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 5RL, 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 9SD, United Kingdom

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A B S T R A C T

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(dimethyldiallylammonium 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 H2O2 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.

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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 Cu²⁺, Ni²⁺ or Co²⁺ [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
Acyl-Coenzyme A synthetase (ACS)

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\begin{align*}
\text{Step 1} & \quad \text{NEFA + Coenzyme A + ATP} \rightarrow \text{Acyl-Coenzyme A + AMP + pyrophosphate} \\
\text{Step 2} & \quad \text{Acyl-Coenzyme A oxidase (ACOD)} \\
& \quad \text{Acyl-Coenzyme A + O}_2 \rightarrow \text{enoyl-CoA + H}_2\text{O}_2 \\
\text{Step 3 (Roche)} & \quad \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + 2,4,6\text{-tribromo-3-hydroxy-benzoic acid (TBHB)} \\
& \quad \text{Peroxidase (POD)} \rightarrow \text{rod dye + 2H}_2\text{O} + \text{HBr} \\
\text{Step 3 (Wako)} & \quad \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + 3\text{-methyl-N-ethyl-N-(betahydroxyethyl)-aniline (MEHA)} \\
& \quad \text{Peroxidase (POD)} \rightarrow \text{blue/purple dye + 3H}_2\text{O}
\end{align*}
\]

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\(_2\)O\(_2\)). 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-(betahydroxyethyl)-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\(_2\)O\(_2\) 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\(_2\)O\(_2\), 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\(_2\)O\(_2\) 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 (\(pI\)) 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(allylamine hydrochloride) (PAH)/poly(sodium 4-styrenesulfonate) (PSS) and poly(dimethyl diallylammonium 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\(_2\)O\(_2\) 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(dimethyl diallylammonium chloride) (PDA) (M\(_{\text{w}}\)=200–250 kDa), sodium dihydrogen phosphate dehydrate, disodium hydrogen phosphate and potassium ferricyanide (\(K_3[\text{Fe(CN)}_6]\)) 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 (Newcastle 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 Dropens (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.
2.2. Layer-by-layer enzyme fabrication of C SPE

A PDA–MWCNT solution was prepared by mixing 3 mg of MWCNTs in 5 ml 1% PDA under sonication for 3 h and the suspension was left overnight for any solid materials to sediment. The top half PDA–MWCNT solution was used. Enzyme electrode (PDA–MWCNT/ACOD) was assembled for acyl-CoA determination and bi-enzyme electrode (PDA–MWCNT/ACS/PDA–MWCNT/ACOD) in is NEFA determination.

To assemble multilayer films (PDA–MWCNT/ACOD) on the electrodes, 15 μl PDA–MWCNT solution was dropped onto the working electrode at 4 °C for 30 min. The electrode was afterward washed with 15 μl PBS twice to remove the excessive amount of PDA–MWCNTs that were not or weakly adsorbed. Thereafter, 15 μl ACOD (4 U/ml) solution was dropped onto the PDA–MWCNT modified electrode at 4 °C for 30 min. The electrode was again washed with 15 μl of PBS twice to complete the first (PDA–MWCNT/ACOD) double layer coating on the electrode. Two double layer of (PDA–MWCNT/ACOD) was assembled on the electrode for the enzyme electrode fabrication. The multilayer film was assembled without drying during each deposition step. The bi-enzyme electrode was prepared in a similar manner by having alternative layers of PDA–MWCNT/ACS and PDA–MWCNT/ACOD. One or four multi layers of (PDA–MWCNT/ACS/PDA–MWCNT/ACOD) were assembled, respectively. The schematic display of the assembly process is shown in Fig. 1. The dispersion of MWCNT in positively charged polycation PDA is possibly due to weak supramolecular interactions between them, which bind electrostatically to the negatively charged (polyanion) acyl-coenzyme A oxidase (ACOD) at pH 7.4, as the isoelectric point of ACOD is 5.5 [37].

2.3. Electrochemical measurements

Electrochemical measurements were carried out by an Autolab potentiotstat–galvanostat (PGSTAT302). Linear sweep voltammetry and chronoamperometry were carried out in 0.1 M PBS with various PAcOa/OAcOa or OA concentrations. Different PAcOa/OAcOa concentration was achieved by stepwise addition of 10 mM PAcOa/OAcOa solution to the cell filled with 0.5 ml PBS. For OA determination on the bi-enzyme electrode, different OA concentration was achieved by dilution of 1 mM OA standard solution in the cell, which was filled with PBS containing ATP and CoA as cofactors for the NEFA acylation reaction (step 1 in Scheme 1). The concentrations of ATP and CoA were both controlled to be 1 mM in solution for each concentration analysis of acyl-CoA/NEFA. Electrochemical impedance measurements were performed to monitor the build-up of films in 5 mM K3[Fe(CN)6] as a redox probe in the frequency range between 0.1 Hz and 100,000 Hz, at a fixed potential of 0.17 V. The amplitude of the alternating voltage was 10 mV. All electrochemical experiments were done at room temperature.

2.4. Validation of the electrochemical detection of NEFA against the colorimetric method

Several concentrations of OA from 0.0 mM to 0.9 mM were measured by the colorimetric method using the commercial NEFA standard solution from Wako [16] and our electrochemical method on the enzyme electrode, respectively. Each concentration was measured three times and the average value was used for obtaining a calibration graph for each method respectively. 0.3 mM and 0.6 mM as two known concentrations of OA were measured three times independently by the colorimetric and the electrochemical method, and the average value obtained from each concentration were used to fit in the calibration curve of each method, respectively.

2.5. Electrochemical detection of NEFA in human plasma on the enzyme electrode

The patient plasma sample provided by Newcastle Medical School was firstly diluted into different concentrations by PBS buffer and then analysed by the Roche kit to determine the NEFA concentrations. The same samples were then analysed by electrochemical method on the (PDA–MWCNT/ACOD/PDA–MWCNT/ACOD) enzyme electrode. The electrochemical measurement include both linear sweep voltammetry and chronoamperometry.

3. Results and discussion

3.1. PAcOa determination on C-SPE with ACOD in solution

Detection of PAcOa, the acylated form of NEFA palmitic acid (PA) (step 1 in Scheme 1) is related to the determination of PA, thus NEFAs. Firstly, the feasibility of PAcOa determination from the enzyme reaction was carried out on a C-SPE having ACOD and PAcOa in PBS solution. Fig. 2a shows the chronoamperometry (CA) (E = 500 mV) for concentrations of PAcOa up to 1.2 mM. A calibration graph (Fig. 2b) was obtained from the current produced at 500 s. Linear increase of currents was observed with increasing concentrations of PAcOa. This oxidation current is associated with H2O2 decomposition to O2. The amount of H2O2 produced from the reaction is proportional to the amount of PAcOa. This result indicates that it is feasible to electrochemically detect PAcOa. Similarly,
electrochemical detection of OACoA, the acylated form of OA, was also achieved (Fig. S1).

3.2. Preparation and characterisation of the polymer–enzyme multilayer modified electrode

The LbL technique was applied to fabricate enzyme electrode with both ACOD and ACS immobilized in PDA polymer. Electrochemical impedance spectroscopy (EIS) was used to monitor the changes in charge transfer resistance ($R_C$) during the assembly of the multilayer films of (PDA–MWCNT/ACOD) or (PDA–MWCNT/ACOD/PDA–MWCNT/ACS) during the modification process. The selection of 0.17 V is due to it being near the equilibrium of reductive and oxidative rates of the $K_d\{Fe(CN)_6\}^2-$ and the redox species would not deplete near the electrode surface during the impedance measurements. Fig. 3 shows the Nyquist plots of electrode modified with various layers of (PDA–MWCNT/ACOD). The diameter of the respective semicircular element corresponds to $R_C$, which was at 1000 $\Omega$ at a bare C-SPE before surface modification; The value increased to 3400 $\Omega$ after one layer of (PDA/MWCNT/ACOD) was deposited on the electrode and it increased further to around 5000 $\Omega$ after two layers. $R_C$ was increased as the number of layers of polymer–enzyme layer increased. This was also observed with the fabrication of bienzyme–polymer layers (PDA–MWCNT/ACOD/PDA–MWCNT/ACS) (data not shown). The charge transfer resistance controls the redox probes electron transfer kinetic process at the electrode interface [38], and $R_C$ depends on the dielectric and insulating features at the electrode/electrolyte interface [39]. The increase of $R_C$ due to the polymer and enzyme deposition confirmed the step-wise assembly of the multilayer films.

3.3. PACoA determination on (PDA–MWCNT/ACOD)${_2}$ multilayer modified C-SPE

Fig. 4 shows the linear sweep voltammograms at the C-SPE electrode modified with (PDA–MWCNT/ACOD)${_2}$ film with different concentrations of PACoA (up to 1.2 mM). The current increased with increasing concentrations of PACoA. A calibration curve obtained at 500 $\Omega$ exhibited a linear correlation ($R^2 = 0.99$) between the PACoA concentration and the oxidation current, which is from the H$_2$O$_2$ produced by PACoA oxidation by ACOD and oxygen. This is in agreement with the solution study in Section 3.1. It indicates that the activity of ACOD was retained within the PDA–MWCNT multilayer, and the enzyme electrode can be used to determine PACoA concentration. OACoA was also determined based on this enzyme electrode in the similar manner (Fig. S2).

3.4. NEFA determination on (PDACNT/ACOD/PDACNT/ACS) modified C-SPE

After the confirmation of PDA–MWCNT–ACOD multilayers functioned for determining PACoA concentrations, enzyme electrodes containing both ACOD and ACS enzymes were fabricated for determination of oleic acid concentrations.

Fig. 5a demonstrated the LSVs obtained from various OA concentrations on C-SPE modified with (PDA–MWCNT/ACOD/PDA–MWCNT/ACS)${_1}$. The calibration of OA (Fig. 5b) obtained at 500 $\Omega$ showed a good linear correlation between the OA concentration and the oxidation current, indicating a promising method for determining NEFA with one operating step. The peak around 370 mV is due to the ACS used from the commercial Wako NEFA determination kit.

To examine the effect of number of polymer–enzyme layers on current response, OA spiking tests on one bienzyme layer of (PDA–MWCNT/ACOD/PDA–MWCNT/ACS)${_1}$ and four bienzyme layers of (PDA–MWCNT/ACOD/PDA–MWCNT/ACS)${_4}$ have been carried out. The result showed that the LbL method produced fine thickness of enzyme–polymer layers. And these layers did not prevent the
mass transport of reactants cascading down the layers to fulfill two-step enzymatic reactions in sequence (Fig. S3). The electrode with four bienzyme layers showed the higher current response than one bienzyme layer electrode both before and after adding OA. This is due to more charges from more number of polymer–enzyme layers, and the increased amount of enzymes present. One bienzyme layer of (PDA–MWCNT/ACOD)/PDA–MWCNT/ACS) is generally adopted as the multilayer assembly condition for NEFA detection in the current study unless otherwise stated, which is the optimal condition for a highly reproducible and rapid enzyme electrode fabrication as well as for effective NEFA detection.

3.5 Validation of the electrochemical detection of NEFA against the colorimetric method

By our electrochemical method and the colorimetric method, respectively, 0.3 mM and 0.6 mM as two known concentrations of OA were measured independently. The currents produced from these two concentrations on the modified electrode show a good fitting in the previously obtained calibration curve by electrochemical method (Fig. 6a) (the error is 2.7% and 1.1%, respectively). This is consistent with the reading obtained by the colorimetric method using the commercial NEFA determination kit from Wako (Fig. 6b) (the error is 5.9% and 1.0%, respectively). The results confirm that reported electrochemical method using multienzyme electrode is highly reliable and reproducible for NEFA determination.

Further work is on going to investigate the effect of interferences and test with serum samples.

3.6 Electrochemical detection of NEFA from human plasma

The enzyme electrode was challenged with human plasma samples for NEFA detection in real application conditions. Samples from one patient has been chosen for the study, and the NEFA concentrations were firstly analysed by the Roche kit, and they were analysed electrochemically using the enzyme electrode. As it is shown in Fig. 7, a linear relationship between the sample concentration and the current (E = 500 mV) is established from the linear sweep voltammograms. The characterisation by chronomperometry showed very consistent results (Fig. S4). The current range obtained from the plasma sample is higher than that from the OA standard measurement (Fig. 5b). This is expected as there

![Image](image-url)
are multiple interferences present in human plasma, which vary in different patients and also with time. Due to different patient profiles, a calibration matrix needs to be established for improved NEFA determination by the enzyme electrode in real applications.

However, it can be expected that the polymer–enzyme layers assembled on the electrode surface can already provide some basic prevention of non-specific binding of other biomolecules (the interferents) compared to bare electrode. Also, one well-known advantage of the LbL technique is the capability of incorporating different polymers for multifunctionalities in the film. Different polymers such as bovine serum albumin, poly(methacrylic acid) and poly(sodium styrene sulfonate) are being investigated as additional coating layers on the present enzyme films, aiming to provide enhanced stability and reduced interferences.

4. Conclusions

An bi-enzyme electrode was fabricated using layer-by-layer configuration containing multilayer of PDA–MWCNT/enzyme composite films for the electrochemical detection of NEFA. The concentration of NEFA was determined by measuring the \( \text{H}_2\text{O}_2 \) oxidation current. As a product of acyl-CoA oxidation for the Beta oxidation route for NEFA, \( \text{H}_2\text{O}_2 \) amount is proportional to the amount of the substrate. The enzymes ACS and ACOD remained good biological activity in the film, and the enzyme electrode exhibited good electrocatalytic activity for NEFA oxidation steps. The fine polymer–enzyme layers produced by LbL method allowed mass transport from the reactant cascading down the layers to accomplish the two-step enzyme reactions. Good linear relationship between NEFA concentrations and the oxidation indicate the promising technique for one-step determination of NEFA for diabetes management. Establishing a calibration matrix proves to be necessary in order to use the bi-enzyme electrode for NEFA determination in real clinical applications. This work provides the technology for a multiplex sensor platform measuring various metabolic biomarkers simultaneously.

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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.snb.2013.09.011.

References

Biographies

Dr Jing Kang obtained her PhD degree in physical chemistry from Free University of Berlin, Germany in 2011 on application of layer-by-layer technique in DNA sensing systems. She joined school of chemical engineering and advanced materials in Newcastle University as a research associate in 2011. Her research interests are bioconjugation systems, self-assembled multilayer thin films, optical and electro-chemical biosensor systems.

Anisah T. Hussain obtained a first class B.A Honours in chemical and pharmaceutical science from the University of Sunderland in 2010. She is currently a PhD student under the supervision of Dr. Eileen Yu, at the University of Newcastle in the chemical engineering and advanced materials department.

Professor Michael Catt (MCatt) is a professor of practice in translation age research at Institute for Ageing & Health (IAH), Newcastle University. He has over twenty years experience in leading technology R&D programmes. He was programme director for the Unilever corporate research ‘Healthy Ageing’ programme from 2005 and had formerly been head of new technology at Unilever’s consumer and clinical in vitro diagnostics company, Unipath. He contributed core IPR and managed the ‘persona’ and ‘clearplan’ instrument development through to successful production and subsequent market support. His current interests focus on the development of in-vitro diagnostics and non-invasive lifestyle measurement for improved lifestyle management.

Professor Michael Trellis (MT) obtained his PhD from University of Sydney, Australia, joined Newcastle University in 2006. He is a clinical physiologist specialised in characterising metabolic changes accompanying disease to observe physiological responses to increased physical activity in health and disease. A significant focus of his research is on how metabolism can be improved in a number of different metabolic disorders (Type 2 diabetes and non-alcoholic fatty liver disease), neuromuscular disease, ageing and in promoting lifelong health and wellbeing.

Dr Barry Haggett is the Head of the Sensor Research Group, Institute of Research in the Applied Natural Sciences (IRANS), University of Bedfordshire. He is a physico-chemist by training and specialises in the development of chemical and biochemical sensing systems. His group works on a range of chemical, biological and physico-chemical sensor devices together with the necessary instrumentation. A core expertise is in the development of disposable devices for industrial applications in process control and environmental monitoring.

Dr Eileen Hao Yu is a lecturer in the School of Chemical Engineering and Advanced Materials at Newcastle University. She obtained her PhD in December 2003 on development of direct methanol alkaline fuel cells. After finishing her PhD, she became a research fellow at the Max Planck Institute for the dynamics of complex technical systems in 2005. She obtained a prestigious EPSRC research fellowship (life science interface) and returned to Newcastle University in 2006. Her research interests include electrocatalysis, electrochemical and bio-materials, and novel bioelectrochemical systems including biological fuel cells and biosensors.