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# Use of immobilized novel dendritic molecules as a marker for the detection of glucose in artificial urine

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ABSTRACT

In this work, novel dendrons (1J and 2J) were prepared as ferrocene core by means of divergent method. And the expanded second generation dendrons including Pd(II) and Pt(II) ions was synthesized from 2J. Synthesized all compounds were characterized by molar conductivity, magnetic susceptibility, thermal analyses (TGA), FT-IR, UV–Vis, $^{1}$H NMR, AAS, LC-MS methods. Then, glucose oxidase (GOx) enzyme was immobilized on them (2J and expanded 2J). Their optimization parameters were determined separately for free enzyme, 2J-immobilized and expanded 2J-immobilized. Immobilized 2J's palladium complex displayed excellent reusability in cycle analysis. Furthermore, determination of glucose in artificial urine was investigated as catalytic. The results showed that presence of glucose in artificial urine may be determined using to immobilized-dendrons. This research is the first example of immobilization of GOx over dendrons with Ferrocene-Stem.

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## 1. Introduction

Nowadays, materials including ferrocene have attracted significant scientific and industrial attention because of their different applications, such as reagents in analytical procedures [1], industrial catalysts [2] and potential drugs. The use of ferrocene and its derivatives as a glucose biosensor can be cited as an example [3]. Junping Dong et al. reported immobilization of glucose oxidase using ferrocene-modified zeolite composite [4]. Chen et al. fabricated a glucose sensor using chitosan-ferrocene/graphene nanocomposite [5]. Moreover, dendritic structures involving ferrocene have been gathering attention due to their ability to do multielectron-transfers. So that, they provide better stability on pH and temperature changes [6]. Although examples on dendritic derivatives structures such as polyamidoamine dendrimers and poly-amino acid dendrimers were studied in the past, aromatic dendritic molecules have been attracting attention recently [7].

In 2001, the aromatic-dendritic compounds were investigated 1,3,5-triazines in dendrimer synthesis by Bert Verheyde [8]. Aromatic dendritic structures have been intensively investigated due to their potential candidacy in enzyme technology. Cheong-Jin Jang et al. reported on aromatic-flexible dendron molecules [9]. They showed that, introduction of a hydrophilic dendritic segment into an extended rigid aromatic segment can lead to a unique self-assembling system. Haridas et al. designed multi-tier dendrimers with an aromatic core [10]. They said that, multi-tier dendrimers can be easily modified at their surfaces to obtain desired biological properties. Over the past three decades, new materials have been prepared for biotechnological applications. In this technology, hard or soft materials are remarkable. While metal oxide particles or graphene have been used as hard materials. Dendrimers and dendrons have been used in biosensor as soft materials [11]. In biotechnological applications, immobilization is important on support of enzymes, proteins and cells. Aromatic dendritic materials are one from used as support in enzyme immobilization. For enzyme immobilization, supports involving ferrocene and its derivatives are important due to high chemical stability, which are electron transfer mediators (e.t.m). E.t.m. is important for reductase enzymes. Glucose oxidase (GOx) is a reductase enzyme and is well-studied, commercially available in medical and industrial applications for glucose sensing.

In this paper, we report the synthesis of aromatic dendritic spread from the Ferrocene core (2J). First and second generations of dendrons including imine (-CH=N-) have been synthesized (Fig. 1B). Following this synthesis, expanded second generations-dendrons including imine complexes ($Pt^{2+}$ and $Pd^{+2}$) with this central core have been synthesized. Then, Glucose oxidase (GOx) was immobilized on 2J, 2J-$CH_3$Pd and 2J-$CH_3$Pt for determine

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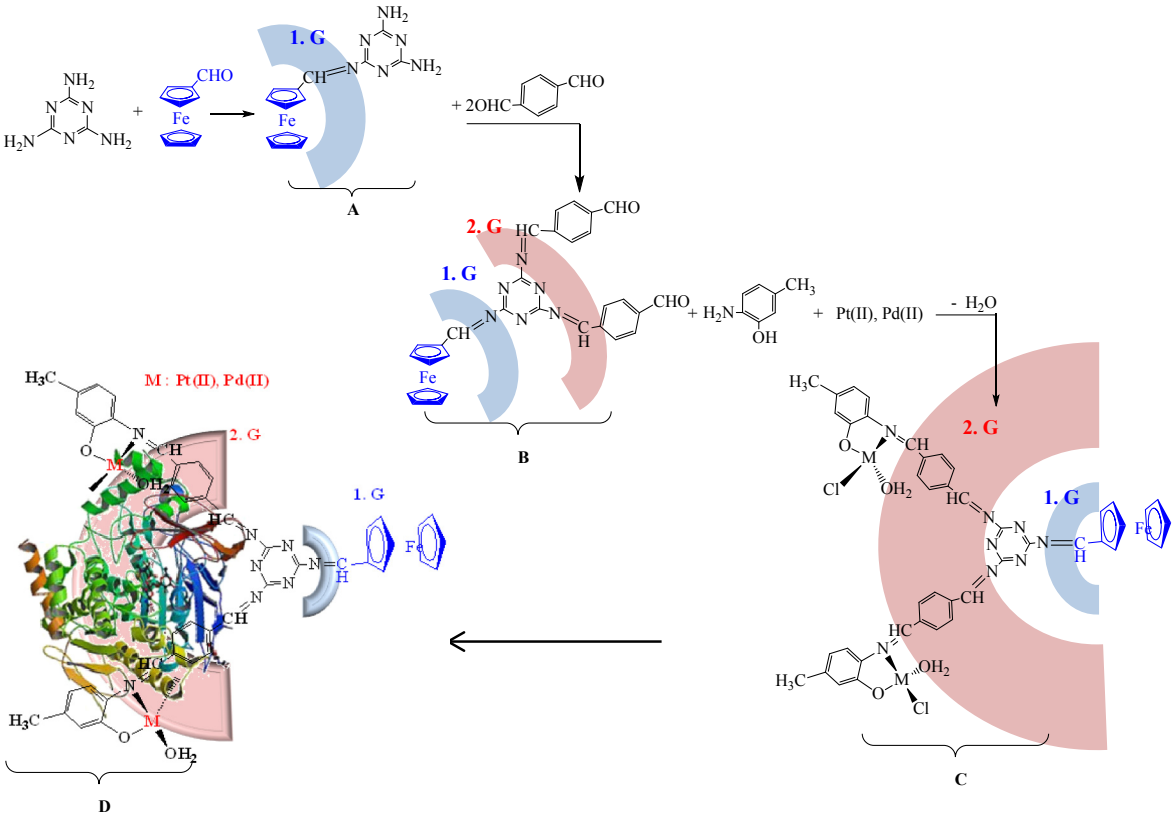



**Fig. 1.** Synthesis rotation of chiral dendrons and their immobilization.

glucose (Fig. 1D). Furthermore, determination of glucose in artificial urine was investigated via immobilized dendrons.

## 2. Experimental

### 2.1. Materials and methods

Infrared spectrum was taken on Bruker Vertex-80 FT-IR spectrometer between 4000 and 400 $cm^{-1}$. $^1H$ NMR spectra were recorded with a Bruker Ultrashield AC300 MHz using TMS as an internal standard and DMSO-$d_6$ as solvent. Electronic spectra were recorded on a UV-1800 ENG240V spectrophotometer in DMSO. The elemental analysis was performed using a LECO-932 CHNS analyzer while the Fe concentrations were determined on an Atomic Absorption Spectrophotometer PerkinElmer 400. Melting points were determined with a Barnstead-Electrothermal-9200 melting point apparatus. The molar conductivities were measured with a inoLab Cond 730 conductivity meter ($1 \times 10^{-3}$ mol $L^{-1}$ in DMSO solution). Magnetic measurements were performed with a Sherwood Scientific magnetic susceptibility balance (Model No: MK 1) at 26 ± 0.1 °C with $Hg[Co(NCS)_4]$ as a calibration.

Ferrocenecarboxaldehyde, melamine, terephthaldialdehyde, 2-Amino-5-methylphenol, $PtCl_2$, $PdCl_2$, toluene, acetone, DMSO, glucose, phenol, 4-aminoantipyrene, horseradish peroxidase were purchased from Sigma-Aldrich. Glucose oxidase from *Aspergillus niger* was purchased from Sigma Chemical Company. Its molecular weight and pI was 160000 Da and 4.2, respectively.

### 2.2. Synthesis of first generation (1J)

To the stirred solution of 0.214 g ($1 \times 10^{-3}$ mol) ferrocenecarboxaldehyde in 20 mL DMF, 0.126 g ($1 \times 10^{-3}$ mol) melamine dissolved in DMF was added dropwise at room temperature. The mixture was stirred for 6 *h* at 80 °C. The complexes were precipitated from solution using acetone and filtered, washed with 1:1 methanol:acetone, and then dried in a vacuum oven. The yield of (1J) was 0.289 g (83%).

### 2.3. Synthesis of second generation (2J)

The second generation dendron including azomethine was synthesized according to Fig. 1A and B. Solutions of 0.322 g ($1 \times 10^{-3}$ mol) (1J) and 0.268 ($2 \times 10^{-3}$ mol) terephthaldialdehyde in 50 mL anhydrous DMF, were added at room temperature. The mixture was stirred for 6 *h* at 80 °C. The complexes were precipitated from solution using acetone and filtered, washed with 1:1 methanol:acetone, and then dried in a vacuum oven. The yield of (2J) was 0.472 g (55%).

### 2.4. Synthesis of (2J-$CH_3$M); M: Pt(II), Pd(II)

The expanded-second generation chiral dendrons were synthesized according to template method (Fig. 1C). General method is that to the stirred solution of 0.554 g ($1 \times 10^{-3}$ mol) of 2J in 20 mL DMF:$C_2H_5OH$ (1:5), 0.246 g ($2 \times 10^{-3}$ mol) 2-Amino-5-methylphenol was added dropwise at room temperature. The mixture was stirred for a hour at 85 °C. $2 \times 10^{-3}$ mol of metal (II) chloride ($PtCl_2$: 0.532 g, $PdCl_2$: 0.355 g) dissolved in DMF (10 mL) was added dropwise to the solution of the mixture, it was then stirred and heated again at 85 °C for 24 *h*. The complexes were precipitated from solution using toluene and filtered, washed with 1:1 toluene-methanol, and then dried in a vacuum oven. Physical

characterization, microanalytical and magnetic susceptibility data of the complexes are given in Table 1.

### 2.5. Immobilization of GOx on (2J), (2J-$CH_3Pd$), (2J-$CH_3Pt$)

0.5 g of 2J, 2J-$CH_3Pd$, 2J-$CH_3Pt$ were placed in a 10 mL water solution of 0.010 $gL^{-1}$ of glucose oxidase at 25 °C in a shaking water bath for 24 *h*. The immobilized dendron was separated and the free enzyme was removed by washing with phosphate buffer (pH: 7.0, 15 mL). The immobilized enzymes were freshly used and then stored at +4 °C. Saturation ratios (s.r) were determined between 97.92 and 98.16% for studied all dendrons, from absorbance value *ca* 507 nm according to the formula given below.

### 2.6. Assay for enzyme activity measurement

Glucose concentration was determined by means of colorimetric method [12]. Effect of pH and temperature on activity of free and immobilized GOx, effect of substrate, storage stability and reusability of immobilized enzyme were determined by using Hasanoglu Ozkan et al. method [13].

### 2.7. Preparation of artificial urine and reusability

Artificial urine prepared by a procedure similar to that in the literature [14]. Then, added 120 mg/dl glucose (Renal Threshold) to artificial urine. For reusability, in a buffer whose pH is 6 has been preferred due to its convenience to people's urine.

## 3. Results and discussion

### 3.1. Characterization of first, second and expanded-second generation chiral dendrons

Analytical data and some of the physical properties of the dendrons, their Pt(II) and Pd(II) complexes are summarized in Table 1. The dendrons are only soluble in DMF and DMSO. The molar conductance values of the dendrons containing Pt (II) and Pd(II) are found to be 15.04,17.64 $\Omega^{-1}cm^2mol^{-1}$ in $10^{-3}$ M DMSO solutions. These dendrons containing Pt (II) and Pd(II) complexes were nonelectrolytic. According to the results of the magnetic moment of dendrons containing Pt (II) and Pd(II), they are diamagnetic. The dendrons containing metal have a square planar geometry.

### 3.2. IR, UV–Visible $^1H$ NMR and LC-Mass spectra of second and expanded-second generation dendron including azomethine

The main IR and UV–visible bands of the first, second and expanded-second generation dendrons are shown in Table 2. IR in first generation dendron including azomethine: 1J spectra show three medium broad band in the region 3433 $cm^{-1}$, 3336 $cm^{-1}$ and at 1633 $cm^{-1}$, assigned to the $\nu_{as}(NH_2)$, $\nu_s(NH_2)$, $\upsilon CH=N_{(Fc)}$ respectively [15]. The IR showed the disappearance of the $NH_2$ bands together with the presence of CH=N band due to reacting the terephthaldialdehyde with amine. It shows that formation of the 2J. IR band of this azomethines in the 1633 $cm^{-1}$ and 1621 $cm^{-1}$ regions are characteristic of $\upsilon(CH{=}N)_{(Fc)}$ and $\upsilon(CH{=}N)_{(melamine)}$ respectively. Furthermore, the appearance of new bands in the 1416 $cm^{-1}$,1211 $cm^{-1}$, 807 $cm^{-1}$ and 541 $cm^{-1}$ regions due to $\delta_{\text{-C-C(rv)}}$, $\delta_{\text{-C-C-H(bv)}}$, $\nu_{\text{-CH (op)}}$ and $\nu_{\text{Fe-Cp(ring)}}$ of ferrocene group, respectively [16].

The IR bands in expanded second generation dendron including azomethine {(2J-$CH_3$M) M: Pt(II) and Pd(II)}: The azomethine bands in the IR spectra of the expanded second generation dendrons appear in the range 1635–1642 $cm^{-1}$, somewhat differently than observed for the second generation dendrons. The bands in the 3065-3057 $cm^{-1}$ and 539-540 $cm^{-1}$, 805-815 $cm^{-1}$, 1240-1242 $cm^{-1}$, 1451-1440 $cm^{-1}$ regions may, respectively, ascribe to υCH aromatic vibrations and ferrocene vibrations. The IR spectra of all expanded second generation dendron exhibit characteristic bands of coordination water at *ca*. 3300, 840 and 770 $cm^{-1}$ assigned to $\nu$(OH), ρr(OH) and $\rho_W(OH_2)$ vibrations, respectively. These observations clearly suggest that the water molecules are coordinated to the metal ion (Fig. 1C). The appearance of weak new bands in the 565–575 and 425-429 $cm^{-1}$ regions due to $\nu$(M-O) and $\nu$(M-N), respectively [16]. On the basis of the i. r. spectral results, it may be deduced that the anion of the dendrons are coordinated to the metal ion as bidentate ligand (Fig. 1C).

The electronic spectra of dendrons in DMSO show bands *ca.* 290 nm are attributed to the azomethine $n\rightarrow\pi^*$(-CH=N) transition. The bands at higher energies (200–250 nm) are associated with the benzene $\sigma\rightarrow\sigma^*$ and $\pi\rightarrow\pi^*$ transition [17]. The d-d bands from the spectra of the Pt(II) complex having low intensities appeared at 579 nm and was assigned to transition in square planar environment around, while the band centered ca 450 nm is assigned to metal to ligand charge-transfer [18]. LC-mass spectroscopy for the given $[M]^+$ 322.2753 (*m/z*: %100) for (1J) and $[M\text{-}2H]^+$ 554.4532 (*m/z*: %100) for (2J). These results indicate that (1J) and (2J) have been synthesized according to the suggested structure (Fig. 1). LC-mass spectroscopy of other all substances were not obtained due to poorly soluble.

The $^1H$ NMR spectrum of (1J) recorded in DMSO-$d_6$ showed the following signals: $NH_2$ and Fc(-CH) proton observed at 5.98 ppm (s, 4H) and 2.89 ppm (s, 5H), 2.73 ppm (m, 2H), 2.59 ppm (m, 1H), 2.29 ppm (m, 1H); respectively. The $^1H$ NMR spectrum of (2J) recorded in same solvent. Aromatic protons observed at 6.9 ppm (s, 4H) and 7.4 ppm (s, 4H). Two –CH=N protons of (2J) observed at

**Table 1**
Analytical data and some of the physical properties of synthesized Dendron.

| Compound | Chemical formula ($M_w$) | Colour/ $\mu_{eff}$, m.p. | Elemental analysis Found (calculated) % | | | | | | Conductance Values (μS/cm) |
|---|---|---|---|---|---|---|---|---|---|
| | | | C | H | N | Fe | Pt | Pd | |
| 1J | $C_{14}H_{14}N_6Fe$ (322.06g) | Light Brown/ D, 160 | 52.13 (52.16) | 5.03 (4.35) | 26.30 (26.08) | 16.79 (17.23) | – | – | 1.01 |
| 2J | $C_{30}H_{24}N_6O_2Fe$ (556.39g) | BlackD, 320* | 62.57 (64.70) | 3.93 (4.31) | 14.17 (15.10) | 9.81 (10.03) | – | – | 1.08 |
| 2J-$CH_3Pt$ | $C_{44}H_{40}N_8O_4FeCl_2Pt_2$ (1261.76g) | Bitter BrownD, >187* | 41.11 (41.85) | 3.63 (3.17) | 9.07 (8.88) | 4.44 (4.43) | 29.90 (30.92) | – | 15.04 |
| 2J-$CH_3Pd$ | $C_{44}H_{40}N_8O_4FeCl_2Pd_2$ (1084.43g) | Dark Brown/ D, 175 | 49.01 (48.69) | 4.09 (3.69) | 10.05 (10.33) | 4.80 (5.15) | – | 19.00 (19.63) | 17.64 |

*decomposition temperature D: diamagnetic.

**Table 2**
Important IR vibration frequencies ($cm^{-1}$) and UV–Visible spectrum values (nm) of synthesized Dendron.

| Compound | $\nu_{as/s(NH2)}$;$\nu_{-O-H/}$ $\nu_{-C-H(aldehyde)/}$ $\nu_{-C=O}$ | $\nu_{-(C-H)alip.}$, $\nu_{-(C-H)arom}$, $\rho_{r(OH)}$, $\rho w_{(H2O)}$ | $\nu_{-CH=N\ (Fc)}/\nu_{-CH=N\ (m)}/\nu_{-CH=N(5\text{-}An)}$, $\nu_{M\text{-}O}$, $\nu_{M\text{-}N}$ | Fc Vibrations, $\nu_{-Fe\text{-}Cp(ring)}$, $\nu_{-C\text{-}H(op)}$,$\delta_{-C\text{-}C\text{-}H(bv)}$ $\delta_{-C\text{-}C(as)}$ | $\lambda_{max}(10^{-4}$ M DMSO) $\sigma\rightarrow\sigma^*$, $\pi\rightarrow\pi^*_{(C=N)}$, $n\rightarrow\pi^*_{(C=N)}$, $d\rightarrow d_{(Fc)}$; (M) |
|---|---|---|---|---|---|
| 1J | 3433/3336; -/-/- | –, -, -, - | 1633/-/-, -, - | 541, 805,1208, 1410 | 204 (1.12), 254 (2.27), 291 (0.86), 446 (0.28); - |
| 2J | -/-;-, 2757-2805/ 1735 | –, 3054, -, - | 1633/1621/-, -, - | 541, 807,1211, 1416 | 207, 257 (1.57), 291 (0.35), 432 (0.15); - |
| 2J-$CH_3$Pt | -/-;3275/ –/ – | –, 3065, 835, - | 1635/1611/1605, 565, 425 | 539, 805,1240, 1451 | 208 (0.36), 258 (2.05), 295 (1.03), 311 (0.92), 451 (0.44); 579 (0.13) |
| 2J-$CH_3$Pd | -/-;3287/ -/ - | -, 3057, 837, 767 | 1642/1625/1611, 575, 429 | 540, 815,1242, 1440 | 208, 260 (1.57), 297 (0.84), 311 (0.78), 402 (0.20); 544 (0.12) |

*$d\rightarrow d_{(Fc)}$: 441 (0.69), op: out of plane, bv: bond vibration, as: asymmetric stretch.

7.95 ppm (s, 1H) and 8.12 (s, 2H). The $^{13}C$ NMR spectra data of the 1J and 2J are not accord due to poorly soluble.

### 3.3. Immobilization studies for biocatalysis second and expanded-second generation dendrons including azomethine

As known, flavin adenine dinucleotide (FAD) is located in active center of glucose oxidase enzyme. When FAD is reduced to $FADH_2$ by taking electron, glucose is oxidized to gluconolactone and then gluconic acid (Fig. 2).

### 3.4. Influence of pH and temperature on the enzyme activity

As known, pH is one of the important parameter capable of altering enzymatic activities. In this study, while 2J has two optimum pH, other all dendrons including azomethine have one. 2J-$CH_3$Pt-GOx has the same pH with free-GOx (pH5), 2J-$CH_3$Pd-GOx has optimum pH 6. For difference in optimum pH, we can say is caused from conformational changes of enzyme or amino acid side chains. As known, the active site of the enzyme contains three amino acid side chains which are His516, Glu412 and His559. This amino acids are important role in catalysis. More clearly, His516 with p*K*a = 6.10 and Glu412 with p*K*a = 4.3 which is hydrogen bonded to His559, with pKa >8. In this study, having optimum pH 4 and 7 of immobilized glucose oxidase on all of studied dendron may be due to the support of Glu412 and His516 residues of enzyme in catalysis [13]. In Table 3 is given p*K*a data of free histidine and glutamine. Histidine has two group including –N atom and their pKa are, 9.18 and 6.10; respectively for amino (-$NH_2$) and imidazole group. The reason of the optimum pH 6 and pH 7 for studied support, hydrogen ions in amino and imidazole groups may be said to be effective.

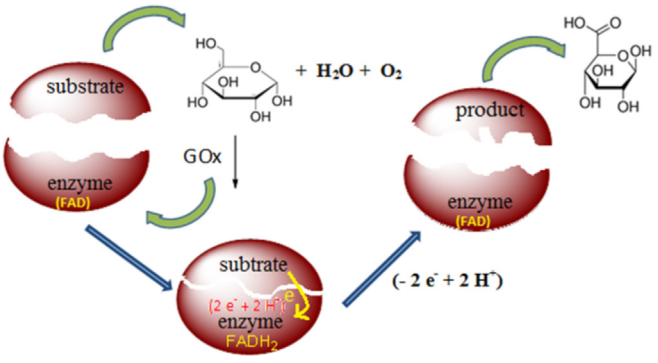



**Fig. 2.** Oxidation scheme of glucose.

The relative activity increases gradually with the temperature while reaching a maximum value at optimum temperature, and then it decreases due to the loss of enzymatic activity what is caused by the denaturation of GOx. Immobilized GOx on 2J-$CH_3$Pd-GOx has shown two optimum temperatures as shown Table 3. Immobilized GOx on dendron including Pd(II) has activity at higher temperature than Pt(II).

### 3.5. Kinetic parameters for free GOx and immobilized glucose oxidase

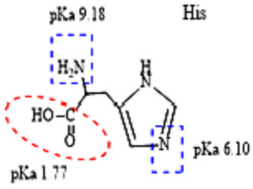
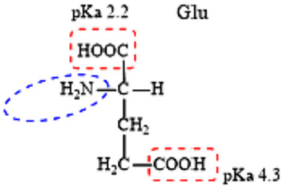
As shown that Table 3, kinetic parameters were studied for free-GOx and immobilized-GOx all optimum at pH (pH: 4.0, 5.0, 6.0 and pH: 7.0) and temperature (40 °C, 60 °C and 80 °C). As presented in Fig. 3, the activities of the immobilized enzymes with various substrate concentrations were plotted as *Lineweaver-Burk* graphs to calculate *V*max and *K*m values [19] and then calculated for immobilized GOx to the 2J and it's derivatives. *K*m value of 2J is lower than expanded second generation dendron molecules. As seen that Table 3, *K*m value of 2J indicates that the enzyme binds as efficiently with the substrate. But, *V*max value of 2J-$CH_3$Pt-GOx is higher than others. In other words, the catalyst rate of 2J-$CH_3$Pt-GOx increased.

### 3.6. Reusability numbers and storage stabilities of immobilized dendrons

The reusability is importance for industrial applications. The immobilized second and expanded-second generation dendrons were used repeatedly 6 times in a hour due to quickly of incubation time. As shown in Fig. 4A, after the twenty use of 2J dendron which is not including metal ion, the immobilized enzyme retained nearly 64.97% of their original activity at determinated optimum conditions. Reusability number of the immobilized GOx showed a significative decreasing in the activity from the first run to until the twentieth run dendron for free metal ion. Reusability of studied all dendrons in artificial urine is shown Fig. 4B.

After 10th use, reusability of 2J-$CH_3$Pd-GOx is the best, because of the pH value of the urine same the pH value of its. Storage stability measurements were conducted in the dry form. Immobilized enzymes exhibited good storage stability for 12 months. After six months of storage, immobilized enzyme lost activity and retained *ca* 85% activity. Especially; for immobilized dendrons reduced activity at the end of the twelfth month.

**Table 3**
Kinetic parameters (*Km*/*V*max; mM/mM $min^{-1}$) and optimum conditons of second and expanded-second generation dendron including azomethine and free GOx, and formula of side chains.

| Symbol of Dendrons | pH | T (°C) | $pK_a$ of His and Glu | Dendron-GOx | | Free enzyme | |
|---|---|---|---|---|---|---|---|
| | | | | *Km* | *V*max | *Km* | *V*max |
| 2J-GOx | 4;<br>7 | 40;<br>60 |  | 10.49;<br>28.79 | 16.86;<br>15.53 | 157.81;<br>8.08 | 36.23;<br>21.83 |
| 2J-$CH_3$Pd-GOx | 6, | 60,<br>80 | | 92.19,<br>60.33 | 23.64,<br>11.29 | 4.40,<br>7.07 | 4.15,<br>4.44 |
| 2J-$CH_3$Pt-GOx | 5 | 60 |  | 122.62 | 79.80 | 138.06 | 111.11 |

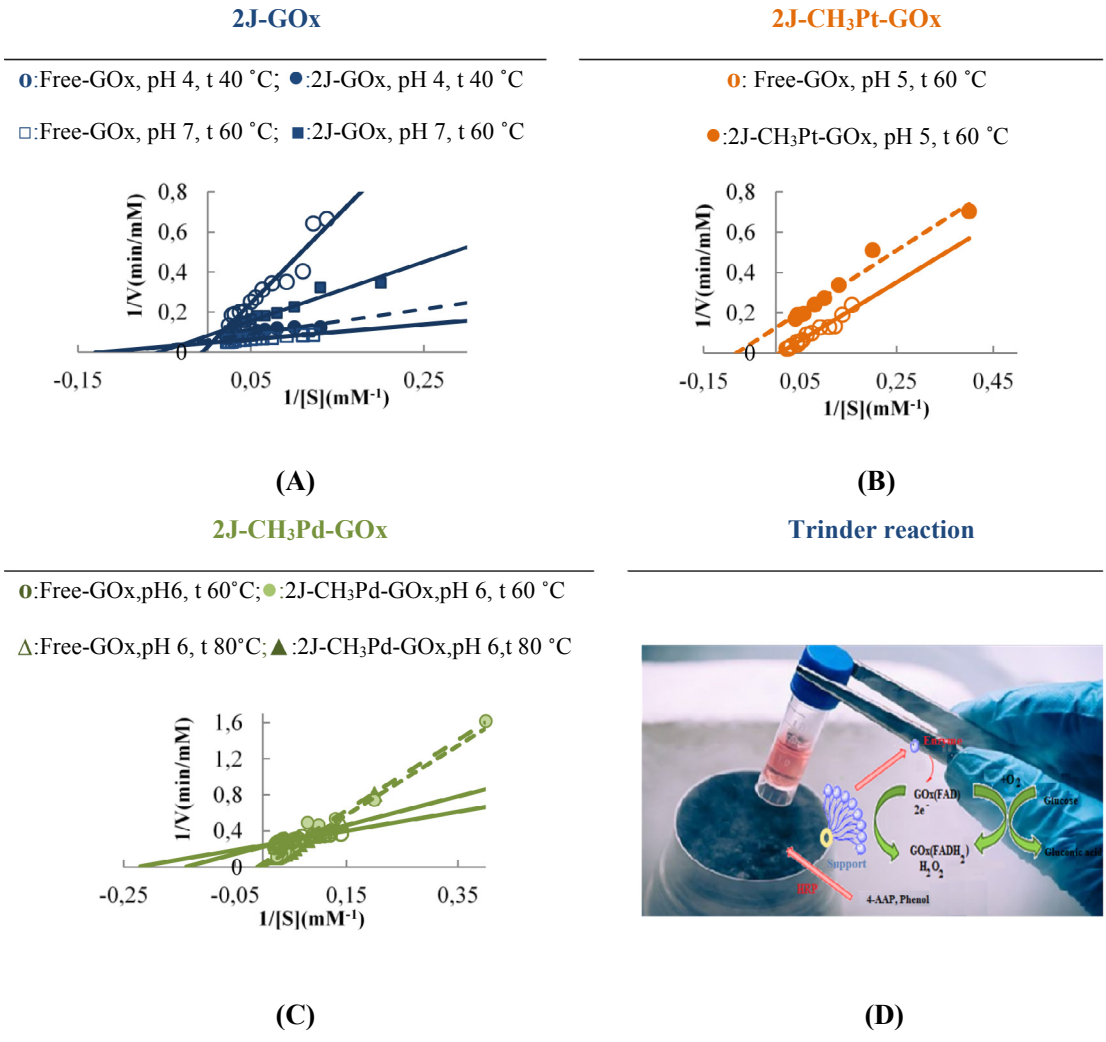



**Fig. 3.** *Lineaweaver-Burk* graphs of immobilized dendrons (A), (B), (C) and Trinder reaction for the determination of glucose (D).

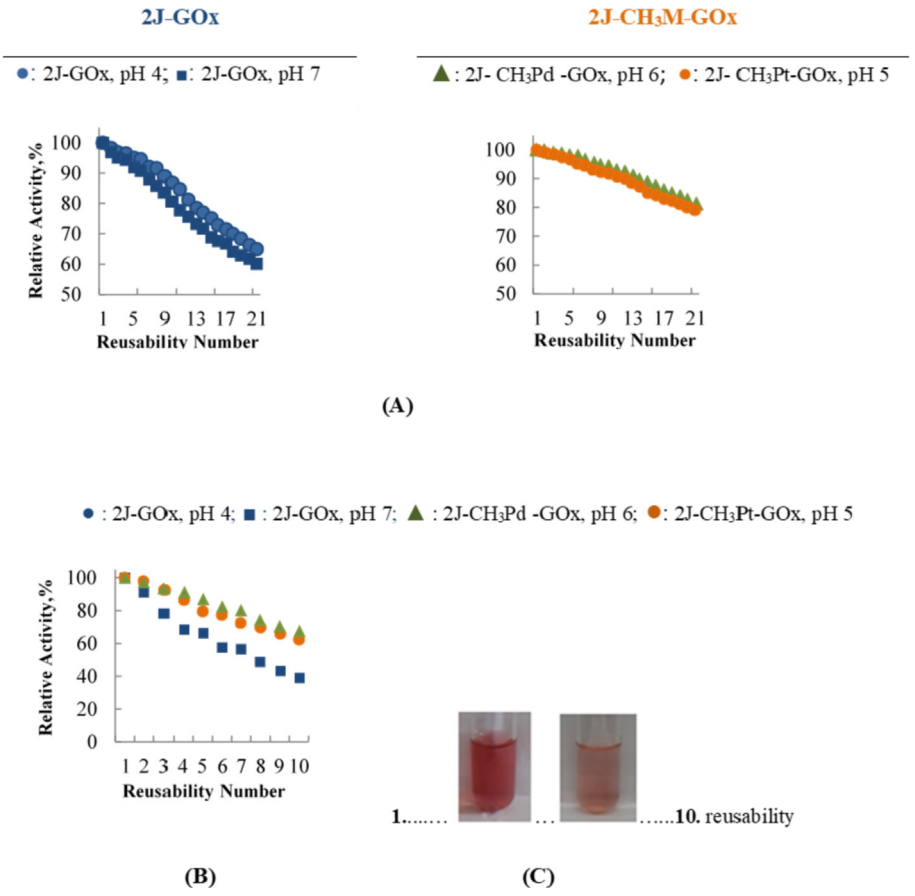



**Fig. 4.** Reusability number of dendron-GOx in buffer solution (A) and artificial urine (B), Image. reusability number of 2J-$CH_3$Pd-GOx (C).

## 4. Conclusions

In this work, novel dendrons based on ferrocene have been presented to investigate the reduction of glucose as catalytic. Ferrocene is a rigid central core that can be mediator to prepare novel dendritic materials. The divergent growth of dendrons gives rise to dendrons 1J and 2J. The second generation dendron has been completely complexed with Pt(II) and Pd(II), as confirmed by different spectroscopic techniques. And then, Glucose oxidase enzyme (GOx) immobilized on them. Glucose oxidase (GOx) immobilization has been successfully fabricated for the detection of glucose. Studied immobilized-dendrons have a wider working range than free enzyme. 2J-$CH_3$M-GOx dendrons (M; Pt(II)/Pd(II)) have shown thermally more stable than 2J dendron. According to kinetic parameters, interaction enzyme-substrate in 2J-dendron is better than 2J-$CH_3$M-GOx dendrons. 2J-$CH_3$Pd-GOx was reused 20 times (in buffer solution) and the residual activity was about 81.35% its initial activity. Furthermore, this is the first study to investigate the determination of glucose in artificial urine (pH 6) by using immobilized-dendron [14]. Among immobilized-dendrons, reusability of 2J-$CH_3$Pd-GOx is better due to optimum pH 6.

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