Affinity Interactions between Phenylboronic Acid-Carrying Self-Assembled Monolayers and Flavin Adenine Dinucleotide or Horseradish Peroxidase

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Abstract: A method is provided for the recognition of glycated molecules based on their binding affinities to boronate-carrying monolayers. The affinity interaction of flavin adenine dinucleotide (FAD) and horseradish peroxidase (HRP) with phenylboronic acid monolayers on gold was investigated by using voltammetric and microgravimetric methods. Conjugates of 3-aminophenylboronic acid and 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester) or 11-mercaptoundecanoic acid were prepared and self-assembled on gold surfaces to generate monolayers. FAD is bound to this modified surface and recognized by a pair of redox peaks with a formal potential of −0.433 V in a 0.1 M phosphate buffer solution, pH 6.5. Upon addition of a sugar to the buffer, the bound FAD could be replaced, indicating that the binding is reversible. Voltammetric, mass measurements, and photometric activity assays show that the HRP can also be bound to the interface. This binding is reversible, and HRP can be replaced by sorbitol or removed in acidic solution. The effects of pH, incubation time, and concentration of H_2O_2 were studied by comparing the catalytic reduction of H_2O_2 in the presence of the electron-donor thionine. The catalytic current of the HRP-loaded electrode was proportional to HRP concentrations in the incubation solution in the range between 5 $\mu g\,mL^{-1}$ and 0.1 $mg\,mL^{-1}$ with a linear slope of 3.34 $\mu A\,mL\,mg^{-1}$ and a correlation coefficient of 0.9945.

Keywords: affinity interactions · boronic acid · glycated molecules · monolayers · quartz crystal nanobalance

Introduction

Boronic acid can form reversible bonds with 1,2- or 1,3-diols to generate five- or six-membered cyclic complexes under mild and easily controllable reaction conditions.[1–4] A number of sugar sensors has been developed on the basis of this property of boronic acid. For example, by measuring fluorescence changes induced by competitive binding of fluorophores and sugars toward boronates, fluorometric sensors for saccharides have been developed.[5–8]A surface plasmon resonance (SPR) sensor for saccharides with a boronic acid membrane–modified thin gold film has been proposed.[9] Furthermore, saccharides and F^- can be detected visually by the redox reaction between ferrocenylboronic acid derivatives and dyes with the appropriate redox potentials.[10] Some sensors have been used for the electrochemical detection of sugar based on the electrochemical sensing of a boronic acid–substituted bipyridine Fe^{II} complex electropolymer,[11] the change of the pK_a value of poly(aniline boronic acid),[12] and the swelling of a polymer boronic acid gel membrane complex.[13] In addition, the immobilized boronic acids in gels can also be used for affinity chromatographic purification and detection of glycoproteins[14–18] and for orientation and reversible immobilization of glycoproteins.[19–21]

The selective reaction of saccharides with the monolayer of boronic acid derivatives at the air–water interface was studied in detail.[22–24] These studies made valuable contributions to the understanding of the compression of monolayers and the interaction between boronic acid derivatives and saccharides at an interface. So far, however, only a few reports have focused on boronic acid self-assembled monolayers (SAMs) for the electrochemical sensing of sugars or glycoproteins. Whitesides et al.[25] described the preparation and the properties of hydrophobic monolayers obtained by the adsorption of ω-mercaptoalkyl boronic acids onto gold substrates. Kanayama and Kitano[19] prepared conjugates by

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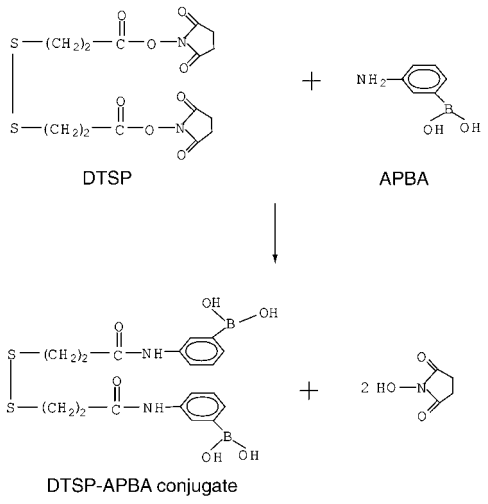
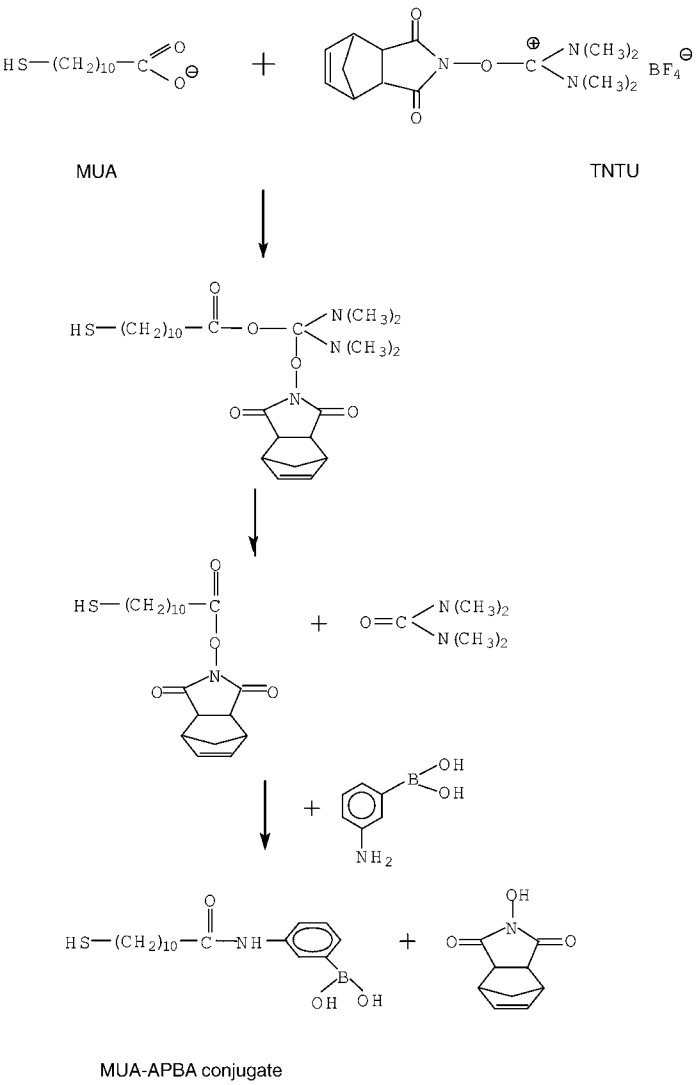
coupling of 3-aminophenylboronic acid with dithiodialiphatic acids. The resulting compounds formed a SAM on both silver and gold colloids and on gold electrodes, in which the boronic acid SAM could display specific sugar recognition. Epoxy-boronate mixed functional monolayers were reported to support the covalent immobilization of peroxidase.[26] In this work, the boronate group was used for the accumulation of the glycoprotein, while it was necessary to stabilize the binding by covalent coupling with the epoxy group. It was characterized by quartz crystal microbalance and atomic force microscopy, and the catalytic response of the immobilized peroxidase to the reduction of H_2O_2 in the presence of thionine was demonstrated. On the other hand, flavoenzymes and NAD(P)$^+$-dependent enzymes were electrically contacted to gold electrodes by reconstitution to FAD, which was bound by affinity interactions to phenylboronic acid monolayers.[27–29] Therefore the SAMs of boronic acid are promising for the preparation of enzyme electrodes for glycoproteins. Moreover, when a glycoprotein enzyme was immobilized onto the boronic acid SAMs through its carbohydrate moiety, which is generally not located in the active site,[30] the immobilizing procedure does not affect the enzyme activity.[31,32] Herein, a simple procedure is described for the reversible formation of cofactor and enzyme monolayers on gold based on the boronic acid–sugar interaction. The conjugates of 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester) (DTSP) or 11-mercaptoundecanoic acid



Scheme 1. Synthesis of DTSP–APBA conjugates.

(MUA) and 3-aminophenylboronic acid (APBA) were prepared (Scheme 1 and 2) and self-assembled on a gold surface and used for the binding of FAD and HRP. Cyclic voltammetry (CV) and quartz crystal nanobalance (QCN) measurements show specific binding of these molecules to the resulting APBA–interface. The HRP binding was studied by comparing the catalytic response of the enzyme-loaded electrode to the reduction of H_2O_2 in the presence of the electron donor thionine.



Scheme 2. Synthesis of MUA–APBA conjugates.

Results and Discussion

Characterization of the phenylboronic acid monolayer: The phenylboronic acid monolayer modified electrode was characterized by the reductive desorption peak associated with the thiol and the degree of blocking of electrode reactions of redox-active species. Figure 1 A shows the cyclic voltammograms of a DTSP–APBA- and a MUA–APBA-modified gold electrode in 0.5 M KOH at 100 mV s^{-1}. The DTSP–APBA-modified electrode displays a reduction peak at −1.065 V, whereas the MUA–APBA-modified electrode shows a reduction peak at −1.184 V. These peaks are attributed to the reductive desorption of the thiolated compounds bound to gold,[33,34] and indicate the presence of the conjugates on the gold surface. The differences in the peak potentials are due to the different lengths of the spacers, which are consistent with the chain length dependence found for alkanethiolate layers.[35] From the integration of the reductive desorption peak, the surface coverage, Γ ($\Gamma = Q/nFA$

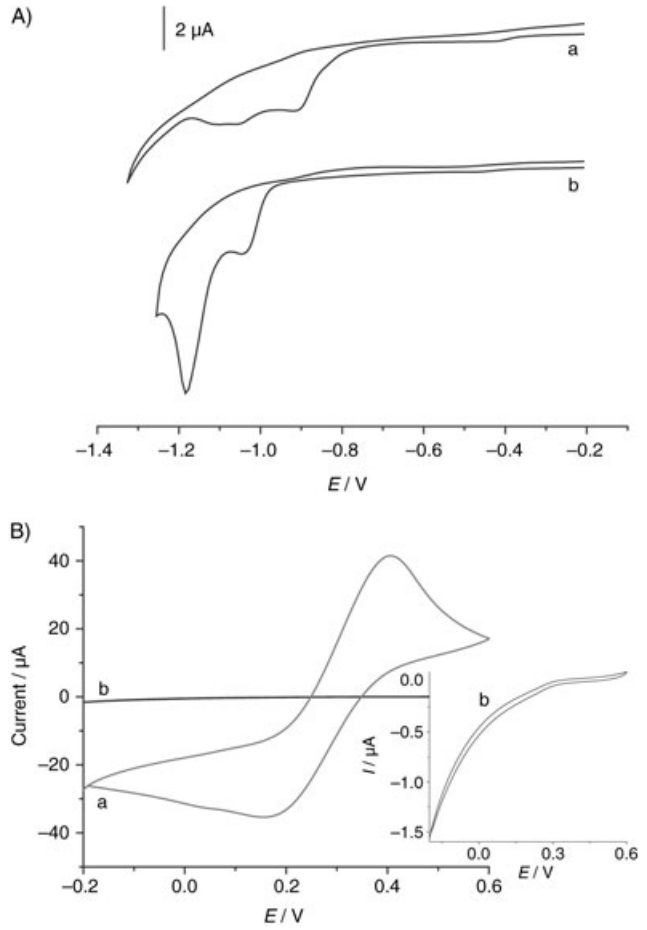


Figure 1. CV of DTSP–APBA (a) and MUA–APBA (b) modified gold electrodes in A) KOH (0.5 M) and B) in 5 mM potassium ferricyanide (in PBS 0.1 M, pH 7.0). The scan rate was 100 mV s^{-1}. The inset in B shows enlargement of the CV of the MUA–APBA-modified electrode in potassium ferricyanide (5 mM) solution.

and A = 0.04 cm^2), is calculated to be 5.7×10^{-10} mol cm^{-2} for DTSP–APBA and 1.6×10^{-9} mol cm^{-2} for MUA–APBA conjugates. The coverage with MUA–APBA is about three times higher than that with DTSP–APBA, indicating that MUA–APBA forms a much more densely packed layer on the gold electrode. The higher coverage with MUA–APBA relative to that with DTSP–APBA is most likely due to the fact that the alkyl chain of MUA is longer than that in DTSP and thus the van der Waals interactions are stronger.[25,36]

Evidence of the more dense packing of the MUA–APBA monolayer is given by the response of the electrode to an electrochemical probe. Figure 1B shows the cyclic voltammogram of DTSP–APBA- and MUA–APBA-modified electrodes to 5 mM potassium ferricyanide ($K_3Fe(CN)_6$) in 0.1 M phosphate buffer solution (PBS). The DTSP–APBA monolayer modified electrode displays a couple of redox peaks of $K_3Fe(CN)_6$, whereas no peaks were observed in the MUA–APBA modified electrode, which shows a very low background current (Figure 1B, curve a and b). Therefore, the formation of the MUA–APBA monolayer totally blocks the electron transfer between ferricyanide and the electrode, indicating again a dense packing of MUA–APBA on the gold electrode.

FAD binding to the APBA monolayer: The APBA monolayer was allowed to interact with FAD to yield the FAD–boronate complex on the electrode by using a binding protocol already used for FAD-coupling to an immobilized pyrroloquinolinquinone–boronate complex.[27] Cyclic voltammograms of the FAD-functionalized electrode were then recorded in PBS (0.1 M, pH 6.5). At this pH, boronate–saccharide bonds are not broken. A quasi-reversible redox-wave with a peak-to-peak separation of 49 mV (at 200 mV s^{-1}; as shown in Figure 2A) was observed, while no peaks appeared in the same potential window with both the bare gold wire electrode and the APBA-DTSP-Au electrode, indicating the presence of the immobilized electroactive FAD molecule in the monolayer. The peak current increased linearly on increasing the scan rate in the range between 10 and

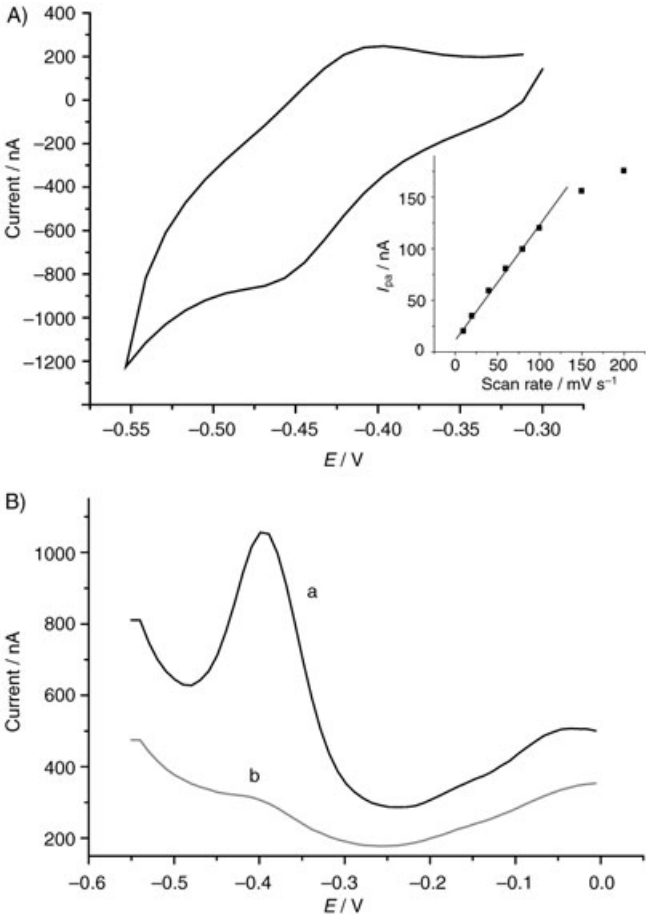


Figure 2. A) CV of FAD-APBA-DTSP-Au at 200 mV s^{-1}. Inset: the oxidation peak current depends on the scan rate. B) Square wave voltammograms of FAD-APBA-DTSP-Au from −0.6 to 0 V at a frequency of 25 Hz and an amplitude of 25 mV before (a) and after addition of fructose (10 mM) (b). Background solution was nitrogen-saturated PBS (0.1 M pH 6.5).

100 mV s^{-1} (see inset in Figure 2A), indicating the electrode reaction was a surface-controlled process. The formal potential of immobilized FAD is −0.433 V, which is comparable with that of the FAD monolayer ($E^0 = -0.49$ V versus SCE, pH 7.0) assembled on gold in a multistep process,[27,28] which was obtained at a slightly higher pH value. The charge produced by the immobilized FAD on the gold surface corresponds to a FAD surface coverage of 4.1×10^{-11} mol cm^{-2} according to the coulometric assay. The surface coverage calculated for a fully packed monolayer of FAD was 9.40×10^{-11} mol cm^{-2} taking the molecule of FAD as a sphere of 3 Å diameter.[37] Thus, we can assume a monolayer coverage on the APBA interface.

Upon addition of fructose to the buffer solution, the peak of FAD disappeared (Figure 2B). Also, the cyclic voltammogram gives no peaks in a fresh buffer (not shown), indicating that no FAD molecule is left on the electrode surface. Furthermore, no peaks were obtained with the APBA–DTSP interface incubated in 1 mM FAD solution in PBS (0.1 M, pH 7.0) containing an additional 10 mM fructose (not shown). However, on incubation of the APBA monolayer modified electrode in 1 mM FAD solution in PBS (0.1 M, pH 7.0) containing 10 mM glucose, electrochemistry of FAD was observed with the resulting electrode, but the peak current was much lower than that without glucose (not shown). The decrease of the peak current indicated a low amount of FAD bound to the APBA interface due to the competition between glucose and FAD. These results show that fructose forms a much stronger interaction with the APBA interface than FAD does. Therefore immobilized FAD on the APBA–DTSP interface can be replaced by fructose, and FAD can not bind in the presence of fructose. In the presence of glucose, FAD can successfully compete and bind to the APBA interface. This is in good agreement with the stability constants between the saccharides and phenylboronic acid reported by Lorand and Edwards[38] (110 M^{-1} for glucose and 4370 M^{-1} for fructose) and the results of Soh et al.[9]

Reaction of the APBA interface with HRP: The APBA interface can interact with peroxidase, which has a degree of glycosylation of about 16.8–21%.[39] The cyclic voltammogram of the HRP-modified Au electrode in PBS (0.1 M, pH 8.0) shows a low background current with a slight increase below −0.13 V due to the reduction of the dissolved oxygen in the buffer (Figure 3, curve a). Upon addition of hydrogen peroxide and thionine to the buffer solution, a reduction current with the shape of a catalytic wave is observed (Figure 3, curve b). The catalytic current for a HRP-APBA-DTSP-Au electrode in the presence of hydrogen peroxide and thionine is 18 times larger than that of an APBA-DTSP-Au electrode without HRP (Figure 3, curve c). These results strongly suggest the presence of peroxidase on the APBA interface. The catalytic reduction current of the HRP-modified electrode may be described by the scheme outlined in Equations (1)–(4).[40]

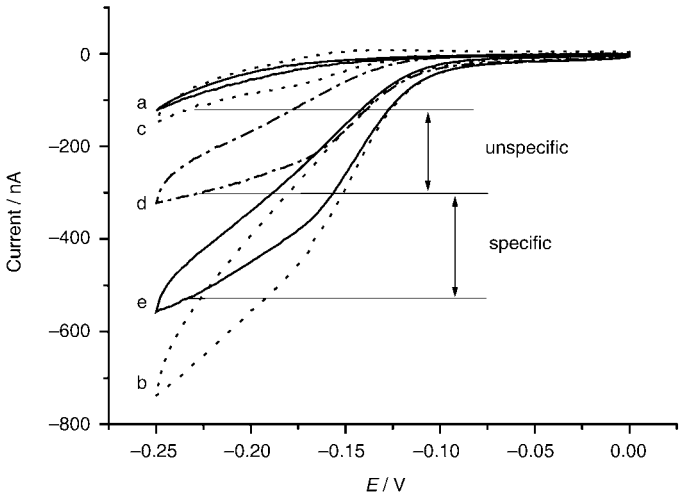


Figure 3. CV of DTSP-APBA-modified gold electrode (a) before and (b) after incubation in HRP (0.5 mg mL^{-1} in 0.1 M pH 8.0 PBS) solution for 4 h in PBS (0.1 M, pH 8.0) containing thionine (10 μM) and H_2O_2 (2 mM) at 5 mV s^{-1}. c) CV of (b) in the same buffer without thionine and H_2O_2. d) Electrocatalytic response of electrodes as in (b) but incubated for 30 min in PBS (pH 5.0, 0.1 M) and e) further incubated in HRP (0.2 mg mL^{-1}) in PBS (pH 8.0, 0.1 M) for 2 h. Specific and unspecific binding is indicated for 0.2 mg mL^{-1} HRP.

$$\mathrm{HRP(Fe^{III})} + \mathrm{H_2O_2} \rightarrow \mathrm{compound\,I} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{compound\,I} + \mathrm{thionine(Red)} \rightarrow \mathrm{compound\,II} + \mathrm{thionine(Ox)^*} \quad (2)$$

$$\mathrm{compound\,II} + \mathrm{thionine(Ox)^*} \rightarrow \mathrm{HRP(Fe^{III})} + \mathrm{thionine(Ox)} \quad (3)$$

$$\mathrm{thionine(Ox)} + 2\mathrm{e^-} + 2\mathrm{H^+} \rightarrow \mathrm{thionine(Red)} \quad (4)$$

The binding of HRP to phenylboronic acid was reversible. (The binding of HRP to the APBA interface included specific and non-specific, and the specific binding was reversible.) When using conditions known to interfere with this binding, such as acidic pH and addition of sugars, protein was partly released. As a result the catalytic current of the HRP-APBA-DTSP modified electrode decreased about 58%, 57%, and 69% of their initial value after 30 min contact with a buffer of pH 5.0 (Figure 3, curve d), with a buffer of pH 8.0 containing 10 mM fructose and a buffer of pH 8.0 containing 10 mM sorbitol (not shown), respectively. The decrease of the catalytic current was due to the release of the specifically bound HRP. The catalytic activity of these electrodes recovered about 70% (Figure 3, curve e), 14%, and 86% of their initial value, respectively, after these electrodes were re-incubated in 0.2 mg mL^{-1} HRP solution in PBS (pH 8.0) for 2 h. Therefore, the binding of HRP to the APBA interface included specific and nonspecific, the specific binding of HRP can be regenerated by sorbitol and acidic buffer.

This strongly suggests that the APBA interface specifically binds the glycosylation sites of the HRP and can be released in acidic solution or be replaced by sorbitol.

When we used MUA–APBA instead of DTSP–APBA, the CV of the resulting electrode also showed a reduction current with the shape of a catalytic wave in the presence of thionine and H_2O_2 (not shown). The catalytic current of the HRP-APBA-MUA-Au electrode is only about 12 times larger than that without HRP. However, the catalytic current of this electrode is smaller than that of the HRP-APBA-DTSP-Au electrode. A possible reason may be the long spacer of MUA–APBA increasing the distance between the protein and the electrode. After immersing the HRP-APBA-MUA-Au electrode in PBS (pH 5.0), and PBS (pH 8.0) with 10 mM sorbitol and 10 mM glucose, respectively, the catalytic current of the reduction of H_2O_2 also decreased considerably, indicating the release of HRP in these solutions. When these electrodes were then incubated in PBS (0.1 M, pH 8.0) containing 0.2 mg mL^{-1} HRP for 2 h, all these electrodes recovered the catalytic activity to the reduction of H_2O_2.

The specific binding of peroxidase to the APBA interface is supported by QCN measurements. Figure 4 shows frequency changes of piezoelectric crystals functionalized with MUA–APBA as a function of the time after injection of 50 μg mL^{-1} HRP into the reaction chamber. The resonance frequency decreases over 10 min (Figure 4, curve a). The amount of HRP on the surface was 45.70 ng (0.89 ng Hz^{-1} for the 10.0 MHz crystals used in this study), which is the total mass change of bound HRP. After injection of sorbitol solution (0.5 M, pH 8.0) into the reaction chamber, the frequency increases, but does not return to its original value, indicating that sorbitol removes only a portion of HRP (not shown). But after this regeneration step only 14.58 ng HRP could be bound (Figure 4 curve b). Repetition of this experiment, results in a similar response (Figure 4 curve c). Assuming that sorbitol removes only the portion of HRP bound through the interaction between the boronic acid and the diol, this reproducible sensor reponse represents the mass change of this specifically bound enzyme.

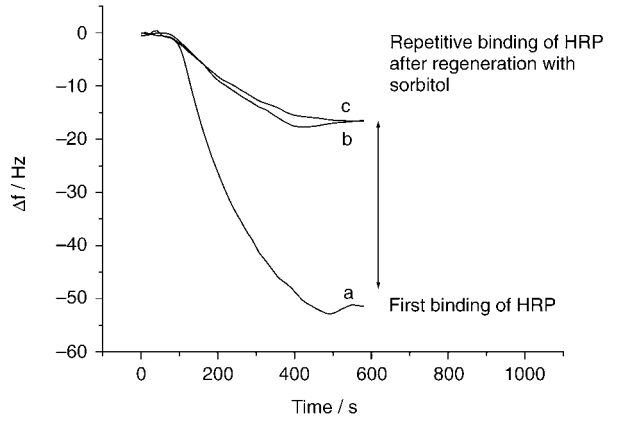


Figure 4. Repetitive measurement of binding of HRP (50 μg mL^{-1}) to a MUA–APBA-modified piezoelectric quartz crystal. Curve a represents the first binding of HRP (total of specific and non-specific binding). Curve b is the response to HRP after regeneration of (a) with sorbitol (0.5 M). Curve c is the repetition of HRP-addition after regeneration with sorbitol (measurement of curve b).

Using 0.1 M PBS (pH 5.0) or 10 mM glucose solution in PBS (0.1 M, pH 8.0) instead of sorbitol, similar responses were obtained (not shown). After injection of fructose (10 mM in 0.1 M pH 7.0 PBS), an increase in frequency with time was also found. However, no rebinding of HRP could be observed by injection of HRP into the reaction chamber (not shown). This is similar to the results obtained with the APBA-modified gold electrodes (see above), because fructose is bound to the APBA interface much stronger than sorbitol, glucose, and HRP.

In addition, bound and released HRP was determined with a photometric activity assay using tetramethyl benzidine as substrate. This measurement revealed that 0.14 nU were released by sorbitol incubation and 0.29 nU were left on the electrode, that is, only about a third of the initial HRP activity could be removed by sorbitol from the electrode.

All these results show that the binding of HRP to the APBA interface includes specific and nonspecific binding. The specific binding of HRP is reversible and can be removed by sorbitol and acidic buffer. As will be shown below this is the basis of a method for the determination of the glycated protein and its substrates.

HRP determination: The catalytic activity of the HRP-modified electrode is influenced by the incubation time, the pH value of the incubation solution, and the concentration of H_2O_2. Figure 5 A shows the effect of incubation time on the catalytic current. In this case, the APBA monolayer was incubated in HRP solution (0.2 mg mL^{-1} in 0.1 M PBS (pH 8.0)) for different times. The catalytic current, measured in PBS (0.1 M, pH 8.0) containing thionine (10 μM) and H_2O_2 (2 mM), was corrected for the reduction current of the enzyme-free APBA monolayer modified electrode. With an increasing incubation time, the catalytic current increased and approached a maximum value.

The pH of the incubation solution greatly affects the enzyme loading and activity on the APBA interface. Accordingly, the catalytic current of the electrode depends on the pH value of the incubation solution. Figure 5 B shows the effect of pH on the catalytic current. In this case, the APBA monolayer was incubated in 0.2 mg mL^{-1} HRP solution in 0.1 M PBS at different pH values for 4 h. However, the catalytic current was then always measured in PBS (0.1 M, pH 8.0) containing peroxide (2 mM) and thionine (10 μM) and was corrected for the reduction current of the enzyme-free APBA monolayer modified electrode. The catalytic current increased with increasing pH during incubation from 5.0 to 9.1 because the formation of the complex between the boronic acid and the *cis*-diol moiety was much faster under alkaline conditions.[41] Considering the lower

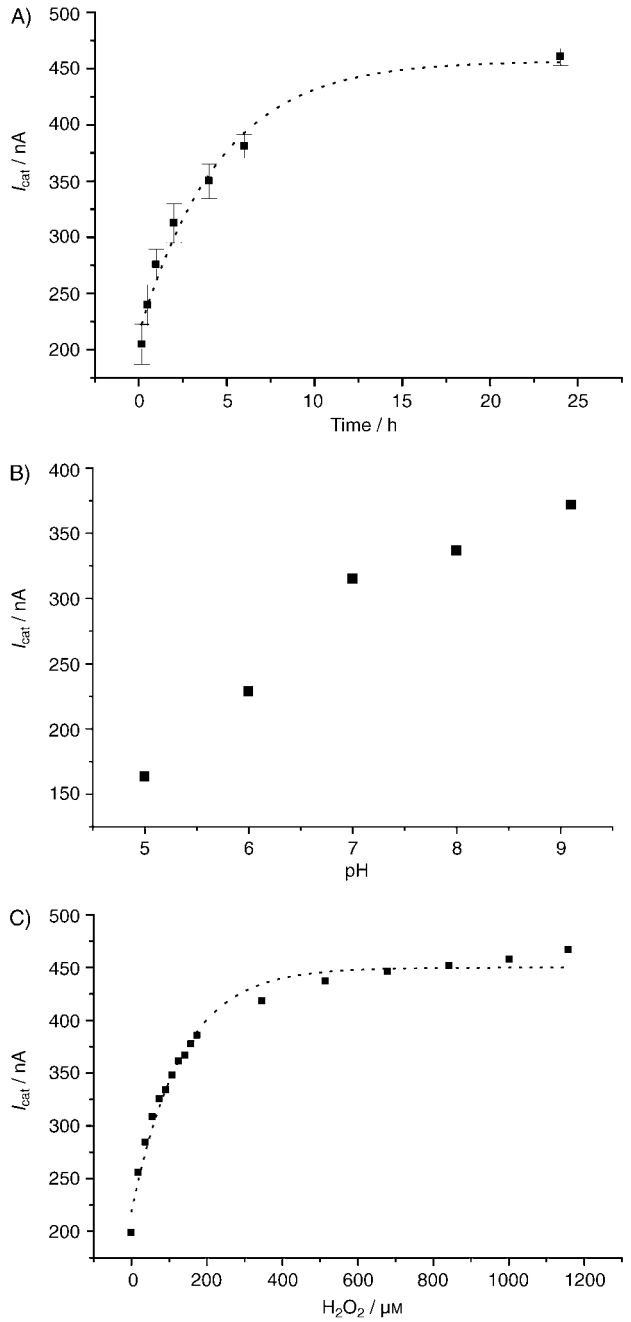


Figure 5. A) Effect of incubation time on the enzyme loading of the APBA-DTSP monolayer using HRP (0.2 mg mL^{-1} in PBS (pH 8.0; 0.1 M)). B) Effect of the pH of the incubation solution on the enzyme loading of the APBA–DTSP monolayer using a HRP (0.2 mg mL^{-1}) in 0.1 M PBS with different pH value for incubation for 4 h. C) Effect of the concentration of H_2O_2 in the measuring solution on the electrocatalytic current of an APBA–DTSP electrode after incubation in HRP (0.2 mg mL^{-1} in PBS (pH 8.0, 0.1 M)) for 4 h. The electrocatalytic current was measured at −0.2 V of the CV of the enzyme loaded electrode in PBS (pH 8.0, 0.1 M) containing thionine (10 μM) and H_2O_2 (2 mM).

stability of HRP at high pH, the optimal pH value of the incubation solution was 8.0.

The amperometric response of the HRP-modified electrode to hydrogen peroxide is shown in Figure 5C. When the H_2O_2 concentration was increased, the response showed a Michaelis–Menten type curve with a K_M^{app} value of 165 μM. The amperometric response increased linearly with increasing H_2O_2 concentration up to 0.18 mM. When the H_2O_2 concentration was more than 0.18 mM, the amperometric response approached a constant value. The optimal peroxide concentration for measuring the catalytic current of the HRP-modified electrode was determined to be 2 mM.

After the stock solution of HRP (3 mg mL^{-1} solution in 0.1 M pH 8.0 PBS) was diluted with buffer solution in different volume ratios, the incubation was performed in the mixed solution (50 μL) at room temperature for 4 h. The catalytic current at −0.2 V increased on increasing the amount of HRP (Figure 6). It can be used for the detection of HRP.

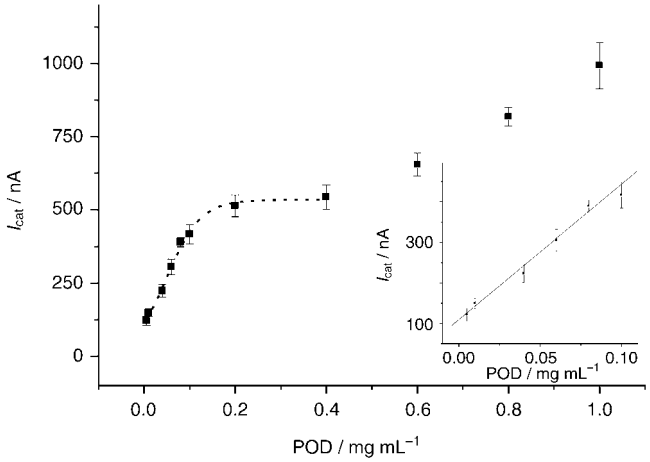


Figure 6. Plot of the electrocatalytic current versus HRP concentration of the incubation solution. Inset: linear calibration curve.

The catalytic current was proportional to the HRP concentration in the range from 5 μg mL^{-1} to 0.1 mg mL^{-1} with a linear slope of 3.34 μA mL mg^{-1} and a correlation coefficient of 0.9945 (inset in Figure 6). Above 0.1 mg mL^{-1} it approaches a total coverage giving a steady value of about 550 nA. The increase of current when the incubation was performed with very high enzyme concentrations may be due to unspecific multilayer formation.

Conclusion

Two kinds of boronic acid derivatives have been self-assembled on gold surfaces for reversible binding of glycated molecules. The resulting interfaces display specific recognition to the *cis*-diols of the target molecules. FAD bound to the surface shows a fast direct redox transformation, whereas HRP displays an electrocatalytic reduction of H_2O_2 in the presence of the electron donor thionine. Both FAD and HRP binding with boronic interface are reversible. This work offers a way to build electrochemical sensors for glycoproteins based on their immobilization on the APBA interface.

Experimental Section

Chemicals: 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester) (DTSP), 11-mercaptoundecanoic acid (MUA), flavin adenine dinudeotide disodium salt dihydrate (FAD), peroxidase (HRP, EC 1.11.1.7, RZ 3.2, 290 purpurogallin units per mg solid, from horseradish), and 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) were obtained from Sigma (Deisenhofen, Germany) and used as received. *N*-Methyl-morpholine (NMM) and 3-aminophenylboronic acid (APBA) were purchased from Fluka (Germany). 2-(5-Norbornene-2,3-dicarboximide)-1,1,3,3-tetramethyluronium tetrafluorborate (TNTU) was from Calbiochem (San Diego, USA), dimethylformamide (DMF) was from Mallinckrodt Baker (Griesheim, Germany). Fructose was provided by Merck (Germany), sorbitol was purchased from Aldrich (Germany). All other chemicals were of reagent grade and were used as supplied. Water purified by a Milli-Q system was used to prepare all solutions. Measurements were performed in phosphate buffer solution (PBS, 0.1 M) with different pH values made of K_2HPO_4 and KH_2PO_4.

Preparation of phenylboronic acid self-assembled monolayer: DTSP (10.1 mg, 0.025 mmol) and APBA (9.3 mg, 0.05 mmol) were dissolved in dimethyl sulfoxide (DMSO, 10 mL) and incubated at room temperature for 90 min to obtain the DTSP–APBA conjugates.

MUA (1.77 mg, 8.0 μmol) and TNTU (2.9 mg, 7.9 μmol) were dissolved in a mixture of NMM (1 μL, 9.6 μmol) and DMF (100 μL) and incubated for 15 min at room temperature. APBA was dissolved in carbonate buffer (0.1 M, pH 9.0) with the final concentration of 10 mM. MUA–TNTU (22 μL) solution and of APBA (200 μL) solution were mixed together and incubated for 90 min at room temperature. The MUA–APBA conjugate thus obtained was stored in a freezer.

Gold wire electrodes (99.99 %, Goodfellow) were pretreated by boiling in KOH (2 M) solution for 2 h. After washing, the gold wires were immersed in "piranha" solution (3:1 concentrated H_2SO_4/30 % H_2O_2) overnight. Prior to modification, the gold wire electrodes were treated with concentrated HNO_3 for 15 min, and rinsed with water. A cyclic voltammogram recorded in H_2SO_4 (1 M) in the potential window from −0.2 to 1.6 V at 100 mV s^{-1} was used to determine the purity and roughness of the electrode surface just before modification. After rinsing thoroughly with water, a clean Au wire electrode (0.5 mm diameter gold wire, geometrical area about 0.04 cm^2, roughness ca. 1.5) was immersed either for 3 h at room temperature in DTSP–APBA solution or for more than 15 h in MUA–APBA solution at 4 °C. The APBA-functionalized electrode was rinsed with water three times. It was then incubated for different times in PBS (50 μL, 0.1 M pH 8.0) containing different amounts of HRP. After rinsing thoroughly with water, the peroxidase-modified electrode (either HRP-APBA-DTSP-Au or HRP-APBA-MUA-Au) was obtained and stored in the same buffer. The electrodes with SAMs of DTSP–APBA were allowed to react with FAD solution (1 mM in 0.1 M pH 7.0 PBS) for 3 h to generate FAD-modified Au electrodes. These modified electrodes were used for two kinds of experiments: 1) the cyclic voltammogram of FAD-functionalized Au electrodes shows the presence of FAD on the APBA interface and the amount of bound FAD. 2) The FAD-functionalized Au electrodes were measured by cyclic and square wave voltammetry in the presence of different sugars to determine whether the binding of FAD was specific.

Electrochemical measurements: Cyclic and square wave voltammetry was performed with a μAutolab (Metrohm/Eco Chemie, Germany/The Netherlands). A conventional homemade three-electrode electrochemical cell, consisting of a modified Au-wire electrode, a Pt-wire auxiliary electrode, and a Ag/AgCl (1 M KCl) reference electrode (Biometra, Germany), was used for all electrochemical measurements. For the FAD-modified Au electrode, prior to measurement, working buffer (1 mL) was carefully bubbled with nitrogen for at least 20 min to remove the dissolved oxygen. All other measurements were carried out in the presence of oxygen.

Photometric activity assay: Photometric measurements were performed with a UV-2501 PC spectrophotometer (Shimadzu; Kyoto Japan).[42] For the measurement of the HRP activity released by sorbitol, the rinsed peroxidase-modified electrode (HRP-APBA-MUA-Au) was incubated in sorbitol solution (340 μL, 0.2 M in 0.1 M PBS (pH 8.0)) for 30 min and then removed. To the remaining solution PBS (600 μL, 0.1 M, pH 6.0), H_2O_2 (30 μL, 10 mM), and TMB (30 μL, 10 mM) in 96 % ethanol were added, rapidly mixed, and the absorbance at 652 nm was measured for 20 min. For the determination of the bound activity, the electrode was dipped into PBS (1 mL, 0.1 M, pH 6.0) containing H_2O_2 (300 μM) and TMB (300 μM) for 20 min, and the absorbance at 652 nm was measured. HRP-free solutions were used as reference.

Quartz crystal nanobalance measurements: The QCN measurements were performed with a MultiLab 3900 piezoelectric instrument (Kitlička, Brno, Czech Republic) controlled by a computer. A software LabTools Version 1.1 (Skládal, Brno, Czech Republic) was used for measurements (theoretical resolution 0.001 Hz), data storage (1 s per point), and evaluation. The optically polished gold sensors (10 MHz, AT-cut, 14 mm in diameter, electrode 5 mm diameter) were purchased from International Crystal Manufacturing Company (Oklahoma, USA). After washing in acetone for 2 h, the sensors were incubated in the conjugate of MUA–APBA in a wet chamber. After 48 h the sensors were washed with distilled water and gently dried with nitrogen and used for the following measurements. The modified sensor was fixed in a thin-layer flow-through cell between two soft rubber O-rings (homemade). Only one side of the sensor was in contact with the flowing solution. All the measurements were performed at room temperature. The working solution, K-phosphate buffer (50 mM pH 7.5), was continuously flowing through the cell. The outflow-tube was connected to a peristaltic pump Minipuls 3 (Gilson, France). A flow rate of 20 μL min^{-1} was used for the experiments. Abimed tubes (∅ 0.2 mm) were used for connections.

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