PROTEIN ADSORPTION AND BACTERIAL ADHESION ON HYBRID STARCH/POLYCAPROLACTONE BLOCK COPOLYMERS

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And approved by

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ABSTRACT OF THE THESIS

PROTEIN ADSORPTION AND BACTERIAL ADHESION ON HYBRID STARCH/POLYCAPROLACTONE (PCL) BLOCK COPOLYMERS

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A wide range of block copolymers were made by changing the variables including polymer molecular weight, ratio of PCL to Starch content, di- or tri- block, linear or branched and chemical modifications on hydrophilic blocks with different chemical group. After the synthesis work, fibrinogen adsorption, *Salmonella* adhesion on 4 different hybrid Starch-PCL-Starch tri block copolymers and 1 poly-(ethylene glycol) (PEG)-PCL-PEG block copolymer coating surfaces were studies while PCL was the control. Besides the protein adsorption and bacterial adhesion assay, atomic force microscopy analysis (AFM), scanning electronic microscopy (SEM) and contact angle measurements were also performed to better understand the polymers' surface properties. The surfaces were prepared by dissolving block copolymers in organic solvent followed by spin coating technique. Preliminary data showed that all the block copolymers have improved protein and bacteria repellency than PCL. Among all the block copolymers, YZ3-38 (with quaternary ammonium group), MC4-38 (substituted

with N,N-diethylaminoethyl ether), MC4-44 (with hydroxypropyl groups) could dramatically improve the protein repellency, and MC4-38 and MC4-44 could significantly reduce the bacterial adhesion, compared with YZ3-14 (without modification on starch end group). MC4-38 even has comparative or better protein and bacteria repellence than PEG/PCL based copolymer (PEG is the most widely utilized protein and bacteria repellency polymer). Moreover, the data on fibrinogen adsorption, *Salmonella* adhesion showed that the ability to reduce fibrinogen adsorption at the copolymers surface correlates with the ability to reduce *Salmonella* adhesion. By evaluating the new copolymer's surface physical, biological properties, this work is able to show the potential protein anti-adsorption and bacteria anti-adhesion properties of the PCL/Starch based copolymer and contribute to the understanding of the connections between protein adsorption, bacterial adhesion, contact angle and surface topography.

ACKNOWLEDGEMENT

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1. INTRODUCTION

Poly (ε-caprolactone) (PCL) is a hydrophobic biodegradable polymer and it is a Food and Drug Administration (FDA) approved material that could be used in human body as a drug delivery device, suture, or adhesion barrier. [1] Owing to its slow degradation rate, a lot of interests have been showed on the long term implantable devices research. [2] However, the application of PCL is limited because there is no main active chemical group which could serve as the hook for further chemical modification. Furthermore, bacteria tend to adhere to hydrophobic surface such as PCL and form biofilms which will often cause infections and inflammatory response in human body. [3] To expand the application of PCL, hydrophilic block polymer which has reactive end group could be grafted onto PCL and the block copolymer could be chemically engineered to fit its application. [4] Starch based material could be excellent candidate since it is hydrophilic, naturally biodegradable and the end group could be chemically functionalized. Grafting the starch molecule onto PCL could increase several independent variables for the molecule architecture, thereby expanding the functionality dramatically. There are several variables included, di- or tri- block, linear or branched starch, different molecule weight, starch end group modification. So the amphiphilic hybrid copolymer could be architecturally engineered to serve different purposes. [5] For example, the copolymer could self assemble upon entering water and form nanosphere with the hydrophobic part inside and hydrophilic part outside and it could be used for drug delivery[6, 7]; it could also form self-assembled monolayer

(SAM) upon annealing after coating on substrate and SAM could serve a series of purposes. [8, 9]

Bacterial contamination, especially in the form of biofilms, is a major concern in the personal care, medical and food industries. When biofilm former, such as *Salmonella* JSG 210 adhere to the surfaces of moist metal, glass or polymers, they produce a polymeric “slime” that provides a continuous matrix for the growth of complex microbial communities. Build-up of these matrices leads to problems such as persistent infections in human body and spoilage in meat and poultry applications. [10, 11] Initial bacterial adhesion, the beginning of biofilm formation, involves van der Waals forces and hydrophobic interactions as well as some electrostatic forces. [12] These interactions may be manipulated by adding hydrophilic binding groups or positive/negative charges to the polymer, in a manner favorably altering the physical-chemical properties of the polymer surface and inhibiting the formation of bacterial biofilm. However, the factors involved in bacterial adhesion have remained elusive and it is thought that a multitude of factors are involved, including surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and surface micro-topography. [12] The adsorption of protein to surfaces could play an important role in bacterial adhesion, as this conditioning of the surface may alter the physical–chemical properties of the surface. Conflicting opinion exists on the ability of surface to resist protein adsorption and bacterial adhesion. [12, 13] It is often assumed that the surface which is more protein adhesive also tends to be more susceptible to bacterial adhesion since protein could be both nutrients and favorable surface

conditioning film for bacteria. [13] However, Whitesides and coworkers observed that there seems to be little or no correlation between the adsorption of protein and the adhesion of cells. [8]

We could synthesize hybrid Starch/PCL block copolymers and improve their protein and bacteria repellency by manipulating the polymer's molecular weight, ratio of PCL to starch content, modifying hydrophilic blocks with different functional groups and changing between di- and tri- blocks. Based on the results from protein adsorption and bacterial adhesion on specified modified block copolymers, further chemical modification on copolymers could be made to increase the protein and bacterial repellent ability. If coated onto the implantable devices or food package films these specially engineered copolymers may significantly reduce protein adsorption and bacterial adhesion, decrease biofilm formation, thereby controlling persistent infections on implant devices and enhancing food safety and food quality.

The testing surfaces have been prepared by dissolving the copolymers in organic solvent followed by spin coating technique. Atomic force microscopy analysis, protein adsorption and contact angle measurements were also performed to better understand the polymers' surface properties. The protein adsorption assay was carried out by flowing fibrinogen (0.3mg/ml) solution on the copolymer surface for 2 hours and then rinsed for 1 hour by PBS solution. The data was recorded and analyzed based on a quartz microbalance with frequency dissipation (QCMD) system. *Salmonella enterica* serovar Typhimurium JSG210, a Gram-negative biofilm bottom former, was grown on the copolymers coated on cover slips which were placed on the

bottom of the micro plate. At predetermined time intervals, viable cells remaining on the polymers were measured with plate count technique by growing attached cells out after washing the unattached cells away from the copolymer surface.

2. LITERATURE REVIEW

2.1. Bacterial Adhesion and Biofilm Formation

Biofilm is an aggregate of microorganism in which cells are stuck to each other and/or to a surface. [14] A top former, such as *Salmonella enterica* serovar Typhimurium MAE52, forms biofilms on the air-liquid interface while a bottom former, such as *S. enterica* JSG210, forms biofilms on a tangible surface. [15] When the planktonic cells find a favorable place, such as place rich in nutrients or away from hazard, they tend to stay and start to produce a polymeric “slime” for their growth. [14] As showed in Fig. 1, when the nutrient is insufficient or the cells in a biofilm face certain threatening factors they could also return to planktonic form. [14]

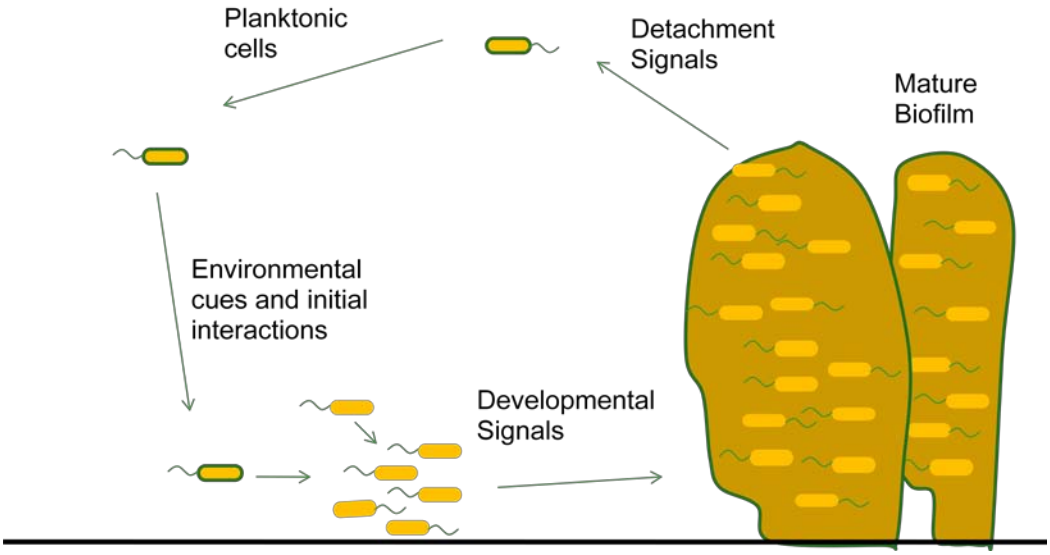


Fig. 1 Model of biofilm development

Individual planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. Cells in the biofilm can return to a planktonic lifestyle to complete the cycle of biofilm development.

Adapted from O'Toole [14]

Bacterial attachment is usually perceived as the beginning of biofilm formation and it typically involves a two-step process. [12] Based on the author’s research, there

is no standard term to describe the bacteria initial attach onto a surface; bacterial attachment, bacterial adhesion, bacterial adsorption are often mixed used. The first step is to transport the bacteria close enough to a surface or to the other bacteria to allow initial attachment, with the forces involved in this initial attachment being van der Waals forces, electrostatic forces and hydrophobic interactions. [12] Irreversible attachment of cells to the surface, described by Dunne [16] is the next step in the attachment process with bacteria locking onto the surface by the production of exo-polysaccharides (EPS). Since biofilms have the most defensive mechanism against hostile environment, the control of bacterial attachment will significantly improve the ability to control biofilm formation in sterile environment. [15]

2.2. Relationship between Protein Adsorption and Bacterial Adhesion

2.2.1. Mechanism of Protein Resistance

The adsorption of protein onto surface is not well understood in mechanism and it is probably caused by a combination of attractive components. [17] Part of the reason is because remarkable differences on adsorption kinetics were exhibited by individual proteins. [18] Chemical properties of the polymer will affect their state of hydration and the repulsive or attractive forces between polymer and water molecule. [19] Poly-(ethylene glycol) (PEG) has been widely used in biomedical devices to decrease protein adhesion. [20, 21] So far we know that inertness is not related to interfacial free energy. The inert surface is hydrophilic; because many hydrophilic surfaces are not inert so a surface must have other characteristics to resist protein

adsorption. [8] Additional inert surfaces are required to test the mechanism of protein resistance and excellent work has been done by many groups. [17, 22, 23]

2.2.2. Argument on Protein Adsorption and Bacterial Adhesion

Previously it is often assumed that protein adsorption is correlated with bacterial adhesion since protein could serve as nutrient for bacteria as well as preconditioning film which will alter the substrate's surface physical and chemical properties. And the ability to resist protein adsorption is believed as a prerequisite for the ability to resist bacterial adhesion. [22, 24] However, Emanuele and coworkers observed that there seems to be little or no correlation between the adsorption of protein and the adhesion of cells. [8] Many works have been done in correlating the relationship between protein adsorption and bacterial adhesion but no conclusion yet. [8, 13, 21-23, 25, 26]

2.2.3. Fibrinogen as a Model to Investigate the Protein Adsorption

Fibrinogen is a commonly used and well established model protein for protein adsorption experiment in QCMD experiment. [26-28] With a comparatively large molecular weight (MW=340kDa for a tetrameric aggregate, pI=5.5), it can readily adsorb onto hydrophobic and charged surface. It is also a major surface protein to initiate coagulation and inflammation; in *Salmonella* caused inflammatory diarrhea, elevated fibrinogen could be observed. It is structurally similar to extracellular matrix protein fibronectin which could facilitate the cells' adhesion to surface since fibronectin could bind with mammalian cells and some bacteria such as *Salmonella.* [29-32] The fibronectin binding proteins on *S. aureus* also facilitate the cell attach to the surface

coated with that protein. Hence, the surface that resistant to fibrinogen adsorption may be used on the application which requires resistance against to certain cell adhesion.

2.2.4. *Salmonella* and its Hazards

Salmonella is a genus of rod-shaped, Gram-negative, predominantly motile enterobacteria with diameters around 0.7 to 1.5 μm and lengths from 2 to 5 μm. *Salmonella* spp. are one of the most important food borne pathogens and studies have discovered that these bacteria are capable of adhering and forming biofilms on different surfaces. [33] *Salmonella typhi* can establish a chronic, asymptomatic infection of the human gallbladder and the infection may be caused by *Salmonella* biofilm formation on gallstones. [34] *Salmonella typhimurium* could adhere to and form biofilms on stainless steel and buna-n rubber, a gasket material commonly used in food processing environments. [35] More than 95% of cases of infections caused by *Salmonella* are food borne and these infections account for about 30% of deaths resulting from food borne illnesses. [33]

2.3. Structure-Property Relationships between Polymers and Adhesion

Historically, the structure-property relationship between chemical structures and protein adsorption and cell adhesion is a difficult subject since there are many influencing factors involved and it was tested on polymeric surfaces which are often chemically and topographically heterogeneous. In 2001, the most systematical research is published by Whitesides' group regarding to the structure-property relationships of surfaces that resist the adsorption of proteins. [17] The use of plasmon

resonance (SPR) spectroscopy and self-assembled monolayers (SAMs) were described to determine the characteristics of functional groups that give surfaces the ability to resist the nonspecific adsorption of proteins from solution. The advantage of SAM is that it is both chemically and topographically homogenous while polymeric film surface is heterogeneous so the SAM could eliminate many other factors and focus on the investigation of structure-property relationship. In Whitesides's work about 48 chemical structures were investigated and in the group of surfaces examined, the following properties were found: they were hydrophilic; they contained groups that were hydrogen-bond acceptors but not hydrogen-bond donors; and they were overall electrically neutral.[17] However, Mrksich found that SAMs presenting mannitol groups are also inert to protein adsorption, although they contain a large number of hydrogen-bond donors. [23] Polysaccharides are also exemption since they contain a lot of hydrogen-bond donors while they are well-known to form inert coatings on synthetic surfaces and on the surfaces of cells. [36] The surfaces coated with derivatives of carbohydrates which also reduce or resist the adsorption of proteins may share similar mechanism or structure as the mannitol-terminated surface. In the same time, we have to realize that this rule is generated by employing SAM and in real application polymeric film is more often used. In polymeric film application more complicated factors are involved such as roughness and micro-topography and the overall repellence depends on which one is the dominating factor.

Poly-(ethylene glycol) (PEG) has been widely used in biomedical devices to decrease protein adhesion. [20, 21] It has been shown that PEG-coated surfaces can

also used to reduce bacterial adhesion such as *Streptococcus, S. epidermidis, P. aeruginosa*. [20, 37]However, as a polyether, PEG has a tendency to autoxidize when exposed to O_2 and transition metals [17]

The inertness of $(EG)_nOR$- terminated surfaces was matched after the development of mannitol-terminated SAMs by Mrksich and co-workers. [8] It not only resist the adsorption of proteins, but also resists the adhesion of certain mammalian cells for longer periods of time than a surface terminated with tri(ethylene glycol). [23]

Quaternary amines are positively charged groups and they are believed to cause cell death by disrupting cell plasma membranes allowing release of the intracellular material. [38] The antimicrobial efficacy of polymer-modified surfaces with a large concentration of quaternary ammonium groups has been showed by Lee and Murata. [38, 39] So specially designed amiphiphilic copolymer with quaternary ammonium group may have substantial antimicrobial activity on Gram-negative bacteria. Furthermore, bacteria are known as negatively charged in neutral pH so the copolymer with quaternary ammonium group may serve as fly paper as shown in Fig. 2. It could attract the bacteria by electrostatic forces then kill them upon attachment by disrupting the cell plasma membranes.

Fig. 2 Fly paper

2.4. Methods Review for Protein Adsorption, Bacterial Adhesion and Biofilm Formation

2.4.1. Epifluorescent Microscopy and Acridine Orange

It is often used as a method to study bacterial adhesion. Acridine Orange (AO) cationic dye, which is a dual-fluorescence nucleic acid stain, could be used to determine the physiological activity and growth stages of cells. It interacts with DNA and RNA through intercalation or electrostatic attractions. When associated with RNA, AO dye fluoresces at a wavelength of 650 nm (orange to red), whereas with DNA it fluoresces at a wavelength of 526 nm (green). It condenses chromatin so that the DNA molecules are packed in a way that does not allow for sufficient acridine orange intercalation so cells in the log phase appear red. But upon entry into the stationary phase the amount of RNA synthesized diminishes, causing the decrease in intensity of the orange color, so cells in the stationary phase are green.

After contact periods, the substrate which is placed on the bottom of the cell suspension is washed twice with peptone water to remove the loosely adhered cells. The wells are then stained with 0.5 ml of 0.0055% acridine orange solution for 30 min, washed in peptone water and air-dried. Observation of biofilm development is conducted with an epifluorescence microscope. Color images with fluorescent microscopy are analyzed using custom-made MATLAB code which produces ratios of the intensities of the green and red components of the image. To evaluate trends in surface cell population development, the data are normalized by the method of moving averages and interpolated with the standard MATLAB functions. [40-42]

2.4.2. LIVE/DEAD® BacLight™ Bacterial Viability Kits

It can be used to study both bacterial adhesion and biofilm formation. The LIVE/DEAD® BacLight™ Bacterial Viability Kits employ two nucleic acid stains—green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO® 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. Live and dead bacteria can be viewed separately or simultaneously by fluorescence microscopy with suitable optical filter sets. The BacLight™ assay has been used on many gram-negative and gram-positive bacteria, mycoplasmas, yeasts, biofilms, and protozoa. [13, 38, 39]

2.4.3. Crystal Violet Staining and Optical Density

It is usually used a method to study biofilm formation. The cell number in biofilms can be quantified by crystal violet staining followed by optical density measurement. Typically 0.1% crystal violet solution (in sterile water) is added to each well which contains biofilm on the bottom of the well and it is incubated for 45 min at room temperature. Unbound dye can be removed by rinsing three times in sterile water. The crystal violet is solubilized by adding 95% ethanol and incubating at 4°C for 30 min. The contents of each well can be then transferred to a sterile polystyrene micro titer plate, and the optical density at 595 nm (OD_{595}) of each well is measured in a micro plate reader. [43, 44] The method is usually used for Gram-positive bacteria since Gram-negative bacteria have cell membrane which may be hard to be penetrated by crystal violet.

2.4.4. Quartz Crystal Microbalance-with Dissipation

It could be used to study for all three adhesion phenomena. Quartz Crystal Microbalance-with Dissipation (QCM-D) is a new quantitative method to investigate surface adsorption phenomena. It could monitor and measure the mass changes in protein adsorption and bacterial adhesion in real time. Quartz crystals were spin-coated with polymer solutions and inserted into the QCM-D instrument. Then, frequency and dissipation will shift as time goes by and the data could be modeled using the software Q-Tools. [26-28]

2.4.5. Other Methods

Parallel flow chamber: The parallel flow chamber is probably the most frequently used design for dynamic bacterial adhesion phenomena. Similar to QCMD, the bacteria suspension is allowed to flow over the coated surfaces, such as cover slips and on the top of chamber there is plate made of glass allowing for microscopic detection. The images can be recorded with a charge coupled device (CCD) camera and processed by an image analyzer. [13, 45]

Plate count: Typically the bacteria would be grown on the substrate which was placed on the bottom of a micro plate or Petri dish containing proper broth for bacteria to grow. At predetermined time intervals, viable cells remaining on the substrate were measured with plate count technique by growing attached cells out after washing the unattached cells away from the substrate. This method could be used for both bacterial adhesion and biofilm formation. The washing step to remove the attached cell is usually done by sonication or vortex. [8, 15, 35]

3. OBJECTIVES

The overall objective is to synthesize a series of hybrid Starch/PCL block copolymers and evaluate their performance on protein adsorption and bacterial adhesion.

To fulfill this objective, we have several sub-objectives to achieve:

(1) Design, synthesize and characterize hybrid Starch-PCL (di) and Starch-PCL-Starch (tri) block copolymers. Create a wide range of block copolymers by varying molecular weight, ratio of PCL to Starch content, di- or tri- block, linear or branched and chemical modifications on hydrophilic blocks with different chemical group.

(2) Identify a series of Starch/PCL based copolymers and chemical modifications which give the surface ability to resist protein adsorption, bacterial adhesion.

(3) Explore the relationship between protein adsorption, bacterial adhesion, contact angle and surface morphology.

4. MATERIALS AND METHODS

4.1. Materials

All organic solvents, if not described individually, were all purchased from Fisher Scientific Inc. (Suwanee, GA, USA) Hexafluoroisopropanol (HFIP) was obtained from DuPont (Wilmington, DE). Phosphate-buffered saline (PBS) was purchased from Sigma (St. Louis, MO). Fibrinogen (Fg) from human plasma was from Calbiochem (La Jolla, CA). 24 Well Tissue Culture Plate was from Falcon (Franklin Lakes, NJ, USA 07417-1886). BHI Plates were from Dickinson & Co. (37 g l^{-1}; BD, Becton, Sparks, MD).

The work for starch block synthesis used Tapon, Hylon VII and Neo Amylose. Tapon and Hylon VII are all brand name and they are acquired from National Starch and Neo amylase is from New Jersey Center for Biomaterials (NJCBM).

TAPON ND® (Amioca 85®) is a high fluidity, acid hydrolyzed waxy hybrid

Hylon VII® is a 70% amylose hybrid

Neo Amylose is a bacteria-generated amylose starch

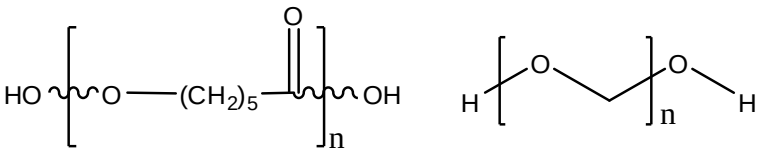
The block copolymers were synthesized in collaboration with the Kohn lab partners NJCBM and the master chart is as below.

Table 1 Chart for evaluated block copolymers

Notebook #	Polymer type	Mn	DS	RMS (nm)	Contact Angle
PCL diol	PCL	80K	N/A	18.5475	78.0
YZ3-14	WM30-PCL85-WM30	114K	N/A	5.481	57.5
YZ3-38	Q_WM30-PCL85-Q_WM30	130K	0.1	13.245	46.5
YZ3-77	PEG20-PCL85-PEG20	99K	N/A	5.543	51.5
MC4-38	WM23-PCL17-WM23	45K	0.25	5.363	28.5
MC4-44	HP-AMII11-PCL17-HP-AMII11	38K	0.1	25.395	39.5

Note:

WM: Waxy Maize Hybrid, AM: Amylose Maize Hybrid, HP: hudroxypropyl groups, Q: quaternary ammonium group, DS: degree of substitution, PEG: poly-(ethylene glycol), AMII: Hylon II, RMS: Root mean square (calculated based on AFM software with scan size of 10 μm × 10 μm)



PCL diol PEG

Fig. 3 Structure of PEG and PCL diol

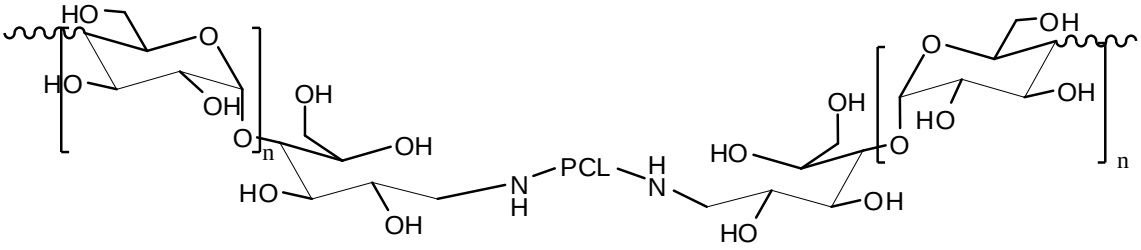


Fig. 4 Chemical structure of general tri block copolymer (e.g. YZ3-14)

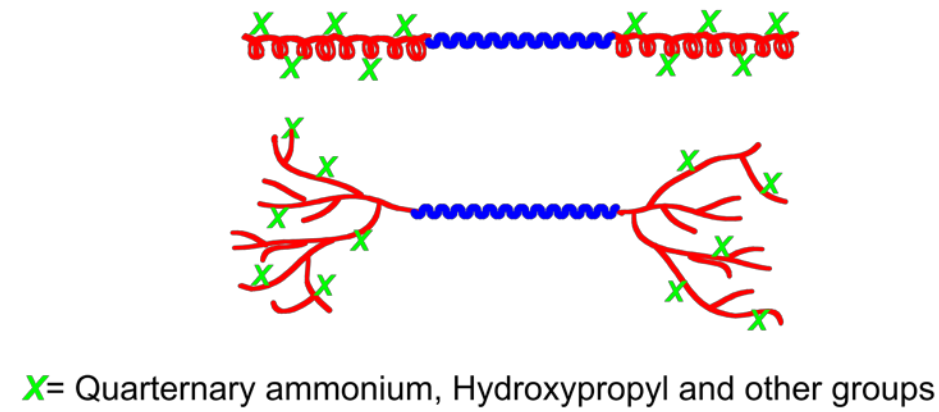
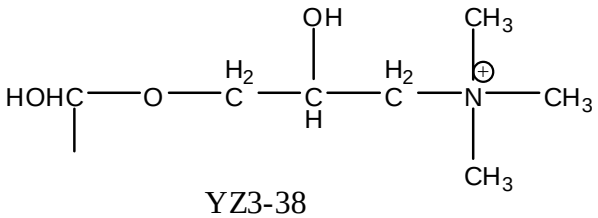
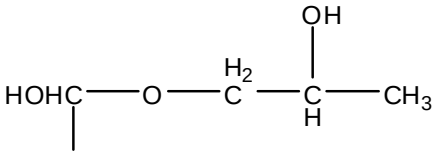


Fig. 5 Linear and branched tri block Starch-PCL-Starch copolymer

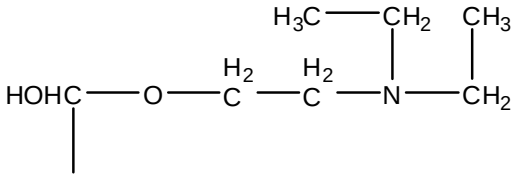
Adapted from Carmine and Yi



YZ3-38



MC4-44



MC4-38

Fig. 6 Chemical structures of end group on YZ3-38, MC4-44 and MC4-38

4.2. Polymer Synthesis

The author participated in the synthesis and characterization for block copolymer YZ2-66 (WM16K-PCL10-WM16), YP1-3 (AM7.5-PCL10K-AM7.5), YZ2-80 (WM30-PCL42-WM30), YP1-9 (Neoamylose4-PCL10-Neoamylose4), YZ2-99 (WM16-PCL13.6) and YZ3-5 (AM9-PCL13.6). However, due to time and priority consideration, the evaluation work for those materials is not described in the thesis. Other materials which are listed in master chart (Table 1) were evaluated.

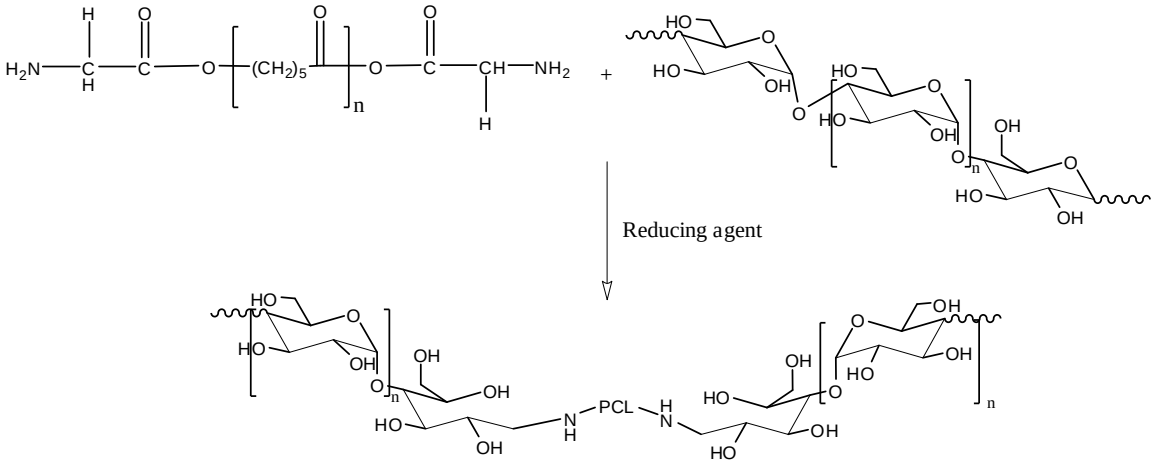


Fig. 7 Synthesis scheme for block copolymer

YZ2-80 (WM30-PCL42-WM30) is used as an example to illustrate the method for synthesis of branched tri block copolymer.

18 g (0.6 mmol) modified TAPON starch from YZ2-21 (Mn=30K) is dissolved in 80 ml DMSO. 8.3 g (0.2 mmol) PCL diamine from YZ2-63 (Mn=42.5K) is dissolved in 140 ml DMF. The two solutions are mixed together in a in a 500 ml reaction flask equipped with a magnetic stirrer, a heating mantle, a condenser and a thermometer. The mixed solution is stirred for 1 hour while being heated to 60°C. 0.26 g (4 mmol) sodium cyanoborohydride is dissolved in 10 ml DMSO to make a 5-shot reagent, 2 ml each. The reagent is added to the reaction mixture in 3 days, two shots per day.

10 ml deionized water is added to the reaction flask to convert excess $CNBH_3$ to HCN. N_2 gas is passed through the flask overnight to remove HCN. The mixture is put to 2500 ml cold acetone to precipitate out polymer product. The precipitation is filtered and dried in a vacuum oven.

YP1-3 (AM7.5-PCL10K-AM7.5) is used as an example to illustrate the method for synthesis of linear tri block copolymer.

18 g (2.4 mmol) modified TAPON starch from YZ2-37 (Mn=7.5K) is dissolved in

80 ml DMSO. 8.0 g (0.8 mmol) PCL diamine acetate from YZ2-51 (Mn=10K) is dissolved in 140 ml DMF. The two solutions are mixed together in a in a 500 ml reaction flask equipped with a magnetic stirrer, a heating mantle, a condenser and a thermometer. The mixed solution is stirred for 1 hour while being heated to 60°C. 1.0 g (16 mmol) sodium cyanoborohydride is dissolved in 10 ml DMSO to make a 5-shot reagent, 2 ml each. The reagent is added to the reaction mixture in 3 days, two shots per day.

18 ml deionized water is added to the reaction flask to convert excess $CNBH_3$ to HCN. N_2 gas is passed through the flask overnight to remove HCN. The mixture is put to 2500 ml cold acetone to precipitate out polymer product. The precipitation is filtered and dried in a vacuum oven.

4.3. Polymer Characterization

Routine polymer characterization included proton nuclear magnetic resonance (^{1}HNMR) spectrometry, measurement of molecular weight by gel permeation chromatography (GPC), dry glass transition temperature by differential scanning calorimetry (DSC) and decomposition temperature by thermo gravimetric analysis (TGA) as described in the literature. [46, 47]

4.4. Surface Preparation

4.4.1. Spin Coating

Before spin coating, the glass cover slips were soaked in 95% ethanol for half an hour in ultrasonic cleaner, followed by rinsing in 95% ethanol, and dried by N_2 one by

one. Cleaned cover slips were coated with different types of polymers. Starch/PCL block copolymer solution were made by dissolving the copolymer in HFIP with a concentration of 0.5% g/ml (w/v), and then filtered by 0.45um filter. (For protein adsorption the solvent is DMSO/DMF=1:2). Spin coating parameters for bacterial adhesion are as follows: RPM: 4000, RH: 8.8%, Time: 30s, Volume: 90ml. Coated cover slips were sterilized in UV zone cleaner for 30 min and waited for bacterial adhesion testing. The method to clean the crystal is described in [28].

4.4.2. Compression Modeling

A. 0.5 g block copolymer sample is loaded on the lower half of the mold covered with PTFE film. Proper shim, e.g. 50μm, 100μm, is set up on the edge of the mold. Another half of the mold is placed on top of the sample.
B. The loaded mold is placed on the Carver press at 50°C. It is held for 2 minutes without pressure so as to soften the polymer.
C. The pressure is increased slowly to 10,000 psi over 1 minute.
D. The 10,000 psi pressure is held for 1.5 minute at 50°C.
E. The pressure is released slowly over 1 minute.
F. The polymer film is removed from the mold.

4.5. Contact Angle Measurement

The static water contact angle measurements were carried out using a VCA Optima XE Dynamic Contact Angle Analyzer (AST Products Inc., Billerica, MA) at ambient condition. The image was recorded by a CCD camera immediately after the

water drop was deposited onto cover slip surface.

4.6. Bacterial Species and Culture Conditions

Salmonella enterica serovar Typhimurium JSG210 is streaked onto brain heart infusion (BHI) plates (37 g l^{-1}) containing 1.85% granulated agar (BD) and incubated overnight at 37°C. Glass test tubes (16×125 mm; Fisher) containing 10 ml of BHI broth were inoculated with a colony isolated from the streak plate and incubated overnight at 37°C. The optical density at 600 nm (OD600) of the overnight culture is measured using a Bio-Rad SmartSpecTM 3000 spectrophotometer. The cultures were then diluted from the overnight culture (10^9 CFU ml^{-1}) for inoculation.

4.7. Bacterial Adhesion and Biofilm Formation Test

Cover slips with different polymer coating were placed on the bottom of the 24 well with sterile forceps. Then fill each well with 1.8 ml BHI broth and aliquoted 0.2 ml diluted inoculum culture to the well (for bacterial adhesion test the inoculation concentration was 10^9 CFU ml^{-1} while for the biofilm formation testing the inoculation concentration was 10^5 CFU ml^{-1}). The plates are incubated at 37°C with aeration for different intervals (for bacterial adhesion the incubation time is 30 minutes while for biofilm formation test the time last to 60 hrs). At predetermined time point the cover slips were removed and washed gently in two Petri dishes containing 30 ml PBS solution on a 3-D rotator for 2 min to remove all unattached cells twice. The cover slips were then placed into glass test tubes containing 4.5 ml PBS and vortexed strongly for a minute at room temperature to remove all attached cells, which were then serially

diluted and plated to do plate count [15, 22].

4.8. Atomic Force Microscopy (AFM) Analysis

Tapping mode AFM images were collected by NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA) equipped with a silicon-etched RTESP7 cantilever (Veeco Nanoprobe, Camarillo, CA) under ambient conditions. The topographies were obtained at the scan size of 10 × 10 μm (data collection at 512 × 512 pixels) and the scan frequency of 0.7535 Hz using a silicon nitride cantilever. The samples were prepared by a spin coating technique on the quartz crystals for protein adsorption experiments and glass cover slips for bacterial adhesion test. Images were analyzed using the software provided by the manufacturer. From the height image, the root-mean-square (RMS) roughness over the scan area of $10\times10um^2$ was calculated.

The samples prepared for bacterial adhesion test were also studied under AFM before strongly vortexed in PBS solution. The cover slips were gently rinsed with deionized water to remove salt crystals, and air-dried before analysis. [48, 49]

4.9. Protein Adsorption

The fibrinogen adsorption was measured by using QCM-D on selected copolymers (Table 1). Quartz crystals were spin-coated with polymer solutions and inserted into the QCM-D instrument. Then, frequency and dissipation shifts versus time curves, induced by sequential adding of protein solutions. The human fibrinogen protein solution was incubated until the binding saturation was reached, proximately 2 hours (as indicated by absence of further significant changes in frequency and

dissipation values) followed by several rinsing steps with protein-free PBS buffer. All experiments were repeated twice and normalized to the fundamental resonant frequency of the quartz crystal (5 MHz). Data were modeled using the software Q-Tools (Q-Sense, Goeteborg, Sweden) and protein adsorption thickness was quantitatively measured. [27].

4.10. Antimicrobial Activity Determination

Antimicrobial test of sectioned portions of the films were performed using a modified ASTM standard: E2149-01 Standard Test Method for Determining the Anti-microbial Activity of Immobilized Antimicrobial Agents under Dynamic Contact Conditions.

Glass test tube containing 5 ml of BHI broth was inoculated with a colony isolated from the streak plate and incubated overnight at 37°C. The cells were diluted with PBS to desired concentration (10^5CFU/ml). The compression molded film with total area of 14cm^2 for each, were cut into small pieces with area of 2 cm^2 and then placed into 50 ml test tubes. Then fill each tube with 4.5 ml PBS solution and aliquoted 0.2 ml pre-diluted inoculum culture to the tube. The test tubes were then incubated at 37°C while being shaken at 300 RPM. The bacterial concentration of solution at the “0” time was determined by performing serial dilutions and plate count techniques. A test tube contained 5 ml cells suspension without film was used as control. Samples were taken after 1 h, diluted appropriately, and plated on BHI plate. The test was carried out twice with 1 specimen each time [38, 39].

5. RESULTS AND DISCUSSION

5.1. Polymer Synthesis and Characterization

A series of block copolymers were made and below is a summary for the block copolymers. We should notice that this summary is different from Table 1 and the evaluation of those materials is not contained in this thesis.

Table 2 Selective chart of block copolymers

Notebook #	Starch Type	Abbreviation	Mn/Mw	Hydrophilic Percentage
YZ2-66	Branched	WM16-PCL16-WM16	51,411/70,807	62%
YZ2-80	Branched	WM30-PCL100-WM30	162,495/228,943	37%
YP1-3	Linear	AM8-PCL16-AM8	27,694/36,506	55%
YP1-9	Linear (Neoamylose)	Neo5-PCL18-Neo5	19,459/32,648	41%
YZ2-99	Branched	WM16-PCL14	28,305/51,693	57%
YZ3-5	Linear	AM9-PCL14	30,929/42,777	29%
YZ3-38	Branched	Q_WM30-PCL85-Q_WM30	129,841/173,027	49%

WM: Waxy Maize Hybrid, AM: Amylose Maize Hybrid

The crude product of YZ2-80 is a light yellow-colored solid material. The yield is 25.6 g, 98.1% theoretically. Below are the results from characterization.

A. NMR analysis

The ^{1}H NMR analysis for the block copolymer product is shown on Fig. 8 . The functional proton shifts both from starch and PCL diamine building blocks can be found on the spectrum, which proves the success of the synthesis.

B. GPC analysis

The GPC analysis for YZ2-80 (Fig. 9) shows the molecular weight distributions of the block copolymer. There is only one major peak with large polydispersity value, with Mn of 103K. After purification with water extraction, the GPC analysis will be

done again to determine the molecular weight.

C. TGA analysis

The TGA analysis for the block copolymer is shown on Fig. 10. It indicates the product contains 8.54% volatile. The degradation of the block polymer starts from 160°C.

D. Water extraction

2.81 g of block polymer product (YZ2-80) was extracted with 150 ml DI water twice, for 24 hours each. The mixture was centrifuged to separate the water soluble and insoluble portions. The water soluble fraction was placed on rota evaporator to remove the water with a dry weight of 1.6g (56.9%wt of total sample). The ^{1}H NMR for the water soluble portion is shown on Fig. 11 . It indicates the soluble phase contains only unreacted starch. The insoluble portion was dried and checked with ^{1}H NMR (Fig. 12). According to NMR integration, only bound starch exists in the purified product. Because of the high molecular weight of Tapon starch for the synthesis (Mn=30K), the proton shifts for the anomeric protons cannot be detected by NMR analysis. The efficiency of block polymerization is 56.3% for YZ2-80 copolymer.

Fig. 14 and Fig. 15 showed the ^{1}H NMR and GPC analyses for purified tri-block copolymer YZ2-80. After purification, Mn/Mw of the triblock copolymer is 162,495/228,943.

Based on the results of water extraction and NMR analyses, the block efficiency for YZ2-80 is 56.3%.

E. Solubility Tests

The purified tri-block copolymer YZ2-80 is fully soluble in DMF and NMP.

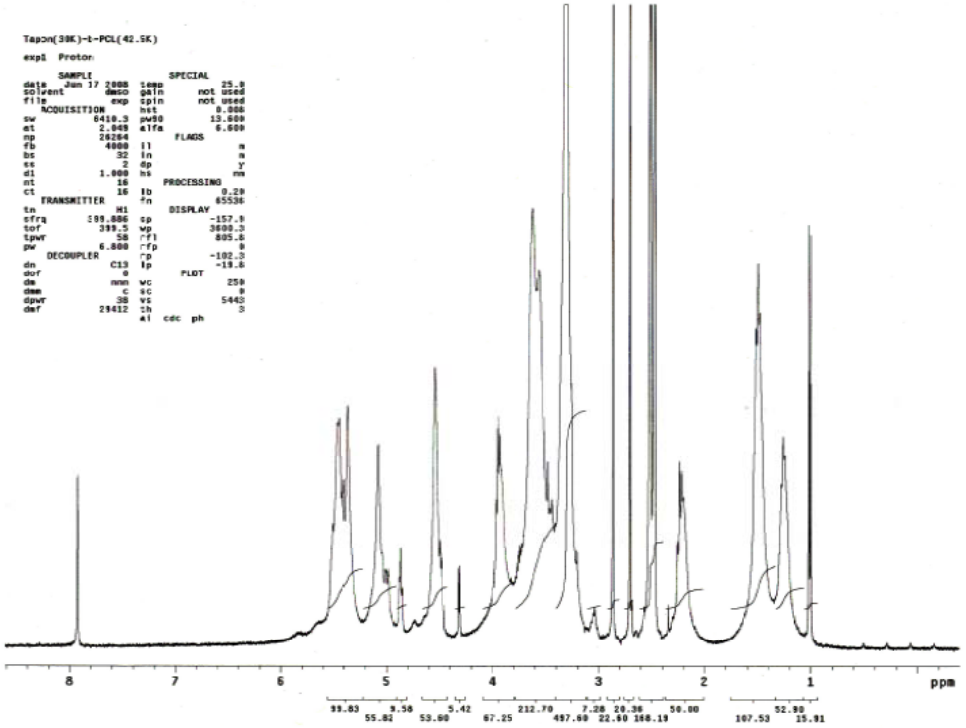
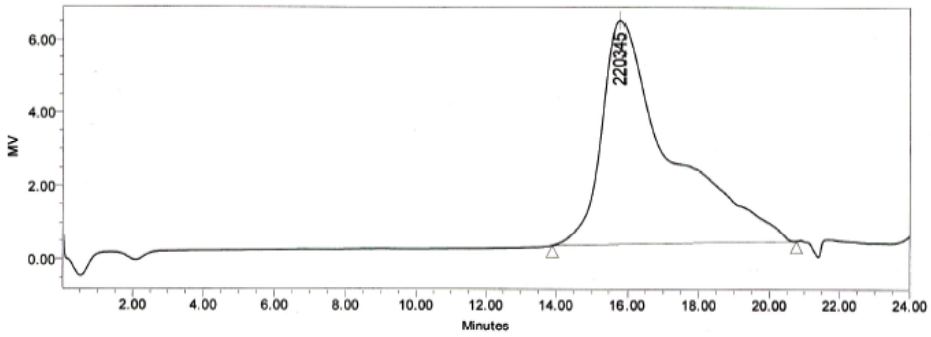


Fig. 8 ^{1}H NMR analysis for YZ2-80

SAMPLE INFORMATION

Sample Name:	YZ2-80 TAPON(30K)-b-PCL(42.5K)	Acquired By:	System
Sample Type:	Broad Unknown	Date Acquired:	6/19/2008 2:26:50 PM EDT
Vial:	2	Acq. Method Set:	AaPSS_032608
Injection #:	1	Date Processed:	6/19/2008 3:19:13 PM EDT
Injection Volume:	20.00 ul	Processing Method:	Aa_PSS032108
Run Time:	24.0 Minutes	Channel Name:	410
Sample Set Name:	zy	Proc. Chnl. Descr.:	



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		103366	163955	220345	213559	250252		1.586162	100000	350000

Fig. 9 GPC analysis for YZ2-80

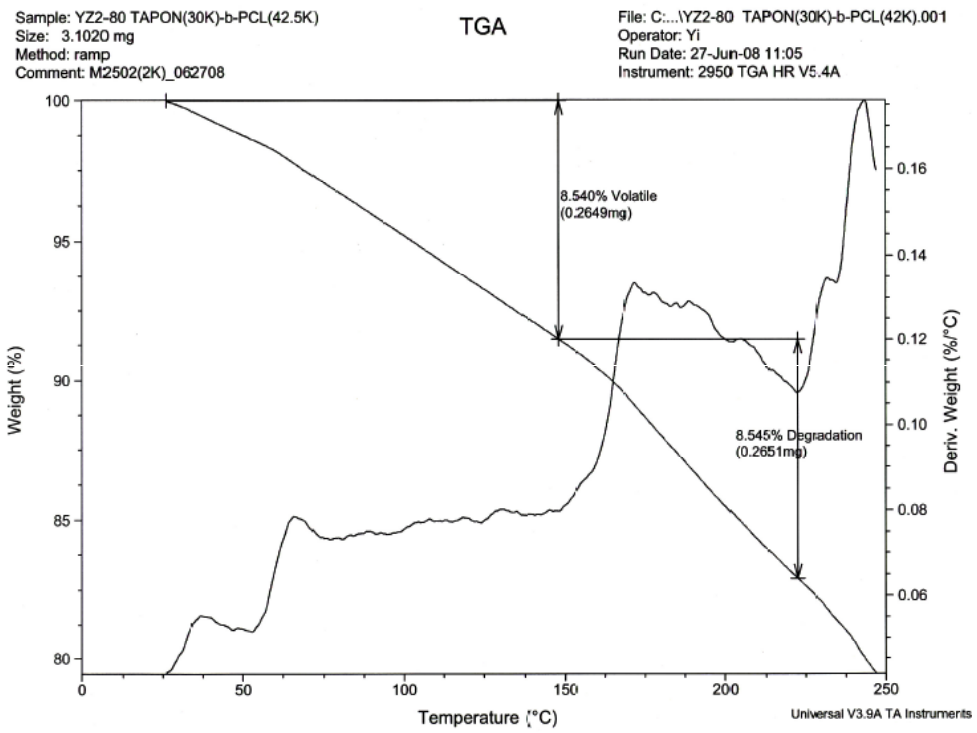


Fig. 10 TGA analysis for YZ2-80

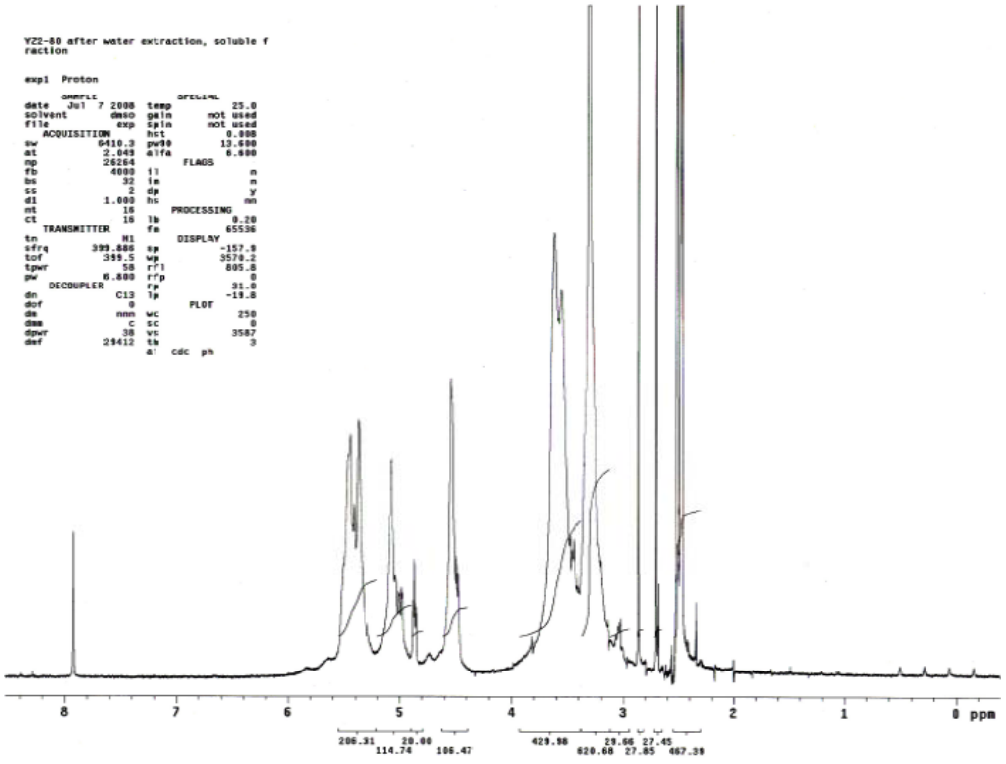


Fig. 11 ^{1}H NMR analysis for water extraction of YZ2-80, soluble fraction

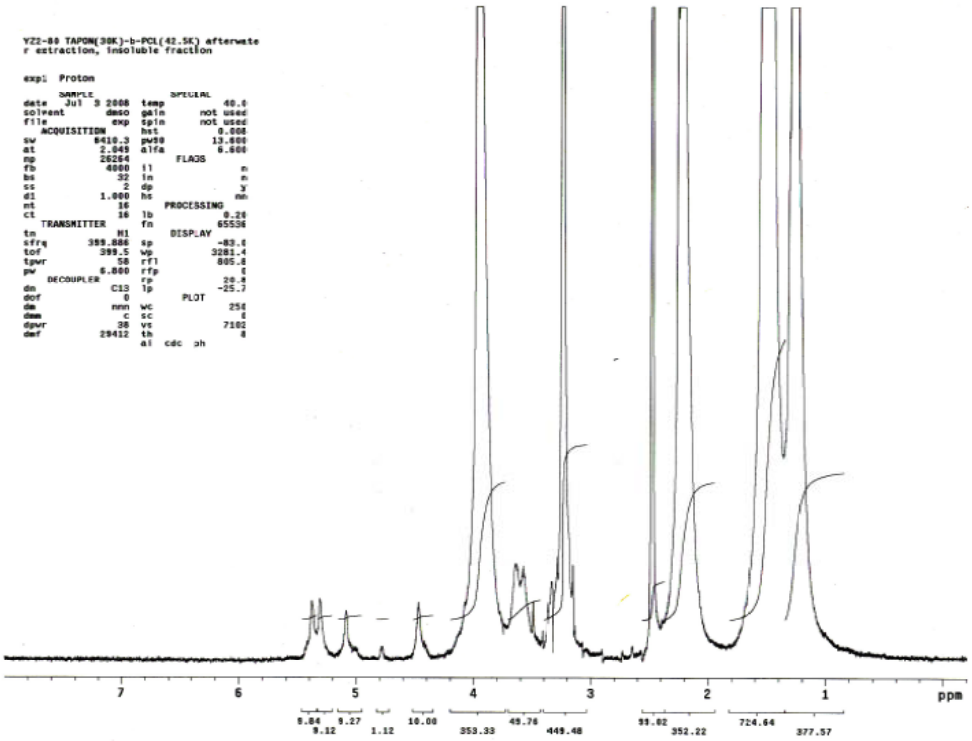
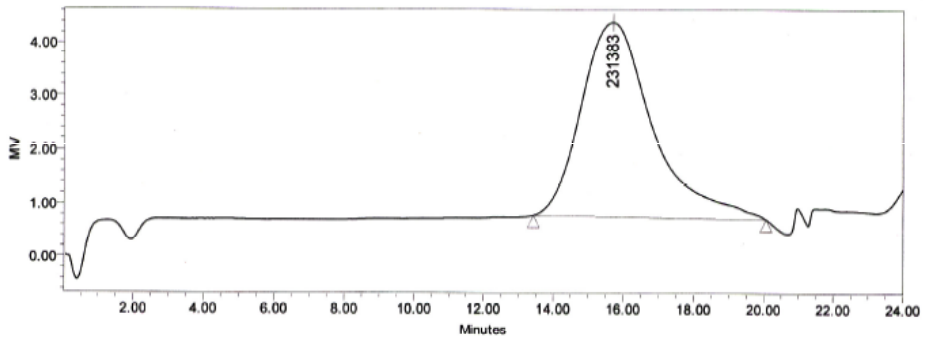


Fig. 12 ^{1}H NMR analysis for water extraction of YZ2-80, insoluble fraction

SAMPLE INFORMATION

Sample Name:	YZ2-80 after w ater extraction	Acquired By:	System
Sample Type:	Broad Unknow n	Date Acquired:	7/7/2008 12:45:07 PM EDT
Vial:	3	Acq. Method Set:	AaPSS_032608
Injection #:	1	Date Processed:	7/7/2008 1:25:12 PM EDT
Injection Volume:	20.00 ul	Processing Method:	Aa_PSS032108
Run Time:	24.0 Minutes	Channel Name:	410
Sample Set Name:	yz 7_7_08	Proc. Chnl. Descr.:	



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		162495	228943	231383	286074	336995		1.408922	100000	350000

Fig. 13 GPC analysis for water extraction of YZ2-80, insoluble fraction

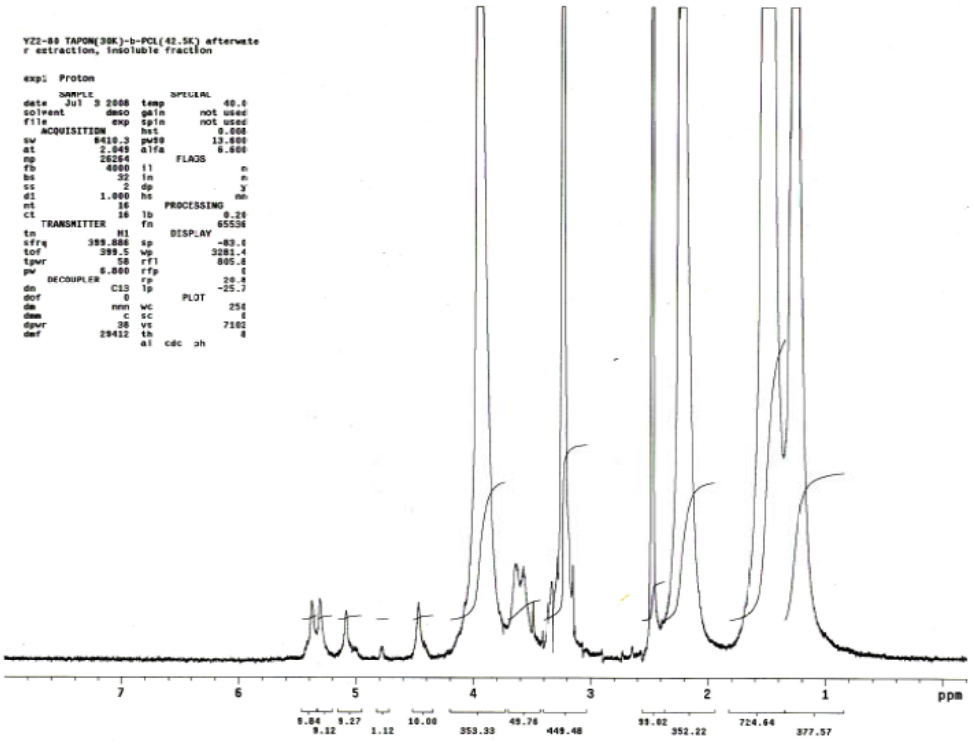
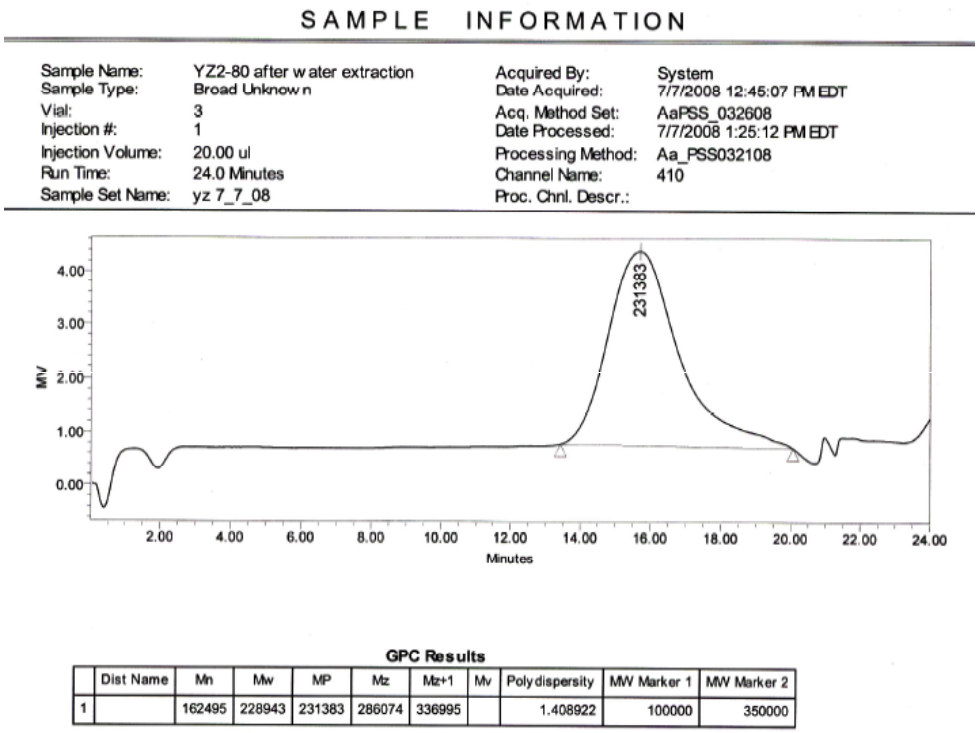


Fig. 14 ^{1}H NMR analysis for YZ2-80, purified



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Polydispersity	MW Marker 1	MW Marker 2
1		162495	228943	231383	286074	336995		1.408922	100000	350000

Fig. 15 GPC analysis for YZ2-80, purified

The solubility tests and particle size analysis for YZ2-66 are listed as below. Those results will help understand the polymer/solvent interaction and the polymer morphology.

A. Solubility Tests

The solubility of YZ2-66 was examined in both pure and mixed solvent systems. The results are listed on Table 3. The data will be used for spin coating and solvent film coating operations.

Table 3 Solubility test for YZ2-66

No.	Solvent	Result	Notes
1	DMSO (6 ml)	dispersion	
2	DMF (6 ml)	solution	
3	NMP (6 ml)	solution	
4	THF (6 ml)	Not soluble	
5	Ethanol (6 ml)	Not soluble	
6	Ethanol/H2O(5 ml/1 ml)	Not soluble	Ethanol added first, then H2O
7	H2O/THF (1 ml/5 ml)	dispersion	Water added first, then THF
8	DMF/H2O(1 ml/5 ml)	dispersion + precipitation	DMF added first, then H2O
9	DMF/THF(1 ml/5 ml)	dispersion + precipitation	DMF added first, then THF
10	NMP/THF(1 ml/5 ml)	dispersion	NMP added first, then THF
11	NMP/H2O(1 ml/5 ml)	light dispersion	NMP added first, then H2O
12	NMP/H2O(3 ml/3 ml)	dispersion	NMP added first, then H2O

Sample size: 50 mg for #1-#11; 500 mg for #12

Concentration: 0.1%wt for #1-#11; 10%wt for #12

B. Particle Sizes

Based on the results in the solubility tests, four solvent systems were selected for measuring the particle sizes using Dynamic Light Scattering (DLS): DMSO, NMP/THF, NMP/H2O, NMP/H2O (High Concentration). The sample solution or dispersions were filtered through 0.45 μm filter before DLS. The results are listed in Table 4 below.

Table 4 Particle size determination for YZ2-66 with DLS

	Solvent System	Mean Diameter	Coefficient Variation	Std. Deviation
1	DMSO	47.1 nm	0.510	24.010 nm
2	NMP/THF	48.0 nm	0.427	25.511nm
3	NMP/H2O	43.0 nm	0.436	18.739 nm
4	NMP/H2O, High Conc.	45.3 nm	0.619	28.013 nm

Notes: sample solutions were filtered through 0.45 um filter before testing.

Concentrations: #1-#3 are 0.1%wt; #4 is 2.5%wt

5.2. Surface Preparation and AFM Analysis

There are four candidate methods for film making for the research on both protein adsorption and bacterial adhesion: solvent casting, spin coating, compression molding and extrusion. The copolymers were synthesized in small quantity and also based on the thermo property it is not suitable for compression molding and extrusion. It is too slow to dry the solvent by using solvent casting so it is not quick enough to make enough surfaces. Since the copolymers are amphiphilic and may self assemble upon annealing, further investigation could be conducted to evaluate the anti-fouling properties of self-assembled coating surface after spin coating. For the current research we are only using the spin coating technique without annealing.

Since it is amphiphilic copolymer and the solubility test was performed to make solutions and it turns out that the polymer is so hard to be dissolved. HFIP is a polar solvent and its strong hydrogen bonding properties enable it dissolve the Starch/PCL copolymers. For bacterial adhesion experiment HFIP was used as the solvent and for protein adsorption experiment the mixed solvent of DMSO/DMF (1:2) was used as the solvent. Based on limited data the coating surface prepared from DMSO/DMF solvent has better protein repellence so the mixed solvent was adopted by protein experiment. Based on AFM observation the HFIP formed very uniform surface and in K. Norrman's review [50] solvent with lower volatility should form more uniform but thinner surface. So we concluded that the surface prepared for protein adsorption and bacterial adhesion experiment should be uniform and compatible with the one from HFIP.

Many groups have reported that roughness is an important factor which could influence bacterial adhesion and greater cell adhesion is often correlated with greater surface roughness. The scratch and pits on material surface with similar size of bacteria also may retain higher amount of cells than those with much larger size than bacteria. Six different polymers all have different topography and roughness. Regarding to roughness, ranking is MC4-44 (25.4nm) > PCL (18.55nm) > YZ3-38 (5.71nm) >= YZ3-77 (5.54nm) >= YZ3-14 (5.48nm) >= MC4-38 (5.36nm) as shown in Table 1. We could find out that among all the polymer coatings, roughness for YZ3-38, YZ3-77, YZ3-14 and MC4-38 are quite comparative, they are about 5.5nm. PCL and MC4-44 have much larger roughness and the RMS for them is 18.55nm and 25.4 nm respectively. In terms of surface micro-topography, YZ3-14 and YZ3-77 are on the early stages in the growth of spherulites and some of the parts are in the beginnings of radial growth in a spherulite of copolymer (Fig. 19 Fig. 20 Fig. 23 and Fig. 24). MC4-38 forms nanometric crystallite and based on the section analysis it also has the smoothest surface. Interestingly, we also found the similar surface morphology for PEO, as shown in Fig. 16, and as we all know PEG has also been known as polyethylene oxide (PEO) or polyoxyethylene (POE). PEG is one of the most known and extensively studied anti-protein repellency material. The similar morphology and good protein repellency property leads us to guess certain morphology-property relationship. For PCL, there are some long cracks on the surface and the width for the crack could range from 1-3 μm and the crack could be as deep as 70 nm (Fig. 17 and Fig. 18). Besides the cracks, there are many “valleys” and “hills” on the surface, the same

situation could also be found on MC4-44.

The static contact angle of glass surface is reported as 26.48 ˚± 0.71 and it is generally regarded as hydrophilic. [51] Surface properties and biocompatibility of solvent-casted PCL films are studied in Williams's paper. [51] In his paper he showed that the contact angle and cell adhesion property will be significantly influenced by the solvent used. Also the coating side which is in contact with glass and the coating side which is in contact with air have different roughness and contact angle. The contact angle for casting film in THF is 101.72 ˚± 1.29. In others' studies the static contact angle of PCL films produced by spin coating has been reported as 78 ˚± 2 [52] and 73 ˚± 2.5 [53], which are quite comparable with our results. In our research, however, spin coating technique is hired so the polymers do not have time to aggregate and the evaluated surface may not be affected dramatically by solvent and coating surface.

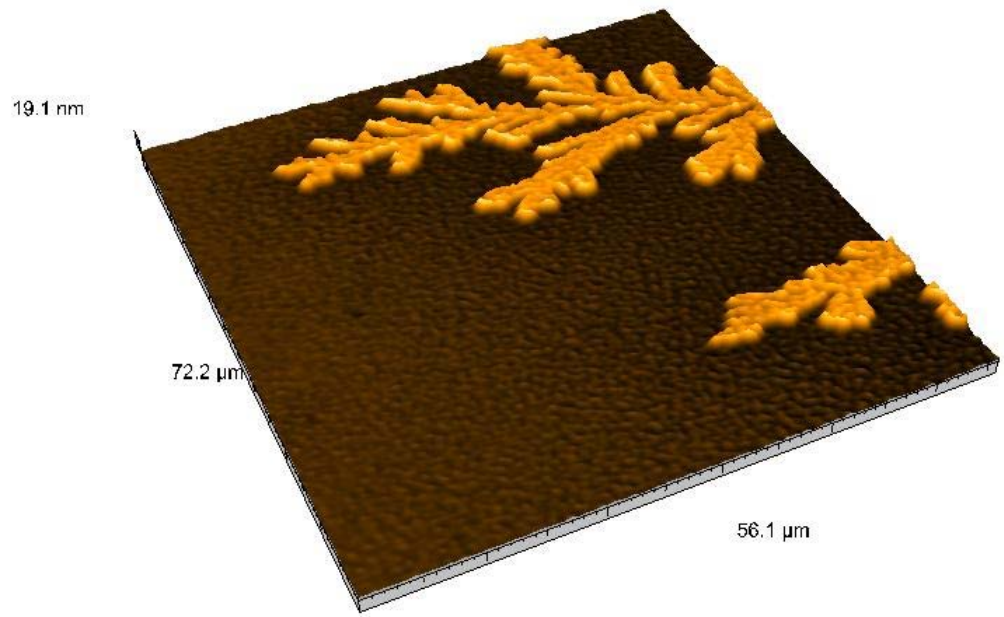


Fig. 16 Polyethylene oxyde (PEO, Mw 4kD) nanometric crystallites (4nm)
Adapted from Wikipedia

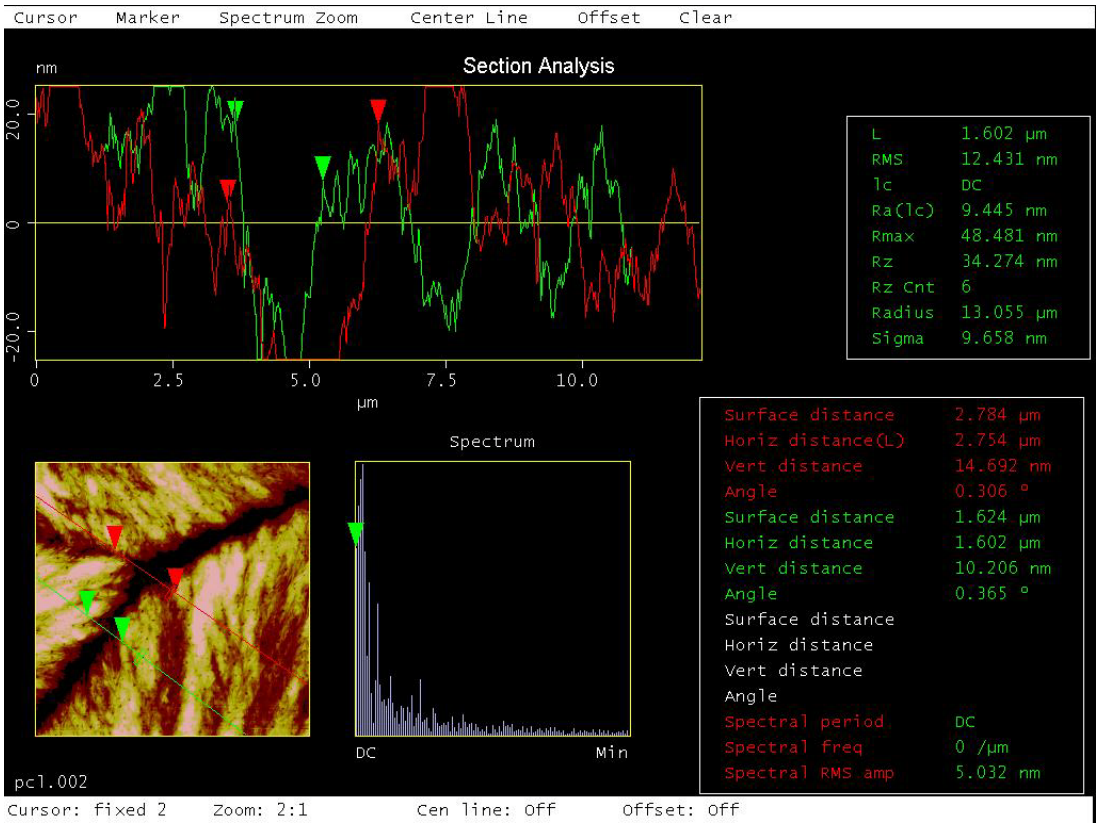


Fig. 17 AFM section analysis of PCL

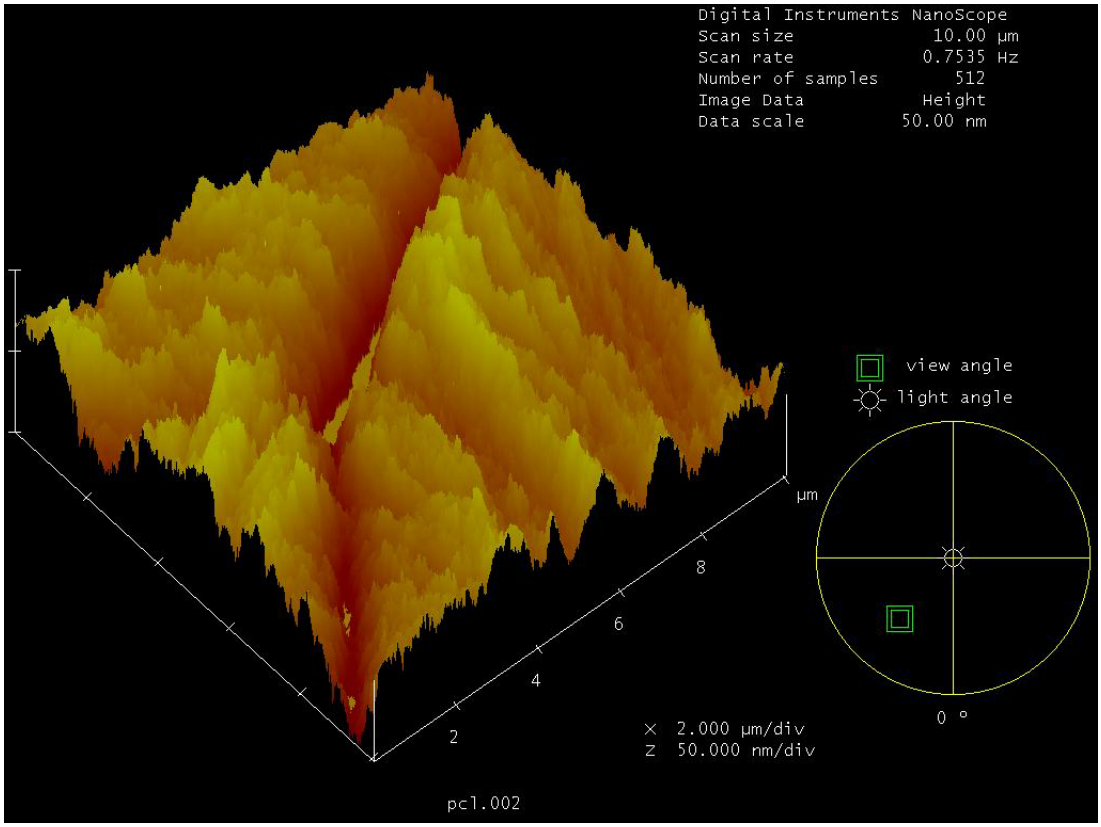


Fig. 18 AFM top view of PCL

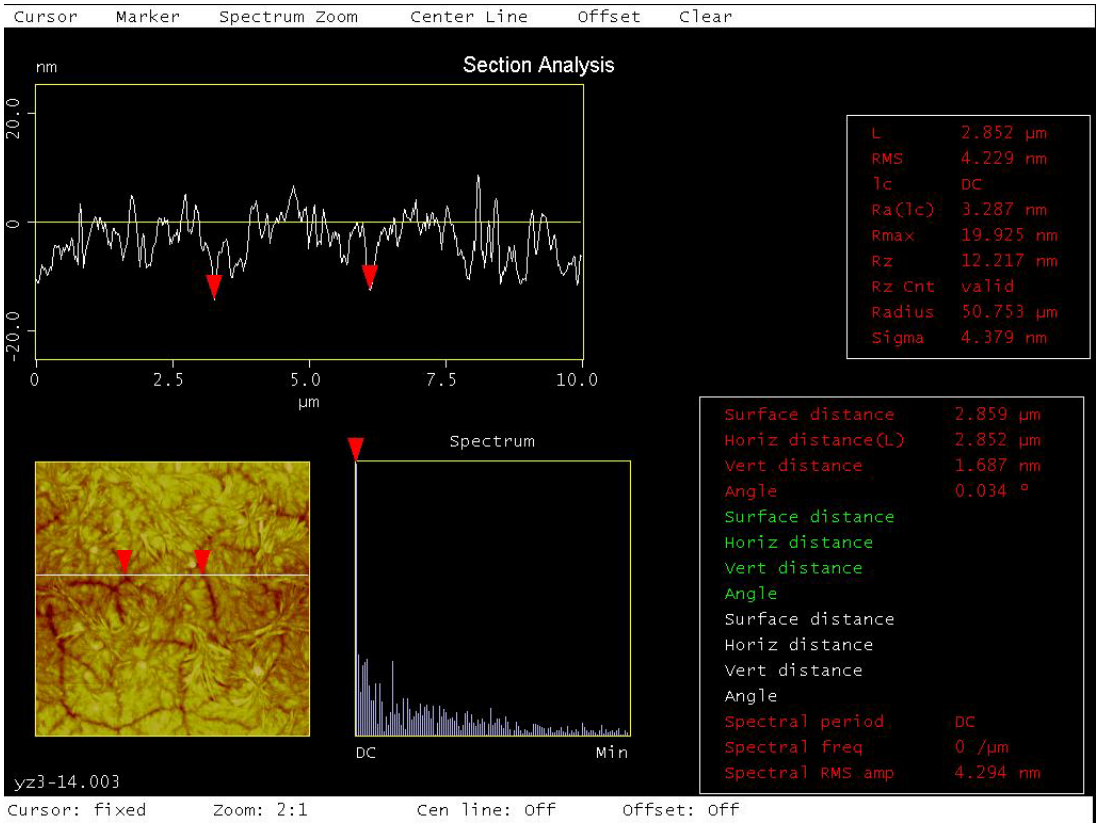


Fig. 19 AFM section analysis of YZ3-14

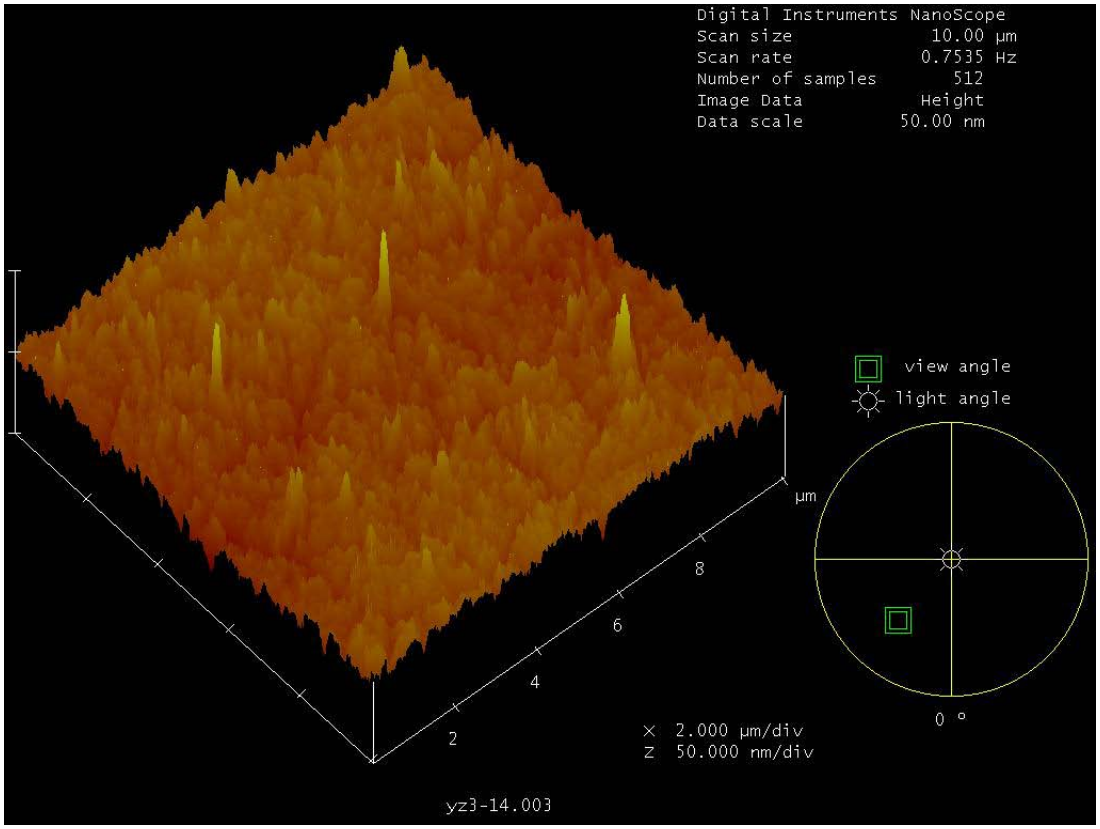


Fig. 20 AFM top view of YZ3-14

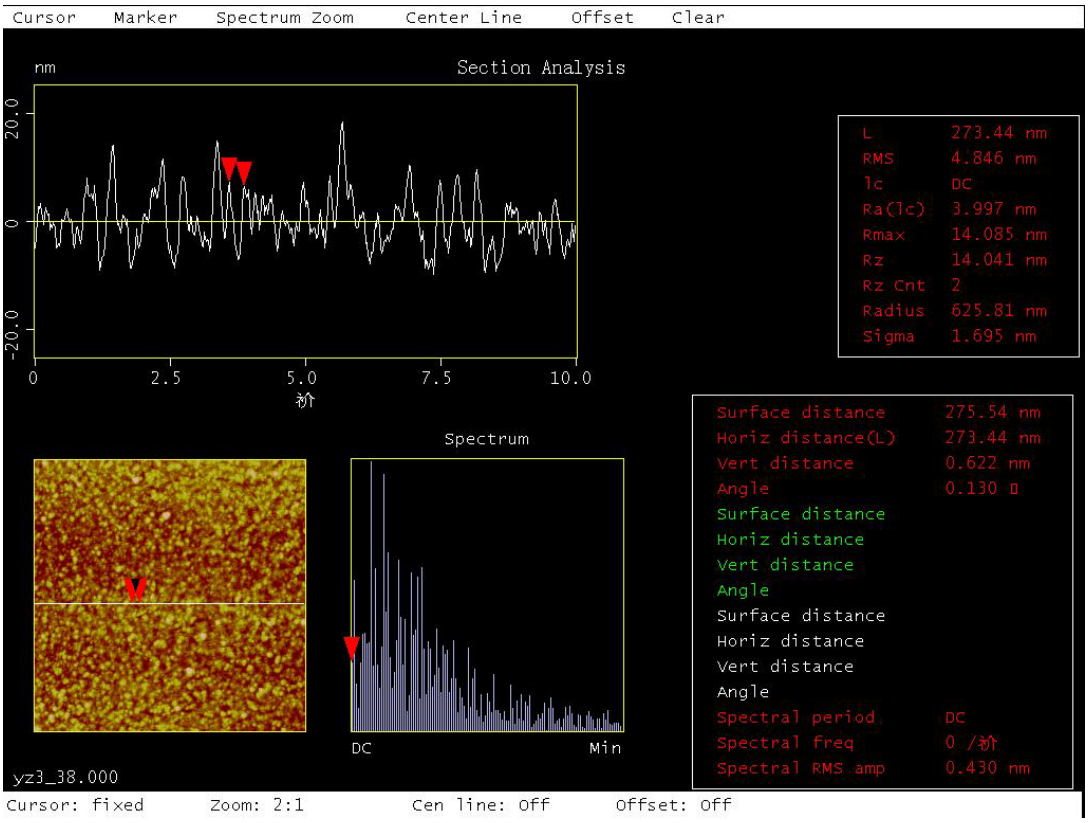


Fig. 21 AFM section analysis of YZ3-38

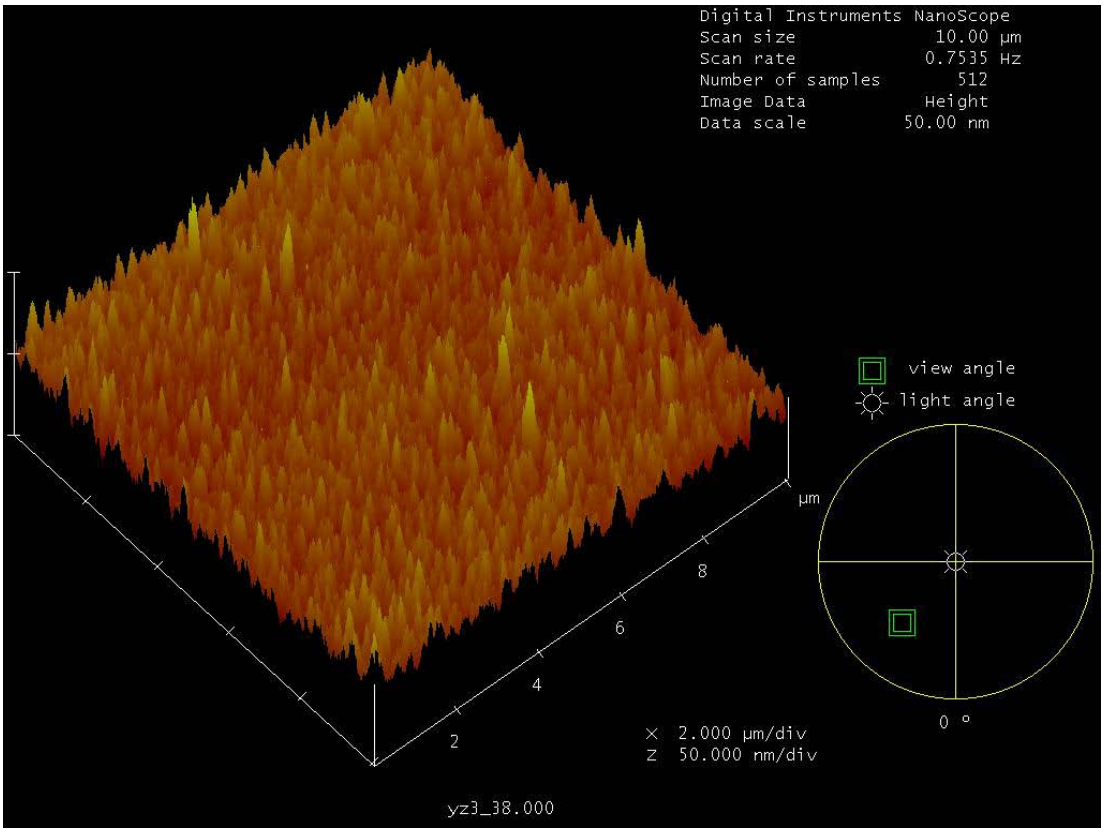


Fig. 22 AFM top view of YZ3-38

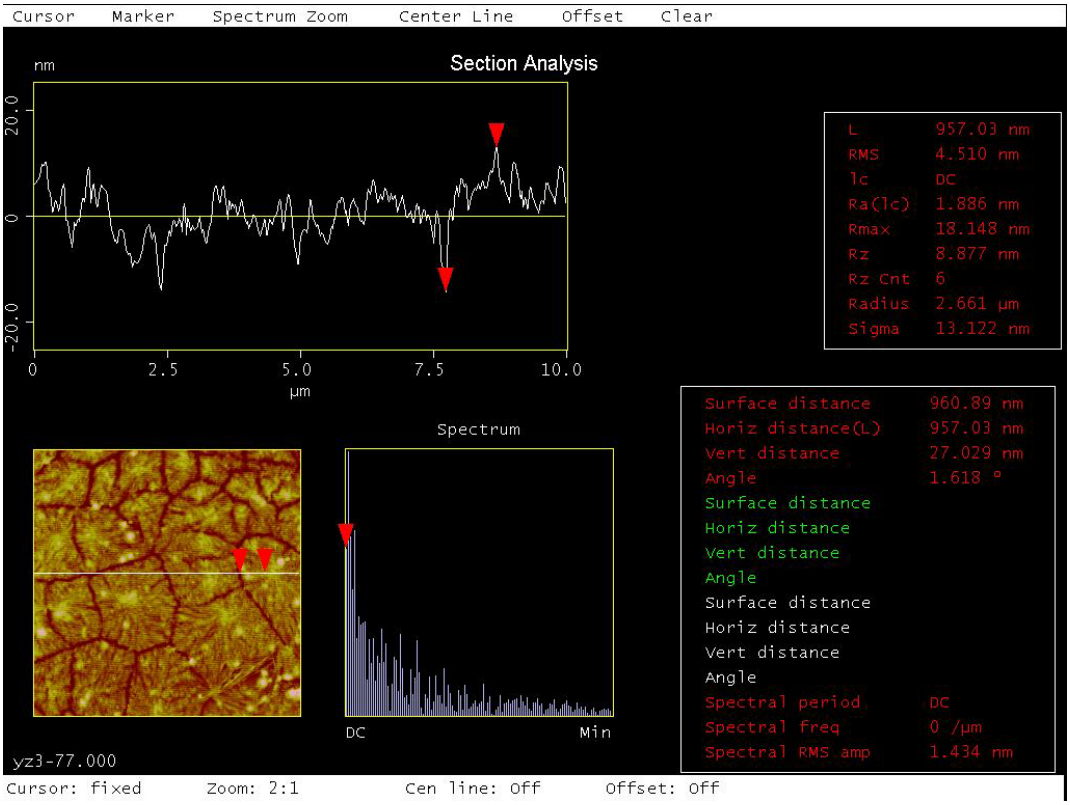


Fig. 23 AFM section analysis of YZ3-77

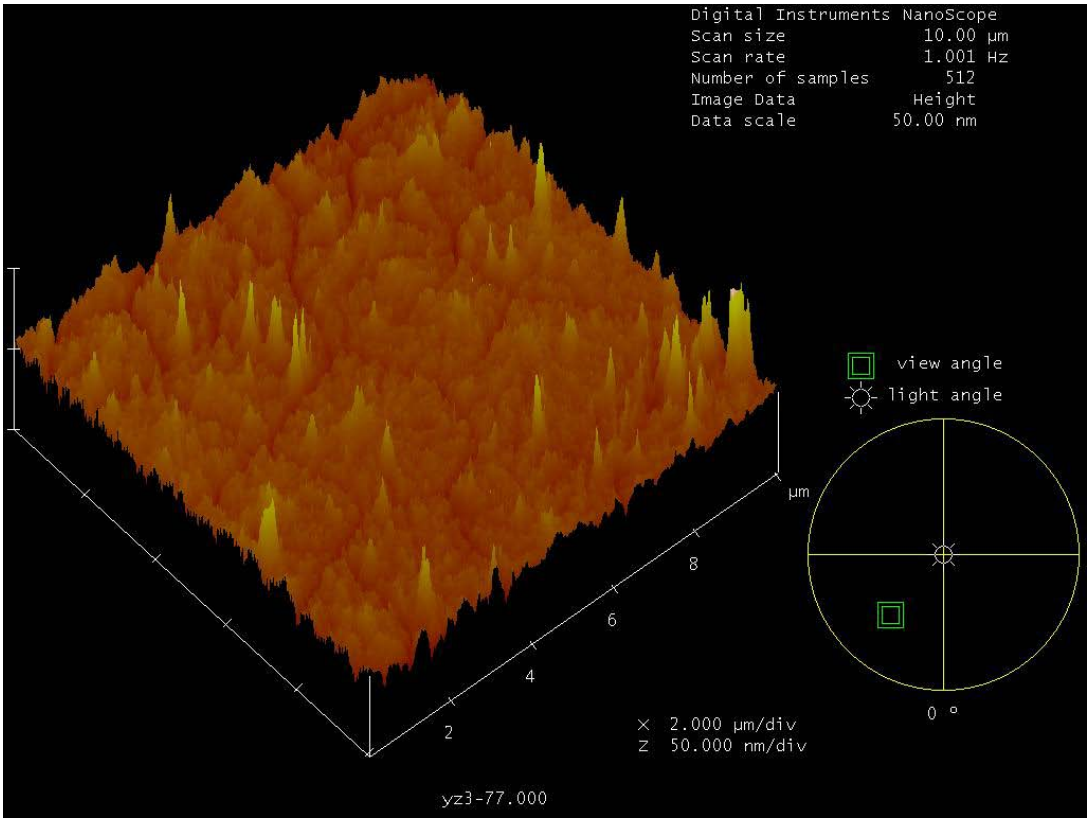


Fig. 24 AFM top view of YZ3-77

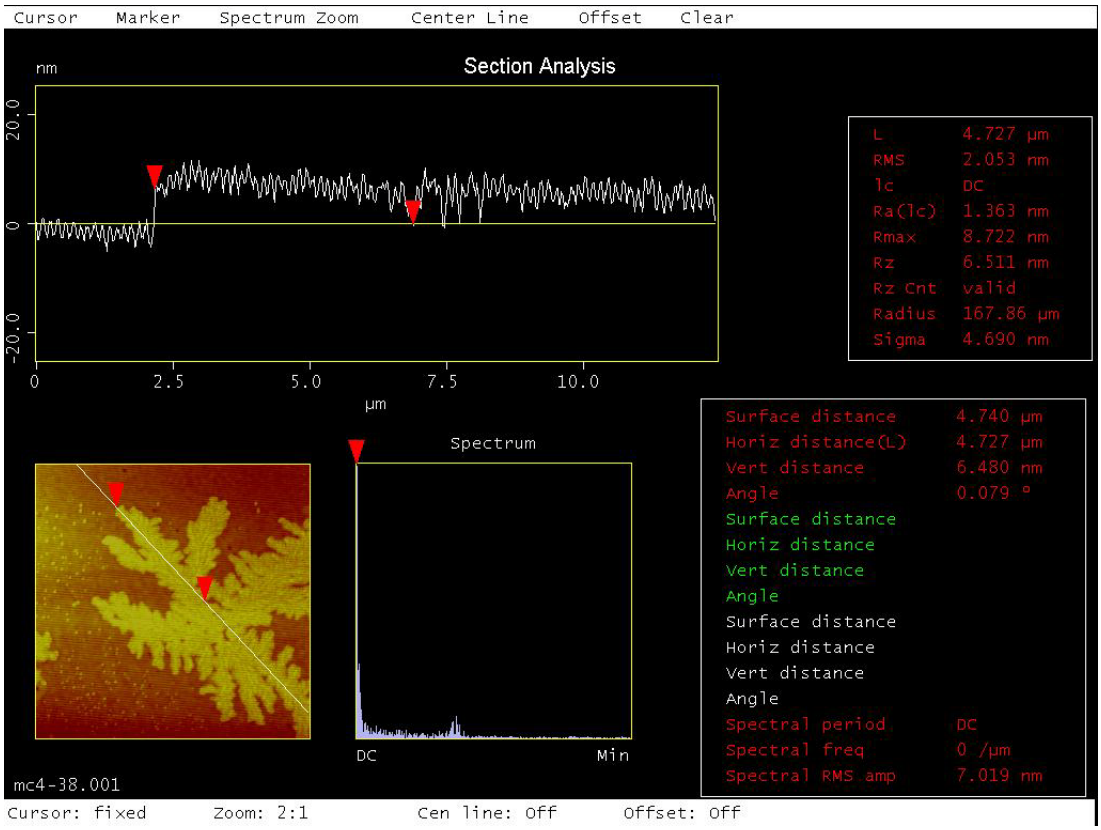


Fig. 25 AFM section analysis of MC4-38

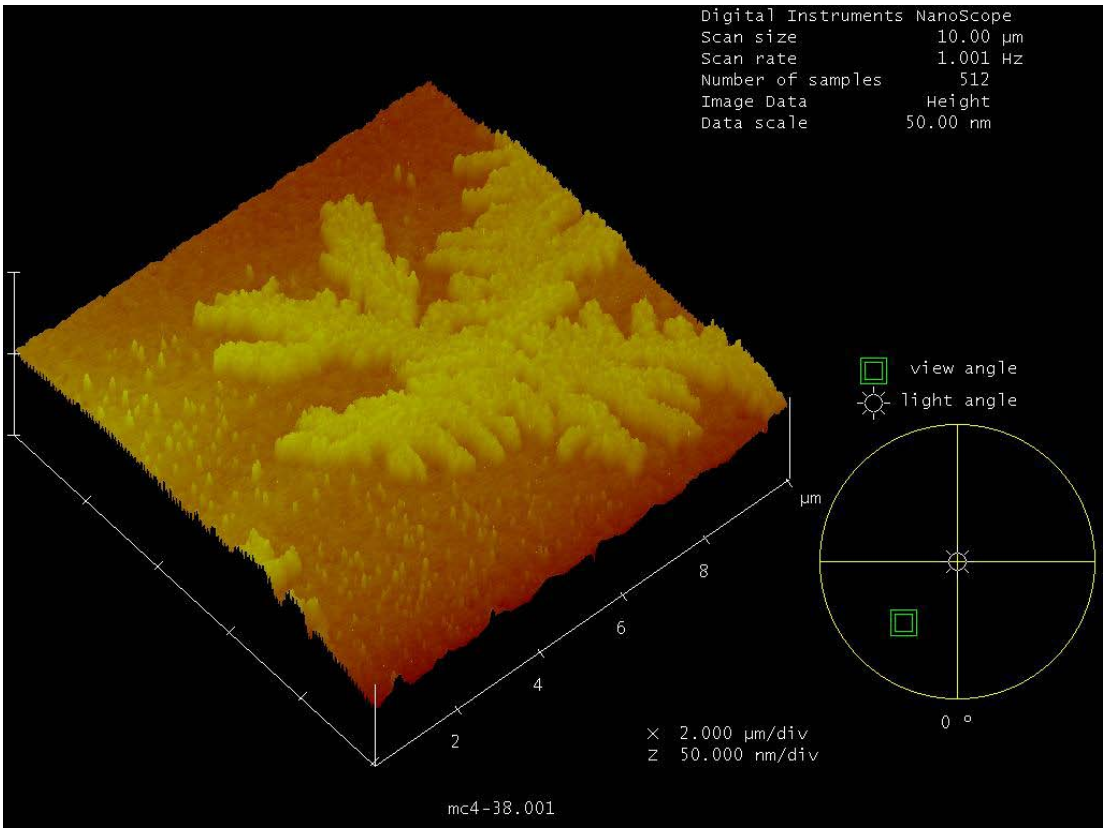


Fig. 26 AFM top view of MC4-38

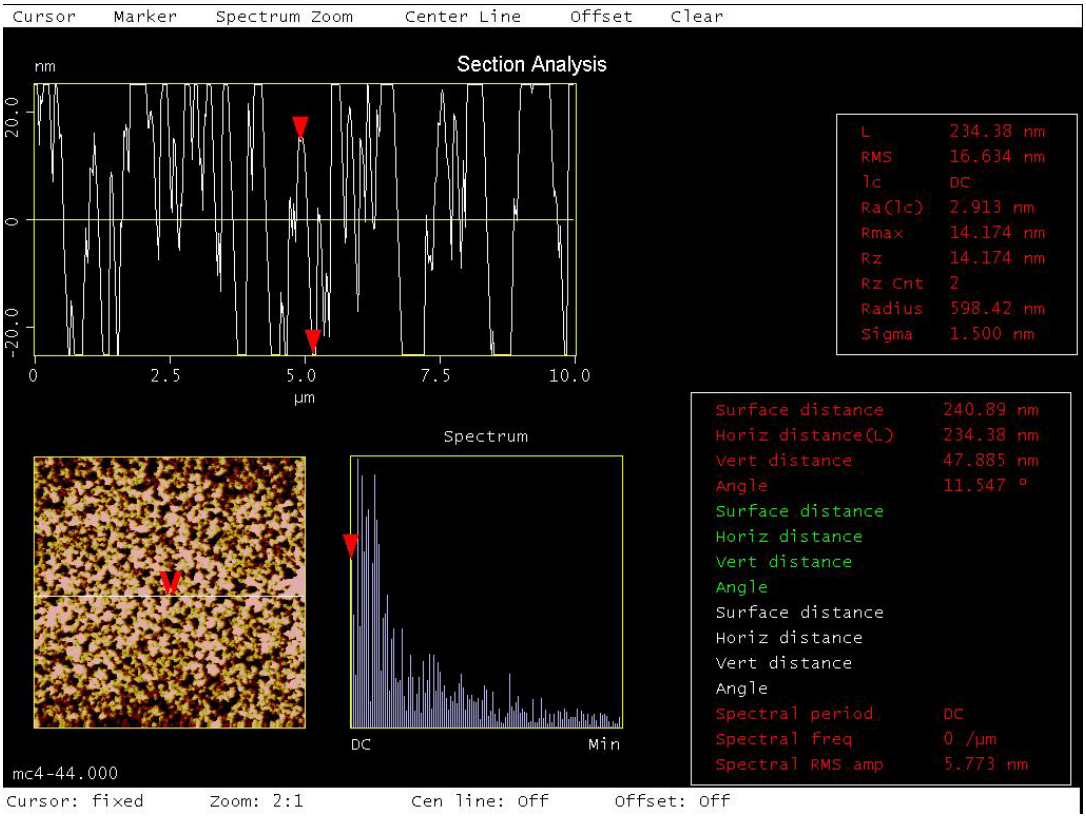


Fig. 27 AFM section analysis of MC4-44

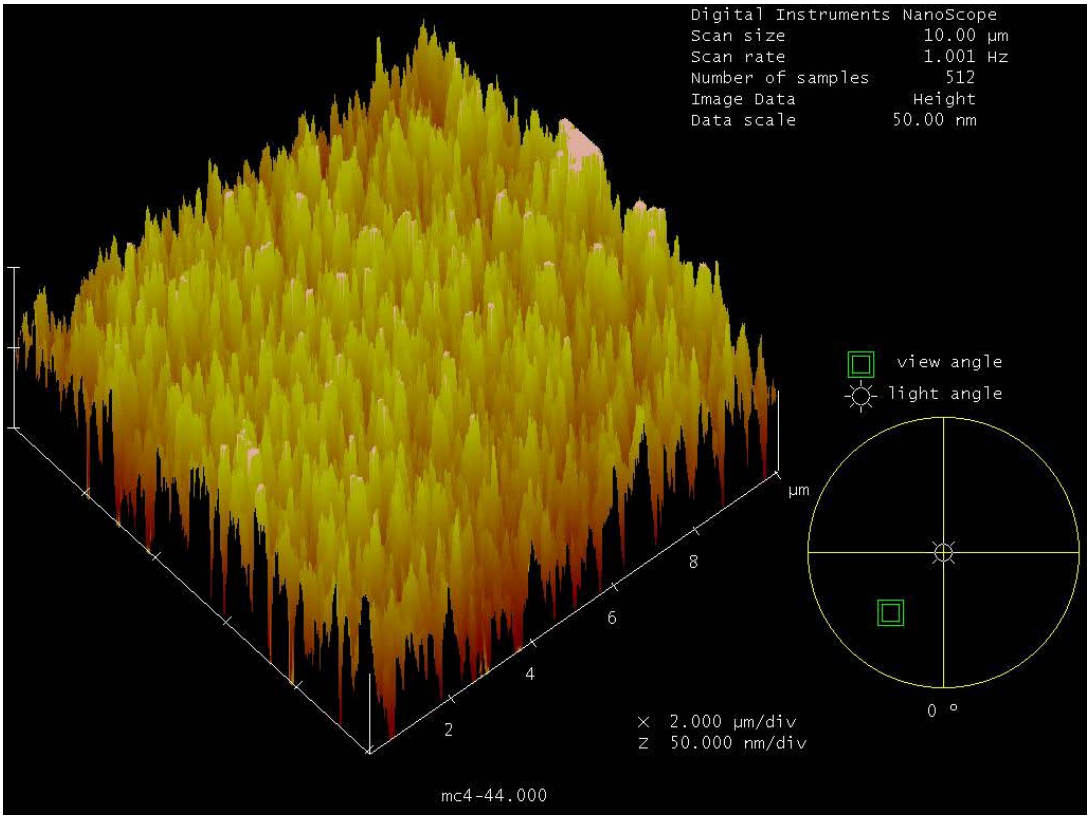


Fig. 28 AFM top view of MC4-44

5.3. Protein Adsorption

It is often assumed that the surfaces that adsorb more cell adhesion proteins (such as fibrinogen), tend to be more bacterial adhesive. We also observed a qualitative correlation between fibrinogen adsorption and *Salmonella* adhesion except YZ3-77. (Fig. 32 The order for fibrinogen adsorption on six different polymers is PCL>YZ3-14>YZ3-77>YZ3-38>MC4-44>MC4-38, while the order for *salmonella* adhesion is PCL>YZ3-14>YZ3-38>MC4-44>YZ3-77>MC4-38. (Fig. 31) However, there is no linear correlation between the two since from Fig. 32 we observed that the CFU value which represents the bacterial adhesion amount on the substrate did not decrease with the protein adsorption number in a manner that allow us to correlate the two in a quantitative way. So we guess there are many influencing factors affecting the bacterial adhesion and among all the influencing factors protein adsorption may be the primary or dominating one. We also observed that there is a very good correlation between contact angle and protein adsorption that the polymers which are more hydrophobic adsorb more cell adhesion proteins. (Fig. 33 So we concluded that the chemical properties of the polymer (such as material type, chemical structure) will determine the physical properties (such as contact angle, morphology) and the physical properties will determine the biological properties (such as protein and bacteria repellency). While the chemical properties are influencing the physical properties and physical property is a reflection of the chemical property. In the same category, such as physical properties, if a dominating factor could be identified to predict the overall performance, the screening work will be much easier. In our case, in terms of physical

properties, without looking at contact angle and other properties, the finest surface has the best protein and bacteria repellency and the roughest surface has the highest protein and bacterial adhesion. We did not find similar observation in publication but surface morphology seems a dominating factors and a good predicting sigh for the biological performance.

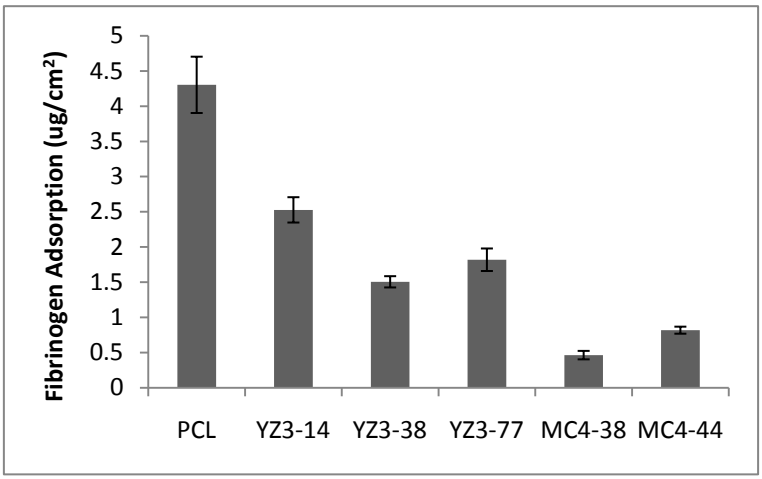


Fig. 29 Fibrinogen adsorption on PCL, YZ3-14, YZ3-38, YZ3-77, MC4-38 and MC4-44, respectively The experiments were performed with duplicate in different days and it is reported as mean±SD.

While Whitesides' group observed that the correlation between nonspecific protein adsorption and bacterial adhesion is little or none, we found the correlation between the two. However, the study done by Whiteside is conducted on SAM and it is a homogenous surface and they examine more than 48 structures. Our research is still in exploratory period and the correlation between protein adsorption and bacterial adhesion may only be true in certain cases.

The chemical modifications for the copolymer seem to be quite effective and for overall performance MC4-38 is the best candidate since it could reduce the adsorption by 9 times for protein adsorption (Fig. 31), and one log reduction for bacterial adhesion, compared with PCL. YZ3-38 and MC4-44 which contain quaternary ammonium and

hydroxypropyl group, respectively, also have improved performance than YZ3-14 which is a non-chemically modified block copolymer. In order to eliminate the other influencing factors in terms of chemical properties, YZ3-38 was synthesized based on YZ3-14 which means the everything remain the same, only quaternary ammonium group was grafted onto the starch building blocks.

The ideal conformation for block copolymer on the substrate is that the hydrophilic part stands up while the hydrophobic part attach on the substrate. (Fig. 30) As we all know, hydrophilic surface is harder for protein to attach since the hydrophilic brush could inhibit protein's adsorption. However, the spin coated film is probably a chemically and topographically heterogeneous surface with both hydrophobic and hydrophilic part on the top. But certain copolymer rich in some chemical groups, such as quaternary ammonium groups, may also be rich in quaternary ammonium groups on the coating surface which will function and resist protein adsorption.

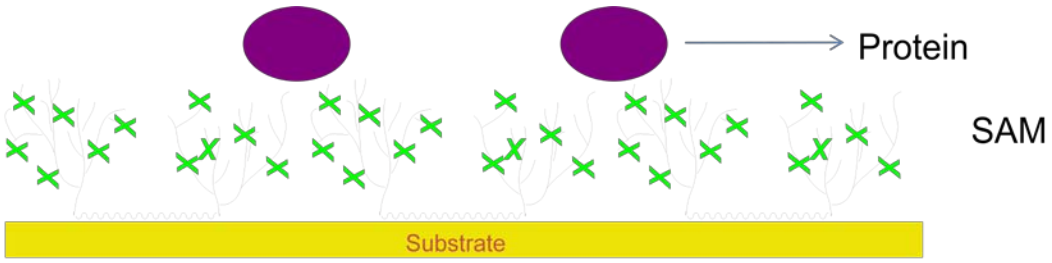


Fig. 30 Ideal bacteria repellency situation

Salmonella is a genus of rod shaped bacteria with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm. Many bacteria may get stuck inside the cracks so it may contribute to the high amount of bacterial adhesion. The same for fibrinogen, with a length around 50 nm and width around 10 nm (Fig. 31), the proteins are very easily got trapped in the "hills and valleys" which in help condition the polymer surface and

attract more bacteria.

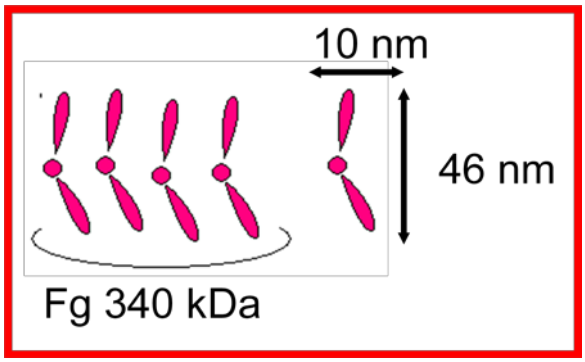


Fig. 31 Structure and size of fibrinogen

Adapted from QCM-D training

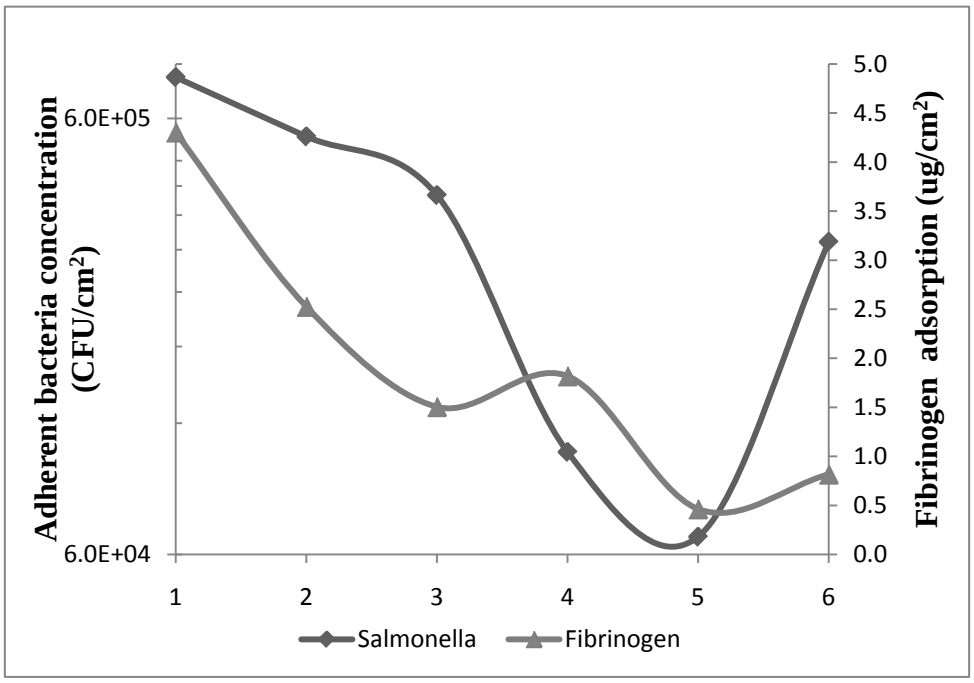


Fig. 32 Correlation between protein adsorption and bacterial adhesion on different copolymers

With 1, 2, 3, 4, 5, 6 refer to PCL, YZ3-14, YZ3-38, YZ3-77, MC4-38 and MC4-44, respectively

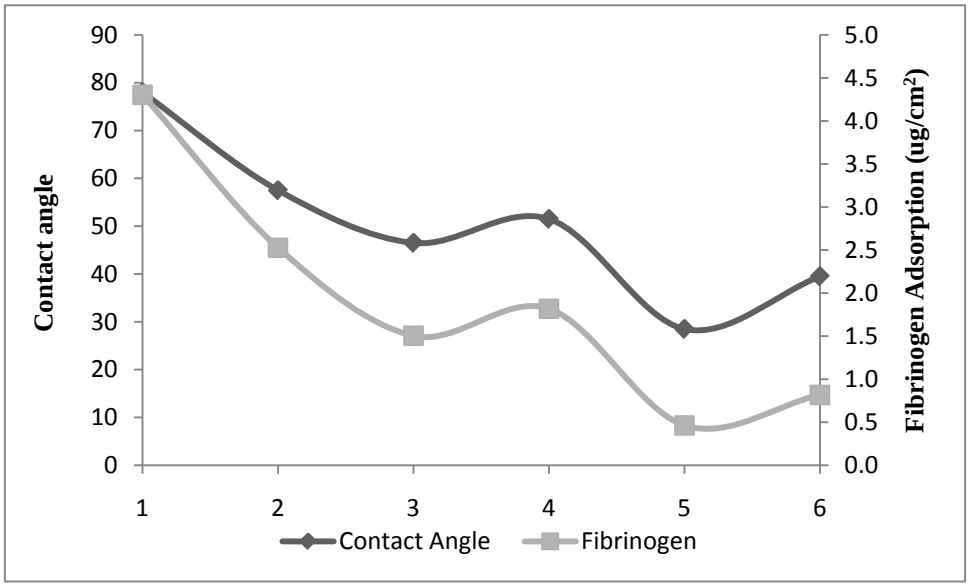


Fig. 33 Correlation between protein adsorption and contact angle on different copolymers With 1, 2, 3, 4, 5, 6 refer to PCL, YZ3-14, YZ3-38, YZ3-77, MC4-38 and MC4-44, respectively

5.4. Bacterial Adhesion

S. enterica JSG210, was grown on the copolymers coated on cover slips which were placed on the bottom of the micro plate. At predetermined time intervals, viable cells remaining on the polymers were measured with plate count technique by growing attached cells out after washing the unattached cells away from the copolymer surface. We use the same assumption that the colony-forming units (CFU) would be proportional to the number of cells adhered on the copolymers. [8] We also improved the washing method by placing the Petri dish on a 3-D rotator instead of using hand washing to wash out the unattached cells. [15]

Extensive research has been carried out on the anti-adhesive behavior and ability to kill bacteria after bacteria adhere to the polymer surface and charged polymers are widely used. In Gottenbos paper it suggests that only positively charged biomaterial surfaces exert an antimicrobial effect on adhering Gram-negative bacteria, but not on Gram-positive ones. [54] YZ3-38 is permanently positively charged with the

quaternary ammonium groups on the starch building block and the degree of substitution is 0.1. As we all know quaternary amines are positively charged groups and they are believed to cause cell death by disrupting cell plasma membranes allowing release of the intracellular material. [38] The antimicrobial efficacy of polymer-modified surfaces with a large concentration of quaternary ammonium groups has been showed by Lee and Murata. [38, 39] We do observed a lower cell adhesion amount on YZ3-38 than YZ3-14 in both long term and short term bacterial adhesion. However, the reason for the lower cell adhesion is unknown; is that because of YZ3-38's killing effect or it is through other mechanism? Further investigation by was did by employing standard test method to determine the anti-microbial activity of immobilized antimicrobial agents under dynamic contact conditions described before. However, no killing effect was found for YZ3-38 since from Table 5 we observed that after shaking for 1 hour, no significant cell reduction was found in the cell suspension with YZ3-38 film in it, compared with the one with YZ3-14 and control.

Table 5 Antimicrobial activity test of block copolymers

It was performed according to the modified ASTM E2149 - 01 standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions

Sample	CFU/ml (average value)	
	Time (0h)	Time(1h)
YZ3-14	8.8E+05	1.3E+06
YZ3-38	9.7E+05	1.4E+06
Inoculum	1.2E+06	2.4E+06

We also conducted AFM analysis for YZ3-14 and YZ3-38 after washing the unattached cell away from polymer substrates after growing the cell on polymer substrates for half an hour. Surprisingly, from AFM images we observed interesting bacterial adhesion phenomena for YZ3-14 and YZ3-38:

There is some excretion surrounding the cell YZ3-38 while no such substance was found for YZ3-14. We guess that it may be extracellular polymeric substances (EPS), also known as exopolysaccharide. If YZ3-38 has killing effect, upon bacteria's contact with the copolymer or after the cell is dead, (in either case), bacteria may excrete EPS to protect itself and after the bacteria is dead secretion will be found outside the cell membrane. [48, 49, 55, 56]

Based on the section analysis for bacterial adhesion, sphere-shaped bacteria are typically found on the YZ3-14 image while rod-shaped bacteria are found for YZ3-38. The height for bacteria on YZ3-14 is around 200nm while the one on YZ3-38 is around 50nm. Bacterial cell are typically negatively charged and the YZ3-38 is positively charged. So we guess the attraction between bacteria and YZ3-38 may change the cell's shape and conformation upon attach on the polymer. Because of the attraction between cells and polymer, the single bacterial cell on YZ3-38 may occupy larger area than the one on YZ3-14.

But whether YZ3-38 has killing effect is still unclear and further investigation is still needed. Considering the low DS of YZ3-38, the charge density may not sufficient to kill bacteria. [38, 39] The other factor to consider is that the polymer substrate for antimicrobial test is from compression molding and the substrate for bacterial adhesion is from spin coating.

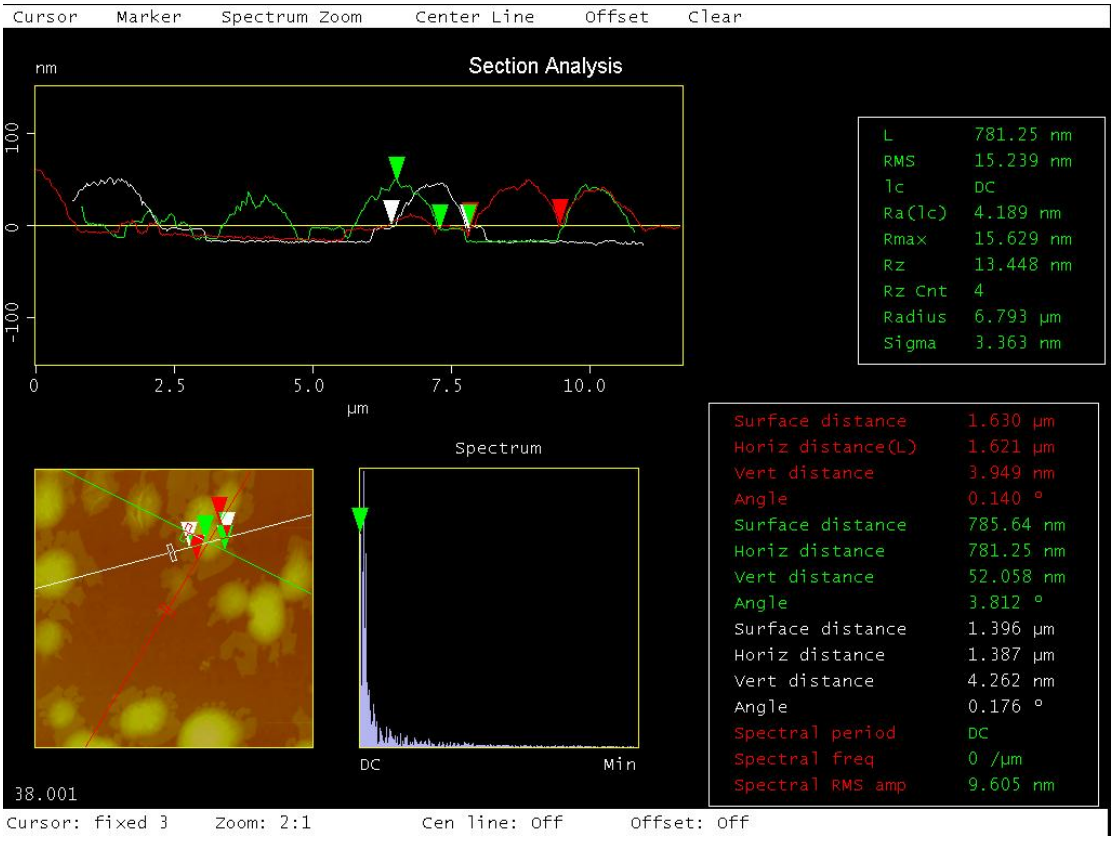


Fig. 34 Section analysis for bacterial adhesion on YZ3-38

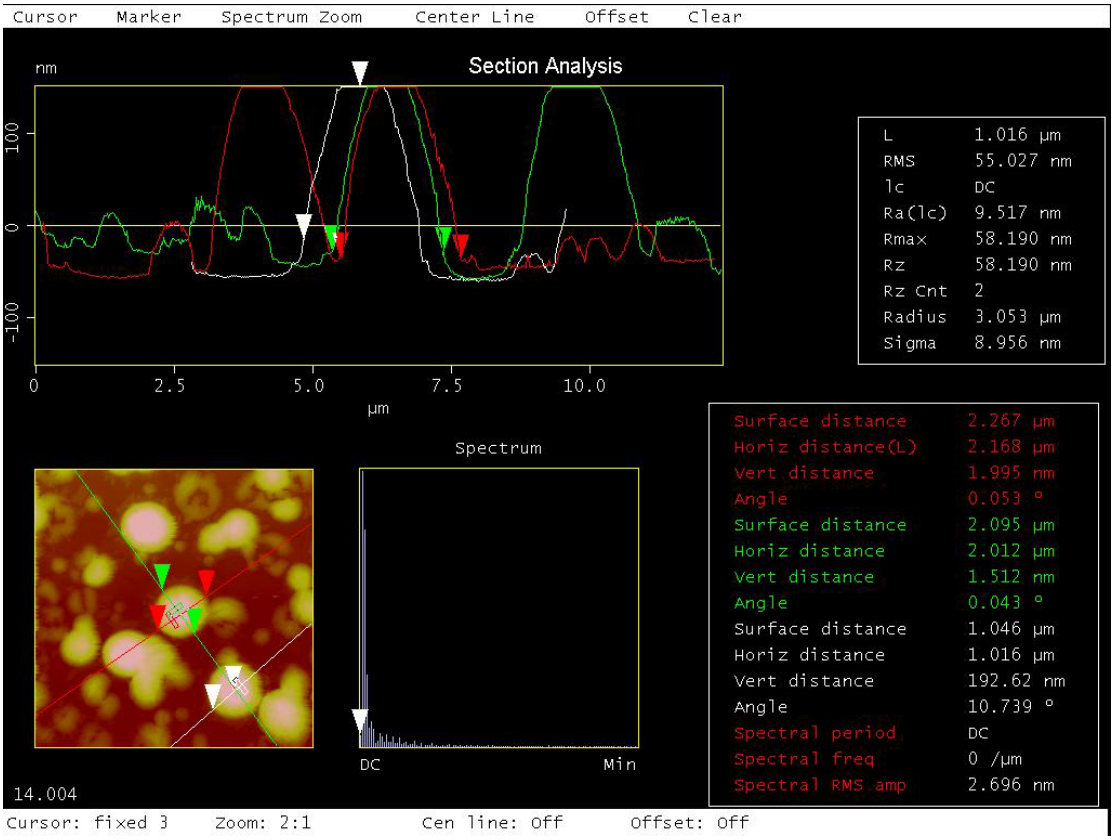


Fig. 35 Section analysis for bacterial adhesion on YZ3-14

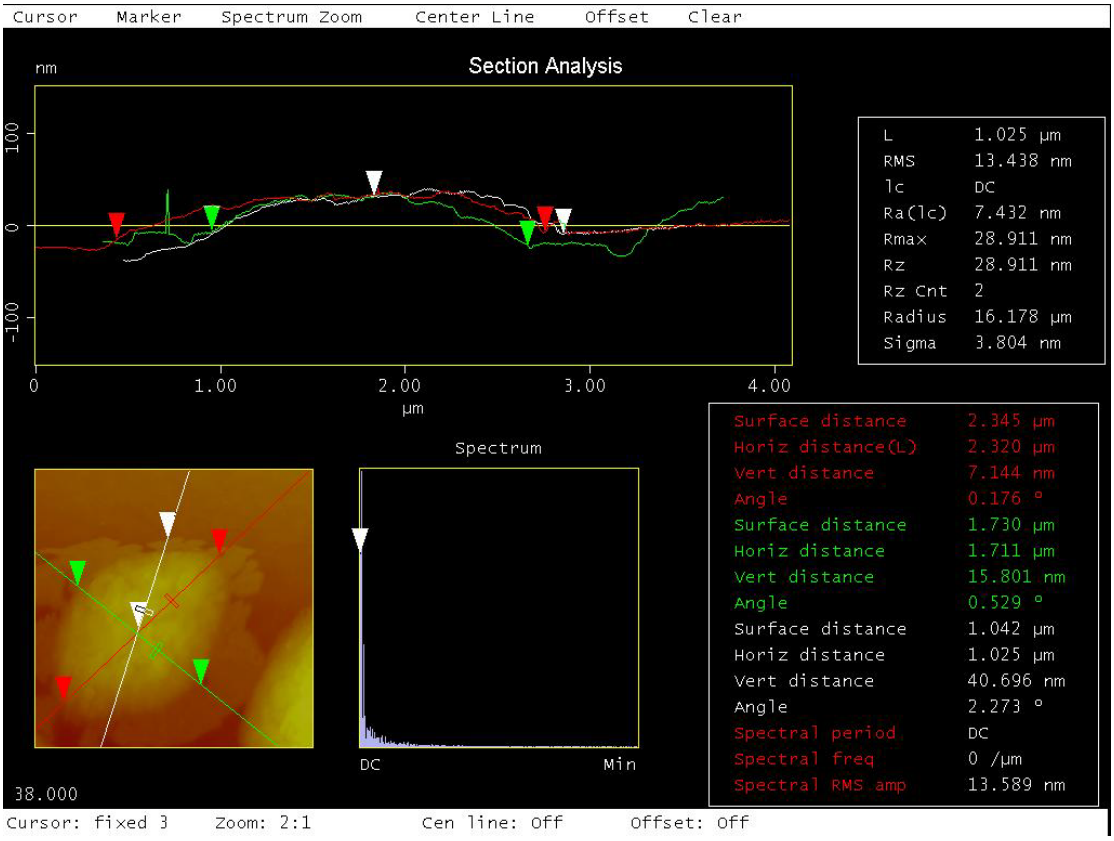


Fig. 36 Section analysis for single bacterial cell adhesion on YZ3-38

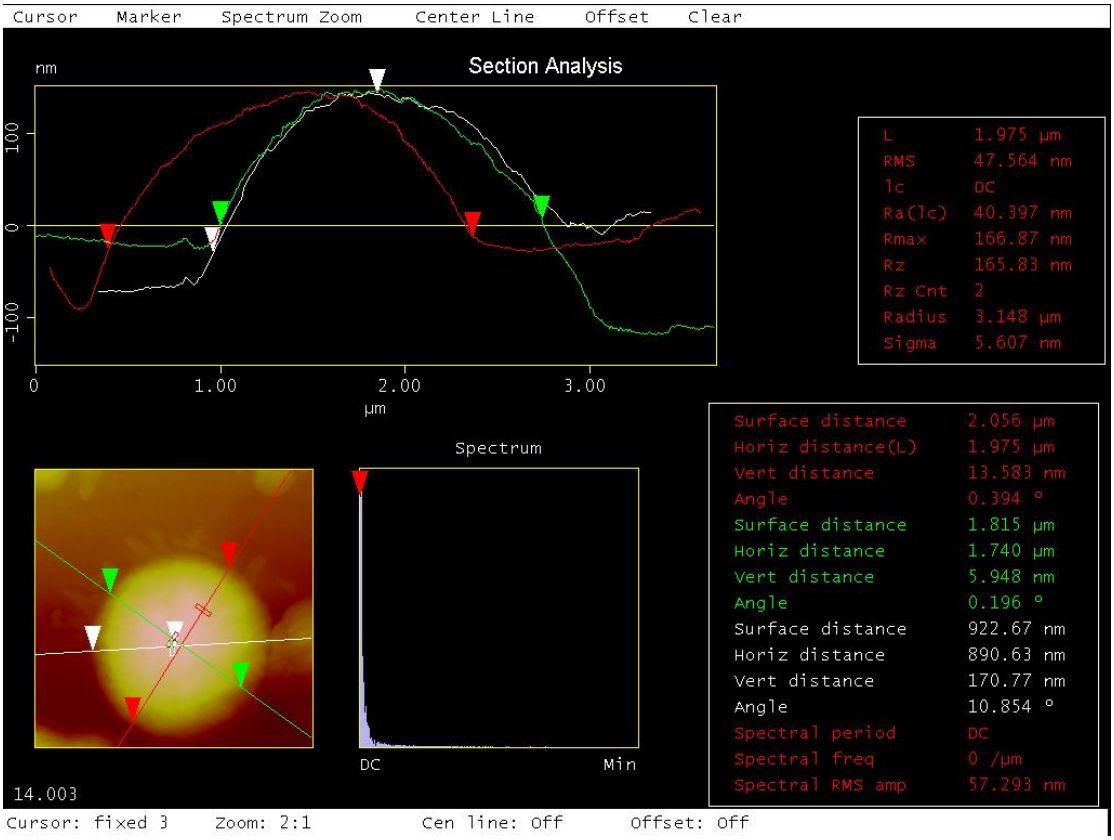


Fig. 37 Section analysis for single bacterial cell adhesion on YZ3-14

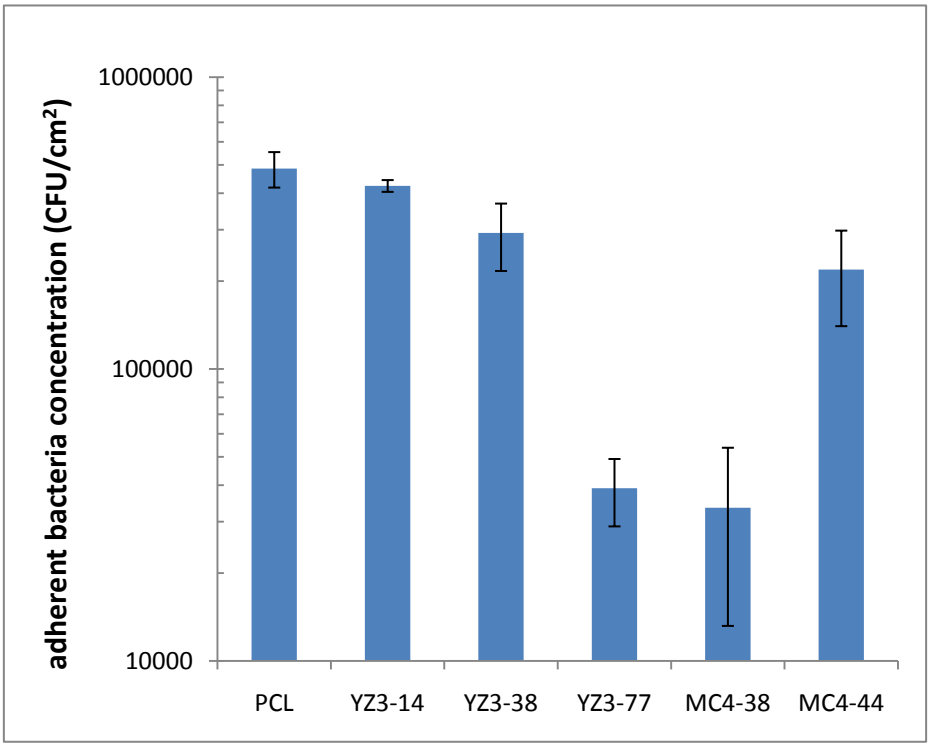


Fig. 38 *S. Enterica* JSG210 adhesion on PCL, YZ3-14, YZ3-38, YZ3-77, MC4-38 and MC4-44 The cells were grown for 30 minutes with initial cell concentration of 10^8 CFU/ml; reported as mean±SD. The experiments were performed with triplicate in three different days with similar results; for clarity, we only plot one set of results.

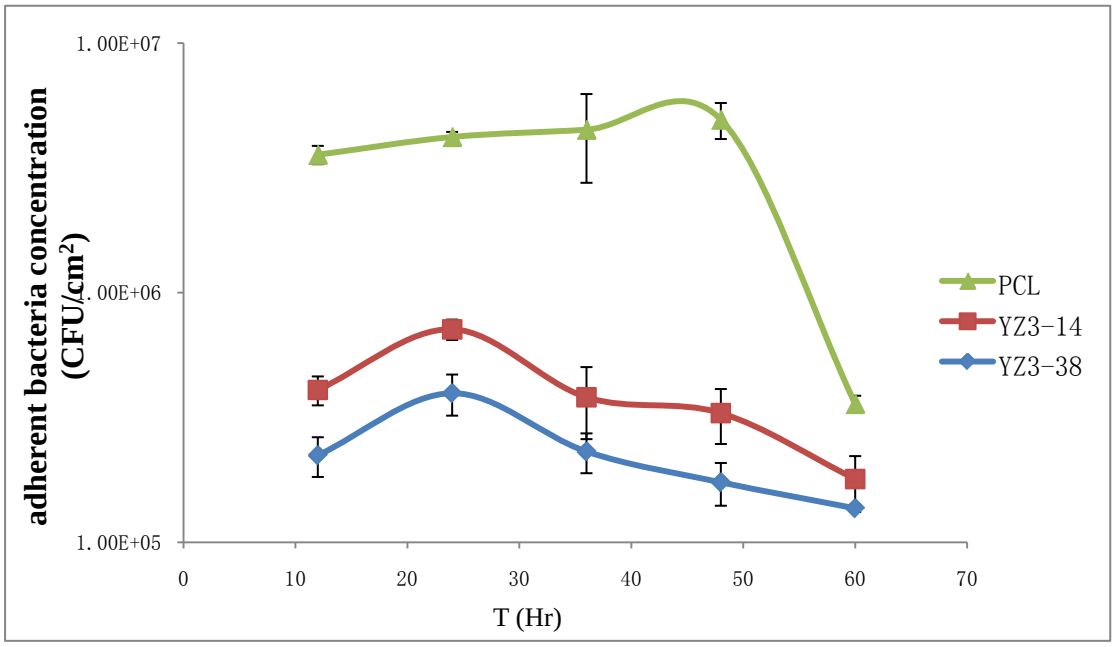


Fig. 39 *S. Enterica* JSG 210 adhesion on PCL, YZ3-14 and YZ3-38.
The cells were withdrawn at predetermined time intervals with initial cell concentration of 10^4 CFU/ml; reported as mean±SD. The experiments were performed in triplicate in three times with similar results; for clarity, we only plot one set of results.

For both short term and long term bacterial adhesion, the results showed that all

Starch/PCL based copolymers have reduced bacterial adhesion, compared with PCL. Some of the copolymers even have comparative or better bacteria repellence than PEG/PCL based copolymer, such as MC4-38. For short term bacterial adhesion, the effect of functional group on bacterial adhesion is discussed previously, together with protein adsorption. (Fig. 38) Longer incubation time could cause the formation of biofilm and in long term bacterial adhesion, the data points for YZ3-14 and YZ3-38 followed the same trend that around 24 hrs the number of adhered cells reached the peak value while number on PCL still have slight increasing until 48 hrs. (Fig. 39) In *Salmonella* growth curve, the planktonic cell reached peak value around 20 hr and maintained stationary phase at least until 60 hrs. (Fig. 40) It seems that the bacteria continued growing or at least maintained the same level on PCL after 24 hrs while the cell number starts to decrease on YZ3-14 and YZ3-38. We did not see the anticipated peak delay on bacterial adhesion on YZ3-14 and YZ3-38 than PCL. So the block copolymers only reduced the bacterial adhesion in number but not delayed the time to reach peak value. Big standard deviation is found in this method which is also used in other group, but quicker and more accurate method should be hired to study the adhesion of bacteria. [8, 22]

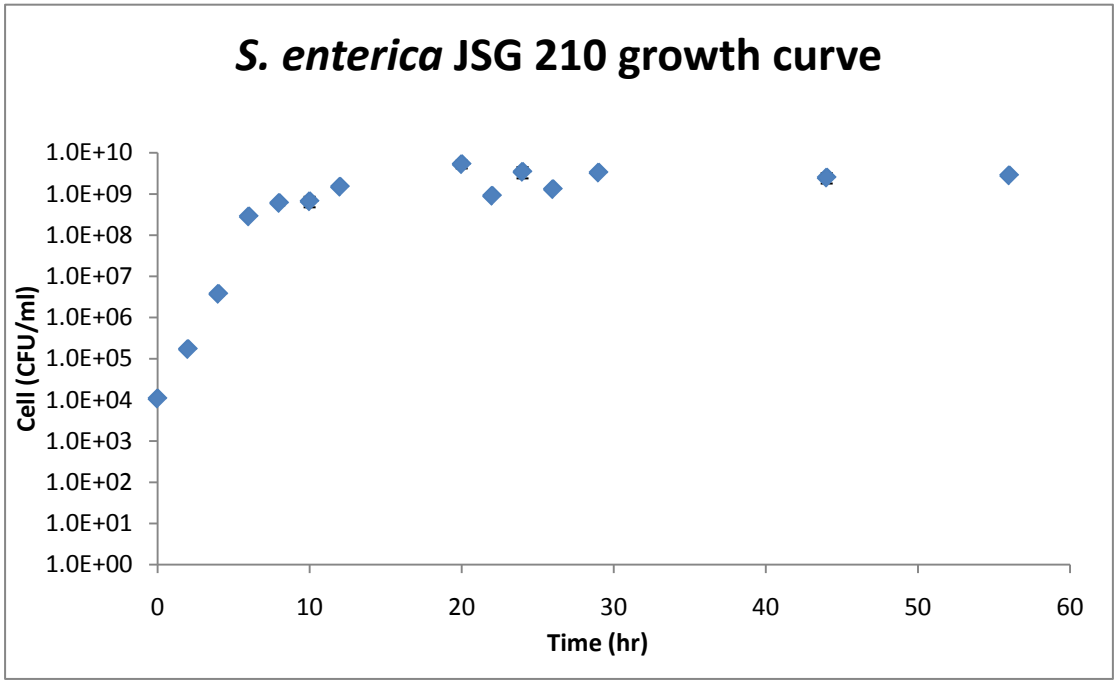


Fig. 40 *S. Enterica* JSG 210 growth curve

6. CONCLUSIONS

A series of hybrid Starch-PCL (di) and Starch-PCL-Starch (tri) block copolymers were synthesized successfully based on the NMR and GPC results. A wide range of block copolymers were made by changing the variables including polymer molecular weight, ratio of PCL to Starch content, di- or tri- block, linear or branched and chemical modifications on hydrophilic blocks with different chemical group.

Preliminary data showed that all the block copolymers have improved protein and bacteria repellency than PCL. Among all the block copolymers, YZ3-38 (with quaternary ammonium group), MC4-38 (substituted with N,N-diethylaminoethyl ether), MC4-44 (with hydroxypropyl groups) could dramatically improve the protein repellency, and MC4-38 and MC4-44 could significantly reduce the bacterial adhesion, compared with YZ3-14 (without modification on starch end group). MC4-38 even has comparative or better protein and bacteria repellence than PEG/PCL based copolymer (PEG is the most widely utilized protein and bacteria repellency polymer).

Moreover, the data on fibrinogen adsorption, *Salmonella* adhesion showed that the ability to reduce fibrinogen adsorption at the copolymers surface correlates with the ability to reduce *Salmonella* adhesion. Surface morphology and contact angle seem to be two indicators for protein and bacteria repellency. Generally speaking, regardless of the chemical structure and modification, the coating surface with finest structure or lowest water contact angle has the lowest protein and bacterial adhesion.

Results suggest that the chemical modification on starch end group on the

copolymers (such as modify starch building block with N, N-diethylaminoethyl ether and hydroxypropyl group) is very effective in reducing the protein and bacterial adhesion. By evaluating the new Starch/PCL based copolymer's surface physical, biological properties, this work is able to show the potential antimicrobial and protein anti-adhesion properties of the Starch/PCl based copolymer and contributing to the understanding of the connections between bacterial adhesion, protein adsorption, hydrophobicity and surface roughness and micro-topography.

7. FUTURE WORK

7.1. Polymer Surface Preparation

To test the effect of annealing on the surface properties: AFM analysis should be conducted both in dry air condition and after withdrawing the substrate from water and protein solution. The purpose is to investigate the effect of annealing on surface topography and protein adsorption and biofilm formation. Upon annealing, SAM of the block copolymers may be formed and the surface property should be significantly different from the surface without annealing. Furthermore, SAM could eliminate the influence of other factors including surface roughness, micro-topography on protein adsorption and bacterial adhesion. So the testing could focus on the correlation between chemical modification on polymer and protein adsorption and/or bacterial adhesion.

7.2. Microbiology Experiment

Gram positive bacteria such as *Staphlococcus epidermidis* should also be tested and compared to the results with Gram negative bacteria. Besides the bacterial adhesion experiment, biofilm formation testing may also be conducted to compare the effect of inhibition on different time period. A more precise and quick testing method to test the bacterial adhesion should be identified and selected. QCMD may be a good candidate for short term bacterial adhesion measurement and crystal violet staining followed by OD measurement may be suitable for biofilm formation.

The mechanism for low bacterial adhesion on YZ3-38 could also be investigated. If YZ3-38 have killing effect, it may act like “fly paper”, attracting bacteria and then

killing them, since bacterial cells generally have a net negative charge on their cell wall at neutral pH while YZ3-38 is permanently positively charged. Higher DS with quaternary ammonium group may increase the killing capability of YZ3-38. The kill effect could be visualized by taking incubated copolymer out of cell suspension and stain with LIVE/DEAD® BacLight™ Bacterial Viability Kits, then observe under epifluorescent microscopy since live cell are stained green while dead cell are stained red.

7.3. Protein Adsorption

Compared with fibrinogen, lysozyme (MW=15 kDa, pI=10.9) is a small protein that is often used as a model in studies of electrostatic adsorption since it is positively charged under the experiment environment we used (pH=7.4). It could help study the repellent capability of the copolymer with quaternary ammonium groups since the copolymer is positively charged. [17]

The attraction between copolymer with quaternary ammonium groups and bacteria may be testified by using Quartz Crystal Microbalance-with Dissipation (QCMD). The adsorption of bacteria onto the copolymer should be slightly enhanced in the beginning than the unmodified “mother” block copolymer since positively charged copolymer should have thicker bacterial adhesion in the beginning.

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