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Capacitive Immunosensor based on Poly-*para*-Phenylenediamine Modified Electrode for HSA Detection

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Abstract

Capacitive immunosensors detect the capacitance change at the electrode-electrolyte interface when the immobilized antibody on the electrode surface binds to the antigen in the sample. One of the important steps for the construction of the capacitive sensor is the immobilization of antibody via an insulating layer on the electrode surface. This layer is generally based on a self-assembled monolayer (SAM) of alkanethiol compounds, however, the self-assembling process requires a relatively long period of time. In this work electropolymerization of *para*-Phenylenediamine (*p*-PD) on the gold electrode surface was investigated as an alternative for the immobilization of antibodies. Anti-human serum albumin (anti-HSA) was immobilized for the capacitive detection of HSA. The concentration of the monomer solution and the number of electropolymerization scan were optimized. Under the optimized conditions, a wide linear range, 1.0×10^{-15} to 1.0×10^{-10} M, was obtained with a very low detection limit of 1.0×10^{-15} M. This sensor could be reused up to 31 times with RSD lower than 3.0%.

Keywords: Capacitive immunosensor, Affinity biosensor, Poly-*para*-phenylenediamine, Electropolymerization

Introduction

Human serum albumin (HSA) is a negatively charged, non-glycosylated globular protein with a molecular weight of 67 kDa. Reported to be the most abundant protein in the body, HSA accounts for 60% of the total proteins in plasma, and is synthesized exclusively in the liver, primarity in the polysomes of hepatocytes (Lu et al., 2014). Clinically, albumin was used in past decades to maintain vascular volume in patients with cirrhosis due to its regulation of oncotic pressure. Today, combined with other therapeutic approaches, the volume-expanding properties of albumin are still believed to be beneficial for patients with cirrhosis. Beside its applications in therapy, HSA is regarded as a standard biomarker, with its levels in serum, urine and saliva serving as diagnostic and prognostic criteria. The normal concentration of HSA in blood serum is 35-50 g/L. In diseased conditions, low levels of albumin in serum (pypoalbuminemia < 30 g/L) may reveal malnutrition, liver disease, nephrosis, gastrointestinal protein loss, shock, edema and cardiovascular disease. On the other hand, high serum levels of albumin (hyperalbuminemia > 55 g/L) (Cieplak et al., 2015).

The detection of HSA using a flow injection capacitive immunosensor relied on the immobilization of anti-HSA via a self-assembled monolayer (SAM) of HSA on the gold electrode. The advantages of SAM is that it can provide a reproducible, ultrathin and well-ordered layer, suitable for the immobilization of biological elements (Ferretti *et al*., 2000; Riepl *et al*., 1999). However, SAM also has some drawbacks. For example, a relatively long time is needed for the fabrication, 12 h of thioctic acid and mercaptosuccinic acid, and 24 h for thiourea (Shervedani and Hatefi-Mehrjardi, 2007). The cleanliness of the electrode surface also affects the quality of the assembled monolayer (Altintas *et al*., 2012). Alternatively, a polymer film modified electrode is an interesting approach because it is a simple and fast method that can provide a homogeneous and chemically stable layer with strong adherence to the electrode surface (Rezaei *et al*., 2015).

In affinity biosensor application, both conducting and non-conducting polymers have been used. However, an electropolymerized non-conducting polymer is more appropriate, because its film thickness is self-controlled during electropolymerization, and a very thin and uniform film can be obtained (Zhang et al., 1996). Non-conducting polymer also have high resistivity (Yuqing et al., 2004) and this is suitable for the capacitive transducer where an insulation electrode surface is required. One interesting polymer is poly-*para*-phenylenediamine (P*p*PD) because there are two amino groups in a monomer unit that can be used to immobilize the sensing probe.

P*p*PD, a non-conducting polymer, is used in this work. P*p*PD presents two amine groups in a monomer unit, a very high surface concentration of reactive groups for the

immobilization of biomolecules (Tran et al., 2003). In addition, P*p*PD film exhibit good stability over a wide pH range (Wu et al., 2005). The deposition of P*p*PD onto a gold electrode surface can be carried out using electropolymerization process where the appropriate potential with the certain number of scan is applied to the gold electrode immersed in the *p*PD monomer solution. The monomers are oxidized to form radical cations, followed by coupling reaction to form oliogomers that eventually lead to the deposition of the polymer on the electrode surface (Peng et al., 2009). From electopolymerization, it allows the reproducible and precise formation of a polymer deposite on the surface of the electrode whatever their size and geometry (Cosnier, 2003). Degree of coverage can be controlled by either the number of the scan of the electropolymerization process or the concentration of the monomer (Pournaras et al., 2008).

Thus, this work reports the capacitive immunosensor detection of human serum albumin. Anti- human serum albumin was immobilized on the gold electrode surface via an electropolymerization P*p*PD film using flow injection system with a highly sensitive capacitive transducer.

Objective

The aim of this work is to study the effect of the P*p*PD formation, i.e., number of scan and concentration of polymer were optimized. The effect of the immobilization, i.e., the time to activate the amine group of the polymer surface was also evaluated. The performance of the P*p*PD modified electrode was tested with HSA.

Literature Reviews

Biosensor can be defined as a device incorporating a biorecognition element connected to a transducer (Eggins, 1996). Biorecognition elements could be enzyme, microorganism, tissue, antibody, DNA, PNA, receptor. These biorecognition elements interact selectively to the target analyte. This is the advantage of biosensor to pick up analyte from a matrix of other molecules. Currently, biosensor play an important role for the detection of trace amount of biological hazards in many fields such as clinical diagnostic, gene mutation, genetically modified organisms or GMOs analysis and environmental monitoring (Manzanares-Palenzuela et al., 2015). This is because biosensor provides reliable results, high sensitivity and selectivity, rapid detection and low cost (Carrara et al., 2010, Tsouti et al., 2010).

Biosensor can be devided into two categories, catalytic biosensor and affinity biosensor. The classification was based on biorecognition elements and analytes.

catalytic biosensor use enzyme, tissue and microorganism as biorecognition element to catalyze the reaction of analyte. The products or detection of substrate were detected. The other category is affinity biosensor where macromolecules and microorganisms are considered suitable analytes. Biorecognition elements employed in affinity biosensor include antibody, cell, DNA and PNA. The binding event was detected by labeling agent or directly by detecting the physical properties change (Eggin, 1996).

Affinity biosensor that used the interaction between antibody and antigen is called immunosensor (Luppa et al., 2001). Antibody is also known as immunoglobulin (Ig). The most common type is immunoglobulin G (IgG). The others are known as IgM, IgA, IgD and IgE. Immunoglobulin has a molecular weight of 150,000 daltons. It is a "Y" shape protein, the arms of the Y are Fabs while the base of Y shape is the Fc portion. An antibody has four polypeptide chains, two heavy chains (H) and two light chains (L), with molecular weight about 50,000 and 25,000 daltons, respectively. The H and L chains are held together by non-covalent forces and covalent interchain disulfide bonds. The binding site of antibody is located at the ends of two arms of Fab unit (Roger, 2000). Interaction between antigen and antibody is based on non-covalent binding such as electrostatic force, hydrogen bonding, hydrophobic and van der waals forces. In immunosensor, either antibody or antigen is immobilized on a transducer surface and the formation of an immune complex between antibody and antigen occurred via affinity interaction. The physical properties change obtaining from the formation of immune complex or the signal generating from labeling agent can be measured with a suitable transducer.

Electrochemical detection of immunosensor is one strategy being explored because an electrochemical device provides a high sensitivity, rapid response, is easy to use, low cost and can be miniaturized (Jayakumar *et al*., 2012). The transduction relies on the conversion of the interaction between antigen and antibody into a useful electrical signal. Electrochemical methods for immunosensor are such as impedance spectroscopy, capacitance measurement and voltammetry. Capacitive device has shown great promise as a direct sensing method for affinity binding due to its sensitivity, simplicity and inexpensive (Berggren and Johansson, 1997; Berggren *et al*., 1999; 2001). Capacitive affinity biosensor is based on the immobilization of a biological recognition element, such as DNA, PNA or antibody as a thin layer on the electrode surface and measure the capacitance change at the electrode-solution interface causes by the binding with an analyte. In a capacitive biosensor, the capacitance at an electrode-solution interface can be described as several capacitors in series (Berggren *et al*., 2001; Berggren and Johansson, 1997). The capacitive affinity biosensor has been applied to detect many analytes. For example, the detection of DNA

(Mahadhy *et al*., 2014), antigen-antibody interaction (Dawan *et al*., 2011) and pesticide (Li *et al*., 2012).

An important step in the development of a immunosensor is the immobilization of antibody on the electrode surface. The accessibility and molecular orientation of the antibody would markedly influence the sensitivity and the detection limit of the sensor. Many immobilization methods have been described, but one of the most useful preparation is covalent binding through molecular self assembly, due to its simplicity and stability. The self-assembled monolayers (SAM) of alkane thiols is often used which allows for a rapid and easy way to covalently immobilized the sensing element as well as provides ordered antibody orientation on the electrode surface. However, the long preparation time of SAMs, the covalent immobilization of biorecognition element onto functionalized polymer surface has received much attention. Electropolymerization of polymer onto transducer surface offers thickness control, reproducibility and uniformity of polymer film. Conducting and non-conducting polymer are extensively used for biosensor application. Conducting polymers have high conductivity while non-conducting polymers are highly resistive. Therefore, the growth of non-conducting polymer is self-limiting and the film formation is much thinner than typical conducting polymer.

Research Methodology

Materials

Human serum albumin (HSA) and glutaraldehyde were purchased from Sigma-Aldrich (Steinheim, Germany). *para*-phenylenediamine (*p*-PD) was from laboratory UNILAB reagent (Sydney-Melbourne, Australia). Polyclonal rabbit anti-human serum albumin (anti-HSA) was from Dako (Denmark). 1-dodecanethiol was obtained from Aldrich (Milwaukee, USA). All buffers were prepared with deionized water treated with a reverse osmosis-deionizing system (Pentair, Inc., USA). Before using, buffers were filtered through a nylon membrane filter (pore size 0.2 μm, 47 mm diameter) (Vertical®, Albet, Spain) and degassed, respectively. Other chemicals were analytical reagent grade and were used as received.

Gold electrode preparation

Gold rod electrodes (99.99% purity) with a diameter of 3.0 mm were cleaned by dipping in piranha solution (conc. H_2SO_4 : 30% H_2O_2 equal to 3:1 %v/v) for 20 min followed by rinsing with distilled water. Then, they were was polished using alumina slurry (5, 1, 0.3 μm), on a smooth polishing cloth until a mirror-like surface was obtained and subsequently washed with distilled water. The gold electrodes were placed in a

plasma cleaner (Model PDC-32G, Harrick, New York, USA) to remove organic and inorganic molecules adsorbed on the electrodes surface.

Antibody Immobilization

In the P*p*PD film modified electrode surface, the monomer of *p*-PD was prepared in 10 mM sodium acetate buffer pH 5.18. The monomer concentration and the number of scans for electropolymerization were optimized simultaneously. Since a 5.0 mM *p*PD in acetate buffer has earlier been used, the monomer concentration closed to 5.0 mM, i.e., 1.0, 5.0, 10 and 15 mM were studied. Electropolymerized was carried out by cyclic voltammetry at 8, 10, 15 and 20 scans using the potential range from 0.0 to 0.8 V vs. Ag/AgCl with a scan rate of 50 mVs^{-1}. The P*p*PD coated electrode was cleaned by rinsing with distilled water and treated with 5.0 % (v/v) glutaraldehyde in 10 mM phosphate buffer pH 7.00 at room temperature for 20 min to activate the aldehyde group. Twenty microlitter of 0.5 mg ml^{-1} anti-HAS was placed on the modified electrode for 24 h at 4^{o}C and washed with 10 mM Tris-HCl buffer pH 7.00. The electrode was finally immersed in 1.0 M ethanolamine pH 8.50 for 20 min followed by 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes on the electrode surface. The electrochemical behavior of each immobilization step was studied by cyclic voltammetry (CV).

Capacitance measurement

Experimental setup for the flow-injection capacitive DNA sensor is shown in Figure 1. The measurement was performed in a three electrode system connected to a potentiostat (Model EA161, EDAQ, New South Wales, Australia). The modified gold electrode was used as working electrode together with a Ag/AgCl reference electrode and a Pt wire counter electrode.

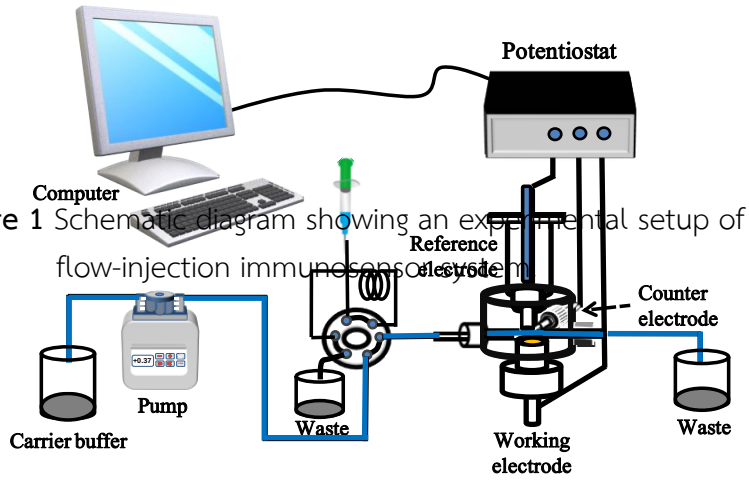

Figure 1 Schematic diagram showing an experimental setup of flow-injection immunosensor system

The transient current obtained from a potential step of 50 mV (1 pulse/min, pulse width 6.4 ms) was sampled and the first 10 points of the current response with sampling rate 40 kHz was used. From the linear least-square fitting of *ln i(t)* versus *t*, value of the capacitance, C_{total}, could be obtained from the slope ($-1/R_sC_{total}$) and R_s can be calculated form intercept ($\ln(u/R_s)$) corresponding to t = 0.

The measuring flow cell had a dead volume of approximately 10 µL. The carrier buffer, 10 mM Tris-HCl buffer pH 7.00, was flowed through the measuring cell using peristaltic pump at a flow rate 50 µL min^{-1} to provide a baseline capacitance. Then, HSA with a volume of 300 µL was injected into the flow system and the affinity binding at the electrode surface was performed at room temperature. The decrease in the total capacitance upon binding due to increase of the dielectric layer thickness described by Equation 1.

$$\frac{1}{C_{total}} = \frac{1}{C_{PpPD}} + \frac{1}{C_{anti-HSA}} + \frac{1}{C_{HSA}} \quad (1)$$

where C_{total} is the total double layer capacitance measured at the electrode-solution interface, C_{PpPD} is the capacitance of the P*p*PD modified layer, $C_{anti\text{-}HSA}$ is the capacitance of the anti-HSA layer, and C_{HSA} is the capacitance of the HSA layer. C_{total} was measured every minute and the results were later plotted as a function of time. The capacitance change (ΔC) could be determine (Figure 2) by Equation 2.

$$\Delta C = C_{before\ binding\ with\ HSA} - C_{after\ binding\ with\ HSA} \quad (2)$$

After that the regeneration solution (300 µL of HCl pH 2.50) was injected into the flow system to break the binding between anti-HSA and HSA. Then, the capacitance returned to its baseline, ready for a new analytical cycle.

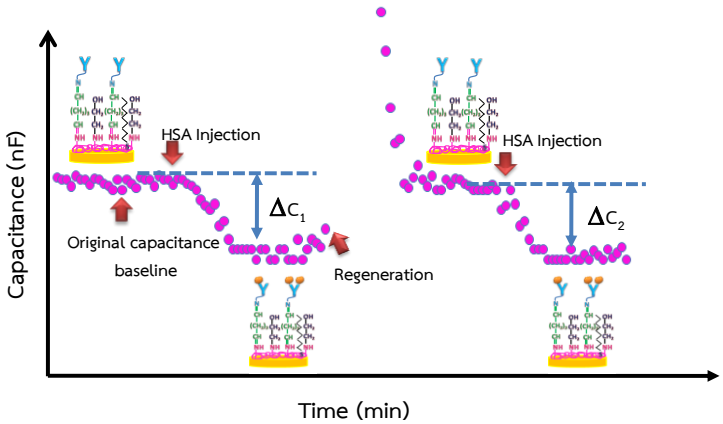

Figure 2 Flow injection capacitive immunosensor system using P*p*PD modified electrode for the detection of HSA.

Results and Discussion

Optimization of the electrode preparation

The degree of insulation of the modified gold electrode after each immobilization step was examined by cyclic voltammetry (Eco Chemie μ-autolab B.V., Utrecht, The Netherlands) with 10 mM $K_3Fe(CN)_6$ for P*p*PD modified electrode in 0.10 M KCl between -0.3 to 0.7 V at a scan rate of 0.1 V s^{-1} *vs* a Ag/AgCl reference electrode. Example of the cyclic voltammograms recorded from the P*p*PD modified electrode with the immobilized anti-HSA is shown in Figure 3. The CVs of the clean gold surface showed large oxidation and reduction peaks (Figures 3(a)). Redox peak decreased when P*p*PD (Figure 3(b)) was coated onto the gold electrode surface. The insulating property of the electrode surface was further increased when avtivaed with glutaraldehyyde and the anti-HSA was immobilized (Figures 3 (c and d)). This indicated that the anti-HSA had been successfully immobilized onto the modified electrode. Then reacted with ethanolamine pH 8.50. This step was to occupy all the remaining aldehyde groups that had not been coupled to the immobilized anti-HSA. After the treatment with the blocking thiol, the electrode surface was completely insulated as shown by the disappearance of the redox peaks (Figure 3(e)). This insulation property is a necessary condition for the non-Faradaic detection of the employed capacitive system.

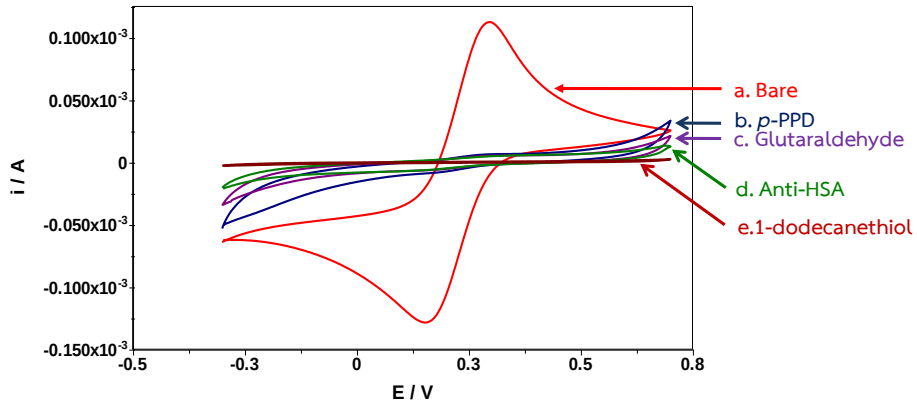

Figure 3 Cyclic voltammograms obtained from (a) bare gold, (b) P*p*PD, (c) glutaraldehyde, (d) Anti-HSA and (e) blocking thiol, 1-dodecanethiol.

Activation time of glutaraldehyde

The amine groups of P*p*PD film were activated by glutaraldehyde to form the aldehyde groups which further formed the amide bond with the amine groups of antibodies. This activation time was evaluated to ensure that all amine groups of P*p*PD were activated. The activation time between 5 and 20 minutes of 5% (v/v)

glutaraldehyde was investigated. The electropolymerization was performed using 5 mM of *p*PD and 10 scans. HSA (1×10^{-15} to 1×10^{-10} M) was analyzed and the sensitivity (nF cm^{-2} log M^{-1}) was calculated and plotted against the activation time (min). From the result, the activation time of 5 min showed the lowest sensitivity because because only a small amount of amine groups were activated resulting in a small amount of immobilized antibodies. Ten minutes of activation time provided the highest sensitivity while at 15 and 20 min the sensitivity was slightly decreased. This is because high glutaraldehyde loading may result in the immobilization of antibodies through multiple binding sites, thus reducing the flexibility (rigidity and binding) and the binding capacity of the antibodies as well as the accessibility of the target analyte to them. Therefore, the activation time of 10 min was chosen as an optimum value and used for the ongoing experiments.

Number of scan and concentration of *para*-phenylenediamine

The number of scan used for the electropolymerization of *p*PD was evaluated using cyclic voltammetry at 8, 10, 15 and 20 scans. The scanning was carried out between 0 and +1.5 V at a scan rate of 50 mVs^{-1}. Number of scan and concentration of *p*PD were then optimized together using one concentration of HSA (1×10^{-12} M). The concentrations of *p*PD were 1, 5 10 and 20 mM. Each concentration was studied with number of scan of 8, 10, 15 and 20.

The amine groups of P*p*PD were then activated with 5% (v/v) glutaraldehyde for 10 min. The electrodes with different number of scan were used to analyze HSA in the flow injection capacitive immunosensor. The capacitance change (nF cm^{-2}) against concentration of monomer is shown in Figure 4. The highest capacitance change was obtained at 10 scans with 5 mM. For the lower scan (8 scan), the lower capacitance change might be the result of the lower number of amine groups resulting in the lesser amount of immobilized antibodies.

Since the capacitive measurement is based on the capacitance change at the electrode/solution interface, a thin and insulated layer is required. The thicker insulating layer may provide a lower capacitance change. When the number of scan was higher (15 and 20 scans) the reduction of the capacitance change was observed. This was because when the number of scan increased the polymer thickness also increased. Moreover, the higher number of amine groups may result in the higher loading of glutaraldehyde. Hence, the immobilization of antibody may occur through multiple binding site, reducing the flexibility and the binding capacity of the antibodies.

Therefore, 5 mM of *p*PD with the number of scan of 10 were then selected as the optimum conditions for P*p*PD electropolymerization.

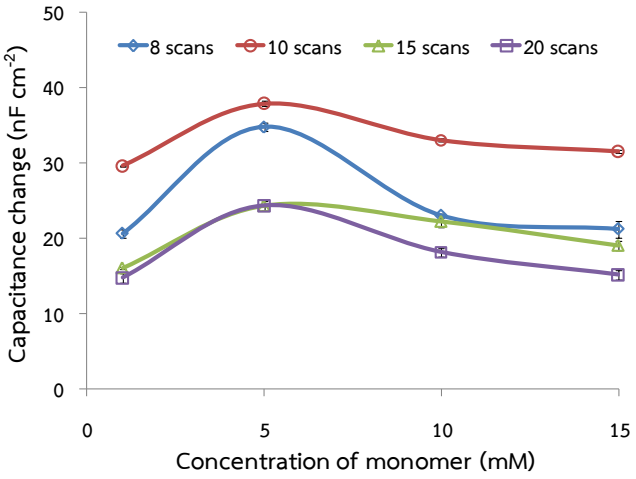

Figure 4 The capacitance change obtained from different concentrations of *p*PD and different number of scan.

Linear range and limit of detection

Under the optimum conditions of P*p*PD preparation, linear range and limit of detection were determined for HSA detection. The flow injection conditions were; flow rate 50 µL min^{-1}, sample volume 300 µL and carrier buffer 10 mM Tris-HCl pH 7.00. The dissociation of HSA can be done using HCl pH 2.50. The linear range of capacitive immunosensor for HSA was tested between 1×10^{-16} and 1×10^{-8} M. Each concentration was analyzed for 3 replications. The calibration curve between concentration of HSA (M) and capacitance change (nF cm^{-2}) showed the linearity between 1×10^{-15} and 1×10^{-10} M. The limit of detection was determined followed IUPAC recommendation 1994, which was found to be 1×10^{-15} M (Figure 5).

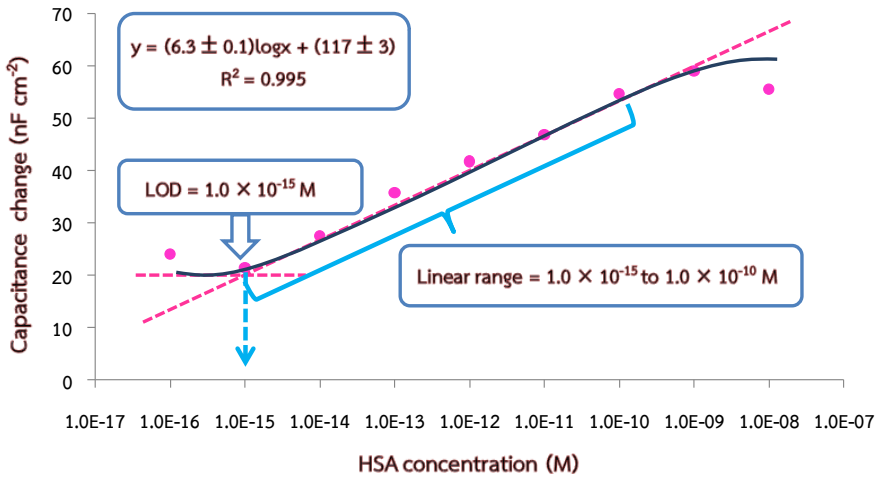

Figure 5 The calibration curve of HSA detection plotted between capacitance change and concentration.

Reproducibility

The reproducibility of capacitive immunosensor was tested by analyzing the same concentration of HSA (1×10^{-12} M). After each injection, the regeneration step was included in the analysis cycle. The residual activity (%) of immobilized antibodies to its antigen after regeneration was calculated.

The residual activity (%) was plotted against the number of injection (Figure 6). For the first 31 injections of HSA, the average residual activity was 96% ± 2% (RSD = 3%). After 32 regeneration cycles, the average residual activity reduced to 88%. The electrode surface was then tested by cyclic voltammetry. A flat voltammogram similar to one obtained after electrode preparation was observed. This confirmed that the film on the electrode surface was not destroyed by the frequent regeneration. The results indicated that the decrease of residual activity after being used several times.

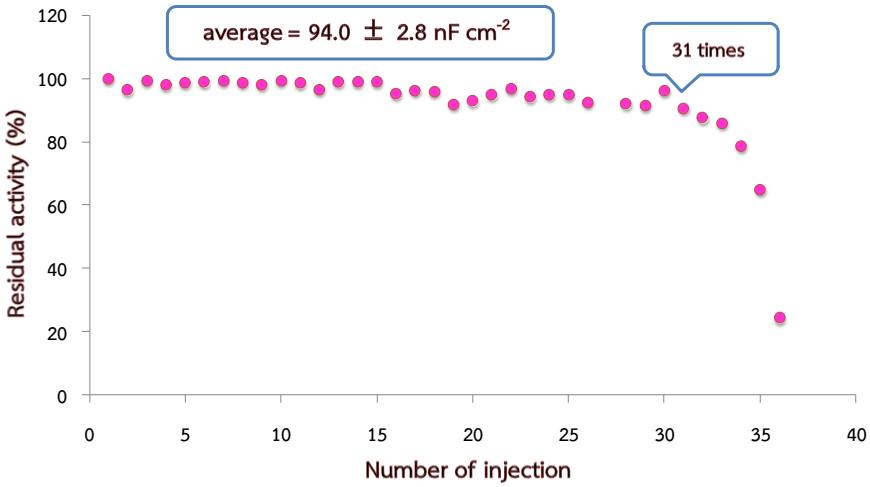

Figure 6 The relationship between residual activity and number of injection.

Conclusions

This work studied the modification of P*p*PD on the electrode surface to be used in the capacitive immunosensor. The electropolymerization of P*p*PD film on the gold electrode was optimized. Anti-HSA and HSA binding was used for the optimization process and the performance of the modified electrode was tested using anti-HSA and HSA interaction. The optimum values of P*p*PD preparation were 10 min of activation time by glutaraldehyde, 10 scans of the electropolymerization with 5 mM of *p*PD. Under these optimum conditions, the modified electrode can be reused up to 31 times. The electropolymerization of the polymer film onto gold surface took only 15 min. This is the advantage over the SAM which needs 12-24 h. The proposed P*p*PD modified electrode would be useful for the electrode preparation when the thin film and high

population of amine groups are needed. Therefore, P*p*PD film is an interesting alternatively material for electrode surface modification in an affinity capacitive immunosensor.

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