rapidly on the cell-based therapies, regenerative medicine, and tissue engineering. For the single cell suspensions injection, the injected cells are expected to remain around the damaged host tissues for the maintenance and recovery of native functions. Nonetheless, the injected cells cannot be preserved around the target tissuein most cases because of difficulties to control the size, shape and location of the injected cells [10]. On the other hand, with the biodegradable scaffolds constructs, cells in the center become necrotic though the cells on the periphery are unimpaired. This is due to the limitedgas diffusion. That is, insufficient delivery of oxygen and nutrients and removal of metabolic waste. Furthermore, strong inflammatory responses are often observed upon biodegradation of the scaffolds. Therefore, a new method base on cell sheet engineering was used to avoid the use of bio-degradable scaffolds for the further development of tissue engineering [7]. Prof. Okano who is professor of Tokyo Women's Medical University has established "cell sheet engineering" as a method for new medical treatments. He discovered that cell sheets construct can be connected to organs or tissues also functionally. Prof. Okano says, “If we could prepare various cell sheets and fully utilize the abilities of the cells, transplantation medicine without donors may be possible” [11].

2.2 Temperatures-Responsive Cell Culture Surfaces

Cell sheet engineering consists of temperature-responsive culture surface, which enables reversible cell attachment/detachment by hydrophobicity control. It allows for a non-invasive harvest of cultured cells as an intact cell sheet containing ECM protein under cell layer [10].

In biomedical material research should concern about interactions between cells and cultured surfaces. Therefore, various cultured surfaces have been developed to control cellular events after they contact to the surface. However, these surfaces are mono-functional and irresponsive to stimuli. Lately, there are many studies about temperature-responsive cell culture surfaces that respond to stimuli such as temperature [12]. Such temperature-responsive surface has been performed by grafting several types of highly hydrophilic polymers. These surfaces are causative to a material’s ‘biocompatibility’ because it is resistant to non-specific protein adsorption and cell adhesion. Moreover, these modify surface are able to maintain the biomaterials functionality for many applications such as membranes, bio-implants and sensors [13].

2.3 Poly (*N*-isopropylacrylamide), PNIPAM

Among the several temperature-responsive polymers, poly(*N*-isopropylacrylamide) (PNIPAM) is a novel temperature-responsive polymer that they utilized for regenerative medicine. It shows a reversible of structure which from coil to globule formation in aqueous solution across its lower critical solution temperature (LCST) at 32°C [14]. The chemical structure of this polymer is consisted of the hydrophobic groups (isopropyl) and hydrophilic groups (amide). Above 32°C, the PNIPAM chains are extended and the amide groups were interacted with water molecules through hydrogen bridges [15]. In particular, temperature-responsive polymers have been prepared using poly (*N*-isopropylacrylamide) (PNIPAM) and its derivative. Typical water contact angles on the surfaces of PNIPAM change reversibly in aqueous solution with temperature. They show

hydrophilic and hydrophobic characteristic at 10 and 37°C, respectively (Figure 2.1). The PNIPAM-grafted surfaces are hydrophobic above the LCST. Cells adhere, spread, and proliferate on the surfaces are similarly to commercial tissue culture dishes (TCPS). When lowering temperature below its LCST, this polymer changes to hydrophilic phase. The cellis spontaneously detach from the surfaces without trypsinization [13]. There are reported that the LCST of NIPAM copolymers can be controlled by adding copolymer, which can lead to very interesting biomedical applications [14].

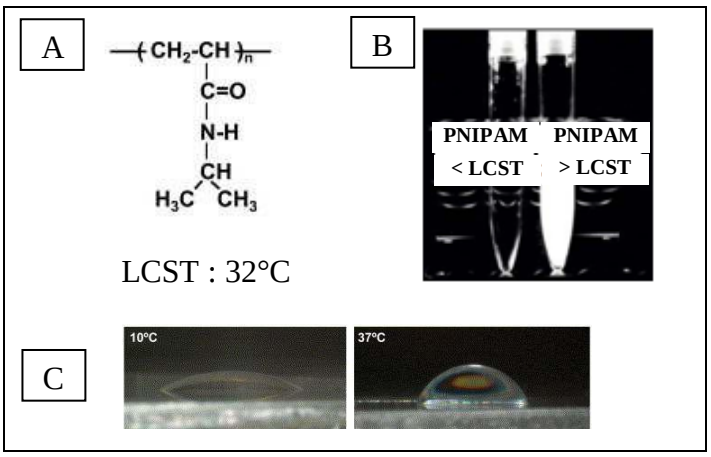


Figure 2.1 Structural formula of Poly *N*-isopropylacrylamide (PNIPAM) (A). It changed in aqueous solution (B) and temperature-dependent wettability (C) [16].

Another important feature about temperature-responsive surface, is the effect of polymer property for cell adhesion and cell detachment such as the thickness and graft amounts of PNIPAM on the surfaces. Cells do not attach to the dish even at 37°C when the PNIPAM grafted is too thick. Because at 37°C, normal TCPS are hydrophobic, complexes that reject water are created by strong hydrophobic bonding in the area of surface contact between polystyrene and PNIPAM. If the grafted surface is thin, the surface is hydrophobic enough to adhesion of the cells as a result of the influence of the interface between polystyrene and PNIPAM. When the PNIPAM layer is thick, cells do not attach to the surface because there is less influence from the interface on the grafted surface [11]. Therefore, the thickness of this polymer grafted surface was studied. The surface wettability changes between lower and above the LCST of PNIPAM are observed for all grafting densities. The temperature-dependent cell adhesion and detachment are only observed on PNIPAM grafted surfaces with 15–20 nm thick. When the grafted layer is thicker than 30 nm, no cell adhesions occur. Mainly, cells adhere on the hydrophobic surfaces better than hydrophilic surfaces [10].

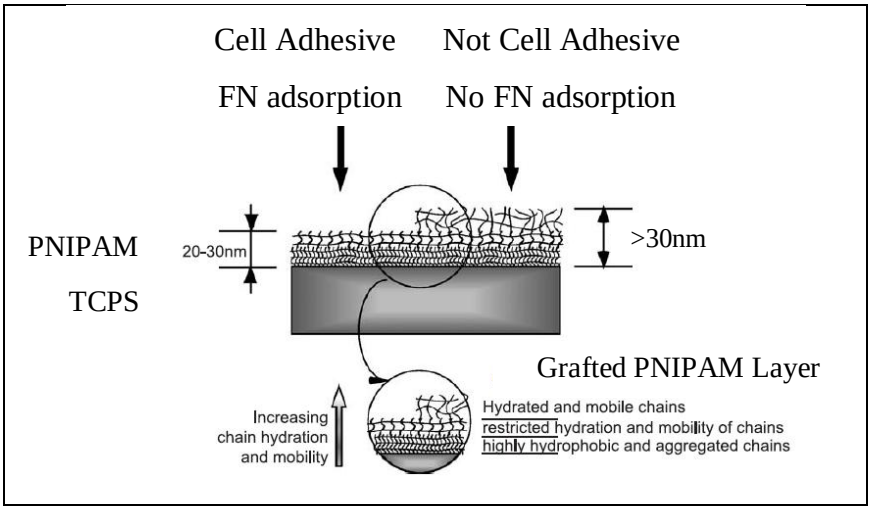


Figure 2.2 Effect of grafted polymer densities on cell adhesion behavior [16].

There must be controlling PNIPAM surface graft density is a critical issue to consistently produce temperature-responsive cell attachment/detachment. The influence of molecular flexibility of grafted chains of PNIPAM layers was shown in Figure 2.2

At 37°C, grafted PNIPAM chains are highly hydrophobic due to strong hydrophobic interaction with the hydrophobic polystyrene interface. These chains aggregate and are immobilized with extended dehydration on TCPS surfaces. This nature of the grafted PNIPAM chains affects the second layer of PNIPAM-grafted chains, which are then also restricted in their mobility, and thus hydration with water molecules is also limited. PNIPAM-grafted surfaces with such restricted chain mobility and limited hydration capabilities are likely to have temperature regulated cell adhesion and adhesion properties. PNIPAM-grafted surfaces with grafted polymer thickness ranging from 20 to 30 nm. The third layer of PNIPAM grafts consists of relatively hydrated and less restricted PNIPAM chains. Graft thicknesses more than 30 nm, no cell-adhesive proteins, interact strongly enough to influence cell attachment [16].

It's obvious that the stronger dehydration and restriction of mobility are obtained between the grafted PNIPAM chain and interface of polystyrene surface. Cell adhesion are observed, thickness is in between 15 and 20 nm. No cell adhesion occurs when grafted PNIPAM layer is thicker than 30 nm [10].

2.4 Poly (*N*-isopropylacrylamide); PNIPAM Grafted Surface Fabrication

The temperature-responsive polymer surfaces were fabricated by grafting poly (*N*-isopropylacrylamide) and its derivatives on the material surfaces. They have been used for regenerative medicine applications [13]. There are different methods to graft PNIPAM on surfaces [6]. Some of polymerization method will be discussed briefly as well.

2.4. 1Radiation-induced Graft Polymerization

Different of polymerization methods have been reported to graft PNIPAM on to surfaces. Radiation-induced grafting methods have been widely used for the surface modification of biomaterials. Many types of reactions can be used, such as UV irradiation, Electron Beam (EB) irradiation, plasma activation and plasma polymerization.

1. Ultraviolet (UV) irradiation

As one of the major techniques developed to achieve surface modification of polymeric materials, UV-induced surface graft polymerization has been applied to improve the surface properties of polymers [17]. Using UV irradiation has been reported that the monomer and photo-initiator were poured into TCPS dishes and irradiated by UV light (365 nm). Grafted PNIPAM was analyzed by X-ray photoelectron spectroscopy (XPS). PNIPAM grafted PS dishes were studied with mouse fibroblast cell lines. The ultraviolet grafted polymerization could potentially be a suitable method for biomaterial surface modification because of its simplicity [18].

Surface graft induced by UV irradiation exhibit some advantages when they were compared with other modification methods. Such as fast reaction rate, low cost of processing, simple equipment, easy industrialization, and maybe the most important, the distribution of grafted chains is limited to a shallow region near the surface [17].

2. Electron Beam (EB) irradiation

The other radiation grafting methods are electron beam (EB) irradiation. Uniformly spread monomer was polymerized and immobilized onto a tissue culture polystyrene (TCPS) dish surface by using the Electron Beam (EB) irradiation. Various types of cultured cells adhere and proliferateon hydrophobic PNIPAM-grafted TCPS surfaces at 37°C. However, they did not attach to hydrated surfaces (hydrophilic) at 20°C. Cells were cultured on hydrophobic PNIPAM-grafted TCPS and detached from hydrophilic surface due to PNIPAM's hydration/dehydration alteration at the LCST (~32°C) [13]. It has been reported that the irradiation by electron beam, the PNIPAM is simultaneously fixed to the surface and polymerized. This technique led to the establishment of cell sheet engineering [11]. The EB irradiation method facilitates the large-scale production of temperature-responsive TCPS. However, it requires the use of expensive equipment [13]. The commercial temperature-responsive polymer, the UpCell™ surface is grafted surface which is based on this technique.

3. Plasma Polymerization

Plasma polymerization is a solvent free and vapor-phase coating method (Figure 2.3). Plasma deposited coatings generally have excellent physical properties. They exhibit conformal, sterile, complete surface coverage and excellent adhesion to different substrates. The plasma-polymerized NIPAM coatings reported thatan excellent retention of the monomer structure after plasma deposition. Subsequently, the phase transition property of PNIPAM is successfully retained in PNIPAM. Excellent chemical and physical properties achieved by plasma coating technique open the exciting prospect of preparing temperature-responsive surfaces [6].

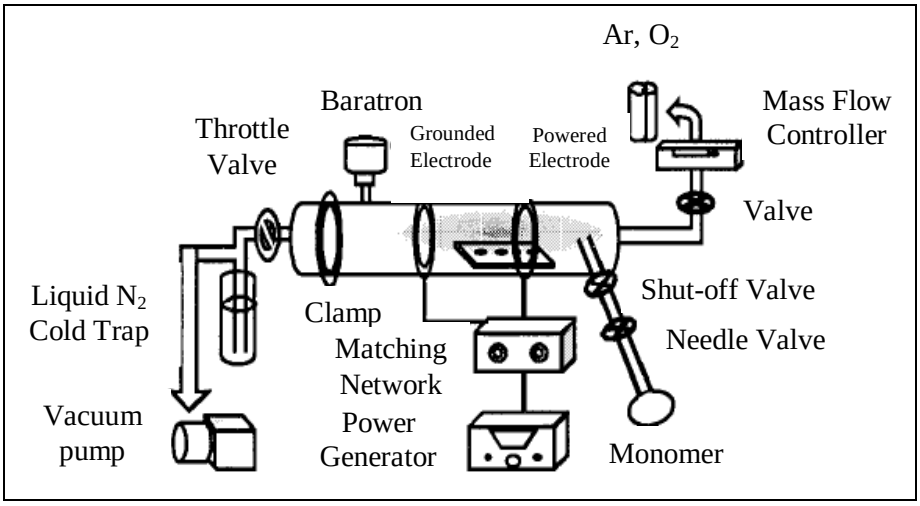


Figure 2.3 Schematic diagram of a radio frequency plasma polymerization reactor [6]

Although, this methodis simplifies a one-step method to fabricate thermally responsive coatings on any substrate but it is not suitable for large-scale production because difficult to control size of polymer [3].

2.4.2 'Grafting to' Approach

The covalent immobilization of polymer chainsonto a substrate surface by coupling reactions ("grafting to" techniques) [17] consists of two procedures that are preparing the polymer with functional groups and reacting the polymer with the solid surface. The advantage of this method is adjustment the polymerization conditions to control the molecular weights of the grafted polymer chains. However, the polymer grafted densities are limited because of the steric effect of the surface functional groups [3].

2.4.3 Surface-initiated Living Radical Polymerization

Atom transfer radical polymerization (ATRP) is a novel technique that has been developed to prepare well-defined polymer brush layers on the surfaces. This method enables the preparation of surfaces with dense polymer brushes from surface-immobilized ATRP initiators. The dense polymer brush layers exhibit specific properties that different from the dilute brush layers were prepared by 'grafting to' or conventional 'grafting from' approaches.

2.5 Surface Characterizations

The Instrumental analysis is important techniques that are used to characterize the PNIPAM-grafted surface. Such surfaces were analyzed by several techniques such as dynamic contact angle measurement, X-ray photoelectron spectroscopy (XPS), time of-flight secondary-ion mass spectroscopy (ToF-SIMS), Atomic Force Microscope (AFM), Attenuated total reflection (ATR/FTIR)[13].

2.5.1 Contact Angle Measurement

The dynamic contact angle was used to study the properties of insoluble PNIPAM surfaces as shown in Table 2.1. The values from this technique are important for supporting cell growth. Nevertheless, the cell adhesion on insoluble PNIPAM-grafted surfaces cannot be described only in terms of surface wettability, because the surfaces are only partially dehydrated at above the LCST. Therefore, we should concern in other

variables such as swelling ratio, molecular mobility, chain density and concentration of the hydrophobic groups [19].

Table 2.1 PNIPAM grafted surface properties by changing its grafting density (Modified from N. Matsuda *et al.* 2007)

Density of grafted PNIPAM (μg cm^{-2})		1.4±0.1	2.9±0.1
Thickness of the grafted		15.5±7.2	29.3±8.4
Contact angle (degrees)	37°C	77.9±0.6	69.5±1.2
	20 °C	65.2±1.2	60.0±0.1
Cell adhesion	37°C	Yes	No adhesion
Cell detachment	20 °C	Yes	Not determined

2.5.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is a quantitative surface analytical tool that sensitive to the atomic composition of a material [20]. The composition of PNIPAM post-cell liftoff surfaces and PNIPAM post-protein adsorption surface were analyzed by this method. However, using XPS are not able to clearly identify which specific protein species are present at the surface [21]. Data from XPS was compared with Time of-flight secondary-ion mass spectroscopy (ToF-SIMS) that it is considered to be a complementary technique.

2.5.3 Atomic Force Microscope (AFM)

The atomic force microscopy is used to measure the property of sample surfaces by sensing the force between a very sharp tip and the sample surface. The advantages of AFM are (a) it generates 3-dimensional surface image, (b) it does not required special sample treatment, (c) it can operate in both air and liquid [22].

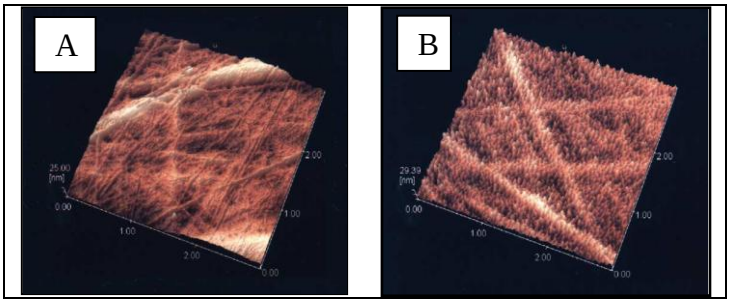


Figure 2.4 AFM images of polystyrene culture dish surfaces (A) and temperature-responsive culture dish surfaces (B) [2]

The AFM images that shown in Figure 2.4. Non-grafted, polystyrene culture dish surfaces and poly(*N*-isopropylacrylamide)-grafted culture dish surfaces were examined in air. Small needle-like pattern can be seen on the grafted surface [2].

2.5.4 Attenuated Total Reflection (ATR/FTIR)

Attenuated total reflection using Fourier Transform Infrared (ATR-FTIR) spectroscopy is considered one of simple, directly, flexible and sensitive in situ infrared technique for substances dissolving in aqueous solutions. Many studies have been done using the sample was pressed to make the pellets using potassium bromide (KBr). Nevertheless, the potassium bromide is difficult to prepare a pellet completely free of contaminating moisture. Therefore, ATR accessory was also combined with KBr method to investigate

the grafted on PS. Attenuated total reflection technique involves the collection of radiation reflected from the interface of the sample. This technique operates by measuring the changes that occur in a totally internally reflected infrared beam when the beam comes into contact with a surface of the sample [23].

2.6 Mechanism of Cell Detachment

The interactions of material surfaces with cells were categorized intopassive and active adhesion. The cell detachment is induced by the hydration of the polymer's chains, followed by an active step, where cells undergo shape changes due to metabolic processes toachieve detachment.The passive phase of the cell detachment was induced by the temperature drop and hydration of PNIPAM chains, effecting initial detachment. This initial detachment stimulates the active phase (shape change) that coordinated by cell metabolic processes [16].

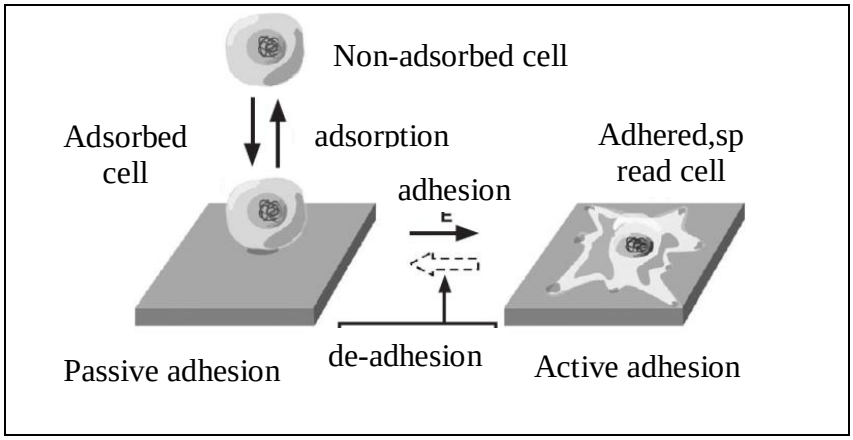


Figure 2.5 Schematic diagrams of materials surfaces and cells interactions [16]

The role of cellular metabolism in the detachment process was supported by observing less detachmentat lower temperatures (4 and 10°C), at which suppressed cell metabolism was observed [24]. When the temperature of culture dishes originally incubated at 37°C is decreased that start to hydrate of polymer chain below 32°C. Although lower temperatures provide more hydrated PNIPAM chains but reduce cell metabolism. This remarkable hydration change initiates cell detachment [25].

This reversible phenomenon is due to the rapid hydration of the polymer on the culture surface during cell culture. When a medium temperature is decreased below the LCST of grafted polymer, the cells lose their flattened morphology. This step, they acquire morphological changes and a rounded appearance (Figure 2.5-2.6) [12]. Therefore, cells are able to float free from the dishes, after mild agitation, without enzymatic treatment, which is usually required for cell detachmentfrom un-grafted TCPS dishes. These morphology changes are due to a reorganization of the cytoskeletal of cells [18].

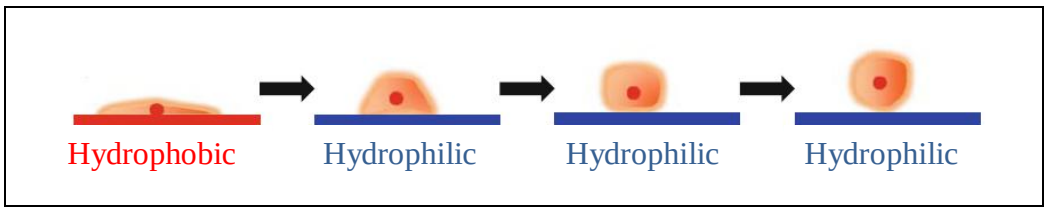


Figure 2.6 Schematic illustration, cells lose their flattened morphology [26]

2.7 Cell Sheet Manipulation

The rapid recovery of single cell and cell sheets is important for maintaining the biological functions and viability of cell sheets. It also leads the reduction of time necessary for the practical assembly of tissue structures. Although, some of cell sheet detachment can be further accelerated by pipetting or agitation but recovered cell sheets are friable because these cells weakened cell-to-cell junctions. Therefore, spontaneous recovery of cell sheet is strongly required [26].

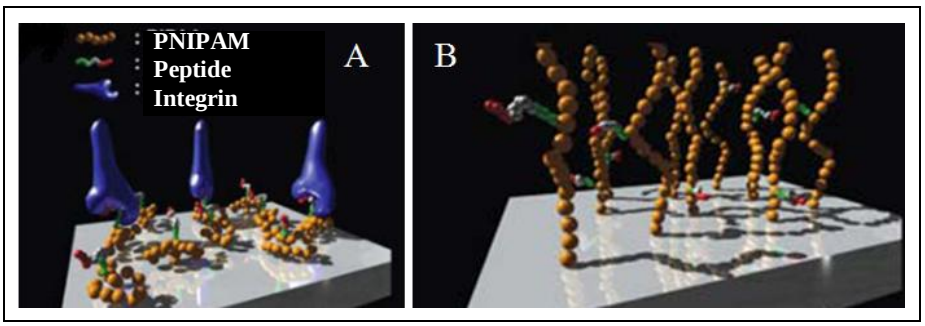


Figure 2.7 Schematic of affinity control between integrin receptors and biomolecule-immobilized the temperature-responsive TCPS on hydrophobic phase (A), and hydrophilic phase (B) [26].

Upon lowering temperature, the grafted PNIPAM is suddenly hydrated and extends outwards to shield the peptides from integrin access, resulting in decreased binding affinity between integrin and peptides, followed by cell detachment from the surface (Figure 2.7) [26]. Optimum temperatures for the cells recovering from PNIPAM grafted surfaces are different in each cell types such as 20°C for endothelial cells and 10°C for hepatocytes cells [10]. According to the mechanism of cell detachment, cells begin to detach from the periphery of the dish surfaces, maintaining cell-cell connections and producing some sheet contractility and supporting membranes (Figure 2.8) [24] such as poly(ethylene terephthalate) membranes, poly(vinylidenedifluoride) (PVDF) membranes or chitin membranes have been used to manipulate the cell sheets without cells agglomeration. The cell sheets can be harvested by using the supporting membranes with physicochemical interaction such as interfacial tension. Cell sheet was transferred to other cell sheets with adhesive effects of ECM that still remain on the basal surface of the recovered cell sheets to construct multilayer cell sheet [10].

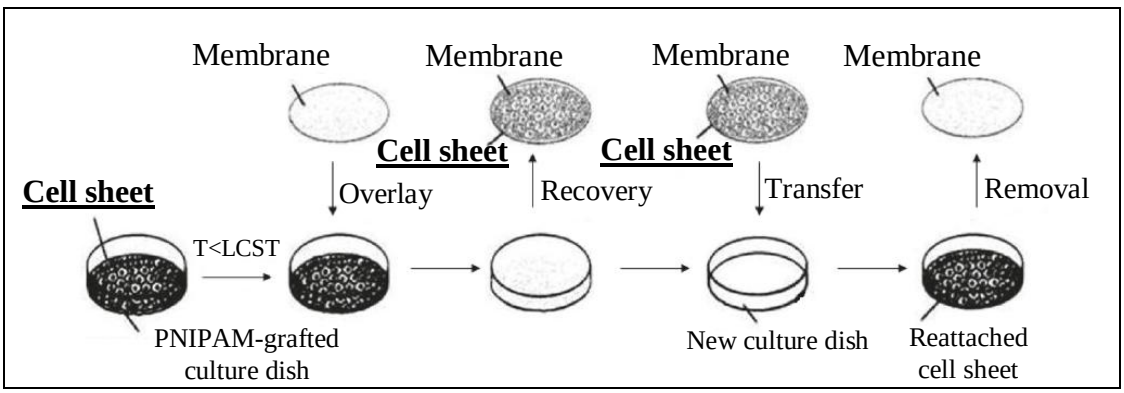


Figure 2.8 Schematic of cell sheets is harvested by membrane supporting [24].

2.8 Applications of Cell Sheet Engineering

Several different cell sheet constructs have been created in vitro using some variation of the cell type. These constructs were then transplanted into *ex vivo*, *in vivo*, and living organisms. They successfully adhered to the native tissue [24]. As the cell attachment/detachment responses on PNIPAM grafted surfaces are important factor for noninvasive recovery of cell sheets for further applications of tissue and organ reconstruction [27]. Therefore, these harvested cell sheets can be used for various tissue reconstructions, including ocular surfaces [28], periodontal ligaments [29], urothelial cell sheets [2], cartilage regeneration [8], cardiac patches [30], bladder augmentation [2] ,and The treatment of diabetes mellitus [31]. In the following sections, we demonstrate a more detailed how these cell sheets can be used in regenerative medicine.

2.8.1 Ocular Surface Reconstruction

The first clinical application of cell sheets harvested from temperature-responsive culture dishes used epidermal cell sheets which used for ocular surface reconstruction. This application used to investigate the possibility of cell sheet for ocular surface reconstruction, using autologous oral mucosal epithelial stem cells. Human epidermal cell sheets prepared using temperature-responsive culture dishes show better adhesion target than similar cell sheets harvested by enzymatic treatment [2]. There have been reported that cultured oral epithelial cells were non enzymatically harvested as transplantable intact cell sheets by reducing culture temperature to 20°C (Figure 2.9). Oral epithelial cells were stratified in three to five cell layers more similar to corneal epithelium than to oral mucosal epithelium.They successfully transplanted autologous tissue engineered epithelial cell sheets fabricated from harvested oral mucosal epithelium in a rabbit model. Cell sheet harvest technology enables fabricationof tissue-engineered epithelial cellsheets that preserve native cells from oral mucosal epithelial cells. Promising clinical capabilities for ocular surface reconstruction are indicated [32].

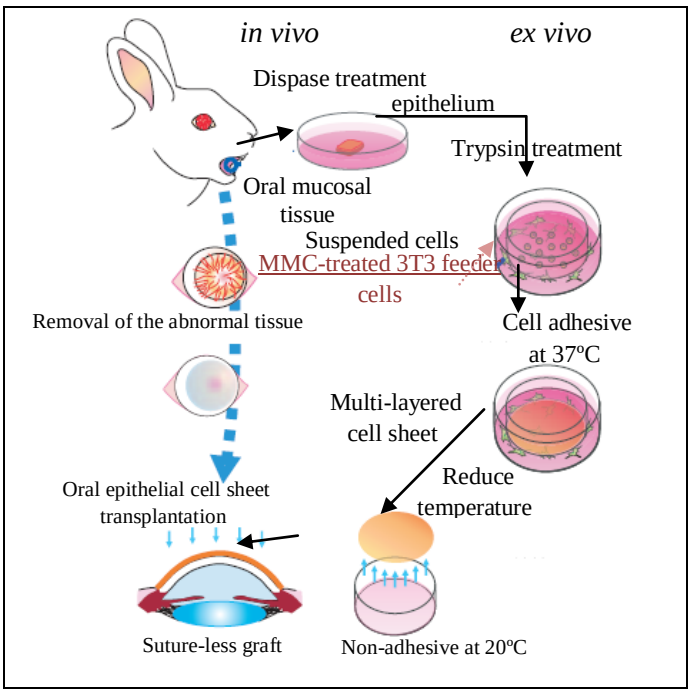


Figure 2.9 Ocular surface reconstruction by transplantation of cell sheets [32]

2.8.2 Periodontal Ligament Cell Sheets

Since conventional methods are insufficient to attain complete andconsistent clinical regeneration of periodontal tissues, patients suffer from periodontitis, halitosis, and tooth loss. Periodontal ligament cell sheet techniques were applied to this problem. Human periodontal ligament cell sheets harvested from temperature-responsive culture dishes were treated into a mesial dehiscence model (Figure 2.10) [2]. Another work, periodontal ligament sheet transplantation reported that six weeks after human periodontal ligament cells were transplanted to animal model. Most of the dentin surfaces showed a newly immature cementum-like tissue formation and periodontal ligament with perpendicular orientation inserted into the newly deposited cementum-like tissue.

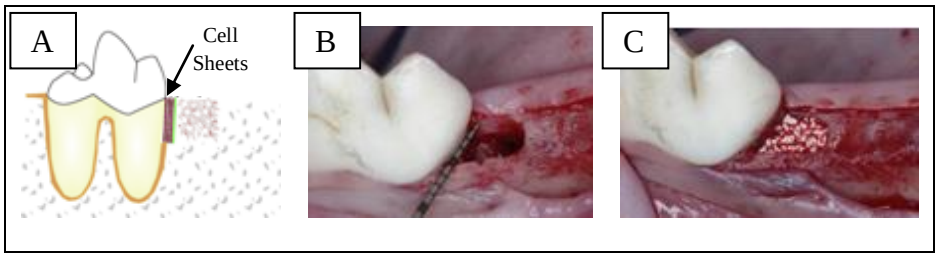


Figure 2.10 Schematic illustration of artificial periodontal defects (A) and animal transplantation were transplant by three-layered PDL cell sheets (B, C) [33].

It is obvious that the multilayered human periodontal ligament cell sheet can be potential to reconstruct the architecture of a periodontal ligament–cementum complex [34].

2.8.3 Urothelial Cell Sheets

The intact urothelial cell sheets were created by using thermo-responsive culturedish[2]. These cell sheets consisted of one to four layers, and contracted slightly after detachment from the culture dish surface. The urothelial cell sheets are grafted on demucosalized gastric flaps, resulting five of eight flaps showed urothelial regeneration in canine model. The cell sheets are attached spontaneously to the flap without any suture or fixation, generating a multilayered urothelium *in vivo* [10].

2.8.4 Cartilage Regeneration

The repair of the partial thickness defect of articular cartilage is requirements for the future treatment of Osteoarthritis. Actually, cell sheet technology might be a useful tool for the treatment of partial-thickness defect of articular cartilage. In culture cell, chondrocyte sheets were adhesive to the partial thickness defect model of porcine articular cartilage. The partial thickness defect model, to which the chondrocyte sheets adhered to the partial thickness defect model that covered by chondrocyte sheets. Another transplantation of cell sheet in cartilage regeneration had been reported. The chondrocyte sheet, synovial cell sheet, and anterior cruciate ligament sheet were studied. They found that chondrocyte sheets have good adhesion to the partial-thickness defect of rabbit articular cartilage [35]. Although the clinical results of chondrocyte implantation for articular cartilage defects have recently improved as a result of advanced techniques based on tissue engineering but problems with cell handling continue to be explained. Chondrocyte sheets relevant to cartilage regeneration can be prepared with the cell sheet technique. However, for clinical application, it is necessary to evaluate the characteristics of the cells in these sheets and to identify their similarities to native cartilage [8].

2.8.5 Myocardial Cell Sheet Tissue Reconstruction

In addition to two and three-dimensional cell sheet manipulation has been used in cardiac tissue engineering. The cell transplantation therapy to repair impaired hearts has inspired further attempts for bioengineering three dimensional heart tissues from cultured cardiac myocytes. Cardiac tissue engineering has also been pursued using conventional technology with biodegradable polymer scaffolds as a temporary ECM. However, the scaffolds is inflexible and significantly hinders the dynamic pulsation of cardiac myocytes [2]. In the development of cardiac tissues for the treatment of severe heart failure, they have layered cardiomyocyte sheets to create patch-like structures. Cardiomyocyte sheets harvested from temperature-responsive culture dishes are cell-dense like the native myocardium. When these layered cardiomyocyte sheets were transplanted into the subcutaneous space, they form a continuous tissue. The transplanted cardiac tissues show spontaneous pulsations that can be observe [30].

2.8.6 Bladder Augmentation

In case of bladder augmentation, the applications are used for treating lithiasis, urinary tract infection, and electrolyte imbalance are often induced. It has beendeveloped a novel augmentation cystoplasty using gastrointestinal flaps and cultured urothelial cell sheets. Gastrointestinal mucosa in the flaps is replaced with urothelial cell sheets, which have been expanded on temperature-responsive culture dishes from an autologous small biopsy. The Urothelial cell sheets spontaneously attach to the demucosalized tissue surfaces completely, without any suturing. It is obvious that Urothelial cell sheets harvested from temperature-responsive culture dishes can be a powerful tool in reconstructive surgery and this versatile technology should prove useful in various surgical reconstructions [2].

2.8.7 The Treatment of Diabetes Mellitus

Tissue engineering base on cell sheet of pancreatic islets has been established for treating insulin-dependent diabetes mellitus. The tissue sheet composed of pancreatic islet cells were used *in vivo* transplantation. Pancreaticislet cell suspensions were obtained from Lewis rats, and plated onto temperature-responsive culture dishes coated with extracellular matrix (ECM) proteins. After the cells reached confluency, islet cells cultured were successfully harvested as a uniformly spread tissue sheet by lowering the culture temperature. The functional activity of the islet cell sheets was confirmed by histological examination and Insulin secretion assay prior to *in vivo* transplantation. They have been reported a method to generate a functional sheet of pancreatic islet cells on coated temperature responsive dishes, which can be subsequently transplanted *in vivo*. This study serves as the foundation for the creation of a novel cell-based therapy for diabetes mellitus to provide patients an alternative method [31].

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

Commercial tissue culture polystyrene (TCPS) dishes (35mm × 10mm) style, treated, non-pyrogenic, sterile were purchased from Corning, New York, USA. Commercial tissue culture 6-well plate and commercial UpCell™ surface were purchased from Thermo Scientific, Denmark. UV lamp (15W, 265nm, without filter), OCA 40 Video-Based Contact Angle Meter, Atomic Force Microscopy (AFM) (Seiko Instrument), Scanning Electron Microscope (SEM; JEOL, model JSM-5410LV), Micro plate reader (TECAN Model InfiniteM200)

3.2 Chemicals

N-isopropylacrylamide (NIPAM) was purchased from Sigma-Aldrich and was re-crystallized at -5°C in *n*-hexans. Acrylamide (AM), *N*,*N*'-Methylenebisacrylamide (MBAAm) and potassium periodate (KIO_4) were purchased from Aldrich and were used without further purification. Dulbecco's Modification of Eagle's Basal Medium (DMEM), Minimum Essential Medium (MEM) Alpha Medium, Trypsin-EDTA, Fetal Bovine Serum (FBS), penicillin-streptomycin (Pt/St), Amphotericin B, Trypan Blue, and Vybant MTT cell proliferation kit, were purchased from Invitrogen (U.S.A.). Dimethyl sulfoxide (DMSO) was purchased from Amresco (U.S.A.). Matrigel basement membrane was purchased from BD science (U.S.A).

3.3 Cell Lines

L929 cell line (Mouse, Fibroblast) was provided by i-Tissue Laboratory, King Chulalongkorn Hospital (Thailand). MC3T3-E1 cell line (Human, Pre-Osteoblast) was provided by Faculty of Medicine, Chulalongkorn University (Thailand). Human Chondrocyte Primary cell was provided by Biomedical Pharmacology Laboratory, Siriraj Hospital (Thailand).

3.4 Preparation of PNIAM-co-AM Grafted Surface

Temperature-responsive polymer, PNIPAM-co-AM was grafted onto TCPS by using UV graft polymerization technique. The grafting conditions such as the post polymerization steps and UV exposure time were varied to give a wide range of grafted surface.The schematic representation of UV grafting method was shown in Figure 3.1. Commercial 6-wells plates or 35mm TCPS were first pre-irradiated by a UV lamp (15W, 265nm, without filter) for 30 minutes to activate the surface. In the following step, 500µl aqueous solution containing 0.0565g monomer NIAM (1mol/l), 0.0367g monomer AM (1.04 mol/l), 1.54×110^{-3}g crosslinker MBAAm (20 mmol/l) and 5.75×10^{-4}g photoinitator KIO_4 (5 mmol/l) was added to each TCPSand left to equilibrate overnight. Aluminum foil was used to cover the surfaces to prevent them from light exposure. After 24 hours, the solution was removed, and these dishes were exposed to the UV light (15W, 265nm, without filter) for another hour or 2 hours. The PNIAM-co-AM grafted TCP dish was dried at room temperature under vacuum condition for 24 hours, resulting in a thick grafted copolymer surface film, or washed with ethanol three times to give a thin grafted copolymer films. The thin grafted TCPS were washed with ethanol to remove any un-reacted monomer. Therefore, the properties of these grafted TCPS were compared.

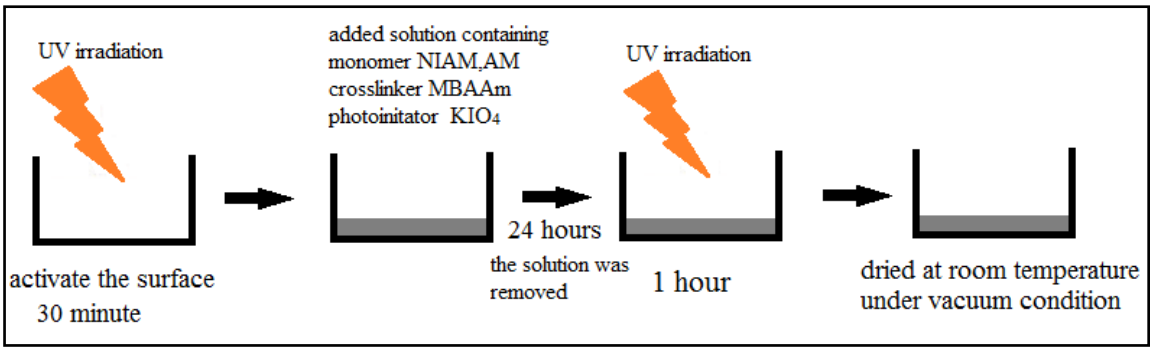


Figure 3.1 Schematic representation of UV grafting method

3.5 Characterization of The PNIAM-co-AM Grafted TCPS

3.5.1 Contact Angle Measurement

The surface dynamics of PNIAM-co-AM grafted surfaces were investigated by a contact angle-measuring device following the sessile drop method. A grafted surface was kept in a temperature-controlled device with the temperature ranging from 10°C to 45°Cto review the sample surfaces hydrophilic/hydrophobic behavior at high and low temperatures. The sample was allowed to equilibrate for 15 minutes before each measurement. The samples were examined at different temperatures and the contact angles were measured at these temperatures. The averaged angle values were calculated from angles that measured with three points of each sample.

3.5.2 Atomic Force Microscopy (AFM)

The main objective of AFM measurements is to determine the thickness of the PNIAM-co-AM grafted surface when temperature is varied and the hydrophobicity and hydrophilicity characteristic of the thin film. AFM measurements of PNIAM-co-AM grafted and ungrafted TCP dishes were performed at 5°C and 45°C in deionized water using Atomic Force Microscopy (AFM) (Seiko Instrument). Samples were hydrated in deionized water and allowed to equilibrate for 30 minutes at each temperature before the surface images were taken using the tapping mode and a scan rate of 1 Hz. The scan area is 5 μm × 5 μm.

3.5.3 Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier Transform Infrared Spectroscopy was carried out using the procedure applied for powder solid sample (Biazar, Zeinali*et al.* 2010). The surface of samples was scratched into powder and was pressed to make the pellets using KBr. The pellets were examined by Fourier Transform Infrared Spectroscopy (FTIR) Thermo Nicolet 6700. The FTIR spectrometer was equipped with KBr beamsplitter.

3.6 Sterilization Study

There are several techniques for PNIAM-co-AM grafted surface sterilization such as Ethylene Oxide (EO) gas sterilization, gamma irradiation, and chemical reagent. In fact, Ethylene Oxide Sterilization and gamma irradiation are costly and suitable to be used with large-scale production. Therefore, PNIAM-co-AM grafted surface in this study was sterilized using acidic alcohol (70%Ethanol, pH 2). The optimal conditions for PNIPAM-co-AM grafted surface sterilization were investigated by incubating L929 cells with the copolymer grafted surface. The copolymer grafted surface was sterilized with 70% Ethanol, pH2 for 0, 30 and 60 minutes and rinsed with sterile PBS. After that L929 cells were seeded onto these surfaces and incubated at 37°C for 72 hours to observe media color, cells morphology and bacterial contamination.

3.7 *In vitro* Toxicity Test of The PNIAM-co-AM Grafted TCPS

3.7.1 Short-Term Assay

Generally, to promote the cell attachment, there is no need to incubate the cells for more than 3 days. Therefore, the short-term assay is suitable to confirm the toxicity of copolymer. Mouse Fibroblast cell line (L929) is cultured in DMEM supplemented with 10% FBS and, Amphotericin B. Mouse Pre-Osteoblast cell line (MC3T3-E1) is cultured in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin (Pt/St) and, Amphotericin B. To prepare for the toxicity experiments, the cells were trypsinized and centrifuged to get the cell pellet. The supernatant was removed. The cell pellet is resuspended in the cell growth medium before cell count. The cell stock was diluted to 2×10^3 cells/well. The cell suspension was transferred to 96 well plates by adding 100 µl of the suspension to the each well.The experimental design is shown in Figure 3.2. The wells in column 2 contained culture media solution, referred to as blank. The spent media at 3, 5, and 7 days were added to the cell culture in column 4, 7, and 10, respectively. The spent media were the cell culture media previously incubated with PNIAM-co-AM grafted copolymer film. Aqueous solution was also added to the edge of the well plate to maintain the culture humidity. The cell viability was expressed as the percentage of the control. The controls are referred to the cells that were cultured on ungrafted surfaces. In case of L929 cell, the spent medium at 3 and 5 days were used in this study.

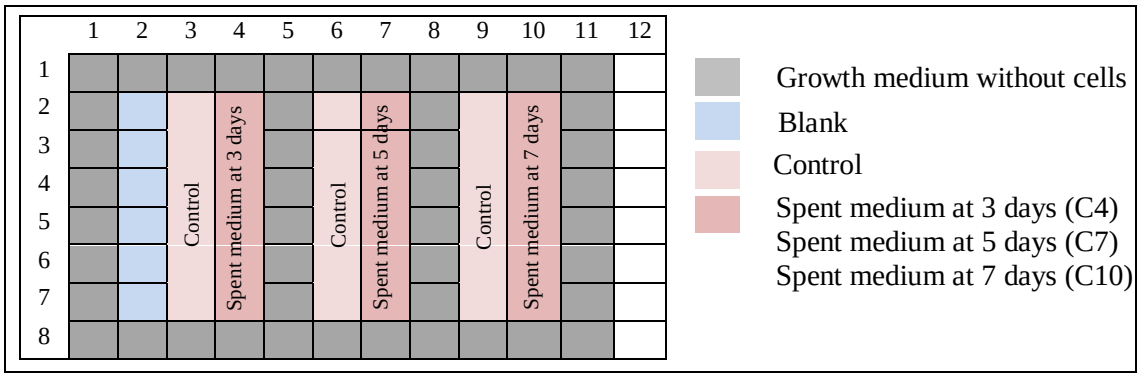


Figure3.2 Schematic representation of three spent medium in 96 well plates

The plates were incubated at 37°C and 5% CO_2 for 24 hours to promote cell attachment to the surfaces. To test for toxicity of the polymer, theold medium were removed from the well and replaced with the spent media. The plates were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 7 days. The cell viability was tested in every 24 hours to observe the changes in cell viability (%). Therefore, there were seven plates that

compose of different spent medium. After the predetermined time point, the supernatant is removed and replaced with the working solution (MTT assay solution in alpha-MEM medium phenol red free). The plates were incubated for 1 hour and protected from light. The formazan salt resulting from the reduction of MTT was dissolved in dimethyl sulfoxide (DMSO). The blue crystals formed in each well were dissolved with DMSO solution. The absorbance from the wells was measured at 570 nm with a microplate reader (Infinite® 200 PRO series).The cell concentration was calculated from the standard curve equation (Appendix) and the cell viability was expressed as percent survival using Equation 3.1.

$$CellViability(\%) = \frac{Cellconcentraionofsample}{Cellconcentrationofcontrol} \times 100 \qquad (3.1)$$

3.7.2 Long-Term Assay

The long-term assay was carried out to focus on the effect of long incubation time on the cell growth and cell viability. The spent medium used in this experiment was alpha MEM medium which was previously incubated on the PNIAM-co-AM grafted for 3, 5, 7, 10 and, 12days in the long-termassay. The cell viability wastested following the same method as in the short-term study. But, the stock of cell solution was diluted to the desire concentration at 1×10^3cells/well to allow the cells reach confluence within 3 days. The cell suspension was transferred to 96 well plates by adding 100 µl of the suspension to the each well. Column 2 and 3 were used as blank and control. The samples in column 4 to 9 were alpha MEM spent medium that was previously incubated with the copolymer film for 3, 5, 7,10 and 12 days, while plain medium was added to the wells in column 1 and 9 and row 1 and row 8 to help maintain the culture cell humidity (Figure 3.3). The plates were incubated at 37°C and 5% CO_2 for 3 days. After that time point, the cell viability wastested following the same method as in the short-term study. Each condition was replicated six times.

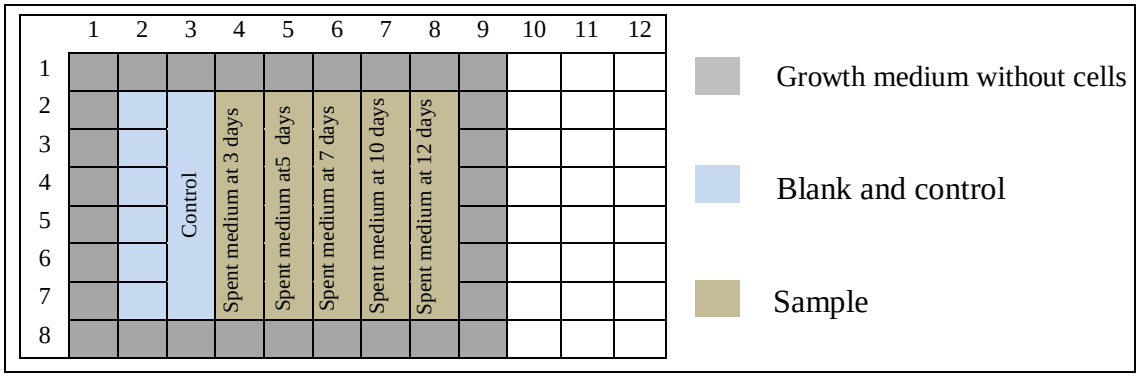


Figure 3.3 Schematic represented five spent medium in 96 well plates.

3.8 Effect of Time and The Low Temperature for Cell Detachment

Since the cell detachment step was performed in a low temperature chamber without CO_2 supplemented, the effects of low temperature and lack of CO_2 on cells need to be investigated. The viability of the cultured cells after incubated at low temperature was investigated by LIVE/DEAD staining. The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224) provides a two-color fluorescence cell viability assay that isbased on the simultaneous determination of live and dead cells. The MC3T3 cells were seeded on the grafted culture dishes at the cell density of 2×10^5 cells/ml and cultured at 37°C for 2 days in alpha-MEM medium. The culture cells were incubated at 10 and 20°C for 30, 60, 90 and 120 minutes. At that time, the cells were stained with LIVE/DEAD assay kit and analyzed with Image J software to determine the cell viability.

3.9 Cells Attachment and Proliferation

L929, MC3T3-E1 and Chondrocyte cells were seeded into a control and sterilized PNIAM-co-AM grafted 6 wells plate. Low (1×10^3 cells/ml) and high (1×10^5 cells/ml) cell densities were used in the single cell detachment and cell sheet detachment studies, respectively.The samples were maintained in DMEM (L929 and Chondrocyte) and α-MEM medium (MC3T3-E1) supplemented with 10% FBS 1% antibiotic (Pt/St and Amp B) at 37°C with 5% CO_2. Then all of the samples were placed in a CO_2 incubator at 37°C for 2-3 days to produce cell-cell junction. After 3 days (for the single-cell detachment study) and 2-3 days (for the cell sheet detachment study), the cell morphology was observed under an inverted optical microscope.

3.10 Single Cell Detachment

L929, MC3T3-E1 and Chondrocyte cells were plated at the cell density of 1×10^3cells/ml and culturedat 37°C. Cells were recovered from the temperature-responsiveculture dishes bylow-temperature treatment,these cells were allowed to detach from the temperature-responsive surfaces in 3 ml of culture media afterincubation at room temperature, 10°C, and 20°C for 60 and 90 minutes (depend on cell type).

3.11 Single Layer Sheet Detachment

L929 and MC3T3-E1 cultured cells were plated at the cell density of 1×10^5cells/ml and cultured at 37°C. Cells were recovered from temperature-responsive culture dishes by low temperature treatment. Non-adherent cells were removed with sterile PBS. Then, the cells were placed in an incubator set at 10°C, 20°C, and 10°C followed by 20°C in order to detach the cell. These cells were allowed to detach from the temperature-responsive surfaces in 3 ml of culture media after incubation for 90 minutes. PVDF membrane was used to transfer cell sheet to new culture surface.

3.12 Double-Layered Sheet Detachment

MC3T3-E1 cells were plated at the cell density of 1×10^5-1×10^6 cells/ml and cultured at 37°C. They were harvested following the same method as in the single layer sheet detachment study. However, these plates were incubated in a low temperature chamber set at 10 (30minutes) and 20°C (60minutes) in order to detach the cells. MC3T3-E1 morphology was observed at 90minutes. Sterile forceps was used to gently detach the cell layer around the edge of the PVDF membrane to prepare for a double layer cell sheet as shown in Figure 3.4.

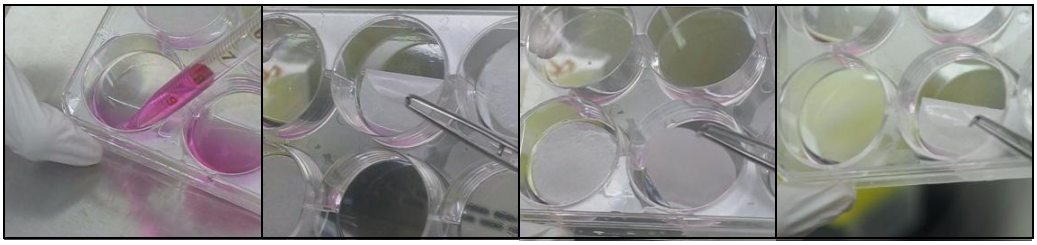


Figure 3.4 The cell sheets were harvested by using PVDF membrane.

3.13Cell Morphology with Scanning Electron Microscope (SEM)

The objective of this study was to learn more about the effect of low temperature on MC3T3-E1 cells morphology. Therefore, the copolymer grafted 35mm TCPS were then cut for specimen preparation. MC3T3-E1 cells were seeded onto the copolymer grafted surface at low (1×10^3cells/ml) and high (1×10^5cells/ml) cell density. The cultured cells were incubated at 37°C (5% CO_2 and 95% air) to promote the cell attachment for 48 hours. Non-adherent cells were removed by washing with PBS and fresh media were added into grafted TCPS. The culture surfaces were incubated at 20°C to detach the cells for 90 minutes. After that, the cell culture was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 2 hours and overnight at 4°C. The specimens were rinsed twice in phosphate buffer for 10 minutes each and in distilled water for 10 minutes. The specimens were dehydrated with a graded series of ethanol (30%, 50%, 70%, 95% for 10 minutes each and absolute ethanol 3 times, 10 minutes each time). Afterwards, the specimens were placed in a critical point dryer (Balzers model CPD 020) before being mounted on SEM specimenstubs and coated with gold in a sputter coater (Balzers model SCD 040). The samples were observed under a scanning electron microscope(SEM; JEOL, model JSM-5410LV).

3.14 Cell Viability

3.14.1 Viability of the re-attached cell sheet

The harvested cells were transferred to new tissue culture polystyrene (TCPS) andprotein coated TCPSsurface for the cells viability testing. The protein coated TCPS were prepared. Briefly, the new TCPS were coated with Matrigel™ (BD Cat.No.356234) on ice at 4°C to overnight. To avoid matrigel from gelling, matrigel was slowly thawed by keeping it on ice and use pre-cooled medium and pipettes to avoid gelling.500 µL of matrigel stock solution was diluted with 28.5 ml pre-cooled alpha-MEM medium in a laminar flow hood. The solution was mixed thoroughly by inverting the tube a few times. The desired 35mm TCPS were coated with 2 ml of diluted matrigel solution to ensure that the surface was completely covered. The surface was coated at room temperature for 1 hour. The remaining diluted matrigel solution was discarded. The coated 35mm TCPS should be used immediately after coating or frozen back at -20°C for future use. Prior to seeding the cells, the coating solution was removed and rinsed with 1×PBS to remove residual ECMs. These TCPS were incubated at 37°C to allow cell attachment.

3.14.2 LIVE/DEAD Staining

The harvested cell sheet was stained with live and dead stain assay kit for cell viability investigation. The proliferation of harvested cells was photographed after the cells were re-grown at 37°C for 24 – 48 hours. The percentage of cell viability was investigated. The image J software was used to quantify live and dead cells based on fluorescent images.

CHARPTER 4 RESULTS AND DISCUSSIONS

4.1 Preparation of Graft PNIAM-co-AM Polymer on PS

In this study, we used UV-radiation or photo-polymerization method to develop a methodto graft PNIAM-co-AM polymer on the polystyrene surface to get the film temperature-sensitive polymer thin film. These surfaces were used to study the cell attachment and detachment. The characteristics of the thermo-responsive surface were analyzed using contact angle measurement and the surface topography of PNIAM-co-AM copolymer layers was measured by atomic force microscopy (AFM) before cultured cells.

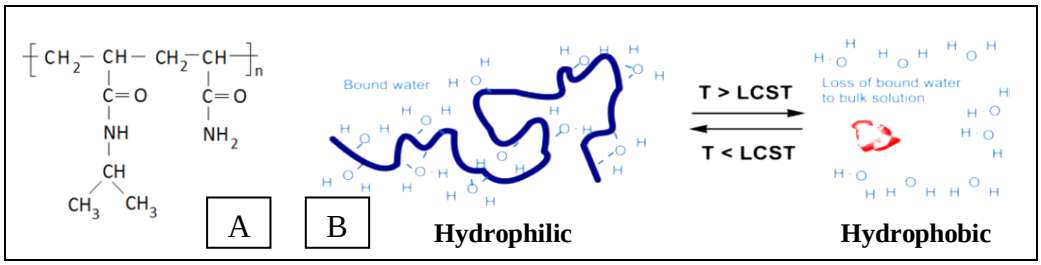


Figure 4.1 Structure of PNIPAM-co-AM polymer; (A) PNIPAM on a substrate above and below its LCST (B).

The hydrophilic/hydrophobic balance occurs in PNIPAM because of existence of the hydrophilic groups (–CONH-) and the hydrophobic groups ($CH(CH_3)_2$) in the side chains. The chemical structure of Poly(*N*-isopropylacrylamide) is consisted of the isopropyl groups (hydrophobic) and amide groups (hydrophilic)[24]. Below the lower critical solution temperature (LCST) at 32°C, the PNIPAM chains are swelled. Then, the amide groups interact with the water molecules through hydrogen bonding (Figure 4.1) [15], allowing the water droplet spreads over the grafted surfaces. Above the LCST, intra-molecular interactions among amide groups turn more favored, and the interactions of PNIPAM chains and water are reduced. The collapsing of PNIPAM chains occurs and the isopropyl groups become more exposed [36], resulting in a more hydrophobic surface.

4.2 Characterization of The PNIAM-co-AM Grafted TCPS

4.2.1 Contact Angle Measurement

Figure 4.2 shows the contact angle of the ungrafted and thin copolymer grafted surface near the LCST. The ungrafted TCPS show a hydrophobic characteristic over the temperature ranging from 20 – 45°C. The contact angles at 20°C and 45°C were 95° and 98°, respectively. For the copolymer grafted surface, the contact angle increased when the temperature was above 32°C. Below 32°C, the contact angle decreased, indicating a more hydrophilic surface. We can determine the LCST of this copolymer grafted surface from the sudden change of the contact angle, which is in the range between 30°C to 35°C because the contact angleswere increase from 57.5° to 74.84°.It has been previously reported that the water contact angles of the PNIAM-grafted surfaces demonstrate relatively large hydrophilic to hydrophobic changes with small temperature increases near 32°C. In contrast, the ungrafted surface showed hydrophobic characteristic, and the surface did not have reversible phase transition behavior with temperature.

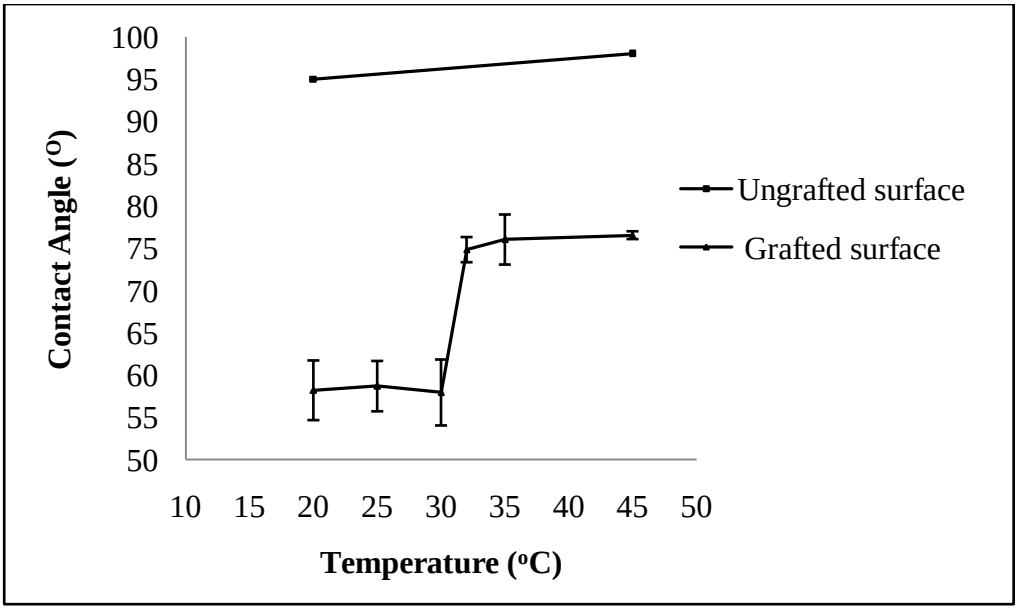


Figure 4.2 Contact angle of the PNIAM-co-AM grafted surface and ungrafted surface versus temperature [37].

As the temperature was increased, the contact angle also increased, demonstrating hydrophobic surface properties above LCST (32°C). A round water droplet was observed on a hydrophobic surface (>32°C), while a flat droplet was observed on a hydrophilic (<32°C) surface were shown in Figure 4.3. This result did not show the relatively large hydrophilic to hydrophobic changes with small temperature increases near 32°C. Therefore, the contact angles near 30-35°C were observed.

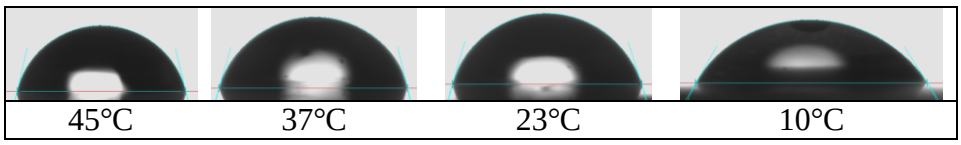


Figure 4.3 Photographs of water-droplet shape at different temperature (Provided by W. Siriwatwechakul and Nguyen Thi Khanh Thuyen, 2011)

4.2.2 Atomic Force Microscopy (AFM)

The AFM technique was analyzed to investigate the surface topography and its mechanical properties of PNIAM-co-AM grafted culture cell surfaces.

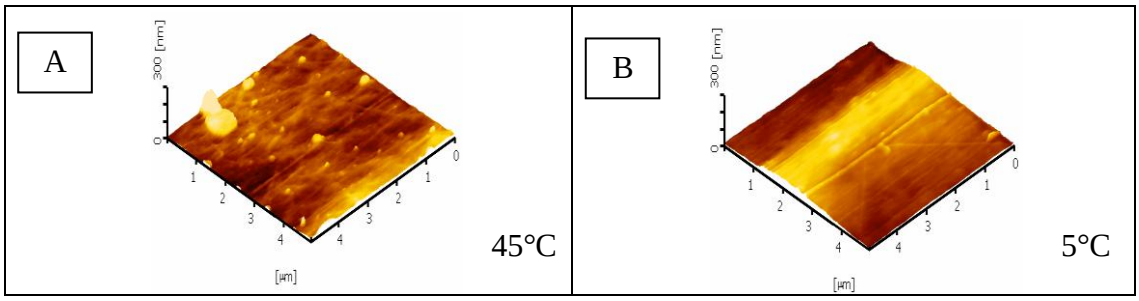


Figure 4.4 AFM images of PNIAM-co-AM grafted TCPS, (A) at 45°C and (B) 5°C [37]

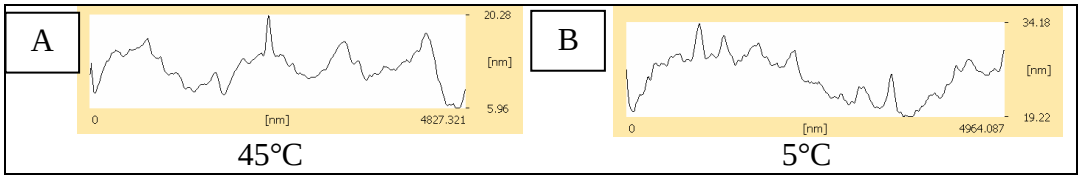


Figure 4.5 Height traces obtained in water of PNIAM-co-AM grafted on TCPS, (A) at 45°C and (B) at 5°C. The height profile is a horizontal line passing 3μm from the front of the picture[37].

Figure 4.4-4.5 shows the AFM topography of PNIAM-co-AM grafted PS surface in water at 45°C and 5°C. The root-mean-square (RMS) roughness of the surface at 45°C is 9.35 nm. The scan area is 5μm × 5μm. When the surface is soaked in water at 5°C, RMS roughness increases to 14.8 nm with the same scan area. This is consistent with the previous study that the RMS roughness of the PNIAM-co-AM grafted polystyrene surface increases with the lowering temperature [41].

4.2.3 Fourier Transform Infrared Spectroscopy

The FTIR results were carried out using the procedure applied for powder solid sample. The surface of the samples was scratched into powder and was pressed to make pellets using KBr. The samples were examined by Fourier transmission infrared spectroscopy (FTIR). The ungrafted polystyrene dish and linear PNIPAM were compared (data not shown).We could not to see the FTIR spectrum of PNIPAM because there wasa lot of noise at the primary amide and secondary amide peaks. This may be because PNIPAM content in the pellet was not sufficient for detection.

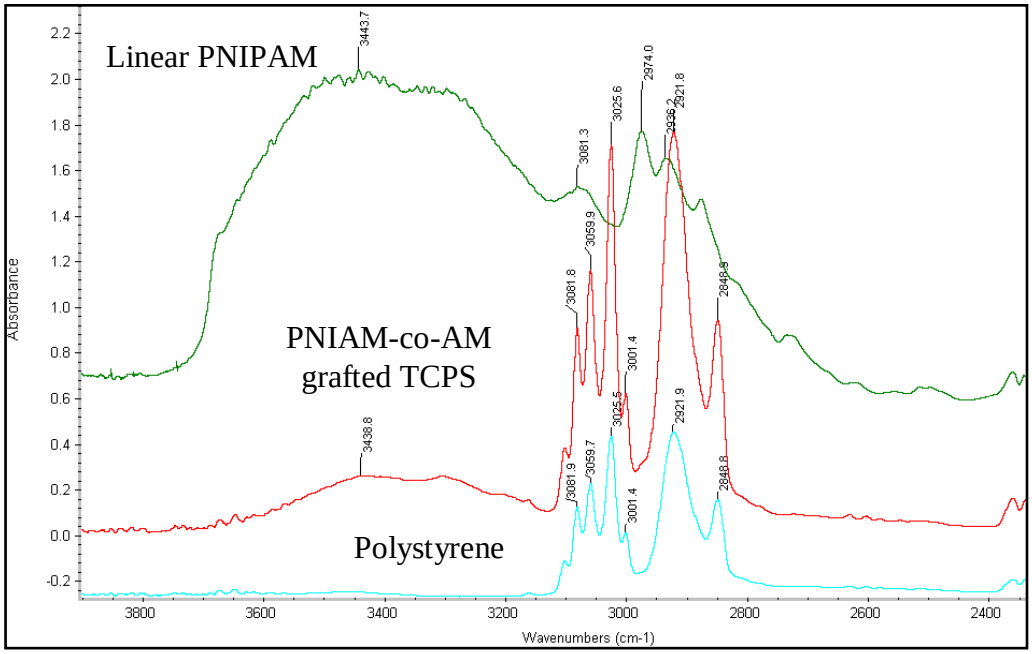


Figure 4.6 FTIR spectra of PNIAM linear, PNIAM-co-AM grafted TCPS, and PS in the wavenumbers from 3800 cm^{-1} to 2400 cm^{-1}(Reproduced from W. Siriwatwechakul and Nguyen Thi Khanh Thuyen, 2011)

The results in Figure 4.6-4.7 show the spectrum of PNIPAM grafted polystyrene dish. There are three peaks that are different from the spectrum of the un-grafted polystyrene dish representing secondary amide I-CONH- (C-O stretching) at 3439cm^{-1}, primary

amide $–CONH_2$ at 1663.1 cm^{-1} and secondary open chain amide at 1551.2 cm^{-1}. These peaks cannot be clearly differentiated from the FTIR spectrum of un-grafted polystyrene but they are found in the FTIR spectrum of linear PNIPAM. It is concluded that PNIPAM was grafted on the surface of polystyrene dish.

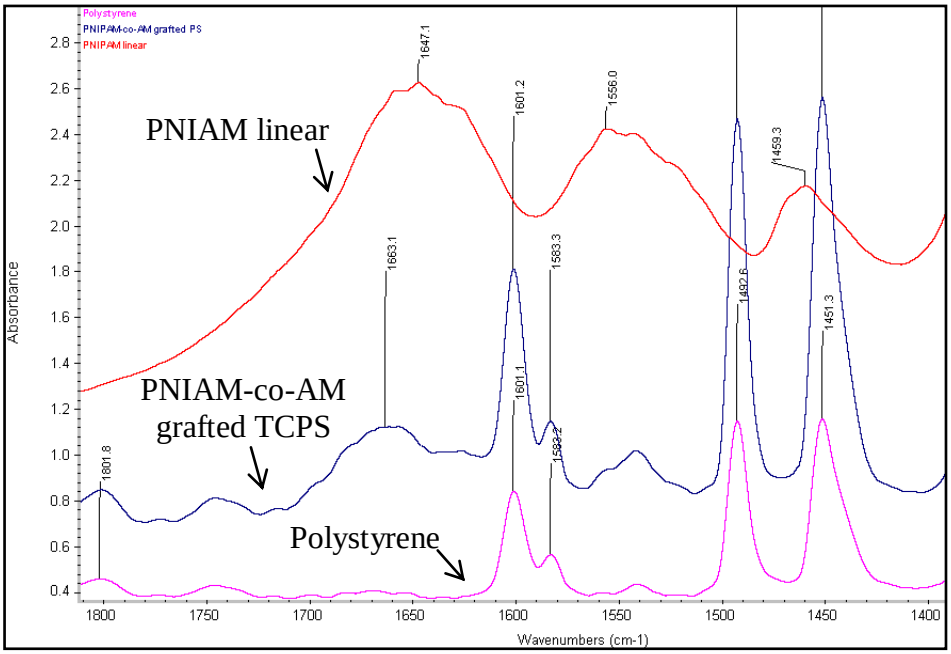


Figure 4.7 FTIR spectra of PNIAM linear, PNIAM-co-AM grafted TCPS, and PS in the wavenumbers from 1800 cm^{-1} to 1400 cm^{-1}(Reproduced from W. Siriwatwechakul and Nguyen Thi Khanh Thuyen, 2011)

Attenuated total reflectance (ATR) accessory was also combined with FTIR spectroscopy to investigate the thin PNIAM-co-AM grafted on PS. The ATR-FTIR spectra in Figure 4.8 and Figure 4.9 show similar results to those of FTIR spectra using KBr except that the peak at 3439cm^{-1} representing primary amide $–CONH_2$. It could be that the thin polymer films of PNIPAM-co-AM on the polystyrene surface hadfewer $–CONH_2$ groups than -CONH- groups. As a result, these $–CONH_2$ groups cannot be seen clearly in the ATR-FTIR spectra.

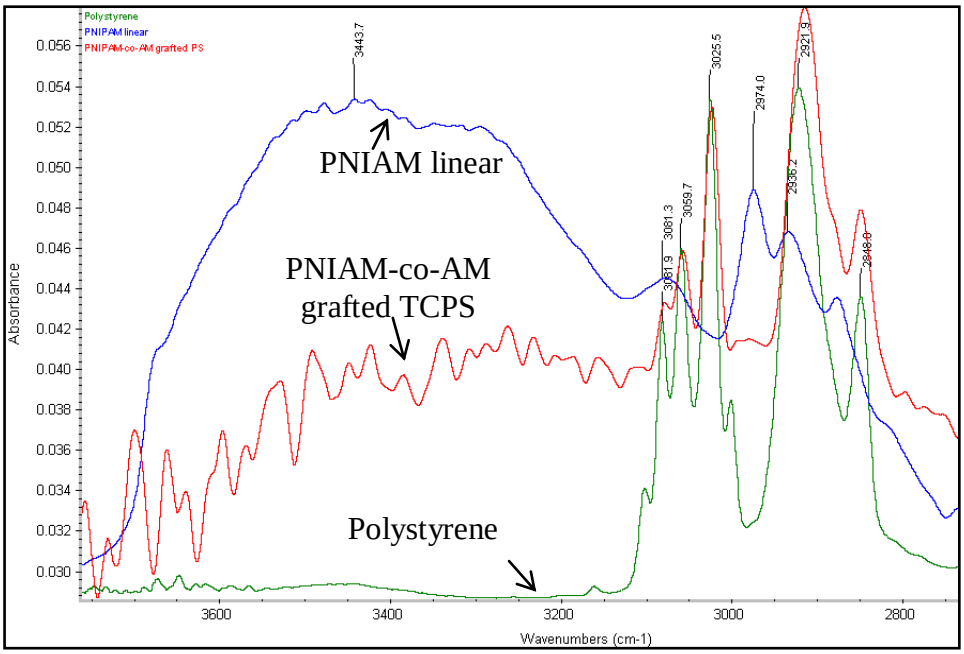


Figure 4.8 ATR-FTIR spectra of PNIAM linear, PNIAM-co-AM grafted TCPS, and PS in the wavenumbers from 3800 cm^{-1} to 2400 cm^{-1}. (Reproduced from W. Siriwatwechakul and Nguyen Thi Khanh Thuyen, 2011)

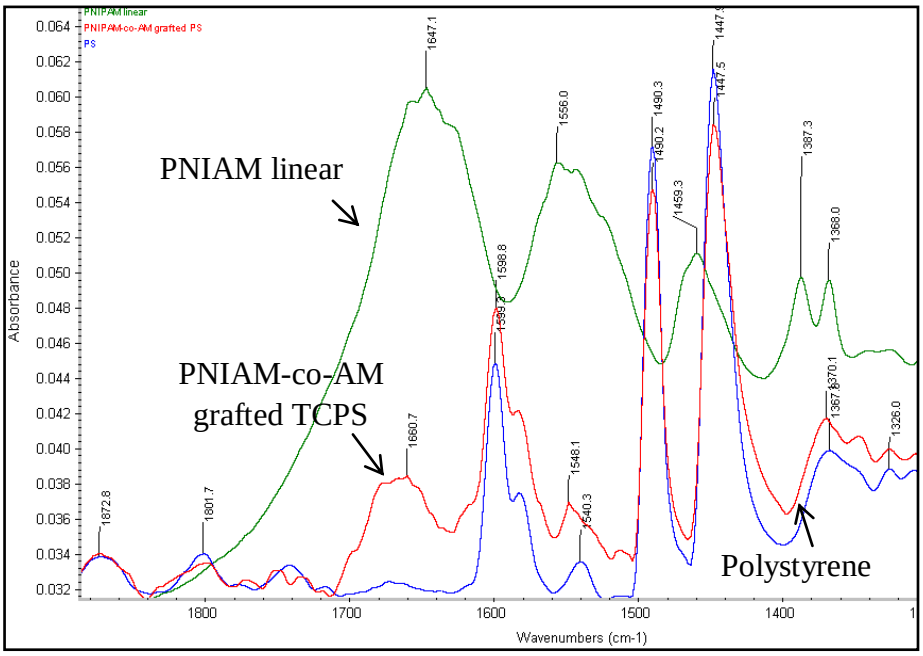


Figure 4.9 ATR-FTIR spectra of PNIAM linear, PNIAM-co-AM grafted TCPS, and PS in the wavenumbers from 1800 cm^{-1} to 1400 cm^{-1}. (Reproduced from W. Siriwatwechakul and Nguyen Thi Khanh Thuyen, 2011)

The FTIR spectra of the grafted and un-grafted dishes showed the existence of this copolymer on the surface. The un-grafted dish did not show the peaks at 3439 cm^{-1} and at

1663.1 cm^{-1} that are different from the FT-IR spectrum of the grafted dish. According to the surface characterization and properties of the PNIAM-co-AM grafted TCPS, PNIAM-co-AM was successfully grafted on the tissue culture polystyrene surfaces. Then, the application of this grafted surface was used in the cell studies that we reported in the next part.

4.3 Sterilization Study

The L929 were seeded onto the copolymer grafted surface that was sterilized with 70% Ethanol, pH = 2 by varying the treatment time from 0, to 30 and to 60 minutes to determine an optimal sterilize condition.The growth of L929 fibroblast cells on the PNIAM-co-AM grafted surfacewas shown in Figure 4.10. We found that L929 cell proliferated well on the non-treated surface or the surface briefly rinsed with 70% ethanol, pH 2. In contrast, L929 cells exhibited low cell density on the grafted surface treated with acidic ethanol for 60 minutes because the residual acidic ethanol may cause cellular damage. From this result, an optimal condition to sterilize the copolymer grafted surface is to wash the surface with 70% ethanol, pH = 2, followed by rinsing with sterile PBS.

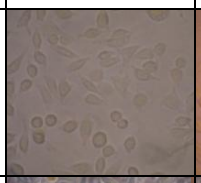
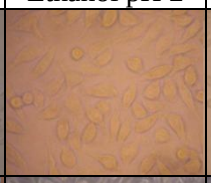
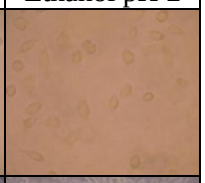
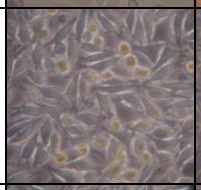
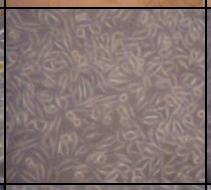
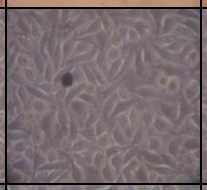
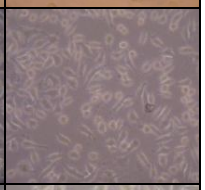
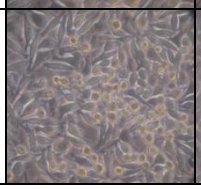
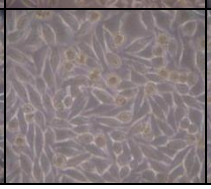
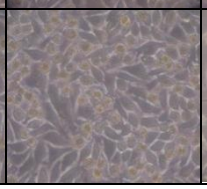
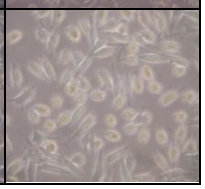
Incubation time	Control	PNIAM-co-AM grafted surface		
		0 min 70% Ethanol pH 2	30min. 70% Ethanol pH 2	60min.70% Ethanol pH 2
24hours				
48 hours				
72 hours				

Figure 4.10 The morphology of L929 cell on the different sterilized condition

4.4 The *In Vitro* Toxicity Test

The biocompatible property of PNIPAM is unknown. It has been shown that the TCPS grafted with PNIPAM leads to limitation of cell culture [14]. Therefore, its toxicity should be examined by MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was selected to determine detrimental intracellular effects on mitochondria and metabolic activity.

The colorimetric MTT test, based on the selective ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into purple formazan relies on intact metabolic activity usedfor screening of cytotoxicity [38]. Changes in cell morphology and the detachment of cells from the TCPS were also used as indicators of cell survival by microscopic investigations.

4.4.1 Short-term assay

There are two types of toxic solutions which are spent mediumat 3 days and 5 days. After L929 cells were incubated at 37°C with the different spent medium for 48 hours, the cells were tested with MTT assay.

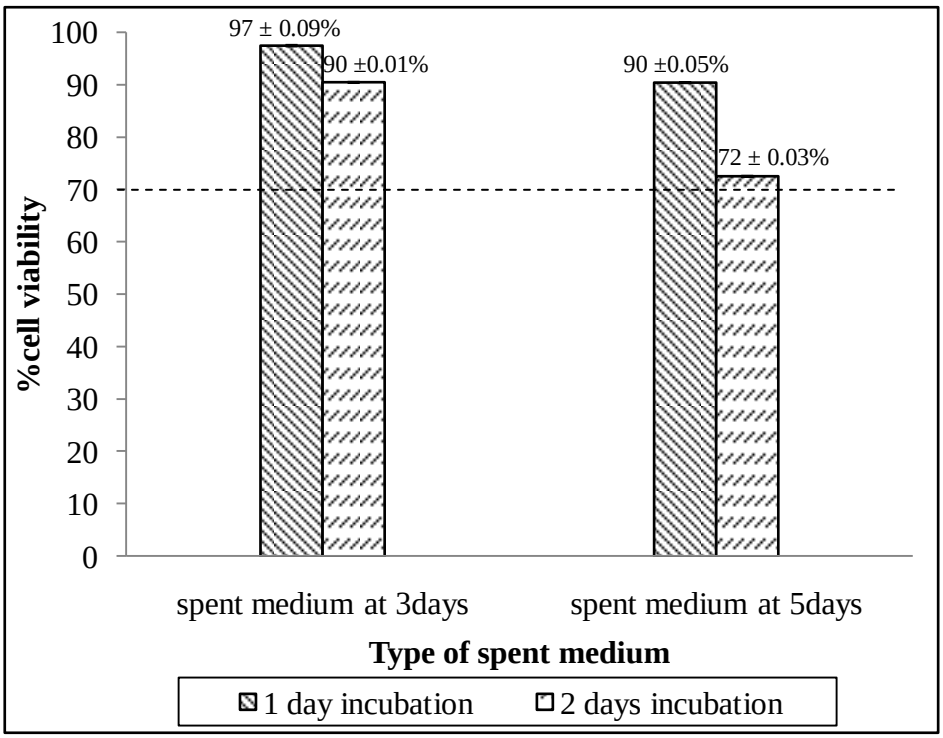


Figure 4.11 *In vitro* cytotoxicity in L929 Cells

The toxicity results of spent medium are shown in Figure 4.11. The L929 cell viability of 3 dayspent medium was 97.47±0.089% (1 day incubation) and 90.42±0.0141% (2 day incubation). For the cell viability of 5 dayspent mediumwas 90.49±0.0463% (1 day incubation) and 72.55±0.0292% (2 day incubation).The dot line in the figure represents the critical point of cytotoxic potential. The international standard ISO 2009 has been reported that if viability is reduced to less than 70 %, it has a cytotoxic potential[39].

The morphology of L929 cells with fresh medium (control) and spent medium (PNIAM-co-AM grafted TCPS) are shown in Figure 4.12. The morphology of L929 cells which incubated with spent mediumfor 2 days showed normal fibroblast-likes tructure similar to that of the control surface. This result suggests that the cells were still alive at the end of 2 days of incubation.

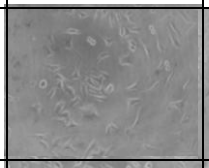
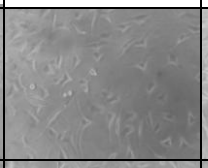
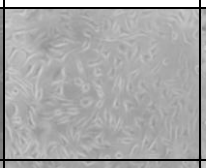
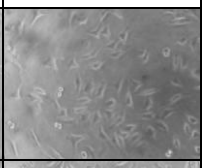
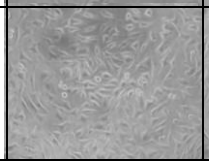
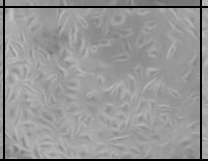
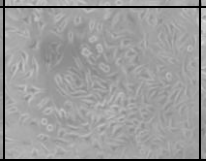
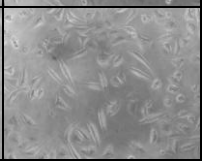
Incubation time	control	3 day spent medium	control	5 day spent medium
1 day				
2 days				

Figure 4.12 The morphology of L929 cells at fresh and spent medium condition

From the short term result, the cytoxicity of PNIAM-co-AM grafted TCPS was non-toxic. However, the toxicity of the copolymer on other cell lines and the long term assay should be investigated.

In case of the other cell line, MC3T3-E1 cell was used to compare with the cytotoxicity in the L929 cell. PNIAM-co-AM grafted TCPS surfaces were incubated with culture medium at different time. Spent medium at each time point was used to incubate with the mouse Pre-Osteoblast MC3T3-E1 cell for MTT testing. The results of the spent medium after incubated with the cell are shown in Figure 4.13. The cell activity of the new culture media was used as a control.Cells were incubated with three different spent media which were 3 day, 5 day, and 7 day spent media. The cell viability of 3 day spent medium was 91.51 ± 0.0377% up to 3 days of incubation.The cell viability of 5 day and 7 day spent mediawere 81.13 ± 0.0064% (up to 5 days of incubation) and 73.73± 0.0171% (up to 7 days incubation).The results show that the cell viability was more than 70% in every condition, indicating that the polymer is biocompatible.

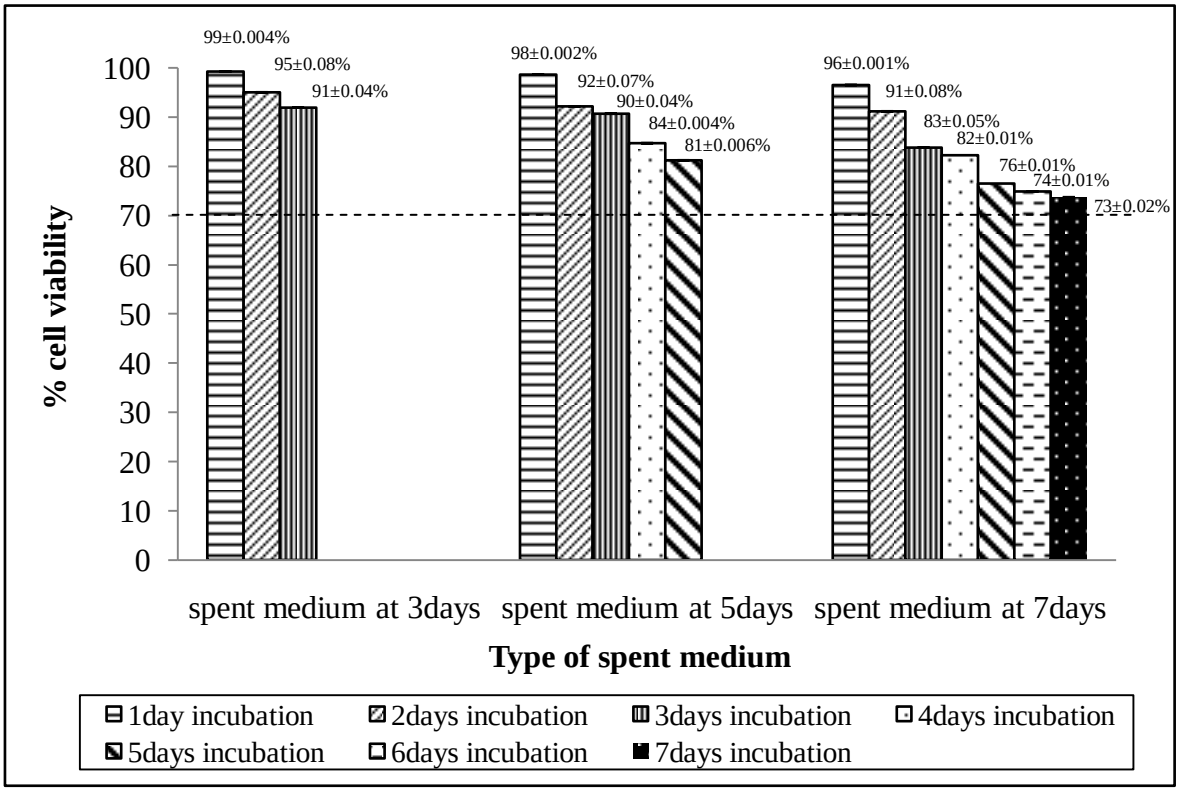


Figure 4.13 *In vitro* cytotoxicity in MC3T3-E1 Cells

4.4.2 Long-term assay

In the long-term assay, we focused on the effect of long incubation time (spent medium at long incubation) on the cell growth and cell viability. The spent media were the cell culture media previously incubated with copolymer film up to 12 days (long term incubation). The cell viability was tested following the same method as in the short-term study. But, the plates were incubated at a normal condition for only 3 days.

The results obtained in the MTT assay demonstrated that the cells cultured on the surfaces in the short-term study (≤7 days) had more activity than those in the long-term study (≥10 days) (Figure 4.14). A decrease in number of living cellsto less than 70 % in long term assay indicated that it had a cytotoxic potential. The mitochondrial activity assays, MTT, are colorimetric assays to measure the activity of enzymes that reduce MTT to formazan dyes, giving a violet color. MTT is a yellow tetrazolium salt, that it is reduced only in living, metabolically active cell mitochondria. After reduction, a violet formazan dye is formed and the amount of living cells can be detecting by fluorescence intensity. This study shows that the reduction of MTT in L929 and MC3T3-E1 cells was decreased by the toxic media.

According to our short-term and long-term studies, PNIAM is considerably biocompatible. However, this polymer may not be suitable for use in long-term culture, possibly due to the accumulation to unknown compounds that maybe toxic to the cells.

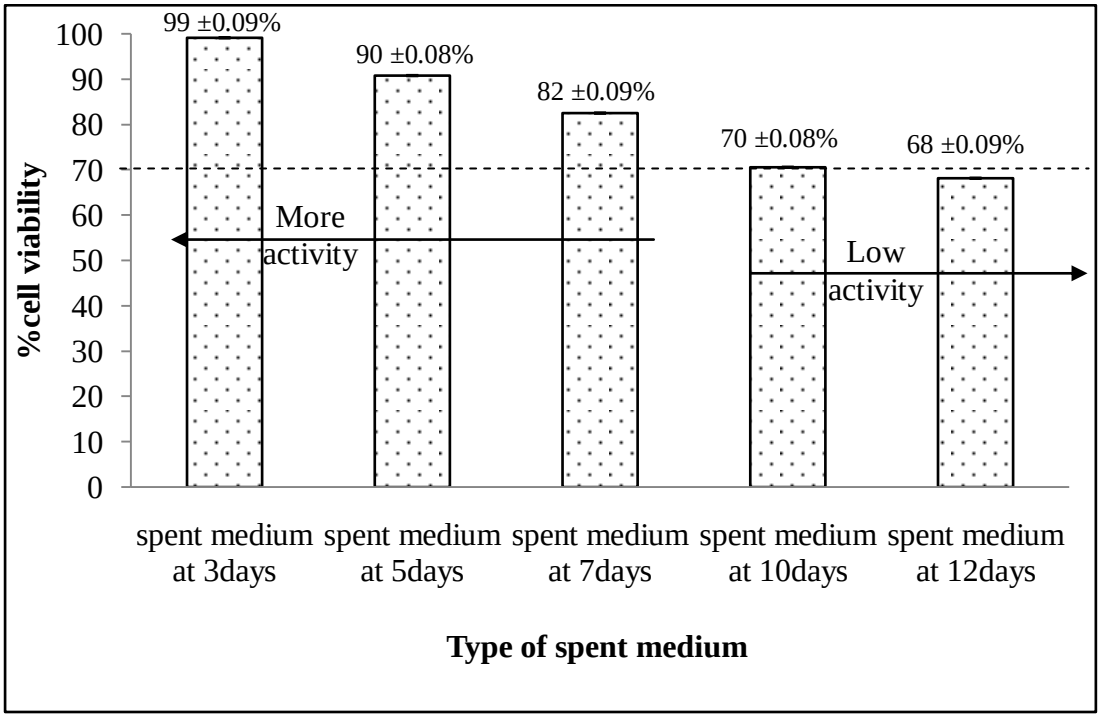


Figure 4.14 Toxicity effects of different toxic on the MC3T3-E1 fibroblasts cells after 72 hours incubation time

4.5 Effect of temperature on cell detachment

This experiment was carried out to study the effect of time and low temperature on the MC3T3-E1 cell cultures. The cell detachment step was performed in a low temperature chamber without CO_2 supplemented.Thus, the effects of low temperature and lack of CO_2 on the cells needed to be investigated. After cells reached confluence, a continuous monolayer cell sheet formed on the surface of the grafted surface. The grown cell sheet was placed at 10 and 20°C. After incubation time of 30, 60, 90 and 120minutes, these cells were stained with the live and dead stain assay kitto determine the cell viability.

The result is shown at different time periods: 30, 60, 90 and 120 minutes. At 10°C, the percentage of cell viability was 98.29% up to 90 minutes of incubation. At the end of 120 minutes incubation, the percentage of cell viability was 97.45% (Table 4.1). At 20°C,the viability of MC3T3-E1 cells was 100% and 98.64% for 90 and 120 minutes of incubation, respectively (Table 4.2). The morphology of MC3T3-E1 cells at those temperatures was shown in Figure 4.15-4.16.

Table 4.1 The percentage of live MC3T3-E1 cells at 10°C

Time (minutes)	Sample No.	Live cells	Dead cells	Total cells	% cell viability	%cell viability (Average)
30	1	414	0	414	100	99.81
	2	525	1	526	99.81	
	3	512	2	514	99.61	
60	1	336	1	337	99.70	99.52
	2	427	5	432	98.84	
	3	389	0	389	100	
90	1	477	10	489	97.55	98.29
	2	369	6	375	98.40	
	3	464	5	469	98.93	
120	1	478	17	495	96.57	97.45
	2	478	10	480	99.58	
	3	431	17	448	96.21	

Table 4.2 The percentage of live MC3T3-E1 cells at 20°C

Time (minutes)	Sample No.	Live cells	Dead cells	Total cells	%cell viability	%cell viability (Average)
30	1	343	0	343	100	100
	2	334	0	334	100	
	3	421	0	421	100	
60	1	494	0	494	100	100
	2	395	0	395	100	
	3	418	0	418	100	
90	1	402	0	402	100	100
	2	357	0	357	100	
	3	425	0	425	100	
120	1	363	2	365	99.45	98.69
	2	335	8	343	97.67	
	3	475	5	480	98.96	

While the percentage of cell viability demonstrates the effect of low temperature chamber without CO_2 supplemented, the live and dead staining of MC3T3 cell can also reveal the cell morphology to confirm live cells in green fluorescence. In this study, we found that the cell viability decreased as the incubation time at low temperature without CO_2 increase.

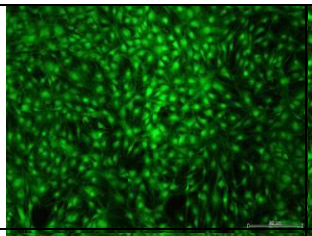
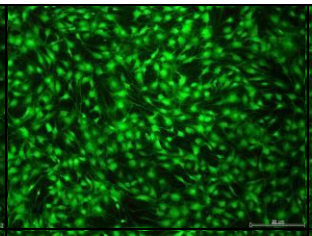
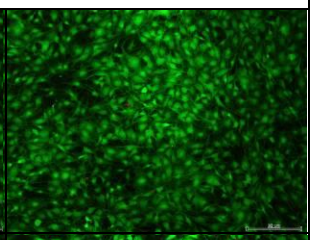
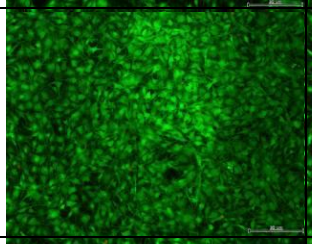
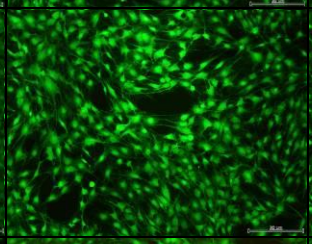
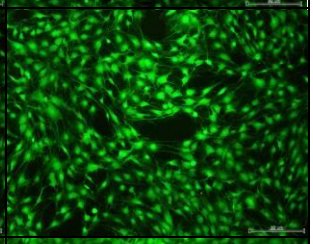
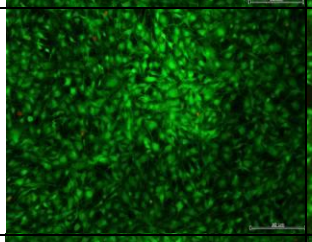
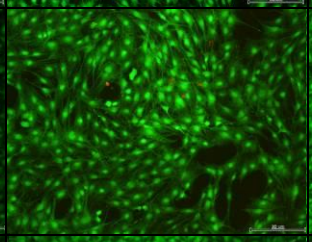
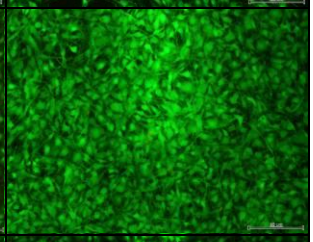
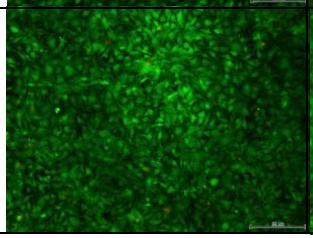
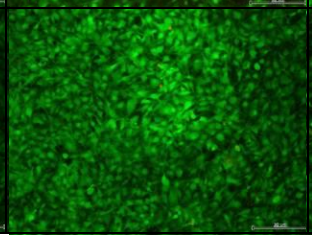
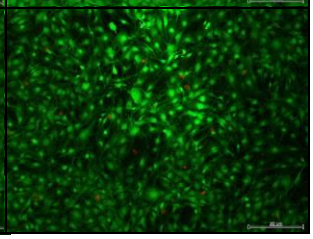
Incubation Time (minutes)	Sample No.1	Sample No.2	Sample No.3
30			
60			
90			
120			

Figure 4.15 The LIVE/DEAD staining of MC3T3-E1 cell sheet on the PNIAM-co-AM grafted surface at 10°C. Scale bar = 100μm

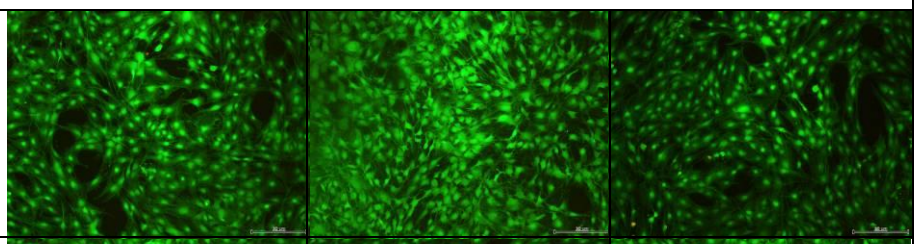
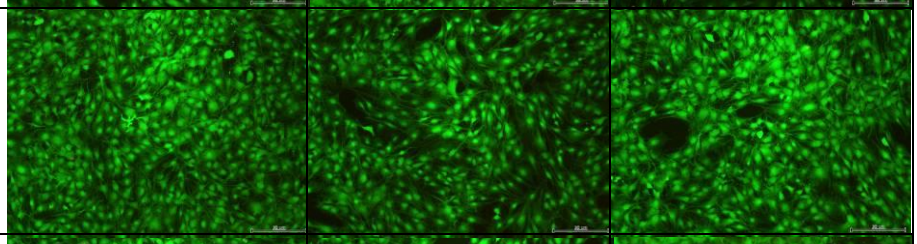
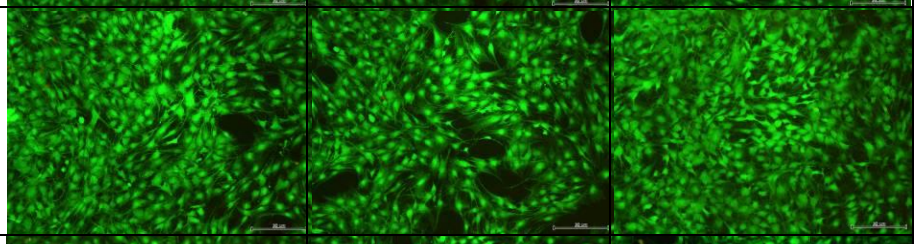
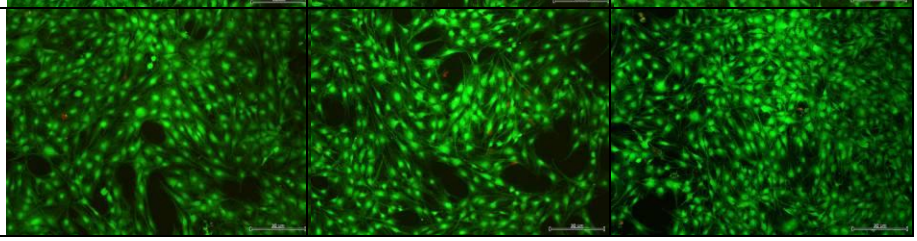
Incubation Time (minutes)	Sample No.1	Sample No.2	Sample No.3
30			
60			
90			
120			

Figure 4.16 The LIVE/DEAD staining of MC3T3-E1 cell sheet on the PNIAM-co-AM grafted surface at 20°C.Scale bar = 100µm

Although these cells were incubated for a long time at 20°C, most of the cells were still alive. At 10°C, the percentages of live cells were slightly less than those at 20°C because at low temperature these cells have lower ATP metabolic rate leading to cell death. ATP metabolism is essential when detaching cells since the cell detachment from the grafted surface requires morphological changes.Active cellular processes or ATP metabolism lead to cells change their morphological which are essential procedure tocomplete cell detachment from hydrated surfaces [25].This result suggests that the cells were still viable more than 97% at 10 and 20°C without CO_2upto 2 hours, indicating that the cells viability was not affected by the low temperature treatment. Therefore, the cell detachment study should not be performed for longer than 2 hours.

4.6 Cell Study

The objective of this study wasto createa protocol to harvesta complete cell sheet.The cell detachment experiments consist mainly of 4 steps. First, cells were cultured at a normal condition (37°C). After that, the confluent cells were harvested using PVDF membrane and agitating the cultured surface with medium. Then, the harvested cells were re-grown on new culture surfaces. Finally, we determine the cell viability of harvested cells by LIVE/DEAD staining.

The study of cell attachment and detachment on the copolymer grafted surface were carried out. The morphology of the cellson Thick, Thin grafting with PNIAM-co-AM, PNIAM-co-AM grafted surface with longer exposure time, the commercial UpCell™ surface, and ungrafted PS were compared. Thick PNIAM-co-AM grafted TCPS refers to the surfaces with residual copolymer, resulting in a thicker film of the copolymer, while thin PNIAM-co-AM refers to the grafted surfaces that were rinsed with ethanol to completely remove the unreacted monomer. These grafted surfaces were exposed to UV for 1 hour. The effect of longer exposure time (2 hours exposure time) on the biological activities of the copolymer film was also investigated. Mouse fibroblast L929, MC3T3-E1, and human chondrocyte cells were well attached and spread on all surfaces and reached 100% confluence at 37°C incubation depending on cell density.

4.7 Single Cell Attachment/Detachment Study

There are three types of the cells, L929, MC3T3-E1, and chondrocyte cells, which were used as cell models. Low cell density was used in the single cell detachment. The cultured cells were maintained at a normal condition in different media depending on the cell type.Then all of the cells were placed in a CO_2 incubator to promote cell attachment. In this section, the efficiency of cell adhesion and the percentage of single cell detachment from the thick and thin PNIAM-co-AM grafted surface were compared.

4.7.1 Single Cell attachment on Thick PNIAM-co-AM grafted TCPS

Mouse fibroblast cell line, L929 and MC3T3-E1 cells were seeded onto thick PNIAM-co-AM grafted surface at a cell density of 1.0×10^3 cells/ml for the single cell detachment study. These cells were incubated at 37°C.

Morphologies of the cultured cells were photographed at 24, 48, and 72 hours after seeding. The cells adhered well on both of the PNIAM-co-AM grafted and ungrafted surfaces as shown in Figure 4.17. L929 and MC3T3-E1 Pre-Osteoblast cells are fibroblast-like cells but the sources of the cell culture media solution and the split ratio are different.The morphology of the cells on the grafted surface showed the typical fibroblast morphology similar to that of the cells cultured on the control ungrafted surface.

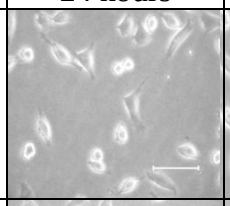
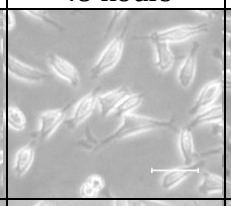
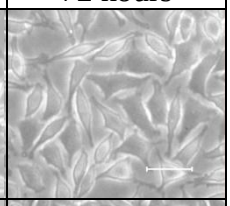
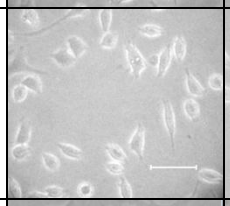
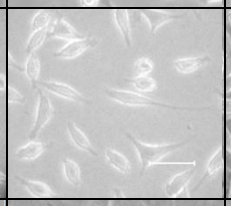
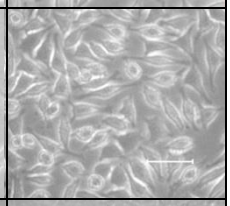
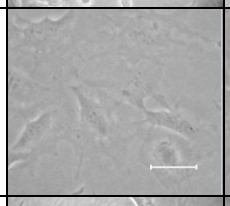
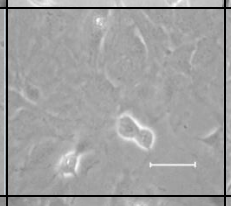
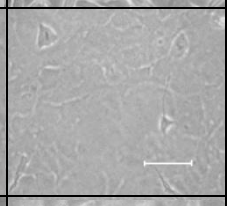
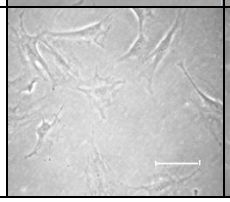
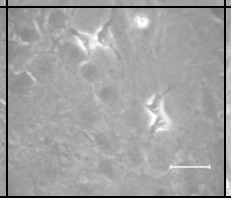
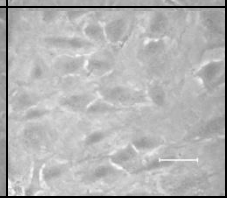
Types of cell and surface	Incubation time		
	24 hours	48 hours	72 hours
L929 cell Control ungrafted surface			
L929 cell PNIAM-co-AM grafted surface			
MC3T3-E1 cells Control ungrafted surface			
MC3T3-E1 cells PNIAM-co-AM grafted surface			

Figure 4.17 The morphology of L929 and MC3T3-E1 cells on the Thick PNIAM-co-AM grafted surface at a cell density of 1.0×10^3cells/ml. Scale bar = 100µm

4.7.2 Single Cell Detachment from Thick PNIAM-co-AM grafted TCPS

The detachment of single fibroblast cells first was performed with L929 cells because this cell line is commonly used in mammalian cell culture. The single cell detachment results were observed using low temperature treatment after 3 - 4 daysin culture at 37°C. These cells were cultured on each surface at a density of 1×10^3 cells/ml and allow to reach 100% confluence attachment.

The first condition that we used to observe the single cell detachment experiment is at room temperature (20-25°C). For low temperature treatment, the confluent cells were transferred to room temperature (20-25°C). After 1, 2 and 3hours of incubation, cell morphology was observed and photographed using an inverted microscope. Ungrafted TCPS was used as a control. In this study, no cell detachment was observed from TCPS within 3 hours. In contrast, individual L929 cells partially changed their shape from a spread to a round form without any enzymatic treatment, and finally detached from the copolymer grafted surfaces, as shown in Figure 4.18. L929 cells changed their shape from afibroblast-like structure to roundcells and floated in the culture medium.

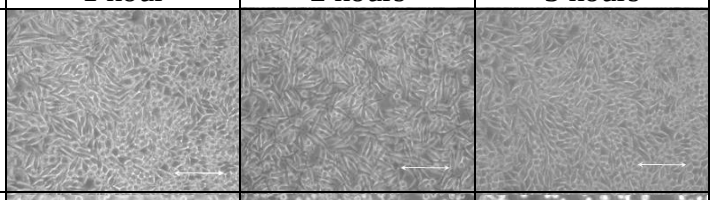
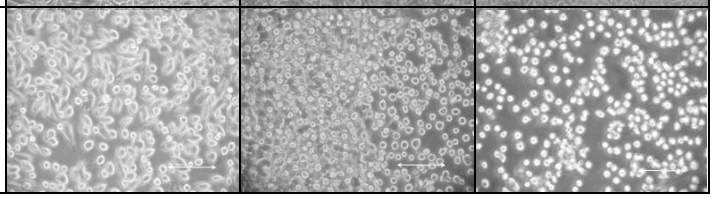
Type of surface	Incubation Time		
	1 hour	2 hours	3 hours
control ungrafted surface			
Thick PNIAM-co-AM grafted surface			

Figure 4.18 Single L929 cells detached from the thick PNIAM-co-AM grafted surface at room temperature (20-25°C). Scale bar = 100μm

A majority of L929 cells detached within 3 hours after incubation at room temperature. Individual cells detached from the grafted dishes because the NIPAM hydration is enhanced when temperature is reduced [25]. Some cells detached from the grafted surface probably due to an inhomogeneous crosslink density. Three hours of incubation was required to detach the cells from the copolymer grafted surface. Long treatment time is not good to re-culture the cells because the lack of CO_2 for a long time significantly reduces the cellular activity and the metabolic rate.In the present study, we found thatmore reduced temperature was required for rapid cell detachment from copolymer grafted dishes. However, the other cell line and low temperature condition without CO_2 for longer treatment time were compared.

The MC3T3-E1 Pre-Osteoblast cell line was another fibroblast cell line used in the cell detachment test. These cells were seeded at a cell density of 1.0×10^3 cells/ml for single cell detachment. Although lower temperatures treatment rendersthe hydration of copolymer chains, the cellular metabolism was also reduced. Moreover, the effects of low temperature on cellular metabolism were different in each cell type. Therefore, an optimum temperature would promote self-detachment of cells from the temperature-responsive surface [25]. In this part, the growth of MC3T3-E1 Pre-Osteoblast cells on both the grafted and un-grafted surface showed good cell adhesion at 37°C. The treatment temperature was dropped to 10°C (below LCST~32°C). The fibroblast cells could be detached partially from the grafted surface after lowering temperature without using enzymes. Both L929 and MC3T3-E1 cells still attached to the control surface at 10°C. In contrast, on the thick PNIAM-co-AM film, L929 cells detached partially within 60 minutes (Figure 4.19), while no MC3T3-E1 cells detached from the surface. It is likely that the temperature and incubation time were not suitable for the cell detachment. Moreover, un-reacted monomer and the chemical residual were not removed from the thick copolymer surface, leading to inhomogeneity onthe surfaces. The thickness of the grafted copolymers were not uniform.

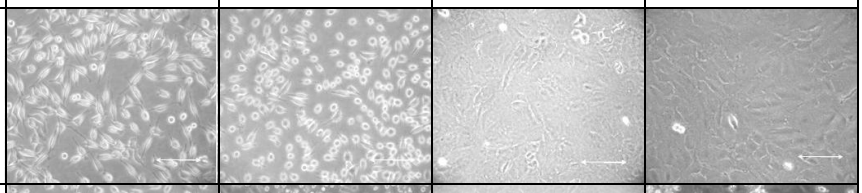
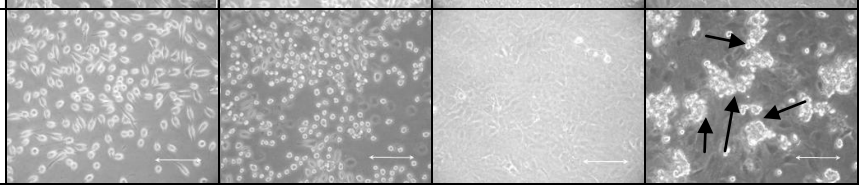
Type of surface	L929 cells		MC3T3-E1 cells	
	30 minutes	60 minutes	30 minutes	60 minutes
Control ungrafted surface				
Thick PNIAM-co-AM grafted surface				

Figure 4.19 Single L929 and MC3T3-E1 cells detached from the thick PNIAM-co-AM grafted surface at 10°C for 60 minutes. Scale bar = 100µm

From these results, we found that the culture cells should be incubated at 37°C to allow for cell adhesion.To detach the cells, the temperature should be decreased to the hydrate phase below 32°C which the copolymer chains started to extend. This significant hydration change initiated cell detachment. However, at 10°C, the release of the cells was not complete. Further cell detachment required consistent temperature-responsive surfaces and low temperature incubation for adherent cells to change their membrane shape, consuming internal metabolic energy [25].

4.7.3 Single Cell attachment on Thin PNIAM-co-AM grafted TCPS

The cell attachment on the thin copolymer surface was investigated. In this study, they were cultured following the same method as in the cell attachment on the thick PNIPAM-co-AM grafted TCPS. Morphologies of L929, MC3T3-E1 were observed at 24, 48, and 72 hours by inverted microscopy after seeding. However, longer incubation time (5 - 6 days) was required for human chondrocyte cell to reach 100% confluence. At 37°C, almost all of the seeded cells wereattached, spread, and proliferated on the thin PNIAM-co-AM grafted similar to those on the non-grafted surface, as shown in Figure 4.20.

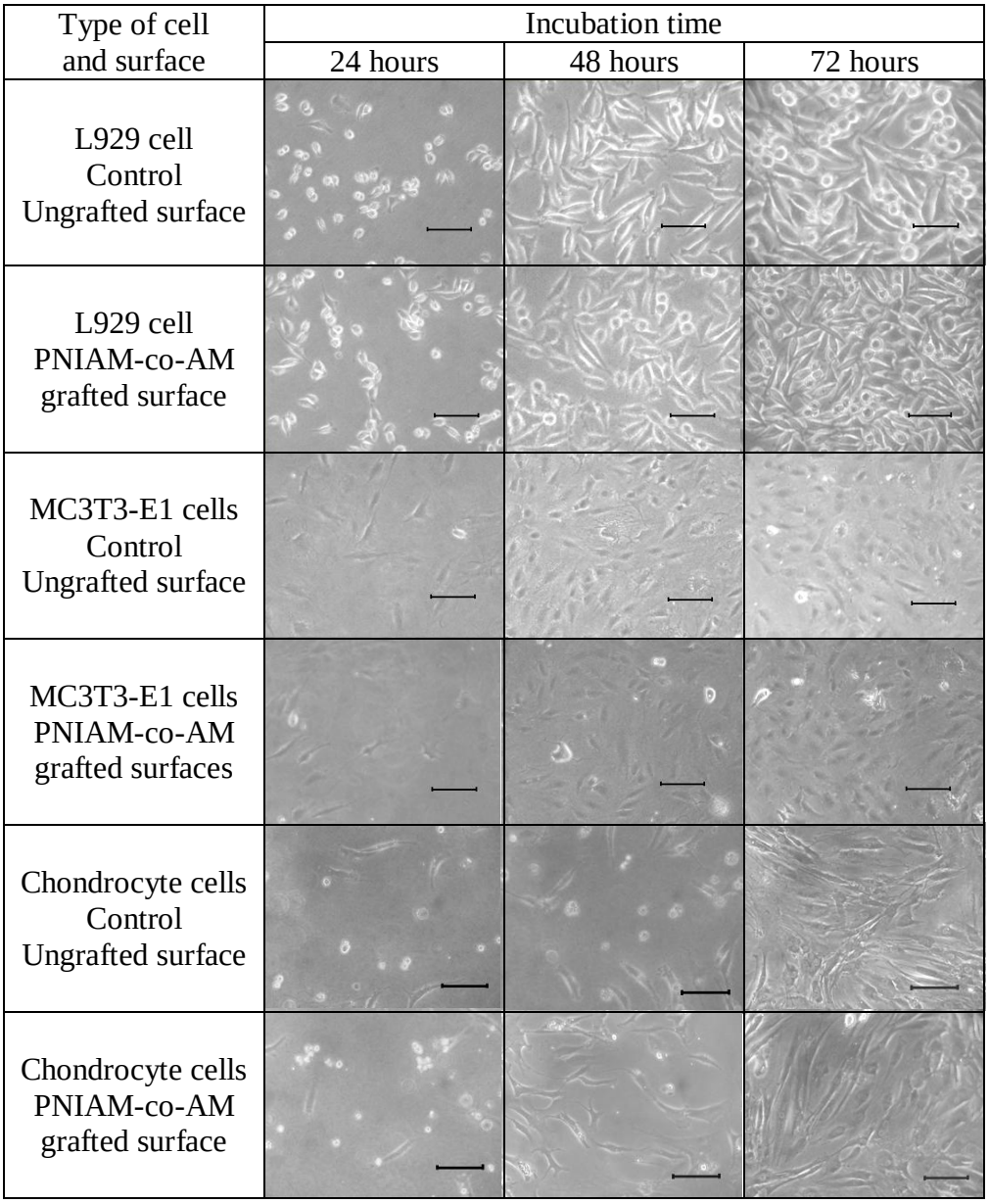


Figure 4.20 The morphology of L929, MC3T3-E1 and chondrocyte cells on the thin PNIAM-co-AM grafted surface at 1.0×10^3 cells/ml. Scale bar = 100 µm

4.7.4 Single Cell Detachment from the Thin PNIAM-co-AM grafted TCPS

A more consistent temperature-responsive surfacethat we used to compare with the results from the previous study wasthe thin PNIAM-co-AM film. The un-reacted monomer was removed from the surface after polymerization. Therefore, the majority of the grafted area was more homogenous than the thick copolymer film. On the other hand, the changes in the wet thickness and hydrophilic/hydrophobic alterations copopolymer grafted surface during phase transitions (~32°C) were in nanoscale, as confirmed by the AFM result. Moreover, the contact-angle changes with temperature were also concerned [13]. We called the new grafted surface as "Thin PNIAM-co-AM grafted surface".The root-mean-square (RMS) roughness of the thin grafted surface at above and lower LCST was consistent with the RMS roughness of the PNIAM-co-AM grafted polystyrene

surface. Thus, the thin copolymer grafted surface was used in the single cell study. The results were shown in Figure 4.21.

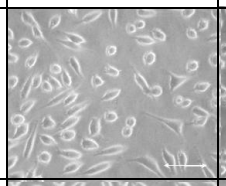
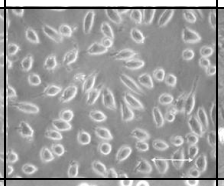
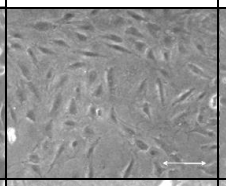
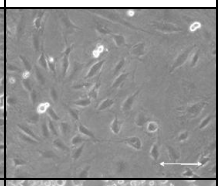
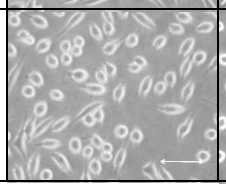
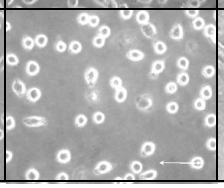
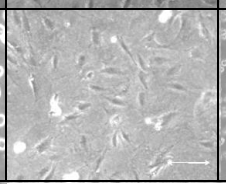
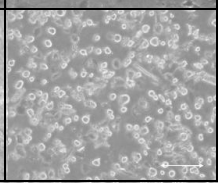
Type of surface	L929 cells		MC3T3-E1 cells	
	30 minutes	60 minutes	30 minutes	60 minutes
Control ungraftedsurface				
Thin PNIAM-co-AM grafted surface				

Figure 4.21 The morphology of L929 and MC3T3-E1 cells on the control and the thin grafted surfaces after lowering temperature to 10°C for 60 minutes. Scale bar = 100µm

From the detachment of individual L929 and MC3T3-E1 cells, thin PNIAM-co-AM grafted surfaces exhibited well defined temperature sensitivity. It was expected that cells cultured on PNIAM-co-AM grafted surfaces could be detached simply by decreasing the temperature from 37°C (hydrophobic) to 10°C (hydrophilic). It was observed that when the culture temperature was reduced to 10°C after 48 hours of incubation at 37°C during which almost all of the seeded cells were attached and spread on those surfaces. The spread cells were rounded and detached from the surfaces.

Figure 4.22 shows the percentage of detached L929 and MC3T3-E1 single cells from the thick and thin PNIAM-co-AM grafted surfaces. For the thick copolymer grafted surface, detached L929 and MC3T3-E1 individual cells were 52.35±13.64% and 54.39±7.81%. On the control surfaces, the detached cells were 3.23 ± 1.26 and 1.09 ± 0.8%, respectively. On the thin consistent copolymer surfaces, the percentage of single cell detachment of L929 and MC3T3-E1 were 77.22 ± 10.28% and 78.25 ± 8.76% but on the control surfaces, were about 10.49± 3.28% and 10 ± 5.76%, respectively.

It can be seen that there were few cells detached from control un-grafted surface because of no surface property alteration, while the cells could detachfrom these copolymer grafted surface by reducing the temperature. This cell detachment is attributed to the formation of an expanded, swollen, and hydrophilic surface of the temperature responsive polymer, which weakened cellular adhesion and resulting in spontaneous cell detachment due to the hydration of PNIAM below its LCST [40].

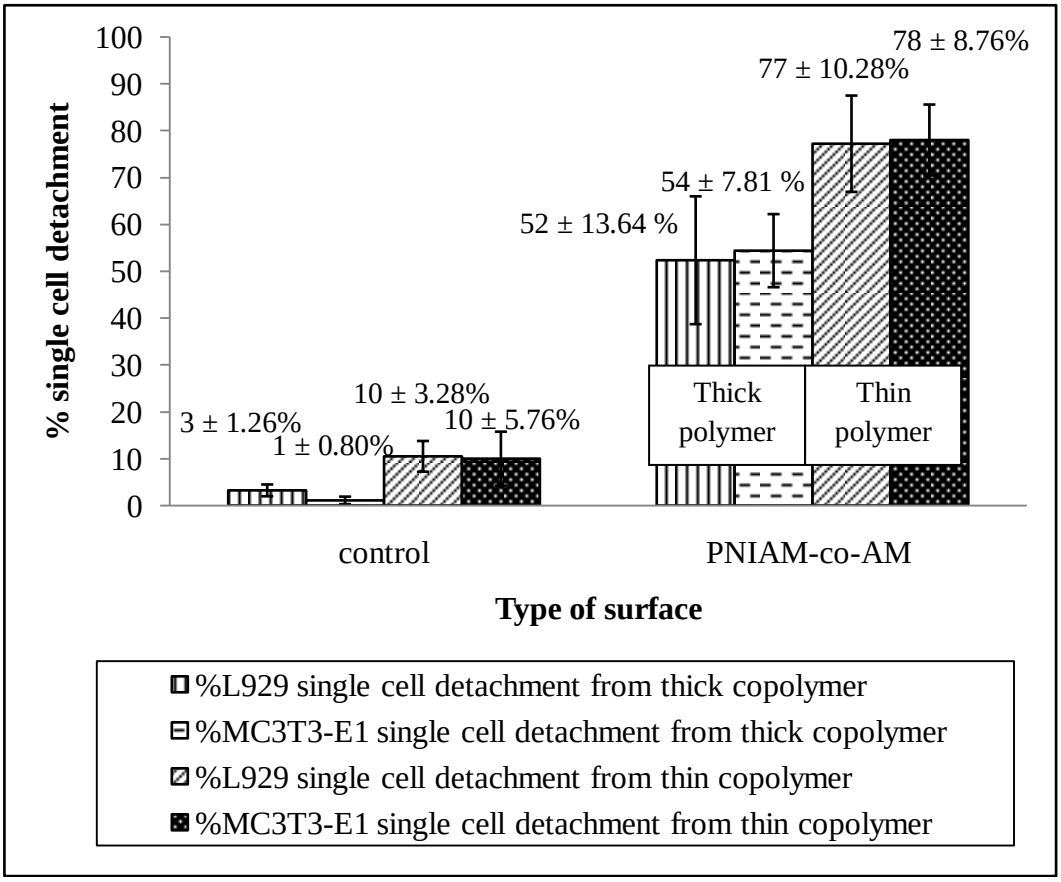


Figure 4.22 The percentage of L929 and MC3T3-E1 single cell detachment at 10°C

One should be noted that the spreadcells on the thinPNIAM-co-AM grafted surfaces showed more individual cell detachment behavior than that on the thick PNIAM-co-AM grafted surfaces. This PNIAM conformation could accelerate the hydration of the material. When the temperature decreased from 37°C to 10°C, the re-swelling of the surfaces occurred due to the hydration of PNIAM [41].

The surface of the thin copolymer was cleaned using ethanol, which they allowed the cells to attach at the normal condition. The cells also detached without using enzymes when the temperature was reduced to 10°C. This property reveals that this type of copolymer has potential as a temperature-responsive culture surface for a cell sheet study.

4.7.5 The retrieval of single cell re-attachment

The harvested cells could be transferred to another surface and re-grown. In this study, we first transferred harvested single L929 and MC3T3-E1cell onto new tissue culture surfaces. The reattachment of single cell detachment showed that the cells in the next cultured after 5 days of incubation maintained the cell viability (Figure 4.23). It can be noticed that either low temperature treatment has not affected the viability of the cells.

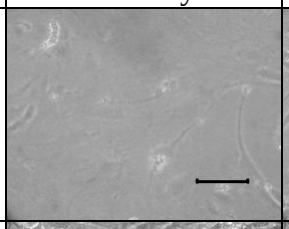
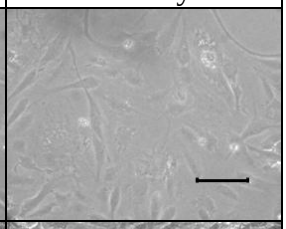
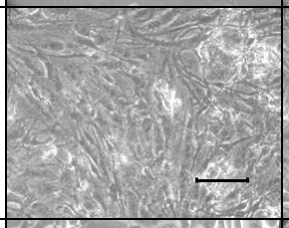
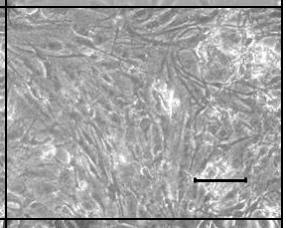
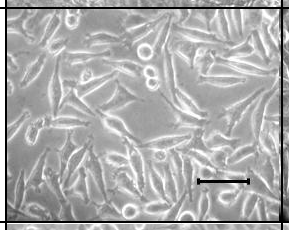
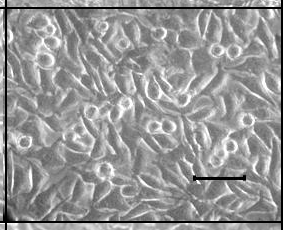
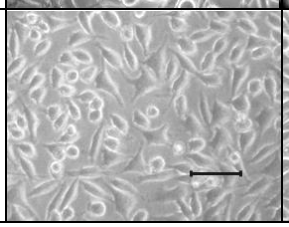
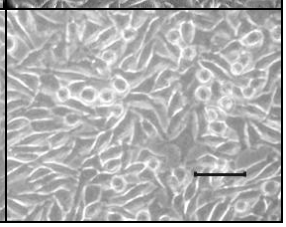
Type of harvested cell	Reattach for 3 days	Reattach for 5days
MC3T3-E1 cells Harvested from thick polymer at 10°C		
MC3T3-E1 cells Harvested from thin polymer at 10°C		
L929 cells Harvested from thick polymer at 10°C		
L929 cells Harvested from thin polymer at 10°C		

Figure 4.23 The morphology of retrieval MC3T3-E1 and L929 cells. Scale bar = 100 µm

4.7.6. Single Chondrocyte Cell Detachment from the Thin PNIAM-co-AM grafted surface

In the case of the human chondrocyte single cell detachment from the thin PNIAM-co-AM grafted surface. The morphology of detached chondrocyte cells on the grafted surface was observed after low temperature at 20°C incubation (Figure 4.24).

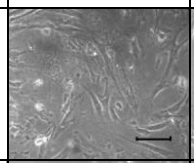
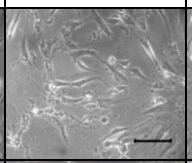
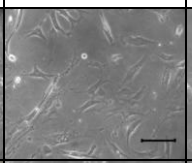
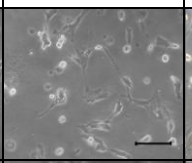
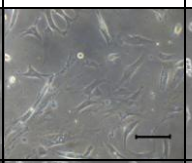
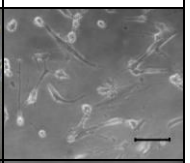
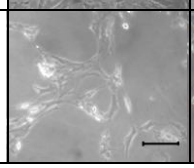
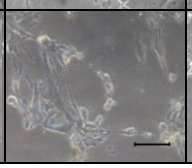
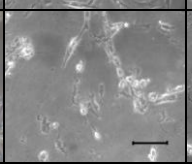
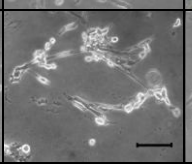
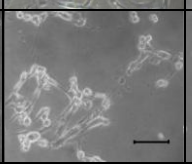
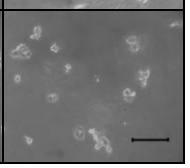
Time	10 minutes	20 minutes	30 minutes	40 minutes	50 minutes	60 minutes
Control						
Grafted surface						

Figure 4.24 The morphology of chondrocyte cell on control and the thin grafted surfaces after lowering temperature to 10°C (30 minutes) followed by 20°C (60minutes). Scale bar = 100µm

In this experiment, the low temperature treatment was adjusted from 10 to 20°C to increase the cell metabolism. The detailswill be described in thestudy of cell sheet detachment. After incubated at 10°C followed by 20°C, at each time point, chondrocyte cells were detached from the grafted surface within 60 minutes. In the following work, weexamine the percentage of the single cell removal with the incubation time from 10 to 60 minutes in the single cell study.

For the thin PNIPAM-co-AM grafted surfaces from which single chondrocyte cells were released, Figure 4.25 shows the percentage of human chondrocyte single cell detachment. The percentage of cell detachment was 92.85±4.13% on the grafted surface. Nevertheless, the cell detachment from the ungrafted surface was 25.77±1.98%. This result suggests the possibility of achieving rapid cell detachment using PNIPAM-co-AM grafted TCPS through which water interacted with the amine group (hydrophilic part) of copolymer, as well as the decrease in temperature after polymer chain was extended, resulting in rapidly pushing the cell away from the surface without enzyme.

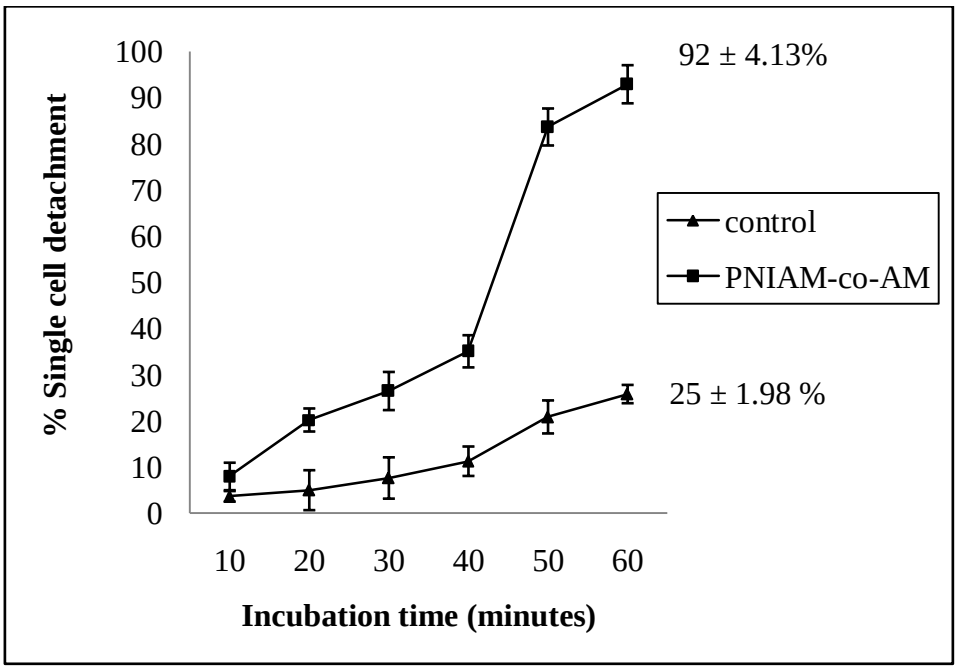


Figure 4.25 The percentage of single chondrocyte cell detachment at (30 minutes) followed by 20°C (60 minutes).

After re-grown, human chondrocyte cell lines showed fibroblast morphology and proliferated well on new TCPS. However, longer incubation time (7 days) was required for reach confluence (Figure 4.26).

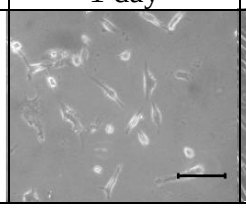
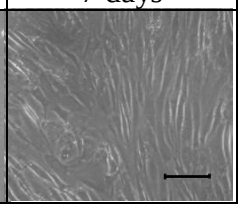
Cell type and incubation time	1 day	7 days
human chondrocyte cell harvested from thin polymer		

Figure 4.26 The morphology of retrieval human chondrocyte cell. Scale bar = 100 µm.

4.8 Cell Sheet Attachment/Detachment Study

The cell sheet study using L929 and MC3T3-E1 cells was explained. The purpose of this section is to find the optimal condition to obtain a complete cell sheet using a culture surface grafted with PNIPAM-co-AM. The percentages of cell sheet detachment from the thick and thin PNIPAM-co-AM grafted TCPS were determined. Therefore, the type of copolymer was confirmed to be applicable for cell sheet detachment. Then, the cell detachment from the selected copolymer grafted surface was compared with the detachment using the commercially available grafted UpCell™ surface. The experiments were summarized as follows.

4.8.1 Cell sheet attachment and detachment from the thick PNIPAM-co-AM grafted TCPS
4.8.2 Cell sheet attachment and detachment from the thin PNIPAM-co-AM grafted TCPS
4.8.3 Mono-layered sheet and double-layered sheets study
4.8.4 Cell sheet attachment and detachment sheet from the PNIAM-co-AM grafted surface with longer exposure time.
4.8.5 Cell sheet attachment and detachment sheet from the commercial UpCell™ surface.

4.8.1 Cell sheet attachment and detachment from the thick PNIPAM-co-AM grafted TCPS

1. Cell attachment/detachment at a cell density of 5.0×10^4 cells/ml

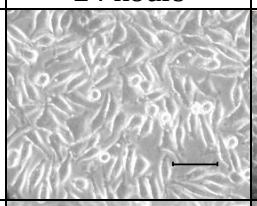
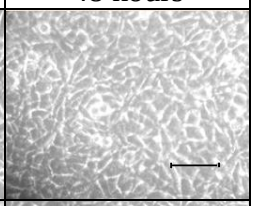
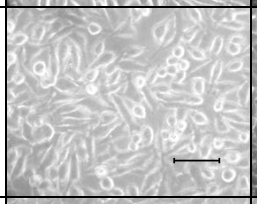
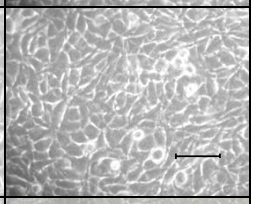
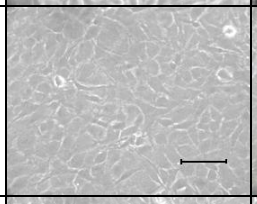
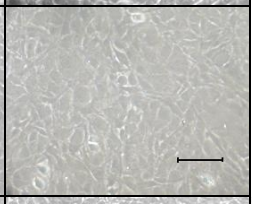
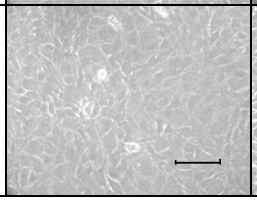
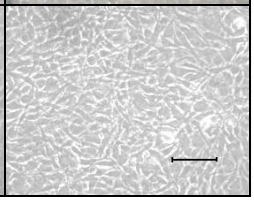
Type of celland surface	Incubation time	
	24 hours	48 hours
L929 cell Control Ungrafted surface		
L929 cell PNIAM-co-AM grafted surface		
MC3T3-E1 cell Control Ungrafted surface		
MC3T3-E1 cell PNIAM-co-AM grafted surface		

Figure 4.27 The morphology of L929 and MC3T3-E1 cells on the thick PNIAM-co-AM grafted surface at 5.0×10^4 cells/ml. Scale bar = 100 µm

In the single cell detachment study, which the cells were seeded at 1.0×10^3 cells/ml results, longer incubation time was required to reach confluence. To shorten the incubation time, higher cell density was used in the cell sheet detachment study.

The purpose of this study is to provide tight cell-to-cell junction for spontaneous detachment. However, the content of the attachment protein should not be too high to cause difficulty in the cell lift-off process. Therefore, the mouse fibroblast L929 and MC3T3-E1 cells at high cell density were seeded on the thick PNIAM-grafted compared with the control surface to a density of 100% confluence.

We found that after 48 hours at 37°C incubation, seeded L929 and MC3T3-E1 cells at a cell density of 5.0×10^4 cells/ml were well attached, spread and, proliferated to 80-100% confluence on the thick grafted surfaces and ungrafted surface (Figure 4.27). The cells were structurally connected to each other by junction proteins and adhered to the surface of the dish with adhesive protein.

1.1 Cell Sheet Detachment from the thick PNIAM-co-AM grafted at 10°C

These culture cells were first incubated at low temperature at 10°C in order to detach the cells. The results show that on the thick PNIAM-grafted surface, L929 and MC3T3-E1 cells detached within 60 minutes (Figure 4.28).

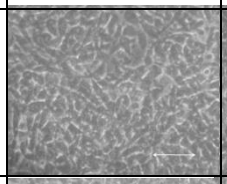
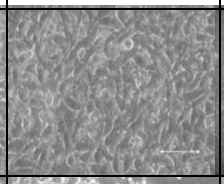
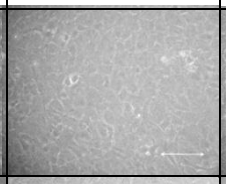
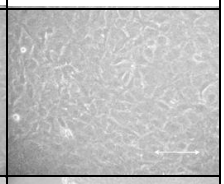
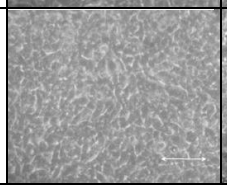
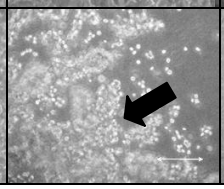
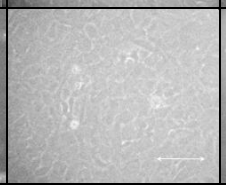
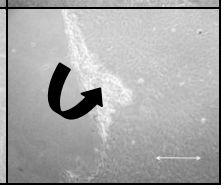
Type of surface	L929 cell		MC3T3-E1 cell	
	30 minutes	60 minutes	30 minutes	60 minutes
Control ungrafted surface				
PNIAM-co-AM grafted surface				

Figure 4.28 The morphology of L929 and MC3T3-E1 cells on the control and the thick PNIAM-co-AM grafted at 10°C for 60 minutes. Scale bar = 100µm

A majority of the cells still attached to the control surface. Both cell types could be detached as individual cells and cell patches were not spontaneously detached. The percentage of L929 and MC3T3-E1 detached cell was 14.03 ± 2.8% and 28.65 ± 4.74%, respectively.

As different cells have different temperature sensitivities for cellular metabolism, the fibroblast-like and pre-osteoblast-like cells require different optimum temperatures for their detachment. At 10°C, they showed minor cell lift off from the thick copolymer surface and there was no spontaneous cell detachment. While, the cells were harvested from the grafted surface, they were quite fragile and prone to breakage because of weakened cell–cell junctions. Moreover, the cell density at over confluence that provided high cell surface protein which led to high forces between cell and grafted surface. These pointswere concerned and proved for optimal cell detachment condition.

Dr. Terno Okano reported that when the surfaces of the culture dishes were hydrated, the cells were affected because they were very sensitive. The success of cell detachment requires an increase in temperature to recover cell metabolism after decreasing the temperature [25]. These demonstrated that the culture dishes were transferred to 10°C, possibly inhibiting the metabolism of cell. Therefore, lowering temperature to 20°C was used, instead of the incubation temperature at 10°C.

1.2. Cell Sheet Detachment from the thick PNIAM-co-AM grafted at 20°C

By reducing the culture temperature to 20°C, the cell sheet detachment at cell density of 5.0×10^4 cells/ml were initially carried out with MC3T3-E1 cells because they showed more tight junction when the temperature was reduced than did L929 cells at 10°C. Then, MC3T3-E1cells were cultured on the control and the thick PNIAM-co-AM grafted surface to a density of 100% confluence. Cell-cell junctions were established. When the culture temperature was reduced to 20°C for 30 and 60 minutes, the cultured cells did not detach at all. The cells could only be detached by gently flushing the surface with culture medium after 90 minutes incubation (Figure 4.29).

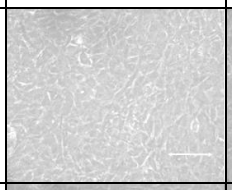
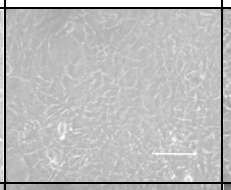
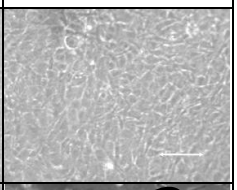
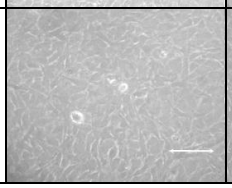
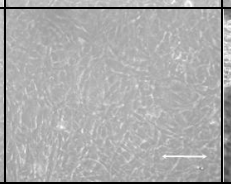
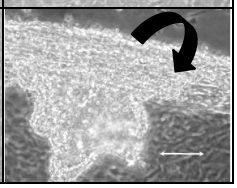
Type of surface	Incubation time		
	30 minutes	60 minutes	90 minutes
Control ungrafted surface			
PNIAM-co-AM grafted surface			

Figure 4.29 MC3T3-E1 morphology (cell density of 5.0×10^4 cells/ml) on the control and the thick PNIAM-co-AM grafted at 20°C for 90 minutes. Scale bar = 100µm

The results show both detached and adherent portions of MC3T3-E1 monolayer. From this result, we can determine the behavior of cell detachment into 2 steps. The grafted copolymer chain becomes hydrated, itsfilm volume expands and the surface becomes hydrophilic phase that leads to the cell release from the structure [42]. In contrast, there were no cells detached from the control surface because of no surface property alteration.In this experiment, the PNIAM-co-AM grafted surface showed both detached and adherent portion of MC3T3-E1 sheet probably because non-homogenous grafting as described in the single cell detachment from the thick copolymer part.

Although, the cells detached from the PNIPAM-grafted surface like a sheet and patch but harvested cells sheet were readily separated by slight agitation and pipetting, probably because there were weak cell-cell junction. However, by lowering the temperature to 20°C in this study, the percentage of cell viability was more than that at 10°C.

2. Cell attachment/detachment at a cell density of 1.0×10^5 cells/ml

In this experiment, the effect of cell density on cell detachment has been evaluated. This study was designed to study the effect of higher cell density and investigate the tight junction between the cells, which might lead to spontaneous cell detachment.

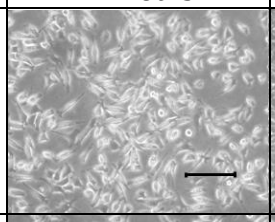
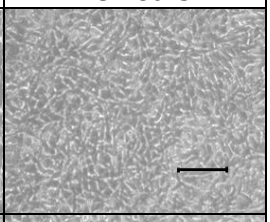
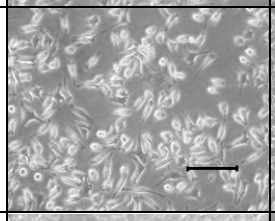
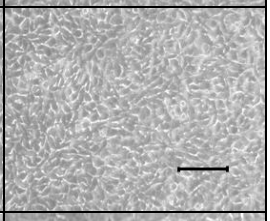
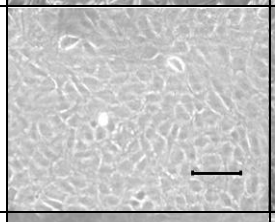
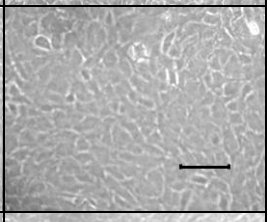
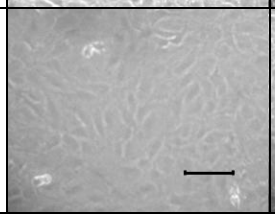
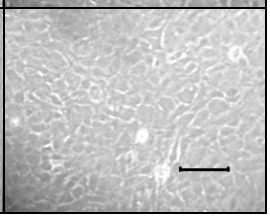
Type of cell and surface	Incubation time	
	24 hours	48 hours
L929 cell Control ungrafted surface		
L929 cell PNIAM-co-AM grafted surface		
MC3T3-E1 cell Control ungrafted surface		
MC3T3-E1 cell PNIAM-co-AM grafted surface		

Figure 4.30 The morphology of L929 and MC3T3-E1 cells (cell density of 1.0×10^5 cells/ml) on the control and the thick PNIAM-co-AM grafted. Scale bar = 100µm

When we increased the cell density to 1.0×10^5cells/ml, after 48 hours, both cell types readily formed strong cell-cell adhesion and exhibited fibroblast characteristic on the thick grafted copolymer similar to those on the control as shown in Figure 4.30. High cell density led to rapid attachment of the cells on to the grafted surface similar to those on the control surfaces.

2.1 Cell Sheet Detachment from the thickPNIAM-co-AM grafted at20°C

The cell sheet detachment study at the cell density of 1.0×10^5 cells/ml at 20°C was done with MC3T3-E1 and repeatedusing L929 cells. According to the cell attachment results, L929 and MC3T3-E1 cells at a cell density of 1.0×10^5 cells/ml rapidly adhered and proliferated on the PNIAM-co-AM grafted surface and control wells plate to a density of 100% confluence. After the cells had reached confluence, below the lower critical solution temperature (~32°C) of polymer, the grafted surface became hydrophilic, due to the transition of the polymer from collapsed globule to extended coil formation. A majority of L929 and MC3T3-E1 cells were slightly detached from the surface like a sheet as shown in Figure 4.31.

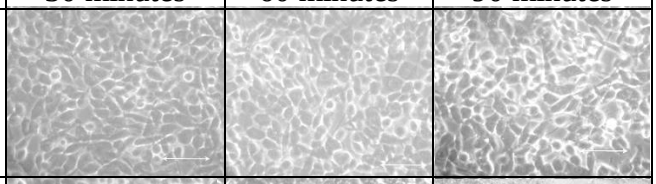
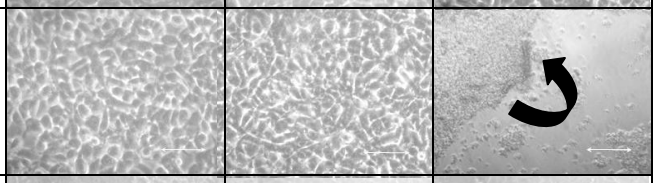
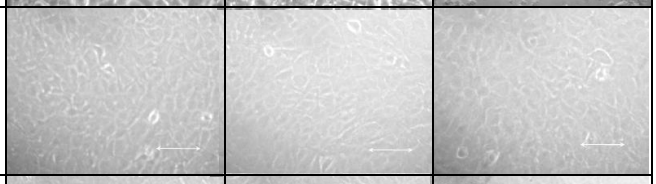
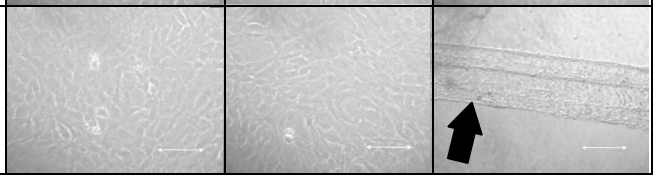
Type of cell	Type of surface	Incubation time		
		30 minutes	60 minutes	90 minutes
L929 cells	Control ungrafted surface			
	Thick PNIAM-co-AM grafted surface			
MC3T3-E1 cells	Control ungraftd surface			
	Thick PNIAM-co-AM grafted surface			

Figure 4.31 The morphology of L929 and MC3T3-E1 cells (cell density of 1.0×10^5 cells/ml) on the control and the thick PNIAM-co-AM grafted at 20°C for 90 minutes. Scale bar = 100µm

The results of L929 detachment show cell detach like a sheet due to strong cell-cell junction more than at a density of 5.0×10^4 cells/ml. However, there were single cells and cell patches observed from the grafted surface because of mild agitation. In contrast, MC3T3-E1 cells were not prone to breakage. They could be recovered as continuous intact monolayer by lowering the culture temperature from 37 to 20°C without digestive enzymes and chelating agents. In addition, we found that an important difference in confluent cultures of cells versus single cells is the formation and maintenance of the cell-to-cell connection in confluent cultures. Cells harvested by trypsinization that used digestion enzyme degraded these cell–cell junctions, producing single cell suspensions. Using thePNIAM-co-AM grafted surface allows temperature reduction to harvest the cells, retaining cell–cell junctions and permitting cell confluence maintenance post-harvest in large area sheets. Furthermore, this cells monolayer maintained basal surface extracellular matrix (ECM) proteins after cell detachment.

4.8.2. Cell sheet attachment and detachment from the thin PNIPAM-co-AM grafted TCPS

The mouse fibroblast cells were cultured following the same method as in the cell attachment on the thick grafted TCPS. Seeded L929 and MC3T3-E1 cells at high cell density were well attached similar to those on the thick surface as described below.

1. Cell attachment at a cell density of 5.0×10^4 cells/ml

To construct a cell sheet, the cell density was increased. L929 and MC3T3-E1 Pre-Osteoblast cells at the cell density of 5.0×10^4 cells/ml were well attached and spread on the modified surfaces. They reached confluency within 48hours of incubation. Figure 4.32 shows 100% confluence and cellular tight junction.

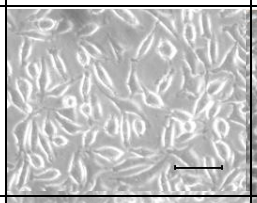
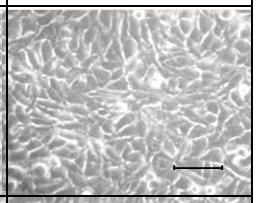
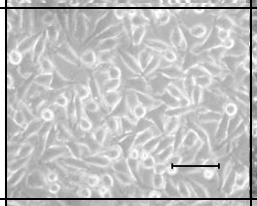
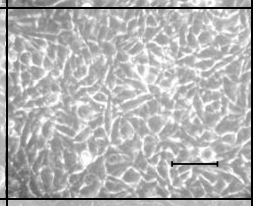
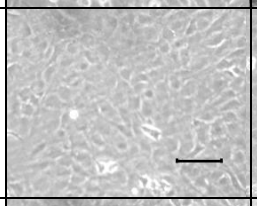
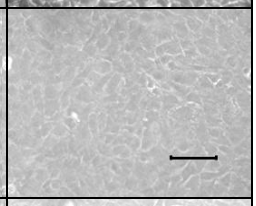
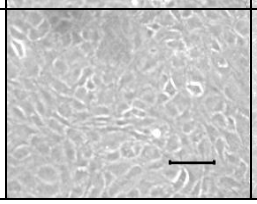
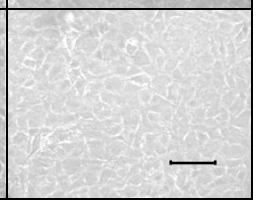
Type of cell and surface	Incubation time	
	24 hours	48 hours
L929 cell Control ungrafted surface		
L929 cell PNIAM-co-AM grafted surface		
MC3T3-E1 cell Control ungrafted surface		
MC3T3-E1 cell PNIAM-co-AM grafted surface		

Figure 4.32 The morphology of L929 and MC3T3-E1 cells (cell density of 5.0×10^4cells/ml) on the control and the thin PNIAM-co-AM grafted. Scale bar = 100µm

The cell attachment and proliferation of the L929 and MC3T3-E1 cells on thick and thin copolymer grafted surface were similar to those on the control or ungrafted surface. However, in the cell detachment study, we found the different results. Although the cell density was increased to 5.0×10^4cells/ml but L929 cells were not able to form a complete cell sheet (data not shown).

2. Cell attachment at a cell density of 1.0×10^5 cells/ml

L929 and MC3T3-E1 cells were plated onto each surface at a density of 1.0×10^5 cells/ml and cultured at 37°C. These cells were well attached and spread on all the sample surfaces within 1 hour incubation at the normal condition. After 48 hours of incubation at 37°C, almost all of the seeded cells were attached and spread on those surfaces (Figure 4.33).

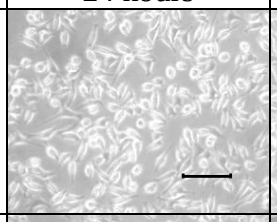
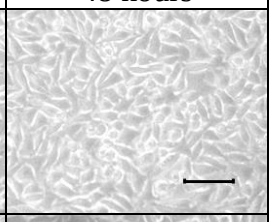
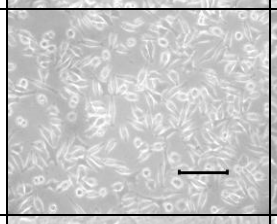
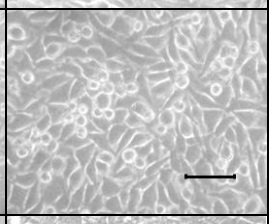
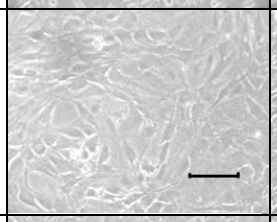
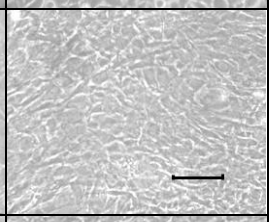
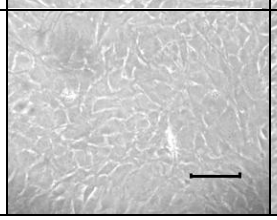
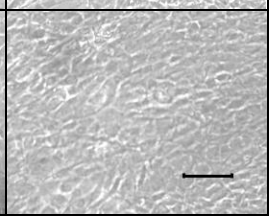
Type of cell and surface	Incubation time	
	24 hours	48 hours
L929 cell Control ungrafted surface		
L929 cell PNIAM-co-AM grafted surfaces		
MC3T3-E1 cell Control ungrafted surface		
MC3T3-E1 cell PNIAM-co-AM grafted surfaces		

Figure 4.33 The morphology of L929 and MC3T3-E1 cells (cell density of 1.0×10^5cells/ml) on the control and the thin PNIAM-co-AM grafted. Scale bar = 100μm

The images clearly show the cells adhered well and began to grow on those grafted surface, indicating good cell compatibility of both copolymer films.

3. Cell Detachment from the Thin PNIAM-co-AM grafted surface at 20°C

On the thick polymer, low temperature treatment at 20°C and a cell density of 1.0×10^5cells/ml led to cell sheet detachment. However, the troubles of cell detachment were that the cellswere not spontaneously detached from the surface. There was some portion of the grafted surface that showed the cell adhesion. Therefore, we went back to the polymer properties to prove the consistence of the grafted surface. According to the detachment results from the thick copolymer study, we suggest that there were some portion of TCPS were not grafted by copolymer and some area there were a lot of non-reacted monomer because we did not remove it out in the step of thick copolymer preparation. In the cell detachment from the thin PNIAM-co-AM grafted surface, we used the same condition as in the thick copolymer grafted surface. L929 and MC3T3-E1 cells on the PNIAM-co-AM grafted surface and control surfacewere cultured to reach 100% confluence. These cells had been cultured at 37°C on the thin copolymer. Only MC3T3-E1 cells were released by lowering the temperature to 20°C and the cell sheet morphology was determined by photographed. The cells morphology was observed at 30, 60, and 90 minutes. The MC3T3-E1 cells spontaneously detached as a cell sheet within 90 minutes. In contrast, the confluence L929 did not detached from the grafted surface (Figure 4.34).

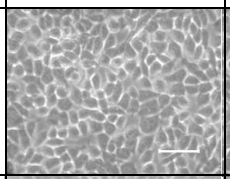
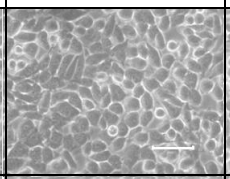
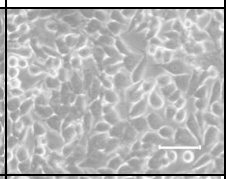
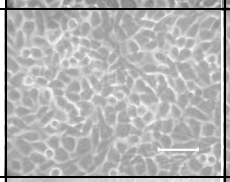
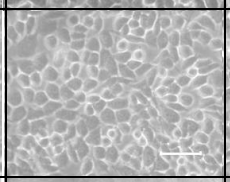
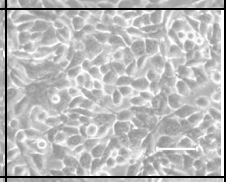
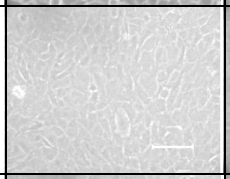
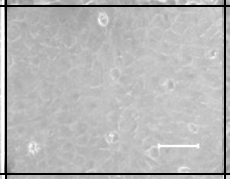
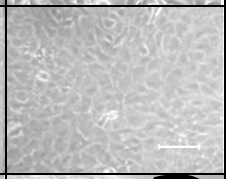
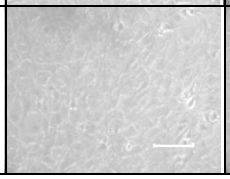
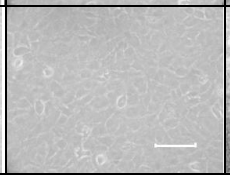
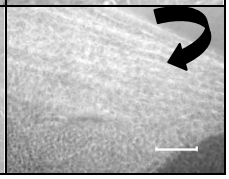
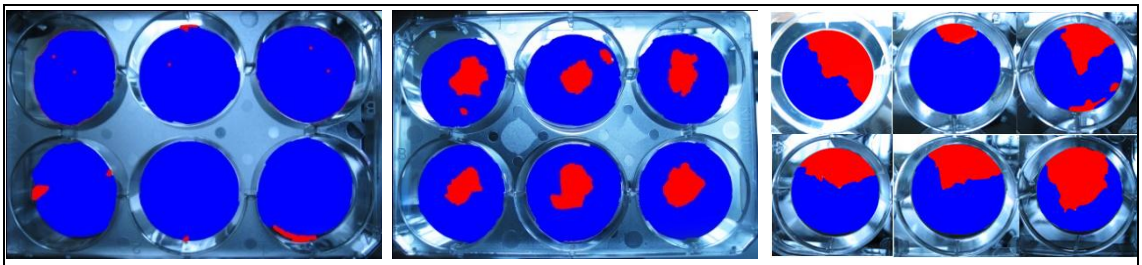
Type of cell	Type of surface	Incubation time		
		30 minutes	60 minutes	90 minutes
L929 Cells	Control ungrafted surface			
	Thin PNIAM-co-AM grafted surface			
MC3T3-E1 cells	Control ungrafted surface			
	Thin PNIAM-co-AM grafted surface			

Figure 4.34 L929 and MC3T3-E1 morphology (cell density of 1.0×10^5 cells/ml) on the control and the thin PNIAM-co-AM grafted at 20°C for 90 minutes. Scale bar = 100µm

The MC3T3-E1 cells sheet started to detach from the sheet edges and moving toward the cell sheet interior, most probably because water penetrated the interface between the cell sheets and grafted dish surfaces only from the edge [41]. The time and suitably low temperature required to recover cell sheet completely from TCPS surface grafted with thin PNIAM-co-AM. In the case of L929 cell, there were no cell detached from the grafted surface probably because of too high cell numbers and the temperature at 20°C is not suitable for them.

From this result, the efficiency of the copolymer grafted surface based on the photographs of the culture surface after cell detachment also was measured (Figure 4.35). Red color isthe cell detachment area. Blue color refers to the remaining attached cells. The control well plate shows the cells still remained on the surface at 20°C for 90 minutes. For thin film grafted surfaces, they were washed with ethanol, allowing the co-polymer to spread across the surface.Then, the copolymer only formed at the periphery. In case of thick film, there are a lot of copolymer occurs at the center of well plate. Un-reacted monomers that were not removed led to inhomogeneity of the grafted surface. Therefore, the detached cells were observed only at the center.



(A) Control well plate | (B) Thick PNIAM-co-AM grafted surfaces | (C) Thin PNIAM-co-AM grafted surfaces

Figure 4.35 The cultured well plates after MC3T3-E1 cell lifted off at 20°C.

The percentages of MC3T3-E1 detached cells from thick, thin and un-grafted surface after temperature reduction to 20°C were compared as shown in Figure 4.36. The MC3T3-E1 cells detached from thick and thin copolymer was 14.69±2.07% and 31.51±9.70%, respectively. Such surfaces exhibited temperature-responsive hydrophilic/hydrophobic alterations with temperature changes to 20°C, resulting in thermally modulated attachment and detachment. On the control surface, the percentage of cell detachment was 0.64±1.16%, which was affected by agitation. When lowering temperature, PNIAM-grafted culture surfaces changed from hydrophobic to hydrophilic because of the hydration of grafted PNIAM polymer, resulting in the release of adherent cells. Therefore, MC3T3-E1 cells detached from the PNIAM-co-AM grafted surface by lowering the temperature, with slight agitation and pipetting. These techniques have a great effect on both cell morphology and cell-cell junction. Although cell sheet detachment can be further accelerated by pipetting or agitation, recovered cell sheets are prone to be friable because of weakened cell–cell junctions.

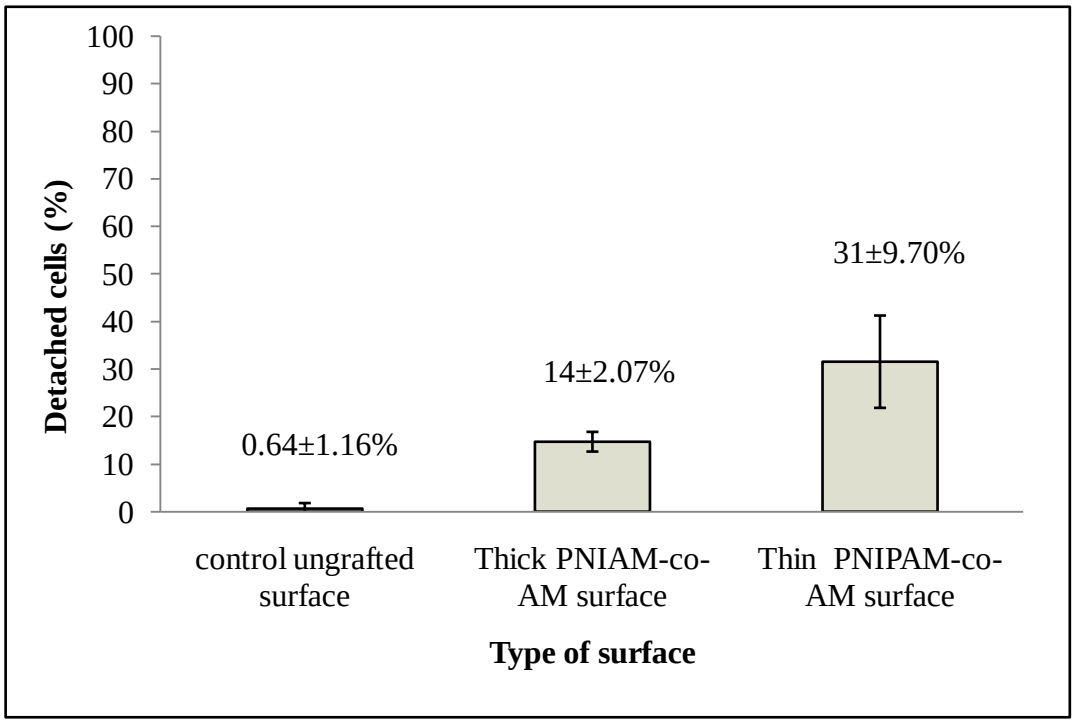


Figure 4.36 The percentage of MC3T3-E1 cell sheet detachment from the thick and thin PNIAM-co-AM grafted surface at 20°C for 90 minutes.

Since, the copolymer and temperature for cell detachment were demonstrated. The MC3T3-E1 sheet exhibited the higher percentage of cell detachment than the thick PNIAM-co-AM grafted TCPS surfaces. The cell sheets formed on PNIAM-co-AM grafted TCPS surfaces detached slowly and gradually, beginning from the sheet edges and moving toward the cell sheet interior, presumably because water penetrated the interface between cell sheets and the PNIAM-co-AM grafted dish surfaces mainly from the periphery of cells [43]. In this study, we did not use PVDF membrane. Thus, harvesting by this method appeared to affect the appearance of the cells to round shape and broke the cell layer into small pieces. Because, cells were linked together by cell junctions and were supported by a matrix which they themselves secreted [44]. If we used the membrane for cell harvesting, this method provides the mechanical strength necessary for the handling of the detached cell layer. Furthermore, harvested cell were held together by cell junctions and the extracellular matrix (ECM) protein deposited by the cells. Therefore, the PVDF membrane was used for further cell sheet harvesting.

4. Viability Test (Proliferation of the harvested cell sheet)

To analyze the growth potential of the retrieved cell construct, the cell sheet was transferred to a new culture dish. The harvested L929 and MC3T3-E1 cells could be transferred to another surface and re-grown. Figure 4.37 shows the proliferation of L929 and MC3T3-E1 cells from the previously transferred cell sheet after re-grown for 48 hours on a new culture dish. This cell layer could adhere to and proliferate on the new culture cell surface, indicating that the harvested cell sheet was healthy.

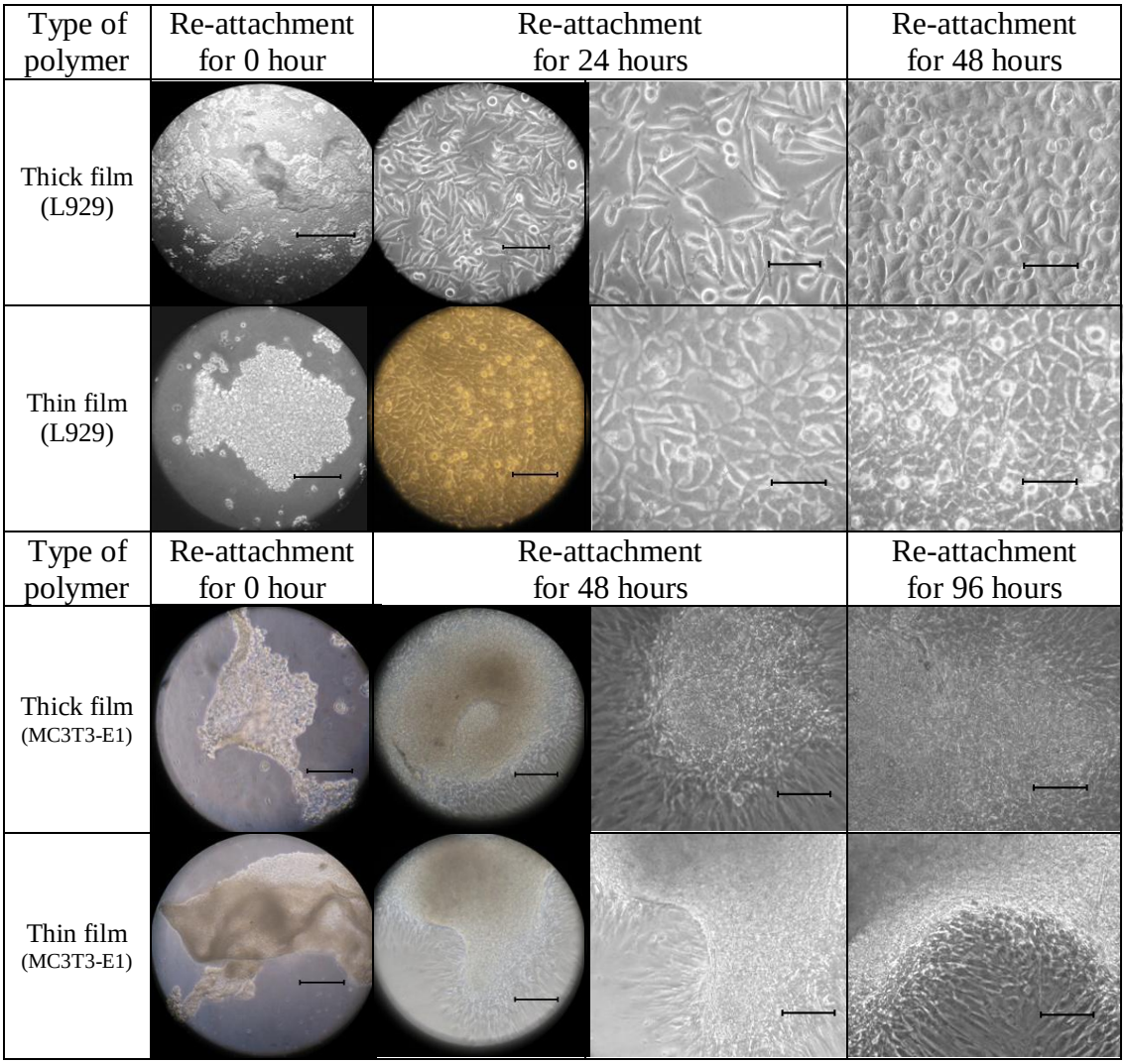


Figure 4.37 The morphology of harvested L929 and MC3T3-E1 cells on new TCPS after 48 and 96 hours incubation, respectively. Scale bar = 100µm

To confirm that the harvested cell sheet was composed of viable cells, the sheet was stained with LIVE/DEAD Assay Kit after the re-attachment. The live-dead stained images of the MC3T3-E1 cells were shown in Figure 4.38.

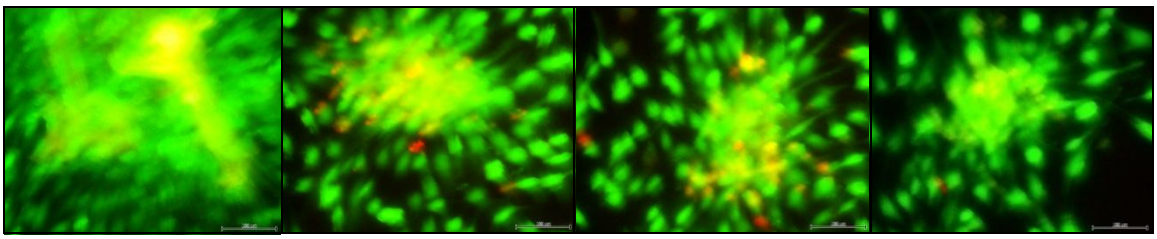


Figure 4.38 The live and dead stained images of MC3T3-E1 sheet after recovery from the thick PNIAM-co-AM grafted TCPS at 20°C. Scale bar = 100µm

After 48 hours (for L929 cell) or 96 hours (for MC3T3-E1 cell) reattached cells were stained with calcein AM and ethidium homodimer (EthD-1). The calcein AM is well preserved within live cells, producing an intense uniform green fluorescence in live cells. In contrast, EthD-1 enters cells with damaged membranes and binding to nucleic acids, thereby producing a bright red fluorescence indead cells.

In this study, the viability of the harvested cells was greater than 74.82±5.20% in green fluorescence (Table 4.3). Dead cells, exhibited red fluorescence, were mostly observed in the middle of the sheet.

Table 4.3 The percentage of the retrieval MC3T3-E1 cells, they were detached from the thick PNIAM-co-AM grafted surface at 20°C.

No.	Dead cells	Live cells	Total cells	Dead cells (%)	Live cells (%)	Live cells (%)
1	11.96	62.86	74.82	15.99	84.01	74.82±5.20%
2	18.31	84.08	102.39	17.88	82.12	
3	47.81	161.94	209.74	22.79	77.21	
4	46.45	109.42	155.87	29.80	70.20	
5	42.28	98.42	140.71	30.05	69.95	
6	42.28	98.42	140.71	30.05	69.95	
7	41.92	108.04	149.96	27.96	72.04	
8	46.53	119.22	165.75	28.07	71.93	
9	34.15	91.02	125.17	27.28	72.72	
10	23.61	83.85	107.47	21.97	78.03	

4.8.3 Mono-Layered Sheet and Double-Layered Sheet Study

1. Mono-Layered Sheet harvesting at 20ºC

In our previous studies, enzyme-free harvest of confluence cultured cell was achieved in MC3T3-E1 cell sheets by lowering culture temperatures to 20°C and flushing the grafted surface with culture media. At this point, temperature-dependent spontaneous cell sheet detachment was investigated on thin copolymer grafted surfaces by using PVDF membrane. A hydrophilically modified poly(vinylidenedifluoride) (PVDF) membrane is used to prevent cell sheets from shrinking and folding after detachment. Moreover, they were also used to construct the mono-layered and double-layered MC3T3-E1 sheet. In fact, cell detachment can also be accomplished without using a membrane. However, the detached cell sheets may shrink, folding, and the detachment process will require more time to allow the cell sheets to detach from the surface without any mechanical help [24].

In this experiment, we also compared the percentage of the cell sheet detached from PNIAM-co-AM grafted culture surface by flushing the surface with culture media and using a PVDF membrane. Mouse fibroblast cells both L929 and MC3T3-E1 cells (1×10^5 cells/well) were studied. In the case of flushing the surface with cultured media (without PVDF membrane), the cell morphology was observed at 30, 60, and 90 minutes. The results were shown in Figure 4.39. L929 cells still adhered onto the control surface after they were incubated at low temperature. We found that some of those adherent L929 cells on the control surface had fibroblast morphology, while somewere round, which may be affected by low temperature treatment and low CO_2. On the copolymer grafted surface, the cells were detached as pieces of the cells within 90 minutes. For the PVDF membrane

harvesting, the old cultured medium was discarded, and then fresh medium was added to prevent cell drying. We used sterile forceps to gently detach the cell layer around the edge of the membrane. Although these cells lifted-off from the grafted surface but there were a lot of harvested cells stuck on the PVDF membrane (data not shown).

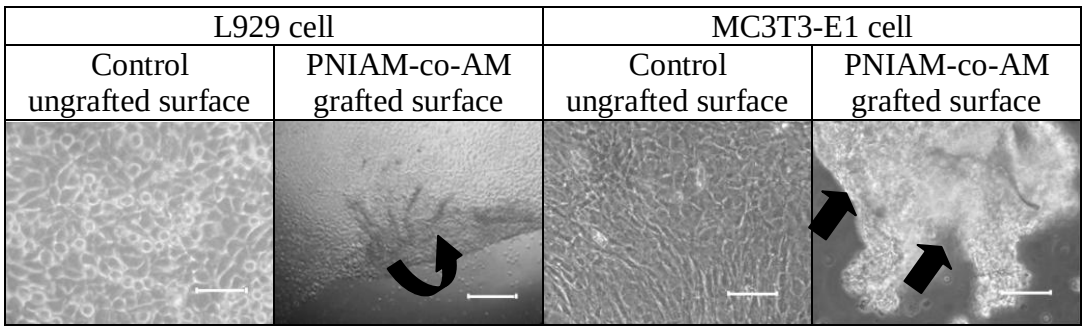


Figure 4.39 The morphology of L929 and MC3T3-E1 cells on the control and the thin PNIAM-co-AM grafted surface at 20°C. They were detached by flushing the surface with culture media. Scale bar = 100µm

Before reducing temperature, the culture medium was decreased in volume and a sheet of PVDF membrane was overlaid onto the cell layer. Becauseof the hydrophilic property of the membrane surface, the membrane rapidly stuck to the apical cell surface. That was difficult to re-attachment of the harvested cell sheet on new culture surface.

Figure 4.40 show culture plates after cell removal, most of the fibroblast cells still remained on the control surface after lowering temperature to 20°C for 90 minutes. The percentage of L929 cells detachment from the thin PNIPAM-co-AM grafted surface by flushing the surface (pipetting method) was 13.84±6.4%. In contrast, the percentage of L929 detachment by using PVDF membrane was 87.63±5.72%. For the MC3T3-E1 cell, the percentage was 30.5±5.6% (without PVDF membrane) and 85.79±8.23% (with PVDF membrane) (Figure 4.41).

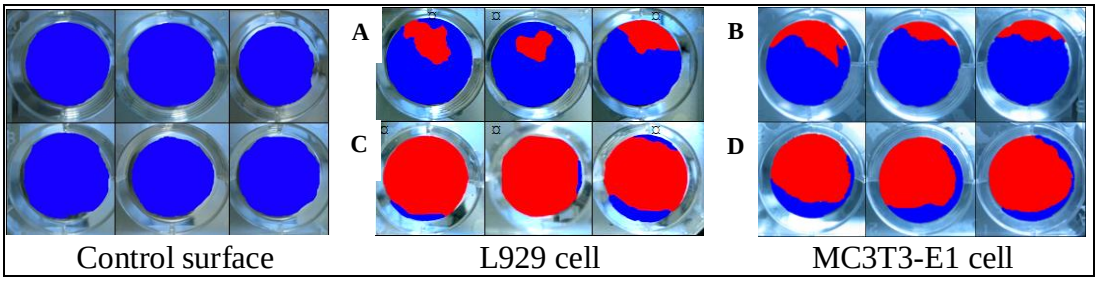


Figure 4.40 Areas of L929 and MC3T3-E1 cells detachment at 20°C
(Red color = detachment area, Blue color = attachment area)
(A, B) Cells were harvested by flushing the surface with culture media.
(C, D) Cells were harvested by using the PVDF membrane.

The percentage of detached cells at low incubation temperature shows that over 80% of the cells remained attached at 20°C after the temperature of the cell culture was decreased from 37°C. It was probably because the temperature at 20°C was not enough to

induce the polymer hydration. The cell layer was not pushed away from the grafted surface at this temperature.

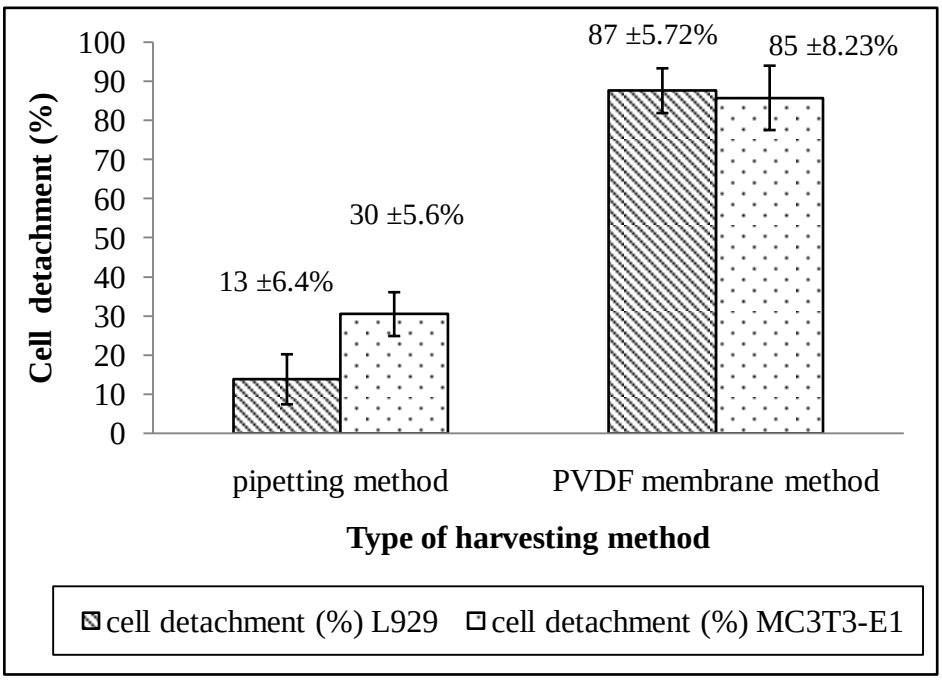


Figure 4.41 The percentage of cell sheet detachment from the thin grafted surfaces by pipetting method and using PVDF membraneat 20°C

These results show that the cell detachment was not directly correlated with the reduced temperature. The grafted copolymer chains were supposed to be hydrated and remained in expanded conformations atlower temperatures resulting in reduced interactions between the cells and grafted surfaces of the cell culture dishes [25].

2. Viability Test (Proliferation of the harvested mono-layered sheet)

The detached mono-layered L929 and MC3T3-E1cell sheets recovered from the thin PNIAM-co-AM grafted surface by using pipetting (no PVDF membrane) and using PVDF membrane at 20°C were stained with live and dead assay kit (Figure 4.42) and the percentage of cell viability were calculated (Table 4.4). It was confirmed that L929 and MC3T3-E1 cell sheets which harvested by using pipeting method or flushing the surface with culture media adhered closely to the new TCPS but the cell sheet was folded. On the other hand, the transfer process by using PVDF membrane was not successful. The cells stuck on the PVDF membrane which led to low cell viability.

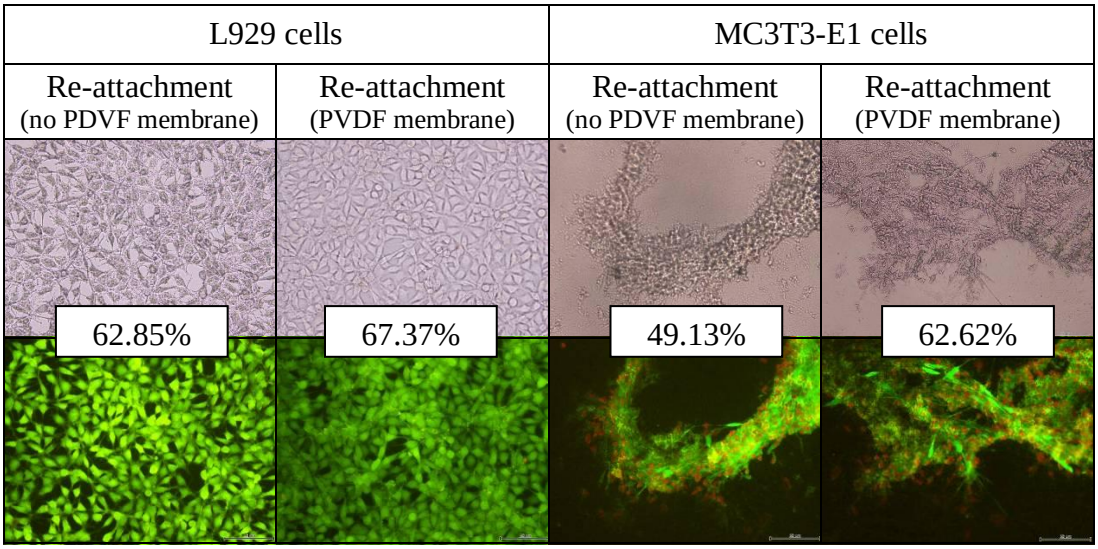


Figure 4.42 Phase contrast (upper) and the live-dead staining (lower) image of recovered L929 and MC3T3-E1 mono-layered sheet on new TCPS after 24 hours reattachment. Scale bar = 100µm

Since the PNIAM-co-AM graft surface supported L929 and MC3T3-E1 cell sheet study. It is demonstrated that under normal culture conditions at 37°C, L929 fibroblast cells proliferate to confluence on the copolymer surface. By reducing the temperature to 20°C for 90 minutes, the cultured cells could be harvested as a sheet. The change in the copolymer surface characteristic enabled the cultured cells to be detached from the dish without digestion enzyme. The harvested cells can be transferred to 35mm TCPS. Most of L929 cells were still alive. The cell viability was calculated to be 62.85±2.06% for without PVDF membrane method and 67.37±0.56% for PVDF membrane harvesting method. Although, cell folding was observed on new TCPS, they also showed cell adhesion and proliferation on each new culture surface.

Table 4.4 The percentage of the retrieval L929 cells, they were detached from the thin PNIAM-co-AM grafted surface at 20°C

Sample No.	Dead cells	Live cells	Total cells	Dead cells(%)	Live cells(%)	Live cells(%)
No PVDF 1	38.83	56.36	95.19	40.79	59.21	
No PVDF 2	62.31	107.75	170.06	36.64	63.36	
No PVDF 3	76.24	134.84	211.08	36.12	63.88	62.85±2.06
No PVDF 4	69.72	121.76	191.48	36.41	63.59	
No PVDF 5	64.08	115.00	179.08	35.78	64.22	
PVDF 1	54.99	108.85	163.85	33.56	66.44	
PVDF 2	55.31	114.58	169.89	32.56	67.44	
PVDF 3	55.31	114.58	169.89	32.56	67.44	67.37±0.56
PVDF 4	45.07	95.59	140.66	32.04	67.96	
PVDF 5	46.43	96.69	143.13	32.44	67.56	

However, the percentage of MC3T3-E1 reattachment was 49.13±4.33% for no PVDF membrane method and 62.62±9.53% for PVDF membrane harvesting method (Table 4.5).

Table 4.5 The percentage of the retrieval MC3T3-E1 cells, they were detached from the thin PNIAM-co-AM grafted surface at 20°C

No.	Dead cells	Live cells	Total cell	Dead cells(%)	Live cells(%)	Live cells %
No PVDF 1	21.71	19.30	41.01	52.94	47.06	
No PVDF 2	49.01	61.34	110.35	44.41	55.59	49.13±4.33
No PVDF 3	40.74	40.67	81.42	50.04	49.96	
PVDF 1	40.56	112.41	152.97	26.51	73.49	
PVDF 2	48.85	58.78	107.63	45.38	54.62	
PVDF 3	42.55	64.52	107.07	39.74	60.26	62.62±9.53
PVDF 4	42.55	64.52	107.07	39.74	60.26	
PVDF 5	33.44	100.06	133.49	25.05	74.95	

3. Mono-Layered Sheet harvesting at 10°C (30 minutes) and 20°C (60 minutes)

Dr. Okano reported that the metabolism of cells was suppressed by decreasing temperature which affected the cell metabolic processes, and the hydration of the culture surface on cell detachments was implicated [25]. In this study, the low temperature incubation at 10°C for 30 minutes followed by a temperature change to 20°C in order to increase cell metabolism were investigated.

The purpose of this study was to construct a single layered MC3T3-E1 Pre-Osteoblast sheet. The control polystyrene surfaces were used to compare with PNIAM-co-AM grafted TCPS. These plates were incubated at 37°C to promote cell attachment. Old medium and non-adherent cells were removed by washing with PBS and fresh medium was added to culture plates. The culture well plates were incubated at 10°C to increase the PNIPAM hydration. After that, the temperature was increased to 20°C in order to allow cellular metabolism accompanying morphological changes. Cell morphology in each well plate was observed by an inverted microscope. We used PVDF membranes to harvest cell layers. For the single and double layer MC3T3-E1 cell sheet detachment, un-grafted plate was also used as a control to confirm that the observed cell detachment depends on PNIPAM hydration.

The control and PNIPAM grafted surfaces were incubated in a low temperature chamber set at 10 (30 minutes) and 20°C (60 minutes) in order to detach the cells. MC3T3-E1 morphology was observed at 90 minutes. By reducing the temperature for 90 minutes, MC3T3-E1 cells still adhered onto the ungrafted surface. They showed fibroblast-like cell morphology. In the case of PNIPAM grafted surface, the cells spontaneously detached when the temperature was reduced below 32°C without the need for enzymes. We used sterile forceps to gently detach the cell layer around the edge of the PVDF membrane. These cells lifted-off from the UV grafted surface but themajority of the harvested cells stuck onto the PVDF membrane, making it difficult to attach onto new TCPS. On the grafted surface, MC3T3-E1 cells detached as a complete single and double layer cell sheet by using PVDF membrane. Spontaneously, the periphery of cell sheets began to detach from the dish surfaces. The overlaid PVDF membrane together with the sheet was peeled off from the well plate with a forceps and transferred to new culture surface.

We found that MC3T3 cells could be detached as continuous layer sheets from the grafted surface. These cells were over-confluent and cell-cell junction that it is firmly established. The percentage of single and double layer cell sheet detachment from the PNIAM-co-AM grafted surface by using PVDF membrane at 10°C (30 minutes) and 20°C (60 minutes) was calculated.

4. Double-Layered Sheet

To mimic a double-layered sheet, a continuous cell layer could be detached from the grafted surface using the supplied PVDF membrane and transferred directly onto another cell sheet. For the double-layered sheet,they were harvested following the same method as in the single-layered sheet detachment study. Two conditions of cell densities at 1×10^5 cells/ml and 1×10^6 cells/ml were investigated.

4.1 Double-Layered Sheet (cell density at 1×10^5 cells/ml)

An important thing, we should be careful to transfer the membrane with the attached cell layer facing downwards to the new layer cell surface and make sure there is no air bubble in between cell-sheet and the membrane. In this study, the percentage of single-layer and double-layered sheet detachment at cell density of 1×10^5 cells/ml were calculated by Image J and was shown in Figure 4.43. After temperature reduction, the MC3T3-E1 cells detached from the grafted surface and became stuck onto the PVDF membrane. On the control well plate, most of the fibroblast cells still remained (Blue color) on the surface after lowering the temperature to 10°C for 30 minutes, followed by 20°C for 90 minutes.

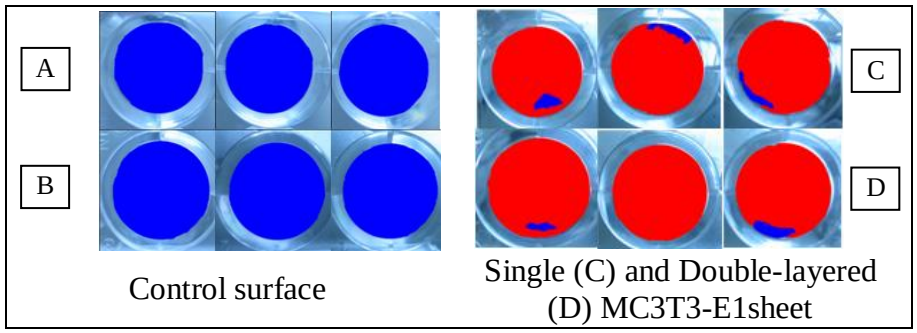


Figure 4.43 Area of cell detachment (cell density at 1×10^5 cells/ml)
(Red color = detachment area, Blue color = attachment area)
(A) Single-layeredsheet detached from control surface
(B) Double-layeredsheet detached from control surface
(C) Single-layeredsheet detached fromcopolymer grafted
(D) Double-layeredsheet detached fromcopolymer grafted

At the end of incubation time, the percentage of the single layer cell detachment was 96.19 ± 0.24% and 97.64 ± 2.69% for the double-layered sheet (Figure 4.44). This result shows that the cell detachment was successful. Cells spontaneously detached from the grafted surface. It has been previously reported thatan incubation time at 10°C (30 minutes) allows PNIPAM chains to hydrate and expand their conformations [25]. However, this alone is not sufficient to induce cell detachment because the temperature is not high enough to allow cellular metabolism and accompanying morphological changes. Therefore, cell detachment is significantly enhanced by increasing the temperature to induce observable cellular shape changes. Even if PNIPAM chains dehydrate slightly by increasing this temperature from 10 to 20°C, metabolic changes of cells at this higher temperature seem to be much more significant than hydration changes of PNIPAM -

grafted chains. The PVDF membranes were used to detach and transfer cell sheets without agglomeration.

By lowering temperature to 10 and 20°C, the culture medium can access the grafted surface from underneath and peripheral to the attached cell sheet, resulting in rapid hydration of grafted PNIPAM molecules and detachment of the cell sheet at 10°C. When the temperature was increased to 20°C, the cell metabolism was active which led to cell lift off from the surface. Detached cell sheets contracted and aggregated after detachment. Therefore, a membrane is necessary to transfer detached cell sheets to new culture surface support as soon as possible to prevent cell sheet agglomeration [45]. We can reveal that cell detachment is controlled not only by the hydration of grafted PNIPAM on the culture dishes but also by active cellular metabolism.

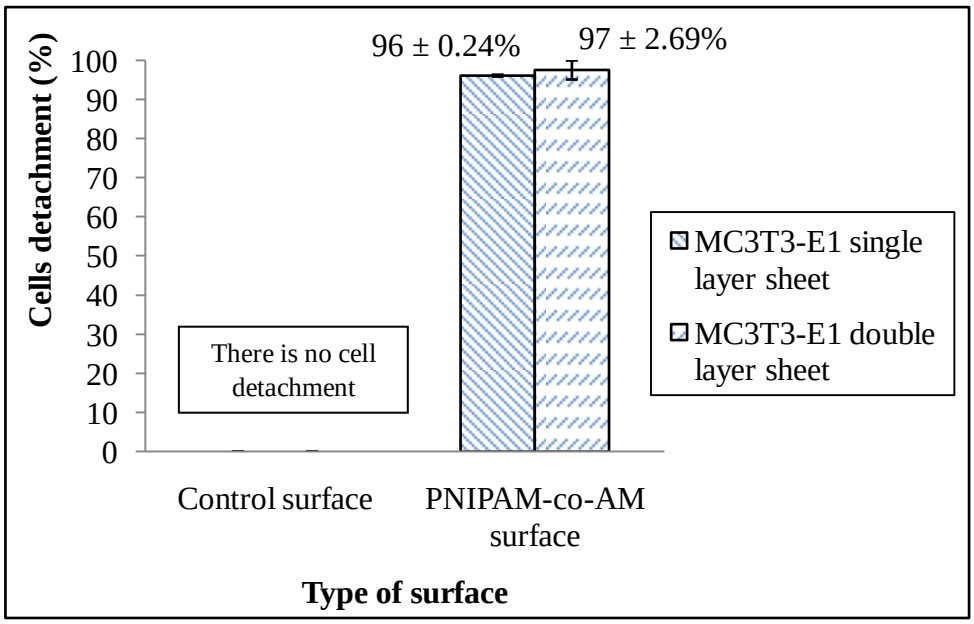


Figure 4.44 The percentage of single and double-layered MC3T3-E1 sheet detachment from the control and the thin PNIAM-co-AM grafted surface by using PVDF membrane at 10°C (30 minutes) and 20°C (60 minutes).

4.2 Viability Test (Proliferation of the harvested double-layered sheet)

To detect whether the transferred cell sheet maintained viability, the transferred double-layered MC3T3-E1 sheet was assessed with fluorescence dye for viability testing after 48 hours of incubation (Figure 4.45 - 4.46). Live–dead stain of the transferred cell sheets was maintained 83.83±4.35 % viability (Table 4.6).

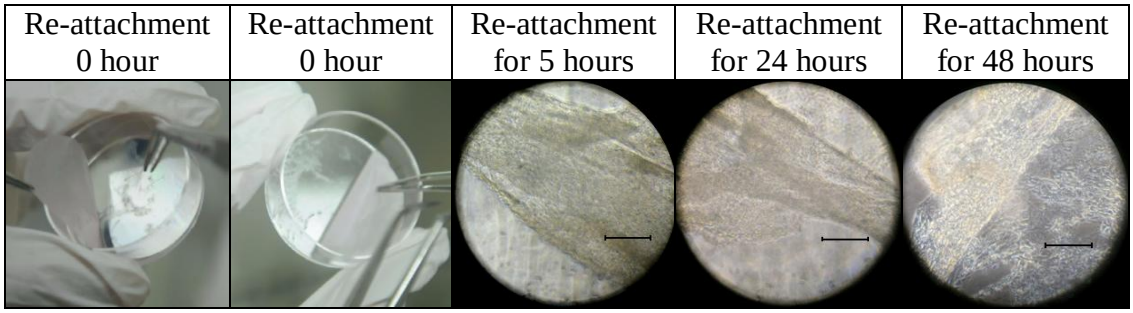


Figure 4.45 Double-layered MC3T3-E1 sheet detach from the PNIAM-co-AM grafted surface by using PVDF membrane at 10°C (30minutes) and 20°C (60 minutes). Scale bar = 100 µm

As a result, by reducing the temperature to 10°C (30 minutes) and 20°C (60 minutes), the MC3T3-E1 cultured cells can be harvested as a sheet without digestion enzyme. The cell sheet was transferred with the help of PVDF membrane but when they reattached to new culture surface, they were damaged by mechanical forces. Therefore, the percent reattachment of the double-layered sheet was not 100% viability.

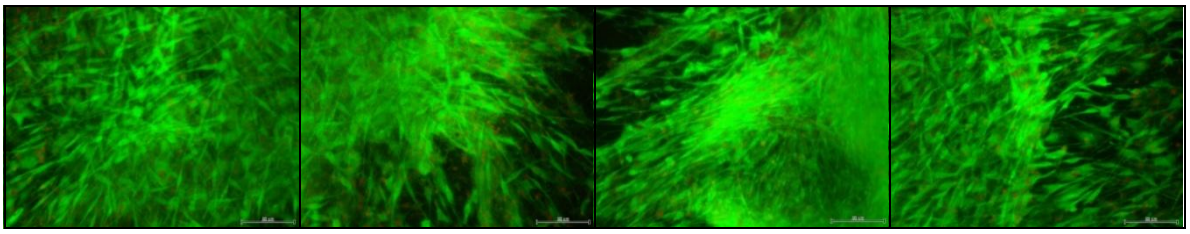


Figure 4.46 Thelive and dead stained images of double-layered MC3T3-E1 sheet after recovery from the thin PNIAM-co-AM grafted TCPS at 10°C (30 minutes) follow by 20°C (60 minutes). Scale bar = 100µm

Table 4.6 The percentage of the retrieval MC3T3-E1 cells single layer sheet, they were detached from the PNIAM-co-AM grafted surface at 10°C (30 minutes) and 20°C (60 minutes).

No.	Dead cells	Live cells	Total cells	Dead cells (%)	Live cells (%)	Live cells (%)
1	16.27	90.35	106.62	15.26	84.74	
2	12.70	57.51	70.21	18.09	81.91	
3	15.87	69.33	85.21	18.63	81.37	
4	13.46	110.88	124.34	10.83	89.17	
5	14.54	95.15	109.69	13.25	86.75	83.83±4.35
6	21.14	103.49	124.63	16.96	83.04	
7	18.28	88.89	107.17	17.05	82.95	
8	48.35	140.32	188.67	25.63	74.37	
9	19.34	106.02	125.36	15.43	84.57	
10	15.29	129.57	144.87	10.56	89.44	

An additional implication of this work relates to mechanisms of cell death. In contrast to the apoptotic mechanism, the obvious disruption of the cells membranes seen here with mechanical reattachment will destroy the cell [40]. The damage to the harvested cell from mechanical harvesting may also impact subsequent studies with reattached cells. Furthermore, the major part of the sheet stuck on PVDF membrane. Therefore, cells reattached by incubation at 37°C before PVDF membrane removal or on the new TCPS coated with extracellular matrix should be more suitable for use in further cell reattachment.

4.3 Double-Layered Sheet (cell density at 1×10^6 cells/ml)

According to the cell detachment study at the cell density of 1.0×10^5 cells/ml, a double-layered sheet spontaneously detached from the grafted surface. Although double-layered sheets could be lifted off from the grafted surface but they stuck on the PVDF membrane.

To overcome this problem, the cell density was increased to the reach the contact-inhibition limit. In addition, the commercial UpCell™ application has reported thatwhen transferring detached cell-sheet to new surface using the membrane, it is important that adherence property of cell-sheet and the membrane is weaker than that of cell-sheet and the new surface.Longer time or some kind of weighing may be necessary to make a cell-sheet firmly attach to the new surface. Additional caution is required on the following points. Moreover, the new TCPS was coated with Matrigel™ (mixture of basement membrane ECM protein) for rapid re-attachment. Therefore, the purpose of this study was to construct a double-layered MC3T3-E1 pre-osteoblast sheet. Cell attachment on PNIPAM-grafted surfaces was observed under a normal condition at 37°C with 5% CO_2 and 95% air. After 72 hours of culture, seeded MC3T3-E1 cells adhered well and showed spreading morphologies all of surfaces as shown in Figure 4.47.

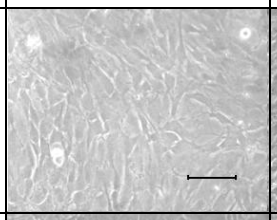
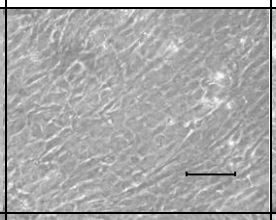
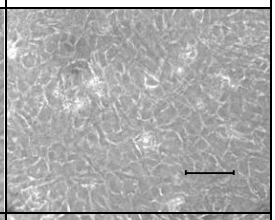
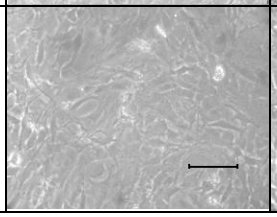
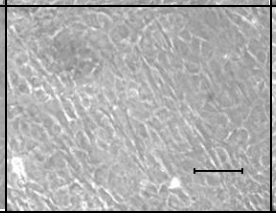
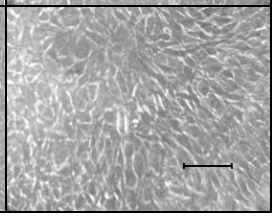
Type of cell and surface	Incubation time		
	24 hours	48 hours	72 hours
MC3T3-E1 cell Control ungrafted surface			
MC3T3-E1 cell PNIAM-co-AM grafted surface			

Figure 4.47 The morphology of MC3T3-E1 cells on the control and the thin PNIAM-co-AM grafted surface at a cell density of 1.0×10^6 cells/ml. Scale bar = 100µm

The cells proliferated on PNIAM-co-AM grafted surface similar to that of the control surface. It was obvious that both of the thick and thin copolymer films supported cell attachment. As described in the attachment study, it is believed that the cell adhesion onto material surface involves two steps. The first step is passive adhesion which is controlled by complex combinations of physicochemical interactions including hydrophobic,

Coulombic, and Van Der Waals forces between the cell membrane and the material surface base on adsorption mechanism. The second step is active adhesion that they have participation of cellular metabolic processes [46]. Attached cells are well-known for changing their shapes and expending metabolic energy in order to stabilize the interface between their membrane and the underlying materials, by both physicochemical and biological mechanisms [25].

According to the attachment results, we demonstrated that the incubation time to create a cell sheet depended on a cell type and cell density. After 48hours in culture, a confluent cell monolayer which had developed tight junctions (TJs) was subjected to single and double-layered cell sheet manipulation. Tight junction (TJs) function is a barrier between apical and basolateral plasma membrane domains (Figure 4.48), preventing the diffusion of solutes through paracellular pathways [47]. TJs comprise an elaborate network of paired strands, which form the so-called 'kissing points' that eliminate the extracellular space. TJ strands also function as a fence between apical and basolateral membrane domains. They are located at the most apical part of lateral membranes [48].

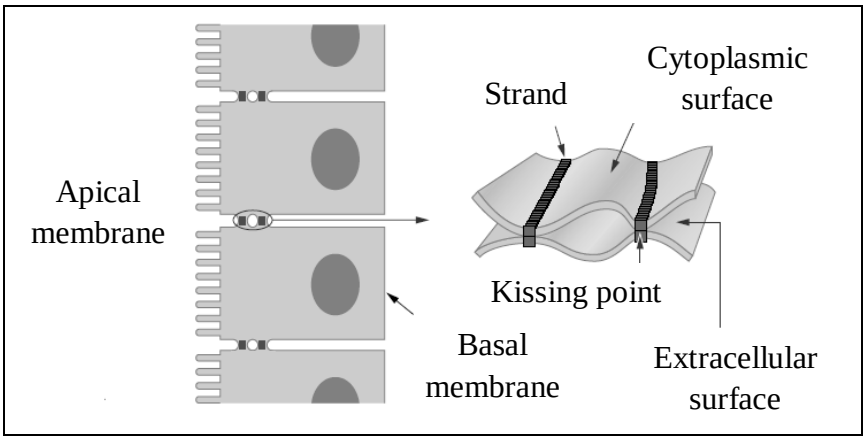


Figure 4.48 Schematic of the tight junction structure

Indeed, cell adhesion is usually accomplished by receptors such as integrin protein, which responds to the adhesion recognition signals. Furthermore, most cells only grow when attached and spread on a solid substrate. As such, cells attach and spread in vitro by either depositing new ECM component or by binding in exogenous ECM. Likewise, if cells are detached from the ECM, they rapidly lose viability and can undergo programmed cell death such as apoptosis [49]. However, the cell attachment images showed the cell morphology onthe different types of copolymer grafted surface. The cell attachment and proliferation were found to be the same on all surfaces, indicating that grafting of PNIAM-co-AM polymers onto TCPS had no effect on the cellular activities. In addition, higher cell density led to a faster growth rate and shorter time for the cells to reach their confluency.

A cell density of 1×10^6 cells/ml was used to construct a double-layer sheet and harvested following the same method as in the previous study. Each culture TCPS was removed from the incubator when the cells reached confluence and then was allowed to be at low temperature. The percentage of single and double-layered sheet detachment from the PNIAM-co-AM grafted surface using PVDF membrane at 10°C (30 minutes) and 20°C (60 minutes) was compared with that of cell density at 1×10^5 cells/ml.

The results are shown in Figure 4.49. The single and double-layered MC3T3-E1 sheets were harvested from the copolymer grafted surface by using PVDF membrane. The single-layered (A) and double-layered sheets (B) were spontaneously detached from the grafted surfaces. The membrane together with the cells were peeled off using forceps and kept on a new matrigel™ coated TCPS, with the cell sheet side facing down. The PVDF membrane was overlaid onto the cell layer. Because of the hydrophilic property of the membrane surface, the membrane rapidly stuck to the apical cell surface by capillary force. Then, the temperature was reduced to 10°C (30 minutes), which was below the LCST resulting in rapid hydration of grafted PNIPAM molecules [50]. At this cell density, the cell sheet was obtainedas a strong cell-sheet.

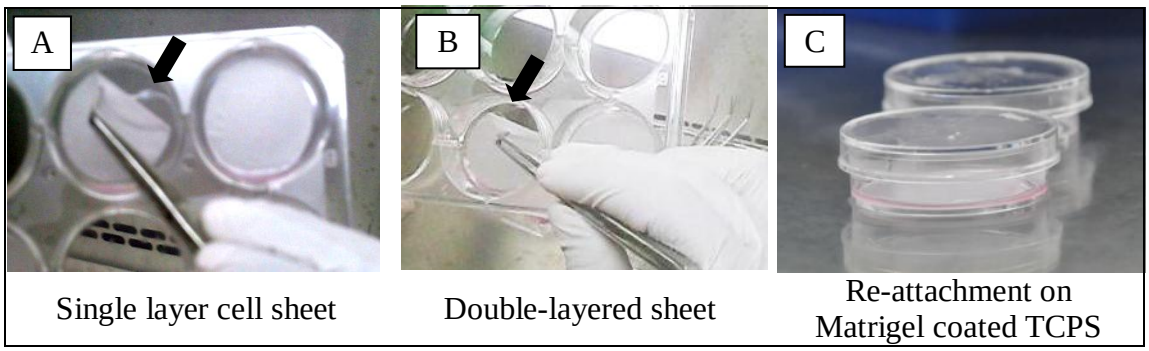


Figure 4.49 Single-layered (A) and double-layered (B) MC3T3-E1 sheets were harvested by using PVDF membrane and re-grown on the Metrigel™ coated TCPS (C).

The result demonstrates that at 10°C, the hydration of PNIPAM between cell and material completely governing copolymer were expended and when the temperature was increased to 20°C, the cell metabolism was not suppressed, leading to successful harvesting of the double-layered sheet from temperature-responsive surfaces [25]. The percentage of cell detachment and area after cell lifted offat cell density 1×10^6cells/ml between control and PNIAM-grafted surface are shown in Figure 4.50 - 4.51.

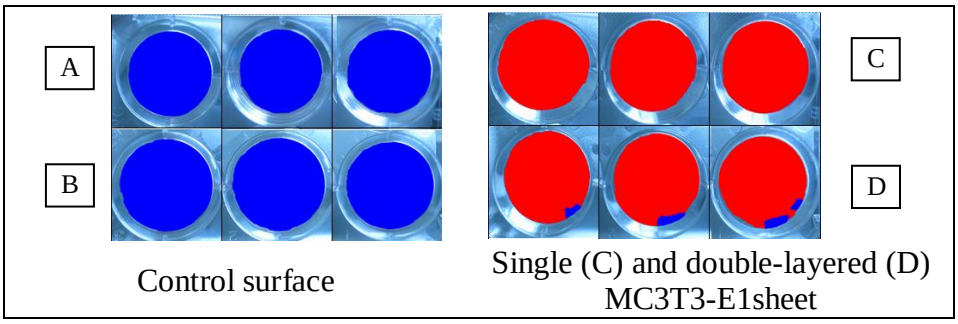


Figure 4.50 Area of cell detachment (cell density at 1×10^6 cells/ml)
(Red color = detachment area, Blue color = attachment area)
(A) Single-layered sheet detachment from control surface
(B) Double-layered sheet detachment from control surface
(C) Single-layered sheet detachment from grafted surface
(D) Double-layered sheet detachment from grafted surface

After harvesting, no cell was observed on the control surface. In contrast, the MC3T3-E1 cells spontaneously detached from the grafted surface. The percentage of single-layered

sheet detachment was 100% and 96.50 ± 2.80% for a double-layered sheet using PVDF membrane. Compared to the control surface, there was no cell detachment at all (data not shown).

The cell sheet detached from the grafted surfaces with intact cell-cell junctions and was easy to reattach onto Matrigel™ coated TCPS. However, there were strong cell–cell interactions that led to cell aggregation. The contraction and aggregation of the detached cell sheets were observed within 30 minutes post detachment. To avoid cell sheet agglomeration, the PVDF membrane support materials were also used in this capacity to detach and transfer cell sheets without agglomeration [51].

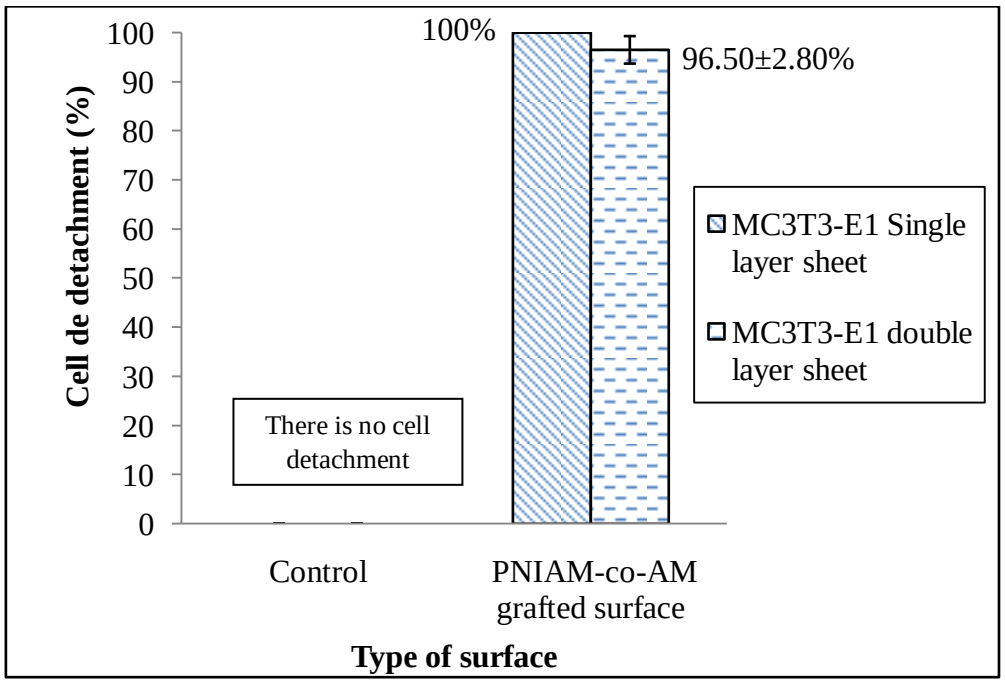


Figure 4.51 The percentage of single and double-layeredMC3T3-E1 sheet detachment at a cell density of 1×10^6 cells/ml from the thin PNIAM-co-AM grafted surface with PVDF membrane at 10°C (30 minutes) and 20°C (60 minutes).

The viability characteristics (live–dead staining) of double-layered MC3T3-E1 sheet cultured on Matrigel™ coated TCPS were analyzed. Observation of cells at 0 hour showed the transfer of intact cell patch to Matrigel™ coated TCPS. The transferred cells on new dishes grew with normal fibroblast-like morphology (Figure 4.52).

Cells were stained with a fluorescence dye for 15 minutes. Stained samples were observed under a fluorescence microscope. To observe whether cell transfer manipulation affects viability, double-layered MC3T3-E1 sheet transferred to new surface and cultured for 48 hours was also subjected to live–dead staining as shown in Figure 4.53.

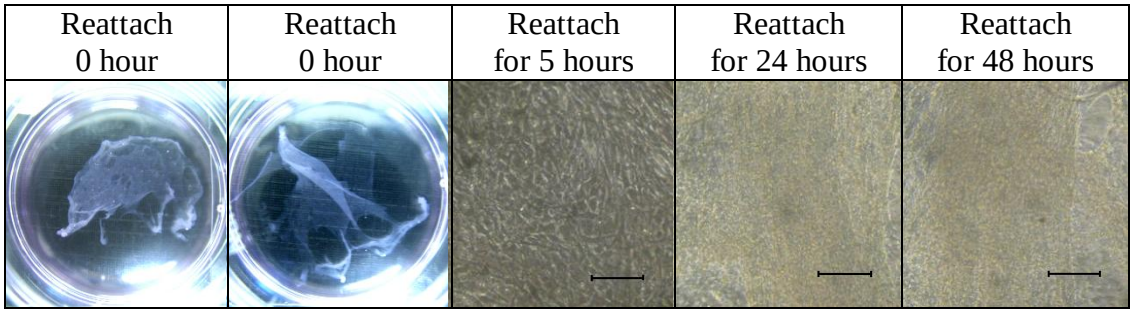


Figure 4.52 The re-attachment of MC3T3-E1 double-layered sheet after 48 hours on new Matigel™ coated tissue culture polystyrene at 37°C. Scale bar = 100µm

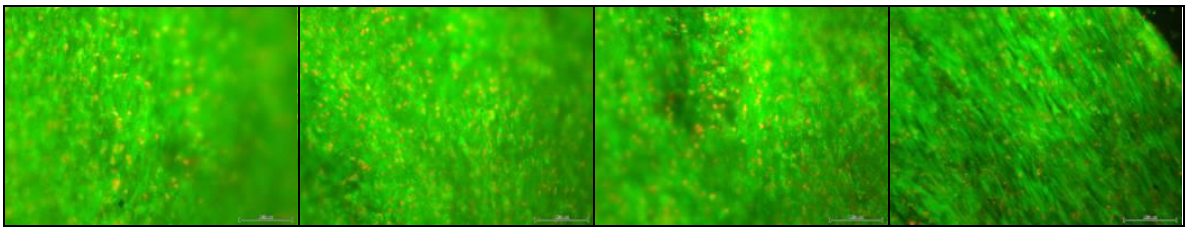


Figure 4.53 Live and dead staining of MC3T3-E1 double-layered sheet at 1×10^6 cells/ml after retrieval from PNIPAM-co-AM grafted surface. Scale bar = 100µm

The percentage of retrieval double-layered MC3T3-E1 sheet based on fluorescence images was 80.80±3.79 % viability (Table 4.7), suggesting that the viability of the double-layer sheet was maintained, although there were some areas of dead cells because of the pack of dense cells. However, at the edge of cell sheet, the cells showed spreading fibroblast morphology similar to the native MC3T3-E1 pre-osteoblast cell.

Table 4.7 The percentage of retrieval MC3T3-E1 double-layered sheet, they were detached from the grafted surface at 10°C (30minutes) and 20°C (60 minutes).

No.	Dead cells	Live cells	Total cell	Dead cells(%)	Live cells (%)	Live cell (%)
1	22.89	118.77	141.66	16.16	83.84	80.80±3.79
2	26.27	115.72	141.99	18.50	81.50	
3	26.75	84.88	111.62	23.96	76.04	
4	29.22	84.84	114.06	25.62	74.38	
5	33.53	151.74	185.27	18.10	81.90	
6	54.41	180.09	234.50	23.20	76.80	
7	27.96	139.39	167.36	16.71	83.29	
8	29.13	167.80	196.93	14.79	85.21	
9	32.26	174.84	207.10	15.58	84.42	
10	20.53	85.60	106.13	19.35	80.65	

The cell sheet collected using the temperature recovery system has been reported to preserve the ECM and adhesive factors on the base and thus readily adhere to other cell

sheets. The present study confirmed that MC3T3-E1 cells could be harvested as sheets and thus made into double-layered by culturing in temperature-responsive TCPS, followed by using the temperature recovery system.

So far, the optimum conditions for complete cell sheet detachment were to use the thin PNIPAM-co-AM grafted TCPS, MC3T3-E1 cells at a cell density of 1×10^6 cells/ml. It is necessary that cells covering entire surface of grafted surface should be over-confluent and cell-cell junction is firmly established before attempting to detach cells as a cell-sheet. When there is only loose connection between cells, it may be difficult to get a strong cell-sheet. By lowering temperature, cultured surface was incubated at 10°C (30 minutes), followed by 20°C (60 minutes), and re-grown on Matrigel™ coated TCPS.

In the next experiment, the PNIPAM-co-AM grafted TCPS with longer exposure time (2 hours apply UV) was used and compared surfaces were exposed to UV light for 2 hours and cleaned with ethanol to remove unreacted copolymer after polymerization.

4.8.4 Cell sheet attachment and detachment sheet from the PNIAM-co-AM grafted surface with longer exposure time (2 hours apply UV).

1. Cell Attachment

In this experiment, cell attachment on the grafted surface with longer exposure time was observed because the characteristic of this copolymer grafted surface exhibited well-defined polymer. Because the FTIR spectra of thin PNIPAM-co-AM grafted surface with longer exposure time (2 hours Apply UV) showed the high existence of this copolymer on the surface (data not shown). The exposure time effect may result in homogeneous polymerization [52]. In this study, the effect of longer exposure time under ultraviolet light on the polystyrene was evaluated. MC3T3-E1 cells were seeded onto the thin PNIAM-co-AM grafted surface with longer exposure time. These plates were incubated at 37°C to promote cell attachment, spreading and proliferation. After 48 hours of culture, seeded MC3T3-E1 cells adhered well and showed their correct morphologies on all surfaces as shown in Figure 4.54. The cell proliferation on PNIAM-co-AM grafted surface was similar to that of the control surface.

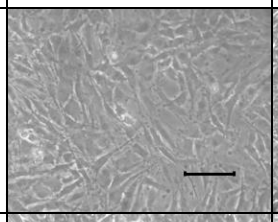
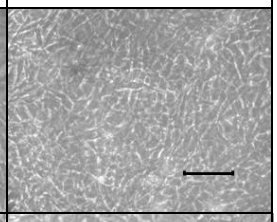
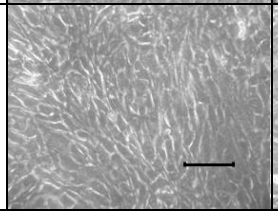
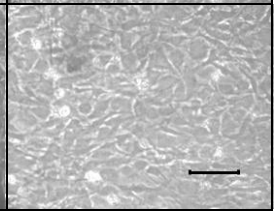
Type of cell and surface	Incubation time	
	24 hours	48 hours
MC3T3-E1 cell Control ungrafted surface		
MC3T3-E1 cell PNIAM-co-AM grafted surface		

Figure 4.54 The morphology of MC3T3-E1 cells on the thin PNIAM-co-AM grafted surface with longer exposure time.

Actually, the cell attachment was slightly suppressed on the thick of grafted surfaces with more than 30 nm. The length of PNIPAM chain, and could not form confluent monolayer cell culture on the whole surface area [10]. In the attachment study, obviously, acrylamide was co-grafted with NIPAM onto TCPS with different conditions (thick film, thin film with 1 hour UV exposure and thin film with 2 hour UV exposure). The copolymer grafted surfaces were tested for cell attachment at 37°C. Thin PNIAM-co-AM grafted surface with longer exposure time allow for cell adhesion. At this temperature, the surface was hydrophobic as indicated by the result of the water contact angle data.The important factor in an animal cell study is cell adhesion. Cells first attach to a surface by pseudopodial extension. Afterward the cells spread to form focal contacts. Thus, cell adhesion plays an important role in tissue and organ formation and in the generation of traction for the migration of cells; it is also important in determining the biocompatibility of materials [49].

2. Cell Sheet Detachment at 20°C

This experiment aims to construct a double-layered MC3T3-E1 pre-osteoblast sheet from the PNIAM-co-AM grafted surface with longer exposure time. It has been previously reported that longer UV exposure time to 2 hours, giving rise to cell adhesion [53]. Thus, MC3T3-E1 cell line was cultured in alpha-MEM medium and was thenincubated at 37°C to promote cell adhesion. For the cell detachment, they were harvested following the same method as in the previous study. However, the temperature was lowered to 20°C to detach the cells.

By reducing the temperature for 60 minutes, MC3T3-E1 cells still adhered onto the un-grafted TCPS surface and showed fibroblast-like cell morphology. In the case of PNIAM-co-AM grafted TCPS with longer exposure time surface, the majority of the cells detached when the temperature was reduced at 20°C without enzymes as shown in Figure 4.55. However, there were majority of harvested cells stuck on the PVDF membrane after re-grown on normal TCPS. The confluent cultured MC3T3-E1 cells were harvested as a layer of the cells retaining cell–cell junction as well as deposited ECM on the basal side.

In this study, the percentage of cell detachment by using pipetting and PVDF membrane method was also compared. For the pipetting method (without PVDF membrane), the cell sheet on the grafted surface was detached. The detachment was possibly caused by incompatibility between the hydrophilic grafted surface at low temperature and the hydrophobic nature of the cells. In contrast, the cells on the ungrafted surface could not be detached using the same procedure because of no surface property alteration with the temperature.

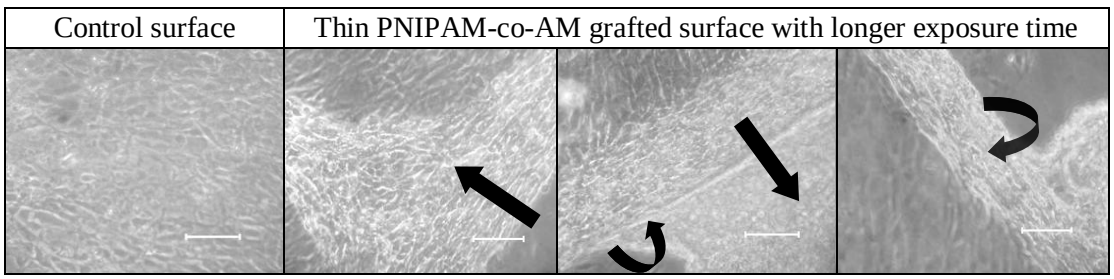


Figure 4.55 The morphology of MC3T3-E1 cell on the control and thin PNIPAM-co-AM grafted surface with longer exposure time at 20°C for 60 minutes by using pipetting method. Sale bar = 100µm

The percentage of detached cells was compared between control and the thin PNIPAM-co-AM grafted surface with longer exposure time. After temperature reduction, on the control well plate, most of the fibroblast cells still remained on the surface after lowering temperature to 20°C for 60 minutes. At the end of the incubation time, the percentage of cell detachment from the culture platewas analyzed (Figure 4.56). The percentage of cell detachment was 25.01 ± 1.13% (without PVDF membrane).

In case of the PVDF membranes, the culture medium could access the grafted surface from underneath and peripheral to the attached cell sheet, resulting in rapid hydration of grafted PNIPAM molecules and detachment of the cell sheet. The percentages of single-layered and double-layered sheet detachment were 87.23 and 87.48%, respectively. However, we also used the PVDF membrane to harvest the cell in control surface. We found that the percentage of cell detachment form thecontrol surface (with PVDF membrane) was 0.82%. It is obvious that there was no the surface property alteration with the temperature.

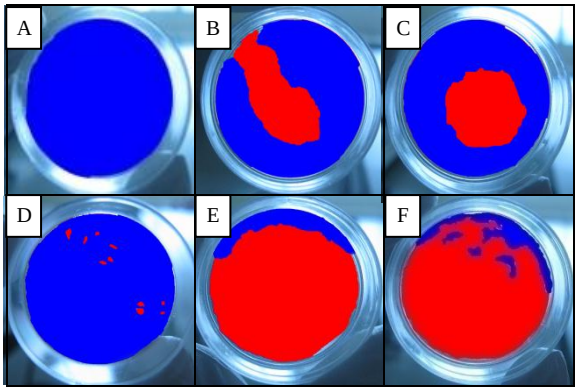


Figure 4.56 Area of cell detachment from the control and grafted surface with longer exposure time after cell sheet were detached at 20°C for 60 minutes. (Red color = detachment area, Blue color = attachment area)

(A) Cell detachment from control surface (without PVDF membrane)

B-C) Cell detachment from grafted surface (without PVDFmembrane)

D) Cell detachment from control surface (with PVDF membrane)

E-F) Cell detachment from grafted surface (with PVDFmembrane)

The harvested MC3T3-E1 cells were observed. The proliferation of the harvested cells sheet was photographed and stained with a fluorescence dye as shown in Figure 4.57.

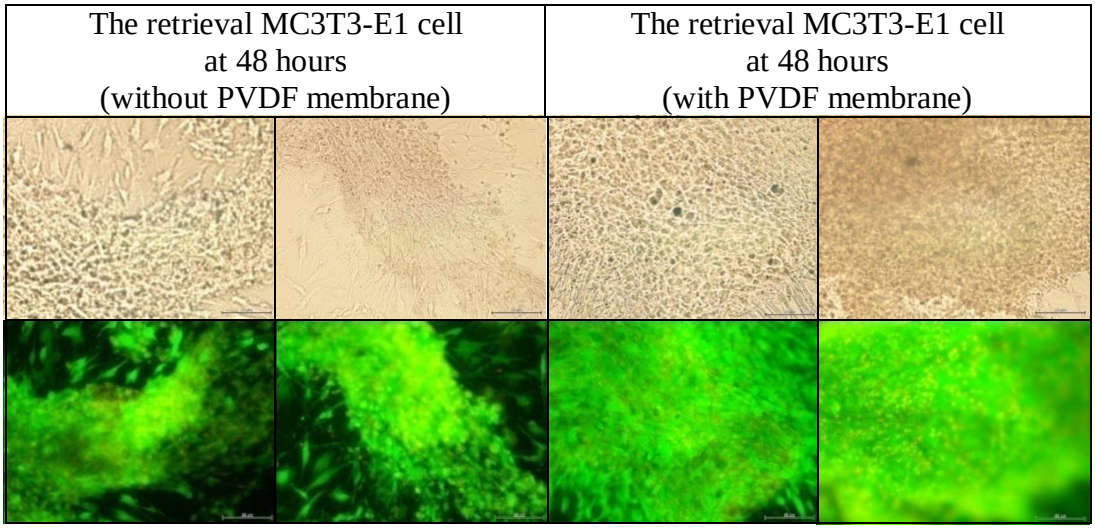


Figure 4.57 Phase contrast and live-dead staining of retrieval MC3T3-E1 sheet grown for 48 hours.Sale bar = 100µm

These harvested cells were incubated at 37°C for 48 hours and were then observed by an inverted microscope to determine the morphology of the re-attached cells. The cell viability of the retrieval MC3T3-E1 cells detached from the PNIAM-co-AM grafted surface with longer exposure time at 20°C (60 minutes) was 80.40±2.37% (without PVDF membrane) 81.35±3.72% (with PVDF membrane) as shown in Table 4.8. This cell layer could adhere to and proliferate on the new TCPS surface, indicating that the double layer sheet was viable.

Table 4.8 The percentage of retrieval MC3T3-E1 double-layered sheet, they were detached from the thin PNIAM-co-AM grafted surface with longer exposure time at 20°C

No.	Dead cells	Live cells	Total cells	Dead cells (%)	Live cells (%)	Live cells (%)
No PVDF 1	29.66	106.72	136.39	21.75	78.25	80.40±2.37
No PVDF 2	32.44	129.55	161.99	20.02	79.98	
No PVDF 3	26.58	103.80	130.39	20.39	79.61	
No PVDF 4	21.35	110.22	131.56	16.23	83.77	
PVDF 1	70.96	206.20	277.16	25.60	74.40	81.35±3.72
PVDF 2	52.14	171.82	223.96	23.28	76.72	
PVDF 3	29.81	118.87	148.68	20.05	79.95	
PVDF 4	33.79	143.29	177.08	19.08	80.92	
PVDF 5	24.48	116.06	140.55	17.42	82.58	
PVDF 6	30.67	143.82	174.48	17.58	82.42	
PVDF 7	37.56	162.42	199.98	18.78	81.22	
PVDF 8	35.56	182.99	218.55	16.27	83.73	
PVDF 9	20.21	140.00	160.21	12.61	87.39	
PVDF 10	26.75	141.78	168.53	15.87	84.13	

3. Cell Sheet Detachment at 10 and 20°C

Since the PNIAM-co-AM grafted surface with longer exposure time at 20°C are successful in providing cell detachment. The percentage of cell detachment at the low temperature to 10 and followed by 20°C was compared withthe results in the previous studies that used the temperature at only 20°C. The operating protocol for collection of a cell-sheet followed the same condition at 20°C. Afterwards, these surfaces were incubated at 37°C to 100% confluence then were incubated at 10°C to increase the PNIPAM hydration. After that, the temperature was increased to 20°C.The cell layers were harvested by PVDF membrane and agitating the surface with culture media. After temperature reduction, we found that MC3T3 cells detached from the grafted surface and the cells still remained on the control surface after lowering temperature to 20°C for 60 minutes.The cells did not come off automatically and needed to be detached by agitating and flushing the surface with media.

At the end of the incubation time, the percentage of cell detachment from the grafted surface was 18.64±2.9% (without PVDF membrane). Whenthe membrane was used to peel off the cell layer from the grafted surfaces, the area and percentage of cell detachment were shown in Figure 4.58 -4.59. On the control well plate, most of the fibroblast cells still remained (Blue color) on the surface after lowering temperature to 10°C (30 minutes) and 20°C (30 minutes). They showed high percentages of single-layered and double-layered sheet detachment by using PVDF membrane of approximately 87.13±4.64% and 89.82±3.66%, respectively.

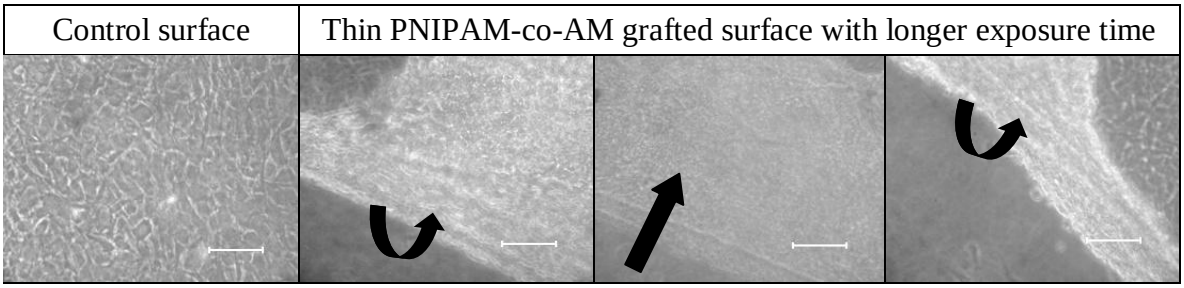


Figure 4.58 The morphology of MC3T3-E1 cell on the control and thin PNIPAM-co-AM grafted surface with longer exposure time at 10°C (30 minutes) and 20°C (30 minutes). Sale bar = 100µm

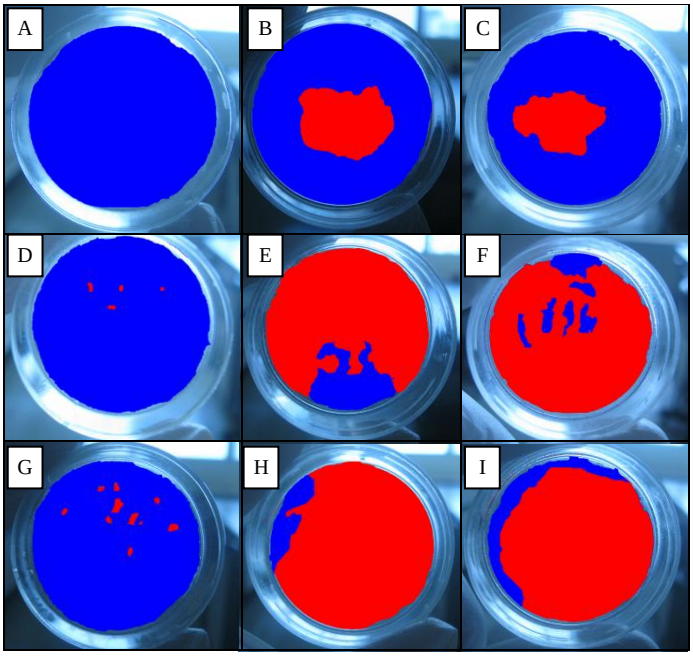


Figure 4.59 Area of cell detachment from the control and grafted surface with longer exposure time after cell sheet were detached at 10 (30 minutes) and 20°C (30 minutes). (Red color = detachment area, Blue color = attachment area)

(A) Cell detachment from control surface (without PVDF membrane)

(B-C) Cell detachment from grafted surface (without PVDF membrane)

(D) Single-layered sheetdetachment from control surface (with PVDF membrane)

(E-F) Single-layered sheetdetachment from grafted surface (with PVDF membrane)

(G) Double-layered sheetdetachment from control surface (with PVDF membrane)

(H-I) Double-layered sheet detachment from grafted surface (with PVDF membrane)

These results were used to compare with the percentage of cell detachment at 20°C (Figure 4.60). The results show that there were quite different in the percentage of detachment area when we used PVDF membrane.

However, the percentage of cell detachment from thethin PNIPAM-co-AM grafted surface with longer exposure time (2 hours apply UV) was less than that of the copolymer with 1 hour UV exposure (approximately 90%), probably because the degree of crosslink affected to the PNIAM chain. A higher degree of crosslink resulted in less extension of the polymer chain [10].

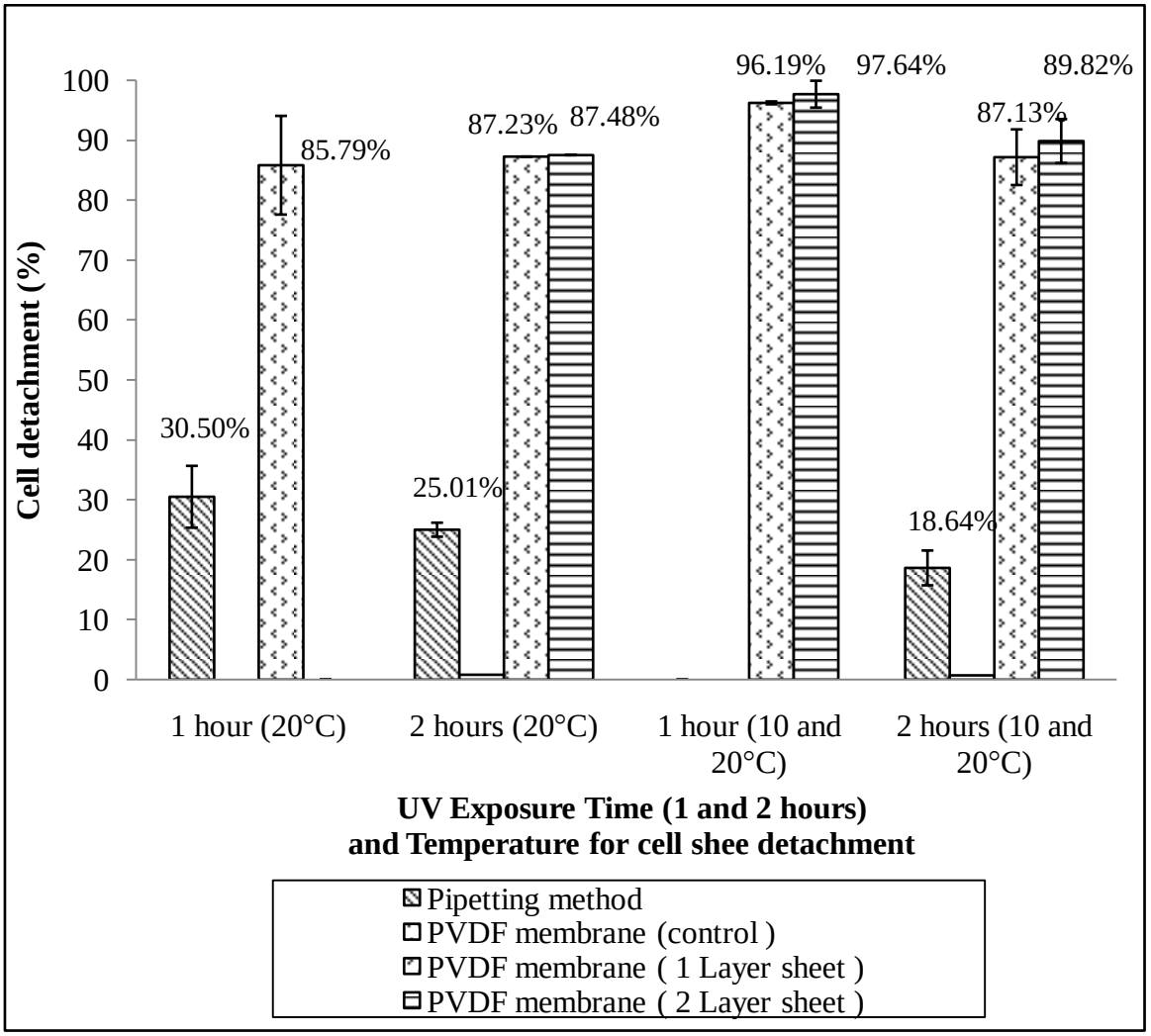


Figure 4.60 The percentage of MC3T3-E1 cell detachment from the thin PNIPAM-grafted surface with longer exposure time at 20°C (60 minutes) and 10°C (30 minutes) follow by 20°C (30 minutes).

By using the thin PNIPAM-grafted surface with longer exposure time, cultured cells could be harvested without enzyme. Phase contrast and live-dead staining was carried out on the normal TCPS surfaces as shown in Figure 4.61.

MC3T3-E1 double-layered sheet could adhere to and proliferate on the new TCPS surface and showed the percentage of live cell were 77.33±11.44% (without PVDF membrane) and 87.39±2.27% (with PVDF membrane)(Table 4.9), indicating that the viability of the double layer sheets was retained. The edge of the double layer showed spreading fibroblast morphology.

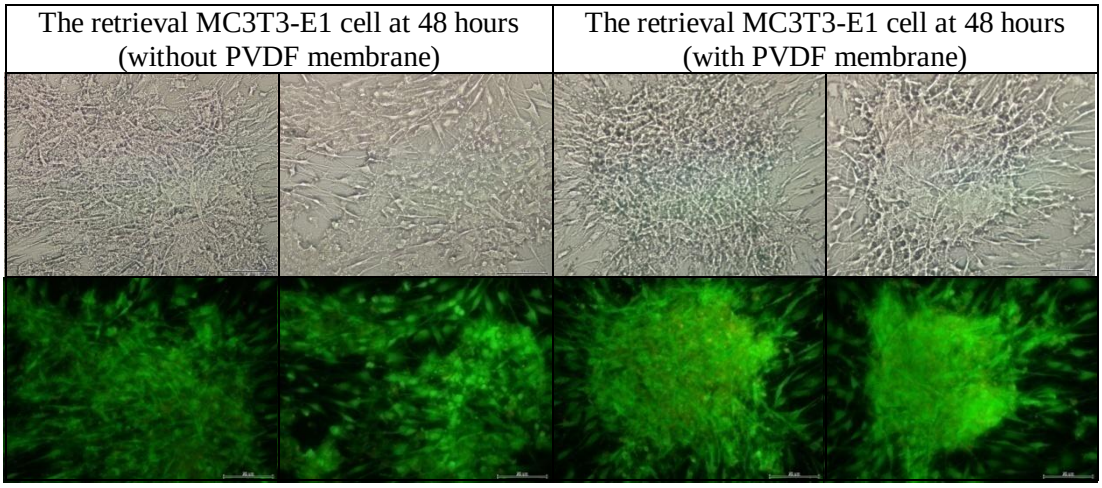


Figure 4.61 Phase contrast and live-dead staining of retrieval MC3T3-E1 sheet grown for 48 hours. Sale bar = 100 µm

Table 4.9 The percentage of retrieval MC3T3-E1 double-layered sheet, they were detached from the PNIAM-co-AM grafted surface with longer exposure time at 10 (30 minutes) and 20°C (30 minutes).

No.	Dead cells	Live cells	Total cell	Dead cells(%)	Live cells(%)	Live cells(%)
No PVDF 1	9.85	74.14	83.99	11.72	88.28	77.33 ±11.44
No PVDF 2	2.97	31.04	34.01	8.73	91.27	
No PVDF 3	38.67	81.05	119.72	32.30	67.70	
No PVDF 4	43.58	99.51	143.09	30.46	69.54	
No PVDF 5	79.80	184.90	264.69	30.15	69.85	
PVDF 1	14.46	78.18	92.64	15.61	84.39	87.39
PVDF 2	11.22	77.86	89.08	12.59	87.41	
PVDF 3	6.77	53.26	60.03	11.28	88.72	
PVDF 4	5.14	47.78	52.92	9.71	90.29	
PVDF 5	7.72	48.01	55.73	13.85	86.15	

4.8.5 Cell sheet attachment and detachment sheet from the commercial UpCell™ surface.

The properties of the cell attachment on thePNIAM-co-AM grafted culture surface synthesized by UV irradiation and that of the commercial grafted culture surface synthesized by electron beam irradiation (UpCell™) were compared. The results of thecell attachment on PNIPAM-grafted surfaces were observed under a normal condition at 37°C as shown Figure 4.62. After 48 hours in culture, seeded L929 and MC3T3-E1 cells adhered well and showed their correct morphologies on all of surfaces. The cell proliferation on the commercial UpCell™ surface was similar to that of the control surface.

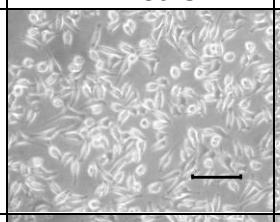
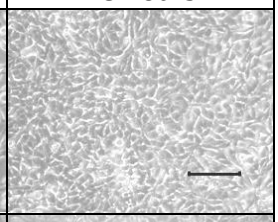
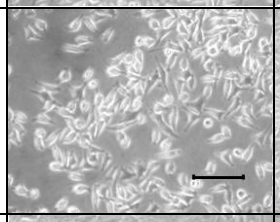
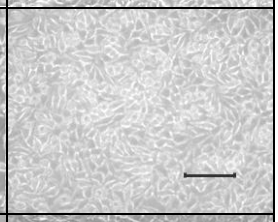
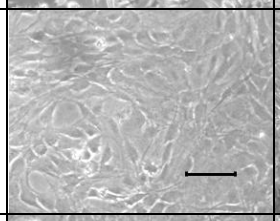
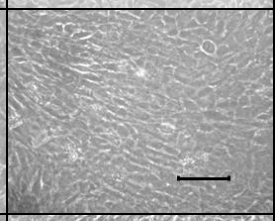
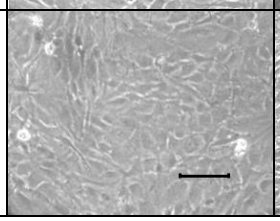
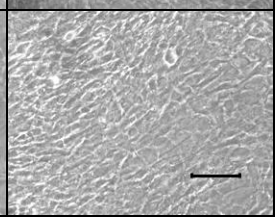
Type of cell and surface	Incubation time	
	24 hours	48 hours
L929 cell Control Ungrafted surface		
L929 cell Commercial Grafted Surface (UpCell™)		
MC3T3-E1 cell Control Ungrafted surface		
MC3T3-E1 cell Commercial Grafted Surface (UpCell™)		

Figure 4.62 The morphology of L929 and MC3T3-E1 cells on the control and the UpCell™ surface. Scale bar = 100 µm

The cell proliferation on UpCell™ surface (EB grafting) was similar to those of the PNIPAM-co-AM grafted surface, and the control surface (un-grafted). These cells rapidly attached to the grafted and ungrafted surfaces and proliferated normally until confluence. They exhibited the fibroblast-like structure. In fact, when cells were cultured to confluence, they connected to each other via cell to cell junction and extracellular matrix (ECM) proteins [54]. Thus, the cell density at 1×10^6 cells/ml showed a strong junction and showed a high-quality cell sheet after detachment as we reported in the cell sheet detachment part.

2. Cell Sheet Detachment from The UpCell™ surface

The properties of the cell sheet detached from the PNIAM-co-AM grafted culture surface synthesized by UV irradiation were compared with those of the commercial PNIAM-co-AM grafted culture surface synthesized by electron beam irradiation (UpCell™). In the cell detachment study, they were harvested following the same method as in the PNIAM-co-AM grafted culture surface synthesized by UV irradiation. L929 Mouse fibroblast cells (1×10^5 cells/ml) and MC3T3-E1 cells were cultured for 2days in DMEM and alpha-MEM medium, respectively. The polystyrene ungrafted surface was used as a control surface. These plates were incubated at 37°C to promote cell attachment.

In the case of L929 cell detachment, non-adherent cells in each well were removed by washing with PBS before adding fresh culture medium into culture plate. The culture well plate was placed in an incubator set at 20°C in order to detach the cells. We found that L929 cells detached from the commercial grafted surface (UpCell™) at 20°C within 10 minutes without PVDF membrane (Figure 4.63 - 4.64). Cell morphology in each well plate was observed by an inverted microscope. Afterwards, we used the PVDF membrane to transfer the detached cells onto new culture surfaces for cell re-grown.

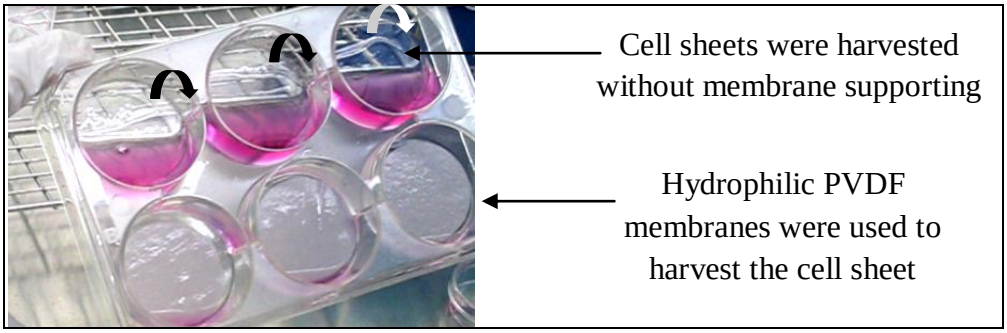


Figure 4.63 L929 sheet spontaneously detached from the commercial UpCell™ surface.

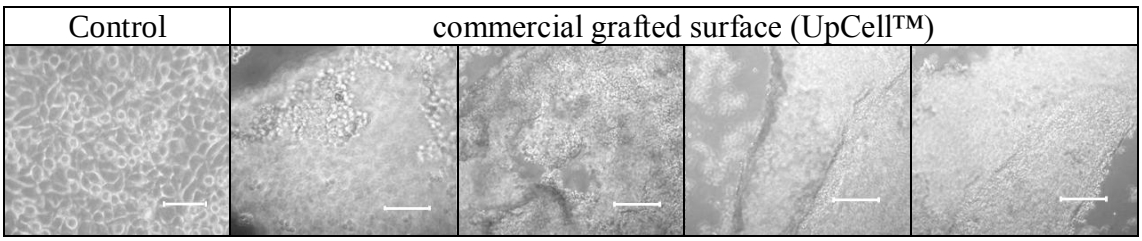


Figure 4.64 The morphology of L929 sheet on the control and the commercial UpCell™ surface. Scale bar = 100 µm

After the temperature reduction, L929 cells detached from the grafted surface and floated and folded in the culture medium. On the control well plate, most of the fibroblast cells still remained on the surface after lowering temperature to 20°C for 10 minutes. On the commercial UpCell™ surface, the percent of cell detachment of commercial surface using pipetting method was more than 95% and 100% when we used PVDF membrane to harvest the cell. However, the cells were attached onto the grafted surface approximately 5%.

After temperature reduction to 20°C, L929 cells detached from the commercial grafted surface within 10 minutes. The L929 sheet was transferred with and without PVDF membrane to a new TCPS for cell viability testing.

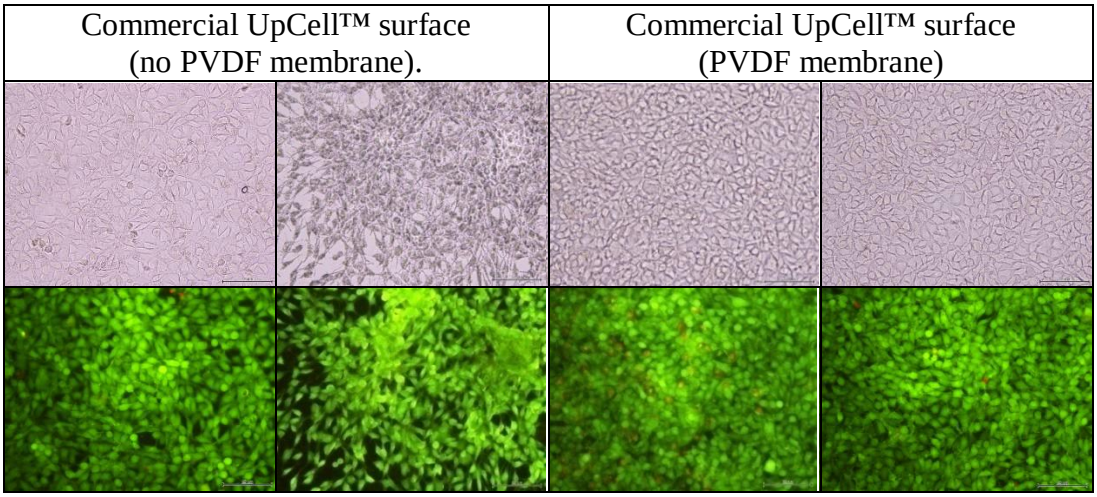


Figure 4.65 Phase contrast andlive dead staining of L929 single-layered sheet after retrieval from commercial UpCell™ surface. Scale bar = 100µm

The percentage of live L929 cells with PVDF harvesting was 66.98±2.90% and 65.61±2.04% (without PVDF membrane) (Table 4.10).

Table 4.10 The percentage of retrieval L929 cell, they were detached from the commercial UpCell™ Surface.

No.	Dead cells	Live cells	Total cells	Dead cells(%)	Live cells(%)	Live cells(%)
No PVDF 2	54.73	101.42	156.15	35.05	64.95	65.61 ±2.04
No PVDF 3	44.34	93.63	137.97	32.14	67.86	
No PVDF 4	58.73	102.92	161.65	36.33	63.67	
No PVDF 5	55.15	115.56	170.71	32.31	67.69	
No PVDF6	72.60	128.24	200.84	36.15	63.85	
PVDF 1	42.48	109.23	151.70	28.00	72.00	66.98 ±2.90
PVDF 3	69.30	124.17	193.47	35.82	64.18	
PVDF 4	51.30	110.11	161.41	31.78	68.22	
PVDF 5	47.43	98.85	146.27	32.42	67.58	
PVDF 6	66.14	128.87	195.01	33.92	66.08	

Another cell line, MC3T3-E1 was also used to confirm that the observed cell detachment depended on PNIPAM hydration. MC3T3-E1 cells were cultured on the control and the commercial UpCell™ surface to a confluent layer. These plates were incubated in a low temperature chamber set at 20°C in order to detach the cells. The morphology of MC3T3-E1 cells was observed under an inverted microscope at 10, 30, 60, and 90 minutes.

We found that MC3T3-E1 cells can be detached as continuous sheets from the commercial UpCell™ surface. Spontaneously, the periphery of MC3T3-E1 cell sheets began to detach from the edge of the culture plate surfaces. Figure 4.66 shows the

overlaid PVDF membrane together with the MC3T3-E1 sheet was peeled off from the well plate with a forceps and transferred to a new culture surface. On the commercial UpCell™ surface, MC3T3-E1 cells detached as a complete cell sheet by using PVDF membrane within 30 minutes as shown in Figure 4.67.

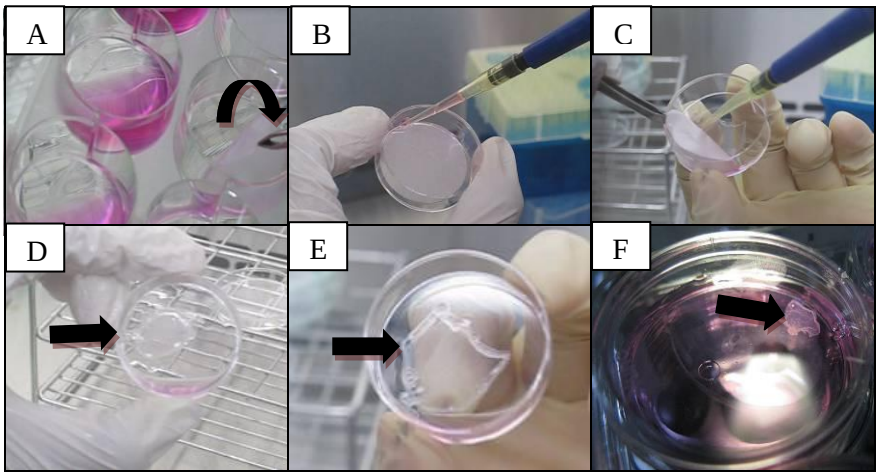


Figure 4.66 MC3T3-E1 cell sheets harvested from the commercial UpCell™ surface.
(A-C) Cultured MC3T3-E1 cell by using a temperature-responsive surface could be released from the dish surface only by reducing the temperature without enzymatic treatment.
(D-E) Cultured MC3T3-E1 cell were harvested as a single layer cell sheet
(F) Cell sheet floated in new culture media.

These results suggest that the cell–cell interactions weresuccessfully established within MC3T3-E1 sheet. Confluent cultured MC3T3-E1 cells were harvested as a single continuous cell sheet retaining cell–cell junction as well as deposited ECM on the basal side. It was clear that the temperature of the commercial UpCell™ surface was reduced to 20°C, the PNPAM layer became very hydrophilic, bound to water, swelled and released adherent cells completely within a few minutes (compared with UV grafting).

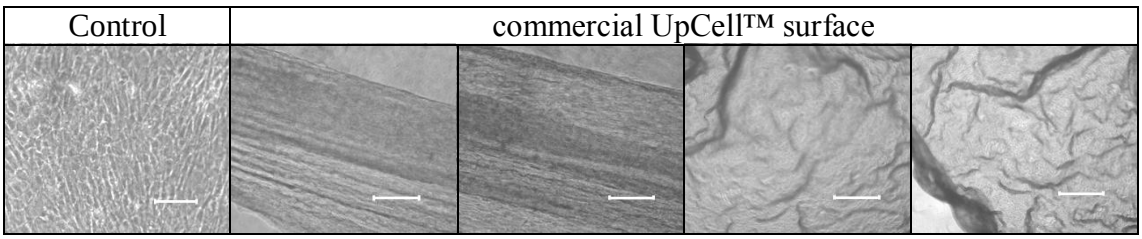


Figure 4.67 MC3T3-E1 cells detached from the commercial UpCell™ surface at 20°C within 30 minutes. Scale bar = 100µm

The surface after L929 and MC3T3 cells detached from commercial UpCell™ surfaces were shown in Figure 4.68. For the control well plate, most of the fibroblast cells still remained (Blue color) on the surface after lowering temperature. The percentage of detached MC3T3-E1sheet was 100% from commercial UpCell™ surface after incubation at 20°C for 30 minutes.

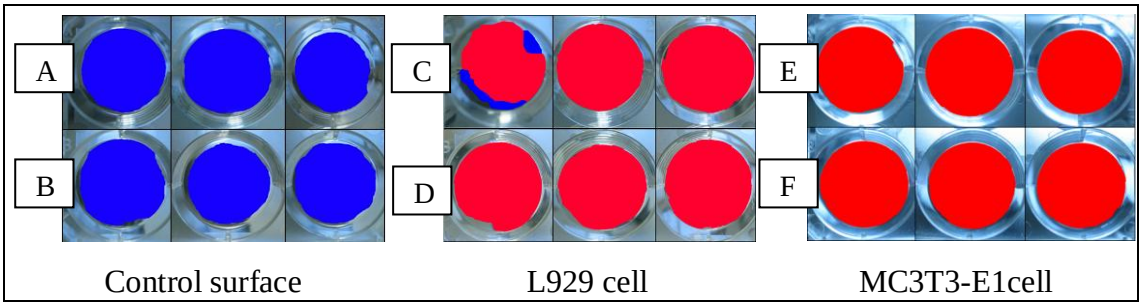


Figure 4.68 Areas of cell detachment from commercial UpCell™ surface. (Red color = detachment area, Blue color = attachment area)
(A) Celldetachment from control surface (without PVDF membrane)
(B) Celldetachment from control surface (with PVDF membrane)
(C) L929 cell detachment from commercial UpCell™ surface (without PVDF membrane)
(D) L929 cell detachment from commercial UpCell™ surface (with PVDF membrane)
(E) MC3T3-E1 sheet detachment from commercial UpCell™ surface(without PVDF membrane)
(F) MC3T3-E1 sheet detachment from commercial UpCell™ surface(with PVDF membrane)

For the MC3T3-E1 cells detachment from the commercial grafted surface by reducing the temperature to 20°C for 30 minutes, MC3T3-E1 cultured cells can be harvested as a sheet without digestion enzyme. The growth of the retrieved cell construct was examined by transferring the cell sheet to a new surface. The single-layered MC3T3-E1 sheet detached from the commercial UpCell™ surface was transferred to new culture surface as the next passage. After 2 days, the harvested MC3T3-E-1 cells could grow, attach, spread, and proliferated on new culture cells. No bacterial contamination was observed (Figure 4.69). After they were re-grown, the percentage of MC3T3-E1 cell viability was more than that of L929 cells, demonstrating that L929 cells were more sensitive more than MC3T3-E1 cells.

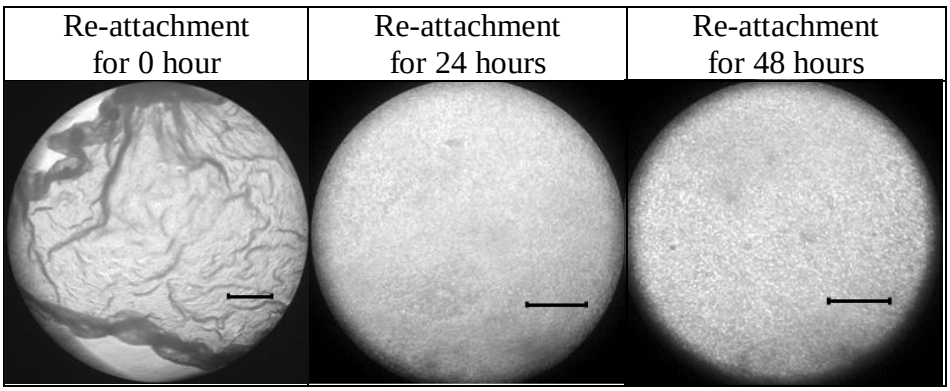


Figure 4.69 The morphology of the single-layered MC3T3-E1 cells on new TCPS after 48 hours incubation. Scale bar = 100 µm

The single-layered MC3T3-E1 cells migrating from the transferred cell sheet to a culture dish were observed after 24 and 48 hours. At 0 hour, the cell sheet construct was floating and folded freely on the new culture surface. The live-dead staining of the retrieved cell sheet shows that cells in the sheet construct maintained high viability (Figure 4.70 and Table 4.11). The percentages of live MC3T3-E1 single layer sheet without and with using PVDF membrane harvesting were 72.96±8.05% and 76.28±0.65%, respectively. It can be noticed that either low temperature treatment or the peeling method did not affected the viability of the cells.

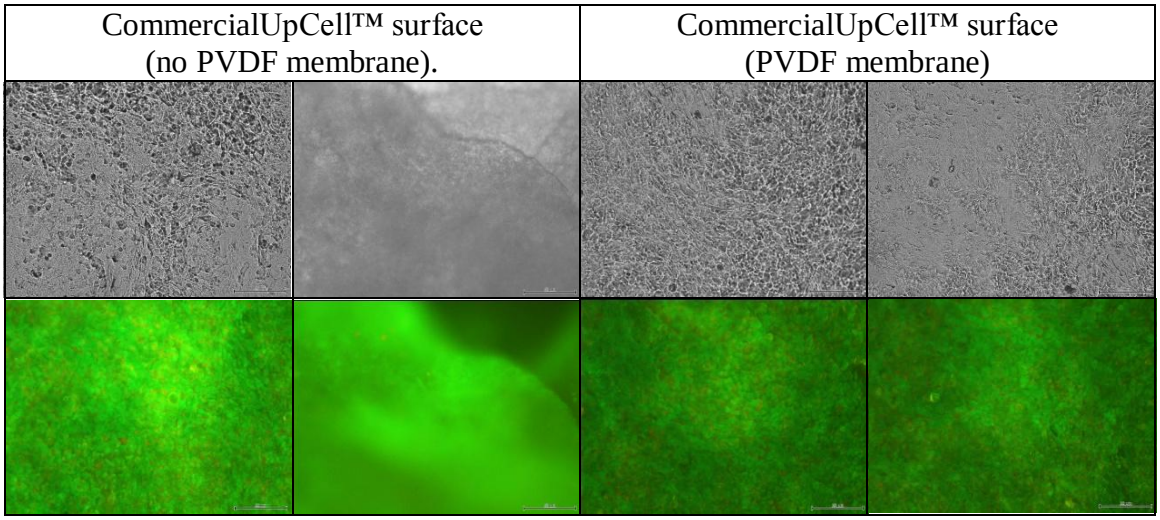


Figure 4.70 Live and dead staining of MC3T3-E1 single-layered sheet after retrieval from commercial UpCell™ surface. Scale bar = 100µm

Table 4.11 The percentage of retrieval MC3T3-E1 single-layered sheet, they were detached from the commercial UpCell™ surface.

No.	Dead cells	Live cells	Total cells	Dead cells(%)	Live cells (%)	Live cells(%)
No PVDF 1	46.65	163.14	209.79	22.24	77.76	
No PVDF 2	51.96	91.03	142.98	36.34	63.66	72.96±8.05
No PVDF 3	48.81	167.69	216.50	22.55	77.45	
PVDF 1	61.40	190.20	251.60	24.40	75.60	
PVDF 2	34.11	110.12	144.22	23.65	76.35	76.28±0.65
PVDF 3	33.41	111.10	144.52	23.12	76.88	

3. Double-Layered Sheet Manipulation with the UpCell™ surface

Double layer sheet harvesting from the UpCell™ surface, and transferring the cell sheet to another cell sheet was investigated. MC3T3-E1 cells were cultured in alpha-MEM medium. These plates were incubated at 37°C to promote cell proliferation. For cell detachment, non-adherent cells in each well were removed by washing with PBS, before adding fresh alpha-MEM medium into a culture plate, and placing the culture well platein an incubator set at 20°C in order to detach the cells.

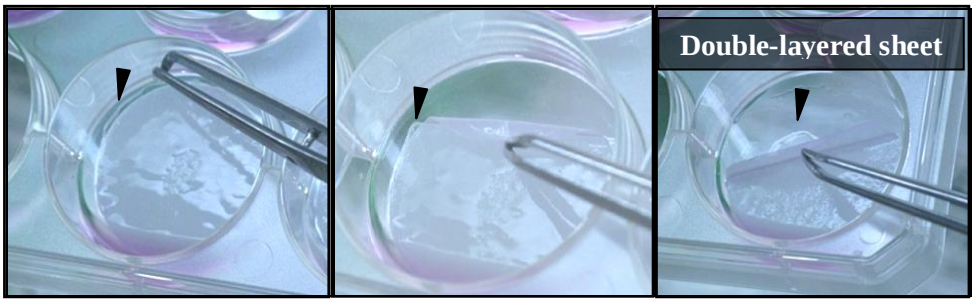


Figure 4.71 Double-layered MC3T3-E1 sheet detached from the UpCell™ surface by using PVDF membrane. Black arrows indicate the direction of detachment.

By reducing the temperature to 20°C at 30minutes, we used sterile forceps to gently detach the double-layered sheet around the edge of the PVDF membrane (Figure 4.71). MC3T3-E1 cellscould be detached as continuous sheets from the UpCell™ surface because when the temperature of the culture was reduced to below 32°C, the PNIPAM layer became very hydrophilic, bound to water, swelled and released adherent cells.

The culture surface after cell was detached shown in Figure 4.72. After temperature reduction, MC3T3-E1 cells detached from the grafted surface. On the control well plate, most of the fibroblast cells still remained on the surface after lowering temperature to 20°C for 30 minutes. The percentage of detached cells from the control was approximately 1.25% (with PVDF membrane), but was 100% from the commercial UpCell™ surface after incubation at 20°C for 30 minutes.

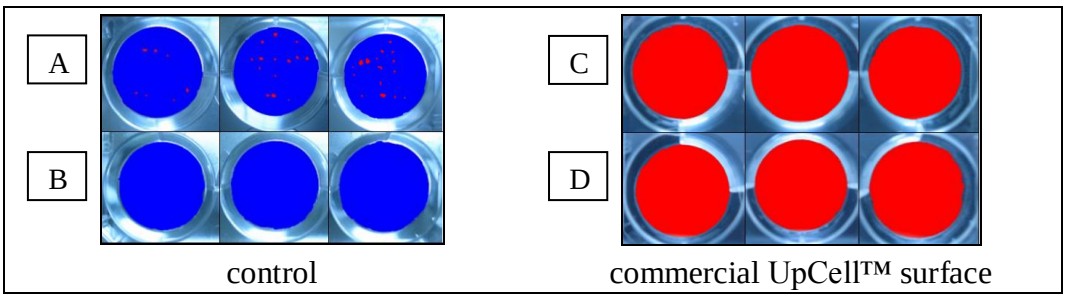


Figure 4.72 Area of cell detachment from the control and commercial UpCell™ surface (with PVDF membrane)
(Red color = detachment area, Blue color = attachment area)
(A) Control single layer sheet
(B) Control double layer sheet
(C) Single-layered MC3T3-E1 sheet detachment
(D) Double-layered MC3T3-E1 sheet detachment

The complete double-layered MC3T3-E1 sheet which was recovered from commercial UpCell™ surface was re-grown on new TCPS. These cells were incubated at 37°C for 48hours. The morphology after retrieval from the grafted surfaces by PVDF membrane was photographed and stained for cell viability testing. We found that, this double-layered sheet was in a good detachment condition as indicated by live and dead stain (Figure 4.73). The percentage of live MC3T3-E1 single layer sheet when they reattached onto new culture surface was 78.49±6.8% (Table 4.12).

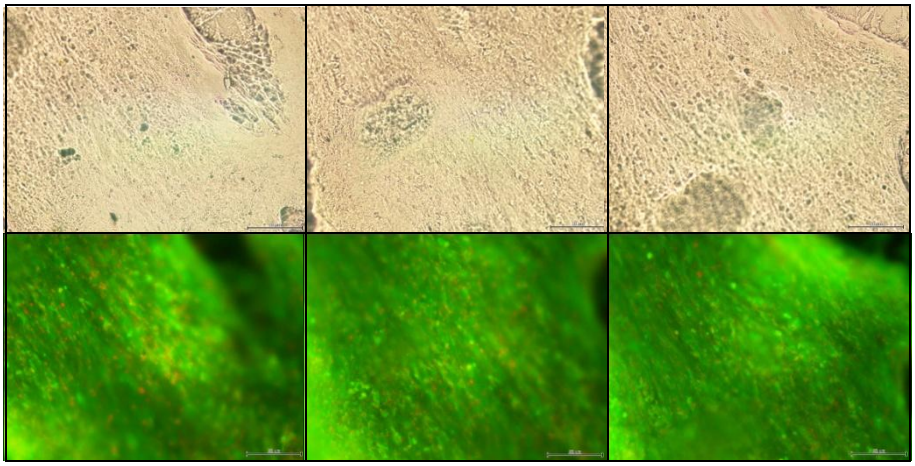


Figure 4.73 The phase contrast (upper) and live/dead stained image (lower) of recovered double-layered MC3T3-E1 sheet that detached from commercial UpCell™ surface at 20°C for 30 minutes. Scale bar = 100 µm

Table 4.12 The percentage of retrieval recovered MC3T3-E1 double-layered sheet detachment from the commercial UpCell™ surface.

No.	Dead cells	Live cells	Total cells	Dead cells(%)	Live cells(%)	Live cells (%)
1	50.94	131.96	182.90	27.85	72.15	
2	44.61	126.45	171.06	26.08	73.92	
3	44.96	132.54	177.50	25.33	74.67	78.49±6.8
4	17.13	100.65	117.78	14.54	85.46	
5	18.80	118.17	136.98	13.73	86.27	

From these results, these cell layers could adhere and proliferate on the new culture cells. Both L929 and MC3T3-E1 cells were transferred into new culture surface. After incubation at 37°C for 48 hours, they showed the high percentage of cell viability. These results obviously described that electron beam irradiation has been proven successful for commercial PNIAM grafting. They showed high percentage of cell detachment within 30 minutes. Therewas no required incubation time to completely detach cell sheets.

4.9 Cell morphology analysis using scanning electron microscope (SEM)

To study the effect of low temperature effect on cell morphology, SEM was used to assess cellular responses when the temperature was changed. The important factors for cell morphology, spreading, and proliferation that greatly depend upon the surface properties of biomaterial is cell adhesion [55]. Therefore, the copolymer property at 37°C is suitable to promote cell attach, spread and proliferation. Under SEM at 37°C, MC3T3-E1 cells revealed fibroblast morphology with similar adhesion pattern of cells at low cell density in comparison with high cells density on grafted TCPS. Highly densed MC3T3-E1 cells on the copolymer grafted surfaces reached confluence with cell-to-cell junction formation at 37°C (Figure 4.74).

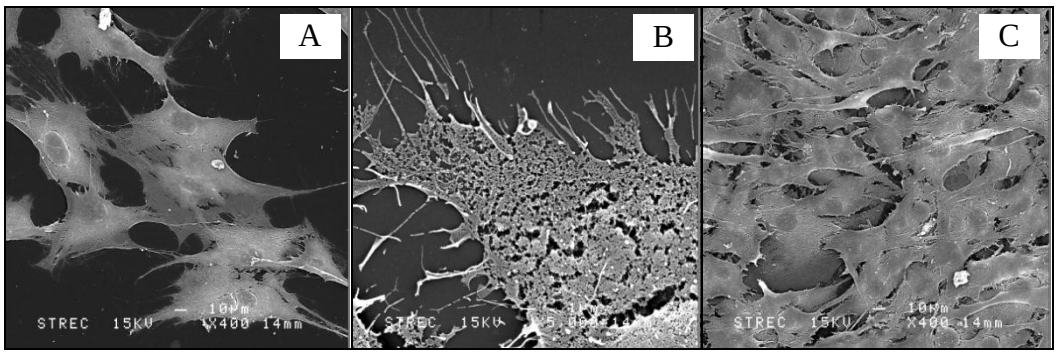


Figure 4.74 SEM image showing the MC3T3-E1 cell morphology at 37°C
(A) MC3T3-E1, low cell density at 37°C, Scale bar = 10µm
(B) MC3T3-E1 cell membrane at 37°C, Scale bar = 1µm
(C) MC3T3-E1, high cell density at 37°C, Scale bar = 10µm

The specimens at low temperature were fixed and observed under SEM. The results were shown in Figure 4.75. At low cell density, the seeded cells were contracted (compared with 37°C) and showed the folding of membrane protein at the edge of cell layer. The cells contracted probably because of the disappearance of anchorage-dependence caused by the surface hydration and the strong cell–cell interactions for the high cell density (cell sheet) [56].

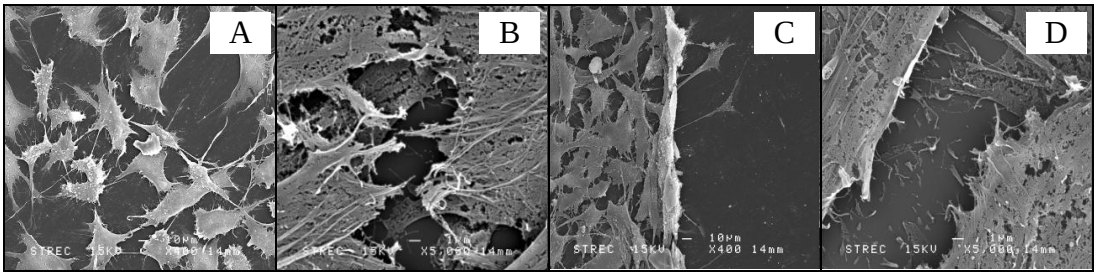


Figure 4.75 SEM image showing the MC3T3-E1 cell morphology at 20°C
(A) MC3T3-E1, low cell density at 20°C, Scale bar = 10µm
(B) MC3T3-E1, low cell density at 20°C, Scale bar = 1µm
(C) MC3T3-E1, high cell density at 20°C, Scale bar = 10µm
(D) MC3T3-E1, low cell density at 20°C, Scale bar = 1µm

CHAPTER 5 CONCLUSIONS

5.1 Conclusions

The temperature-responsive polymer, PNIAM-co-AM was successfully grafted on the polystyrene culture surfaces. The hydrophilic-hydrophobic transition of the grafted surfaces was confirmed by AFM, contact angle measurement. FTIR and ATR-FTIR results showed the spectra of copolymer grafted dish on the surface. The in vitro cytoxicity of the grafted surface was non-toxic.The mouse fibroblast L929, mouse pre-osteoblast MC3T3-E1, and human chondrocyte cells were adhered and proliferated on the grafted surface similar to control surface at 37°C. The recovery of these cells by low-temperature treatment, the cells could be detached as single cells and cell sheet without digestion enzyme. A protocol to harvest mono-layered and double-layered sheets from the thermo-responsive polymer, PNIAM-co-AM, grafted surface for construct complete sheet and further cell sheet applications was archived (Appendix C).

The cells were cultured over-confluency and cell-cell junction was firmly established at 37°C on the PNIAM-co-AM surface. When the temperature of the culture was reduced to 10°C (30 minutes), and 20°C (60 minutes), the grafted copolymer became very hydrophilic, bound to water, extended and released the adherent cells as a sheet. The amount of medium was 100 µL to be added when detaching the cell sheet from 3.5 cm PNIAM-co-AM surface using membrane to prevent cells from drying. When transferring detached cell sheet to another sheet using the hydrophilic PVDF membrane, it is important that some kind of weight may be necessary to make the cell sheet firmly attach to the new surface. Because the extracellular matrix (ECM) under the cultured cells was harvested with the cells, cell sheets were normally adhesive to other cell sheets for double-layered or multilayer sheet.

The low-temperature lift-off technique destructively harvested the cell layer at a low cell density into single cells and most of the underlying ECM, whereas slight agitating the surface with culture media had deleterious effects on the morphological appearance of cells being harvest. The harvested cells could be transferred to other tissue culture dishes to promote cell sheet regeneration. These characteristics suggest that PNIAM-co-AM grafted TCPS have potential as biomaterials for cell sheet engineering.

5.2 Recommendations

Then, further studies, There are some steps to explain, and clarifying the double layer that were harvested. This study requires other cell lines and primary cells to compare the cell sheet manipulation using a temperature–responsive surface that grafting by UV irradiation.

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APPENDIX A

THE PERCENTAGE OF CELL DETACHMENT

Table A. 1 The percentage of L929 single cells detachment from the thin PNIAM-co- AM grafted surface at 10°C.

PNIPAM No.	L929 cell attachment	L929 single cells detachment	Total cells	L929 single cells detachment (%)	L929 single cells detachment (%)
1	10	26	36	72.22	
2	6	28	34	82.35	
3	8	14	22	63.64	
4	9	22	31	70.97	
5	3	34	37	91.89	77.22
6	6	34	40	85.00	±10.28
7	12	19	31	61.29	
8	5	38	43	88.37	
9	9	27	36	75.00	
10	5	22	27	81.48	
Control No.	L929 cell attachment	L929 single cells detachment	Total cells	L929 single cells detachment (%)	L929 single cells detachment (%)
1	79	7	86	8.14	
2	83	10	93	10.75	
3	95	5	100	5.00	
4	62	9	71	12.68	
5	74	11	85	12.94	10.49
6	81	9	90	10.00	±3.28
7	57	8	65	12.31	
8	73	13	86	15.12	
9	98	6	104	5.77	
10	86	12	98	12.24	

Table A. 2 The percentage of MC3T3-E1 single cell detachment from the thin PNIAM-co-AM grafted surface at 10°C.

PNIPAM No.	MC3T3-E1 cells attachment	MC3T3-E1 single cells detachment	Total cells	MC3T3-E1 single cells detachment (%)	MC3T3-E1 single cells detachment (%)
1	20	124	144	86.11	
2	38	128	166	77.11	
3	49	98	147	66.67	78.25±8.76
4	45	126	171	73.68	
5	19	135	154	87.66	
Control No.	MC3T3-E1 cells attachment	MC3T3-E1 single cells detachment	Total cells	MC3T3-E1 single cells detachment (%)	MC3T3-E1 single cells detachment (%)
1	95	9	104	8.65	
2	84	15	99	15.15	
3	89	18	107	16.82	10±5.76
4	98	4	102	3.92	
5	104	6	110	5.45	

Table A. 3 The percentage of L929 single cell detachment from the thick PNIAM-co-AM grafted surface at 10°C.

PNIPAM No.	L929 cell attachment	L929 single cells detachment	Total cells	L929 single cells detachment (%)	L929 single cells detachment (%)
1	156	106	262	40.46	
2	194	137	331	41.39	
3	79	168	247	68.02	52.35±13.64
4	102	150	252	59.52	
Control No.	L929 cell attachment	L929 single cells detachment	Total cells	L929 single cells detachment (%)	L929 single cells detachment (%)
1	328	10	338	2.96	
2	278	13	291	4.47	3.23
3	302	7	309	2.27	

Table A. 4 The percentage of MC3T3-E1 single cell detachment from the thick PNIAM-co-AM grafted surface at 10°C.

PNIPAM No.	MC3T3-E1 cell attachment	MC3T3-E1 single cells detachment	Total area	MC3T3-E1 single cells detachment (%)	MC3T3-E1 single cells detachment (%)
1	95	175	270	64.81	
2	102	106	208	50.96	
3	79	98	177	55.37	54.39±7.81
4	102	136	238	57.14	
5	89	69	158	43.67	
control	MC3T3-E1 cell attachment	MC3T3-E1 single cells detachment	Total area	MC3T3-E1 single cells detachment (%)	MC3T3-E1 single cells detachment (%)
1	167	2	169	1.18	
2	158	1	159	0.63	
3	184	3	187	1.60	1.09±0.80
4	97	2	99	2.02	
5	186	0	186	0.00	

Table A.5 The percentage of MC3T3-E1 sheet detachment from the thick and thin PNIAM-co-AM grafted surface at 20°C.

Total area (Control)	MC3T3-E1 cell detachment area	MC3T3-E1 cell detachment (%)	MC3T3-E1 cell detachment (%)
651405	534	0.08	0.64±1.16
818129	356	0.04	
789122	4940	0.63	
648086	364	0.06	
804917	563	0.07	
714240	21201	2.97	
Total area (Thin film)	MC3T3-E1 cell detachment area	MC3T3-E1 cell detachment (%)	MC3T3-E1 cell detachment (%)
3511620	1485516	42.30	31.51±9.70
2202240	198824	9.03	
2587300	724941	28.02	
2541864	771336	30.35	
1792949	526064	29.34	
3135304	1568493	50.03	
Total area (Thick film)	MC3T3-E1 cell detachment area	MC3T3-E1 cell detachment (%)	MC3T3-E1 cell detachment (%)
602716	93181	15.46	14.69±2.07
667444	77575	11.62	
610956	90901	14.88	
599252	76912	12.83	
667492	113492	17.00	
653180	106859	16.36	

Table A.6 The percentage of cell sheet detachment from the thin PNIAM-co-AM grafted surfaces by using pipetting and PVDF membrane.

Sample.	Total area	L929 detachment area	L929 Cell detachment (%)	L929 Cell detachment (%)
No PVDF 1	1869658	394238	21.08610238	
No PVDF 2	1885888	216419	11.475708	13.84±6.39
No PVDF 3	2324872	208476	8.967203356	
PVDF 1	2443860	2018061	82.57678427	
PVDF 2	2205018	2069313	93.84562847	87.62±5.72
PVDF 3	2266000	1959175	86.45962048	
Sample	Total area	MC3T3-E1 detachment area	MC3T3-E1 Cell detachment (%)	MC3T3-E1 Cell detachment (%)
No PVDF 1	2377227	640311	26.93520644	
No PVDF 2	2398380	886508	36.96278321	30.50±5.60
No PVDF 3	2296344	633902	27.60483621	
PVDF 1	2345140	1847700	78.78847318	
PVDF 2	2208740	2095452	94.87092188	85.79±8.23
PVDF 3	2304692	1929769	83.73218634	

Table A. 7 The percentage of single and double layer cell sheet detachment from the thin PNIAM-co-AM grafted surface by using PVDF membrane at 10°C (30minutes) and 20°C (60minutes) cell density at 1×10^5 cells/ml.

Type of layer and Well No.	Total area	average	MC3T3-E1 cell attachment area	MC3T3-E1 cell detachment area	average	MC3T3-E1 cell detachment (%)
1Layer No.1	2621544	2624253.333	103062	2518482	2520959	96.19±0.24
	2638696		103402	2535294		
	2612520		103418	2509102		
1Layer No.2	3404448	3368657.333	133492	3270956	3235265	
	3355632		133392	3222240		
	3345892		133292	3212600		
1Layer No.3	3633880	3606887	127179	3506701	3479688	
	3593384		127208	3466176		
	3593397		127210	3466187		
2Layer No.1	2904176	2888489.333	51446	2852730	2836609	97.79±2.69
	2894888		51646	2843242		
	2866404		52550	2813854		
2Layer No.2	1938361	1942500.333	0	1938361	1942500	
	1952010		0	1952010		
	1937130		0	1937130		
2Layer No.3	2185256	2177354.667	105136	2080120	2072145	
	2169496		105336	2064160		
	2177312		105156	2072156		

Table A. 8 The percentage of single and double layer cell sheet detachment from the thin PNIAM-co-AM grafted surface by using PVDF membrane at 10°C (30minutes) and 20°C (60minutes), cell density at 1×10^6 cells/ml

Cell sheet	Total area	Average	Detachment area	Average	%cell detachment
1 Layer 1	1168824 1222244 1193680	1194916	1168824 1222244 1193680	1194916	100
1 Layer 2	1248429 1253510 1307960	1269966.33	1248429 1253510 1307960	1269966.33	100
1 Layer 3	1362382 1362232 1349884	1358166	1362382 1362232 1349884	1358166	100
2 Layer 1	948574 915387 821788	895249.67	933038 903245 803547	879943.33	98.29
2 Layer 2	1015271 1098296 968531	1027366	985299 1079273 954242	1006271.33	97.95
2 Layer 3	1088181 1087994 1027322	1067832.33	1016057 998349 973667	996024.33	93.28

Table A. 9 The percentage of cell detachment from the PNIPAM-grafted surface with longer exposure time at 20°C.

Sample No.	Total area	attachment area	Detachment area	%cell detachment	%cell detachment
control	2030776	2030776	0	0	0
	2045932	2045932	0	0	
	2038372	2038372	0	0	
Grafted surface 1	1323152	989735	333417	25.19869221	25.82
	1320985	978767	342218	25.90627448	
	1302859	959337	343522	26.36678259	
Grafted surface2	2084148	1518305	565843	27.14984732	24.21
	2053560	1588239	465321	22.65923567	
	2038372	1572919	465453	22.83454639	
PVDF control	4376896	4340470	36426	0.832233619	0.81
	4300416	4266165	34251	0.796457831	
	4311376	4275943	35433	0.821848987	
Grafted surface1 layer	2090610	266488	1824122	87.25309838	87.23
	2086616	264534	1822082	87.32234393	
	2063828	265329	1798499	87.14384144	
Grafted surface2Layer	1996766	275235	1721531	86.21596121	87.48
	1979002	207137	1771865	89.53325969	
	1986386	264067	1722319	86.70615882	

Table A.10 The percentage of cell detachment from the PNIPAM-grafted surface with longer exposure time at 10 and 20°C.

No.	Total area	Cell attachment	Cell detachment	% cell detachment	% cell detachment
Control no PVDF 1	2390540	1937191	453349	18.96	17.77
Control no PVDF 2	2932760	2446362	486398	16.58	
Control PVDF 1	1922117	1917661	4456	0.23	0.72
Control PVDF 2	1743851	1722699	21152	1.21	
1 Layer 1	2630066	424817	2205249	83.84	87.13
1 Layer 2	2753517	263759	2489758	90.42	
2 Layer 1	2448165	185738	2262427	92.41	89.82
2 Layer 2	2066858	263818	1803040	87.23	

APPENDIX B

STANDARD CURVE

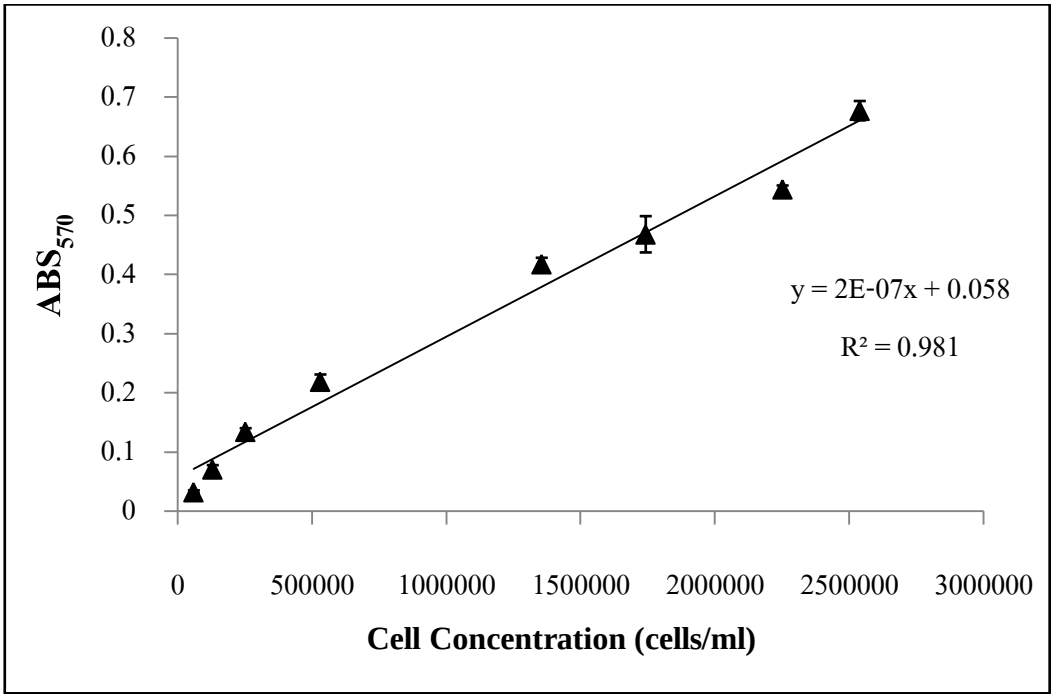

Figure B. 1 MC3T3-E1 cells standard curve

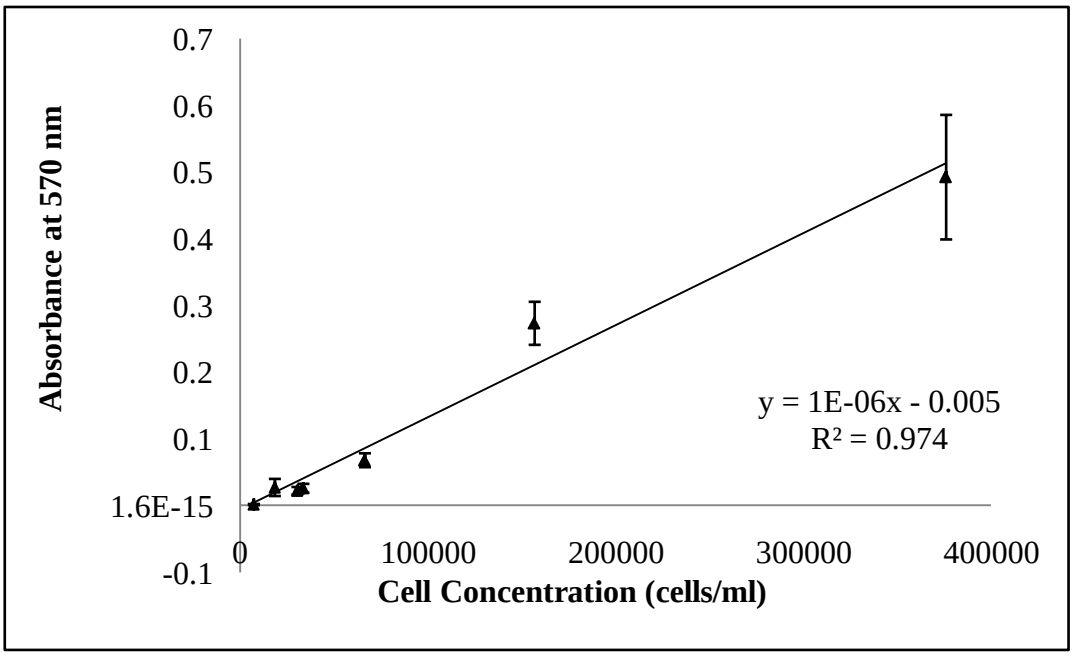

Figure B. 2 L929 cells standard curve

APPENDIX C

A PROTOCAL TO HARVEST CELL SHEET

A protocol to harvest mono-layered and double-layered sheets from the PNIAM-co-AM grafted surface.

Storage

The culture plate should be stored at room temperature (20–25°C) and out of direct sunlight.

Cell attachment study

1. The PNIPAM-co-AM grafted culture plate was sterilized by using 70% ethanol, pH2 follow by sterile PBS.
2. Low cell density was used in the single cell detachment and high density for cell sheet detachment study. Therefore, attachment time for cells may vary depending on cell type and cell density.
3. Observations cell morphology under an inverted microscope should also be kept short in order to prevent cooling.

Detachment of single cell

1. Culture cells on the PNIPAM-co-AM grafted culture plate to a density of less than 70% confluence.
2. Place the PNIPAM-co-AM grafted culture plate in low temperature chamber set at 10°C (30 minutes) and 20°C (60 minutes) in order to extend polymer chain and detach the cells, respectively.
3. Cells should detach spontaneously within 90 minutes, depending on the cell type.
4. Cells that fail to detach spontaneously can be detached by gently flushing the surface with culture medium.

Detachment and transfer of cell sheet

1. Culture cells on the PNIPAM-co-AM grafted culture plate to over confluent layer.
2. Remove old culture medium.
3. Add fresh medium approximately 50 µl per well to prevent the cells from drying out.
4. Gently place the PVDF membrane on top of the cell layer. Avoid air bubbles between the membrane and the cell layer.
5. Place the PNIPAM-co-AM grafted culture plate at 10°C for 30 minutes and follow by 20ºC for 60 minutes (detachment time may vary depending on cell type).
6. Use sterile forceps and gently detach the cell layer around the edge of the PVDF membrane. Grasp under the membrane and cell layer with the forceps and carefully withdraw from the PNIPAM-co-AM grafted culture plate.
7. Transfer the PVDF membrane with the attached cell layer facing downwards to the Matrigel™ coated surface (or another cell layer for double-layered sheet).
8. Gently withdraw the membrane from the cell layer using the forceps.
9. Add fresh medium (approximately 2 ml per dish) and leave it undisturbed at 37°C for at least 30-60 minutes to allow for direct attachment of the cell layer to the new surface.
10. The morphology of the harvested cell was observed under inverted microscope after 24 hours incubation.

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