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Development of immunoassay for C-reactive protein with chronoamperometric detection

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Title (English)

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Abstract

Electrochemical immunoassays are a promising tool for fast and sensitive detection of biomarkers in blood. Detection is based on antibody-antigen interaction and quantitative measurement is accomplished by an enzyme reaction that generates a measurable current when a substrate is added. In this report, I present my work of developing an electrochemical immunoassay for detection of C-reactive protein (CRP) from plasma samples. The assay was based on a sandwich enzyme linked immunosorbent assay (ELISA) on disposable screen printed gold working electrodes. The best assay performance gave stable results in the range of 0.3-3 ng/ml CRP. The average current and reproducibility levels of each measurement varied between different experiment rounds, but a clear correlation between CRP-concentration and current levels was always shown. The results in this report give good prospects for further assay development.

Keywords

Electrochemical immunoassay, C-reactive protein, sandwich ELISA, screen printed electrodes

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Development of immunoassay for C-reactive protein with chronoamperometric detection

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Populärvetenskaplig sammanfattning

En biomarkör är en mätbar biologisk indikator som används för att påvisa ett visst biologiskt tillstånd. Biomarkörer har en stor betydelse inom sjukvården där de i hög utsträckning används för att diagnostisera patienter. Ett exempel på en biomarkör är C-reaktivt protein (CRP). Koncentrationen av CRP i blodet stiger drastiskt vid en inflammation, men förhöjda nivåer av CRP kan också vara ett tecken på risk för hjärt- och kärlsjukdom. Eftersom att CRP, precis som de flesta andra biomarkörer, finns löst i blodplasman så kan mängden CRP mätas via ett enkelt blodprov.

Många tekniker finns idag för att mäta förekomsten av biomarkörer. Det finns dock ett stort behov av snabbare och känsligare testmetoder som skulle bidra till att tidigare och mer exakta diagnoser kan ställas. En sådan metod skulle kunna vara elektrokemisk utläsning av immunoassays. I denna teknik används antikroppar, en typ av proteiner som binder till sitt antigen med hög specificitet. Antigenet kan till exempel vara en biomarkör. När antikropparna bundit till antigenet detekteras mängden antigen via en kemisk reaktion som avger ström. Denna ström är proportionell mot mängden inbundet antigen och mäts sedan med hjälp av elektroder. I detta arbete presenteras framtagandet av en elektrokemisk immunoassay för detektion av CRP.

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Contents

Abbreviations

1-NP	1-Naphtyl phosphate
4-APP	4-Aminophenyl phosphate
ALP	Alkaline phosphatase
BSA	Bovine serum albumin
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DPV	Differential pulse voltammetry
ELISA	Enzyme linked immunosorbent assay
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
PBS	Phosphate-buffered saline
SAM	Self-assembled monolayer
SPE	Screen printed electrode
SPGE	Screen printed gold electrode
SWV	Square wave voltammetry
TMB	3,3′,5,5′-Tetramethylbenzidine

1. Introduction

Sensitive testing and rapid diagnosis are of crucial importance when it comes to treating and preventing a wide range of illnesses, not least cardiovascular diseases (CVD)[1]. By measuring cardiac markers in blood one could prevent both decease and suffering among patients. C-reactive protein (CRP) has until recently been considered to be only a general biomarker indicating infections and other inflammatory events. Nowadays, CRP is proved to be an important factor also when predicting CVD[1]. Rapid and sensitive detection of biomarkers like CRP has not only the potential to enable earlier diagnosis and thereby safe lives, it can also save a great deal of money and time for the healthcare system. When resources are stretched, the need of discriminating between patients with life-threatening conditions and manageable ones is crucial.

Even if many of the recently developed techniques for biomarker detection have reported a great sensitivity, not many of them could easily be transferred to a point of care systems[2]. A lot of these test are also time-consuming, which in a case of CVD can be fatal since the time window of chance to treat the patient often is limited[2]. A way to circumvent these problems could be to use electrochemical immunoassays. These types of assays utilize enzyme-linked antibodies to electrochemically detect antigens with help of disposable electrodes. Recent publications have reported a limit of detection below 2.5 ng/ml CRP in plasma samples and results have been measured within minutes[2,3]. These assays also have the advantage of being small and easy to handle, making potential incorporation into public healthcare possible. Thanks to its sensitivity and quickness, the technique has the potential of improving modern healthcare.

However, electrochemical immunoassays are at this point rarely used for diagnostic purposes. Even though these types of assays show a great potential, the technique still suffers from drawbacks. The biggest issue among scientists and engineers struggling to develop electrochemical assays are problems with low reproducibility between the measurements (Professor Leif Nyholm, personal communication). There are many potential causes for these issues, which makes the development of a fully functional electrochemical immunoassay a potentially challenging task. Still, recent publications show that the task is attainable, which indicates a promising future for the technique [2,3].

1.1 Project description

Ginolis AB in Uppsala, Sweden is a company specialised in developing disposable test platforms for the medtech industries. Electrochemical immunoassays are of great interests for Ginolis since the company recently has developed other techniques which potentially can be integrated with these types of assays. The project is aimed to increase Ginolis' understanding of electrochemical immunoassays in order to determine whether this technique is a suitable candidate for future product expansions.

The project will include the design and assemblage of an electrochemical immunoassay. The design will be based on what is presented in recent literature. Because of its clinical relevance and relatively high abundance in plasma samples, CRP will be used as model analyte in this feasibility study.

1.1.1 Projects demands

- Detection of CRP in the range of 1- 200 µg/ml
- Assay of sandwich-type

1.1.2 Projects wishes

- Utilization of gold electrodes
- Higher sensitivity than what is acquired for CRP-detection

2. Background Theory

2.1 C-reactive protein

C-reactive protein (CRP) is a plasma protein that participates in the immune systems response to inflammation and tissue damage[2]. CRP is defined as an acute phase protein; its synthesis increases highly within hours after infection or tissue injury[1]. During this time, the CRP concentration in plasma can increase up to a 1000-fold[1]. This has for a long time been utilized for clinical purposes. Nowadays, CRP is also linked to cardiovascular disease (CVD)[2]. Small rises of CRP levels are associated with risk of upcoming major cardiovascular events. Both the American Heart Association and Centers for Disease Control and Prevention are recommending that patients with an intermediate risk of CVD should have their CRP levels measured[1]. For CVD, the clinical references for risk ranges from ≤ 1 µg/ml for low risk, 1-3 µg/ml for moderate risk and ≥ 3 µg/ml for high risk[2].

CRP is built up by five identical subunits which are symmetrically arranged around a central pore[4], see figure 1. The protein is therefore often described as a pentraxin[4]. Each subunit is around 23 kDa in size and is folded into two antiparallel beta sheets, formed into a so called jelly roll topology[4]. Each subunit has a binding site for phosphocholine, which is a phospholipid intermediate that takes part in the immunologic response chain[4]**.** The binding site consists of two calcium ions placed next to a hydrophobic patch[4].

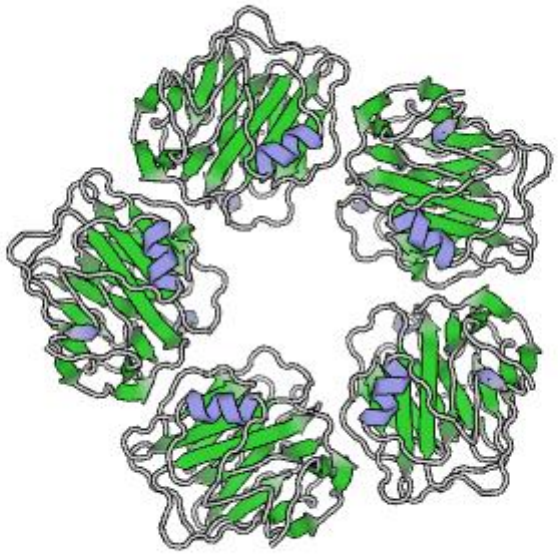


Figure 1. Physical structure of human C-reactive protein. The protein is composed of five identical subunits which consist of anti-parallel beta sheets showed in green and one alpha helix showed in blue. Copy right free, PDB ID:1B09, Thompson *et al*., 1999.

2.2 ELISA

2.2.1 General technique

The enzyme linked immunosorbent assay (ELISA) is a biochemical method used to detect and quantify proteins or other antigens in complex mixtures with the help of antibodies. The technique was first presented in 1971 and it has become widely used since its introduction[5]. The possibility to raise antibodies against almost any target molecule combined with the sensitivity and simplicity of the technique are some of the reasons for its popularity. Many variants of ELISA have been developed[5], however, all variants share the same basic steps:

1. **Coating/Capture**: The antigen is immobilised to a solid support. Normally, the antigen is placed in polystyrene microplate wells[5]. In this direct immobilisation, the adhesion of the antigen to the plastic surface occurs through passive adsorption[4]. Indirect immobilisation of the antigen can also be performed through the use of antigen-specific capture antibodies which are immobilised to the surface of the well before the antigen is added[5].
2. **Plate blocking:** After the plate has been washed, a non-reactive protein is added to cover any unsaturated plastic[5]. This is done to avoid any unspecific binding of proteins or antibodies in later steps.
3. **Detection:** An enzyme-conjugated antibody targeting the antigen is added to the well. It is also possible to use a secondary antibody[5]. In this case, the first antibody, called primary, is non-conjugated and binds to the antigen. A conjugated, secondary antibody is then added[5]. This antibody will bind to the Fc-region of the primary antibody[5].
4. **Signal measurement:** Upon addition of the appropriate enzymatic substrate, the chemical reaction will induce a measurable signal which is proportional to the amount of bound antigen in the sample[5]. The signal is most often a colour change which can be measured by spectrophotometry[6].

2.2.2 Sandwich ELISA

In the sandwich ELISA approach, the antigen is immobilised by a capture antibody[5]. After the addition of the detection antibody, the antigen becomes "sandwiched" between two antibody layers. This type of ELISA is considered both sensitive and robust; the specificity increases greatly since the antigen must be recognized by two different antibodies for signal to occur[5,7], see figure 2. Another advantage with this variant is that only the antigen becomes immobilised rather than the whole set of proteins in the sample[5]. This is especially useful when working with complex samples.

2.2.3 Direct and indirect detection

Direct detection, where the detection antibody is labelled with a signalling molecule, is generally faster than the indirect detection[5]. The indirect detection involves a labelled secondary antibody which binds to the primary antigen-binding antibody, see figure 2. The use of an indirect approach has the advantage of signal amplification due to multiple bindings of labelled antibody per antigen molecule[7]. A disadvantage of the indirect approach is the risk of cross-reactivity with the capture antibody, which is eliminated when using the direct approach[5,7].

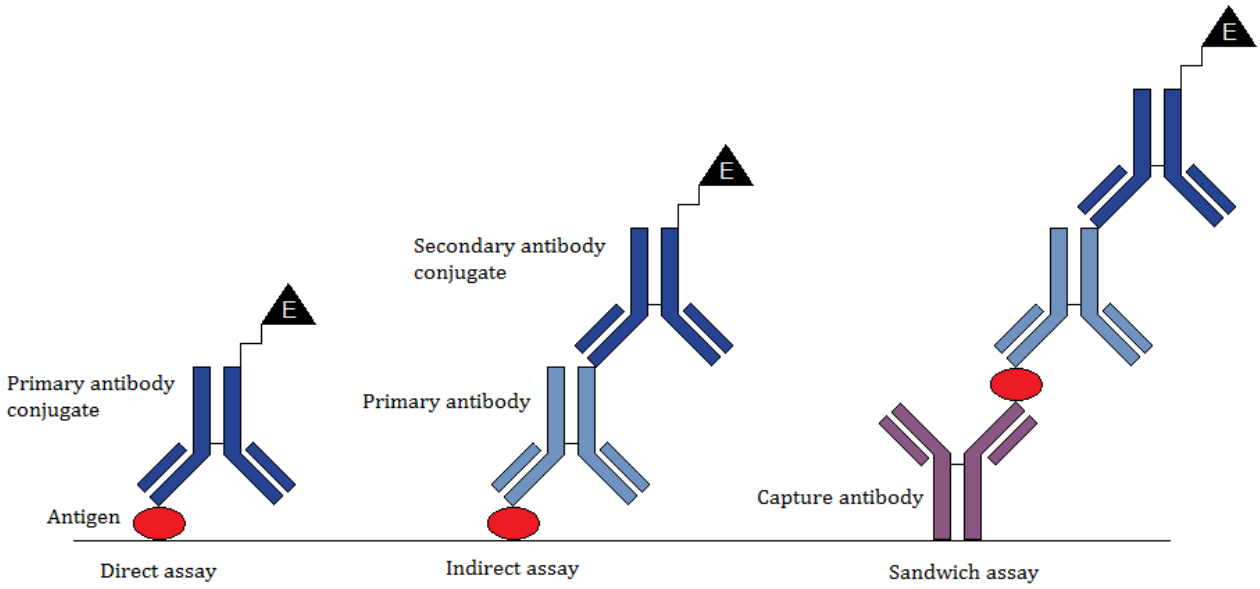


Figure 2. Three different ELISA approaches. In the direct assay, the primary antibody is labelled with an enzyme. In the indirect assay, a labelled secondary antibody binds to a non-labelled primary antibody. In the sandwich approach, the antigen is immobilised to a capture antibody, leaving the antigen "sandwiched" between two antibody layers after incubation with the detection antibody. Modified from "Overview of ELISA", 2015.

2.2.4 Competitive ELISA

In the competitive ELISA approach, two versions of the antigen are used[7]. The antigen in the sample is mixed with a labelled version of the same antigen into the same well[7]. The well has been pre-coated with immobilised capture antibodies[7]. The two antigens will then compete for the binding of the limited amount of capture antigen[7]. This will give a reversed relationship between the amount of antigen in the sample and the label induced signal; the more unlabelled antigen in the sample, the weaker the signal. This ELISA technique is common to use when the antigen has only one epitope[7]. Few epitopes is normally due to small antigen size[7].

2.3 Electrochemical immunoassays

2.3.1 Overview

The technique behind electrochemical immunoassays is very similar to a traditional ELISA. The major difference is the measurement step; the enzyme reaction will generate a measurable current instead of a colour change as in ELISA. When developing electrochemical immunoassays, it is most common to use the sandwich method for antigen capture[6]. The capture antibodies are in the electrochemical approach therefore immobilised to a working electrode instead of polystyrene microplate wells[6], see figure 3. The next step of the procedure is blocking. Here, any unsaturated parts of the electrode surface are covered with a non-reactive protein to prevent any unspecific binding of proteins or antigen. The antigen is then added to the assay. This is followed by the addition of enzyme-conjugated detection antibodies. Upon the addition of an appropriate substrate under a constant electric potential, the enzyme reaction will generate an electroactive product[6]. The current arising from the reaction can be measured by voltammetric or amperometric techniques[6].

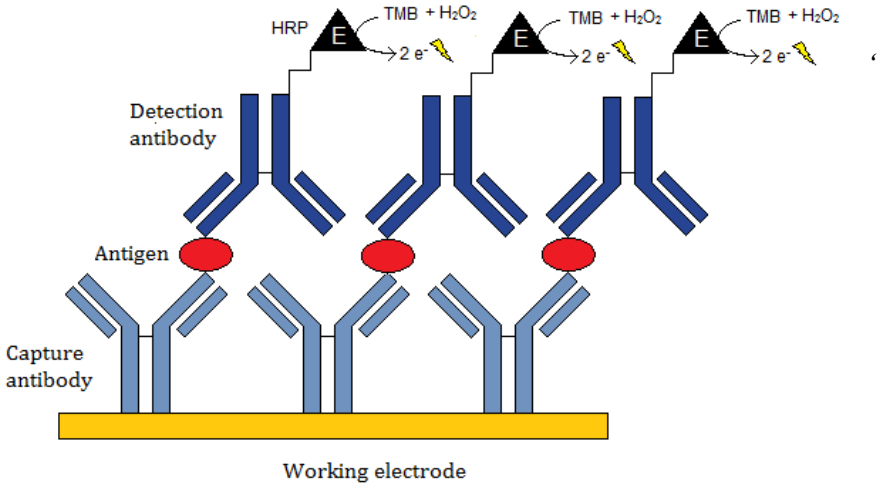


Figure 3. Electrochemical immunoassay of sandwich type. Electrochemical immunoassays are very similar to common ELISA; the first step includes immobilisation of capture antibodies to a working electrode. Unsaturated parts of the electrode are blocked and the antigen is then added. This is followed by the addition of enzyme-conjugated detection antibodies. Upon the addition of the appropriate enzyme substrate, the chemical reaction will generate a detectable current. In the figure, the enzyme is represented by HRP (Horseradish peroxidase) and the substrate is represented by 3,3′,5,5′-Tetramethylbenzidine (TMB) and H_2O_2. The working electrode will act as a sensor surface. The electrode is connected to a potentiostat, which measures the current.

2.3.2 Electrochemical immunoassay vs. common ELISA

Even though ELISA is a popular and rather simple analytical method to use for quantification and detection of proteins or other antigens, the technique suffers from several drawbacks. This includes e.g. the fact that a minimum sample volume is required to accomplish a certain level of performance as well as the risk of getting false signals from complex coloured enzyme products[6]. Big and power-intense equipment is often also needed for common ELISA techniques[6]. Electrochemical detection offers many advantages compared to ELISA; the measurement step is generally faster, the potential limit of detection is lower and the sensitivity of the method is not dependent on sample volume[3]. The possibility to miniaturize electronics also offers great possibilities to work with minimal sample volumes as well as making the measurement step simpler compared to common ELISA[6]. Low cost and large scale production of electrodes gives a great advantage for high throughput analysis[6].

2.3.3 Enzymatic labels and substrates

An important step when developing an electrochemical immunosensor is the choice of enzyme and substrate. Just as in ELISA techniques, horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are the two most used enzymes[6]. This is mainly due to the fact that these enzymes are very easy to obtain and have a long history of application within common ELISA[6]. However, the most essential feature of an enzyme used in electrochemical techniques is the ability to produce an electroactive product which can be measured through e.g. voltammetric or amperometric techniques. This applies for both the HRP and the ALP enzyme[6].

HRP and other peroxidases catalyse the oxidation of a wide range of substrates, e.g. different forms of benzidine and other aromatic amines[8]. The by far most used substrate for HRP is 3,3′,5,5′-Tetramethylbenzidine (TMB) together with H_2O_2[6]. In contrast to other benzidine compounds, TMB is not cancerogenic[8]. HRP-catalysed oxidation of TMB will result in a

diimine product caused by a two-electron transfer[8]. The H_2O_2 will act as an oxidation agent in the reaction[8]. The reaction is shown in figure 4.

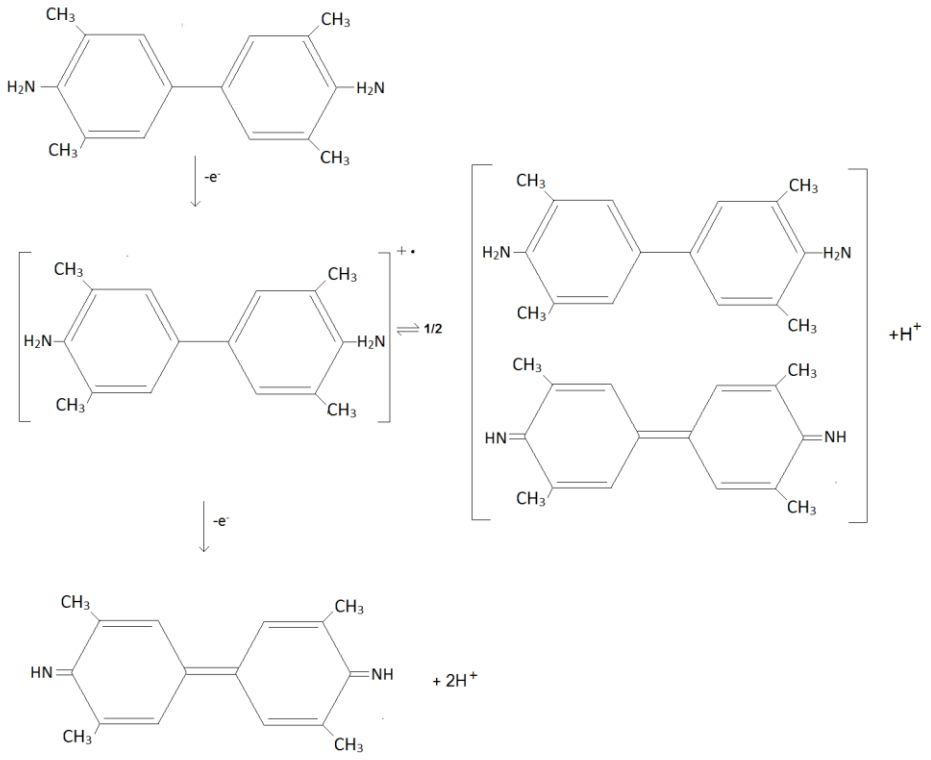


Figure 4. The HRP catalysed oxidation of TMB. The figure shows the horseradish peroxidase-catalysed 2-electron oxidation of 3,3′,5,5′-Tetramethylbenzidine. The two-step reaction will end in the production of a diamine. Modified from Josephy *et al.*, 1982.

An advantage of using HRP instead of ALP is the fact that peroxidases are small molecules (~40 kDa), which means that HRP rarely will cause any steric hindrance in the interaction between the antigen and antibody[9]. HRP-conjugated antibodies are also more commercially available than ALP-conjugates antibodies.

The ALP enzyme can hydrolyse a great range of different phosphate esters[9]. Two common substrates for ALP are 1-naphtyl phosphate (1-NP) and 4-Aminophenyl (4-APP)[9]. Upon hydrolysis, these substrates will turn in to their electroactive forms; 1-napthol and 4-aminophenol, respectively[9]. The electroactive forms will undergo oxidation under application of a small voltage[10]. The reactions are shown in figure 5.

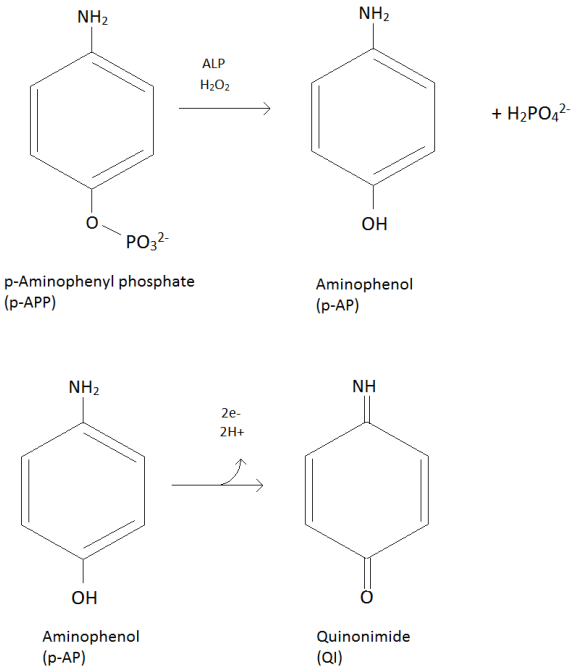


Figure 5. The ALP-hydrolysis of p-aminophenyl phosphate and the oxidation of the hydrolysed aminophenol. Alkaline phosphatases will cause hydrolysis of phosphate esters. The substrate p-aminophenol will hydrolyse to aminophenol, which can undergo a 2-electron oxidation to quinonimide. Modified from Yin *et al.*, 2011.

One of the ALP drawbacks is its size; ALP is approximately twice the size of HRP (~86 kDa)[9]. This will give a risk of causing steric hindrance in closely packed antigen-antibody interactions[9]. Another disadvantage with using ALP is the need of specific buffers; the enzyme will be inactivated by inorganic phosphates, acidic pH and chelating agents[9].

2.3.4 Electrodes

Electrochemical assays utilize a three-electrode system. The system comprises a working electrode, a reference electrode and an auxiliary electrode[11]. The working electrode, to which the capture antibodies are immobilised, acts as the sensor surface during the measurements[4]. The function of the reference electrode is to establish an electric potential which will act as potential reference during the measurements[11]. The reference electrode must therefore have a known and stable potential[6]. The function of the auxiliary electrode is to provide a circuit for the current arising from the chemical reaction occurring at the working electrode[11]. The auxiliary electrode is sometimes called counter electrode. The only requirement for this electrode is that it must be an electrical conductor[11]. The current arising from the enzymatic reaction is then measured by a device called potentiostat through amperometric or voltammetric techniques[11].

The choice of electrodes is essential when developing an electrochemical immunoassay. The electrodes will affect the sensitivity of the assay and the possibility to utilize different antibody immobilization techniques[6]. For the electrochemical assays to be of practical use, the electrodes must be disposable. If conventional, reusable electrodes would be used, these would have to be washed and cleaned very carefully several times between each measurement[6]. This would be time consuming and require a lot of chemicals. Conventional

electrodes would also have to be immersed in a large volume of buffers and other reagents[6], which would be expensive and also require a lot of reagents. Another disadvantage with using the same electrode more the once is the risk of surface fouling and carry-over effects[12]. Conventional electrodes are though very good from an electrochemical point of view[6] and can therefore be used for proof of principle, but not for optimized immunosensors.

The most common type of electrodes used in electrochemical biosensor-applications is screen printed electrodes (SPEs)[6]. These electrodes are produced by printing different types of "ink" onto a planar plastic or ceramic support[6]. The "ink" is often composed of inert metals or different versions of carbon, mixed with a binding agent[6]. SPEs have a low production cost and are possible to mass produce[6]; the usage of SPEs is therefore very cost efficient. The electrodes are commercially available, but they can also be made rather easily. Another great advantage with SPEs is the small size of the electrodes, which enabled all the analytical steps to be performed in one drop.

Gold is often the preferred material for the working electrode since the material has proven to be the superior material in terms of both immobilisation of capture antibodies and generation of signal[2]. The stability, chemical inertness, good electrical conductivity and the corrosions resistance is what makes gold and ideal transducer material[12]. The fact that gold is easily modified also makes it an ideal candidate for covalent immobilisation of the capture antibodies or other molecules[12]. Due to its stable electrode potential, silver/silverchloride is most often used as reference electrode[6]. The material of the counter electrode has the least requirements, the most common materials for this electrode are carbon and platina[6].

2.3.5 Immobilisation of capture antibody

In contrast to ELISA, the immobilisation of the antigen to solid support greatly affects the sensitivity of the measurement when working with electrochemical detection[6]. This is due to the fact that the measurement step in the electrochemical immunoassay takes place at the interface between the electrode and the working solution were the enzymatic reaction occurs[6]. In common ELISA, the measurement step occurs in a homogenous solution through the use of absorbance measurement at the selected wavelength[6]. The immobilisation of the capture antibody in common ELISA is therefore only important in the sense of the interaction between the antibody and antigen, but does not affects the measurement step itself[6].

Passive adsorption

The most simple, straight forward and common immobilisation technique used when working with electrochemical immunosensors is passive adsorption. This approach is very similar to the one used in common sandwich ELISA; the capture antibodies are simply added to the working electrode surface. Upon incubation, the antibodies will be adsorbed to the surface through electrostatic interactions[6].

The advantage of immobilising the recognition complex directly onto the electrode surface is that it will favour the diffusion of the electroactive enzyme products on to the electrode[6], which act as the sensing surface. The more electroactive products sensed by the electrode, the more sensitive the technique. However, the immobilisation of antibodies directly onto the surface will widely affect the interaction between the antibody and antigen[6]. Use of passive adsorption will lead to uncontrolled orientation and random placement of the capture antibodies[6]. This will give non-optimal interaction between the antibody and antigen[6],

causing fewer interactions to occur and thereby compromising the sensitivity of the technique.

Self-assembled monolayers

A number of strategies have been developed in order to avoid the problem of random and uncontrolled orientation of the recognition element. The most common[6] approach is to create self-assembled monolayers (SAMs) by thiol chemistry. In this immobilisation technique, the capture antibodies are coupled to the highly structured monolayer[13], giving an optimal positioning of the capture antibody for the antigen interaction, see figure 6. Another advantage with SAMs is that it often also gives an optimal spacing between the capture antibodies and thereby to a well-behaving electrochemical surface[13].

Thiols are composed of a carbon-bounded sulfhydryl[13]. Sulphurs are known for its reactivity against noble metals such as gold, silver and platina[13]. Gold is the most used electrode material in SAM-utilizing electrochemical sensors[2]. This is because of the fact that gold and sulphur compounds make an exceptionally strong covalent bond[13]. The S-Au bond forms a densely packed, stable and highly ordered monolayer[13]. The strength of this covalent bond depends on the fact that both gold and sulphur has a "soft" nature[13], in this context meaning big and polarizable. The mechanism behind the monolayers is not yet fully understood[13]. However, it is believed that the covalent bond between gold and thiol compounds is formed by the charging and discharging of H_2[13]. The process of creating these monolayers is very simple; the layer forms itself spontaneously on the metal surface upon an open-circuit potential[13]. There are however a number of factors that can affect the quality of the monolayer:

- **Concentration of the thiol solution.** The formation of an ordered monolayer requires a rather dilute thiol solution. A high concentration of thiol favours multilayer formation[13].
- **Interactions between adsorbate molecules.** The compactness of the monolayers is dependent on van der Waals interactions between the aliphatic chains of the thiols[13]. Longer chains lead to stronger intermolecular interactions and therefore to more ordered and stable SAMs[13]. Short aliphatic chains lead to liquid-like layers[13].
- **The nature of the adsorbate**. As previously mentioned; the strongest binding is accomplished through the use of thiol compounds and gold[13].

The use of SAMs in electrochemical immunoassays requires a linking agent which at one end is connected to the thiol and at the other end contains a functional group which is connected to the capture antibody[13]. The functional group of the linking agent is most often a carboxylic acid or an amine[13]. The SAM is therefore said to be functionalized when the linking agent is attached[13]. The capture antibody can either bind directly to the functionalized thiol by covalent links or it can be attach indirectly by the help of a bridging molecule, which most often is a glutaraldehyde[6,13].

Despite the advantages mentioned, the use of SAMs in electrochemical immunoassays has some limitations. A requirement for all supplementary compounds used in electrochemical applications is the need of stability under a wide range of potentials[13]. Adsorbed thiols will be reductively desorbed at potentials more negative than -1.4 V[13]. This means that the sulphur at this potential will pick up an electron and the covalent thiol-gold bond will break. At potentials above -0.8 V, the adsorbed thiols will undergo an oxidative desorption[13]. In this case, the sulphur will lose an electron[13], giving the same effect on the thiol-gold bond as the reduction. The potentials at which the thiols undergoes oxidative or reductive desorption can however vary; the process is dependent on e.g. chain length[13]. Another limitation with SAMs is the fact that electrochemical immunoassays require a conductive interface where the electroactive products are formed and measured[13]. From this perspective, short aliphatic chains are preferable, since this will favour the electron transfer caused by the enzymatic reaction[13]. Short aliphatic chains of the thiol can however compromise the quality of the SAM, making the interface less stable[13]. The most used thiols for electrochemical application is cysteamine and 3-mercaptopropanoic[13].

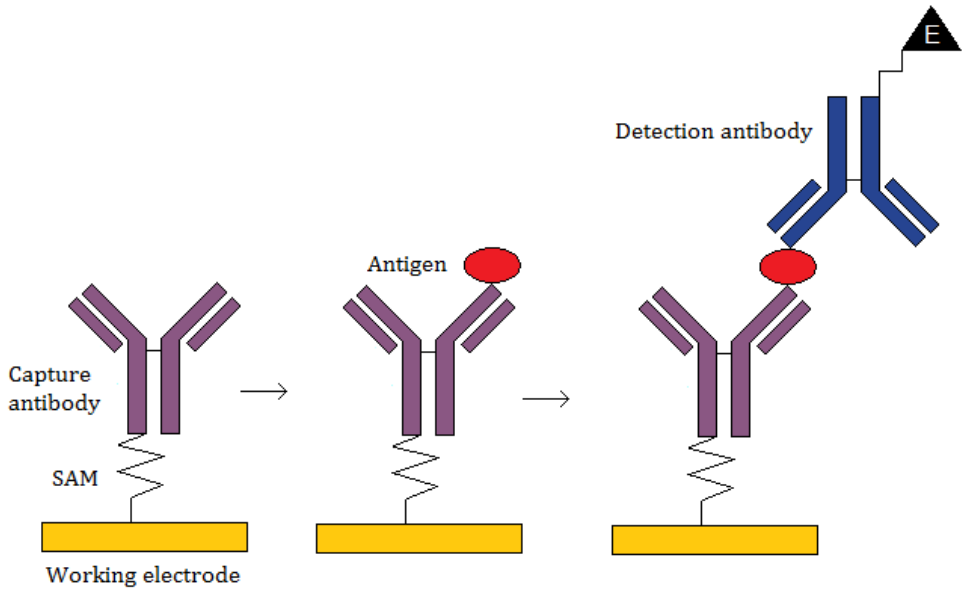


Figure 6. The formation of a self- assembled monolayer on a gold surface. The Y-shaped capture antibodies is immobilised to the electrode by the help of a self-assembled monolayer (SAM). This gives good orientation of the capture antibody, making the interactions with the antigen more optimal. The addition of the enzyme-conjugated antibodies and substrate follows the standard procedure

2.3.6 Electrochemical detection

Chronoamperometry, differential pulse voltammetry (DPV) and square wave voltammetry (SWV) are the most common detection techniques used when developing electrochemical immunoassays[11]. The principle behind all these techniques are the same: the current arising from the enzymatic reaction is measured as a function of time while an electric potential is applied between the working and the reference electrode[11]. In traditional chronoamperometry, the potential is stepped once at the start of the measurement and is kept constant during the rest of the measurement[11]. The potential step can also be excluded completely. In differential pulse voltammetry, the potential is stepped multiple times during the measurement[11]. In square wave voltammetry, the potential is swept linear in time[11]. DPV and SWV are especially useful when you want to investigate multiple potential areas. Instruments capable

of handling all these techniques are commercially available and can be accessible in both bench-top and portable forms[6].

3. Materials

3.1 Reagents

Carbonate-bicarbonate buffer (0.05 M, pH 9.6), phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4) in tablet form, phosphate buffered saline with 0.05% TWEEN©20 (PBS-TWEEN©20, 0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) in dry powder form, skimmed milk protein in powder form, bovine serum albumin (BSA, 200 mg/ml) and C-reactive protein (CRP, 2.1 mg/ml) from human plasma were purchased from Sigma- Aldrich (Stockholm, Sweden). Goat IgG, rabbit polyclonal anti-goat IgG HRP-conjugated antibody and substrate reagents (stabilized hydrogen peroxide and stabilized tetramethylbenzidine) were purchased from R&D Systems (Abingdon, UK). Mouse monoclonal anti human CRP [C6] antibody and mouse monoclonal anti human CRP [C2] HRP- conjugated antibody were purchased from Abcam (Cambridge, UK). NaCl and TWEEN©20 was a gift from Olink Bioscience AB.

3.2 Equipment

A µStat 200 Bipotentiostat and screen printed gold electrodes (220BT) with a gold working electrode (ø4 mm), gold counter electrode and a silver reference electrode were purchased from DropSens (Llanera, Spain), see figure 7. The software used for monitoring and analysing the results was DropView 200. Micropipettes were purchased from Eppendorf (UK).

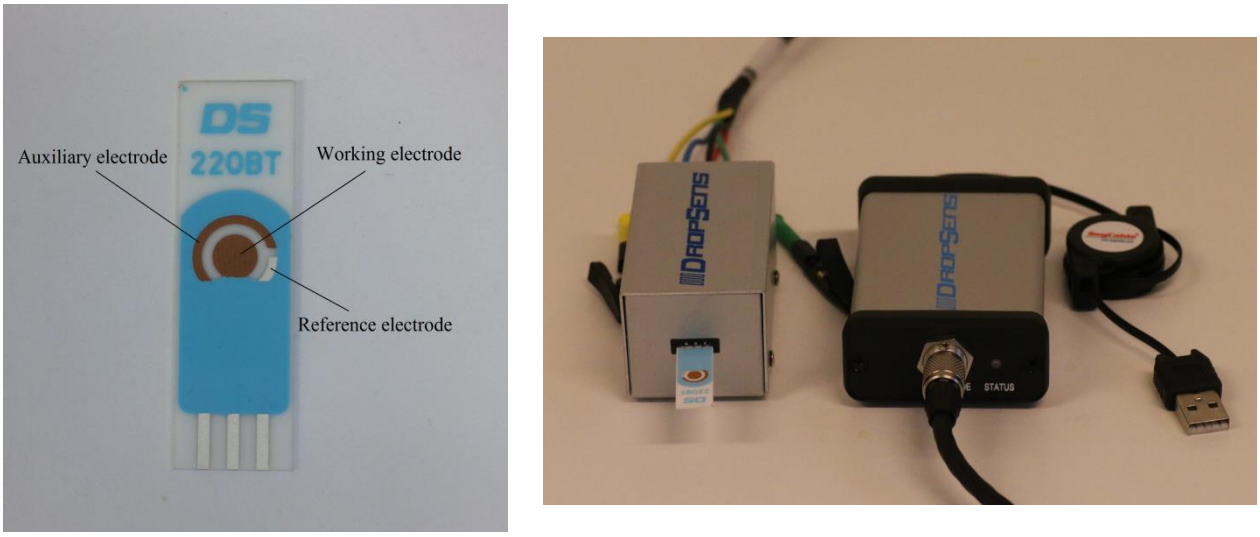


Figure 7. Equipment used for electrochemical detection. To the left: A screen printed gold electrode (220BT). To the right: A µStat 200 Bipotentiostat with a screen printed gold electrodes (220BT) inserted.

4. Methods

The experiments were adapted from a procedure first mentioned in an article by Salam and Tothill, 2009. In this study, an electrochemical immunosensor was used for detection of *Salmonella typhimurium*.

4.1 Equations

The standard deviation (σ) was calculated according to:

$$\sigma = \sqrt{\frac{\sum(x-\overline{x})^2}{(n-1)}}$$

Here, x is measured value, $\overline{x}$ is the mean value of the measurements and n is the number of measurements.

The calibration curves for the goat IgG and CRP experiments were constructed by a standard linear model:

$$y = kx + m$$

The coefficient of variation (CV) was calculated according to:

$$CV = \frac{\sigma}{|\overline{x}|}$$

4.2 Negative control

Screen printed electrodes were covered with 50 µl 1:10 skimmed milk in PBS or 50 µl 200 mg/ml bovine serum albumin in duplicates and were allowed to incubate for 2 h at 37°C in a humidity chamber. The electrodes were then carefully washed for 2 min in PBS-TWEEN 20 using a magnet stirrer and a 100 ml beaker. The electrodes were dried using gentle N_2-flow. 20 µl 1.0 µg/ml HRP-conjugated antibody diluted in 1:40 skimmed milk protein in PBS was added to the electrodes. Both HRP-conjugated rabbit anti-goat IgG antibody and HRP-conjugated anti-human CRP antibody were used. The antibody covered electrodes were incubated for 30 min at 37°C in a humidity chamber. After incubation, the electrodes were washed again in a similar manner as described above and dried using N_2-flow. The electrodes were connected to a µStat 200 bipotentiostat and the measurements started by adding 75 µl substrate reagent containing TMB and H_2O_2. The measurements were carried out for 200 s at -200 mV. Blank measurements were done by adding 75 µl substrate reagents to empty electrodes and measure the reaction under the same conditions as described above.

4.3 Experiments using goat IgG

Capture antibody immobilization was carried out using passive adsorption. The working electrode of the screen printed electrodes was covered with 10 µl 30 µg/ml capture antibody (goat IgG) diluted in 0.05 M carbonate-bicarbonate buffer. The antibody-covered electrodes were incubated for 2 h at 37°C in a humidity chamber. The electrodes were then carefully washed for 3 min in PBS-TWEEN 20 with using a magnet stirrer and a 100 ml beaker. The electrodes were dried using gentle N_2-flow. The surfaces of the electrodes were blocked with

50 µl 1:10 skimmed milk in PBS for 30 min at 37°C in a humidity chamber. All electrodes were then washed in a similar manner as described above and dried using N_2-flow. 20 µl of different concentrations (0.1, 0.3 and 1.0 µg/ml) of the HRP-conjugated detection antibody (rabbit anti-goat IgG) diluted in 1:40 skimmed milk in PBS were deposited to the electrodes in triplicates. The antibody covered electrodes were allowed to incubate for 30 min at 37°C in a humidity chamber. The electrodes were washed again in a similar manner as described above and dried using N_2-flow. The electrodes were connected to a µStat 200 bipotentiostat and the measurements were carried out for 200 s at -200 mV. The measurements started by the adding of 75 µl substrate reagent containing TMB and H_2O_2.

A calibration curve was constructed according to a standard linear model, where the concentration of the detection antibodies was plotted as a function of the mean current. The trend line equation was used to calibrate the detection antibody concentrations. The new, calibrated concentration values were used to calculate the CV and σ-values for all concentrations of the detection antibody.

4.4 Experiments using CRP

The experiments including CRP and anti-human CRP antibodies were carried out using a sandwich approach. The capture antibody immobilization was again done by passive adsorption. The working electrode was covered with 20 µl 30 µg/ml capture antibody (mouse monoclonal [C2] anti-human CRP) diluted in 0.05 M carbonate-bicarbonate buffer. The electrodes were incubated overnight at 4°C in a humidity chamber. The electrodes were then carefully washed for 3 min in PBS-TWEEN 20 using a magnet stirrer and a 100 ml beaker. The electrodes were dried using gentle N_2-flow. The surfaces of the electrodes were blocked with 50 µl 1:10 skimmed milk in PBS for 1 h at 37°C in a humidity chamber. The electrodes were washed in a similar manner as described above and dried using N_2-flow. To enhance the selectivity, the CRP and the HRP-conjugated detection antibodies (mouse monoclonal [C6] anti-human CRP) were premixed in 1:40 dilution of skimmed milk in 150 mM NaCl with 0.05 % TWEEN 20. The mix was incubated at room temperature for 1 h before being added to the electrodes. Different concentrations of both the CRP and detection antibodies were tested. The CRP-antibody complexes were deposited on the electrodes in triplicates and the electrodes were incubated for 2 h at 37°C in a humidity chamber. The electrodes were washed and dried in the same manner as described above. The electrodes were then connected to a µStat 200 bipotentiostat and the measurements started by adding 75 µl substrate reagent containing TMB and H_2O_2. The measurements were carried out for 200 s at -200 mV. Data was collected at 100 s.

A linear calibration curve was constructed for each round of measurement. The curves were created by plotting the CRP-concentrations as a function of the mean current values. The trend line equations were used to calibrate the corresponding CRP-concentrations.

4.5 System stability tests

System stability tests were done by adding a mix of 25 µl 0.1 µg/ml HRP-conjugated rabbit anti-goat IgG antibody and 50 µl substrate reagents on empty electrodes. The antibody was diluted in both 150 mM NaCl and PBS at different measurement rounds. Some of the electrodes were baked in 120°C for 30 min and rinsed with PBS-TWEEN 20 before the measurement. The reaction was measured for 200 s at -200 mV. Standard deviation and CV were calculated based on the measured data.

5. Results

5.1 Negative control

In order to test the binding specificity of the HRP-conjugated antibodies, the electrode surface was covered with BSA and skimmed milk protein in two separate experiments. The electrodes were then incubated with HRP-conjugated anti-goat IgG antibodies or HRP-conjugated anti-human CRP antibodies. Since the enzyme-conjugated antibodies only should bind to its antigen, the antibodies should be rinsed off during the washing step. A blank measurement with only substrate regents on empty electrodes was made to compare to. The blank measurements gave a small current signal of 0.0052 µA at 50 s. The measurement results from the electrodes incubated with BSA or skimmed milk protein and enzyme conjugated antibodies are showing very little or no difference in current compared to the blank measurements. The results can be seen below in figure 8 and exact current values can be seen in table 1 in the appendix.

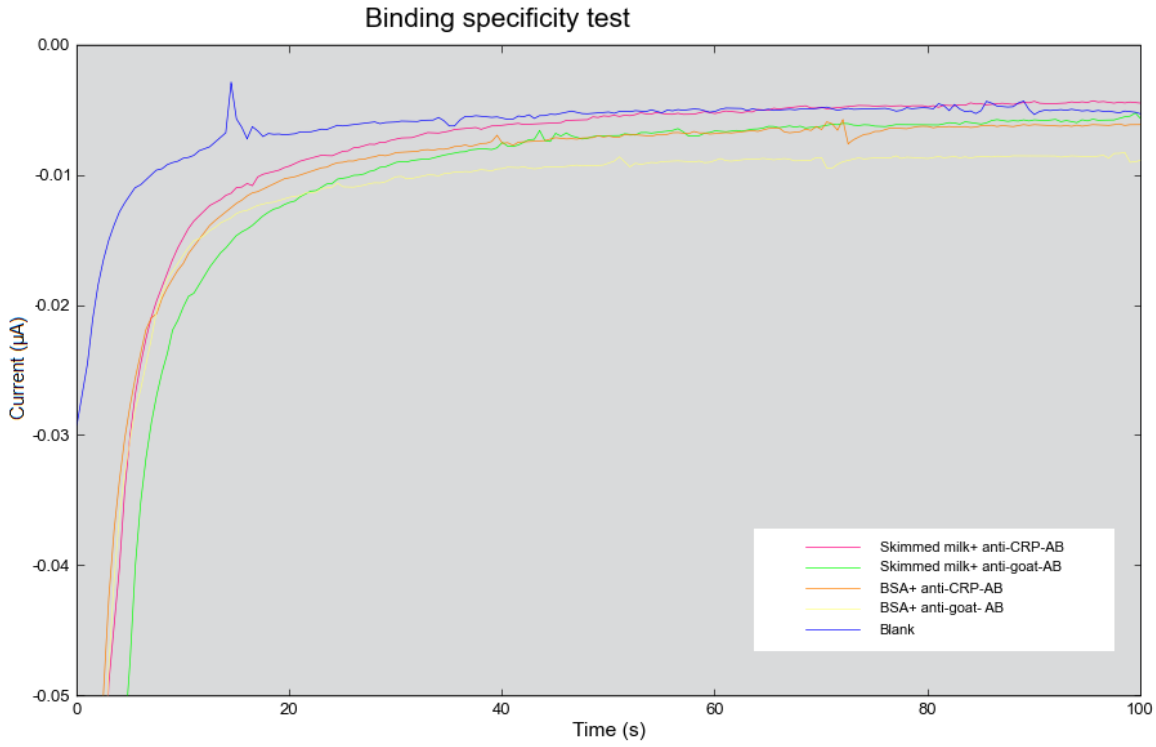


Figure 8. Binding specificity test of HRP-conjugated anti-goat IgG and anti-human CRP antibodies. The surfaces of screen printed electrodes were covered with skimmed milk protein or BSA before they were incubated with 1 µg/ml HRP-conjugated antibodies. A blank measurement with only substrate regent on empty electrodes was made to compare the measurement data to. The results are showing no significant current above the blank measurement from the electrodes incubated with BSA or skimmed milk protein and HRP-conjugated antibodies.

5.2 Experiments using goat IgG

The first assay experiments with antibodies immobilised to the electrode surface were carried out using only goat IgG and rabbit anti-goat IgG HRP-conjugated antibodies without any CRP. These experiments were done in order to develop a standard procedure for the

technique and to identify any potential problems before applying the system to CRP and anti-CRP antibodies in order to reduce the cost of the assay.

Goat IgG was immobilized to the electrode surface by passive adsorption. The same concentration of goat IgG was used in every experiment (30 µg/ml), but the HRP-conjugated anti-goat IgG antibody was varied (0.1, 0.3 and 1 µg/ml). To be able to investigate the reproducibility and to calculate the standard deviation (σ) and coefficient of variation (CV) for the assay, every experiment was performed in triplicates. The measurement results can be seen in figure 9. The graphs are showing distinct current levels when different concentrations of HRP-conjugated antibodies are used. The graphs are also showing relative little difference in current between the triplicates of the same concentrations of the HRP-conjugated antibodies. Precise current values can be found in table 2 in the appendix.

The measurement data was calibrated according to a linear calibration curve, and the calibrated values were used to calculate the CV and σ-values for the experiment. The calculation results and the calibration curve are presented in figure 10. The CV-values are very low for the experiments using 0.3 and 3 µg/ml HRP-conjugated antibody, but very high for the measurement using 0.1 µg/ml detection antibody. This is probably due to that this measurement is out of the measurement range. The fairly low σ-value still indicates a fairly low variation between the triplicates.

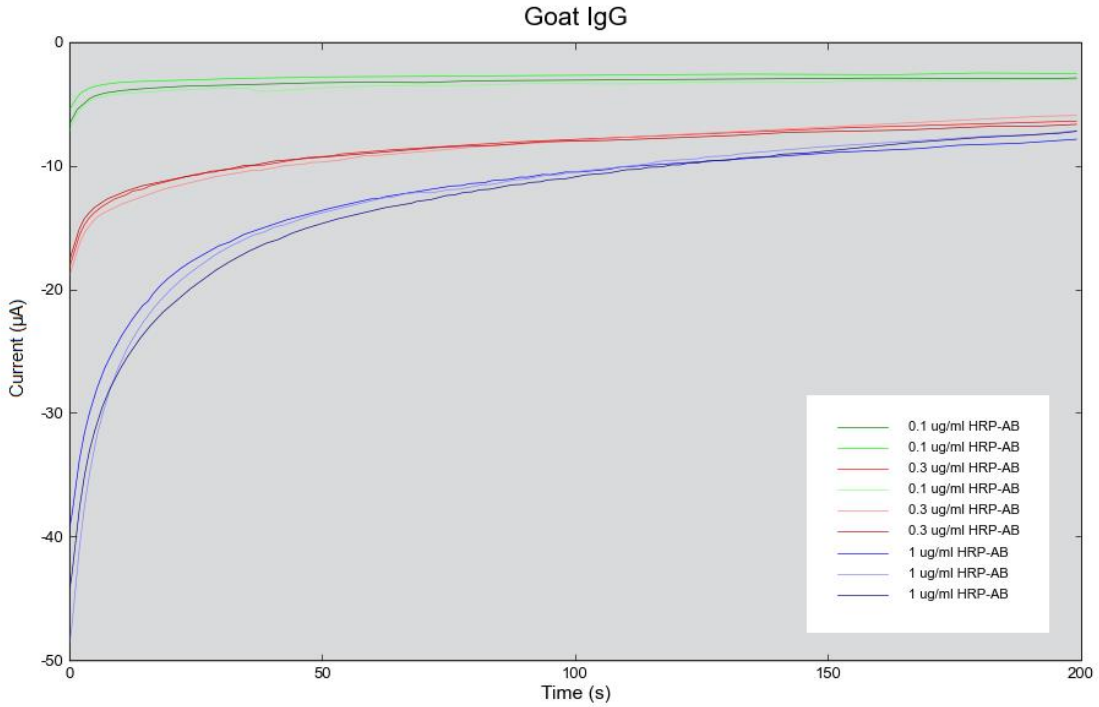
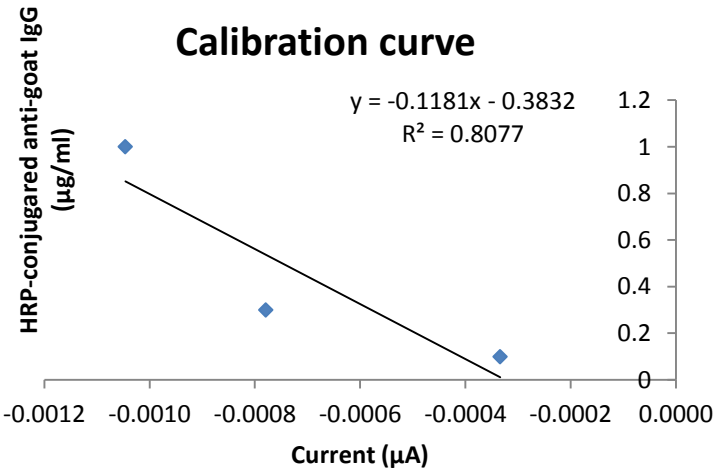


Figure 9. Measurement data from the initial electrochemical immunoassay using only immobilised goat IgG and HRP-conjugated anti-goat IgG antibodies. The same concentration (30 µg/ml) of goat IgG was immobilised by passive adsorption to nine screen printed electrodes. Three different concentrations of HRP-conjugated antibodies were tested (0.1, 0.3 and 1 µg/ml). Each concentration was measured in triplicates.



Added HRP-AB (µg/ml)	Average (µg/ml)	σ	CV
0.1	0.0113	0.0368	327.13%
0.3	0.5373	0.0222	4.14%
1	0.8511	0.0256	3.01%

Figure 10. Linear calibration curve, σ and CV for the experiment using 30 µg/ml immobilised goat IgG and 0.1, 0.3 and 1 µg/ml HRP-conjugated detection antibody. The concentrations of the HRP-conjugated anti-goat IgG antibodies are plotted against the measured mean current values. The trend line equation of the calibration curve was used to calculate the CV and σ, shown in the table to the right.

5.3 Experiments using CRP

Since the experiments using goat IgG gave fairly good and reproducible results, CRP was added to the assay. The electrodes were covered with 30 µg/ml capture antibody. To enhance the selectivity, the CRP and detection antibody was premixed before they were added to the electrodes. All electrodes were prepared in duplicates or triplicates. The concentration of the detection antibody was set to 0.5 µg/ml, but different concentrations of CRP were tested, ranging from 0.3 ng/ml to 3 µg/ml. The results were varying in terms of reproducibility; some experiments gave distinct, stable and almost identical data for each replicate. The assay at its best gave excellent results down to 0.3 ng/ml CRP. The measurement graph from this experiment can be seen in figure 11. The exact current values are presented in table 3 in the appendix. The calibration curve, CV and σ are shown in figure 12. The CV-values for this round of experiments were very low, indicating a very good reproducibility between each measurement replicate.

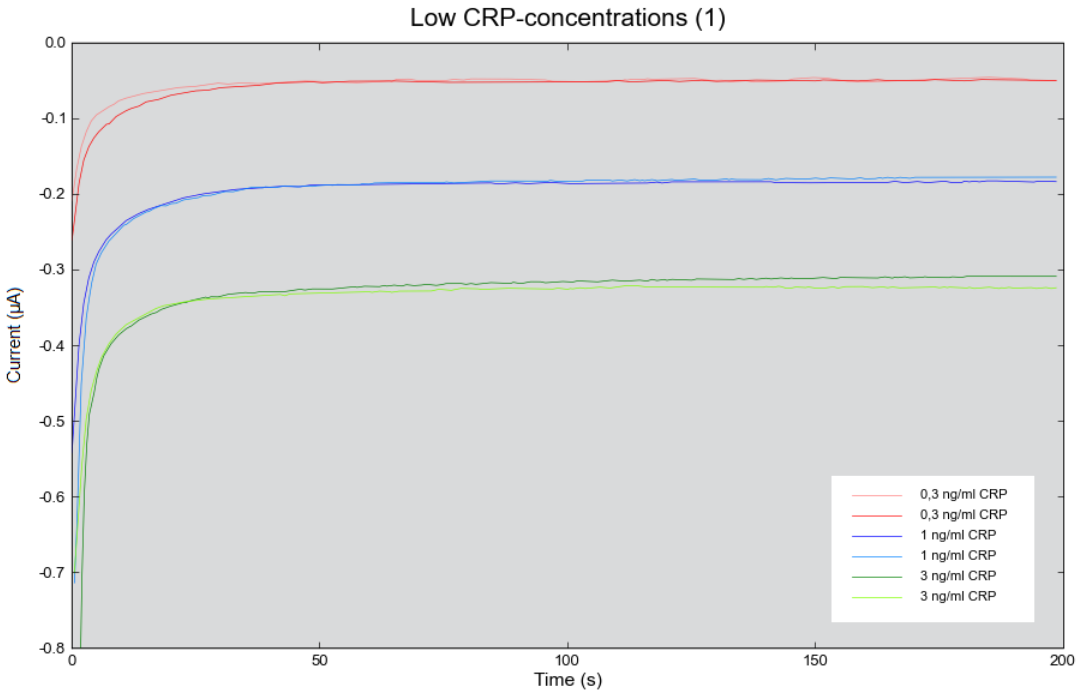
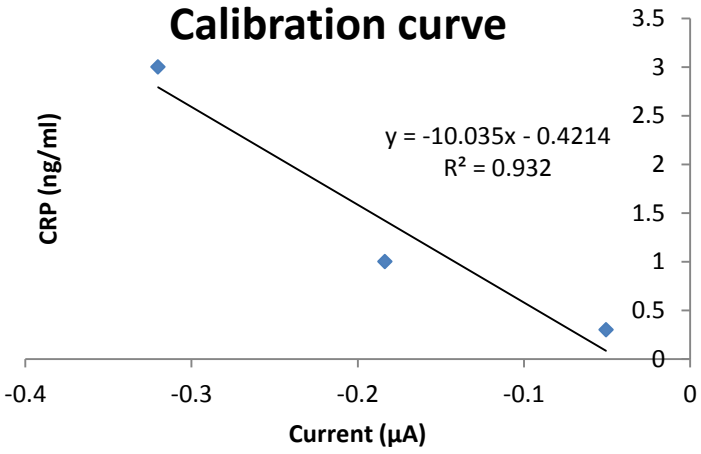


Figure 11. CRP-test with 30 µg/ml capture antibody, 0.5 µg/ml detection antibody and CRP-concentration of 0.3, 1 and 3 ng/ml, first try. Each electrode was prepared in duplicates. The results are very stable and show almost no difference in current between the replicate measurements.



Added CRP (ng/ml)	Average (ng/ml)	σ	CV
0.3	0.0864	0.0043	4.93%
1	1.4220	0.0199	1.40%
3	2.7919	0.0653	2.34%

Figure 12. Linear calibration curve, σ and CV from the first measurements with 0.3, 1 and 3 ng/ml CRP, 30 µg/ml capture antibody and 0.5 µg/ml detection antibody. CRP-concentrations are plotted against the measured mean current values. The trend line equation was used to calibrate the measured CRP concentrations and to calculate the CV and σ, presented in the table to the right.

Most of the following rounds of CRP-experiments showed less reproducibility between each measurement replicate, despite being performed according to the exact same protocol. This can be seen in figure 13, where the experiment with promising results in figure 11 was

repeated. This time, triplicates of the CRP concentration of 0.3, 1 and 3 ng/ml were used. The reproducibility of this experiment was poorer and the average current was lower. Just as for the previous experiments, a calibration curve was constructed and the trend line equation was used to calibrate the measured values. Calibration curve, CV and σ for this experiment are shown in figure 14. All current values are presented in table 4 in the appendix.

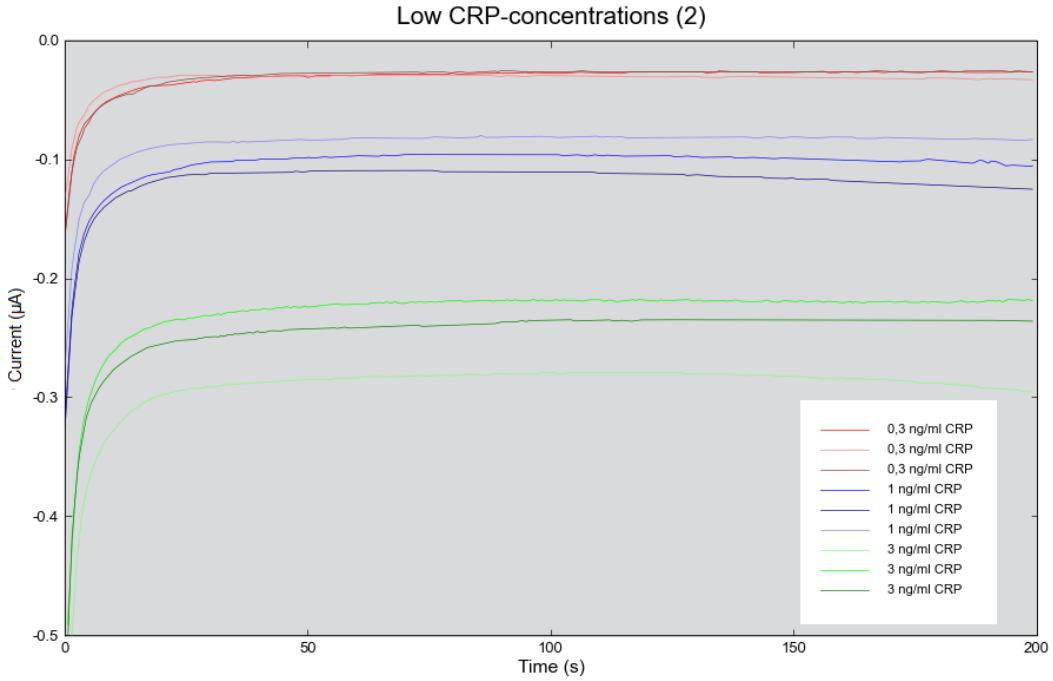
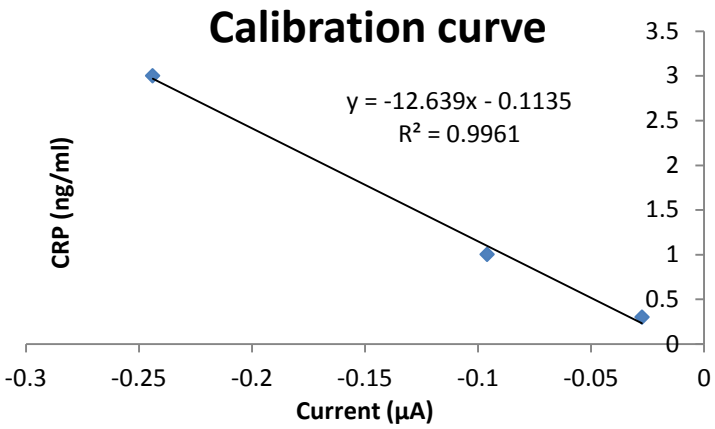


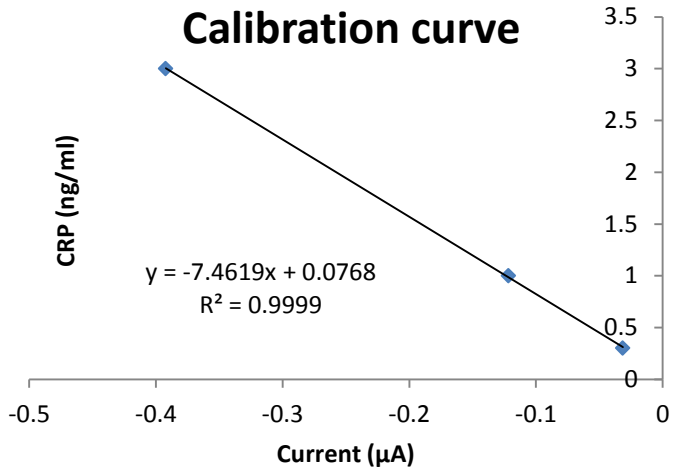
Figure 13. Second round of assay experiment using 30 µg/ml capture antibody, 0.5 µg/ml detection antibody and 0.3, 1 and 3 ng/ml CRP. Triplicates of the same CRP-concentrations were measured. The experiments have been performed according to the same protocol as the experiments in figure 11, but the results in this experiment show less reproducibility and lower current values.



CRP (ng/ml)	Average (µA)	σ	CV
0.3	0.2324	0.0267	11.49%
1	1.0990	0.1896	17.25%
3	2.9687	0.3949	13.30%

Figure 14. Linear calibration curve, σ and CV from the second measurement round with 0.3, 1 and 3 ng/ml CRP, **30 µg/ml capture antibody and 0.5 µg/ml detection antibody**. CRP-concentrations are plotted against the measured mean current values. The trend line equation was used to calibrate the measured CRP-concentrations and to calculate the CV and σ, presented in the table to the right.

The same experiments were repeated several times according to the same protocol using the same concentrations of CRP. The CV varied from very low, like the ones of the first round of experiments in figure 12, to mediocre, like the ones in the second round of the same experiment shown in figure 14. Looking only at the individual experiments, the CV is within a reasonable low range. This means that the reproducibility between the replicates within the same round of experiment is fairly high, and sometimes even very good. However, the average current measured from each round of experiment varies quite a lot. This means that the joined CV from all the experiments preformed at different times are high. This can be seen in figure 15, where the calibration curve, combined CV and σ are presented. Precise data from all experiments preformed under the same conditions using 0.3, 1 and 3 ng/ml CRP can be seen in table 5 in the appendix.

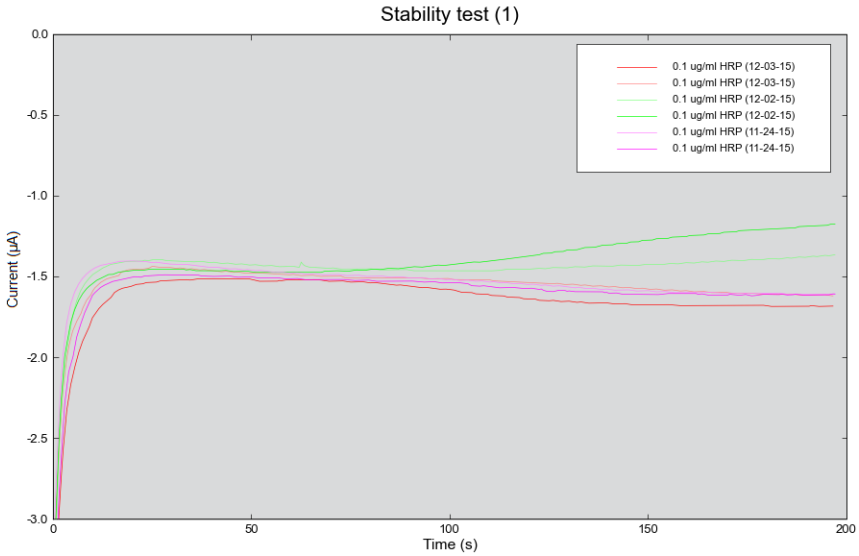


CRP (ng/ml)	**Average (µA)**	**σ**	**CV**
0.3	0.3114	0.1025	32.93%
1	0.9848	0.2609	26.50%
3	3.0038	1.3860	46.14%

Figure 15. Calibration curve, CV and σ based on the average current from all experiments using 0.3, 1 and 3 ng/ml CRP. CRP-concentrations are plotted against the measured mean current values from all experiments preformed according to the same protocol. The trend line equation was used to calibrate the measured CRP-concentrations and to calculate the combined CV and σ, presented in the table to the right.

5.4 Stability tests

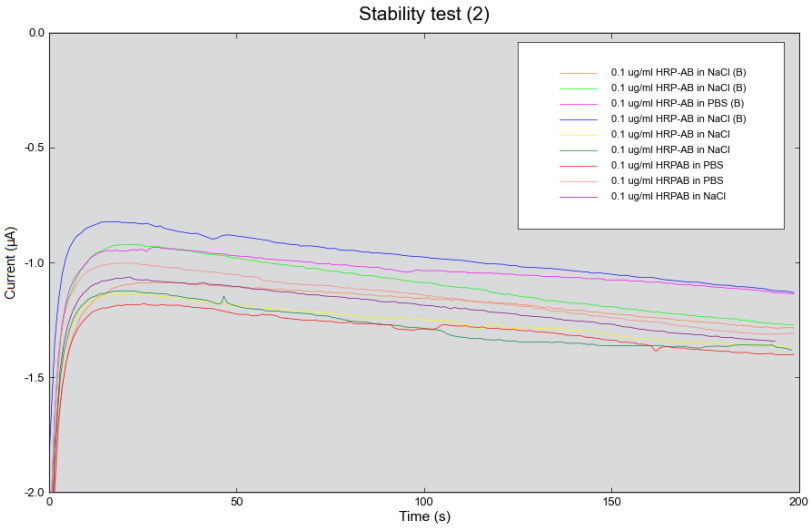
In order to test the stability and reproducibility of the assay, the reaction between the HRP-enzyme and the TMB/H_2O_2 substrate was measured on empty electrodes using a mix of 25 µl 0.1 µg/ml HRP-conjugated anti-goat IgG antibodies and 50 µl substrate reagent. Two rounds of stability tests were made, the first one with electrodes from a mix of different batches, and the second round with electrodes form only one batch used late in the project. The experiments were carried out in duplicates several times during different days. The results are showing fairly reproducible results before 100 s into the measurement. After 100 s, the curve starts to thrust to higher or lower current values. Data was therefore collected at 80 s. Results can be seen in figure 16. All current values are presented in table 6 in the appendix.



HRP-conc. (µg/ml)	Average (µA)	σ	CV
0.1	-1.4941	0.0341	2.2%

Figure 16. Enzyme measurements with 25 µl HRP-conjugated anti-goat IgG and 50 µl substrate reagents on empty electrodes. The stability and reproducibility of the assay was tested by mixing enzyme-conjugated antibodies and substrate reagents and measure the reaction of empty electrodes. The results are reproducible before 100 s.

For the second round of stability tests, electrodes from the same batch were used. The experiments were carried out in several replicates and data was collected at 100 s. The measurement data showed both higher variation between the replicates and lower current compared to the previous stability test; the current varied from -0.9782 µA to -1.2881 µA. The measurement graph, CV and σ is shown is figure 17. To make sure that the difference in current was not a result from external conditions, the experiment was performed with the antibodies diluted in both NaCl and PBS at separate measurement times. Some electrodes were also baked in 120°C and washed with PBS-TWEEN 20 to remove any potential particle contamination before the measurement. However, the low reproducibility and low current still remained. Precise values can be seen in table 7 in the appendix.



HRP-conc. (µg/ml)	Average current (µA)	σ	CV
0.1	-1.1551	0.1089	9.4%

Figure 17. Stability measurements with 25 µl HRP-conjugated anti-goat IgG and 50 µl substrate reagents on empty electrodes from the last stability test. The stability and reproducibility of the assay was tested by mixing enzyme-conjugated antibodies and substrate reagents and measure the reaction of empty electrodes. The results show a variation in current between – 0.9782 µA to -1.2881 µA, a fairly high variation considering the fact that the enzyme reaction is measured on empty electrodes.

6. Discussion

Electrochemical immunoassays have a great possibility of being a top-notch detection technique; the potential detection limit is low, measurement is fast and the required equipment is small and compact. But, as the scientific community already has stated, it is challenging to get reproducible results when you are developing these types of immunoassays.

6.1 Negative control and experiments using goat IgG

Figure 8 shows that the background current in the system is negligible. The same figure also shows that none of the detection antibodies bind to proteins other that its antigen at any relevant level. This is because the rise in current in the negative control test is nearly zero compared to the blank measurement. The small current detected in the specificity test could be a consequence of non-complete washing of the electrodes. The enzyme-conjugated antibodies could also have adsorbed to the electrode surface if they came in touch with a spot not blocked by the BSA or skimmed milk.

The experiment shown in figure 9, where goat IgG was used, demonstrates that it is possible to get reproducible results from the developed assay. It also indicates that the applied electric potential, incubation times and other physical factors are suitable for the assay. Figure 10 shows that the CV-values of the measurements are very low, with the exception of the experiment were 0.1 µg/ml detection antibody was used. The extremely high CV at 327.13 % is probably due to that this measurement is out of the measurement range. The σ-value for this measurement is still reasonably low, indicating that the variation between the replicates is low despite its high CV.

6.2 CRP-experiments and stability tests

Even though the assay with goat IgG gave promising results, the development of the CRP-assay was not as straightforward. The experiment presented in figure 11 shows excellent result. Here, the highest CV in the experiments is 4.93 %, which is extremely low. Even though I followed the exact same protocol for all the next coming CRP-experiments, both the average current and reproducibility between the measurement replicates varied for each experiment round. Figure 14 shows that the second round of assay experiment, with the same CRP-concentrations, gave CV-values of up to 17.2 %. Even if this CV is reasonable low for an assay at this stage in development, it is still much higher compared to the previous experiment. What is more interesting is however the fact that the average current varied so much between the different measurement rounds. Then mean current for the same CRP-concentrations seem to fluctuate between different current levels for experiment rounds performed at different days. This is also the main reason for the high CV-values of the combined data from all experiments put together: high difference in the average current gives high standard deviation and thereby also high CV.

The initial stability test showed in figure 16 indicates that the measurements of the enzyme reaction actually gave reproducible results. The thrust in current towards higher or lower current values could be due to steric hindrance from the conjugated antibodies or other diffusion related reasons. However, since this problem did not occur in any of the assay experiments, I do not consider it a problem. The stability test applied on the last batch of the electrodes showed in figure 17 gave very different results in terms of both current levels and reproducibility. The results were very surprising; it indicates that the system itself has an

inbuilt instability. Unless the reproducibility issues are not due to steric hindrance from the antibodies conjugated with the enzyme, the variations are probably due to the electrodes. Steric hindrance is probably not the cause since the same enzyme conjugated antibodies gave both stable and higher current levels in the first round of the stability test. Also, if steric hindrance was the problem; it is reasonable to assume that the measurement signal would have been shakier.

6.3 Possible explanations for the reproducibility issues

I tried a number of different approaches in attempt to increase the reproducibility of the assay. This includes baking the electrodes in 120°C for 30 min and rinse with PBS-TWEEN 20 to remove any particle contamination from the electrodes before the capture antibodies were added. I also tried to remove all phosphate from the chemicals which at some point came in touch with the antibodies, since phosphate sometimes can make antibodies precipitate in solution. Since all liquid samples simply were added to the electrodes by pipetting, I tried to delimit the active part of the electrodes by the use of a hydrophobic tape to see if the problems were caused by diffusion in the liquid droplets. I tried to wash the electrodes with different techniques and with different intensities and I tried to vary a number of other physical factors such as incubation times and amount of substrate. Still, none of the attempts increased the reproducibility of the experiments. Only one aspect did actually increase the stability; the initial assay with goat IgG did not give reproducible results until I stopped rinsing the electrodes with deionized H_2O after washing in PBS-TWEEN 20. This is probably due to the fact that deionized H_2O, because it does not contain any ions, does not have any electric conductivity.

Considering the fact that the average current levels for the different CRP-concentrations varied so much between each round of experiment, it is possible that the variations are caused by difference in electroactive area of the electrodes. An electroactive area is the part of the electrodes that can conduct electric current. As stated in the background section of this rapport, it is possible to passivate the electrode by modifying its surface. The same risk applies for chemicals; species adsorption on the electrodes can give variations in electroactive area (personal communication, Professor Leif Nyholm). This is especially true for surfactants found in most detergents like PBS-TWEEN 20. However, many successful electrochemical immunoassays have been developed using detergents like PBS-TWEEN 20 in the washing steps[2,3]. Species adsorption of other chemicals can of course also be the cause for a potential change of the electroactive area. It is possible that e.g. compounds of the substrate reagents can be adsorbed to the surface. Unfortunately, the content of the substrate reagent used are made confidential from the reseller. Differences in electroactive area caused by the substrate reagents could also explain the variations in the stability test since the instability in that case would remain when the enzyme reaction is measured in empty electrodes. Also, the fact that the assay with goat IgG became stable after I stopped rinsing the electrodes in deionized H_20 supports the theory that the electrode surface is highly affected by chemicals. If species adsorption is the main cause for the measurement variations, it is also reasonable to assume that physical factors like air humidity and temperature also can contribute to the variations. This could explain why the same experiment repeated at different days sometimes gave different results.

Another possible reason for the variation in the results is the surface structure. The electrodes in the experiments are cured at 150°C, a fairly low temperature giving the electrodes a somewhat rough surface. It is possible that this type of surface structure is less optimal for the

electron transfer occurring in the oxidation reaction, or that the variation in surface structure between different electrodes are affecting the measurement result. This could also explain why the second round of stability test gave much poorer results in terms of reproducibility and average current levels compared to the earlier stability tests, since different batches of electrodes were used. The electrodes from the batch used in the late stability test could have been from an especially bad batch due to mistakes made in the production affecting the electrode surface.

As stated in the background section, immobilisation of the capture antibody to the electrode surface by passive adsorption will cause random and sometimes suboptimal positioning of the antibodies. This will affect the interaction between the antigen and the antibodies and thereby affect the amount of antigen detected. This could explain the fact that the assay using only goat IgG and enzyme conjugated anti-goat antibodies gave less variation in the measurement results compared to the CRP-assay; when an antigen and a sandwich approach are utilized, positioning of the capture antibodies in the electrodes surfaces becomes more important.

6.4 Optimisation

All experiments described in this report showed a clear correlation between CRP-concentration and current levels, even if the reproducibility issue remains. The developed assay was also always able to distinguish between different CRP-levels from the same experiment. Since the assay sometimes showed excellent stability and sensitivity, I do believe that it is possible to circumvent the reproducibility problems.

In order to optimize the assay, it is essential to investigate which chemicals that possibly could passivate the electrodes and thereby cause variations in electroactive area. A way to both control environmental factors like air humidity and other physical factors like incubation time and exact amount of chemicals added could be to place the electrodes in some sort of flow cell. To fully optimize the assay, it is also necessary to investigate different type of electrode surfaces, both rough and smooth ones to see which one is the most optimal for the enzyme reaction.

A way to circumvent the potential problems with variations in surface structure and random positioning of the capture antibodies would be to modify the electrode surface with self-assembled monolayers, whose procedure is described in the background section of this rapport. Chemical modification of the electrode surface will potentially also decrease any unspecific species adsorption, but as stated before; any chemical modification of the electrode surface will in itself also risk passivating the electrode.

The data presented in this report is based on a quite low number of experiments. A way to get more precise and robust data would of course be to do more measurements of the same CRP-concentrations. It would also be interesting to lower the CRP-concentrations and to measure at closer concentration intervals to see how it would affect the CV. However, the project time was limited, and due to the mistake of not investigating the measurement range earlier in the project, some of the measurements preformed were out of the measurement range.

The calibration curve used is this project is linear. This could of course be optimised to suit the measurement data better, but for an assay development at this early stage, I consider a linear model to give good enough indication of the variation in the system.

7. Acknowledgements

I would like to thank my supervisor Björn Ekström for great guidance and support during this whole project. I would also like to thank Anna Palls, Niklas Rogeman and Max Ekström at Ginolis for all the help and support along the way. Thanks also to the rest of the crew at Ginolis for being so welcoming and helpful. Special thanks to Professor Leif Nyholm at Uppsala University for great scientific advice. I would also like to thank my scientific reviewer Ove Öhman for his guidance and great inputs.

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9. Appendix

Table 1. Current values form the binding specificity test with HRP-conjugated anti-goat IgG and anti-human CRP antibodies and skimmed milk/BSA. Data were collected at 100 s.

Protein	Antibody	Current (µA)
None	None	0.0052
Skimmed milk	Mouse anti-human CRP	0.0055
Skimmed milk	Rabbit anti-goat IgG	0.0069
BSA	Mouse anti-human CRP	0.0070
BSA	Rabbit anti-goat IgG	0.0090

Table 2. Data from the electrochemical immunoassay using 30 µg/ml immobilised goat IgG and 0.1, 0.3 and 1 µg/ml HRP-conjugated detection antibody. Data were collected at 100 s.

	Replicate 1	Replicate 2	Replicate 3	
HRP-AB (µg/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Average (µA)**
0.1	-2.6805	-3.075	-3.303	-3.0321
0.3	-7.9678	-7.568	-7.8396	-7.7918
1	-10.8713	-10.836	-10.605	-10.6051

Table 3. Measured data from the first CRP-experiments using 30 µg/ml capture antibody, 0.5 µg/ml detection antibody and CRP- concentration of 0.3, 1 and 3 ng/ml. Data were collected at 100 s.

CRP-conc. (ng/ml)	Current (µA)	Current (µA)	Average (µA)
0.3	-0.0503	-0.0509	-0.0506
1	-0.1823	-0.1851	-0.1837
3	-0.3156	-0.328	-0.3202

Table 4. Measured data from the second CRP-experiments using 0.3, 1 and 3 ng/ml. Data were collected at 100 s.

	Replicate 1	Replicate 2	Replicate 3	
CRP-conc. (ng/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Average (µA)**
0.3	-0.025	-0.0271	-0.0296	-0.027
1	-0.0808	-0.0962	-0.1108	-0.0960
3	-0.2183	-0.236	-0.2787	-0.239

Table 5. Measured data from all assay experiments with 0.3, 1 and 3 ng/ml CRP. All data were collected at 100 s. Experiments are performed at separate occasions.

	Rep. 1	**Rep. 2**	**Rep. 3**	**Rep. 4**	**Rep. 5**	**Rep. 6**
CRP-conc. (ng/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**
0.3	-0.0503	-0.0509	-0.0254	-0.0271	-0.0296	-0.0183
1	-0.1823	-0.1851	-0.0808	-0.0962	-0.1108	-0.1166
3	-0.3156	-0.3248	-0.2183	-0.2346	-0.2787	-0.6378

	Rep. 7	**Rep. 8**	**Rep. 9**	
CRP-conc. (ng/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Average (µA)**
0.3	-0.0185	-	-	-0.0314
1	-0.1208	-0.0986	-0.104	-0.1217
3	-0.7622	-0.3846	-0.3737	-0.3923

Table 6. Stability measurements with 25 µl HRP-conjugated anti-goat IgG and 50 µl substrate reagents on empty electrodes from mixed batches. Data were collected at 80 s.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate**
HRP-conc. (µg/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current**
0.1	-1.5338	-1.5236	-1.5053	-1.91

	Replicate 5	**Replicate 6**			
HRP-conc. (µg/ml)	**Current (µA)**	**Current (µA)**	**Average (µA)**	**σ**	**CV**
0.1	-1.56	-1.531	-1.91	0.031	2.2%

Table 7. Measurement data and calculations for 25 µl HRP- conjugated anti- goat IgG and 50 µl substrate reagents on empty electrodes from batch two. Data were collected at 100 s.

	Replicate 1	Replicate 2	Replicate 3	Replicate	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9
HRP-conc. (µg/ml)	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**	**Current (µA)**
0.1	-0.9782	-1.0337	-1.0873	-1.137	-1.152	-1.188	-1.28	-1.283	-1.2881

HRP-conc.(µg/ml)	**Average current (µA)**	**σ**	**CV**
0.1	-1.1551	0.1089	9.%