พอลิเมอร์บรัชตอบสนองต่ออุณหภูมิที่เตรียมโดยการดัดแปรหลังพอลิเมอไรเซชันของพอลิเพนทะฟลูออโรเฟนิลอะคริเลตสำหรับการเตรียมแผ่นเซลล์

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THERMORESPONSIVE POLYMER BRUSHES PREPARED BY POST-POLYMERIZATION MODIFICATION OF POLY(PENTAFLUOROPHENYL ACRYLATE) FOR CELL SHEET PREPARATION

Miss Metawee Bunwanna

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เมธาวี บุ่นวรรณา : พอลิเมอร์บรัชตอบสนองต่ออุณหภูมิที่เตรียมโดยการดัดแปรหลังพอลิเมอไรเซชันของพอลิเพนทะฟลูออโรเฟนิลอะคริเลตสำหรับการเตรียมแผ่นเซลล์ (THERMORESPONSIVE POLYMER BRUSHES PREPARED BY POST-POLYMERIZATION MODIFICATION OF POLY(PENTAFLUOROPHENYL ACRYLATE) FOR CELL SHEET PREPARATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น, 43 หน้า.

ในงานวิจัยนี้สนใจตรึงโคพอลิเมอร์บรัชของพอลิ(เพนทาฟลูออโรเฟนิลแอคริเลต-โค-เอ็นไอโซโพรพิลแอคริลาไมด์) หรือ P(PFPA-co-NIPAM) บนพื้นผิวกระจกด้วยวิธีการ “grafting from” หรือ “grafting onto” สำหรับวิธีการ “grafting from” เริ่มจากการเตรียม PPFPAตรึงบนพื้นผิวด้วยการทำปฏิกิริยาพอลิเมอไรเซชันริเริ่มจากพื้นผิวด้วยกลไกแบบ reversible addition−fragmentation chain transfer (RAFT) polymerization ตามด้วยการดัดแปรหลังพอลิเมอไรเซชันด้วยการทำปฏิกิริยากับไอโซโพรพิลแอมีน (IPA) สำหรับวิธีการ “grafting onto” ทำได้โดยการนำ PPFPA ที่สังเคราะห์ได้ในสารละลายด้วย RAFT polymerization ไปดัดแปรหลังพอลิเมอไรเซชันด้วยการทำปฏิกิริยากับ IPA ก่อนนำไปกราฟต์บนแผ่นกระจกที่มีหมู่แอมิโน การพิสูจน์เอกลักษณ์ด้วยฟูเรียร์ทรานฟอร์มอินฟราเรดสเปกโทรสโกปี, เอ็กซ์เรย์โฟโตอิเล็กตรอนสเปกโทรสโกปี และการวัดมุมสัมผัสกับน้ำพิสูจน์ยันยันความสำเร็จในการเตรียมโคพอลิเมอร์บรัช ซึ่งสัดส่วนของโคพอลิเมอร์สามารถควบคุมได้โดยความเข้มข้นของ IPA และเวลาในการทำปฏิกิริยา จากการทดลองพบว่าการดัดแปลงอย่างต่อเนื่องของหมู่ PFPA ที่เหลือในโคพอลิเมอร์กับคอลลาเจนซึ่งเป็นโปรตีนที่ช่วยในการยึดเกาะของเซลล์ ส่งเสริมให้เกิดการเพิ่มการยึดเกาะของเซลล์ขณะทำการเลี้ยงเซลล์ได้ นอกจากนี้ยังทำปฏิกิริยาไฮโดรไลซิสของ P(PFPA-co-NIPAM)บรัช เพื่อเตรียมเป็นโคพอลิเมอร์ของพอลิแอคริลิกแอซิดและพอลิไอโซโพรพิลอะคริลาไมด์ P(AA-co-NIPAM) บรัช จากนั้นจึงศึกษาการนำโคพอลิเมอร์ทั้งสองชนิดไปเป็นซับสเตรทในการเตรียมแผ่นเซลล์ งานวิจัยนี้แสดงให้เห็นว่าพื้นผิวที่มีหลายหน้าที่และตอบสนองต่ออุณหภูมินี้สามารถนำไปใช้สำหรับการเตรียมแผ่นเซลล์ซึ่งเป็นประโยชน์ต่อการใช้งานทางด้านวิศวกรรมเนื้อเยื่อ

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METAWEE BUNWANNA: THERMORESPONSIVE POLYMER BRUSHES PREPARED BY POST-POLYMERIZATION MODIFICATION OF POLY(PENTAFLUOROPHENYL ACRYLATE) FOR CELL SHEET PREPARATION. ADVISOR: ASSOC. PROF. VORAVEE HOVEN, Ph.D., 43 pp.

Here in this research, polymer brushes of poly(pentafluorophenyl acrylate-*co*-N-isopropylacrylamide) or P(PFPA-*co*-NIPAM) were fabricated on glass substrates by either “grafting from” or “grafting onto” methods. For the “grafting from approach”, the surface-grafted PPFPA were first prepared by surface-initiated reversible addition−fragmentation chain transfer (RAFT) polymerization followed by post-polymerization modification with isopropylamine (IPA). Alternatively, PPFPA synthesized by RAFT polymerization in solution underwent post polymerization modification with IPA prior to being grafted onto the aminated glass substrates. Characterization by Fourier transform-infrared spectroscopy (FTIR), x-ray photoelectron spectroscopy (XPS) and water contact angle measurements proved the successful formation of the copolymer brushes, of which composition can be controlled by IPA concentration and reaction time. Tandem modification of the remaining PFPA units in the surface-grafted copolymer brushes with collagen type I, a cell adhesion promoter, was found to enhance adhesion of Keratinocyte cell upon cell culturing. Hydrolysis of P(PFPA-*co*-NIPAM) brushes were also performed to yield P(AA-*co*-NIPAM) brushes. Both copolymer brushes were then evaluated as substrates for cell sheet preparation. This study has suggested that this multifunctional and thermoresponsive platform can be used for cell sheet fabrication which is beneficial for tissue engineering applications.

Field of Study: Petrochemistry and Polymer Science Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

AA	: Acrylic acid
ACVC	: 4,4′-azobis(4-cyanovaleric acid)
AFM	: Atomic force microscopy
APTES	: (3-Aminopropyl)triethoxysilane
ATRP	: Atom transfer radical polymerization
DCC	: Dicyclohexylcarbodiimide
DMAP	: 4-Dimethylaminopyridine
°C	: Degree celcius
CAPs	: Cell adhesion promoter
CO_2	: Carbon dioxide
CPD	: 4-cyanopentanoic acid dithiobenzoate
CTA	: Chain transfer reagent
D_2O	: Deuterium monoxide
DP	: Degree of polymerization
ECs	: Endothelial cells
ECM	: Extracellular matrix
EDC	: 1-(3-Dimethyllaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	: Ethylenediaminetetraacetic acid
FT-IR	: Fourier transform infrared spectroscopy
g	: Gram
GPC	: Gel permeation chromatography
h	: Hour
HPC	: Hematopoietic progenitor cells
HUVEC	: Human umbilical vein endothelial cells
IPA	: Isopropylamine
RAFT	: Reversible addition – fragmentation chain transfer
RI	: Refractive index

LCST	: Lower critical solution temperature
Min	: Minute
mL	: Milliliter
mM	: Millimolar
Mn	: Number average molecular weight
MW	: Molecular weight
NHS	: *N*-Hydroxysuccinimide
nm	: Nanometer
NMR	: Nuclear magnetic resonance
PAA	: Poly(acrylic acid)
P(AA-*co*-NIPAM)	: Poly(acrylic acid-*co*- *N*-isopropylacrylamide)
PDEGMEMA	: Poly(diethylene glycol methyl ether methacrylate)
PDI	: Polydispersity index
PFPA	: Pentafluorophenyl acrylate
PNIPAM	: Poly(*N*-isopropylacrylamide)
P(NIPAM-*co*-CIPAM)	: Poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide)
PPFPA	: Poly(pentafluorophenyl acrylate)
P(PFPA-*co*-NIPAM)	: Poly(pentafluorophenyl acrylate-*co*-*N*-isopropylacrylamide)
SPR	: Surface plasmon resonance
TEA	: Triethylamine
THF	: Tetrahydrofuran
TMS	: Tetramethylsilane
UCST	: Upper critical solution temperature
W	: Watt
XPS	: X-ray photoelectron spectroscopy
μg	: Microgram
μL	: Microliter
θ_A	: Dynamic advancing contact angle
θ_R	: Dynamic receding contact angle
δ	: Chemical shift

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Thermoresponsive polymers belong to a class of smart materials that have the ability to alter their physical properties in response to temperature change. Poly(*N*-isopropylacrylamide) (PNIPAM) is the most well-known in its class, exhibiting a lower critical solution temperature (LCST) of about 32°C in water. Below the LCST, PNIPAM forms an expanded structure and soluble in solution. In contrast, PNIPAM forms denser globular structure and becomes insoluble in the solution once the temperature is raised above the LCST [1,2]. Since its LCST is close to body temperature, PNIPAM is currently recognized as valuable materials for a variety of biomedical applications e.g. drug delivery, biomolecules separation, and tissue engineering [3,4,5].

A tissue-like cellular monolayer or cell sheet has become one of the most effective platforms for tissue engineering because they can be directly transplanted to host tissues without the demand for biodegradable scaffolds which are often the cause of inflammatory response. The direct transplantation has been recently applied to several diseased organs e.g. eye, heart and kidney [6]. To prepare this platform, the thermoresponsive PNIPAM has been developed as cell-culture substrates for cell sheet preparation. At above the LCST, cultured cells can adhere and proliferate on PNIPAM surface. When reducing temperature below the LCST, cell sheet can be harvested intact with associated extracellular matrix (ECM) without EDTA or enzymatic treatment [7,8,9].

To improve properties of the thermoresponsive brushes, terminal functionalization and synthesis of PNIPAM containing more active functional group such as carboxyl group have been reported by Okano and coworkers [10,5,11]. For example, modification of PNIPAM chain end with hydrophilic maleimide group and maleimide derivatives demonstrated that maleimide and its derivatives can improve cell adhesion property of thermoresponsive surfaces, while maleimide derivative containing carboxyl group exhibited the ability to promote cell sheet detachment

within 30 min after low temperature treatment. In addition, the immobilization of biomolecules for enhance cell adhesion property through carbodiimide chemistry of carboxyl groups in PNIPAM brushes has been reported. 3-Mercaptopropionic acid was used as chain transfer agent for polymerization of NIPAM providing carboxyl group at chain end. It was reported that the terminal carboxyl groups of PNIPAM brushes showed the ability to immobilize protein and promote cell adhesion and detachment of surfaces.

Immobilizing cell-specific biomolecules is one of effective ways to improve cell adhesion property of materials. Such strategy cannot be directly applied to PNIPAM, of which its amide side chains are hydrolytically stable, unless complicated synthetic techniques are employed [5,11,12]. Poly(pentafluorophenyl acrylate) (PPFPA) is an attractive precursor polymer containing active pentafluorophenyl (PFPA) moieties in its side chains. The active PPFPA also provide a good hydrolytic stability and good solubility in a wide range of organic solvents [13,14]. The active PFPA groups can react with hydrophilic amine compounds including isopropylamine (IPA) under mild conditions via post-polymerization modification yielding the thermoresponsive PNIPAM [15]. In principle, these highly reactive ester groups should readily available for interacting with N-terminus of protein/peptide rendering effective biomolecule immobilization [16,17].

Here in this research, active PPFPA grafted substrates were prepared by either "grafting from" or "grafting to" methods via RAFT polymerization and converted to thermoresponsive PNIPAM brushes by post-polymerization modification with IPA The composition of P(PFPA-*co*-NIPAM) can be controlled by IPA concentration and reaction time. The resulting surface-grafted copolymer brushes of P(PFPA-co-NIPAM) were then immobilized with collagen type I, a cell adhesion promoter (CAPs). PAA brushes have been shown to have the capacity to resist cell adhesion and promote cell detachment property [5,18]. Because the pentafluorophenyl esters can be transformed to acrylic acid under suitable hydrolytic condition, another series of surface-grafted polymer brushes based on poly(acrylic acid-co- *N*-isopropylacrylamide) or P(AA-*co*-NIPAM) were also prepared by hydrolysis of P(PFPA-*co*-NIPAM). Both collagen type I immobilized P(PFPA-co-NIPAM) and P(AA-*co*-NIPAM) brushes were investigated as thermoresponsive substrates for cell

sheet fabrication. This research aims to demonstrate the versatility of active-ester containing PPFPA as precursor polymer capable of undergoing tandam post-polymerization with the right choice of active molecules and yield multifunctional polymeric platform for tissue engineering applications.

1.2 Objectives

1. To prepare the thermoresponsive PNIPAM and P(PFPA-*co*-NIPAM) brushes by post-polymerization modification of PPFPA brushes obtained from either "grafting from" or "grafting to" as cell culture materials for cell sheet preparation.
2. To immobilize collagen type I onto the reactive P(PFPA-*co*-NIPAM) brushes to improve cell adhesion and detachment properties of the substrates.

1.3 Scope of investigation

The stepwise investigation was carried out as follows:

1. Literature survey for related research work.
2. Synthesis and characterization of P(PFPA-*co*-NIPAM) containing various compositions of PNIPAM by post-polymerization modification of PPFPA.
3. Preparation of PNIPAM brushes by either "grafting from" or "grafting to" method to determine the optimal thickness for cell sheet preparation.
4. Preparation and characterization of P(PFPA-*co*-NIPAM) brushes containing various composition of PNIPAM obtained from either "grafting from" or "grafting to" method.
5. Preparation and characterization of P(AA-*co*-NIPAM) and collagen type I immobilized P(PFPA-*co*-NIPAM) brushes.
6. Investigation of cell sheet preparation using the developed substrates.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Thermoresponsive polymers

Thermoresponsive polymers belong to a class of smart materials that have the ability to alter their physical properties in response to temperature change. There are two main types of thermoresponsive polymers divided by their solubility property. The first type is polymer that their solubility depends on a lower critical solution temperature (LCST) while the solubility of the other depends on an upper critical solution temperature (UCST). LCST and UCST are the critical temperature *below* and *above* which the polymer and solvent are completely miscible for all compositions [1,19]. The thermoresponsive polymers have been used for a wide range of biomedical applications such as sensors, drug delivery, gene delivery and tissue engineering [3,20].

Poly(*N*-isopropylacrylamide) (PNIPAM) is one of the most well-known thermoresponsive polymers. PNIPAM exhibits LCST behavior in water at about 32°C. For example, PNIPAM solution below 32°C is a clear, homogeneous solution while the solution above 32°C appears cloudy. Below the LCST, the coiled structure is favored. This structure allows a maximum interaction between the polymer and water. In systems where strong hydrogen bonding is possible, such interactions lower the free energy of dissolution considerably. At higher temperatures, the hydrogen-bonding effect weakens. As a consequence, the entropy controlled “hydrophobic effect”, the tendency of the system to minimize the contact between water and hydrophobic surfaces increases [19]. However, because LCST of PNIPAM is close to body temperature, PNIPAM has often been used for biomedical applications.

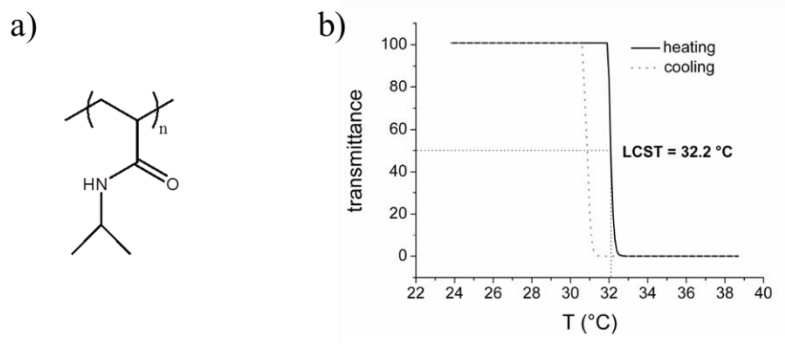


Figure 2.1 Structure (a) and LCST curve [21] (b) of PNIPAM.

2.2 Cell sheet preparation

Because of the problems from 2 traditional tissue engineering methods namely, isolated cell injections causing of uncontrollable of size, shape, location of the injected cells and biodegradable scaffolds-based technologies to fabricate three-dimensional tissue formation often causing host inflammatory response, cell sheet technology has been invented to eliminate these problems [7]. Cell sheet can be prepared from the thermoresponsive cell culture dishes using the changing of temperature to harvest cell sheet. The obtained cell sheets maintain their associated extracellular matrix (ECM) andnative-tissue functions due to the absence of trypsinization process. Thus cell sheets can be directly transplanted to the injured hosttissues without using sutures or additional adhesive agents, such as fibrin glue [8]. Thermoresponsive PNIPAM brushes has been studied and developed as cell culture material for cell sheet preparation because its LCST is close to incubated temperature at 37°C. At the incubated temperature, the surfaces exhibit hydrophobic property because PNIPAM chains extensively dehydrate and collapse on the surfaces resulting in cell adhesion and growth. While reducing the temperature below the LCST, PNIPAM chains are highly hydrated and the surfaces change to hydrophilic. At low temperature, the mobility of PNIPAM chains is the cause of cell sheet detachment. However, cell adhesion and cell detachment properties are controlled by polymer film thickness and grafted density. Many researches have studied about the optimum thickness of PNIPAM brushes for cell sheet preparation.

In 2004, Okano *et al*. [22] prepared two types of PNIPAM surface to elucidate the influential factors for cell adhesion and detachment. The two types of PNIPAM-

grafted surfaces characterized by infrared spectroscopy revealed that amounts of the grafted polymers were 1.4 ± 0.1 and 2.9 ± 0.1 μg/cm^2, respectively. These two surface types are abbreviated as PIPAAm-1.4 and PIPAAm-2.9, respectively, Thicknesses of grafted PNIPAM surface were determined to be 15.5 ± 7.2 and 29.5 ± 8.4 nm for PNIPAM-1.4 and PNIPAM-2.9 using atomic force microscopy (AFM) technique. Because of the more hydrophobicity of the PNIPAM-1.4, Bovine carotid artery endothelial cells (ECs) could adhere and proliferate on it to form confluent cell monolayers. Cell sheet was harvested by temperature decrease from 37 to 20 °C. On the contrary, ECs difficultly adhered to the surfaces of PNIPAM-2.9 because the grafted polymers at the outermost interfaces have more polar characteristics than the PNIPAM-1.4 even at 37 °C. This investigation has demonstrated that the thickness and amount of PNIPAM grafted on surface have significant influence on surface properties.

In 2008, Mizutani *et al*. [23] prepared surface-grafted PNIPAM brushes with different thickness to study the effect of thickness on cell sheet preparation. Bovine carotid artery endothelial cells (ECs) were also used for this investigation. It was found that smaller amount of grafted PNIPAM and PNIPAM layer thickness provided a larger number of adhered cells on the surfaces. However, no adherent cells were observed on the PNIPAM brushes with thickness more than 60 nm. On the other hand, the cell sheet easily detached from PNIPAM brushes with thickness of 20 – 50 nm. While PNIPAM brushes with thickness of 2 – 10 nm spent a long time for cell sheet detachment.

In 2009, Fukumori *et al.* [2] studied the effect of PNIPAM grafting density for cell adhesion and detachment properties. PNIPAM-grafted substrates were prepared by electron beam irradiation with different grafting density.Grafted density and thickness of the PNIPAM brushes were determined by ATR-FTIR and AFM, respectively. The grafted thickness and amount of polymer increased with the initial monomer concentration. In this work, PNIPAM brushes with grafted density of about 2 μg/cm^2 were optimal for the temperature-responsive cell adhesion/detachment control.

To improve the properties of cell culture surfaces, the modifications of PNIPAM brushes by modification of chain end, synthesis of copolymer based PNIPAM, and immobilization of biomolecules have been reported.

In 2009, Oezyuerek, *et al*. [12] synthesized glyco-block copolymer based PNIPAM which combine both thermoresponsive and heparin-like functionality. The copolymers synthesized from PNIPAM and glucose units were covalently fixed onto glass substrates by low pressure plasma cross-linking. This work showed the successful preparation of biomimetic surfaces with dual functionalities of thermoresponsive and heparin-like characteristics. The copolymer surfaces exhibited the ability to enhance celladhesion of Human umbilical vein endothelial cells (HUVEC) and hematopoietic progenitor cells (HPC).

In 2011, Takahashi *et al.*[5] prepared PNIPAM brushes and modified the terminal groups to improve cell adhesion and detachment properties of PNIPAM brushes. The PNIPAM grafted surfaces fabricated via surface-initiated reversible addition−fragmentation chain transfer (RAFT) polymerization gave dithiobenzoate (DTB) groups at PNIPAM chain end. The DTB groups were converted to various functional groups providing various types of cell culture materials. In this study, PNIPAM brushes modified with carboxyl groups showed the acceleration of cell adhesion because carboxyl groups have ability to enhance protein adsorption. The carboxyl groups also enhance hydrophilic property of surfaces that causes the rapid detachment of cell sheets after low temperature treatment.

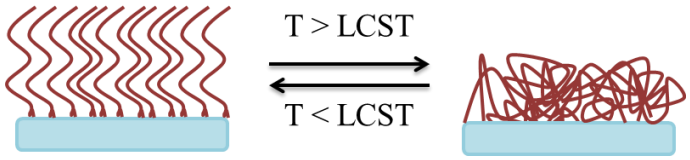


Figure 2.2 Conformational changes of the PNIPAM brushes at below and above LCST.

In 2011, Gao *et al.* [24] synthesized poly(*N*-isopropylacrylamide)-*b*-poly(acrylic acid) (PNIPAM-*b*-PAA) brushes via surface initiated atomic transfer radical polymerization (ATRP). In this study, RGD peptide was covalently

immobilized onto the PAA units by carbodiimide chemistry to accelerate cell attachment property of the substrates. In addition, BSA-FITC was also grafted onto the PAA block, as observed directly by fluorescence microscopy. *In vitro* cell culture confirmed that the PNIPAM-*b*-PAA-g-RGD surface, combining the thermal responsive and cell affinity properties, could well regulate the cell adhesion and detachment by simple alteration of temperature.

In 2013, Arisaka*et al*. [25] fabricated poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) [P(NIPAM-*co*-CIPAM)] grafted surfaces to immobilize heparin through carboxyl groups in the copolymer using EDC/NHS as coupling agent. The resulting heparin-functionalized thermoresponsive surfaces were investigated for cell sheet preparation. The results demonstrated that these modified surfaces were able to promote cell adhesion and proliferation of mouse fibroblast (NIH/3T3) cells and the confluent cells detached themselves as a contiguous cell sheet by changing temperature.

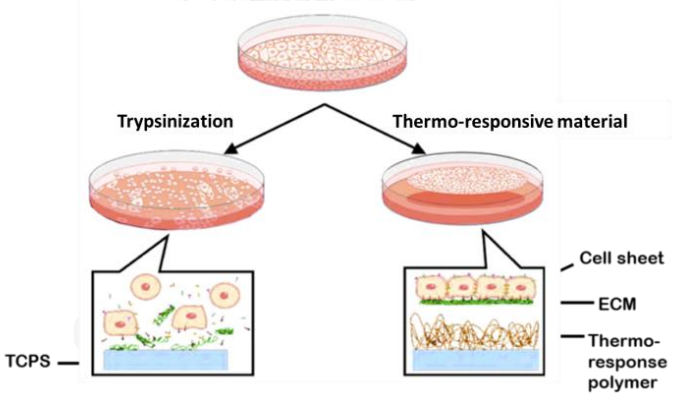


Figure 2.3 The traditional method (left) and using thermoresponsive material (right) for cell harvesting. **[**26**]**

2.3 Active ester polymer

Active ester polymers having multiple reactive units in polymer chains are the attractive precursors for multifunctional polymer preparation. Among the active precursors, poly(pentafluorophenyl acrylate) (PPFPA) has been recently introduced as alternative to the traditional succinimide based active ester polymers [27]. PPFPA exhibits a high reactivity with a good hydrolytic stability and soluble in common organic solvents. The active PPFPA can react with amines under mild conditions via

post-polymerization modification yielding functionalized polyacrylamide derivatives. By the reaction with isopropylamine (IPA), PPFPA can be converted to the thermoresponsive PNIPAM. Because of the controllable of IPA concentration and reaction time, P(PFPA-*co*-NIPAM) with various composition can also be obtained. This attractive polymer has been used in peptide synthesis, polymer chemistry and surface chemistry as reported in the following publications.

In 2009, Kessler *et al.* [16] fabricated reactive surface containing the active PFP groups which can react with amines or specific binding sites for protein adsorption forthe applications in future biosensor design. In this research, the binding of specific molecules such as, biotin, L-thyroxine, and folic acid were used to immobilize on the surfaces through reactions with the PFP groups. After successful attachment of designated molecules, streptavidin, pre-albumin, and folate-binding protein could be immobilized, respectively. The covalent attachment of these specific molecules onto the thin reactive surface and protein assembly process could be monitored by surface plasmon resonance (SPR) *in situ*. The conjugation of proteins could be characterized using AFM, FTIR, fluorescence spectroscopy, and ellipsometry. This simple method to obtain smooth homogeneous protein layers may be useful for further biosensor applications.

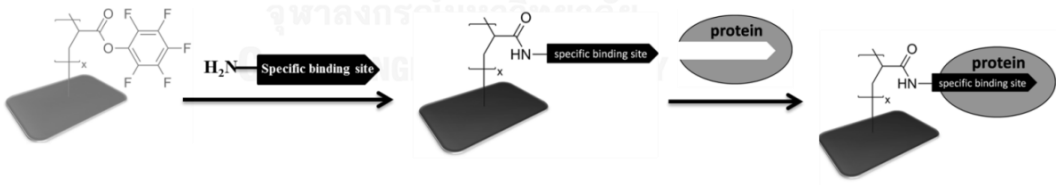


Figure 2.4 The specific binding site immobilized surfaces for protein adsorption. [16]

In 2009, Wiss *et al.* [28] successfully synthesized hybrid triblock copolymer via activated ester chemistry from poly(diethylene glycol methyl ether methacrylate) (PDEGMEMA) and a collagen-like peptide. The stimuli-responsive polymer, PDEGMEMA, was synthesized via RAFT polymerization using pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) as chain transfer agent (CTA). The active PFP groups at polymer chain ends reacted with amine groups at both the N- and C-termini of collagen-like peptide. The resulting PDEGMEMA-b-collagen-b-

PDEGMEMA triblock copolymer exhibited the expected collagen triple-helical structure, suggesting opportunities to sequentially drive self-assembly behavior of the triblock via simple changes in temperature.

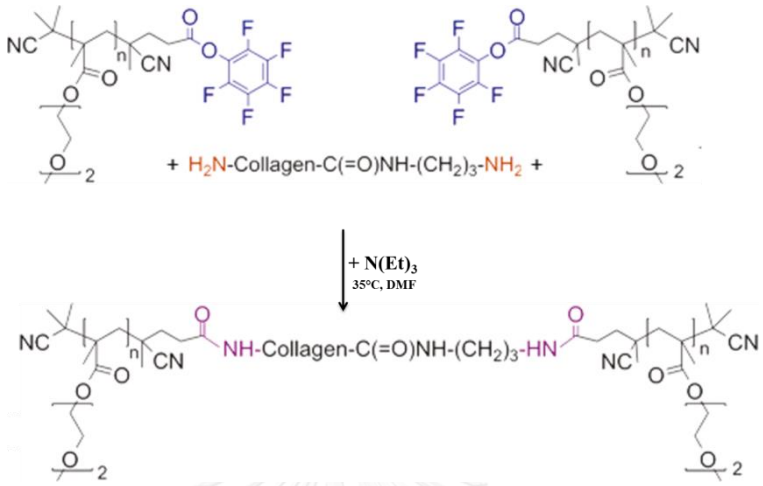


Figure 2.5 Synthesis of a hybrid triblock copolymer via activated ester chemistry from PDEGMEMA and a collagen-like peptide. [28]

In 2012, Günay *et al.* [15] studied the reactivity of poly(pentafluorophenyl methacrylate) (PPFMA) brushes prepared via RAFT polymerization towards post-polymerization modification with a variety of amines. To confirm the success of this reaction FTIR, x-ray photoelectron spectroscopy (XPS), and water contact angle measurement were used to characterize the surfaces after post-polymerization modification. In addition, the change in thickness of polymer brushes after the reaction was also determined.

In 2012, Choi *et al.* [29] prepared patterned reactive PPFPA brushes fabricated by RAFT polymerization using dithiobenzoic acid benzyl-(4-ethyltrimethoxylsilyl) ester as the surface chain transfer agent (S-CTA). UV irradiation of the S-CTA-modified substrates led to a selective degradation of S-CTA in the exposed areas and gave patterned activated polymer brushes. Conversion of the patterned polymer brushes with amino-spiropyrans and 5-((2- aminoethyl) amino) naphthalene-1-sulfonic acid through post-polymerization modification resulted in patterned light-responsive and fluorescent polymer brush films, respectively.

In 2012, Deng *et al.* [17] fabricated cell culture material containing alkyne and the active PFP groups to immobilize multiple biomolecules. Cyclic argine-glycine-aspartic acid (cRGD) adhesion peptide and epidermal growth factor (EGF) were immobilized through alkyne–azidecycloaddtion ("click" chemistry) and active ester–amine reaction, respectively. Human umbilical vein endothelial cells (HUVEC) were used in this work. The result demonstrated that the surfaces immobilized with both cRGD and EGF showed higher capacity to induce the adhered cells than surfaces immobilized only with EGF.

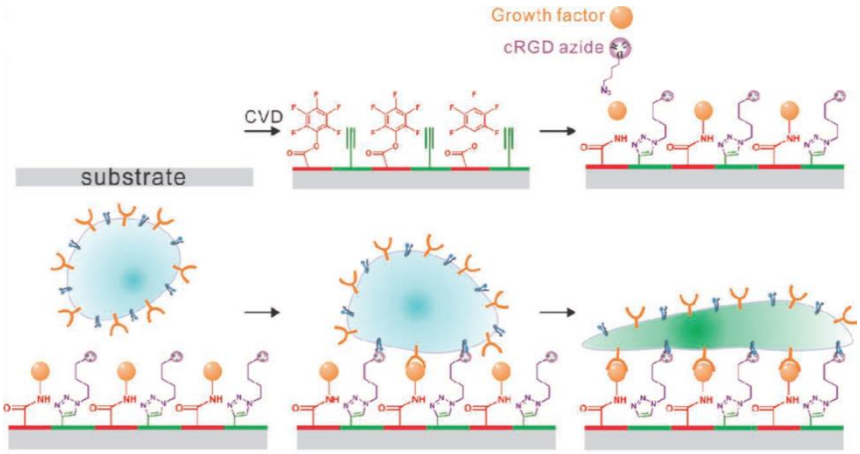


Figure 2.6 Co-immobilization of cRGD and EGF on the reactive surfaces to improve cell adhesion property. [17]

CHAPTER III

EXPERIMENTAL SECTION

3.1 Materials

Acrylic acid (AA), 4-dimethylaminopyridine (DMAP), 4,4′-azobis(4-cyanovaleric acid) (ACVA), and 4-cyanopentanoic acid dithiobenzoate (CPD) (chain transfer agent or CTA) were obtained from Aldrich (USA). AA was purified by vacuum distillation. Pentafluorophenol, acetone, toluene, hexane, ethanol, dichloromethane, 1,4-dioxane and tetrahydrofuran (THF) were purchased from Merck (Germany). Dicyclohexyl carbodiimide (DCC), phosphate buffered saline pH 7.4 (PBS), 3-aminopropyltriethoxysilane (APTES), triethylamine (TEA), isopropylamine (IPA) were obtained from Sigma-Aldrich (USA). Glass slides were supplied by S.E. SUPPLY LTD PART. Pentafluorophenyl acrylate (PFPA) was synthesized according a published procedure [29]. Keratinocyte cells and collagen type I were purchased from STEMCELL Technologies. Ultrapure distilled water that was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step (18.2 MΩ cm resistance).

3.2 Equipment

3.2.1 Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H NMR spectra were recorded in CF_3COOH/D_2O using Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts (δ) were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra were recorded in KBr discs with a FT-IR spectrometer (Nicolet, USA), model Impact 410, with 32 scans at resolution 4 cm-1. A frequency of 400-4000 cm^{-1} was collected by using TGS detector.

3.2.3 Gel permeation chromatography (GPC)

PPFPA was characterized by GPC using Waters 600 controller chromatograph equipped with HR1 and HR4 columns (Waters, USA) at 35 ^{o}C and refractive index (RI) detector (Waters, USA, model 2414). THF was used as mobile phase with a flow rate of 1.0 mL/min. A calibration curve was obtained using polystyrene standards.

3.2.4 Water contact angle measurement

Wettability of the modified surfaces were determined by water contact angle measurement using a contact angle goniometer (Ramé-Hart, Inc., USA, model 100-00), equipped with a Gilmont syringe and a 24-gauge flat-tipped needle. All of the measurements were carried out in air at ambient temperature. The maximal contact angle (advancing contact angles (θ_A)) and the minimal contact angle (receding water contact angle (θ_R))are recorded and averagedfrom 5 measurements on different area of each sample.

3.2.5 X-Ray photoelectron spectroscopy (XPS)

The surface composition was 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°.

3.2.6 Atomic force microscopy (AFM)

The thickness of polymer brushes was recorded with Scanning Probe Microscope (Veeco, USA, model NanoScope®IV). Measurements were performed in air using tapping mode. Silicon nitride tips with a resonance frequency of 267-295 KHz and a spring constant 20-80 N/m were used.

3.3 Experimental Procedure

3.3.1 Preparation of P(PFPA-*co*-NIPAM) by post-polymerization modification

PPFPA was first synthesized via RAFT polymerization. PFPA (1.48 mL, 1M), CPD (6.29, 13, 50 mg, 2.5, 5, 20 mM) and ACVA (0.79, 1.58, 6.31 mg, 0.31, 0.62, 2.5 mM) were dissolved in 1,4-dioxane (9 mL). The solution was heated up to 70ºC for 20 h and then cooled down to room temperature before precipitated in

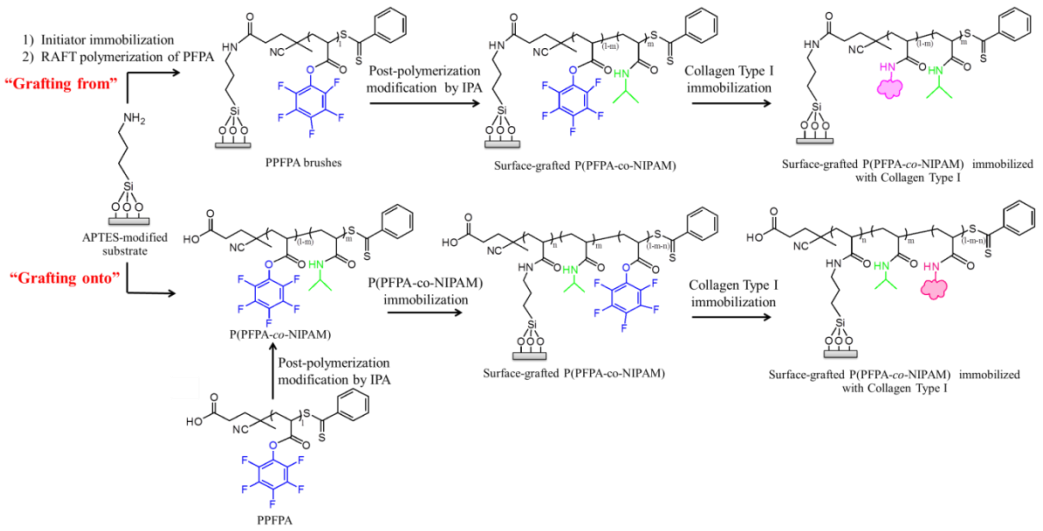
methanol. Purified PPFPA was obtained as pink powder after re-precipitation twice from polymer solution in 1,4-dioxane into methanol.

P(PFPA-*co*-NIPAM) was then prepared by post-polymerization modification of PPFPA with IPA at 45ºC. The mole equivalent of IPA to PFPA unit in the PPFPA was varied in a range of 0.25-1. The reaction time was also varied to determine the kinetics of post-polymerization modification. At the end of modification period, the obtained (co)polymer product was precipitated in hexane or diethyl ether.

3.3.2 Preparation of surface-grafted (co)polymer brushes

Glass coverslips were cleaned by air plasma cleaner (Harrick, USA, model PDC-32G) with power of 18 W for 5 min prior to use. APTES was immobilized on the cleaned glass substrates via vapor-phase deposition method. APTES (100 µL) was added to a reaction vial containing the cleaned glass substrates and then heated to 80 °C. After 72 h, the APTES-modified substrates were rinsed with toluene, acetone and DI water, respectively.

The active PPFPA was grafted on glass surfaces via "grafting from" and "grafting onto" methods (**Scheme 3.1**). For the "grafting from" method, the APTES-modified substrates were immersed in 15 mL of DMF containing ACVA (0.16 g, 37.5 mM), DCC (0.15 g, 47.0 mM) and DMAP (6.86 mg, 3.74 mM) at room temperature for 20 h under nitrogen atmosphere to yield initiator-immobilized substrates. The substrates were rinsed thoroughly with DMF and ethanol. The initiator-immobilized substrates were immersed in 1,4-dioxane containing PFPA (1.48 mL, 1 M) and CTA (2.5, 5, 20 mM) and then heated to 70 °C for 20 h under nitrogen atmosphere. The resulting surface-grafted PPFPA were rinsed thoroughly with 1,4-dioxane. Free PPFPA formed simultaneously in solution was also recovered by precipitation with methanol. The surface-grafted PNIPAM and P(PFPA-*co*-NIPAM) were then obtained by post polymerization modification of the surface-grafted PPFPA. Briefly, the surface-grafted PPFPA were immersed in 10 mL of THF containing IPA (8.52 µL, 10 mM) and TEA (13.94 µL, 10 mM) at room temperature for 5, 10, 30 and 60 minutes then rinsed with THF and acetone, respectively.



Scheme 3.1 Preparation of surface-grafted polymer brushes by "grafting from" and "grafting onto" methods followed by collagen type I immobilization.

For the "grafting onto" method, APTES-modified substrates were immersed in 1,4-dioxane containing 0.5 M of P(PFPA-co-NIPAM) synthesized by post-polymerization modification of PPFPA at room temperature. After 16 h, the substrates were rinsed with 1,4-dioxane.

To prepare P(AA-co-NIPAM) grafted surface, glass substrates bearing P(PFPA-co-NIPAM) brushes obtained from both "grafting from" and "grafting onto" method were immersed in milli-Q water containing 0.5 M of TEA at room temperature. After 20 h, the substrates were rinsed thoroughly with milli-Q water.

3.3.3 Protein immobilization

The P(PFPA-*co*-NIPAM) brushes with various %PNIPAM composition were immersed in PBS buffer (pH 7.4) containing collagen type I (10 µg/mL) and stirred for 12 h at 4ºC. The substrates were rinsed with PBS buffer and Milli-Q water, respectively.

3.3.4 Cell sheet preparation

Keratinocyte cells were cultured in keratinocyte Growth Medium on tissue culture dishes at 37^{o}C, 5% CO_2. After 10 days, keratinocyte cells were trysinized

andseeded at $1x10^4$ cells/cm^2 onto the desired glass substrates, followed by incubation at 37 ºC. After reaching confluency, the adherent cells were observed by optical microscope (ZEISS, Germany, model Axio Oserver Z1 Motorized) and harvested by decreasing temperature from 37ºC to 25ºC.The amount of adherent cells observed by optical microscope on each substrate was used to study cell adhesion property of the substrates. For cell detachment property of the substrates, size of the detached cell sheet on each substrate at the same time was used for this evaluation.

CHAPTER IV

RESULTS AND DISCUSSION

In this chapter, the results are divided into four sections. The first section explains about the synthesis of the thermoresponsive poly(pentafluorophenyl acrylate-*co*-*N*-isopropylacrylamide) (P(PFPA-*co*-NIPAM)) prepared by post-polymerization modification of poly(pentafluorophenyl acrylate) (PPFPA) with isopropylamine (IPA) and the ability to control the composition of copolymer by IPA concentration and reaction time. The second section shows the preparation and characterization of P(PFPA-*co*-NIPAM) brushes fabricated via "grafting from" and "grafting onto" approach. The third section focuses on the development of copolymer brushes for cell sheet preparation. The various %PNIPAM composition of those copolymer brushes were used to immobilize cell adhesion protein such as collagen type I and used to provide poly(acrylic acid-co-*N*-isopropylacrylamide) (P(AA-*co*-NIPAM)) through the hydrolysis of P(PFPA-*co*-NIPAM). The last section presents the determination of the appropriate polymer film thickness and the investigation of developed copolymer brushes for cell sheet preparation.

4.1 Preparation of P(PFPA-*co*-NIPAM) by post-polymerization modification

PPFPA with different MW was synthesized via RAFT polymerization. The MW and polydispersity index (PDI) were characterized by ^{1}H NMR and GPC, respectively. All monomer content (degree of polymerization) was calculated from relative ratio between integral of protons of PPFPAat position a (δ=3.1) and integral of the aromatic protons of dithiobenzoate group (position e, δ=7.4-7.9) at the chain end of PPFPA using **equation 4.1**. Then, the molecular weight of the PPFPA could be calculated using **equation 4.2** (^{1}H NMR spectra are shown in appendix). The data shown in **Table 4.1** indicated that the polymerization was well-controlled for the targeted 50 and 200 degree of polymerization (DP) with narrow PDI and the MW being close to the expected MW. Less control over the polymerization of higher

targeted DP (400) was observed as indicated by the PDI being significantly deviated from 1.0.

$$\text{PPFPA content (unit)} = \frac{\text{integral of H}(\delta=3.1)}{\text{integral of H}(\delta=7.4-7.9)/5} \quad (4.1)$$

$$\text{Mn of PPFPA} = (\text{PPFPA content (unit)} \times \text{Mn of PFPA}) + \text{Mn of RAFT agent} \quad (4.2)$$

Table 4.1 Molecular weight information of PPFPA synthesized by RAFT polymerization

Targeted DP	**Theoretical M_n**	**M_n determined by ^{1}H NMR**	**PDI determined by GPC**
50	11,905	10,238	1.37
200	47,622	50,003	1.24
400	95,244	112,507	1.72

Effects of IPA concentration and reaction time on the extent of post-polymerization modification of PPFPA in solution were determined in order to obtain appropriate conditions that gave P(PFPA-*co*-NIPAM) with varied PNIPAM composition. PPFPA having MW of 112,507 was used for this investigation. As evaluated by FT-IR (**Figure 4.1**), %conversion from PPFPA to PNIPAM or %PNIPAM composition in the resulting P(PFPA-*co*-NIPAM) can be calculated from a relative intensity ratio of amide C=O stretching of PNIPAM at 1650 cm^{-1} to that of ester C=O stretching of PPFPA. For example, treating PPFPA with 0.25 eq. IPA for 16 h gave 30% conversion meaning that 30% of PPFPA was converted to PNIPAM whereas 70% PPFPA remained unreacted. In other words, such condition yielded P(PFPA-co-NIPAM) with 30%PNIPAM composition. As demonstrated in **Figure 4.2**, %PNIPAM composition can be varied as a function of both IPA concentration and reaction time.

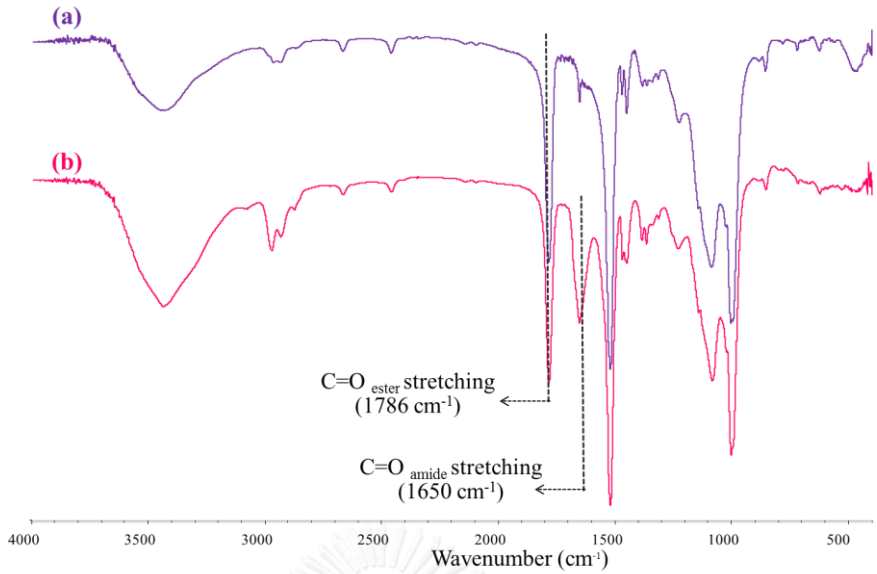


Figure 4.1 FTIR spectra of PPFPA (a) before and (b) after a reaction with 0.25 eq. IPA for 16 h.

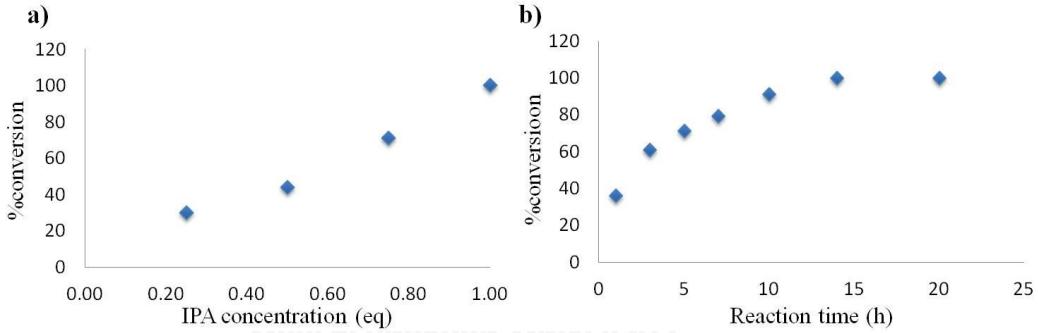


Figure 4.2 %Conversion from PPFPA to PNIPAM in solution upon post-polymerization modification with IPA as determined by FT-IR as a function of: (a) IPA concentration for 16 h, (b) reaction time using 1 eq. IPA.

4.2 Preparation and characterization of surface-grafted (co)polymer brushes

Surface-grafted P(PFPA-*co*-NIPAM) brushes were fabricated by two methods (**See Scheme 3.1** for detail). The first method was based on "grafting from" approach which began with surface-initiated RAFT polymerization of PFPA from initiator-immobilized substrates (having Θ_A/Θ_R of 80.1°/43.7°) that resulted in surface-grafted PPFPA followed by post-polymerization modification with IPA. Formation of the

surface-grafted P(PFPA-*co*-NIPAM) brushes was monitored by water contact angle measurements. As shown in **Figure 4.3**, the water contact angle rapidly decreased from 98°/67° of the surface-grafted PPFPA to 90/56^{o} after 5 min of exposure to 10 mM of IPA. Decreasing of water contact angle continued and reached a minimum of 84/52^{o} after 20 min. The water contact angle remained unchanged despite the longer reaction time suggesting that the PPFPA was completely transformed into PNIPAM. This set of data have demonstrated that the surface-grafted P(PFPA-*co*-NIPAM) brushes with varied PNIPAM composition can be prepared by post-polymerization modification of the surface-grafted PPFPA brushes initially prepared by "grafting from" approach.

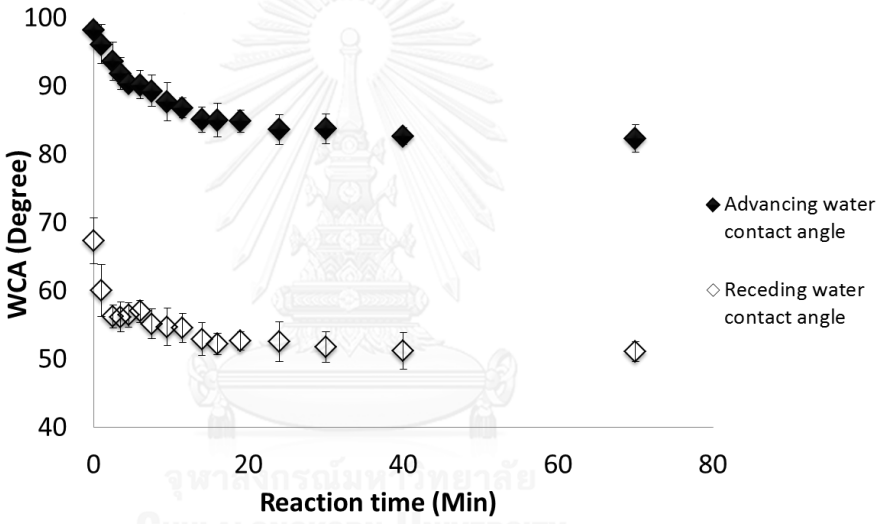


Figure 4.3 Kinetics of post-polymerization modification of the surface-grafted PPFPA brushes prepared by "grafting from" approach after exposure to 10 mM of IPA.

The ability to control the extent of post-polymerization modification of PPFPA by IPA in solution by varying IPA concentration (as shown in **Figure 4.2(a)**) allows for the preparation of P(PFPA-*co*-NIPAM) with varied %PNIPAM via the second method based on "grafting onto" approach. This can be accomplished by a reaction between remaining PFP groups in the copolymers after the first post-polymerization modification with IPA and amino groups on the surface of the

APTES-modified substrates. A series of P(PFPA-*co*-NIPAM) having of 30, 45, 59, 78 %PNIPAM obtained from post-polymerization modification described in section 4.1 were selected for this investigation.

As shown in **Table 4.2**, the surface-grafted P(PFPA-*co*-NIPAM) brushes became more hydrophilic with lower water contact angles as a function of %PNIPAM. Notably, similar water contact angle values were observed for both the surface-grafted PPFPA and PNIPAM prepared by the "grafting onto" method suggesting their comparable surface properties to those prepared by "grafting from" method despite their different preparation methods.

Table 4.2 Water contact angle datas of the surface-grafted (co)polymer brushes prepared by "grafting onto" approach.

Surfaces	**Water contact angle (Degree)**
APTES-modified	84.7/50.4
PPFPA brushes	99.5/64.0
P(PFPA-*co*-NIPAM) brushes	
- 45% PNIPAM	92.4/50.1
- 59% PNIPAM	89.7/46.1
- 78% PNIPAM	82.4/52.3
PNIPAM brushes	77.1/33.9

The surface grafted P(PFPA-*co*-NIPAM) brushes prepared by this "grafting onto" approach were also characterized by FT-IR analysis. The characteristic peaks assigned to ester C=O stretching of PPFPA and amide C=O stretching of PNIPAM appeared at 1786 and 1650 cm^{-1}, respectively. The relative intensity between the former and latter peak became correspondingly decreased as the %PNIPAM in the copolymer increased from 30 to 78%.

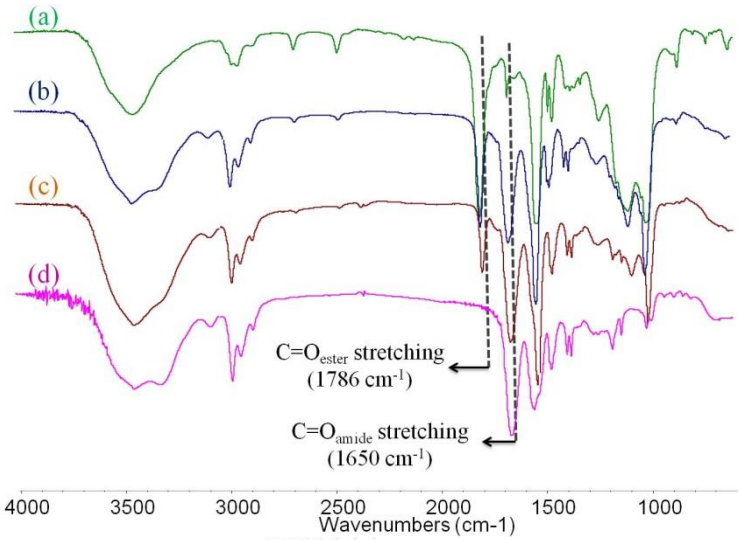


Figure 4.4 FTIR spectra of the surface-grafted (co)polymer brushes prepared by "grafting onto" approach: (a) PPFPA, P(PFPA-*co*-NIPAM) with %PNIPAM of (b) 45 and (c) 78, and (d) PNIPAM.

To identify the varied copolymer composition (PPFPA vs PNIPAM) on the surface-grafted P(PFPA-*co*-NIPAM) brushes, XPS analysis has been performed on two series of substrates:one prepared by the "grafting from" approach and the other prepared by "grafting onto" approach.In the case of "grafting from" approach of which XPS data are shown in **Table 4.3**, fluorine composition correspondingly decreased as a function of reaction time with IPA suggesting the PFP groups were eliminated as a result of post polymerization modification. The decreasing F/N ratio indicated the greater %PNIPAM as the reaction time increased. As anticipated, the %F as well as F/N ratio also decreased as the %PNIPAM in the copolymer used for "grafting onto" the APTES-modified substrates increased.

Table 4.3 Atomic compositionof thesurface-grafted P(PFPA-*co*-NIPAM) brushes prepared by "grafting from" approach by post-polymerization modification of the surface-grafted PPFPA in 10 mM of IPA for different period of time.

Reaction time with IPA (min)	Atomic composition (%)						Atomic ratio
	C	N	O	F	Si	S	F/N
0	61.10	2.59	23.79	5.67	6.39	0.46	2.189
5	60.06	3.66	25.87	2.55	7.41	0.46	0.697
10	61.42	4.77	25.30	1.43	6.78	0.30	0.300
30	61.35	3.95	26.15	0.55	7.6	0.33	0.140
60	58.59	4.93	27.02	0.46	8.43	0.57	0.093

Table 4.4 Atomic compositionof thesurface-grafted P(PFPA-*co*-NIPAM) brushes prepared by "grafting onto" approachfrom P(PFPA-co-NIPAM) obtained from post-polymerization modification of PPFPA with varied equivalence of IPA for 16 h

%PNIPAM	Atomic composition (%)						Atomic ratio
	C	N	O	F	Si	S	F/N
30	62.22	2.31	25.87	1.44	7.69	0.48	0.623
45	56.71	3.15	28.69	1.63	9.00	0.82	0.517
59	61.53	2.64	25.39	1.21	8.84	0.38	0.458
78	54.92	4.03	30.21	1.05	9.08	0.72	0.261

4.3 Determination of graft density and thickness of the surface-grafted polymer brushes

It has been reported by Okano and co-workers [23] that the thickness of surface-grafted PNIPAM exhibits a strong influence on its thermoresponsiveness and therefore determines its success in subsequent application for cell sheet fabrication.

Grafting quantity of PPFPA on glass substrates can be first determined by FT-IR. As the base substrate was glass, a strong adsorption of Si–O was observed at 1000 cm^{-1} and the characteristic peak of PPFPA is C=O appeared in the region of 1786 cm^{-1}. The peak intensity ratio of Si-O and C=O can be used to determine the amount of PPFPA grafted onto surfaces using a calibration curve prepared by a known amount of PPFPA cast on APTES-modified glass surfaces (**Figure 4.5**).

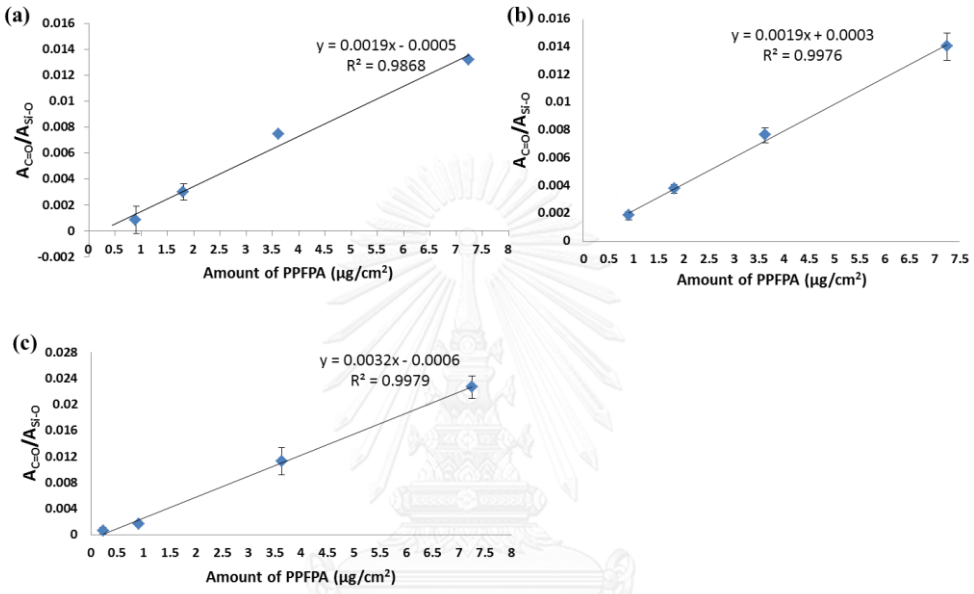


Figure 4.5 Calibration curvesof PPFPA casted on glass substrates for grafted amount determination; DP of (a) 50, (b) 200, and (c) 400.

As outlined in **Table 4.5**, the grafted quantity of PPFPA on the glass surface proportionally increased as a function of molecular weight of PPFPA. Apparently, grafting amount of the surface-grafted PPFPA prepared by “grafting onto” approach was smaller and less dependent on the PPFPA molecular weight than that obtained via “grafting from” approach. The “grafting onto” method is known to yield low graft density. This is mainly because the incoming polymer chains usually suffer entropic barrier due to the crowding of the initially grafted polymer chains that inhibits further insertion of the polymer chains. The limitation becomes more problematic for polymer with high molecular weight. As determined by AFM, the thickness values of the surface-grafted PNIPAM obtained after post-polymerization modification agree

well with the PPFPA grafted amount previously quantified by FT-IR. The greater the grafted quantity of PPFPA yielded thicker PNIPAM layer. The thickness of the PNIPAM can be more broadly and efficiently tuned by the "grafting from" method than the "grafting onto" method.

Table 4.5 Grafting amount of the surface-grafted PPFPA and thickness of the surface-grafted PNIPAM prepared by both "grafting from" and "grafting onto" approach

Targeted DP	Grafted amount of PPFPA[a] (μg/cm^2)		Thickness of PNIPAM[b] (nm)	
	Grafting from	Grafting onto	Grafting from	Grafting onto
50	1.18±0.23	1.28±0.07	3.76±0.09	5.05±0.48
200	3.54±0.20	1.66±0.05	20.89±3.54	8.18±1.45
400	4.48±0.20	1.95±0.14	34.64±7.14	12.50±3.10

[a]estimated by FT-IR analysis

[b]determined by AFM measurements

4.4 Hydrolysis and collagen immobilization of the surface-grafted P(PFPA-*co*-NIPAM)

To highlight the benefit of having active PFP groups available in the grafted copolymer structure that are capable of undergoing tandem post-polymerization modification, two reactions were performed. Firstly, the surface-grafted P(PFPA-*co*-NIPAM) brushes obtained from both "grafting from" and "grafting onto" methods were subjected to hydrolysis to convert the PFP to carboxyl groups. The extent of this subsequent modification was monitored by water contact angle measurements. Data shown in **Table 4.6** indicated that the hydrolyzed surface-grafted P(PFPA-*co*-NIPAM) was more hydrophilic with lower water contact angle than its counterpart, P(PFPA-*co*-NIPAM) implying the presence of more hydrophilic carboxyl groups in the copolymer structure after hydrolysis.It was also found that the grafted P(PFPA-*co*-NIPAM) with greater PPFPA composition would give the copolymer with higher carboxyl group content upon hydrolysis. The second modification which is quite

relevant to the application of this developed thermoresponsive platform for cell sheet fabrication is based on collagen type I immobilization. Collagen type I is known to promote cell adhesion and growth. Upon collagen immobilization, all substrates became highly hydrophilic with advancing water contact angles in a range of 42-67^{o} depending on the initial PPFPA composition. The larger content of active PPFPA apparently allows for more of collagen type I to be immobilized.

Table 4.6 Water contact angledata of thesurface-grafted P(PFPA-*co*-NIPAM) brushes after hydrolysis and collagen immobilization.

Grafting Methods	Conditions	**Advancing Water contact Angle (Degree)**		
		P(PFPA-*co*-NIPAM)	Hydrolyzed P(PFPA-*co*-NIPAM)	Collagen Type I immobilized P(PFPA-*co*-NIPAM)
Grafting From	Reaction time with IPA(min)			
	0	96.49 ± 1.75	84.32 ± 3.72	59.14 ± 6.28
	5	88.05 ± 1.97	73.87 ± 1.99	46.82 ± 6.06
	10	84.20 ± 2.33	74.33 ± 4.46	53.63 ± 2.50
	30	87.40 ± 2.09	68.01 ± 0.69	61.24 ± 4.50
	60	78.33 ± 3.04	71.91 ± 0.33	63.27 ± 7.62
Grafting Onto	% PNIPAM (eq)			
	30	94.07 ± 1.25	54.00 ± 5.31	41.74 ± 3.33
	45	92.36 ± 4.07	63.89 ± 4.40	48.85 ± 1.31
	59	89.43 ± 1.92	57.19 ± 5.59	56.91 ± 1.45
	78	84.80 ± 1.55	77.88 ± 1.92	66.73 ± 0.72

The success of hydrolysis was also confirmed by FT-IR analysis. As shown in **Figure 4.6**, the characteristic peak assigned to ester C=O stretching of PPFPA completely disappeared and replaced by C=O stretching of COOH of PAA at 1710 cm^{-1}. The relative intensity between the peak at 1710 cm^{-1} and amide C=O stretching of PNIPAM became correspondingly decreased as the %PNIPAM in the copolymer increased.

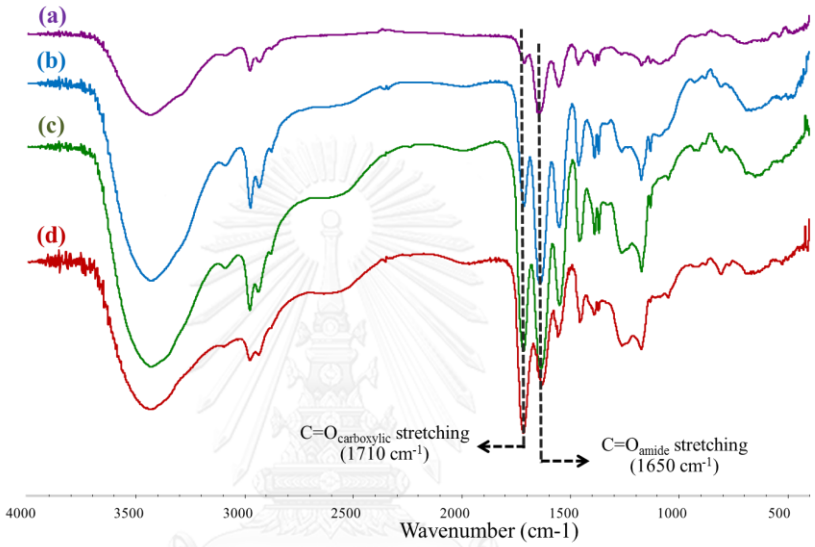


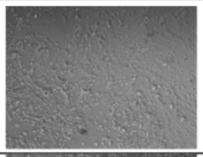
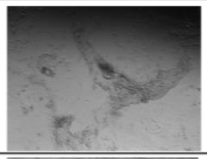
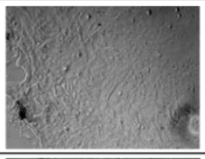
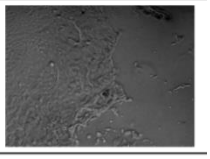
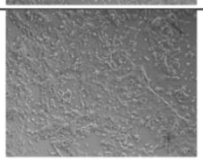
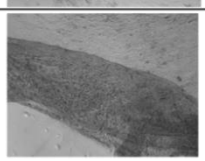
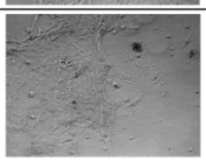
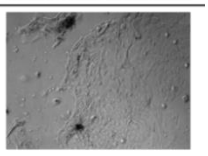
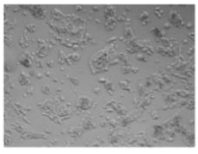
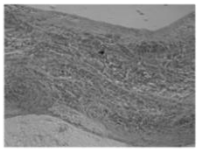
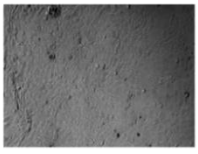
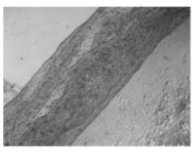
Figure 4.6 FTIR spectra of hydrolyzed P(PFPA-*co*-NIPAM) with %PNIPAM of (a) 78, (b) 59, (c) 45, and (d) 30.

4.5 Cell sheet fabrication

Because the thickness of thermoresponsive brushes is an important factor for cell adhesion and cell detachment properties, different MW of PNIPAM brushes prepared by post-polymerization modification of PPFPA brushes by both "grafting from" and grafting onto" methods were investigated. In cell culture process, the thermoresponsive substrate which is generally based on PNIPAM has to be coated with cell adhesion promotors (CAPs) such as collagen, laminin or fibronectin before cell seeding to promote cell adhesion and growth. After incubated at 37°C, the adherent cells were observed to study cell adhesion property of the grafted polymer brushes. As demonstrated in **Table 4.7**, PNIPAM brushes prepared via "grafting onto" method showed the impressive result for cell adhesion property. Because of the

smaller thickness, keratinocyte cells easier adhere and reach confluency on the surface-grafted PNIPAM brushes prepared via “grafting onto” method (having thickness of 5.1-12.5 nm) than the surface-grafted PNIPAM brushes prepared via “grafting from” method (having thickness of 3.8-34.6 nm). Although all of PNIPAM brushes prepared via “grafting onto” method exhibited the good property for cell adhesion, cell sheet could only be harvested from PNIPAM brushes with DP of 400 (entry 3, last column, Table 4.7). Since the swelling and mobility of the PNIPAM chains on the surface-grafted PNIPAM brushes with DP 50 and 200 having low thickness were limited (5.1 and 8.2 nm, respectively), the adherent cells could not detach from these surfaces as a cell sheet. A similar behavior was also observed for the surface-grafted PNIPAM brushes prepared by “grafting from” approach having DP of 50 with thickness of 3.8 nm. These results strongly suggest that there is a minimum thickness of PNIPAM layer required for this cell sheet harvesting application.

Table 4.7 Microscopic photographs of keratinocyte cells sheet preparation on the surface-grafted PNIPAM brushes with various MW prepared by post-polymerization modification of the surface-grafted PPFPA brushes obtained from “grafting from” and “grafting onto” methods.

DP	Grafting From		Grafting Onto	
	Adhesion	Detachment	Adhesion	Detachment
50				
200				
400				

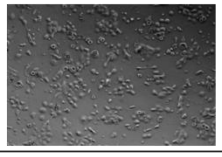
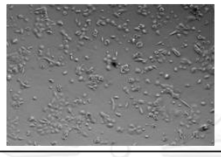
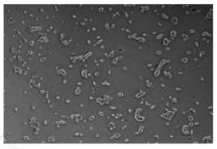
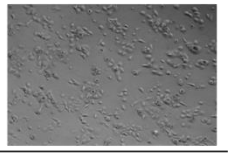
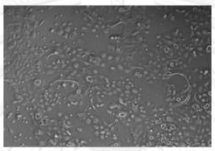
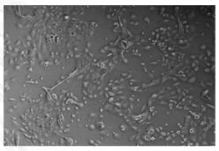
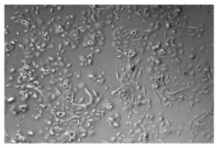
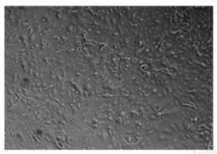
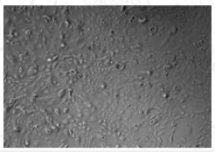
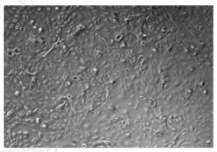
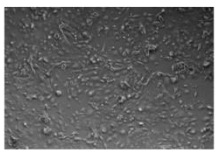
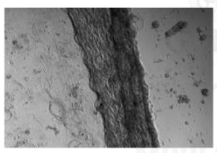
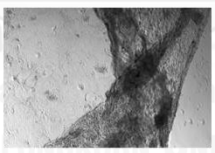
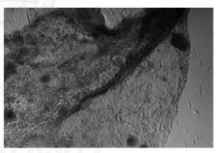
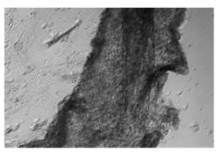
In contrast, the surface-grafted PNIPAM brushes prepared by “grafting from” method showed the good property for cell sheet detachment. Although keratinocyte

cells difficultly adhered and reached confluency when the thickness of PNIPAM increased, the cell sheet can easily detach after low temperature treatment because of the mobility of long PNIPAM chains on the surface-grafted PNIPAM of higher DP with thicker PNIPAM layer. As shown in **Table 4.7**, the surface-grafted PNIPAM brushes with DP of 400 fabricated by "grafting from" and "grafting onto" approaches could be used to provide keratinocyte cell sheets. Therefore PPFPA with DP of 400 was selected for next investigation.

To further investigate the potential use of the developed thermoresponsive platform for cell sheet preparation, the surface-grafted P(PFPA-*co*-NIPAM) brushes with various PNIPAM composition prepared by "grafting from" and "grafting onto" approach were employed for collagen type I immobilization. The active pentafluorophenyl ester groups in the copolymer are readily available for covalent immobilization with collagen type I. Because collagen type I was covalently immobilized on the surface grafted P(PFPA-*co*-NIPAM) brushes, keratinocyte cells could easily adhere without CAPs coating process. As shown in **Table 4.8**, the collagen type I immobilized P(PFPA-*co*-NIPAM) brushes with various PNIPAM composition prepared by "grafting from" method demonstrated that PPFPA brushes immersed in IPA solution with less reaction time showed more amount of adherent cells because there was more collagen type I on the surfaces. Therefore the PPFPA brushes reacted with IPA for 5 minutes that yield P(PFPA-*co*-NIPAM) brushes with highest remaining PFP moieties and thus highest immobilized collagen type I quantity showed the best property for cell adhesion while the smallest amount of adherent cells were observed on the PPFPA surfaces reacted with IPA for 60 min that gave P(PFPA-*co*-NIPAM) brushes with lowest remaining PFP moieties and thus lowest immobilized collagen type I quantity . For cell sheet harvesting step, the keratinocyte cell sheet could be obtained from all of collagen type I immobilized P(PFPA-*co*-NIPAM) substrates but it is difficult for cell sheet to detach from the PPFPA brushes reacted with IPA for 5 minutes . Moreover, the residual cells were observed on cell culture substrates because thermoresponsive property of substrates was limited by a little PNIPAM composition in the copolymer. PPFPA brushes reacted with IPA for the longer reaction time provided more composition of PNIPAM in copolymer. Thus

cell sheet could be easily harvested without the residual cells from the PPFPA brushes reacted with IPA for 60, 30 and 10 min, respectively.

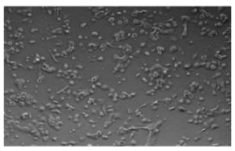
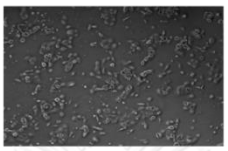
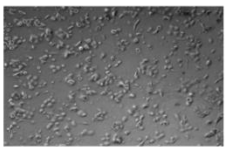
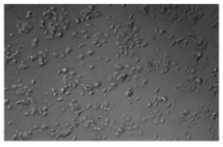
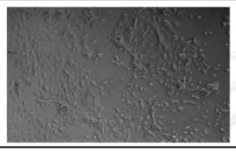
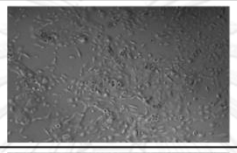
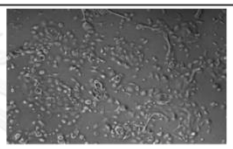
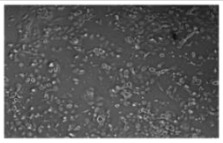
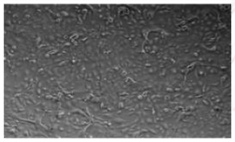
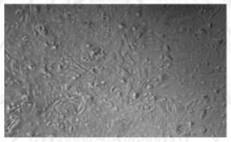
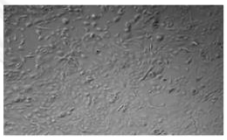
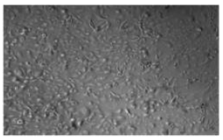
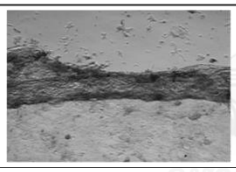
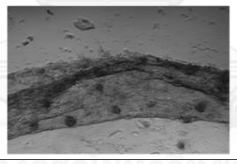
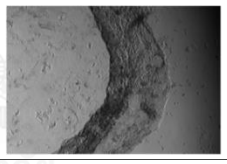
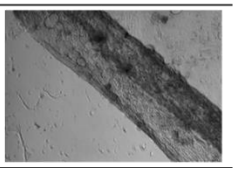
Table 4.8 Microscopic photographs of keratinocyte cells adhesion and detachment on collagen type I immobilized P(PFPA-*co*-NIPAM) brushes having varied %PNIPAM fabricated via "grafting from method".

	Reaction time in 10mM IPA			
	5 min (77 %PNIPAM)	10 min (90 %PNIPAM)	30 min (N/A)	60 min (N/A)
Day1				
Day2				
Day3				
cell sheet				

The similar results were observed from the collagen type I immobilized P(PFPA-*co*-NIPAM) brushes prepared by "grafting onto" method (**Table 4.9**). Thermoresponsive substrates containing less %PNIPAM composition exhibited the ability to promote cell adhesion property but cell sheet detachment process was slow. On the other hand, the substrates containing more % PNIPAM composition showed the detachment of cell sheet in a short time after low temperature treatment. As shown in **Table 4.9**, there are more adherent cells on collagen type I immobilized P(PFPA-NIPAM) with 30% PNIPAM surfaces than copolymer surface with 45, 59 and 78% PNIPAM, respectively. Although the adhesion of cells on the surface-grafted

P(PFPA-*co*-NIPAM) with 78% PNIPAM surfaces was slow, the detachment of cell sheet rapidly occurred without residual cells on substrates.

Table 4.9 Microscopic photographs of keratinocyte cells adhesion and detachment on collagen type I immobilized P(PFPA-*co*-NIPAM) brushes fabricated via "grafting onto method".

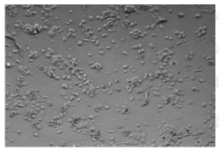
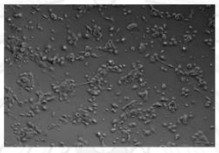
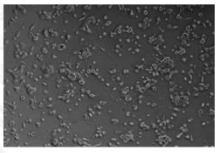
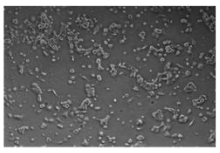
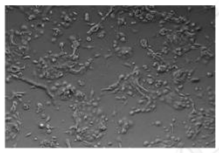
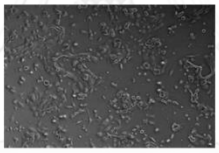
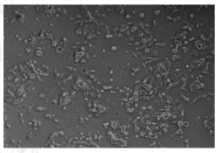
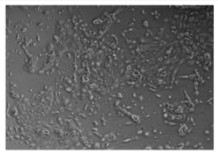
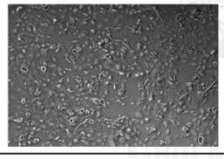
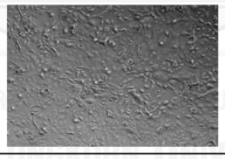
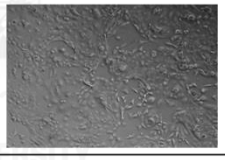
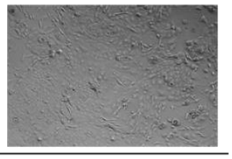
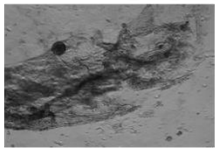
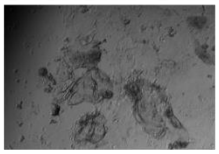
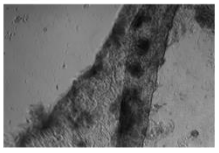
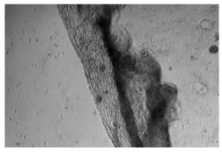
	P(PFPA-*co*-NIPAM) with 30% PNIPAM	P(PFPA-*co*-NIPAM) with 45% PNIPAM	P(PFPA-*co*-NIPAM) with 59% PNIPAM	P(PFPA-*co*-NIPAM) with 78% PNIPAM
Day1				
Day2				
Day3				
cell sheet				

This study indicated that the amount of collagen type I immobilized on copolymer surface through active PFP groups, improved cell adhesion property of the thermoresponsive surfaces. However, the % PNIPAM composition in copolymer was also important for cell sheet detachment after temperature reduction.

In addition, the resulting P(AA-*co*-NIPAM) brushes with various % PAA in copolymer obtained from hydrolysis of P(PFPA-*co*-NIPAM) were investigated to study the effect of hydrophilic groups for cell sheet preparation. The hydrolyzed P(PFPA-*co*-NIPAM) brushes prepared by "grafting from" and "grafting onto" method showed poor property of cell adhesion (**Table 4.10-4.11**). The process of cell growth and confluency reaching spent too much time when compared with the collagen type I immobilized P(PFPA-NIPAM) surfaces. For the P(AA-*co*-NIPAM) brushes prepared via "grafting from" method, the composition of PAA in copolymer was controlled by

reaction time of PPFPA brushes with IPA solution. Thus the surface-grafted PPFPA brushes reacted with IPA for a short time provided high density of PAA in the copolymer surfaces. The difficulty of cell adhesion and spreading is shown in **Table 4.10** and the hydrolyzed P(PFPA-*co*-NIPAM) brushes obtained from reaction of PPFPA with IPA for 5 min showed the worst cell adhesion property.

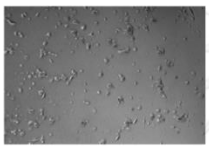
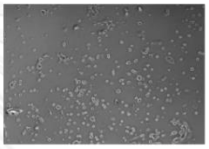
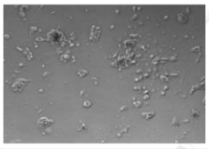
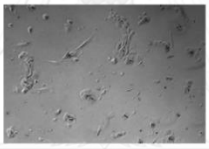
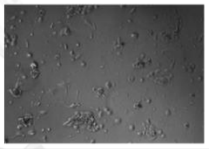
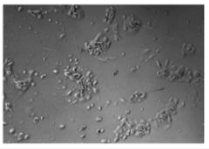
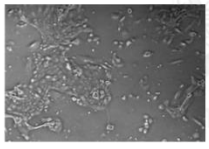
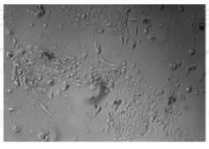
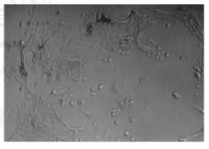
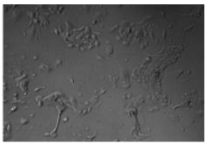
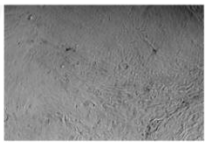
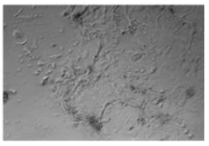
Table 4.10 Microscopic photographs of keratinocyte cells adhesion and detachment on hydrolyzed P(PFPA-*co*-NIPAM) brushes fabricated via "grafting from" method.

	Reaction time in 10mM IPA			
	5 min (77 %PNIPAM)	10 min (90 %PNIPAM)	30 min (N/A)	60 min (N/A)
Day1				
Day4				
Day6				
cell sheet				

For the hydrolyzed copolymer brushes obtained from reaction of the surface-grafted PPFPA with IPA for 60 min, cells could easier adhered and spread on the surfaces because most of the copolymer content is PNIPAM. In this experiment, the keratinocyte cell sheet was obtained from the copolymer brushes prepared by reaction of PPFPA in IPA solution with reaction time 30 and 60 min only because there was enough PNIPAM composition to perform thermoresponsive property. However, detachment of adherent cell on the other surfaces could be observed but the adherent

cell could not detach as a cell sheet. Hydrolyzed P(PFPA-*co*-NIPAM) brushes prepared via "grafting onto" method were investigated for cell sheet preparation and the result shown in **Table 4.11** demonstrated that cell sheet couldn't detach from all % PAA composition of copolymer brushes. Although keratinocyte cells could adhere onto the substrates, it couldn't detach after low temperature treatment because the "grafting onto" method provided low polymer film thickness.

Table 4.11 Microscopic photographs of keratinocyte cells adhesion and detachment on hydrolyzed P(PFPA-*co*-NIPAM) brushes fabricated via "grafting onto method".

	P(PFPA-*co*-NIPAM) with 30% PNIPAM	P(PFPA-*co*-NIPAM) with 45% PNIPAM	P(PFPA-*co*-NIPAM) with 59% PNIPAM	P(PFPA-*co*-NIPAM) with 78% PNIPAM
Day1				
Day4				
Day6				
cell sheet				

CHAPTER V

CONCLUSION AND SUGGESTIONS

PPFPA with well-controlled molecular weight was synthesized by RAFT polymerization. As monitored by FT-IR, post-polymerization modification of PPFPA yielded P(PFPA-*co*-NIPAM) of which PNIPAM composition can be varied as a function of both IPA concentration and reaction time.

Surface-grafted P(PFPA-*co*-NIPAM) brushes were fabricated by two methods. The first method relied on "grafting from" approach, involving surface-initiated RAFT polymerization of PFPA from initiator-immobilized substrates followed by post-polymerization modification with IPA. The second method was based on "grafting onto" approach which was accomplished by a reaction between PFP groups in P(PFPA-*co*-NIPAM) previously obtained from post-polymerization modification of PPFPA and amino groups on the surface of the APTES-modified substrates. The success of grafting by both methods was verified by contact angle measurements, FT-IR and XPS analyses. Results from FT-IR and AFM analyses indicated that the "grafting onto" approach gave the surface-grafted polymer brushes with lower grafted amount and thickness than those prepared by "grafting from" approach.

The remaining PFP units on the surface-grafted P(PFPA-*co*-NIPAM) brushes prepared by both methods were readily available for tandem post-polymerization modification. Hydrolysis of the surface-grafted P(PFPA-*co*-NIPAM) brushes yielded surface-grafted P(AA-*co*-NIPAM) brushes. Whereas the attachment of collagen type I to the surface-grafted P(PFPA-*co*-NIPAM) brushes can be effectively done without having to use coupling agent.

An optimum targeted DP that gave surface-grafted PNIPAM having the ability to prepare keratinocyte cell sheet was 400. Both hydrolyzed- and collagen type I-immobilized surface-grafted P(PFPA-*co*-NIPAM) brushes with the same targeted DP prepared by both "grafting from" and "grafting onto" were also tested for their applicability for cell sheet preparation. It was found that cell adhesion property was proportionally improved on collagen type I immobilized P(PFPA-*co*-NIPAM) as a

function of %PPFPA composition. On the other hand, the substrates containing lower %PPFPA composition showed better characteristic for cell detachment. While the hydrolyzed surface-grafted P(PFPA-*co*-NIPAM) brushes with high %PPFPA were not suitable for cell sheet preparation because their highly hydrophilic surfaces resisted the adhesion and proliferation of cells. This study has demonstrated that multifunctional thermoresponsive platform for cell sheet fabrication can be developed from the surface-grafted PPFPA brushes via the "grafting from" and "grafting onto" approach.

For future work, it is necessary to quantitatively determine the number of adhered cells on surfaces at different incubation time as well as cell adhesion and detachment profile of keratinocyte cells on the developed substrates.

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APPENDIX

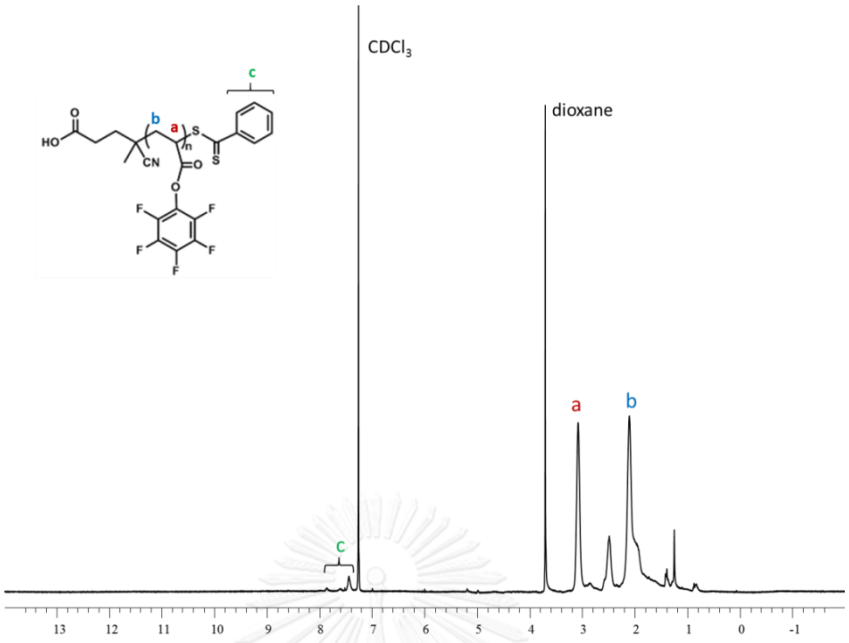

Figure A-1. ^{1}H NMR spectrum of poly(pentafluorophenyl acrylate) (PPFPA) with DP 50.

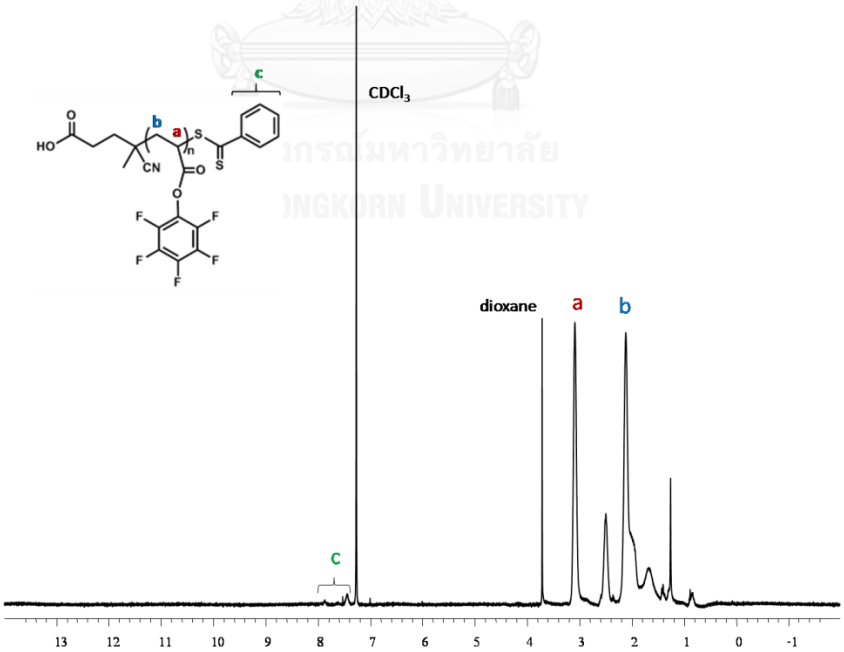

Figure A-2. ^{1}H NMR spectrum of poly(pentafluorophenyl acrylate) (PPFPA) with DP 200.

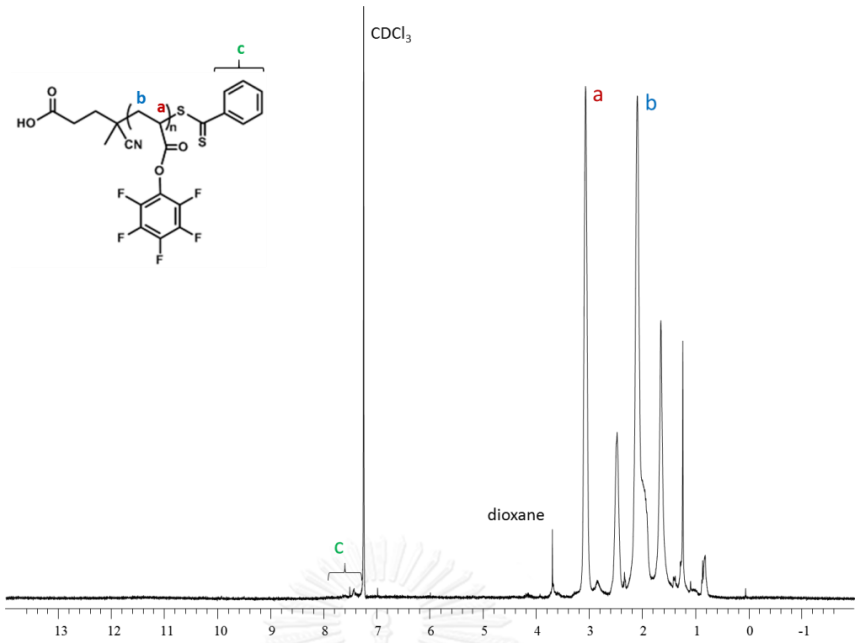

Figure A-3. ^{1}H NMR spectrum of poly(pentafluorophenyl acrylate) (PPFPA) with DP 400.

VITA

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