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Design Biocatalyst: the Influence of the Nature of the Carrier on the Catalytic Properties of Immobilized Glucose Oxidase

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Mesoporous silica, alumina and zirconia, were utilized as appropriate support for the formation of magnetite nanoparticles by in-situ crystallization and GOx immobilization. Magnetic and nonmagnetic supports were functionalized with amino groups and using glutaraldehyde as a linker. A comparative study showed that the nature of the carrier and the presence of magnetite nanoparticles all affect the catalytic properties of biocatalysts and their stability. Biocatalysts based on immobilized glucose oxidase demonstrated high catalytic activity (preservation of the activity of the free enzyme up to 98 %) and stability in D-glucose oxidation to D-gluconic acid at pH 4-8 and temperature of 30-60 °C.

1. Introduction

Immobilized native enzymes called biocatalysts received considerable attention due to their successful applications in a number of reactions including syntheses of pharmaceuticals, their intermediates, vitamins and dietary supplements (Dwevedi, 2016). They are also utilized in remediation processes of polluted water, air and soil by efficient removal of persistent organic pollutants (Brena et al., 2006). It is noteworthy that immobilized enzymes have an advantage over native enzymes such as 100 % selectivity (Drout et al., 2019), and at the same time, can be recovered from catalytic reactions (Bilal et al., 2020). They show much higher stability compared to native enzymes in the broad pH (Cui et al., 2018) and temperature range (Virgen-Ortiz et al., 2017). Nowadays, the key challenges in the field of immobilized enzymes are the increased enzyme loading on support to enhance the biocatalyst efficiency, the protection of the activity of the native enzyme, and the facile isolation of biocatalysts (Devine et al., 2018). Overcoming these challenges will allow one to improve the target product quality and reduce the price tag of the process (Dwevedi, 2016). The immobilized enzyme employed in the industry is glucose oxidase (GOx, EC 1.1.3.4). Due to its low cost and good stability, GOx is efficiently used for biosensors to determine blood glucose content. In a commercial organic synthesis, GOx is utilized for the preparation of D-gluconic acid. D-gluconic acid and its salts are widely used as dietary supplements (to control acidity) and components of pharmaceuticals (Pal et al., 2016).
The most universal method to preserve the enzyme activity and selectivity is a covalent attachment to functionalized supports, allowing one to control the enzyme surface density. Hierarchical porous supports (polymers, metal oxides) showed advanced properties due to large surfaces areas (Sulman et al., 2019) and variations of pore sizes (Kumari et al., 2020), as well as magnetic recovery when magnetic nanoparticles (NPs) are incorporated in the pores (Matveeva et al., 2021). The opportunity to achieve certain crowding of enzymes in pores (similar to crowding in cells) can further boost the biocatalyst activity (Chapanian et al., 2014).
As one of the most promising nanostructured materials as versatile carriers for enzyme immobilization, magnetic nanoparticles (MNPs) (Marino et al., 2021) have gained special attention due to their important characteristics, including high specific area, elevated enzyme loading ability, controllable particle size, modifiable surface, and most importantly, easily, and rapid separation from solutions by applying an external

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magnetic field. It has been reported that surface coating or modification of MNPs with a variety of complex materials, such as polymers, silica, Metal-Organic Frameworks (MOFs), and carbon-based materials, might allow to improve some properties and provide potential industrial exploitability (Martinez et al., 2022). The various immobilization methods and biocatalytic activity were performed for various non-magnetically and magnetically responsive supports to immobilize peroxidases. Conclusively, magnetically separatable peroxidases show more stability towards extreme temp. and pH conditions and can be used for repeated cycles than free and non-magnetically separatable peroxidase. (Khalid et al., 2022).
For the immobilization of glucose oxidase on magnetic silica, the application of the GOx on SiO_2-coated Ni-Co ferrite nanocomposites can be noted in recent publications (Nasir et al., 2021). This study used a sol-gel auto-combustion approach to make silica (SiO_2)-coated Ni-Co ferrite nanocomposites that were used as a platform for enzyme immobilization. The immobilized GOx exhibited increased catalytic stability and after 10 times of reduction, it still had 69 % catalytic activity. Overall, the immobilized GOx displayed higher operational stability than the free enzyme under severe circumstances and was easily recovered by magnetic separation (Nasir et al., 2021).
Based on the literature data in this work, it was interesting to synthesize magnetic carriers based on mesoporous oxides in the pores of which magnetite nanoparticles were formed by in-situ crystallization. The novelty of the study consists in the comparison of the catalytic properties of GOx immobilized on magnetic and non-magnetic carriers. In this paper, we report GOx immobilization on (i) parent oxides (SiO_2, Al_2O_3, ZrO_2), (ii) magnetic oxides (Fe_3O_4-SiO_2, Fe_3O_4-Al_2O_3, Fe_3O_4-ZrO_2), which are modified by amino groups on the surface and then by glutaraldehyde to create aldehyde moieties for the easy GOx attachment. Magnetically recoverable biocatalysts developed displayed the higher catalytic activity in D-glucose oxidation to D-gluconic acid compared to GOx immobilized on parent oxides.

2. Experimental

2.1 Materials

Iron (III) nitrate nonahydrate (98 %), ammonium hydroxide solution (28 %), glycerol (≥ 99.5 %), silica gel (99 %, 6 nm, 200-425 mesh), D-glucose (99.5 %), glucose oxidase lyophilized powder (*Aspergillus niger*, 174.9 U/mg), glutaraldehyde solution (GA, 25 %) and phosphate-buffered saline (PBS buffer) were purchased from Sigma-Aldrich and used as received. 3-Aminopropyltriethoxysilane (APTES 98 %), and tetraethyl orthosilicate (TEOS, 99 %), were purchased from Fluka and used without purification. Ethylene glycol (99.5 %), succinic acid (99 %), and urea (99 %) were all purchased from TCI and used without purification. Ethanol was received from Pharmco-Aaper and used as received.

2.2 Synthesis of SiO_2-Fe_3O_4, Al_2O_3-Fe_3O_4, ZrO_2-Fe_3O_4

Magnetically separated oxides were obtained by in-situ crystallization of Fe_3O_4 nanoparticles in mesopores of the parent oxide. SiO_2-Fe_3O_4 was synthesized using a modified procedure. In a typical experiment, 2.5 g of silica gel and 0.03 mL of the ammonium hydroxide solution (28 %) were stirred in 10 mL of deionized water for 12 h, separated by bench-top centrifugation at 4,000 rpm and then washed five times with water, using centrifugation to remove supernatant. Then treated silica was added to 10 mL of the ethanol solution containing 2.0 g of $Fe(NO_3)_3 \times 9H_2O$ (5 mmol), stirred for 24 h and then allowed to air dry at room temperature upon stirring. The resulting powder was mixed with 70 drops of EG and placed in a ceramic boat, which was then heated in a tube furnace under argon at 350 °C for 6 h with a heating rate of 2 °C/min. After cooling, the sample was ground into a fine powder with a mortar and pestle. The powder was washed five times in acetone using magnetic separation with a permanent magnet and then dried. The samples Al_2O_3-Fe_3O_4 and ZrO_2-Fe_3O_4 were synthesized analogously.

2.3 Functionalization with APTES

The sample of support (SiO_2, Fe_3O_4-SiO_2, Al_2O_3, Fe_3O_4-Al_2O_3, ZrO_2, Fe_3O_4-ZrO_2) 0.3 g was suspended in 8 mL of the APTES aqueous solution. For the preparation of this solution, glacial acetic acid was added to 10 mL of water until pH 4 was reached. Then 8 mL of the acidified water was mixed with 0.8 mL of APTES and used to suspend the support. After that, 4 mL of glycerol was added. The reaction was carried out under stirring at 90 °C for 5 h. Then, the support was washed with water (three times) and methanol (five times) and dried in a vacuum oven overnight.

2.4 Attachment of GA and GOx

The dry modified support (0.10 g) was added to 20 mL of glutaraldehyde (GA) solution in the PBS buffer and stirred for 1 h. To prepare the GA solution, 0.08 mL of 25 % GA in water was mixed with 20 mL of the PBS buffer at pH 7.0. The support functionalized with GA was magnetically separated and washed with water five

times. At the same time, 10 mg of GOx were incubated and stirred in 20 mL of the PBS buffer for 1 h. The GOx solution was added to the GA functionalized support and stirred for 1 h. Then the biocatalyst was magnetically separated. The samples Al_2O_3-GOx, SiO_2-GOx, Fe_3O_4-Al_2O_3-GOx, Fe_3O_4-SiO_2-GOx, ZrO_2-GOx, Fe_3O_4-ZrO_2-GOx were synthesized.

2.5 Biocatalyst activity assays

D-glucose oxidation was carried out at atmospheric pressure in the temperature range of 30 - 60 °C, in the pH one of 4-8 in a double-jacketed three-neck round-bottom flask equipped with a gas inlet, overhead stirrer(80 rpm), and a reflux condenser, serving as a gas outlet. The reaction temperature was maintained by circulating a heating medium in the flask jacket. After reaching the required temperature, 15 mL of the 0.1 M PBS buffer (pH 6.0), 10 mg of D-glucose and 0.11 g of the biocatalyst were loaded in the flask and kept stirring for 60 min. Oxygen with a feeding rate of 440-450 mL/min was used as the oxidant. Each experiment was replicated three times. The error of the experimental determination of relative activity was 3.8 %. After the reaction, the catalyst was separated with a rare-earth magnet and the reaction mixture was analyzed using HPLC, UltiMate 3000 (ChromaTech, Russia) equipped with a refractometer detector and a ReproGel H Column (500x10 mm, number of theoretical plates 160,000). The H_2SO_4 solution (9 mM) was used as eluent at the 0.5 mL/min rate for 30 min at the eluent pressure of 6.5 kPa and the column temperature of 250 °C. Pure D-gluconic acid was used for product identification.

The amount of GOx attached was evaluated determining immobilization coefficient (IC) (Nguyen et al., 2017):

$$IC = (C_0\text{-}C_1)/C_0 \times 100\ \% \quad (1)$$

where C0 is the initial GOx amount (μg/mL) and C1 is the GOx amount (μg/mL) in the filtrate after immobilization and the biocatalyst magnetic separation. The amount of GOx in the solution before and after immobilization was determined via the activity in the D-glucose oxidation, considering that in all experiments the initial GOx activity was 174.9 U/mg.

The relative activity for biocatalysts was calculated from the formula:

$$R(\%) = \frac{A_0 - A_i}{A_0} \times\ 100\ (\%) \quad (2)$$

where R is the relative activity (%), A_0 is the D-glucose concentration before the reaction, and A_i is the D-glucose concentration after the reaction. (Zhao et al., 2018).

In order to show that the enzyme keeps immobilized and does not pass into solution (when studying stability), a standard method (Memon et al., 2018) of determination GOx relative activity by monitoring the oxidation of 2,2'-azino-bis(3-ehylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) at 415 nm was used.

2.6 Characterization

Electron-transparent specimens for transmission electron microscopy (TEM) were prepared by placing a drop of a sample suspension onto a carbon-coated Cu grid. Images were acquired at an accelerating voltage of 80 kV on a JEOL JEM1010 transmission electron microscope. X-ray powder diffraction (XRD) patterns were collected on an Empyrean from PANalytical. X-rays were generated from a copper target with a scattering wavelength of 1.54 °A. The step size of the experiment was 0.02.Magnetic measurements were performed on a Quantum Design PPMS-14 magnetometer using the system with DC measurement capabilities. The sample (50 mg) was placed in a standard gelatin capsule.

3. Results and Discussion

To synthesize magnetic supports (Fe_3O_4-SiO_2, Fe_3O_4-Al_2O_3, Fe_3O_4-ZrO_2), we used mesoporous silica, and alumina (Jaquish et al., 2018) and zirconia (Haskell et al., 2020) with 6 nm pores, forming iron oxide NPs in the pores. A typical TEM image and XRD pattern of Fe_3O_4-SiO_2 are presented in Figure 1. The TEM and EDS map data show an even distribution of magnetic nanoparticles in the pores of the support. The size of magnetite nanoparticles was 2-2.5 nm. The XRD pattern (Figure1b) shows a broad reflection around 22° two theta which is assigned to amorphous silica as well as a set of sharper Bragg reflections whose intensity and positions are typical of spinel (magnetite, Fe_3O_4). The TEM image and XRD pattern of magnetic alumina are presented in Figure 2. The TEM image shows a porous material with small NPs inside the pores. The XRD pattern is complicated due to the crystallinity of parent alumina. It shows a set of reflections (notated in black) which can be assigned to γ-Al_2O_3 as well as several (partially overlapping) reflections which can be ascribed to spinel Fe_3O_4.

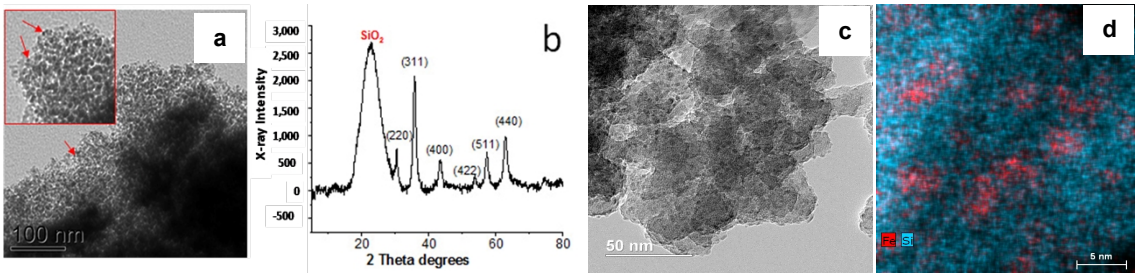

Figure 1: TEM image (a, c) and XRD pattern (b) of Fe_3O_4, EDS map for the Fe-Si mix (d) for the 6nm-SiO_2-Fe_3O_4 sample

Figure 3 shows representative TEM images of ZrO_2 and Fe_3O_4-ZrO_2 based on the same zirconia. Both images look nearly identical because the high electron density of ZrO_2 makes iron oxide NPs poorly visible. A few possible small iron oxide NPs are indicated by red arrows in the inset of Figure 3b. The XRD pattern of Fe_3O_4-ZrO_2 shows a set of reflections which can be ascribed to spinel Fe_3O_4.

To assess the composition of magnetic silica, alumina, zirconia and the oxidation state of iron, the XPS measurements were carried out for Fe_3O_4-SiO_2, Fe_3O_4-Al_2O_3 and Fe_3O_4-ZrO_2. For all three samples, the state of iron was estimated as magnetite.

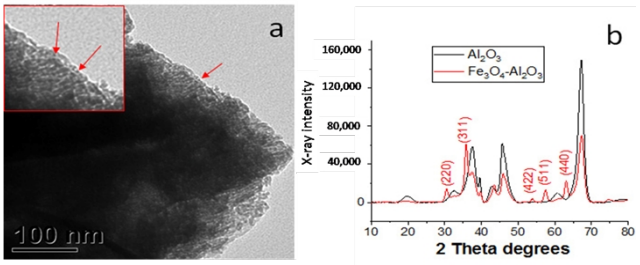

Figure 2: TEM image (a) and XRD pattern (b) of Fe_3O_4-Al_2O_3. Inset in (a) shows a higher magnification image. Red arrows indicate iron oxide NPs

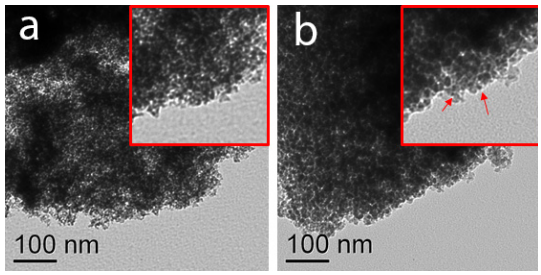

Figure 3: TEM images of ZrO_2 (a) and Fe_3O_4-ZrO_2 (b)

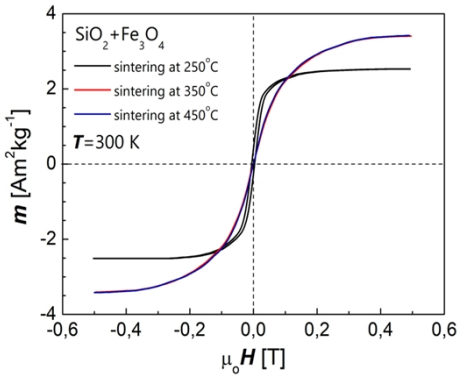

Figure 4: Hysteresis loop of Fe_3O_4-SiO_2

The magnetization curves of Fe_3O_4-SiO_2 (Figure 4) show a mean saturation magnetization of ~3.0 emu/g (Fe_3O_4 NPs formation temperature 350 and 450 °C) which is consistent with small magnetic NPs. Nevertheless, due to the cooperative interactions of numerous NPs, the material is magnetically recoverable in water and buffers within 60 s. At the synthesis temperature of magnetite NPs 250 °C, the magnetization had a lower value of about 2 emu/g. Stronger magnetic properties are probably associated with a change in the structure or chemical composition of the composite when heated to 350 °C.

To covalently attach GOx to the magnetic support, the latter was first functionalized with amino groups using APTES, then reacted with the excess of GA, followed by the reaction of aldehyde groups of the GA linker with amino groups of GOx. To assess the amount of immobilized GOx, i.e. immobilization coefficient, IC, we evaluated the catalytic activity of the supernatant after the enzyme attachment and the catalyst separation(calculation according to equation 1). According to these data, both parent silica and alumina attach

~65 % of GOx calculated from the total amount used for functionalization. For the supports containing magnetic NPs, attached GOx increases to 70 %. For zirconia, the immobilization coefficient was 70 % and for Fe_3O_4-ZrO_2 – the 76 %. Immobilization efficiency is slightly higher for magnetic samples which could be ascribed to the magnetite NP contribution.

All the developed biocatalysts have been studied in D-glucose oxidation in the pH range of 4-8 (Figure 5 shows only the maximum and minimum values). To characterize the catalyst behavior, we used the relative activity parameter (calculation according to equation 2), which allows one to assess the biocatalyst activity (Figure5a). The dependence of the relative activity for native GOx shows a sharp maximum at 100 % at pH 6, while at the higher and lower pH values the relative activity sharply decreases. For all immobilized GOx biocatalysts developed in this work, the relative activities are high in the whole pH range. The relative activity at pH 6 for Fe_3O_4-Al_2O_3-GOx, Fe_3O_4-SiO_2-GOx, Fe_3O_4-ZrO_2 –GOx is 89 %, 95 % and 98 % accordingly. At the same time, at pH4, the relative activity of the free GOx is 20 %, while the activity of the Fe_3O_4-SiO_2-GOx is 82 %. Similarly, we observe at pH 8, the relative activity of the free GOx is only 30 %, while the Fe_3O_4-SiO_2-GOx shows 81 % relative activity.

The dependence of the biocatalyst relative activity(calculation according to equation 2) on the temperature is presented in Figure 5b. The free GOx shows the maximum activity at 40 °C, followed by a sharp decrease in the product yield, indicating a loss of enzyme activity. In the case of immobilized GOx, we observed a lower relative activity by 2-20 % at 40 °C (depending on the support) as compared to that of native GOx. With an increase in temperature to 60 °C, the immobilized GOx becomes more stable and the Fe_3O_4-Al_2O_3-GOx, Fe_3O_4-SiO_2-GOx, Fe_3O_4-ZrO_2–GOx show 84, 91 and 76 % relative activity accordingly. The comparison of magnetic biocatalyst with nonmagnetic analogues demonstrates the increase of the immobilized GOx relative activity in the presence of Fe_3O_4 NPs, which is attributed to an intrinsic enzyme-like activity of magnetite, enhancing the activity of the immobilized GOx and creating a synergetic effect.

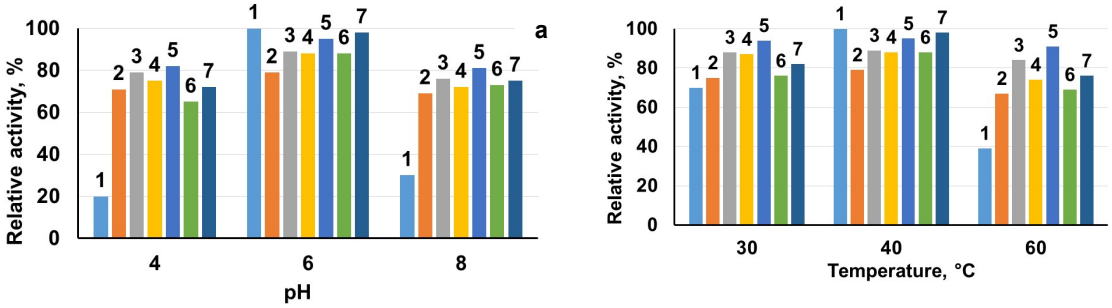

Figure 5: Influence of pH at 40 °C (a) and temperature at pH 6 (b) on the relative activity of free and immobilized GOx. There are designations: 1 – free GOx, 2- Al_2O_3-GOx, 3 - Fe_3O_4-Al_2O_3-GOx,4 - SiO_2-GOx, 5 - Fe_3O_4-SiO_2-GOx, 6 - ZrO_2-GOx, 7 - Fe_3O_4-ZrO_2–GOx

To test the stability of biocatalysts in repeated use, we carried out ten successive experiments of the D-glucose oxidation in the optimal conditions (pH 6 and 40°C). Data show that for parent alumina, silica and zirconia the relative activity decreases by 30, 24 and 14 %, after ten successive experiments. For biocatalysts based on magnetic alumina, silica and zirconia, the relative activity decreases only by 13, 11 and 7 %, after the same number of consecutive reactions, demonstrating much higher catalyst stability. The enzyme immobilized on mesoporous oxides showed higher catalytic activity and stability under harsh conditions compared to the native enzyme. Magnetic biocatalysts turned out to be more active than non-magnetic ones, which is explained by the influence of magnetite nanoparticles possessing enzyme-like activity.

4. Conclusions

We designed highly efficient magnetically recoverable enzymatic catalysts by tethering GOx via a GA linker to magnetic mesoporous silica, alumina and zirconia. The biocatalysts were characterized using a combination of physicochemical methods and tested in D-glucose oxidation to D-gluconic acid. The formation of Fe_3O_4 NPs with a size of 2-2.5 nm was observed in mesopores of all three oxides. A comparison of magnetic and parent silica, alumina and zirconia-based biocatalysts reveals that the incorporation of magnetite NPs in the support increases catalytic activity. At the same time, silica and zirconia-based catalysts are more active than their alumina-based analogues. An increase in the catalytic activity of biocatalysts based on a magnetic carrier occurs due to the enzyme-like properties of iron oxide. The high stability of Fe_3O_4-SiO_2-GOx and Fe_3O_4-ZrO_2–GOx after easy magnetic separation coupled with the exceptional activity in a broad pH and temperature range makes these catalysts promising for practical applications.

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