Glucose Oxidase Immobilized on Magnetic Zirconia: Controlling Catalytic Performance and Stability

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ABSTRACT: Here, we report the structures and properties of biocatalysts based on glucose oxidase (GOx) macromolecules immobilized on the mesoporous zirconia surface with or without magnetic iron oxide nanoparticles (IONPs) in zirconia pores. Properties of these biocatalysts were studied in oxidation of D-glucose to D-gluconic acid at a wide range of pH and temperatures. We demonstrate that the calcination temperature (300, 400, or 600 °C) of zirconia determines its structure, with crystalline materials obtained at 400 and 600 °C. This, in turn, influences the catalytic behavior of immobilized GOx, which was tentatively assigned to the preservation of GOx conformation on the crystalline support surface. IONPs significantly enhance the biocatalyst activity due to synergy with the enzyme. At the same time, neither support porosity nor acidity/basicity shows correlations with the properties of this biocatalyst. The highest relative activity of 98% (of native GOx) at a pH 6−7 and temperature of 40−45 °C was achieved for the biocatalyst based on ZrO2 calcined at 600 °C and containing IONPs. This process is green as it is characterized by a high atom economy due to the formation of a single product with high selectivity and conversion and minimization of waste due to magnetic separation of the catalyst from an aqueous solution. These and an exceptional stability of this catalyst in 10 consecutive reactions (7% relative activity loss) make it favorable for practical applications.

INTRODUCTION

Enzymes are biological macromolecules which function as catalysts in biochemical processes in nature. It is worth noting that enzymatic catalysts possess a number of advantages including selectivity in mild reaction conditions compared to metal-containing catalysts.1,2 Biocatalysts based on immobilized enzymes received considerable attention, especially in the last 10 years, due to higher stability of immobilized enzymes versus native enzymes at various pH and temperatures.3,4−7 This and the possibility of separation from reactions solutions for repeated use allow for enzyme conservation. The major challenges in biocatalysis are preservation of native enzyme activity, high loading of enzymes on a support, and facilitated separation of catalysts to name a few.8 It is reported that the support structure and morphology can strongly influence the enzyme properties.9,10 We believe that the above challenges can be met by developing magnetic porous supports, allowing for high enzyme loading and easy magnetic separation from reaction solutions, while a covalent attachment via a linker should prevent denaturing. However, to the best of our knowledge, such studies are very limited and include immobilization of lipase to be used in ester hydrolysis and β-lactamase utilized in the hydrolysis of penoxymethyl penicillin,11,12 as well as our preceding paper on immobilized glucose oxidase (GOx) on mesoporous silica and alumina containing magnetite nanoparticles (NPs) for D-glucose oxidation to D-gluconic acid.13 D-Gluconic acid and its salts are utilized in pharmaceuticals and food industries as well as in the production of household products.14,15 We demonstrated the best catalytic results in D-glucose oxidation for a magnetic silica support,13 but we also observed its significant attrition because porous silica is brittle.16 It is well known that zirconia is much more attrition-resistant than silica,17,18 and it contains much stronger Lewis acid sites,19,20 which could influence the catalytic reaction with the enzyme via ionization of its amino acid residues.

Macro- and mesoporous zirconia has been previously employed for attachment of enzymes.21 For example, porous microtubes based on ZrO2 stabilized by yttria were used for...
bacteria filtration due to immobilized lysozyme as an antibacterial enzyme. Adsorption of α-amylase on zirconia led to partial conformational changes of the enzyme as well as lower activity compared to the native enzyme. Macroporous zirconia has been utilized for immobilization of lipase for testing in hydrolysis of p-nitrophenyl palmitate with, however, a significant loss of activity compared to native lipase. On the other hand, lipase encapsulation in zirconia obtained by sol–gel reaction allowed for lipase stabilization in a wider temperature range. Lipase immobilization on hydrophobically modified ZrO₂ NPs resulted in highly enantioselective catalysts.

Mesoporous zirconia, similar to mesoporous silica and alumina, would be an appropriate support for the formation of iron oxide NPs (IONPs) and enzyme immobilization, yet providing a higher attrition resistance. However, mesoporous zirconia is not commercially available. In this work, magnetic mesoporous zirconia was prepared by templating over mesoporous silica, followed by template removal, and then IONP incorporation into the pores. It is reported that the ZrO₂ calcination temperature strongly influences the crystal structure and acidity of the zirconia support, thus allowing us to explore the influence of these parameters on the performance of immobilized GOx in D-glucose oxidation. We demonstrate that the highest GOx loading and catalytic activity are observed for zirconia calcined at 600 °C and containing IONPs due to synergy of unperturbed GOx conformation on the highly crystalline surface and enzyme-emulating properties of iron oxide.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of GOx Support.** To synthesize mesoporous zirconia and to study the influence of its structure on the biocatalyst properties, we utilized a templating approach via preparation of a zirconia replica of mesoporous silica using the condensation of ZrOCl₂ ×
This was followed by calcination at different temperatures (300, 400, and 600 °C) to vary the zirconia structure. The silica template was removed by washing with base. IONPs were formed in zirconia pores, utilizing procedures developed for magnetic silica and alumina.

Figure 1 shows representative transmission electron microscopy (TEM) images of ZrO₂ prepared at 600 °C and magnetic zirconia (denoted IO-ZrO₂) based on the same zirconia. Both images look nearly identical because the high electron density of ZrO₂ makes iron oxide NPs poorly visible. A few possible small iron oxide NPs are indicated by red arrows in the inset of Figure 1b. It is noteworthy that all ZrO₂ samples prepared at different temperatures look identical and therefore are not shown.

Figure 2 displays X-ray diffraction (XRD) patterns of ZrO₂ prepared at different temperatures. The 300 °C treatment results in amorphous ZrO₂ (Figure 2a). The treatment at 400 °C (Figure 2b) mainly leads to the tetragonal structure (t-

Figure 3. STEM dark-field image (a) and EDS maps of IO-ZrO₂-600 for Fe (b), Zr (c), O (d) and superposition of Fe and Zr (e) and Fe, Zr, and O (f). Scale bar is 100 nm.

Figure 4. Liquid N₂ adsorption–desorption isotherms (a,c) and pore sizes distributions (b,d) of ZrO₂-600 and IO-ZrO₂-600s.
ZrO2). The distinction between cubic and tetragonal phase is difficult however as peak positions overlap. The pattern of ZrO2 prepared at 600 °C (Figure 2c) contains reflections ascribed to both monoclinic (m-ZrO2, 62%) and t-ZrO2 (or cubic, 38%). The XRD pattern of IO-ZrO2-400 (Figure 2d) looks very similar to that of the parent material, ZrO2-400 (Figure 2b) displaying mainly t-ZrO2. Because the strongest reflections of iron oxide spinel overlap with those of t-ZrO2, we chose to zoom in the area between 47 and 63° 2θ (see inset in Figure 2d), where the least overlap takes place. A very weak reflection at ~57° 2θ can be associated with spinel, indicating that iron oxide NPs are either amorphous or very small.

The magnetization curves of IO-ZrO2-400 (Figure S1, Supporting Information) show a low saturation magnetization of ~2.2 emu/g which is consistent with small IONPs. Nevertheless, because of cooperative interactions of numerous NPs, the material is magnetically recoverable in water and buffers within 60 s (Figure S2, the Supporting Information).

The high-resolution (HR) Zr 3d XPS (X-ray photoelectron spectroscopy) spectrum of IO-ZrO2-600 presented in Figure S3a (Supporting Information) exhibits two peaks at 182.4 and 184.8 eV, which is consistent with the spectrum of non-modified ZrO2. The HR Fe 2p XPS spectrum of IO-ZrO2 (Figure S3b, Supporting Information) displays a major peak with a binding energy (BE) of 711.5 eV, which is typical for iron oxides. The spectrum can be deconvoluted into ten peaks including two peaks for Fe2+ at 709.91 and 723.36 eV, four peaks for octahedral (711.51 and 725.05 eV) and tetrahedral (713.51 and 727.01 eV) Fe3+ components, and four peaks for satellites. A shake-up satellite at ~715 eV belongs to Fe2+ whereas a satellite at ~720 eV is the convolution of both octahedral and tetrahedral Fe3+. The presence of this satellite indicates the formation of Fe3O4 along with Fe2O3. It is worth noting that for magnetic silica and alumina, solely magnetite was formed, indicating that ZrO2 promotes further oxidation of FeO4 to γ-Fe2O3 despite otherwise identical conditions (inert atmosphere and the presence of ethylene glycol as reducing agent). See Tables S1 and S2 (Supporting Information) for fitting parameters.

To evaluate the IONP distribution in ZrO2, we carried out mapping for IO-ZrO2-600 using scanning TEM (STEM) energy dispersive spectroscopy (EDS). Figure 3 shows that the Fe, Zr, and O maps are similar in shape to the STEM dark-field image of the IO-ZrO2-400 piece, revealing that IONPs are evenly distributed through ZrO2. The superposition of Fe/Zr and Zr/Fe/O maps validate this conclusion.

Liquid N2 adsorption measurements were used to evaluate porosity of supports (Figure 4 and Table S3, Supporting Information). The adsorption—desorption isotherms of ZrO2-600 and IO-ZrO2-600 (Figure 4) are type IV, which is common for materials containing mesopores. Porosity of ZrO2 supports depends on the calcination temperature (crystal structure of ZrO2), decreasing with the increase of the temperature. For all supports, the porosity and pore diameter (presented for the ZrO2-600/IO-ZrO2-600 pair in Figure 4) decrease after the IONP incorporation which is consistent with our preceding studies.

**GOx Immobilization on Zirconia.** Immobilization of GOx macromolecules (see Scheme S1, Supporting Information) on IO-ZrO2 (a similar procedure was used for nonmagnetic ZrO2 supports) was performed according to the protocol presented in Scheme 1. First, the support was modified with amino groups using APTES followed by the reaction with the attachment of glutaraldehyde (GA) linker. Next, aldehyde groups of GA were reacted with GOx amino groups.

In order to determine the amount of immobilized GOx on nonmagnetic and magnetic supports, we tested the GOx activity before the attachment to the support and the activity of the supernatant after the biocatalyst separation. Immobilization coefficient (IC) values presented in Table S4 (Supporting Information) indicate that they mainly depend on the ZrO2 calcination temperature, with the highest values for ZrO2-600 (70%) and IO-ZrO2-600 (76%) and the lowest values for ZrO2-300 (52%) and IO-ZrO2-300 (55%). At the same time, differences between the samples treated and 400 and 600 °C (both crystalline supports) are minor. In addition, immobilization efficiency is slightly higher for magnetic samples which could be ascribed to the IONP contribution. A similar increase of IC was observed for Fe3O4-SiO2 (70%) compared to SiO2 (65%).

Considering the complexity of these biocatalysts, we cannot directly assess any structural or conformational changes of GOx upon immobilization. However, this assessment can be carried out indirectly using the biocatalyst relative activity in comparison with that of native GOx and assuming that the high relative activity of the biocatalyst indicates an unperturbed structure of immobilized GOx.

**Catalytic Properties of Immobilized GOx. Influence of pH.** The value of pH is one of the crucial factors in enzymatic reactions because it determines ionization of amino acids in the active site. D-Glucose oxidation with the biocatalysts carried out indirectly using the biocatalyst relative activity in comparison to that of native GOx and assuming that the activity in comparison to that of native GOx in identical conditions (Figure 5a and Table S5, Supporting Information). The native GOx dependence exhibits a sharp maximum (reaches 100%) at pH 6 with a strong decrease of the relative activity at the higher and lower pH values. For zirconia-based biocatalysts with immobilized GOx, the relative...
activities are the same at pH 6 and 7 and slowly decrease with increasing or decreasing pH, indicating a greater tolerance toward pH variations compared to that by native GOx. It is well documented that GOx immobilization leads to retaining relative activity in a broad pH range, which is consistent with our data. Figure 5a also shows that the ZrO2 calcination temperature influences the catalysis, although the differences between the samples treated at 400 and 600 °C are not pronounced. In addition, the relative activities of IONP-containing samples are higher compared to parent ZrO2 samples, revealing the promoting influence of iron oxide most likely due to its enzyme-like activity. However, the smallest differences are observed for the ZrO2-300-GOX/IO-ZrO2-300-GOX pair. The highest relative activity of 98% at pH 6 and 7 was detected for IO-ZrO2-600-GOX.

**Influence of Temperature.** To assess the temperature influence on the relative activities of the biocatalysts, the temperature was varied in the 30–70 °C range at pH 6, allowing best comparison with native GOx. Figure 5b (see also Table S6, Supporting Information) shows that native GOx displays maximum activity at 40 °C, but the activity sharply decreases at higher temperatures, most probably due to denaturation. The biocatalysts with immobilized GOx show better tolerance toward temperature changes, although at 40 °C the activities are lower than those of the native enzyme most likely due to a loss of mobility and reactivity because of the GOx attachment to the support. Again, the best results were obtained for IO-ZrO2-600-GOX with a relative activity of 98% at 40–45 °C. At 45 °C, the activity of IO-ZrO2-600-GOX is 5% higher than that of native GOx. Such stabilization of GOx upon immobilization is consistent with the literature data, but surpasses the best examples by at least 3–5% at 45–50 °C. Therefore, IO-ZrO2-600-GOX shows an outstanding relative activity in a robust range of pH and temperatures. The GOx immobilization allows D-glucose oxidation in a wide range of pH and temperatures with a high product yield, making it beneficial for industrial production and process sustainability.

**Key Parameters Influencing the Catalytic Performance.** The question arises, what are the key factors influencing the activity of immobilized GOx? As was already discussed above, the incorporation of IONPs into ZrO2 supports enhances the relative activity of immobilized GOx most likely due to their own enzyme-like activity. At the same time, the activity differences within nonmagnetic and magnetic biocatalysts still require an explanation. Textural properties of supports could influence both enzyme loading and activity. However, the liquid N2 adsorption–desorption data presented in Figure 4 and Table S3 (Supporting Information) show that changes in the BET surface area and pore diameter are inconsistent with the data on loading and relative activities (Figure 5 and Table S4, Supporting Information). Indeed, the highest surface areas are observed for supports treated at 300 °C, whereas after GOx immobilization, these biocatalysts display lowest catalytic activities. It is noteworthy that pore diameters for ZrO2-600 and IO-ZrO2-600s are ~6 and 4.5 nm, respectively (Figure 4), which is below the GOx hydrodynamic diameter of 7.6 nm. This should result in GOx macromolecules located outside of pores, thus, undermining the porosity influence. The other possible factor is the acidity or basicity of the support. It was demonstrated that Lewis (LAS) and Brønsted (BAS) acid sites on the support may influence the activity of immobilized enzymes. To evaluate acidity and basicity of all supports utilized in this work, we used DRIFTS for the adsorption of CD3CN and CCl3D, respectively (see the Supporting Information). To better understand the relationship between the biocatalyst properties and their LAS and BAS strengths, the DRIFTS band shifts are summarized in Table 1. BAS acidity and basicity do not change upon incorporation of IONPs into ZrO2-300, whereas LAS acidity disappears. Considering that catalytic activities of ZrO2-300 and IO-

![Figure 5](https://dx.doi.org/10.1021/acsomega.0c01067)

**Table 1. DRIFTS Band Shifts Associated with CN and OH for ZrO2-Based Supports**

| support     | LAS CN shift, cm⁻¹ | BAS CN shift, cm⁻¹ | BAS OH shift, cm⁻¹ |
|-------------|---------------------|--------------------|-------------------|
| ZrO2-300    | 47                  | 11–18              | 167               |
| IO-ZrO2-300 | 47                  | 10–18              | 172               |
| ZrO2-400    | 47                  | 11–18              | 167               |
| IO-ZrO2-400 | 47                  | 9                  |                   |
| ZrO2-600    | 47                  | 11–18              | 66                |
| IO-ZrO2-600 | 47                  | 8                  |                   |
| Fe3O4-SiO2  | 49                  | 19                 | 156               |
ZrO$_2$-300 are nearly the same, there is no correlation between LAS and the catalytic performance, whereas values of BAS acidity and basicity are consistent with the relative activities for the ZrO$_2$-300-GOx/IO-ZrO$_2$-300-GOx pair. A similar correlation is also observed for ZrO$_2$-400/IO-ZrO$_2$-400 and ZrO$_2$-600/IO-ZrO$_2$-600 pairs where a decrease of BAS acidity is consistent with the increase of the catalytic activity for magnetic supports. Thus, it could be suggested that for the ZrO$_2$ based supports, acidity negatively influences the GOx catalytic performance.

On the other hand, the acidity of Fe$_3$O$_4$−SiO$_2$ studied in our preceding paper is significantly higher than that of IO-ZrO$_2$-600 and very close to the acidity of ZrO$_2$-300 (Table 1), whereas the relative catalytic activity of Fe$_3$O$_4$−SiO$_2$−GOx (95%) under the same conditions is significantly higher than that of ZrO$_2$-300-GOx (78%). We conclude that neither acidity nor basicity is a key factor in determining the biocatalyst activity. We propose that the relative activity differences within nonmagnetic and magnetic biocatalysts (Tables S5 and S6, Supporting Information) are better explained by the crystal order of the ZrO$_2$ surface versus its amorphous character. Indeed, the surface of t-ZrO$_2$ (obtained at 400 °C) or mixed t-ZrO$_2$/m-ZrO$_2$ (obtained at 600 °C) could be better suited for preservation of the GOx native conformation upon immobilization than a disordered surface of amorphous ZrO$_2$ (obtained at 300 °C). To the best of our knowledge, there are no reports on the direct influence of the ZrO$_2$ crystal structure on the enzyme activity, which does not exclude such an influence.

**Kinetic Parameters.** To gauge the efficiency of immobilized GOx and to understand the essential characteristics determining this efficiency, we utilized the maximum reaction rate ($V_{\text{max}}$) and the Michaelis constant ($K_m$) as the crucial kinetic parameters. It is established that higher $V_{\text{max}}$ indicates a faster reaction rate, whereas lower $K_m$ reflects a higher affinity between the enzyme and substrate. The $V_{\text{max}}/K_m$ ratio is utilized to compare the catalytic performance in a particular enzyme−substrate pair. In our case, to obtain these parameters, we plotted the dependences of the reaction rate ($\nu$) versus the substrate concentration ([S]) using the Michaelis–Menten equation. The key parameters of this equation were obtained from the Lineweaver–Burk graphs following eq 1:

$$\frac{1}{\nu} = \frac{K_m}{V_{\text{max}}} \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$

The results for free and immobilized GOx are summarized in Table 2.

Table 2. Kinetic Parameters of the Oxidation of D-Glucose to D-Gluconic Acid

| catalyst            | $K_m$ mM | $V_{\text{max}}$ μM/min | $V_{\text{max}}/K_m$ $10^3$, min$^{-1}$ |
|---------------------|---------|--------------------------|-----------------------------------------|
| GOx                 | 78      | 173                      | 2.2                                     |
| ZrO$_2$-300-GOx     | 133     | 88                       | 0.7                                     |
| ZrO$_2$-400-GOx     | 125     | 113                      | 0.9                                     |
| ZrO$_2$-600-GOx     | 115     | 151                      | 1.3                                     |
| IO-ZrO$_2$-300-GOx  | 124     | 104                      | 0.8                                     |
| IO-ZrO$_2$-400-GOx  | 113     | 148                      | 1.3                                     |
| IO-ZrO$_2$-600-GOx  | 105     | 164                      | 1.6                                     |
| Al$_2$O$_3$−GOx     | 140     | 43                       | 0.3                                     |
| SiO$_2$−GOx         | 132     | 77                       | 0.6                                     |
| Fe$_3$O$_4$−Al$_2$O$_3$−GOx | 125 | 104 | 0.8 |
| Fe$_3$O$_4$−SiO$_2$−GOx$^a$ | 118 | 152 | 1.3 |

$^a$This work.

**Biocatalyst Reusability in Repeated Experiments.** To assess reusability of the biocatalysts in the repeated use, ZrO$_2$-600-GOx and IO-ZrO$_2$-600-GOx were tested in ten consecutive experiments at pH 6 and a temperature of 40 °C. ZrO$_2$-600-GOx was separated using centrifugation, whereas IO-ZrO$_2$-600-GOx was magnetically separated after each catalytic reaction using a rare earth magnet (for 60 s) and used in the next experiment.

Figure 6 demonstrates that the relative activity of ZrO$_2$-600-GOx decreases by 14% after ten successive experiments, whereas for IO-ZrO$_2$-600-GOx, the decrease of the relative activity is only 7%, exhibiting much better activity retention. Comparison with our preceding work, in the case of GOx immobilized on silica and alumina, the relative activity decreased by 24 and 30%, respectively. For magnetic silica and alumina, the decrease was 11 and 13%, respectively, after ten consecutive reactions. This shows better reusability of the ZrO$_2$-based biocatalysts. It is worth noting that we observed no catalyst attrition after ten consecutive reactions with either ZrO$_2$-600-GOx or IO-ZrO$_2$-600-GOx, ascribed to the higher attrition resistance of ZrO$_2$. However, additional stabilization of the biocatalysts containing IONPs should be attributed to the stabilizing/activating influence of iron oxide as the enzymatic activity enhancer. It is also possible that the presence of IONPs in the ZrO$_2$ pores has a stabilizing influence on the GOx macromolecule conformation, further improving reusability.
Long-Term Incubation Stability. To test a long-term incubation stability of native GOx, ZrO$_2$-600-GOx, and IO-ZrO$_2$-600-GOx, the samples were incubated at 50 °C for 120 h. As shown in Figure 7, native GOx lost more than 94% of its enzymatic activity after only 6 h, whereas IO-ZrO$_2$-600-GOx and ZrO$_2$-600-GOx preserved 82 and 74% of the relative activity, respectively. After 120 h of incubation, IO-ZrO$_2$-600-GOx showed 31% of the relative activity compared to a complete loss of the activity of native GOx. Similar results were obtained at 37 °C. A comparison with the literature data shows that at 50 °C the stability of IO-ZrO$_2$-600-GOx significantly exceeds those demonstrated for enzymes immobilized on DNA-coated IONPs.

![Graph](image_url)

Figure 7. Long-term incubation stability at 50 °C.

CONCLUSIONS

We developed novel biocatalysts by immobilization of GOx macromolecules on ZrO$_2$ and IO-ZrO$_2$ supports, where the latter have an important advantage of fast magnetic separation. A careful examination of the structure of supports as well as their textural and acidic-basic properties allowed us to determine key parameters influencing the catalytic performance of biocatalysts. We demonstrate that both loading and catalytic activity in d-glucose oxidation to d-gluconic acid are higher for biocatalysts based on crystalline supports compared to amorphous supports, which is tentatively attributed to the influence of the support surface on the GOx conformation. At the same time, neither porosity nor support acidity/basicity shows a correlation with catalytic properties, indicating that they are not crucial parameters for these ZrO$_2$-based catalysts. Yet, the incorporation of IONPs significantly increases the relative activity which we assign to synergy between the GOx activity and enzyme-like activity of IONPs. This effect was barely observed for a pair of amorphous supports (ZrO$_2$-300/IO-ZrO$_2$-300), revealing that the crystalline structure is the most important parameter of ZrO$_2$-based supports in controlling the biocatalyst properties. The kinetic parameters such as $K_{m}$, $V_{max}$ and the $V_{max}/K_{m}$ ratio reveal that IO-ZrO$_2$-600-GOx possesses the highest affinity to d-glucose and the highest catalytic activity in comparison with other catalysts. The exceptional IO-ZrO$_2$-600-GOx reusability is attributed to high attrition resistance of ZrO$_2$ as well as the presence of IONPs, making this catalyst promising for immobilization of other enzymes and for practical applications.

Experimental Part. Materials and characterization are reported in the Supporting Information.

Synthesis of Mesoporous Zirconia. Mesoporous ZrO$_2$ was synthesized following the procedure reported elsewhere for ordered mesoporous ZrO$_2$ but using a different template. In a typical experiment, 1.0 g of silica with 6 nm pores was added to 6.0 g of an aqueous solution containing 1.8 g of ZrOCl$_2$ × 8H$_2$O. The solution was stirred until silica was completely suspended. The mixture was then dried in an oven at 100 °C for 1 h in order to evaporate the solvent. The completely dried powder was transferred to a ceramic boat and calcined from room temperature to 300, 400, or 600 °C with a heating rate of 1 °C/min and maintained at this temperature for 5 h. The silica template was removed by stirred ZrO$_2$ in a 2 M sodium hydroxide solution for 12 h and then the product was washed with water three times, using centrifugation to separate the resultant solid. Finally, it was washed with ethanol and dried at 80 °C for 1 h.

Synthesis of Magnetic Mesoporous Zirconia. IO-ZrO$_2$ was obtained following a modified procedure reported elsewhere for magnetic silica. In a typical experiment, 0.48 g of iron(III) nitrate nonahydrate in 2.4 mL of ethanol was added to 0.6 g of ZrO$_2$ and stirred for 12 h in order to completely evaporate the solvent. Seventy drops of ethylene glycol (EG) were added to the solid sample and the latter was transferred to a ceramic boat. The boat was placed in a tube furnace, heated under argon at 2 °C/min to 250 °C and kept at this temperature for 6 h. The solid was washed multiple times with acetone until the supernatant was colorless using separation with a magnet each time. After that, the product was dried in a vacuum oven for 3 h.

Functionalization with Amino Groups. IO-ZrO$_2$ or ZrO$_2$ (0.3 g) was mixed with an APTES (3-aminopropyl)triethoxysilane) aqueous solution (8 mL). The APTES solution was prepared by mixing glacial acetic acid with 10 mL of water until reaching a pH of 4. After that, 0.8 mL of APTES was mixed with 8 mL of acidic water and 4 mL of glycerol for suspending the support. The reaction was performed at 90 °C under stirring for 5 h, after which the product was washed three times with water and five times with methanol. The product was dried in a vacuum oven for 12 h.

Attachment of Glutaraldehyde and GOx. IO-ZrO$_2$-NH$_2$ or ZrO$_2$-NH$_2$ (0.1 g) was suspended in the solution (20 mL) of GA in phosphate-buffered saline (PBS) and kept stirring for 1 h. The solution of GA was prepared by mixing 0.08 mL of 25% GA in water with 20 mL of the PBS buffer with pH 7.0. The GA-functionalized support was isolated (magnetically or by centrifugation) and washed multiple times with water. Meanwhile, GOx (10 mg) was mixed with 20 mL of the PBS buffer and stirred for 1 h. The GA-modified support was added to the GOx solution and stirred for 1 h, after which the biocatalyst was separated. The calculation of the amount of immobilized GOx was carried out using an initial GOx concentration in the buffer and the concentration of GOx left in the buffer solution after the immobilization. To assess the amount of immobilized GOx, we determined the IC:

$$IC = \left( \frac{C_0 - C_1}{C_0} \right) \times 100\%$$

where $C_0$ and $C_1$ are the GOx amounts (μg/mL) in the filtrate before and after immobilization (and biocatalyst removal), respectively. These amounts were found, estimating the activity.
in the d-glucose oxidation and taking into account that in all experiments the initial GOx activity is 174.9 U/mg.

Testing of Biocatalyst Activity. Oxidation of d-glucose to d-gluconic acid was performed in a double-jacketed three-neck round-bottom flask equipped with an overhead stirrer, gas inlet, and a reflux condenser working as gas outlet. The reaction was carried at 30–70 °C and atmospheric pressure. The flask jacket was used to circulate a heating liquid to maintain the reaction temperature. After the desired temperature was reached, 0.11 g of the biocatalyst, 10 mg of d-glucose, and 15 mL of the 0.1 M PBS buffer (pH 6.0) were added and stirred for 1 h, after which oxygen was introduced (a feeding rate of 440–450 mL/min). After completion of the reaction, the catalyst was isolated, whereas the reaction mixture was analyzed using HPLC, UltiMate 3000 (ChromaTech, Russia) containing a refractometer detector and a ReproGel H Column (500 × 10 mm, NTP 160,000). A H2SO4 solution (9 mM) was used as the eluent at a rate of 0.5 mL/min for 30 min at an eluent pressure of 6.5 kPa and a column temperature of 250 °C. Pure d-gluconic acid was used for the product identification, considering that selectivity of the process is 100%.

The biocatalyst activity (A, μM(Gl)÷(mg (GOx) × min)) was calculated using eq 3:

\[ A = \frac{\text{Gl}_t}{[\text{GOx}] \times IC \times V \times t} \] (3)

where [GOx] is the amount (mg) of native or immobilized GOx, IC is the immobilization coefficient, V is the volume of the reaction solution, t is the reaction time, and Glt is the amount of d-glucose (mmol) reacted. It is noteworthy that the reaction time was 60 min in all experiments.

The biocatalyst relative activity was quantified using formula 4:

\[ R = \frac{A}{A_0} \times 100\% \] (4)

where A0 and A are the activities of native GOx and immobilized GOx, respectively.

The influence of pH on the activities of free GOx and biocatalysts was assessed in two buffers (0.1 M): sodium-acetate (pH 3.0–5.5) and sodium-phosphate (pH 6.0–8.0). To determine the influence of the temperature, GOx and biocatalysts were assayed in the 30–70 °C temperature range. The long-term incubation stability of native GOx, IO-ZrO2-600-GOx, and ZrO2-600-GOx were determined by storing in the PBS buffer (50 mM, pH 6.0) at 37 or 50 °C.

The maximum rate (Vmax) and the Michaelis constant (Km) were calculated by determining the initial reaction rates for native and immobilized GOx varying d-glucose concentrations in the PBS buffer (3.8–380 mM, pH 6.0) at 40 °C. The dependence of the d-glucose oxidation rate on the d-glucose concentration was defined by the Michaelis–Menten model.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01067.

Materials, characterization, magnetic properties, XPS, BET, GOx immobilization, catalytic data, and DRIFTS (PDF)

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the Russian Science Foundation (project 17-19-01408). V.M. and A.S. thank the Russian Foundation for Basic Research (grants 18-08-00468 and 18-38-00159). We also thank the IU Nanoscale Characterization Facility for access to the instrumentation as well as NSF grant #CHE-1048613 which funded the Empyrean from PANalytical. IU Bloomington XPS facility was funded by NSF MRI grant (NSF DMR 1126394).

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