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Bioelectrocatalytic sensor for triglycerides in human skin sebum based on enzymatic cascade reaction of lipase, glycerol kinase and glycerophosphate oxidase

Chi Yong Jeong, Yong Duk Han, Jae Ho Yoon, Hyun C. Yoon *

Department of Molecular Science and Technology, Ajou University, Yeongtong-gu, Suwon 443-749, South Korea

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ABSTRACT

We report the development of an electrochemical biosensor for the quantification of triglycerides in human skin sebum, based on a multienzyme cascade reaction. The presence of excessive triglycerides in human sebum is one of the leading causes of various skin ailments. However, to the best of our knowledge, no bioelectrocatalytic approach for the quantification of sebum triglycerides has been made. In order to develop triglyceride biosensor, we fabricated a multienzyme-associated electrode incorporating lipase, glycerol kinase, and glycerophosphate oxidase. Enzymes were deposited by electrostatic force and further stabilized *via* crosslinking between enzymes and polymer matrices. The enzyme-modified biosensing electrode maintained its bioelectrocatalytic activity for five days. An additional constraint was the limited solubility of sebum triglycerides in aqueous electrolytes, impeding the analysis. To address this issue, triglyceride samples were prepared in the form of micelles, enabling efficient sample preparation for biosensor signaling. Calibration tests revealed that the designed assay had a detection range of 15–200 mg/dL of micellar triglyceride, which covered the required determination range. The developed biosensing approach was successfully used to determine triglyceride concentrations in real sebum samples of unknown triglyceride content.

1. Introduction

Human skin contains various organs, such as sebaceous glands, sweat glands, hair, and nails. The sebaceous glands, found throughout the skin, are appendages of the hair follicle that do not exist at hairless regions such as the palms of the hands and soles of the feet. Sebaceous glands are lodged in the corium and their size is inversely proportional to the diameter of the hairs connected to glands (Downie et al., 2004; Smith and Thiboutot, 2008). Sebaceous glands secrete an oily and waxy mixture called sebum that is composed of triglycerides, wax, squalene, and other lipid components (Zouboulis, 2004; Zouboulis et al., 2008).

Sebum secreted by the follicle forms a lipid layer on the surface of human skin to provide a barrier against infection, ultraviolet light, and drying (Thiele et al., 1999). Sebum secretion, however, may cause several skin ailments, such as acne and seborrheic dermatitis (Mastrolonardo et al., 2003). Skin ailments are caused by a number of factors, but the primary concern with sebum secretion is an excess of sebum release, followed by clogging of the hair follicle. Abnormal sebum secretion gives rise to sebum accumulation, which causes partial anaerobic conditions in the follicle in which some anaerobic skin flora may proliferate (Youn, 2010). Most skin flora is non-pathogenic, but some (*e.g. Propionibacterium acnes*) are capable of metabolizing triglycerides in human sebum and irritating the skin, triggering skin ailments (Thiboutot, 1996; Toyoda and Morohashi, 2001) that are often accompanied by pain resulting from comedo, papule, nodules, and rash. Skin ailments may also cause permanent scars, physical distress, and severe mental anguish, such as humiliation and depression (Yosipovitch et al., 2007). For these reasons, prediction of skin ailments would be useful in improving patient care. Thus, the determination of triglyceride concentrations in human skin sebum would be a useful measure of microorganism growth and related skin disease.

A triglyceride (TG) is an ester derived from glycerol and fatty acids that can be hydrolyzed into a monoglyceride and a fatty acid chain by lipase reaction (Xu, 2000). TGs have been conventionally analyzed using gas chromatography (Paik et al., 2009), high performance liquid chromatography (Lu et al., 2009), nuclear magnetic resonance (Robosky et al., 2008), and enzymatic colorimetric assay (Biggs et al., 1975). However, conventional methods

* Corresponding author. Tel.: +82 31 2192512; fax: +82 31 2192394.
E-mail address: hcyoon@ajou.ac.kr (H.C. Yoon).

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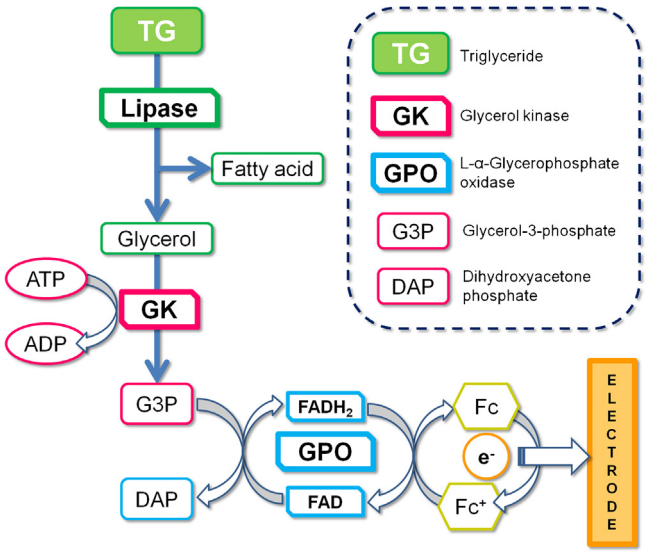

Fig. 1. Schematic diagram for signal amplification by successive multi-enzyme reactions.

require complex instruments that are heavy when handling and often complicated for unskilled persons to operate. Also, most of the conventional detection strategies for TGs have focused on the detection of TG in blood, solubilized in aqueous phase like lipoproteins (Reis et al., 2009), rather than the detection of sebum TG which is water-insoluble. As such, there is a need to develop on-site analytical methods that could easily and rapidly be applied to sebum TG detection for field diagnosis. In this study, we developed a bioelectrocatalytic electrochemical biosensor to determine human sebum TG concentrations based on a multi-enzyme cascade reaction. A tri-enzyme system, comprising lipase, glycerol kinase (GK), and glycerophosphate oxidase (GPO), was employed for biosensor fabrication. Lipase metabolizes TG under physiological conditions, while GK and GPO participate in the metabolism of lipase products and flavin adenine dinucleotide (FAD)-dependent bioelectrocatalytic signaling (Narang and Chauhan, 2011; Narang et al., 2013a,b).

The schematic in Fig. 1 depicts the signaling strategy employed in this study, and summarizes how an electrochemical signal is generated according to the TG concentrations and is then amplified by a multi-enzyme cascade reaction. The electrochemical signal is generated by the electron transfer of ferrocenemethanol (Fc) as an electron-shuttle in the electrolyte (Won et al., 2007; Song et al., 2012). Regardless of the type of fatty acid chain present, a TG substrate is decomposed into glycerol and free fatty acid by lipase catalysis (Mead et al., 2002). The generated glycerol is then converted into glycerol-3-phosphate (G3P) by GK in the presence of adenosine triphosphate (ATP). G3P is oxidized to dihydroxyacetone phosphate (DAP) by GPO, and the FAD group in GPO is changed to $FADH_2$, which is the reduced form of FAD (Claiborne, 1986). Finally, $FADH_2$ transfers its electrons into oxidized Fc, which is free in the electrolyte, to reproduce the electrochemical signal (Han et al., 2011, 2012). Using this strategy, the quantification of TG is accomplished by registering the electrocatalytically amplified signal through a tri-enzymatic cascade reaction. In addition, the limited solubility of human sebum TG in aqueous phase was another cause for concern regarding the sampling and preparation of sebum specimens for TG analysis. To enable efficient bioelectrocatalytic sensing, we prepared and analyzed TG samples in the form of micelle emulsions using detergent. Details are reported herein.

2. Materials and methods

2.1. Chemicals and instruments

Poly-L-lysine (PLL), G3P, glycerol, ATP disodium salt, and Triton X-100 were purchased from Sigma. Ferrocenemethanol (Fc, 97%) was obtained from Aldrich. Hexane (HPLC grade) and glutaraldehyde (25% solution) was purchased from Sigma–Aldrich. Lipoprotein lipase (from *Pseudomonas* sp.), GK (from *Cellulomonas* sp.), and GPO (from *Pediococcus* sp.) were obtained from Toyobo. Magnesium chloride was purchased from Duchefa Biochemie. Liquid-phase TG (clinical TG reagent) was obtained from Verichem Laboratories Inc. Solid-phase TG mix was purchased from Supelco. Bis(sulfosuccinimidyl) suberate (BS^3) was obtained from Pierce. Phosphate-buffered saline (PBS, pH 7.2) solution containing 0.1 M sodium phosphate and 0.15 M sodium chloride was prepared using deionized and double-distilled water (DDW).

Electrochemical measurements were carried out using an Electrochemical Analyzer 660D (CH Instruments). We employed a standard three-electrode system with a platinum wire auxiliary, a silver/silver chloride (Ag/AgCl) reference, and an evaporated gold working electrode. The effective area of the employed gold working electrode was 0.28 cm^2. As a reference analysis device, a Reflotron® Plus (Roche Diagnostics) was used. Determination of the TG micelle diameter was accomplished by using Zeta-potential & Particle size Analyzer ELSZ-2 (Otsuka Electronics).

2.2. Verification of multi-enzyme cascade reaction for triglyceride analysis

To verify the catalytic activity of GPO, GPO solution was prepared by dissolving 50 units of GPO in 1 mL of electrolyte (3.2 mM $MgCl_2$ and 0.2 mM Fc in 50 mM PBS containing 0.2% (w/v) Triton X-100 (PBST)). As an enzyme substrate, G3P was dissolved in PBST. After the 1:1 mixing of GPO enzyme solution and substrate solution, electrochemical analysis was conducted at potentials between 0 mV and 400 mV (*vs.* Ag/AgCl reference electrode) under a potential sweep rate of 60 mV/s.

For confirmation of the GK–GPO enzyme cascade reaction, a bi-enzyme (GK and GPO) solution was prepared by dissolving 50 units of GK and GPO in 1 mL of electrolyte (3.2 mM $MgCl_2$ and 0.2 mM Fc in PBST). As enzyme substrate, PBST-based solutions with a predetermined concentration of glycerol mixed with 3.6 mM ATP were prepared and used. To confirm the GK–GPO enzyme cascade reaction, enzymes and glycerol solutions were mixed at a ratio of 1:1. The final concentration of electron transferring mediator (Fc) was 0.1 mM, and this condition was applied all the following electrochemical experiments. Electrochemical analysis was conducted at potentials between 0 mV and 400 mV (*vs.* the Ag/AgCl reference electrode) under a potential sweep rate of 60 mV/s.

To ascertain the lipoprotein lipase, GK and GPO cascade reaction, a multi-enzyme solution was prepared by dissolving 50 units of each enzyme into 1 mL of electrolyte (3.6 mM ATP, 3.2 mM $MgCl_2$ and 0.2 mM Fc in PBST). Substrate solution was made by diluting aqueous TG samples in PBST buffer. Electrochemical measurement was performed in the same way as the GPO and GK-GPO enzyme cascade reactions. Every standard curve for each substrate was created using anodic current at the potential of 300 mV (*vs.* Ag/AgCl reference electrode) of the second cyclic voltammetric cycle.

2.3. Selection of the enzyme immobilization method

We prepared an evaporated gold surface on a silicon wafer as a substrate for the immobilization of enzymes, as well as for electrochemical signaling. Prior to the modification process, the gold surface was cleaned with a piranha solution composed of

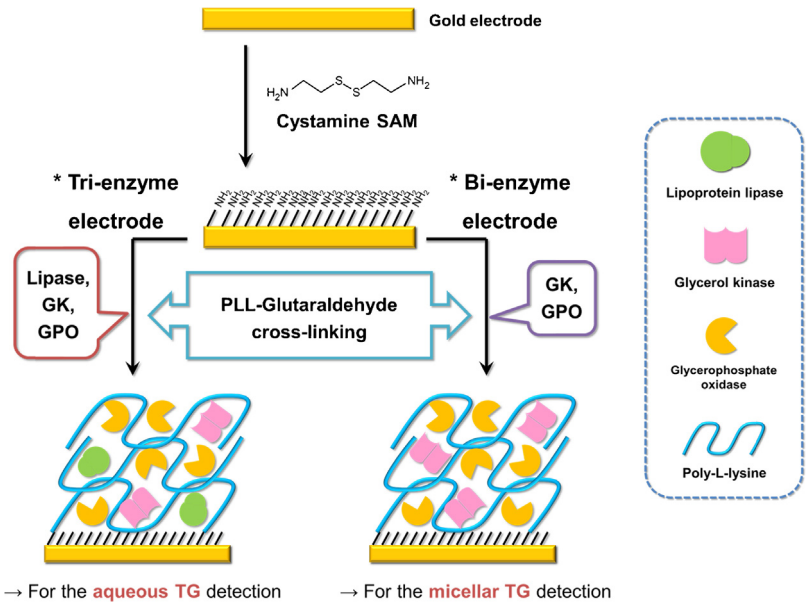

Fig. 2. Schematic illustration of the fabrication procedures used to functionalize tri-enzyme modified electrodes for aqueous triglyceride detection (left) and bi-enzyme associated electrodes for micellar triglyceride detection (right).

sulfuric acid and 30% hydrogen peroxide solution at a 4:1 ratio. (*Piranha solution reacts violently with most organic materials and must be handled with extreme care.*) After cleaning the surface with DDW, the electrodes were functionalized by testing four different methods, described as follows, and the immobilization performance was evaluated. The first immobilization method utilized 3,3-dithiodiproponic acid bis-N-hydroxysuccinimide ester (DTSP), which forms a self-assembled monolayer (SAM) on the gold surface. The DTSP self-assembled monolayer was constructed by dipping the electrodes into a 5 mM DTSP solution in dimethyl sulfoxide (DMSO) for 2 h. After formation of the self-assembled monolayer, the electrodes were rinsed with DMSO, followed by sequential rinsing with absolute ethanol and then water. For GPO immobilization, 150 μL of GPO (66 U/mL of enzyme in 50 mM PBS) solution was applied to the electrode surface for 30 min and washed using PBS. The second approach of immobilization used cystamine monolayer and glutaraldehyde crosslinking. Cleansed electrodes were dipped in a 5 mM cystamine dichloride solution in DMSO to functionalize the electrode surface. After 2 h, electrodes were rinsed with DMSO, followed by absolute ethanol and water. Then, 150 μL of 0.2% glutaraldehyde solution was placed on the electrodes for 1 h, and 150 μL of GPO (66 U/mL in 50 mM PBS) solution was applied for 30 min, followed by washing with PBS. The third method employed cystamine and a bis(sulfosuccinimidyl) suberate (BS^3) chemical linker molecule. The process for immobilization was the same as that described for the second method, except that the cross-linker used was BS^3 instead of glutaraldehyde. The final method utilized PLL and glutaraldehyde for the entrapment of GPO within the cross-linked polymeric network. For immobilization, 0.7 mg of PLL was dissolved into 300 μL of 50 mM PBS containing GPO (66 U/mL). As a crosslinker, 20 μL of glutaraldehyde (0.2%) was added to the mixed PLL-GPO solution. The mixture was placed on the electrode surface, where a cystamine self-assembled monolayer was previously formed. Following enzyme immobilization, electrochemical analyses were conducted using 10 mM G3P solution as the enzyme substrate in the presence of 0.1 mM Fc as an electron mediator.

2.4. Fabrication of multienzyme-associated electrodes

Evaporated gold electrodes were cleaned with piranha solution and cleansed by DDW. (*Piranha solution reacts violently with most organic materials and must be handled with extreme care.*) The electrodes were immersed in DMSO containing 5 mM cystamine for 2 h to functionalize the electrode surface. The enzyme immobilization process was then accomplished with either tri-enzyme or bi-enzyme immobilization. For tri-enzyme immobilization, 0.7 mg of PLL and the three enzymes (3.8 U of lipase, 2.6 U of GK and 14.1 U of GPO) were dissolved in PBS (300 μL, 50 mM). As a cross-linker, 20 μL of glutaraldehyde (0.2%) was added to the prepared solution. The mixture was then immediately deposited onto the electrode surface for 2 h. After immobilization, the electrode was rinsed with PBS buffer and stored. For bi-enzyme immobilization, the same protocol was employed except for the inclusion of lipase as shown in Fig. 2. The tri-enzyme modified electrode (left, Fig. 2) was utilized for the TG analysis in the solution phase, while bi-enzyme associated electrode (right, Fig. 2) was used for the sensing of TG in a micellar form.

2.5. Electrochemical detection of triglycerides in aqueous phase

Aqueous TG samples were prepared by diluting a clinical TG reagent to the required concentrations, ranging from 60 mg/dL to 300 mg/dL with PBST buffer containing electron transferring mediator (Fc), metal cation ($MgCl_2$) and coenzyme (ATP). Final concentrations of each electrolyte in the prepared aqueous TG samples were 0.1 mM for Fc, 1.6 mM for $MgCl_2$ and 1.8 mM for ATP. Voltammetric measurements were conducted under 60 mV/s scan rate using the tri-enzyme-immobilized working electrode. A calibration curve for aqueous TG was generated based on the amplified anodic currents sampled at 300 mV (*vs.* Ag/AgCl) from respective voltammograms.

2.6. Micellization of triglycerides

The purchased TG reference mix was dissolved in 2 mL of hexane containing 0.2% (w/v) Triton X-100 by sonication. After complete dissolution, 800 μL of TG solution was transferred into a vial and mixed with 4.2 mL of hexane containing 0.2% (w/v) Triton X-100. The sample was dried under nitrogen gas, and 5 mL of PBST buffer was injected into the vial and sonicated to prepare micellar TG (Burns and Roberts, 1981; Sun et al., 2007; Kim et al., 2009; Zhu et al., 2013). By using Reflotron® Plus system, TG concentrations

of the individual micelle samples were evaluated. The hydrodynamic size of obtained TG micelles was determined using the electrophoretic light scattering (ELS) method.

2.7. Electrochemical analysis of micellar triglycerides

Micellar TG samples were prepared by diluting TG micelle mixtures to the required concentration with PBST buffer (TG:PBST = 3:1). The electrolyte used for signal generation consisted of 11.9 U/mL lipase, 1.6 mM $MgCl_2$, 1.8 mM ATP, and 0.1 mM Fc. Voltammetric measurements and data analysis were accomplished by using the same procedure as for aqueous TG.

2.8. Analysis of human skin sebum samples: real specimen testing

Sebum samples were taken from both sides of the face, the forehead, and the nose regions of ten male volunteers. Before sample collection, the skin was cleansed by washing with soap and dried. To collect human sebum TG, lipid-absorbing polymeric film (Sebutape® S100, CuDerm Co.) was attached to the washed facial regions for 30 min, after which it was detached from the skin using tweezers and placed into a glass vial. A volume of 1 mL of hexane containing 0.2% (w/v) Triton X-100 was added to the vials, and samples were sonicated for 10 s. The extract was dried under nitrogen gas and re-suspended in 1 mL of PBST buffer, resulting in micelle formation. Quantification of human sebum TG was conducted using the procedures as described for micellar TG.

3. Results and discussion

3.1. Verification of the enzyme cascade reaction for triglyceride bioelectrocatalysis

The enzymatic cascade reaction with lipase, GK, and GPO, in the presence of peroxidase and color developing reagents, has been employed to colorimetrically determine TG concentrations. However, to the best of our knowledge, few electrochemical enzymatic approaches exist for the quantification of TGs. For this reason, we attempted to verify whether the enzyme cascade reaction of lipase, GK, and GPO is suitable for bioelectrocatalytic analysis and, further, to utilize the developed method for the analysis of actual human skin sebum samples. A proof-of-concept analysis of the tri-enzyme cascade reaction for bioelecrocatalysis was first conducted using solution-based voltammetric tests.

The schematic in Fig. 3 illustrates the enzyme cascade reaction that was employed, and the electrochemical signals registered for different steps of cascade reaction in solution phase were plotted. First, the schematic illustration of the GPO reaction is presented in the innermost box of Fig. 3 (blue line). In this test, G3P was used as a substrate for GPO, which generates an anodically amplified electrochemical signal in the presence of Fc electron-mediator. The GPO enzyme reaction was analyzed by cyclic voltammetric method, and signals were sampled and plotted by applying various concentrations of G3P. According to the calibration curve of the GPO reaction (right, Fig. 3), the electrochemical signal increases in proportion to the concentration of G3P. The middle box of Fig. 3 (magenta line) indicates the bi-enzymatic cascade reaction of GK and GPO. With the bi-enzymatic reaction, the glycerol substrate was first converted to G3P by GK catalysis in the presence of ATP. The generated G3P was then used in the amplification of the electrochemical signal by GPO in the presence of Fc electron-mediator. The calibration result of this experiment (middle, Fig. 3) showed the relation between the electrochemical signal and the concentration of applied glycerol. Finally, the tri-enzyme cascade reaction of lipase, GK and GPO was tested as shown in the outermost box of Fig. 3 (green line). In this sequential enzymatic reaction, target TG was first decomposed into glycerol and three molecules of free fatty acids by the lipase reaction. Generated glycerol was then used for signal generation and amplification following the previously mentioned GK and GPO reaction. The calibration curve (left, Fig. 3) suggests that the electrochemical signal was generated as intended, and that the signal level from the calibration results was proportional to the concentration of TG up to 80 mg/dL. Based on the results from solution phase experiments, we confirmed that the TG concentration is measurable using the electrode by employing tri-enzyme associated cascade reaction and bioelectrocatalytic signaling.

3.2. Electrochemical analysis of aqueous triglycerides using multienzyme electrode

After observing the feasibility of bioelectrocatalytic TG analysis with the solution phase test, we moved to the fabrication of enzyme-associated electrode. To select a method for enzyme immobilization on the electrode surface, we compared two major types of immobilization mechanisms: covalent bonding and entrapment. The covalent bonding method was directly used to form covalent bonds between amine groups on the enzyme surface (epsilon-amine from lysine residue) and chemical reagents reactive to amines (DTSP, glutaraldehyde and BS^3). On the other hand, an enzyme entrapment method was conducted using a PLL polymer backbone and glutaraldehyde crosslinking reagent, resulting in PLL matrix. During the crosslinking process, enzymes were entrapped in the PLL matrix and indirectly immobilized on the electrode surface due to the electrostatic force between the positively charged PLL matrix and negatively charged enzymes possessing isoelectric points (pI) lower than pH 7. Although there exists the possibility of direct covalent linking of enzyme with glutaraldehyde during the crosslinking reaction, we reacted higher amount of PLL comparing to the enzyme to minimize the unwanted reaction. Fig. S1 (*Supplementary Data*) demonstrates the registered electrochemical signal difference between the enzyme immobilization methods, showing the change in the amount of actively (enzymatically as well as bioelectrocatalytically) immobilized enzymes. When covalent bonding was employed to immobilize GPO on the electrode surface by using self-assembled monolayers containing amine-reactive functional groups such as DTSP, glutaraldehyde and BS^3, the electrochemical signal was only weakly amplified. However, the enzyme electrode prepared by using the entrapment method significantly amplified the electrochemical signal (Fig. S1). According to these results, the covalent bonding method was not able to immobilize sufficient amount of GPO, or the immobilized GPO molecules were partly unable to conduct bioelectrocatalytic reaction to amplify the electrochemical signal from steric limitations. This reasoning is supported by previous reports regarding GPO immobilization by employing matrix-based adsorption and/or crosslinking rather than the direct covalent bonding (Ghica and Brett, 2006; Minakshi and Pundir, 2008; Narang et al., 2010; Pundir et al., 2010; Monosik et al., 2012).

To assess the amount of GPO which could be immobilized on the electrode by PLL-mediated entrapment method, a protein quantification based on the absorbance at 280 nm was conducted with the PLL–GPO–GA mixture solution before and after the immobilization. As shown in Fig. S2, 45% of GPO in the PLL–GPO–GA mixture solution was immobilized on the electrode by the entrapment process. In addition, the GPO-modified electrode maintained its bioelectrocatalytic activity over 70% for five days when the electrode was stored in PBS at 4 °C. Based on this result, we adopted the entrapment method for the immobilization of enzymes, concerning the activity of signal generating enzyme GPO.

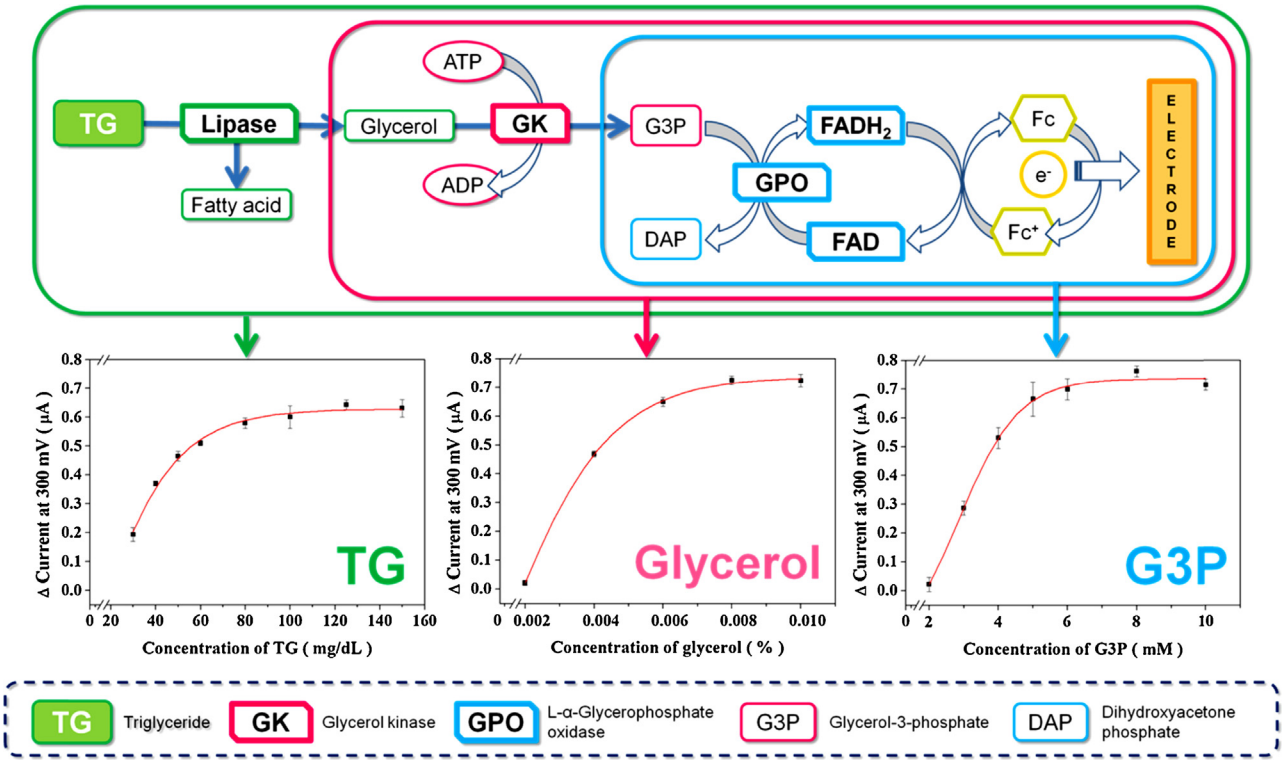

Fig. 3. Verification of the multi-enzyme reaction cascade in solution phase. Each graph depicts the signal amplified by enzyme reaction *vs.* concentration of each substrate. Measurements were performed in the presence of 0.1 mM Fc, 1.8 mM ATP, and 1.6 mM $MgCl_2$ in a 50 mM PBS (pH 7.2) under a scan rate of 60 mV/s. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

Fig. 4 shows the calibration curve obtained from different concentrations of TG samples in aqueous electrolyte using the tri-enzyme immobilized electrodes. The anodic currents were registered at +300 mV *vs.* Ag/AgCl from the second voltammetric cycle. The electrochemical analysis of single TG sample was accomplished within 30 s. As shown in the calibration curve, signals were elevated linearly in proportion to the aqueous TG concentration and leveled off around the sample concentration of 100 mg/dL. With the result, we could confirm that the electrochemical analysis of TG by using the enzyme cascade reaction followed by the GPO electrocatalysis was possible. Then, we moved to the electrochemical analysis of skin sebum TG as the target analyte of the study, having analytical issues still not answered such as low solubility and wide determination range, comparing to the aqueous-phase TG sensing.

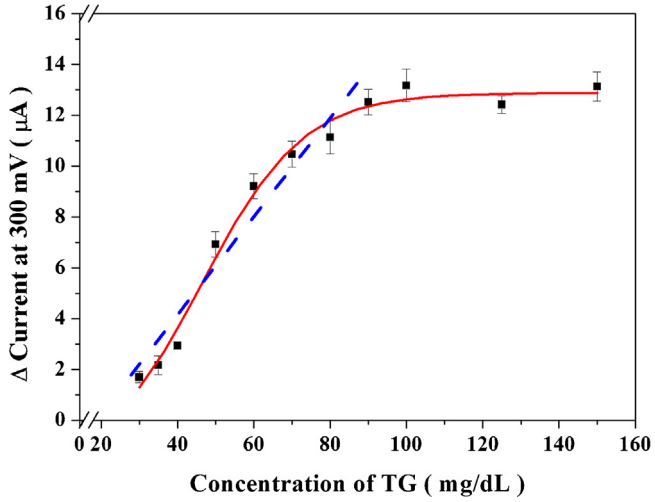

Fig. 4. Calibration curve of aqueous triglycerides using a tri-enzyme immobilized electrode. The curve was depicted by an anodic current sampled at 300 mV in the second voltammetric cycle.

3.3. Quantification of sebum triglycerides using human specimens

An enzymatic assay must be performed in aqueous conditions because the majority of enzymes, as well as their essential cofactors, are unstable in the presence of an organic solvent. Also, the substrate of enzyme lipase should be in a certain molecular structure across water–oil interface, such as micelle or emulsion drop, to be consumed by the lipid–hydrolysis activity of lipase (Burns and Roberts, 1981; Sun et al., 2007; Kim et al., 2009; Zhu et al., 2013). For this reason, the pretreatment and emulsification of TGs are necessary when quantifying human sebum TG that is not readily soluble in aqueous solution. Therefore, TG samples from human skin sebum as well as standards were prepared in the form of detergent micelles utilizing TX-100 as an emulsification reagent. Fig. S3(A) describes methods for the preparation of micellar TG. The TG was simply dissolved in hexane containing surfactant, and its dry matter was easily soluble in the aqueous phase, as opposed to those dissolved without surfactant. After preparation, the mean hydrodynamic diameter of the synthesized TG micelles which were evaluated by electrophoretic light scattering (ELS) analysis was around 1010 nm. ELS volume distribution result for the obtained TG micelles was shown in Fig. S3(B). The polydispersity index (PDI) of micelle, indicating the extent of micelle size distribution, was determined as ~0.4 by the ELS measurement. Generally, when PDI value of the particle is lower than ~0.7, we can assume that the micelle exhibits uniform size distribution. Also, the prepared TG micelles maintained their structural stability for five days at room temperature.

To confirm the concentration of the prepared micellar TG, we undertook a conventional enzymatic colorimetric assay using

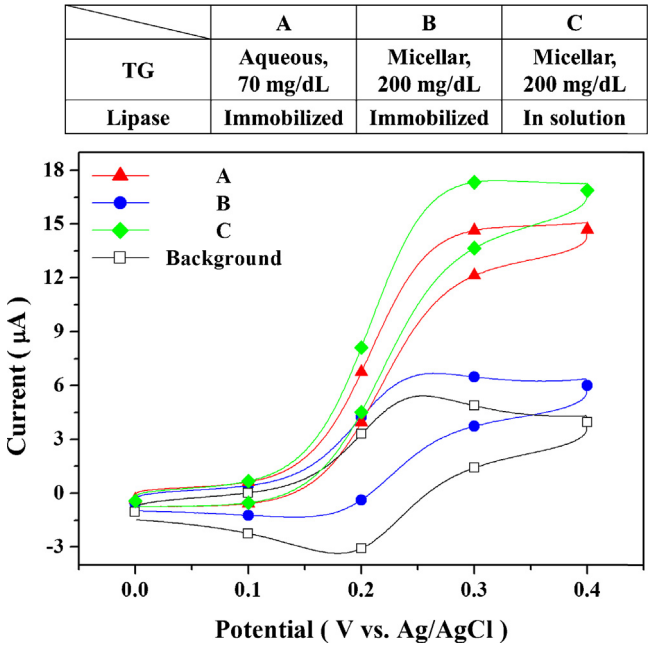

Fig. 5. Changes in the cyclic voltammograms dependent on experimental conditions used. The electrochemical signal was amplified by a cascade enzyme reaction of aqueous triglycerides on the tri-enzyme immobilized electrode (A, red), whereas micellar triglycerides could not activate the amplification of electrochemical signal (B, blue). By using a bi-enzyme immobilized electrode with the lipase in electrolyte, micellar triglycerides could generate the amperometric signal amplification (C, green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Reflotron® Plus Triglyceride. When the sample was applied to the Reflotron® Plus triglyceride test strip, tri-enzyme cascade reaction of lipase, GK, GPO generated H_2O_2. The produced H_2O_2 was catalyzed by horseradish peroxidase (HRP) in the presence of a redox indicator, 4-(4-dimethylaminophenyl)-5-methyl-2-(3,5-dimethoxy-4-hydroxyphenyl)-imidazole dihydrochloride. By measuring the oxidized redox indicator (λ_{max} = 642 nm) on the test strip, the concentrations of TG in applied samples could be evaluated. Fig. S3(C) shows correlations between the theoretical concentration of micellar TG (*x*-axis) and the measured concentration of micellar TG from enzymatic colorimetric assay (*y*-axis). This linear relationship indicates that it was possible to measure micellar TG using enzymes. However, unlike aqueous TG, we were not able to analyze micellar TG with tri-enzyme immobilized electrodes for quantification. We conducted tests under several different conditions to verify the cause of this observation.

As shown in Fig. 5, aqueous TG was capable of amplifying signal currents through enzyme reaction on the tri-enzyme immobilized electrode surface (voltammogram A, red line), whereas micellar TG could not (B, blue line). This result was caused by the size problem of the synthesized TG micelle which restricts the approaching of substrate (TG micelle) to the enzyme lipase that is located inside the PLL matrix. By comparing with the sizes of lipoproteins such as very low density lipoprotein (VLDL, ~50 nm) and chylomicron (75–1200 nm) which are major TG transferring molecules in human serum (Wood et al., 2006; Savorani et al., 2010), the sizes of prepared micelle (mean dia. 1010 nm) were belong to the relatively large ones. The tested aqueous TG samples which were made up of small sized-lipoproteins were able to easily penetrate in the PLL matrix and interact with the lipase inside. On the contrary to this, synthesized TG micelles could not reach to the lipase in the PLL matrix on the enzyme electrode. To eliminate this limitation, lipase was positioned not in the PLL matrix but added to the electrolyte solution unlike the other enzymes (GK and GPO), which were immobilized on the electrode surface. With this configuration, highly amplified electrochemical signals were again observed as shown in Fig. 5C, supporting that the lipase in solution could react with TG in detergent micelles.

Fundamentally, in the digestive organs, the enzymatic reaction of lipase is initiated by the contact of enzyme to the emulsified lipid micelles such as bile salt-based micelles and lipoproteins (Burns and Roberts, 1981; Sun et al., 2007; Kim et al., 2009; Reis et al., 2009; Zhu et al., 2013). Based on the reasoning, free lipases in aqueous solution would easily interact with TG micelles and hydrolyze the TG into fatty acid and glycerol. From the amplified voltammetric signal shown in Fig. 5C, metabolites produced from TG by the lipase reaction could participate in the cascade GK reaction and biocatalysis with GPO at the bi-enzyme associated electrode. Thus, based on the result of the experiment, detection system which was made up of bi-enzyme (GK and GPO) electrode and free lipase was chosen for the detection of water-insoluble TG samples including micelle TG and skin sebum TG sample.

To verify the applicability for real sample detection, we measured human sebum using the developed TG sensing system. Fig. 6(A) depicts the extraction and analysis of TG from human skin sebum. Micellar sebum samples contained various lipid compounds, but electrochemical signal amplification only occurred in the presence of TG by the enzyme cascade reaction. Therefore, the developed system was able to selectively determine the concentration of TG in human skin sebum.

The electrochemical signal alterations according to the changes of concentration of standard micellar TG, human sebum samples and micellar TG-spiked sebum samples were shown in Fig. 6(B). First, a standard calibration curve for the standard micellar TG samples was prepared (red line). Compared to the aqueous TG analysis, the calibration curve for the TG micelles exhibited an increased linear range (0–200 mg/dL) while with lower signal intensity. It could be assumed that the difference in calibration results according to the sample type was originated from the change in diffusion distance of glycerol to the GK/GPO-modified electrode. Contrary to the aqueous TG biosensing electrode, where all the enzyme cascade reactions occur just above electrode surface and the diffusion distance is negligible, glycerol generation from the micellar TG analysis mainly occurs on the TG micelle in bulk solution. Therefore, for the subsequent enzyme reactions including the conversion of glycerol into G3P and the oxidation of G3P, the generated glycerol in bulk solution should be diffused to the enzyme-modified electrode surface. Therefore, the extended diffusional distance would reduce the G3P generation, and the alteration in the ratio of G3P generation to the G3P consumption would induce the decrease in signal magnitude and increases in the apparent linear range as shown in Fig. 6(B). In the range from 0 to 200 mg/dL of TG micelle, where the calibration curve exhibited linearity, the limit of detection (LOD), which was calculated as three times the standard deviation (SD) of the background signal, was 15 mg/dL. With the standard micellar TG samples, the coefficient of variation (CV = SD/mean × 100), obtained from calibration tests, was approximately 6.7% and the practical dynamic detection range was from 15 to 200 mg/dL of micellar TG concentration (blue dashed line).

The obtained results from electrochemical detection of 10 sebum real samples and 3 TG micelle-spiked sebum samples were depicted on the micellar TG calibration curve of Fig. 6(B). According to the increase in TG concentration of each sebum samples which were evaluated by Reflotron® Plus assay, electrochemical signals were also increased in the dynamic detection ranges for micellar TG. For thirteen sebum-based samples, the relative accuracy of the proposed biosensing system compared to the conventional method (Reflotron® Plus) was calculated as 80%.

Because we have tested sebum samples from healthy volunteers, whole dynamic detection range was not covered by real

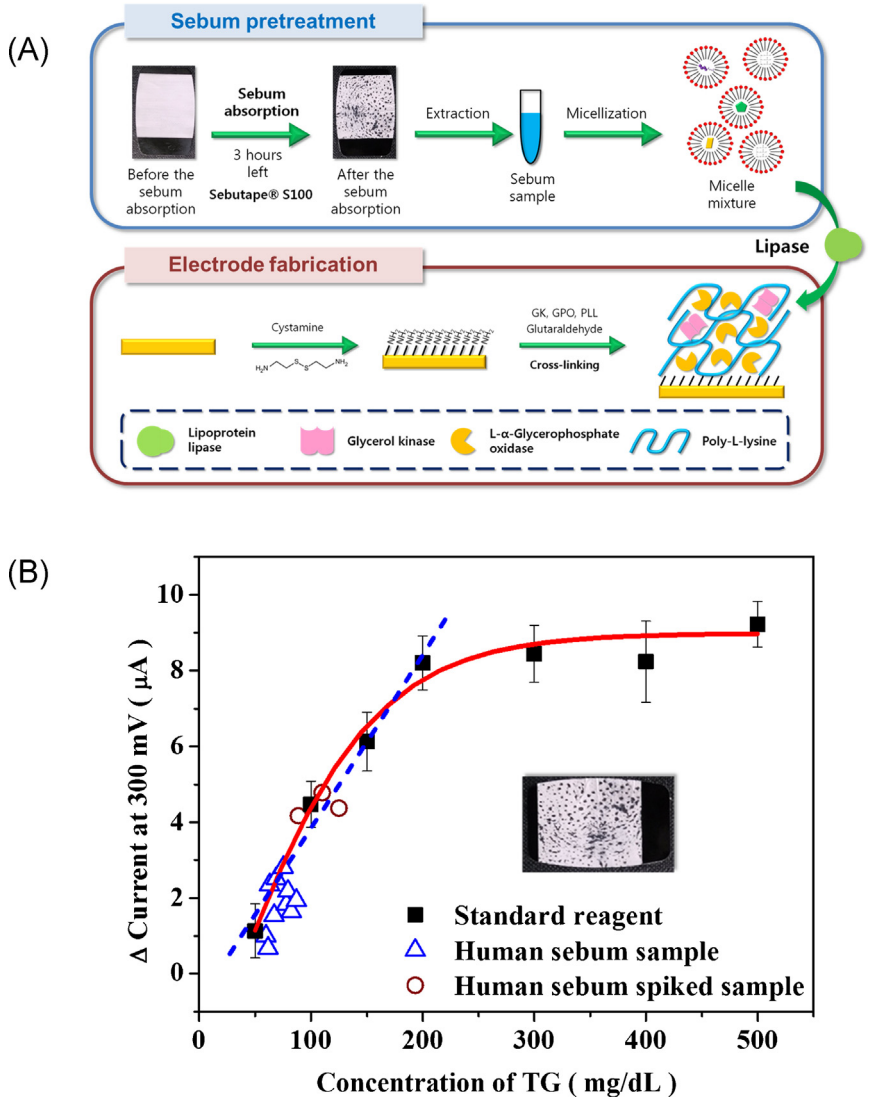

Fig. 6. (A) Experimental procedures for quantitative analysis of human sebum triglycerides. (B) Quantitative analysis data for standard micellar triglyceride samples (filled square), human sebum triglyceride samples (open triangle) and micellar triglyceride-spiked sebum samples (open circle) using the developed biosensing configuration. Dashed line represents the linear regression according to the standard micelle TG sample analysis. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

sample data. The validation of this biosensing strategy for the diagnosis of skin ailments, exhibiting abnormally increased TG level, will be achieved through collaboration with dermatologists. However, the obtained results from this study signify that the developed biosensing system would be used as a field device to quantify human sebum TG.

4. Conclusion

In this study, we developed a TG electrochemical biosensor using enzyme cascade reactions, and successfully used this system to detect human sebum TG. Currently, various analytical methods such as HPLC, GC–MS, and NMR are used for the determination of human sebum TG concentrations. However, these conventional methods use complicated and expensive instruments that must be operated by skilled technicians. The significant merit of the biosensor developed in this study is its applicability, since this electrochemical system rapidly detects samples, may be simply miniaturized to a smaller size and easily operated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.01.036.

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