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Evaluation of non-specific binding suppression schemes for neutravidin and alkaline phosphatase at the surface of reticulated vitreous carbon electrodes

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ABSTRACT

Non-specific binding (NSB) of high-molecular-weight proteins onto electrode surfaces can complicate the application of electroanalytical techniques to clinical and environmental research, particularly in biosensor applications. We present herein various strategies to modify the surface of reticulated vitreous carbon (RVC) electrodes to suppress non-specific binding of biomolecules onto its surface. Non-specific binding and specific binding (SB) of two enzyme conjugates, neutravidin-alkaline phosphatase (NA-ALP) and biotinylated alkaline phosphatase (B-ALP), and also neutravidin itself, were studied using hydroquinone diphosphate (HQDP) as an enzyme substrate for ALP inside the pores of RVC electrodes that had been subjected to various modification schemes. The extent of NSB and SB of these biomolecules inside RVC pores was assessed by measuring the initial rate of generation of an electroactive product, hydroquinone (HQ), of the enzyme-catalyzed reaction, using linear scan voltammetry (LSV) for HQ detection. Electrodes functionalized with phenylacetic acid and poly(ethylene glycol) (PEG) showed low NSB and high SB (when biotin capture ligands were included in the modification scheme) in comparison with unmodified electrodes and RVC electrodes modified in other ways. A simple sandwich bioassay for neutravidin was performed on the RVC electrode with the lowest NSB. A concentration detection limit of $52 \pm 2\,\text{ng mL}^{-1}$ and an absolute detection limit of 5.2 ± 0.2 ng were achieved for neutravidin when this assay was performed using a 100 μL sample size.

1. Introduction

Non-specific binding (NSB) of proteins onto sensor surfaces is a well-known problem which can lead to false positive or negative results. Many useful biosensor applications are affected by NSB signals. For example analyte measurements in whole blood, blood plasma, or serum are often affected by NSB of serum proteins on the sensor surface [1,2], and NSB of immunoglobulins onto sensor surfaces greatly influences the specific antibody–antigen binding kinetics [3–5]. In nucleic acid bioassays suppression of NSB of DNA onto sensor surfaces is essential for achieving sequence-specific DNA detection [6–8]. Minimizing NSB of molecules to maintain a measurable and minimal signal-to-noise ratio in a sensor is therefore one of the most important goals in sensor technology.

Protein adsorption onto solid surfaces is influenced by the physical and chemical characteristics of the surface as well as the sample under test. Many chemical and biological molecules tend to adsorb on surfaces through hydrophobic and/or charge interactions [9–11]. The rate and extent of protein adsorption onto a sensor surface can be altered by changing the surface chemistry of the sensor, modifying protein concentration and also varying ionic strength, pH and temperature of buffer solutions under test. One common way of blocking protein adsorption is through the use of commercially available blocking buffers such as Superblock RTM, sea block buffer, blocker casein, fish skin gelatin, and bovine serum albumin (BSA). These blocking buffers create surface functional groups which minimize the unwanted adsorption of proteins. For example, a commonly used method for reducing NSB in tests like ELISA is through the use of milk protein or BSA [12–15]. A concentration of 2–5% of BSA protein in solutions to be analyzed is often good enough to block NSB of many analytically important target molecules. Another common approach for reducing NSB is to chemically functionalize a sensor surface, for example by grafting polymers onto the sensor surface [16–18]. Sensor surfaces modified with polyethylene glycol films and with hydrogel networks such as poly(methyl methacrylate) (PMMA), poly(hydroxyethyl acrylate), carboxymethylated-dextran, cross-linked cellulose and polyethyleneimine show an ability to block protein adsorption [19–23]. Surface modification with highly negatively charged molecules such as poly(acrylic acid) and carboxylate dendrimers has also been used to suppress NSB of negatively charged proteins and DNA [24,25].

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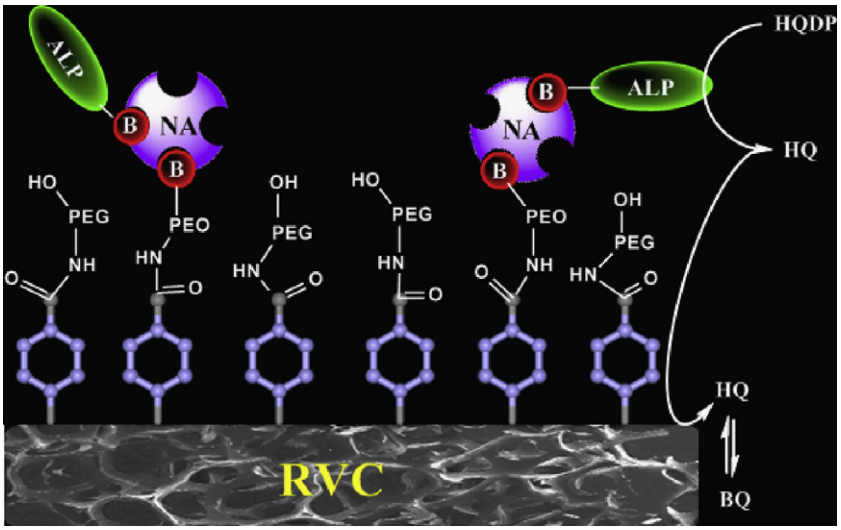


Scheme 1. Neutravidin bioassay on RVC electrode modified with phenylacetic acid and PEG.

In this work we present and discuss various strategies for modifying reticulated vitreous carbon (RVC) electrode surfaces to suppress non-specific binding while also promoting specific binding of proteins, particularly neutravidin. One advantage of using RVC in bioassays is that RVC provides a high surface area per volume of analyte solution, which facilitates better analyte capture via surface-bound bioaffinity agents. Another advantage is that RVC provides a low resistance to fluid flow which facilitates surface washing steps which are nearly always required in bioassays. Use of RVC also prevents diffusive losses of redox-active analytes or redox-active molecules generated via labeling [26–30]. This latter attribute can allow for accumulation of redox-active species in a confined space, thereby improving detection limits for analyses that involve detection of redox-active molecules. But, like glassy carbon, the surface of RVC is hydrophobic which tends to promote protein adsorption. Therefore to accomplish bioassays with RVC, it is necessary to modify the surface of RVC in such a way that it will reduce or prevent non-specific binding but at the same time facilitate specific binding of analyte molecules onto the surface.

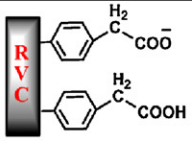
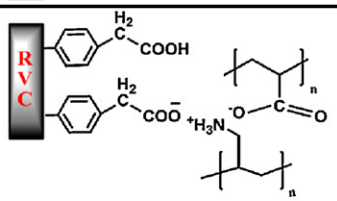
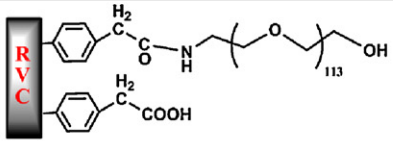
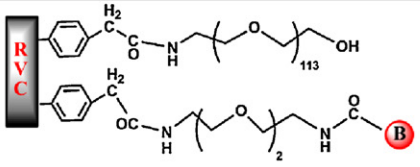
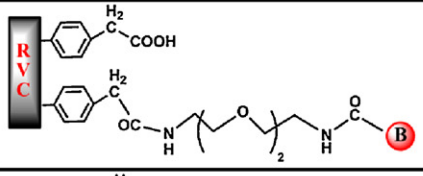
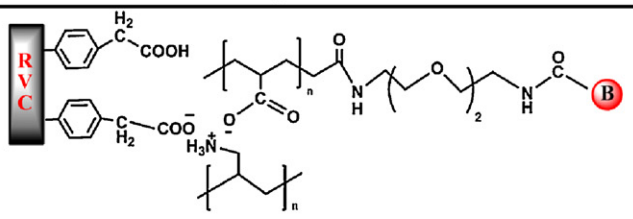
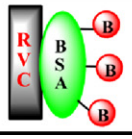
Nine different RVC electrode modification schemes were tested in this work to assess their relative properties for suppressing NSB. Some of the modification schemes included surface grafting of biotin groups to also allow for specific binding of avidin-containing molecules. These electrodes were then subjected to adsorption of neutravidin (NA), neutravidin-conjugated alkaline phosphatase (NA-ALP) and biotin-conjugated alkaline phosphatase (B-ALP), to test the extent of non-specific and specific binding. For cases involving ALP, the amount of ALP bound onto the RVC surface was assessed by measuring the initial rate of conversion of hydroquinone diphosphate (HQDP, an ALP enzyme substrate) into hydroquinone (HQ) by surface-bound enzyme. The initial rate was determined from analysis of multiple linear scan voltammograms obtained following exposure of the electrode to a solution of HQDP. Since the initial rate of HQ generated is directly proportional to the amount of enzyme present, it is possible to compare non-specific and specific binding of ALP-containing molecules onto RVC surfaces by measuring the initial rate of HQ production. The modified RVC electrode with lowest non-specific binding was then used as a platform for a neutravidin sandwich bioassay, using biotinylated ALP as an enzyme label to detect captured neutravidin. A schematic representation of this neutravidin sandwich bioassay is illustrated in Scheme 1.

2. Experimental

2.1. Materials, chemicals and instruments

Reticulated vitreous carbon (RVC, 100 ppi) foam used for making electrodes was purchased from ERG Materials and Aerospace Corp. Oakland, CA. All chemicals were used as received from the manufacturer, unless otherwise mentioned. Bovine serum albumin (BSA), biotinylated bovine serum albumin (B-BSA), neutravidin (NA), biotin-conjugated alkaline phosphatase (B-ALP), neutravidin-conjugated alkaline phosphatase (NA-ALP), biotinyl-3,6-dioxaoctanediamine (biotin-PEO-amine, M.W. 374.50), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Pierce Biotechnology. Hydroquinone (HQ), potassium ferricyanide and ruthenium hexamine trichloride were obtained from VWR and poly(acrylic acid sodium salt) (PAA, M.W. ~50,000 Da) and poly(allylamine hydrochloride) (PAH, M.W. ~70,000 Da) were obtained from Aldrich. Amine-terminated polyethylene glycol (PEG-amine, M.W. ~5000 Da) was obtained from Nektar Therapeutics (previously Shearwater Corp). Water was purified using a Millipore MilliQ Ultrapure Water Purification System. Reaction buffer (pH 8) consisted of 200 mM Tris, 150 mM NaCl and 10 mM $MgCl_2$. MES buffer (pH 6) was prepared by dissolving 50 mM of 2-(*N*-morpholino)ethanesulfonic acid and 500 mM NaCl. All electrochemical experiments were performed using a CH Instruments Inc. model CHI 660A electrochemical workstation with aqueous Ag/AgCl as a reference electrode and Pt wire mesh as a counter electrode unless otherwise mentioned. 4-amino phenylacetic acid diazonium tetrafluoroborate salt was synthesized according to a published procedure [31]. Hydroquinone diphosphate (HQDP) was also synthesized by modifying two published procedure [32,33].

The pipette-type RVC electrodes were prepared from RVC foam using a syringe needle, shrink tube, gold wire and a micropipette tip. A cylinder (2 mm diameter × 5 mm height) of 100 ppi RVC foam was cut using a 12 gauge syringe needle. Electric contact was made by wrapping a gold wire (0.12 mm diameter) around the RVC cylinder. The RVC cylinder was then connected to a micropipette tip using polyolefinic heat-shrink tubing. The internal surface area of the RVC electrodes used in this work was approximately $1.3 \pm 0.2\ cm^2\ mg^{-1}$ of RVC foam and the internal free volume of the electrodes (i.e. volume of solution that could be held inside the electrode) was $\sim 10 \pm 1\ \mu L$.

I	Bare RVC	
II	Adsorption of BSA on RVC	
III	Modification with phenyl acetic acid	
IV	Layer by layer adsorption of polyelectrolytes on type III	
V	Immobilization of PEG-amine on type III	
VI	Immobilization of B-PEO-amine on type VI	
VII	Immobilization of B-PEO-amine on type III	
VIII	Immobilization of B-PEO-amine on type IV	
IX	Adsorption of B-BSA on RVC	

Scheme 2. Modification of RVC electrodes with different molecules (type I to type IX).

2.2. Fabrication of RVC electrodes for non-specific and specific binding studies

Nine RVC electrode types were fabricated to study non-specific and specific binding of various enzyme-containing molecules. The RVC electrodes type I to type V were modified so as to prevent NSB, and electrodes type VI to type IX were modified so as to prevent NSB while also allowing for SB of targets via biotin–avidin chemistry. The structure of all modified electrodes is illustrated in Scheme 2. The fabrication and characterization of these electrodes are described below.

Type I: The type I electrode is a control that is not functionalized in any directed way. The electrode was pre-soaked in tris buffer for 5 min prior to exposure to B-ALP or NA-ALP enzyme solutions as described in Section 2.4.

Type II: The type II electrode was modified with BSA by simple adsorption. The electrode was incubated in 1 mg mL^{-1} of BSA in tris buffer for 30 min followed by rinsing with tris buffer.

Type III: The type III electrode was prepared by functionalizing the RVC surface to produce exposed phenylacetic acid groups. The RVC electrode was first rinsed with anhydrous acetonitrile and dried under nitrogen. The electrode was then modified by electrochemically reducing 5 mM of 4-amino phenylacetic acid tetrafluoroborate diazonium salt in anhydrous acetonitrile containing 0.1 M $N(Bu)_4BF_4$. The diazonium salt solution was purged with nitrogen for 15 min to remove dissolved oxygen prior to electrode modification. The reduction was accomplished by applying a 5-segment cyclic potential scan starting at 0.2 V followed by three forward scans up to −1.2 V and two backward scans to 0.6 V, at a scan rate of 50 mV s^{-1}. The electrode was then sonicated in acetonitrile to remove any adsorbed material and rinsed thoroughly with acetonitrile followed by rinsing with water.

Type IV: The type IV electrode was made using layer-by-layer (LBL) adsorption of polyelectrolytes onto the surface of a type III electrode. Poly(allylamine hydrochloride) (PAH) and poly(acrylic acid sodium salt) (PAA) were dissolved in pure water at a concentration of 1 mg mL^{-1}. The negatively charged type III RVC electrodes were used for polyelectrolyte deposition beginning with the polycation by immersing the electrode in PAH solution for 15 min, followed by a wash with water for 5 min, and then immersion in the PAA solution for 15 min, followed by a wash for 5 min. Multilayer assemblies consisting of four layers each of PAA and PAH were prepared by consecutive adsorption of both polyelectrolytes.

Type V: The type V electrode was modified by covalent attachment of polyethylene glycol onto the RVC surface. The electrode was first functionalized with phenylacetic acid groups as described for type III electrode. The surface carboxylic acid groups were then activated by incubating the functionalized RVC electrode in 25 mM EDC and 25 mM NHS in MES buffer (pH 7) for 4 h. The covalent binding of amine-terminated PEG was carried out by treating the activated surface carboxylic acid groups with 1 mg mL^{-1} of PEG-amine in MES buffer for 2 h.

Type VI: The type VI electrode was first functionalized with activated phenylacetic acid groups in a manner similar to that described above for type V electrodes. The activated surface carboxylic acid groups were then allowed to react with a solution of 1 mg mL^{-1} of biotin-PEO-amine and 1 mg mL^{-1} PEG-amine in MES buffer for 2 h.

Type VII: The type VII electrode was prepared in a manner similar to type VI with biotin-PEO-amine but without the PEG-amine.

Type VIII: The type VIII electrode was first modified with polyelectrolyte multilayers as described for type IV electrode. The carboxylic acid groups of the outermost poly(acrylic acid) layer on the RVC electrode were then activated in 25 mM EDC and 25 mM NHS in MES buffer for 4 h. The activated surface carboxylic acid groups on the RVC electrode were then allowed to react with 1 mg mL^{-1} of biotin-PEO-amine in MES buffer for 2 h.

Type IX: The type IX electrode consists simply of an adsorbed layer of biotinylated BSA. An otherwise bare RVC electrode was incubated in a solution containing 1 mg mL^{-1} of biotin-BSA and 1 mg mL^{-1} of BSA in tris buffer for 30 min. The electrodes were rinsed with buffer and used for further experiments.

2.3. Characterization of modified RVC electrodes

Electrodes modified with charged groups were characterized by their ability to block cyclic voltammetric oxidation/reduction of two charged redox probe molecules, $K_3Fe(CN)_6$ and $Ru(NH_3)_6Cl_3$. Electrodes having negatively charged surfaces (types III, V, and IV when the last polyelectrolyte layer is poly(acrylic acid)) were effective in blocking oxidation of $Fe(CN)_6^{4-}$, whereas electrodes having positively charged surfaces (types IV when the last polyelectrolyte layer is poly(allylamine) were effective in blocking reduction of $Ru(NH_3)_6^{3+}$. Hydroquinone oxidation/reduction was unimpeded at all nine electrode types studied in this work.

2.4. Non-specific and specific binding of NA-ALP and B-ALP onto modified RVC electrodes

NA-ALP and B-ALP enzyme conjugate solutions were prepared at a concentration of 50 μg mL^{-1} in tris buffer (pH 8). The modified RVC electrodes were always rinsed with tris buffer and dried under a brief flow of nitrogen gas prior to exposure to enzyme solutions. NSB of enzyme conjugates onto RVC electrodes was studied by incubating one set of type I–V electrodes in NA-ALP solution and a second set of type I–V electrodes in B-ALP solution for 30 min each. The electrodes were rinsed thoroughly with tris buffer and water and used for further studies. Specific binding of NA-ALP via direct biotin-neutravidin binding was studied on type VI–IX RVC electrodes. The electrodes were allowed to react with 50 μg mL^{-1} solution of NA-ALP in tris buffer for 30 min, followed by rinsing with buffer and water. For specific binding of B-ALP, the type VI–IX electrodes were first allowed to react with 25 μg mL^{-1} of neutravidin in tris buffer for 30 min, followed by rinsing with buffer to remove any adsorbed neutravidin. The enzyme conjugate B-ALP was then immobilized onto these RVC electrodes by incubating the electrodes in 50 μg mL^{-1} solution of B-ALP in tris buffer for 30 min. The electrodes were then further tested with enzymatic substrate HQDP to test for catalytic activity of enzymes that were attached onto the RVC surface.

2.5. Enzyme substrate reaction on modified RVC electrodes

After specific and non-specific binding of NA-ALP and B-ALP onto RVC electrodes was accomplished as described above, the electrodes were subjected to further testing using ALP-catalyzed hydrolysis of HQDP as an indicator of the presence of ALP on electrode surfaces. The modified electrodes were exposed to a 1 mM solution of HQDP in tris buffer and catalytically generated HQ was detected using a series of linear scan voltammograms (LSV). A potential range of −0.3 V to 0.7 V was used in LSV scans at a scan rate of 100 mV s^{-1} with a quiet time of 10 s prior to the start of each individual LSV scan. A continuous method for 10 scans was set up in such a way that each scan (including the 10 s quiet time) was automatically initiated immediately after the previous scan was completed. The time for the HQ oxidative peak to appear was noted in real time using a digital timer. The time when the electrode was dipped into the solution was set as the reference zero time. For LSV, the increasing peak current with time was recorded at HQ peak potential (E_p) and plotted against time. The initial rate of change of HQ peak current was then calculated from the initial slope of a linear region of LSV curve, and the value was compared for all modified electrodes.

2.6. Neutravidin sandwich bioassay

A series of type VI electrodes was used to perform sandwich bioassays with neutravidin as analyte. Neutravidin solutions were prepared in a concentration range from 1 μg mL^{-1} to 20 μg mL^{-1} in tris buffer. Each of a series of type VI electrodes was incubated in a different neutravidin solution for 30 min each. The electrodes were then rinsed thoroughly with tris buffer followed by incubation in 50 μg mL^{-1} of B-ALP solution for 30 min, followed by exposure to a 1 mM HQDP solution to test for the amount of bound ALP via the catalytic conversion of HQDP to HQ using LSV as described above.

Table 1
The initial rate of change of peak current of HQ generated by catalytic conversion of 1 mM HQDP. The values were obtained by calculating the initial slope of increasing peak current of HQ with time.

RVC electrode type	NA-ALP Initial rate of change of peak current of HQ (A min^{-1})			B-ALP Initial rate of change of peak current of HQ (A min^{-1})		
	RVC1	RVC2	Average	RVC1	RVC2	Average
I	3.75×10^{-7}	3.30×10^{-7}	3.53×10^{-7}	3.96×10^{-7}	4.31×10^{-7}	4.14×10^{-7}
II	9.25×10^{-7}	1.25×10^{-6}	1.09×10^{-6}	1.04×10^{-6}	1.28×10^{-6}	1.16×10^{-6}
III	2.18×10^{-7}	2.06×10^{-7}	2.12×10^{-7}	2.91×10^{-7}	2.54×10^{-7}	2.73×10^{-7}
IV	5.03×10^{-7}	4.87×10^{-7}	4.95×10^{-7}	–	7.75×10^{-7}	7.75×10^{-7}
V	9.25×10^{-8}	8.95×10^{-8}	9.10×10^{-8}	8.63×10^{-8}	7.25×10^{-8}	7.94×10^{-8}
VI	1.82×10^{-5}	2.01×10^{-5}	1.92×10^{-5}	1.85×10^{-5}	2.08×10^{-5}	1.97×10^{-5}
VII	2.35×10^{-5}	2.18×10^{-5}	2.27×10^{-5}	2.16×10^{-5}	2.83×10^{-5}	2.50×10^{-5}
VIII	3.17×10^{-6}	–	3.17×10^{-6}	2.79×10^{-5}	4.45×10^{-6}	1.62×10^{-5}
IX	4.06×10^{-5}	4.83×10^{-5}	4.45×10^{-5}	5.10×10^{-5}	4.86×10^{-5}	4.98×10^{-5}

3. Results and discussion

All enzyme assays measure either consumption of substrate or production of product over time. The time-course of product formation of an enzyme-catalyzed reaction can be followed via a plot of product concentration versus time. At the start of the reaction, the product concentration increases linearly with time but at later times the curve starts to level off due to depletion of substrate and eventually the concentration of product reaches a plateau. It has been observed that fitting the full time-course data of product formation is difficult, as the process cannot be easily reduced to a simple equation that expresses product concentration as a function of time [34,35]. The most widely used method to approximate the reaction progress curve is defined by differential equations or by an implicit equation. But this method is complicated and requires use of complex mathematics and assumptions that may not be realistic [36,37]. Recently another more approximate method has been used to fit the data of enzyme-catalyzed reaction progress curves using a non-linear least squares fit [38]. The equation used in this method is as follows:

$$I = \frac{I_{max}\, t}{(t_{1/2} + t)} \quad (1)$$

where I is the LSV peak current in amperes, which is proportional to the concentration of product generated at a given time (t), I_{max} is the saturation value of I which is achieved at long times when substrate has been fully converted into product, and $t_{1/2}$ is the time required to elicit half of the maximum current response. This equation describes the data in a way that is consistent with the enzyme rate law but it is not directly derived from the law. We have used this method in the present work to estimate the initial rate of product formation from the growth in peak current with time for a series of LSV's acquired at electrodes at which an enzyme-catalyzed reaction produces a redox-active product.

In this work we modified RVC electrodes with different functional groups in order to study non-specific and specific binding of two enzymes conjugates, NA-ALP and B-ALP, onto the electrodes. The amount of enzyme bound to each electrode surface was compared by measuring the initial rate of HQ generation. It is assumed that the initial rate of HQ production is proportional to the initial rate of increase of the HQ peak current detected at each RVC electrode. For each electrode HQ peak current was recorded at +0.32 V with time. The recorded data (I versus t) for each electrode were fitted using Eq. (1) to obtain the parameters I_{max} and $t_{1/2}$. The initial rate of product generation was then obtained by calculating slope of the tangent to the early region of the curve. The tangent was approximated by fitting a straight line to the portion of the curve near zero time. To fit a straight line we generated current values for a time interval close to zero using the parameters derived from Eq. (1).

The initial rate values for HQ generation for all the electrode types from Scheme 2 are given in Table 1 for adsorption of both NA-ALP and B-ALP. In each case two independent measurements were made, and the results of both measurements and their average values are reported. On type I to type V electrodes, the enzyme conjugates were adsorbed non-specifically and hence the amount of enzyme present on these electrode surfaces was comparatively lower than the amount of specifically bound enzyme conjugates on type VI to type IX electrodes. These data provide an overview of all the experiments; the following sections discuss individual experiments in more detail.

Before modifying the RVC electrodes, the NSB of enzyme conjugates was studied on unmodified electrodes (type I). Fig. 1a presents a series of linear scan voltammograms which show the growth of HQ peak current with time on a type I electrode following exposure to a 1 mM HQDP solution. The peak current at +0.32 V increases with time, indicating the catalytic conversion of HQDP into HQ by non-specifically adsorbed enzyme inside the RVC electrode pores. The initial HQ generation rate was obtained from the slope of the initial region of the line in Fig. 1b. The initial rate values for NSB of NA-ALP and B-ALP are given in Table 1. The reason for non-specific binding of enzymes onto type I RVC is most likely due to hydrophobic interactions between carbon surface and enzymes [39].

The type II electrode was modified with BSA and NSB of NA-ALP and B-ALP was studied in a similar manner as described for type I electrode. The HQ peak current and the initial rate of HQ generation on this electrode are given in Figs. 2 and 3 respectively. The initial rate value on type II electrodes was higher than those obtained for type I electrodes. The higher NSB on type II electrodes could possibly be due to electrostatic interactions between surface-bound BSA and the enzyme conjugates [40]. The type III RVC electrode modified with phenylacetic acid groups showed lower initial rate values compared to those obtained on type I and type II electrodes (Figs. 2 and 3b), which indicates that comparatively less enzyme was adsorbed onto this surface. At pH 8, the surface phenylacetic acid groups are deprotonated (pKa = 4.28) and the RVC surface is negatively charged. Also the net charges on neutravidin (pI ~6.3) and alkaline phosphatase (pI ~5.7) are negative at this pH. Adsorption of neutravidin or alkaline phosphatase onto this electrode is comparatively low due to repulsion between negatively charged surface carboxylic acid groups and negatively charged biomolecules. Also the carboxylate groups on the RVC electrode make the surface relatively hydrophilic, thus adsorption of proteins on this surface via hydrophobic mechanisms is expected to be diminished compared to that on unmodified and BSA coated electrodes [41]. However, NSB on type III electrodes was not entirely suppressed, and one possible reason for this finding is that the electrode surface might not completely be covered with the phenylacetic acid layer and some part of RVC electrode surface might be exposed to enzymes.

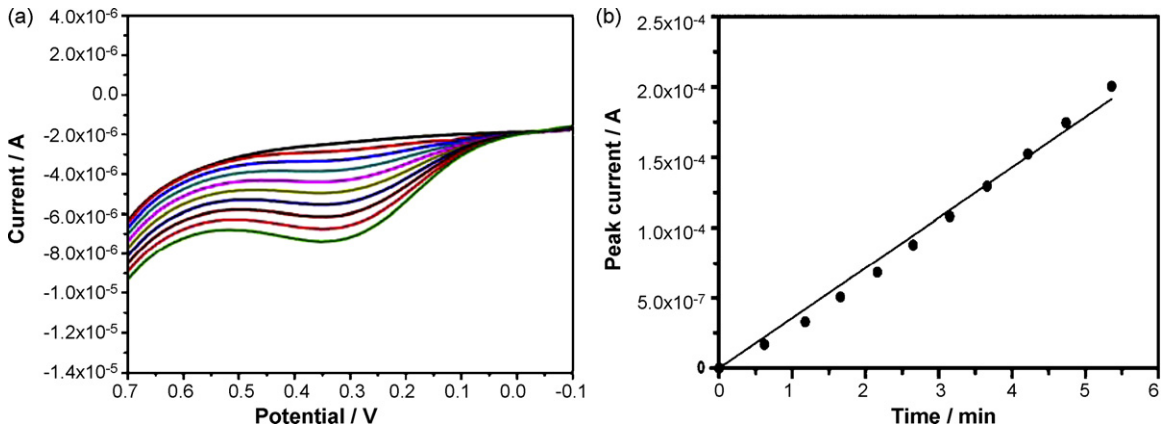


Fig. 1. (a) Linear scan voltammetry of HQ generated by hydrolysis of 1 mM HQDP in tris buffer (pH 8) by an enzyme, NA-ALP adsorbed non-specifically on the type I RVC electrode. (b) The HQ peak current recorded at 0.32 V (a) with time.

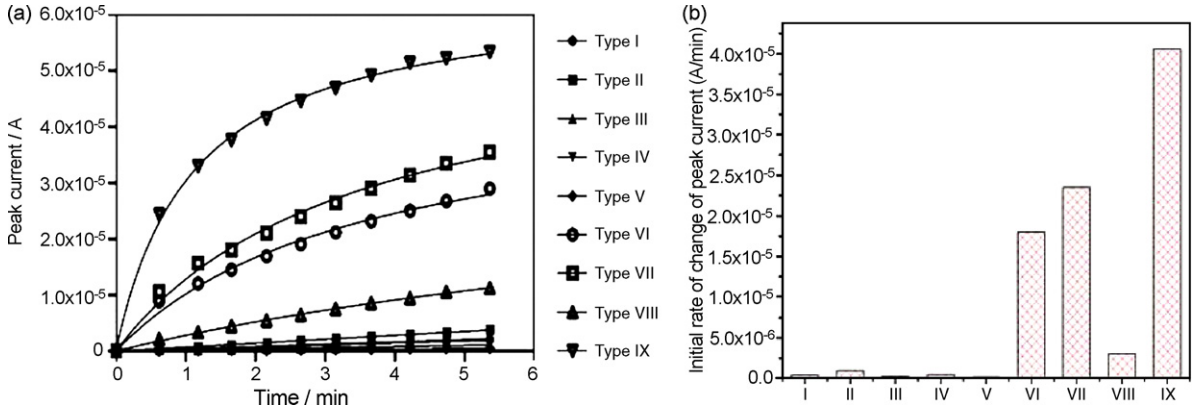


Fig. 2. Comparison of peak currents of catalytically generated HQ with time on RVC electrodes with non-specific (type I–V) and specific (VI–IX) binding of enzyme NA-ALP (b) The initial rate of change of HQ peak current obtained from the initial slope of curves in (a).

To achieve a more complete coverage with a negatively charged layer, another approach was used to modify RVC electrode. Polyelectrolytes with negatively charged functional groups are known to suppress adsorption of negatively charged oligonucleotides [42] and other proteins [43]. The RVC electrode was modified by layer-by-layer adsorption of poly(allylamine) (PAH) and poly(acrylic acid) (PAA) as shown in Scheme 2 for the type IV electrode. PAH/PAA was chosen for the multilayer build-up because it has been shown to be bio-inert towards cell attachment [44]. However, it was observed that the rate of HQ generation was higher on the type IV electrode as compared to type I and III electrodes (Figs. 2 and 3). Two mechanisms are possible for non-specific adsorption of negatively charged enzymes onto a negatively charged PAA layer. The first is that patches of positive charges on proteins could behave similarly to multivalent counterions of the polyelectrolyte layer and thus the protein becomes adsorbed onto the negatively charged surface via the positively charged regions on the protein [45,46]. The protein molecules can interact with polyelectrolytes via their positive charges even if the net charge on the protein molecule is negative. A second possibility is that there could be adsorptive interactions between proteins and the inner positively charged PAH layer. Even if the outer layer of the polyelectrolyte multilayer is

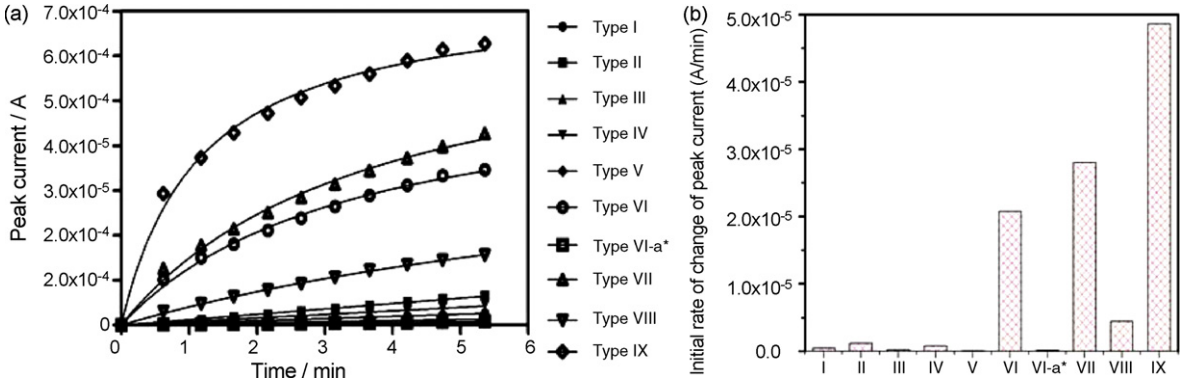


Fig. 3. (a) Comparison of peak currents of catalytically generated HQ with time on RVC electrodes with non-specific (type I–V and VI-a*) and specific (VI–IX) binding of enzyme B-ALP. (b) The initial rate of change of HQ peak current resulted on type I–IX electrodes. The type VI-a* electrode represents the non-specific adsorption of B-ALP onto the type VI electrode in the absence of neutravidin analyte.

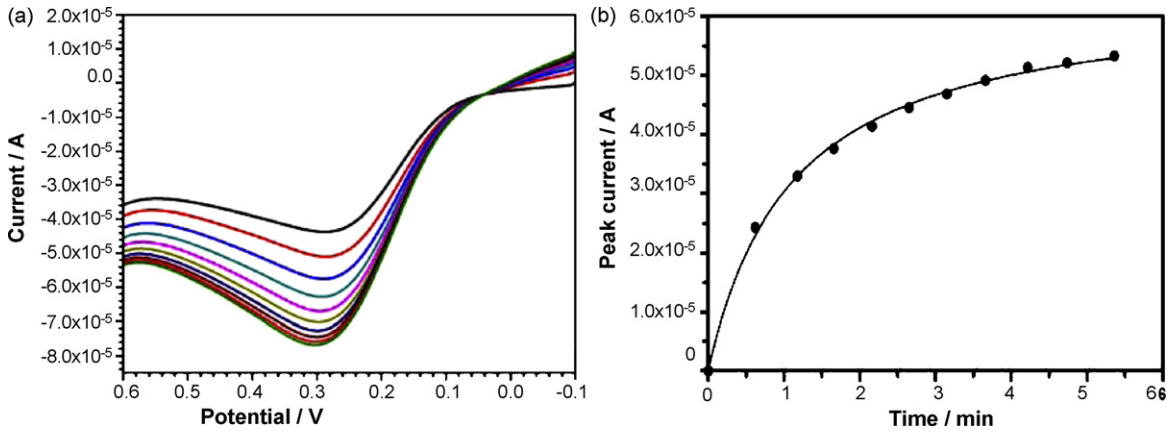


Fig. 4. (a) Linear scan voltammetry of HQ generated by hydrolysis of 1 mM HQDP in tris buffer (pH 8) by an enzyme, NA-ALP specifically coupled to biotinylated BSA on the type IX RVC electrode. (b) Increasing peak current of generated HQ recorded at +0.32 V a) with time.

terminating with a negatively charged polymer, some positively charged groups of the inner polyelectrolyte layer can still emerge and interact with negatively charged proteins [47]. Also, although experimental data showed the formation of multilayers on the RVC electrode (tested using redox probes), it is possible that the entire electrode surface was not covered with the polyelectrolytes due to the porous nature of RVC (compared to a flat planar carbon). The exposed RVC surface may still be subject to NSB of proteins.

The type V electrode was obtained by covalent attachment of polyethylene glycol (PEG) oligomers onto the RVC electrode. PEG is known for its ability to suppress protein adsorption due to its neutral hydrophilic groups [48–54]. It is believed that PEG uses "steric repulsion" and a hydration layer via hydrogen bonding around the PEG molecule to block NSB of proteins [55,56]. The initial rate of production of HQ on this electrode was much lower than those observed for type I, II, III and IV electrodes (Figs. 2 and 3b), indicating that much less enzyme was bound to the PEG-coated RVC surface. From these findings it was concluded that the type V electrode was most effective in suppressing NSB of NA-ALP and B-ALP and was further used in our subsequent studies of neutravidin bioassays.

On type VI to type IX electrodes, NA-ALP and B-ALP were bound specifically (Scheme 2) through formation of a neutravidin–biotin complex. Fig. 4b shows the plot of HQ peak current versus time on a type IX electrode where NA-ALP is specifically bound onto the B-BSA-coated RVC electrode. The HQ peak current increases with time linearly in the early-time region however it quickly becomes non-linear even at relatively short times. The saturation of peak current at later times is attributable to depletion of HQDP substrate. As the concentration of enzyme conjugate on this electrode was high due to specific binding, more substrate was converted at a given time compared to type I to type V electrodes. Similar behavior was observed on type VI to type VIII electrodes indicating that specific binding of enzyme on these electrodes has occurred (Figs. 2 and 3a). The initial rates of HQ generation on type VI to type IX electrodes are given in Figs. 2 and 3b for NA-ALP and B-ALP respectively. The type IX electrode showed the highest signal which was attributed to the relatively large number of biotin groups (9 mol biotin/mole of BSA) available on the electrode surface for binding. The type VIII electrode with polyelectrolyte multilayers, however showed relatively low initial rate values indicating that the specific binding of enzymes on this surface did not occur to a very great extent. One possible cause for this behavior is that after activating or modifying the outer PAA layer, it may no longer be negatively charged because the acid groups have been converted to amides. In such a case the outer functionalized PAA layer could just rinse away. The initial rate values for type VI and VII electrodes differ marginally indicating that the modification with PEG groups did not greatly affect the specific binding of enzyme conjugates on the type VI electrode. After comparing all the electrodes, the type VI electrode was selected as a best platform for further studies of RVC-based sandwich bioassay because it has the lowest non-specific binding of NA-ALP and B-ALP and relatively high specific binding of NA-ALP and NA (as indicated by subsequent labeling of captured NA with B-ALP.

A neutravidin sandwich bioassay was carried out using a series of type VI electrodes. Neutravidin, a deglycosylated form of avidin, was used as analyte and B-ALP was used as an enzyme label to allow for detection of captured analyte. Five different type VI electrodes were treated with five different solutions containing different con-

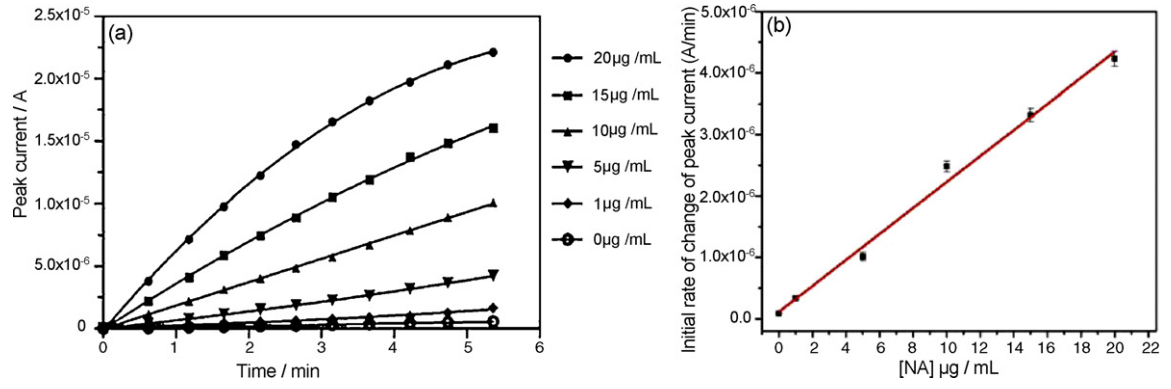


Fig. 5. (a) Plot of HQ peak current obtained from LSV plotted against time for concentrations of neutravidin analyte (0–20 µg mL^{-1}) in tris buffer at a scan rate of 100 mV s^{-1}. (b) The initial rate of HQ generation plotted against various concentrations of neutravidin.

centrations of neutravidin and the rate of conversion of HQDP to HQ by surface-immobilized B-ALP was then studied at each electrode. Fig. 5a show a series of plots of peak current of generated HQ with time for electrodes exposed to solutions containing different concentration of neutravidin. The initial rate of change of HQ peak current was calculated from the initial slope for all five concentrations and a calibration curve was obtained by plotting the initial slope values against neutravidin concentration (Fig. 5b). The initial rate values increase with increasing concentration of neutravidin. With increasing neutravidin concentration, the number of B-ALP molecules captured on the surface increases, and thus the amount of HQ generated at a given time varies with the amount of B-ALP on the electrodes. The calibration curve for neutravidin concentration was found to be linear having the equation $y = 2.13 \times 10^{-7}x + 6.51 \times 10^{-8}$ ($R^2 = 0.993$) where y is initial rate, and x is neutravidin concentration.

A control experiment in the absence of neutravidin shows a very low initial rate value (8×10^{-8} A min^{-1}) indicating that substantial binding of B-ALP occurs only in the presence of neutravidin analyte (Fig. 3b, type VI-a*). The lower concentration detection limit for this assay was estimated using the equation DL = $(3\sigma_{blank})/m$, where σ is the standard deviation for the blank (3.67×10^{-9} A min^{-1}) and m is the slope of the calibration curve line of Fig. 5b which is equal to $2.13 \pm 0.09 \times 10^{-7}$ A min^{-1} μg^{-1} mL^{-1}. The limit of detection for neutravidin on type VI RVC electrodes was thus estimated to be 52 ± 2 ng mL^{-1} and the absolute detection was 5.2 ± 0.2 ng for the sample size of 100 μL used for this analysis.

It is instructive to compare the analytical figures of merits for the present assay with those of previously described assays. The detection limit of our type VI RVC assay for neutravidin is close to that of a recently reported voltammetric detection method for avidin [57,58], however it is lower than that reported for the colorimetric quantitation of avidin in a competitive inhibition method [59] and in a sandwich bioassay [60,61].

The sensitivity of the RVC-based electrodes studied in this work could be affected by several factors. One important factor is the ability to reproduce the RVC electrodes for each experiment. Since RVC is porous in nature, an inhomogeneous pore distribution can result in uneven coating and/or mechanical instability of the electrode. Another important factor is possible variation in contact resistance between the gold wire and the RVC foam that could result in an uneven potential distribution across the electrode. Therefore, care should be taken while fabricating the RVC electrodes to have consistent size and volume and only the electrodes with low resistance (<50 Ω) should be use for the experiments.

4. Conclusion

For electrodes of type I to type V, the initial rate of HQ generation was much lower compared to that observed on type VI to type IX electrodes. The initial rate values were attributed to non-specific and specific binding of NA-ALP and B-ALP on RVC electrodes modified in different ways. Both phenylacetic acid and polyethylene glycol-based electrode modification schemes were successful in suppressing the non-specific binding of the NA-ALP and B-ALP enzymes conjugates on RVC electrodes. The electrode modified with PEG (type VI) showed the lowest non-specific binding and also comparatively high specific binding when some biotin was included in the electrode modification method to serve as a capture agent for neutravidin. The initial rate of HQ generation versus neutravidin concentration in a neutravidin sandwich assay was linear over the neutravidin concentration range studied. The lower concentration detection limit for neutravidin in this assay was 52 ± 2 ng mL^{-1} and absolute detection of 5.2 ± 0.2 ng of neutravidin was achieved in a 100 μL sample.

References

[1] D.J. Fabrizius-Homan, S.L. Cooper, Journal of Biomaterials Science, Polymer Edition 3 (1992) 27.
[2] A. Welle, M. Grunze, D. Tur, Journal of Colloid and Interface Science 197 (1998) 263.
[3] A. Sadana, Z. Chen, Biosensors and Bioelectronics 11 (1996) 17.
[4] Z. Chen, A. Sadana, Biophysical Chemistry 57 (1996) 177.
[5] A. Sadana, Engineering Biosensors, Academic Press, San Diego, 2002, p. 221.
[6] A. Sassolas, B.D. Leca-Bouvier, L.J. Blum, Chemical Reviews 108 (2008) 109.
[7] E. Palecek, M. Fojta, Talanta 74 (2007) 276.
[8] N.L. Rosi, C.A. Mirkin, Chemical Reviews 105 (2005) 1547.
[9] P. Vadgama (Ed.), Surfaces and Interfaces for Biomaterials, 2005.
[10] C.F. Wertz, M.M. Santore, Langmuir 17 (2001) 3006.
[11] C.F. Wertz, M.M. Santore, Langmuir 15 (1999) 8884.
[12] M. Steinitz, Analytical Biochemistry 282 (2000) 232.
[13] L.M. Bonanno, L.A. DeLouise, Biosensors and Bioelectronics 23 (2007) 444.
[14] A.M. Makoto Ide, T. Miyahita, Bulletin of the Chemical Society of Japan 74 (2001) 1335.
[15] T.T. Huang, J. Sturgis, R. Gomez, T. Geng, R. Bashir, A.K. Bhunia, J.P. Robinson, M.R. Ladisch, Biotechnology and Bioengineering 81 (2003) 618.
[16] J.-F. Masson, T.M. Battaglia, M.J. Davidson, Y.-C. Kim, A.M.C. Prakash, S. Beaudoin, K.S. Booksh, Talanta 67 (2005) 918.
[17] K.L. Prime, G.M. Whitesides, Science (Washington, DC, United States) 252 (1991) 1164.
[18] J. Ladd, Z. Zhang, S. Chen, J.C. Hower, S. Jiang, Biomacromolecules 9 (2008) 1357.
[19] T.W. Cha, V. Boiadjiev, J. Lozano, H. Yang, X.Y. Zhu, Analytical Biochemistry 311 (2002) 27.
[20] O. Andersson, A.a. Larsson, T. Ekblad, B. Liedberg, Biomacromolecules 10 (2009) 142.
[21] S. Loefaes, B. Johnson, Journal of the Chemical Society, Chemical Communications (1990) 1526.
[22] S.D. Carrigan, M. Tabrizian, Langmuir 21 (2005) 12320.
[23] R. Schlapak, D. Armitage, N. Saucedo-Zeni, M. Hohage, S. Howorka, Langmuir 23 (2007) 10244.
[24] R. Benters, C.M. Niemeyer, D. Drutschmann, D. Blohm, D. Woehrle, Nucleic Acids Research 30 (2002) e10/1.
[25] A. Krieg, T. Ruckstuhl, S. Seeger, Analytical Biochemistry 349 (2006) 181.
[26] J. Wang, Electrochimica Acta 26 (1981) 1721.
[27] J.M. Friedrich, C. Ponce-de-León, G.W. Reade, F.C. Walsh, Journal of Electroanalytical Chemistry 561 (2004) 203.
[28] M. Khayyami, M.T. Pérez Pita, N. Peña Garcia, G. Johansson, B. Danielsson, P.-O. Larsson, Talanta 45 (1998) 557.
[29] H.J. Wieck, G.H. Heider Jr., A.M. Yacynych, Analytica Chimica Acta 158 (1984) 137.
[30] N. Pena, M. Romero, F.J.M. De Villena, A.J. Riviejo, J.M. Pingarron, Electroanalysis 11 (1999) 85.
[31] C. Bourdillon, M. Delamar, C. Demaille, R. Hitmi, J. Moiroux, J. Pinson, Journal of Electroanalytical Chemistry 336 (1992) 113.
[32] M.S. Wilson, R.D. Rauh, Biosensors and Bioelectronics 19 (2004) 693.
[33] C. Bieniarz, C.A. Kernag, J.W. Kosmeder, P.M. Rodgers, J. Wong, Enzyme-catalyzed metal deposition for the enhanced in situ detection of immunohistochemical epitopes and nucleic acid sequences, (Ventana Medical Systems, Inc., USA). Application: US, 2004, p. 19 pp.
[34] R.G. Duggleby, Methods in Enzymology 249 (1995) 61.
[35] I.A. Nimmo, G.L. Atkins, The Biochemical Journal 141 (1974) 913.
[36] B. Perdicakis, H.J. Montgomery, J.G. Guillemette, E. Jervis, Analytical Biochemistry 332 (2004) 122.
[37] H.N. Fernley, European Journal of Biochemistry 43 (1974) 377.
[38] A.K. Rao, S.E. Creager, Analytica Chimica Acta 628 (2008) 190.
[39] G. Raffaini, F. Ganazzoli, Computer Simulations of Liquid Crystals and Polymers (2005) 203.
[40] B.S. Chang, R.R. Mahoney, Biotechnology and Applied Biochemistry 22 (1995) 203.
[41] A.D.R. Alison, J. Downard, Electroanalysis 7 (1995) 376.
[42] E.P. Kartalov, M.A. Unger, S.R. Quake, BioTechniques 34 (2003) 505.
[43] J. Dai, G.L. Baker, M.L. Bruening, Analytical Chemistry 78 (2006) 135.
[44] J.D. Mendelsohn, S.Y. Yang, J.A. Hiller, A.I. Hochbaum, M.F. Rubner, Biomacromolecules 4 (2003) 96.
[45] C. Fleck, H.H. von Grunberg, Physical Review E: Statistical, Non-linear, and Soft Matter Physics 63 (2001) 061804/1.
[46] C.F. Wertz, M.M. Santore, Langmuir 18 (2002) 706.
[47] G. Ladam, C. Gergely, B. Senger, G. Decher, J.-C. Voegel, P. Schaaf, F.J.G. Cuisinier, Biomacromolecules 1 (2000) 674.
[48] C.D. Heyes, A.Y. Kobitski, E.V. Amirgoulova, G.U. Nienhaus, Journal of Physical Chemistry B 108 (2004) 13387.
[49] F. Frederix, K. Bonroy, G. Reekmans, W. Laureyn, A. Campitelli, M.A. Abramov, W. Dehaen, G. Maes, Journal of Biochemical and Biophysical Methods 58 (2004) 67.
[50] L.S. Roach, H. Song, R.F. Ismagilov, Analytical Chemistry 77 (2005) 785.
[51] T. Tamura, T. Terada, A. Tanaka, Bioconjugate Chemistry 14 (2003) 1222.
[52] A.J. Downard, A. Bin Mohamed, Electroanalysis 11 (1999) 418.
[53] B. Guo, J.-I. Anzai, T. Osa, Chemical and Pharmaceutical Bulletin 44 (1996) 860.

[54] N. Marme, H. Lee, A. Friedrich, C.-W. Park, J. Fiore, D.J. Nesbitt, J.-P. Knemeyer, Proceedings of SPIE 6862 (2008) 686212/1.
[55] L. Li, S. Chen, J. Zheng, B.D. Ratner, S. Jiang, The Journal of Physical Chemistry B 109 (2005) 2934.
[56] J. Zheng, L. Li, H.-K. Tsao, Y.-J. Sheng, S. Chen, S. Jiang, Biophysical Journal 89 (2005) 158.
[57] K. Sugawara, S. Hoshi, K. Akatsuka, Fresenius Journal of Analytical Chemistry 358 (1997) 755.
[58] R. Kizek, M. Masarik, K.J. Kramer, D. Potesil, M. Bailey, J.A. Howard, B. Klejdus, R. Mikelova, V. Adam, L. Trnkova, F. Jelen, Analytical and Bioanalytical Chemistry 381 (2005) 1167.
[59] Z. Gan, R.R. Marquardt, Journal of Biochemical and Biophysical Methods 39 (1999) 1.
[60] W.C. Mak, Y. Li, W.K. Lau, D. Trau, Electroanalysis 16 (2004) 156.
[61] E.A. Bayer, H. Ben-Hur, M. Wilchek, Analytical Biochemistry 154 (1986) 367.