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BACTERIAL IMPRINTED POLYMER: INNOVATION FOR *LEPTOSPIRA* DIAGNOSTIC KIT

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Applied Polymer Science and Textile Technology
Department of Materials Science
Faculty of Science
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Thesis Title	BACTERIAL IMPRINTED POLYMER: INNOVATION FOR *LEPTOSPIRA* DIAGNOSTIC KIT
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พอลิเมอร์ลอกแบบโมเลกุลเป็นฟังก์ชันนอลพอลิเมอร์ที่ได้รับความสนใจในงานวิจัยจำนวนมาก เนื่องจากสมบัติในการจดจำและจับกับโมเลกุลที่ถูกลอกแบบลงบนพอลิเมอร์นั้นๆ ในงานวิจัยนี้จึงมีความสนใจที่จะท้าทายกระบวนการลอกแบบโดยใช้แบบทีเรียที่มีรูปร่างซับซ้อน ดังนั้นเราจึงใช้แบคทีเรียก่อโรคฉี่หนูเป็นต้นแบบ เนื่องจากแบคทีเรียก่อโรคฉี่หนูมีลักษณะเฉพาะเป็นเกลียว พรีพอลิเมอร์ของพอลิเมทิลเมทาคริเลตโคไฮดรอกซีเอทิลเมทาคริเลตถูกสังเคราะห์สำเร็จด้วยโฟโตพอลิเมอไรเซชันโดยใช้เบนโซฟีโนน และไตรเอทิลลามีน ภายใต้การฉายแสงยูวี ($\lambda \approx$ 250 นาโนเมตร) จากนั้นพรีพอลิเมอร์ที่ได้ถูกหมุนเคลือบลงบนกระจกปิดสไลด์ และถูกประทับด้วยแม่แบบแบคทีเรียก่อโรคฉี่หนูแล้วนำไปฉายด้วยแสงยูวีเป็นเวลา 24 ชั่วโมงเพื่อให้ได้ฟิล์มพอลิเมอร์ลอกแบบ หลังจากนั้นฟิล์มพอลิเมอร์ลอกแบบที่ได้ถูกล้างด้วยสารละลายโซเดียมไฮดรอกไซด์ความเข้มข้นร้อยละ 3 โดยน้ำหนักภายใต้คลื่นเสียงความถี่สูงเพื่อกำจัดแม่แบบออกจากแผ่นฟิล์ม ฟิล์มพอลิเมอร์ลอกแบบที่ได้ถูกศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราดและกล้องจุลทรรศน์แรงอะตอม จากผลการศึกษาพบว่าแบคทีเรียก่อโรคฉี่หนูถูกลอกแบบลงบนพื้นผิวของแผ่นฟิล์มพอลิเมอร์โดยยังคงโครงสร้างที่เป็นเกลียวของแบคทีเรียไว้ได้

ภาควิชา	วัสดุศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา	วิทยาศาสตร์พอลิเมอร์ประยุกต์และเทคโนโลยีสิ่งทอ	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SURAPHUN AUNGWEROJANAWIT: BACTERIAL IMPRINTED POLYMER: INNOVATION FOR *LEPTOSPIRA* DIAGNOSTIC KIT. ADVISOR: ASST. PROF. WANPEN TACHABOONYAKIAT, Ph.D., CO-ADVISOR: ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D., ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., pp.

Molecular imprinted polymer is a functional polymer that has gained attention in many researches due to its abilities to recognize and bind the molecules which are imprinted onto the polymer. In this study, we aimed to challenge the imprinting technique using more complex morphology of bacteria. Thus, we used *Leptospira* as the template, since; *Leptospira* has a unique spiral structure. Prepolymer of poly (methyl methacrylate)-co-(hydroxyethyl methacrylate) was successfully synthesized via photopolymerization using benzophenone and triethylamine under UV radiation ($\lambda \approx 250$ nm). The prepolymer was then spin-coated onto the glass coverslip and stamped with *Leptospira* template then cured under UV light for 24 hour to obtain the imprinted polymer (IP) film. IP film was then washed with 3%(w/v) sodium hydroxide solution under sonication to remove the bacterial template. The IP film was observed by field emission electron microscope and atomic force microscope. The results showed that *Leptospira* were successfully imprinted onto the polymer film surface and maintain the spiral structure of the bacteria.

Department:	Materials Science	Student's Signature .....
Field of Study:	Applied Polymer Science and Textile Technology	Advisor's Signature .....
		Co-Advisor's Signature .....
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Chapter 1
Introduction

1.1 Introduction

Leptospirosis is a well-known zoonosis disease which is caused by spirochaete bacteria called *Leptospira.* Carriers of the bacteria can be wild animals or domestic animals. Leptospirosis can be spread via the carriers' urine. The infection of the disease in human can occur by directly contact with the diseased animals or by contact with the water or mud that contaminated with the bacteria [1, 2]. The diagnosis of the leptospirosis can be done in various ways such as observing under microscopy (dark field microscopy, immunofluorescence), cell culture, serology or serodiagnosis by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) and molecular diagnosis by polymerase chain reaction (PCR) [3]. However, these methods are having some disadvantages such as low sensitivity as in microscopy method, time consuming and complicated procedure [4]. Thus, we interested in develop the diagnosis method to be more efficiency and easy to use by using molecular imprinted polymers (MIPs) as a screening materials.

MIPs are functional polymers that function as biorecognition system which have an ability to recognize the molecule, hence, fabrication of the MIPs become potential materials to be developed as a stable biomimetic sensor [5] that can separate and distinguish the substances or other usage in drug delivery system [6]. MIPs are able to bind or trap the substances without creating the covalent bonds between polymers and the substances [7]. The fabrication of MIPs can be done using composition of monomer, initiator, crosslinker and template. The polymerization process will bind the template on the polymer surface. The removal of the template will leave imprinted pattern of the template on polymer surface which is the

effective sites that can rebind the molecules similar to the template [8]. However, the efficiency of the MIPs still has a challenging in sizes, structures and sensitivity of the template. Thus, there are many studies that tried to imprinted various type of molecule range from small to large molecules, for example, glycosides, steroids, vitamins, drugs, proteins and even viruses or bacteria [7].

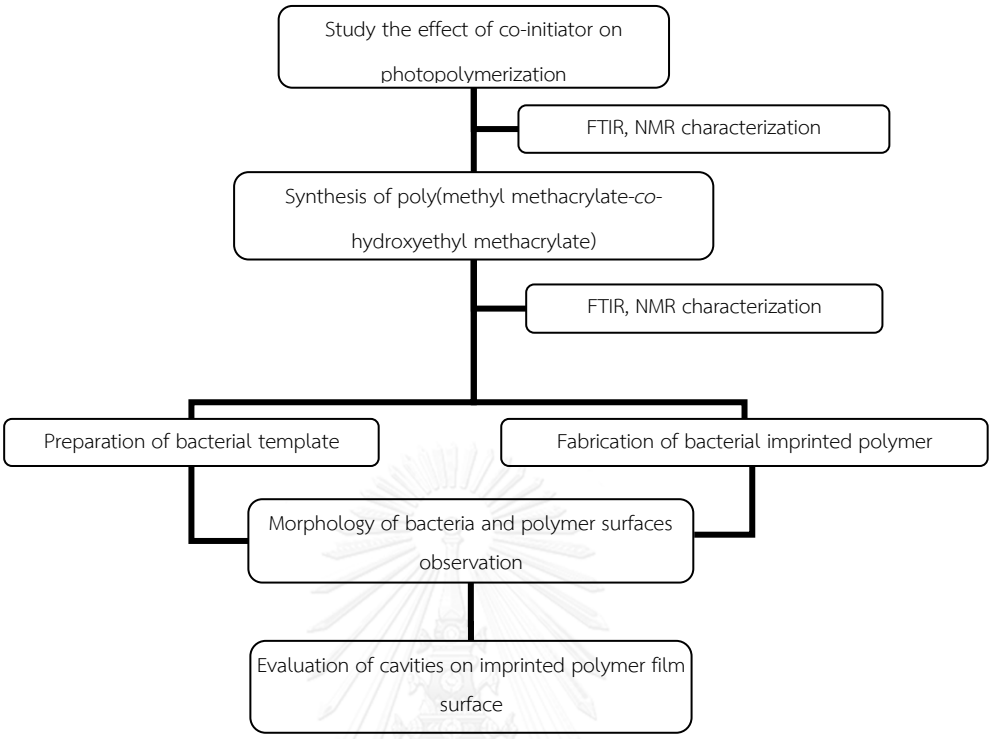
In our study, we intended to use MIPs as a new method for *Leptospira* detection using the ability of MIPs which can separate and distinguish the substances or molecules. MIPs can be prepared by imprinting the *Leptospira* onto the polymer surface. The morphology of imprinted polymer surface was observed with field emission scanning electron microscope (FE-SEM) and atomic force microscope (AFM). The condition to fabricate polymer matrix was also studied to indicate the proper method for preparing MIP.

1.2 Objectives

1. To study the proper condition of photopolymerization of poly(methyl methacrylate-*co*-hydroxyethyl methacrylate) with benzophenone and triethylamine

2. To investigate the feasibility of fabrication of molecular imprinted polymer for bacteria having complex morphology

1.3 Experiment flow chart



Chapter 2
Literature Review

2.1 Leptospirosis

Leptospirosis is a well-known zoonosis (the disease that can be transferred from animal to human) which causes by bacteria called *Leptospira* spp. Leptospirosis can be spread through the carriers' urine both from wild and domestic animals. Human can be infected by directly contact with the bacteria or contact with the contaminated mud, soil or water [1, 3]. Clinical symptoms can be various and easily misdiagnosed as aseptic meningitis, hepatic disease, influenza or unknown origin fever [2, 9]. Therefore, medical examinations are mostly based on laboratory tests. There are many common methods for leptospirosis diagnosis such as dark field microscopy and immunofluorescence which are direct examination that cheap and easy methods but taking a risk of false positive results. Since; the observation needs the specialists. The result errors might come from interfering of cell debris and other artifacts. Also, it is difficult to distinguish the pathogenic bacteria from non-pathogenic bacteria [4, 10]. Microscopic agglutination test (MAT) is one of the serological diagnosis relies on antibody detection. MAT is performed by incubating patient serum with various serovars of leptospires and observed the agglutination. Serovars or serotypes are bacterial classification in each species from their found antigen. The serovar that reacts with patient serum is supposed to be the infecting serovar. [11, 12]. The other method is polymerase chain reaction (PCR) which is one of the gene amplification methods. PCR enables the amplification of specific DNA fragment using DNA primers as templates which specify DNA that needed to be amplified. The PCR system requires primers, nucleotides and DNA polymerase in order to amplify the DNA [13, 14]. PCR is sensitive, specific and rapid technique which

has been successfully in detection of various microorganisms relies on specific DNA detection.

2.2 Molecular imprinted polymers (MIPs)

Molecular imprinted polymers or MIPs are functional polymers which are used for biorecognition system due to their ability to be able to trap the molecules, which have the same shape as their cavity on polymer surfaces, without having covalent bond between the polymer and the molecules (Fig. 2.1). MIPs can be achieved by using monomer, initiator and crosslinker. The template will be stamped on to the prepolymer matrix and then polymerized. The removal of template will leave cavities onto the polymer matrix's surface [5, 7, 8]. There have been many studies used small molecules as the template such as glycoside [15], steroids [16, 17], or drugs [18, 19]. There are also some studies that used macromolecules such as proteins [20-22], viruses [23] or bacteria [24] as the template.

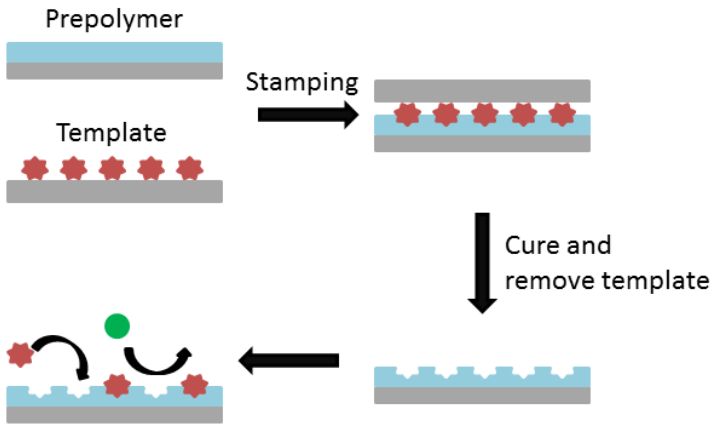


Figure 2.1 Molecular imprinted polymer

In Wangchareunsuk et al. study [25], they tried to develop MIPs to be able to detect Influenza A virus using copolymer of acrylamide, methacrylic acid, methyl methacrylate, and vinyl pyrrolidone. *N,N*-(1,2-dihydroxyethylene) was used as

crosslinker and 2,2'-azobis(isobutyronitrile) was used as an initiator. The pre-copolymer was first prepared by heating at 70°C for 40 minutes. The template stamp was prepared by dropping of influenza A virus cell suspension onto glass plate and keeping for sedimentation at 4°C for 30 minutes. Excess media was spun off from the glass plate. The prepared pre-copolymer was spin-coated on quartz crystal microbalance (QCM) electrodes. The template was then stamped onto the spin-coated prepolymer, then polymerized under UV light overnight. The template was removed from polymer surface, then washed with 10% hydrochloric acid and stirred in water at 45°C for 3 hrs. to remove cells. The cavities were found on the surface of imprinted polymer in the range of 80-120 nm which was the same size of influenza A virus. The sensitivity test was measured by observing the changes in frequency of oscillation characteristic of quartz crystal, since, the frequency has a linear relationship with the mass that absorbed onto the crystal surface. The QCM's result showed that the imprinted polymer had much higher frequency change than non-imprinted polymer. The frequency change ratio between imprinted polymer and non-imprinted polymer was at least five times. It could be concluded that the imprinted polymer had higher ability to trap virus cell than non-imprinted polymer. The selectivity test was also measured by frequency change of QCM by using various subtypes of influenza A such as H1N3, H5N3, H1N1, and H6N1. The result showed that the imprinted polymer had the highest selectivity to the subtype of virus that used as the template.

In the other study of Schirhagl et al. [26], three types of cyanobacteria, Synechococcus OS-B' (Syn OS-B'), Synechococcus elongates PCC 7942 (Syn 7942), and Synechocystis PCC 6803 (6803), were used as the templates to make a MIPs. Poly(dimethylsiloxane) (PDMS) was used as the polymer matrix for imprinting. In this study, they also interested in effect of cell cavities alignment to the binding ability of imprinted polymer; thus, random alignment and defined alignment of cells were

prepared. For random alignment of cells, template stamp was prepared by spreading cell suspension onto microscope slide and maintaining at 4°C for 30 minutes and then spinning off to remove excess buffer. For defined alignment of cells, microscope slide was coated with 0.01% polylysine solution and attached with temporary channel. A directional flow of cell suspension was employed. The excess suspension and the channel were removed to obtain the well-aligned cell template. Imprinted polymer was prepared by using PDMS diluted with cyclohexane and spin-coated on the microscope slide. The coated microscope slide was then pre-cured at 80°C for 4 minutes. The cell template was stamped onto the prepolymer and finally cured at room temperature overnight. Imprinted polymer was washed with distilled water and sonicated for 5 minutes to remove the cells off. The cell morphological cavities were then generated onto imprinted polymer surface. The result from atomic force microscopy (AFM) showed rod-shape cavities on polymer surface. The imprinted polymer can be reused by wash with 0.01% polylysine solution. Accumulation of cells on the imprinted polymer surface was tested. The cell numbers were counted by using the combination of charge-coupled device (CCD) camera and inverted microscope. It was found that cells were accumulated on MIP surface and saturated after reach the given volume at around 500 µl with $2x10^4$ cells. From selectivity tests, imprinted polymer had higher ability to capture the cells that used as template than others cells about 3-4 times. By mixing two cell suspensions together, the result also showed that imprinted polymer had ability to distinguish one kind of cells from another. The alignments of cell cavities also affect to capture ability of imprinted polymer. The MIP with alignment of cell imprinted parallel to the flow showed the highest ability to capture the cells, whereas that of with alignment of cell imprinted perpendicular to the flow showed lowest ability to capture the cells.

2.3 Photopolymerization of benzophenone and co-initiator

Polymerization can be divided into various type depending on the mechanism of polymerization [27].The easiest way to fabricate polymer matrix for imprinting polymer is the use of photopolymerization. Photopolymerization is a polymerization system that can be initiated by light (photoinitiate). Normally, monomers and oligomers are not sensitive to light exposure, thus, the presence of photoinitiator is required. However, in some case, monomers or oligomers are modified with specific structure that can absorb light [28].

Benzophenone or diphenylmethanone (BP) is a common and easy-to-find type II phototoinitiator (Fig. 2.2) that has been used in various applications such as flavor ingredient, fragrance enhancer, perfume fixative and UV curing agent [29].

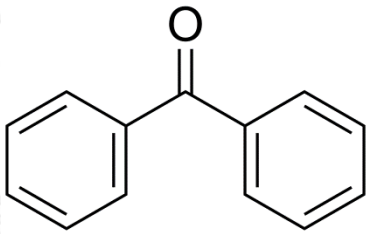


Figure 2.2 Chemical structure of benzophenone

BP can be initiated into radical species by light at 350 nm or lower with maximum absorption around 250 nm. However, as a type II photoinitiator, BP cannot directly initiate monomer in polymerization process, thus, co-initiator is required. BP cannot directly initiate monomer due to its sterical hindrance and delocalization of electron from phenyl group. Nevertheless, BP can receive hydrogen radical from co-initiator such as ether, amine, alcohol or thiol, thus, reactive center is generated on co-initiator instead [30, 31].

Triethylamine or *N,N*-diethylethanamine (NEt_3) (Fig. 2.3) is an alkyl amine that has been used as solvent, catalyst or source of amine and acid-binding agent for chemical reaction [32].

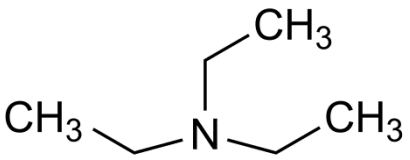
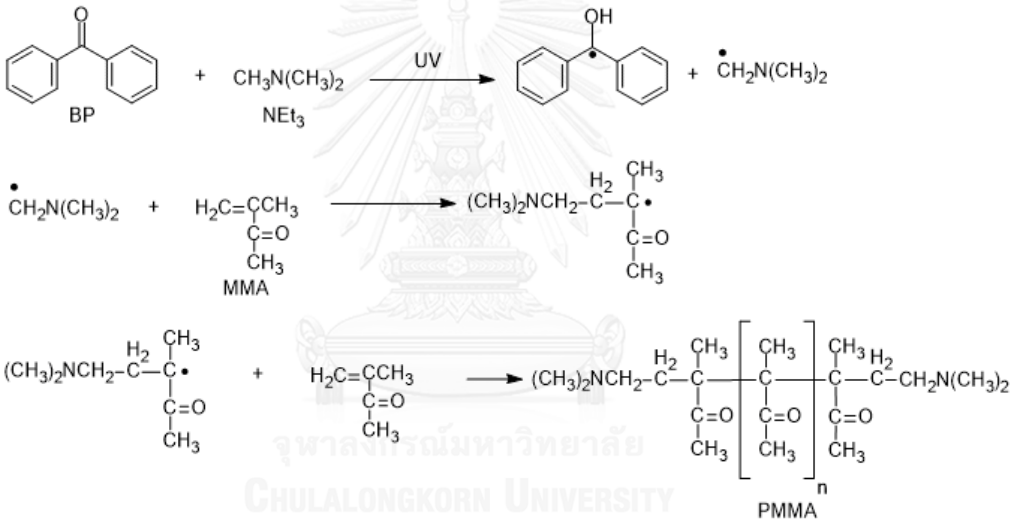


Figure 2.3 Chemical structure of trimethylamine

NEt_3 has been used widely as a co-initiator for type II photoinitiator. Photoinitiator can abstract proton from NEt_3 and generate the radical species on NEt_3 instead. The mechanism between type II photoinitiator (benzophenone) and NEt_3 has been purposed by Temel et al., Sandner et al. and Erjian et al. [31, 33, 34] as shown in Scheme 2.1



Scheme 2.1 Mechanism of BP/NEt_3 in photopolymerization

2.4 Methyl methacrylate and 2-Hydroxyethyl methacrylate monomers

In this study, methyl methacrylate and 2-hydroxyethyl methacrylate monomers were selected to fabricate as a polymer matrix for imprinting process. Methyl methacrylate or methyl 2-methylprop-2-enoate (MMA) is one of a common vinyl ester monomer (Fig. 2.4) that has been used to synthesize poly(methyl methacrylate) (PMMA).

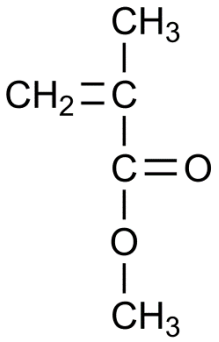


Figure 2.4 Chemical structure of methyl methacrylate monomer

PMMA has a high transparency, acceptable photo stability with good mechanical properties and also has resistance toward acid and alkaline hydrolysis. Moreover, PMMA is also inert to chemical reaction [35, 36]. PMMA can be used in various fields of applications such as optical [37], drug releases [38], screening and separations [39, 40].

2-Hydroxyethyl methacrylate or 2-hydroxyethyl 2-methylprop-2-enoate (HEMA) is also one of the common vinyl ester monomer used to synthesize poly(2-hydroxyethyl methacrylate) (PHEMA). PHEMA has high transparency and clarity with good mechanical properties similar to PMMA [41, 42].The most outstanding property of PHEMA is its hydrophilicity which mainly originated from hydroxyl group in its structure (Fig. 2.5).

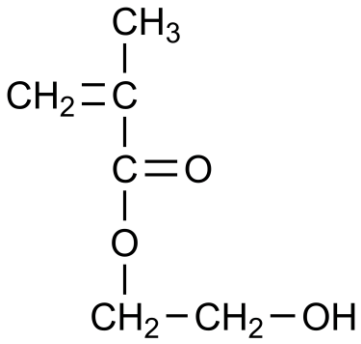


Figure 2.5 Chemical structure of 2-hydroxyethyl methacrylate monomer

PHEMA has been used in many fields of applications such as dentistry [43], immobilization of biomolecules [44, 45], drug delivery system [46] while the main application of PHEMA is optical lenses [42].

In addition, copolymer between MMA and HEMA was synthesized to reach the desirable properties since MMA has higher modulus while HEMA has higher elongation. Also, HEMA can lower glass transition temperature of MMA which result in more flexible in poly(MMA-*co*-HEMA) [47]. Thus, in this study, we interested to find the proper condition for copolymerization via photopolymerization between MMA and HEMA with BP as initiator and NEt_3 as co-initiator. Polymerization process will be carried under UV radiation and nitrogen atmosphere to produce prepolymer that appropriate to be used as the polymer matrix for imprinting the molecule.

Chapter 3
Experiments

3.1 Materials and equipment

Methyl methacrylate (MMA) monomer, hydroxyethyl methacrylate (HEMA) monomer and benzophenone (BP) were purchased from Sigma Aldrich (Darmstadt, Germany). Triethylamine (NEt_3) was bought from Fluka (Belgium). Formalin was purchased from Sigma Aldrich (Darmstadt, Germany). Polyethylene terephthalate (PET) films were purchased from Thai Kolon Co., Ltd (Bangkok, Thailand). Round-coverslips (10mm diameter) were purchased from Menzel (Braunschweig, Germany). UV lamp (Germicidal lamp, λ_{max}=254 nm) was bought from narwar City's Spark Group Company Limited (CSG) (Bangkok, Thailand). *Leptospira interrogans* serovar Pomona bacteria (strain from Khon kaen University) were received from Department of Microbiology, Faculty of Medicine, Chulalongkorn University. All of the chemicals were used without further purification.

3.2 Experiments

3.2.1 Study of the effect of co-initiator on photopolymerization of MMA and HEMA by bulk polymerization

To study the effect of NEt_3 on photopolymerization, MMA and HEMA monomer were used at mole ratio of 1:1 (1.07 ml (10 mmol) : 1.22 ml (10 mmol)). With total monomers of 20 mmol, 90 mg (0.5 mmol, 2.5 %mole/mole of total monomers) and 360 mg (2 mmol, 10% mole/mole of total monomers) of benzophenone were added and followed by addition of triethylamine at 0, 0.5, 1, 1.5, 2 equivalent mole of benzophenone respectively (Table 3.1). The mixtures were purged with nitrogen gas for 1 min and then polymerized under UV light for 5 hrs. The products were obtained by precipitation in hexane.

Table 3.1 Amounts of initiator and coinitiator used in photopolymerization with 1:1 mole ration between MMA and HEMA

Run No.	Mole ration of monomers	MMA:HEMA (ml)	BP(mg)	NEt_3(µl)
1	50:50	1.07:1.22 (10:10 mmol)	90 (0.5mmol)	0
2				35 (0.25 mmol)
3				70 (0.5 mmol)
4				105 (0.75 mmol)
5				140 (1.0 mmol)
6			360 (2.0mmol)	0
7				280 (2.0 mmol)
8				560 (4.0 mmol)

3.2.2 Synthesis of poly(MMA-co-HEMA)

The amounts of BP and NEt_3 were chosen from the previous experiment at mole ratio of 1:2 (0.5:1 mmol) and added into monomer mixtures. Mole ratios between MMA and HEMA were varied as follows 100:0, 95:5, 90:10, 85:15, 80:20 and 0:100 with total amount of monomer at 20 mmol as shown in the Table 3.2. The mixtures were purged with nitrogen gas for 1 minute and then polymerized under UV light for 5 hrs. The products were obtained by precipitation in hexane.

Table 3.2 Amounts of monomers used in photopolymerization

Run No.	Mole ratio of monomer	MMA		HEMA		BP(mg):NEt3(ul)
		mmol	ml	mmol	ml	
9	100:0	20.0	2.13	0	0	90:140 (0.5:1 mmol)
10	95:5	19.0	2.04	1.0	0.13	
11	90:10	18.0	1.92	2.0	0.25	
12	85:15	17.0	1.81	3.0	0.37	
13	80:20	16.0	1.71	4.0	0.49	
14	0:100	0	0	20.0	2.43	

3.2.3 Preparation of *L. interrogans* template

L. interrogans suspension at concentration of 1×10^8 cells/ml was fixed with 2.5 %(v/v) formalin in phosphate buffer saline solution for 2 hrs. Fixed cell suspension was centrifuged to remove formalin solution. The fixed cell was washed and redispersed with absolute ethanol. The cell suspension in ethanol was drop onto PET film (10x10 mm) thoroughly at 20 µl ($2x10^6$ cells), 40 µl ($4x10^6$ cells), 60 µl ($6x10^6$ cells) and 80 µl ($8x10^6$ cells), dried at room temperature and used as bacterial cell templates.

3.2.4 Fabrication of *L. interrogans* imprinted polymer

Prepolymerization of poly(MMA-*co*-HEMA) was prepared with the monomer mole ratio of MMA : HEMA of 90:5. Briefly, 2.04 ml (19 mmol) of MMA monomer and 0.13 ml (1 mmol) of HEMA monomer were mixed together then 90 mg (0.5 mmol) of BP and 140 µl (1 mmol) of NEt_3 were added into the mixed monomers. The mixed solution was purged with N_2 gas and prepolymerized under UV radiation at λ_{max} **of 254 nm** for 5 hrs to obtain the prepolymer. The obtained prepolymer was spin-coated on round-cover glass (10mm in diameter) at 2500 rpm for 30 seconds. The prepared *L. interrogans* template (step 3.3) was then stamped on the prepolymer coated cover glass and then cured under UV light overnight to complete the polymerization. After polymerization, PET film was peeled off and the obtained imprinted polymer films were washed with 0.75 M sodium hydroxide solution under sonication to remove the bacterial cells, washed again with distilled water and dried at room temperature.

3.2.5 *L. interrogans* morphology and polymer surfaces observation

L. interrogans cell suspension was filtered with polycarbonate filter and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 2 hours then wash with phosphate buffer solution and distilled water to remove glutaraldehyde. The fixed cell was washed with ethanol at 30%, 50%, 70%, 95% and 100% and dried with critical point dryer. The fixed *L. interrogans* cell, *L. interrogans* cell template, non-imprinted and *L. interrogans* imprinted polymer films were coated with platinum for 20 sec and observed with field-emission scanning electron microscope (FE-SEM) (JEOL JSM-7001F) using SEM and GB-HIGH (Gentle beam) mode with electron beam at 2 to 5 eV.

3.2.6 Evaluation of ultrathin cavities on imprinted polymer film surface

Non-imprinted and *L.interrogans* imprinted (at 1×10^{8} cells/ml, 80µl bacterial suspension) polymers were observed with atomic force microscope (AFM) (SPA 400, SII NanoTechnology Inc., Tokyo, Japan) using non-contact mode. All measurements were performed in air, at room temperature and atmospheric pressure using silicon cantilevers (HQ: NSC15/ALBS, 125 lm in length). Typical scan rate during recording was 1.99 Hz using scan heads with a maximum range of 100 µm× 100µm. The image analysis has been performed with the 7.20 Version NanoScope V software.

Chapter 4
Results and discussions

4.1 Synthesis and characterization of poly(MMA-*co*-HEMA)

Methyl methacrylate (MMA) and hydroxyethyl methacrylate (HEMA) are normally polymerized by radical polymerization or bulk polymerization via radical initiators such as azobisisobutyronitrile (AIBN), hydrogen peroxide and so on. In order to activate the initiator into radical, heat is required. Bulk polymerization has several advantages over other polymerization such as simple system, polymerizing pure polymer, highly viscous prepolymer usages. However, the reaction is exothermic and carried out in an absence of solvent to distribute heat throughout the system. Thus, it is very difficult to control the properties of the obtained polymers such as molecular weight, molecular weight distribution and so on. Wide range of molecular weight of polymer is produced. Chain transfer agents are normally used to control the molecular weight and molecular weight distribution.

An alternative way to avoid heating and broad molecular weight distribution, photopolymerization of PMMA or PHEMA was considered. BP was used as type II photoinitiator that required co-initiator such as trimethylamine (NEt_3) that can readily have hydrogens abstracted, then, reactive center is generated on co-initiator instead.

Owing to the brittleness of PMMA, HEMA was selected to copolymerize with MMA in order to prepare Poly(MMA-*co*-HEMA) film with less brittleness, since PHEMA shows lower T_g than PMMA, thus, In this study, the effect of co-initiator to polymerization was investigated by preliminary measuring % yield. The condition to polymerize poly(MMA-*co*-HEMA) with BP and NEt_3 were studied by fixing mole ratio of MMA and HEMA to be 1:1, whilst varying mole ratios of BP to NEt_3 from 1:0.5 to 1:2. Poly(MMA-co-HEMA) was successfully polymerized using BP as initiator and NEt_3

as co-initiator. The %yields of the obtained copolymer were calculated (1) and shown in Table 4.1.

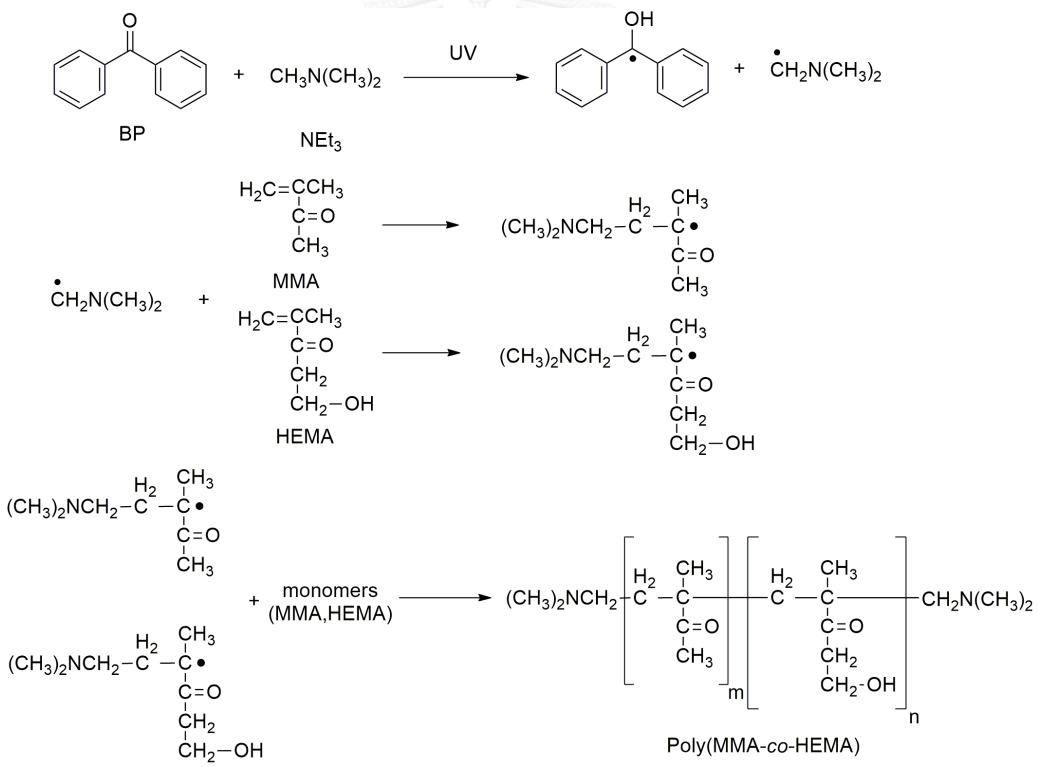
$$\%\text{yield} = \frac{obtained\ copolymer\ (g)}{total\ monomer\ (g)} \times 100 \qquad (1)$$

Table 4.1 %yield of copolymer with various mole ratios of initiator and co-initiator

Run No.	MMA:HEMA (ml)	BP(mg)	NEt_3(µl)	%yield
1	1.07:1.22 (10:10 mmol)	90 (0.5mmol)	0	N/A
2			35 (0.25 mmol)	N/A
3			70 (0.50 mmol)	N/A
4			105 (0.75 mmol)	1.4
5			140 (1.0 mmol)	3.1
6		360 (2.0mmol)	0	N/A
7			280 (2.0 mmol)	49.5
8			560 (4.0 mmol)	63.9

From the results, the polymerizations occurred with the presence of NEt_3 while the increasing in the amount of NEt_3 increased %yield of the obtained copolymer. The comparative studies of excess amount of BP were also studied (run 6, 7 and 8). It was clearly shown that polymerization does not occurred in the absence of NEt_3 even with the excess amount of benzophenone in run 6. It was indicated that the presence of benzophenone alone cannot initiate the polymerization. This may be due to the bulky structure of benzene ring in benzophenone structure that causes the steric hindrance and prevents the initiation of polymerization [31]. Moreover, with the mole of NEt_3 less than or equal to BP, run 2 and 3, the polymerizations did not take place. This also supported that fact that reactive centers were generated

onto co-initiator to activate the polymerization. The less mole of NEt_3 than BP might not generate enough active species for polymerization. On the other hand, with the mole of NEt_3 higher than or equal to BP, run 7 and 8, the polymerization took place. Therefore, the amount of reactive species on the co-initiator was high enough for the polymerization. With the equivalent mole of NEt_3 and BP, run 3 and run 7, the higher the addition of initiator and co-initiator gave rise to the higher amount of reactive species to initiate polymerization. The mechanism of photopolymerization of PMMA using BP and NEt_3 as initiator and co-initiator was predicted as shown below (Scheme 4.1)



Scheme 4.1 Mechanism of poly(MMA-*co*-HEMA) photopolymerization using BP and NEt_3

BP and NEt_3 at 1:2 mole ratio (run 5) was selected as the condition for copolymerization between MMA and HEMA due to its easy to control and operate than at higher amount of BP and NEt_3 (run 7 and 8). Even though, at higher amount of BP and NEt_3 resulted in higher %yield, the viscosity of prepolymer is relatively hard to control since the change in viscosity is fast. The viscosity of prepolymer plays an important role during spin coating process in the thin film fabrication. The higher viscosity, the harder film fabrication and resulted in an uneven thin film. The polymerizations were carried out with various ratios of monomer (MMA) and comonomer (HEMA).

Table 4.2 %yield of copolymer from various mole ratios of monomer and comonomer

Run No.	Mole ratio of monomer	MMA		HEMA		BP(mg):NEt3(ul)	%yield
		mmol	ml	mmol	ml		
9	100:0	20.0	2.13	0	0	90:140 (0.5:1 mmol)	6.2
10	95:5	19.0	2.04	1.0	0.13		10.3
11	90:10	18.0	1.92	2.0	0.25		10.3
12	85:15	17.0	1.81	3.0	0.37		14.9
13	80:20	16.0	1.71	4.0	0.49		21.0
14	0:100	0	0	20.0	2.43		38.9

From the results, homopolymer of PHEMA gave the highest %yield at 41.3% while homopolymer of PMMA gave the lowest %yield at 5.4%. Also, it was clearly shown that the increasing of HEMA monomer resulted in an increasing of %yield as can be seen in Table 4.2. The copolymer of MMA and HEMA was characterized by fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. From FTIR results, -OH stretching appeared at 3350-3550 cm^{-1} both in PHEMA and poly(MMA-*co*-HEMA) and –C=O stretching was found at 1710 cm^{-1} in PMMA, PHEMA and poly(MMA-co-HEMA). CH stretching was also found at 2850-

3150 cm^{-1} (Fig. 4.1). However, the FTIR results cannot distinguish the differences between PHEMA and poly(MMA-*co*-HEMA) this is due to the similarity of chemical structures between PMMA and PHEMA that showed the similar peaks.

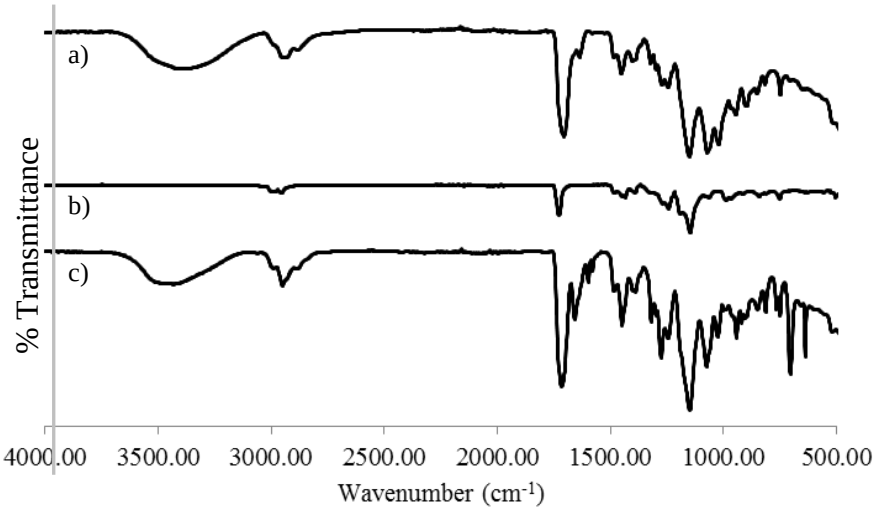


Figure 4.1 FTIR spectra of a) PHEMA, b) PMMA and c) poly(MMA-co-HEMA)

Poly(MMA-*co*-HEMA) from run 10 was selected and characterized with ^{1}H NMR as shown in Fig. 4.2 The signal of methyl proton from –OC**H**$_3$ in MMA is occurred at 3.63 ppm while the signal of –CH_2C**H**$_2$OH and –C**H**$_2CH_2$OH in HEMA can be found at 3.82 ppm and 4.07 ppm respectively. Composition of HEMA in copolymer from ^{1}H NMR data is 5.7% which was calculated from integral area of the –OC**H**$_3$ and –C**H**$_2CH_2$OH signal using the equation (2)

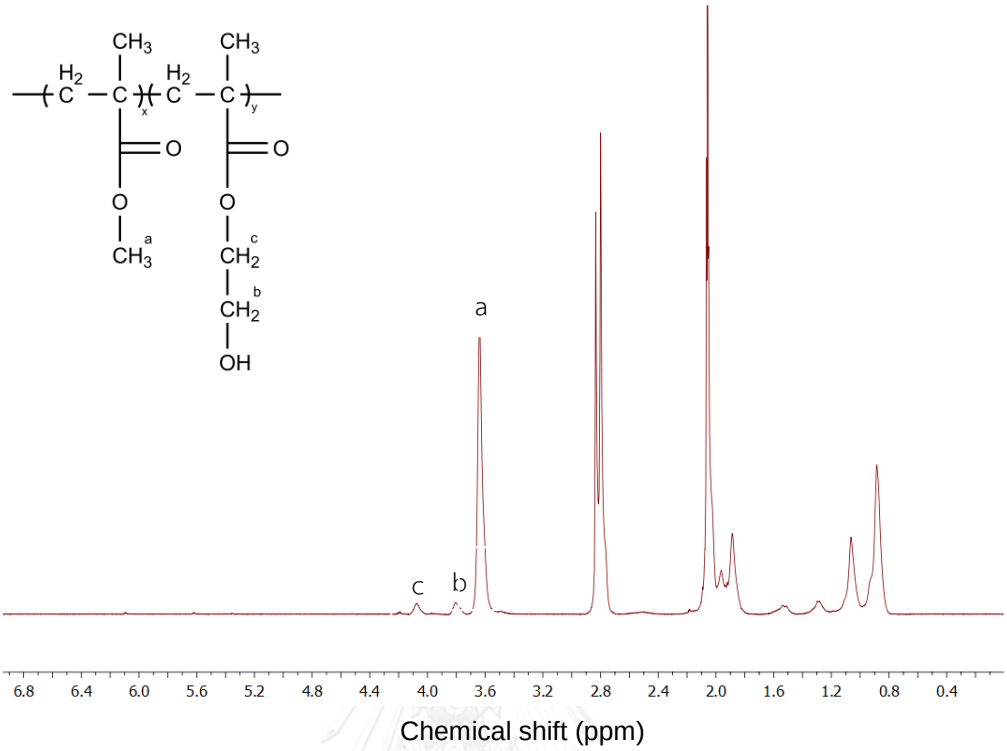


Figure 4.2 ^{1}H NMR of poly(MMA-co-HEMA) from run no.10 with mole ratio of MMA to HEMA of 95:5

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100 \quad (2)$$

Table 4.3 Percentage of HEMA in poly(MMA-*co*-HEMA)

Run No.	Mole ratios of monomers	Percentage of HEMA
4	50:50	55.4
5	50:50	55.4
7	50:50	52.9
8	50:50	55.2
10	95:5	5.7
11	90:10	13.0
12	85:15	18.4
13	80:20	24.8

From Table 4.3, The percentage of HEMA in poly(MMA-*co*-HEMA) was calculated from the integral area from NMR spectra showed that the composition of HEMA in copolymer was slightly higher than the supposed amount in the reaction. This can be concluded that HEMA have a higher reactivity than MMA. Reactivity value of MMA (r_1) was reported at 0.75 while reactivity of HEMA (r_2) was at 1.5 [48]. Since, reactivity of monomers that have value higher than 1 make monomer to favorable polymerized with themselves and also the reactivity that lower than 1 cause monomer to favorable polymerize with other monomer, thus, the copolymerization between MMA and HEMA resulted in HEMA composition is higher than the supposed amount. It also can be concluded that copolymer has a random structure.

Poly(MMA-*co*-HEMA) run 10 was selected to use as polymeric matrix to fabricate molecular imprinting polymer because at higher content of HEMA the obtained film easily peel off from glass substrate. This might be caused from the

higher amount of hydroxyl group presence in copolymer since the surface of glass also has the hydroxyl group which may lead to the lower adhesion between copolymer and glass surface. Therefore, it is not suitable to use copolymer with higher HEMA composition to prevent the peeling off of copolymer.

4.2 Preparation of *L. interrogans* template

PET film was selected to use as a substrate for bacterial template due to its resistant to UV light and also its flexibility and low polarity which made it easy to peel off from the imprinted polymeric film. In Figure 4.3, the morphology of *L. interrogans* was compared between untreated bacteria cell (Fig. 4.3a) and the bacterial cell on PET substrate (preparing at 20µl ($2x10^6$ cells) bacterial suspension (Fig. 4.3b). This Figure showed that the morphology of bacterial cell had no changes after prepared on PET film as template and the cell still maintained spiral structure which is the unique characteristic of *L. interrogans*.

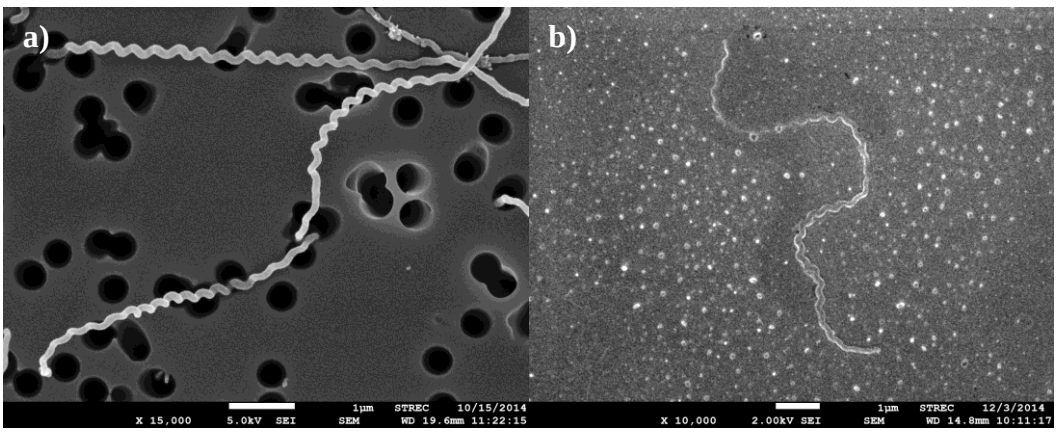


Figure 4.3 FESEM images of a) *L. interrogans* untreated cells (×15,000) and b) *L. interrogans* cell template attached on PET film (×10,000).

4.3 Fabrication of *L. interrogans* imprinted polymer

The prepolymer of poly(MMA-*co*-HEMA) was successfully copolymerized between MMA and HEMA under UV radiation using BP as initiator and NEt_3 as co-initiator prior to gel point. The prepolymer was casted into thin film by spin coating, then, the bacterial template was mildly place onto the prepolymer thin film. The obtained poly(MMA-*co*-HEMA) film was more tough and flexible than homopolymer of PMMA film. *L. interrogans* was successfully imprinted on the poly(MMA-*co*-HEMA) film surface and removed by using sodium hydroxide solution under sonication. The surface morphology of bacterial cells imprinted onto poly(MMA-*co*-HEMA) film and non-imprinted poly(MMA-*co*-HEMA) film were observed with FESEM (Fig. 4.4). From the results, non-imprinted poly(MMA-*co*-HEMA) film showed a smooth surface with small bubble holes (Fig. 4.4a) whilst bacterial cells were found to be imprinted onto poly(MMA-co-HEMA) film (Fig. 4.4b).

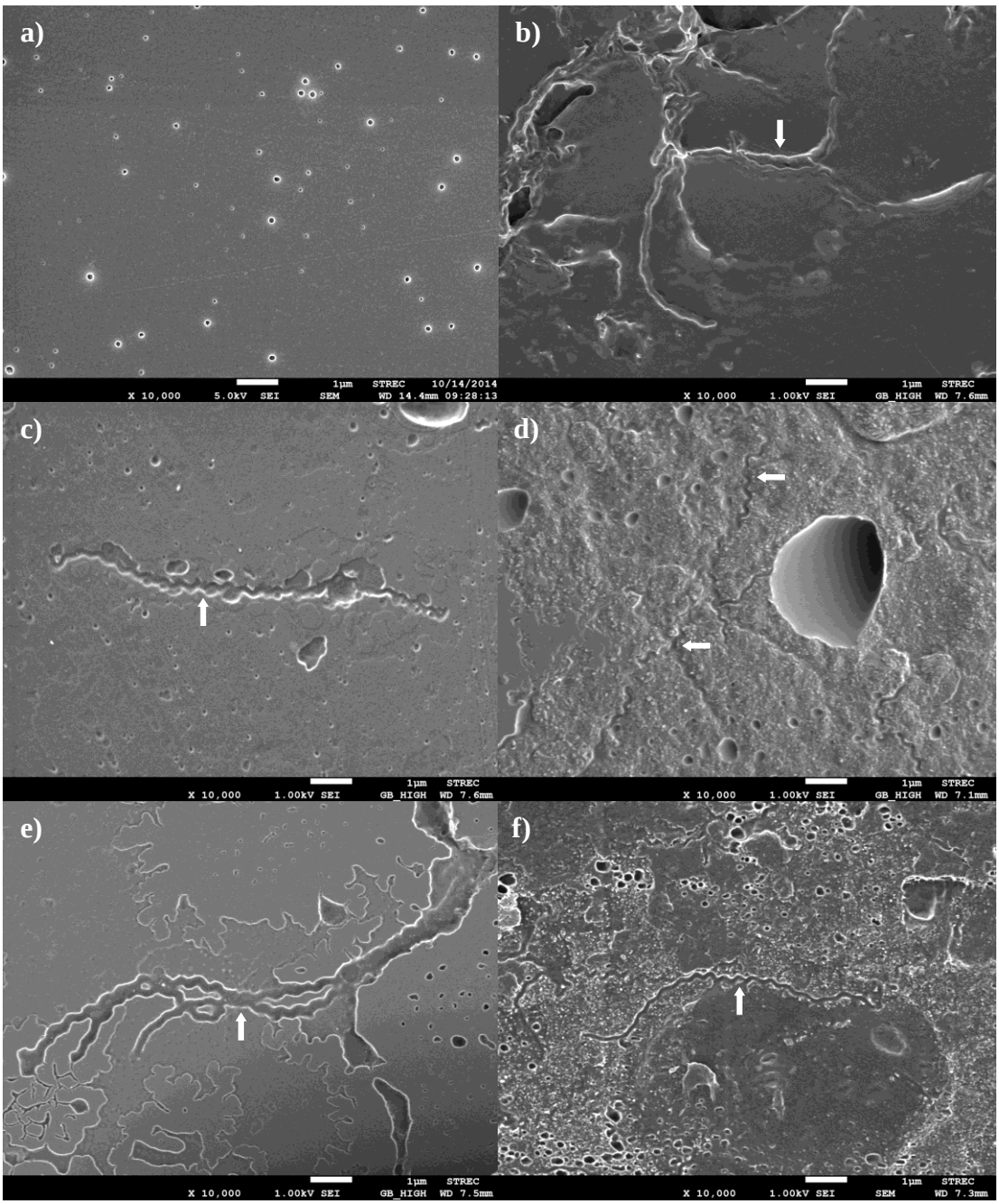


Figure 4.4 FESEM images of (a) Poly(MMA-*co*-HEMA) film surface (non-imprinted polymer surface), (b) bacterial cells image imprinted on polymer film ($2x10^6$ cells) and bacterial cavities after removal of bacterial templates with bacterial number of c) $2x10^6$ cells d) $4x10^6$ cells e) $6x10^6$ cells f) $8x10^6$ cells.

After the removal of bacterial cells from the imprinting polymer film surface, the cavities can be clearly seen on the polymer film surface (Fig. 4.4c-f). The obtained cavities clearly showed the spiral-like structure. These spiral-like structures had the same size as *L. interrogans*. Thus, *L. interrogans* was proved to be imprinted onto the polymer surface while the imprinting process did not alter the spiral-like structure which was a unique characteristic of *L. interrogans*. Moreover, an increasing in the amount of bacterial cells from $2x10^6$ cells to $8x10^6$ cells had showed no difference in the cavities. However, at the higher amount of bacterial cells, the numbers of cavities were found in many places while at the lower amount of bacterial cells, the numbers of the cavities found during observation were significantly lower compared to the above.

Although bacterial cells were clearly imprinted on the polymer film surface, due to the long body of *L. interrogans*, overlaps and entanglements between the bacterial cells were also found (Fig. 4.5a-b). However, the spiral-like cavities still can be clearly seen as the cavities of cells overlap or entanglement.

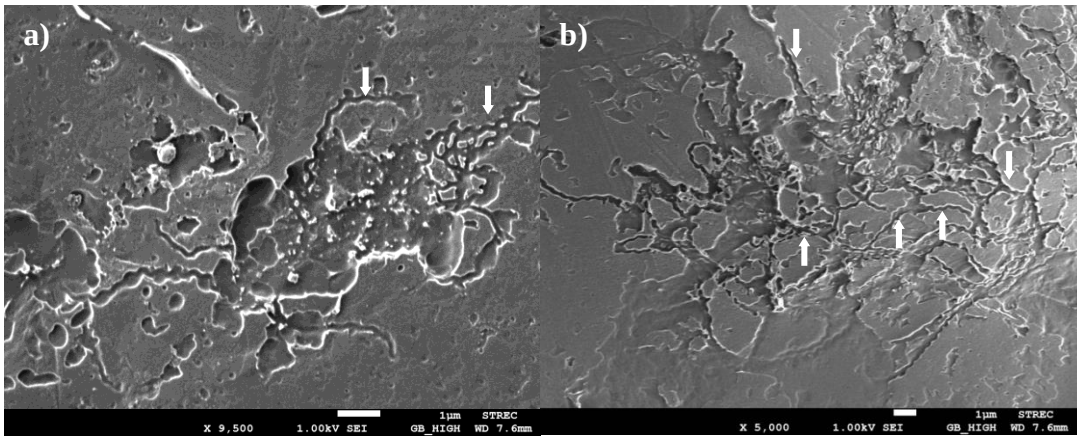


Figure 4.5 Overlaps and entanglements of the bacterial cells at a) $2x10^6$ cells (×9,500) and b) $8x10^6$ cells (×5,000)

The cells overlaps and entanglements might affect the binding ability of the imprinted polymer. Since, it's form a large cavity that might lead to non-specific binding which resulted in lower sensitivity and selectivity properties.

4.4 Evaluation of ultrathin *L. interrogans* cavities

The bacterial imprinted poly(MMA-*co*-HEMA) film using bacterial template of $8x10^6$ cells and non-imprinted polymer film surfaces were observed with AFM to measure the depth of the cavities. Non-imprinted film showed a smooth surface while *L. interrogans* imprinted film showed rougher surface along with the bacterial cavities (Fig. 4.6). The cavities of cells entanglement or overlap as well as the individual bacterial cavities were found (Fig. 4.6b-c).

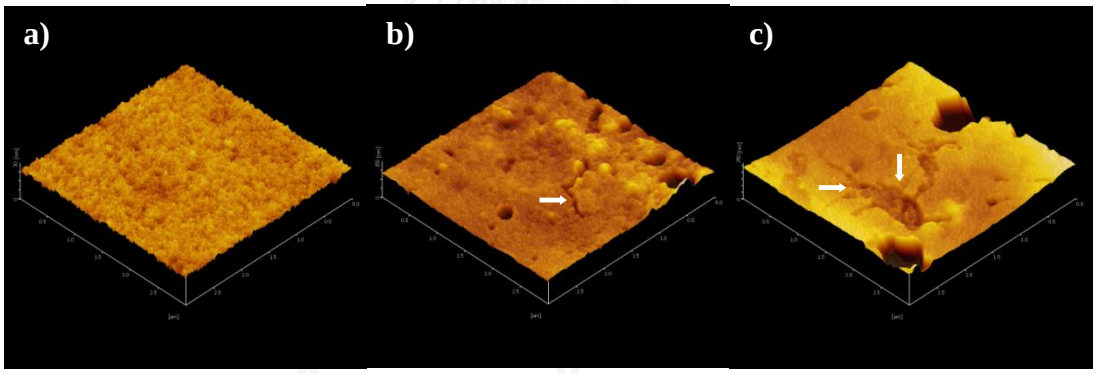


Figure 4.6 3D-AFM images of a) non-imprinted film b) individual cell cavity c) entanglement and overlapping cavity.

The cavities depths range from 30-50 nm either cavities of individual cells or of cells entanglement while the width of the individual bacteria cavities were range from 50-70 nm. This could be concluded that the entanglement or overlapping of *L. interrogans* during imprinting process does not affect the depth of the bacterial cavities. *Leptospira* imprinted polymer was successfully fabricated. However, the sensitivity and selectivity are still unknown. Further investigation of sensitivity and selectivity are required in order to clarify the limitation of the imprinted polymer.

Chapter 5
Conclusions

Poly(MMA-co-HEMA) was successfully synthesized via photopolymerization using BP as initiator and NEt_3 as co-initiator. The presence of NEt_3 had significant effect on the polymerization yield while BP alone cannot initiate the polymerization. Also, the amount of HEMA composition has an effect on the polymerization yield due to the reactivity of monomer.

L. interrogans were successfully prepared into the template and successfully imprinted onto poly(MMA-*co*-HEMA) film. The obtained imprinted film showed the spiral-like cavities which is the unique structure of the *Leptospira*. The results indicated that MIP can be prepared using bacterial cells with complex morphology while maintaining the original or unique structures of the bacterial cells that used as the template. However, in *L. interrogans* case, the overlaps or entanglements of bacterial cells owing to their long spiral bodies, leading to the cavities of cells overlap or entanglement.

Future Aspects

1. The imprinted polymer will be further investigated in terms of binding and recognition efficiency to find the limitation of imprinted polymer.
2. Molecular weight (M_n, M_w) and polydispersity index (PDI) of copolymer will be investigated.

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APPENDIX

Appendix A

%yield of poly(MMA-*co-HEMA*)

Table A1. %yield of copolymer with various mole ratios of initiator and co-initiator

Run No.	MMA:HEMA (ml)	BP(mg)	NEt_3(µl)	Total monomer (g)	Copolymer (g)	%yield
1	1.07:1.22 (10:10 mmol)	90 (0.5mmol)	0	2.30	N/A	N/A
2			35 (0.25 mmol)		N/A	N/A
3			70 (0.5 mmol)		N/A	N/A
4			105 (0.75 mmol)		0.0330	1.4
5			140 (1.0 mmol)		0.0712	3.1
6		360 (2.0mmol)	0		N/A	N/A
7			280 (2.0 mmol)		1.1343	49.5
8			560 (4.0 mmol)		1.4636	63.9

Table A2. %yield of copolymer from various mole ratios of monomer and comonomer

Run No.	Mole ratio of monomer	MMA		HEMA		BP(mg):NEt3(ul)	Total monomer (g)	Copolymer (g)	%yield
		mmol	ml	mmol	ml				
9	100:0	20.0	2.13	0	0	90:140 (0.5:1 mmol)	2.00	0.1235	6.2
10	95:5	19.0	2.04	1.0	0.13		2.02	0.2093	10.3
11	90:10	18.0	1.92	2.0	0.25		2.04	0.2106	10.3
12	85:15	17.0	1.81	3.0	0.37		2.06	0.3076	14.9
13	80:20	16.0	1.71	4.0	0.49		2.08	0.4369	21.0
14	0:100	0	0	20.0	2.43		2.43	0.9458	38.9

$$\%\text{yield} = \frac{obtained\ copolymer\ (g)}{total\ monomer\ (g)} \times 100$$

Appendix B

FTIR Spectra

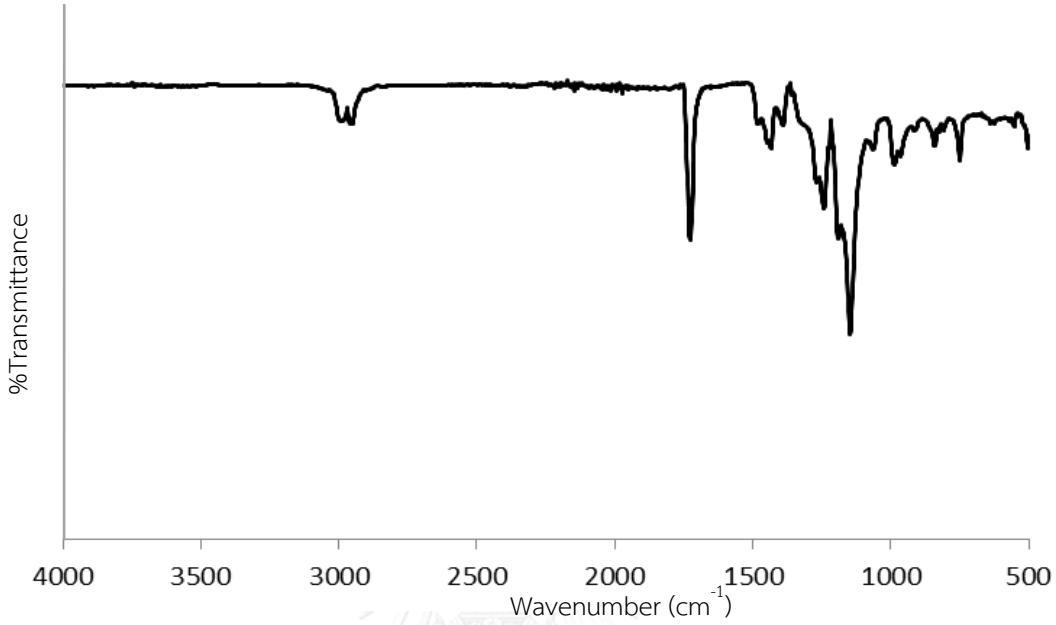

Figure B1 FTIR Spectrum of poly(methyl methacrylate)

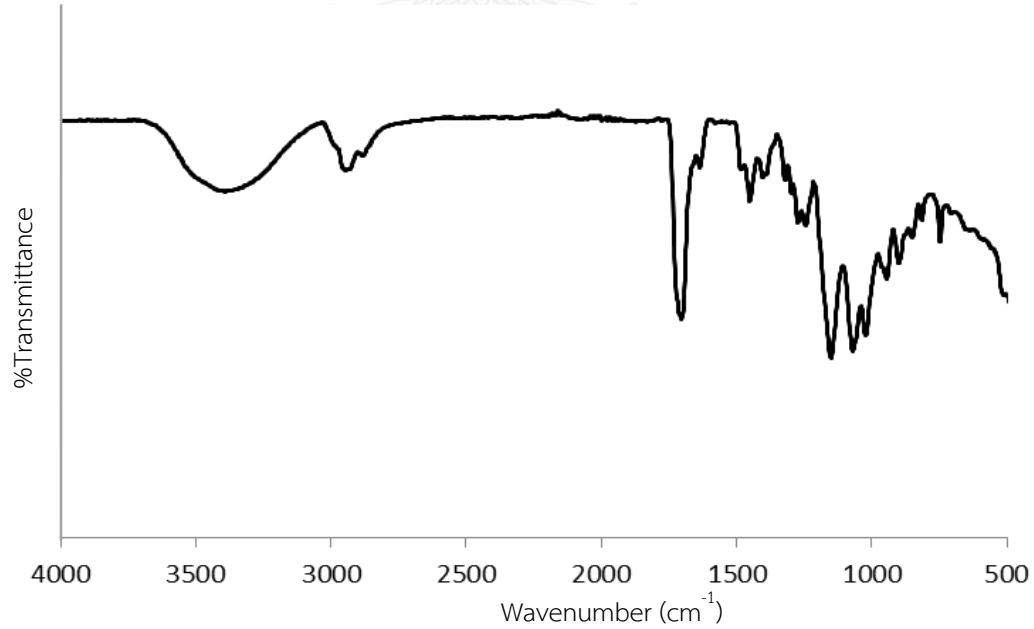

Figure B2 FTIR Spectrum of poly(hydroxyethyl methacrylate)

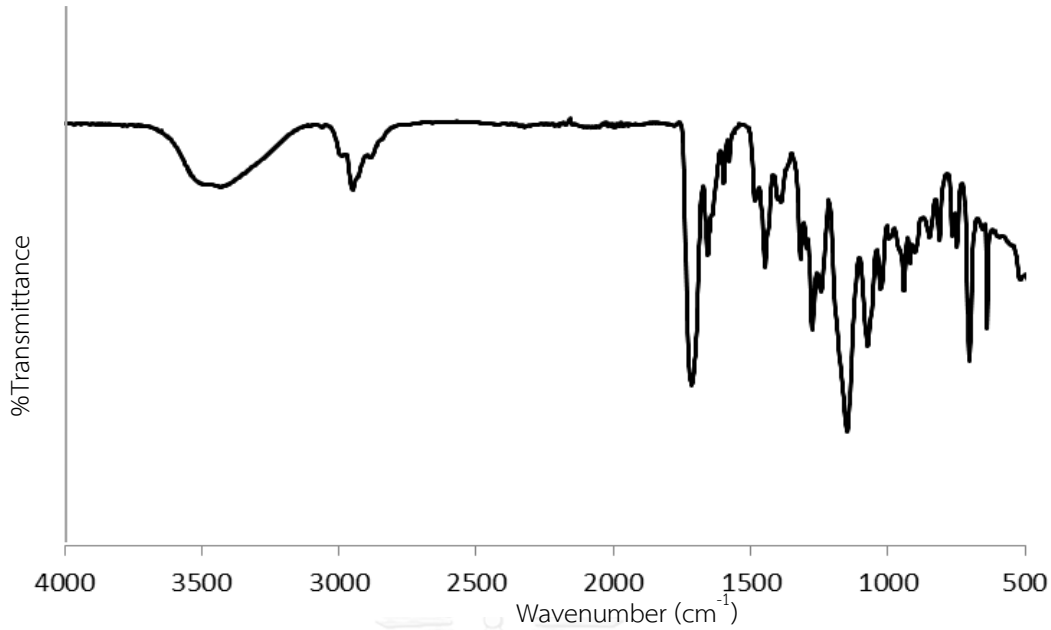

Figure B3 FTIR Spectrum of poly(MMA-*co*-HEMA)

Appendix C

^{1}H NMR Spectra

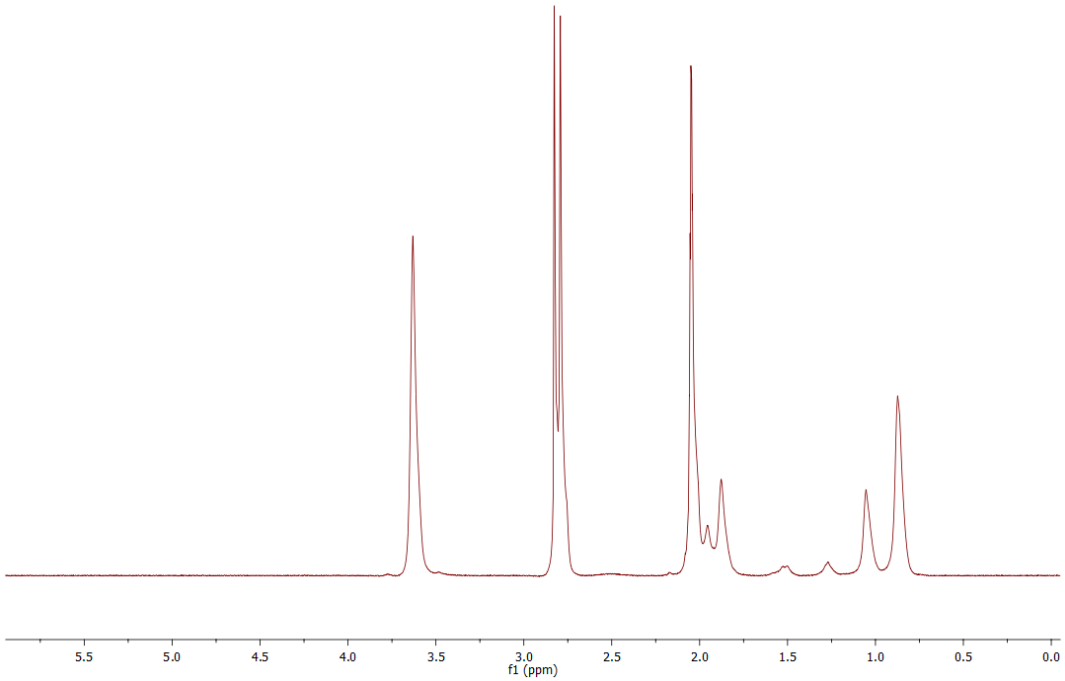

Figure C1 ^{1}H NMR spectrum of poly(methyl methacrylate)

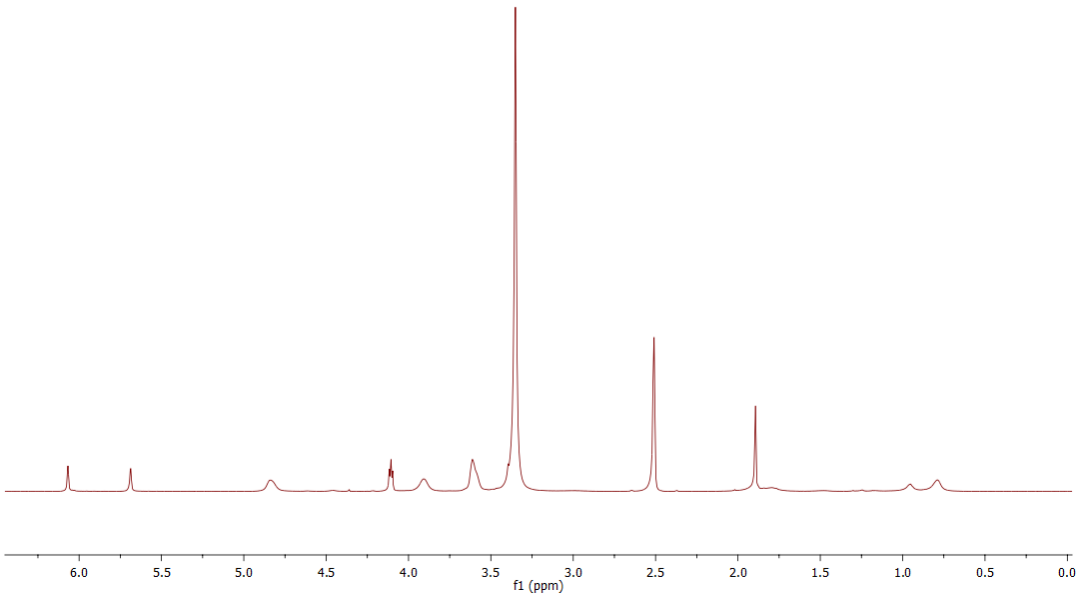

Figure C2 ^{1}H NMR spectrum of poly(hydroxyethyl methacrylate)

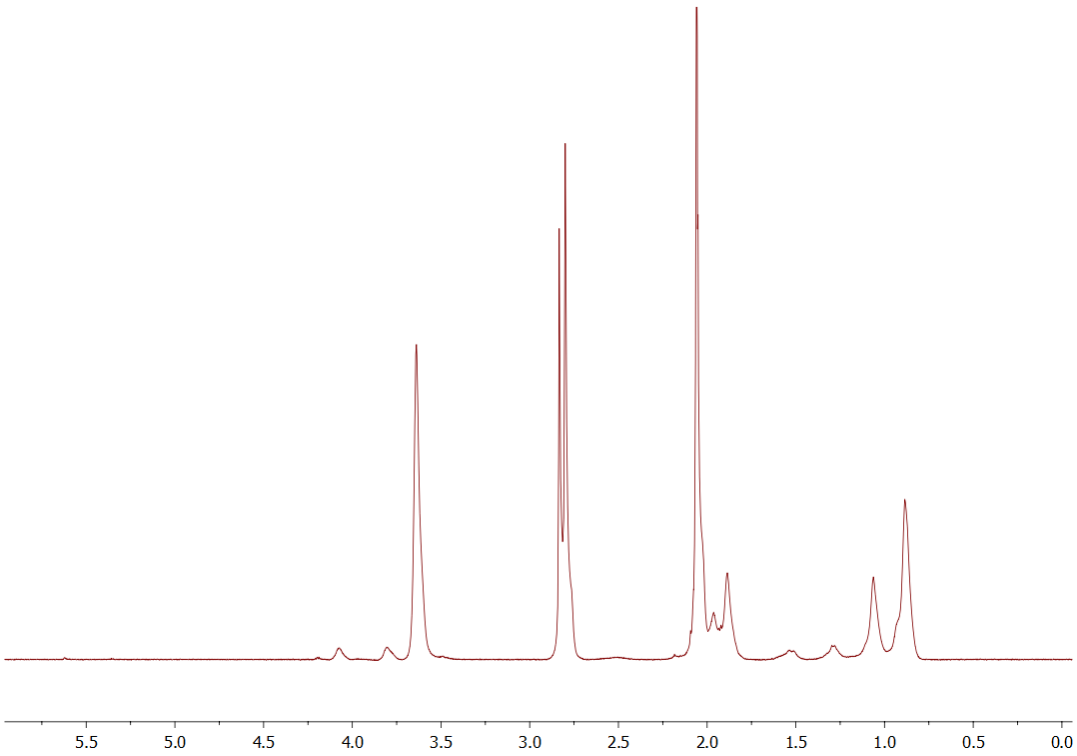

Figure C3 ^{1}H-NMR spectrum of poly(methyl methacrylate-co-hydroxyethy methacrylate) with the mole ratio of 95:5 (run 10).

Appendix D

^{1}H NMR Spectra and Percentage of HEMA composition in poly(MMA-*co*-HEMA)

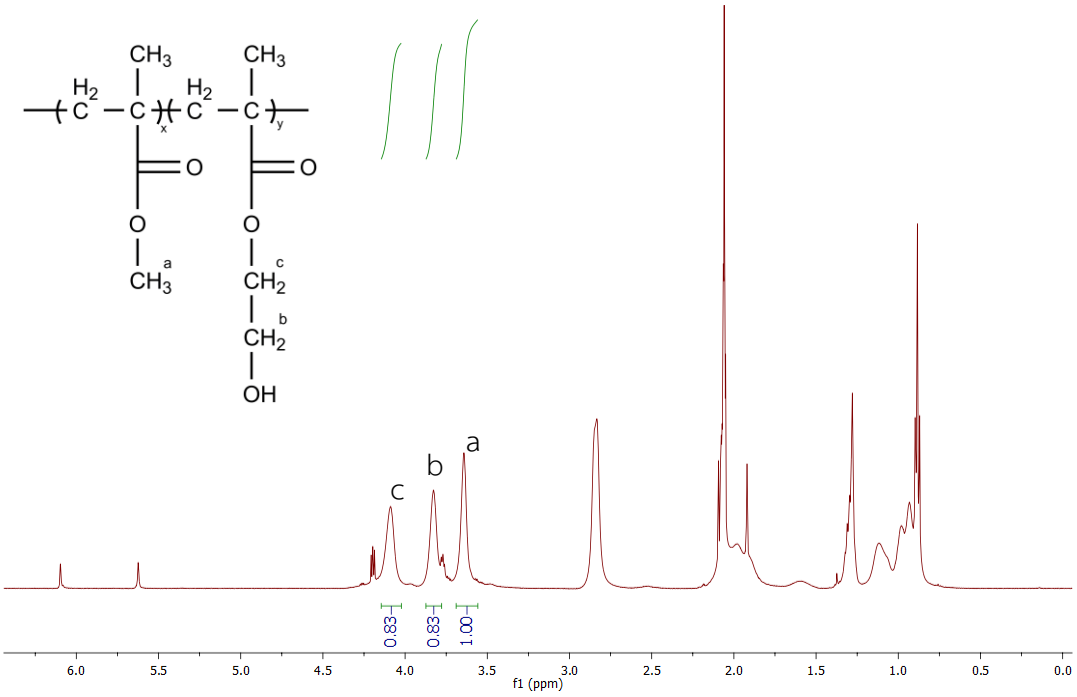

Figure D1 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.4)

Percentage of HEMA $= \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$

$= \frac{0.83/2}{(0.83/2)+(1/3)} \times 100$

= 55.4%

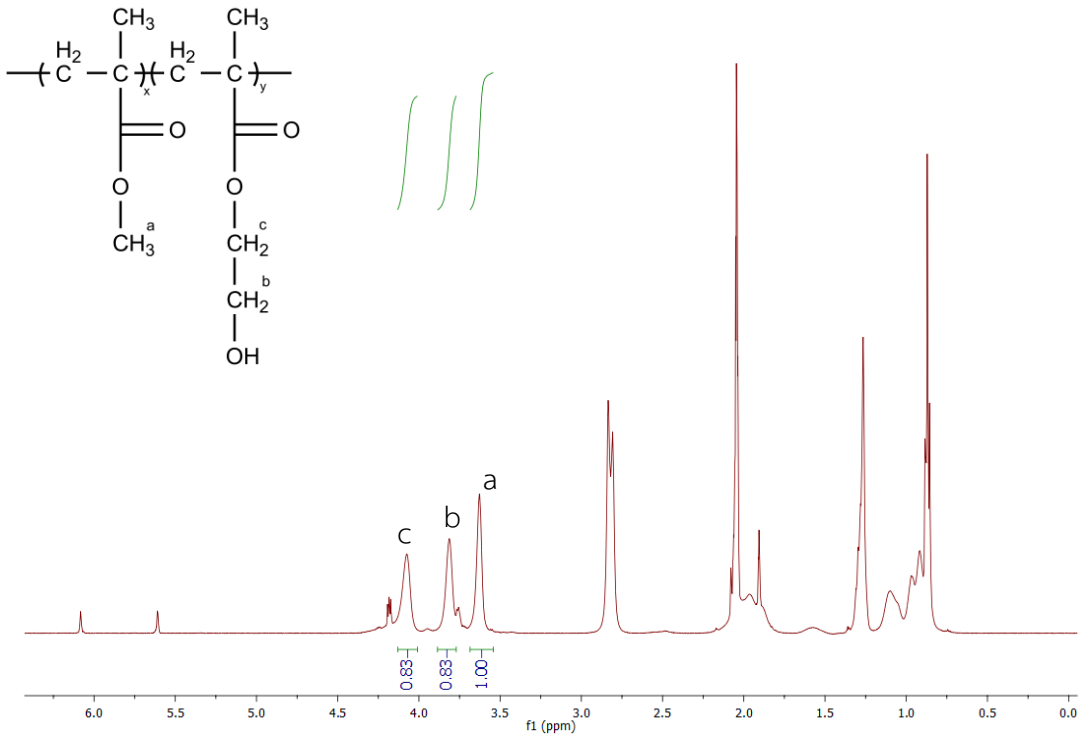

Figure D2 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.5)

Percentage of HEMA $= \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$

$= \frac{0.83/2}{(0.83/2)+(1/3)} \times 100$

= 55.4%

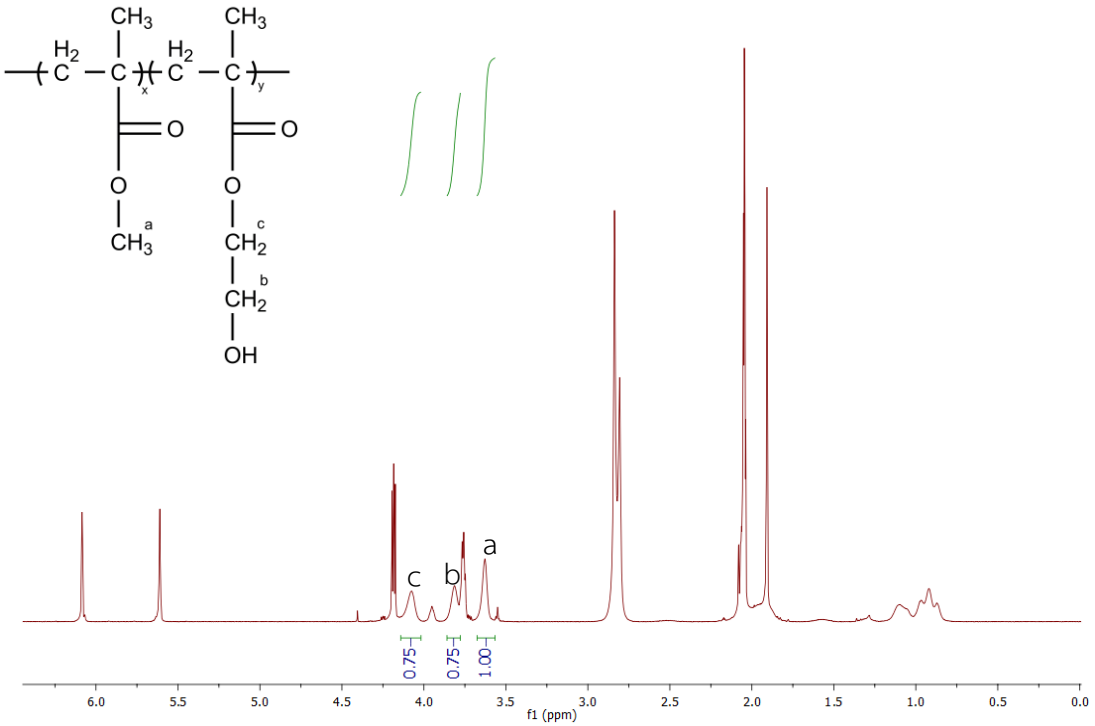

Figure D3 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.7)

Percentage of HEMA $= \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$

$= \frac{0.75/2}{(0.75/2)+(1/3)} \times 100$

= 52.9%

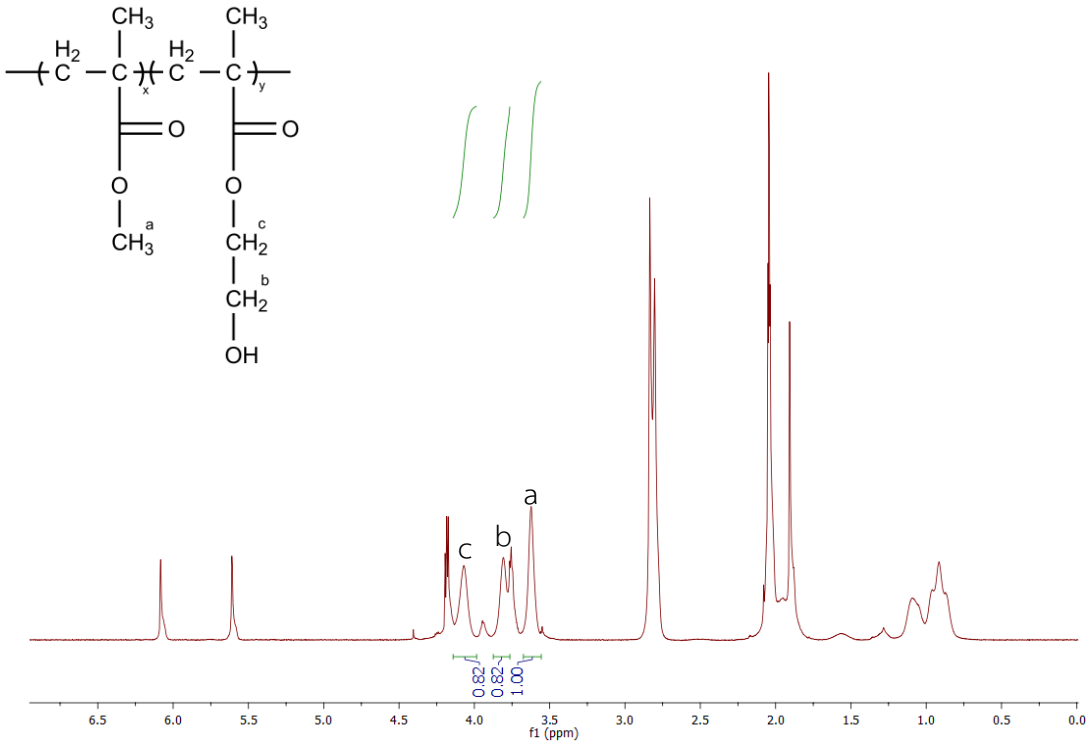

Figure D4 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.8)

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$$

$$= \frac{0.82/2}{(0.82/2)+(1/3)} \times 100$$

$$= 55.2\%$$

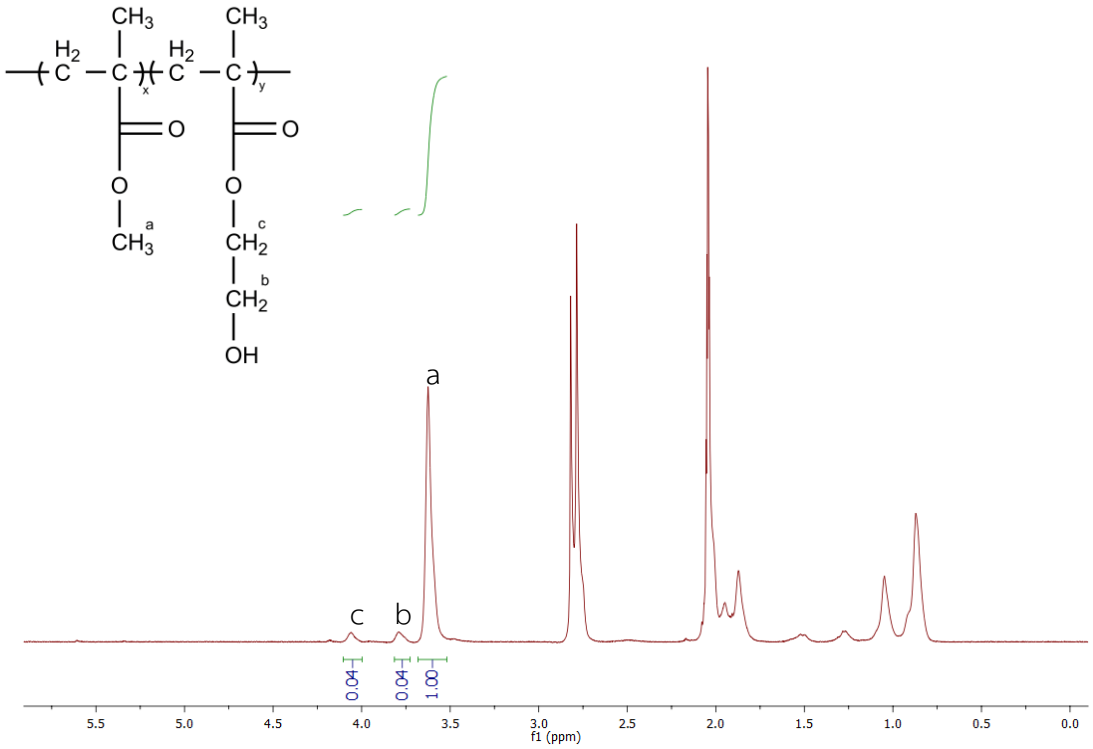

Figure D5 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.10)

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$$

$$= \frac{0.04/2}{(0.04/2)+(1/3)} \times 100$$

$$= 5.7\%$$

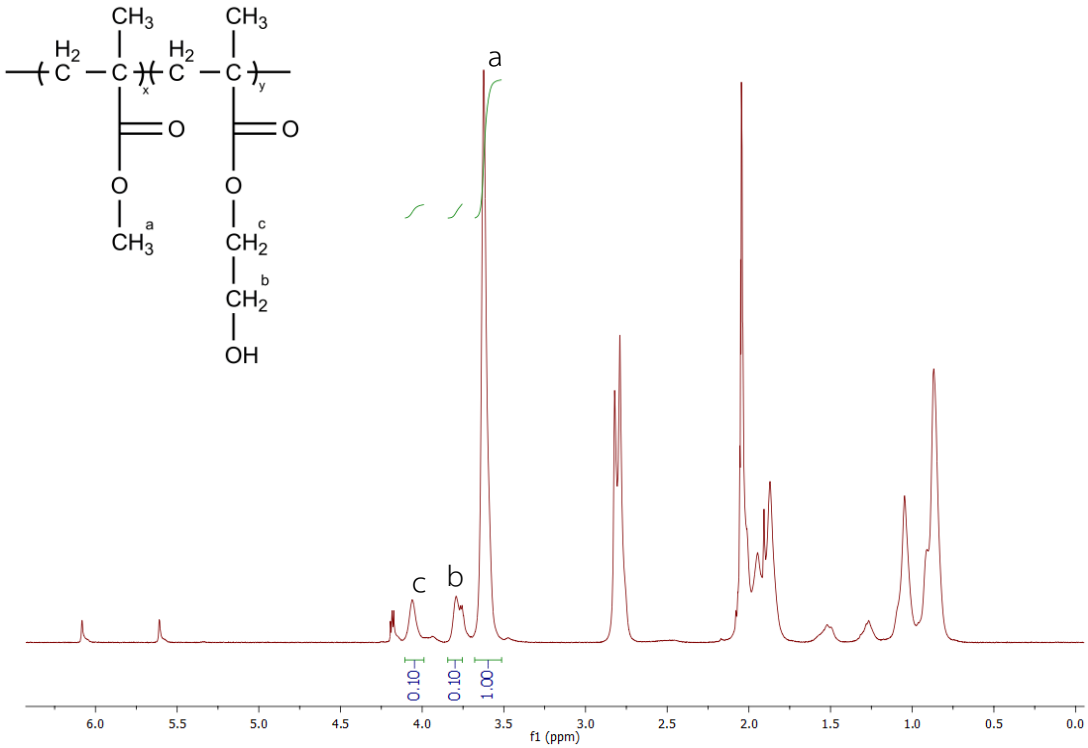

Figure D6 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.11)

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$$

$$= \frac{0.10/2}{(0.10/2)+(1/3)} \times 100$$

$$= 13.0\%$$

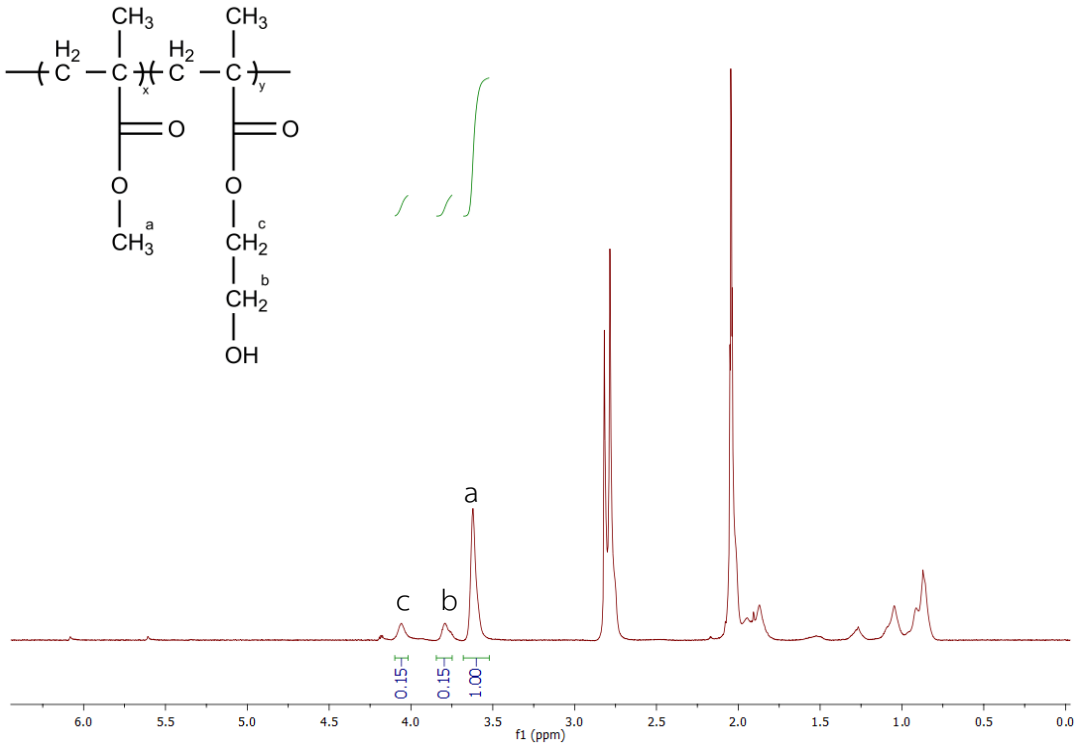

Figure D7 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.12)

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$$

$$= \frac{0.15/2}{(0.15/2)+(1/3)} \times 100$$

$$= 18.4\%$$

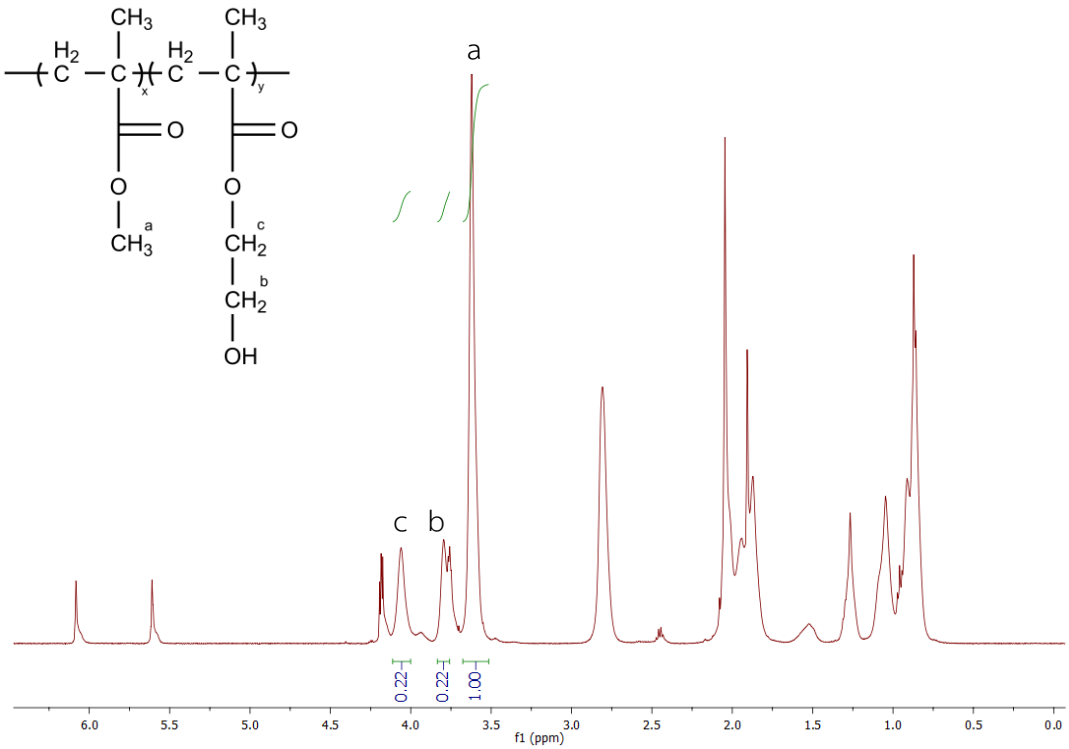

Figure D8 ^{1}H NMR spectrum of poly(MMA-*co*-HEMA) (run no.13)

$$\text{Percentage of HEMA} = \frac{\int(c)/2}{(\int(c)/2)+(\int(a)/3)} \times 100$$

$$= \frac{0.22/2}{(0.22/2)+(1/3)} \times 100$$

= 24.8%

Appendix E

AFM image of non-imprinted and imprinted polymer film surface

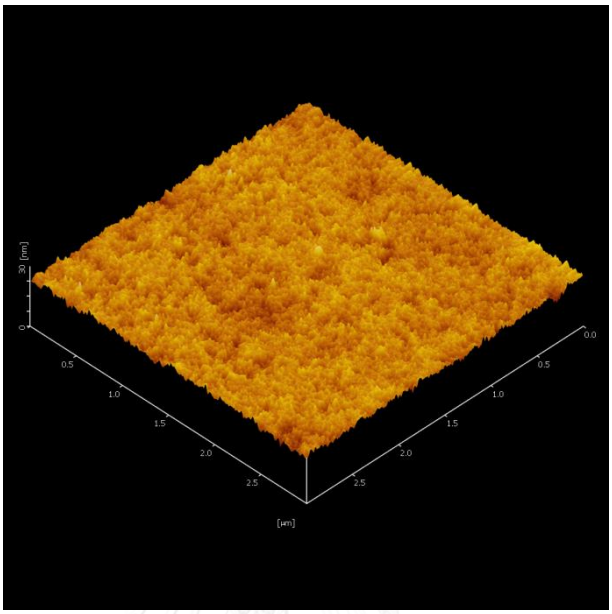

Figure E1 3D image of AFM of non-imprinted polymer film surface

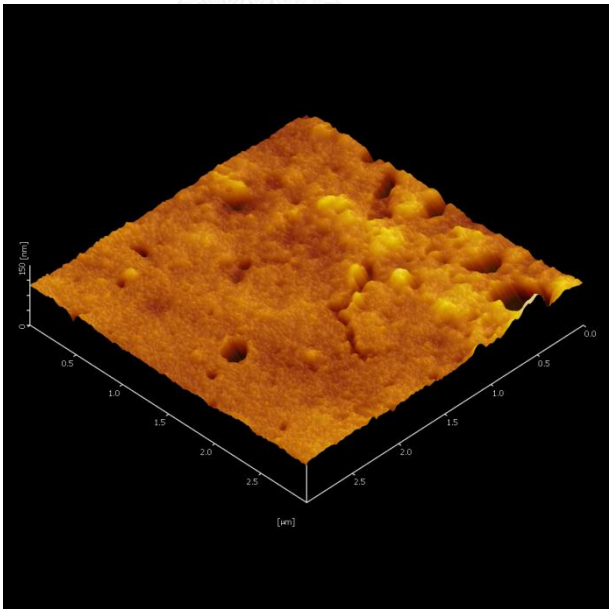

Figure E2 3D image of AFM of *L.interrogans* imprinted polymer film surface with individual cell cavity

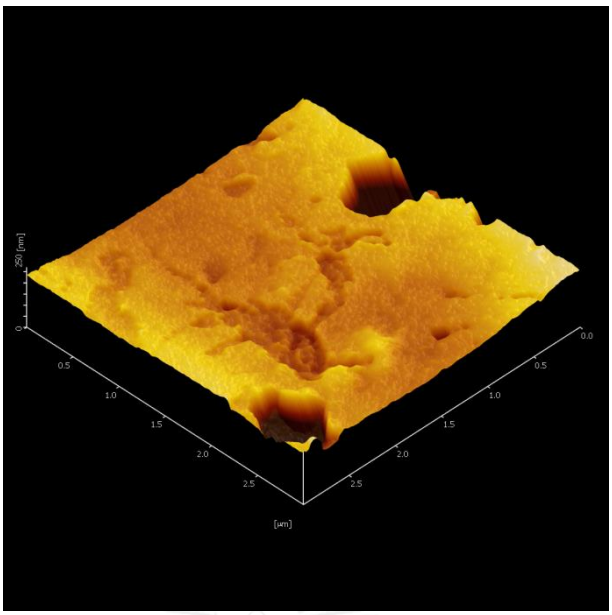

Figure E3 3D image of AFM of *L.interrogans* imprinted polymer film surface with overlap or entanglement cell cavity

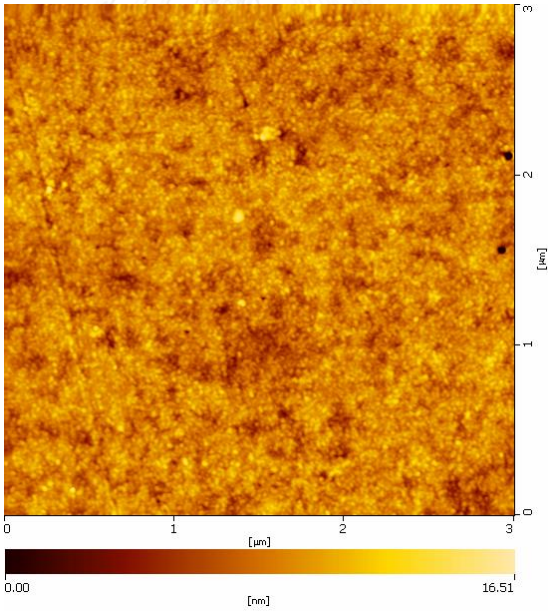

Figure E4 2D image of AFM of non-imprinted polymer film surface

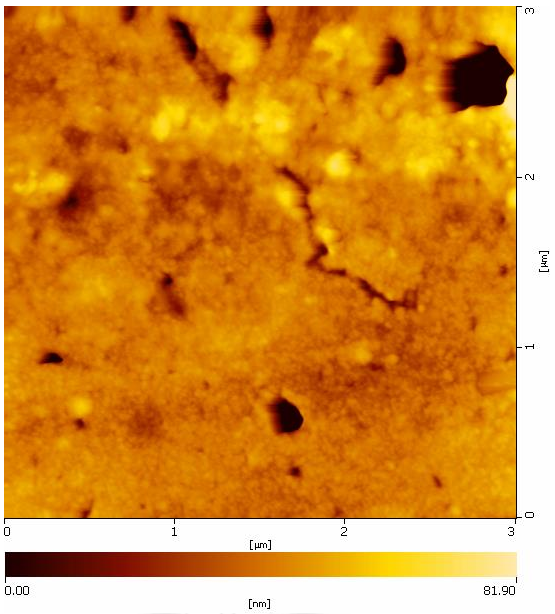

Figure E5 2D image of AFM of *L.interrogans* imprinted polymer film surface with individual cell cavity

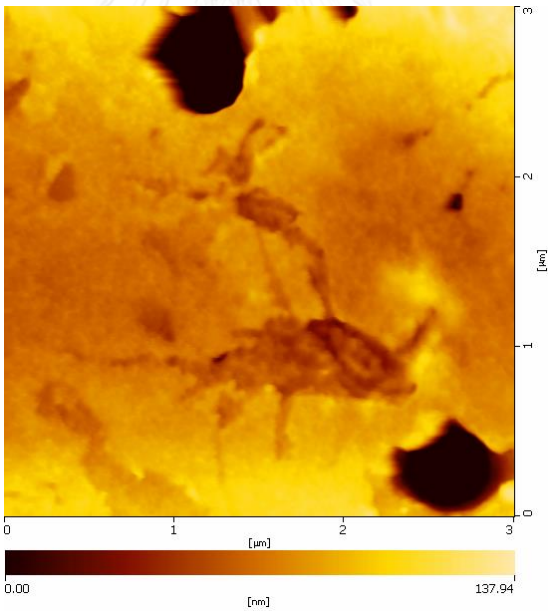

Figure E6 2D image of AFM of *L.interrogans* imprinted polymer film surface with overlap or entanglement cell cavity

Appendix F

Measurement of depth and width of cavities

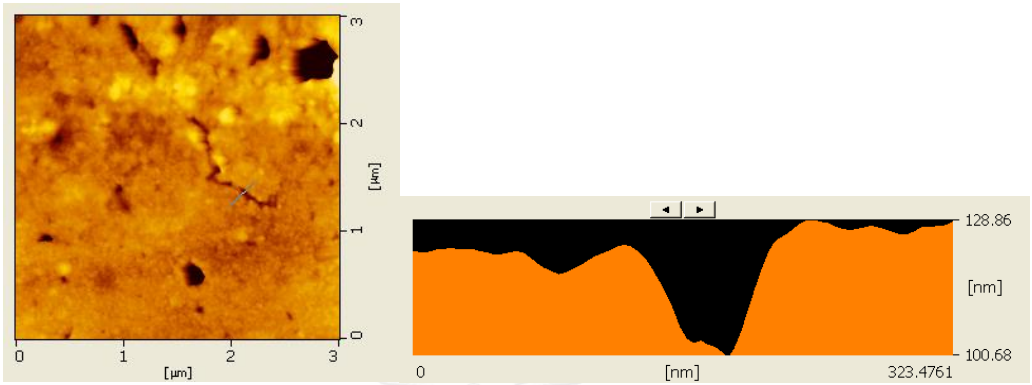

Figure F1 2D image of AFM of *L.interrogans* imprinted polymer film surface with individual cell cavity and measurement data (Position 1)

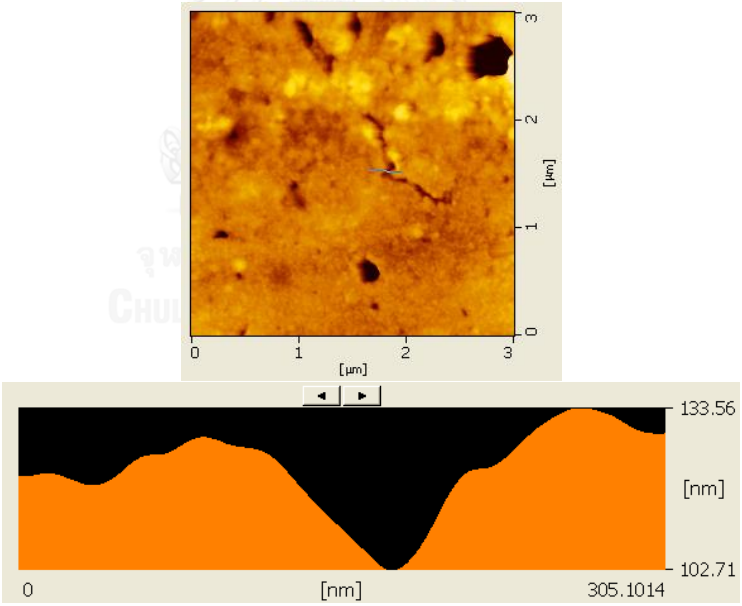

Figure F2 2D image of AFM of *L.interrogans* imprinted polymer film surface with individual cell cavity and measurement data (Position 2)

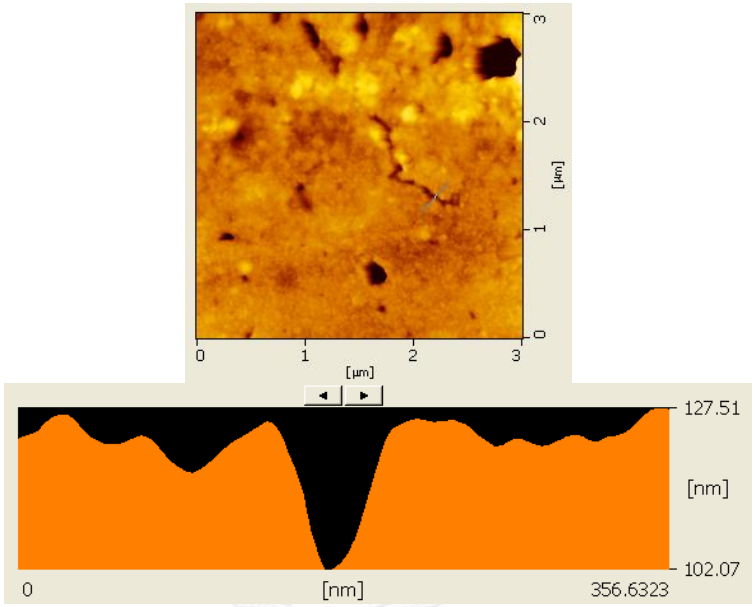

Figure F3 2D image of AFM of *L.interrogans* imprinted polymer film surface with individual cell cavity and measurement data (Position 3)

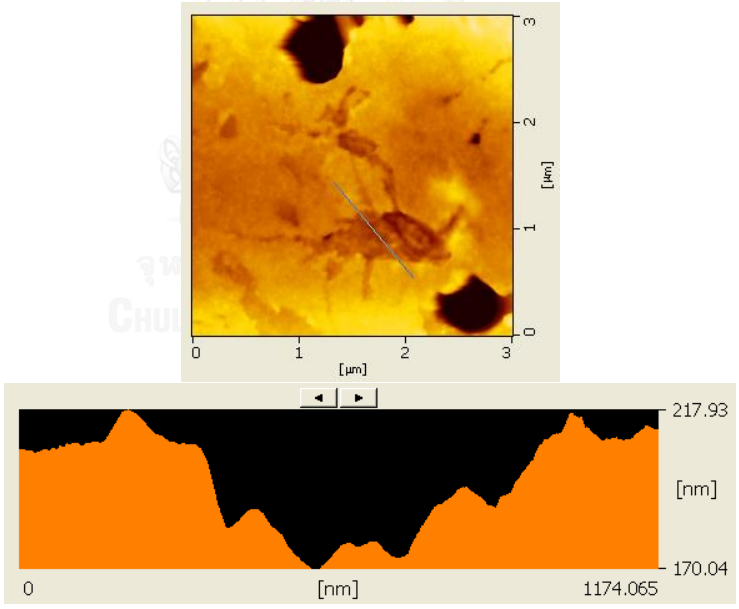

Figure F4 2D image of AFM of *L.interrogans* imprinted polymer film surface with overlap cell cavity and measurement data (Position 1)

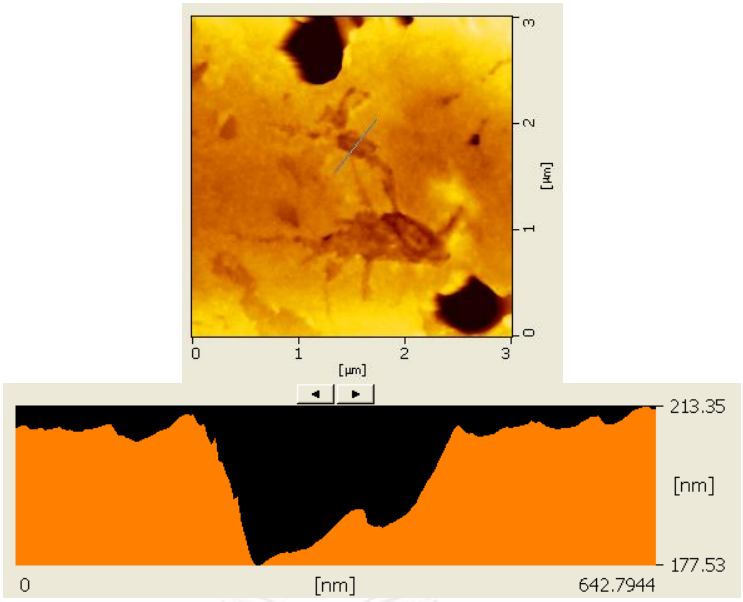

Figure F5 2D image of AFM of *L.interrogans* imprinted polymer film surface with overlap cell cavity and measurement data (Position 2)

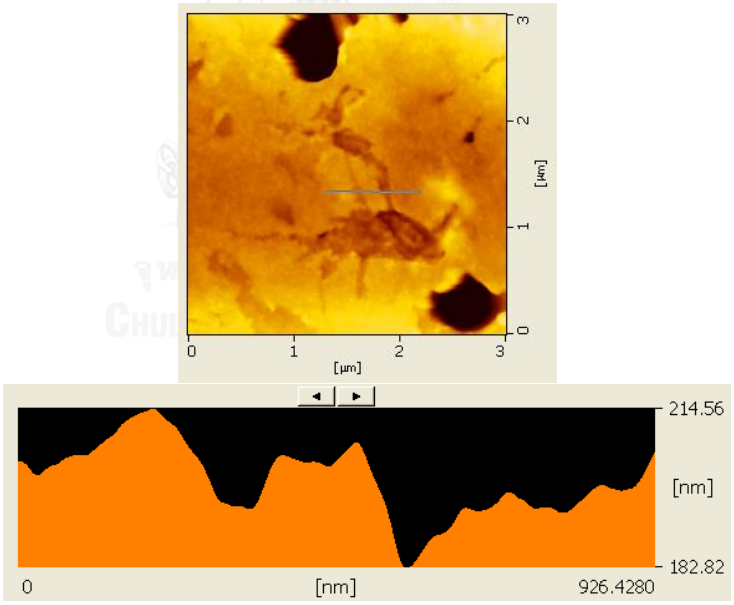

Figure F6 2D image of AFM of *L.interrogans* imprinted polymer film surface with overlap cell cavity and measurement data (Position 3)

VITA

Mr. Suraphun Aungwerojanawit was born in Bangkok, Thailand on March 16, 1991. He was graduated with Bachelor's Degree of Science in Materials Science (Polymer and Textiles) from Faculty of Science, Chulalongkorn University in 2012. He was granted the commemorate the 72nd anniversary of his Majesty King Bhumibala Aduladeja scholarship from Graduate School, Chulalongkorn University to continue his Master Degree. Then, he graduated with Master Degree in the field of Applied Polymer Science and Textile Technology at the Department of Materials Science, Faculty of Science, Chulalongkorn University in 2016.

Publications:

International Proceedings

S. Aungwerojanawit, W. Tachaboonyakiat, "Synthesis of Poly(MMA-co¬-HEMA) using photopolymerization with benzophenone and triethylamine by bulk polymerization", Proceedings International Polymer Conference of Thailand, Pathumwan Princess Hotel, Bangkok, Thailand. June 30 – July 1, 2016, pp. 177-180.

International Conferences

1. S. Aungwerojanawit, K. Patarakul, A. Sereemaspun, W. Tachaboonyakiat, "Bacterial imprinted polymer: challenging in imprinting using complex morphology bacteria", The 252nd American Chemical Society National and Exposition (ACS International meeting), Pennsylvania Convention Center, Philadelphia, Pennsylvania, USA. August 21 – 25, 2016.

2. S. Aungwerojanawit, W. Tachaboonyakiat, "Synthesis of Poly(MMA-co¬-HEMA) using photopolymerization with benzophenone and triethylamine by bulk polymerization", The 6th International Polymer Conference of Thailand (PCT6), Pathumwan Princess Hotel, Bangkok, Thailand. June 30 – July 1, 2016.

3. S. Aungwerojanawit, W. Tachaboonyakiat, "Preparation and Morphological Study of Chitin Nanoparticles", The 4th International Polymer Conference of Thailand (PCT4), Pathumwan Princess Hotel, Bangkok, Thailand. March 20 – 21, 2014.

4. S. Aungwerojanawit, W. Tachaboonyakiat, "Preparation of Cholesterol Modified Chitin for Drug Carrier", The 3rd International Polymer Conference of Thailand (PCT3), Pathumwan Princess Hotel, Bangkok, Thailand. March 28 - 29, 2013.

International Internship

1. "Project to Form a Hub for Human Resources development and New Industry Creation building a sustainable society through highly interactive, cooperative educational research with Pacific Rim countries (Pacific Rim Program)", Nagaoka University of Technology (NUT), Nagaoka, Niigata, Japan. January 9 – March 31, 2015.