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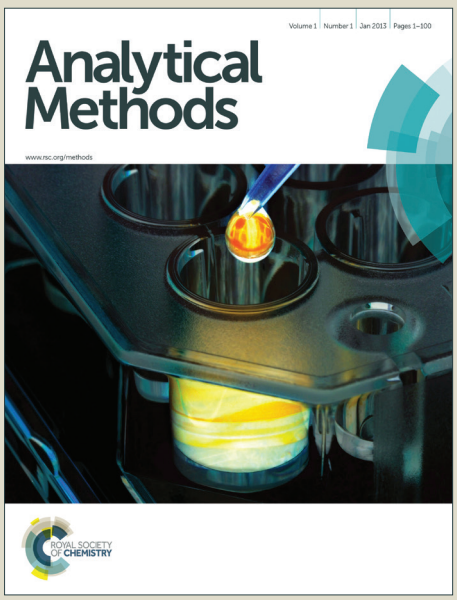

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Photoelectrochemical glucose biosensor in flow injection analysis system based on glucose dehydrogenase immobilized on poly-hematoxylin modified glassy carbon electrode

Didem Giray Dilgin[a,b*], H. İsmet Gökçel[b]

[a,b]Çanakkale Onsekiz Mart University, Biga Vocational School, Biga Çanakkale-Turkey

[b]Ege University, Science Faculty, Department of Chemistry, 35100 Bornova-İzmir, Turkey

Abstract

In this study, a photoelectrochemical glucose biosensor based on electropolymerized Hematoxylin film onto poly-amidoamine (PAMAM) dendrimers adsorbed glassy carbon electrode (Poly-HT/PAMAM/GCE) was presented. After the immobilization of glucose dehydrogenase (GDH) onto the poly-HT/PAMAM/GCE, photoelectrochemical biosensing of glucose was investigated using cyclic voltammetry and amperometry in flow injection analysis (FIA) system dependent on the NAD^+/NADH redox couple-dehydrogenase enzyme. The linear range was from $1x10^{-5}$ M to $1x10^{-3}$ M with the sensitivity of 0.76 μAmM^{-1} and detection limit of 3.0 μM without irradiation in FIA system. After the irradiation, the linear range was from $5x10^{-6}$ M to $1x10^{-3}$ M with the sensitivity of 1.90 μAmM^{-1} and a detection limit of 1.5 μM. When the results obtained from the irradiation of electrode surface compared with the reaction without irradiation, the sensitivity and the detection limit increased around 2.5 and 2.0 folds, respectively. The photoelectrochemical biosensor showed a good performance with high upper detection limit, acceptable repeatability and selectivity providing a rapid alternative method for monitoring biomolecules and extending the photoelectrochemical determination in FIA system. The proposed electrochemical and photoelectrochemical biosensor was succesfully applied to determination of glucose in real samples. It was indicated that the results obtained from this study will provide the basis for a further development for future studies in these directions.

Keywords: Photoelectrochemical biosensor, flow injection analysis, hematoxylin, NADH, glucose dehydrogenase.

***Corresponding Author** **E-mail:** didemgiray79@hotmail.com

1. Introduction

Chemically Modified Electrodes (CMEs) have attracted considerable attention in the development of new electrochemical sensors and biosensors, because CMEs offer advantages such as high sensitivity, selectivity, reduced interference effect, preconcentration of target species, etc.[1-3] An important application area of CMEs is investigation of electrocatalytic oxidation of Reduced β-Nicotinamide adenine dinucleotide (NADH) and construction of biosensors for some biologically important compounds that depend on NAD^+/NADH redox couple and dehydrogenase enzymes[4-7]. The biosensing of molecules using dehydrogenase enzymes requires a highly sensitive NADH transducer, because the transformed signal of the biosensor is based on the detection of enzymatically generated NADH.

However, direct electrochemical oxidation of NADH to NAD^+; at the bare electrode surface is highly irreversible and takes place at high overpotential (between 0.5 and 1 V dependent on electrode type) and is accompanied by rapid poisoning of the reaction because the electrode surface is fouled by adsorption of its oxidation products and intermediate radicals[4,8]. In order to overcome these problems, modification of the electrode surface with redox mediators has been extensively used. Various modified electrodes have been prepared with many redox mediators such as azine type dyes (phenothiazine, phenoxazine, phenazine), quinolic compounds (flavonoids, phenolic acids, catecholic compounds), nitrofluorenones, flavine and adenine derivatives, some transition metal complexes and some conducting polymers[4-6]. Modified electrodes prepared with various redox mediators have also been used for the construction of electrochemical biosensors for many biologically important molecules based on dehydrogenase enzymes[6,9-13].

Recently, many scientists have focused on photoelectrochemical sensors, which are a newly developed analytical device based on the photoelectrochemical properties of electrode or mediators. Because the combination of CMEs and photoelectrochemistry exhibits more sensitive results than that of CMEs without irradiation electrode surface. Poly-Toluidine Blue (poly-TB)[14], poly-Methylene Blue (poly-MB)[15], poly-Hematoxylin (Poly-HT)[16,] poly-Neutral Red (Poly-NR)[17] modified glassy carbon electrodes (GCE), a new polymeric phenothiazine modified graphite electrode[18], Graphene-TiO_2 nanohybrids modified GCE[19], Dopamine/nanoporous TiO_2 modified Indium Tin Oxide (ITO) electrode[20], and poly(4,4' diaminodiphenyl sulfone)/nanoTiO_2 composite film ITO electrode[21] have been used for the photoelectrocatalytic oxidation of NADH. In these studies, a significant enhancement in the current for the oxidation of NADH was observed when the working electrode surface was

irradiated by a light source. In addition, a photoelectrochemical glucose biosensor based on NAD^+/NADH redox couple and glucose dehydrogenase enzyme has been constructed by using the Quantum dot modified Au electrode[22] and Thionine (Th) cross-linked multi-walled carbon nanotubes (MWNTs) and Au nanoparticles (Au NPs) multilayer functionalized ITO electrode[23]. These studies showed that the sensitivity of a photoelectrochemical sensor was better than an electrochemical sensor.

Another useful approach in electrochemical sensors and biosensors is the usage of Flow Injection Analysis (FIA) with CMEs in electrochemical techniques[24,25]. Because FIA has some advantages for routine analytical determinations such as very limited sample consumption, short analysis time based on a transient signal measurement in a flow-through detector, and an on-line carrying out difficult operations of separation, chemical conversion of analyses into detectable species. Electrocatalytic oxidation of NADH and biosensors that depend on NAD^+/NADH redox couple-dehydrogenase enzymes has also been investigated in FIA with redox mediator-modified electrodes[15,16,26-30].

It is concluded that the combination of CMEs, photoelectrochemistry and FIA can be useful for electrocatalytic oxidation of NADH and biosensor dependent on NAD^+/NADH redox couple-dehydrogenase enzyme. When we take into consideration the advantages of this combination, the developed photoelectrochemical sensor and biosensor offers advantages such as i) good selectivity (CMEs have good selectivity properties), ii) good sensitivity (the sensitivity of photoelectrochemical sensors is generally better than electrochemical sensors), and iii) fast and economical analysis (FIA exhibits fast analysis and lower cost due to less consumption of reactive). Therefore, an investigation on photoelectrocatalytic oxidation of NADH and the construction of photoelectrochemical glucose biosensor dependent on NAD^+/NADH redox couple-glucose dehydrogenase enzyme in the FIA system have been proposed using glucose dehydrogenase immobilized on poly-Hematoxylin modified glassy carbon electrode in this study.

2. Results and Discussion

2.1. Electropolymerization of HT on PAMAM/GCE surface

Prior to enzyme immobilization, redox mediator HT, which shows a good electrocatalytic and also photoelectrocatalytic effect towards the oxidation of NADH[16] was electropolymerized onto PAMAM/GCE. Figure 1 shows the typical cyclic voltammograms of

the polymer film growth during the electropolymerization of 0.3 mM HT solution in 0.1 M phosphate buffer solution (PBS, pH 7.0) containing 0.1 M $NaNO_3$. $NaNO_3$ was used as 0.1 M in the supporting electrolyte during all the electropolymerization process due to the catalytic effect of the NO_3^- anions on the electropolymerization process[31]. In the first cycle of cyclic voltammograms of HT (Fig.1, black line), one oxidation peak was observed at about 385 mV with a shoulder at 285 mV, which is attributed to the oxidation of monomeric catechol groups to quinone The reduction peak was also formed more specifically at about -480 mV. In the second cycle, the oxidation peak current decreased. After the third cycle, the oxidation peak current began to grow slowly with increasing cycle numbers and peak potential was shifted to about 310 mV. Cathodic peak of HT at about -480 mV increased gradually with increasing scan cycles (Fig. 1). These results demonstrated that the polymeric film of HT could be formed on GCE. In our previous study[16], similar discussions and electropolymerization mechanism was reported for electropolymerization of HT on GCE. The electropolymerized HT film was also prepared on GCE at the same conditions (data not shown). However, it was observed that increase of monomeric peaks by increasing the scan cycle at PAMAM/GCE was found to be better than at GCE. This result shows that PAMAM dendrimer has a good surface for electropolymerization of HT.

Fig. 1

2.2. Cyclic voltammetric studies for photoelectrochemical biosensing of glucose

Electrocatalytic and photoelectrocatalytic oxidation of glucose at GDH based poly-HT modified GCEs were investigated using cyclic voltammetric techniques. Firstly, the electrochemical and photoelectrochemical response of dehydrogenase immobilized PAMAM modified GCE (GDH/PAMAM/GCE without poly-HT) to glucose was investigated by recording cyclic voltammograms. For this, cyclic voltammograms of GDH/PAMAM/GCE were recorded in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ in the absence and in the presence of 10 mM glucose at 5 mV s^{-1} scan rate, respectively. The obtained cyclic voltammograms are given in Figure 2A. In the first voltammograms (Fig. 2A/a), no peak was observed because the supporting electrolyte did not include any substrate (glucose). When the substrate was added to supporting electrolyte, a reversible peak at about 760 mV at GDH/PAMAM/GCE (Fig. 2A/b) was observed, which was attributed to the oxidation of enzymatically produced NADH to NAD^+ at PAMAM modified GCEs (Reactions 1 and 2).

When the electrode surface was irradiated with the light source, the peak current increased a little (Fig. 2A/c).

$$Glucose + NAD^{+} \xrightarrow{GDH} NADH + Glucanolactone \quad (1)$$

$$NADH \longrightarrow NAD^{+} + H^{+} + 2e^{-} \quad (2)$$

Fig. 2

After polymerization of HT, GDH was immobilized onto poly-HT/PAMAM/GCE. The electrochemical and photoelectrochemical response of GDH/Poly-HT/PAMAM/GCE to glucose was also investigated by cyclic voltammetric technique. The above mentioned (for GDH/PAMAM/GCE) cyclic voltammetric procedures for biosensing and photobiosensing of glucose were also repeated for this electrode. The obtained cyclic voltammograms are given in Figure 2B. In the first cyclic voltammogram, only an anodic peak at about 210 mV with a shoulder at 120 mV was observed in the presence of NAD^{+} (Fig. 2B/a). These peaks can be attributed to oxidation of polymeric form of HT. In the presence of glucose, the current of this peak increased, which is attributed to the electrocatalytic oxidation of enzymatically produced NADH to NAD^{+} (Fig. 2B/b). When the electrode surface was irradiated with the light source, a large enhancement in this peak current was observed (Fig. 2B/c). If it was compared the peak potential of glucose at GDH/PAMAM/GCE (about 760 mV, see Figure 2A/b) with that at the GDH/poly-HT/PAMAM/GCE (about 300 mV, Fig. 2B/b), the overpotential for the electrochemical oxidation of NADH produced by enzymatic reaction of glucose decreased by 460 mV at GDH/poly-HT/PAMAM/GCE. Moreover, the peak current at 300 mV (GDH/poly-HT/PAMAM/GCE) attributed to electrocatalytic oxidation of NADH increased with irradiation of electrode surface (Fig. 2B/c). The increase in the electrocatalytic peak current of NADH with light depends on excitation of poly-HT on the electrode surface and its excited form can more rapidly react with NADH. Thus, it can be concluded that irradiation of the surface of/GDH/ poly-HT/PAMAM/GCE causes a faster photoelectrochemical oxidation of NADH than that of electrochemical. The mechanism can be explained by reactions between 1 and 4. According to equation 4, the increasing oxidation current of poly HT(red) which was produced dependent on the concentration of enzymatically produced NADH was observed upon illumination. However, in the cyclic voltammograms of GDH/poly-HT/PAMAM/GCE in the presence of 10 mM NAD+ with irradiation of electrode surface, a small enhancement on peak current of HT was observed (see supplementary file, Figure S1), indicating that a big

enhencemnent on peak current in the presence of glucose is dependent on NADH (indirectly glucose) concentration.

$$NADH + poly\text{-}HT^{*}_{(Ox)} \longrightarrow NAD^{+} + poly - HT^{*}_{(Red)} \quad (3)$$

$$poly - HT_{(Red)} \longrightarrow poly\text{-}HT_{(Ox)} + 2e^{-} + 2H^{+} \quad (4)$$

These results showed that the photoelectrochemical biosensor dependent on NAD^{+}/NADH redox couple and dehydrogenase enzyme can be constructed for glucose detection. A similar result was also obtained for Thionine modified multiwalled carbon nanotube and gold nanoparticles functionalized indium tin oxide electrode[23]. However, in this cited study FIA system was not used. As distinct from this, the proposed method in present study also includes photoelectrochemical biosensor in FIA system.

2.3. Photoamperometric detection of glucose in FIA system

2.3.1. Optimization of the Experimental Parameters

After the investigation of electrochemical and photoelectrochemical biosensing of glucose using cyclic voltammetric technique, it was also investigated using amperometric techniques in FIA system. In order to obtain the best amperometric and photoamperometric response of GDH/Poly-HT/PAMAM/GCE towards glucose in FIA system, the effect of the applied potential and flow rate on the current of 0.8 mM glucose containing 10 mM NAD^{+} was investigated by recording fiagrams. Firstly, the applied potential was optimized under the conditions of 1.1 mL min^{-1} flow rate, 100 µL sample loop, 10 cm tubing length and 0.1 M PBS (pH 7.0) containing 0.1 M KCl as carrier stream. Then, the current-time curves were recorded at various applied potential. After a steady background current was obtained, 0.8 mM glucose containing 10 mM NAD^{+} was injected into the carrier stream. The amperometric and photoamperometric currents were measured from current-time curves obtained at various applied potential. The current-time curves of GDH/Poly-HT/PAMAM/GCE for 0.8 mM glucose containing 10 mM NAD^{+} obtained at various applied potential were shown in Figure S2/A (see supplementary file). In addition, Figure S2/B (see supplementary file) shows the plot of electrocatalytic and photoelectrocatalytic currents of glucose versus the applied potential. The best currents for electrocatalytic and photoelectrocatalytic oxidation of NADH produced by

enzymatic reaction of glucose in FIA system were found at about 300 mV. In addition, the current values obtained from the photoamperometric method were about 50-40% higher than that obtained from amperometric method. Thus an applied potential of 300 mV was selected as optimum in order to minimize interference effects.

In the second optimization step, the effect of flow rate on electrocatalytic and photoelectrocatalytic currents of glucose was investigated. For this purpose, the current-time curves were recorded at various flow rates using GDH/Poly-HT/PAMAM/GCE in 0.1 M PBS (pH 7.0) containing 0.1 M KCl using 100 µL sample loop and 10 cm tubing length. In these conditions, the obtained fiagrams for 0.8 mM glucose containing 10 mM NAD^+ at various flow rates were shown in Figure S3/A shows and the plot of the electrocatalytic and photoelectrocatalytic currents versus flow rate were shown in Figure S3/B (see supplementary file).

As can be seen in thse figures, the maximum peak current was observed at the lowest flow rate, 0.125 mL, because, biosensors and photoelectrochemical biosensors could find enough time for the occurrence of enzymatic reaction and also photoexcitation of mediator in the low flow rate. The peak currents decreased by increasing the flow rate from 0.125 mL min^{-1} to 2.2 mL min^{-1}. Thus, the lowest flow rate, 0.125 mL min^{-1} was selected as optimum flow rate even though sample frequency is very low.

2.3.2. Calibration curve and amperometric response in FIA

To establish that a reliable analytical response could be achieved for the glucose, under optimized conditions using a GDH/Poly-HT/PAMAM/GCE, a calibration study was carried out over the range $5x10^{-6}$ – $1x10^{-2}$ M glucose concentration, with two injections of each concentration being made via a 100 µL sample loop. Figure 3 shows the fiagrams for amperometric and photoamperometric FI responses to various concentrations of glucose. Although the peak currents increased depending on glucose concentration for both the amperometric and the photoamperometric methods, the responses of photoamperometric method were higher than those of amperometric in all concentrations.

Both amperometric and photoamperometric currents at various concentrations of glucose were also recorded using GDH/PAMAM/GCE (without HT) in the optimized conditions. Figure S4 (see supplementary file) shows current-time curves obtained from GDH/PAMAM/GCE for various glucose concentrations. No enzymatically produced NADH

oxidation peaks were observed until 8 mM glucose concentration. Only small peaks were observed up to 8 mM glucose. Thus, it can be concluded that the using of electropolymerization of HT in the electrode modification emphasizes its importance in especially photoamperometric measurements.

Fig. 3

Figure 4A shows a plot of catalytic current versus glucose concentration. From this figure, a linear relationship between the glucose concentration and the peak current was obtained over the concentration range 1×10^{-5} – 1×10^{-3} M and $5\text{x}10^{-6}$ – 1×10^{-3} M by the amperometric FIA and the photoamperometric FIA method, respectively, at the GDH/Poly-HT/PAMAM/GCE (Figure 4B). The linearity of these methods is described by the following equations:

i (μA) = 0.76c (mM) + 0.0035 (R^2 = 0.995)

i (μA) = 1.90c (mM) + 0.0027 (R^2 = 0.998)

for amperometric and photoamperometric studies, respectively, where i is the peak current and c is the concentration of glucose. When these equations are compared in terms of their slopes, it is clear that the sensitivity of the photoelectrocatalytic FIA procedure is better than that of the amperometric method and the ratio of improvement is about 2.5. The limit of detection (LOD) was calculated as 3×10^{-6} and 1.5×10^{-6} M glucose for amperometric and photoamperometric glucose biosensors, respectively, based on $3s_b/m$ where s_b is the standard deviation of the blank response and m is the slope of the calibration curve. Limit of quantification (LOQ) was calculated as 1×10^{-5} and 5×10^{-6} M glucose for amperometric and photoamperometric glucose biosensors, respectively, based on $10s_b/m$.

Fig. 4

The precision of electrochemical and photoelectrochemical biosensor was investigated by making 5 repeat injections of 3×10^{-4} and 1×10^{-3} M glucose solution. The RSD for electrochemical and photoelectrochemical biosensors were calculated to be 1.8% and 2.3% for $3.0\text{x}10^{-4}$ and 1.4% and 3.5% for $1\text{x}10^{-3}$ M, respectively. These results indicate GDH/Poly-HT/PAMAM/GCE has very good repeatability for electrochemical and photoelectrochemical biosensing of glucose.

The apparent Michaelis–Menten constant K_m, which depicts the enzyme-substrate kinetics of biosensor, can be calculated from the Lineweaver–Burk equation: $1/I_{ss} = (K_m/I_{max})(1/C_s) + 1/I_{max}$, where C_s is the substrate concentration, I_{ss} is the steady-state current and I_{max} is the maximum current measured under substrate saturation[32]. From the curve of the I_{ss}^{-1} versus C_s^{-1} (see supplementary file, Figure S5), based on the experimental date from Fig. 4A, the apparent Michaelis–Menten constant K_m and the maximum current response I_{max} were estimated to be 1.12 mM, 3.5 μA/mM for amperometric method and 4.4 mM, 5.13 μA/mM for amperometric method, respectively. The value of K_m agrees well with the reported value of K_m for GDH modified electrodes for example 1.767 mM for carbon nanotube-ionic liquid-chitosan-GDH modified GCE[33] is much lower than the reported values of K_m for some constructed biosensors (4.53 and 3.09[34], 12[35] and 6.7[36] mM) dependent on GDG and NAD^+/NADH redox couple. Smaller K_m values show that the electrochemical and photoelectrochemical biosensors possess higher biological affinity to glucose and have a superior enzymatic activity.

2.3.3. Investigation of interfering substances

There are various species that interfere with glucose detection in the real samples, including ascorbic acid (AA), dopamine (DA), uric acid (UA), L-cysteine (L-Cyst) and other monosaccharides such as galactose, saccharose, and fructose. With this aim, the fiagrams of glucose in the presence of these interfering compounds without irradiation were recorded in the optimum conditions. Figure S6 (see supplementary file) shows the obtained fiagram of $3x10^{-4}$ M glucose in the presence of possible interfering compounds with various concentrations. As can be seen, in the presence of AA, DA, UA and L-Cyst, serious interferences were occurred with the amperometric enzyme glucose sensors because their oxidation potentials were close to that of glucose (300 mV at GDH/Poly-HT/PAMAM/GCE). It was generally reported that, using the Nafion membrane effectively removes the interference of AA because it is negatively charged and repels negatively charged organic species[37]. However, it is difficult to remove the interference caused by neutral or positively charged, very small organics, such as DA and UA, etc. Consquently, these interfering species cannot be removed using only a Nafion membrane. Another possible way to eliminate the interference effect of AA, is to use ascorbate oxidase[38]. While ascorbate is selectively oxidized to dehydroascorbate in the presence of ascorbate oxidase and molecular oxygen, NADH produced by enzymatic reaction was not influenced by this medium.

In order to remove the interfering effect of these compounds, very small amount of lead(IV) acetate as an oxidizing agent can be added to Nafion layer due to preoxidation reaction of these interfering compounds before they reach the electrode surface[37].

On the other hand, no interference in the presence of other monosaccharides including galactose, saccharose and fructose was observed because the GDH is very selective for glucose against other monosaccharides. In order to reduce the interfering effect of AA, DA, UA and L-Cyst, the fiagrams of $3x10^{-4}$ M glucose were also recorded in the absence and in the presence of these interfering compounds at applied potential of 100 mV. Although interference effect of UA and L-Cyst was reduced, the reducing of interfering effect of AA and DA has not been achieved in this potential also. On the other hand, the peak current of glucose at 100 mV decreased about two folds in comparison with 300 mV.

2.3.4. Storage stability of the biosensor

The storage stability of the biosensor has been studied over a period of 15 days. The sensor was stored in 0.1 M PBS (pH 7.0) at 4°C when not in use. The electrocatalytic and photoelectroctalytic current responses could maintain about 85% and 50% of the initial signal, respectively. The first reason of decrease in photoelectrochemical biosensor responses was the effect of light on dehydrogenase enzyme. The other reason was defects and deformations occurring on modified electrodes day by day.

2.3.5. Real sample analysis

In order to show the practical applicability of the proposed biosensor, two real samples (human blood serum and commercial dextrose solution) were selected for determination of glucose as described in literature[39]. 250 μL of blood serum samples were diluted to 5 mL with 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ and 1 M KCl. Glucose detection was performed by spiking a known volume and concentration of glucose standard solution into the diluted serum samples in order to obtain various concentrations, and by measuring of the amperometric and photoamperometric response of electrode in FIA system. Commercially supplied dextrose solution (including 5% glucose) was used as a second real sample. For the determination of glucose, dextrose solution was diluted 555 times with 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ and 1 M KCl. Thus diluted dextrose solution containing about 0.5 mM glucose was obtained. Glucose detection was also performed for this sample as described

human serum sample. The results for the recovery test were given in Table 1. It can be seen that acceptable recoveries were obtained for spiked glucose in blood plasma and commercial dextrose solution samples.

3. Experimental

3.1. Apparatus

Cyclic voltammetric and chronoamperometric measurements were performed at room temperature in a traditional three electrode system. A platinum wire was used as the counter electrode, an home-made Ag/AgCl (saturated KCl) electrode as the reference electrode and a PTFE-shrouded GCE (MF2040 Bioanalytical system, 3 mm diameter) as the working electrode. However, an Ag/AgCl (0.1 M KCl) was used as reference electrode for the chronoamperometric measurements in FIA system. All electrochemical experiments were carried out using two voltammetric instruments, Compactstat Electrochemical Interface (Ivium Technologies, Eindhoven, Netherlands) and Autolab PGSTAT302N Potentiostat/Galvanostat. In order to record cyclic voltammograms during photoelectrochemical experiments, a fiberoptic illuminator 250 W halogen bulb with Foi-5 Light Guide (Titan Tool Supply Inc., USA) was used to irradiate the electrode surface within a home-made photoelectrochemical cell, which was constructed of Teflon[16]. A Perkin Elmer Lambda 35 Uv-Vis Spectrometer was used for measuring the absorbance of the NADH solutions at 340 nm. **.** The pH of the buffer solutions was measured using a Hanna HI 221 pH-meter with a combined glass electrode (Hanna HI 1332).A Bandelin Sonorex RK 100H Ultrasonic bath was used for cleaning procedure of the GCEs before their modification. Deionized water supplied by a Milli-Q device (Millipore, Bedford, USA) throughout all experiments. In order to perform FIA experiments, an eight-channel Ismatec, Ecoline peristaltic pump with polyethylene tubing (0.75mm i.d.), and a Rheodyne 8125 sample injection valve were used.

3.2. Chemicals

Glucose dehydrogenase enzyme from Pseudomonas sp. (GDH, 338.7 U/mg), β-Nicotinamide adenine dinucleotide sodium salt from saccharomyces cerevisiae ($C_{21}H_{26}N_7NaO_{14}P_2$, $NaNAD^+$, MW: 685.41 g mol^{-1}), Bovine serum albumin (BSA),

glutaraldehyde (GA, d: 1.061 g mL^{-1}, MW: 100.12 g mol^{-1}, 25% w/w in water) and PAMAM Dendrimer, ethylenediamine core, generation 4.0 (d: 0.813 g mL^{-1}, MW: 14214.17 g mol^{-1}, 10% w/w in methanol) were supplied from Sigma-Aldrich (St. Louis, USA). D-Glucose, Reduced β-Nicotinamide adenine dinucleotide, disodium salt (MW: 709.40 g mol^{-1} $C_{21}H_{27}N_7Na_2O_{14}P_2$, $NADHNa_2$), Hematoxylin, (HT, $C_6H_{14}O_6$),) were supplied from Merck (Steinheim, Germany). Other used chemicals such as HNO_3 (65%, 1.39 g mL^{-1}), HCl (30%, 1.15 g mL^{-1}), H_3PO_4 (85%, 1.71 g mL^{-1}) KCl, $NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$, NaOH, $NaNO_3$, methanol and ethanol were purchased from Merck or Sigma-Aldrich Companies and they were also of analytical reagent grade.

Human blood plasma samples were collected from the Hospital of Canakkale Onsekiz Mart University in Turkey. Commercial dextrose solution (5%=277.5 mM glucose) was purchased a local drugstore. The experiments related with human serum sample were approved and conducted according to the guidelines of Canakkale Onsekiz Mart University (Turkey) ethics committee.

3.3. Immobilization of enzyme

PAMAM dendrimers have attracted increasing attention in recent years because of their unique structure and interesting properties. A highly branched dendritic macromolecule, PAMAM, possesses a unique surface with multiple chain ends, and the number of surface groups can be precisely controlled by choosing the appropriate synthetic generation. For example, the fourth-generation (G4) PAMAM with a particle size of ca 5 nm possesses 64 surface amine groups per particle with excellent solubility in water[40]. In addition, the PAMAM dendrimer represents a friendly environment for the immobilization of enzymes, and it is stable and capable of generating high power density compared to other immobilization methods. Thus, in the enzyme immobilization procedure, G4-PAMAM was used.

Prior to enzyme immobilization, GCEs were mechanically polished using a BAS-polishing kit with aqueous 1.0, 0.3 and 0.05 μm alumina (Al_2O_3) slurry on a polishing cloth for three min to a mirror-like surface. After they were washed with deionized water, they were cleaned by sonication in ethanol and deionized water for 5 min, respectively.

In the enzyme immobilization procedure, firstly an aliquot of 4 μL 2 mg mL^{-1} PAMAM in methanol was cast onto the polished and cleaned bare GCE. After PAMAM was dried at room temperature for about 10 min, a polymer film of HT was prepared by recording

successive (20 cycle) cyclic voltammograms of 0.3 mM HT on GCE in the potential range between −0.5 and 2.0 V in the 0.1 M PBS (pH 7.0) containing 0.1 M $NaNO_3$ at 100 mV s^{-1} . Then Poly-HT/PAMAM/GCE washed with deionized water and dried under Ar atmosphere and the glucose dehydrogenase (GDH) enzyme were immobilized directly on poly-HT/PAMAM/GCE by the cross-linking method using glutaraldehyde (GA). For this aim, firstly 5 mg mL^{-1} GDH and 1% BSA, which were prepared in PBS, was mixed in a ratio 1:1 and then 5 µL of the mixture and 4 µL of GA (20 mM) were cast on poly-HT/PAMAM/GCE, respectively. Finally, this electrode was dried at 4°C in a refrigerator for at least 4 h. A schematic representation of the preparation of enzyme modified electrodes is given in Figure 5. Enzyme was immobilized via crosslinking of aldehyde group of GA with amino groups of enzyme and PAMAM (Enzyme-GA-PAMAM) in which a Schiff Bases between two ammino groups and aldehyde groups were produced (Enzyme-N=CH-$(CH_2)_3$-CH=N-PAMAM). Poly-HT film is formed between branches of PAMAM, thus many NH_2 groups can be free for croslinking of GDH on PAMAM via GA.

Fig. 5

3.4. Electrochemical Procedure

Electrocatalytic and photoelectrocatalytic oxidation of glucose at this GDH based poly-HT/PAMAM/GCE was investigated using cyclic voltammetric techniques. Firstly, cyclic voltammogram of GDH-poly-HT/PAMAM/GCE was recorded in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ in the potential range between −0.8 and 0.8 V vs. Ag/AgCl at a scan rate of 5 mV s^{-1} in the absence of glucose. In order to see response of biosensor towards glucose, cyclic voltammograms of modified electrodes were recorded in the same conditions but in the presence of 10 mM glucose. The cyclic voltammograms in the same conditions were also recorded for bare GCE, poly-HT/GCE, poly-HT/PAMAM/GCE.

Finally, photoelectrochemical biosensor studies were performed by using the home-made photoelectrochemical cell[16]. In the photoelectrocatalytic experiments, the cyclic voltammograms of GDH/poly-HT/PAMAM/GCE were recorded under the above mentioned same conditions (supporting electrolyte: 0.1 M PBS (pH 7.0), scan rate: 5 mV s^{-1}, potential range: from -0.8 to 0.8 V in the absence and presence of glucose), but under irradiation of the working electrode surface by the fiberoptic illuminator with 250 W halogen bulb. The

constructed photoelectrochemical biosensor for glucose detection at GDH/poly-HT/PAMAM/GCE is schematically shown in Figure 6.

Fig. 6

3.5. Photoelectrochemical biosensor studies in FIA system

Previously constructed the new home-made photoelectrochemical flow cell and FIA diagram[16] were also used for studies of electrocatalytic and photoelectrocatalytic oxidation of glucose in FIA system. In all FIA experiments, 0.1 M PBS (pH 7.0) containing 0.1 M KCl was used as carrier solution. After GDH/poly-HT/PAMAM/GCE had been inserted into a flow cell, electrochemical and photoelectrochemical responses of glucose were monitored dependent on glucose concentration. For this, after a steady-state background current of supporting electrolyte under optimum conditions, which were optimized in the FIA procedure section, was obtained, the various concentrations of glucose containing 10 mM NAD^+ were injected into the system and the current–time curves were recorded. The current–time curves were also recorded for the photoamperometric FIA study by irradiation of electrode surface throughout experiment. In all FIA experiments, three injections were made for each NADH standard solution. Same experiments were also repeated for bare GCE for comparision. All supporting electrolytes were deaerated by allowing highly pure argon to pass through for 5 min before the electrochemical experiments.

4. Conclusion

In this study, the constructing photoelectrochemical glucose biosensor in FIA system was proposed. Although, photoelectrochemical biosensor dependent on NAD^+/NADH redox couple-dehydrogenase enzymes[23,24] has been reported, according to our search of the literature, photoelectrochemical biosensor in FIA system have not been reported, yet. When the electrochemical biosensor was performed, the anodic current increased linearly with the glucose concentration over the range from $1.0x10^{-5}$ to $1.0\ x10^{-3}$ M with the sensitivity of 0.76 μA mM^{-1} and detection limit 3.0 μM. After the irradiation, the linear range of the photoelectrochemical biosensor was from $5x10^{-6}$ to $1.0x10^{-3}$ M with the sensitivity of 1.90 μAmM^{-1} and detection limit 1.5 μM. Compared with the reaction without irradiation, the sensitivity and the detection limit increased around 2.5 and 2.0 folds, respectively. The

various analytical detection parameters such as detection potential of glucose, linearity ranges and calculated LOD were compared with other modified electrodes in previously published reports for the biosensing of glucose. The results are illustrated in Table 2. As can be seen, the electrocatalytic detection potential of glucose (DP) at the GDH/poly-HT/PAMAM/GCE was better than DP of 5-[2,5-di (thiophen-2-yl)-1H-pyrrol-1-yl]-1,10-phenanthroline iron(III) chloride modified screen printed carbon electrode (SPCE). Although the DP data reported for the modified electrodes were better than the GDH/poly-HT/PAMAM/GCE, the LOD and linearity range of proposed electrode was better than that of the compared modified electrodes.

As a result, this study represented the successful dehydrogenase-based electrochemical and photoelectrochemical biosensors in FIA system at GDH/poly-HT/PAMAM/GCE. Glucose in the commercial dextrose solution and human serum sample was successfully determined with proposed electrochemical and photoelectrochemical biosensor. The biocatalytical performance of the biosensor was greatly improved by the photovoltaic effect of the dye using as mediator.

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Table 1. The results of recovery studies for glucose dterminaiton in real samples (n=3)

Sample	Spiked (mM)	FIA Amperometric			FIA Photoamperometric		
		Found (mM)	Recovery	RSD %	Found (mM)	Recovery	RSD %
Human serum	0.25	0.26 ± 0.01	105.2	4.0	0.26 ± 0.03	104.6	7.9
	0.50	0.49 ± 0.02	98.7	3.1	0.52 ± 0.04	103.3	7.3
	1.00	1.01 ± 0.03	101.3	2.5	1.02 ± 0.06	102.3	6.0
Dextrose solution	Labelled claim (%5=277.5 mM)	277.7 ± 4.5	-	-	282.3 ± 6.0	-	-

Table 2. Comparison of analytical parameters obtained from GDH/poly-HT/PAMAM/GCE with different electrodes in the literature for electrochemical biosensing of glucose dependent on NAD^+/NADH redox couple and dehydrogenase enzyme.

Electrode type	**Method**	**DP**	**LR**	**LOD**	**Ref.**
Th cross-linked MWCNTs and Au NPs multilayer functionalized ITO electrode	AMP	0.2 V *vs.* Ag/AgCl	10-2560 µM	5 µM	23
	AMP under irradiation		1-3250 µM	0.7 µM	
MdB modified SPCE	FIA AMP	0.05 V *vs*. Ag/AgCl	75-30.000 µM	-	28
CNTs-IL/GCE	CV	0.25 V *vs*. Ag/AgCl	20-1000µM	9 µM	33
FAD-MWCNT nanocomposite electrode	AMP	0.05 V *vs.* Ag/AgCl	70-620 µM (for *Asp. oryzae*) 50-660 µM (for *Asp. sp*)	4.15 µM 4.45 µM	34
Poly-NB modified SWCNT/GCE	AMP	0.05 V *vs*. Ag/AgCl	10-8500 µM	5 µM	35
Au modified SWCNT-a nanothin PPF	AMP	0.2 V *vs*. Ag/AgCl	4900-19.000 µM	-	36
FePhenTPy modified SPCE	AMP	0.55 V *vs*. C electrode	30-600 mg/dL (17-330 µM)	12 mg/dL (6.7 µM)	37
CNTP modified with Os –redox polymers	AMP	0.2 V *vs*. Ag/AgCl	Upto 800 µM	10 µM	41
NB SWCNT modified GCE	AMP	0 V *vs*. SCE	100-1700 µM	0.3 µM	42
Poly-TB on graphite electrode	AMP	-	50-3000 µM	-	43
GDH/poly-HT/PAMAM/GCE	FIA AMP	0.3 V *vs.* Ag/AgCl	10-1000 µM	3 µM	This work
	FIA AMP under irradiation		5-1000 µM	1.5 µM	

FePhenTPy: 5-[2,5-di (thiophen-2-yl)-1H-pyrrol-1-yl]-1,10-phenanthroline iron(III) chloride, PPF: Plasma polymerized film, SPCE: Screen printed carbon electrode, LR: linearity range, DP: Detection potential, LOD: Limit of detection, CNTP: Carbon nanotube paste, AMP: Amperometry, NB: Nile Blue, SWCNT: Single walled carbon nanotube, IL Ionic liquid, TB: Toluidine blue, FAD:Flavine adenine dinucleotide, ITO: Indium tin oxide, NPs: Nanoparticles, GDH: Glucose dehydrogenase.

Figure Captions

Figure 1. Repetitive cyclic voltammograms (20 cycles) of 0.3 mM HT at PAMAM/GCE in 0.1 M PBS (pH 7.0) containing 0.1 M $NaNO_3$ at 100 mV s^{-1} in the range of -0.5 to 2.1 V *vs.* Ag/AgCl (black and blue lines are first and last cycles, respectively).

Figure 2. Cyclic voltammograms of A) GDH/PAMAM/GCE and B) GDH/poly-HT/PAMAM/GCE in the presence of 10 mM NAD^+ and a) in the absence, b) in the presence of 10 mM glucose without irradiation and c) b with irradiation. Supporting electrolyte: 0.1 M PBS (pH 7.0); scan rate: 5 mV s^{-1}.

Figure 3. Current-time curves of glucose with different concentrations in the range from 0.005 mM to 10 mM glucose containing 10 mM NAD^+ using GDH/Poly-HT/PAMAM/GCE in FIA system (Carrier stream: 0.1 M PBS (pH 7.0) containing 0.1 M KCl, applied potential: 300 mV; flow rate: 0.125 mL min^{-1}; sample loop: 100 μL; transmission tubing length: 10 cm).

Figure 4. **A)** Dependence of the catalytic currents on glucose concentration, **B)** calibration curve for ■) amperometric and ●) photoamperometric analysis of glucose using GDH/Poly-HT/PAMAM/GCE in FIA system.

Figure 5. Schematic representation of enzyme immobilization procedure.

Figure 6. Schematic representation of the photoelectrochemical biosensor.

Figure 1

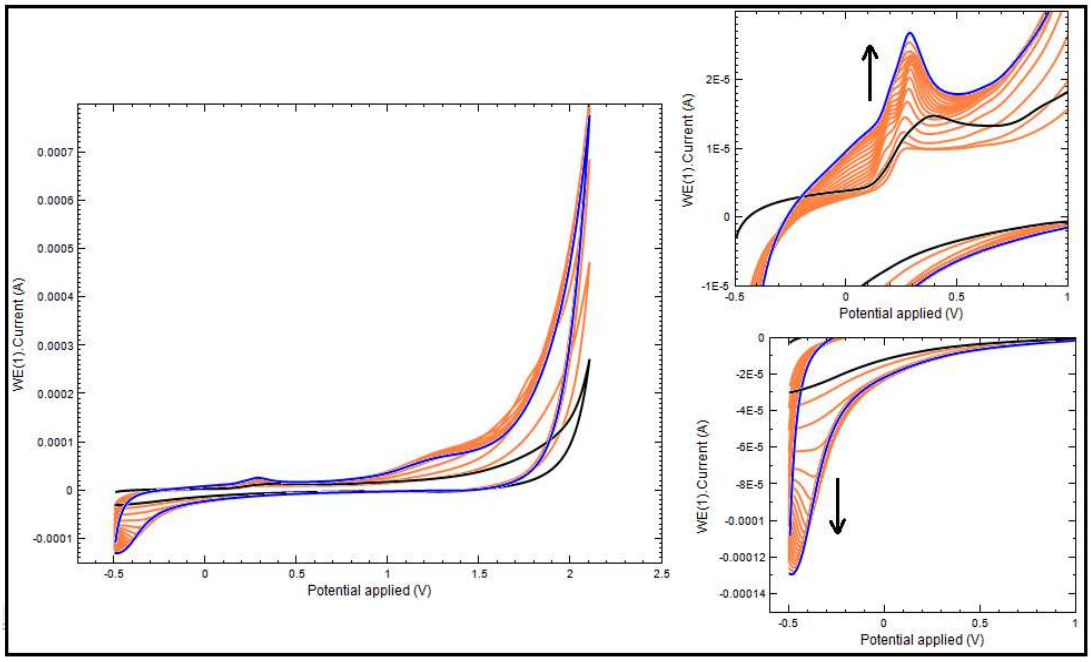

Figure 2

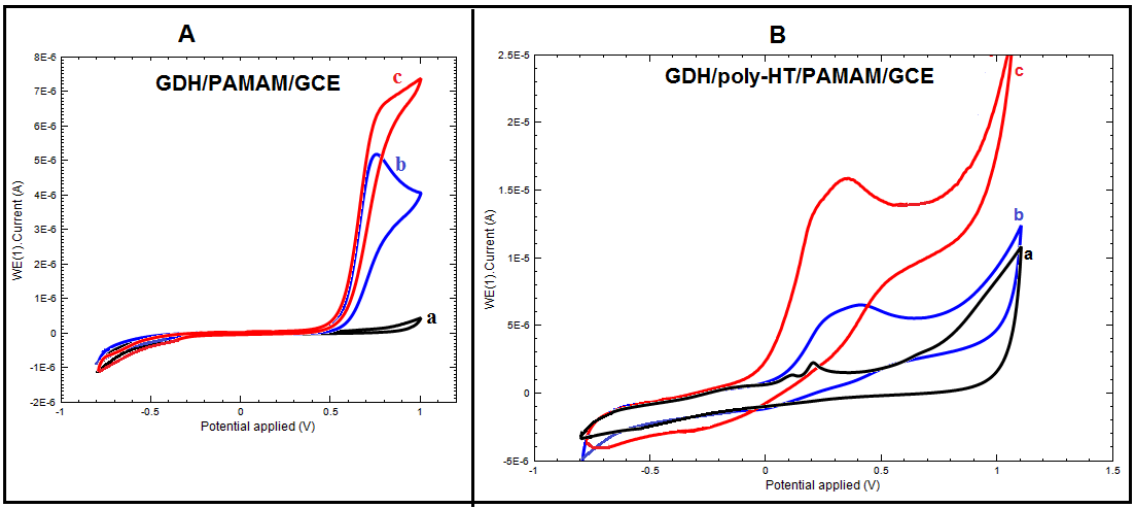

Figure 3

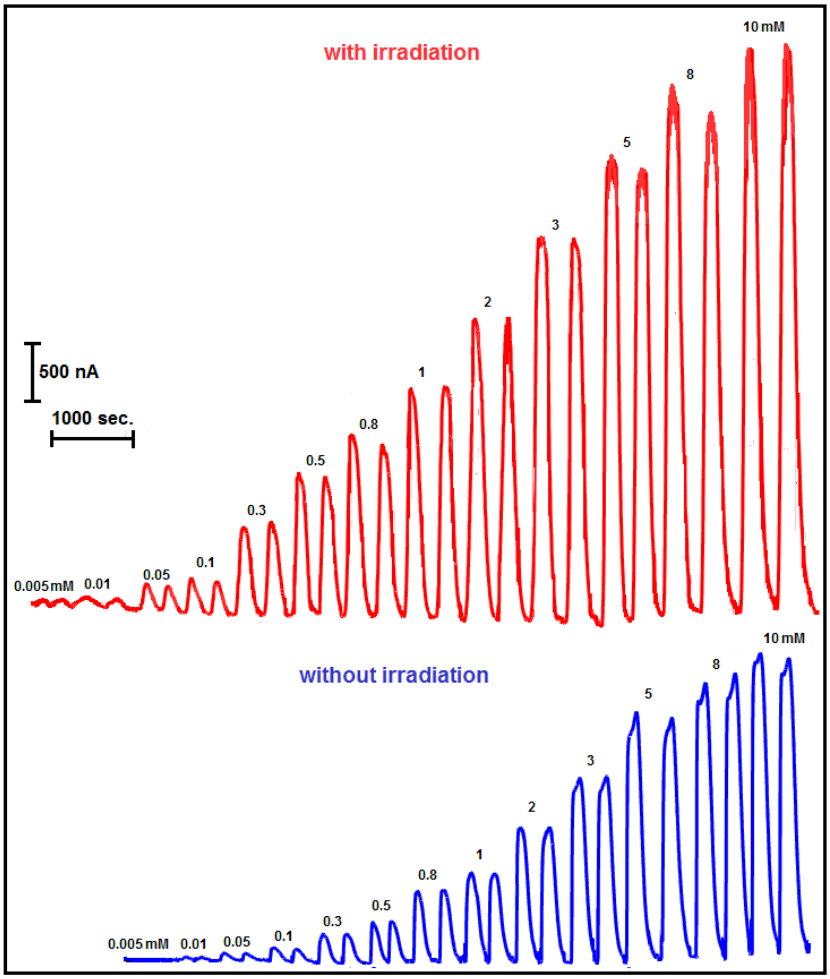

Figure 4

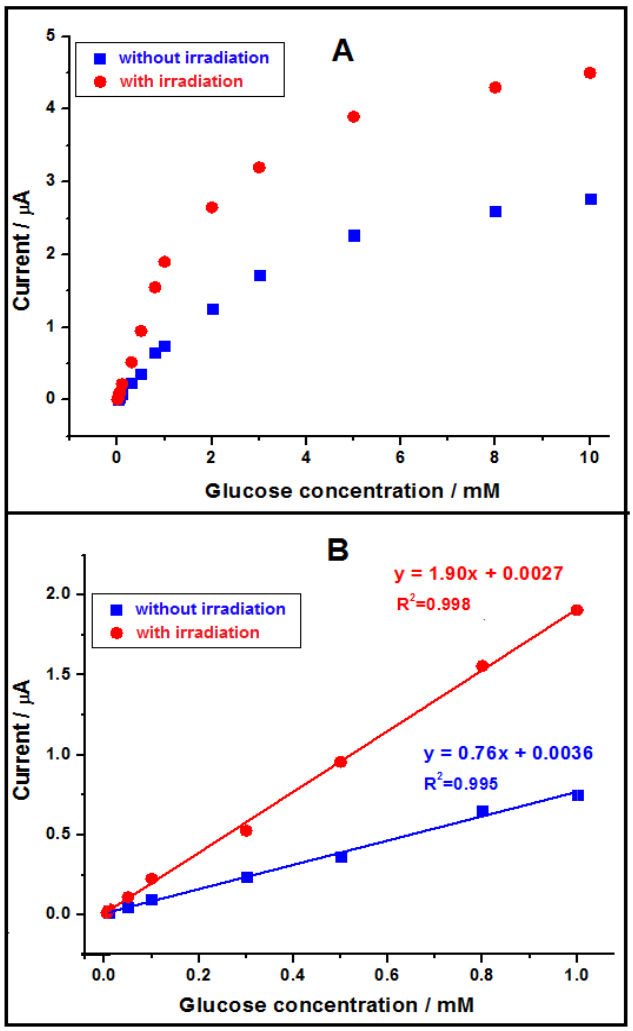

Figure 5

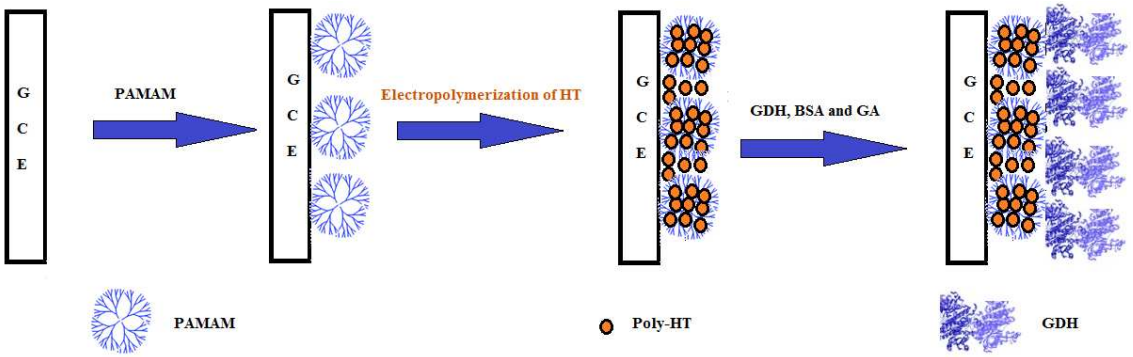

Figure 6