Development of Mevalonic Acid Biosensor Using Amperometric Technique Based on Nanocomposite of Nicotinamide Adenine Dinucleotide and Carbon Nanotubes

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This study reports the development of an amperometric biosensor for quantification of mevalonic acid (MA), which is the first intermediate of HMG-CoA reductase in the isoprenoids biosynthesis pathway and therefore a useful indicator of HMGR activity. This method offers important advantages over previous reports because no radiolabeled substrates or expensive techniques are required, and time of analysis is relatively short. Self-assembled NAD+ onto multiwall-carbon nanotubes (MWCNTs) was synthesized for a biosensing system. Adsorption of NAD+ on MCWNTs was characterized by X-ray photoelectron spectroscopy (XPS) technique. This biosensor was constructed by modifying a screen-printed carbon electrode (SPCE) with NAD+/MWCNTs nanocomposite. The electrochemical and electrocatalytic behaviors of the modified electrode were studied using amperometry and cyclic voltammetry (CV). The resulting biosensor demonstrated great electrocatalytic activity, good stability and fast response to MA. At the NAD+/MWCNTs-modified SPCE, the catalytic currents are linearly proportional to the concentrations of MA in the wide range from 10 μM to 140 μM with a limit of detection down to 5 nM (S/N = 3), and the biosensor exhibited a sensitivity of 18.3 μA/μM. We measured the interference effect on the MA analysis and the results demonstrated its imperviousness to the effects of haemoglobin, bilirubin and serum albumin.

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Measurement of mevalonic acid (MA) is of great interest in health monitoring as it is the first intermediate of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) and appears to be a good indicator of HMGR activity. HMGR plays a crucial role in regulating sterol biosynthesis. Its activity is shown as the rate-limiting step in the mevalonate pathway in the isoprenoid metabolism pathway1,2 and thus is a prime target for the drug development. Biofluid levels of MA can be used as a biomarker for many diseases. For example, the reduction of plasma concentrations and urinary excretion of MA levels is an indirect measure of decreased cholesterol levels. For treatment of hypercholesterolemia, the statin class of drugs are used to block HMGR activity resulting in inhibition of MA synthesis.3,4 Moreover, cholesterol biosynthesis deficiencies cover a heterogeneous group of disorders. Mevalonate kinase is an enzyme located proximally in the pathway of cholesterol and nonsterol isoprene biosynthesis. Its allelic defects can cause hyperimmunoglobulinemia D syndrome (HIDS) and Mevalonic aciduria (MVA). The diagnosis of these diseases can be performed by determining MA concentration in urine followed by enzyme activity determination or mevalonate-5-phosphate (MVAP) detection by isotope dilution UPLC-MS/MS.5 Interestingly, monitoring MA levels in biofluids allows a better understanding of the drug pharmacodynamics, especially with respect to response to statin therapy due to any interindividual variations, and may allow for improvements in patient management and treatment regimes.6 Furthermore, inhibition of HMGR may be useful for treatment of many diseases other than hypercholesterolemia.7–12 For example, elevated levels of HMGR activity was reported in rapidly proliferating human cancer cells13 indicating that tumor malignancy may be regulated by this enzyme. MA is not only an intermediate in the biosynthesis of sterol,12 but also isoprenoids,13–16 which constitute a large class of secondary metabolites that are important in industries, for instance, steroids, anti-oxidants, flavors, fragrances, and pharmaceuticals including the anti-marial artemisinin.17 Therefore, detection of MA level is significant since it is involved with many applications.18

A number of methods for measuring serum, plasma, or urinary MA have been reported. These methods involve enzymatic method,19 enzyme immunoassay,20 radioimmunoassay,21,22 GC-MS methods23–25 and LC-MS/MS assay.26–31 However, their disadvantage is due to the expense of equipment, extensive sample preparation, time consuming, complications as well as radioactive materials. Hence, there is a need for developing a new method, which should be rapid, sensitive, specific, cost effective and preferable at the point of sample collection. From our preliminary report,12 we demonstrated the cyclic voltammetry behavior of modified electrode which successfully showed characteristic of an efficient catalysis to MA. For this work, we aim to further develop a NAD+/MWCNTs–based amperometric biosensor through noncovalent attachment of NAD+/MWCNTs nanocomposite on a screen-printed carbon electrode (SPCE) to create innovative disposable biosensor, which is more reliable than the conventional techniques to measure MA concentration. Carbon nanotubes (CNTs) are useful as immobilization substances as they offer high surface area, superior electrical conductivity remarkable mechanical strength, and good chemical stability.32 New types of sensor and biosensors based on nanotubes have been developed by immobilizing molecules and biomolecules on CNTs. A few papers reported the preparation of NAD+ and CNTs composite modified electrode for biosensing32–36 and biofuel cell applications.37,38 To address these issues, electrochemical measurements are utilized to illustrate significant functions provided by nanocomposite, electrochemical reaction catalysis, and the electron transfer enhancement between SPCE surfaces and nanocomposite. The main challenge in developing a sensor for determining MA was the use of NAD+/MWCNTs nanocomposite modified screen-printed carbon electrode since NAD+ acts as a cofactor in the reaction of MA with CoA which is then converted to HMG-CoA (see Scheme 1).

Materials and Methods

Reagents and materials.—β-Nicotinamide adenine dinucleotide ion (NAD+), mevalonic acid (MA) and coenzyme A (CoA) were purchased from Sigma Aldrich (USA). Multi-wall carbon nanotubes

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Scheme 1. Conversion of MA to HMG-CoA by HMG-CoA reductase.\textsuperscript{19} HMGR converts MA and CoA to HMG-CoA with NAD\textsuperscript{+} as a cofactor. HMGR also converts HMG-CoA to MA with NADH as a cofactor.

(MWCNTs) (purity > 95%, diameter 20–40 nm, length 10–30 μm) were obtained from Cheap Tubes Inc Co. Ltd. (USA). Membrane filters (mixed cellulose esters, hydrophilic, pore size 0.45 μm, diameter 47 mm) was purchased from Millipore. 0.1 M phosphate buffer saline (PBS) pH 7.0 was prepared with 0.1 M Na₂HPO₄ and 0.1 M NaH₂PO₄ containing additional 0.1 M KCl. All solutions were prepared with deionized water (DI). MA and CoA stock solutions were prepared in 0.1 M PBS pH 7.0 and stored at 4°C.

Apparatus.—X-ray photoelectron spectroscopy (XPS, PHI VersaProbe II) was carried out at the SUT-NANOTEC-SLRI Joint Facility, BL5.2: SUT-NANOTEC-SLRI, Synchrotron Light Research Institute. The excitation energy was 1486 eV using an Al K\textsubscript{α} source. The diameter of the irradiated area was 100 micron.

All electrochemical experiments were carried out with \( \mu \)-Autolab modular electrochemical system (Eco Chemie Ultecht, The Netherlands). This instrument was operated using the GPES program (Eco Chemie). The electrochemical experiments were performed with a SPCE from Quasense. The SPCE consisted of a carbon working electrode and a reference electrode (an Ag/AgCl ink was used for screen-printed reference electrode). The three-electrode system was employed with a bare and modified carbon working electrode (GC) as working electrode (3 mm x 5 mm), an Ag/AgCl (saturated Sodium chloride) as reference electrode and counter electrode. A 0.1 M phosphate buffer was used as a supporting electrolyte. All solutions were deoxygenated by pure nitrogen for at least 15 min. The system can perform various electrochemical measurements including cyclic voltammetry and chronoamperometry.

Preparation of the NAD\textsuperscript{+}/MWCNTs nanocomposite modified electrode.—Firstly, 10 mg NAD\textsuperscript{+} and 2 mg MWCNTs were dispersed in 10 mL DI water. Then, NAD\textsuperscript{+} was attached to the surface of MWCNTs by sonicating for 2 hours at room temperature. The mixture solution was then filtered with the membrane filter and washed with DI water several times to remove non-absorbed NAD\textsuperscript{+}. After that, the NAD\textsuperscript{+}/MWCNTs nanocomposite was redispersed in 250 μL DI water and kept at 4°C. The NAD\textsuperscript{+}/MWCNTs nanocomposite modified SPCE was fabricated by dropping 5 μL NAD\textsuperscript{+}/MWCNTs nanocomposite on the surface of SPCE. The modified electrode was dried for 8 hours and stored at 4°C until use.

HMG-CoA reductase activity.—HMG-CoA reductase specific activities at various HMG-CoA concentrations were measured using HMG-CoA Reductase Kit CS1090 (Sigma-Aldrich, St. Louis, MO) with some modifications from the protocol provided by the manufacturer. The concentration of the purified human enzyme stock solution (Sigma) was 0.50–0.70 mg protein/mL. To measure HMG-CoA reductase specific activity under defined assay conditions, the assay was performed in a 96-well plate in a final volume of 0.2 mL with HMG-CoA substrate ranging from 0.2–10 μM, NAD(P)H was used at a constant final concentration of 40 μM. The reactions were initiated (time 0) by the addition of 2 μL of the catalytic domain of human recombinant HMG-CoA reductase. The rates of NAD(P)H consumed were monitored at room temperature every 20 sec for up to 30 mins by scanning spectrophotometrically the decrease in absorbance at 340 nm, using a Tecan Infinite M200 plate reader. Results were expressed as specific activity of the enzyme (μmol of MA produced/min/mg protein).

Results and Discussion

Adsorption of NAD\textsuperscript{+} on MWCNTs.—To investigate NAD\textsuperscript{+}/MWCNTs adsorption, the XPS measurement was focused on the nitrogen and phosphorus which represented the adenine, nicotinamide and phosphate groups in NAD\textsuperscript{+}. XPS is a surface sensitive technique which allows the quantifying of elemental composition near the surface region. Thus, this technique is usually employed to identify oxidation state and type of the element in the interested materials. The P 2p and N 1s XPS spectra of the NAD\textsuperscript{+}/MWCNTs nanocomposite with curve fittings are shown in Figure 1.

Fine scan for N 1s displayed a broad and asymmetric feature due to contributions from different nitrogen species (Figure 1A). Peak fitting analysis exhibited two binding energies at 398.1 and 401.2 eV, which could be ascribed to the C-N and O=C-N bonds, respectively. The N 1s spectra of the NAD\textsuperscript{+}/MWCNTs nanocomposite with curve fittings are shown in Figure 1B. The N 1s spectra were monitored at room temperature every 20 sec for up to 30 mins by scanning spectrophotometrically the decrease in absorbance at 340 nm, using a Tecan Infinite M200 plate reader. Results were expressed as specific activity of the enzyme (μmol of MA produced/min/mg protein).
to be 80.9/19.1 which was consistent with NAD\(^+\) molecular structure. The measurement of P 2p was further performed to confirm the NAD\(^+\)/MWCNTs adsorption. The single type of phosphorous at the binding energies of 134.0 eV (2p3/2) and 134.8 eV (2p1/2) was consistent with phosphorus of the phosphate (Figure 1B).

**Electrochemical properties of NAD\(^+\)/MWCNTs modified SPCE.**—The electrochemical behaviors of NAD\(^+\)/MWCNTs nanocomposite were investigated using cyclic voltammetry. Figure 2 shows the cyclic voltammograms (CVs) of different modified SPCE in 0.1 M PBS pH 7.0 at a scan rate of 50 mV/s. The results show that no peaks appeared at bare SPCE (curve a) and MWCNTs-SPCE (curve b) in potential range between −0.4 to 0.4 V vs. Ag/AgCl. Comparing with the bare SPCE, the effect of the MWCNTs-SPCE exhibited higher capacitive background current, which can be attributed to the properties of carbon nanotubes which increases the conductivity and surface area of the modified electrode. Obviously, immobilized NAD\(^+\) with MWCNTs (curve c), a pair of redox peaks was clearly observed at 0.122 V vs. Ag/AgCl and −0.092 V vs. Ag/AgCl corresponding to the oxidation and reduction peak, respectively. This result indicated a surface-confined electron transfer of NAD\(^+\) adsorbed onto MWCNTs. The surface concentration (\(N_{ads}\)) of NAD\(^+\) on MWCNTs modified electrode can be determined based on the Laviron’s equation \(^1\) as Equation 1:

\[
\frac{1}{I_p} = \frac{n^2 F^2 v A T}{4 R T} = \frac{n F Q_v}{4 R T}
\]

where \(I_p\) is the peak current, \(v\) is the scan rate, \(R\) is the gas constant, \(T\) is the temperature, \(Q\) (9.36 × 10\(^{-5}\) C) is the charge obtained by integrating the cathodic peak at low voltage scan rate (10 mV/s), \(A\) (0.045 cm\(^2\)) is the area of the working electrode, \(n\) is the number of electron transfer, \(F\) is the Faraday’s constant, which indicated that high amount of NAD\(^+\) was loaded on MWCNTs.

The heterogeneous electron transfer rate constant (\(k_s\)) and the charge-transfer coefficient (\(\alpha\)) could be calculated from the peak-to-peak separation (\(\Delta E_p\)) according to the Laviron’s equations for diffusionless thin-layer voltammetry derived by Laviron\(^1\) which are as follows:

\[
E_{pc} = E^0 + \frac{RT}{n F} \ln v
\]

\[
E_{pa} = E^0' + \frac{RT}{(1 - \alpha) n F} \ln v
\]

\[
\log k_s = \alpha \log (1 - \alpha) + (1 - \alpha) \log \alpha - \frac{RT}{n F V} = \frac{\alpha (1 - \alpha) n F \Delta E_p}{2.3 R T}
\]

The plot of the \(E_{pc}\) and \(E_{pa}\) versus logarithm of the scan rates yielded two straight lines with the slopes equal to 2.3RT/\((1 - \alpha)nF\) and \(-2.3RT/nF\) for the cathodic and anodic peaks, respectively. From the slopes and using Equation 4, the values of \(\alpha\) and \(k_s\) were estimated to be 0.5 and 2.05 s\(^{-1}\), respectively. It was suggested that this nanocomposite may facilitate the electron transfer reaction as MWCNTs promote electron transfer between NAD\(^+\) and electrode surface.

**Electrocatalytic behavior of NAD\(^+\)/MWCNTs-SPCE to MA.—** Cyclic voltammetry was used to investigate the electrocatalytic responses of NAD\(^+\)/MWCNTs-SPCE in the absence and presence of MA and CoA in 0.1 M PBS pH 7.0 at a scan rate of 20 mV/s. Figure 3A shows the CVs response to none, MA, CoA and MA + CoA obtained from the bare SPCE (curve a, b, c and d, respectively) and MWCNTs-SPCE (curve e, f, g and h, respectively). Unsurprisingly, there was no electrocatalytic activity observed after addition of MA (curve b and f), CoA (curve c and g) and MA + CoA (curve d and h) to the bare and MWCNTs-SPCE.

Figure 3B shows the CVs of NAD\(^+\)/MWCNTs-SPCE in the absence (curve i), the presence of MA (curve j), of CoA (curve k) and of MA + CoA at different concentrations (curve l, m and n). A typical pair of redox peak of NAD\(^+\)/MWCNTs-SPCE (curve i) was obtained in the absence of MA and CoA. When only MA (curve j) or CoA (curve k) was presented, no catalytic current response to MA was observed and the redox peaks showed almost the same intensity. After addition of 20 μM (curve l), 50 μM (curve m) and 145 μM (curve n) of MA to the solution containing 150 μM CoA, the significant oxidation peak currents increased proportional with the increasing concentration of MA while the reduction peak current decreased, demon-strating the electrocatalytic activity of nanocomposite toward MA concentration.
As mentioned, NAD$^+$ can be reduced to NADH using CoA as cofactor. This catalytic reaction cannot occur without NAD$^+$. However, the oxidation peak potential shifted positively may be due to oxidation product from a two-step reaction of enzyme cycling which occurred at an electrode surface.

In the first reaction (Equation 5a), the reduction of NAD$^+$ to NADH help converting MA to mevaldehyde as an intermediate. Then, mevaldehyde reacts with CoA to form mevaldyl CoA in the equation 5b. In the second reaction, mevaldyl CoA reacts with NAD$^+$ and, consequently, HMG-CoA and NADH are produced, respectively (Eq. 6). The process is illustrated as follows:

$$\text{MA} + \text{NAD}^+ \rightarrow \text{Mevaldehyde} + \text{NADH} \quad [5a]$$

$$\text{Mevaldehyde} + \text{CoA} \rightarrow \text{Mevaldyl CoA} \quad [5b]$$

$$\text{Mevaldyl CoA} + \text{NAD}^+ \rightarrow \text{HMG} - \text{CoA} + \text{NADH} \quad [6]$$

**Optimized experimental conditions.**—Effect of applied potential.—The applied potential was investigated in order to determine an optimal operational condition for MA measurement. Plot of chronoamperometric current versus working potential of NAD$^+$/MWCNTs-SPCE to 50 nM MA in 0.1 M PBS pH 7.0 containing 150 μM of CoA (% RSD ≤ 6.99, n = 3) is shown in Figure 4A. Steady-state currents changed with increasing applied potentials from +0.1 to +0.3 V vs. Ag/AgCl. However, further increase of applied potentials from +0.3 to +0.6 V vs. Ag/AgCl led to reduction of steady-state currents due to the decrease in driving force of the oxidation of NADH at higher potentials. Hence, the potential of +0.3 V vs. Ag/AgCl was selected as the optimized monitoring potential due to its good sensitivity, also avoiding enzyme inactivation.

**Effect of CoA concentration.**—The amperometric current responses of the sensor depended on CoA concentrations because it is used as a mediating cofactor for MA detection. The cofactor might play an important role as an acceptor of electrons generated and transformed to NAD$^+$. Therefore, the influence of CoA concentrations on the NAD$^+$/MWCNTs-SPCE to MA was evaluated. The amperometric responses for various concentrations of CoA ranging from 37.5 to 300 μM were investigated when 50 nM MA was used and the results are shown in Figure 4B.

With increasing CoA concentrations, the amperometric responses of the sensor increased from 37.5 μM to 150 μM. Higher CoA concentrations gave rise to a decrease of the measured current that can be attributed to the inhibitory effect produced by high cofactor concentrations. Hence, the CoA concentration at 150 μM was chosen as the optimal cofactor concentration in this experiment.

**Amperometric response of MA detection.**—The analytical performance of NAD$^+$/MWCNTs-SPCE and the determination of MA was carried out with amperometric method. The current response of the sensor was investigated under the optimal experimental conditions with injections of MA to the PBS containing 150 μM CoA at the applied potential of +0.3 V vs. Ag/AgCl (Figure 5).
After additions of 35 μL of freshly prepared MA at different concentrations (0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 200 nM) to the solution, the oxidation current response of the sensor increased proportionally to MA concentration. This could be attributed to NAD⁺ on MWCNTs and coenzyme A as a cofactor. The results showed that current response increased with increasing MA concentrations from 10 to 200 nM (%RSD = 4.18, n = 3). Calibration curve of the sensor is shown as inset in Figure 5. The response to MA displayed a good linear relationship in a range from \(10 \times 10^{-9}\) M to \(14 \times 10^{-8}\) M, and the sensitivity of this sensor corresponding to the linear range was 18.3 μA/mM. The linear regression equation was \(y = 0.183x - 0.907\), with correction coefficient of 0.997, and the limit of detection (LOD) was 5 nM at a signal to noise ratio of 3.

The reaction mechanism of the sensor summarized as follows:

\[
\text{MA} + \text{CoA} + 2\text{NAD}^+ \rightarrow \text{HMG-CoA} + 2\text{NADH} \quad [7]
\]

\[
2\text{NADH} \rightarrow 2\text{NAD}^+ + 2\epsilon^- + 2\text{H}^+ \quad [8]
\]

MA reacts with NAD⁺ and CoA to form HMG-CoA and NADH. The NAD⁺ can then be recycled at the electrode leading to an increase in its oxidation current. The sensors showed excellent analytical performance, high stability, high sensitivity and high catalytic activity due to the properties of these nanocomposites which could promote the electron transfer to electrode surface as shown in Figure 6.

**Interferences study.**—Hemoglobin (Hb), bilirubin and serum albumin are coexisting compounds with MA in biological systems and could result in inaccurate determinations of MA. The interferences effect was studied by amperometry at the applied potential of +0.3 V vs. Ag/AgCl in 0.1 M PBS pH 7.0. Figure 7A shows the current response of 50 nM MA compared with 100 μM hemoglobin, 100 μM bilirubin and 0.5 mM serum albumin to the NAD⁺/MWCNTs modified electrodes. In the presence of Hb, bilirubin and serum albumin, the current signal had no significant changes, indicating that the presence of Hb, bilirubin and serum albumin did not interfere with MA determination. These results confirmed that this sensor displayed good selectivity for the determination of MA.

**Reproducibility and stability analysis of the MA sensor.**—The reproducibility of the electrode was determined using ten electrodes made independently. After each determination, the sensor showed an acceptable reproducibility with the relative standard deviation (RSD) of 5.93% for the current response of 100 nM MA concentration indicating that the sensor had good reproducibility (Figure S3). The long-term stability was also investigated. The storage stability of the sensor was examined by checking relative response currents of 100 nM MA every week over the test period of 4 weeks. After 4-week
storage, the sensor showed retained activity of 76.97% of its original response as shown in Figure S4.

**Measuring of HMG specific activity at various HMG-CoA concentrations.**—The HMG assay kit from Sigma was used to determine HMG specific activity at HMG-CoA concentrations ranging from 0.2–100 μM. The kit is designed for the detection of HMG activity and based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NAD(P)H by the catalytic subunit of HMG in the presence of the substrate HMG-CoA.

Reaction scheme of HMG:

\[ \text{HMG-CoA} + 2\text{NAD}(P)H + 2\text{H}^+ \rightarrow \text{MA} + 2\text{NAD}(P)^+ + \text{CoA} \]

The specific activities of HMG at various substrate concentrations were calculated according to the manufacturer’s protocol (see supporting protocol 1).

HMG converts 1 mole of HMG-CoA using 2 moles of NAD(P)H into 1 mole of MA therefore MA quantification can be used to determine HMG specific activity. Figure 7B illustrates HMG specific activity at various substrate concentrations measured at room temperature. At 200 nM MA concentration, the specific activity was calculated to be 4.87 nmol MA/min/mgP, which means 1 mg of HMG can convert 4.87 nmol of HMG-CoA to 4.87 nmol of MA. This value is the lowest measurable specific activity obtained from this method. However, the detection range of our method is much lower, i.e. 10–120 nM, if our method is used to measure a sample containing unknown MA concentration and the \( I_o = 23.6 \mu \text{A} \) which falls in the plateau range (I reaches steady state), we can only conclude that the specific activity is approximately 4.87 nmol MA/min/mgP at MA concentrations range from 140–200 nM. This is because the kit is based on spectrophotometric detection thus cannot detect such small change in absorbance due to oxidation of NAD(P)H by the catalytic subunit of HMG at very low substrate HMG-CoA concentrations and resulting in such high error at low MA concentrations.

The HMG Assay Kit is an important tool for the basic research of cholesterol and other related metabolic pathways. The assay is typically used to screen for different inhibitors/activators of the purified catalytic subunit of the enzyme. The ability to measure MA concentrations at a lower range may be crucial to the development of new inhibitors/activators of the enzyme as it allows detection near the normal physiological range which are reported to be 1.0–11.2 ng/mL (6.7–75.6 nM) in plasma and 12.28 ± 2.54 ng/mL (82.9 ± 17.1 μM) in serum. However, the commercially available enzymatic assay is based on spectrophotometric detection thus is not suitable for detection of trace amounts of MA in biological samples.

**Conclusions**

This research is the first report of using amperometric method to measure the MA, which can represent the HMG activity. The disposable biosensor was developed and prepared based on noncovalent-linking of NAD+/MWCNTs nanocomposite coated on SPCE, with NAD+ acting as a cofactor. Importantly, the fabrication of this biosensor offers many advantages compared to other complicated immobilization procedures such as no requirements for specific reagents, very simple preparation and modification of electrode. This biosensor showed good electrochemical characteristics and performances with a specificity, sufficiently sensitive method for the detection and quantification of MA. Finally, as the detection range of our technique is also within the physiological range, it may be useful for not only clinical applications but also for improvements in treatment regimes and patient management.

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