Recombinant Anti BNP-SCFV Production in *Escherichia coli* and its Application for the Detection of Heart Failure by Electrochemical Immunosensor using Screen-Printed Carbon Electrode-Gold Nanoparticles (SPCE-GNP)

Gaffar Shabarni*, Hartati Yeni Wahyuni, Susanti Rini and Subroto Toto
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang, INDONESIA
*shabarni.gaffar@unpad.ac.id

Abstract
Heart failure is a medical emergency when the left ventricle gets expanded and this further stimulates the secretion of the BNP hormone into the blood. BNP can be used as diagnostic and prognostic marker for patients with heart failure. The concentration of BNP in a patient's blood can be detected through the interaction of BNP with Anti BNP-SCFV antibody fragment. In this study we reported the detection of BNP by using Anti BNP-SCFV-based immunosensor. Recombinant Anti BNP-SCFV was produced in the periplasm of Escherichia coli host. Furthermore, Anti BNP-SCFV was immobilized on the surface of carbon electrode, coated with gold nanoparticles (Screen-Printed Carbon Electrode-Gold Nanoparticles = SPCE-GNP).

The result showed that Anti-BNP-SCFV with molecular weight of 28 kDa was successfully produced in E. coli. Standard BNPs with concentration range from 1 pg/mL to 10.000 pg/mL were tested on SPCE-GNP-Anti BNP-SCFV immunosensor to determine their sensitivity and detection limit. The detection limit of Anti BNP-SCFV immunosensor was 1.03 pg/mL. Measuring of BNP concentration in normal blood serum by using Anti BNP-SCFV immunosensor showed that the BNP concentration in the normal blood was 0.55 pg/mL. Measurement of serum sample added with BNP showed that the recovery value was 93.5%. Anti BNP-SCFV immunosensor with SPCE-GNP electrode can be used to detect BNP levels in the blood serum.

Keywords: Recombinant Anti BNP-SCFV, heart failure, BNP, immunosensor.

Introduction

B-type natriuretic peptide (BNP) is a 32-amino acid peptide primarily secreted by the cardiac ventricles in response to increased myocardial wall stress due to volume overload or higher end-diastolic pressure inside the ventricle. BNP levels rise primarily in the presence of left ventricular dysfunction[1]. BNP has been recognized as a powerful cardiovascular biomarker for a number of disease states specifically heart failure[2,3]. BNP can be used to guide the therapy of heart failure and determine the patient's stage of disease. A large amount of data supports extending the use of BNP from a diagnostic marker to a prognostic marker[4-6].

BNP is produced as a 134 amino acid pre-pro-BNP peptide that is cleaved soon after secretion to yield the 108 amino acid pro-BNP. Further cleavage produces the biologically active 32 amino acid BNP peptide (BNP1-32) which contains a 17 amino acid ring closed by a disulfide bond and also the linear 76 amino acid N-terminal peptide (NT-pro-BNP1-76)[7]. The biologically active BNP1-32, the pro-BNP and the NT-pro-BNP1-76 can be measured in the plasma. Among cardiovascular biomarkers, BNP and NT-pro-BNP have emerged as powerful diagnostic tools for acute heart failure and as screening tools for detecting left ventricular systolic and diastolic dysfunction. BNP has estimated half-life of 20 min compared to 90–120 min for NT-pro-BNP[8].

In most cases, BNP and NT-pro-BNP levels are higher in patients with heart failure than people who have normal heart function. The normal concentration of BNP in the blood is less than 30 pg/mL and NT-pro-BNP is less than 125 pg/mL[9].

The level of BNP in blood plasma can be measured by point of care Triage Meter Plus assay (Biosite Diagnostic) that uses immune-fluorescence technology. Approximately 250 µL of whole blood was used as sample. This analysis can measure BNP concentration between 5-5000 pg/ml[10]. A BNP immunosensor using a microfluidic device equipped with portable surface plasmon resonance (SPR) spectroscopy achieved a detectable concentration range from 5 pg/ml to 100 ng/ml, covering clinical and pathological levels of BNP in blood[11]. NT-pro-BNP determination by using electrochemiluminescence (ECL) imunosensor demonstrated a quite wide linear range of 0.01–100 pg/mL[12].

Recombinant antibodies are highly specific detection probes in research, diagnostics and have emerged over the last two decades as the fastest growing class of therapeutic proteins[13]. A wide variety of recombinant antibody formats have been engineered, the most popular being the single chain variable fragment (SCFV) that consists of antibody variable domains connected by a flexible linker[14]. Antibody fragment against

BNP (Anti BNP-SCFV) is suitable to be used as diagnostic tool for BNP detection due to its small size compared to whole antibody[15].

Electrochemical biosensors have a unique place in the determination of hormones due to simplicity, sensitivity, portability and ease of operation. Unlike chromatographic techniques, electrochemical techniques used do not require pre-treatment[16]. Screen-printed electrodes were modified with nanomaterials to enhance the sensitivity of sensing devices which have been widely exploited. Among them, gold nanoparticles have been extensively used due to their unique properties such as large surface area, good biocompatibility and excellent catalytic and electronic property[17].

GNPs work in increasing the electroactive surface area which enhances the electron-transfer between the electrode and the analyte[18].The basic principle of electrochemical biosensors is the chemical reaction between immobilized biomolecule and target analyte produces or consumes ions or electrons which affects measurable electrical properties of the solution such as electric current or potential[19].

Anti BNP-SCFV has been expressed in methilothropic yeast *Pichia pastoris* and has been used as biosensor for detection of BNP by using planar type electrode. This electrochemical immunoassay exhibited excellent analytical performance with a detection limit of 1 fg/ml and a wide linear detection range from 1 to 10,000 fg/ml[15].

Escherichia coli have been successfully employed to generate genetically-engineered Anti BNP-SCFV antibody. The recombinant Anti BNP-SCFV was expressed in the cytoplasm of *E. coli*. However, most of the expressed SCFV (approximately 73.4%) formed insoluble inclusion bodies[20]. In the previous study, we used *E. coli* extracellular expression system to express our recombinant Anti BNP-SCFV and secreted it to periplasmic space[21]. However, Anti BNP-SCFV expressed in the periplasm still contains another protein. In this study we reported the production and isolation of Anti BNP-SCFV from the periplasm of *E. coli* and its application for the detection of BNP using immunosensor.

Hartati et al[22] used monoclonal Anti BNP and streptavidin-biotin system to immobilize the antibody to the surface of SPCE electrode. The detection of BNP using this immunosensor gives the linear range between 0,01-100 ng/mL and detection limit of 3,3 pg/mL. In this study, to improve the sensitivity of immunosensor we used nanogold modified SPCE. The use of small size recognition molecule (Anti BNP-SCFV) hopefully will increase immunoaffinity.

Material and Methods

Material: *E. coli* BL21[Anti BNP-SCFV] was from Gaffar et al.[21] The growth medium used was: Luria Bertani (0.5% yeast extract, 1% tryptone, 1% NaCl, 2% bacto agar). All materials for growth media were purchased from Oxoid. HRP labelled Anti BNP, 3,5,3',5'- tetrametyl benzidine (TMB) and BNP antigen was purchased from Abcam, UK. All chemicals used in the experiments were purchased from Sigma Aldrich.

Apparatus: Differential pulse voltammetry was carried out using potensiostat Metrohm® μAutolab type III connected to PC with NOVA software. The SPCEs-GNP electrode used consists of an Ag/AgCl as reference electrode, platinum electrode as an auxiliary electrode and carbon electrode with nanogold particle (0.5 mm) as working electrode (Dropsens).

Production and Isolation of Anti BNP-SCFV from *E. coli*
E. coli BL21 [Anti BNP-SCFV] was used to express Anti BNP-SCFV following the procedure of Gaffar et al[21]. Anti BNP-SCFV was isolated from *E. coli* peripheral by using osmotic shock method and TSE method (Tris-Sucrose-EDTA), following the procedure of Quan et al[23]. Twenty microliter of protein fraction was analyzed by SDS-PAGE. Protein band were detected using Coomassie brilliant blue dye. Protein concentration was measured following Lowry method[24].

Detection of BNP by Anti BNP-SCFV Immunosensor
Immobilization of the Anti BNP-SCFV: A solution of 20 μL Anti BNP- SCFV (50 μg/mL in 0,01 M PBS buffer) was immobilized on SPCEs-GNP electrode. The SPCEs-GNP-Anti BNP-SCFV was incubated overnight at 4°C. The electrode surface was washed by immersing into PBS (0.01 M; pH 7.4). Later, non-specific bindings were blocked by incubating the electrode surface with BSA 1% for 15 minutes[22].

Analysis of immobilized immunoreagents: The attachment of immunoreagents on SPCE-GNP was measured by observing cyclic voltammograms from redox activity of electroactive species 10 mM $[Fe(CN)_6]^{3-}$ in 0,001 mM KCl. Measurement was performed after addition of each immunoreagent at potential ranging from -0,6V to –0,6V with scanning rate at 50 mV/sec.

Optimization of Anti BNP-SCFV concentration: Anti BNP-SCFV with various concentrations (50, 100, 150, 200 and 250 μg/mL) was immobilized on the surface of SPCEs-GNP and incubated for 60 minutes. Non-binding electrode surface was blocked by incubating the electrode with BSA 1% for 15 minutes. Furthermore, 20 μL of BNP antigen (1 ng/mL) prepared in 0.01 M PBS was added onto the electrodes surface and left to react with the Anti BNP-SCFV capture antibody for 1 h. Afterwards, the surface was washed by immersing electrode into PBS (0.01 M; pH 7.4).

Furthermore, 20 μL of 5 μg/mL HRP-labelled Anti BNP detection antibody was dropped and incubated for 60 minutes[20]. The electrodes were washed prior to electro-chemical measurement. Immunosensor measurement was

done by an addition of 50 µL TMB (1:1000) as substrate and it was allowed to react for 10 minutes. Immunosensor responses were measured by differential pulse voltammetric by scanning at 50 $mV \cdot s^{-1}$ and potential ranging from −0.6 V to 1.0 V. The schematic method for BNP detection by immunosensor was is shown in figure 1.

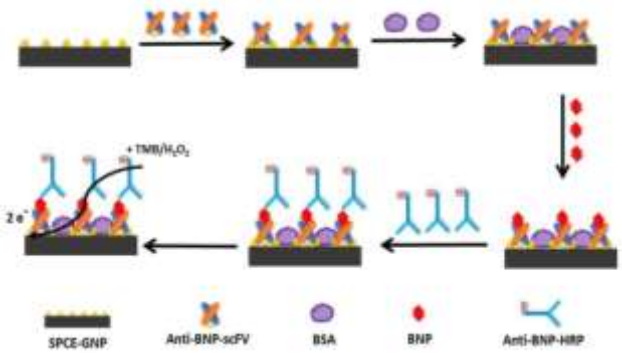

Figure 1: Schematic protocol of BNP determination by electrochemical immunosensor using SPCE-GNP.

Calibration curve and LOD determination: BNP with various concentrations (10^{-2},10^{-3},10^{-4}, 10^{-5} and 10^{-6} µg/mL) were added onto SPCEs-GNP electrode that has been immobilized with optimum concentration of Anti BNP-SCFV. Immunosensor responses were measured as mentioned above.

Measurement of serum BNP: Serum was separated from whole blood by centrifugation. Only 20 µL serum was needed for measurement. Serum with an addition of 10 pg/mL BNP was used to calculate the recovery value. Concentration of serum BNP was calculated based on calibration curve.

Results and Discussion

Production and isolation of Anti BNP SCFV from *E. coli* periplasm: Anti BNP SCFV was produced on the periplasm of *E. coli* by using signal peptide of *E. coli* OmpA protein[20]. However, the result showed that periplasm still contains another protein (figure 2). Periplasmic proteins are estimated to constitute only ~4–16% of the total cellular protein in *E. coli*. To isolate only periplasmic protein, we used two methods: osmotic shock and TSE extraction procedure[22]. Contamination of periplasmic protein extraction is cytosolic protein that was released due to cell lysis during the repetitive pelleting and resuspension steps of the osmotic shock procedure.

Sucrose in the extraction buffer increases the extracellular osmolality causing the cells to shrink and release water and periplasmic contents into the surrounding medium. Sucrose can go through the outer membrane to enter the periplasmic space but is unable to penetrate the inner membrane. The presence of sucrose at the water–inner membrane interface presumably helps to stabilize the membrane and therefore prevents cell lysis[24]. SDS-PAGE characterization (figure 2) shows that Anti BNP-SCFV with molecular weight of 28 kDa was succesfully expressed into pheriplasmic space of *E.coli*. Figure 2 also shows that isolated protein by using TSE method contains less contamination compared to osmotic shock method.

Immunosensor Responses of Anti BNP-SCFV: Cyclic voltamogram from redox activity of electroactive species of 10 mM $[Fe(CN)_6]^{3-}$ in 0,001 mM KCl after addition of each immunoreagent is shown in figure 3.The decrease in peak current after immobilization of Anti BNP-SCFV means that a part of SPCE-GNP surface has been covered by Anti BNP-SCFV. Therefore, it caused the impediment of electron transfer between $[Fe(CN)_6]^{3-}$ and electrode. It also occured after the addition of BSA, BNP and Anti BNP-HRP. This means that the addition of immunoregents caused the electroactive species $[Fe(CN)_6^{3-}$to become far away from electrode surface which consequently blocked the electron transfer.

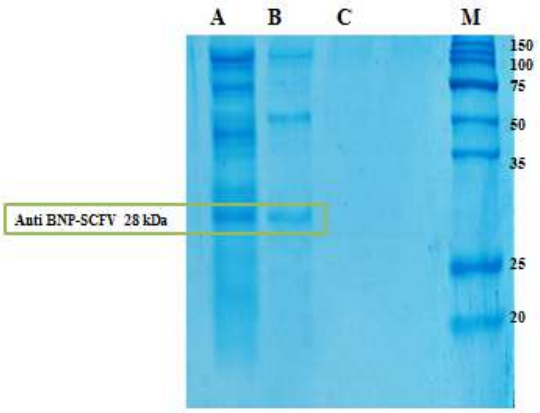

Figure 2: SDS-PAGE characterization of periplasm protein. (M) protein marker; (A) periplasm protein isolated with osmotic shock method and (B) periplasm protein isolated with TSE method.

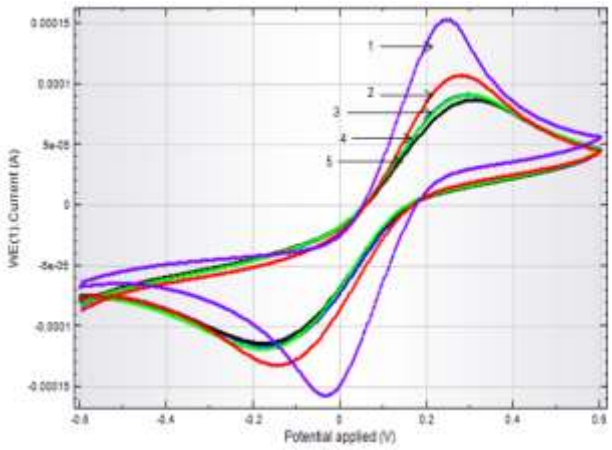

Figure 3: Cyclic voltammogram of SPCEs-GNP with addition of 10 mM $K_3[Fe(CN)_6]$ in 100 mM KCl with scan rate 50 mV/s. (1) Bare SPCEs-GNP in 0.01 M PBS pH 7.4. (2) SPCEs-GNP-Anti BNP-SCFV (3) SPCEs-GNP-Anti BNP-SCFV-BSA (4) SPCEs-GNP-Anti BNP-SCFV-BSA-BNP (5) SPCEs-GNP-Anti BNP- SCFV-BSA-BNP-Anti BNP-HRP.

The most important criterion in the immobilization of functional biomolecules onto solid-state surfaces is to maintain the biological activity while retaining the 3D native structures[26]. The immobilization of Anti BNP-SCFV onto the functionalized surfaces of SPCE-GNP was achieved via physical absorption. GNP works on increasing the electroactive surface area which enhances the electron-transfer between the electrode and the analyte.

Although the non-specific immobilization of biomolecules on solid-state surfaces may be the rapid, simple and cheap method, such non-specific immobilization of biomolecules on a solid-state surface often leads to highly random orientation of the immobilized biomolecules and it may induce significant conformational change to the native 3D structure of biomolecules, thus causing severe loss of biological activity[26]. However, using small factional molecule such as Anti BNP SCFV will reduce conformational changes.

Anti BNP-SCFV optimum concentration: Various concentrations of Anti BNP-SCFV were tested to measure the optimum concentration which gives higher peak current. Figure 4 shows that 150 µg/mL of Anti BNP SCFV gives the higher peak current. Anti BNP-SCFV with higher than 150 µg/mL gives lower peak current. Our previous study used biotinilated Anti BNP capture antibody and the optimum concentration is 5 µg/mL[22]. Higher concentration of Anti BNP-SCFV was needed, may be because anti BNP-SCFV is a small molecule, but it may also be because the anti BNP-SCFV produced in the periplasm of *E. coli* was mixed with another periplasmic protein.

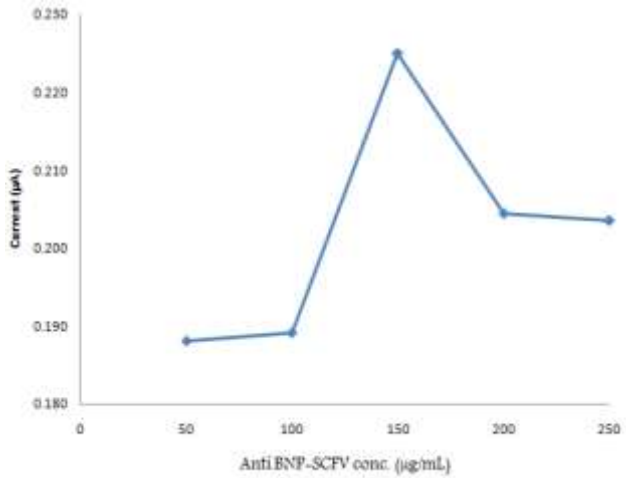

Figure 4: Optimum concentration of Anti BNP-SCFV

Calibration curve and limit of detection: Figure 5 shows that the calibration graph for oxidation peak was linear from 1 to 10.000 pg/mL and obeyed the equation:

Y = 9,958x + 0,1365

where y and x are the peak current (µA) and BNP concentration (pg/mL), respectively. The correlation coefficient (r) was 0.9926. Detection limit was measured by using the equation of Y= 4.933 x + 0,0950 (BNP concentration 0-10 pg/mL) with r^2 value as 0,998. The detection limit was estimated as 1,03 pg/mL.

Anti BNP-SCFV immunosensor was used to measure BNP concentration in normal blood serum. We found that the concentration of BNP was 0,55 pg/mL. Recovery value was measured by addition of 10 pg/mL standard BNP into normal serum and we found that the recovery value was 93,5%.

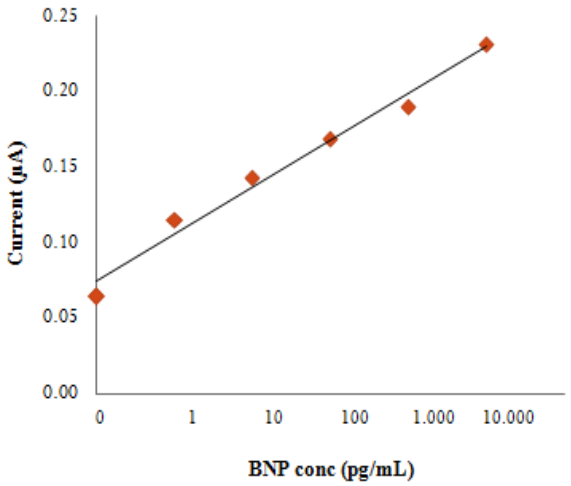

Figure 5: Calibration curve of BNP concentration

Since many factors can elevate BNP, interpreting blood levels of BNP can be complex. Most experts now agree that it is appropriate to use two cut-off values in assessing BNP levels-a lower value that reliably excludes heart failure and a higher value that accurately confirms heart failure. A BNP value of less than 100 pg/mL essentially rules out heart failure whereas values greater than 400 pg/mL indicate a 95% likelihood of heart failure. Values between 100 pg/mL and 400 pg/mL warrant further investigation. A suggested "normal" range for BNP is 0.5-30 pg/mL[9].

The most important use of natriuretic peptides is in helping to establish the diagnosis of heart failure (HF) in a patient in the urgent care setting within whom the diagnosis is uncertain. The reference values of BNP are different to exclude or confirm a diagnosis of heart failure. These values also depend on age and gender and are higher in elderly persons and women[9].

The good immunoaffinity of Anti BNP-SCFV was attributed to the smaller size of Anti BNP-SCFV than that of the whole antibody. Indeed, the small size of Anti BNP-SCFV facilitates its highly concentrated immobilization on the electrode surface.

Conclusion

Recombinant Anti BNP-SCFV with molecular weight BM of 28 kDa was successfully produced in the periplasm of *E.*

coli. Anti BNP-SCFV immunosensor with SPCE-NGP electrode can be used to detect BNP concentration with the detection limit of 1,03 pg/mL. Measurement of serum samples with addition of standard BNP showed that the recovery value was 93,5 %.

Acknowledgement

The authors acknowledge the financial support from Penelitian Unggulan Perguruan Tinggi (PUPT) the Ministry of Research, Technology and Higher Education of the Republic of Indonesia and Academic Leadership Grant Program, Universitas Padjadjaran.

References

1. Leto L., Testa M. and Feola M., Correlation between B-type Natriuretic Peptide and Functional/Cognitive Parameters in Discharged Congestive Heart Failure Patients, *Int. J. of Endocrin.*, **2015**, 1-7 **(2015)**

2. Maalouf R. and Bailey S., A Review on B-type Natriuretic Peptide Monitoring: Assays and Biosensors, *Heart Fail. Rev*., **21**, 567–578 **(2016)**

3. Grewal J., Chandavimol M. and Ignaszewski A., B-type Natriuretic Peptide: A New Marker for Congestive Heart Failure, *British Columbia Medical Journal*, **46(1)**, 24-29 **(2004)**

4. Harrison A., Morrison K. and Krishnaswamy P., B-type Natriuretic Peptide Predicts Future Cardiac Events in Patients Presenting to the Emergency Department with Dyspnea, *Ann. Emerg. Med*., **39**, 131-138 **(2002)**

5. Berger R., Huelsman M. and Strecker K., B-type Natriuretic Peptide Predicts Sudden Death in Patients with Chronic Heart Failure, *Circulation*, **105**, 2392-2397 **(2002)**

6. Maeda K., Tsutamoto T. and Wada A., High Levels of Plasma Brain Natriuretic Peptide and Interleukin-6 after Optimized Treatment for Heart Failure are Independent Risk Factors for Morbidity and Mortality in Patients with Congestive Heart Failure, *J. Am. Coll. Cardiol*., **36**, 1587-1593 **(2000)**

7. De Lemos J.A., McGuire D.K. and Drazner M.H., B-type Natriuretic Peptide in Cardiovascular Disease, *Lancet*, **362**, 316–322 **(2003)**

8. Vanderheyden M., Bartunek J. and Goethals M., Brain and Other Natriuretic Peptides: Molecular Aspects, *Eur. J. Heart Fail*., **6**, 261–268 **(2004)**

9. O'Hanlon R., O'Shea P., Ledwidge M., O'Loughlin C., Lange S., Conlon C., Phelan D., Cunningham S. and McDonald K., The Biologic Variability of B-type Natriuretic Peptide and N-terminal pro-B-type Natriuretic Peptide in Stable Heart Failure Patients, *J. Card. Fail*., **13(1)**, 50-5 **(2007)**

10. McDonnell B., Hearty S., Leonard P. and O'Kennedy R., Cardiac Biomarkers and the Case for Point-of-care Testing, *Clinical Biochem*., **42**, 549–561 **(2009)**

11. Kurita R., Yokota Y., Sato Y., Mizutani F. and Niwa O., On-chip Enzyme Immunoassay of a Cardiac Marker using a Microfluidic Device Combined with a Portable Surface Plasmon Resonance System, *Anal. Chem*., **78**, 5525–5531 **(2006)**

12. Zhang H., Han Z., Wang X., Li F., Cui H., Yang D. and Bian Z., Sensitive Immunosensor for N-Terminal Pro-brain Natriuretic Peptide Based on N-(Aminobutyl)-N-(ethylisoluminol)-functionalized Gold Nanodots/Multiwalled Carbon Nanotube Electrochemiluminescence Nanointerface, *ACS Appl. Mater. Interfaces.*, **7(14)**, 7599–7604 **(2015)**

13. Frenzel A., Hust M. and Schirrmann T., Expression of Recombinant Antibodies, *Front Immunol*., **4**, 217 **(2013)**

14. Bach H., Mazor Y., Shaky S., Shoham-Lev A., Berdichevsky Y., Gutnick D.L. and Benhar I., *Escherichia coli* Maltose-binding Protein as a Molecular Chaperone for Recombinant Intracellular Cytoplasmic Single Chain Antibodies, *J. Mol. Biol*., **312**, 79–93 **(2001)**

15. Maeng B.H., Choi J., Sa Y.S., Shin J.H. and Kim Y.H., Functional Expression of Recombinant Anti-BNP SCFV in Methylotrophic Yeast *Pichia pastoris* and Application as a Recognition Molecule in Electrochemical Sensors, *World Journal Microbiol. Biotechnol*., **28(3)**, 1027-1034 **(2012)**

16. Bahadır E.B. and Sezgintürk M.K., Electrochemical Analyses for Hormone Analyses, *Biosensor and Bioelectronics*, **68**, 62-71 **(2015)**

17. See W.P., Nathan S. and Heng L.Y., A Disposable Copper (II) Ion Biosensor Based on Self-assembly of L-cysteine on Gold Nanoparticle-modified Screen-Printed Carbon Electrode, *Journal of Sensors*, **2011**, 1-5 **(2011)**

18. Farghali R.A. and Ahmed R.A., Gold Nanoparticles Modified Screen Printed Carbon Electrode for Voltammetric Determination of Sildenafil Citrate (Viagra) in Pure Form, Biological and Pharmaceutical Formulations, *Int. J. Electrochem. Sci*., **10**, 1494 – 1505 **(2015)**

19. Monosík R., Miroslav S. and Ernest S., Biosensors-classification, Characterization and New Trends, *Acta Chimica Slovaca*, **5**, 109-120 **(2012)**

20. Bu D., Zhou Y., Tang J., Jing F. and Zhang W., Expression and Purification of a Novel Therapeutic Single-chain Variable Fragment Antibody against BNP from Inclusion Bodies of *Escherichia coli*, *Protein Expression and Purification*, **92**, 203–207 **(2013)**

21. Gaffar S., Aji S.M.N., Hartati Y.W., Ishmayana S. and Subroto T., Expression of Recombinant Antibody Fragment, Anti BNP-SCFV on the Periplasm of *Escherichia coli* for the Detection of Heart Failure, *Molekul*, **12(1)**, 30–36 **(2017)**

22. Hartati Y.W., Daniswara M.H., Nurmalasari R., Gaffar S. and Subroto T., An Electrochemical Immunosensorfor the Detection of B-type Natriuretic Peptide (BNP) Based on Double Sandwich ELISA Using Screen Printed Carbon Electrodes, *Molekul*, **13(1)**, 1-8 **(2018)**

23. Quan S., Hiniker A., Collet J.F. and Bardwell J.C.A., Isolation of Bacteria Envelope Proteins, *Methods in Molecular Biology*, **966**, 359-66 **(2013)**

24. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall J.R., Protein Measurement with the Folin Phenol Reagent, *Journal of Biological Chemistry*, **193**, 265-275 **(1951)**

25. Strauss G. and Hauser H., Stabilization of Lipid Bilayer Vesicles by Sucrose during Freezing, Proc. Natl. Acad. Sci. USA, **83**, 2422–2426 **(1986)**

26. Kim D.C. and Kang D.J., Molecular Recognition and Specific Interactions for Biosensing Applications, *Sensors*, **8**, 6605-6641 **(2008)**.