Title	オープンサーキットポテンシャルを用いた超高感度分子検出
Author(s)	Charoenkitamorn, Kanokwan
Citation	
Issue Date	2018-03
Type	Thesis or Dissertation
Text version	ETD
URL	http://hdl.handle.net/10119/15333
Rights	
Description	Supervisor:高村　禅，マテリアルサイエンス研究科，博士

氏　　　　　名	Kanokwan CHAROENKITAMORN
学位の種類	博士(マテリアルサイエンス)
学位記番号	博材第 450 号
学位授与年月日	平成 30 年 3 月 23 日
論文題目	Ultra-Sensitive Molecular Detection Using Open Circuit Potential
論文審査委員	主査　高村　禅　北陸先端科学技術大学院大学　教授
	芳坂　貴弘　同　教授
	平塚　祐一　同　准教授
	赤堀　誠志　同　准教授
	山口　孝浩　金沢大学　准教授

論文の内容の要旨

1. Background

The detection of biomolecules, especially protein biomarkers, plays a crucial role in clinical application to prevent and control epidemic diseases. Conventionally, optical-based sandwich-type immunosensors have been used, however, these methods suffer from several drawbacks such as poor sensitivity, complexity, and lab-intensive instruments [1]. As an alternative to the optical-based method, the electrochemical-based detection has attracted much attention for the development of portable smart immunosensors due to its high sensitivity, simplicity, low-cost, and portability [2]. Among electrochemical techniques, amperometry and voltammetry are frequently used to screen various kinds of analytes with high sensitivity. Although the amperometry and voltammetry methods can provide limit of detection (LOD) in the pg/mL range [3], the scanning of potential makes the whole system complicated in design and operation. Therefore, the development of simple electrochemical method for detection of protein biomarker is desired. In this regard, open circuit potential (OCP) appears as a suitable technique since it simply measures the voltage difference between working electrode immersed in medium solution and a suitable reference electrode without application of neither potential nor current to the system. Compared to the voltammetry and amperometry, the OCP technique possesses several main advantages such as spontaneous measurement of the electrode potential built by electrochemical reactions on the electrode surface, and easily acquiring multiple electrode potentials at once. With such benefits from the mentioned background, OCP is suitable for simple detections of protein biomarkers [4, 5].

In this work, we proposed a novel electrochemical immunosystem, metal nanoparticles labeled electrochemical immunoassay of hCG detection. Gold nanoparticles (AuNPs) and platinum nanoparticles (PtNPs) were used as electrochemical labels for development of OCP based detection. First, the proof of concept of using OCP with AuNPs for hCG detection with relatively high sensitivity was confirmed. However, AuNPs based OCP detection requires the application of both oxidation and reduction potentials to achieve detectable signal, which makes it not simplified as OCP method should be. Then, we considered PtNPs based OCP detection. The good electrocatalytic property of hydrazine on PtNPs surface can circumvent the complicated preoxidation and reduction processes during measurement. As a result, the change of signal was simply observed without any applied external power source. This work demonstrates a novel application of OCP to highly-sensitive biomarker detection, which can be applied to low-cost, simplified, and miniaturized diagnostic systems. Moreover, this proposed method shows potential use for isolating and counting single molecule. To do that, it is necessary to find a suitable material and method for fabrication of high-density nano-micro electrode array. As primary experiments, carbon-based materials, i.e., AZ5214E photoresist and SAL601-SR2 electron beam resist wereused as source materials to fabricate conducting carbon film via a pyrolysis process.. It was found that the pyrolyzed SAL601-SR2 shows the possibility to fabricate dot pattern array at sub-micrometer scale.

2. Aim

The aims of this work are to prove the concept of OCP method for biomarker (hCG) detection using metal NPs and to simplify OCP based method for protein biomarker detection. Finally, the fabrication of micro-nano electrode was studied for the multiplex application.

3. Experimental

3.1 Fabrication of pyrolysis photoresist carbon electrode (PPCE)

In this study, the SiO_2 (100 nm)/p^+-Si was used as a substrate. Prior to use, the substrate was thoroughly rinsed by acetone and DI water, followed by a soft-bake at 150 ^oC. Remained organic substances were removed by using O_2 plasma ashing (O_2 30 sccm, RF power 15 W for 3 min). To create carbon film, AZ5214E photoresist was used as a carbon source. First, OAP was spin-coated on the substrate at 3000 rpm for 30 s, and baked at 110 ^oC for 3 min as an adhesion-promoting agent between the substrate and the photoresist. After that, AZ5214E was spin coated at 6000 rpm for 60 s, and baked at 90 ^oC for 10 min. Two coatings were used to obtain the desired thickness of PPCE, which is around 600 nm. The pyrolysis was performed in a furnace with a quartz tube flushed by forming gas (95%N_2 + 5%H_2,) for 15 min at room temperature. Under continuous gas flow, the temperature was increased from room temperature to 700 °C with a heating rate of 10 ^oC/min, then held at 700 °C for 1 h, and finally cooled down to room temperature.

3.2 Preparation of the sandwich-type immunoassay

Screen printed carbon electrode (SPCE) and PPCE were used as working electrode for immobilization of immunocomplex. The primary antibody was immobilized directly on working electrode array surface at 4 ^oc for 12 hours. After that, blocking solution (1% BSA) was incubated at 4 ^oC for 12 hours. Between each step, electrode was rinsed using blank PBS. The sample solutions consisting of various concentrations of hCG were applied onto immunosensors at room temperature for 30 min. After rinsing with PBS, either AuNPs-labeled hCG antibody or PtNPs-labeled hCG antibody was introduced onto the surface at room temperature for 30 min, and rinsed with blank PBS. The process of the sandwich-type immunoassay is shown in Figure 1.

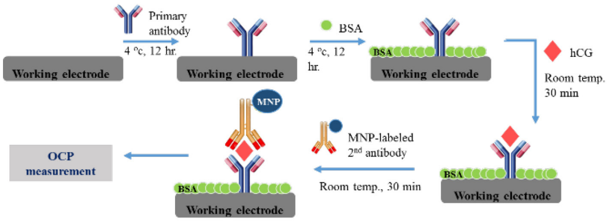

Figure 1. Schematic illustration of preparation of sandwich-type immunosystem

3.3 OCP measurement

The OCP method was used to measure the hCG concentration after preparing the sandwich-type immunosensor. For AuNPs based OCP detection, the number of AuNPs at secondary Mab was dependent on the hCG concentration and directly detected in 0.1 M HCl. Figure 2 shows the process of detection procedure. The preoxidation and reduction processes were applied in the detection procedure to obtain the direct attachment of AuNPs at electrode surface, followed by electrical detection using OCP. The concentration of hCG detected was related to the amount of AuNPs at the electrode surface after the reduction.

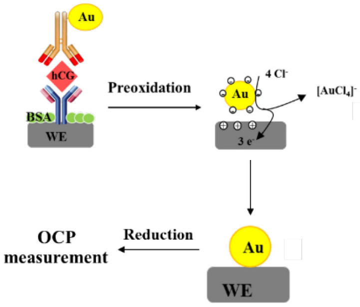

Figure 2. The detection procedure of AuNPs-based OCP with preoxidation and reduction processes

For PtNPs based OCP detection, hydrazine solution was added to sandwich-type immunocomplex at electrode surface followed by OCP measurement immediately without application of preoxidation and reduction processes as shown in the Fig. 3.

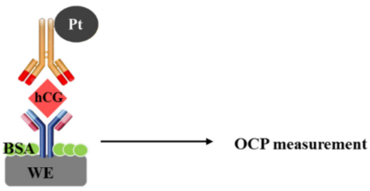

Figure 3. The detection procedure of PtNPs-based OCP without preoxidation and reduction processes

3.4 Fabrication of nano-micro pattern of pyrolyzed SAL601-SR2 film

First, OAP was spin coated at 4000 rpm for 30 s, then baked on hot plate at 110 ^{o}C for 3 min. After that, SAL601-SR2, negative EB resist, was coated at 4000 rpm for 60 s, and baked at same condition. The dot patterns were written using EBL system at different beam currents and dose times. After EB exposure, the sample was post baked at 100 ^{o}C for 5 min, and developed in MF-319 for 6 min followed by water rinsing for 3 min to remove unexposed area. Finally, the patterns of EB resist were pyrolyzed at 700 ^{o}C for 1 hr under mixture of 5%H_2 and 95%N_2 atmosphere with heating rate of 10^{o}C/min. Optical microscope and SEM were used to investigate obtained pattern before and after pyrolysis.

4. Results and discussion

4.1 AuNPs based OCP detection

The OCP method was used to measure the hCG concentration after preparing the sandwich-type immunosensor on SPCE and PPCE surface. The number of AuNPs at secondary Mab is dependent on the hCG concentration and can be directly detected in 0.1 M HCl. The different amounts of AuNPs on electrode surface affects to the catalytic activities towards proton in the solution that result in the change of OCP signal. Without preoxidation and reduction processes, the OCP signal was not significantly changed with respect to the hCG concentrations. This could be attributed to the poor electrocatalysis of proton by AuNPs in the acid solution. The immunocomplexes, consisting of primary Mab, hCG, and AuNPs-labeled secondary Mab, created a space between AuNPs and the electrode surface that prevented electron transfer of electrocatalytic process. Thus, AuNPs in the solution could not induce the change of OCP signal due to the loss of their catalytic activities. To overcome this problem, the preoxidation and reduction processes were applied in the detection procedure to obtain the direct attachment of AuNPs on electrode surface, followed by electrical detection using OCP. Compared to the procedure without the preoxidation and reduction, the preoxidation and reduction processes effectively facilitated the detection.

The detection procedure consists of three processes including preoxidation process, diffusion step, and reduction process. The parameters of these processes were studied because these factors can effect to the analytical results. A summary of optimal condition is shown in table 1. Under the optimal condition, the wide linearities were observed in the range of 0.05 to 10 ng/mL for SPCE, and 0.7 to 5 ng/mL for PPCE. Good linearity value with correlation coefficient (r^2) > 0.99 was obtained. LODs were found to be 0.079 and 0.1 ng/mL for SPCE and PPCE, respectively.

Table 1. The optimal detection procedure of AuNPs based OCP detection

Parameters	**Potential (V)**	
	SPCE	**PPCE**
Preoxidation	1.2 V, 60 s	1.2 V, 30 s
Diffusion tim	240 s	180 s
Reduction	-0.2 V, 30 s	- 0.4 V, 30 s

4.2 PtNPs based OCP detection

The number of PtNPs at secondary Mab was dependent on the hCG concentration and directly detected in 1 mM hydrazine solution. The different amounts of PtNPs on electrode surface affected to the catalytic activities towards the oxidation of hydrazine that resulted in the change of OCP signal as shown in Figure 4. The potential was shifted to negative direction with increasing of hCG concentration. The results indicated that the oxidation of hydrazine effectively occurred at the Pt surface due to its high electrocatalytic activity. Such characteristic enables the OCP-based hCG detection without any preoxidation and reduction steps.

Figure 4. Schematic illustration of the electrocatalysis of hydrazine by PtNPs

Using SPCE as working electrode, pH of buffer solution and concentration of hydrazine were optimized on SPCE. Under optimal condition of 1 mM hydrazine in phosphate buffer pH 6.0, the linearity was observed in the range of 0.5 – 10 ng/mL. The LOD was found to be 0.28 ng/mL.

For using PPCE as working electrode, it was found that PtNPs based OCP detection of PPCE cannot show obvious difference in signals at various concentrations of hCG.However, it was successful to distinguish the surface in the presence and absence of hCG. This success suggests the possibility to use this technique for the development of single molecule separation and counting.

Our proposed PtNPs based method shows simpler electrochemical detection procedure than those obtained from the AuNPs based method with relatively high sensitivity and good reproducibility.

4.3 Fabrication of micro-nano carbon dot on pyrolzed SAL601-SR2 film

The fabrication of carbon dot in micro- nano scale was studied. The size of electrode is estimated from the immobilization density of PtNPs on PPCE. It was found that the pattern was expanded by pyrolysis (figure 5).

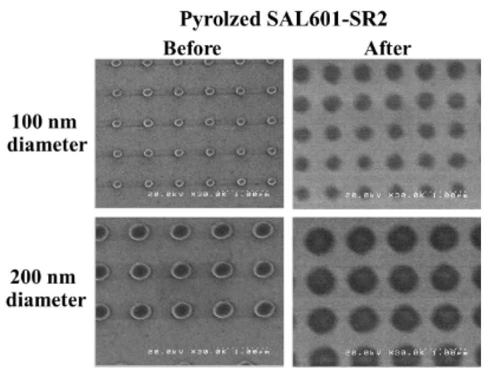

Figure 5. The obtained pattern before and after dry etching at beam current of 1 nA and dose time of 0.6 µs/dot.

The expansion about 2 times from original size was observed. This method is able to fabricate carbon dot with the smallest of 186 nm in diameter. Currently, the proposed method was successful in the sub-micrometer fabrication and could realize the desired dot's size of 300 nm.

5. Conclusion

In this work, a novel electrochemical immunosystem, metal nanoparticles labeled OCP-based immunoassay of hCG detection was successfully developed. For AuNPs based OCP detection, the pre-oxidation and reduction processes were found to have significant effect on the sensitivity of the proposed system since they enabled catalytic activities of AuNPs. However, this method requires the application of both oxidation and reduction potentials to achieve detectable signal, which makes it not simplified as OCP method should be. Therefore, the new simple electrochemical immunoassay based on PtNPs was developed. The good electrocatalytic property of hydrazine on PtNPs surface can circumvent the complicated preoxidation and reduction processes during measurement. Therefore, the proposed detection scheme offers simplicity and high electrochemical sensitivity for hCG detection using PtNPs-labeled immunocomplex, which can be extended to a simplified and miniaturized electrochemical system for clinical diagnosis.

Finally the fabrication of micro-nano sized electrode was studied to approach the application for single molecule detection. The direct pyrolysis of SAL601-SR2 EB resist after patterning to obtain carbon dot was performed. This method is capable of fabricating sub-micrometer carbon dot patterns.

This work demonstrates the simplification of electrochemical assay for protein biomarker detection and possibility to fabricate sub-micron electrode array for single molecule isolating and counting application.

References

[1] F. Ricci, G. Adornetto, G. Palleschi, A review of experimental aspects of electrochemical immunosensors, Electrochimica Acta, 84 (2012) 74-83.

[2] Y. Wan, Y. Su, X. Zhu, G. Liu, C. Fan, Development of electrochemical immunosensors towards point of care diagnostics, Biosensors and Bioelectronics, 47 (2013) 1-11.

[3] K. Idegami, M. Chikae, K. Kerman, N. Nagatani, T. Yuhi, T. Endo, E. Tamiya, Gold Nanoparticle-Based Redox Signal Enhancement for Sensitive Detection of Human Chorionic Gonadotropin Hormone, Electroanalysis, 20 (2008) 14-21.

[4] H.A. Videla, Manual of Biocorrosion, CRC press, Inc., Florida, USA, 1996.

[5] M. Ciobanu, J.P. Wilburn, N.I. Buss, P. Ditavong, D.A. Lowy, Miniaturized Reference Electrodes Based on Ag/AgiX Internal Reference Elements. I. Manufacturing and Performance, Electroanalysis, 14 (2002) 989-997.

論文審査の結果の要旨

本論文は、高感度バイオセンサに必要とされる抗原抗体反応を用いた被測定物の定量を、オープンサーキットポテンシャル（OCP）測定により実現する手法の開発に関するものである。

従来 1 ng/ml以下の濃度帯のバイオマーカの測定には抗原抗体反応が用いられ、その簡便な定量法として電気化学的方法が開発されてきた。その多くは特異性と感度の観点から

電圧を変化させながら電流を測るボルタンメトリが用いられてきた。しかし多項目測定のために、複数の電極を配置した場合、お互いの電極が干渉し、ボルタンメトリはうまく機能しない問題点があった。一方OCPは電圧測定のみであり、複数の電極の信号を干渉せずに高速に測定が可能である。本論文では、金属ナノ粒子の触媒作用を用い、抗原の量によりOCPが変化する系を構築し、OCPを用いた抗原抗体反応の定量方法を確立した。

第1章では、従来の電流測定型の電気化学バイオセンサについて説明し、特に多項目測定を行う場合の問題点について言及し、本研究の課題を明らかにした。

第2章では、抗原抗体反応をOCPの差として検出するために、二次抗体に修飾した金属ナノ粒子の触媒作用を用いた新しい反応系を提案した。また材料の選定、様々な最適化と不安定要因の解析を行い、印刷カーボン電極上で金ナノ粒子を酸化し、還元処理を経た後にOCPを測定することで、モデルバイオマーカとして用いたhCGを、従来法と遜色なく高感度に測定できる手法を確立した。

第3章では、白金ナノ粒子とヒドラジンを用いることで、酸化還元処理なしで抗原抗体反応の量をOCPの変化として測定できる系を開発した。これにより電極に電圧を印加する必要が全くなくなり、測定デバイス、および測定手順の大幅な簡略化が可能となった。

第4章では、OCPを用いて、実際に多数の抗原抗体反応を検出するための、電極材料およびそのパターニング方法の開発を行った。第3章までは印刷カーボン電極を用いていたが、これはあまり微細な構造が作れないため、電極数に限界がある。よって、微細なパターニングが可能で、かつ大きなOCP変化が観察できる電極材料の選定とそのパターニングプロセス開発を行った。パターニングしたレジスト材料を炭化処理することで、サブμmのサイズまで微細可能でOCP変化が得られる電極の開発に成功した。

以上、本論文は、金属ナノ粒子を用いて抗原抗体反応をOCP測定により検出する手法を発案・構築し、その安定性に関連する要因を明らかにし、またこれを多数の独立した電極上で測定するための基礎を確立したものであり、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。