ผิวพอลิสไตรีนกราฟต์ด้วยพอลิ(*เอ็น*-ไอโซโพรพิลอะคริลาไมด์)บรัช

นางสาวพิริยา ไชยล้อม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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POLYSTYRENE SURFACE GRAFTED WITH POLY(*N*-ISOPROPYLACRYLAMIDE) BRUSHES

Miss Piriya Chailom

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Chemistry

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Thesis Title	POLYSTYRENE SURFACE GRAFTED WITH POLY(*N*-ISOPROPYLACRYLAMIDE) BRUSHES
By	Miss Piriya Chailom
Field of Study	Chemistry
Thesis Advisor	Associate Professor Voravee Hoven, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Associate Professor Voravee Hoven, Ph.D.)

.....Examiner
(Associate Professor Sumrit Wacharasindhu, Ph.D.)

.....External Examiner
(Assistant Professor Punnama Siriphannon, D.Eng.)

พิริยา ไชยล้อม : ผิวพอลิสไตรีนกราฟต์ด้วยพอลิ(*เอ็น*-ไอโซโพรพิลอะคริลาไมด์)บรัช (POLYSTYRENE SURFACE GRAFTED WITH POLY(*N*-ISOPROPYLACRYLAMIDE) BRUSHES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น, 39 หน้า.

เตรียมฟิล์มพอลิสไตรีนที่มีรูพรุนได้จากการขึ้นรูปสารละลายผสมระหว่างพอลิสไตรีน (น้ำหนักโมเลกุล = $3x10^6$ ดาลตัน) กับบล็อกโคพอลิเมอร์ของพอลิสไตรีนและพอลิอะคริลิกแอซิด (PS_{130}-*b*-PAA_{12}) ในอัตราส่วน 9:1 โดยน้ำหนักบนแผ่นกระจกโดยวิธี breath figure จากการวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดพบว่าแผ่นฟิล์มมีลักษณะเป็นรูพรุนและมีการจัดเรียงตัวเป็นระเบียบคล้ายรังผึ้ง โดยมีเส้นผ่าศูนย์กลางของรูพรุนเท่ากับ 7.67±0.69 ไมโครเมตร จากนั้นนำฟิล์มพอลิสไตรีนที่มีรูพรุนมาเคลือบด้วยพอลิโดปามีนที่ดัดแปรให้มีหมู่โบรโมเอสเทอร์ (p-DA-BiBBr) ซึ่งจะทำหน้าที่เป็นตัวริเริ่มปฏิกิริยาสำหรับการสังเคราะห์พอลิเมอร์บรัชของพอลิ(*เอ็น*-ไอโซโพรพิลอะคริลาไมด์) (PNIPAM) ที่มีสมบัติตอบสนองต่ออุณหภูมิด้วยปฏิริยา activators regenerated by electron transfer for atom transfer radical polymerization (ARGET ATRP) ยืนยันความสำเร็จในการเคลือบ p-DA-BiBBr และการกราฟต์ PNIPAM บนฟิล์มพอลิสไตรีนที่มีรูพรุนด้วยการวัดมุมสัมผัสของน้ำ แอทเทนนูเอทเตทโททอลรีเฟรกชันฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปี และเอ็กซ์เรย์โฟโตอิเล็กตรอนสเปกโทรสโกปี จากการวิเคราะห์ด้วยเทคนิคโปรตอนนิวเคลียร์แมกเนติกแรโซแนนซ์สเปกโทรสโกปีและเจลเพอร์มีเอชันโครมาโทกราฟีพบว่า PNIPAM ที่สังเคราะห์ได้ในสารละลายมีจำนวนหน่วยซ้ำประมาณ 300 จากการวิเคราะห์ด้วยเทคนิคอิลลิปโซเมทรีพบว่าชั้นของ PNIPAM มีความหนาประมาณ 18.23 นาโนเมตร ซึ่งเป็นความหนาเพียงพอที่จะนำไปใช้เป็นสับสเตรทในการศึกษาการเกาะและการหลุดออกของเซลล์ จากการศึกษาเบื้องต้นพบว่ามีเปอร์เซนต์การหลุดออกของเซลล์เคราติโนไซต์ 12 เปอร์เซนต์ จากฟิล์มพอลิสไตรีนที่มีรูพรุนที่กราฟต์ด้วย PNIPAM เมื่อลดอุณหภูมิลงจาก 37 เป็น 20 องศาเซลเซียส ซึ่งเป็นอุณหภูมิที่ต่ำกว่าอุณหภูมิสารละลายวิกฤตล่างของ PNIPAM อย่างไรก็ตามเปอร์เซนต์การหลุดออกของเซลล์จากแผ่นฟิล์มที่มีรูพรุนดังกล่าวไม่แตกต่างอย่างมีนัยสำคัญกับแผ่นฟิล์มผิวเรียบกราฟต์ด้วย PNIPAM (10 เปอร์เซนต์)

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PIRIYA CHAILOM: POLYSTYRENE SURFACE GRAFTED WITH POLY(*N*-ISOPROPYLACRYLAMIDE) BRUSHES. ADVISOR: ASSOC. PROF. VORAVEE HOVEN, Ph.D., 39 pp.

Porous polystyrene (PS) film was prepared by casting a mixed solution of polystyrene (Mw=3x10^6 Daltons) and block copolymer of polystyrene and poly(acrylic acid) (PS_{130}-*b*-PAA_{12}) in weight ratio of 9:1 on glass substrate via breath figure method. As monitored by scanning electron microscopy, the casted film was porous and exhibited ordered honeycomb pattern with pore diameter of 7.67±0.69 μm. This porous PS film was then coated with polydopamine modified with bromoester group (p-DA-BiBBr) that functions as initiator for growing thermoresponsive poly(*N*-isopropylacrylamide) brushes via activators regenerated by electron transfer for atom transfer radical polymerization (ARGET ATRP). The success of p-DA-BiBBr coating and PNIPAM grafting on porous PS film was verified by water contact angle measurements, Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and x-ray photoelectron spectroscopy (XPS). As evaluated by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and gel permeation chromatography (GPC), the synthesized PNIPAM in solution had a degree of polymerization around 300. The thickness of the PNIPAM layer was found to be 18.3 nm as estimated by ellipsometry. This thickness should be suitable for cell attachment and detachment studies. From preliminary investigation, it was found that approximately 12% of attached keratinocyte cells at 37°C were detached from PNIPAM-grafted porous PS film when the temperature was decreased to 20°C, below LCST of PNIPAM, for 2 h. However, such percentage of cell detachment was not significantly different from that of PNIPAM-grafted flat PS film (10%).

Department: Chemistry Student's Signature .....
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LIST OF ABBREVIATION

ATR-FTIR	Attenuated total reflectance- Fourier transform infrared
BiBBr	2-Bromoisobutyryl bromide
CS_2	carbon disulfide
DMSO-d_6	deuterated dimethyl sulfoxide
DA	dopamine
EtOH	ethanol
GPC	Gel permeation chromatography
Mw	molecular weight
Mn	number average molecular weight
ppm	part per million
PAA	poly(acrylic acid)
PNIPAM	poly(*N*-isopropylacrylamide)
PDI	polydispersity index
PS	polystyrene
^{1}H NMR	proton nuclear magnetic resonance
SEM	Scanning electron microscopy
XPS	X-ray photoelectron spectroscopy

CHAPTER I
INTRODUCTION

1.1.Introduction

1.1.1. Poly(*N*-isopropylacrylamide) and its application for cell sheet fabrication

Poly(*N*-isopropylacrylamide) (PNIPAM) is a well-known thermo-responsive polymer synthesized from *N*-isopropylacrylamide. PNIPAM exhibits reversible phase transition in aqueous solution at lower critical solution temperature (LCST) of 32°C. Below LCST, PNIPAM is hydrated and exists in a form of expanded chains which can dissolve in water. Above LCST, PNIPAM chains would expel water molecules to form internal hydrogen bonding, and become compact chain and hydrophobic [1] (**Figure 1.1**). With its thermoresponsive phase transition, PNIPAM has been widely used as material for biomedical application such as drug delivery [2-4], biomolecule separation [5], and modified substrate for cell sheet engineering [6-9].

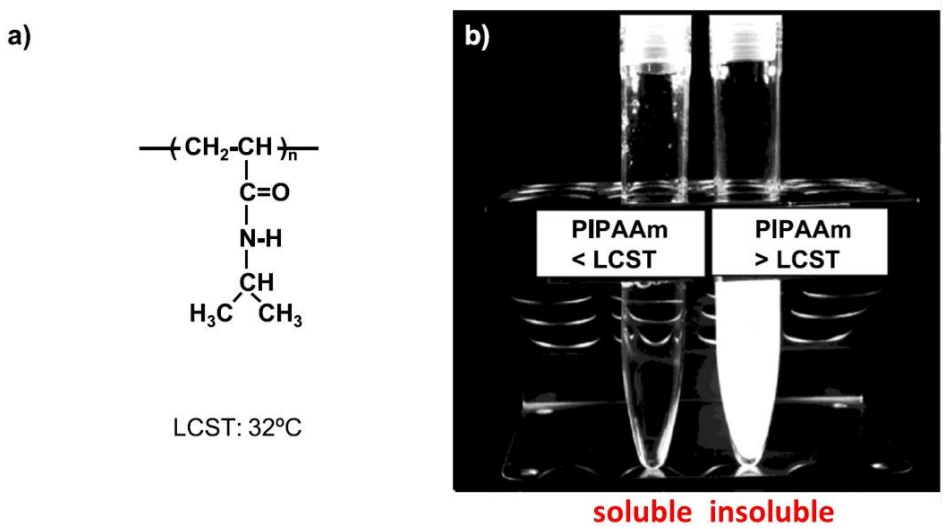


Figure 1.1 Structure (a) and thermoresponsive change in aqueous solution (b) of PNIPAM [1].

Cell sheet engineering has been developed for damaged tissue repair as an alternative to injection of single cell suspensions due to its difficulties in control of size

and position of injected cells. Cell sheet can also be used without biodegradable scaffolds which can cause inflammatory response to reconstruct various types of tissues such as bone [10], cornea [11], and heart [12]. Cell sheet can be prepared using thermoresponsive PNIPAM as cell culture substrate. At 37 °C which is cell culture temperature, PNIPAM exhibits hydrophobic property that allows cell to adhere and spread on the substrate to form cell sheet. By reducing temperature to 20 °C, PNIPAM turns hydrophilic and becomes hydrated. The mobility of PNIPAM chains cause detachment of cell sheet from the substrate without having to use digestive enzyme, allowing cell sheet with extracellular matrix (ECM) to remain intact, as shown in **Figure 1.2** [13].

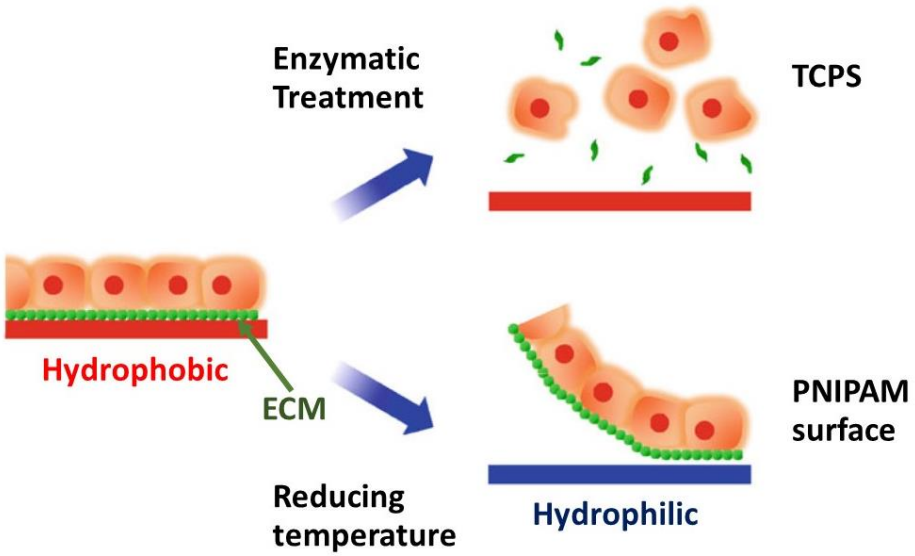


Figure 1.2 Preparation of cell sheet by thermo-responsive substrate.

In recent years, PNIPAM has been grafted on various types of surface and it was found that the thickness of polymer layer and porosity of the substrate have effects on cell adhesion and detachment.

In 2004, Akiyama *et al*. [14] grafted PNIPAM on tissue culture polystyrene (TCPS) surface by electron beam polymerization method. They synthesized PNIPAM-grafted surfaces with two different graft density of 1.4 ± 0.1 $\mu g/cm^2$ and 2.9 ± 0.1 $\mu g/cm^2$ which resulted in PNIPAM layers with the thickness of 15.5 ± 7.2 nm and 29.5 ± 8.4 nm, respectively. Cell adhesion and detachment on these surfaces were investigated using

Bovine carotid artery endothelial cells (ECs) as a model. With the thickness of PNIPAM at 15.5 ± 7.2 nm, cells were shown to effectively adhere and proliferate to form cell sheet on the surface. And when the temperature was decreased from 37 ℃ to 20 ℃, cell sheet could be recovered from the surface. At higher thickness (29.5 ± 8.4 nm), however, cells did not adhere on the surface at 37 ℃ due to hydrophilicity of the outermost portion of PNIPAM. It was suggested that as the distance from TCPS interfaces increase, the PNIPAM chains become more mobile resulting in their increasing ability to be hydrated. This result demonstrated that control of the thickness of polymer on the surface was an important factor in promoting cell adhesion.

In 2008, Li *et al*. [15] prepared PNIPAM brushes on silicon wafer via surface-initiated atom transfer radical polymerization (ATRP) with various thickness by controlling polymerization time. Cell adhesion and detachment on the surfaces were investigated using HepG2 cells as model. It was found that cells can both adhere and detach from material when the temperature was changed on the surface having PNIPAM thickness within a range of 20-45 nm. The surface with thinner layer had tightly packed PNIPAM chains which could not be hydrated and remains hydrophobic, causing cells to remain attached. The surface with thicker layer, on the other hand, had loosely packed PNIPAM chains that could be easily hydrated similar to the previous example, which deteriorated cell adhesion.

In 2008, Mizutani *et al*. [16] synthesized PNIPAM brushes with different thickness on polystyrene (PS) substrates via surface-initiated ATRP after coating the PS surfaces with 4-vinylbenzyl chloride as surface-initiator layer. Cell adhesion and cell detachment on all of grafted-PNIPAM thicknesses were then investigated with Bovine carotid artery endothelial cells (ECs) as a model. The results showed similar trend to the prior examples in that cells could adhere the most on grafted-PNIPAM substrate with thin PNIPAM layer and the adhesion decreased with the increase of PNIPAM layer thickness. For cell detachment, it was found that the time required for cell detachment increased when PNIPAM layer thickness. The cells detached from the surface with PNIPAM thickness of 20 – 50 nm were significantly lower than those with PNIPAM layer thickness 2 – 10 nm.

In 1999, Kwon *et al*. [17] grafted PNIPAM on TCPS dishes and porous poly(ethylene terephthalate membrane having pore diameter of 0.45 μm via electron beam irradiation. ECs cell sheet could be harvested from the porous membrane at 20 ℃ in 30 min while it took 75 min for the PNIPAM-grafted TCPS. It was believed that porosity of the PET membrane allowed water to reach and hydrate PNIPAM more easily, resulting in rapid detachment of cell sheet.

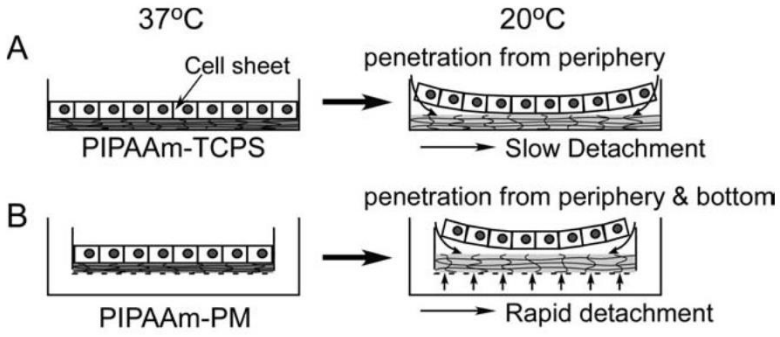


Figure 1.3 Processing of cell detachment with different substrates (a) TCPS (b) porous membrane (PM).

In 2014, Oh *et al*. [18] reported grafting of PNIPAM on PS nanofibrous mat fabricated by electrospinning technique and PS dish via electron beam method. Cell adhesion and detachment were investigated with human fibroblast cells as a model. Similar to the previous example, rapid cell detachment was observed in the case of PS nanofiber mat due to an increase of PNIPAM hydration by gaps between fibers in the same principle in porous membrane.

1.1.2. Preparation of porous material by breath figure method

In this research, we are interested in grafting PNIPAM brushes on porous PS surface which is the same material as tissue culture plate while the porous surface can be prepared by breath figure (BF) method that is easy, quick and inexpensive. By evaporating highly volatile solvent in moist atmosphere, water vapor can condense into microdroplets due to evaporative cooling. These microdroplets serve as a template for the polymer solution to form ordered array of honeycomb pattern [19-20].

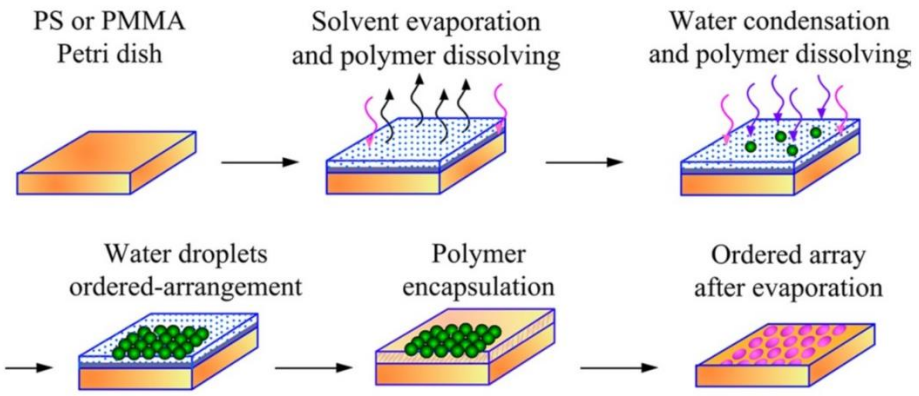


Figure 1.4 Preparation of ordered honeycomb structures on substrate by using BF method [19].

To facilitate formation of honeycomb pattern, amphiphilic block copolymer can be added to help stabilize the microdroplets and prevent them from aggregation before polymer pattern is set. Examples of porous film prepared from block copolymer of polystyrene and poly(acrylic acid) (PS-*b*-PAA) via BF method include the following:

In 2008, Wang *et al*. [21] prepared ordered microporous thin film by casting solution of PS-*b*-PAA in ratio of 2:1 in tetrahydrofuran (THF) onto glass slide under high humidity state. The relative humidity of air and concentration of block copolymer are influencing factors in controlling the pore size. Diameter of pores increases with the increase of humidity due to the high humidity allow large water droplet to form while impede the rate of solvent evaporation. As for block copolymer concentration, the number of pores and pore sizes were found to decrease when the concentration of polymer solution increased due to high viscosity of the solution slowing down the growth of the droplets and preventing them from packing tightly.

In 2013, Li *et al*. [22] fabricated highly ordered microporous films via BF method by casting the solution of PS-*b*-PAA in different solvents including carbon dioxide (CS_2) and chloroform ($CHCl_3$) on glass slide with different concentrations. The result showed that average pore diameter was 0.64-1.79 μm with the pore sizes decreasing when block copolymer concentration increased similar to the previous example. Moreover, the boiling point of solvent also affected the pore size, with solution in highly volatile CS_2 (46 °C) formed pattern with smaller pore size than the solution in $CHCl_3$ (61 °C).

This could be explained by the fact that solvent with lower volatility would take longer to evaporate and allow more time for the water droplets to form.

1.1.3. Surface grafting of polymer

Covalent attachment seems to be the most effective method for grafting PNIPAM onto polymer surface. The typical strategies of covalent attachment are "grafting to" and "grafting from" (**Figure 1.5**) [23]. In the "grafting to" method, polymer brushes are attached onto substrate by reaction of the terminal functional group of the polymer molecules and the appropriate functional groups on the substrate. Although the general procedure for the "grafting to" method is simple, the resulting polymer brushes usually have low grafting density due to steric hindrance of the polymer chains preventing additional polymer chains from grafting onto the surface. Another method, "grafting from" or surface-initiated polymerization (SIP), is performed first by immobilizing the initiator on substrate followed by growing polymer chains, which gives high grafted-polymer density [24]. To control the molecular weight of the grafted polymer, "controlled" polymerization such as atom transfer radical polymerization (ATRP) and reversible addition–fragmentation chain transfer (RAFT) polymerization can be used. In this research, the polymerization method used must be compatible with the polystyrene (PS) surface. PS has glass transition temperature (Tg) at 100 °C which can be problematic for RAFT method which requires high temperature to activate the initiator, since high temperature can cause the porous PS substrate to lose its physical integrity. As such, ATRP is chosen for this work due to its ability to activate the polymerization at room temperature.

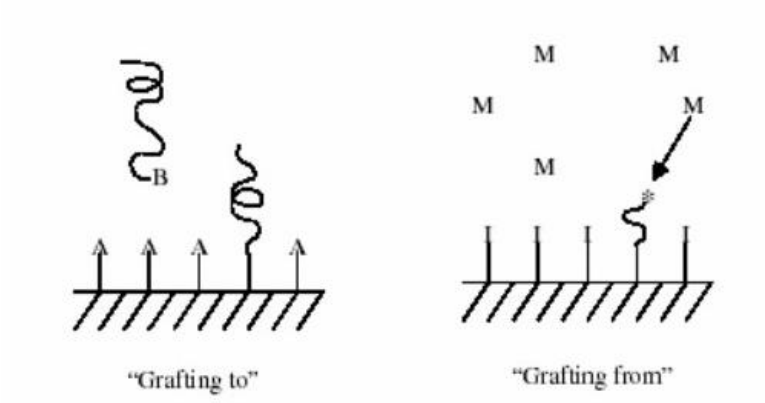


Figure 1.5 Preparation of polymer brushes via covalent attachments: "grafting to", "grafting from".

ATRP is a radical polymerization controlled by the equilibrium of redox reaction between alkyl halide and active metal complex catalyst such as copper(I) ion coordinated to nitrogen ligands. This mechanism of reaction requires an excess amount of copper catalyst to generate alkyl radicals to compensate for the loss of chain end functionality due to side reactions and keep the level of catalyst high enough to continue the reaction. The disadvantage of this reaction is that copper catalyst must be removed in order to minimize the toxicity that may affect the biocompatibility if the material is to be used for biomedical or biomaterials applications. To reduce the required concentration of catalyst, reducing agent can be added to help regenerate the catalyst in the process called "activators regenerated by electron transfer" for atom transfer radical polymerization (ARGET ATRP) as shown in **Figure 1.6** This process required only a minute quantity of catalyst concentration and can be done in limited amount of air so the reaction does not require prior de-oxygenation.

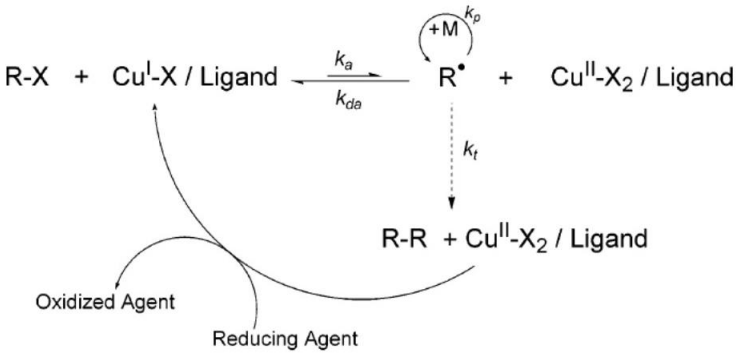


Figure 1.6 Mechanism of ARGET ATRP process [25].

In 2012, Shivapooja *et al*. [26] reported synthesis of PNIPAM brushes on silicon wafer substrate via ARGET ATRP at ambient temperature by using (3-trimethoxysilyl) propyl 2-bromo 2-methylpropionate as surface-immobilized initiator, low amount of copper(II) bromide ($CuBr_2$) as catalyst, *N*,*N*,*N*',*N*",N"-pentamethyldiethylenetriamine (PMDETA) as ligand, and two reducing agents including tin(II) ethylhexanoate and ascorbic acid. The presence of elemental composite signaling of PNIPAM in x-ray photoelectron spectroscopy (XPS) spectra was used to confirm the success of grafting of PNIPAM brushes on substrate via ARGET ATRP.

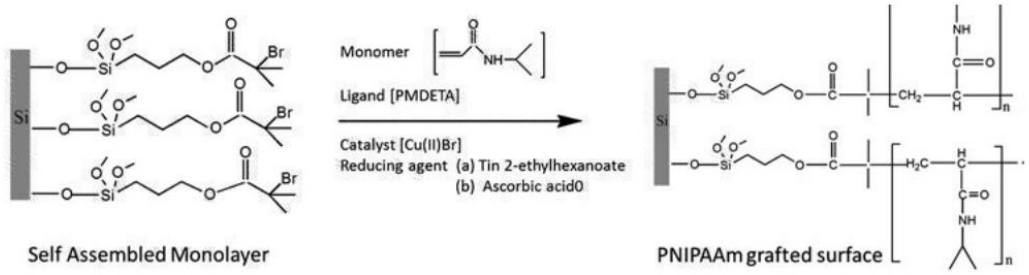


Figure 1.7 Synthesis of PNIPAM brushes on silicon wafer by surfaced-initiated ARGET ATRP.

Several attempts to immobilize the initiator on the hydrophobic PS substrate has been reported as shown below.

In 2008, Mizutani *et al*. [16] coated poly(4-vinylbenzyl chloride) layer as surface initiator on polystyrene surface, followed by polymerization of PNIPAM brushes via ATRP.

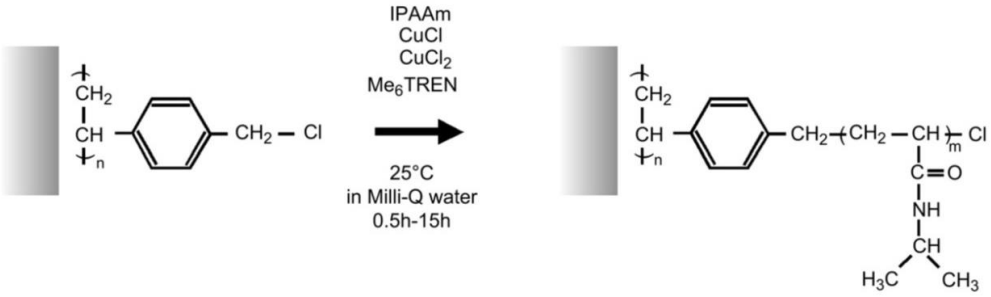


Figure 1.8 Preparation of PNIPAM brushes on surface via ATRP by using poly(4-vinylbenzyl chloride) as initiator layer.

The disadvantage of this method is that poly(4-vinylbenzyl chloride) must be synthesized in addition to other steps which is time consuming.

In 2008, Mizutani *et al*. [27] reported synthesis of PS via ATRP by using 11-(2-bromoisobutyrate)-undecyl-1-phosphonic acid as initiator immobilized on titanium. ATR-FTIR spectra exhibited absorption band corresponding to functionality of PS that confirmed the success of PS grafted on surface from the initiator.

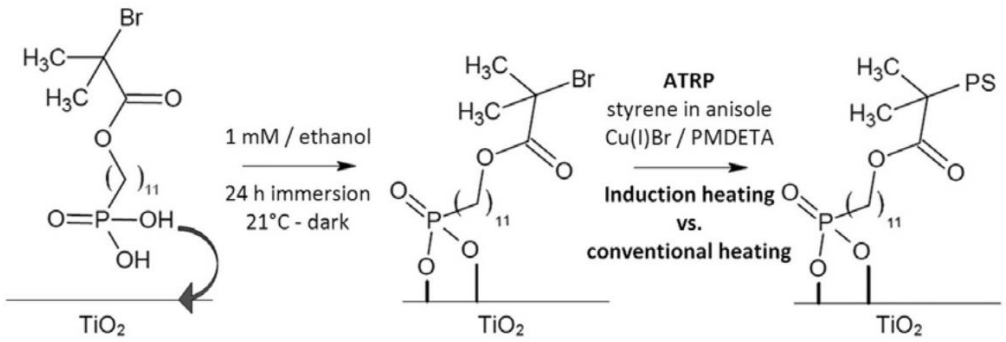


Figure 1.9 Synthesis of PS on titanium modified with 11-(2-bromoisobutyrate)-undecyl-1-phosphonic acid as ATRP initiator.

From the previous work ,one of the interesting method to immobilize initiator on surface is coating substrate with modified polydopamine as initiator layer for surface-initiated polymerization (SIP), followed by formation of PNIPAM brushes from surface-initiator via ARGET ATRP.

Polydopamine has been known as universal coating material that can be deposited on a wide variety of substrates including hydrophobic substrate such as glass, Aluminium (Al), foil, steel and polystyrene as shown in **Figure 1.10** [28]. The polymerization of dopamine is a simple and rapid reaction that can be done at mild pH in aqueous solution in one-step. Coated material is shown to have biocompatibility and can be used in biomedical applications.

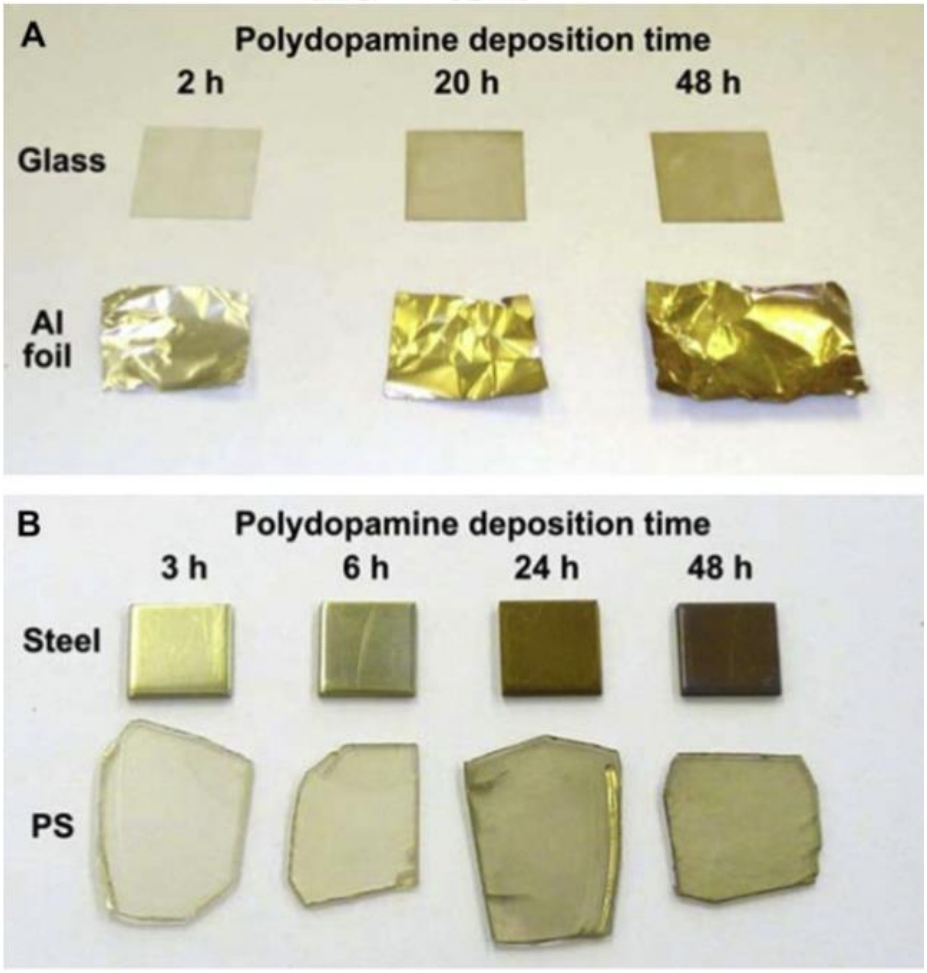


Figure 1.10 Photographs of polydopamine coated on various substrates with different deposition time exhibiting increasing of brown color of polydopamine layer which can be increased as a function of deposition time.

In recent years, there have been reports on modified polydopamine as initiator for surface-initiated ATRP.

In 2011, Zhu *et al*. [28] synthesized modified polydopamine as initiator for surface-initiated polymerization through the reaction of dopamine monomer with 2-bromoisobutyryl bromide (BiBBr) which was then deposited on silicon wafers, PS chips, aluminium foil and stainless steel chips. After that, poly(methyl methacrylate) (PMMA) brushes were polymerized onto the surface by ARGET ATRP. The presence of PMMA signal on ATR-IR spectra indicated the success of PMMA formation on surface and confirmed that modified polydopamine could be used as surface-initiator and could be coated on a variety of substrates.

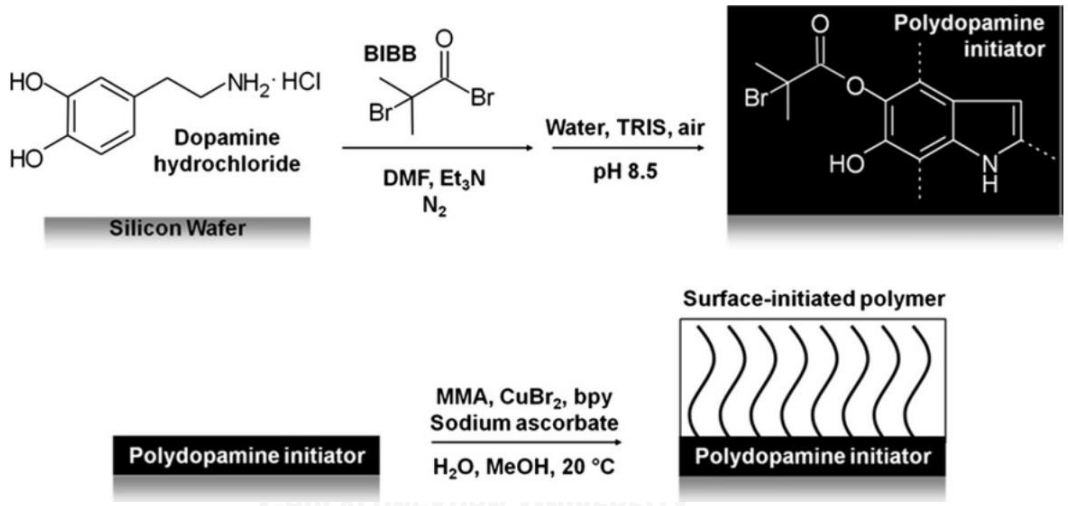


Figure 1.11 Fabrication of modified polydopamine layer for surface-initiated ARGET ATRP of PMMA brushes.

In 2015, Ginic-Markovic *et al*. [29] reported the synthesis of bromomacroinitiator from the reaction of dopamine hydrochloride and BiBBr to coat on polyamide layer followed by polymerization of sulfobetaine monomer for anti-fouling application. This material showed significant reduction of protein and bacterial attachment as compared with unmodified substrate. This result demonstrated the success of synthesized sulfobetaine polymer via ARGET ATRP from surface modified with BiBBr-modified polydopamine.

Herein this research, we are interested to synthesize PNIPAM brushes on porous styrene surface that was prepared by casting the mixed solution of PS and block copolymer of PS-*b*-PAA via breath figure method. Then, this surface was coated with modified polydopamine bearing bromoester group (p-DA-BiBBr) for surface-initiated polymerization of NIPAM via ARGET ATRP. It is anticipated this material can be used as substrate for cell adhesion and detachment. And the porous structure should promote easy cell detachment.

1.2. Objectives

1.2.1. To prepare and characterize porous polystyrene surface.

1.2.2. To graft PNIPAM brushes onto porous polystyrene surface and investigate the material as substrate for cell adhesion and cell detachment.

1.3. Scopes of investigation

The stepwise investigation was carried out as follows:

1.3.1. To study the related literature reviews.

1.3.2. To prepare and characterize porous polystyrene film by breath figure method.

1.3.3. To coat modified polydopamine as surface-initiator on the porous polystyrene film.

1.3.4. To synthesize and characterize the PNIPAM brushes grafted on the porous PS film by surface-initiated polymerization via ARGET ATRP.

1.3.5. To evaluate PNIPAM brushes grafted-porous PS film as a substrate for cell adhesion and detachment.

CHAPTER II
MATERIALS AND METHODS

2.1.Materials

Ethanol was purchased from RCl Labscan. Styrene, copper(II) bromide ($CuBr_2$) and ethyl 2-bromoisobutyrate (EBiB) were purchased from Fluka. Triethylamine (TEA) was purchased from Merck. Dopamine hydrochloride and tin(II) 2-ethylhexanoate ($Sn(EH)_2$) were purchased from Sigma. 2-Bromoisobutyryl bromide (BiBBr) was purchased from Aldrich. Anhydrous tetrahydrofuran (THF), anhydrous *N,N'*dimethylformamide (DMF), carbon disulfide (CS_2), 4,4'-azobis(4-cyanovaleric acid) (ACVA), *N,N,N',N",N'"*-pentamethyldiethylenetriamine (PMDETA), 4-cyanopentanoic acid dithiobenzoate (CPADB), tris(hydroxymethyl)aminomethane (Tris), styrene, acrylic acid (AA) and *N*-isopropylamide (NIPAM) were purchased from Sigma-Aldrich. All monomers were purified before used. Styrene was purified by passing through basic alumina column. AA was purified by vacuum distillation. NIPAM monomer was re-crystallized from hexane and then dried under vacuum. Deionized (DI) water was purified by Millipore Milli-Q system that involves osmosis, ion exchange and a filtration steps.

2.2.Equipments

2.2.1. Scanning electron microscopy (SEM)

SEM images were recorded by using a JEOL Model JSM-6400 scanning electron microscope (USA) and analyzed with an accelerating voltage of 15 kV. Pore sizes of PS porous substrate were measured and averaged by using a SemAfore 5.21.

2.2.2. Water Contact Angle measurements

Water contact angle values of the modified substrates were measured by using a contact angle goniometer (Ramé-Hart, Inc., USA, model 100-00), equipped with a

Gilmont syringe and a 24-gauge flat-tipped needle. Dynamic advancing and receding angles were recorded while water was added on and withdrawn from the drop, respectively. The reported angle is an average of 5 measurements on different area of each sample.

2.2.3. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of modified substrates were recorded with a FT-IR spectrometer (Nicolet), model system 6700 with using DLa TGS detector. The spectra were collected at 64 scans at a resolution of 4 cm^{-1} by using diamond ATR IR accessory.

2.2.4. X-Ray photoelectron spectroscopy (XPS)

Elemental compositions of the modified substrates were characterized by x-ray photoelectron spectroscopy (XPS) on a Scienta ESCA 200 spectrometer (Uppsala, Sweden) with Al Kα x-rays. All the XPS data were collected at a takeoff angle of 90°.

2.2.5. Ellipsometry

Thickness of the grafted polymer layer on silicon wafer was measured by using a J.A. Woollam M-2000X-KMy (USA) spectroscopic ellipsometer.

2.2.6. Nuclear magnetic resonance spectroscopy (NMR)

^1H NMR spectra were recorded in DMSO-d_6 using Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz with 32 scans.

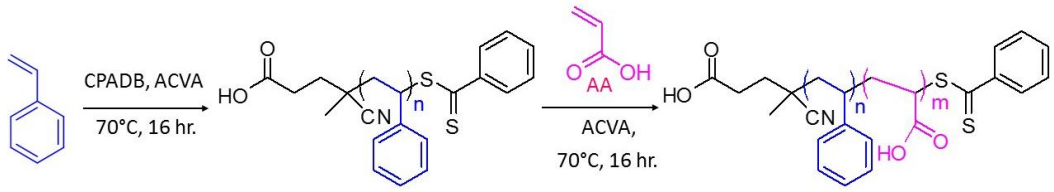
2.2.7. Gel permeation chromatography (GPC)

Molecular weight of polymer was evaluated by GPC using Waters 600 controller chromatograph equipped with HR1 and HR4 columns (Waters, USA) at 35 °C and

refractive index (RI) detector (Waters, USA, model 2414). THF was used as eluting with a flow rate of 1.0 mL/min. A calibration curve was obtained using polystyrene standards.

2.3.Experimental procedures

2.3.1. Synthesis of block copolymer of polystyrene and poly(acrylic acid) (PS-*b*-PAA)



PS-*b*-PAA was synthesized via two-steps with a sequential reversible addition-fragmentation chain transfer (RAFT) polymerization of styrene and AA. Firstly, PS-macro initiator was synthesized by mixing of styrene (10 mL, 6.5×10^{-2} mol), CPADB (0.084 g, 3.0×10^{-4} mol) and ACVA (0.021 g, 7.5×10^{-5}mol). The mix solution was purged with nitrogen gas for 15 min and then stirred at 70 ℃ for 18 h. The crude solution was cooled down and then dissolved in THF. The polymer solution was precipitated in ethanol and dried under vacuum at ambient temperature. Secondly, diblock copolymer was performed via RAFT polymerization of AA from PS-macroinitiator. PS (2 g, 1.9×10^{-2}mol) and ACVA (0.108 g, 3.8×10^{-4} mol) were dissolved in anhydrous DMF (5 mL) followed by an addition of AA (1.96 mL, 2.8×10^{-2} mol). The solution was purged with nitrogen gas for 15 min. then stirred at 70 ℃ for 14 h. The polymer solution was dialyzed in DMF for 1 day and DI water for 3 days, respectively. And then, PS-*b*-PAA was obtained as white foam-like after freeze drying.

2.3.2. Preparation of porous polystyrene substrate by breath figure method

The mixed solution of 2.25 g PS (MW = $3x10^6$ Da) and 250 mg block copolymer (PS-*b*-PAA) was dissolved in 25 mL CS_2. Mixed polymer solution with concentration of 10% w/v was cast onto glass petri dish (9 cm in diameter) in a closed plastic box under 90% relative humidity generated by passing air through water at 26 °C. After 1 h, the porous film was successfully formed by condensation of water droplets while CS_2 was evaporated. Porous polystyrene film was dried at room temperature and cut to small circles (1.5 cm in diameter) for further polymer grafting.

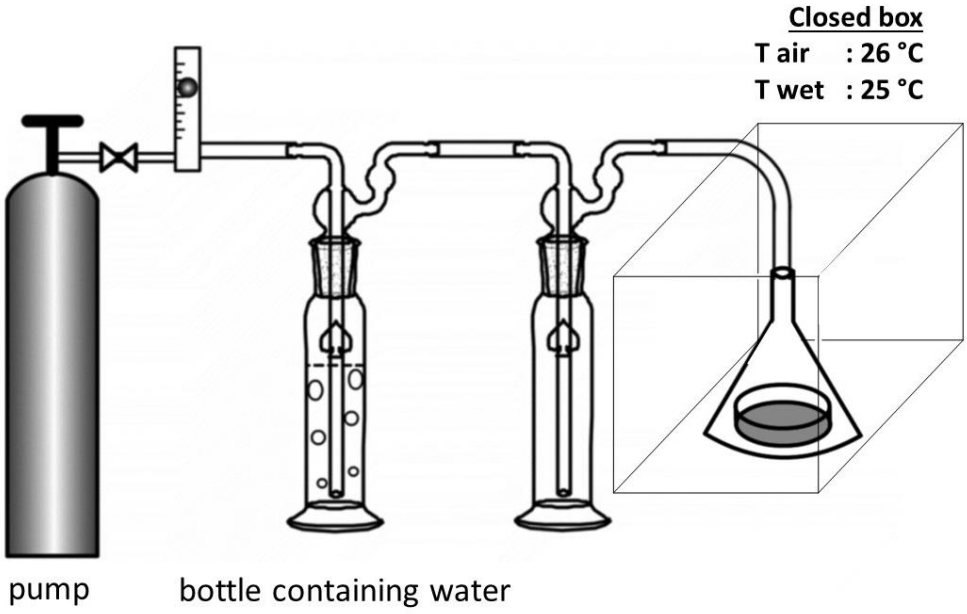


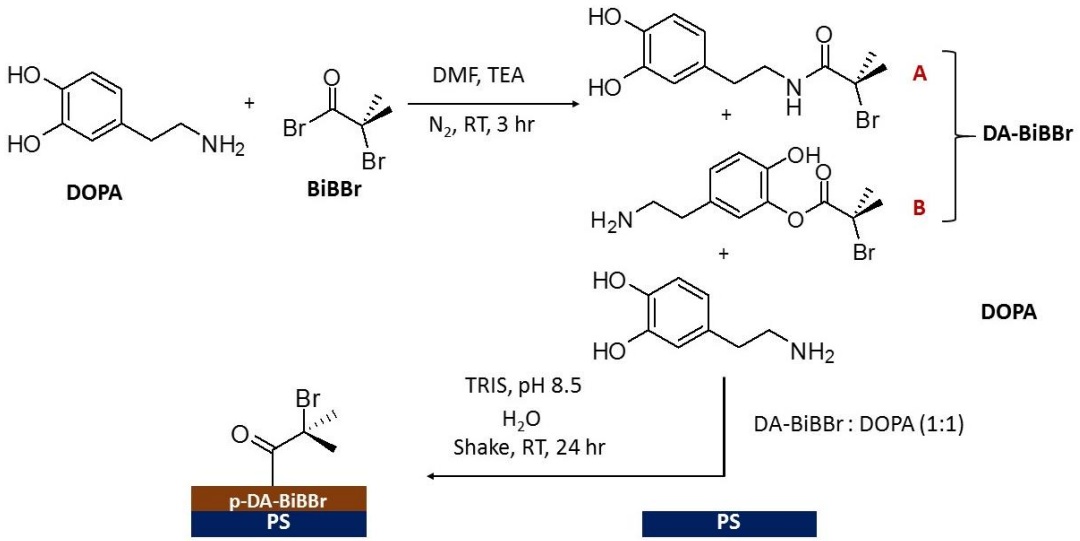
Figure 2.1 Setup for porous film casting using method modified from ref [20]

2.3.3. Coating of modified polydopamine (p-DA-BiBBr) on porous polystyrene substrate

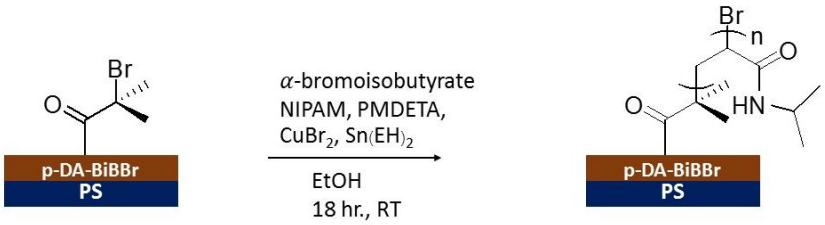
Synthesis of modified dopamine (DA-BiBBr) was done following the procedure described by Gicnic-Markovic *et al*. [29]. Dopamine hydrochloride (DOPA) (120 mg, $6.3x10^{-4}$mol) was purged with nitrogen gas for 5 min, followed by an addition of DMF (6 mL), TEA (44 µL, $3.2x10^{-4}$ mol) and BiBBr (39 µL, $3.2x10^{-4}$ mol) under nitrogen atmosphere. The solution was stirred at room temperature for 3 h. The crude product

which should contain 1:1 moile ratio of DOPA and DA-BiBBr was used in the next step without purification.

Porous PS films obtained from 2.3.2 were rinsed with DI water and ethanol then dried with nitrogen gas, followed by air plasma cleaner (Harrick, USA, model PDC-32G-2) with power of 18 W for 5 min. After the plasma treatment, the porous PS films were placed into the bottom of 24-well plates. 1 Millilitre of solution of the crude product having 1:1 moile ratio of DOPA and DA-BiBBr in TRIS buffer solution (40 mM, 40 mL, pH 8.5) was pipetted in each well. And then, the well plate was put on a shaker and shaked at speed 220 rpm at ambient temperature in open air for 24 h. After that, the porous PS films were removed from the wells and rinsed twice with DI water followed by drying under vacuum. The brown color of p-DA-BiBBr coating can be clearly observed on the porous PS films. The p-DA-BiBBr formed in solution was then freeze-dried and characterized by ATR-FTIR.



2.3.4. Surface grafting of PNIPAM brushes on porous polystyrene substrate by surface-initiated polymerization via ARGET ATRP



NIPAM monomer (2.829 g, 2.5×10^{-2} mol) was dissolved in ethanol in a glass vial having a magnetic stirred bar followed by an addition of the mixed solution of $CuBr_2$ (0.14 mg, 6.3×10^{-4} mol) and PMDETA (3 µL, 1.4×10^{-5} mol) in 1 mL ethanol. The mixture was stirred at ambient temperature for approximately 10 min until it became homogeneous. EBiB (9.17 µL, 6.3×10^{-5} mol) as a sacrificial initiator was added to the vial. The porous PS films coated with p-DA-BiBBr obtained from 2.3.3 were fixed in a slotted hollow glass cylinder and put into the glass vial containing the mixed solution which was then sealed with rubber septum. A mixed solution of $Sn(EH)_2$ (123.46 µL, 3.8×10^{-4} mol) as reducing agent, PMDETA (5 µL, 2.4×10^{-5} mol) as ligand and ethanol (1 mL) prepared in a separated vial was added via syringe to the reaction vial containing the PS porous films. The volume of air above the solution in the vial was fixed at 17 mL. The polymerization was done at ambient temperature for 18 h. After that, the reaction was stopped by opening the vial to air. The porous PS films were removed then rinsed thoroughly with ethanol and dried under vacuum. Free PNIPAM formed in solution was purified by dialysis in ethanol for 1 day and DI water for 3 days at 4°C and then dried by freeze drying.

2.3.5. Determination of cell adhesion and detachment by MTT assay

Keratinocyte cells were cultured in Dulbecco's Modified Eagle Medium supplemented with Nutrient Mixture F-12 (DMEM/F12), 10% fetal bovine serum (FBS), 1% L-glutamine, 1% Peniciline-streptomycin, hydrocortisone 100 ng/mL, $NaHCO_3$ 25 ng/mL, Insulin 5 µg/mL, Epidermal growth factor (EGF) 20 ng/mL on tissue culture dishes which contain 3T3 fibroblast cells as progenitor cell on substrate. After 10 days,

keratinocyte cells were digested with Trypsin enzyme and seeded at $1x10^5$ cells/well onto sterilized samples placed at the bottom of 24 well-plates. And 500 µL of Dulbecco's Modified Eagle medium was then pipetted into each well, followed by incubation for 24 h at 37 ºC. After that, medium was removed and a 500 µL solution of 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in cell medium was then pipetted into each well. After incubation for 3 h, 500 µL of isopropanol was pipetted into each well for overnight at room temperature. Optical density of the obtained solution in each wells were measured by using microplate reader at wavelength at 570 nm. After that, cell detachment was investigated by decreasing temperature from 37 to 25ºC for 1 h and 2 h. and optical density was measured at wavelength at 570 nm.

CHAPTER III
RESULTS AND DISCUSSION

In this chapter, the results are divided into four sections. In the first section, synthesis of block copolymer (PS-*b*-PAA), fabrication and characterization of porous PS film are explained. The second section is dedicated to the synthesis and characterization of modified polydopamine (p-DOPA-BiBBr) as well as its coating on porous PS films. The third section shows the preparation and characterization of surface-grafted PNIPAM brushes prepared by surface-initiated polymerization via activators regenerated by electron transfer for atom transfer radical polymerization (ARGET ATRP). The last section focuses on the evaluation of cell adhesion and detachment on the PNIPAM-grafted PS substrates.

3.1.Preparation and characterization of porous polystyrene substrates

Block copolymer of polystyrene and poly(acrylic acid) (PS-*b*-PAA) was synthesized via sequential reversible addition fragmentation chain transfer (RAFT) polymerization of styrene and AA. AS evaluated by GPC, the first block of PS which acts as the macroinitiator exhibited Mn of 13548 Daltons with PDI of 1.30. After copolymerization, the Mn of PS-*b*-PAA appeared to be 14473 Daltons with PDI of 1.43. The additional 925 Daltons from the second block of PAA led to the estimation of copolymer composition of 130 to 12. The synthesized block copolymer can be designated as PS_{130}-*b*-PAA_{12}.

Porous PS films were then prepared via breath figure method by casting 10%w/v solution in CS_2 of PS or PS (Mw= $3x10^6$ Da) mixed with PS_{130}-*b*-PAA_{12} (9:1 w/w) on glass substrates. After CS_2 and water were evaporated, porous PS films were formed in both cases as verified by SEM analysis. SEM images shown in **Figure 3.1** suggested that porous PS film fabricated from the pure PS solution exhibited pores with much larger diameter (45.01±5.52 μm) than that fabricated from the mixed solution between

PS and PS-*b*-PAA (7.67±0.69 μm). The film obtained from the mixed solution exhibited well-ordered honeycomb pattern. In principle, the pore size of the film should correspond with the diameter of water droplets condensed from the moisture in air during breath figure process. It seems that small quantity of PAA in the PS_{130}-*b*-PAA_{12} introduced into the mixed polymer solution can promote multiple nucleation of small water droplets on the film so that a large number of small pores can be simultaneously created. The substrate with this pore dimension is appropriate for further grafting with PNIPAM brushes to be used for cell sheet preparation because it is smaller than the size of keratinocyte cells (10 μm) [30].

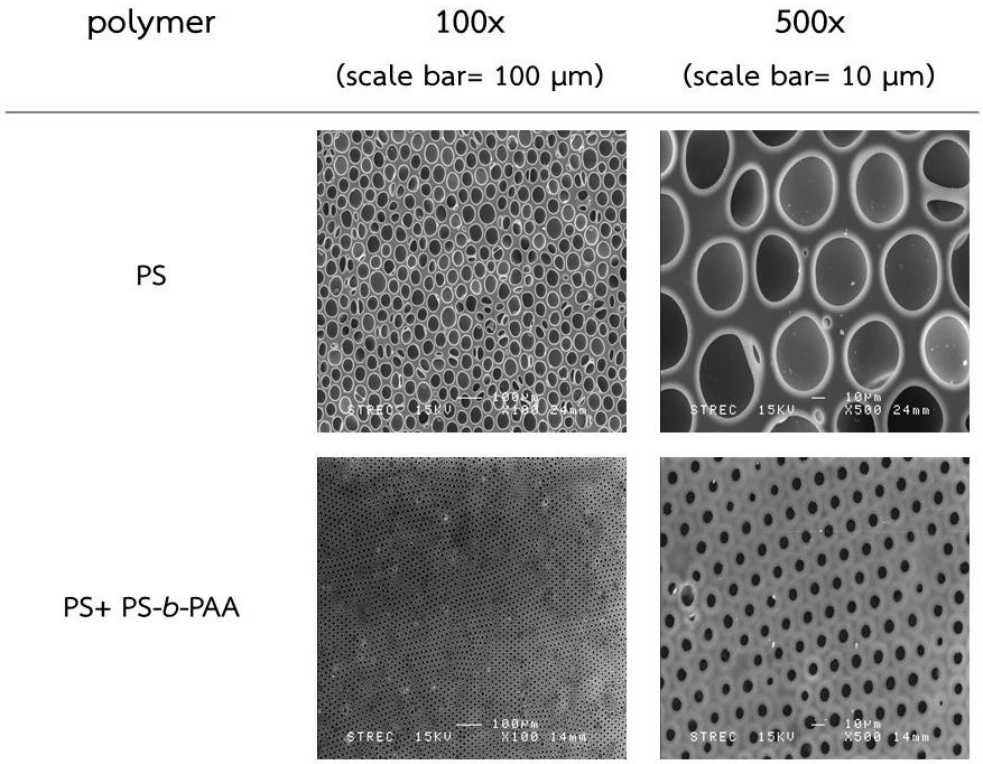
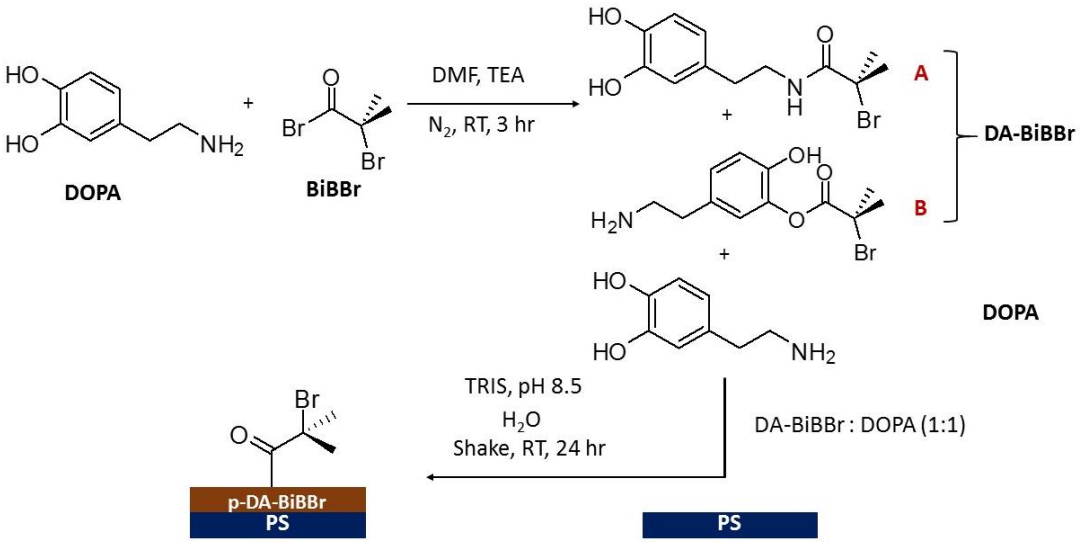


Figure 3.1 SEM images of porous PS films fabricated by breath figure method from a solution of PS and a mixed solution of PS and PS_{130}-*b*-PAA_{12}.

3.2. Preparation and Characterization of porous polystyrene substrates coated with p-DOPA-BiBBr

Dopamine modified with bromoester group (DA-BiBBr) that can later function as an initiator for growing polymer brushes was synthesized by a substitution reaction between dopamine (DOPA) and 2-bromoisobutyryl bromide (BiBBr) that yielded two products (A and B). It should be emphasized that only 0.5 mole equivalent of BiBBr to DOPA was used for the modification. For this reason, the product obtained should contain DA-BiBBr (combined product A and B) along with the unreacted DOPA in a mole ratio of 1:1. This crude product was directly used for surface coating of the modified polydopamine (p-DA-BiBBr) on porous PS film without purification.



Scheme 3.1 Synthesis of modified dopamine (DA-BiBBr) and subsequent coating of the modified polydopamine (p-DA-BiBBr) on porous PS film.

The p-DA-BiBBr formed in solution was characterized by ATR-FTIR technique. The spectrum of modified polydopamine (p-DA-BiBBr) shown in **Figure 3.2** exhibited a strong intensity at 1650 cm^{-1} and a shoulder at 1705 cm^{-1} assignable to amide C=O and ester C=O stretching, respectively, suggesting that both product A and B (**Scheme 3.1**) were simultaneously formed. The fact that the signal intensity of amide C=O stretching is much higher than that of the ester C=O stretching implied that the majority of the product is A.

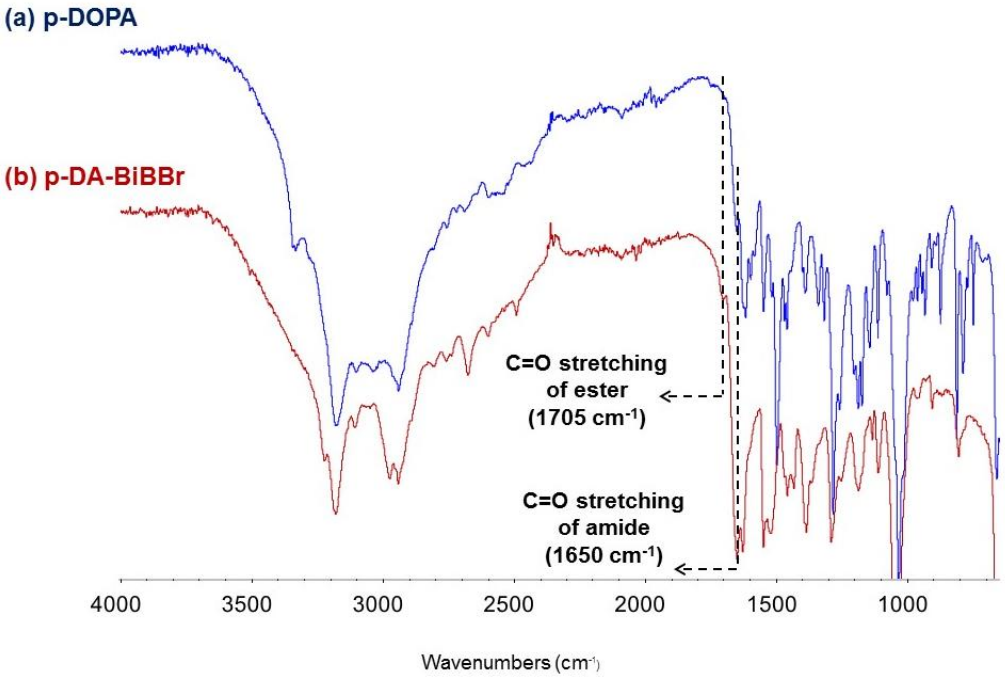


Figure 3.2 ATR-IR spectra of polydopamine (p-DOPA) and modified polydopamine (p-DA-BIBBr).

Morphology of the porous PS film both before and after coating with p-DOPA-BiBBr was characterized by SEM as shown in **Figure 3.3**. The SEM micrographs obtained from 500x magnification suggested that porosity of the PS film was not affected by the coating of p-DA-BiBBr initiator layer. By increasing the magnification to 10000x, small white aggregates with size of 0.13±0.01 μm scattered all over the surface were observed which can be used as an evidence of p-DA-BiBBr coating.

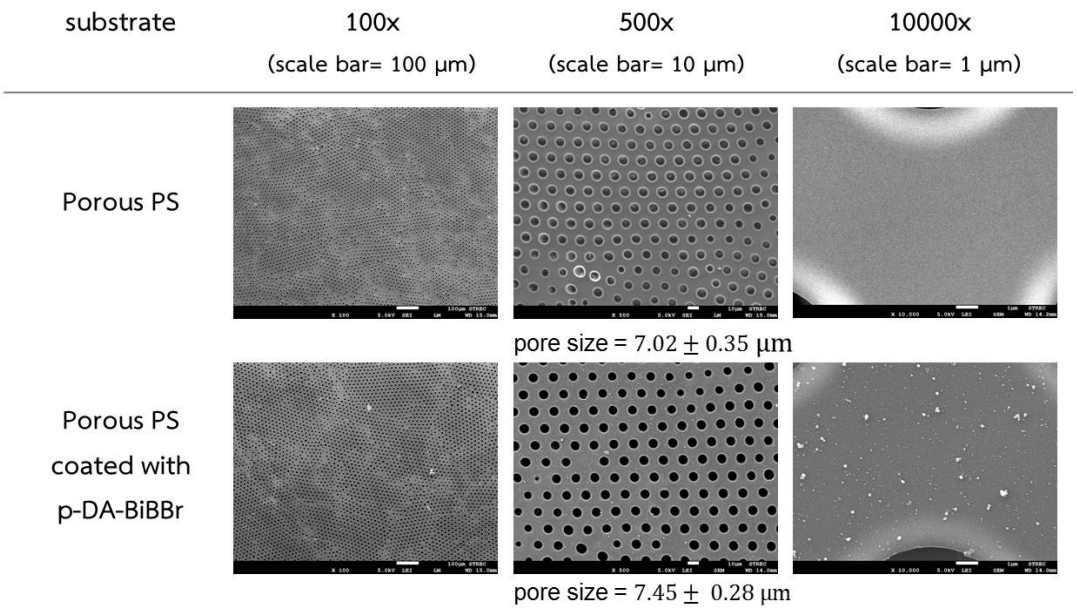
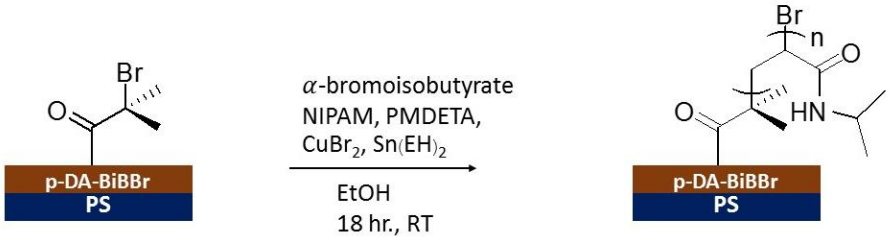


Figure 3.3 SEM images of porous PS film before and after coating with p-DA-BiBBr.

3.3. Grafting and Characterization of PNIPAM brushes on porous polystyrene substrates

The PNIPAM brushes were grown from the p-DA-BiBBr layer coated on porous PS film by surface initiated polymerization via ARGET-ATRP mechanism as shown in **Scheme 3.2**.



Scheme 3.2 Formation of PNIPAM brushes from the p-DA-BiBBr coated on porous PS film via surface-initiated ARGET-ATRP.

Morphology of the porous PS film after surface grafting with PNIPAM brushes via surface-initiated ARGET ATRP is shown in **Figure 3.4**. At 10000x magnification, larger white aggregates with size of 0.31±0.36 μm, as compared with those of the porous PS

film coated with p-DA-BiBBr appeared all over the surface suggesting that there was larger macromolecules of PNIPAM brushes deposited on the p-DOPA-BiBBr layer.

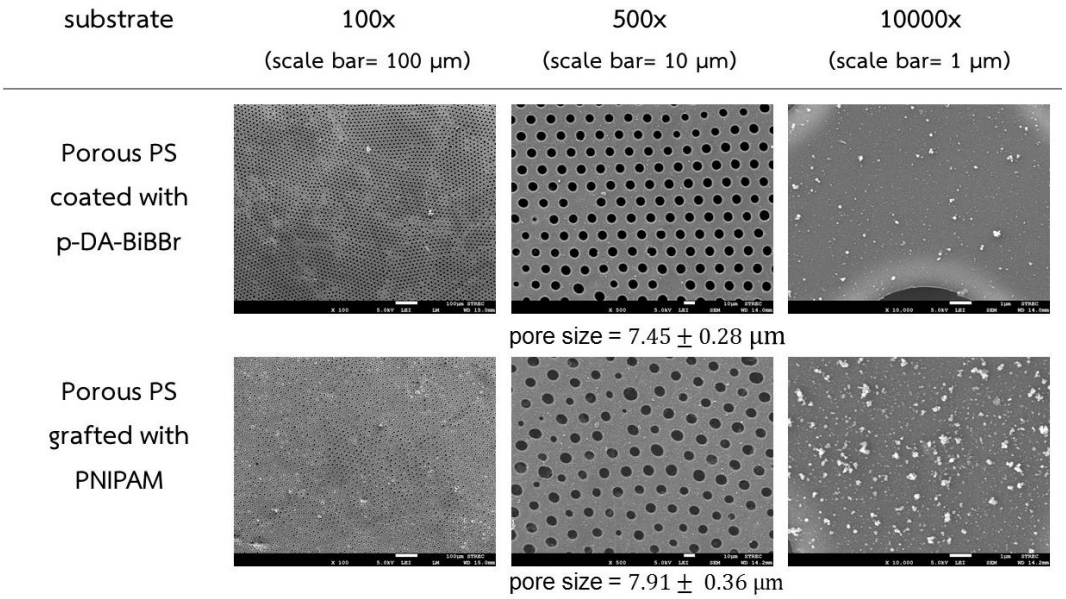


Figure 3.4 SEM images of porous PS film before and after grafting with PNIPAM brushes.

The dynamic water contact angles of the porous PS substrates after stepwise modification are shown in **Table 3.1**. Porous PS film possessed high advancing contact angle (θ_A) indicating hydrophobic polystyrene (PS) is dominated at solid/air interface but much lower receding contact angle (θ_R) was detected implying that the hydrophilic carboxyl groups from the incorporated PS-*b*-PAA can rearrange and became dominated at the solid/liquid interface once the surface was hydrated during receding contact angle measurement. After coating with the p-DA-BiBBr layer, the surface became less hydrophobic with lower advancing contact angle and unmeasurable receding angle due to the presence of hydroxyl, amine and amide group from p-DA-BiBBr. After grafting with PNIPAM brushes, the surface turned to be quite hydrophilic at ambient temperature (25 °C). Once the temperature was raised to 38°C, above LCST of PNIPAM (32°C), the surface became more hydrophobic, verifying thermoresponsive property of the surface-grafted PNIPAM. The extremely high contact angle hysteresis of all

substrates (θ_A-θ_R) may be explained as a result of water droplet pinning on the microporous surfaces.

Table 3.1 Water contact angle data of modified porous PS substrates.

Substrate	Water Contact Angle (°)	
	Advancing (θ_A)	Receding (θ_R)
Porous PS	110.7 ± 3.6	64.2 ± 2.4
Porous PS coated with p-DA-BiBBr	76.8 ± 1.6	N/A
Porous PS grafted with PNIPAM brushes	24.7 ± 1.7 (25 °C)	N/A
	62.6 ± 1.2 (38 °C)	N/A

N/A = unmeasurable

The emergence of amide C=O stretching peak at 1655 cm^{-1} in the ATR-FTIR spectra of porous PS films after p-DA-BiBBr coating and PNIPAM grafting (**Figure 3.5**) implied that the surface of porous PS films were successfully coated with the initiator layer of p-DA-BiBBr and grafted with PNIPAM brushes.

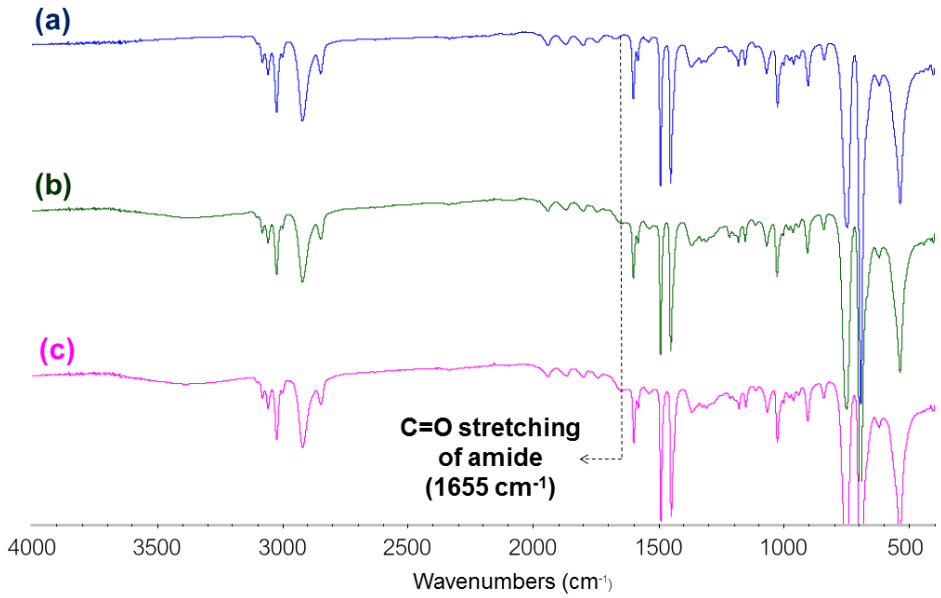


Figure 3.5 ATR-FTIR spectra of porous PS film (a) before and after (b) p-DA-BiBBr coating and (c) PNIPAM grafting.

In order to confirm the existence of PNIPAM brushes on the surface, the porous PS substrates were also analyzed by x-ray photoelectron spectroscopy (XPS), the results of which are shown in **Table 3.2**. The porous PS substrate showed the signal of both carbon and oxygen atoms. The surface coated with p-DA-BiBBr exhibited composition of nitrogen and oxygen indicating the success of p-DA-BiBBr coating. Further increase of nitrogen and oxygen composition after grafting PNIPAM brushes confirmed the presence of PNIPAM chains on the PS substrates.

Table 3.2 Atomic composition of porous PS substrates before and after surface modification as analyzed by XPS.

Substrate	Atomic Composition (%)			
	C	O	N	Br
Porous PS	75.49	14.00	0.17	0.10
Porous PS coated with p-DOPA-BiBBr	74.30	20.81	4.31	0.07
Porous PS grafted with PNIPAM brushes	66.29	24.29	4.48	0.12

Considering XPS narrow scan analysis of C1S peak of modified PS substrates in **Figure 3.6**, major contribution of all substrates comes from C-H peak with binding energy of 285 eV. The porous PS exhibited peak at 287 eV which corresponded to carbon linked to two oxygen atoms of O=C-OH in PAA of the block copolymer. The surface coated with p-DOPA-BiBBr exhibited peak at 287.5 eV with higher intensity implying there were greater number of carbon atoms connected to 1-2 heteroatoms having higher electronegativity which should be in the form of O-C=O, N-C=O and C-Br in this case. After growing PNIPAM brushes from the initiator layer, the peak at binding energy of 288 eV which should mainly be originated from N-C=O became even more intense confirming that there was PNIPAM brushes grafted on the surface.

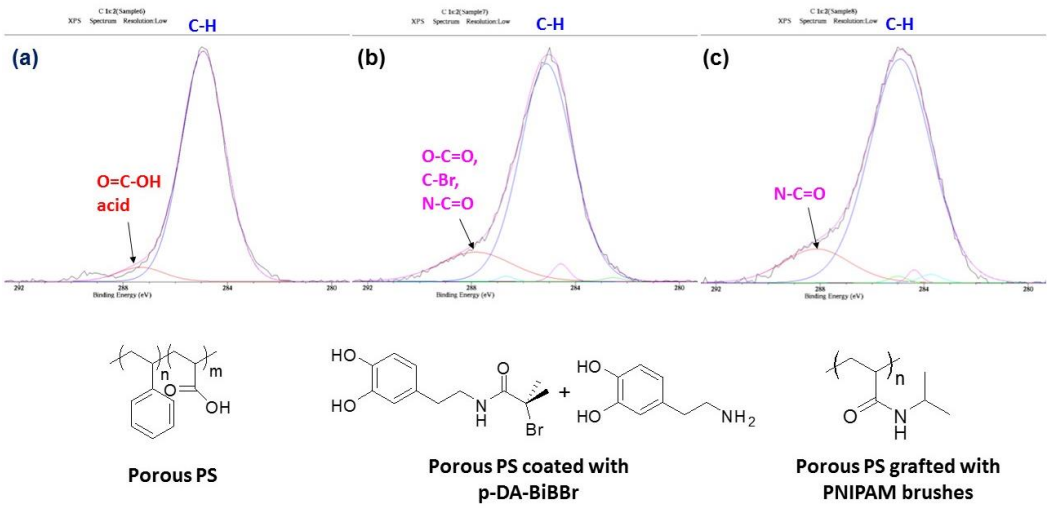


Figure 3.6 XPS narrow scan of C1S spectra of porous PS film (a) before and after (b) p-DA-BiBBr coating and (c) PNIPAM grafting.

From previous researches, reported the thickness of PNIPAM brushes grafted on surface influence thermoresponsiveness of PNIPAM for using as substrate in cell sheet preparation. Previously, it was found that the thickness of PNIPAM grafted layer in a range of 15-50 nm can effectively promoted cell attachment and detachment [14-16]. To make sure that PNIPAM grafted layer would be suitable for cell sheet culture, the thickness of p-DA-BiBBr layer as surface initiator and grafted-PNIPAM layer was determined by ellipsometry. Due to the roughness of grafted porous PS surface and similar refractive index of PS and PNIPAM as both were organic layers, ellipsometry could not be used to directly measure the thickness of PNIPAM layer on porous PS film. To solve this problem, PNIPAM layer was grafted onto a silicon wafer substrate which is extremely smooth. The data showed the thickness of p-DA-BiBBr alone was 61.76±1.21 nm and the thickness p-DA-BiBBr layer combined with grafted PNIPAM brushes was 79.99±1.43 nm. The calculated thickness of grafted-PNIPAM can be estimated to be18.23 nm. This thickness value of PNIPAM layer falls in the suitable range to be used as substrate for cell sheet preparation.

Free PNIPAM formed by the free initiator (“sacrificial” initiator) in solution was also characterized for its chemical structure by ^{1}H NMR of which spectrum is displayed

in **Figure 3.7**. The peaks appearing at 7.0-7.5(*b*), 3.8(*s*), 2.0(*b*), 1.2-1.6(*b*) and 1.0(*s*) ppm can be assigned to –N<u>H</u>-, (CH_3)C<u>H</u>N-, -$C\underline{H}_2$-C<u>H</u>- of polymer backbone and –$C\underline{H}_3$, respectively. This information confirmed that PNIPAM was successfully synthesized.

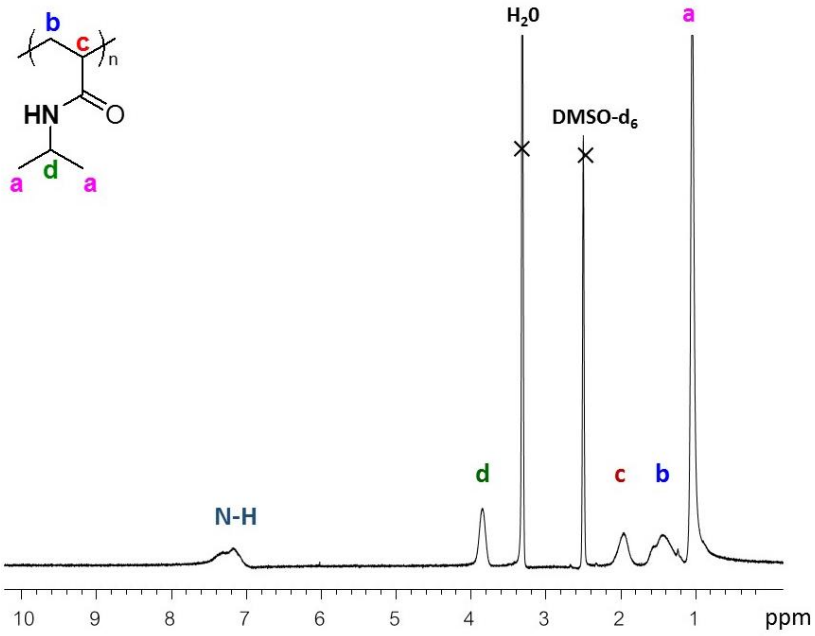


Figure 3.7 ^{1}H NMR spectrum of PNIPAM in DMSO-d6.

GPC analysis of free PNIPAM formed in solution had Mn of 32817 Da and PDI of 1.99 which is lower than the target molecular weight (Mn= 45264 Da). Degree of polymerization (DP) was calculated to be 290. The synthesized PNIPAM had high molecular weight distribution (PDI of 1.99) suggesting that ARGET ATRP is not well-controlled. This is not un-expected given that the target molecular weight is relatively high.

3.4.Cell attachment and detachment studies by MTT assay

In cell culture study, cell attachment and detachment was preliminarily tested to observe thermoreponsiveness of the surface-grafted PNIPAM brushes before being used for cell sheet preparation.

MTT assay was employed for determination of %cell adhesion after incubation at 37 ℃. The temperature was then decreased to 20 ℃ for 1 h and 2 h to observe cell detachment.

In this research, optical density (OD) of the solution of product from MTT assay was measured by using microplate reader at wavelength at 570 nm. Percentage of cell adhesion was calculated from the ratio between OD of the sample and OD of the PS substrate which was used as a control at the same temperature (equation 3.1)

$$\% \text{ cell adhesion} = \frac{OD_{sample}}{OD_{PS}} \text{ X } 100 \qquad (3.1)$$

% Cell adhesion of PNIPAM-grafted porous PS film and PNIPAM-grafted PS film is displayed in **Figure 3.8**. After incubated at 37 ℃ for 24 h, PNIPAM-grafted PS film seems to be a better substrate in supporting cell adhesion than the PS film which was used as a control (%cell adhesion > 100%) . In contrast, PNIPAM-grafted porous PS film exhibited lower percentage of cell attachment (80%).

For cell detachment study, the temperature was decreased from 37 ℃ to 20 ℃ for 1 and 2 hr, PNIPAM-grafted porous PS film and PNIPAM-grafted PS showed decreasing % cell adhesion remained on the surface implying there was cell detachment as a result of thermoresponsive property of the surface-grafted PNIPAM layer. Nevertheless, the magnitude of cell detachment was still small and the impact of porosity cannot be realized.

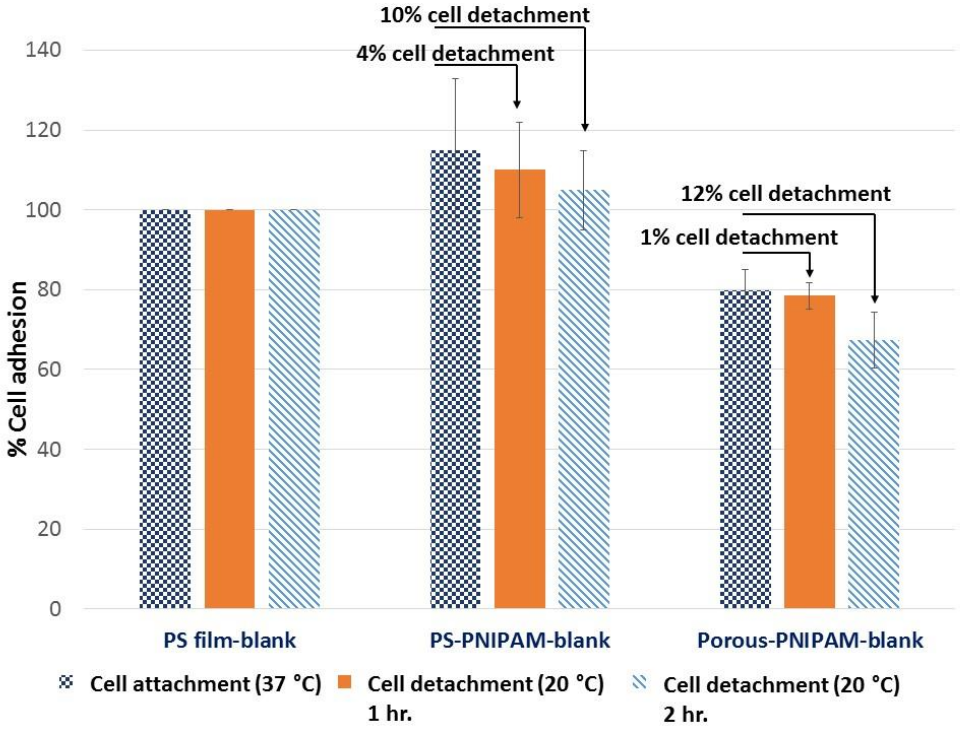


Figure 3.8 % Cell adhesion on PS films with and without surface-grafted PNIPAM and porous PS film grafted with PNIPAM at different temperature.

CHAPTER IV
CONCLUSION AND SUGGESTIONS

In this research, the porous polystyrene film was prepared by casting 10%w/v solution in CS_2 of PS (Mw= $3x10^5$ Da) mixed with PS_{130}-*b*-PAA_{12} (9:1 w/w) on glass substrates. As characterized by SEM analysis, the film exhibited well-ordered honeycomb pattern with pore diameter of 7.67±0.69 μm. The porous PS film was then coated with polydopamine modified with bromoester group (p-DA-BiBBr) which would function as surface-initiator for polymerization of NIPAM via ARGET ATRP. The success of p-DA-BiBBr coating and PNIPAM grafting on porous PS film was confirmed by ATR-FTIR and XPS analysis. Thermoresponsive property of the grafted PNIPAM layer was verified by water contact angle measurements. The PNIPAM-grafted porous PS film became more hydrophobic with higher contact angle as the temperature was increased from ambient temperature (25°C) to 38°C. According to ellipsometric analysis, the grafted PNIPAM layer has a thickness of 18 nm, the value falls in a suitable range to be used for cell attachment and detachment investigation.

From preliminary tests with keratinocyte cells, PNIPAM-grafted PS film seems to be a better substrate in supporting cell adhesion than the PS film which was used as a control (%cell adhesion > 100%). In contrast, PNIPAM-grafted porous PS film exhibited lower percentage of cell attachment (80%). When the temperature was decreased from 37 °C to 20 °C, both PNIPAM-grafted porous PS film and PNIPAM-grafted PS film showed decreasing % cell adhesion remained on the surface implying there was cell detachment as a result of thermoresponsive property of the surface-grafted PNIPAM layer. Nevertheless, the magnitude of cell detachment was still small (10-12% after 2 h) and the impact of porosity cannot be realized.

So far, we have demonstrated that PNIPAM brushes can be successfully and conveniently grafted on PS substrates using modified polydopamine as the initiator layer under mild condition at ambient temperature. The PNIPAM thickness is reasonably high enough so that it exhibits thermoresponsiveness. Nevertheless, the porous film with pore diameter being close to cell size (keratinocyte = 10 μm) may

not be appropriate feature for thermoresponsive cell sheet fabrication. Different approaches for generating porous material that yield smaller and controllable pore diameter should be explored. Further investigation on cellular responses in terms of attachment and detachment are also necessary.

REFERENCES

1. Heskins, M.; Guillet, J. E., Solution Properties of Poly(N-isopropylacrylamide). *Journal of Macromolecular Science: Part A - Chemistry* **1968,** *2*, 1441-1455.
2. Nakayama, M.; Okano, T., Intelligent Thermoresponsive Polymeric Micelles for Targeted Drug Delivery. *Journal of Drug Delivery Science and Technology* **2006,** *16*, 35-44.
3. Tan, L.; Liu, J.; Zhou, W.; Wei, J.; Peng, Z., A Novel Thermal and pH Responsive Drug Delivery System Based on ZnO@PNIPAM Hybrid Nanoparticles. *Materials Science and Engineering: C* **2014,** *45*, 524-529.
4. Yadavalli, T.; Ramasamy, S.; Chandrasekaran, G.; Michael, I.; Therese, H. A.; Chennakesavulu, R., Dual Responsive PNIPAM–Chitosan Targeted Magnetic Nanopolymers for Targeted Drug Delivery. *Journal of Magnetism and Magnetic Materials* **2015,** *380*, 315-320.
5. Nagase, K.; Hatakeyama, Y.; Shimizu, T.; Matsuura, K.; Yamato, M.; Takeda, N.; Okano, T., Hydrophobized Thermoresponsive Copolymer Brushes for Cell Separation by Multistep Temperature Change. *Biomacromolecules* **2013,** *14*, 3423-3433.
6. Okano, T.; Kikuchi, A.; Sakurai, Y.; Takei, Y.; Ogata, N., Temperature-Responsive Poly(N-isopropylacrylamide) as a Modulator for Alteration of Hydrophilic/Hydrophobic Surface Properties to Control Activation/Inactivation of Platelets. *Journal of Controlled Release* **1995,** *36*, 125-133.
7. Okano, T.; Yamada, N.; Sakai, H.; Sakurai, Y., A Novel Recovery System for Cultured Cells Using Plasma-Treated Polystyrene Dishes Grafted with Poly(N-isopropylacrylamide). *Journal of Biomedical Materials Research* **1993,** *27*, 1243-1251.
8. Tsuda, Y.; Kikuchi, A.; Yamato, M.; Sakurai, Y.; Umezu, M.; Okano, T., Control of Cell Adhesion and Detachment Using Temperature and Thermoresponsive Copolymer Grafted Culture Surfaces. *Journal of Biomedical Materials Research Part A* **2004,** *69A*, 70-78.
9. Yamato, M.; Akiyama, Y.; Kobayashi, J.; Yang, J.; Kikuchi, A.; Okano, T., Temperature-Responsive Cell Culture Surfaces for Regenerative Medicine with Cell Sheet Engineering. *Progress in Polymer Science* **2007,** *32*, 1123-1133.

10. Puelacher, W. C.; Vacanti, J. P.; Ferraro, N. F.; Schloo, B.; Vacanti, C. A., Femoral Shaft Reconstruction Using Tissue-Engineered Growth of Bone. *International Journal of Oral and Maxillofacial Surgery* **1996,** *25*, 223-228.
11. Nishida, K.; Yamato, M.; Hayashida, Y.; Watanabe, K.; Maeda, N.; Watanabe, H.; Yamamoto, K.; Nagai, S.; Kikuchi, A.; Tano, Y.; Okano, T., Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. *Transplantation* **2004,** *77*, 379-385.
12. Masuda, S.; Shimizu, T.; Yamato, M.; Okano, T., Cell Sheet Engineering for Heart Tissue Repair. *Advanced Drug Delivery Reviews* **2008,** *60*, 277-285.
13. Kumashiro, Y.; Yamato, M.; Okano, T., Cell Attachment–Detachment Control on Temperature-Responsive Thin Surfaces for Novel Tissue Engineering. *Annals of Biomedical Engineering* **2010,** *38*, 1977-1988.
14. Akiyama, Y.; Kikuchi, A.; Yamato, M.; Okano, T., Ultrathin Poly(N-isopropylacrylamide) Grafted Layer on Polystyrene Surfaces for Cell Adhesion/Detachment Control. *Langmuir* **2004,** *20*, 5506-5511.
15. Li, L.; Zhu, Y.; Li, B.; Gao, C., Fabrication of Thermoresponsive Polymer Gradients for Study of Cell Adhesion and Detachment. *Langmuir* **2008,** *24*, 13632-13639.
16. Mizutani, A.; Kikuchi, A.; Yamato, M.; Kanazawa, H., Preparation of Thermoresponsive Polymer Brush Surfaces and Their Interaction with Cells. *Biomaterials* **2008,** *29*, 2073-2081.
17. Kwon, O. H.; Kikuchi, A.; Yamato, M.; Sakurai, Y.; Okano, T., Rapid Cell Sheet Detachment from Poly(N-isopropylacrylamide)-Grafted Porous Cell Culture Membranes. *Journal of Biomedical Materials Research* **2000,** *50*, 82-89.
18. Oh, H. H.; Ko, Y.-G.; Uyama, H.; Park, W. H.; Cho, D.; Kwon, O. H., Fabrication and Characterization of Thermoresponsive Polystyrene Nanofibrous Mats for Cultured Cell Recovery. *BioMed Research International* **2014,** *2014*, 9.
19. Huang, C.; Kamra, T.; Chaudhary, S.; Shen, X., Breath Figure Patterns Made Easy. *ACS Applied Materials & Interfaces* **2014,** *6*, 5971-5976.
20. Zheng, Y.; Kubowaki, Y.; Kashiwagi, M.; Miyazaki, K., Process Optimization of Preparing Honeycomb-Patterned Polystyrene Films by Breath Figure Method. *Journal of Mechanical Science and Technology* **2011,** *25*, 33-36.
21. Wang, C.; Mao, Y.; Wang, D.; Qu, Q.; Yang, G.; Hu, X., Fabrication of Highly Ordered Microporous Thin Films by PS-b-PAA Self-Assembly and Investigation of their Tunable Surface Properties. *Journal of Materials Chemistry* **2008,** *18*, 683-690.

22. Li, X.-Y.; Zhao, Q.-L.; Xu, T.-T.; Huang, J.; Wei, L.-H.; Ma, Z., Highly Ordered Microporous Polystyrene-b-Poly(acrylic acid) Films: Study on the Influencing Factors in their Fabrication via a Static Breath-figure Method. *European Polymer Journal* **2014,** *50*, 135-141.
23. Zhao, B.; Brittain, W. J., Polymer Brushes: Surface-Immobilized Macromolecules. *Progress in Polymer Science* **2000,** *25*, 677-710.
24. Braunecker, W. A.; Matyjaszewski, K., Controlled/living Radical Polymerization: Features, Developments, and Perspectives. *Progress in Polymer Science* **2007,** *32*, 93-146.
25. Wang, J.-S.; Matyjaszewski, K., Controlled/"Living" Radical Polymerization. Atom Transfer Radical Polymerization in the Presence of Transition-Metal Complexes. *Journal of the American Chemical Society* **1995,** *117*, 5614-5615.
26. Shivapooja, P.; Ista, L. K.; Canavan, H. E.; Lopez, G. P., ARGET–ATRP Synthesis and Characterization of PNIPAAm Brushes for Quantitative Cell Detachment Studies. *Biointerphases* **2012,** *7*, 32.
27. Barthélémy, B.; Devillers, S.; Minet, I.; Delhalle, J.; Mekhalif, Z., Induction Heating for Surface Triggering Styrene Polymerization on Titanium Modified with Atrp Initiator. *Journal of Colloid and Interface Science* **2011,** *354*, 873-879.
28. Zhu, B.; Edmondson, S., Polydopamine-Melanin Initiators for Surface-Initiated ATRP. *Polymer* **2011,** *52*, 2141-2149.
29. Ginic-Markovic, M.; Barclay, T.; Constantopoulos, K. T.; Al-Ghamdi, T.; Blok, A.; Markovic, E.; Ellis, A. V., A Versatile Approach to Grafting Biofouling Resistant Coatings from Polymeric Membrane Surfaces Using an Adhesive Macroinitiator. *RSC Advances* **2015,** *5*, 63017-63024.
30. Barrandon, Y.; Green, H., Cell size as a determinant of the clone-forming ability of human keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America* **1985,** *82*, 5390-5394.

APPENDIX

Table A1 OD at wavelength 570 nm of all substrates after incubated 37 ℃ for 24 hr.

sample	PS film	PS film grafted with PNIPAM	Porous PS film grafted with PNIPAM
1	0.77571737	0.81341037	0.65434437
2	0.76846637	0.99859337	0.72960137
3	1.00394337	1.11603337	0.64911037

Table A2 OD at wavelength 570 nm of all substrates after decreasing temperature from 37 ℃ to 20 ℃ for 1 hr.

sample	PS film	PS film grafted with PNIPAM	Porous PS film grafted with PNIPAM
1	0.99673647	1.12442647	0.75698547
2	0.95743847	0.98306647	0.82223147
3	1.07255647	1.22256647	0.79557947

Table A3 OD at wavelength 570 nm of all substrates after decreasing temperature from 37 ℃ to 20 ℃ for 2 hr.

sample	PS film	PS film grafted with PNIPAM	Porous PS film grafted with PNIPAM
1	1.29744703	1.40356703	0.95266203
2	1.18847703	1.18093703	0.8015360
3	1.30054703	1.38792703	0.79601403

VITA

Miss Piriya Chailom was born on March 13th, 1991 in Chiang rai, Thailand. She graduted a Bachelor degree of Science, majoring in Chemistry from Naresuan University in 2013. In the same year, she was started Master's degree with a major course as Organic Chemistry, Department of chemistry, Faculty of Science, Chulalongkorn University and finished her study in the academic year of 2016.

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