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Modified gold surfaces by poly(amidoamine) dendrimers and fructose dehydrogenase for mediated fructose sensing

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ABSTRACT

An electrochemical biosensor for detection of fructose in food samples was developed by immobilization of fructose dehydrogenase (FDH) on cysteamine and poly(amidoamine) dendrimers (PAMAM)-modified gold electrode surface. Electrochemical analysis was carried out by using hexacyanoferrate (HCF) as a mediator and the response time was 35 s at +300 mV vs. Ag/AgCl. Moreover, some parameters such as pH, enzyme loading and type of PAMAM (Generations 2, 3 and 4) were investigated. Then, the FDH biosensor was calibrated for fructose in the concentration range of 0.25–5.0 mM. To evaluate its utility, the FDH biosensor was applied for fructose analysis in real samples. Finally, obtained data were compared with those measured with HPLC as a reference method.

1. Introduction

Fructose is an important monosaccharide in food and clinical samples [1]. A selective, fast, simple and low cost method for fructose determination is desirable, for this aim various biosensors using immobilized enzymes have been applied in batch or in flow mode. To analyze fructose in food and beverages, several electrodes such as gold [2], carbon paste [3], Pt [1,4], graphite [5,6], epoxy graphite [7] and GCE [8] have been modified by using fructose dehydrogenase by means of various immobilization techniques. Fructose dehydrogenase (FDH; EC1.1.99.11) is a membrane-bound oxidoreductase, which catalysis oxidation of fructose to 5-keto-D-fructose [9–11]. FDH was first detected by Yamada et al. in *Gluconobacter cerinus* [9,12,13]. FDH has both a pyrroloquinoline/quinine (PQQ) redox site and a few heme c sites like other membrane-bound dehydrogenases of *Gluconobacter* such as alcohol dehydrogenase and aldehyde dehydrogenase [14]. Active center of FDH is based on PQQ [3]. So that use of FDH is advantageous due to the fact that oxygen is not necessary to regenerate the catalytic center of the enzyme [15]. Nowadays, nanotechnology and nanomaterials are enormous chance to develop an efficient novel tool for food and bioprocess industry [16].

As immobilization materials, dendrimers are nanomaterials with highly branched, three dimensional uniform structures. Central core, branches and surface groups are main components of their dendritic architectures [17]. The use of dendrimers has been attractive subject in some areas such as drug delivery, gene therapy, highly sensitive analytical devices because of their nanosize (in the range 2.5 to10 nm) and ease of functionalization of end groups [18–20]. The well-known dendrimers are poly(propylene imine), poly(amidoamine) and poly(benzyl ether) dendrimers [21]. Moreover, amino-terminated amphiphilic poly(amidoamine) dendrimers (PAMAM) with NH_3 core, is a mix of polyamides and amines and have ellipsoidal or spheroidal shape [19,22]. PAMAM is a specific family of dendritic polymers which can be used in biomedical applications such as carriers for the delivery of drugs and DNA or oligonucleotides because of their different terminal groups [23–25]. Also, immunoconjugates of boronated PAMAM and monoclonal antibody have been applied as an efficient anti-cancer reactive. PAMAM has been employed as magnetic resonance imaging contrast agent to improve the quality of the clinical diagnostics [26]. PAMAM has also been used as stabilizer of the gold particles [22]. On the other hand, PAMAM-modified electrodes have great promise for various biosensing applications, for instance, DNA detection methods based on PAMAM were previously reported by different groups [27–29]. Moreover, Chen et al. reported the nitrite biosensor by the immobilization of cytochrome *c* (Cyt *c*) on glassy carbon electrode via PAMAM–chitosan nanocomposite [30]. In another study, acetylcholinesterase and choline oxidase were co-immobilized onto the Au electrode by means of PAMAM dendrimer for the pesticide analysis [31]. Use of PAMAM for the immobilization of alcohol dehydrogenase (ADH) onto carbon cloth platforms and its application as bioanode in ethanol/O_2 biofuel cells was carried out by Forti et al. [32]. Additionally, Horseradish peroxidase

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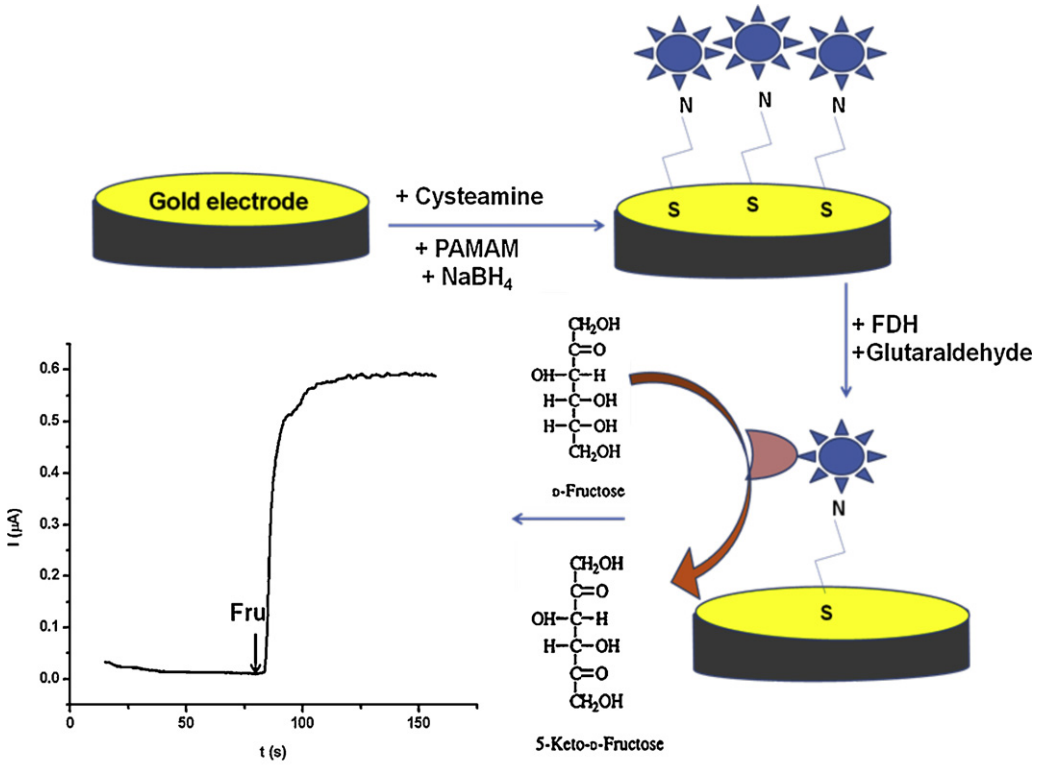

Scheme 1. Schematic representation of FDH/PAMAM biosensor construction including biosensor response current after addition of fructose in reaction medium.

(HRP), glucose oxidase (GOx), pyranose oxidase (PyOx) and alcohol oxidase (AOx) were immobilized on to Au gold surface modified by PAMAM/cysteamine [33–36].

In this study, a mediated fructose biosensor was prepared. FDH was immobilized on Au electrode after PAMAM modification. As well as characterization studies, optimization and application of FDH/PAMAM biosensor for fructose analysis in the real samples were carried out.

2. Materials and methods

2.1. Materials

Fructose dehydrogenase (FDH from *Gluconobacter* sp., 1.9 mg solid, 131 U/mg), fructose, cysteamine hydrochloride, and sodium borohydride were from Fluka (Steinheim, Germany), Poly(amidoamine) dendrimers (PAMAM, 75% amino and 25% [*N*-(2-hydroxydodecyl)] surface groups; 25% C12 Generation 4.0 dendrimer, 10 wt.% in methanol; 25% C12 Generation 3.0 dendrimer, 20 wt.% in methanol and 25% C12 Generation 2.0 dendrimer, 20 wt.% solution in methanol) and glutaraldehyde solution (25%, v/v) were purchased from Sigma–Aldrich (Dorset, UK).

2.2. Instruments

Cyclic voltammetric and amperometric measurements were carried out on PalmSens Electrochemical Measurement System. A three-electrode cell (10 mL) was used with the modified gold electrode (Au; BASI, USA) as a working electrode, a silver chloride (Ag/AgCl; Metrohm) as a reference electrode, and a platinum electrode (Metrohm) as a counter-electrode. Cyclic voltammetric experiments were performed in unstirred acetate buffer (pH 4.5; 50 mM).

2.3. Fabrication of FDH/PAMAM biosensor

The gold surface was initially polished with alumina polishing suspension (Baikowski International Corporation, 0.05, 0.1, 0.3, 1.0 and 3.0 μm). Then, it was cleaned in 0.5 M H_2SO_4 solution by cyclic voltammetry between 0 and +1.5 V until a reproducible response was obtained. The polished gold electrode was subsequently immersed in 0.1 M cysteamine for 30 min, glutaraldehyde solution (5.0% in sodium phosphate buffer; pH 7.0, 50 mM) for 30 min, PAMAM dendrimer (1.0%; in 50 mM sodium phosphate buffer; pH 7.0) for 1 h and $NaBH_4$ solution (5.0 mM) for 30 min, respectively [34–36]. After each step, the electrode surface was fully rinsed with distilled water. Finally, glutaraldehyde (10 μL; 1.0% in 50 mM pH 7.0 phosphate buffer) and FDH (1.0 μL; 12.4 U) solutions were dropped on the PAMAM modified gold electrode surface. In this case, the cross-linkages between amino groups of PAMAM and aldehyde groups of glutaraldehyde and then the cross-linkage between another aldehyde group of glutaraldehyde and amino group of the enzyme were occurred. After the surface was dried at room temperature (1 h), the FDH/PAMAM biosensor was washed with water to remove unbound enzyme and excess of glutaraldehyde. The schematic representation of FDH/PAMAM biosensor fabrication was shown in Scheme 1. The FDH/PAMAM biosensors were stored in acetate buffer (pH 4.5; 50 mM) at 4 °C when not in use. Daily prepared electrodes were used during the experiments.

2.4. Measurements

All measurements were performed at room temperature in an open vessel filled with the vigorously stirred 5.0 mM acetate buffer solution, pH 4.5. Increasing concentrations of fructose were adjusted by adding definite volumes of the stock solution of 1.0 M fructose (in pH 4.5, 50 mM acetate buffer). The biosensor response were evaluated via the following steps: Firstly, the FDH/PAMAM

biosensor was placed into the 10 mL of acetate buffer solution (pH 4.5, 50 mM) including 5.0 mM HCF as a mediator and the amperometric background current (base line) signal (85 s) was registered at +0.3 V vs. Ag/AgCl. Then a standard solution of fructose was injected to the reaction medium by using a micropipette and the steady-state current signal was registered as μA in 35 s. After measurements, the biosensor surface and reaction vessel were washed with water.

FDH catalyzes the following reaction in which 5-keto-D-fructose was produced [37]:

$$\text{d-fructose} + \text{FDH(PQQ)} \rightarrow \text{5-keto-d-fructose} + \text{FDH(PQQH}_2\text{)}$$

$$\text{FDH(PQQH}_2\text{)} + 2\text{K}_3[\text{Fe(CN)}_6] \rightarrow \text{FDH(PQQ)} + 2\text{K}_4[\text{Fe(CN)}_6] + 2\text{H}^+$$

$$2\text{K}_4[\text{Fe(CN)}_6] \rightarrow 2\text{K}_3[\text{Fe(CN)}_6] + 2\text{e}^-$$

During the enzymatic reaction, PQQ localized in active center was reduced to $PQQH_2$ [38]. And in the presence of HCF mediator, the enzyme was re-oxidized and Fe^{3+} is reduced to Fe^{2+}. The oxidation of Fe^{2+} on the electrode surface could be followed by amperometrically at +0.3 V. The current differences due to the enzymatic reaction are proportional to the concentrations of fructose in the reaction medium. The trials were repeated at least three times, and the mean values of measurements are presented with the standard deviations or relative standard deviations.

2.5. Sample application

Various beverages, such as cherry juice, orange juice, peach juice, fizzy and energy drink, were purchased from the local markets. Fructose concentration in real samples was determined in four replicates on the basis of the calibration curve for the standard fructose solution. To test the results measured by the FDH/PAMAM biosensor, HPLC analysis was performed using HP-Chemstation from Agilent (Karlsruhe, Germany) with a refractive index detector (HPLC-RID). HPLC column (GL Sciences Inc. Inertsil NH_2 5.0 μm (4.6 I.D × 250 mm), Japan) was used for the chromatographic separation of fructose at 30 °C. Injection volume was 20 μL. The mobile phase was sulphuric acid (5.0 mM) [34,35]. The flow rate was 0.6 ml/min. Initially, standard curve for fructose was plotted (0.25–5.0 mg/mL for fructose). After dilution with mobile phase and filtration through membrane filter (pore size; 0.20 μM), samples were applied to the column and then fructose concentrations were calculated using calibration plot.

3. Results and discussion

The preparation of FDH/PAMAM biosensor for electrochemical detection of fructose was described in this study. In order to examine the electrochemical activity of FDH/PAMAM biosensor, cyclic voltammograms were taken in pH 4.5, 50 mM acetate buffer. Fig. 1 shows the cyclic voltammograms (CV) of HCF at the gold electrode modified with FDH/PAMAM. The current increased with the elevated scan rates from 5.0 to 100 mV/s (Fig. 1A). As shown in Fig. 1B, when 15.0 mM fructose was added in to reaction medium, higher oxidation peak was observed. It is showed that fructose can be oxidized by FDH and PAMAM modified gold electrodes is good alternative to immobilize FDH.

3.1. Optimization of FDH/PAMAM biosensor

The effect of pH, enzyme amount and type of dendrimer were tested to find the optimal conditions for fructose detection. First of

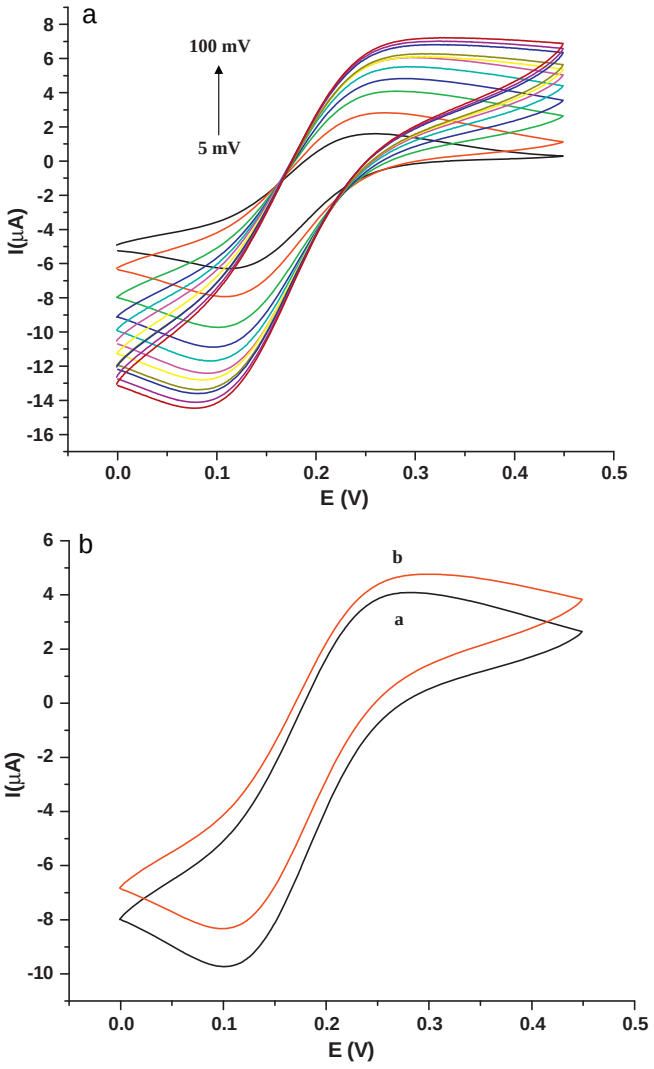

Fig. 1. Cyclic voltammograms of FDH/PAMAM biosensor at different scan rates. (A and B) Effect of fructose (a: in the absence and b: in the presence of 5.0 mM fructose) (in acetate buffer, 50 mM, pH 4.5).

all, the effects of various pHs (pH 4.0–5.5, 50 mM acetate buffers) on the FDH/PAMAM biosensor response were tested. The sensor performance was not been strongly influenced by pH in the range studied (4.0–4.5), but a decrease on the sensor sensitivity was investigated at pH 5.0 and 5.5. The pH profile in Fig. 2 shows that the optimal pH is 4.5. For free FDH, optimum pH is 4.0–5.0 [39], after immobilization of FDH, structure of PAMAM did not change optimum pH of enzyme.

The effect of enzyme loading on the current response of FDH/PAMAM biosensor was investigated in the range from 6.2 U to 24.9 U of FDH. As shown in Fig. 3, the current gradually increased with the increase of FDH value from 6.2 to 12.4 U. However, when the FDH further increased to 24.9, the current did not increase significantly. So 12.4 U FDH was used to prepare the biosensors in all experiments.

The FDH/PAMAM was assembled on the gold surface via cross-linkage between the amine groups of PAMAM and enzyme. Number of free amine groups of the dendrimer is important for the efficient cross-linkages. When going from the core to the surface, the numbers of branching points (cascade points) are called the dendrimer generation [19]. Dendrimers have a lot of vulnerable free surface amino groups per molecule (12 in PAMAM G2, 24 in G3 and 48 in G4). To examine the effect of the surface amine groups, Generations

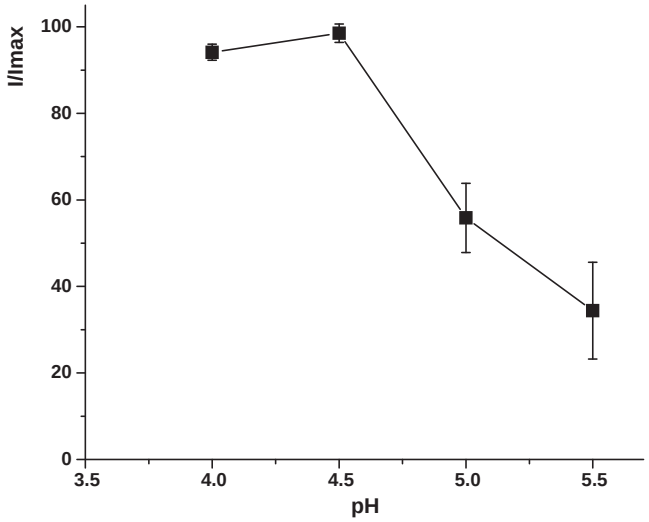

Fig. 2. Effect of pH on the biosensor response (50 mM, sodium acetate buffers at pH 4.0, 4.5, 5.0, 5.5; 0.3 V, [Fru]; 5.0 mM, error bars show S.D.).

4, 3 and 2 PAMAM was used to prepare PDH/PAMAM biosensor. PAMAM G4, G3 and G2 are dendrimers having 48, 24 and 12 primary amines as a surface group, respectively. Using same amounts of different type dendrimer (Generations 4, 3 and 2), enzyme biosensors were fabricated. To test the effect of PAMAM generation, linear graphics to fructose was obtained and results were displayed in Fig. 4. FDH biosensors formed with PAMAM G4 dendrimer showed higher currents than other generations. So, PAMAM G4 dendrimer was selected for consequent experiments.

The morphology of FDH/PAMAM modified Au electrodes was imaged by Scanning Electron Microscopy (SEM), (Fig. 5). Use of Generation 2 PAMAM dendrimer as an immobilization material caused the formation of holes.

3.2. Analytical characteristics

Under the optimized conditions, FDH/PAMAM biosensor was calibrated for fructose. The relationship between the substrate concentration and the current was examined in the range of 0.25–5.0 mM fructose (Fig. 6, inset: calibration plot for fructose). For five successive detection of 1.0 mM fructose, the standard deviation

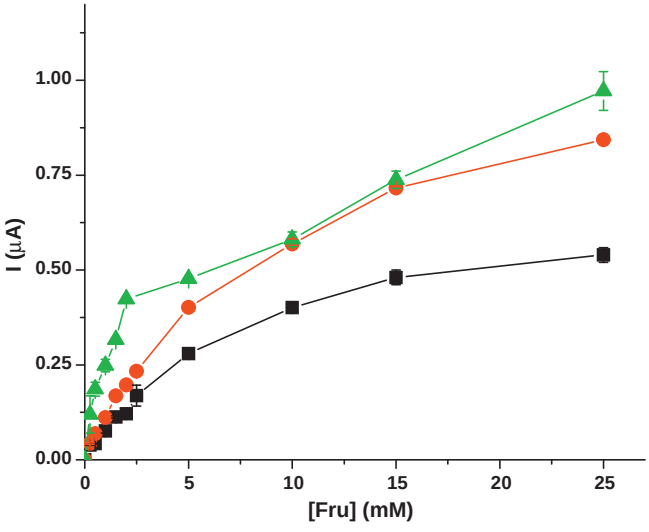

Fig. 3. Effect of enzyme loading on the biosensor response (in sodium acetate buffer, 50 mM, pH 4.5; 0.3 V, error bars show S.D.; ■, 6.2 U; ●, 12.4 U; ▲, 24.9 U).

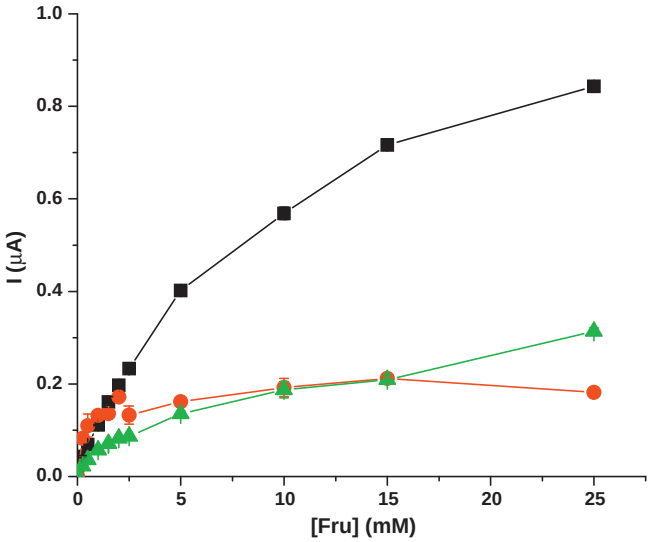

Fig. 4. Effect of generation number of PAMAM on the biosensor response (in sodium acetate buffer, 50 mM, pH 4.5; 0.3 V, error bars show S.D.; ▲,Generation 2 PAMAM; ●, Generation 3 PAMAM; ■, Generation 4 PAMAM).

(SD) and coefficient of variation (cv, %) were ±0.04 and 4.5%, respectively. The RSD value for the electrode to electrode reproducibility was searched for three biosensors and calculated as 4.6%. Therefore, it can be claimed that the good repeatability and reproducibility were obtained for FDH/PAMAM biosensors.

The stability and lifetime of the fabricated biosensor was also tested by consecutive measurements of the response to fructose standard (1.0 mM). FDH/PAMAM biosensor retained 70% of its initial activity at the end of the 20th measurement (after 5 h). When the biosensor was stored at 4 °C, FDH/PAMAM biosensor was reused without loosing of its activity during 7 days. The response sensitivity dropped to 85% of its initial value over the 10 days. This decrease was attributed that FDH is unstable enzyme [1].

In the literature, several FDH biosensors based on platinum, glassy carbon and carbon paste electrodes have been reported. The comparison of some analytical characteristics of the previously reported FDH biosensors and the proposed biosensor was given in Table 1.

In order to evaluate the selectivity of the FDH/PAMAM biosensor, the amperometric response of fructose (0.5 mM) and other species, such as ascorbic acid (0.5 mM), 3-acetamidophenol (0.5 mM) and uric acid (0.5 mM) were recorded. The relative responses were 10%, 5% and 0.8% for ascorbic acid, 3-acetamidophenol and uric acid when the response to fructose was 100%, respectively. As shown, these species did not interfere during detection of fructose.

3.3. Sample application

The proposed FDH/PAMAM biosensor was successfully applied to the analysis of fructose in various types of beverages. The samples were analyzed under optimal experimental conditions in three replicates. Fructose content was calculated applying the calibration plot method. Additionally, HPLC-RID was used as a reference method and chromatogram of 5.0 mM fructose (elution time 5.73 min) was shown in Fig. 7A. Linear range for fructose was obtained between 0.25 and 5.0 mM (Fig. 7B).

The calculated results using FDH/PAMAM biosensor were compared with that obtained by HPLC-RID. The determined contents and recovery of the assays were given in Table 2. Good recovery yields confirmed the accuracy of the developed FDH/PAMAM biosensor.

Table 1
Comparison of analytical characteristics of various FDH-modified electrodes reported in literature.

Electrode configuration	Immobilization method	Mediator	Working potential (mV)	Response time	Analytical performance			Ref.
					Linear range for fructose	Variation coefficient	Operational stability	
CCG[a]-modified GCE	Adsorption on CCG	–	500 mV (vs. Ag/AgC1)	–	–	–	–	[40]
Pt-SPGE[b]	In a PEI[c] and PCS[d] hydrogel	HCF[e]	400 mV (vs. Ag/AgC1)	18–25 s	3–13 mM	–	Decrease after 10–15 measurements	[1]
MWCNTs/Pt[f]	Adsorption	DET[g]	−150 mV (vs. Ag/AgCl/KCl)	–	5–35 mM	–	–	[41]
AgNP/AgE[h]	Adsorption	DET	−100 mV (vs. Ag/AgCl/NaCl)	–	–	–	–	[42]
BP-HOPG[i]	Adsorption	DET	−100 mV (vs. Ag/AgCl/KCl)	–	–	–	–	[4]
GCE[j]	On a Fc-CA[k]	Fc[m]	300 mV (vs. SCE)	75 s.	–	–	–	[43]
GCE	On a Fc-CA	Fc	250 mV (vs. SCE)	45–75 s	–	–	50% decrease after 9 h	[6]
Pt DE[n]	PPY[p]	HCF	230 mV (vs. SCE)	8 s	0.1–0.8 mM	0.68%	–	[39]
Pt DE	PPY[p]-HCF[e]	HCF	250 mV (vs. SCE)	15 s	0.1–0.8 mM	–	–	[44]
CPE	PDAR[s]	HCF	390 mV (vs. Ag/AgC1)	–	10 μM–1 mM	–	40% decrease after 1 day	[37]
CPE	PDAR	[t]TCNQ	210 mV (vs. Ag/AgC1)	–	10 μM–0.8 mM	–	90% decrease after 1 day	[37]
CPE	Entrapment	Os(bpy)2Cl$_2$[v]	100 mV (vs. Ag/AgC1)	–	0.220 mM (batch) 0.5–15 mM (FIA)	–	No significant Decrease after 4 h.	[13]
Pt	With BSA and glutaraldehyde on immobilon AV membrane (covered with a polycarbonate membrane)	HCF	380 mV	–	1.0 μM–1.0 mM	≤2%	50% decrease after 3 months (in DEAE-dextran + lactitol)	[4]
SPGE[y]	BSA and glutaraldehyde	Phenazine methansul-phate	70 mV(vs. Ag pseudo reference electrode)	30 s	0.05–0.5 mM	5%	10% decrease after 15 days	[5]
Au	PAMAM[z]	HCF	300 mV (vs. Ag/AgC1)	35 s	0.25–5.0 mM	4.5%	30% decrease after 5 h	This work

[a] CCG: carbon cryogels.
[b] Pt-SPGE: platinum tip of a screen printed graphite electrode.
[c] PEI: polyethylenemine.
[d] PCS: poly(carbamoylsulphonate).
[e] HCF: hexacyanoferrate.
[f] MWCNTs/Pt: multiwalled carbon nanotube modified platinum electrodes.
[g] DET: direct electron transfer.
[h] AgNP/AgE: alkane-thiol-modified silver nanoparticles/silverelectrodes.
[i] BP-HOPG: basal-plane, highly oriented pyrolytic graphite.
[j] GCE: glassy carbon electrode.
[k] Fc-CA: ferrocene-embedded cellulose acetate membrane.
[m] Fc: ferrocene.
[n] Pt DE: platinum disc electrode.
[p] PPY: polypyrrole.
[s] PDAR: electropolymer film of 1,3 phenylene diamine-resorcinol; CP: carbon paste electrode.
[t] TCNQ: tetracyanoquinodimethane.
[v] Os(bpy)2Cl$_2$: osmium polymer.
[y] Screen printed graphite electrode.
[z] PAMAM: poly(amidoamine) dendrimer.

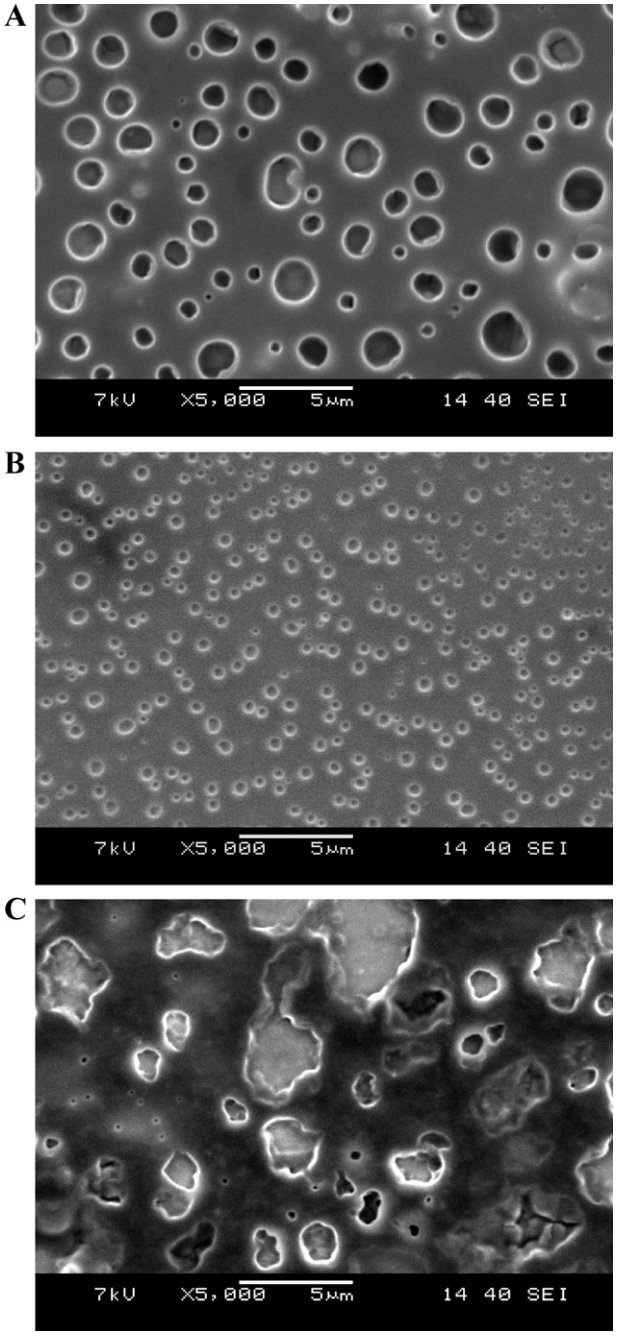

Fig. 5. SEM images of FDH/PAMAM dendrimers modified electrodes (A: Generation 4 PAMAM; B: Generation 3 PAMAM; C: Generation 2 PAMAM).

Table 2
Results for fructose analysis in real samples by FDH biosensor and HPLC.

Sample	Fructose (g/100 ml)[a]		Recovery %
	FDH/PAMAM	HPLC-RID	
Cherry juice	9.04 ± 0.19	8.93 ± 0.02	101
Orange juice	6.53 ± 0.04	6.71 ± 0.00	97
Peach juice	9.55 ± 0.13	9.10 ± 0.06	105
Fizzy	12.17 ± 0.12	11.64 ± 0.02	104
Energy drink	12.53 ± 0.46	12.425 ± 0.01	101

[a] All measurements were repeated three times and reported as average ± standard deviation.

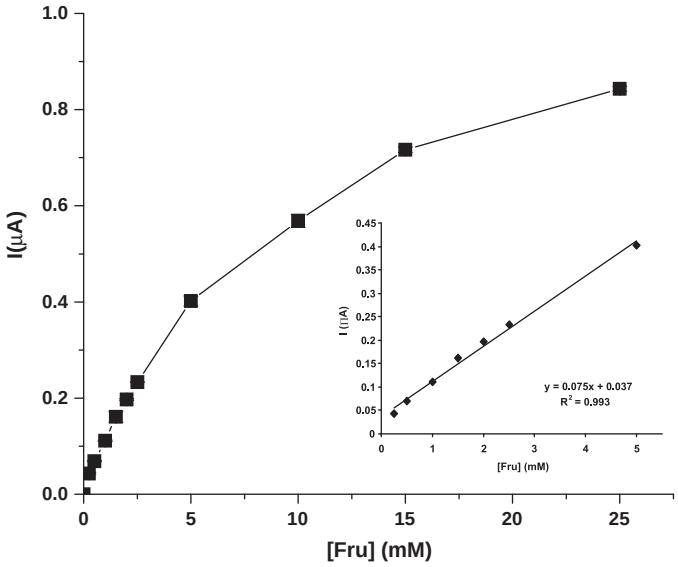

Fig. 6. Effect of fructose concentration on the biosensor response (inset: calibration plot of the FDH/PAMAM biosensor for fructose) (in sodium acetate buffer, 50 mM, pH 4.5; 0.3 V, error bars show S.D.).

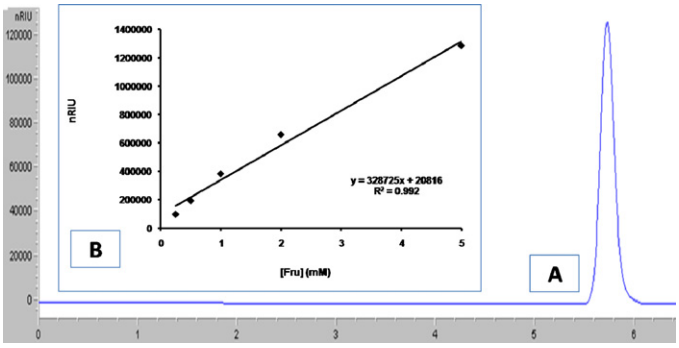

Fig. 7. (A) HPLC peak and (B) the corresponding fructose calibration plot of the HPLC.

4. Conclusion

A sensitive and cost effective detection method for detection of fructose based on the FDH/PAMAM biosensor was demonstrated in this study. The response time taken for the analysis of each sample was short. The analyses performed on the calibration graph for fructose and real samples demonstrated that the system could successfully detect fructose in all the samples. In these conditions the samples did not produce any positive or negative interference. Also the present and our previous studies with PAMAM have shown the importance of dendrimers on biosensor performance such as stability and reproducibility.

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