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Electrochemical detection of non-esterified fatty acid by layer-by-layer assembled enzyme electrodes

Jing Kang[a,*,1], Anisah T. Hussain[a,1], Michael Catt[b], Michael Trenell[c], Barry Haggett[d], Eileen Hao Yu[a,*]

[a] School of Chemical Engineering and Advanced Materials, Newcastle University, Newcastle upon Tyne NE1 7RU, United Kingdom
[b] Institute of Ageing and Health, Newcastle University, Newcastle upon Tyne NE4 5PL, United Kingdom
[c] Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne NE1 7RU, United Kingdom
[d] Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton LU2 8DL, United Kingdom

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ABSTRACT

In this study, detection and measurement of non-esterified fatty acids (NEFA) concentration has been achieved by electrochemical method in one operation step. Multilayer films of poly(dimethyldiallyammonium chloride) (PDA) wrapped multi-wall carbon nanotubes (MWCNTs) and two enzymes acyl-CoA synthetase (ACS) and acyl-CoA oxidase (ACOD) were assembled on a carbon screen printed electrode by the layer-by-layer (LbL) immobilization. The fine polymer–enzyme layers produced by the LbL method, allowed mass transport from the reactant cascading down the layers to accomplish the two-step enzyme reactions. The polymer–CNTs and enzyme modified electrode exhibited good electrocatalytical property retaining enzyme activity. Linear increase of anodic current from H_2O_2 produced from NEFA oxidation was observed with the increasing concentrations of oleic acid. These results indicate a promising technique for a simple, rapid one-step determination of NEFA for diabetes management.

1. Introduction

Diabetes poses a major and growing health and socio-economic burden on society [1]. With an increase in people being diagnosed with type 2 diabetes (T2D), there should be more ways to monitor not only the blood glucose levels but also the other metabolism biomarkers associated with T2D. It is increasingly understood that diabetes is a disorder of energy metabolism involving both sugar and fat utilisation. Patients with T2D often show a higher level of non-esterified fatty acids (NEFA), associated with increased insulin resistance (IR) and poor glucose disposal rate (GDR) [2,3]. NEFA is an important skeletal muscle fuel source. The change in plasma NEFAs is a useful indicator of rate of lipolysis. NEFAs are about 10% of the total blood fatty acids, usually with the physiological concentration range of 0.1–1.8 mmol/l [4]. Chronically elevated NEFA concentrations in T2D may be involved in β-cell dysfunction and apoptosis [5]. Plasma NEFA concentration is used as a diagnostic marker for identification of the people at greater risk for developing T2D prior to the appearance of insulin resistance and insulin secretion defects. This is of particular importance in providing early diagnoses of T2D, assessing the degree of myocardial infarction and other obesity-related illnesses. Existing diabetes management promotes self-monitoring of blood glucose for disease management and secondary prevention [6]. The monitoring of the changes of both sugar and NEFA during metabolic processes would provide a more accurate means of diagnosis, and subsequently, more effective means of disease prevention and management.

NEFA detection in blood can be dated back to the late 1950s [7,8], the methods developed during this time were either based on colorimetric titration of fatty acids in the presence of a pH indicator [9] or spectrophotometric or radiochemical measurements of complexes of fatty acids with divalent metal ions such as Cu^{2+}, Ni^{2+} or Co^{2+} [10]. These methods, however, were time-consuming and showed a lack of good sensitivity. Other methods using different spectroscopic measurements, such as liquid chromatography–mass spectrometry and fourier transform infrared have been developed over the past decades [11,12].

There have been very few reports on electrochemical detection of NEFA in plasma or blood serum [13,14]. Karube et al. has reported the first electrochemical NEFA sensor that was based on the enzyme reactions utilizing acyl-CoA synthetase (ACS) and acyl-CoA oxidase (ACOD) [14]. They monitored dissolved oxygen consumption by the two sequential reactions catalyzed by the enzymes immobilized in photo-cross-linkable poly(vinyl alcohol) resin. A linear correlation between current decrease and 0.3–2.6 mM oleic or palmitic

* Corresponding authors. Tel.: +44 (0)1417013265; fax: +44 191 2225292.
E-mail addresses: jingkang.jk@gmail.com (J. Kang), eileen.yu@ncl.ac.uk (E.H. Yu).
[1] These authors have contributed to this manuscript equally.

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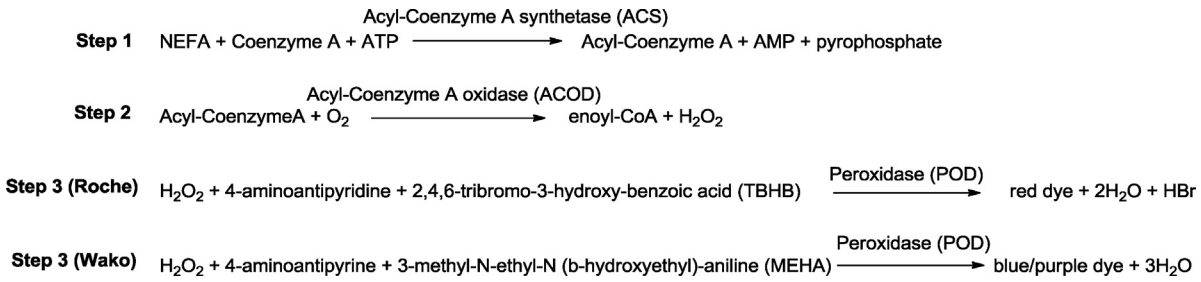

Scheme 1. Reaction schemes of the enzymatic colorimetric method used by Roche and Wako [15,16].

acid was observed. However, such 'signal-off' method lacks of high specificity due to many potential interferences that can cause decrease of current upon sample injection. Also, their immobilization method relied on the enzyme entrapment within layers of membranes.

To date, there are two commercial in-vitro enzymatic colorimetric assays available for quantitative determination of NEFA in serum samples. They are manufactured by Wako diagnostics and Roche for detection of oleic acid and palmitic acid, respectively [15,16]. Both methods are based on the study published by Sakaru et al. [17]. It relies on the first conversion of NEFA to acyl-coenzyme A (Acyl-CoA) in the presence of acyl-CoA synthetase (ACS) (Scheme 1). The acyl-CoA produced is oxidised by acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide (H_2O_2). In the presence of peroxidase (POD), the hydrogen peroxide formed yields a red or blue/purple pigment by quantitative oxidation condensation with TBHB or 3-methyl-*N*-ethyl-*N*-(betahydroxyethlyl)-aniline (MEHA), which can be quantified by UV-spectrometer at defined wavelength.

These methods are expensive, time consuming and can only be operated in the laboratory. Therefore, NEFAs are not routinely assessed except within specific clinical study contexts despite their importance in diabetes research and disease management. If H_2O_2 produced from step 2, acyl-CoA oxidation by ACOD at the presence of oxygen, can be detected electrochemically, it should be possible to determine NEFA concentration electrochemically from the amount of H_2O_2, similar to the colorimetric method. This could provide a coherent method to current electrochemical based glucose biosensor to develop a multiplex biosensor platform for simultaneous measurement of different metabolic biomarkers.

This study will look at the electrochemical detection of H_2O_2 from the reactions in Scheme 1, using oleic acid (OA) as the NEFA of interest. It was chosen as the NEFA of investigation in this study as it is one of the most abundant plasma NEFAs and it's functional uptake in both muscle and liver are the same as that of total NEFA [18].

Layer-by-layer (LBL) technique is one of the most promising methods for surface modification and biomolecule immobilization because of its simplicity, versatility and wide range of materials that can be used for film assembly [19,20]. The method is mostly based on electrostatic assembly of oppositely charged polyelectrolytes to a charged solid surface. It has been widely used for functional coating, materials encapsulation, biosensing and other applications [21–24]. Since enzymes are also polyelectrolytes, whose charge depends on the isoelectric point (*Ip*) of the protein and the solution pH, they can be easily immobilized into the LbL film without complex chemical reactions and dehydration. In this way, the electrostatically deposited enzymes can remain good biological activity [25]. However, the commonly used polyelectrolytes in the LbL technique such as poly(allyalmine hydrochloride) (PAH)/poly(sodium 4-styrenesulfonate) (PSS) and poly(dimethyldiallyammonium chloride) (PDA)/polymethacrylic acid (PMAA) are usually electrically nonconductive, which would be a limiting factor for them to be used in bioelectronic devices [23,26].

Carbon nanotubes (CNTs) have attracted great attention as functional materials for preparation of enzyme electrodes and biosensors due to their unique electrocatalytic properties [27–30]. PDA is a strong polycation that can be well adsorbed on carbon or gold electrodes [31,32]. It has been reported to have good affinity to CNTs and gold nanoparticles (GNPs) [33]. The conductive PDA composite can be used to immobilize enzymes in the LbL manner for electrochemical biosensor applications [34,35].

In this work, PDA–MWCNT composites were employed for LbL fabricating enzyme electrode containing two enzymes: ACS and ACOD on the carbon screen printed electrodes (C-SPE); the enzyme modified electrode were used for NEFA detection through measuring the oxidation current of H_2O_2 produced from enzyme reactions. The multilayers were chosen in the order of polymer/ACOD/polymer/ACS, to keep consistent with the enzymatic reaction order. Good linear correlation between NEFA concentration and H_2O_2 oxidation currents was obtained, indicating one step NEFA detection achieved. This provides a solid base for further development of a multiplex sensor platform.

2. Experimental

2.1. Materials

Oleoyl coenzyme A lithium salt (OACoA), palmitoyl coenzyme A lithium salt (PACoA), coenzyme A sodium salt hydrate (CoA), adenosine 5′-triphosphate disodium salt hydrate (ATP), poly(dimethyldiallyammonium chloride) (PDA) (M_w = 200–250 kDa), sodium dihydrogen phosphate dehydrate, disodium hydrogen phosphate and potassium ferricyanide ($K_3[Fe(CN)_6]$) were purchased from Sigma-Aldrich (Dorset, UK). 1 mM Oleic acid (OA) was the Wako NEFA standard solution purchased from Wako HR-series NEFA-HR(2) enzymatic NEFA assay kit (Neuss, Germany); acyl-CoA synthetase (ACS) was also supplied from this assay kit in a solid mixture with CoA, ATP and a few other reagent (R1a) [16]. The enzyme solution was made by dissolving the R1a reagent in PBS with an ACS concentration of 4 U/ml. ACOD was purchased as pure enzyme from Wako Chemicals GmbH (Germany). Optical validation work was done using the Roche Free fatty acids, Half-micro test from Roche diagnostics (Penzberg, Germany). The patient plasma samples were provided by Newcastle Medical School (Newcaslte upon Tyne, UK). MWCNTs with inner diameters of 20–50 nm and outer diameters of 70–200 nm were obtained from Applied Sciences Inc. (Ohio, USA).

The C-SPEs (model DRP-C110) were from the company Dropsens (Oviedo, Spain)[36]. The electrodes have a diameter of working electrode of 0.40 cm and an area of 0.13 cm^2. The reference electrode is silver/silver ion (Ag) and the counter electrode is carbon.