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Mediator effect of luteolin on electrooxidation of NADH

Melike BİLGİ KAMAÇ1*, Gulshat. GYLYJOVA2

Abstract

The effects of luteolin (LU) as a new mediator on the electrooxidation of the reduced nicotinamide adenine dinucleotide (NADH) were investigated and developed a new disposable NADH sensor. Firstly, screen printed carbon electrodes (SPCE) modified with gold nanoparticles (AuNP), and then, LU was deposited on SPCE modified with AuNP (SPCE/AuNP) using cyclic voltammetry. Electrochemical behavior of LU on SPCE/AuNP was investigated and, the redox proses of LU on SPCE/AuNP was found to be adsorption controlled. The number of cycles was optimized for the electrochemical deposition of LU and determined to be 15 cycles. LU modified SPCE/AuNP (SPCE/AuNP/LU) was found to show the electrocatalytic effect on electrooxidation of NADH and acts as a mediator. Working potential and pH were optimized for electrochemical detection of NADH with the proposed SPCE/AuNP/LU sensor and found to be +0.225 V and 7.25, respectively. The proposed NADH sensor showed a well linear response from 15.90 to 925 μM with a detection limit of 4.57 μM and a sensitivity of 11.19 μAmM⁻¹. The repeatability of the NADH sensor was tested +0.225 V with 50 μM NADH solution. The relative standard deviation (RSD) was calculated as 3.68% (for n=10). The operational stability studies have shown that the initial amperometric response of sensor to NADH decreased by 62.1% at the 30th day. The analysis of NADH in artificial human serum samples was successfully performed with SPCE/AuNP/LU sensor.

Keywords: NADH, luteolin, gold nanoparticles, redox mediator, human blood serum

1. INTRODUCTION

The studies on the electrooxidation of the reduced nicotinamide adenine dinucleotide (NADH) have attracted great attention because it is a vital coenzyme found in the human body [1, 2]. NADH is involved in many metabolic reactions, such as stimulating brain functions, providing energy support for the immune system [3]. In enzyme
experiments, analysis of NADH is usually done [4, 5]. The analysis of NADH is also important as NAD\(^+\) coenzyme is used in dehydrogenase-based biosensors. The direct electrooxidation of NADH in bare electrodes (C, Au, Pt etc.) occurs at high overpotentials. Irreversible inactive forms of NADH are formed at high overpotential and accumulate on the electrode surface, causing electrode contamination, which results in background currents that cause interference in real samples [6, 7]. Modified electrodes with various mediators [8-11] are widely used to reduce the electooxidation potential of NADH and the interference effect.

Antioxidant that has quinone-hydroquinone (Q/QH\(_2\)) redox pairs in the structure are also used as mediators in the electrooxidation of NADH. A caffeic acid modified glassy carbon electrode (GCE) [12, 13] and screen-printed electrode modified with carbon black nanoparticle (SPCE/CB) [14] were used for determination of NADH. GCE modified with chlorogenic acid was used for electrooxidation of NADH [15]. Dilgin et al used pencil graphite electrode (PGE) modified with quercetin for electrocatalytic oxidation of NADH [16]. In recently work, SPCE modified with RA was used for amperometric determination of NADH [17]. Luteolin (LU), 3,4,5,7-tetrahydroxyflavone, is one of the most important flavonoid compounds that has an electroactive catechol group in the B ring [18, 19]. Therefore, the electrochemical behavior of LU has been widely studied and sensors have been prepared for its electrochemical determination [20-25]. The structure of the LU is given in Figure 1. In the literature, no studies have been reported that the use of modified electrode with LU used for electrooxidation of NADH. The aim of this work is to examine the mediation effects of LU for the electrooxidation of NADH and to develop a new NADH sensor.

Screen printed carbon electrodes (SPCEs) have been reported to be widely used in the development of sensors and biosensors as they are suitable for point-of-care and are disposable [26-31]. In this study, bare SPCEs were used and modified with gold nanoparticles and LU for the preparation of a new, simple, practical, and disposable NADH sensors. Moreover, the SPCE/AuNP/LU electrode was used for the accurate determination of NADH in artificial human serum sample.

2. MATERIALS AND METHODS

2.1. Materials

LU, AuNP solution (10 nm), NADH and artificial human blood serum sample were purchased from Sigma-Aldrich. KH\(_2\)PO\(_4\), K\(_2\)HPO\(_4\), KCl were obtained from Merck. All solutions were prepared with deionized water supplied by a Millipore Milli-Q Direct Q-3 device. Phosphate buffer solution (PBS) were prepared using K\(_2\)HPO\(_4\), KH\(_2\)PO\(_4\) and KCl. NADH and LU were prepared in 50 mM PBSs (in 0.1M KCl).

2.2. Instrumentation

Screen-printed carbon electrodes (SPCEs, DRP110) were purchased from DropSens, Spain. The reference electrode is silver, while the auxiliary and working electrode are carbon, and they are deposited on a ceramic support. All electrochemical experiments were carried out using DropSens, Bipotentiotstate/Galvanostate (µSTAT 400) with DropView software. The measurements of pH were applied with a Mettler
Toledo pH-meter. Amperometric measurements were performed under stirred conditions.

2.3. Preparation of SPCEs modified with LU and AuNP

Electrochemical pretreatment to SPCEs to before use was performed by applying 1.8 V potential for 300 seconds in PBS (pH 7.5) \[11, 31\]. The 10 μL of AuNPs solution was deposited onto the surface of SPCE and was left to dry at 20°C. Then LU was electropolymerized on modified electrode (SPCE/AuNP) by potential cycling at a scan rate of 50 mV s\(^{-1}\). The potential was cycled between -0.2 to +1.0 V for 15 cycles in 1 mM LU (in 50 mM pH 7.0 PBS) \[32\]. The electrode (SPCE/AuNP/LU) was rinsed in the PBS.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behavior of LU at SPCE/AuNP

Figure 1 shows cyclic voltammogram of 1 mM LU in PBS (pH 7.0) at SPCE/AuNP electrode. It is seen that the reversible redox couple of LU at 10 mV.s\(^{-1}\) are +0.204 (Epa) and +0.168 V (Epc). While Epa corresponded to the electrooxidation of the 3',4'-dihydroxy substituent on the ring-B, Epc is corresponding reduction peak of the formed 3',4'-diquinone \[20, 21, 25\]. Epa values of LU reported in the literature are given in Table 1. When the literature data are examined, it is understood that the Epa value of LU observed in our study was much lower. The reason for the Epa value of LU being observed at a lower potential in this study may be due to electrochemical pretreatment procedure applied to SPCEs \[17\]. This result is an advantage for using LU as a mediator in the electrochemical analysis of NADH.

![Figure 1 CV of SPCE/AuNP in 1 mM LU at 50 mM pH 7.0 PBS containing 0.1M KCl (E: from -0.1 V to +0.5V, scan rate: 10 mV s\(^{-1}\))](image)

Table 1

| Electrode formulation       | Epa (V) | References |
|-----------------------------|---------|------------|
| GCE                         | +0.410  | 20         |
| MWCNT/GCE                   | +0.450  | 21         |
| GCE                         | +0.336  | 22         |
| PDDA-G-CNTs/β-CD/GCE        | +0.400  | 23         |
| PDDA-RGO/GCE                | +0.400  | 24         |
| In\(_2\)O\(_3\)NPs/GCPE     | +0.459  | 25         |
| SPCE/AuNP                   | +0.204  | In this study |

The difference between the anodic and cathodic peak potentials at scan rate of 10 mV.s\(^{-1}\) was calculated as 36 mV. ΔEp value is close to 30 mV which is reaction mechanism of a reversible two-electron \[17, 20, 21, 25\]. Figure 2 (a) shows CVs of 1 mM LU at SPCE/AuNP electrode with different scanning rates. The CVs show that it is apparently a well-defined redox pair and, both anodic and cathodic peak currents are linear with the scan rate (Figure 2 (b)). This indicates that the redox proses of LU on SPCE/AuNP is adsorption controlled \[20, 21, 25\].
Electrochemical characterization of SPCE/AuNP/LU

In order to deposition of LU on SPCE/AuNP, the cyclic voltammetry was carried out between -0.2 to +1.0 V for 15 cycles in a solution containing 1 mM LU, 50 mM pH 7.0 PBS (0.1 M KCl). The cyclic voltammograms recorded by electropolymerization of LU on SPCE/AuNP show anodic and cathodic peaks indicating the typical Q/QH\textsubscript{2} redox couple (Figure 3). With the increase in the number of cycles, anodic and cathodic peak currents increased, while the relative increase rate in peak currents decreased.

Similar results were reported for the caffeic acid [12-14], chlorogenic acid [15] and rosmarinic acid [17].

To examine the effect of the number of cycles on the electrodeposition of the LU, cyclic voltammetry was applied to the SPCE/AuNP modified with LU in different cycle numbers (5, 10, 15 and 20 cycles). Figures 4 (a, b, c, d) show that the cyclic voltammograms at various scan rates in PBS (pH 7.0) for SPCE/AuNP modified with LU in different cycle numbers (5, 10, 15 and 20 cycles), respectively. The peak currents are linear with the scan rate (Figures 4 (e, f, g, h)). This indicate that the electrochemical proses is an adsorption-controlled and LU is deposited on AuNP modified SPCE.
Figure 4 CVs of SPCE/AuNP modified with LU (a) 5, (b) 10, (c) 15 and (d) 20 cycles in 50 mM PBS pH 7.0 (in 0.1M KCl) at various scan rates. The scan rate is 10 mV s$^{-1}$ for the innermost CV and increases to 25, 50, 75, 100, 125 mV s$^{-1}$ for outer; influence of the scan rate on the peak currents (e) 5, (f) 10, (g) 15 and (h) 20 cycles.

To determine the optimization of the number of cycles on the deposition of the LU at SPCE/AuNP, the average electroactive surface concentration of LU on SPCE/AuNP, $\Gamma$, was calculated using the following equation [33]:

$$I_p = \frac{[(n^2F^2A\nu)/(4RT)] \Gamma}{\Gamma}$$

(1)

where $I_p$ is the peak current (A), $n$ is the number of electrons transferred, $F$ is the Faraday constant (96485 C mol$^{-1}$), $\nu$ is the scan rate (V.s$^{-1}$), $A$ is the electroactive surface area of the electrode (cm$^2$), $R$ is the gas constant (8.314 J K$^{-1}$ mol$^{-1}$), $T$ is the temperature (293.15 K). To calculate the $A$ of the SPCE/AuNP/LU electrode, the cyclic voltammetry was applied to SPCE/AuNP/LU electrode in 1 mM K$_3$Fe(CN)$_6$ (in 0.1MKCl) at various scan rates (10 mV s$^{-1}$ to 125 mV s$^{-1}$). (Figure not shown). Using the Randles-Sevcik equation [34] and slope of I vs. $\nu^{1/2}$ plot (Figure not shown) the $A$ of the SPCE/AuNP/LU electrode was calculated to be 0.175 cm$^2$. The $\Gamma$ values of SPCE/AuNP modified with LU for 5, 10, 15 and 20 cycles, using values of $A$ and the equation (1), were calculated (1.22×10$^{-10}$, 1.50×10$^{-10}$, 1.78×10$^{-10}$ and 1.59×10$^{-10}$) mol cm$^{-2}$, respectively. The highest $\Gamma$ value was obtained with the SPCE/AuNP electrode modified with LU for 15 cycles. Therefore, it was decided that 15 cycles are optimum for the deposition of LU. Further studies have been carried out to confirm the determination of optimization of the number of cycles. For this purpose, amperometric determination of NADH with these modified electrodes was performed at +0.3 V. The current vs concentration graphs of the NADH analysis are given in Figure 5. Table 2 show the sensitivities and correlation coefficient values of graphs in Figure 5. As the number of cycles increased from 5 to 15, the slope of the current concentration graphs increased, while the slope decreased in the 20th cycle due to the increase in film thickness and, as a result, decreased diffusion. The highest sensitivity and correlation coefficients are observed at SPCE/AuNP electrode modified with LU for 15 cycles. Therefore, for further studies, SPCE/AuNP electrode were modified with LU for 15 cycles.
Table 2
Values of sensitivities and correlation coefficients of graphs in Figure 5

| Number of cycles | Sensitivity/ (μA.mM⁻¹) | Correlation coefficients |
|------------------|-------------------------|-------------------------|
| 5                | 2.61 ± 0.009            | 0.9947                  |
| 10               | 5.76 ± 0.011            | 0.9990                  |
| 15               | 6.80 ± 0.006            | 0.9997                  |
| 20               | 4.51 ± 0.013            | 0.9932                  |

The cyclic voltammogram of bare SPCE, SPCE/AuNP and SPCE/AuNP/LU electrodes in 5 mM K₃Fe(CN)₆/K₄Fe(CN)₉ (in 1 M KCl) are given in Figure 6. The anodic peaks currents (Ipa) and cathodic peak currents (Ipc) increased by modification of SPCE with AuNP and, then LU. The highest values of Ipa and Ipc are obtained in SPCE/AuNP/LU electrode.

3.3. Electro catalytic oxidation of NADH at SPCE/AuNP/LU

To see the electrocatalytic effect of the SPCE/AuNP/LU electrode toward electrooxidation of NADH, the CVs were performed in the absence and presence of 1 mM NADH in 50 mM pH 7.0 PBS containing 0.1 M KCl at 50 mV s⁻¹ (Figure 7 (a) and (b)). With the presence of 1 mM NADH at +0.212 V, the oxidation peak current increased remarkably, from 0.92 μA to 33.35 μA, while the cathodic peak current at +0.172 V slightly increased, from 0.76 μA to 5.46 μA. The Epa of NADH at the SPCE/AuNP/LU was observed at +0.212 V, while NADH was oxidized at +0.492 V and +0.326 V at the bare SPCE and SPCE/AuNP, respectively (Figure 7 (c) and (d)). According to these results, LU has an electrocatalytic effect on the oxidation of NADH and acts as a mediator [6-10]. LU was used for the first time in this study as a mediator in the electrooxidation of NADH.
Cyclic voltammograms of SPCE/AuNP/LU in the absence (a) and in the presence (b) of 1 mM NADH and the CVs of 1 mM NADH at bare SPCE (c) and SPCE/AuNP (d) (50 mM pH 7.0 PBS containing in 0.1 M KCl; scan rate: 50 mV s\(^{-1}\))

The increase of anodic peak current in the presence of NADH indicates that NADH is oxidized by diffusing from the solution to the electrode surface, whereas the LU on the surface is reduced. The reaction can take place as the following reactions:

\[
\text{LU}_{\text{red}} \rightarrow \text{LU}_{\text{ox}} + 2\text{H}^+ + 2\text{e}^- \quad (2)
\]

\[
\text{LU}_{\text{ox}} + \text{NADH} + \text{H}^+ \rightarrow \text{LU}_{\text{red}} + \text{NAD}^+ \quad (3)
\]

\[
\text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2\text{e}^- \quad (4)
\]

The increase of anodic peak current in the presence of NADH indicates that NADH is oxidized by diffusing from the solution to the electrode surface, whereas the LU on the surface is reduced. The reaction can take place as the following reactions:

\[
\text{LU}_{\text{red}} \rightarrow \text{LU}_{\text{ox}} + 2\text{H}^+ + 2\text{e}^- \quad (2)
\]

\[
\text{LU}_{\text{ox}} + \text{NADH} + \text{H}^+ \rightarrow \text{LU}_{\text{red}} + \text{NAD}^+ \quad (3)
\]

\[
\text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2\text{e}^- \quad (4)
\]

Figure 8 (a) shows the cyclic voltammograms recorded in 1 mM NADH at different scan rates (10 to 100 mV s\(^{-1}\)) on SPCE/AuNP/LU. The plot of the anodic peak currents obtained from these CVs against the square root of the scan rate is linear (Figure 8 (b)). This result show that electrochemical proses of the electrocatalytic oxidation of NADH at SPCE/AuNP/LU is a diffusion controlled. Using the slope of Figure 8 (b), the diffusion coefficient of 1mM NADH on SPCE/AuNP/LU was calculated to be 4.49 × 10\(^{-9}\) cm\(^2\) s\(^{-1}\) using Randless-Sevcik Equation [34]. A Plot of the anodic peak potential (Epa) and the natural logarithm of scan rate (lnv) for the electrocatalytic oxidation of 1 mM NADH at SPCE/AuNP/LU is given in Figure 8 (c). The electron transfer coefficient (α) for the electrocatalytic oxidation of NADH at SPCE/AuNP/LU electrode was calculated from the following equation [35]:

\[
\text{Ep} = \frac{(RT)}{(2\alpha F)} \ln \nu + \text{constant} \quad (5)
\]

where Ep is the anodic or cathodic peak potential. Using the slope of Figure 8 (c), the α was calculated to be 0.654 for 1mM NADH.

Figure 8 (a) Cyclic voltammogram of 1 mM NADH in PBS (pH 7.0) on SPCE/AuNP/LU at different scan rates from 10 to 100 mV s\(^{-1}\), (b) Plot of anodic peak currents vs square root of scan rate, (c) The variation of anodic peak potentials vs natural logarithm of scan rate.
3.4. Amperometric detection of NADH with SPCE/AuNP/LU

To obtain the best sensor performance of SPCE/AuNP/LU towards NADH, the experimental conditions which are working potential and pH were optimized. Firstly, the amperometric currents for NADH electrooxidation at a fixed concentration of 100 µM with different pH (6-8) were measured at +0.300 V. According to the current vs pH graph given in Figure 9, the highest current was observed at pH 7.25, thus the optimum pH was determined to be 7.25.

Figure 9 Current vs pH graph for the electrooxidation of 100 µM NADH at +0.300 V in PBSs of different pH

NADH analysis with SPCE/AuNP/LU were performed by amperometric detection in 50 mM phosphate buffer solution (pH 7.25) at various potentials (+0.225 V to +0.325 V) for the optimization study of the working potential. The current vs concentration graphs were obtained from the amperograms given in Figure 10. Table 3 show the sensitivities and correlation coefficient values of graphs in Figure 10. The optimum working potential is selected to be +0.225 V, due to the highest sensitivity and correlation coefficient are observed at this potential.

Table 3 Values of sensitivities and correlation coefficients of graphs in Figure 10

| Working potential/(V) | Sensitivity / (µA.mM⁻¹) | Correlation coefficients |
|-----------------------|--------------------------|-------------------------|
| +0.225                | 7.96 ± 0.005             | 0.9995                  |
| +0.250                | 3.85 ± 0.026             | 0.9988                  |
| +0.275                | 2.95 ± 0.018             | 0.9910                  |
| +0.300                | 6.92 ± 0.006             | 0.9984                  |
| +0.325                | 5.79 ± 0.023             | 0.9969                  |

Amperometric analysis of NADH was applied with SPCE/AuNP/LU sensor under stirring at +0.225 V in PBS (pH 7.25). After the stable current was reached, 25 µM NADH was injected successive to the electrochemical cell and the response of current was recorded by the potentiostat. The obtained amperogram for amperometric determination of NADH is given in Figure 11 (a). The measured current values with each injection of NADH against concentration values are plotted in Figure 11 (b). The sensitivity of the proposed NADH sensor in the range of 25 µM to 925 µM is found to be 11.19 µA.mM⁻¹. The limit of detection and quantification (3σ/m) were calculated to be 4.77 µA.mM⁻¹ and 15.90 µA.mM⁻¹, respectively. For the repeatability of the SPCE/AuNP/LU sensor, NADH analysis was performed at +0.225 V with 50 µM NADH.
solution. The relative standard deviation (RSD) was calculated as 3.68% (for n=10). The linear range was determined to be 25 μM - 925 μM. The comparison of the analytical performance of the proposed NADH sensor with the other recently reported NADH sensors is given in Table 4.

Table 4 Analytical performance comparison of the proposed SPCE/AuNP/LU with the other reported antioxidant mediated amperometric NADH sensors

| Modified electrode | LR/ (µM) | LOD/ (µM) | References |
|--------------------|----------|-----------|------------|
| GCE/CA             | 50–1000  | –         | 12         |
| SPCE/CB/CA         | –        | 1.6       | 14         |
| GCE/CGA            | 100–1000 | –         | 15         |
| PGE/QH₂            | 0.3–100  | 0.15      | 16         |
| SPCE/RA            | 18.7-220 | 5.62      | 17         |
| SPCE/AuNP/LU       | 25-925   | 4.77      | This work  |

CGA: chlorogenic acid, CA: caffeic acid, QH₂: quercetine, RA: rosmarinic acid, LU: luteolin, GCE: glassy carbon electrode, PGE: pencil graphite electrode, CB: carbon black nanoparticles, SPCE: screen-printed carbon electrode, AuNP: gold nanoparticles

To examine the operational stability of the NADH sensor, the currents of 50 µM NADH solution in PBS (pH 7.25) were measured at +0.225 V over a period of 30 days (n=3). The percent relative current values are compared as a measure of the response of SPCE/AuNP/LU sensor toward NADH for 30 days (Figure 12). The first day current response of the proposed sensor to NADH decreased by 1.50% on the 2nd day, by 13.8% on the 5th day, by 29.5% on the 10th day and by 62.1% on the 30th day. Although the operation stability of the proposed NADH sensor decreased by 62.1% on the 30th day compared to the first use, the response was received even on the 30th day with the disposable manufactured SPCEs. The presence of AuNP and natural antioxidant LU in the electrode formulation improved the operational stability of the NADH sensor.

Figure 11 (a) Amperometric current response of SPCE/AuNP/LU sensor to addition of 25 µM NADH in PBS (pH 7.25) at +0.225 V, (b) Calibration curve vs NADH concentration

Figure 12 %Relative current response vs time plot over 30 day for amperometric detection of NADH in PBS (pH 7.25) with SPCE/AuNP/LU sensor
3.5. Application of NADH sensor of human serum sample

The proposed NADH sensor based on SPCE/AuNP/LU is used for the analysis of NADH in artificial human serum samples. 100 µM NADH was spiked into the artificial human blood serum sample diluted 1:100 with pH 7.4 buffer solution and amperometric determination of NADH with SPCE/AuNP/LU. The experimentally calculated NADH concentration was determined to be 103.1 ± 0.18 and recovery was to be 103.1%.

4. CONCLUSION

The new disposable, simple and practical amperometric NADH sensor, easily prepared by electrodeposition of LU on the SPCE/AuNP electrode, was defined for the first time in this study. SPCE/AuNP/LU exhibits electrocatalytic effect on electrooxidation of NADH due to LU acts as a mediator. The electrooxidation potential of NADH was reduced by the effect of the LU mediator. Optimum working potential and pH of the NADH sensor were found to be +0.225 V and 7.25, respectively. The developed NADH sensor showed a good linear response in the range between 25 and 925 µM with a detection limit of 4.57 µM and sensitivity of 11.19 µAmM⁻¹. The relative standard deviation (RSD) was calculated as 3.68% (for n=10). The operational stability studies showed that the developed NADH sensor responds even on the 30th day although SPCE was produced for single use. This may be considered to be due to the presence of natural antioxidant LU and AuNP in the electrode formulation. Moreover, the developed SPCE/AuNP/LU sensor demonstrated very attractive features for the target NADH in human serum samples in terms of sensitivity (recovery was to be 103.1%). The NADH sensor mediated with LU presented a new methodology that had simplicity, disposable, practical, storage stability, and potential to perform the accurate determination of the target analyte in serum samples.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

This study is the undergraduate thesis of Gulshat GYLYJOVA, and the idea of the study belongs to supervisor that is Melike BİLGİ KAMAÇ. Melike BİLGİ KAMAÇ had a great contribution in providing support for the study, preparing and developing the NADH sensor, conducting the experiments, interpreting the findings and writing the undergraduate thesis. Gulshat GYLYJOVA contributed to the experimental studies.

The Declaration of Ethics Committee Approval

The authors declare that this document does not require an ethics committee approval or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the article and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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