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Detection of enzyme-catalyzed polysaccharide synthesis on surfaces

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Abstract
Strategically important cellular components, such as the cell wall and the starch granule, present surfaces during their biosynthesis and degradation. The enzymology of such surfaces is experimentally challenging and goes well beyond classical solution-state analyses. The kinetics of surface catalysis is complex but tractable. A number of approaches to monitor surface catalysis are reviewed and each is suited to a different biological problem. Particular attention is paid to a method we have recently developed for quantitatively monitoring polysaccharide synthesis on a surface in real time using surface plasmon resonance spectroscopy. This method has many attractive features with the potential to tackle both biological and industrial problems.

Keywords: *Enzyme, surface, catalysis, polysaccharide, surface plasmon resonance spectroscopy, kinetics*

Introduction

Many biological phenomena involve surfaces that need to be biosynthesized, modified and degraded. The enzymology of biologically and strategically important surfaces, such as cell walls and starch granules, is poorly understood. For example, although the solution-state enzymology of starch biosynthesis has been extensively studied (Ball & Morell 2003), such *in vitro* studies rarely take into account the fact that the starch granule is both an insoluble substrate and an insoluble product that would present quite different surfaces to enzymes during the assembly of its highly organized architecture. To illustrate the importance of solid surfaces in this system, we have observed that granule-bound starch synthase I is activated in the presence of amylopectin crystallites, consistent with its role in synthesizing amylase within a solid amylopectin matrix (Edwards et al. 1999). This observation can be rationalized as allosteric enzyme activation by a phase change of amylopectin from solution state to the solid state, thus presenting a new solid surface. The disassembly of the starch granule, so important in fermentation for biofuels and other traditional uses, clearly also involves enzyme–surface interactions. Enzymic surface degradation of cellulose likewise has great potential (Lynd et al. 2002). Surface enzymology is gaining importance in many other commercial applications, such as in the detergent, paper and food industries (Foose et al. 2007), as well as in biocatalysis in terms of screening and synthesis (Halling et al. 2005; Laurent et al. 2008a).

The present review addresses the methods used to study surface enzymology, with a particular emphasis on the surface plasmon resonance (SPR) spectroscopy method we have developed (Clé et al. 2008), together with a general discussion of the kinetics associated with surface modification by enzymes.

Monitoring surface biocatalysis

There are few options available to study the kinetics of surface enzymology in real time. A number of methods have been reported to allow surface catalysis to be monitored; however, these have focused mostly on hydrolysis rather than synthesis. For example, glucan hydrolysis with glucoamylase, phosphorylase b, isomalto-dextranase or α-amylase has been quantitatively monitored using a quartz-crystal microbalance (Nishino et al. 2004a,b; Sasaki et al.

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2008). In addition, xyloglucan hydrolysis by an endo-xyloglucanase (Nordgren et al. 2008) and cellulose degradation by cellulases (Josefsson et al. 2008) have been monitored using this method. There is only one example of polysaccharide synthesis being monitored on a surface using this approach. Phosphorylase was used in the reverse reaction to drive amylopectin extension (Murakawa et al. 2007).

Starch hydrolysis by amyloglucosidase has been monitored electronically using single-walled carbon nanotubes as semiconducting probes in field-effect transistors (Star et al. 2004). Protease activity at surfaces has been monitored using ellipsometry (Foose et al. 2007). Esterase activity has been measured electrochemically on surfaces in real time (Nayak et al. 2007; Yeo & Mrksich 2003). Even systems allowing the UV detection of collagenase activity on surfaces have been developed (Gaspers et al. 1995). These latter approaches could, in principle, be used to monitor surface synthesis but no examples appear to have been reported.

Other surface enzymology approaches can be very powerful, but do not usually provide real-time measurements (Halling et al. 2005). Such systems are typically based on mass spectrometry (Ban & Mrksich 2008; Laurent et al. 2008b; Zhi et al. 2008), radiolabels (Matsuo et al. 1992; Donovan et al. 1999; Shipp et al. 2008), lectin affinity (Bryan et al. 2004; Houseman & Mrksich 2002; Plath et al. 2006; Park & Shin 2007), antibody detection (Yu et al. 1995), fluorescence affinity systems (Blixt et al. 2008) and bead-based systems (Halling et al. 2005). In some applications where there is a small change in mass after enzymatic conversion, such methods may be the ones of choice. However, they do not lend themselves well to polysaccharide synthesis monitoring in real time.

One of the most widely used surface-based kinetic techniques in biology is SPR spectroscopy (Rich & Myszka 2006). This is almost always used to address reversible binding events, but there are a few limited studies that have addressed surface enzymology. One of the earliest examples is the monitoring of DNA polymerase activity (Nilsson et al. 1995), but real-time signals associated with DNA synthesis were obscured by those associated with protein binding to the DNA template, precluding real-time quantification and necessitating end-point measurements. However, real-time detection of reverse transcriptase activity has since been reported (Buckle et al. 1996). The activity of an α-1,4-galactosyltransferase–maltose binding protein fusion has been detected on a maltotriose-carrying glycolipid membrane using SPR spectroscopy (Nagahori et al. 2003). However, the product was only transiently bound to the enzyme, precluding quantitative kinetics, and, in any case, the surface itself was not one of the co-substrates for the enzyme. Fructosyltransferase activity has also been monitored using SPR spectroscopy (Shemesh & Steinberg 2006; Jabbour et al. 2007). However, the enzyme was again (covalently) immobilized on the chip, the product was not covalently bound to the surface, and the surface itself was not one of the co-substrates. The same issues also apply to a system where polyhydroxyalkanoates are enzymically synthesized on surfaces (Kim et al. 2006). However, there is one recent example of SPR spectroscopy being used to monitor bacterial cell wall peptidoglycan synthesis with tethered lipid bilayer surfaces acting as the substrate (Spencelayh et al. 2006). In addition, we have recently demonstrated the possibility of using SPR spectroscopy to quantitatively measure the enzymic synthesis of surface polysaccharide in real time (Clé et al. 2008) and this is described below.

Real-time detection of surface polysaccharide synthesis using surface plasmon resonance spectroscopy

The model system (Clé et al. 2008) that we developed (for cartoon see Figure 1) involved a carboxymethyl dextran surface (Löfås & Johnsson 1990) and the transglucosidase alternansucrase (E.C. 2.4.1.140) from *Leuconostoc mesenteroides* NRRL B-1355 (Côté 2002). This enzyme catalyses the transfer of glucose from sucrose to acceptors, including carboxymethyl dextran, at their non-reducing ends. The new polymer contains predominantly alternating α-1,6 and α-1,3 links with 10% α-1,6 branching with branch lengths of two or three glucosyl units (for cartoon see Figure 2 (Côté 2002)). A co-injection of both enzyme and sucrose over the surface led to an increase in the SPR response (Figure 3). The rate of this increase was a function of the enzyme concentration (e.g. 1 response unit (RU) s^{-1} μg^{-1} mL) and linearly dependent on the extent of enzyme bound to the surface. At the end of the co-injection, the enzyme slowly dissociated from the surface and an injection of salt removed any remaining enzyme. The difference between the final and initial signal reflected how much new polysaccharide material

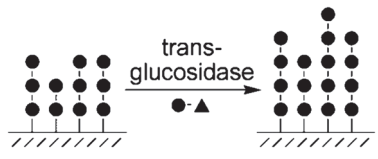

Figure 1. A cartoon showing the enzyme-catalyzed extension of a linear oligoglucan surface using the donor substrate, sucrose.

Figure 2. A cartoon depicting the structure of alternan, a glucan containing predominantly alternating α-1,6 and α-1,3 links with 10% α-1,6 branching with branch lengths of two or three glucosyl units. The single reducing end is on the extreme left.

was present on the surface. Again, this was a function of enzyme concentration (e.g. 40 RU μg^{-1} mL) and linearly dependent on the extent of enzyme bound to the surface. The events underlying each part of the sensorgram are described in more detail below.

It was possible to use this method to determine the K_m for sucrose, which was indistinguishable from that obtained using a solution-state assay based on fructose release (3.3±0.6 mM). Furthermore, it was possible to determine the turnover number of the enzyme on the surface. This was possible because one can measure the rate of signal increase (RU s^{-1}) and calculate the amount of enzyme bound (RU) at the end of each co-injection (see below). Although this gives a rate in s^{-1}, the SPR spectroscopy response is a function of refractive index and these are different for dextran and protein (refractive index increments d*n*/d*c* of dextran and protein are 0.15 and 0.18 mL g^{-1}, respectively (Stenberg et al. 1991)). The rate can therefore be corrected for this difference. Finally, knowing their molecular masses (162 and 225 000 Da for glucoside units and enzyme, respectively), the rate can be adjusted to give the surface turnover number (i.e. the number of glucose units transferred to the surface per enzyme active site in s^{-1}). This was determined to be about half that of k_{cat} in solution. It is noteworthy that this calculation depends neither on the type of initial surface nor on specific SPR spectroscopy responses because ratios are taken in each step which cancels out any specific responses one may wish to invoke. This indicated that about half of the enzyme was bound to the surface in an unproductive mode. This was likely due to some electrostatic interactions between the enzyme and the anionic surface at the pH used.

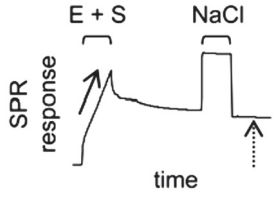

Figure 3. A typical SPR spectroscopy sensorgram of the enzyme-catalyzed extension of a surface. A co-injection of enzyme (E) and donor substrate (S) allows the rate of extension of the surface to be monitored in real time during the latter part of the co-injection (solid arrow). A salt wash removes any enzyme still bound after the injection, allowing the level of surface extension to be determined (dashed arrow).

It was possible to confirm the presence of new polysaccharide material on the surface in several ways. Concanavalin A is a lectin that binds most strongly to mannose units at non-reducing ends. However, it also binds strongly to glucosyl units at the non-reducing ends of dextrans (Goldstein et al. 1965). This lectin bound much more strongly to the modified surface, consistent with the addition of new material that had short branches presenting more non-reducing ends. Atomic force microscopy revealed the presence of additional soft amorphous material on the modified surface. One could also observe the new material with the naked eye due to the differential dispersion of light of the modified portion of the chip surface. IR signals consistent with a glucan could also be detected, but these were weak given a surface thickness of only <400 nm.

Glucansucrases have often been used to modify small molecule acceptors in solution to generate novel oligosaccharide products (Monchois et al. 1999). Acceptors are typically extended by only a few glucose units giving high yields of specific oligosaccharide products. It therefore may seem surprising that extensive surface extension is observed. However, the reaction conditions are very different. In a typical solution-state reaction, the donor and acceptor concentrations (e.g. 10 and 1 mmol dm^{-3}, respectively) are much greater than the enzyme concentration (e.g. 100 nmol dm^{-3}). In addition, each acceptor molecule could only be extended by ten glucose units on average but even this is not observed because there is a competition with the normal reaction involving sucrose alone. By contrast, when the enzyme and sucrose at these concentrations are presented to a surface acceptor at a density of ~50 pmol dm^{-2}, the enzyme is clearly in large excess over the acceptor. Furthermore, a typical 20 μL co-injection will contain 234 nmol of sucrose and yet the amount of glucose units that are transferred to the surface (0.6 mm^2) is estimated to be <7 pmol. Therefore, the acceptor will encounter enzyme much more often and yet the acceptor reaction will account for <0.003% of the sucrose available. Therefore the enzyme can extend acceptors by many more glucose units under these conditions than in solution because both the enzyme and donor are in such large excess over the acceptor on a surface.

Features of a typical sensorgram

A number of overlapping events are observed when monitoring surface polysaccharide synthesis using SPR spectroscopy (Figure 3). During an injection, there can be a bulk refractive index change if

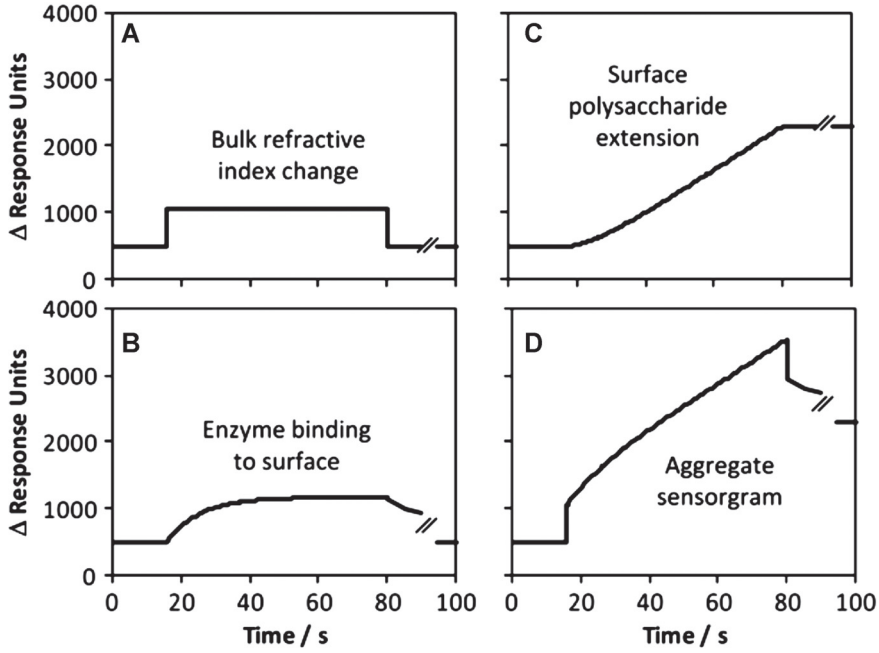

Figure 4. An SPR spectroscopy sensorgram of the enzyme-catalyzed extension of a surface consists of several components. The first three panels show the theoretical individual contributions of bulk refractive index changes (A), the binding of enzyme to the surface (B) and polymer synthesis on a surface (C) during an injection of enzyme and donor substrate (16–80 s). The final signal shown (90–100 s) corresponds to that after all enzyme has dissociated from the surface. The fourth panel (D) shows the observable sensorgram, which is the sum of each of the three individual contributions. These theoretical sensorgrams were generated using DynaFit© (Kuzmič 1996), the latter one fitting well to experimental data.

components have a high refractive index and/or are at a high concentration (Figure 4A). With our model system, the high concentration of the donor substrate sucrose gives such a bulk response. In principle, high enzyme concentrations could also contribute but this is not the case in our system because enzyme-only controls in the concentration range used do not give bulk responses. The injection of enzyme will lead to its association with the surface, if there is any affinity between the two (Figure 4B). This is essential for surface synthesis because only formally bound enzyme could extend a surface. The association rate will be a function of both the association and dissociation rate constants and reflect the rate at which equilibrium binding occurs according to Langmuir-type kinetics. In our case, control experiments show that binding does reach equilibrium during each co-injection because shorter or longer co-injections gave the same final level of enzyme binding. At the end of the injection, the enzyme dissociates with a rate that reflects the dissociation rate constant only. This is because the contents of the flow cell above the chip surface are continually being replaced by running buffer without enzyme after each injection. A salt wash has been established to remove the remainder of the enzyme. Formally bound enzyme, in the presence of the donor substrate, will then be able to catalyze surface synthesis of polysaccharide (Figure 4C). The rate of surface synthesis must be a function of the level of productively bound enzyme. Therefore, the rate of synthesis will increase during the enzyme association phase and reach a steady state when equilibrium binding of enzyme to surface has been attained. At the end of the co-injection, catalysis will stop even if some enzyme remains bound because no more sucrose will flow over the chip surface. The observable sensorgram will be the sum of each of these three signals (Figure 4D).

One can calculate the signal associated with bound enzyme at the end of each co-injection (Figure 4B) by subtracting those associated with the bulk refractive index change (Figure 4A) and the extra surface polysaccharide (Figure 4C) from the signal measured at the end of each co-injection (Figure 4D). This is very important because the K_d of the enzyme for the surface could be affected by the presence of the donor substrate, precluding obtaining meaningful information from control injections of enzyme alone. In our case however, sucrose had no effect on the K_d of the enzyme for the surface, allowing a K_m for sucrose to be determined with confidence. The ideal enzyme concentration range to use is one where it is well below the K_d for the surface. Indeed, the lower the better because then the extent of binding will be pseudo-linearly dependent on bulk

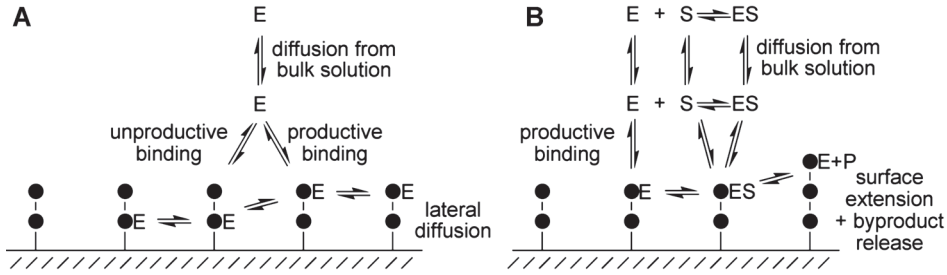

Figure 5. The kinetics of surface extension by an enzyme with a donor co-substrate. Enzyme (E) must first diffuse from bulk solution towards the surface before it can bind (A). Binding can be either productive or unproductive as far as the potential for catalysis is concerned. Once bound to the surface, there is the potential for lateral diffusion. The donor substrate (S) must also diffuse from bulk solution before it can bind to enzyme already associated with the surface (B; unproductive binding modes have been omitted for clarity). It could also bind to the enzyme in bulk solution. A ternary complex consisting of enzyme, substrate and surface in a productive binding mode would lead to catalysis giving surface extension and byproduct release (i.e. release of the leaving group of the donor substrate). Mechanisms, such as the ping-pong type, that do not require a ternary complex will apply in some systems. The subsequent steps will depend on whether the enzyme is either processive or distributive, with dissociation of enzyme from a surface molecule between catalytic cycles from a given chain being either infrequent or frequent, respectively.

enzyme concentration (Gutiérrez et al. 2004; Nayak et al. 2007). By contrast, enzyme concentrations above the K_d for the surface will approach surface saturation and give synthesis rates that will be insensitive to enzyme concentration.

The kinetics of surface enzymology

The kinetics of enzyme catalysis involving a surface substrate is more complex than those in solution. Let us consider a system where a surface is extended by an enzyme that requires a donor co-substrate. The enzyme must first diffuse through bulk solution towards the surface before it can bind (Figure 5A). If the rate of enzyme association to the surface is very rapid, there is the potential for mass transport limitation of this process (Fang et al. 2005). Indeed, a high surface density of the acceptor substrate makes such rate limitation more likely. This is not the case in our model experimental system. In any case, mass transport limitation can be alleviated with fast flow rates. Furthermore, the important phase of the co-injection sensorgram is when enzyme–surface binding has reached equilibrium and not the association phase itself (Kim et al. 2002; Wegner et al. 2004). The binding of the enzyme to the surface could be either productive or unproductive in terms of the potential for catalysis. It is also possible for lateral diffusion to occur across the surface (Gaspers et al. 1994; Trigiante et al. 1999; Lieto et al. 2003; Roy et al. 2005), which could either be kinetically beneficial or not, depending on the nature of the system.

In order for catalysis to occur, the donor substrate also needs to be present (Figure 5B). The enzyme could associate with the donor substrate in bulk solution or on the surface. Figure 5B illustrates an example of where a ternary complex is required for catalysis. Surface extension can then occur with the release of the leaving group of the donor substrate. With a ping-pong type of mechanism, the leaving group would be released before productive binding of the enzyme to the acceptor surface. Either way, there is the possibility of mass transport limitation, this time of donor substrate approaching the surface. Again, there is no evidence for this in our model experimental system because, for example, the sucrose K_m curves do not deviate from ideal behavior. Such rate limitation would only occur when high levels of enzyme were surface-bound and/or the enzyme had a high turnover rate. If such mass transport limitation were an issue, it could nevertheless be alleviated using high flow rates, low enzyme concentrations or non-saturating donor substrate concentrations.

The subsequent steps will be system-dependent. For example, the enzyme may dissociate from the surface molecule it has just extended and bind elsewhere (either through lateral diffusion or via bulk solution (Lagerholm & Thompson 2000)), giving a distributive mode of extension. Alternatively, it may reside on a single surface molecule for several catalytic cycles in a processive manner. Either way, if the nature of the surface is changed either chemically or physically by the enzyme, there could be an effect on the rate of surface extension. However, this will often be short-lived because several catalytic cycles are likely required before a signal is observed. An exception will be with enzymes that give extended branching. This would give a rate of surface synthesis that would always increase with time as more non-reducing ends are both generated and extended.

One feature of the SPR spectroscopy method presently employed is the continuous flow of solution through the flow cell above the chip surface. This has several advantages. Mass transport-limited association of enzyme and co-substrate to the surface can be overcome by increasing the flow rate through the

flow cell as described above. A very important additional benefit is that the concentration of the bulk solution constituents remains constant during an injection. This is because they are continually being replaced even if they bind to the surface or get consumed at any given moment. This makes analyzing such data much more tractable because synthesis rates will become linear as soon as surface enzyme binding reaches equilibrium. It is possible to model our experimental data to help deconvolute the observable co-injection sensorgrams. To this end, we have used DynaFit© (Kuzmič 1996) to simulate and fit such experimental data (for example, see Figure 4). In order to do this, a feature enabling both enzyme and substrate bulk concentrations to remain fixed during a co-injection was added to v4 of the software.

We observed the suppression of k_{cat} on a surface in our experimental system. There have been other reports that enzymes turn over slower on a surface than in solution (Fang et al. 2005; Halling et al. 2005). The two most common explanations are two-dimensional lateral transfer diffusion rate limitation and an equilibrium between unproductive and productive binding (Gaspers et al. 1994; Trigiante et al. 1999; Lieto et al. 2003; Lee et al. 2005; Roy et al. 2005). The latter seems most likely in our system because the enzyme could bind to the carboxylmethyl dextran chip in three possible modes: electrostatically to the carboxymethyl groups, perhaps in a non-productive mode to the dextran main chain, or productively to a non-reducing end of the dextran. Indeed, it would appear that electrostatic interactions assist in increasing local surface concentrations of enzyme, but there may be a price to pay in the net rate of turnover.

There have been a number of studies that have considered putting surface enzyme kinetics into an algebraic framework that is usually based on a combination of Michaelis–Menten-type (noting that the enzyme is usually in excess of surface substrate rather than the other way around) and Langmuir-type kinetics (Gutiérrez et al. 2002, 2005; Lee et al. 2006). Various models have been derived but these have been system-specific. For example, systems can involve either closed cells (Nayak et al. 2007) or flow cells (Lee et al. 2005). Surfaces can present substrates that are transformed to product in one discrete step (Nayak et al. 2007) or several steps when polymers are either degraded (Nishino et al. 2004a,b) or extended with a donor substrate in solution (Murakawa et al. 2007). There are also ways in which mass transport limitation could be taken into account (Schuck & Minton 1996; Myszka 1997; Myszka et al. 1998; Schuck et al. 1998). In the case of surface polymer substrates, degradation would have an absolute limit but extension would not; only the ability to monitor it could be limited. It is not always possible to generate an algebraic solution to complex kinetic schemes, so kinetic simulation software would be the method of choice. We intend to develop a detailed model to simulate our system and help establish what depresses k_{cat} on a surface. A complete description may require an ability to independently measure enzyme binding and polymer synthesis. Others have used SPR imaging together with SPR fluorescence to address this point in other systems (Kim et al. 2002; Fang et al. 2005; Lee et al. 2005).

Prospects

We have already established that the SPR spectroscopy method of monitoring alternansucrase activity on a surface is as sensitive as the solution-state assay based on measuring fructose release (Clé et al. 2008). The sensitivity of the SPR spectroscopy method would be dependent on surface density. The type of chip used in our model experimental system (Biacore® CM5 chip) has a surface density of carboxymethyl dextran chains of ~5 fmol mm^{-2} (Stenberg et al. 1991; Liedberg et al. 1993) and, incidentally, the density of bound enzyme in a typical experiment is less than this. An estimate of the detection limit of our system using such a chip is ~1 pmol of glucosyl units per mm^2, which is equivalent to the net addition of 200 glucosyl units per chain. If one assumes the average footprint of a glucosyl unit on a homogeneous flat surface to be ~0.5 nm^2 (based on a hydrodynamic volume of 166 Å with close square packing (Fioretto et al. 2007) that is consistent with other estimates (Stenberg et al. 1991)), this would give 0.58 ng mm^{-2} or 3.6 pmol mm^{-2}. There is therefore scope to generate surface densities of non-reducing ends much higher than those exhibited by a carboxymethyl dextran chip. A carboxymethyl dextran chip presents a three-dimensional hydrogel of ~100 nm thickness, where its reducing ends would be distributed throughout the hydrogel. By contrast, an oligosaccharide substrate bearing non-reducing ends on a flat surface would be as close as possible to the chip surface where responses are maximal. If the specific responses on such a flat surface were similar to those on a carboxymethyl dextran chip surface, the sensitivity of this method could be increased significantly allowing only a few net glucosyl transfers per chain to be detectable.

The SPR spectroscopy method could be used to tackle a wide range of biological and industrial problems, such as cell wall or starch synthesis and degradation, provided that care is taken in the design of each experiment. There are few other approaches available to tackle surface enzymology (other than

the complementary quartz-crystal microbalance and ellipsometry approaches), but flow-based systems have considerable advantages. The preparation of appropriate substrate surfaces can take advantage of recent developments in carbohydrate array technologies (Ratner et al. 2004; Coullerez et al. 2006; Larsen et al. 2006; Shin et al. 2007; Turnbull & Field 2007; Laurent et al. 2008a). Indeed, one could use these developments to generate screens for surface substrates or novel enzymes.

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