DISPOSABLE CYSTEINE BASED ELECTROCHEMICAL IMPEDANCE BIOSENSOR FOR SKIN SENSITIZATION ANALYSIS

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A thesis submitted in fulfilment of the

requirements for the award of the degree of

Master of Engineering (Bioprocess)

Faculty of Chemical Engineering

Universiti Teknologi Malaysia

OCTOBER 2014

Dedicated to my beloved parents, bestfriends, and myself

ACKNOWLEDGEMENT

First of all, Alhamdulillah. I would like to express my gratitude to Allah SWT for all of His gifts and strengths for me to complete this thesis. I want to dedicate this thesis to my parents, Noh Mohammad and Nor Azizan Agus, and my siblings for all their prayers, patience and full support. Because of them, I have the courage to go through the entire ordeal in completing my thesis.

I wish to verbalize my most sincere gratitude for my lovely supervisor, Assoc. Prof. Dr. Azila Abd. Aziz, that has provided me with the outmost support, understanding and kindness. I also like to express my gratitude to all of my examiners, technical staffs of Faculty Chemical Engineering and Mr. Abdul Rahim from Faculty of Science.

Subsequently, I would like to express my gratefulness to the PSM students, Syahidah and Azirah for helping me to understand this research and to my bestfriends, Noraayu, Norazila and Fatihah Hayati who have always given me the support and endless motivation to continue my studies. Besides that, to Mrs. Norhayati Mohamed Noor and all staffs in the Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia, thank you for sharing with me the knowledge and kindly gave me the permission to use their apparatus and laboratory. Also, thanks to my labmates, Mastura, Wahida and Sakura who helped me during the experiments. Last but not least, a bunch of thank you to all of my friends that contributed their favors directly or indirectly in helping me to finish my thesis and gave a lot of strength to me. Thank you very much.

ABSTRACT

Nowadays, the personal care and cosmetics market is one of the largest markets in the world. However, some potential cosmetic ingredients may cause skin sensitization. Animal testing is deemed as a perfect but controversial solution to skin sensitization analysis. The European Union (EU), which has the most stringent and protective regulations for cosmetic has agreed to ban all tests related to animals. This has wide ranging implications for cosmetic companies worldwide as a cosmetic product which has been successfully registered in the EU can be easily registered worldwide. Thus, *in chemico, in silico* or *in vitro* alternative methods for the prediction of skin sensitization need to be introduced. The main purpose of this work was to investigate the performance of an impedance skin sensitizer biosensor obtained using self-assembly of cysteine on screen printed carbon electrode (SPCE) modified with gold nanoparticles (AuNPs). The basis of the biosensor developed in this work was that the conjugation of allergen to cysteine-AuNPs on SPCE would result in the commencement of the skin sensitization process. A biosensor with good reproducibility (relative standard deviations of 8.43 %) and sensitivity was obtained when 50 mM of cysteine was deposited on the AuNPs and left for 24 hours on the SPCE. The biosensor managed to successfully differentiate between water soluble mild, medium and strong sensitizers based on the values of the changes in charge transfer resistance (ΔRct). Different allergen concentrations did not significantly affect ΔRct readings (the range studied was between 10 to 90 mM). The biosensor in this research work was found to have the potential to be successfully used as an alternative method to animal testing for the detection of skin sensitizers.

ABSTRAK

Pada masa kini, pasaran produk penjagaan diri dan kosmetik adalah salah satu pasaran yang terbesar di dunia. Walau bagaimanapun, beberapa bahan yang berpotensi digunakan dalam kosmetik boleh menyebabkan perengsaan kulit. Ujian haiwan dianggap sebagai penyelesaian yang sempurna bagi analisis perengsaan kulit tetapi ia adalah penyelesaian yang kontroversial. Kesatuan Eropah yang mempunyai peraturan yang paling ketat untuk kosmetik telah bersetuju untuk mengharamkan semua ujian yang berkaitan dengan haiwan. Ini memberi implikasi yang besar kepada syarikat kosmetik di seluruh dunia kerana produk kosmetik yang berjaya didaftarkan di Kesatuan Eropah akan mudah didaftarkan di seluruh dunia. Oleh itu, kaedah alternatif 'in *chemico'*, '*in silico*' atau '*in vitro*' untuk ramalan perengsaan kulit perlu diperkenalkan. Tujuan utama penyelidikan ini adalah untuk mengkaji prestasi biosensor perengsa kulit berasaskan impedans yang diperoleh dengan kaedah pembentukan sendiri lapisan sisteina pada karbon elektrod dicetak skrin (SPCE) yang diubahsuai dengan menggunakan nanopartikel emas (AuNPs). Asas biosensor yang dibangunkan dalam kerja ini adalah konjugasi alergen dengan sisteina-AuNPs akan memulakan proses perengsaan kulit. Biosensor yang mempunyai kebolehulangan yang baik (sisihan piawai relatif 8.43 %) dan kepekaan yang baik telah diperoleh apabila 50 mM sisteina diletakkan di atas lapisan AuNPs dan ditinggalkan selama 24 jam. Biosensor ini berjaya membezakan antara bahan perengsa larut air yang dikelaskan sebagai perengsa yang rendah, sederhana dan kuat berdasarkan nilai perubahan rintangan pemindahan cas (ΔRct). Kepekatan alergen yang berbeza tidak memberi kesan yang ketara kepada bacaan ΔRct (julat kepekatan yang dikaji ialah diantara 10 hingga 90 mM). Biosensor dalam penyelidikan ini mempunyai potensi untuk digunakan dengan jayanya sebagai kaedah alternatif kepada ujian haiwan untuk mengesan perengsa kulit.

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LIST OF ABBREVIATIONS

AC	-	Alternation current
ACD	-	Allergic contact dermatitis
AuNPs	-	Gold nanoparticles
C	-	Capacitors
CE	-	Counter electrode
CIR	-	Cosmetic ingredient review
CPE	-	Constant phase element
CTFRA	-	Cosmetic, toiletries and fragrance
DC	-	Direct current
DPRA	-	Direct peptide reactivity assay
E	-	Extreme
EE	-	Epidermal equivalent
EEC	-	European economic community
EIS	-	Electrochemical impedance spectroscopy
EU	-	European union
FESEM	-	Field scanning electrons microscoping
FDA	-	Food and drug administration
FRA	-	Frequency response analysis

GHS	-	Globally harmonized system
HcLAT	-	Human cell line activation test
HET-CAM		Hen’s egg test chorio-allantoic membrane
HPLC	-	High-performance liquid chromatography
HSA	-	Human serum albumin
IFRA	-	International fragrance association
INRA		Institute national de la recherche agronomique
ISE	-	Ion selective electrode
IUPAC	-	International union of pure and applied chemistry
LLNA	-	Local lymph node assay
LVMH	-	Louis vuitton moët hennessy
M	-	Moderate
MUSST	-	Myeloid u937 skin sensitization test
n	-	Roughness of the electrode
NS	-	Non-sensitizer
NLLS	-	Nonlinear least squares
PBS	-	Phosphate buffer saline
S	-	Strong
SAM	-	Self –assembled monolayer
SPCE	-	Screen printed carbon electrode
SPR	-	Surface plasmon resonance
Rct	-	Charge transfer resistance
RE	-	Reference electrode
r-LLNA	-	Reduced local lymph node assay

Rs	-	Solution resistance
RSD	-	Relative standard deviation
W	-	Warburg impedance
w	-	Weak
WE	-	Working electrode

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CHAPTER 1

INTRODUCTON

1.1 Background of Study

Cosmetics and personal cares products are always referred to as any substance used to beautify, cleanse and promote attractiveness of the human body. Although cosmetics and personal cares products can help to beautify the body, they can also cause skin allergies or skin sensitization. Certain ingredients used for the production of cosmetic products such as fragrance and preservatives can act as allergens or substances that can trigger allergic reaction or skin sensitization. Hence, to ensure that cosmetics are safe for the consumers, safety analysis of cosmetic ingredients should always be performed (Basketter *et al*., 2008).

Skin sensitizers are substances that are able to elicit an allergic response following contact with the skin. Allergic contact dermatitis is the second common occupational illness with 10 % to 15 % cases reported throughout the world. Toxicity testing using animals is usually conducted to ensure that cosmetics and personal cares product are free from dangerous toxics that can cause eye, skin and other irritations.

However, animal testing is controversial, particularly for cosmetic development, and a lot of efforts have been expended to replace animal test in cosmetics. Regarding skin sensitization, one validated alternative method is the mouse local lymph node assay (LLNA) (Alexandre *et al*., 2011). The LLNA is a predictive test to identify chemicals that have potential to cause skin sensitization by using guinea pig and human data (Ryan *et al.*, 2000). LLNA is an *in vivo* method but with potential to reduce the number of animals required to assess the allergenic contact sensitizing activity and offers a substantial refinement of the way in which the animals are used (Basketter and Kimber, 2006). LLNA for skin sensitization testing was performed until March 2013.

Animal testing of cosmetic ingredients was completely banned in Europe starting from March 2013. Non-animal methods have been introduced such as cell culture analysis, microorganism studies, human skin recombinants, and embryonic testing for prediction of skin sensitization (Chew and Maibach, 2006). However, a validated non-animal method is not yet available for assessing skin sensitization. At present, there are a few non-animal test methods, such as direct peptide reactivity assay (DPRA), Keratinosens^{TM}, human cell line activation test (hCLAT) and myeloid U937 skin sensitization test (MUSST) which measures the induction of protein markers associated with dendritic cell maturation *in vivo* following exposure to the chemicals. The results are expected to be out in late 2013 or early 2014. It has been anticipated however that, it will take at least another 3-5 years for the full replacement of the currently used animal models to assess sensitization (Adler *et al*., 2011).

Some researchers published *in vitro* methods based on skin dendritic cell lines and keratinocytes (Ashikaga *et al*., 2006; Ryan *et al*., 2007). Schoeters *et al.* (2007) investigated whether different gene expression patterns in dendritic cells are relevant for prediction of chemicals sensitizing potential using microarray technology.

Other than that, some alternative methods to replace animal testing are based on electrophilic assays. The electrophilic properties of allergens may enable reaction with skin nucleophiles to form macromolecular immunogens (Aptula *et al*., 2005). The reactivity of nucleophiles might then be used as a skin sensitizer screening tool. The strongest potential nucleophiles in proteins are the sulfhydryl group of cysteine, e-amino group of lysine and imidazole group of histidine (Divkovic *et al*., 2003). Recently published methods are by: 1) using human serum albumin (HAS) reactivity to find the types of amino acids modified and the nature of haptenation by exact mass shift determination and sequencing, 2) using glutathione for non-enzymatic thiol reactivity combined with *in vitro* toxicity measurement (Aptula *et al*., 2006) and 3) using HPLC for synthetic peptides reactivity (Gerberick *et al*., 2004).

Incorporation of lysine into the peroxidase peptide reactivity assay for skin sensitization assessments was reported by Troutman *et al*. (2011). The researchers reported that practical quantitative *in chemico* reactivity assay for screening contact allergens had been incorporated into the liquid chromatography for reactivity assessments of hapten and pre-/pro-hapten chemical sensitizers. On the other hand, investigation of pro-hapten skin sensitizer peptide reactivity using a peroxidase-peroxide oxidation system was investigated by Gerberick *et al*. (2009). The work showed the potential to merge an enzyme-mediated approach by using hydrogen peroxide and horseradish peroxidase for assessing the skin sensitization potential of pro-haptens.

In 2010, Institute National de la Recherche Agronomique (INRA) researchers collaborated with Louis vuitton moet hennessy (LVMH) to produce potential biosensors that use surface plasmon resonance (SPR) technology to assess the risks of skin sensitization by chemical substances for the first response studies (Achilleos *et al*., 2009). SPR is used to measure the interaction between analyte and ligand immobilized on the surface of metallic layer based on the variations in the connection’s refractive index when the analyte interacts with the ligand (David Myszka, 1999).

SPR biosensors technology involves label-free binding interactions between an analyte in solution and an immobilized ligand, being monitored directly by changes in refractive index at the biosensors surface. The researchers proved the applicability of SPR in characterizing small molecule interactions: drug–protein interactions (Touil *et al*., 2005), antigen–antibody interactions (Aizawa *et al*., 2007), inhibitor–enzyme interactions (Stenlund *et al*., 2006) and small molecule–nucleic acid interactions (Cao *et al*., 2007).

SPR can also measure equilibrium analysis in affinity and enthalpy. The kinetic measurements by SPR also generated real-time binding data that matched to the studies of binding kinetics (Van der Merwe, 2001). However, SPR has several disadvantages. One of them is long response time to analyze the analyte. SPR is not suitable for concentration measurement because it requires long equilibration period. It also has low sensitivity, high regeneration time, high-cost detection, blockages or air bubbles occurrence during extended experiments and others (Francis Markey, 1999).

Electrochemical impedance spectroscopy (EIS) would be one of the alternative methods to solve the problems faced by SPR. EIS is rapidly developing due to their low-cost detection, high sensitivity, simplicity and miniaturization compared to SPR (Daniel and Pourmand, 2008). Using very small amplitude voltage signals, EIS can measure electrochemical events without disturbing the properties of the analytes. Besides, EIS can provide repeatable and accurate measurements of surface conditions such as the adsorption and desorption process at the electrode surface. Furthermore, EIS allows complex biorecognition events to be probed in a sensitive, simple and label-free strategy (Chen *et al*., 2011). Label-free biosensor is a direct detection measurement of the biological interaction and do not required labeling for detection.

Chemical allergens can react with proteins to produce stable covalent bonds that can results in skin sensitization. Chemical allergens are electrophilic and can

react with nucleophilic amino acids (peptide) such as histidine cysteine, and lysine. Divkovic *et al*. (2003) reported that the strongest potential nucleophiles in proteins are the imidazole group of histidine, the Ɛ-amino group of lysine, and the sulfhydryl group of cysteine. In this work, EIS has been proposed as a method that can be utilized to probe the haptenation process between the chemical allergen and the nucleophilic amino acid, thus making it a potential method that can be used in a skin sensitization biosensor.

Cysteine cannot be immobilized directly to screen printed carbon electrode (SPCE). Alternatively, gold nanoparticles (AuNPs) can be used as the first layer on SPCE to immobilize the self assembled cysteine (Pooi See *et al*., 2011). In this work, comparison between AuNPs electrodeposition and growth method was performed to modify the SPCE. Gold electrodeposition is a process that uses electrical current to reduce gold solution to form a gold coating on an electrode. It mostly changes the surface properties of an electrode and is equivalent to the electroplating process. The synthesis of AuNPs by seeding growth approach method in the presence of cysteine as a reducing agent and surface additive is a recent popular study. The size of AuNPs contributes to the size of the nucleation site in which decreasing the AuNPs size resulted in increasing the size of the nucleation sites. The AuNPs can be fabricated and grown through slow diffusion onto the surface electrode (Pranjal Chandra *et al*., 2013).

1.2 Problem Statements

Due to the limitation in using animal models for the testing of the safety of cosmetic ingredients, a potential skin sensitizer biosensor based on EIS was proposed in this study. Haptenation process was expected to bring about a measurable change in impedance. No biosensor of this kind has been proposed before, thus this study aimed to prove this concept.

Electrochemical measurements are typically analyzed using conventional three cell electrodes cell. However, for miniaturization purpose, SPCE can be used to replace the conventional electrode cell for developing biosensors. This is because it is disposable and is cost effective (Loaiza Oscar *et al.*, 2011). In this work, cysteine was chosen as the nucleophilic amino acid for the main biorecognition element for the biosensor. Cysteine is more reactive than lysine and histidine to screen allergens against the amino acid in direct binding assay (Achilleos *et al.*, 2009). Unfortunately, cysteine cannot be immobilized directly on to SPCE. Alternatively, AuNPs are used as the first layer on SPCE and cysteine was then immobilized on top of the AuNPs (Pooi See *et al.*, 2011).

Proper immobilization of AuNPs onto the SPCE will influence the surface area and particle size of AuNPs (Dolati *et al.*, 2011). By using proper additive in the AuNPs deposition method, the surface area of AuNPs on SPCE will be increased and the particle size will be smaller.

1.3 Objective

The main purpose of this work was to develop a label-free impedance biosensor based on cysteine for skin sensitization studies.

1.4 Hypothesis

The Rct for different classes of skin sensitization allergen will produce different values. The high classes of skin sensitization allergen will produce high Rct

compared to medium classes of skin sensitization allergen while low classes of skin sensitization will result in the lowest value for Rct.

1.5 Scopes of the Research

Below is the scope of this work:

I. Preparation of a label-free impedance biosensor. Modifying SPCE with AuNPs and cysteine, the effects of cysteine concentration and deposition time of the immobilization process of SPCE were investigated.

II. Investigations on the characteristics of a label-free impedance biosensor in terms of biosensor stability, surface characterization of the SPCE and reproducibility of the label-free impedance biosensor.

III. Determination of a label-free impedance biosensor performance including the effect of contact time of allergens, concentration of allergen, different classes of sensitization of allergen towards cysteine and skin sensitization analysis of extract A and B.

1.6 Rational and Significant

This biosensor will become a contribution to humankind and can act as an essential tool to detect the presence of potential allergen in cosmetic. The biosensor in this research is more identical to a diagnostic kit in terms of its qualitative measurements. This biosensor is also environmental-friendly and can provide detection at low-cost.

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