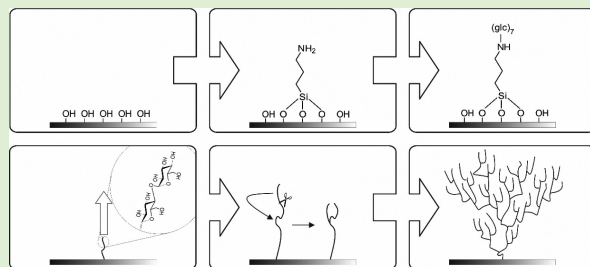


Utilization of Glycosyltransferases for the Synthesis of a Densely Packed Hyperbranched Polysaccharide Brush Coating as Artificial Glycocalyx

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ABSTRACT: Densely packed polysaccharide brushes consisting of α -D-glucose residues were grafted from modified silicon substrates. Potato phosphorylase was herein used to grow linear polysaccharide chains from silicon tethered maltoheptaose oligosaccharides using glucose-1-phosphate as donor substrate. The combined use of potato phosphorylase and *Deinococcus geothermalis* branching enzyme resulted in a hyperbranched brush coating as the latter one redistributes short oligosaccharides from the $\alpha(1\rightarrow4)$ -linked position to the $\alpha(1\rightarrow6)$ -linked position in the polysaccharide brush. The obtained grafting density of the brushes was estimated on 1.89 nm^{-2} while the thickness was measured with ellipsometric techniques and determined to be between 12.2 and 20.2 nm.



INTRODUCTION

The exterior of endothelial blood vessel cells is covered with a highly hydrated coating known as the glycocalyx. The glycocalyx consists of a complex mixture of (macro)molecules, including proteins, glycolipids, glycoproteins, and proteoglycans, and shields the vascular wall from direct exposure to blood flow.^{1–3} Next to many other functions it possesses antiadhesive properties in order to prevent the nonspecific adhesion of plasma proteins and blood cells.

Implantable devices can suffer from undesirable protein, cell, and bacterial adhesion which can ultimately lead to biofilm formation and an inflammatory response including thrombosis coagulation and infection.^{4,5} Polymer brushes containing poly(ethylene glycol) (PEG) have been a topic of interest in the preparation of protein-resistant surfaces.^{6–11} However, PEG brushes lack the ability to be adequately functionalized since it has only one end-group and the oxidative–thermal stability is limited in most biochemically relevant solutions.^{12–15} Another problem associated with engineering a polymer brush coating is obtaining a high surface density which is needed to give a good protection against undesirable adhesion. A possible solution to overcome these problems is biomimicking the glycocalyx on the surface of (implantable) devices by means of a glycosylated brush coating.^{15–20}

Moreover, a polysaccharide brush coating can overcome the problems associated with PEG brushes as the many hydroxyl groups of the glucose residues can be functionalized. Furthermore, when using a (hyper)branched polysaccharide brush, the brushes cover a larger surface area than linear (PEG) chains.

Here we describe the surface modification of a silicon (Si) wafer using enzymes from the glycosyltransferase family in order to grow a hyperbranched α -glucan brush coating similar

to glycogen. The residual hydroxyl groups of the glucose residues can eventually be sulfonated in order to obtain a negatively charged brush, just like the endothelial glycocalyx.^{6,21,22} The enzyme-catalyzed surface modification is demonstrated on Si wafers but can easily be extended to other surfaces.

The regio- and stereoselective properties of potato phosphorylase (PP) and the microbial *Deinococcus geothermalis* branching enzyme (GBE_{DG}) were used to construct a hyperbranched polyglucan consisting of $\alpha(1\rightarrow4)$ -linked glucose residues with branches at the $\alpha(1\rightarrow6)$ linkage, leaving the other hydroxyl groups of the glucose residues available for further modification. The combined catalysis of hyperbranched polysaccharides was performed previously in solution and yielded polysaccharides with a predefined molecular weight and a degree of branching of 11%.^{23–25} Potato phosphorylase was herein able to catalyze the formation of linear polysaccharide chains starting from maltoheptaose (G7) with glucose-1-phosphate (G1P) as donor substrate. GBE_{DG} catalyzes the formation of $\alpha(1\rightarrow6)$ branch points by the hydrolysis of an $\alpha(1\rightarrow4)$ glycosidic linkage from the growing polysaccharide. Subsequent inter- or intrachain transfer of the hydrolyzed oligosaccharide to a C6 hydroxyl position resulted in a branch point.²⁶ Here we use the combined action of both enzymes to prepare a (hyperbranched) polysaccharide brush coating on a silicon wafer.

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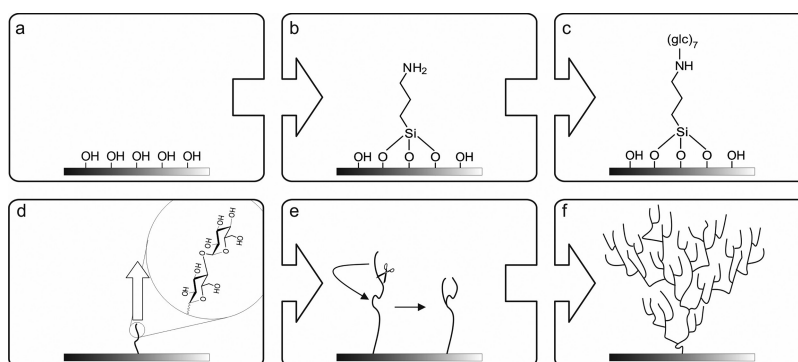


Figure 1. (a) Oxidized and clean Si wafer, (b) aminosilanized wafer, (c) maltoseptase functionalized surface, (d) enzyme-catalyzed growth of a linear chain by PP, (e) catalytic action of GBE_{DG}, and (f) resulting hyperbranched polysaccharide after the combined biocatalysis.

EXPERIMENTAL SECTION

Materials. Double polished silicon wafers were purchased from TOPSIL (Frederikssund, Denmark). Toluene (Labscan) was freshly distilled from sodium, and DMSO (Labscan) was distilled from CaH₂. Sodium cyanoborohydride (NaCNBH₃), 3-aminopropyltriethoxysilane (APTES), and anthraldehyde (all purchased from Aldrich) were used as received. α -D-Glucose-1-phosphate (G1P) and 3-(*N*-morpholino)-propanesulfonic acid (MOPS) were purchased from Sigma and used as received. Water was purified with a Milli-Q system from Millipore.

Surface Preparation and Modification. The wafers were cut in pieces of 10 mm \times 20 mm, ultrasonically rinsed with ethanol and dichloromethane, and finally submerged in a hot piranha solution. After 1 h, the substrates were extensively rinsed with Milli-Q water and sonicated with methanol and toluene. The cleaned surfaces were immediately silanized as described in the following section.

Aminosilanization. The aminosilanization process was carried out in a 2% (v/v) APTES solution in freshly distilled toluene at room temperature in a shaking incubator. After 1.5 h the substrates were rinsed with toluene and placed in a Soxhlet apparatus for 24 h to remove the excess of APTES. Finally, the substrates were baked at 120 °C for 30 min.

Synthesis of Maltoseptase. The donor substrate maltoseptase was obtained by the acid-catalyzed hydrolyses of β -cyclodextrin. A more detailed procedure can be found elsewhere.²⁵

Anchoring Maltoseptase. The amino-functionalized wafers were immersed in a DMSO solution containing 1% (v/v) acetic acid, 10 mg mL⁻¹ maltoseptase, 2.5 mg mL⁻¹ NaCNBH₃, and 4 Å molecular sieves. The reductive amination was carried out at a temperature of 60 °C for 3 and 7 days in a shaking incubator. Substrates were after reaction rinsed and sonicated with Milli-Q water and ethanol.

Spectrophotometric Determination of the Amine Density. 50 mg (242.4 μ mol) of anthraldehyde was dissolved in 50 mL of distilled DMSO containing 12.5 μ L of acetic acid. The amine bearing surfaces were immersed in 2 mL of the above-described solution, and 4 Å molecular sieves were added. The samples, incubated at 60 °C for 20 h, were afterward rinsed with DMSO, Milli-Q water, and dry methanol and dried in a vacuum. Hydrolysis of the surface bound imines was realized by immersing the substrates in 1 mL of Milli-Q water containing 0.8% (v/v) acetic acid. This solution was heated to 30 °C for 30 min. The absorbance of the solution was measured at a wavelength of 262 nm. A calibration curve of anthraldehyde in water containing 0.8% (v/v) acetic acid was made in the range 27–1300 nM. Absorptions were measured at a wavelength of 262 nm.

Isolation and Purification of the Glycosyltransferases. The isolation and the purification of potato phosphorylase²⁵ as well as the cloning, expression, and purification of the GBE_{DG} can be found elsewhere.²⁶

Typical Enzyme Catalyzed Synthesis of (Hyperbranched) Glucan Brushes. A maltoseptase-functionalized wafer was immersed in a buffered solution containing G1P (250 nM), PP (5 U mL⁻¹), and GBE_{DG} (250 U mL⁻¹). Citrate buffer (pH 6.2, 50 mM) was used when PP was utilized, and a MOPS buffer (pH 8.0, 50 mM) was used in the

case of a tandem polymerization. The immersed wafer was, depending on the enzymes used, incubated for 3 days at 37 °C (tandem polymerization) or 38 °C (PP) in a shaking incubator. Afterward, the substrates were thoroughly rinsed with Milli-Q water, dried with a stream of air, and stored *in vacuo*.

X-ray Photoelectron Spectroscopy (XPS). XPS spectra were recorded on a Surface Science Instruments SSX-100 photoelectron spectrometer with a monochromatic Al K α X-ray source ($h\nu = 1486.6$ eV). Measurements were carried out at a photoelectron takeoff angle of 35° with respect to the sample surface. The resolution of the survey scans was set to 4, and the acquisition of C_{1s} signal was done at constant pass energy of 50 eV. All spectra are the averaged results of four measurements. Data analysis was performed with the software package Winspec 2.09. The elemental compositions were calculated with the following relative sensitivity factors: O_{1s}: 2.49; N_{1s}: 1.68; C_{1s}: 1; Si_{2s}: 1.03; Si_{2p}: 0.90.

Spectroscopic ellipsometry was performed on a VASE VB-400 ellipsometer in the range 400–1000 nm. The angle of incidence was varied between 74° and 76° with an interval of 1°. The software package WVASE32 was used to make a model consisting of different layers with characteristic values for the refractive indices. A Cauchy dispersion layer was used to determine the thickness of the APTES, maltoseptase, and polysaccharide layer. A refractive index of 1.336²⁸ was used for maltoseptase and the α -glucan brush and a refractive index of 1.465 for APTES.

RESULTS AND DISCUSSION

In our previous study we combined the catalytic action of potato PP and GBE_{DG} to grow hyperbranched polysaccharides from maltoseptase in solution.²⁵ Here, we use the same glycosyltransferases to grow hyperbranched polysaccharide brushes from a modified silicon surface. Since PP needs a short oligosaccharide of at least three glucose residues as acceptor substrate,²⁷ maltoseptase was covalently bonded to a silicon wafer. The maltoseptase-functionalized surface was obtained by an aminosilanization procedure followed by a reductive amination with the reducing end-group of maltoseptase. The resulting maltoseptase-functionalized wafer was able to start the phosphorylase driven chain elongation while GBE_{DG} could introduce branch points by transferring short oligosaccharides to the C6 positions of silicon bound polysaccharides. The complete process of the surface modification is schematically depicted in Figure 1.

The modifications to the Si surface prior to the enzyme-catalyzed synthesis are required for a high-density brush coating and were thoroughly evaluated with ellipsometry and XPS measurements. First, APTES was tethered to the Si wafer in order to amine functionalize the surface. The efficiency of the aminosilanization procedure was measured by coupling

anthraldehyde to the free available surface bound amine groups. Subsequent hydrolysis of anthraldehyde in a known volume of water followed by UV absorption measurements gave the concentration of anthraldehyde which reflects the amount of amine groups on the surface.²⁹ The amine density was determined on $2.8 \text{ amines nm}^{-2}$ and fits well with values found in the literature.^{29,30} The amount of amines per square nanometer predetermines the maximum attainable polymer brush density and is an important parameter since brushes are only formed when the anchored polymer to polymer distance is smaller as the radius of gyration of the free polymer chain. The chemical composition of the amine-functionalized wafer was, after Soxhlet extraction, examined with XPS (see Figure 2).

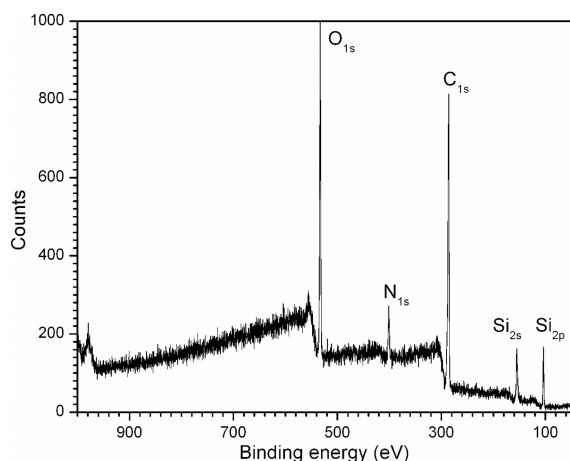


Figure 2. XPS wide scan of APTES grafted Si wafer.

Signals from the N(1s) and C(1s) core levels from the APTES coating were detected as well as signals from Si(1s) and Si(2p) which originate from the Si wafer and proves that the APTES molecules are after extraction still present at the silicon surface.

Moreover, the APTES originated C(1s) signal was deconvoluted in Figure 3 into three signals which were attributed to carbon atoms in different chemical environments.

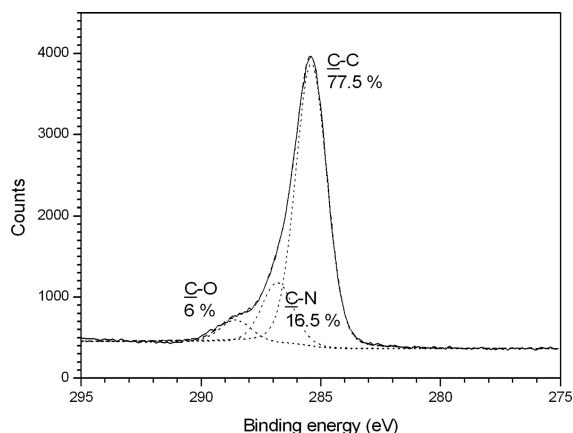


Figure 3. C(1s) core level region of the silicon wafer after aminosilanization.

The 6% contribution of the C–O signal indicates that there are still unreacted ethoxy groups present. However, a value of 6% is acceptable and not expected to have consequences for further procedures. Subsequently, maltoheptaose was anchored

to the amine-functionalized substrate by reductive amination. The reducing end of maltoheptaose reacts herein with the anchored amine, resulting in an imine linkage. Reduction with NaCNBH₃ results the secondary amine which couples the maltoheptaose moiety to the Si wafer.^{31–33} The layer thickness of maltoheptaose as well as the SiO₂ and APTES was measured on five independent wafers with ellipsometry and fitted with a Cauchy dispersion model (see Table 1). The measured

Table 1. Thickness of the the SiO₂, APTES, and Maltoheptaose Layers

sample	SiO ₂ thickness (nm)	APTES thickness (nm)	G7 thickness (nm)
1	3.606 ± 0.0224	4.661 ± 0.0206	3.388 ± 0.12
2	3.875 ± 0.0234	4.114 ± 0.0211	2.817 ± 0.0451
3	3.266 ± 0.0209	4.529 ± 0.0195	3.412 ± 0.0452
4	3.614 ± 0.0202	4.792 ± 0.0239	3.138 ± 0.0583
5	3.733 ± 0.0242	4.740 ± 0.0180	4.743 ± 0.0842
average	3.62 ± 0.23	4.57 ± 0.27	3.50 ± 0.74

maltoheptaose thickness of $3.5 \pm 0.7 \text{ nm}$ is in good agreement with the length of one maltoheptaose molecule. The (virtual) bond length of one glucose residue is 4.2 \AA .³⁴ Taking 4.2 \AA as unity, maltoheptaose would have a maximum length of $0.42 \times 7 = 2.9 \text{ nm}$. Since maltoheptaose can take up to 10% of (atmospheric) water, thicker layers can be observed. The average density profile (σ) of the tethered maltoheptaose can now be calculated by applying eq 1

$$\sigma = L\rho N_a/M_n \quad (1)$$

where L is the layer thickness as obtained with spectroscopic ellipsometry (3.5 nm), ρ is the density of maltoheptaose (1.0386 g mL^{-1}),³⁵ N_a is the constant of Avogadro, and M_n is the molecular weight of maltoheptaose (1153 g mol^{-1}), yielding a grafting density of 1.90 nm^{-2} . This means that 68% of the previously anchored APTES molecules reacted with maltoheptaose. This conversion was reached after 3 days of reaction. Increasing the reaction time to 7 days did not yield higher grafting densities, suggesting that the maximum grafting density is attained after 3 days.

Taking the maltoheptaose-functionalized wafers as starting material for the enzyme-catalyzed surface-initiated polymerization, polysaccharide brush coatings were grafted from solid supports. Linear as well as hyperbranched brushes were synthesized depending on the enzyme system used. The maltoheptaose-functionalized wafers were immersed in a buffered solution containing the donor substrate, G1P, and the glycosyltransferases. Incubation for 3 days at 37 or 38°C yielded the desired polyglucan brush coating as proven with ellipsometric data. A hyperbranched polysaccharide brush coating was obtained by adding PP and GBE_{DG} while a linear polysaccharide brush coating was obtained after incubation with PP alone. Since PP needs a short oligosaccharide of at least three glucose residues as acceptor substrate,²⁷ polymerization occurs only at the surface and not in solution. An important feature of a polymer brush coating used for antibacterial purposes is, next to the grafting density, the thickness. The thickness of the layer was therefore determined with ellipsometric measurements. The corresponding degree of polymerization was estimated from the layer thickness of the linear polysaccharide brush coatings by using the molecular geometries of amylose V as determined by X-ray diffraction.³⁶

Immel et al. determined the pitch of the helical structure of amylose V on 8.05 Å with six glucose residues per turn.³⁶ By determining the layer thickness, the amount of pitches and the glucose residues can be calculated. For the sake of simplicity, the tilt angles of the chains are neglected (90°; perpendicular to the surface). In reality, the tilt angle is equal to or smaller than 90°, resulting in a higher degree of polymerization. The polymer brush thicknesses of linear amylose brushes as well as hyperbranched brushes are displayed in Table 2. The amount

Table 2. Polyglucan Brush Thickness and the Corresponding Degree of Polymerization

type of brush	incubation time (days)	thickness (nm)	no. of pitches	DP
linear	3	16.719 ± 0.1440	20.8	125
linear	3	20.171 ± 0.0405	25.1	150
linear	3	15.214 ± 0.0314	18.9	113
linear	3	19.435 ± 0.0557	24.1	144
linear	3	19.654 ± 0.0700	24.4	146
hyperbranched	3	16.903 ± 0.0432		
hyperbranched	3	12.223 ± 0.0242		

of pitches and the corresponding degree of polymerization were calculated for the linear brushes. Although this is not possible for the hyperbranched brushes, a similar degree of polymerization is expected. The degree of branching of the polysaccharide brushes is expected to be at most 11% but may be lower due to the restricted space.

The glycosyltransferase driven reaction was fed with a 1000-fold excess of the donor substrate G1P, making sure that the brushes could grow to completeness. A control experiment with a 1000-fold excess of G1P in solution showed that about 70% of the G1P was consumed, corresponding to a DP of over 765. However, by anchoring maltoheptaose to a solid support, the accessibility of the primer to the active site of PP is strongly hindered, and brushes with a lower degree of polymerization were formed. Prolonged incubation of the wafers did not result in an increased layer thickness, suggesting that the obtained layer thickness is limited to ~20.2 nm. Similar layer thicknesses were observed for the hyperbranched brushes as synthesized by the combined action of PP and GBE_{DPG}. It is likely that the degree of polymerization of the hyperbranched brushes is comparable to the linear brushes since layer thicknesses are comparable. The degree of branching of the brushes is limited to 11% but may be lower due to a difficult to reach active site of GBE_{DPG} since the acceptor polysaccharide is fixed to the silicon wafer and less mobile.

CONCLUSIONS

A system is presented to graft a polysaccharide brush coating via enzymatic pathways. The resulting polysaccharide coating can be seen as a rudimentary glycocalyx that can be functionalized to tailor the specific needs. Ellipsometric analysis of the brush coatings showed layer thicknesses in the range 12.2–20.2 nm. Depending on the chosen enzyme system, both linear and hyperbranched polymers were grafted. The exact degree of branching could not be determined but is limited to 11%. The use of potato phosphorylase (PP) resulted in linear amylose-like brushes while the combination of PP/GBE_{DPG} yielded hyperbranched brushes. The degree of polymerization of the brush polymers was estimated from ellipsometric data and is limited to ~150 glucose residues.

Steric hindrance at the surface and a difficult to reach active site of the glycosyltransferases might cause this. In the future we hope to obtain more detailed data of the polyglucan brushes via XPS measurements.

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