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4-Amino-1-naphthylphosphate as a substrate for the amperometric detection of alkaline phosphatase activity and its application for immunoassay

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Abstract

Immunosensors and biochemical array detection systems based on electrochemical transducers have many advantages such as low detection limit, fast response, simple design and ease of miniaturization. However, further development of such sensors will depend on the availability of suitable substrates that can be converted by a labeling enzyme to an electrochemically active product. Here, we report the synthesis of 4-amino-1-naphthylphosphate and it's application as a new substrate for alkaline phosphatase. The electrochemical and enzymatic properties of this compound were investigated and compared with the properties of other aromatic 1,4-dihydroxy and 1,4-hydroxy-amine derivatives. The product of the enzyme reaction was 4-aminonaphthol, which was rapidly converted in the presences of air to 1,4-iminonaphthoquinone. This compound could then be detected in an amperometric flow injection assay (AFIA) with −200 mV versus Ag/AgCl potential application. The analytical range for mouse IgG, in an alkaline phosphatase amplified sandwich immuoassay with amperometric detection, was 0.01–100 μg ml^{-1}.

Keywords: Amperometry; Flow injection assay; Alkaline phosphatase; Immunoassay; Biosensors

1. Introduction

Research into biochemical assays systems is increasingly focusing on the development of biosensors [1–3] and array detection [4,5]. The biosensor technology is mostly aimed at developing simple and economical devices that can be used in clinical practice, environmental monitoring and process control [6]. The development of biochemical array detection systems is directed towards high throughput screening were a large number of assays can be performed in parallel.

In these devices the biochemical reaction is performed in a confined space on the device and, in the case of biosensors, directly linked to the transducer that will convert the biochemical signal to an electronic signal. The transducers are usually photometric transducers that will respond to changes in absorption or fluorescence. However, in the case of biosensors electrochemical transducers are often preferred [1,2]. These will offer the advantages of low detection limit, fast response, simple design and ease of miniaturization. However, commercial devices based on electrochemical transducers are mostly limited to enzyme sensors such as the glucose sensor [7] where the enzyme reaction is linked to the reduction or oxidation of an electrochemical mediator. Immunoreactions and other types of bioaffinity reactions are usually monitored with the help of some suitable fluorescent labeling molecule or a reaction catalyzed by a labeling enzyme that will produce a photoactive compound from a photo-inactive substrate. Electrochemical reactions are not much used for immunoassays in part due to the limited availability of suitable labeling molecules and substrates that can be electrochemically activated in an enzyme reaction. Photometry is also commonly used to monitor reactions in biochemical array systems. Such arrays can be addressed at multiple wavelengths to monitor more than one molecular label simultaneously [8,9].

The future development of electrochemical immunosensors and biochemical arrays based on electrochemical transducers depends on the availability of suitable substrates that can be converted by a labeling enzyme to an electro-

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chemical active product. To fulfill the requirements of good sensitivity, minimum interference and fast response, the substrate should be chemically stable and electrochemically inactive at the potential used for detection. The electrochemically active product should also be detectable at relatively low potential with rapid electron transfer to the electrode (minimum overpotential).

Horseradish peroxidase (HPR), alkaline phosphtase, and to a lesser extend glactosidase, are the most commonly used labeling enzymes for immunassay applications. Horseradish peroxidase has some advantage for electrochemical imunosensors, such as small size (Mw = 44.000) and high turnover rate of the enzymatic reaction. Electrochemical detection of the catalytic reduction of hydrogen peroxide can then be done with the help of a suitable electrochemical mediator. However, the practical development of immunosensors employing this enzyme limited by the fact that the substrate, hydrogen peroxide, is chemically unstable in the presence of organic material and a very aggressive reagent.

Alkaline phosphatase, has also the advantages of high turnover rate and relatively small size (Mw = 140.000). The selection of suitable substrates for the electrochemical detection of alkaline phosphates activity that fulfill the criteria given above is though rather limited. Phenyl phosphate [10], naphthyl phosphate [11–13], some ferrocenyl aminophenyl phosphate derivatives [14] and some indolyl phosphate derivatives [15] have been investigated for application in electrochemical immunosensing systems. However, these substrates have some limitations regarding the electrochemical properties of the product, such as high potential for detection [10], non-reversible electrochemical reaction and fouling of the electrode [12] or that parent compound is not electrochemically inactive at potential that can be used for detection [11].

Aromatic 1,4-dihydroxy and 1,4-hydroxy-amine derivatives can be converted to the corresponding quinones or iminoquinones in a reversible electrochemical reaction. Substrates which can be enzymtically converted to produce this type of electrochemically active compounds have been used in immunoassays with electrochemical detection and for immunosensors. Kulys et al. [16] and others [17,18] have reported 4-aminophenyl phosphate (4APP) as a suitable substrate when alkaline phosphatase is used as labeling enzyme and we have reported that 4-aminophenyl-β-L-galactopyranoside [19] can be used as substrate when glactosidase is used as labeling enzyme. 4-Aminophenol (4AP) which is produced by the enzyme reactions, has favorable electrochemical properties for application in biosensors. It can be detected at potentials where the parent compound is electrochemically inactive, and it can be used in immunoreaction with high sensitivity [17,19]. We have also reported synthesis and application of 4-hydroxynaphthyl-1-phosphate as substrate for alkaline phoshpatase [20]. 1,4-Dihydroxynaphthol, the product of the enzymatic reaction, has lower redox potential than 4AP and is spontaneously converted to the 1,4-naphthoquinone in the presence of oxygen. This compound can be electrochemically detected through reduction current. Here we report further investigations of this type of compounds. We have now synthesized 4-aminonaphthyl-1-phosphate and investigated the electrochemical properties and it's potential application in electrochemical immunoassay systems.

2. Experimental

2.1. Reagents

The reagents used were: 4-amino-1-naphthol-HCl (4AN) and 10% palladium on activated carbon from Sigma Aldrich Chemical Company Inc. (USA) and di-*t*-butyl dicarbonate from ICN biochemicals (USA). All other chemicals were either of reagent or analytical grade. Biochemicals and immunochemical used were: Alkaline phosphatase (P-5521), Mouse IgG (I-5381), goat anti-mouse IgG (M-6898) and goat anti-mouse IgG alkaline phosphatase conjugate (A-1293) form Sigma Aldrich Chemical Company Inc.

2.2. N-t-butoxycarbonyl-4-amino-1-naphthol (Boc-4AN)

4-Amino-1-naphthol HCl (4 g, 18.4 mmol) was dissolved in 40 ml of methanol that had been deoxygenated and saturated with nitrogen. Di-*t*-butyl dicarbonate (8.8 g, 40.5 mmol) and triethylamine (3.6 ml, 25.8 mmol) were then added and the solution stirred overnight, under nitrogen stream. The dark oily residue obtained after evaporation of the solvent was purified by silica gel column chromatography (Silica gel 60 form Merck, Germany) using chloroform:ethyl acetate (90:10) as eluent. The isolated product recrystalized from chloroform to give Boc-4AN as white crystals (1.7 g, 36%), mp 184–185 °C. ^{1}H NMR (250 MHz, DMSO): δ 1.45 (s, 9H), 6.81 (d, 1H, J = 8.1 Hz), 7.21 (d, 1H, J = 8.1 Hz), 7.40–7.53 (m, 2H), 7.84 (d, 1H, J = 7.7 Hz), 8.13 (d, 1H, J = 8.4 Hz), 8.81 (s, 1H), 10.08 (s, 1H). ^{13}C NMR (60 MHz, DMSO): δ 28.27, δ 78.38, δ 107.31, δ 122.25, δ 122.90, δ 123.85, δ 124.55, δ 124.74, δ 125.14, δ 126.00, δ 130.29, δ 151.12, δ 154.74.

2.3. Pyridinium N-t-butoxycarbonyl-4-amino-naphthyl-1-phosphate (Boc-4ANP)

Eight hundred and fifty milligram of Boc-4AN (3.3 mmol) was dissolved in 10 ml of pyridine. The solution was cooled to 0 °C and phosphoroxychloride (8.0 ml, 8.6 mmol) was added dropwise. Pyridinium chloride, which precipitated as a white material from the solution, was removed by filtration after 6 h reaction. The solution was then poured slowly into 15 ml of water and the solvent removed by evaporation. The residue was dissolved in EtOH/acetone (80/20) mixture. Precipitation was achieved by adding water and the product was

filtrated from the solution. The final compound was isolated as pyridinium salt, (0.68 g, 50% yield), mp 235–238 °C. ^{1}H NMR (250 MHz, DMSO): δ 1.44 (s, 9H), δ 7.35 (dd, 1H, $J = 8.3$ Hz, 1.4 Hz), δ 7.43 (d, 1H, $J = 8.2$ Hz), δ 7.52–7.57 (m, 5H), δ 7.87–8.01 (m, 2H), δ 8.07–8.16 (m, 1H), δ 8.64 (d, 1H, $J = 5.9$ Hz), δ 9.13 (s, 1H). ^{13}C NMR (60 MHz, DMSO): δ 28.2, δ 78.83, δ 114.59 (d, $J = 2.9$ Hz), δ 121.87, δ 122.25, δ 123.04, δ 124.74, δ 125.92, δ 126.25, δ 126.82 (d, $J = 5.9$ Hz), δ 129.47, δ 130.01, δ 138.60, δ 144.67 (d, $J = 6.8$ Hz), δ 147.81, δ and 154.30.

2.4. 4-amino-1-naphthylphosphate (4ANP)

Three hundred and sixty milligram of Boc-4ANP (0.86 mmol) was stirred into $CHCl_3$ which was kept at 0 °C on an ice water bath. Four milliliters of trifluoroacetic acid were then added dropwise to the solution. After 2 h reaction the solution removed by evaporation under reduced pressure. The white solid was collected and washed with 5 ml of MeOH by filtration. One hundred anf eighty-nine milligram of 4ANP (91% yield) was obtained by this procedure, mp 249–251 °C. ^{1}H NMR (250 MHz, D_2O, NaOH): δ 6.90 (d, 1H, $J = 8.1$ Hz), δ 7.27 (dd, 1H, $J = 8.1$ Hz, 1.51 Hz), δ 7.54–7.59 (m, 2H), δ 7.90–7.94 (m, 1H), δ 8.27–8.31 (m, 1H). ^{13}C NMR (60 MHz, D_2O, NaOH): δ 113.68, δ 117.34 (d, $J = 3.00$ Hz), δ 123.67, δ 125.81, δ 127.52, δ 128.07, δ 128.19, δ 130.30 (d, $J = 5.2$ Hz), δ 138.61, δ 145.82 (d, $J = 6.7$ Hz)

2.5. Cyclic voltammetry

Cyclic voltammetry (CV) was done with a HAP 151 Potentiostat/Galvanostat (Hokuto Denko Ltd., Japan), using a glassy carbon working electrode, Ag/AgCl reference in saturated KCl solution and Pt counter electrodes. The compounds were dissolved deoxygenated 0.1 M Tris, pH 9.0, 0.5 mM $MgCl_2$ (Tris).

2.6. Amperometric flow injection analysis

We used a liquid chromatography system, and a Pulsed Amperometric Detector (PAD-2) from Dionex (USA) with a glassy carbon working electrode and Ag/AgCl reference cell. Water 501, HPLC pump (Millipore, USA) was used in the system with the flow of 0.3 ml/min. A 0.1 M Tris buffer, pH 9.0 or 0.1 M phosphate buffer (PB), pH 7.0, was used as carrier solution. To increase the backpressure in the system a CarboPac PA1, guard (10–32), P/N 43096 S/N 43096 column was placed between the pump and the 20 μl sample injection loop. The PAD system was set to constant potential and the current signal generated by electrochemical reaction of the sample was recorded with an Omni Scribe, series D5000 recorder (Bausch & Lomb). The peak currents were determined and corrected by subtracting the residual current signal obtained when pure buffer was injected. The response time on PAD-2 was set on 0.3 s.

2.7. Investigation of the alkaline phosphates reaction

The enzyme and substrate (4ANP) were diluted to a suitable concentration in the Tris buffer. The reaction was initiated by mixing 150 μl of the enzyme solution with 150 μl of the substrate solution in an immunoassay plate well. The reaction was stopped after 30 min incubation at room temperature (19–21 °C), by adding 80 μl of pH 5.5, 0.4 M phosphate buffer (PB5.5). Sample was drawn from the stopped solution and injected into amperometric flow injection assay (AFIA) system using phosphate buffer as carrier solution.

2.8. Immunoassay for Mouse IgG with amperometric detection

A 96-well Nunc (Denmark) Maxisorp plate was coated with goat anti mouse IgG, diluted to 25 μg ml^{-1} in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6, for 1.5 h at room temperature. Unspecific binding was blocked by 0.5 h treatment with 5% casein in pH 7.5 0.15 M NaCl, 15 mM NaN_3, 0.1 M phosphate buffer (PBS). The plate was washed three times with 0.15 M NaCl, 15 mM NaN_3 (NACL). The mouse IgG standards, diluted into PBS containing 0.1% tween (PBS-Tween), were incubated for 2.5 h and the plate was then washed three times with NACL. The goat anti mouse alkaline phosphatase conjugate was diluted 1:2000 in pH 7.6 0.15 M NaCl, 2 mM ZnCl, 15 mM NaN_3, 4 mM MgCl, 25 mM Tris (Tris–NaCl) buffer containg 1% casein, and incubated on the plate for 1 h. The plate was then washed three times with NACL. Then 240 μl of the substrate solution (1 mM ANP in Tris buffer) was added to each well and the reaction stopped after 30 min by addition of 80 μl of PB5.5. Samples were drawn from the wells and analyzed by AFIA.

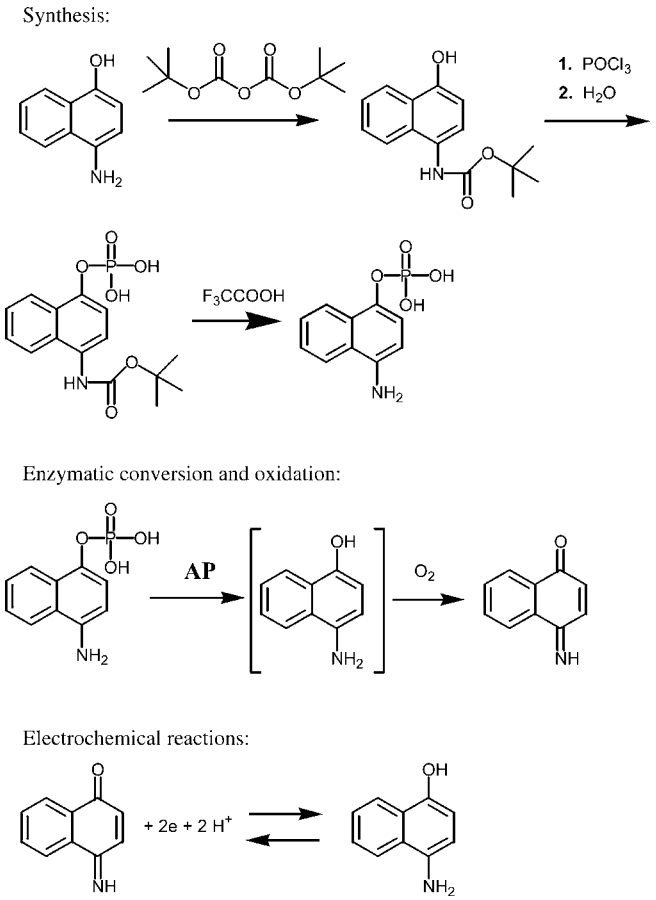
3. Results and discussion

3.1. Synthesis and purification

The synthesis procedure for 4ANP (Scheme 1) was comparable to what has previously been reported for 4-hydroxynaphthyl phosphate (4HNP) [20], except that we used *t*-butyloxy-carbonyl protection strategy rather than the benzyloxy-carbonyl protection group strategy. The presence phosphate group could be detected by the appearance of a coupling doublet in the ^{13}C NMR spectrum. The absence of any extra peaks in the NMR spectra indicated that product was free of any organic contaminants. 4-Aminophenylphosphate (4APP) was also synthesized by same procedure as previously reported [21].

3.2. Cyclic voltammetry

The cyclic voltammetry (Fig. 1a) of 4AN showed that electron transfer was rapid ($\Delta E = 64$ mV) and that the redox potential (E_0) at pH 9 was −200 mV versus Ag/AgCl.



Scheme 1. The chemical, electrochemical and enzymatic reactions.

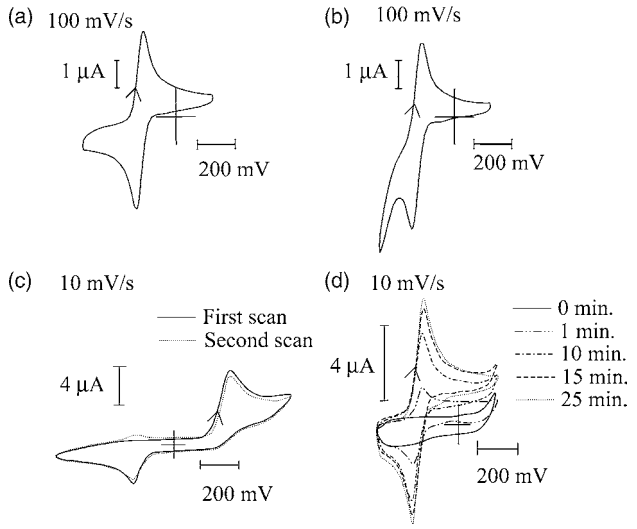


Fig. 1. Cyclic voltametry: (a) 0.1 mM 4AN in a nitrogen purged pH 9.0, 0.5 mM $MgCl_2$ 0.1 M Tris buffer (b) 0.1 mM 4AN in the same buffer saturated with air (c) 1 mM 4ANP in the same buffer purged with nitrogen (d) 1 mM 4ANP in the same buffer, before (0 min) and after addition of 0.02 U/ml of alkaline phosphatase. A glassy carbon working electrode and Ag/AgCl reference electrode were used in all cases and the scan rate was 10 mV/sec.

Under the same conditions the redox potential for 4AP was −25 and −309 mV for DHN. In the presence of air 4AN is oxidized to the corresponding iminoquinone. The peak anodic current decreased and the peak cathodic current increased when cyclic voltametry was performed in air saturated solutions (Fig. 1b). The anodic/cathodic current ratio was 0.7 for 100 mV s^{-1} scan rate and 0.6 for 20 mV s^{-1} scan rate. The ratio was 1.0 for nitrogen purged solutions. This showed that, when oxygen was present, there was significant oxidation of 4AN in the few seconds time required for one scan.

4AN, 4AP and DHN have similar electrochemical properties with relatively rapid electron transfer and they can also be reversibly converted from oxidized to reduced form. It is therefore possible amplify signals based on the on these compounds by electrochemical or enzymatic recycling [22–24] and furthermore they can be separately detected in a homogenous system due to differences in reduction potential.

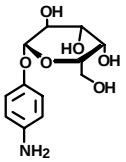
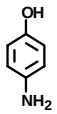
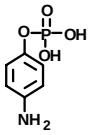
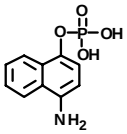
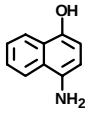
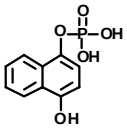
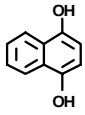
CV of 4ANP revealed an oxidation peak at 300 mV in the first forward scan (Fig. 1c). In the return scan and the second forward peaks at −240 and −160 mV could be observed, which is characteristic for the presence of 4AN in oxidized and reduced form. These observations were then consistent with the interpretation that 4ANP is oxidized at 300 mV to produce 1,4-naphthoiminoquinone (4NIQ, Scheme 1), the oxidized form of 4AN. The CV could also be used to confirm that 4AN is the product of enzymatic conversion of 4ANP by alkaline phosphatase (Fig. 1d).

The oxidation of 4ANP was observed at 500 mV above the redox potential for 4AN, thus detection of the product of the enzymatic reaction (4AN) would be possible at potential below 100 mV versus Ag/AgCl without any interference from the electrochemical reaction of 4ANP. The results of the electrochemical investigations of 1,4-aromatic hydroxylamine and 1,4-aromatic dihydroxy type of compounds are summarized in Table 1. From this table can obtain some information about the general properties of this type of compounds. The redox potential for 4AN is between what is observed for the phenyl analogue (4AP) and the dihydroxy analogue (DHN). In every case oxidation peak for the enzyme substrate was observed at approximately 500 mV above the redox potential for the corresponding electrochemically active product.

3.3. Detection of AN, enzyme kinetics and immunoassay

The 4AN concentration was determined by amperometric flow injection analysis (AFIA). Standard solutions of 4AN were made in Tris buffer and injected into the carrier solution. Various carrier buffers where investigated but no oxidation current could be observed. However, at potential below −100 mV and reduction current was observed. Thus, it could be established that 4AN is rapidly converted to the corresponding 4NIQ in Tris buffer. When Tris was used as carrier solution the blank signal, for injection of pure

Table 1
Properties of enzyme substrates, suitable for immunosensing with electrochemical detection, and their corresponding 1,4-aromatic products

Substrate	E_{ox} (mV)[a]	K_m (mM)	k_{cat} (s^{-1})	Product[b]	E_o (mV)[a]	$t_{1/2}$ (h)	Reference
 4APG	627 (pH 7.0)	0.18	94	 4AP	130	>4	[19]
 4APP	480 (pH 9.0)	0.23	2270		−25	0.5	This work
 4ANP	300 (pH 9.0)	2.5	1050	 4AN	−200	1.0[c]	This work
 4HNP	235 (pH 9.0)	ND[d]	ND	 DHN	−309	>4[c]	[20]

[a] Vs. Ag/AgCl reference electrode in 0.1 M phosphate buffer in case of 4APG and 4AP and Tris in the case of 4APP, 4ANP and 4HNP.
[b] Enzymatic conversion with alkaline phosphatase in the case of 4APP, 4ANP and 4HNP and with glactosidase in the case 4APG.
[c] For the corresponding naphthoiminoquinone and naphthoquinone.
[d] Not determined.

buffer, was about 50% of the signal for 50 μM 4AN standard. When 0.1 M phosphate buffer was used as carrier this ratio was 20%. With this buffer there was also less variation between injections. The PB buffer was therefore used as carrier solution in the present work. The reduction signal was detectable below −50 mV potential and it reached the maximum at −300 mV (Fig. 2). Some increase in the baseline current, due to reduction of H^+, is observed at this low potential. At −200 mV the current signal was 80% of the maximum signal at −300 mV but at the higher potential there was less variation between injections. The −200 mV potential was therefore used for subsequent investigations. A linear response to the injection of 4AN samples up to 150 μM concentration with 1500 nA current response (Fig. 3). The response decreased with time when 4AN was incubated in the Tris buffer, with 50% reduction in 1 h (Table 1). The stability depended on the buffer system. This time ($t_{1/2}$) was 2 h in pH 9, borate buffer. Like in the case of 4AP a much higher stability was observed pH 7, with $t_{1/2} = 23$ h. Slow oxidation of 4AN takes place at alkaline pH, whereas 4AN and DHN are immediately converted to the corresponding iminoquinone and quinone respectively. These compounds are not as susceptible to oxidation and should therefore be relatively stable at alkaline pH. However the investigation shows that naphthoquinone is more stable than the corresponding iminoquinone. Considering the stability of the of product of the enzymatic conversion of 4ANP, the reaction times were set to 30 min or less.

Investigation of the enzyme kinetics of the showed that the K_m value for the bulkier 4ANP substrate was more than 10

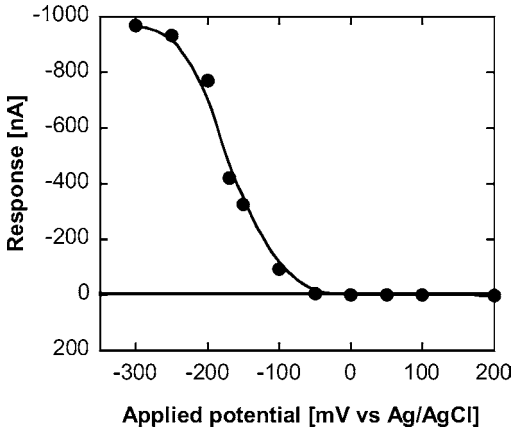


Fig. 2. Potential (Ag/AgCl reference electrode) vs. signal for 4AN in a 0.1 M phosphate buffer system with pH 7. Each point is an average of three injections of 0.1 mM, 4AN sample and the response is corrected for background.

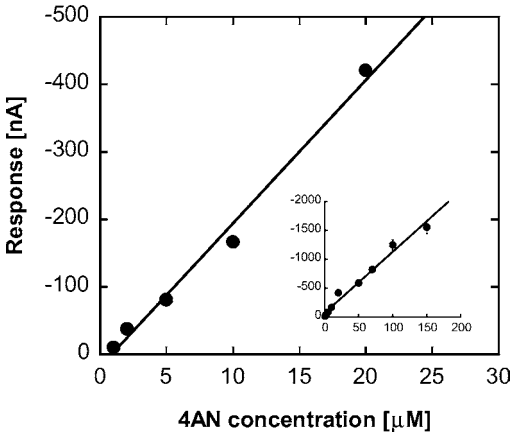


Fig. 3. Calibration curve for 4AN in AFIA. Each point is the average of four injections. The sample volume was 20 μl. The carrier solution was 0.1 M phosphate buffer, pH 7.0. The applied potential was −200 mV vs. Ag/AgCl.

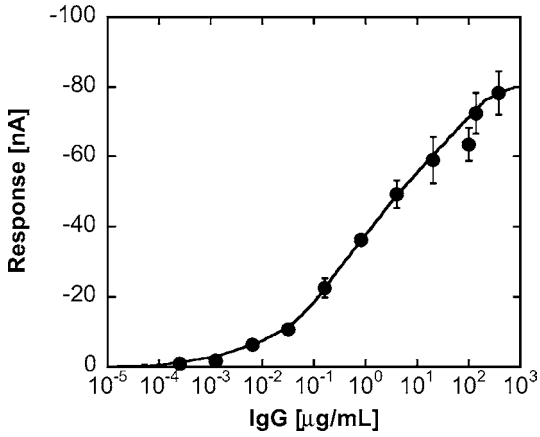


Fig. 5. Immunoassay for mouse IgG with amperometric detection. Each point is the average of four injections. The sample volume was 20 μl. The carrier solution was 0.1 M phosphate buffer, pH 7.0. The applied potential was −200 mV vs. Ag/AgCl.

times the value observed for the 4APP (Table 1). As would be expected there was less difference in the k_{cat} values. The k_{cat} for the alkaline phosphatase substrates were also 10 times higher than for the galactosidase substrate (4APG). Higher amplification can therefore be achieved in immunassay systems were alkaline phosphatase is used as labeling enzyme. The enzyme constants for 4HNP have not been determined.

Fig. 4 shows the calibration curve for alkaline phosphatase using 4ANP as substrate. The substrate concentration used was 1 mM. Although the reaction rate at this concentration was about 1/3 of the maximum reaction rate (V_{max}) the response was linear. The detection limit, with signal twice the standard deviation was 20 pM for 30 min reaction.

The application of 4ANP for amperometic immunoassay for Mouse IgG was investigated (Fig. 5). The 4ANP concentration was 1 mM and 30 min reaction time was used for the enzymatic amplification. Thus, 57 μg of 4ANP was used in each well The detection range was 0.01–100 μg ml^{-1} and the detection limit for s Mouse IgG in the immunoassay system was approximately 0.006 μg ml^{-1}.

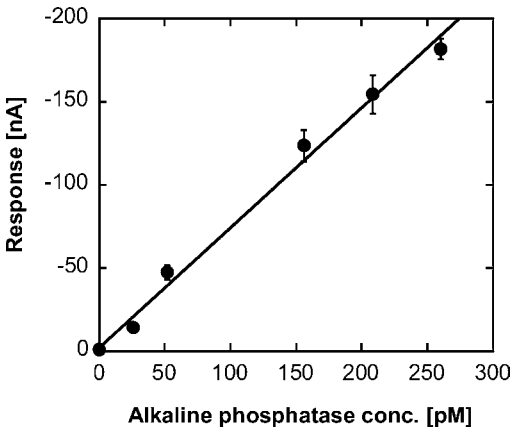


Fig. 4. Calibration curve for alkaline phosphatase determined by the AFIA method after 30 min reaction, with 1 mM 4ANP concentration. Each point is the average of three injections. The sample volume was 20 μl. The carrier solution was 0.1 M phosphate buffer, pH 7.0. The applied potential was −200 mV vs. Ag/AgCl.

4. Conclusion

4ANP can be used as a substrate for amperometric detection of alkaline phosphatase activity and is also suitable substrate for application in advanced and homogenous immunosensing systems. The product of the enzyme reaction can be detected at relatively low potential and there should therefore be minimal interference from any oxidizable components of biological fluids, such as ascorbic acid. Array detection system with 4APG and 4ANP would allow separate detection of galactosidase and alkaline phosphatase amplification based on the differences in redox potential of the products of the enzymatic reaction. Furthermore the air oxidation of 4AN, the product of the enzyme reaction, offers a novel approach to recycling of the product for electrochemical detection.

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