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Immobilization of GOx on Trp / Trp-Fc Functionalized Nanospheres: Improved of Reusability and Stability

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Abstract

In the study, new nanospheres support containing ferrocene were synthesized and characterized, then the properties of nanospheres as biocatalysis were researched. Novel support was prepared from (Aminomethyl)polystyrene (AMP), tryptophan (Trp) and ferrocene aldehyde (Fc) and the enzymatic properties of immobilized glucose oxidase enzyme (GOx) were investigated. Modified polystyrene was characterized IR spectra, Gel permeation chromatography and Scanning electron microscopy. Ferrocene-tagged nanospheres-GOx shows high reusability and storage capacity and fast incubation time determination of glucose. After 12 months, immobilized GOx to the AMP-Trp and AMP-Trp-Fc retained ca 20 % and 40 % for their original activity, respectively. High activities were found for modified polymers AMP-Trp-Fc retains more than 35 % of the initial activity after fifteenth successive cycles, which is a perfect performance.

1. INTRODUCTION

In recent years, investigation on chemically modified nanospheres has been extensively studied due to their small size and large surface area [1]. Nanospheres are solids whose size vary from 10 nm to 100 nm. It is well known that the advantages in using this kind of materials in drug delivery and tissue engineering [2]. Furthermore, chemically modified nanospheres are the useful to improve the immobilization [3].

Nanospheres act as very effective support materials for enzyme immobilization, because of their ideal characteristics for stabilizing the key factors that define biocatalysts efficiency, including high volue ratio surface area and influential enzyme loading [4-7]. A lot of articles on immobilization of enzymes on different nanospheres have been published earlier [8,9].

In this work, Glucose oxidase (GOx) enzyme was immobilized on the nanospheres. It is known, GOx is redox enzyme and it catalyzes the oxidation of glucose to gluconic acid [10-12]. GOx enzyme has been extensively used in fabrication of determination for glucose. GOx contains flavin adenine dinucleotide (FAD). FAD is a redox cofactor. In the catalysis reaction, FAD works as electron acceptor and then it is reduced to $FADH_2$. Generally, redox mediators are used for ensure more effective electron transport to the active center of the enzyme [13, 14]. As a mediator, ferrocene (Fc) is a good choice due to its reversibility and generation of stable redox states. Fc has played an important role in chemical and biological processes because of it shows good electron transfer kinetics [15].

The aim of this work is to evaluate the effect of ferrocene-containing immobilized nanospheres on kinetic parameters.

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2. EXPERIMENTAL

2.1. Materials and Methods

Glucose oxidase (β-D-glucose: oxygen-l-oxidoreductase, EC 1.1.3.4) from Aspergillus niger was purchased from Sigma Chemical Company (SIGMA, 49180). Its molecular weight and pI was 160,000 Da and 4.2, respectively. (Aminomethyl)polystyrene (AMP), tryptophan (Trp) and ferrocene aldehyde (Fc) were purchased from Sigma (St. Louis, MO). All the other chemicals used in this work were provided by Sigma-Aldrich and used without further purification. IR spectra were recorded on a Nicolet 6700 GA-FTIR instrument in KBr pellets. The GPC measurements were recorded on a Waters 1500 Series Gel permeation chromatography (GPC). Elemental analyses were carried out with a LECO, CHNS-932 instrument at Bozok University. Scanning electron microscopy of the Au coated compounds was done by using a JEOL JEM 100 CX II scanning electron microscope (JEOL, Peabody, MA, at Gazi University).

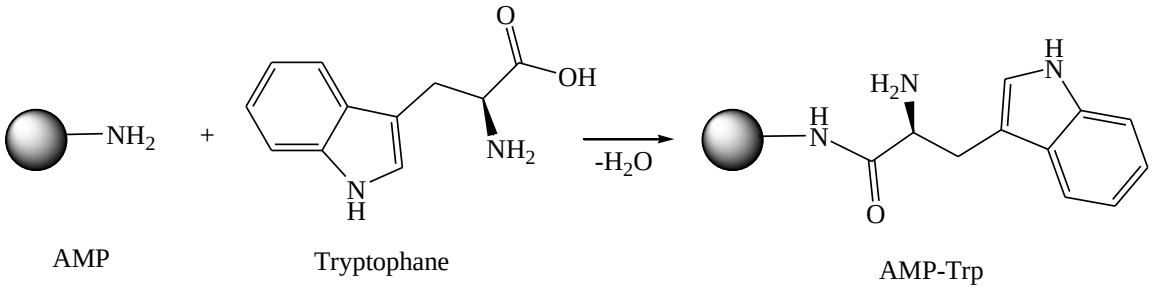
2.2. Preperation of Nanospheres Attached Tryptophan(*AMP-Trp)* and Ferrocenylidene-Tryptophan (*AMP-Trp-Fc)*

2.2.1. Synthesis of AMP-Trp

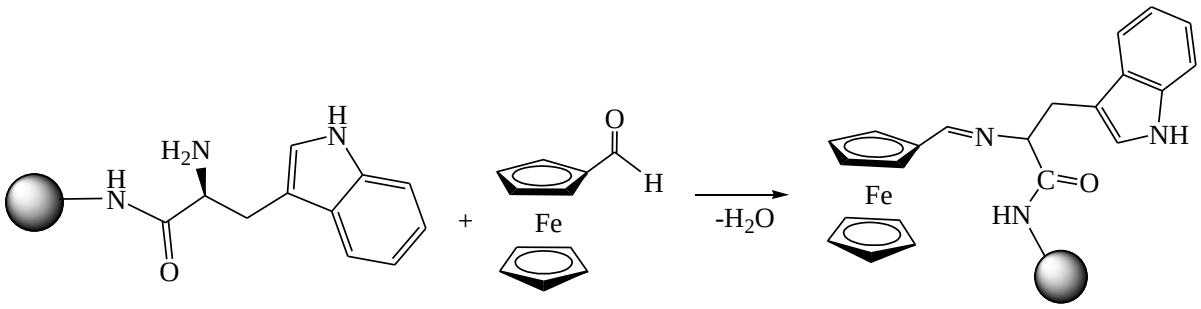
The polymeric *AMP-Trp* was prepared by reacting of (aminomethyl) polystyrene (AMP) (1 g, 50-100 mesh, 2.0 mmol/g $–NH_2$ loaded, 1 % cross-linked (Aldrich)) in hot DMF (15 mL) with tryptophane (1.0 mmol) in EtOH (10 mL). Tryptophane solutions was slowly added by the drop wise on amine solutions while stirring through 30 min. Then the reaction mixture was boiled and stirred under a reflux condenser *ca*. 3 *h*, at 70 °C. After the mixture cooling to room temperature, modified polymer was poured into the aceton and washed by adding acetone. The resulting clear yellow product was filtered and dried in the oven and kept with desiccator over anhydrous $CaCl_2$ (Figure 1A).

2.2.2. Synthesis of AMP-Trp-Fc

AMP-Trp (7 mmol) in hot DMF (10 mL) to a solution of ferrocene (7 mmol) in DMF (10 mL) while stirring. The stirring was continued until the solution was refluxed *ca*. three hours later room temperature and under N_2(g). Then mixture was poured into the acetone (30 mL). The resulting solid was collected from acetone (AMP-Trp-Fc). The solid was filtered and then it was dried. It kept with desiccator over anhydrous $CaCl_2$ (Figure 1B) [16].



(A)



(B)

Figure 1. *Synthesis of AMP-Trp (A) and AMP-Trp-Fc (B)*

2.3. Immobilization of GOx on AMP-Trp and AMP-Trp –Fc

The polymers (AMP-Trp and AMP-Trp-Fc) (0.5 g) 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 8 *h*. The immobilized polymers were 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 were determined as 92.43 % for AMP-Trp and 96.48 % for AMP-Trp-Fc, from absorbance value in 507 nm.

2.4. Assay for Enzyme Activity Measurement

A colorimetric method based on Trinder's reaction was used for the determination of glucose concentration [17]. The reaction was started by adding 20 mg glucose on the immobilized support. Then glucose is enzymatically oxidized to gluconic acid in the presence of glucose oxidase and hydrogen peroxide occurs. The following reaction; hydrogen peroxide reacts with 4-aminoantipyrene (4-AAP) and phenol to form pink colored quinoneimine dye (at 507 nm) (Figure 2). This mixture was removed after incubating the reaction mixture at 20 °C for 20 min under continues stirring. Finally solution was transferred quartz cuvette for measurement.

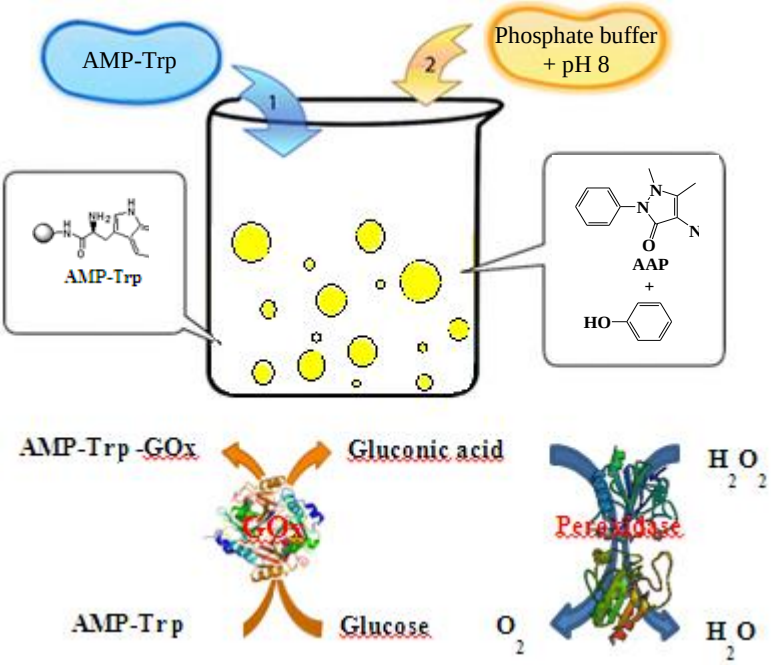


Figure 2. *Trinder reaction of immobilized enzyme and shematic image reactants*

2.5. Effect of pH and Temperature on Activity of Free and Immobilized GOx

Optimum pH for free and immobilized glucose oxidase were determined by measuring the activities of free and immobilized enzymes in buffers of different pH values ranging from 3.0 to 9.5. The buffers used were pH: 3.0-4.0 (CH_3COONa=CH_3COOH); pH: 5.0 (NaH_2PO_4=H_3PO_4); pH: 6 (Na_2HPO_4=NaH_2PO_4); pH: 7.0-9.0 (NaH_2PO_4=Na_2HPO_4); pH: 9.0 ($Na_2B_4O_7$=NaOH). In temperature studies, free and immobilized enzymes were incubated in the reaction mixtures at different temperatures ranging from 20 to 90 °C. The activities of free and immobilized enzymes were plotted against respective temperature.

2.6. Effect of Substrate

To determine the extent at which immobilization affects the enzyme activity, K_m and V_{max} were determined at optimum pH 6 and 8, optimum temprature 30, 40 and 70 °C [17]. Free and immobilized enzyme were incubated with different substrate concentrations (0.5–50 mM) in phosphate buffer of pH 6, 8 and they were assayed for enzyme activity at 30, 40 and 70 °C recommended temperature for enzyme assays.

2.7. Storage Stability and Reusability of Immobilized Enzyme

Storage stability experiments were carried out to determine the stabilities of immobilized enzymes after storage in dry conditions at +4 °C during the 12 months. The enzyme activity was measured every 30 days. Observed results are compared to the initial activities. To evaluate the reusability, the glucose oxidase immobilized polymeric supports were also washed with buffer solution after every run and reintroduced into a fresh solution. Reaction cycles under the conditions (pH 6.0 and 8.0 at room temperature) described previously were performed. The enzyme activity was measured at every 15 min.

3. RESULTS AND DISCUSSION

3.1. Characterization of Support Nanosphere

Polydispersity index (PDI) and some of the physical properties of all studied polymers were given in Table 1. Molecular weight distribution (Mn) and average molecular weight (Mw) were determined by gel permeation chromatography (GPC) [18]. According to GPC, modified polymers have a narrow molecular weight distribution (PDI: 1.84 and 2.04 for AMP-Trp and AMP-Trp-Fc, respectively). SEM images of AMP-Trp and AMP-Trp-Fc polymers are shown in Figure 3. SEM images of studied polymers were not markedly different from each other. These images indicates that the protection sphere-structures are of AMP-Trp and AMP-Trp-Fc polymers.

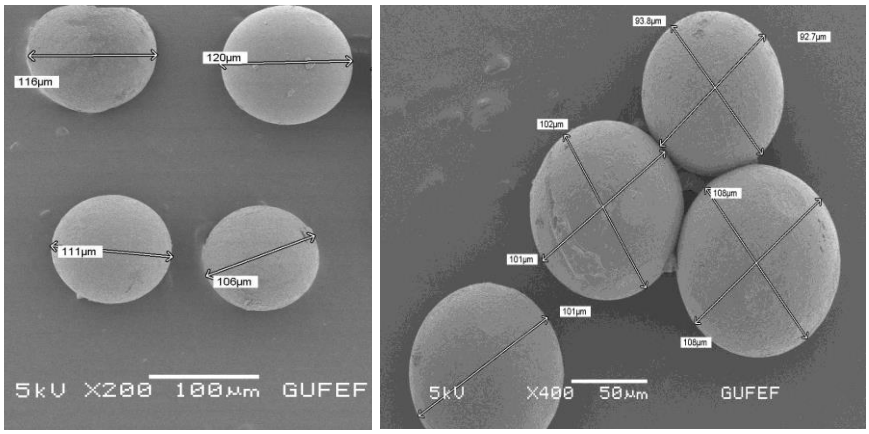


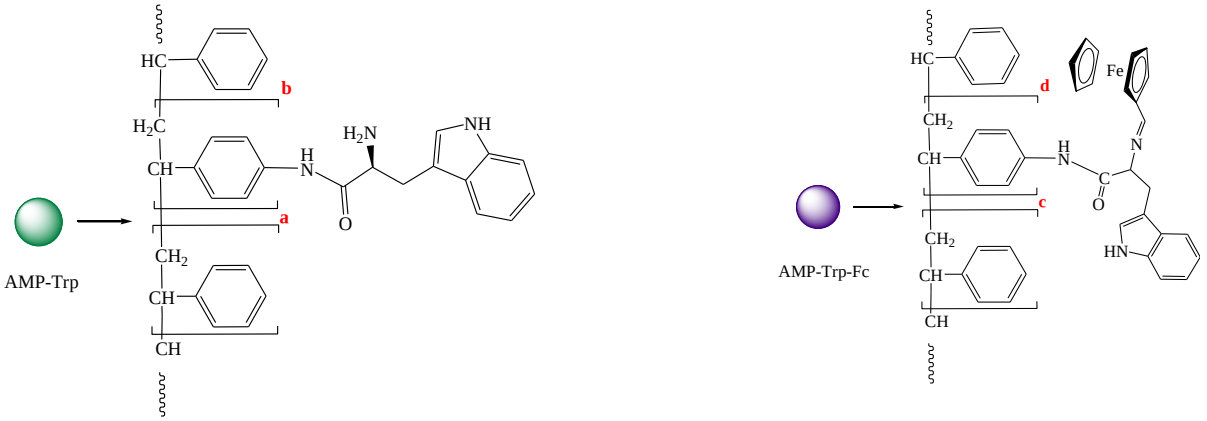
Figure 3. *Sem images of AMP-Trp and AMP-Trp-Fc*

3.2. IR Spectra of Ligand (AMP-Trp) and Ferrocene-Tagged Nanomaterial (AMP-Trp-Fc)

The characteristic peak of IR spectra of AMP-Trp and AMP-Trp-Fc support polymers are given in Table 1. IR spectra of AMP-Trp shown three medium broad band in the region 3440, 3390 and 2990 cm^{-1} assigned to the $\upsilon NH_{(sym,asym)}$, υ(CH) aromatic, respectively [19]. Three overtone peaks showed in *ca.* 1941, 1872, 1802 cm^{-1} for support coordination polymers. IR bands in the *ca.* 3010 and 2950 cm^{-1} regions are characteristic of υ(CH) aromatic, υ(CH) aliphatic for AMP-Trp and AMP-Trp-Fc. Furthermore, 490 and 1385/1420 cm^{-1} are characteristic of $\upsilon_{ring\text{-}metal}$ and $\upsilon(C\text{-}C)_{Fc}$, respectively. Imine band was observed in 1648 cm^{-1} for AMP-Trp-Fc.

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

Compound Colour		IR Spectra					Elemental analysis Found (calculated)%			
		ν_{NH2}	$\nu_{(C\text{-}H)aliph.}$	$\nu_{(C\text{-}H)arom.}$	$\nu_{CH=N}$	$\upsilon_{(C\text{-}C)Fc}$	C	H	N	Fe
AMP-Trp (**a**=5, **b**=1) *M_w:825	Yellow	3440 3390	2900	2990	-	-	85.86 (85.82)	7.11 (7.15)	5.13 (5.09)	- -
AMP-Trp-Fc (**c**=8, **d**=1) *M_w: 1332	Orange	3400	2952	3010	1648	1385	84.58 (84.62)	6.86 (6.83)	3.12 (3.15)	4.19 (4.20)



*Mw: Weight average molecular weight (values are according to elemental analysis). Mw and Mn: according to GPC.

3.3. Immobilization Studies

In this study, glucose is oxidized to gluconic acid and flavin adenine dinucleotide (FAD), which is a prostetic group in enzyme structure, FAD is reduced to $FADH_2$ by taking electron. $FADH_2$ in enzyme is oxidized by giving its electrons to oxygen in solution to make the reaction reversible. Thus, enzyme comes to its previous form (Figure 2). The maximum activity was obtained at pH 6.0 and 8.0 for the immobilized enzyme [20]. The amount of loaded enzyme per gram of polymer found according to saturation ratio (s.r.).

3.4. Immobilized GOx Influence of pH and Temprature on the Enzyme Activity

It is known, the pH is one of the important parameter capable of altering enzymatic activities in aqueous solution. Immobilization of enzyme is likely to result in conformational changes of enzyme or amino acid side chains resulting in a variation of optimum pH. AMP-Trp-Fc-GOx has optimum pH= 6, while all AMP-Trp has pH= 8. They are illustrated in Figure 4. It is known that the active site of the enzyme contains three amino acid side chains that they are intimately involved in catalysis (His516 with pKa= 6.9, Glu412 with pKa= 3.4 and His559, with pKa>8). The protonation of each of these residues have a

strong influence on all rate constants in the catalytic mechanism [20]. It is known that histidine has three pKa which are 1.77, 9.18 and 6.10, respectively for carboxylic acid (-COOH), amino (-NH_2) and imidazole group (Figure 4). The reason of the optimum pH= 8 for studied support, hydrogen ions in amino and imidazole groups may be said to be effective. So, base form is predominantly effective at pH= 8.

The effect of temperature on the activity of immobilized GOx is shown in Figure 4. Immobilized glucose oxidase on AMP-Trp-GOx has shown one optimum temperature (40 °C) and AMP-Trp-Fc-GOx has shown two optimum temperatures (30, 70 °C) as shown Table 3. The results show that, immobilized GOx enzyme become more stable against heat and denaturing agents even different conditions.

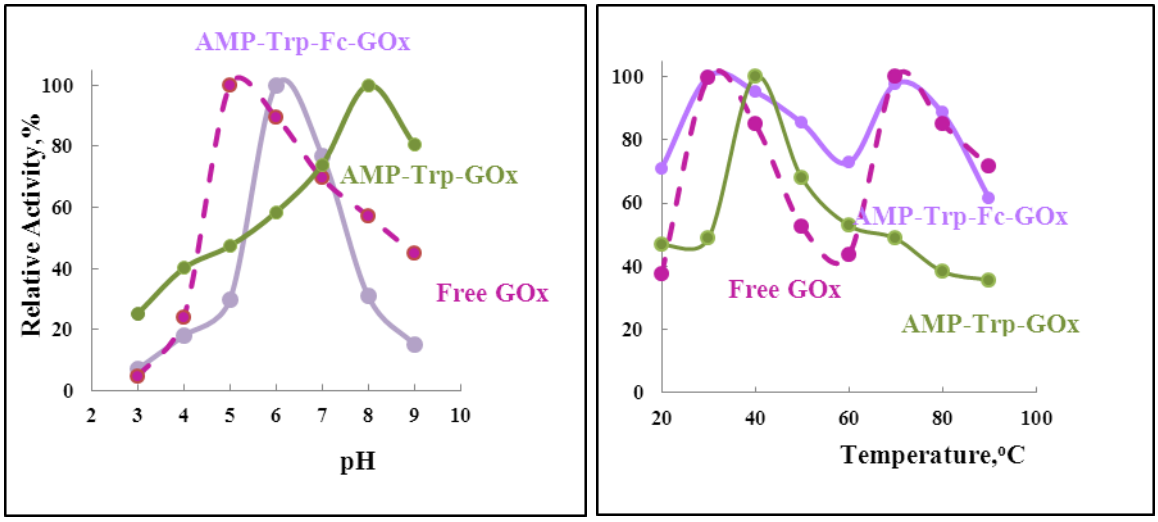
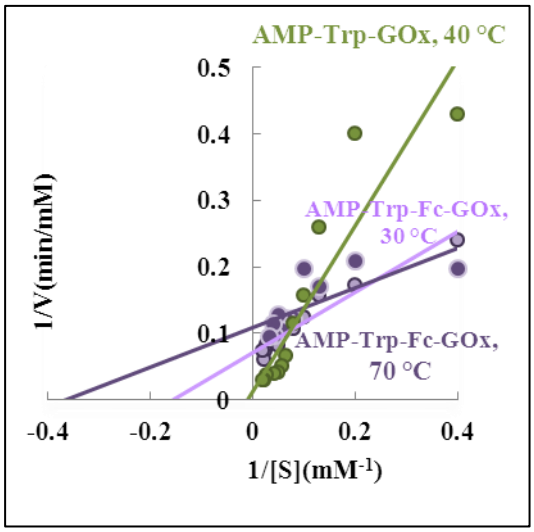


Figure 4. *Effect of pH and temperature on enzyme activity of studied polymers*

3.5. Kinetic Parameters for Free GOx and Immobilized Glucose Oxidase

The activities of the free and immobilized enzymes with various substrate concentrations were plotted as Lineweaver–Burk graphs to calculate V_{max} and K_m values (Figure 5). The V_{max} value defines the maximum velocity when all of enzyme is saturated with substrate. K_m, the substrate concentration at which an enzyme reaches ½ V_{max} reflects the effective characteristic of the enzyme and depends upon both partitioning and diffusion.

Kinetic parameters were studied for free GOx and immobilized glucose oxidase all optimum at pH (pH 6.0 and 8.0) and temperature (30, 40 and 70 °C). The effect of the substrate concentration on the reaction rate was studied using varying initial concentrations (0.5–50 mM) of β-D-glucose substrate. The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) of immobilized GOx and free GOx were calculated from the *Lineweaver-Burk* plots (Figure 5). K_m and V_{max} values for immobilized GOx: for the AMP-Trp support, pH 8, 40 °C, K_m: 92.26 mM, V_{max}: 74.07mM.min.$^{-1}$ and for the AMP-Trp-Fc support, pH 6, 30 °C, K_m:6.50 mM, V_{max}: 14.22 mM.min.$^{-1}$; 70 °C K_m:2.75 mM, V_{max}: 9.17 mM.min.$^{-1}$



Working Conditions		Sphere	Supports
pH,	t (°C)	(AMP-Trp)-GOx	(AMP-Trp-Fc)-GOx
8.0,	40	92.26 / 74.07	-
6.0,	30	-	6.50 / 14.22
6.0,	70	-	2.75 / 9.17

Figure 5. *Kinetic parameters (K_m / V_{max}) for (AMP-Trp)-GOx and (AMP-Trp-Fc)-GOx*

3.6. Storage Stability and Reusabilty

The storage stability of an enzyme is one of its most important characteristics. Enzymes often lose their activities during the storage. Free and immobilized enzymes were stored in a dark bottle at +4 °C for 12 months. After the first month, free enzyme activity decreased, however, the activity of the immobilized enzymes was preserved even after 12 months. These results indicate that the immobilized GOx retains its high enzymatic activity which was very important for the preperation of the proposed enzyme support in low-cost application. After 12 months, immobilized GOx to the AMP-Trp and AMP-Trp-Fc retained *ca* 20 % and 40 % for their original activity, respectively. High activities were found for modified polymers (Figure 6).

The reusability was tested because of its importance for repeated applications in a batch reactor [21]. The immobilized polymers were used repeatedly two times in a hour due to very quickly of incubation time. After the 15th use of AMP-Trp and AMP-Trp-Fc, the immobilized enzyme retained nearly 20 % and 35 % of their original activity, respectively.

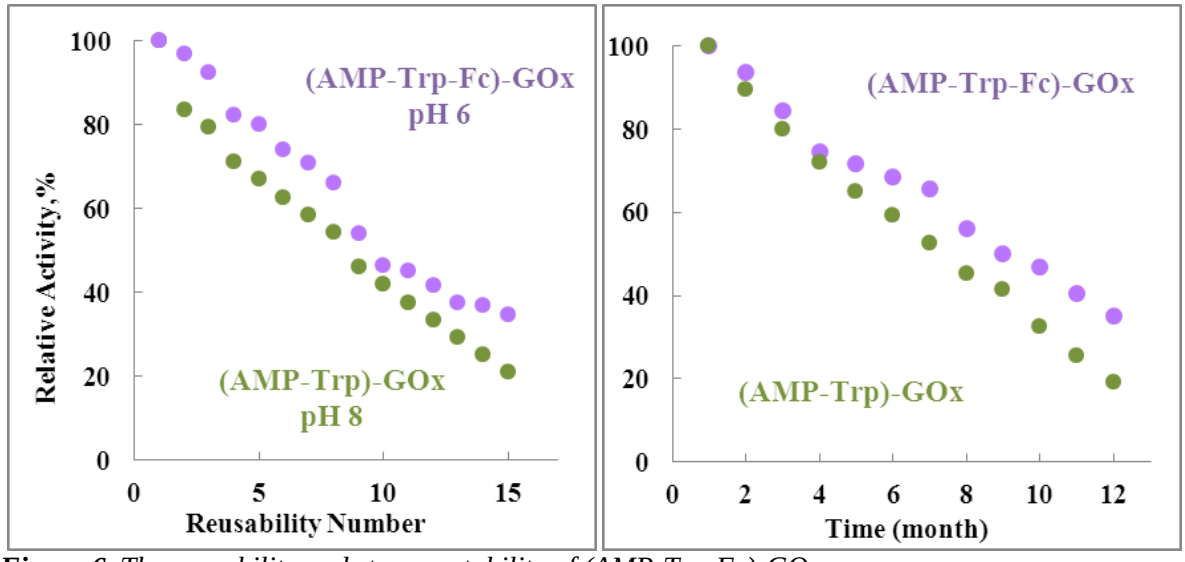


Figure 6. *The reusability and storage stability of (AMP-Trp-Fc)-GOx*

4. CONCLUSIONS

In conclusion, we have optimized a procedure to covalently immobilize the enzyme glucose oxidase on novel polimeric nanospheres. The amine and carbonyl groups in the structure of the polymeric support contribute to the overloading of the GOx enzyme to support. According to the results obtained, the reproducibility after immobilization on the support polymer containing Fc was found to be quite high. These results showed that the polymeric support prepared from ferrocene (Fc) and (aminomethyl)polystyrene (AMP) may be choice as a biosensor for glucose detection. Furthermore both of supports can also be used to investigate the biocatalytic properties of other enzymes.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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