



Electrochemical Impedimetric Immunosensor for the Detection of C-Reactive Protein using ThioPhenylboronic Acid Modified Surface

A. O. ADESINA¹, P. N. MASHAZI²

¹Department of Chemical Science, Adekunle Ajasin University AkungbaAkoko Ondo State Nigeria

²Department of Chemistry Rhodes University South Africa

*Corresponding author E-mail address: abiola.adesina@aaau.edu.ng, adesina_abiola50@yahoo.com

Abstract

Keywords:

C-reactive protein Immunosensor, Electrochemical Impedance Spectroscopy, Immunoassay, 4-mercaptophenylboronic acid

A self-assembled monolayer of 4-mercaptophenylboronic acid modified electrode was used for the detection of C-reactive protein using direct and sandwich assay format. Monoclonal mouse anti-Human CRP antibody (mAb) was used as the capture probe on the thiol-modified surface using the affinity boronate ester interaction. A polyclonal anti-CRP antibody was used as the detection antibody for signal enhancement in the sandwich assay. Cyclic voltammetry and electrochemical impedance spectroscopy were used to investigate the fabrication steps of the immunosensor and immunochemical reaction between the surface-bound antibody and the antigen. The impedance change due to antibody-antigen interaction at the surface of the immunosensor was utilized for the detection of CRP. The linear detection range of this immunosensor for both assays ranged from 10-100 ng.ml⁻¹. The best fit for both assays were found to be $y=0.0088x - 0.0536$ with R^2 0.9982 for the direct assay and $y= 0.0139x - 0.0335$ with R^2 0.9997 for the sandwich assay. The detection limit for the direct and sandwich assay were 9.75 and 6.23 ng.ml⁻¹.

Accepted: 27 June 2019

© Science Research Annals. All rights reserved.

INTRODUCTION

Cardiovascular diseases (CVD) is the leading cause of death world-wide according to World Health Organisation (Altintas et al., 2014; Ross, 1999). Coronary artery disease is responsible for about half of the death occurring from CVD. Coronary heart disease also known as ischemic heart disease is the narrowing or blockage of the coronary arteries usually caused by atherosclerosis. Atherosclerosis has to do with the blockage or interruption of blood flowing through the walls of the arteries as a result of build-up of fatty deposit called plaque. C-reactive protein (CRP) is a well-known biomarker for inflammation which can be used to assess the risk of

cardiovascular disease since atherosclerosis is associated with chronic inflammatory process (EHN, 2008; Mazer and Rabbani, 2004; Meyer et al., 2007). C-reactive protein (CRP) is a pentraxin family of protein secreted in the liver in response to interleukin 6 (IL-6) and the concentration increases about 1000 fold in response to injury, inflammation and tissue death (Quershi et al., 2009). It is a well-known biomarker for inflammation and tissue damage (Libby et al., 2002). CRP has been identified as the most specific biomarker for inflammation that independently predict the risk of myocardial infarction (Hansson, 2005). The American Heart Association (AHA) and the Centre for Disease Control and Prevention (CDC) reported the

evaluation of CRP concentration associated with CVD risk. Individuals with CRP concentration <1 ng/ μ l, 1-3 ng/ μ l, >3 ng/ μ l were associated with low, moderate and high risk of cardiovascular disease (Hu et al., 2006; Verma and Yeh, 2003; Yang et al., 2009). Therefore monitoring the concentration of CRP in the body and quantifying the amount present may help in reducing or predicting the risk of cardiovascular disease (CVD). In the clinical laboratories, enzyme-linked immunosorbent assay (ELISA) is a major method used for quantifying the concentration of CRP (Clarke et al., 2005; Dominici et al., 2004; Parra et al., 2005). Other available methods include the use of immuno-turbidimetric and immuno-nephelometric technologies which requires automated clinical instruments (Mali et al., 2009). Although ELISA is a sensitive technique, it is time consuming expensive, bulky equipment are needed for analysis and also required highly-skilled personnel for performing the analysis and results interpretation (Ding et al., 2013). This requirement makes ELISA and other laboratory-based techniques not suitable for diagnosis in low resource settings.

The quantification and the detection of CRP using electrochemical immunoassay is the major alternative to the clinical diagnostic of CVD. This method provide realistic opportunities to overcome the challenges of traditional ELISAs and other laboratory based diagnostic techniques (Kadir and Tothill, 2010; Salam and Tothill, 2009; Tothill, 2009). Electrochemical immunosensors has received much attention in the field of clinical diagnosis owing to its higher analytical efficiency as well as the unique advantages of high sensitivity, fast response, portability, low cost, simple instrument and ease of miniaturization and point-of-care applications (Mazloun-Ardakani et al., 2014; Wang et al., 2010; Zhang et al., 2011)

Electrochemical impedance spectroscopy (EIS) is a useful tool for sensing the formation of antibody-antigen interaction on the electrode surface by probing the features of the interfacial properties (Vogt et al., 2016). EIS is a non-destructive and highly informative technique which requires no labelled secondary antibody for signal generation (Chowdhury et al., 2017; Repo et al., 2002). The results are direct as the affinitive interactions between the antibody and the antigen can be directly detected using the change in charge transfer resistance (R_{CT}) (Bogomolova et al., 2009; Rodriguez et al., 2005).

We hereby report a simple and effective method for site-specific and self-oriented immobilization of antibodies. The immunosensor fabrication was achieved through the immobilization of the capture antibody (monoclonal mouse anti-human CRP) on the electrode surface via affinity boronate ester interaction for the detection of CRP biomarker.

METHOD AND MATERIALS

Materials and reagents

Monoclonal mouse anti-human CRP (MCA5880G, mAb capture antibody), polyclonal goat anti-human CRP (1707-0189G, pAb detection antibody), native human CRP (1707-2029) were purchased from AbDSerotech. 4-aminophenylboronic acid (APBA), glucose (blocking reagent), absolute ethanol (EtOH), potassium ferrocyanide $K_4Fe(CN)_6$, Potassium ferricyanide $K_3Fe(CN)_6$, were purchased from Sigma Aldrich. Sulphuric acid, potassium chloride, hydrogen peroxide (30%) were purchased from ACE chemicals (South Africa). Phosphate buffer saline (PBS, 10mM, pH 7.4) containing monobasic potassium phosphate, sodium chloride, and dibasic potassium phosphate was used. Ultrapurewater with a resistivity of 18.2 M Ω .cm obtained from Milli-Q water purification system (Millipore Corp, Bedford, USA) was used throughout the experiment.

Equipment

All electrochemical and impedance spectroscopy experiments were carried out using the AUTOLAB PGSTAT 302N Electrochemical Work-station interfaced to a proline desktop computer equipped with a 1.10 version NOVA. A three-electrode system was used for all the electrochemical measurement. The electrochemical data was collected using a gold disk electrode as working electrodes ($r=0.8$ mm) purchased from BASi (USA), platinum wire as counter electrode and silver/silver chloride Ag/AgCl) 3M NaCl solution as reference electrode. All cyclic voltammetry (CV) and Electrochemical Impedance Spectroscopy were recorded in of 2.0 mM $[Fe(CN)_6]^{3-/4-}$ redox couple in PBS buffer. CVs were recorded between -0.2 to 0.6 V Ag/AgCl) 3M NaCl. Electrochemical impedance Spectroscopy (EIS) experiments were recorded in the frequency range between 10 kHz to 100 mHz at the half-wave potential ($E_{1/2}$) of 2.0 mM $[Fe(CN)_6]^{3-/4-}$ redox couple for bare gold with an amplitude 5 mV rms sinusoidal modulation.

Fabrication of Au-MPBA-Mab/glucose immunosensor

Prior to modification, the polycrystalline Au electrodes were polished sequentially with 1.0, 0.3 and 0.03 μ m alumina slurry on micro cloth pads, rinsed with water and sonicated for 3 min after each stage of polishing. The polished electrodes were then treated with piranha solution (concentrated $H_2SO_4:H_2O_2$, v/v 3:1) for 3min and subsequently washed with Milli-Q water. Finally, the electrodes were electrochemically cleaned by cycling between -0.2 and 1.5 V vs (Ag/AgCl (3 M KCl) at 0.1 V/s in 0.5 M sulphuric acid until reproducible voltammograms of a cleaned gold electrode was obtained. The clean gold electrode was then rinsed with ethanol and dried in an argon atmosphere.

The gold surface was modified using self-assembly (SAM) monolayer method by immersing in an absolute ethanol solution containing 4-mercaptophenylboronic acid (MPBA, 5.0 mM) for 24 hours. The modified gold surface was represented as Au-MPBA. The Au-MBA electrode was rinsed with water and ethanol. To immobilize the capture antibody (mAb), the Au-MPBA SAM was exposed to 10 μl mouse anti-human CRP pH 7.4 solution (30 μgml^{-1}) at 4 $^{\circ}\text{C}$ for 24 hours to give Au-MPBA-mAb. The antibody modified gold electrode was washed with PBS (pH 7.4) to remove unbound and physically adsorbed antibody from the electrode surface. The unreacted boronic sites were blocked by reacting with glucose solution (30 μgml^{-1}) at room temperature for 2 hours to give the sensing gold surface, Au-MPBA-mAb/glucose. The Au-MPBA-mAb/glucose was stored at 4 $^{\circ}\text{C}$ before use.

Assay procedure for detection of CRP using Au-MPBA-mAb/glucose.

To access the analytical performance of the Au-MPBA-mAb/glucose immunosensor, 20 μl of CRP antigen concentrations ranging from 10 to 400 ngml^{-1} in PBS (pH 7.4) was applied to the electrode surface and incubated for 1 hour at room temperature. Afterwards the electrode was further incubated with 20 μl CRP polyclonal antibody signal amplification.

All measurements were performed in triplicates. The change in total charge transfer resistance (R_{CT}) for each concentration was calculated from equation (1).

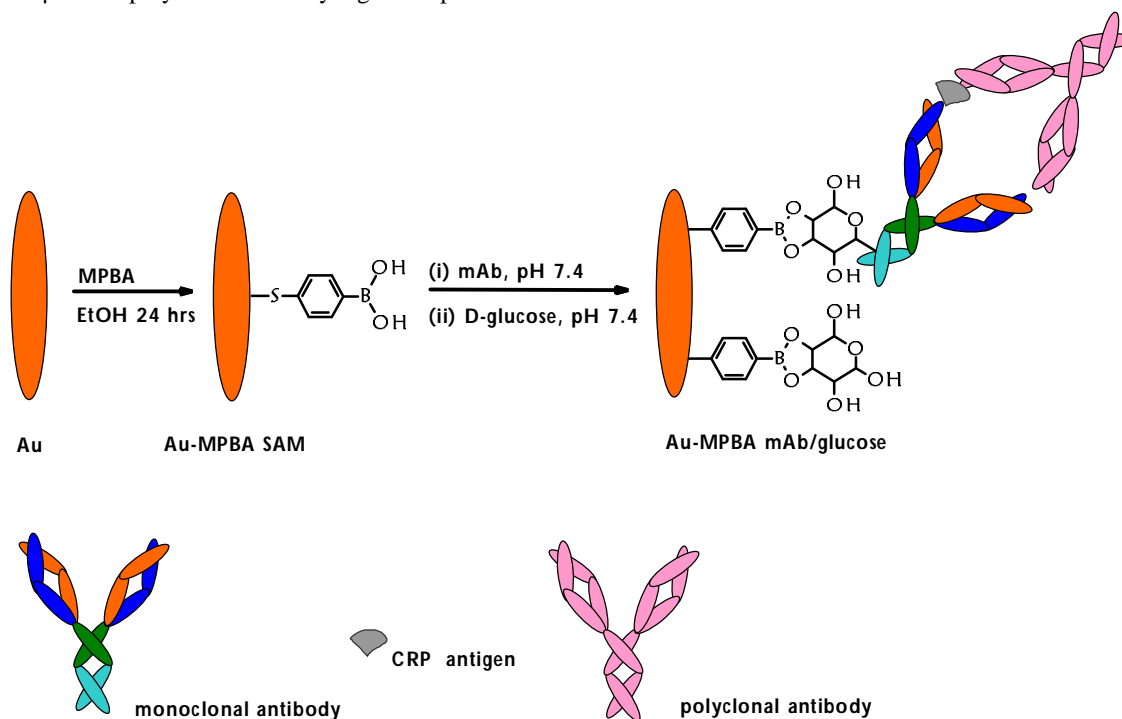
$$\Delta R_{CT} = R_{Ab-Ag} - R_{Ab}$$

Where A_b is the charge transfer resistance of the Au-MPBA-mAb/glucose before the incubation with the detection antibody and R_{Ab-Ag} is the value of the Au-MPBA-mAb/glucose after the incubation with the CRP antigen.

RESULTS AND DISCUSSION

Surface modification using MPBA and biomolecules

The fabrication of the CRP sensing gold surface was modified following the step-by-step procedure shown in Scheme 1 and as described in the experimental section above. The method followed was chosen as it will allow for oriented immobilization of the monoclonal mouse anti-Human CRP antibody (mAb) via site-specific glycosylation. The unreacted 1,2-diol reactive surfaces from the Au-MPBA SAM was blocked with glucose. After the fabrication steps, the immunosensors is represented as Au-MPBA-mAb/glucose. The step-by-step modification of gold surfaces was characterized using electrochemistry.



Scheme1- Step-by-step by modification of gold surface and the immobilization of monoclonal anti-human CRP antibody

Electrochemical characterization of Au-MPBA-mAb/glucose immunosensor

Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) were used for the characterization of the fabricated immunosensor. The CV of the bare electrode (Figure 1(ai)) exhibited a reversible redox peak due to $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with peak to peak separation of (ΔE) of 0.075 V. Figure 1 (a(ii)) shows a slight increase in the peak to peak separation ($\Delta E=0.081$ V) of the self-assembled monolayer of 4-mercaptophenylboronic acid (Au-MPBA SAM) which confirms the successful modification of the electrode. A further increase in peak to peak separation ($\Delta E = 0.104$ V) was observed with the Au-MPBA-mAb/glucose electrode which is an indication of glucose.

of the blocking behaviour of the immobilized antibody (Figure 1 (a(iii))).

Electrochemical impedance spectroscopy (EIS) measurements (Nyquist plot representation) in Figure 1(b) was further used to investigate the different fabrication stages of the immunosensor. The bare electrode (Figure 1(b)(i)) exhibits a small semicircle at high frequency with a charge transfer resistance of 505 Ω . An increase in the charge transfer resistance was observed in Figure 1(b)(ii) with the Au-MPBA SAM modified electrode (1.08 k Ω). The increase in R_{CT} (2.44 k Ω) observed with Au-MPBA-mAb/glucose electrode (Figure 1b(ii)) is as a result of the slow rate of electron transfer which is attributed to the insulating property of the antibody and

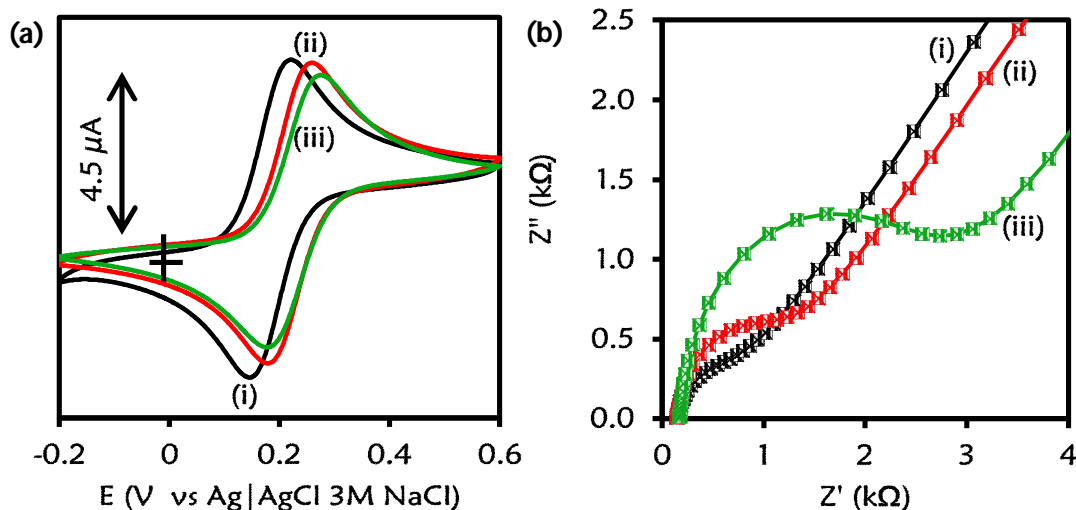


Figure 1: (a) CV and (b) EIS of (i) bare Au, (ii) Au-MPBA SAM and (iii) Au-MPBA-mAb/glucose in (1:1) 2 mM $\text{K}_3/\text{K}_4\text{Fe}(\text{CN})_6$ solution containing 0.1 M KCl.

Detection of CRP

To evaluate the antibody-antigen complexation between anti-CRP monoclonal antibody and CRP antigen. The Au-MPBA-mAb/glucose electrode was exposed to different concentrations of the antigen (1 - 400 $\text{ng}\cdot\text{ml}^{-1}$) in a direct and sandwich immunoassay format. EIS was used to monitor the change in charge transfer resistance (ΔR_{CT}) of the Au-MPBA-mAb/glucose electrode. The Nyquist plot for both assay is presented in Figure 2. The diameter of the semicircle of the Nyquist plot increases with increase in antigen concentration which was an indication of increase in charge transfer resistance. The linear concentration for both assay ranged between (1-100 $\text{ng}\cdot\text{ml}^{-1}$), it was found that the charge transfer resistance decreases

at higher concentrations (200-400 $\text{ng}\cdot\text{ml}^{-1}$). This observation is attributed to the fact that all the available antigen binding site on the surface of the sensor was fully occupied. Hence, more antigen are competing for limited binding site on the surface of the sensor (Songjaroen et al., 2016). Thereby reducing the binding efficiency between the immobilized anti-CRP mAb and the CRP antigen.

The best fit for the calibration curve for the direct assay was $\Delta R_{CT} = 0.0088 [\text{CRP-antigen}] - 0.0536$ and R^2 0.9982 ($n=3$). The regression line of $\Delta R_{CT} = 0.0139 [\text{CRP-antigen}] - 0.0335$ with R^2 0.9997 ($n = 3$) was obtained for the sandwich immunoassay. The sensitivity of the direct immunoassay was 0.438 $\text{k}\Omega\cdot\text{ng}^{-1}\cdot\text{ml}\cdot\text{cm}^2$ and 0.691

$k\Omega \cdot ng^{-1} \cdot ml \cdot cm^{-2}$ for the sandwich immunoassay. The observed increase in the sensitivity of the sandwich immunoassay was attributed to the enhanced signal generated by the pAb as observed in **Fig. 2(b')**.

The limit of detection and limit of quantification (LOD and LOQ) were calculated using the IUPAC

methods of $3 SD/m$ and $10 SD/m$ respectively. Where m is the slope of the calibration curve and SD is the standard deviation of the blank measurement without the presence of the analyte. The LOD and LOQ were calculated to be 9.75 and $32.51 ng ml^{-1}$ for the direct assay and 6.23 and $20.77 ng ml^{-1}$ for the sandwich assay.

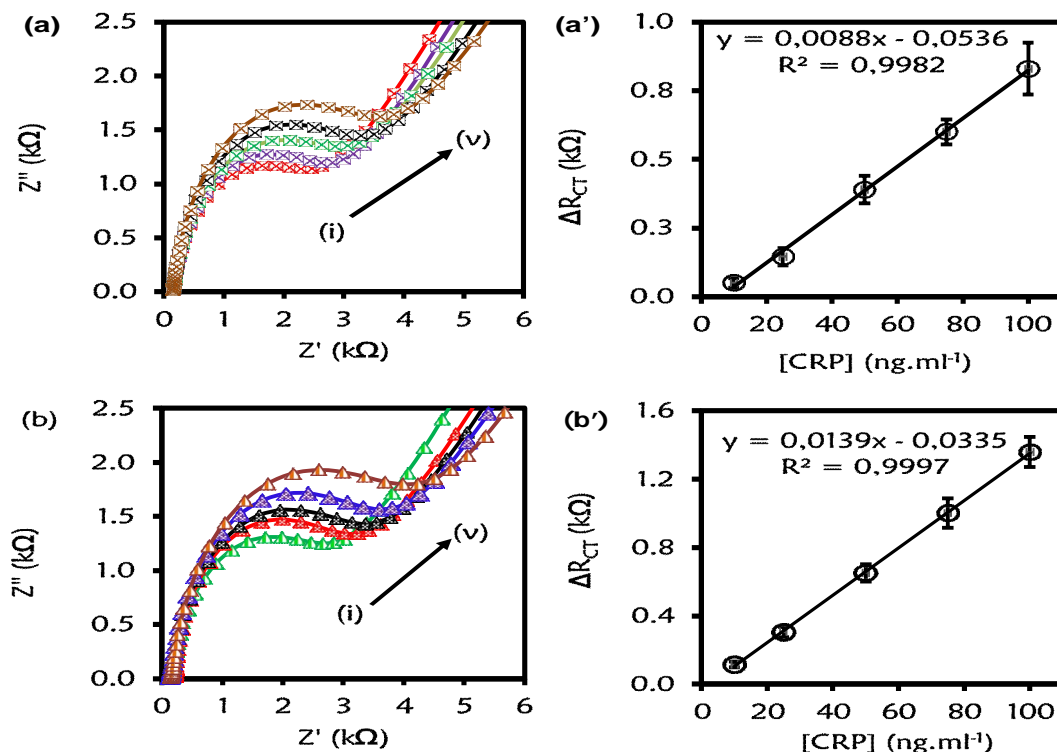


Figure 2(a) and (b)Nyquist plots and (a') and (b') the corresponding calibration curves at varied CRP-antigen concentrations (i) $10 ng \cdot ml^{-1}$ (ii) $25 ng \cdot ml^{-1}$ (iii) $50 ng \cdot ml^{-1}$ (iv) $75 ng \cdot ml^{-1}$ (v) $100 ng \cdot ml^{-1}$ for the (a) direct assay (b) sandwich immunoassays

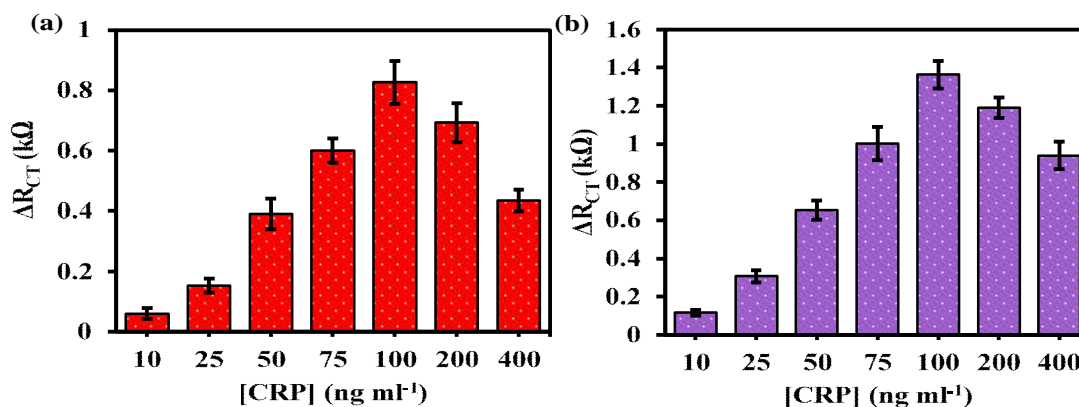


Figure 3: Chart showing the change in charge transfer resistance (ΔR_{CT}) at different antigen concentrations at higher concentrations for the (a) direct assay and (b) sandwich assay.

Comparison of Analytical Performance of CRP Immunosensor

The performance of the immunosensor was compared with some of the immunosensor previously reported in the literature as shown in **Table 1**. The obtained result (LoD) was better than some of those previously reported in **Table 1**. The LoD (6.23 ng.ml⁻¹) of the fabricated immunosensor was better than the impedimetric immunosensor reported by Gupta et al. (LoD, 11 ng.ml⁻¹), Puri et al. (LoD, 30 ng.ml⁻¹). The lower LoD could be attributed to the oriented immobilization strategy employed in this work. However, the developed immunosensors reported by Chen et al., Zhou et al., and Lv et al. gave a better LoD than the one

reported in this work (**Table 1**). The lower limit of detection reported by Zhou et al. and Lv et al. could be attributed to the incorporation of quantum dot labelled detection probe. The fluorescence labelled technique requires additional time and quite expensive than the label-free method in this work. Hence the developed immunosensor provides a simple low-cost technique for the detection of CRP-antigen.

Table 1: Comparison of analytical performance of CRP immunosensors

Immunoassay	Linear range (ng.ml ⁻¹)	LoD (ng.ml ⁻¹)	References
Amperometry	up to 100	2.6	(Fakanya and Tohill, 2014)
SWASV	0.5 – 200	0.22	(Zhou et al., 2010)
EIS	45 - 5840	30	(Puri et al., 2010)
EIS	0.1 – 20	0.1	(Chen et al., 2008)
Fluorescence	1.56 – 400	0.46	(Lv et al., 2017)
EIS	-	11	(Gupta et al., 2014)
Impedimetric	10 - 100	6.23	This work

Legend: EIS – Electrochemical Impedimetric Spectroscopy, SWASV – Square Wave Anodic Stripping Voltammetry

CONCLUSION

A sensitive electrochemical immunosensor for the detection of CRP was developed. The immunosensor is promising towards label-free detection of CRP which guarantee the reliable detection of CRP for clinical application. The simple method of the fabrication of the immunosensor and the enhanced signal generated from the polyclonal antibody increased in the sensitivity of the immunosensor. The sandwich immunoassay showed a better analytical performance than the direct immunoassay. The limit of detection of the fabricated immunosensor falls within the clinical diagnostic range of CRP.

REFERENCES

- Altintas, Z., Fakanya, W.M. and Tohill, I.E. (2014). Cardiovascular disease detection using bio-sensing techniques. *Talanta* 128, 177–186.
- Bogomolova, A., Komarova, E., Reber, K., Gerasimov, T., Yavuz, O., Bhatt, S. and Aldissi, M. (2009). Challenges of electrochemical impedance spectroscopy in protein biosensing. *Analytical Chemistry* 81, 3944–3949.
- Chen, X., Wang, Y., Zhou, J., Yan, W., Li, X. and Zhu, J.-J. (2008). Electrochemical Impedance Immunosensor Based on Three-Dimensionally Ordered Macroporous Gold Film. *Analytical Chemistry* 80, 2133–2140.
- Chowdhury, A., Singh, P., Bera, T.K., Ghoshal, D. and Chakraborty, B. (2017). Electrical impedance spectroscopic study of mandarin orange during ripening. *Journal of Food Measurement and Characterization* 11, 1–11.
- Clarke, J.L., Anderson, J.L., Carlquist, J.F., Roberts, R.F., Horne, B.D., Bair, T.L., Kolek,

- M.J., Mower, C.P., Crane, A.M., Roberts, W.L. and Muhlestein, J.B. (2005). Comparison of differing C-reactive protein assay methods and their impact on cardiovascular risk assessment. *American Journal of Cardiology* 95.
- Ding, P., Liu, R., Liu, S., Mao, X., Hu, R. and Li, G.(2013). Reusable gold nanoparticle enhanced QCM immunosensor for detecting C-reactive protein. *Sensors and Actuators, B Chemical* 188, 1277–1283.
- Dominici, R., Luraschi, P. and Franzini, C. (2004). Measurement of C-reactive protein: Two high sensitivity methods compared. *Journal of Clinical Laboratory Analysis*18, 280–284.
- EHN, (2008). *European Heart Network - European Cardiovascular Disease Statistics 2008* 1–113.
- Fakanya, W.M. and Tothill, I.E. (2014). Detection of the inflammation biomarker C-reactive protein in serum samples: Towards an optimal biosensor formula. *Biosensors* 4, 340–357.
- Gupta, R.K., Periyakaruppan, A., Meyyappan, M. and Koehne, J.E. (2014). Label-free detection of C-reactive protein using a carbon nanofiber based biosensor. *Biosensors & Bioelectronics*59, 112–119.
- Hansson, G.K. (2005). Mechanisms of disease: Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine*352.
- Hu, W.P., Hsu, H.-Y., Chiou, A., Tseng, K.Y., Lin, H.-Y., Chang, G.L. and Chen, S.-J. (2006). Immunodetection of pentamer and modified C-reactive protein using surface plasmon resonance biosensing. *Biosensors & Bioelectronics* 21, 1631–1637.
- Kadir, M.K.A. and Tothill, I.E. (2010). Development of an electrochemical immunosensor for fumonisins detection in foods. *Toxins (Basel)*. 2, 382–398.
- Libby, P., Ridker, P.M. and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* 105, 1135–1143.
- Lv, Y., Wu, R., Feng, K., Li, J., Mao, Q., Yuan, H., Shen, H., Chai, X., and Li, L.S. (2017). Highly sensitive and accurate detection of C-reactive protein by CdSe/ZnS quantum dot-based fluorescence-linked immunosorbent assay. *Journal of Nanobiotechnology* 15, 35.
- Mali, B., Armbruster, D., Serediak, E. and Ottenbreit, T. (2009). Comparison of immunoturbidimetric and immunonephelometric assays for specific proteins. *Clinical Biochemistry* 42, 1568–1571.
- Mazer, S.P. and Rabbani, L.E. (2004). Evidence for C-reactive protein's role in (CRP) vascular disease: Atherothrombosis, immunoregulation and CRP. *Journal of Thrombosis and Thrombolysis* 17, 95–105.
- Mazloum-Ardakani, M., Hosseinzadeh, L., Khoshroo, A., Naeimi, H. and Moradian, M. (2014). Simultaneous determination of isoproterenol, acetaminophen and folic acid using a novel nanostructure-based electrochemical sensor. *Electroanalysis* 26, 275–284.
- Meyer, M.H.F., Hartmann, M., Krause, H.-J., Blankenstein, G., Mueller-Chorus, B., Oster, J., Miethe, P. and Keusgen, M. (2007). CRP determination based on a novel magnetic biosensor. *Biosensors & Bioelectronics* 22, 973–979.
- Parra, M.D., Tuomola, M., Cabezas-Herrera, J. and Cerón, J.J. (2005). Use of a time-resolved immunofluorometric assay for determination of canine C-reactive protein concentrations in whole blood. *American Journal of Veterinary Research*66, 62–66.
- Puri, N., Tanwar, V.K., Sharma, V., Ahuja, T., Biradar, A.M. and Rajesh (2010). Electrochemical characterization of surface modified gold electrode for the detection of C-reactive protein. *International Journal of Integrative Biology* 9, 1–5.
- Quershi, A., Gurbuz, Y., Kang, W.P. and Davidson, J.L. (2009). A novel interdigitated capacitor based biosensor for detection of cardiovascular risk marker. *Biosensors & Bioelectronics* 25, 877–882.
- Repo, T., Paine, D.H. and Taylor A.G.* (2002). Electrical impedance spectroscopy in relation to seed viability and moisture content in snap bean (*Phaseolus vulgaris* L.). *Seed Science Research*12, 17–29.
- Rodriguez, M.C., Kawde, A.-N. and Wang, J. (2005). Aptamer biosensor for label-free impedance spectroscopy detection of proteins based on recognition-induced switching of the surface charge. *Chemical Communications* 4267–4269.

- Ross, R. (1999). Atherosclerosis — An Inflammatory Disease — NEJM. *New England Journal of Medicine* 1999, 340115-126.
- Salam, F. and Tothill, I.E. (2009). Detection of Salmonella typhimurium using an electrochemical immunosensor. *Biosensors & Bioelectronics* 24, 2630–2636.
- Songjaroen, T., Feeny, R.M., Mensack, M.M., Laiwattanapaisal, W. and Henry, C.S. (2016). Label-free detection of C-reactive protein using an electrochemical DNA immunoassay. *Sensing and Bio-Sensing Research* 8, 14–19.
- Tothill, I.E. (2009). Biosensors for cancer markers diagnosis. *Seminars in Cell and Developmental Biology* 20, 55–62.
- Verma, S. and Yeh, E.T.H. (2003). C-reactive protein and atherothrombosis--beyond a biomarker: an actual partaker of lesion formation. *American Journal of Physiology Regulatory Integrative and Comparative Physiology* 285, R1253-NaN-R1258.
- Vogt, S., Su, Q., Gutiérrez-Sánchez, C. and Nöll, G. (2016). Critical View on Electrochemical Impedance Spectroscopy Using the Ferri/Ferrocyanide Redox Couple at Gold Electrodes. *Analytical Chemistry* 88, 4383–4390.
- Wang, J., Yuan, R., Chai, Y., Cao, S., Guan, S., Fu, P. and Min, L. (2010). A novel immunosensor based on gold nanoparticles and poly-(2,6-pyridinediamine)/multiwall carbon nanotubes composite for immunoassay of human chorionic gonadotrophin. *Biochemical Engineering Journal* 51, 95–101.
- Yang, Y.-N., Lin, H.-I., Wang, J.-H., Shiesh, S.-C. and Lee, G.-B. (2009). An integrated microfluidic system for C-reactive protein measurement. *Biosensors & Bioelectronics* 24, 3091–3096.
- Zhang, B., Tang, D., Liu, B., Chen, H., Cui, Y. and Chen, G. (2011). GoldMag nanocomposite-functionalized graphene sensing platform for one-step electrochemical immunoassay of alpha-fetoprotein. *Biosensors & Bioelectronics* 28, 174–180.
- Zhou, F., Lu, M., Wang, W., Bian, Z.P., Zhang, J.R. and Zhu, J.J. (2010). Electrochemical immunosensor for simultaneous detection of dual cardiac markers based on a poly(dimethylsiloxane)-gold nanoparticles composite microfluidic chip: A proof of principle. *Clinical Chemistry* 56, 1701–1707.