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A new coccolith modified electrode-based biosensor using a cognate pair of aptamers with sandwich-type binding

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ABSTRACT

In this study, we report a cognate pair of the aptamer-based sandwich-type electrochemical biosensor for type 2 diabetes biomarker (Vaspin) using coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE). The coccolith derived from E. huxleyi used in this study were known to be highly-structured microparticles with many nano-sized pores. The CME-SPGE was successfully fabricated by drop-casting coccoliths, followed by Au sputtering and electrodeposition of Au. On this CME-SPGE electrode, the sandwich-type electrochemical aptasensor was fabricated by using a cognate pair of aptamers. The morphological, electrochemical characteristics and the performances of both the CME-SPGE and the completely fabricated sandwich-type aptasensor were investigated by SEM, EDAX, cyclic voltammetry, and chronoamperometry. Due to the synergic effect of a cognate pair of aptamers on CME-SPGE, this newly developed sandwich-type electrochemical biosensor for Vaspin showed high specificity, and good sensitivity with a limit of detection (LOD) of 298 pM, along with more widen the linear range. To the best of our knowledge, this is the first report about the use of a coccolith modified electrode with a cognate pair aptamer resulting in sandwich-type binding in an electrochemical biosensor. With the advantages of using highly-structured biomineral microparticles and a cognate pair of aptamers, this new study may pave the innovative way to design a novel sandwich-type electrochemical aptasensor platform.

1. Introduction

To deliver the high quality of life for individuals by providing decentralized diagnostics, the development of robust, cost-effective, and miniaturized electrochemical biosensor is essential. Notably, the modification of the electrode surface with the advanced materials has been known to be one of many ways to fabricate a highly sensitive and selective biosensor (Sarma et al., 2009; Sekretaryova et al., 2016; Walcarius et al., 2013).

Recently, there is growing interest in using biominerals produced by unicellular algae, a kind of marine organisms. The intricate 3D structures and delicate patterns of biominerals from marine organisms are the prominent features for diverse applications (Davis et al., 2013; Kröger and Brunner, 2014). Such ready-made inorganic marine structures can be utilized as promising materials in biosensors, photonic device or microfluidics, owing to characteristically unique nanometer-sized pores and channels inside (Mizukawa et al., 2015; Shen et al., 2017; Yu et al., 2010). Furthermore, the difficulty of producing the similar hierarchical structure of minerals makes marine-derived biominerals more attractive for applications.

Among biominerals, coccolithophores (coccoliths) are highly-structured microparticles with a lot of nano-sized pores and consisted with $CaCO_3$ have several advantages, such as large surface to volume ratio, mechanical properties, and thermal stability (Skeffington and Scheffel, 2018). However, the coccoliths had received less attention due to their undesired properties which originate from calcite, such as low electrical conductivity, dissolution at low pH, and little functional groups (Volodkin et al., 2004; Skeffington and Scheffel, 2018). These limitations, however, could be overcome by coating the surface with metal or organic materials like silica.

Aptamers are well-known bioreceptors that can bind to a broad range of targets with high affinity and specificity. The unique properties like the ease of modification, thermal stability, and low-cost make aptamers promising molecular probes for a biosensor (Kim et al., 2016; Seo and Gu, 2017a). Especially, a pair of aptamers, like antibody-based sandwich assay, can also easily be used in a sandwich-format which will, in turn, improve the analytical performance and the reliability of the biosensor. However, there have been few studies about the cognate

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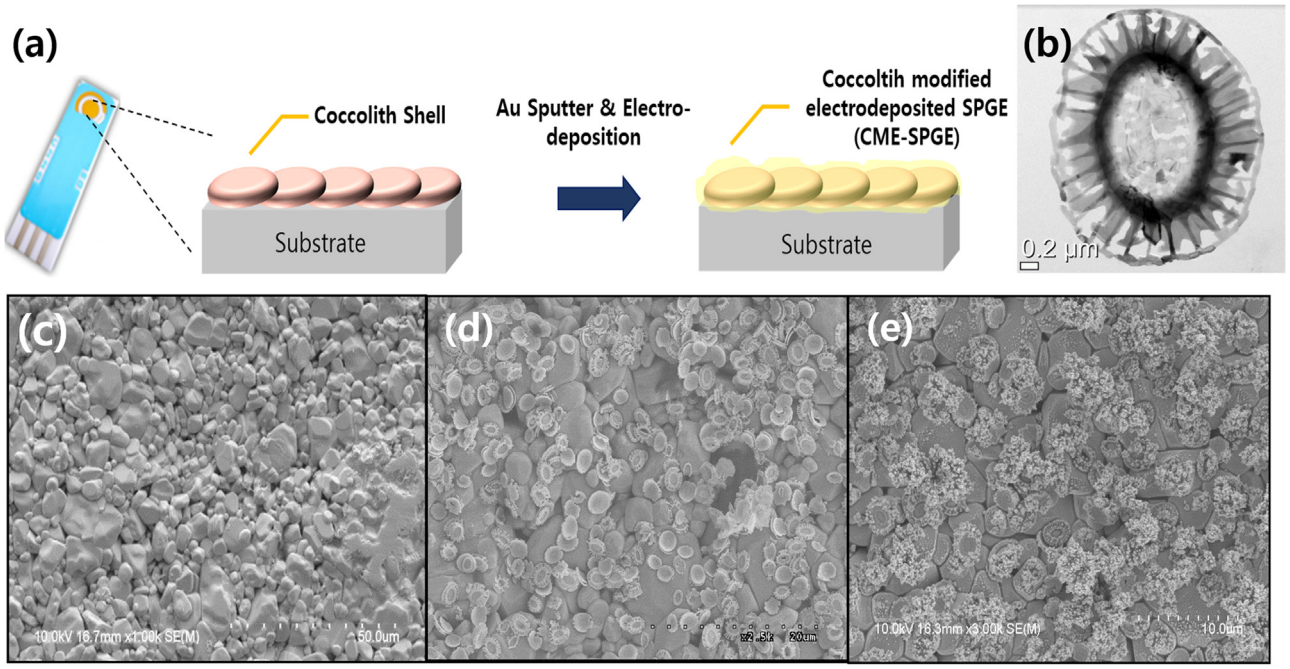


Fig. 1. (a) Diagrams of the preparation of the coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE). (b) The TEM image of coccolith shell produced by *Emiliania huxleyi.* The SEM images of (c) control screen-printed gold electrode (control SPGE), (d) the coccolith modified on the screen-printed gold electrode (CM-SPGE), and (e) the coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE), obtained at each step respectively.

aptamer duo-based sandwich-type assays or biosensors, except for thrombin (Jo et al., 2017; Zhang et al., 2015, 2018).

Recently, our research group reported a few successful developments of the cognate pair of aptamers (Nguyen et al., 2016; Park et al., 2014), including a cognate pair of aptamers (V_1 and V_{49} aptamers) that binds to two different epitopes of the target, Vaspin (Raston and Gu, 2015). In this study, we report the development of a sandwich-type electrochemical biosensor using a cognate pair of aptamers utilized on the coccolith modified electrodeposited screen-printed gold electrode (CME-SPGE) for the first time. The coccolith, which is the $CaCO_3$ shell of the *Emiliania huxleyi,* was used, and the Vaspin, which is known to be a biomarker protein related to type 2 diabetes in human (Blüher, 2012; Heiker, 2014), was used as a model target in this study. The morphological characteristics of the resulting electrodes were tested by scanning electron microscope (SEM) and energy dispersive spectrometer (EDAX). The electrochemical properties of the resulting electrode and the analytical performance of the successfully developed sandwich-type electrochemical aptasensor were evaluated by using cyclic voltammetry and chronoamperometry. To the best of our knowledge, this is the first report on the fabrication of coccolith modified electrode and its application in a cognate pair of aptamers-based sandwich-type electrochemical biosensor.

2. Experimental

2.1. Materials

Potassium chloride, 6-Mercapto-1-hexanol, Tris(2-carboxyethyl) phosphine hydrochloride, potassium hexacyanoferrate(III), sodium chloride, sulfuric acid, Gold(III) chloride trihydrate, potassium nitrate, 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB) were purchased from Sigma-Aldrich and used without any additional purification. Avidin-HRP was purchased from Thermo Scientific and diluted with 1xPBS. The Vaspin binding aptamers were synthesized from GenoTech Corp. (Daejeon, Korea) with the following sequence:

V_1 Aptamer (V_1APT): 5′-HS-$(CH_2)_6$- CGT ACG GAA TTC GCT AGC TGA TGG TGT GGC GGG GGC GGC CTG GGG CGG GCC GCC GAT GGG ATC CGA GCT CCA CGT G- 3′

V_{49} Aptamer (V_{49}APT): 5′-biotin-TTT TTT TTT TCG TAC GGA ATT CGC TAG CGG TGG CTC TAG GGC CTA TCG TTG CGC CGA CGG ATC CGA GCT CCA CGT G- 3′

2.2. Apparatus and electrochemical measurements

Electrochemical experiments were conducted using an Autolab PGSTAT30 (Ecochemie, The Netherlands). The analysis of the recorded data was done by GPES 4.9 software. Screen-printed gold electrode (SPGE) (C220BT, DropSens, Spain), consisting of a gold working electrode, gold counter electrode, and silver pseudo-reference electrode was used for fabricating the coccolith modified electrodeposited screen-printed gold electrode (CME-SPGE) and for the comparison of the analytical performance of the sandwich-type electrochemical aptasensor with CME-SPGE. Cyclic voltammograms were recorded at a scan rate of 100 mVs^{-1} in 5 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl. Amperometry signals were measured at a potential of − 0.10 V vs. the Ag pseudo-reference electrode for 60 s.

2.3. Preparation of coccolith shells

Coccolith-bearing *Emiliania huxleyi* strain CCMP371 was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA). The culture of *E. huxleyi* was maintained at 20 °C under irradiance of 40 μmol s^{-1} m^{-2} (12:12 h light: dark). The culture was continuously agitated by orbital shaker at 130 rpm. Culture medium was prepared in artificial seawater (ASW) supplemented with f/2 nutrients (without added Si). Coccoliths were isolated from the calcifying *E. huxleyi* strain CCMP371. Cells were harvested at late exponential phase by centrifugation (2, 170 × *g*, 10 min). The cells were resuspended with 1 ml of autoclaved distilled water followed by addition of 2 ml of methanol and 2 ml of chloroform. Then the solution

containing tubes were vortexed for 30 s at full speed and centrifuged at 2, 170 ×*g* for 10 min. The chloroform phase was carefully removed right after the centrifugation. By adding 2 ml of methanol, additional centrifugation was performed. The coccoliths were resuspended in methanol and stored at − 20 °C for the further experiment (Guillard and Ryther, 1962).

2.4. Preparation of coccolith modified electrodeposited SPGE (CME-SPGE)

The fabrication of coccolith modified electrodeposited on screen printed gold electrode (CME-SPGE) is shown in Fig. 1a. First, the working electrode of SPGE was erased by the aqua-regia solution and rinsed thoroughly with deionized water. Next, 1 µl of coccolith shell (45 mg/ml) was applied to the substrate, where the working electrode was located, by the drop-casting method, and dried by a hot plate. Thereafter, same diameter (4 mm) of the gold working electrode was formed by sputtering the gold onto the coccolith modified surface (Gas pressure: 3 mTorr, 100 w, Ar gas 21 sccm; Base pressure: 2.1×10^{7} Torr) using the mask. The prepared coccolith modified SPGE (CM-SPGE) was treated by cycling the potential between 0 and + 1.2 V in 0.5 M sulfuric acid. Finally, electrodeposition of pretreated electrodes was performed in a $HAuCl_4$ solution containing 0.1 M KNO_3 by applying a constant potential of − 1.2 V (vs Ag pseudo-reference electrode) for 70 s. We optimized the coccolith concentration and electrodeposition times to 45 µg and 70 s, respectively (Fig. S2 and S3). The optimized conditions were further used for other experiments unless mentioned otherwise. For the control experiment, control screen-printed gold electrode (control SPGE) was also prepared by sputtering gold after erasing the working electrode of SPGE without modifying with coccolith.

2.5. Preparation of the sandwich-type electrochemical biosensor using a cognate pair of aptamers and assay procedure

The preparation processes for the sandwich-type electrochemical aptasensor were given in Scheme 1. Prior to the immobilization of the aptamers, cleaned CME-SPGEs were prepared by electrochemically upon treatment of potential cycling between 0 to + 1.2 V in 0.5 M H_2SO_4 solution (Hoare, 1984). Then, Cleaned CME-SPGEs were rinsed thoroughly with ultra-pure water and dried under the N_2 gas stream. A 10 µl of 1 µM capture probe solution (V_1APT), which was reduced by 20 mM of TCEP, was dropped onto the surface of CME-SPGE and incubated for 12 h. Subsequently, it was thoroughly washed with deionized water to remove unbound V_1APT. After that, 2 mM of MCH solution was treated for 60 min to prevent the non-specific adsorption. After the washing step, the electrodes were stored in 10 mM PBS (pH 7.6) for further use. Signaling probe (V_{49}APT@HRP) was prepared by conjugating V_{49}APT and avidin-HRP for 60 min at room temperature. Subsequently, the resulting solution was purified by an amicon filter (MWCO 100 kDa). The assay procedure for the detection of Vaspin was conducted as follows. Ten ul of different concentration of the Vaspin solution in buffer and serum was incubated on the previously prepared electrode and incubated for 60 min. It was rinsed with 1 × BB (pH 7.6) for 10 s. Next, 10 µl of V_{49}APT@HRP (1 µg mL^{-1}) was applied and incubated for 60 min. After washing the unbound signaling probes, 40 µl of TMB solutions was applied, and chronoamperometry was measured. Human serum (from human male AB plasma, Sigma Aldrich, USA) was diluted up to 100-fold. All the incubation procedures were carried out by keeping the electrode in a petri dish to prevent evaporation of the solutions and performed at room temperature.

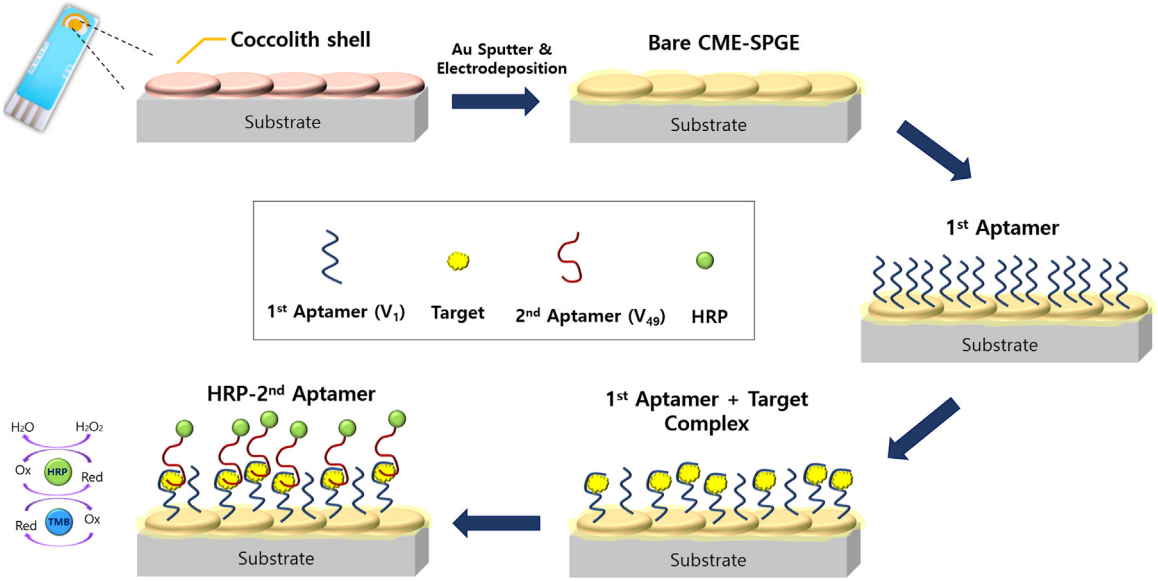
2.6. Characterization

The morphological and elemental analysis of the control screen-printed gold electrode (control SPGE), coccolith modified SPGE (CM-SPGE), and coccolith modified electrodeposited SPGE (CME-SPGE) were investigated by SEM (SEM; SU-70, Hitachi Co. Ltd, Tokyo, Japan) equipped with energy-dispersive X-ray spectroscopy (X-Max^N 50 mm^2, Horiba Ltd., Kyoto, Japan). The SEM was used to get the high-resolution images. The transmission electron micrograph of the coccolith shell was obtained by Tecnai F20 G^2 transmission electron microscope.

3. Results and discussion

The Scheme 1 illustrates the entire process in the preparation of the coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE) and the use of it as a cognate pair of aptamer-based sandwich-type electrochemical aptasensor for the type 2 diabetes biomarker, Vaspin.

Since the coccolith has low electrical conductivity and little functional groups, we have overcome this limitation by using the gold sputtering process and electrodeposition to fabricate the electrode with



Scheme 1. Schematic illustration of the preparation of the coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE) and a cognate pair of aptamer-based sandwich-type electrochemical aptasensor.

the increased surface area. Furthermore, the utilization of a cognate pair of aptamers in a sandwich-type binding to the target could provide an enhanced analytical performance of this aptasensor, compared to the use of the single aptamer alone (Chen et al., 2014; Pei et al., 2013; Wang et al., 2009). To fabricate this sandwich-type electrochemical aptasensor, V_1 Vaspin aptamer (V_1APT) was immobilized onto the CME-SPGE, and its secondary aptamer (V_{49}APT) was conjugated with HRP as a reporter. Upon the capturing of the target Vaspin in samples by the primary aptamer V1 immobilized on the CME-SPGE, the electrochemical signaling current, mediated by HRP on TMB/H2O2, is proportionally increased with the increased binding of the secondary aptamer, V49, to the target. Therefore, using a CME-SPGE and a cognate pair of aptamers could allow improved detection of the target analyte via the sandwich-type binding to the target (Abbaspour et al., 2015; Gong et al., 2012; Seo and Gu, 2017b).

3.1. Morphological characterization of the coccolith modified SPGE

Fig. 1a shows the fabrication process of the coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE). First, the coccolith, which has unique microstructure as shown in Fig. 1b, was uniformly applied to the substrate of the screen-printed electrode, in which the working electrode has been erased. By applying the coccolith, we could exploit the natural complex 3D structure of the coccoltih for the increase of the electrode surface. Subsequently, Au was sputtered to make coccolith modified SPGE (CM-SPGE).

Fig. 1c shows that the control SPGE has a planar electrode surface. After modifying with coccolith shown in Fig. 1d, the CM-SPGE electrode surface can be seen uniformly covered with coccolith shells, and intact microstructure of coccolith was maintained. In fact, the coccolith shells were almost uniformly distributed throughout the electrode surface with the diameter of around 4 μm, and it provided the highly-structured and rough surface to the electrode. This highly-structured electrode surface could increase its surface area significantly.

Since CM-SPGE seemed to have several layer differences on the electrode surface which lead to forming the disconnected gaps under the gold layers, which could disrupt the electrochemical current flow, this CM-SPGE should be modified by one more step, which is called electrodeposition. Therefore, as a final step, electrodeposition of gold was performed to fabricate the coccolith modified electrodeposited SPGE (CME-SPGE). As shown in Fig. 1e, we could observe the electrodeposition of Au on the surface of the CM-SPGE, resulted in CME-SPGE. To characterize the elemental composition of this CME-SPGE, Energy-dispersive X-ray Spectroscopy (EDX) analysis was performed (Fig. S1). As we expected, a characteristic Au peak was detected from the CME-SPGE, specifying successful electrodeposition of gold on the surface of the coccolith modified electrode.

3.2. Electrochemical properties of the coccolith modified electrodeposited SPGE

The electrochemical properties of the coccolith modified electrodeposited SPGE (CME-SPGE) were characterized by performing cyclic voltammetry in 0.5 M H_2SO_4 solution and 5 mM $[Fe(CN)6]^{3-/4-}$ solution containing 0.1 M KCl, respectively. In order to characterize the electroactive surface area of electrodes, the formation of oxide monolayer and its reduction on electrode surface using 0.5 M sulfuric acid was measured by the standard method of CV analysis used for the electrochemical characterization of gold electrodes (Jia et al., 2007). The potential was cycled between 0 and + 1.2 V, gold oxide formed above + 0.8 V and reduction peak was situated around + 0.5 V. The charge, related to the reduction of the gold oxide layer, is corresponding to the real surface area of the electrode.

As shown in Fig. 2a, the intensity of the reduction peak of CME-SPGE was increased, compared to the bare SPGE, indicating that the electroactive surface area of the CME-SPGE was increased about 3.8-fold. It is mainly due to the roughened surface provided by the coccolith shells and the reconnected gaps in the gold layers by the electrodeposition. Meanwhile, the intensity of the reduction peak of coccolith modified SPGE (CM-SPGE) was not much increased, compared to the bare SPGE. As mentioned above, it is likely due to the disconnected gaps in the gold layers after Au sputter process.

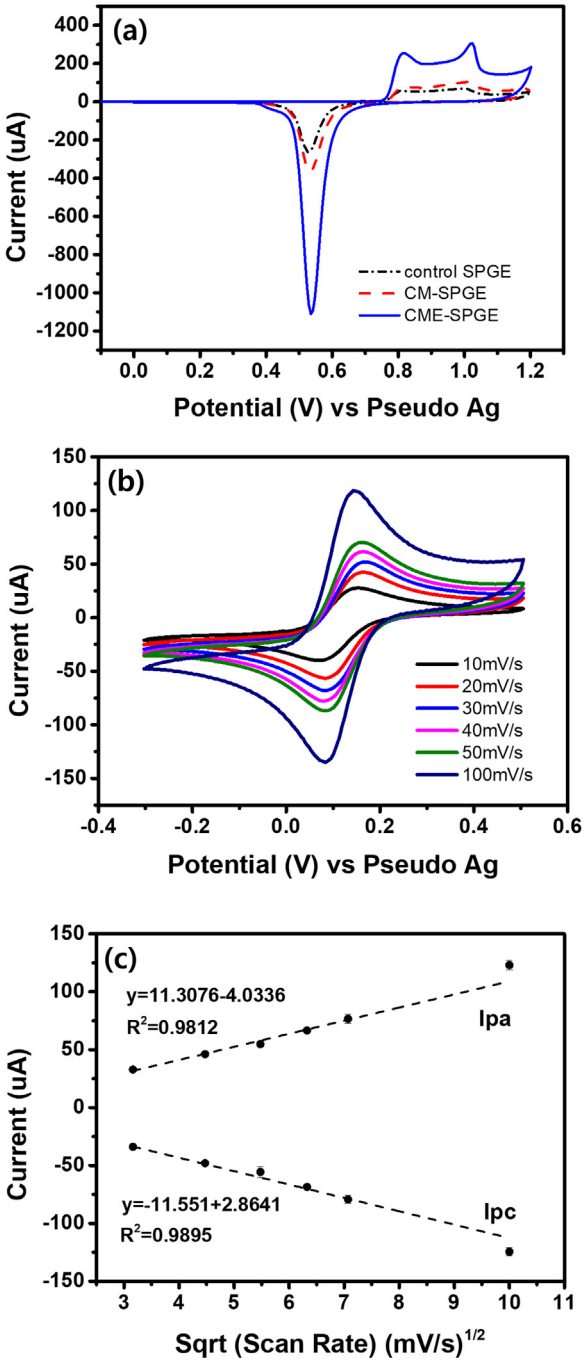


Fig. 2. Cyclic voltammograms of (a) control screen-printed gold electrode (bare SPGE), the coccolith modified screen-printed gold electrode (CM-SPGE), and the coccolith modified electrodeposited screen-printed gold electrode (CME-SPGE), respectively, obtained at 100 mVs^{-1} in 0.5 M H_2SO_4 solution; (b) Cyclic voltammograms of CME-SPGE at various scan rates from 10 to 100 mVs^{-1} in 5 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl. (c) Plots of Anodic peak current (Ipa) and Cathodic peak current (Ipc) versus square root of scan rates.

Therefore, CME-SPGE was used for all the experiments, including the fabrication of the sandwich-type electrochemical aptasensor. The interfacial electrochemical behavior of the CME-SPGE was

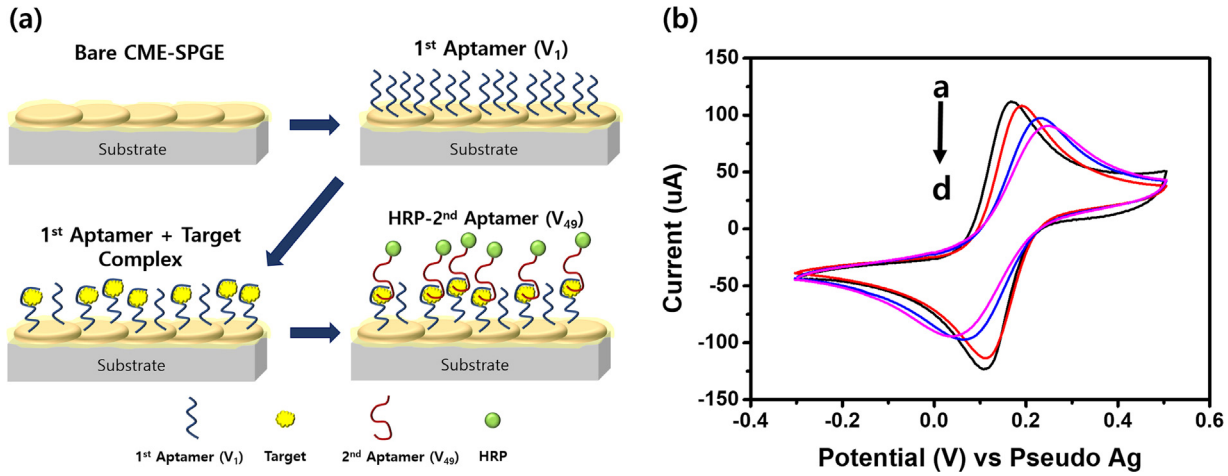


Fig. 3. (a) Diagram of the assembling steps for developing the sandwich-type electrochemical biosensor using a cognate pair of aptamers on CME-SPGE. (b) The Cyclic voltammograms of each modification step obtained at the sensor surface at 100 mVs^{-1} in 5 mM $K_3[Fe(CN)_6]$ solution, containing 0.1 M KCl (from a to d: bare CME-SPGE, V_1APT/CME-SPGE, Vaspin/V_1APT/CME-SPGE, V_{49}APT@HRP/Vaspin/V_1APT/CME-SPGE).

characterized using the redox reaction of $[Fe(CN)6]^{3-/4-}$ to determine the electron transfer rate by a CV analysis (Wang, 2006). Fig. 2b shows the cyclic voltammograms of the CME-SPGE at various scan rates (10–100 mV s^{-1}). As shown in Fig. 2c, cathodic and anodic peak currents were increased linearly with an increase of square root of scan rates. The constant peak-to-peak separation (ΔEp) and the linearity indicates a diffusion controlled process.

3.3. Characteristics of the successfully developed sandwich-type electrochemical aptasensor

The resulting coccolith modified electrodeposited on the screen-printed gold electrode (CME-SPGE) was used for the fabrication of a cognate pair of aptamers based sandwich-type electrochemical biosensor.

Using CME-SPGE, the electrochemical aptasensor binding to the target in a sandwich format was prepared, as shown in Fig. 3a. The V_1 aptamer (V_1APT) was immobilized onto CME-SPGE through the covalent bonding, and it was used as a recognition element for the target, Vaspin. The HRP conjugated V_{49} aptamer (V_{49}APT) was used as a signaling probe for a chronoamperometric response. The changes of the electrode surface by the modification with the V_1 aptamer (V_1APT), Vaspin, and the HRP conjugated V_{49} aptamer (V_{49}APT@HRP) were monitored using cyclic voltammetry (CV) technique. Fig. 3b shows cyclic voltammograms of bare CME-SPGE, V_1APT/CME-SPGE, Vaspin/V_1APT/CME-SPGE, and V_{49}APT@HRP/Vaspin/V_1APT/CME-SPGE. The bare CME-SPGE displayed a high faradaic peak current which shows a fast electron-transfer process (Fig. 3b, curve a). Upon the immobilization of V_1APT onto the CME-SPGE, the charge of aptamer interfered the electron-transfer resulting in a significant decrease in faradaic peak currents along with increased peak-to-peak potential separation (ΔEp) (Fig. 3b, curve b). When the target protein, Vaspin, was applied to the V_1APT /CME-SPGE, peak currents were decreased, and peak separation was increased significantly (Fig. 3b, curve c). Finally, V_{49}APT@HRP was applied to the captured Vaspin on the electrode to form the aptamer-target-aptamer sandwich-type formation (Fig. 3b, curve d). The decrease of the peak current along with the increase of peak separation was observed, indicating that this sandwich-type electrochemical aptasensor is successfully working.

3.4. Analytical performance of the sandwich-type electrochemical biosensor using a cognate pair of aptamers

The specificity of this newly developed aptasensor was also verified by testing with four counter targets, such as Nicotinamide phosphoribosyltransferase (Nampt), Human serum albumin (HSA), Resistin, and Adiponectin, respectively (Fig. 4a). It is clear, from Fig. 4a that only the target protein (Vaspin) showed a significant amperometric response change, while the other counter targets exhibited minor responses, indicating the excellent selectivity of this sandwich-type electrochemical biosensor using a cognate pair of aptamers for Vaspin.

The feasibility of a newly developed cognate pair of the aptamer-based sandwich-type electrochemical biosensor using CME-SPGE in comparison with commercial screen-printed gold electrode (SPGE) was studied by testing the different concentrations of Vaspin in buffer and serum. The chronoamperometric responses at different concentrations of target Vaspin for both CME-SPGE and SPGE in the buffer were measured, and its resulting calibration plots are shown in Fig. 4. As can be seen in Fig. 4c and d, CME-SPGE showed enhanced response compared to the SPGE. The limit of detection (LOD) was found to be 298 pM and 345.2 pM for CME-SPGE and SPGE, respectively, calculated by using blank + 3 standard deviations (SDs). The sensitivity was improved to 1.16-fold for Vaspin detection. Additional experiments were conducted to investigate the analytical performance of a cognate pair of the aptamer-based sandwich-type electrochemical biosensor using both CME-SPGE and SPGE in human serum condition. When serum was applied, significant decrease in electrochemical currents were measured on both CME-SPGE and SPGE, compared to the buffer condition. It is likely due to the complex matrices in serum affecting the electrode (Pei et al., 2014). However, the CME-SPGE showed much higher signals compared to the SPGE (Fig. 4e). The LOD was estimated to be 2.58 nM for CME-SPGE and 7.72 nM for SPGE. Therefore, the detection sensitivity was improved to 3-fold by using CME-SPGE compared to SPGE in serum condition. The improved analytical performance of this newly developed sandwich-type electrochemical aptasensor, compared to the aptasensor using the commercially available SPGE, could be explained by the surface properties of CME-SPGE which has an increased electroactive surface area, due to the coccolith shells and the electrodeposition, leading to affect the diffusion of electrolytes, analytes and so on.

Additionally, the analytical performance of CME-SPGE was compared with previously reported studies, as shown in Table. S1. This newly developed sandwich-type electrochemical aptasensor using a cognate pair of aptamers on CME-SPGE showed a comparable detection limit to SPR platform and Enzyme-Linked Antibody-Aptamer Sandwich (ELAAS) assay (Lee et al., 2012; Raston and Gu, 2015). Although the sandwich-type SPR-based sensing platform showed the highest analytical performance in terms of a LOD, the SPR method is relatively

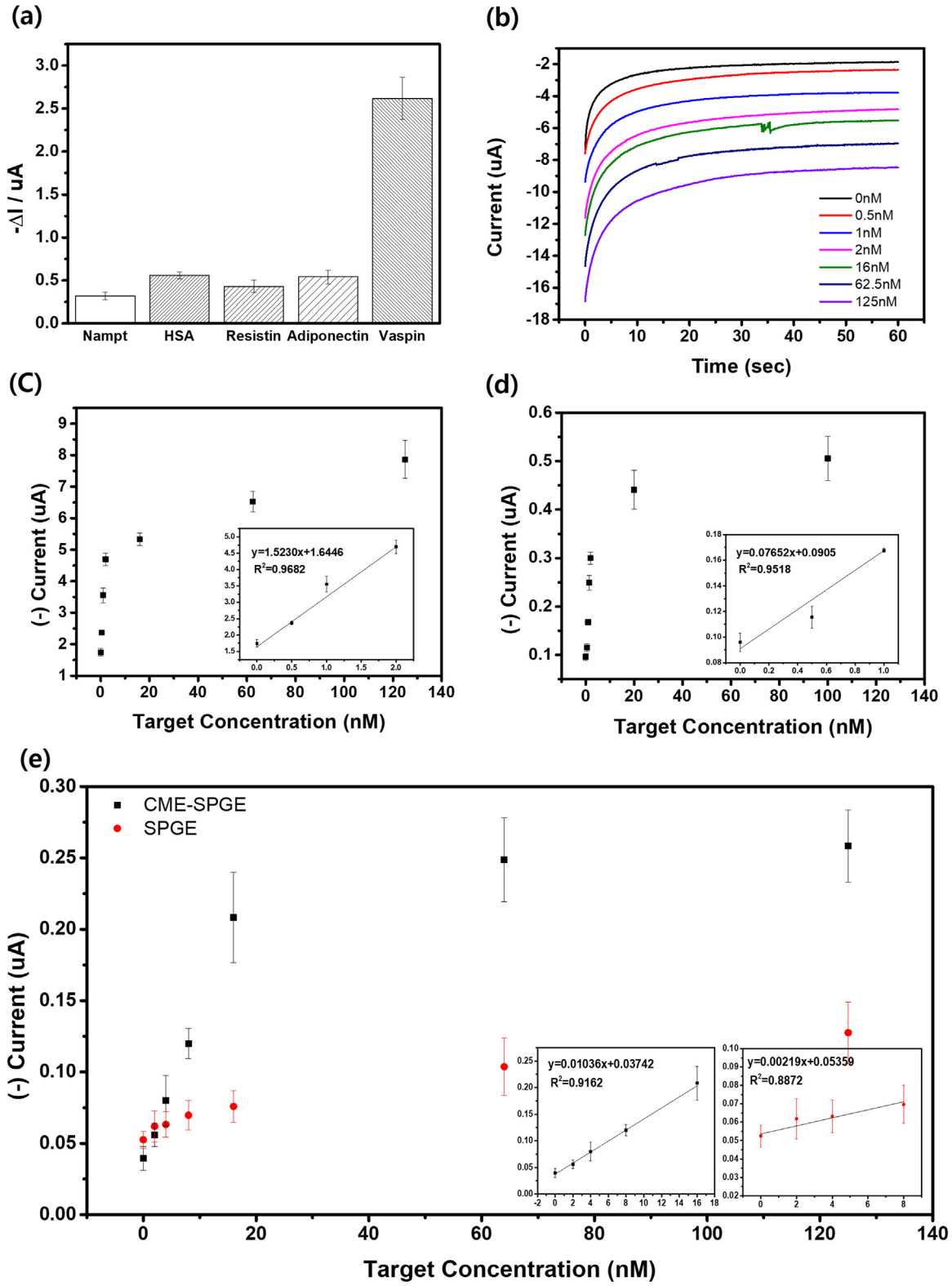


Fig. 4. (a) The electrochemical signals of this newly developed cognate pair of aptamer-based biosensor obtained at the addition of different counter targets (Nampt, HSA, Resistin, and Adiponectin). Chronoamperometric responses of at various concentrations of Vaspin (b), and its corresponding calibration curve (c) using CME-SPGE in buffer condition. (d) The calibration curve of the Vaspin detection using SPGE in buffer condition. (e) The calibration curves of Vaspin detection using CEM-SPGE and SPGE in human serum condition.

complicated to be applied to the point of care system which requires high-end instruments, compared to the electrochemical biosensors. Therefore, our newly developed cognate pair of the aptamer-based sandwich-type electrochemical biosensor using CME-SPGE could be a useful biosensor for type 2 diabetes biomarker detection.

4. Conclusion

In conclusion, we have successfully developed a sandwich-type electrochemical biosensor using a cognate pair of aptamers on coccolith modified electrodeposited screen-printed gold electrode (CME-SPGE). The CME-SPGE was characterized by SEM, EDAX, and cyclic voltammetry. By using a cognate pair of aptamers on CME-SPGE, the sandwich-type electrochemical aptasensor for Vaspin was successfully developed with high specificity and good sensitivity with a limit of detection of 298 pM With the advantages of using nano-structured biominerals and a cognate pair of aptamers, this will open up new possibilities for developing a novel sandwich-type electrochemical aptasensors.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.08.021.

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