



SYNTHESIS OF AMYLOSE AND AMYLOPECTIN BRUSHES ON SILICON WAFER SURFACE

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ABSTRACT

Polysaccharides are polymer from nature that has attracted new interest as advanced materials due to its biocompatibility, structure-forming capacity, and environmentally friendly property. One of its application fields is to synthesis polymer brushes from amylose and amylopectin. Polymer brushes can be obtained after three steps of reaction: modifying the surface of silicon wafers with (3-aminopropyl)trimethoxysilane (APTMS), grafting maltoheptaose as a primer to silicon surface, and enzymatic polymerization of the primer. To understand polymers property on silicon wafer surface, three different glucose-1-phosphate (G1P) concentrations have been used. According to IR spectrum and XPS data, amylose and amylopectin brushes have been successfully synthesized but cannot be distinguished. The roughness of the layer on the surface was determined by using AFM but it still cannot be concluded whether the concentration of G1P has an effect towards it. The thickness of the layer on the surface has been calculated but can only be applied to amylose brushes. Kinetics measurements showed that amylopectin wafers always grow faster than amylose wafers for every G1P concentration used. Determination of surface density of the amine group concludes that the amount of APTMS has been attached to silicon wafer was 1.86 molecule/nm² meanwhile maltoheptaose gives value of 0.45 molecule/nm².

Keywords: amylose, amylopectin, polymer brushes, enzymatic polymerization.

INTRODUCTION

Polysaccharides are polymer from nature that have attracted new interest as advanced materials due to its biocompatibility, chirality, structure-forming capacity, and environmentally friendly property. Its application field come from liquid crystalline polymers, selective membranes, sensor matrices, to bioactive and biocompatible materials such as sugar based surfactant, drug delivery molecule, and polymer brushes [1-5]. Synthesis of well-defined polysaccharides backbones in a molecule is difficult because it needs perfect control of regio and stereochemistry in glycosylation reactions. One of the alternative ways is to use enzyme as biocatalyst [6]. Enzymatic polymerization can be done by using two enzyme: phosphorylase and glycogen branching enzyme. Linear polysaccharides such as amylose can be synthesized using phosphorylase meanwhile branched polysaccharides like amylopectin can be synthesized using both phosphorylase and branching enzyme. Amylose is a polymer which has long linear α (1 \rightarrow 4) linkages to join each glucose units while amylopectin also has α (1 \rightarrow 6) linkages as branching point where short amylose chains are connected together. The brushes were synthesis to mimic glycocalyx, the external region of cell membrane dominated by glycosylated molecules. Glycocalyx has non adhesive property that prevent undesirable non-specific adhesion of molecules and cells [7]. This research was focused to synthesis amylose and amylopectin brushes on silicon wafers surface with three different glucose-1-phosphate concentrations to understand polymer properties on the surface such as layer thickness and roughness. This biomimetic glycocalix-like surface can be used as coating agents on the surface of synthetic organ to prevent bacterial growth.

MATERIALS AND METHOD

Materials

Silicon wafers were purchased from Topsil. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, HCl, NaOH, p-xylene, ethanol, citric buffer, 4 Å molecular sieve, chloroform, H_2SO_4 , H_2O_2 , ammonium molybdate solution, acetate solution were purchased from Merck. D_2O , (3-aminopropyl)trimethoxysilane (APTMS), NaCNBH_3 were purchased from Aldrich. Methanol and toluene for washing silicon surface were purchased from Lab Scan. Glacial acetic acid was from Acros Organics and metol solution from Fluka. β -cyclodextrin, MOPS buffer, α -D-glucose-1-phosphate disodium salt hydrate, and 9-anthraldehyde were purchased from Sigma-Aldrich. Phosphorylase was isolated from potato and *Deinococcus geothermalis* glycogen branching enzyme obtained from Center for Carbohydrate Bioengineering RuG, The Netherlands. Deionized water was purified through a Milli-Q purification system and distilled water purified by a reverse-osmosis system. DMSO, anhydrous toluene for synthesis of amino functionalized silicon wafer, and anhydrous methanol for determination of surface density of the amine group were all used after purification.

Instrumentals

Ultraviolet/Visible (UV/Vis) absorbance data were obtained from a Pye Unicam SP8-200 spectrometer. Proton Nuclear Magnetic Resonance (^1H NMR) spectrum was measured with a Varian VXR-300 (300 MHz) spectrometer. Infra Red (IR) spectra were recorded on a Bruker IFS 66V/S spectrometer. Atomic Force Microscopic (AFM) data were obtained using a Veeco Digital Instruments EnviroScope spectrometer version 5.30. X-ray Photoelectronic Spectroscopy (XPS) spectra were recorded using an SSX-100 (Surface Science Instruments) photoelectron spectrometer with a monochromatic Al K α X-ray source ($h\nu = 1486.6$ eV). The base pressure in the spectrometer was 1×10^{-9} Torr during the measurements.

Synthesis of Maltoheptaose from β -cyclodextrin

Maltoheptaose was synthesized via acid catalyzed ring cleavage of β -CD [8]. β -CD (477.70 g) was dissolved in 1900 mL 0.01 M HCl and refluxed for 2 hours. The solution was neutralized by 19 mL 1 M NaOH indicated by color change from colorless to pale yellow, confirmed by pH paper showing scale 7. The solution was stored in the refrigerator (4 °C) overnight. The regenerated β -CD was collected by filtration and was saved for the next run. The remaining solution was saturated with the excess of p-xylene and refluxed using heating mantle for 1.5 hours. β -CD/p-xylene complex crystals were precipitated by storing the solution in the refrigerator overnight and then centrifuged for 30 min. This step was repeated two times to make sure that β -CD/p-xylene complex has been removed completely from the solution. The remaining solution was saturated by rotary evaporator and refluxed with addition of p-xylene for 1 hour in oil bath. The remaining crystals of β -CD/p-xylene complex were precipitated by storing the solution in the refrigerator overnight and separated by cold centrifugation (5 °C) for 30 min. The solution was then precipitated drop wise in cold ethanol. The resulting white powder of maltoheptaose was dried in the oven for several days and characterized by ^1H NMR using D_2O as a solvent.

Calibration Curve for Standard Solution of Phosphate

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (68.995 mg) was dissolved in 50 mL distilled water. Phosphate solution was added to 10 mL vials according to Table 1. Fiske and Subbarow method [9] was used to determine total amount of phosphate in solutions. 0.5 mL metol solution and 1.0 mL ammonium molybdate solution were added to these vials. The solutions were mixed with distilled water. After 10 minutes, 2 mL acetate solution was added. Measurement of the solutions on UV-VIS apparatus at wavelength 716 nm was done after 30 minutes.

Table 1 Concentration and aliquot for standard solution of phosphate

Solution	Concentration (mmol/L)	Stock Solution (μL)
1	0	0
2	0,01	10
3	0,02	20
4	0,04	40
5	0,06	60
6	0,08	80
7	0,1	100
8	0,15	150
9	0,2	200
10	0,3	300

Si Surface Cleaning

This is based on Jeroen Lujten method with some modifications [10]. Si wafers were cut into slides around $1.05 - 1.4 \text{ cm}^2$. These wafers were sonicated in methanol and chloroform, each for 15 min respectively, followed by immersion in piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ 7:3 v/v). After 1 hour, the wafers were rinsed with DI water several times and dried at room temperature.

Synthesis of Amino Functionalized Si Surface

The clean wafers were immersed into 5 mL anhydrous toluene containing APTMS (10% v/v) under nitrogen atmosphere at 25°C for 1 hour. APTMS molecules can make 1, 2, or 3 bonds with Si surface and the reactions can not be controlled. After silanization, the wafers were washed with toluene, methanol, and DI water respectively. Residual methoxy functionalities can be removed by immersion of the wafers in DI water overnight at room temperature. Si wafers were then dried at room temperature and characterized by IR and AFM [11].

Grafting of Maltoheptaose on Amino Functionalized Si Surface

The amino functionalized Si wafer was immersed into 5 mL distilled DMSO containing glacial acetic acid (1% v/v), NaCNBH_3 (50 mg), maltoheptaose (50 mg), and molecular sieves (4 \AA) in a vial. The mixture was heated at 50°C for 4 days. The resulting maltoheptaose functionalized wafer was then rinsed with DI water several times followed by sonification in DI water for 2 min and dried at room temperature. The wafers were characterized by IR and AFM.

Enzymatic Polymerization of Maltoheptaose on Si Surface

Concentration of α -D-glucose-1-phosphate disodium salt hydrate (G1P) solution was diversified to know its effect on layer thickness and roughness. Three different concentrations of G1P (0.029 g/mL, 0.23 g/mL, 0.425 g/mL) in 50 mM MOPS buffer pH 7 (20 mL) were mixed with 400 μ L (34.4 U/mL) potato phosphorylase. Each solution was divided to two parts. One part was used to synthesis amylose functionalized Si wafers while the other part was used to synthesis amylopectin functionalized Si wafers by adding 10 μ L (700 U/mL) GBE. Maltoheptaose functionalized wafers were immersed into the solutions at 37 °C. The progress of the reactions was observed with Fiske and Subbarow method by sampling out the solution each time for 40 μ L. After the reactions finished, the wafers were washed with DI water several times, immersed in DI water overnight, and then dried at room temperature. The wafers were characterized by IR, AFM, and XPS.

Determination of Surface Density of the Amine Group

Stock solution was made by dissolving 0.4 mg 9-anthraldehyde in 1 L distilled water containing 0.8% v/v acetic acid. Aliquots were then taken to 10 mL vials according to Table 2. and measured on UV-VIS apparatus at wavelength 262 nm. Amino functionalized and maltoheptaose functionalized Si wafers were immersed into anhydrous methanol (20 mL) containing 9-anthraldehyde (40 mg), glacial acetic acid (10 μ L), and 4 Å molecular sieve under nitrogen atmosphere for 5 hour at 60 °C. Imine groups were formed after the reaction. Si wafers were washed with methanol and sonicated in methanol for 1 min. The wafers were then dried under N₂ atmosphere. Hydrolysis of the imine groups was performed in distilled water (5 mL) containing acetic acid (0.8% v/v). The solution was heated for 30 min at 30 °C. UV measurement at 262 nm was done by sampling out the solution for 2 mL [12].

Table 2 Concentration and aliquot for standard solution of 9-anthraldehyde

Solution	Concentration (mmol/L)	Stock Solution (μ L)
1	0	0
2	3.879×10^{-5}	200
3	4.849×10^{-5}	250
4	5.818×10^{-5}	300
5	6.788×10^{-5}	350
6	7.758×10^{-5}	400
7	9.697×10^{-5}	500
8	1.164×10^{-4}	600
9	1.455×10^{-4}	750
10	1.648×10^{-4}	850
11	1.842×10^{-4}	950

RESULTS AND DISCUSSION

Synthesis of Maltoheptaose from β -cyclodextrin

The yield of maltoheptaose that has been synthesized is 7% (33.184 g). The ^1H NMR spectrum in Figure 1 shows that it still contains impurities like β -CD and ethanol. β -CD impurity was expected to be less because it has been complexed with p-xylene while impurity from ethanol can be removed by heating maltoheptaose in the oven. The β -CD impurity must be avoided because it can inhibit potato phosphorylase activity so that enzymatic polymerization cannot occur. To know whether enzymatic polymerization still can occur, an experiment to determine degree of polymerization of maltoheptaose from β -CD was conducted.

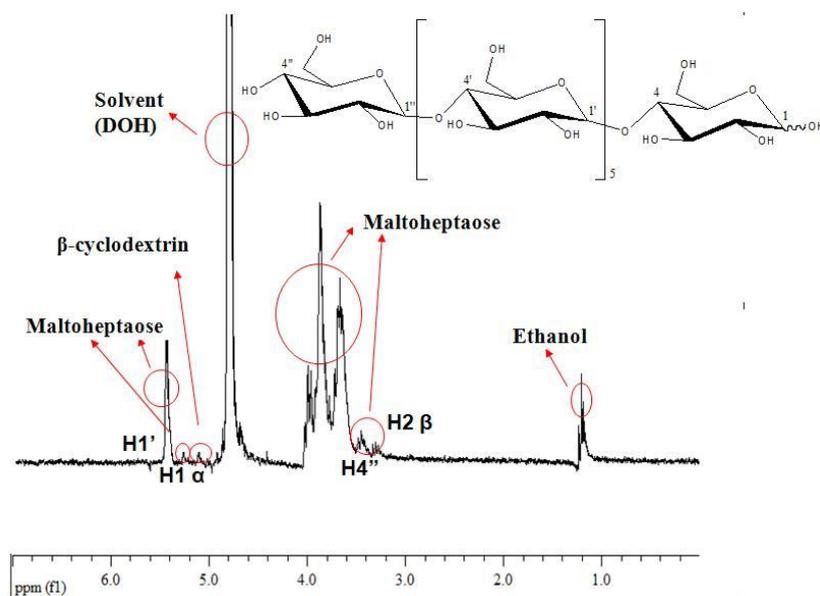


Figure 1 ^1H NMR Spectrum of maltoheptaose from β -cyclodextrin

Enzymatic Polymerization of Maltoheptaose from β -cyclodextrin

According to Figure 2, enzymatic polymerization of maltoheptaose from β -CD still can occur quite well. Fiske and Subbarow method has been used to monitor reaction progress. Since the amount of inorganic phosphates has been release during the reaction equals with the amount of glucose units attached to primer, the degree of polymerization (\overline{DP}) can also be obtained as described in Figure 2.

From this result, maltoheptaose that has been synthesized from β -CD can be used for the next steps without further purification.

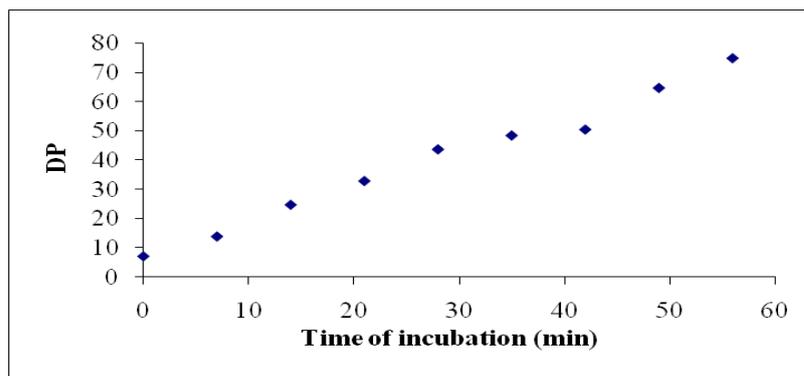


Figure 2 Degree of polimerization of maltoheptaose from β -cyclodextrin

Infra Red Spectroscopy

IR spectroscopy was used to identify functional groups formed during the reactions. From Figure 3, it can be concluded that amylose and amylopectin can not be distinguished by IR spectroscopy. This is because they have the same functional groups such as: C – N stretching (m,w; 1250 cm^{-1}), N – H bending (m,s; 1650 cm^{-1}), and free O – H groups (br; 3250 cm^{-1}). From the broad peak of the free O – H groups, it still can be expected that there are some sugar attach on the surface.

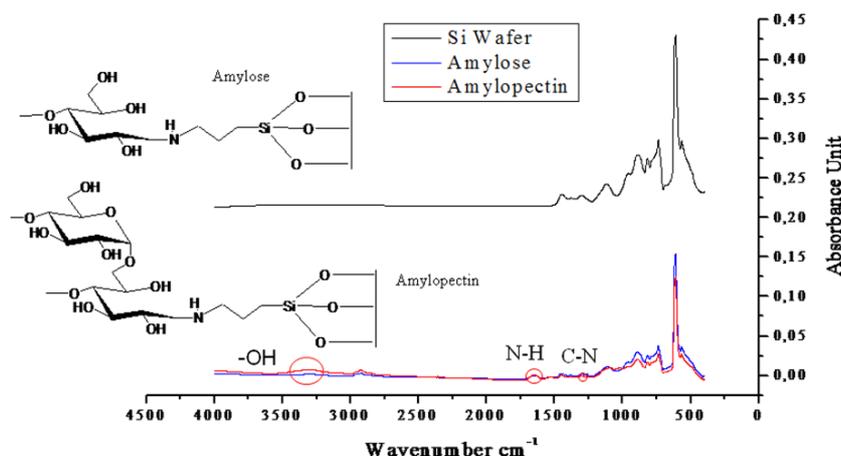


Figure 3 Infra red spectrum of amylose and amylopectine wafer

X-ray Photoelectron Spectroscopy

XPS was used to know the elemental composition of the surface. It was expected that amylose and amylopectin can be distinguish with XPS because of the difference in peak shiftness. From Figure 4 to Figure 6, it can be seen that the peak shiftness between amylose and amylopectin are not very different so these two compounds still can not be distinguished. These results are different from comparison data as can be seen in Figure 7. In this comparison data, amylose and amylopectin are distinguishable from its big peak shiftness. This difference might be caused by the less amount of

amylose and amylopectin on the surface. To understand XPS result from amylose and amylopectin brushes better, additional experimental need to be investigated further.

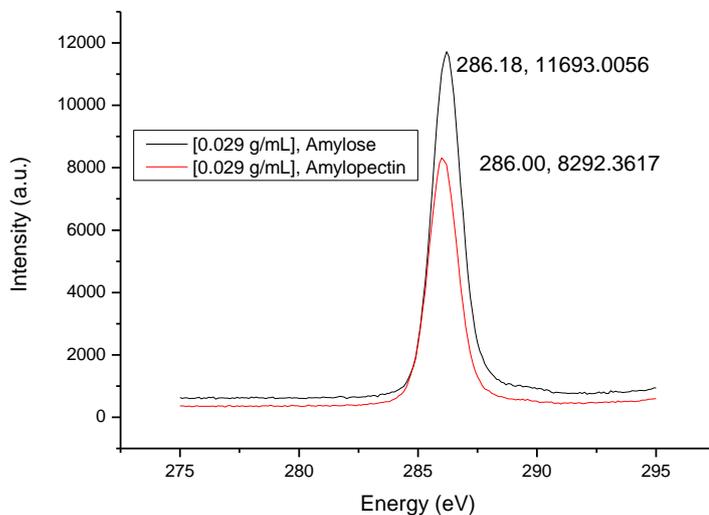


Figure 4 X-ray photoelectron spectroscopy spectrum for less concentrated G1P in solution

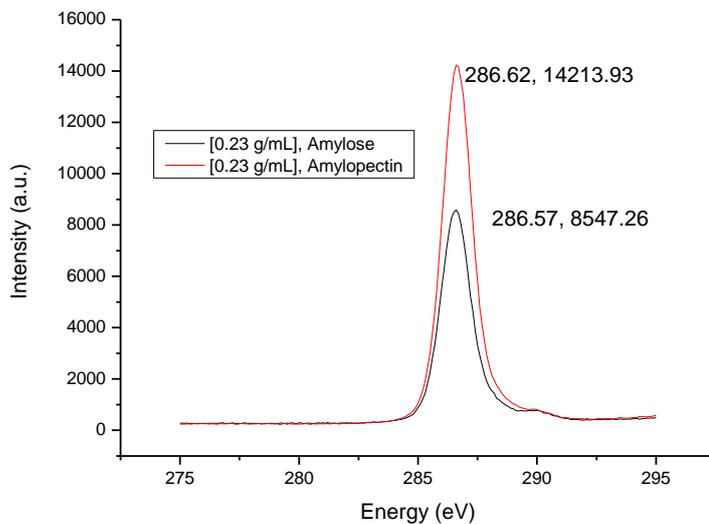


Figure 5 X-ray photoelectron spectroscopy spectrum for middle concentrated G1P in solution

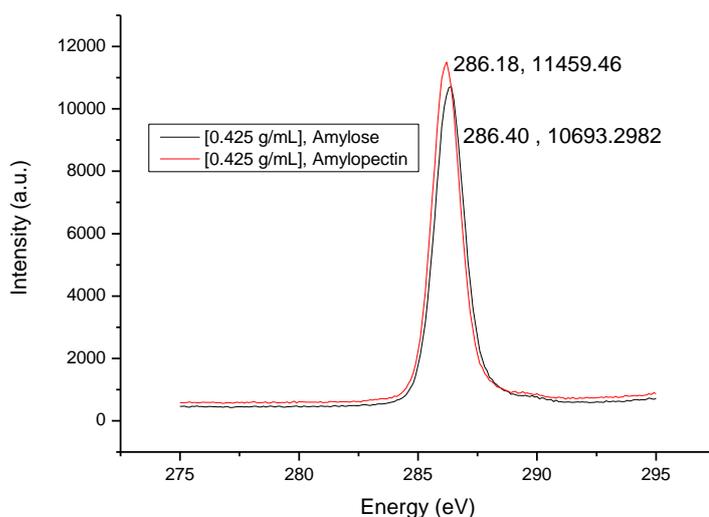


Figure 6 X-ray photoelectron spectroscopy spectrum for high concentrated GIP in solution

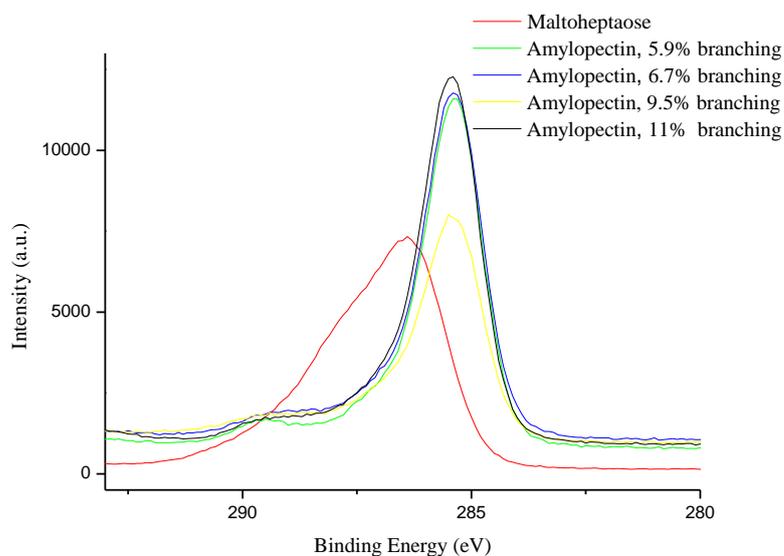


Figure 7 Comparison data for x-ray photoelectron spectroscopy spectrum of amylose and amylopectin

Atomic Force Microscopy

AFM data was used to know the roughness of the surface. It is being proposed that amylose brushes have more roughness than amylopectin brushes because it can not cover the surface homogeneously. Amylopectin is able to cover the surface homogeneously due to its branching property. The illustration of the surface roughness can be seen in Figure 8. From the AFM result in Table , the theory of surface roughness between amylose and amylopectin still can not be proved because there is no trend in the results. To understand surface property better, the thickness of the layer that has been formed on the surface also must be determined. The thickness of the layer was determined using AFM by scratching the surface and measuring its height difference between

scratched and unscratched surface but further research is needed to interpret the results. The thickness of the layer can also be calculated from data of kinetics and the amount of maltoheptaose on the surface. The result is presented in Table 1. It is assumed that maltoheptaose on the surface has V_H polymorph form so it consists of six glucose residues for each turn and the average axial spacing per turn is 0.805 nm. This calculation can not be applied to amylopectin brushes because it has branched chain that should be considered. It is expected that concentration of G1P in solution has linear correlation with surface layer thickness. If G1P concentration is high, the amount of glucose attach to maltoheptaose will increase resulting very thick surface layer. This expectation can not be seen in the result because the kinetics data from the very saturated G1P solution was not too good. It is proposed that amylose chain has a tendency to grow lower than amylopectin. The linear chain of amylose might lies flat on the surface while amylopectin grows higher because it has branched chains. The amount of maltoheptaose molecules attached on the surface is low so the density of sugar chain is also low. This can make amylopectin chain grows freely without interference from other chains. To understand this surface property better, another information about chains alignment is needed.

Table 3 Mean roughness of amylose and amylopectin functionalized wafer

[G1P] g/mL	Amylose (nm)	Amylopectin (nm)
0.029	6.873	5.691
0.23	4.710	8.951
0.425	10.413	2.569

Table 4 Layer thickness for amylose functionalized wafer

[G1P] g/mL	Amylose (cm)
0.029	0.0285
0.23	0.0525
0.425	0.0151

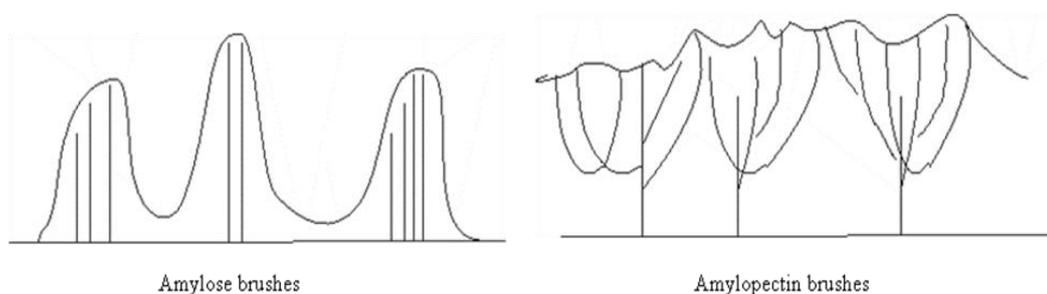


Figure 8 Proposed surface roughness of amylose and amylopectin brushes

The layer thickness calculation results may not be very accurate because it is possible that secondary amines on the surface are attached to another maltoheptaose so the thickness will become less than the results. The chain length for every maltoheptaose chain might be different resulting inhomogeneity in surface layer thickness. Ellipsometry method must be conducted to know the layer thickness more precisely.

The Kinetics of Enzymatic Reaction on Silicon Wafer Surface

Kinetics of the reaction were determined using Fiske and Subbarow method. The color of the solutions was observed before and after enzymatic polymerization. At the beginning, all the solutions were colorless but in the end it become milky with white solid precipitates. This unhomogenous white milkysolution was tested using iodine reagent giving dark blue color. From this results, it can be concluded that the solution was contaminated with amylose. This amylose was expected come from the grafting step where some maltoheptaose might not be chemically attach to the surface and not be completely removed after cleaning procedure. All results are similar for each solutions except for amylopectin wafer in less concentrated G1P solution (0.029 g/mL) which did not give blue color after iodine test. This contamination might affect kinetics result and percentage of G1P converted during the reaction. From Figure 9, amylopectin functionalized wafers always grow faster for every G1P concentration. This is because with branching enzyme, there will be more site for glucose unit from G1P to attach on non reducing end of the primer. The higher concentration of G1P, the faster glucose molecules attach to maltoheptaose so that the amount of phosphate that has been release will also increase. For less concentrated G1P solution, the curves for amylose and amylopectin have a plateau after some period of time but not for other solutions. This is because the buffer can not maintain its pH around 7 anymore if G1P in the solution is too high so that phosphorylase and branching enzyme did not work properly. The effect of G1P concentration towards the pH of MOPS buffer is shown in Figure 10.

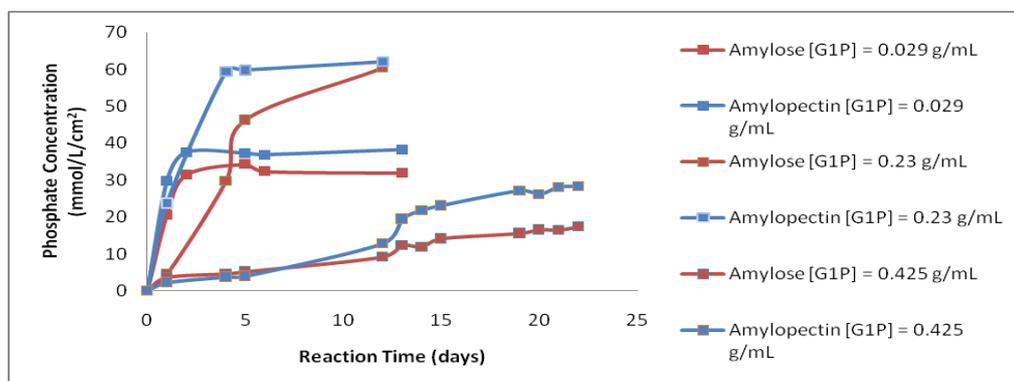


Figure 9 The kinetics of enzymatic reactions on silicon wafer surface

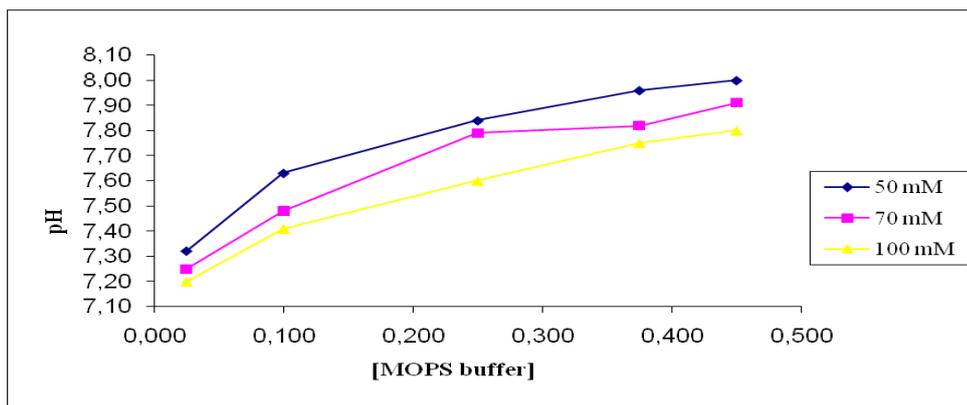


Figure 10 The effect of G1P concentration to the ability of MOPS buffer to maintain its pH

Determination of Surface Density of the Amine Group

As has been shown in Figure 11, 9-anthraldehyde will make an imine bond with amine groups on the surface. The resulting compound has a lot of chromophors thus can be detected using UV-Vis spectroscopy. To determine the surface density of amine group, it is assumed that one amine group will attach to one molecule of 9-anthraldehyde. It is also assumed that not every amine group is attached to maltoheptaose so there are still some 9-anthraldehyde molecules that can react with amine group. With these assumptions, the amount of initial maltoheptaose that has attached on the surface are calculated. Absorbance of amino and maltoheptaose functionalized wafer were 0.087 and 0.080 respectively. With some calculations, the amount of APTMS that has been attached to silicon wafer was 1.86 molecule/nm² meanwhile for maltoheptaose, it gives value of 0.45 molecule/nm². In literature, it was reported that surface density of the amine groups was around 2.4 – 2.5 molecule/nm². These different values were caused by the difficulty in dissolving 9-anthraldehyde in water so the exact concentration of 9-anthraldehyde standard solution was difficult to determine. From the amount of maltoheptaose attached on the surface, the degree of polymerization of amylose and amylopectin brushes can be determined as being shown in Figure 12.

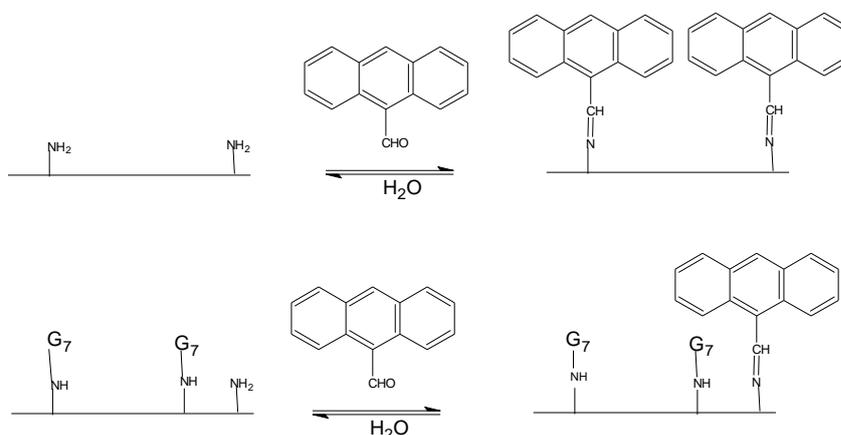


Figure 11 Reaction scheme between 9-anthraldehyde and amine groups on the surface

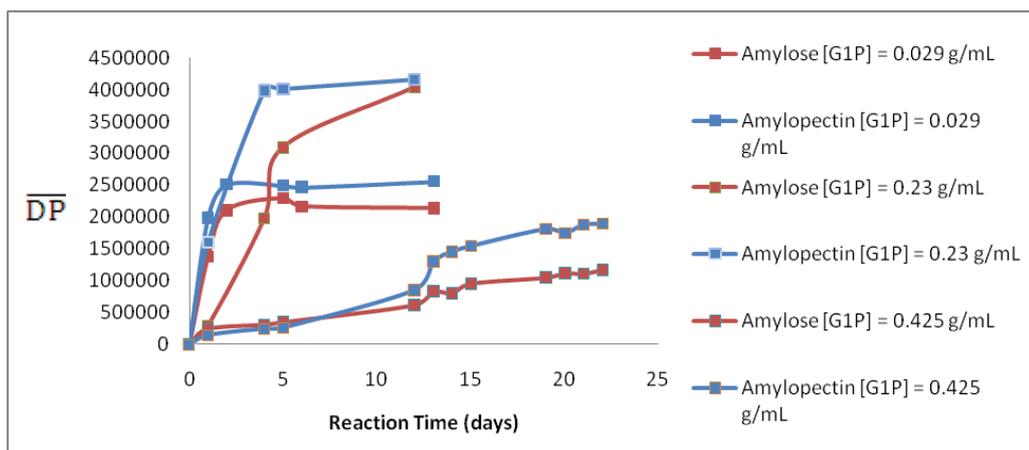


Figure 12 Degree of polymerization amylose and amylopectin

CONCLUSION

The Synthesis of amylose and amylopectin brushes can be done via enzymatic polymerization by using phosphorylase and branching enzyme. The roughness of the layer has been determined by using AFM but it still can not be concluded whether the concentration of G1P has an effect towards it. The thickness of the layer has been calculated but can only be applied to amylose brushes because amylopectin brushes has branched chain that should be considered. Therefore G1P effect cannot be determined. From kinetic measurement, amylopectin functionalized wafers seem to always grow faster than amylose functionalized wafers for every G1P concentration. Determination of surface density of the amine group concludes that the amount of APTMS has been attached to silicon wafer was 1.86 molecule/nm² meanwhile for maltoheptaose, it gives value of 0.45 molecule/nm².

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REFERENCES

- [1] Hill, K., and Rhode, O. (1999), Sugar-based surfactants for consumer products and technical applications, *Lipid - Fett*, 101, 25.
- [2] Immel, S. (2005), *Flexible and Rigid Non-glucose Cyclooligosaccharides: Synthesis, Structure, and Properties*, Doctoral Disertation, Technische Universität Darmstadt, 1, 4.

- [3] Kobayashi, S., Sakamoto, J., and Kimura, S. (2001a), In vitro synthesis of cellulose and related polysaccharides, *Progr Polym. Sci.*, 26, 1526 - 1527.
- [4] von Rybinski, W., and Hill, K. (1998), Alkyl polyglycosides - properties and applications of a new class of surfactants, *Angew. Chem Int. Ed.*, 37, 1329.
- [5] Zhao, B., and Brittain, W. J. (2000), Polymer brushes: surface-immobilized macromolecules, *Prog. Polym. Sci.*, 25, 678 - 680, 692 - 693.
- [6] van der Vlist, J., Reixach, M. P., van der Maarel, M., Dijkhuizen, L., Schouten, A. J., and Loos, K. (2008), Synthesis of Branched Polyglucans by the Tandem Action of Potato Phosphorylase and *Deinococcus geothermalis* Glycogen Branching Enzyme, *Macromol. Rapid Commun.*, 29, 1293.
- [7] Holland, N. B., Qiu, Y., Ruegsegger, M., and Marchant, R. E. (1998), Biomimetic engineering of non-adhesive glycocalyx-like surfaces using oligosaccharide surfactant polymers, *Nature*, 392, 799.
- [8] von Braunmühl, V., Jones, G., and Stadler, R. (1995), Enzymatic grafting of amylose from poly(dimethylsiloxanes), *Macromolecules*, 28, 17, 22.
- [9] Fiske, C. H., and Subbarow, Y. (1925), The colorimetric determination of phosphorus, *J. Biol. Chem.*, 66, 375-400.
- [10] Luijten, J., Vorenkamp, E. J., and Schouten, A. J. (2007), Reversible helix sense inversion in surface-grafted poly(beta-phenethyl-L-aspartate) films, *Langmuir*, 23, 10772-10778.
- [11] Howarter, J. A., and Youngblood, J. P. (2006), Optimization of silica silanization by 3-aminopropyltriethoxysilane, *Langmuir*, 22, 11142-11147.
- [12] Zhao, J., Li, Y., Guo, H., and Gao, L. (2006), Relative surface density and stability of the amines on the biochip, *Chin. J. of Anal. Chem.*, 34, 1235-1238.