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Square wave voltammetric detection of *Anthrax* utilizing a peptide for selective recognition of a protein biomarker

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ABSTRACT

A short chain peptide (16mer) has been successfully utilized for the selective electrochemical detection of the protein biomarker, protective antigen (PA), for the diagnosis of *Anthrax*. The major motivation of using a peptide instead of an antibody for the development of a biosensor is that there are advantages associated with the smaller size, better biological stability and easy synthesizability of a peptide. PA-selective peptide was synthesized and conjugated on a binding layer previously immobilized onto gold electrode. The same sensing scheme using a conventional antibody instead of a peptide was also tested for the purpose of comparison. Since the size of the peptide is approximately 1–2 orders of magnitude smaller than that of the antibody, a more populated immobilization of peptide on the sensing layer is possible and will eventually lead to improved sensitivity for PA detection. The selectivity of the peptide-based sensor was evaluated by observing the reduction peaks of samples containing PA with different concentrations of bovine serum albumin (BSA). The resulting responses were interference-free from BSA. The results of this study demonstrate the strong analytical potential of a peptide-based biosensor for diverse applications, especially in disease diagnosis where detection of a specific protein biomarker is particularly demanding.

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1. Introduction

The detection of *Anthrax* has been a popular research topic in analytical chemistry due to the lethal impact of *Anthrax* on health as well as for the establishment of homeland security from bio-terrorism (Hicks et al., 2004; Collier and Young, 2003; Dixon et al., 1999). *Anthrax* induces diverse biomarkers, including dipicolinic acid (DPA) and proteins, as well as gene and spores that can be detected using various bio-analytical methodologies as summarized in Table 1. To achieve the required selectivity, various phases selective to the corresponding target analytes can also be incorporated into the analysis when necessary. Among the analytical schemes in Table 1, PCR (Polymerase Chain Reaction)-based measurements are the most reliable since they provide the required sensitivity and selectivity; however, PCR has limitations when a fast, simple and cost-effective analytical method with minimal or no sample pretreatment is desired. In this situation, both optical and electrochemical-based sensing schemes are preferable; however, optical-based measurements still require a photon detector and/or a necessary light source that could result in a bulky and cost-demanding analytical system.

Electrochemical-based measurement is a more practical choice for the development of a miniaturized and cost-effective sensor for the detection of *Anthrax*; however, only a few researchers have reported using electrochemical schemes. The protective antigen (PA: 83 kDa), an *Anthrax* toxin, was selectively measured using an electrochemical sandwich-type enzyme-linked immunosorbant assay (Aguilar and Sirisena, 2007). For the gene-based detection of *Anthrax*, a specific B. *anthracis* gene sequence was probed using the capture probe (oligonucleotide) by combining differential pulse voltammetry (DPV) and electrochemical impedance spectrometry (EIS) (Kara et al., 2008). In other research, the oxidation of *para*-aminophenol generated from the catalytic hydrolysis by specific cell markers (alpha- and beta-glucosidase, released into the medium upon phase-induced cell lysis) was amperometrically measured (Yemini et al., 2007). Although diverse electrochemical researches have been accomplished for the detection of *Anthrax* as described above, the utilization of a peptide for the selective capture of an *Anthrax* protein biomarker in a liquid phase has not been reported. The use of peptide has been only limited to the capture of *Anthrax* spores in air as shown in Table 1. An electrochemical detection of *Anthrax* protein biomarkers in a liquid such as serum

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Table 1
Summary of publications relevant to the detection of *Anthrax*.

References	Biomarker used	Analytical method used	Material used for selectivity	Limit of detection	Linear response range
Kara et al. (2008)	Gene	DPV and EIS	Oligonucleotide	20 pM	1–1000 ng/mL
Aguilar and Sirisena (2007)	PA	AM	Antibody	0.05 μg/mL	0.05–10 μg/mL
Yemini et al. (2007)	CME	AM	-	10 spores/mL	–
Hao et al. (2009)	Spore	QCM	Antibody	10^3 spores/mL	10^3–7×10^8 spores/mL
Tang et al. (2009)	PA	Fluorescence	Antibody	1 ng/mL	1–100 ng/mL
Park et al. (2006)	Spore	Fluorescence	Peptide	–	–
Williams et al. (2003)	Spore	Fluorescence	Peptide	–	–
Castro and Okinaka (2000)	DNA	Fluorescence	Nucleic acid	–	–
Hurtle et al. (2004)	Gene	RT-PCR-F	Oligonucleotide	–	–
Bell et al. (2002)	DNA	RT-PCR-F	–	–	–
Makino et al. (2001)	Spores	RT-PCR-F	–	–	–
Levine et al. (2002)	DNA	PCR	–	–	–
Makino et al. (1993)	DNA	PCR	–	–	–
Boyer et al. (2007)	LF	MS	Antibody	0.05 ng/mL	0.05–400 ng/mL
Zhang and Appella (2007)	DNA	MS	Nucleic acid	–	–
Krebs et al. (2006)	Spore	MS	–	–	–
Cable et al. (2007)	DPA	Luminescence	Tb^{3+}	–	–
Bruno and Kiel (1999)	Spore	ECL	Aptamer	–	–
Gatto-Menking et al. (1995)	Spore	ECL	Antibody	10^2 spores/mL	–
Bell et al. (2005)	DPA	SERS	–	1 μg/mL	1–50 μg/mL
Zhang et al. (2005)	Spore	SERS	Physically separated	2.6×10^3 spores/0.2 μL	2.6×10^3–4×10^5 spores/0.2 μL
Acharya et al. (2007)	Spore	LT	Peptide	–	–
Wang et al. (2006)	PA	AFM	Antibody	1 ng/mL	1–1000 ng/mL

Abbreviations: differential pulse voltammetry (DPV); protective antigen (PA); cell-marker enzyme (CME); lethal factor (LF); dipicolinic acid (DPA); amperometry (AM); electrochemical impedence spectrometry (EIS); quartz crystal microbalance (QCM); polymerase chain reaction (PCR); real-time polymerase chain reaction and fluorescence (RT-PCR-F); mass spectrometry (MS); electrochemiluminescence (ECL); surface enhanced Raman scattering (SERS); light transmission (LT); atomic force microscopy (AFM).

has wide applicability in the diagnosis of *Anthrax* for poultry as well as human.

In this paper, we developed a novel peptide-based biosensor to selectively recognize PA in a liquid phase using square wave voltammetry (SWV). The peptide replaces the function of an antibody for the selective binding of biomarkers with valuable practical advantages. First, the structure of the peptide is more stable than that of the antibody under changing experimental conditions such as pH, ionic strength, and temperature, where an antibody could be easily denatured (Shigaki et al., 2007; Houseman et al., 2002; Han et al., 2008). Second, its size is much smaller than that of an antibody (~1–2 orders approximately); therefore, more populated immobilization of peptide can be achieved on a sensing layer, potentially leading to improved sensitivity (Uttamchandani et al., 2003). Finally, short peptides can be easily synthesized and mass-produced, allowing for their use when high throughput analyses are required (Kim et al., 2006).

The PA-selective peptide sequence, HKHAHNYRLPASGGKK, was chosen using traditional peptide screening in which phage display methods were utilized (Kim et al., 2006). Initially, the receptor (peptide or antibody) binding layer was formed on the surface of a gold electrode and then the peptide was conjugated to the surface. Using this sensor, square wave voltammograms of samples containing different concentrations of PA were acquired. The same procedure was also performed in parallel after the conjugation of a conventional antibody in place of peptide for the purpose of comparing the sensitivity and the limit of detection. Finally, the selectivity of the proposed method was demonstrated by evaluating the resulting electrochemical responses when various concentrations of bovine serum albumin (BSA) were present in the sample solutions.

2. Experimental

2.1. Chemicals and instrumentation

All necessary chemicals 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP), 11-mercapto-1-undacanol (MUD, 97%), 0.5 M KOH (volumetric standards), acetone and phosphate buffered saline (PBS: pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) were purchased from Sigma–Aldrich. As mentioned in the introduction, the PA-selective peptide sequence (HKHAHNYRLPASGGKK) was found using traditional peptide screening (phase display) and the resulting binding affinity of the peptide (K_d value) was 4.06 ± 0.33 pM (Kim et al., 2006). The peptide was commercially synthesized at Peptron, Inc. (385-19, Doryong-Dong, Yusung-Gu, Daejeon, 305-340, Korea).

All the measurements in this study were performed at room temperature using a standard cell with three electrode arrangements in which Ag/AgCl and Pt wire were used as reference and counter electrodes, respectively. The potential measurement was performed relative to the Ag/AgCl reference electrode and all electrochemical data were recorded using a potentiostat (eDAQ, Model PowerLab/4SP, Denistone East, NSW 2112, Australia).

The surface of the gold electrode was polished with waterproof abrasive paper and then rinsed by distilled water before activation. It was further cleaned using cyclic voltammetry by continuous scanning from −200 to 1600 mV in 0.5 M H_2SO_4, at a scan rate of 200 mV/s until a reproducible scan was obtained.

2.2. Overall procedure for sensing layer preparation

Fig. 1 summarizes the overall procedure for the preparation of a sensing layer on gold electrode. First, a self-assembled monolayer (SAM) of DSP is formed at the gold surface via the reduction of the disulfide group. A gold electrode with a clean surface was immersed in a 5 mM DSP solution in acetone for 1.5 h. Next, the surface was rinsed with acetone and incubated in a 1 mM MUD solution in ethanol to passivate empty sites on the gold surface, thereby minimizing or eliminating the possibility of nonspecific binding of either peptide or antibody. Then, both the polyclonal IgG antibody (rabbit) and the PA-specific peptide were separately conjugated onto the top of SAM of DSP through the reaction between the succinimidyl group of the monolayer and the terminal amino group of the antibody or peptide (Kerman et al., 2008). The parallel experimental scheme serves as a straightforward comparison of the analytical performances of the two receptor molecules. After the preparation of both modified electrodes using either the peptide or antibody, square wave voltammograms of the samples containing

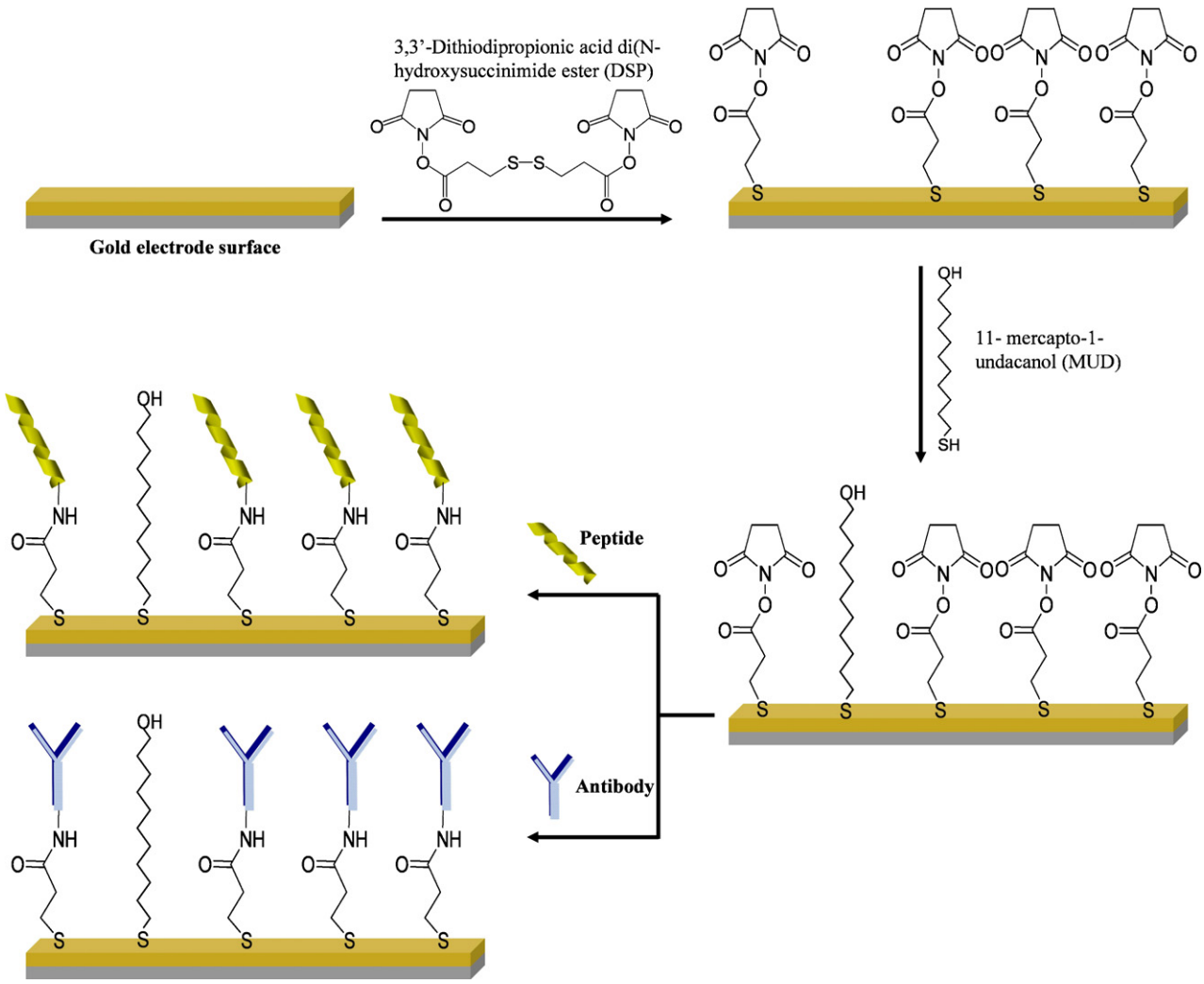

Fig. 1. Graphical descriptions of the preparation of the sensing layer using both peptide and antibody.

various concentrations of PA were collected and the corresponding reduction peaks were analyzed. It should be noted that the size of the peptide in Fig. 1 has been exaggerated for visual presentation, since the actual size of the peptide is ~75 times smaller than that of the antibody.

3. Results and discussion

3.1. Identification of peptide or antibody conjugation on the receptor-binding layer

Cyclic voltammetry was initially used to investigate the permeability of a given redox couple through the receptor-binding layer. We used 5 mM $[Fe(CN)_6]^{3-}$ as a redox species and the corresponding CVs were collected at three different stages of sensing layer preparation; the receptor-binding layer, peptide bound to the receptor-binding layer and antibody bound to the receptor-binding layer. For the purpose of comparison, CV corresponding to the bare gold electrode was also measured. The resulting CVs are shown in Fig. 2. The typical symmetric duck-shape CV of $[Fe(CN)_6]^{3-}$ is clearly observed when the bare electrode is used (a). Compared to the CV of $[Fe(CN)_6]^{3-}$ at the bare gold electrode, the Faradaic current decreases when the receptor-binding layer is formed (b). The layer of DSP and MUD works as an insulator by prohibiting the transport of the redox species. When the peptide is conjugated onto the receptor-binding layer, the transport of the redox species is almost limited and no distinct peaks of $[Fe(CN)_6]^{3-}$ are observed (c). Similarly, no current is observed when the antibody is conjugated to the receptor-binding layer (d). As previously mentioned, the size of the antibody is much larger than that of the peptide; therefore,

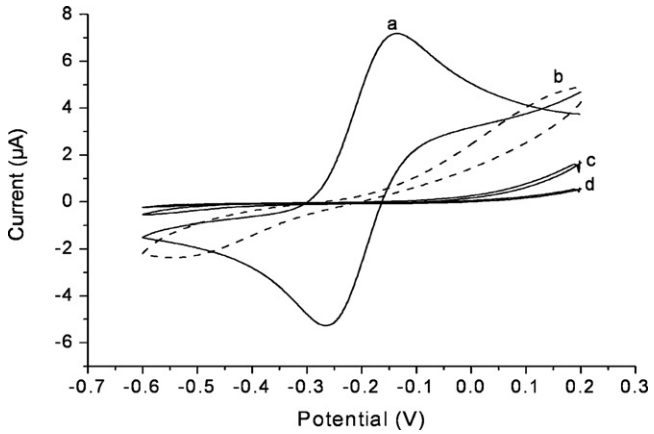

Fig. 2. Cyclic voltammograms of 5 mM $K_3[Fe(CN)_6]$ acquired using a) bare gold electrode, b) only receptor-binding layer immobilized electrode, c) peptide-conjugated and d) antibody-conjugated electrode.

the resulting antibody-conjugated layer works as a stronger insulator (Limbut et al., 2007; Berggren et al., 2001). Consequently, when PA recognizing receptors (peptide and antibody) are finally conjugated, any electroactive species present in the sample will not cross the sensing layer. This ensures no interfering response when a sample contains other electroactive species.

3.2. Optimization of incubation time

For the quantitative detection of PA, we used square wave voltammetry where the reductive desorption peak of the sensing layer can be utilized. If the peak height changes from the selective binding of PA with different concentrations, the proposed scheme will work as a successful electrochemical sensor. Initially, we examined the square wave voltammogram (SWV) of only the receptor-binding layer on the gold electrode. The measurement was performed in a PBS buffer of pH 7.3. In the first scan, the reductive desorption peak clearly appeared at −1180 mV, but this peak disappeared in the third scan. This observation shows the desorption of the receptor-binding layer. Based on several previous studies, the potential of the reductive desorption peak depends on its length as well as structure of the self-assembled monolayer (Love et al., 2005). When peptide or antibody is conjugated onto the receptor-binding layer, a shift in the reduction potential occurs.

The sensitivity of the proposed measurement is governed by the efficient conjugation of the peptide to maximize surface coverage. We expected that an increased population of peptides would increase the binding probability for PA. As described above, the reduction potential peak will also vary when different amounts of peptide are conjugated to the receptor-binding layer. Fig. 3 shows the SWVs collected for different incubation times for PA-specific peptide to the receptor-binding layer. The peaks are magnified in the same figure for clear visual presentation. As the incubation time increases from 40 to 120 min (40 (a), 60 (b), 80 (c), 100 (d) and 120 min (e)), the reduction peak shifts in the positive direction and the corresponding peak areas increase. No significant change in the peak potential is observed after 100 min of incubation. The observed trend indicates that the peptide almost fully covers the sensing layer by allowing enough incubation time (100 min in this case).

To characterize the surface coverage, we examined the variation of charge density (Q, C/cm^2) as a function of incubation time for both peptide and antibody. It is reasonable to expect that the

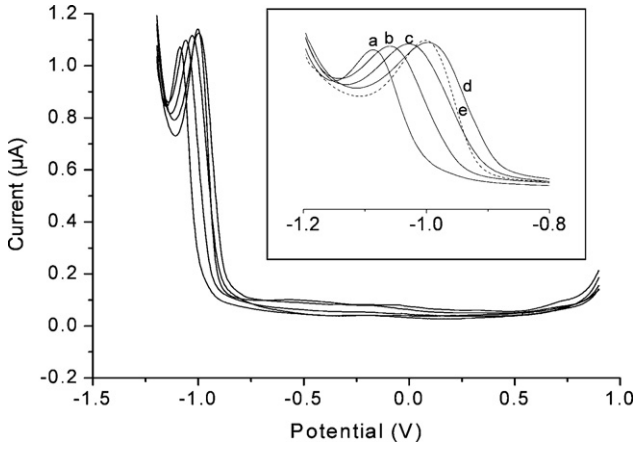

Fig. 3. SWVs with five different incubation times (40 (a), 60 (b), 80 (c), 100 (d) and 120 min (e)) of peptide to allow for conjugation onto the receptor-binding layer. The reductive desorption peaks are enlarged in the figure for clear visual identification. SWVs were recorded under the conditions below; square wave amplitude: 25 mV, sample period: 10 ms, scan rate: 75 mV/s (scan followed cathode direction) and frequency: 20 Hz.

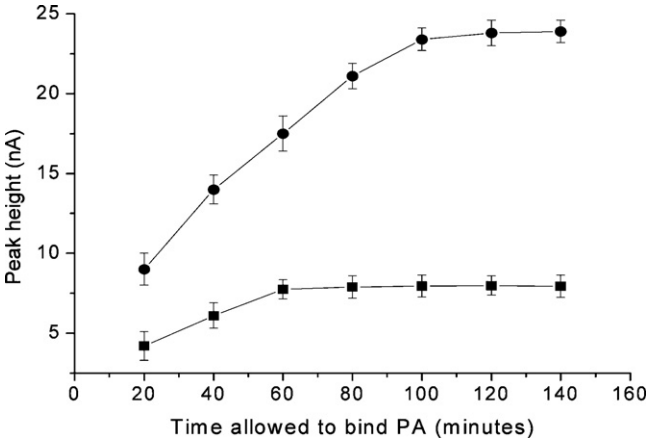

Fig. 4. Variation in peak heights of 100 pM PA in PBS buffer as a function of time allowing for PA to bind onto the peptide (circle)- and antibody (square)-conjugated sensing layer (b).

required incubation time for an antibody to fully cover the surface is shorter than that for a peptide, since it is considerably larger in size. In the case of peptide conjugation, no significant increase in charge density is observed after incubation for 100 min, and this trend is in accordance with the variations in the reduction peak as shown in Fig. 3. The maximum coverage (charge density) of antibody on the receptor-binding layer occurs at 60 min of incubation, which is shorter than that for peptide. Since the size of the antibody is ~1–2 orders larger than that of the peptide, fewer antibodies can be conjugated to a given surface area, resulting in a shorter incubation time.

To determine the optimal incubation time for allowing PA to bind onto the sensing layer, we investigated the variation in peak heights of 100 pM PA in PBS buffer as a function of incubation time for PA to bind with receptor molecules. Peak height was calculated by subtracting the resulting peak from that of a blank (no PA conjugated on the receptor-binding layer). Fig. 4 shows the resulting variations in the peak heights of PA when peptide (circle) and antibody (square) conjugated sensing layers are used. Three independent measurements were accomplished to generate the plot. The peak after the binding of PA was observed at −30 and −100 mV for peptide- and antibody-conjugated sensing layers, respectively (shown in Fig. 5). After allowing PA to bind to the peptide-conjugated sensing layer for 100 min, the peak height was nearly at the maximum. This trend is analogous to the variation of charge density as a function of incubation time. A more sensitive measurement would be possible with a peptide-conjugated sensing layer due to the denser surface coverage, which enables an increase in PA binding. A longer binding time is necessary to allow the capture of more PA by the peptide. Finally, for further analysis, the peak heights were measured at 100 and 60 min after immersing the peptide- and antibody-conjugated sensors, respectively, into the samples.

3.3. PA detection

Using PA sensors with either peptide or antibody, we measured samples containing PA in the 40–120 pM range with an interval of 20 pM and recorded the resulting peak intensities. The resulting peak variations as well as calibration curves for each case are shown in Fig. 5(a) (peptide-conjugated sensor) and Fig. 5(b) (antibody-conjugated sensor). The y-scales are the same for both plots. From bottom to top SWVs, the corresponding PA concentrations are 40, 60, 80, 100 and 120 pM PA, respectively. At a given concentration, four independent measurements were performed

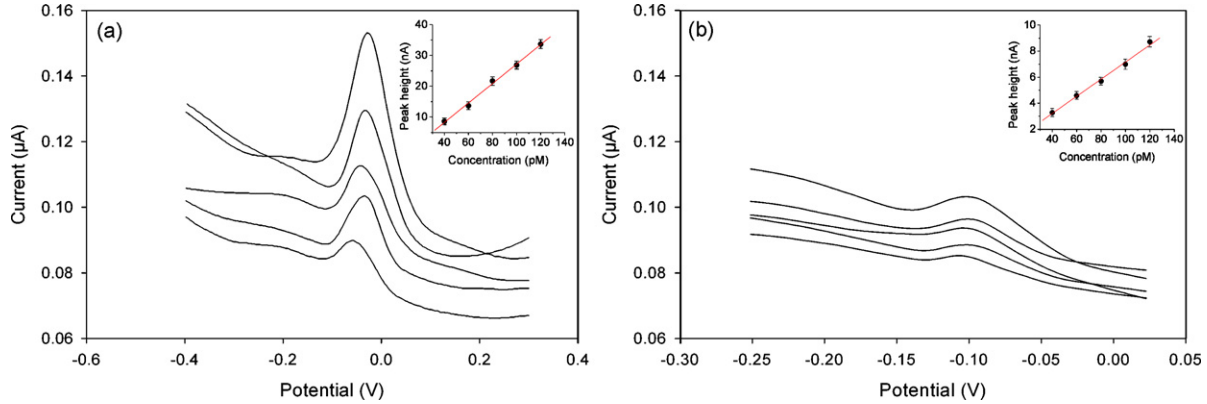

Fig. 5. SWVs of five different concentrations (40, 60, 80, 100, 120 pM) of PA selectively captured by peptide (a) and antibody (b) in the sensing layer. The corresponding calibration curve using peak height is also shown in this figure. SWVs were recorded under the conditions below; square wave amplitude: 25 mV, sample period: 10 ms, scan rate: 75 mV/s (scan followed cathode direction) and frequency: 20 Hz.

by preparing four individual sensing layers. In both cases, the peak intensity increases according to the variation in concentration, while the magnitude of intensity variation is larger for the peptide-conjugated sensor because its denser conjugation increases the binding of PA.

The responses of each sensor were analyzed by performing regression analysis. The resulting slope (sensitivity) and correlation coefficient (R^2) for the peptide-based detection were 0.316 ± 0.019 (nA/pM) and 0.998; while, 0.066 ± 0.005 (nA/pM) and 0.996 for the antibody-based detection, respectively. Both responses are linear over the concentration range, but the measurement using the peptide is approximately five times more sensitive than that using antibody based on a direct comparison of the slopes in both cases. Additionally, the better sensitivity of the peptide-based sensor results in a lower limit of detection (LOD), 5.2 pM; while, 16.4 pM for the antibody-based sensor.

3.4. Selectivity test

To show the practical applicability of a peptide-based sensor, the issue of selectivity should be clearly addressed. If a peptide responds to proteins other than PA, a positive error occurs. To demonstrate selectivity, we initially investigated the SWVs of samples containing only BSA (Bovine Serum Albumin, 40–160 pM, 40 pM interval) without PA. Obviously, no significant peaks were observed, indicating no selectivity for BSA. We also analyzed the peaks from 100 pM PA in the presence of 40–160 pM (40 pM interval) BSA. The resulting peak heights were similar in magnitude to those of 100 pM PA without BSA. The average peak height of these four measurements (even in the presence of BSA) was 26.7 ± 0.3 nA, while the peak intensity of 100 pM PA (without BSA) in the calibration was 26.9 nA. This result clearly demonstrates the superior selectivity of the peptide-based sensor. The same experiment was also performed using the antibody-based sensor, and no noticeable interference from BSA was also observed.

4. Conclusion

We have developed a peptide-based electrochemical sensor for the selective detection of PA as a biomarker for *Anthrax*. The use of peptide in the development of a biosensor is advantageous over the use of a conventional antibody due to the denser conjugation of peptide on a sensing layer, which can lead to improved sensitivity. Since diverse protein biomarkers have recently been studied for use in the diagnosis of human disease such as prostate specific antigen (biomarker of prostate cancer), sensitive and selective detection of these biomarkers is necessary. Therefore, we strongly believe that the proposed peptide-based sensor has strong potential and a bright future in the development of cost-effective and miniaturized electrochemical sensors for the diagnosis of diseases. Additionally, a peptide-based sensor is versatile and flexible since any peptide specific to a target protein can be easily synthesized and conjugated onto an electrode surface for selective detection.

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