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Carbohydrate-Modified Electrode Surfaces Sensitive to β-Glucosidase Enzyme Activity

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A surface modification which renders a carbon electrode sensitive to β-glucosidase enzyme activity is reported. 2-Nitro-4-(4-diazophenoxymethyl)-phenyl β-D-glucopyranoside was prepared in four steps from the known starting material 2-nitro-4-benzyloxy 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside. A film was formed by the electrochemical deposition of the aromatic diazonium salt 2-nitro-4-(4-diazoophenoxymethyl)-phenyl β-D-glucopyranoside onto a glassy carbon electrode surface, as judged by the change in the oxidation potential for the test analyte ascorbic acid in the cyclic voltammogram (CV). This carbohydrate derivative incorporated a self-immolative linker to physically distance the sugar from the electrode surface and make it more accessible to the active site of the enzyme. A +0.341 V change in the oxidation potential for ascorbic acid was observed in the CV for a modified electrode following treatment with the retaining β-glucosidase from *Agrobacterium* sp. (Abg). Deposition of either 2-nitro-4-diazophenyl-β-D-glucopyranoside or 4-diazophenyl-β-D-glucopyranoside, both of which lacked the self-immolative linker, produced electrode surfaces that were relatively insensitive to Abg, demonstrating the importance of the distance from the electrode surface provided by the linker. This work represents an important step towards the development of a sensor that could be used to detect glycosidase activity.

Keywords: glycosidase; surface modification; carbon electrode; self-immolative linker; cyclic voltammetry;

ABBREVIATIONS:
ascorbic acid (AA); *tert*-butoxycarbonyl (Boc); cyclic voltammogram (CV); retaining β-glucosidase from *Agrobacterium* sp. (Abg); Sweet Almond β-glucosidase (SAB).

1. INTRODUCTION

The surface modification of an electrode can influence its sensitivity, selectivity, and resistance to problems such as surface fouling. One method for derivatizing the surface of a carbon electrode involves the electrochemical deposition of aryl diazonium salts through reduction of the diazo group, followed by the formation of a covalent bond to the carbon surface through a radical reaction. [1, 2] One advantage of this method is that many different substituted aromatic rings can be used, meaning the electrode surface can be modified with a wide variety of functionalities. [3-7]

Glycosidases are the hydrolytic enzymes responsible for cleaving glycosidic bonds, or bonds to the anomeric center of a carbohydrate. These enzymes play roles in a number of important industrial processes, including pulp and paper processing as well as biofuel development and refining. The function or dysfunction of this class of enzyme has been shown to play a role in a number of diseases, including HIV/AIDS, [8] cancer, [9] Parkinson's disease, [10] and metabolic diseases such as Gauchers disease. [11, 12] Methods for the selective and sensitive detection of glycosidase activity could provide valuable research tools and diagnostic techniques, especially for the detection of glycosidase activity in complex biological samples.

Radi *et al.* have reported a sensor for alkaline phosphatase activity through the attachment of an enzymatic substrate to the surface of a carbon electrode. [13] Detection of enzyme activity was achieved by observing changes in the cyclic voltammogram (CV) for the modified surface of the electrode before and after incubation with a solution containing the enzyme. The enzyme hydrolyzed the phosphate group from the 2-aminophenol ring, which itself had been covalently attached to the electrode surface using diazonium chemistry. Enzymatic hydrolysis of the 2-aminophenyl phosphate yielded a 2-aminophenol surface monolayer, which was electrochemically detected by oxidation to the quinoneimine as shown in Fig. 1. The authors also demonstrated that the observed current increased in response to increasing concentrations of alkaline phosphatase, showing that their system can both detect and quantify enzyme activity.

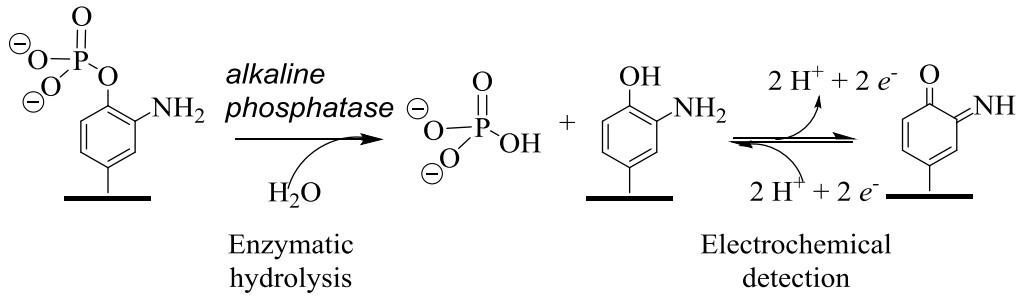


Figure 1. Electrochemical detection of alkaline phosphatase activity described by Radi *et al.*: following enzymatic cleavage of the phosphoryl group from the electrode surface, the 2-aminophenol/2-quinoneimine redox pair is electrochemically detected and quantified. Adapted from [13].

We became interested in the idea of fixing a biomolecule to a carbon electrode surface to generate films with unique properties, that could be further modified by a suitable enzyme. One potential application for such a film would be in an electrochemical sensor for glycosidase activity through the detection of the surface modification. Thus, we decided to investigate whether a glycosylated carbon electrode surface sensitive to glycosidase activity could be generated by reaction with carbohydrate derivatives (Fig. 2), whose enzymatic removal could be detected electrochemically. However, we were concerned that the strategy previously described[13] might not be widely applicable to all classes of hydrolytic enzymes, including glycosidases, because of the close proximity between the substrate recognition element (in the previous instance, the phenyl phosphate group) and the electrode surface. Having the substrate positioned close to the surface of the electrode means the surface itself may act as a physical barrier, preventing access of the substrate to the enzyme's active site. Additionally, conformational changes in the enzyme induced by close proximity to the electrode surface could also attenuate enzymatic activity.

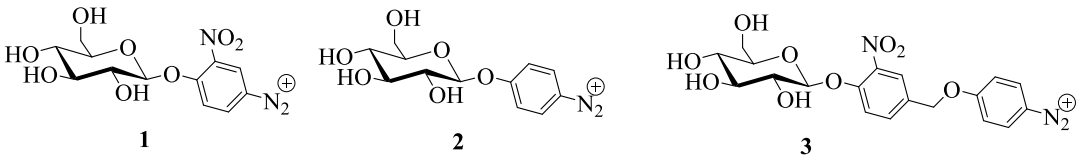


Figure 2. Carbohydrate-containing aryl diazonium compounds prepared and used to modify carbon electrodes.

To address this potential concern, we decided to borrow a strategy from pro-drug therapy and investigate the use of a self-immolative linker. In pro-drug therapy, a biologically inactive derivative of a drug is introduced into an organism, and it is often through the action of an enzyme at a desired location (for example, at the site of a tumour in anti-cancer therapy) that the pro-drug becomes activated to release the active form of the drug. [14, 15] However in many cases, the direct attachment of the drug to the recognition element prevents efficient processing by the enzyme due to steric clashes between the bulky drug and the enzyme active site. [16] To address this, chemists designing pro-drugs have developed self-immolative linkers that, following hydrolysis of the drug conjugate, spontaneously decompose to rapidly release the free drug as represented in Fig. 3. More generally, a self-immolative linker should be useful in any system needing physical distance between an enzyme recognition element and a reporter molecule that becomes active only after being released from the conjugate. The advantage of using linkers is that they are often tolerated in an enzymatic active site, and can be incorporated into substrate derivatives that are efficiently processed. Indeed, self-immolative linkers have already been employed in the development of FDA-approved antibody derived pro-drugs which are activated by proteases, [17] as well as several sugar derivatives which are processed by glycosidases. [18-21]

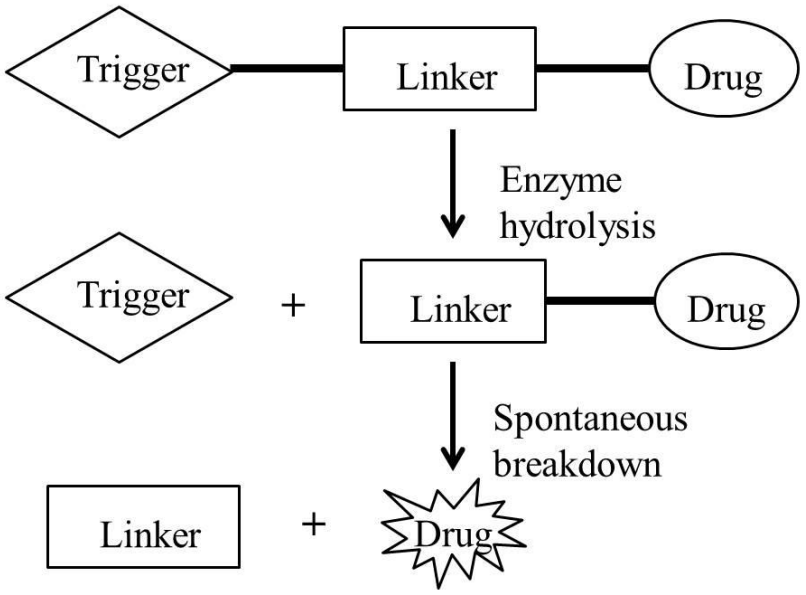


Figure 3. Schematic representation of the use of a self-immolative linker in pro-drug therapy. The drug is biologically inert in the initial complex containing both linker and trigger. Following enzymatic hydrolysis of the trigger, the linker rapidly decomposes spontaneously to leave the active form of the drug.

To test the utility of incorporating such a linker into a carbohydrate-modified electrode coating, one of our carbohydrate derivatives incorporated a self-immolative linker to physically distance the carbohydrate recognition element from the electrode surface and make it more accessible to the enzyme. We opted to introduce a 3-nitro-4-hydroxybenzyl alcohol linker between the carbohydrate and the aromatic ring bonded to the electrode surface, which decomposes through the formation of a quinonemethide as shown in Fig. 4 to leave a free phenol on the electrode surface. We chose this linker because it is readily synthesized, and aromatic rings are often well tolerated in the active sites of many glycosidases (for example, see reference [22]) making it more likely that this surface would be recognized by an enzyme. As well, the nitro group increases the leaving group ability of the linker, thereby increasing the rate of enzymatic hydrolysis as is typical for artificial substrates of glycosidases. We hypothesized that the free phenol formed by the action of the enzyme on the electrode surface could be indirectly detected by monitoring changes in the peak potential for an analyte in solution. If this general approach could generate a sensor of enzyme activity such as that described by Radi *et al.*, [13] it could detect the presence of glycosidase activity in complex biological samples while requiring minimal sample preparation. This could have eventual applications in disease diagnosis and discovery of new enzymes in environmental samples.

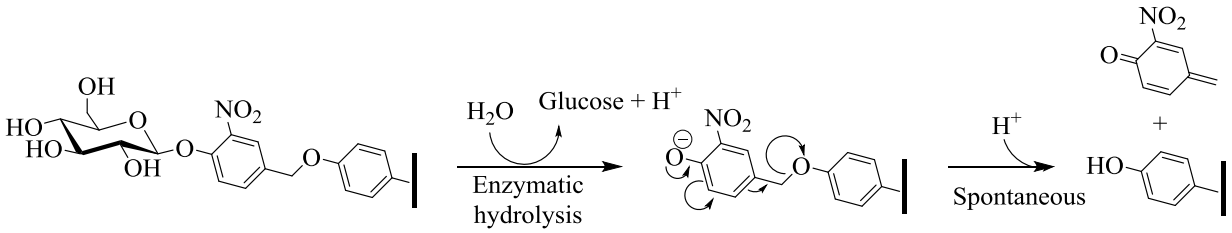


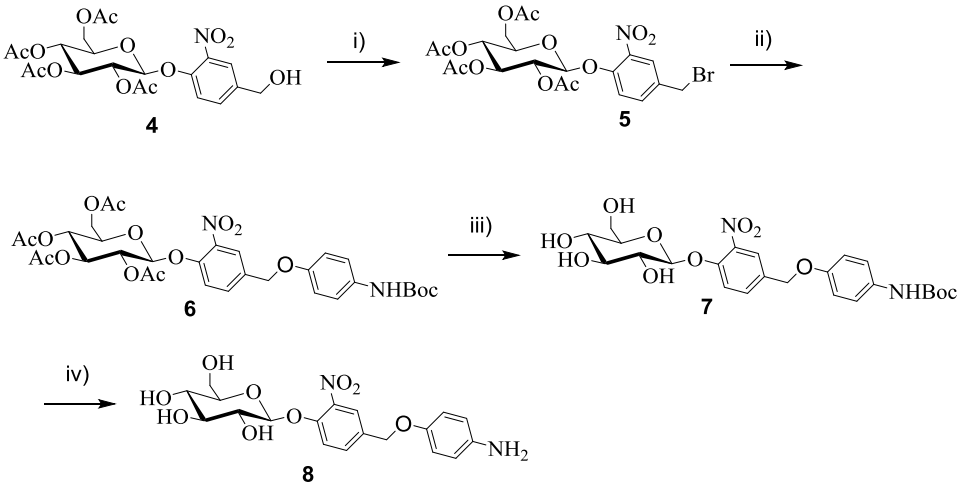
Figure 4. Mechanism for the modification of the derivatized electrode surface by a β-glucosidase. Following enzymatic removal of the glucosyl residue, the self-immolative linker spontaneously fragments to generate a quinone methide and the phenol ring covalently attached to the electrode.

In this paper, we describe the synthesis of **3**, and our efforts to bond diazonium salts **1-3** to the surface of a carbon electrode. Furthermore, we report the results of treatment of the modified surfaces with strong aqueous acid or two β-glucosidases, as measured by changes in the CV for the oxidation of ascorbic acid (AA) before and after treatment of the modified surface. Finally, we demonstrate the need for a self-immolative linker to yield improved recognition of the carbohydrate-modified surface by a β-glucosidase. In addition to creating a novel surface sensitive to glycosidase activity, this work represents an important step in the development of an electrochemical sensor for glycosidase activity.

2. MATERIALS AND METHODS

2.1 Synthetic procedures

2.1.1 General synthetic procedures



Scheme 1. Synthesis of **8**. Reagents and conditions: i) benzotriazole, thionyl chloride, KBr (82%); ii) *N*-Boc-4-hydroxyaniline, K_2CO_3 (67%); iii) K_2CO_3, MeOH/H_2O (89%); iv) TFA, CH_2Cl_2 (99%).

General Procedures. Proton (1H) and carbon (13C) NMR spectra were acquired at the Lakehead University Instrumentation Laboratory (LUIL) on a Varian Unity Inova 500 MHz spectrometer in DMSO-d6 with TMS as the internal standard, where J (coupling constant) values are estimated in hertz (Hz). Thin layer chromatography (TLC) and silica gel column chromatography were performed using TLC silica gel 60 F254 (EMD) and SiliaFlashP60

(SiliCycle), respectively. Synthetic reactions were monitored by TLC visualized with a UV lamp followed by charring with ammonium molybdate. All reagents and solvents were purchased from Fisher Scientific or Sigma-Aldrich and used without further purification.

*2.1.2 Preparation of 2-nitro-4-bromomethylphenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**5**)*

To a solution of 2-Nitro-4-benzyloxyphenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside [23] (**4**, 1.28 g, 2.56 mmol, 1.00 eq.) in anhydrous dichloromethane (25 mL), benzotriazole (305 mg, 2.56 mmol, 1.0 eq.) and thionyl chloride (205 μL, 2.81 mmol 1.10 eq.) were added under argon and allowed to stir for 5 minutes at room temperature. Potassium bromide (457 mg, 3.84 mmol, 1.50 eq.) and anhydrous dimethylformamide (40 mL) were then added and allowed to stir for 1 hour at room temperature. The solution was diluted with water (50 mL) and the organics extracted with ethyl acetate (300 mL), washed with water (1 x 100 mL) dried with brine (1 x 100mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was then purified by recrystallization from hot ethyl acetate and solids washed with cold ethyl acetate to give pure **5** as a white powder (1.18 g, 2.10 mmol, 82 %). ^{1}H NMR (500 MHz, D6-DMSO) δ 1.96-2.06 (m, 12H), 3.35 (s, 1H), 4.15 (dd, J = 12.3, 2.4 Hz, 1H), 4.23 (dd, J = 12.3, 5.4 Hz, 1H), 4.30 (ddd, J = 10.0, 5.4, 2.5 Hz, 1H), 4.83 (s, 1H), 5.05 (t, J = 9.7 Hz, 1H), 5.10 (dd, J = 9.8, 8.0 Hz, 1H), 5.41 (t, J = 9.7 Hz, 1H), 5.70 (d, J = 8.0, 1H), 7.44 (d, J = 8.7 Hz, 1H), 7.80 (dd, J = 8.8, 2.3 Hz, 1H), 8.03 (d, J = 2.2 Hz, 1H). ^{13}C NMR (500 MHz, D6-DMSO) δ 20.67, 20.75, 20.86, 20.96, 44.69, 45.00, 45.01, 61.95, 68.23, 70.48, 71.64, 72.08, 98.48, 118.53, 125.56, 133.77, 135.19, 140.53, 148.61, 169.26, 169.76, 170.07, 170.48.

*2.1.3 Preparation of 2-nitro-4-(4'-tert-butoxycarbonylaminophenyl)-phenyl 2,3,4,6-tetra-O-acetyl β-D-glucopyranoside (**6**)*

To a solution of **5** (500 mg, 0.889 mmol, 1.00 eq.) in anhydrous dimethylformamide (20 mL), potassium carbonate (540 mg, 3.91 mmol, 4.40 eq.) and *N*-Boc-4-aminophenol (744 mg, 3.56 mmol, 4.00 eq.) were added and allowed to stir under argon at room temperature for two days in the dark. The solution was diluted with water (25 mL) and the organics extracted with ethyl acetate (200 mL), washed with water (1 x 100 mL), dried with brine (1 x 100mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was then recrystallized from hot diethyl ether, and the resulting crystals washed with cold diethyl ether to give **6** as a pink powder (408 mg, 0.591 mmol, 67 %). ^{1}H NMR (500 MHz, D6-DMSO) δ 1.46 (s, 9H), 1.96-2.08 (m, 12H), 3.35 (s, 1H), 4.14, (dd, J = 12.4, 2.4 Hz, 1H), 4.23 (dd, J = 12.2, 5.3 Hz, 1H), 4.29 (ddd, J = 10.0, 5.3, 2.4 Hz, 1H), 5.02-5.13 (m, 4H) 5.41 (t, J = 9.6 Hz, 1H) 5.68 (d, J = 8.0 Hz, 1H), 6.91-6.95 (m, 2H) 7.36 (d, J = 8.2 Hz, 2H), 7.44 (d, J = 8.7

Hz, 1H), 7.77 (dd, 8.8, 2.1 Hz, 1H), 7.98 (d, J = 2.0), 9.18 (s, 1H). ^{13}C NMR (500 MHz, D6-DMSO) δ 20.67, 20.75, 20.86, 20.95, 28.58, 28.62, 45.00 ,61.97, 68.11, 68.26, 70.51, 71.60, 72.11, 98.59, 115.41, 118.36, 119.98, 119.99, 120.00, 124.30, 133.41, 133.56, 133.87, 140.66, 148.30, 153.37, 153.48, 169.27, 169.76, 170.06, 170.48. LRMS (ESI) m/z: 713.2 $[M + Na]^+$ Calculated for $C_{32}H_{38}N_2O_{15}Na$: 713.2170

*2.1.3 Preparation of 2-nitro-4-(4'-tert-butoxycarbonylaminophenyl)-phenyl β-D-glucopyranoside (**7**)*

Potassium carbonate (900 mg, 6.51 mmol, 6.2 eq.) was added to a solution of **6** (725 mg, 1.05 mmol, 1.0 eq.) in methanol (80 mL) and water (20 mL) and allowed to stir at room temperature for 1 hour in the dark. The solvents were removed under reduced pressure and low temperature (35 °C) and azeotroped with chloroform to assist in the removal of water. The residue was dissolved in ethyl acetate and methanol (4:1) and filtered through a short plug of silica (4:1, ethyl acetate to methanol) to remove the inorganic salts, leaving pure **7** as a cream colored solid (490 mg, 0.938 mmol, 89 %). ^{1}H NMR (500 MHz, D6-DMSO) δ 1.46 (s, 9H), 3.17-3.30 (m, 3H), 3.34-3.43 (m, 2H), 3.48 (dd, J = 11.7, 5.7 Hz, 1H), 3.69 (d, J = 11.8 Hz, 1H), 5.06 (s, 2H), 5.12 (d, J = 7.4 Hz, 1H), 6.91-6.95 (m, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.45 (d, J = 8.8 Hz, 1H), 7.69 (dd, J = 8.8, 2.2 Hz, 1H), 7.93 (d, J = 2.1 Hz, 1H), 9.17 (s, 1H). ^{13}C NMR (500 MHz, D6-DMSO) δ 28.63, 60.97, 68.24, 69.85 ,73.50 ,77.10 ,77.68 ,79.18 ,100.71 ,115.41, 117.49, 119.99, 120.01, 120.03, 120.04, 120.06, 124.31, 131.48, 133.50, 133.82, 140.23, 149.52, 153.38, 153.58. LRMS (ESI) m/z: 521.2 $[M - H]^-$ Calculated for $C_{24}H_{29}N_2O_{11}^-$: 521.1777

*2.1.4 Preparation of 2-nitro-4-(aminophenyl)-phenyl β-D-glucopyranoside (**8**)*

A solution of trifluoroacetic acid in anhydrous dichloromethane (15 mL, 1:1) was cooled to 0°C on ice before being added to **7** (396 mg, 0.758 mmol, 1 eq.) and allowed to stir for 10 minutes on ice. Diethyl ether was then added to precipitate the product. The residue was then purified through trituration with diethyl ether to give **8** as a white powder (402 mg, 0.750 mmol, 99 %). ^{1}H NMR (500 MHz, D6-DMSO) δ 3.17-3.30 (m, 3H), 3.41 (ddd, J = 9.7, 5.7, 2.0 Hz, 1H), 3.69 (dd, J =11.7, 1.8 Hz, 1H), 5.13 (d, 7.3 Hz, 1H), 5.15 (s, 2H), 7.11-7.15 (m, 2H), 7.28-7.31 (m, 2H), 7.46 (d, J = 8.9 Hz, 1H), 7.71 (dd, 8.8, 2.2 Hz, 1H), 7.96 (d, J = 2.2 Hz, 1H). ^{13}C NMR (500 MHz, D6-DMSO) δ 45.00, 60.98, 68.40, 69.87, 73.49, 77.08, 77.69, 100.68, 116.38, 117.54, 124.50, 124.61, 125.55, 130.89, 133.96, 140.22, 149.64, 157.70. LRMS (ESI) m/z: 423.1 $[M + H]^+$ Calculated for $C_{19}H_{23}N_2O_9^+$: 423.1398

2.1.5 General method for preparation of diazonium salts

The aromatic amine (~0.5 mmol) and sodium nitrite (1.5 mmol) were dissolved in 10 mL of 2 mM HCl. The resultant solution was stirred for 1 h at 0^{o}C before being used directly in the coating procedure described below.

2.2 Electrochemical methods

2.2.1 Instrumentation and electrode polishing

Cyclic voltammetry was performed using a Pine Instrument Company bipotentiostat (Model AFCBP1) and AfterMath™ software. All tests were carried out against a Ag|AgCl reference electrode and Pt counter electrode. A three-electrode cell was used for all electrochemical experiments.

Prior to derivatization, glassy carbon electrodes were polished in three steps with distilled water slurries of 1.0, 0.3 and 0.05 μm Buehler Micropolish II® alumina powder against a Buehler microcloth®. After each polish, the electrodes were sonicated in distilled water for 15 min. The electrodes were then sonicated in dry acetonitrile or anhydrous ethanol for 15 min, thoroughly rinsed with distilled water, dried under a stream of nitrogen gas and used immediately for electrochemical testing.

2.2.2 Electrochemical testing

Testing of the bare, derivatized, or enzyme-treated glassy carbon electrodes was carried out in three different solutions: 0.1 M phosphate buffer at pH 7.0; ascorbic acid (200 μM, 2 mM, or 200 mM) in 0.1 M phosphate buffer at pH 7.0; or 1 mM potassium ferricyanide in distilled water. All tests were carried out over 2 cycles. Initial potential = 0 mV; vertex potential = 800-1200 mV; final potential = 0 mV; scan rate = 5-10 mV/s; electrode range = 1 μA (phosphate buffer), 10-30 μA (ascorbic acid) or 2.5 μA (potassium ferricyanide). Prior to testing, all electrochemical solutions were sparged with nitrogen gas for 2 min.

2.2.3 Coating of glassy carbon electrodes with diazonium salts

A bare glassy carbon electrode was placed in an electrochemical cell containing a solution of the freshly prepared diazonium salt. The electrode was reacted with the diazonium salt over 10 cycles (initial potential = 0 mV; vertex potential = –1100 mV; final potential = 0 mV; scan rate = 5 mV/s; electrode range = 10 μA). For the reduction of the aromatic nitro group on 1, electrodes were then reacted with 0.1 M H_2SO_4 over 10 cycles (initial potential = 0 mV; vertex potential = –1100 mV; final potential = 0 mV; scan rate = 5 mV/s; electrode range = 10 μA).

2.3 Enzymological methods

2.3.1 General enzymological method

Sweet Almond β-glucosidase (SAB) was purchased from Sigma, and was used without any further purification. The β-glucosidase from *Agrobacterium* sp. was prepared as previously described. [22]

2.3.2 Pre-treatment of carbohydrate-modified electrodes

Carbohydrate-modified glassy carbon electrodes underwent one of two pre-treatments prior to electrochemical analysis:

1. Sweet Almond β-glucosidase: Derivatized electrodes were incubated in freshly prepared solutions (between 0.004 mg/mL and 1.8 mg/mL) of Sweet Almond β-glucosidase in 50 mM phosphate buffer at pH 7.0 for 15-30 min at 37^{o}C.
2. β-Glucosidase from *Agrobacterium* sp.: Derivatized electrodes were incubated in freshly prepared solutions (between 0.0005 mg/mL and 0.0267 mg/mL) of Abg in 50 mM phosphate buffer at pH 7.0 for 15-30 min at 37^{o}C.

3. RESULTS AND DISCUSSION

3.1 Synthesis of aryl diazonium salts

The syntheses of both **1** and **2** were relatively straightforward by treatment of the known precursor amines 4-amino-2-nitrophenyl-β-D-glucopyranoside [24] and 4-aminophenyl-β-D-glucopyranoside [25] with $NaNO_2$ under acidic conditions. The synthetic route for the preparation of **8** is shown in Scheme 1.

Briefly, the known alcohol 4 [23] was treated with benzotriazole, thionyl chloride and potassium bromide to furnish the intermediate bromide **5** (82%), which was subsequently coupled to *N*-*tert*-butoxycarbonyl-4-aminophenol (Boc-4-aminophenol) to yield the protected precursor **6** in 67% yield. Deprotection of the *O*-acetyl protecting groups using potassium carbonate in methanol furnished **7** (89%). Finally, removal of the Boc protecting group using trifluoroacetic acid (TFA) provided the free aniline **8** (99%) which was then treated under the standard diazotization conditions to produce the desired compound **3**, which was used without further purification or characterization.

Our attempts to produce **8** using *N*-carboxybenzyl (Cbz), *N*-acetyl or *N*-fluorenylmethyloxycarbonyl (Fmoc) protecting groups were unsuccessful: removal of the Cbz group using H_2 and Pd/C led to undesired concomitant reduction of the nitro group despite previous reports of successful deprotections on similar compounds, [24] while attempts to remove the acetyl protecting group led to decomposition. The *N*-Fmoc-protected phenol did not efficiently couple to bromide **5** and yielded only products arising from decomposition during the coupling step.

*3.2 Electrochemical deposition of **1**-**3** on carbon electrodes*

The protocol for the grafting of **1**-**3** was adapted from that previously described. [13] Briefly, the carbon electrode was immersed in a solution containing the freshly prepared aromatic diazonium salt and the film was formed by electrochemical reduction of the diazonium salt to form a phenyl radical. The phenyl radical rapidly reacts with the carbon surface to covalently link the two and form the film. Following the grafting of **1**, the electrode was placed in an acidic solution and the aromatic

nitro group was electrochemically reduced to an amine. As compound **2** lacks a nitro group, it was not subjected to this second electrochemical reduction. For compound **3**, it was important that the nitro group remain intact as the presence of nitro groups generally make aromatic rings better substrates for glycosidases, [22] so electrodes modified with 3 also did not have their nitro groups electrochemically reduced.

Electrodes with **1**-**3** grafted onto their surfaces were placed in a solution containing either 200 μM (for **1** or **2**) or 200 mM AA (for **3**), used as a test analyte, and their CVs recorded. Note that electrodes coated with **3** required a higher concentration of AA to observe reasonable currents. A single potential cycle of 0.000 V to +1.200 V revealed an oxidation peak for AA at the potential values shown in Table 1 for a bare carbon electrode, or the same electrode with **1**-**3** grafted onto its surface. A comparison of the potentials for the modified electrode to the same electrode before modification shows a shift in the oxidation potential for electrodes modified with **1** or **2**, showing that the surface had been modified by the formation of a film. Our attempts to measure these oxidation potential differences using potassium ferricyanide instead of AA gave inconsistent results (data not shown), suggesting that AA was a better test analyte for monitoring the surface of these particular modified electrodes. Electrodes treated with **3** in most cases did not show a substantial change in the potential compared to the bare electrode, but the dramatic decrease in observed current (from 1.9 mA for the bare electrode compared to 0.011 mA for the treated electrode) clearly indicated a change in the surface of the electrode.

The exact nature of the films formed on the electrode surfaces following electrochemical deposition of compounds 1-3 have not yet been characterized, so the relative coating densities of the different carbohydrate derivatives are not known. However, by monitoring the changes in the AA oxidation potential for an electrode with a particular coating, the degree of enzymatic remodelling of that surface could be qualitatively judged. We hypothesize that these changes in peak potential arise from enzyme-catalyzed trimming of the carbohydrate residues from the electrode surfaces that in turn alter the accessibility of the surfaces towards AA. Whether these changes are arising from a different mechanism for the oxidation reaction, for example electron tunneling through the film versus electrocatalysis, [26] cannot be determined from the present experiments.

*3.3 Testing sensitivity of electrode surfaces modified with **1**- **3** using Sweet Almond β-glucosidase (SAB)*

Sweet Almond β-glucosidase (SAB) is a commercially available retaining β-glucosidase that is frequently used as a model enzyme for proof-of-principle studies. [27-29] We initially tried using electrodes modified by the grafting of diazonium salt **1** onto their surface for the detection of SAB, as this closely modelled the method used by Radi *et al.* in their successful detection of alkaline phosphatase. [13] In their system, the enzymatic loss of the phosphoryl group led to the exposure of the 2-aminophenol surface, which was detected by the presence of the 2-aminophenol/2-quinoneimine redox pair on the electrode using differential pulse voltammetry. Our attempts to detect SAB activity with an electrode modified with 1 using a similar method were unsuccessful. Following exposure of the electrode to a 0.004 mg/mL solution of SAB for 15 minutes at 37 °C, no 2-aminophenol/2-

quinoneimine redox pair could be detected in the CV. Analysis of the enzyme-treated electrode in the 200 μM AA test solution showed no change in the oxidation potential peak for AA in the CV, indicating that the surface had not been altered by the enzyme.

Table 1. Measured oxidation potential peak using cyclic voltammetry for either 200 μM AA (using **1** or **2**) or 200 mM AA (using **3**), with electrodes modified by deposition of **1**, **2** or **3**. Note that the same electrode was used for each series of measurements reported in a row.

Surface Modification	Potential of Bare Electrode (V)	Potential of Modified Electrode (V)	Potential After Treatment With SAB (V)	Potential After Treatment With Abg (V)	Potential after Treatment with 6 M HCl (V)	Change in Potential after Treatment (V)
1	+0.380	+0.798	+0.799	-	-	+0.001
2	+0.351	+0.781	+0.795	-	-	+0.014
2	+0.341	+0.512	-	+0.552	-	+0.040
3	+0.600	+0.630	+0.652	-	-	+0.022
3	+0.586	+0.571	-	+0.912	-	+0.341
3	+0.064	+0.288	-	-	+0.551	+0.263

Since we were concerned that the inability of SAB to recognize and cleave the β-glucosyl residue from the surface was owing to the enzyme being unable to properly bind to the carbohydrate residue on the surface, we decided to try introducing a self-immolative linker into the system. The advantage of such a linker was that it would distance the glucosyl residue from the surface and potentially allow it to reach into the active site of the enzyme, while also spontaneously decomposing to leave only a single aromatic residue on the electrode surface following enzymatic removal of the sugar. However, this meant that relying on direct detection of a 2-aminophenol residue on the electrode surface as our reporter following enzymatic hydrolysis would not be possible, since the nitro group in the 3-nitro-4-hydroxybenzyl alcohol linker would also react when the nitro group on the reporter was electrochemically reduced. Therefore, we decided to abandon attempts to directly monitor the modification of the electrode surface using the detection of the 2-aminophenol/2-quinoneimine redox pair, and instead opted to pursue the indirect detection of surface modification by observing changes in the oxidation potential for AA before and after exposure of the electrode to a solution containing the enzyme. Compound **2** was prepared and used to modify the electrode surfaces, since it would leave behind a phenol film similar to that generated by compound **3** following removal of the sugar residue from either. This would allow a means to directly test the effect of the self-immolative linker on the sensitivity of the electrode surface to β-glucosidase activity.

When an electrode modified through the deposition of **2** on its surface was treated with a solution containing SAB, measurement of the oxidation potential of AA before and after treatment with enzyme yielded a similar result as that obtained when using the electrode modified with **1**: there was only a very small difference in oxidation potential (+0.014 V) for AA when using the electrode before and after treatment with SAB. This suggested that the surface modified with **2** was also

relatively insensitive to the presence of the enzyme, and is consistent with the hypothesis that the sugar residue was physically inaccessible to the enzymatic active site.

To test this hypothesis, we created a surface film on an electrode using diazonium salt **3** which incorporated the 3-nitro-4-hydroxybenzyl alcohol linker to distance the carbohydrate from the electrode surface. This modified electrode was used to measure the oxidation potential of AA both before and after exposure to a solution containing SAB. A very small difference in the oxidation potential was observed (+0.022 V), which strongly suggested that the carbohydrate was not being efficiently recognized and hydrolyzed by SAB to produce the anticipated phenol film on the electrode.

*3.4 Enzymatic remodelling of electrode surfaces modified with **2** and **3** using the retaining β-glucosidase from Agrobacterium sp. (Abg)*

Since electrodes modified with either **2** or **3** did not appear to be efficiently recognized by SAB, we decided to try using another enzyme: the retaining β-glucosidase from *Agrobacterium* sp. (Abg). Abg is a well-studied model enzyme that has been useful in studying the mechanism of retaining β-glycosidases and is known to be highly active towards a variety of artificial substrates. [22] More importantly, the specific activity of a solution containing Abg was higher than that for a similar concentration of SAB when using 4-nitrophenyl- β-D-glucopyranoside as a substrate (data not shown). When the electrode modified by a film derived from diazonium derivative **2** was used to measure the oxidation potential of AA before and after incubation of the electrode in a solution containing Abg for 30 minutes at 37 °C, a shift in the oxidation potential of +0.040V was observed (Fig. 5). While this change in potential was still very small, it represented an almost three-fold larger change compared to the similar experiment which employed SAB for which a shift in oxidation potential of only +0.014 V was observed.

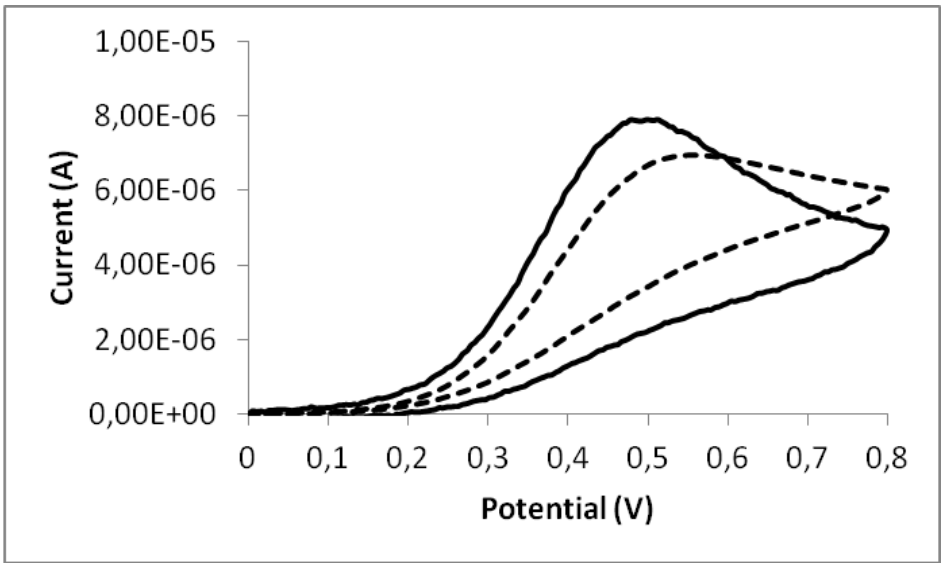


Figure 5. CV of electrodes modified by the grafting of **2** onto their surfaces used to measure the oxidation potential of a 200 μM AA solution before incubation with Abg (solid line) and after incubation (dashed line).

When an electrode modified by the deposition of a film of **3** was used to measure the oxidation potential of AA before and after incubation in a solution containing Abg, a shift in the oxidation potential of +0.341 V was observed (Fig. 6). This dramatic change in potential demonstrates that the enzyme had successfully hydrolyzed the sugar off the surface, and that the self-immolative linker had collapsed leaving a phenol film bonded to the surface of the electrode. The large shift in oxidation potential observed for an electrode modified with **3** (+0.341 V) compared to a similar electrode modified with **2** (+0.040 V) demonstrated that the surface was considerably more sensitive to Abg when the self-immolative linker was present. This drastically improved surface recognition by the enzyme must arise from the greater accessibility of the carbohydrate residue when the self-immolative linker places the carbohydrate further from the electrode surface. This in turn means a greater proportion of the carbohydrate residues have been hydrolyzed off the surface, and thus the surface has been modified to a much larger degree as measured by the greater change in oxidation potential for AA. The magnitude of the shift in oxidation potential was further shown to be dependent on the concentration of Abg. Incubation with a 0.00267 mg/mL solution of Abg led to a change in potential of the oxidation peak of +0.126 mV, while incubation with a 0.0267 mg/mL solution yielded a potential change of +0.341 V, clearly demonstrating that the change was a direct reflection of enzymatic activity.

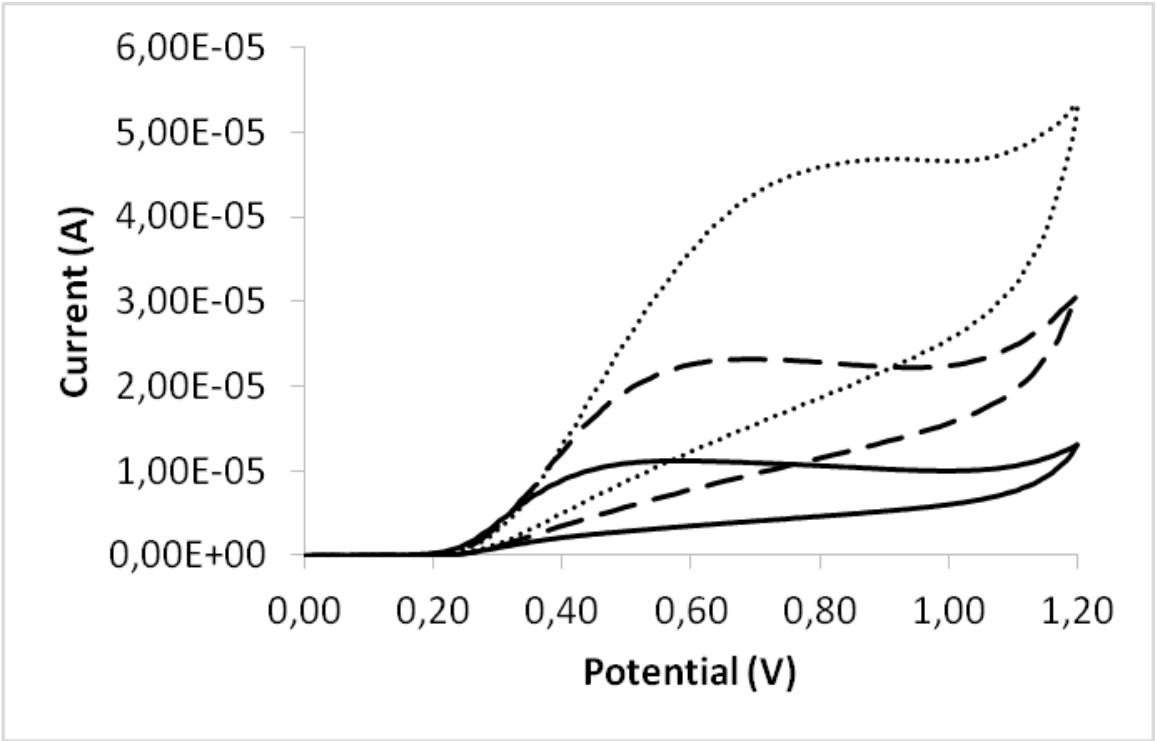


Figure 6. CV of electrodes modified by the grafting of **3** onto their surfaces used to measure the oxidation potential of a 200 mM AA solution, before incubation with Abg (solid line), after incubation with 0.00267 mg/mL Abg (dashed line), and after incubation with 0.0267 mg/mL Abg (dotted line).

*3.5 Acid-catalyzed cleavage of carbohydrate residues from the electrode surfaces modified with **3***

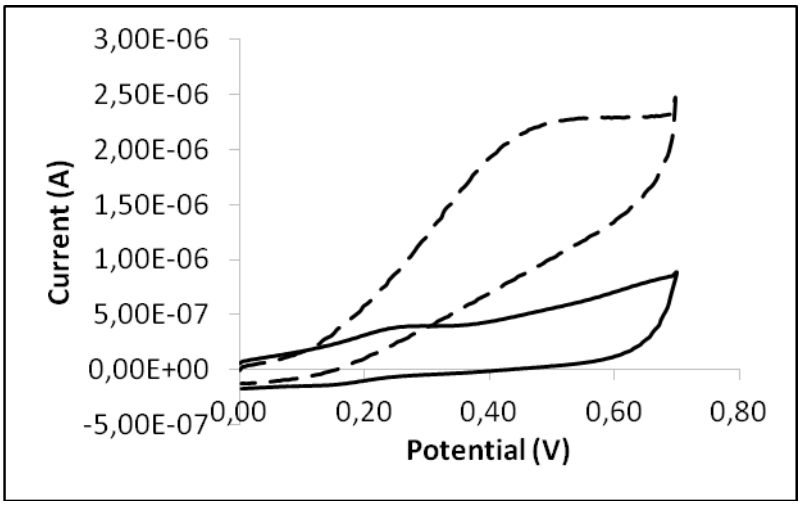


Figure 7. CV of electrodes modified by grafting of **3** onto their surfaces used to measure the oxidation potential of a 2 mM AA solution before incubation with aqueous acid (solid line), and after incubation (dashed line).

To further prove that the observed change in oxidation potential was arising from loss of the carbohydrate residues, an electrode modified with 3 on its surface was incubated with 6 M HCl at 80 °C for 15 minutes. These reaction conditions should hydrolyze the acetal bond at the anomeric center to expose the self-immolative linker, which subsequently decomposes to leave the free phenol attached to the electrode surface. As can be seen in Fig. 7, treatment of the coated electrode in acidic aqueous conditions led to a +0.263 V change in oxidation potential in the CV for a 2 mM AA test solution. Since treatment with both the enzyme or with aqueous acid leads to a similar shift in the oxidation potential of AA, this shows that both are causing a similar loss of the surface carbohydrates, followed by spontaneous decomposition of the self-immolative linker.

4. CONCLUSIONS

We report here the synthesis of two novel compounds and the electrochemical deposition of three diazonium derivatives (**1**-**3**) onto the surface of a carbon electrode, one of which (**3**) incorporates a self-immolative linker. A shift in the potential for the oxidation peak in the CV for AA and a change in the current were used as evidence for the formation of a film on the surface of the electrode when comparing the potential for the bare and modified electrode. Further evidence for the presence of a sugar-containing film on the surface of the electrode was provided by treatment of the modified electrode with two different β-glucosidases: the commercially available retaining β-glucosidase SAB, and the recombinant retaining β-glucosidase Abg. The surfaces modified by electrochemical reduction in the presence of **1**, **2**, or **3** which were subsequently exposed to SAB did not show substantial changes in the CV, indicating that the enzyme had not removed the sugar from the surface. Treatment

of the electrode surface modified by the deposition of **2** with a solution containing Abg led to a very small change in the oxidation potential peak in the CV. However, the electrode surface which had been modified by the electrochemical deposition of **3** showed a substantial change in the CV when exposed to a solution containing Abg, indicating that the sugar residue had been recognized by the enzyme and efficiently cleaved from the surface. The importance of the self-immolative linker for enzymatic recognition was demonstrated by the difference in susceptibility of the surface modified by either **2**, which lacks a self-immolative linker and showed little response to treatment with Abg, and the surface modified by **3**, which contains a self-immolative linker and was sensitive to treatment with Abg. This demonstrated that the linker was serving the intended role of distancing the sugar residue from the electrode surface and allowing efficient recognition in the active site of the enzyme. Furthermore, treatment of the modified electrode with aqueous acidic conditions caused a similar shift in the oxidation potential for the AA solution, demonstrating that both enzymatic and non-enzymatic acidic conditions were causing a similar change to the electrode surface. From the data presented here, it was not possible to determine the exact structure of the film formed, and future experiments will attempt to fully characterize the exact nature of the surface modification.

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