Fast electrochemical detection of anti-HIV antibodies: coupling allosteric enzymes and disk microelectrode arrays.

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Abstract

Here a novel electrochemical method for the rapid detection of anti-HIV antibodies in serum is presented. The novelty lies in the combination of allosteric enzymes and coulometry to yield a fast, simple and reliable HIV diagnostic method. We have used a previously developed β-galactosidase enzyme that is efficiently activated by anti-HIV antibodies directed against a major B-cell epitope of the gp41 glycoprotein. When these antibodies bind the enzyme, the 3D conformation changes positively affecting the performance of the active site and, consequently, the enzyme activity is stimulated. Using 4-aminophenyl β-D-galactopyranoside (PAPG) as substrate yields p-aminophenol (PAP), which is reversibly oxidised at a very mild potential, ca. 0.37 V vs. Ag/AgCl over a range of electrode materials within the working pH range of β-galactosidase. In the present case, photolithographically produced microelectrode arrays resulted in a detection limit of 4 μM for 4-aminophenol (PAP). The presence of anti-HIV antibodies results in enzyme activity increases above 50% which, combined with the sensitivity and response time afforded by the microelectrode arrays, allowed for the diagnosis of HIV in sera samples within an hour.

Keywords: Disk microelectrode arrays; beta-galactosidase; amperometry of 4-aminophenol, coulometry; allosteric enzymes; anti-HIV antibodies.
1 Introduction

The timely diagnostic of infectious diseases is key in clinical and veterinary medicine. Over the past 20 years, the human immunodeficiency virus (HIV) has been among the most studied emerging viruses and a high number direct and indirect of methods have been developed to detect its infection [5]. With direct methods, viral components (e.g. nucleic acids, proteins) are detected by antigen-ELISA, [6] immunoperoxidase/peroxidase-immunoperoxidase staining, [7, 8] complement-fixation test, [9] immunofluorescence or Polymerase chain reaction (PCR) based methods [10-14], with Antibody-ELISA and agar-gel immunodiffusion being the most common [2]. Regardless of this, Significant efforts are being invested in the discovery of new strategies to detect infections with better sensitivity, reliability and test performance [2-4], and new methods are continuously being developed to either improve existing assays or to make new detection procedures available [1]. Portable and rapid detection platforms are particularly necessary in geographical regions lacking adequate medical facilities, and in these regions biosensing devices offer a promising alternative to conventional analytical methods [15, 16].

Allosteric enzymes can be used as efficient biocomponents as their activity is modulated by the specific recognition of target peptides, in general distant from the active site, by specific anti-peptide antibodies [17]. The construction of allosteric enzymes involves the identification of an antigenic peptide from the pathogen and its insertion using protein-engineering methods onto appropriate permissive sites in an enzyme. This offers intriguing possibilities in the fast and ultra-fast molecular diagnosis of infectious diseases because the allosteric reaction can be monitored in enzymatic assays and after short reaction times in simple homogeneous assays. Among the different enzymes suitable to be used as allosteric sensors [17], *Escherichia coli* β-galactosidase (β-D-galactoside hydrolase, E.C. 3.2.1.23) was chosen as a suitable enzyme to be used as an allosteric sensor as it hydrolyzes
lactose and synthetic analogues such as 4-aminophenyl β-D-galactopyranoside (PAPG), a substrate yielding p-aminophenol (PAP), a well-known electroactive product [18-20]. We are using this enzyme-substrate system in combination with microdisk electrode arrays [21] to develop a rapid and sensitive method to detect anti-HIV antibodies. To summarize, a small amount of modified β-galactosidase HisNF795gpC was incubated in parallel in a serum test sample and a blank. Following these incubations, PAPG was added and the allosteric activations in the samples were compared to each other via the direct oxidation of PAP at a microdisk electrode array. HIV positive human sera generated PAP at a significantly higher rate, demonstrating the feasibility of this electrochemical assay in real samples.

2 Experimental

2.1 Chemical reagents and instrumentation

All chemicals used were analytical grade and were used as received from Sigma-Aldrich without any further purification. These were: potassium chloride, potassium hexacyanoferrate (II), 4-aminophenol (PESTANAL grade, 99.9% purity), β-galactosidase, 4-Aminophenyl β-D-galactopyranoside, Bovine serum albumin (BSA) and magnesium sulphate heptahydrate. For the antibody detection experiments, a specially engineered β-galactosidase was used [22]. Buffer Z was prepared using commercial PBS tablets (Invitrogen) reaching a 0.1 M concentration and completed to 1 mM MgSO₄ and 20 mM KCl. Solutions were prepared using deionised water of resistivity not less than 18 MΩ cm⁻¹. All the material used was cleaned with bleach between experiments. The pH of the solutions was controlled using a METROHM 827 pH Lab meter with temperature correction. Human sera samples were generously provided by Dr. Miguel Angel Martínez (Fundació IRSI Caixa) and treated anonymously during all the procedures to make them untraceable. Seroconversion in HIV-infected individuals had been previously
determined by conventional routine immunoassays. Patients’ consent was given and approval of local ethical committee was obtained.

Autolab PG12 potentiostat controlled by GPES 4 software running on a Windows XP based PC. A BAS (West Lafayette, IN, USA) 1.6 mm diameter gold electrode was used for the preliminary voltammetric study of 4-aminophenol. The analytical work was then performed with a series of disk microelectrode arrays as described below. A 5 cm long, 3 mm diameter glassy carbon rod was used as auxiliary electrode and an Ag/AgCl (3M KCl) Metrohm biotrode® was used as reference electrode. The temperature of all solutions was controlled by means of a jacketed cell connected to a thermostatic bath.

2.2 Cloning, expression, purification and activity assay of HisNF795gpC

The construction of his-tagged recombinant β-galactosidase HisNF795gpC, displaying HIV B-cell epitope from the gp41 envelope glycoprotein, was achieved adding a removable His6 tag and a Tobacco Etch Virus protease cleavage site at the N-terminal end of NF795gpC [22] by polymerase chain reaction. The specific DNA band obtained from pNF795gpC was subcloned into the NcoI and EcoRI unique restriction sites of pJLA602 [23] resulting in pHisNF795gpC which encodes engineered β-galactosidase under the control of the cI857-repressed lambda pL and pR strong promoters.

Protein HisNF795gpC was produced in E. coli MC4100 by standard procedures [22]. Cells were spun down and re-suspended in buffer Z supplemented with 500 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol and protease inhibitor cocktail tablets (Complete EDTA-free from Roche Applied Science). After sonication, the clarified supernatant was loaded on a nickel column (1 ml HiTrap chelating HP column, GE Healthcare) equilibrated with the same buffer. The column was washed with the loading buffer and the protein was eluted with a 10-300 mM imidazole
gradient. Positive protein fractions were detected by a miniaturised $\beta$-galactosidase activity assay in ELISA microtiter plates using ortho-nitrophenyl $\beta$-D-galactopyranoside (ONPG) as substrate. Pooled positive protein fractions were dialysed against buffer Z. Protein concentration was obtained by spectrophotometry measurements at 260 and 280 nm and the enzymatic activity was determined by published variations of the Miller’s method [22].

2.3 Electrode Fabrication and Characterisation

The thin film microelectrode arrays used in this study have been thoroughly described elsewhere [24, 25]. These arrays are made up of 128 microdisks, 10 micron in diameter, arranged in a cubic lattice with an inter-centre separation of 100 microns. The electrodes were first cleaned with ethanol and then electrochemically activated in 0.1 M KCl applying a series of potential pulses from 0 V to -2 V vs. Ag/AgCl (3M KCl). The electrodes were then characterized by cyclic voltammetry in 1mM ferrocyanide solutions. Analysis of the limiting currents using the approach described in [26] at a range of scan rates enabled the elucidation of the number of active microdisks in the array throughout the experiments. This operation was repeated after each measurement to determine the degree of microelectrode passivation. Although electrode performance was generally affected by the adsorption of proteins present in the samples, it was possible to recover the full response of the arrays following a new cycle of electrochemical activation in an electrolyte solution.

3 Results and Discussion

Electrochemistry of 4-aminophenol

The detection of anti-HIV antibodies is indirectly reported by the oxidation of 4-aminophenol, PAP, which is produced by $\beta$-galactosidase from 4-Aminophenyl-$\beta$-D-galactopyranoside, PAPG. [27, 28] The electrochemistry of PAP is well known and
numerous literature reports deal with the subject. [20, 27, 29-32] The advantage of using a PAP related substrate lays in that PAP is reversibly oxidised at a mildly positive potential ($E_o$ ca. 0.3 V vs. Ag/AgCl (3M KCl)), causing next to no electrode fouling (unlike $\alpha$-aminophenol, $p$-aminophenol is reported not polymerise upon oxidation at an electrode [33]). Unfortunately, the oxidation product is unstable and is known to undergo hydrolysis over a wide range of pHs but specially under very acidic conditions [34]. This makes the oxidation of PAP a classical example of the EC mechanism. [29].

$k_2$ is a first order constant around 0.020 s$^{-1}$ [29] at pH=1.18. According to this, the follow-up $C$ step should not be an issue in our case since all our solutions were buffered at a pH=7.5, where the hydrolysis step is believed to be slower. On the other hand, we found 4-aminophenol to adsorb weakly on our gold working electrodes. Having said this, adsorption was not found to be detrimental to the overall performance of the enzymatic assay, and any remains on the electrodes could be easily removed by rinsing with DMF. Figure 1 shows a sample cyclic voltammogram of 0.5 mM PAP at pH=7.5 at 50 mVs$^{-1}$ over a 128 microdisk microelectrode array. An apparent diffusion coefficient of $(7.44\pm0.91) \times 10^{-10}$ m$^2$ s$^{-1}$ was estimated by analysis of the oxidation peak currents at a macroelectrode. This is in good agreement with an estimated value of $8.60 \times 10^{-10}$ m$^2$ s$^{-1}$, obtained by application of the Wilke-Chang semiempirical approximation [35]. Although electrochemical reversibility is an interesting feature in itself for amperometric biosensors, what makes it even more attractive is the fact that its parent enzyme substrate, PAPG, is not electroactive at the detection potential for aminophenol. From the voltammetry, we decided to subsequently use 0.37 V vs.
Ag/AgCl (3M KCl) as working potential for the amperometric detection of PAP under diffusion control. The detection limit for 4-aminophenol was determined from the limiting currents measured at 100 mV s⁻¹ at four calibrated 128 disk microelectrode arrays. This scan rate ensured diffusional independence between micro disks [36, 37], and a sample calibration plot is provided in Figure 2. We found this limit for PAP to be 4 µM using the propagation of errors approach [38] as it is more realistic than the 1.8 µM obtained from application of IUPAC’s 3σ method [39].

**Electrochemical detection of wild type β-galactosidase activity**

The 4-aminophenol formed by activity of β-galactosidase is readily oxidised at the electrode surface. This was ensured from a series of control experiments. These consisted in separate electrochemical studies of the starting materials alone. Figure 3.a shows that neither the substrate nor β-galactosidase alone brought about any significant currents at the working potential over a span of 20 minutes. However, when the two were mixed together, 4-aminophenol was immediately generated. The increase of this product was linear during the early stages of the process, which enabled the study of the enzyme kinetics. The dependency upon substrate concentration was performed at a β-galactosidase concentration of 2 µg ml⁻¹ and different substrate amounts (0.05, 0.10, 0.25 and 0.50 mg ml⁻¹). We measured the output current for 30 minutes, which enabled us to monitor the rate of product formation. Enzyme activity, measured from the initial slope, was proportional to the concentration of substrate introduced in the system. Figure 3.b depicts the response obtained over time and the slopes calculated for the different initial substrate concentration. Using these results the parameters governing the system kinetics were elucidated assuming a Michaelis-Menten type mechanism [40]. Data analysis yielded $K_m$ and $k_{cat}$ values of $4.3±0.1 \times 10^{-4}$ mol l⁻¹ and $0.08±0.05$ s⁻¹, respectively.
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**His-NF795gpC: detection of anti-HIV antibodies in sera**

We used mutant versions of the *Escherichia coli* β-galactosidase that are enzymatically responsive to antibodies against human immunodeficiency virus 1 (HIV-1) [22]. These custom-made enzymes, when used as biosensing tools, showed specificities and sensitivities comparable to conventional antibody detection immunoassays [22]. Solving the structural rationale for the sensing mechanics [41-46] has permitted to optimize the performance of the engineered proteins by dramatically shortening the reaction time [47] and reaching signal:background ratios higher than 10 [48, 49]. The improved versions of the allosteric sensing reactions permit the use of these molecular sensors in high-throughput sera analysis [50].

Given that His-NF795gpC is more unstable than the wild type enzyme, measurements were conducted in buffer Z containing 1% (w/v) BSA. However, electrochemical monitoring of enzyme activity was still feasible at the microelectrode array. Figure 4 shows that the current decayed after going through a maximum at higher enzyme activities. This was thought to be due to the depletion of the diffusion layer at the microelectrode array after the quantitative consumption of the enzyme substrate at higher enzyme levels. The initial concentration of substrate was close to 1 mM, which might have eventually led to the production of 1 mM aminophenol. The magnitude of the peak current, near 300 nA, would correspond to an aminophenol concentration in that range, considering Figures 1 and 2. Other factors affecting the current response were microelectrode passivation, caused by adsorption of protein, and perhaps enzyme inhibition. Although we cannot rule out enzyme inhibition, we believe it was negligible under our experimental conditions and that the current decay can simply be explained by a combination of substrate consumption and electrode passivation.

The oxidation current arising from PAP formation was recorded following addition of the substrate into the electrochemical cell. Incubation of His-NF795gpC with
positive sera enhanced its activity by over 56% in all cases studied. In the assays carried out in real samples, 2 µg L⁻¹ of enzyme were incubated for 45 minutes with a 1/60 final dilution of a sera pool from HIV-1 infected individuals. Figure 5 shows the results of the main experiment, consisting on incubating the enzyme with a bank of serial dilutions from two distinct sera coming from HIV-infected and HIV-negative individuals, respectively, in the presence of HisNF795gpC. For each condition, three measurements were conducted to obtain the mean and the corresponding error bars. We used combined standard deviations at 95% confidence as the measure of error† [51]. Antibody recognition resulted optimum for a serum dilution of 1/320. The activity increase was not enhanced any further beyond this point of serum concentration. Coulometry was found to be more reliable than measuring the initial velocity of the enzymatic reaction or current intensity after 20 minutes of measurement, showing better accuracy and being able to operate under lower enzyme and substrate concentrations. The current decays observed in the presence of higher protein concentrations are likely due to electrode fouling rather than enzyme inhibition. The activity enhancement in the positive serum samples is also clear. Table 1 gives the values of the initial velocities

† Since all standard deviations are due to the testing method, it is possible to combine them to obtain a parameter that quantifies the variability of the latter. Combining standard deviations does not mean to average standard deviations, since that would not account for the fact that different samples may contain a different level of antibodies in them. The way to calculate the combined standard deviation is:

\[ CSD = \sqrt{\frac{\Sigma (df^2)(s^2)}{\Sigma df}} \],

where \( df \) represents the degrees of freedom, and \( s \) is the standard deviation of each data set. The combined standard deviation is a better estimation of the population standard deviation because it has more degrees of freedom. Thus, when calculating confidence intervals, the value of Student’s \( t \) parameter can be significantly decreased. The result of this approach is that lower detection limits can be reached, and is of great help in samples at the lower concentration range where very small slope or current plateau changes are produced. Reproducibility could be achieved because re-activation and characterisation of the arrays was conducted between measurements.
of PAP production before and after incubation with the serum. Although a bigger activity change was observed for the low enzyme concentration (72% vs. 56%), we chose to work with 2µg ml\(^{-1}\) enzyme concentration in subsequent experiments because the resulting currents were easier to measure.

Three parameters were compared: (i) the initial velocity of the enzymatic reaction, determined during the first five minutes of operation, (ii) signal intensity reached after 20 minutes of measurement and (iii) total charge passed throughout the experiment. The three graphs are depicted in figure 5a-c. As the figure shows, for 2 µg ml\(^{-1}\) HisNF795gpC, the antibody recognition was optimum for a serum dilution of 1/320. In fact the activity increase was not enhanced any further beyond this point of serum concentration. Current losses were observed both in the infected sample and in the blank above this optimum serum concentration. This effect has been explained above as a combination of substrate depletion and electrode fouling, and it does not prevent the feasibility of the measurement. Among the three approaches to determine the presence of anti-HIV antibodies, the coulometric (figure 5c) seems the most reliable, followed by the amperometric mode and finally the initial slope. Although the initial slope method may be the quickest, it is also less sensitive than the others. The amperometric method is more sensitive, but it requires high enzyme and substrate concentrations. Besides, if the substrate were quantitatively consumed, as it happened in our case, the measurement might be subject to relatively large errors. Despite the fact that in our long chronoamperometric measurement we may have partially lost the current amplification feature of microelectrode arrays, the fact is that electrical noise pickup and capacitive currents were negligible compared to faradaic response. The coulometric method, on the other hand, showed better accuracy and it could operate under low enzyme and substrate concentrations. Given that charge accumulates over time, the fact that the currents are in the low nanoampere range may not necessarily prevent a successful measurement if the reaction is left to proceed for a few minutes.
4 Conclusions and Further Prospects

We were able to measure the presence of anti-HIV antibodies in serum by measuring enzyme activity of an allosteric β-galactosidase over p-aminophenyl β-D-galactopyranoside using disk microelectrode arrays to directly oxidise the p-aminophenol produced in the reaction. The novelty of this research stems from the use of major technological advances in allosteric enzymes and microelectrode arrays, which enable the successful electrochemical detection of p-aminophenol in blood serum, indicating HIV infection. HIV infection is detected by measuring the difference in enzyme activity between a sample and a blank, with a positive sample producing a higher yield of the electroactive product p-aminophenol.

Methodology improvements may be generated from a thorough optimisation of the chemical conditions such as pH, salinity, protein and substrate concentrations, and better microelectrode design may lead to a shorter analysis time. Also better-engineered microsystems could produce a more cost effective process by reducing the quantity of reagents required and, more importantly, thereby reducing the cost of disposal. Interestingly, the recent finding that allosteric sensing is maintained in immobilized enzyme versions [48] offers new possibilities to develop optimized platforms holding the sensing reaction, so that an integrated micro-electrochemical cell - able to handle sample volumes in the order of microlitres - may turn this method into a true competitor to current ELISA and Western blot assays.

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Tables

**Table 1**

<table>
<thead>
<tr>
<th>Condition tested</th>
<th>Initial velocity (linear part slope) / A s(^{-1})</th>
<th>Initial velocity increase after serum addition.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum and substrate; no β-gal (control)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>0.86 µg ml(^{-1}) β-gal ; incubation without serum</td>
<td>4.84x10(^{-11})</td>
<td>72.1 %</td>
</tr>
<tr>
<td>0.86 µg ml(^{-1}) β-gal ; incubation with serum</td>
<td>8.33x10(^{-11})</td>
<td></td>
</tr>
<tr>
<td>2µg ml(^{-1}) β-gal ; incubation without serum.</td>
<td>4.00x10(^{-10})</td>
<td>56.2 %</td>
</tr>
<tr>
<td>2µg ml(^{-1}) β-gal ; incubation with serum.</td>
<td>6.25x10(^{-10})</td>
<td></td>
</tr>
</tbody>
</table>

**Caption to tables**

Table 1: Increase of the engineered β-Gal activity after incubation with HIV-antibody containing sera.
Caption to figures

Figure 1: Cyclic voltammetry of 0.5 mM 4-aminophenol (PAP) in a phosphate buffered solution at a gold microelectrode array composed of 128 microdisks of 5 mm radius and inter-central distance of 100 microns arranged in a square lattice.

Figure 2: Typical calibration plot resulting from the measurement of the oxidation current of 4-aminophenol at a gold microelectrode array. The detection limit (see inset) is ca. 4 µM.

Figure 3: (a) Chronoamperograms recorded at the microelectrode array at a constant potential 0.37 V. in solutions containing (X) the substrate alone at 0.25 mg.ml⁻¹, (▲) β-galactosidase alone at 2µg.ml⁻¹ or (●) both the substrate (0.25 mg.ml⁻¹) and the enzyme (2 µg.ml⁻¹) in buffer Z. (b) Evolution of the slopes obtained from the linear part of the curves corresponding to substrate additions to a solution containing 0.2 µg.ml⁻¹ of β-galactosidase and fitting to the corresponding Michaelis-Menten-type rate law.

Figure 4: Chronoamperograms measured at 0.37 V vs. Ag/AgCl (3M KCl) at two different HisNF795gpC concentrations after a 45-minute incubation at 28ºC in the presence or absence of a 1/60 final dilution serum containing anti-HIV antibodies. Substrate concentration was 0.25 mg.ml⁻¹. 2µg.ml⁻¹ HisNF795gpC concentration with (■) or without (□) contaminated serum and a 0.86 µg.ml⁻¹ HisNF795gpC with (▲) or without (Δ) serum. A negative control with serum but no HisNF795gpC was also performed (●).

Figure 5: Intensity obtained from (a) initial slope values, (b) final current and (c) total charge passed after 20 minutes applying 0.37 V. The signals correspond to the activity of HisNF795gpC in contact with 0.25 mg.ml⁻¹ substrate following 45
minutes incubation at 28°C with anti-HIV antibodies positive serum (◼) or negative serum (□).

References