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Towards On-site Determination of Secretory IgA in Artificial Saliva with Gold-Linked Electrochemical Immunoassay (GLEIA) Using Portable Potentiostat and Disposable Printed Electrode

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Abstract

Mental stress is closely connected with our physical and mental wellness. Therefore, stress measurement can contribute to assess our lifestyle and increase our quality of life. In this paper, we detect the secretory immunoglobulin A (sIgA), which is the candidate of salivary stress markers, with original electrochemical immunoassay: gold-linked electrochemical immunoassay (GLEIA). This biosensor is based on a sandwich-type immunosensor and adopts the electrochemical method to detect the reduction peak from Au nanoparticles linked to the secondary antibody. GLEIA is convenient and cost-effective that only requires a low sample volume (10 μL). In addition, the GLEIA show high sensitivity and selectivity. We obtained the linear response to relate the concentration of sIgA (10–300 ng/mL) in D-PBS buffer with the artificial saliva which includes salivary inorganic salt and typically glycoprotein (mucin). Furthermore, we obtained acceptable selectivity in the various solution with salivary proteins such as α-amylase, human serum albumin, immunoglobulin G (IgG), lysozyme, and mucin. In the future, we try to detect the sIgA in real saliva for on-site stress measurement using GLEIA and to integrate the various immunosensors for stress markers in saliva.

Keywords SPCE · sIgA · Electrochemical biosensor · Gold nanoparticles · Immunoglobulin A · Stress marker · Immunosensor

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Introduction

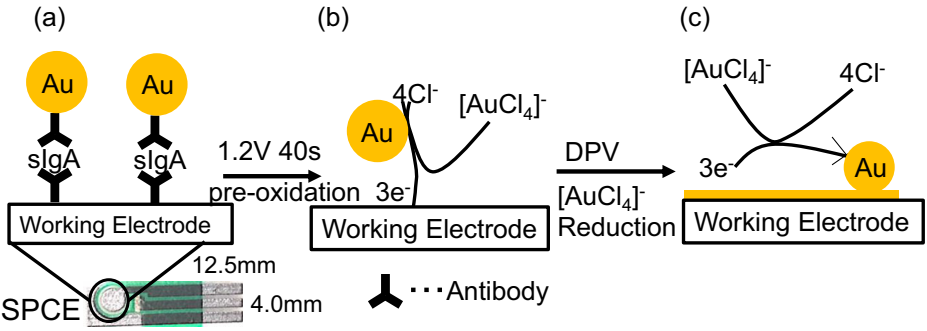
Mental stress is associated with various diseases and physical disorders. In particular, depression may be linked with stress [1, 2]. Therefore, the stress measurement is important information that can contribute to the prevention of mental illness such as depression. Conventionally, mental stress is evaluated by subjective diagnosis with psychological tests such as the profile of mood test (POMS) and Cornell Medical Index (CMI). However, these tests lack accuracy because it is subjective. The objective measurement systems are needed. As a more direct and objective method, the biochemical method to evaluate from stress markers in body fluids, which are serum, urine, and saliva, is focused by various researchers. The serum which contains typical stress markers (adrenaline and noradrenalin [3, 4]) cannot be collected non-invasively. In contrast, saliva can be collected non-invasively when necessary. One of the stress marker, salivary secretly immunoglobulin A (salivary sIgA), is considered by many researchers [5–10]. Christopher and co-workers reported that salivary sIgA does not only form the first line of immune defense but also both acute and protracted stress appeared for impact on sIgA [8]. Salivary sIgA levels are also associated with chronic pain [9] but not acute pain [10].

However, the stress measurement from salivary stress markers is not well established, because the salivary stress markers have many shortcomings. For example, the salivary sample has circadian rhythm [11] and blood contamination [12], and the concentration range of the salivary component differs from person to person. To establish the stress measurement, we think that an individual must indicate the stress level by obtaining numerous data continuously throughout the day. Therefore, the measurement method which can be suitable for this condition is needed. As a detection method, electrochemical biosensors have many great characteristics that include high sensitivity, specification, and ease for integration with other devices and portability for utilization at point-of-care testing (POCT). In addition, they are widely used due to their simplicity, and miniaturization of the device is relatively easy. In particular, the screen-printed carbon electrode (SPCE) is used for the sensors aiming at the POCT, because it shows various benefits as cost-effective, disposability, miniaturization, and easy for electrode design [13]. For example, DNA sensor [14], enzyme-based biosensor [15], electrochemiluminescent sensor [16], heavy metal sensor [17], and immunosensor [18–20] can be used at POCT.

The gold-linked electrochemical immunosensor (GLEIA) which is one of the SPCE-based electrochemical immunosensor was used for the determination of the antigen. The detail of GLEIA is sandwich-type immunoassay and its protocol is slightly the same as previously reported by our group [18–20]. To describe briefly (see Scheme 1), after the recognition reaction between the surface-immobilized primary antibody and antigen, the captured antigen was sandwiched with a secondary antibody that was labeled with Au nanoparticles. Au nanoparticles were exposed to a pre-oxidation process at 1.2 V for 40 s, which was subsequently followed with a reduction scan on the same surface using differential pulse voltammetry (DPV). DPV is one of the electrochemical detection techniques and is one of the methods to record the current between base potential and pulse potential, and base potential is not constant but sweeps in a certain direction.

Therefore, DPV suppresses the background current and shows high sensitivity. We show the DPV condition and profile in Fig. S1.

The current intensity obtained from Au reduction shows a linear relationship with antigen concentration. The GLEIA method shows various advantages suitable for stress measurement



Scheme 1 Illustration of the gold-linked electrochemical immunoassay. **a** The Primary antibody is immobilized on the working electrode in the screen-printed working electrode, and the sandwich-type immunoreaction is performed on the electrode. **b** The potential at 1.2 V is applied in 2M KCl solution for the pre-oxidation of Au nanoparticles. **c** The differential pulse voltammetry is performed for the reduction of gold chloride

as compared with other IgA detection methods. For example, GLEIA is suitable for on-site measurement because all device set is minimal containing PC, portable potentiostat, and SPCE (see supporting photograph 1) which may be used anywhere and anytime. On the other hand, measurement techniques, such as enzyme-linked immunosorbent assay (ELISA) [21], surface plasmon resonance (SPR) [22], and localized SPR [23], are not suitable for on-site measurement, because they need unportable apparatus to detect the sensor signals. Regarding SPR, Hemmi and co-workers reported compact disk-type SPR for IgA detection [24]. However, despite the fact that the human salivary IgA level is approximately microgram per milliliter orders [5–10], the dynamic range is 0.2–1.0 mg/mL of IgA.

In this paper, we describe that GLEIA is suitable for on-site and daily measurement of salivary sIgA. For that reason, we determinate sIgA in artificial saliva and standard solution using GLEIA and investigate the cross-reactivity with typically salivary proteins such as α-amylase (AAm), human serum albumin (HSA), immunoglobulin G (IgG), lysozyme (Lys), and mucin (glycoprotein) [25].

Experimental

Reagents

Anti-IgA (α), human, goat poly, A80-102A, and purified human IgA, P80-102, is purchased from Bethyl Laboratory (Montgomery, USA). A colloidal solution of Au nanoparticles of diameter 60 nm was purchased from British Biocell International Ltd. (Cardiff, UK). $NaH_2PO_4{\cdot}2H_2O$, Na_2HPO_4, NaCl, KCl, KH_2PO_4, urea, Na_2SO_4, NH_4Cl, $CaCl_2{\cdot}2H_2O$, $NaHCO_3$, polyethylene glycol (PEG: Mw = 20,000), lysozyme from egg white, and pH = 7.5: D-PBS (−) buffer were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium azide (NaN_3), human serum albumin (HSA), and α-amylase (AAm) were purchased from Nakarai Tesque (Kyoto, Japan). Bovine serum albumin (BSA) and immunoglobulin G (IgG) from human serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mucin from bovine submaxillary glands was purchased from MP Biomedicals, LLC (Santa Ana, CA,

USA). All inorganic reagents and urea are reagent grade, and artificial saliva is prepared with Milli-Q water (18.2 MΩ cm).

Instruments

A potentiostat, miniSTAT101, was purchased from BioDevice Technology (Ishikawa, Japan). Disposable electrochemical printed (DEP)-chip (DEP-EP-N) which has an integrated working electrode (2.64-mm^2 diameter), counter electrode, and Ag/AgCl reference electrode, with a total length of 11 mm, were also purchased from BioDevice Technology. UV-visible spectrophotometer (UV-2550) was purchased from Shimazu (Kyoto, Japan). Centrifuge 5417R was purchased from Eppendorf (Hamburg, Germany).

Preparation of Au Nanoparticle-Labeled Secondary Antibody

After the 0.9 mL of Au nanoparticle solution was mixed with the 0.1 mL of phosphate buffer (Na_2HPO_4/NaH_2PO_4, 50 mM, pH = 7.5), the 50 μg/mL of anti-IgA to dissolve the 5 mM of phosphate buffer (pH = 7.5) adds the Au nanoparticle solution and kept for 10 min at room temperature. Hereinafter, it is called Au conjugate. Then, 0.1 mL of 10% BSA in PBS buffer and 0.05 mL of 1% PEG in PBS buffer were added in Au conjugate. Au and anti-IgA conjugates were collected by centrifugal operation (8000 rcf for 15 min at 4 °C). After the centrifugal, Au and anti-IgA conjugates were suspended in 1 mL of the preservation solution (1% BSA, 0.05% PEG 20000, 0.1% NaN3, and 150 mM NaCl in 20 mM Tris-HCl buffer, pH 8.2) and collected again in the same manner. For the stock solution, Au and anti-IgA conjugates were suspended in the preservation solution and the optical density was adjusted to $OD_{520} = 6$.

The Au and anti-IgA conjugates were diluted by 3 times with 50 w/v of trehalose ($OD_{520} = 2$) and dripping 5 μL of this solution in the immunomodule. Then, the immunomodule was dried in a vacuum condition for 5 min. Dried immunomodule was stored at 4 °C until use.

Immobilization of Primary Antibody on the Working Electrode

For the immobilization of the primary antibody onto the carbon electrode surface, 2 μL of anti-IgA solution at 50 μg/mL in PBS was dropped onto the surface. After incubation at room temperature for 1 h, excess antibodies were eliminated with an air gun. For the suppression of non-specific adsorption, 10 μL of blocking solution (1% BSA in PBS) was incubated in all electrode surface at room temperature for 1 h. After the incubation, the blocking solution was eliminated by an air gun. As a result, BSA adsorbed on the uncoated parts of the electrode surface and helped prevent the further adsorption of interfering biomolecules on these vulnerable sites. Anti-IgA-immobilized immunosensor was stored at 4 °C until use.

Antibody-Antigen Reaction on the Working Electrode and Electrochemical Determination of Au Nanoparticles

Different concentrations of the IgA antigen solution (0, 10, 60, 150, 300 ng/mL) were made by diluting in PBS. For the detection of antibody-antigen reaction, 10 μL of IgA solutions is dropped in prepared immunomodule and mixed for 10 s. After 1 min, 1.4 μL

of the solution was placed on immunosensor to incubate for 1 h at room temperature in the closed box with damped cotton (for keeping the humidity to prevent the volatilization of the sample), after rinsing with PBS and eliminating the PBS solution with an air gun. The direct redox reaction was performed in 2M KCl solution (20 μL) covering the entire three-electrode zone at room temperature.

The pre-oxidation of Au nanoparticles was performed at a constant potential 1.2 V for 40 s, immediately followed by DPV, while scanning the potential range from 0.6 to 0.1 V with a step potential of 4.0 mV, pulse amplitude of 50 mV, and a pulse period of 0.2 s. These DPV conditions were determined by our previous reports [18]. The potentials were recorded against the Ag/AgCl electrode.

For the verification of the reduction peak, the DPV response with Au nanoparticles was obtained by the following protocol. First, the various concentrations of Au nanoparticles (OD_{520} = 1, 0.5, 0.25, 0.125, 0.0625) were dropped on the working electrode (surface treated with primary antibody and blocking protein) and dried completely. Second, 20 μL of 2M KCl solution was dropped on the working electrode. Finally, the electrochemical measurement was carried out with the same condition of GLEIA.

Evaluation of the Cross-reactivity with Salivary Proteins

The cross-reactivity is evaluated with salivary mainly proteins [25] such as immunoglobulin G, albumin, α-amylase, lysozyme, and mucin. The sample solution is prepared with the 2 types of IgA concentration (300 or 0 ng/mL) and proteins respectively in the D-PBS solution. The protein concentration is as follows: 13 ng/mL of IgG, 170 ng/mL of HAS, 90 mU/mL of AAm, 100 ng/mL of Lyz, and 1.1 μg/mL of mucin. Mucin is the main glycoprotein; it was added in the same amount as the total protein. All protein concentration is obtained by dilution salivary concentration with 1000 times. Furthermore, the calibration curve in artificial saliva was also determined. Artificial saliva is an aqueous solution containing human salivary electrolytes. Reagents used for artificial saliva and their concentrations are as follows: 2.15 mM NaCl, 12.9 mM KCl, 4.81 mM KH2PO4, 0.33 mM urea, 2.37 mM Na2SO4, and 0.5 wt% of mucin.

Results and Discussion

Electrochemical Detection of IgA Using GLEIA

The differential pulse voltammogram with IgA solution is shown in Fig. 1a. It was compared with the direct redox response of Au nanoparticles on the protein-modified electrode (Fig. 1b). The *y*-axis shows the current (μA) and the *x*-axis shows the potential (mV). For visualization, the starting point of the voltammogram is adjusted. Since the peak current intensity is calculated using the software to draw a tangent before and after the peak point, the background current actually gave no significant influence to calibration.

The voltammogram is already matched but the reduction peak expressed around 0.5 V (a) and 0.4 V (b). It may be caused by the Au nanoparticle condition. Regarding Fig. 1a, Au nanoparticle is diffused in the KCl solution; on the other hand, Au nanoparticle is immobilized on the electrode through the antigen and antibody. Therefore, the reduction peak around 0.4 V can be obtained from Au nanoparticles immobilized from the

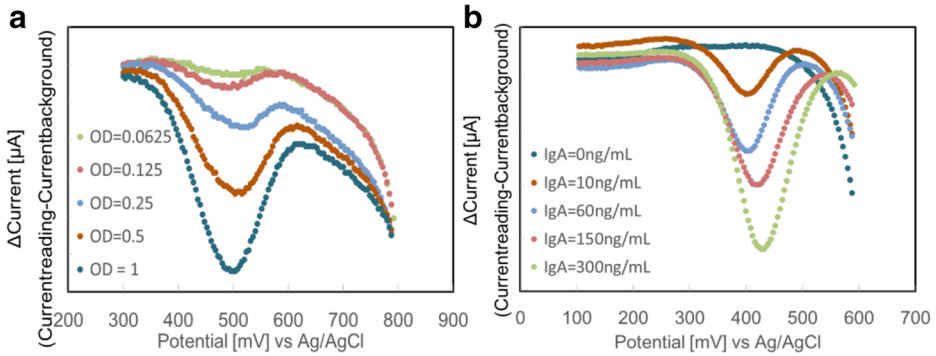


Fig. 1 Differential pulse voltammogram on protein-modified working electrode of SPCE. **a** The reduction response of Au nanoparticles. The pre-oxidation was applied with the potential, at 1.2 V, and 40 s. **b** The reduction response of the Au nanoparticles immobilized on secondary antibody after sandwich immunoreaction in D-PBS buffer

antibody-antigen reaction on the electrode, and the peak current is increased with IgA concentration relativity.

Figure 2 shows the calibration curve from the reduction peak. The *y*-axis shows the peak intensity and the *x*-axis shows the concentration of IgA. The linear range is 10–300 ng/mL, and the correlation coefficient (R^2) of the calibration curve for this relationship was 0.9834.

Determination of IgA in Artificial Saliva

Figure 3 shows the differential pulse voltammogram of GLEIA with IgA in artificial saliva (a) and calibration curve (b). The voltammogram is already the same as Fig. 1b; therefore, the antibody-antigen reaction is not hindered in artificial saliva. In addition, the calibration curve shows the linear range within 10–300 ng/mL and the correlation coefficient (R^2) of the calibration curve for this relationship was 0.963. The peak current intensity is decreased from a standard solution (Fig. 2). It may prevent the antigen-antibody reaction from artificial saliva in which pH is nearly 8.5. Generally, the antibody shows the best affinity in pH = 6.5~8.4 conditions. However, these results show that GLEIA is suitable for salivary IgA detection because the salivary pH is mainly neutral.

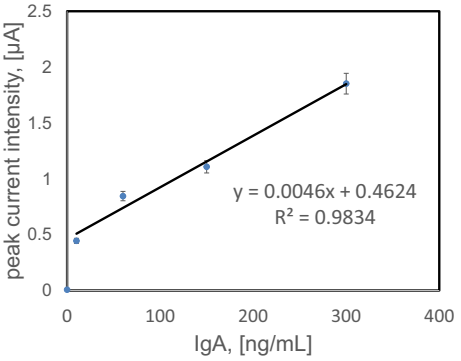


Fig. 2 Calibration curve of sIgA determination with the reduction peak current intensity of Au nanoparticles in D-PBS buffer. The error bar indicates the relative standard deviation of three measurements ($n = 3$)

The Cross-reactivity of GLEIA for IgA Detection

We investigate the cross-reactivity of GLEIA because real saliva has various proteins or glycoprotein. We chose the following proteins as interferents: α-amylase (AAm), human serum albumin (HSA), immunoglobulin G (IgG), lysozyme (Lys), and mucin which are mainly proteins in saliva [25]. These concentrations are indicated in the "Experimental" section. We evaluate the selectivity of the immunosensor by comparing it with the reduction peak between IgA = 300 ng/mL or 0 ng/mL and interference protein (Fig. 3). We acknowledge GLEIA shows the signal increasing within the existence of interference proteins. This may be the affinity of antibody and non-specific reaction between antibody and the proteins. More importantly, we recognize that the reduction peak is increasing with the concentration of sIgA in the existence of interference proteins. Therefore, GLEIA can be applied for stress measurement using salivary sIgA that can be used in daily life anywhere and anytime whenever necessary (Fig. 4).

We also compared the electrochemical method including potentiometric, impedance, and amperometric techniques. The organic field-effect transistors (OFETs) [26] and electrochemical impedance spectroscopy (EIS) [27] need lab-scale experimental; they are not suitable for the condition which we assume. On the other hand, the amperometric method [28, 29] including GLEIA can be suitable for that condition, because they may use common equipment such as a potentiostat (portable or not portable) and carbon electrode. We summarize sensor characteristics between GLEIA and other methods in Table 1. GLEIA is compatible with both high sensitivity and portability. Moreover, the linear range is within the nanogram per milliliter order. In the actual measurement, the salivary sample will be diluted for 1000 times, because the salivary sIgA concentration is in 100 μg/mL order [5–11]. In addition, the dilution will be beneficial to avoid non-specific reaction with other salivary proteins.

In conclusion, we aim to apply for the on-site and convenient stress measurement with salivary sIgA using the original electrochemical immunosensor such as gold-linked electrochemical immunoassay (GLEIA) which makes use of the portable potentiostat and the

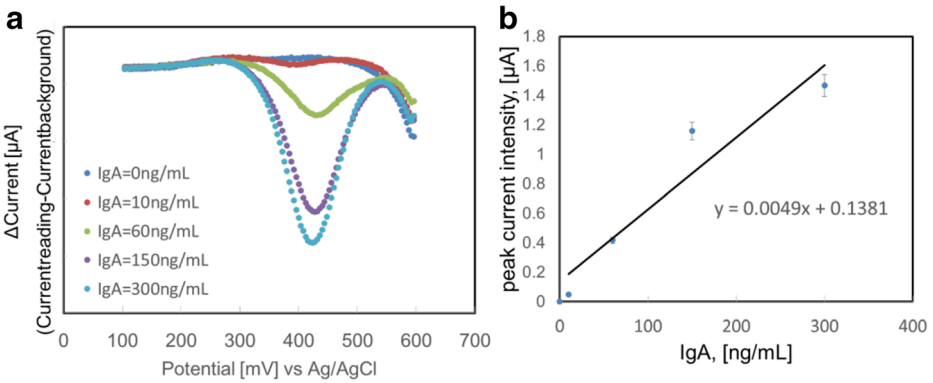


Fig. 3 **a** Differential pulse voltammogram of the Au nanoparticles immobilized on secondary antibody after sandwich immunoreaction in artificial saliva. **b** The calibration curve of sIgA determination with the reduction peak current intensity of Au nanoparticles in artificial saliva. The error bar indicates the relative standard deviation of three measurements ($n = 3$)

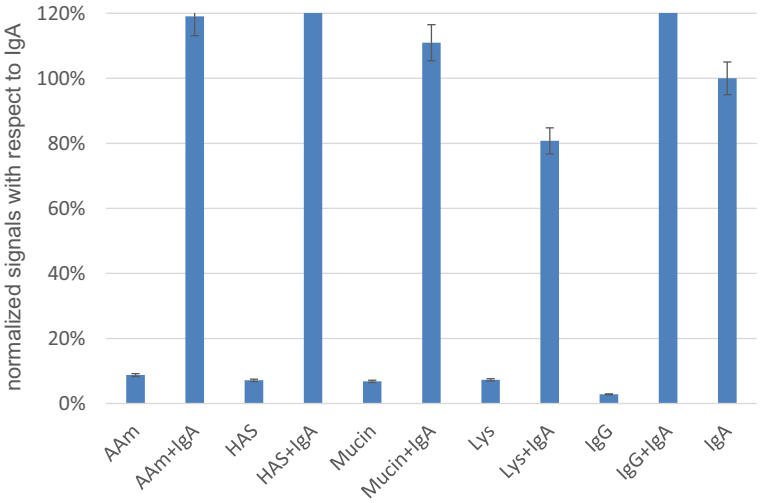


Fig. 4 The cross-reactivity respect to interference proteins. The sample solutions are prepared with the 2 types of IgA concentration (300 or 0 ng/mL) and proteins respectively in D-PBS buffer. The protein concentration is as follows: 13 ng/mL of IgG, 170 ng/mL of HAS, 90 mU/mL of AAm, 100 ng/mL of Lyz, and 1.1 μg/mL of mucin. All protein concentration is obtained by dilution salivary concentration with 1000 times

disposable screen-printed carbon electrode (SPCE). For this purpose, we determinate the sIgA in artificial saliva which reflects the salivary inorganic salts, urea, glycoprotein, and standard D-PBS buffer. In addition, we investigate the cross-reactivity of GLEIA with salivary proteins. From the results, GLEIA shows sufficient performance for the on-site determination of salivary sIgA. The linear range is 10–300 ng/mL in artificial saliva and D-PBS buffer respectively, and the correlation coefficient (R^2) of the calibration curve for this relationship was 0.983 and 0.963. Following the salivary concentration of sIgA which is in microgram per milliliter order, the linear range by GLEIA is highly sensitive. The cross-reactivity shows that the signal is increasing with the existence of interference proteins. However, the signal is significantly in the presence of sIgA with a linear relationship to the concentration. Therefore, GLEIA can be suitable for on-site stress measurement in our assumption.

Table 1 Comparison with our sensor and other sIgA determination methods

Sensing platform/materials	Dynamic range	Portability	Reference
ELISA	2.5–600 μg/mL	Difficult	[21]
Surface plasmon resonance (SPR)	0.4–50 μg/mL	Possible	[22]
Localized SPR	10–1000 ng/mL	Difficult	[23]
Organic field-effect transistors (OFET)	3–50 μg/mL	Difficult	[26]
Electrochemical impedance spectroscopy (EIS)	0.01–100 ng/mL	Difficult	[27]
Sandwich-type immunoassay using HRP-labeled secondary antibody	125–4000 ng/mL	Possible	[29]
Co-polymerized dopamine and carbon nano-onion-based electrochemical sensing	250–2500 ng/mL	Possible	[28]
GLEIA method	10–300 ng/mL	Possible	This work

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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