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Immobilization of Glucose Oxidase on Magnetic Nanoparticles Modified with Chitosan and Sodium Tripolyphosphate

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In the last decades, the utilization of chitin-containing wastes has become an urgent task. The current work is aimed at studies on the use of chitosan (one of the main chitin components) for the preparation of magnetically separable biocatalysts. A multicomponent biocatalyst based on glucose oxidase (GOx) immobilized on Fe_3O_4 nanoparticles modified with chitosan and sodium tripolyphosphate was synthesized. The carboxyl groups of GOx were pre-activated with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Fourier-transform infrared spectroscopy and low-temperature nitrogen physisorption proved successful modification of the magnetically separable support with a fine layer of chitosan. The presence of target functional groups on the support surface was also confirmed. The activity and stability of the biocatalyst were investigated in the oxidation reaction of D-glucose to D-glucono-δ-lactone. The immobilized biocatalyst showed slightly lower activity than that for the native enzyme. However, the immobilized enzyme can be easily separated from the reaction mixture by an external magnet and reused practically without activity loss. The ratio of the biocatalyst components providing maximum activity and stability was determined. It has been shown that the immobilization of GOx by the method described above results in an expansion of the operating range of pH and temperatures by 15-20 % compared to the native enzyme. The synthesized biocatalyst can be used to produce gluconic acid and determine the concentration of D-glucose in various fluids.

1. Introduction

Glucose oxidase (EC 1.1.3.4, GOx) is a dimeric flavoprotein of the «glucose-methanol-choline oxidoreductase» (GMC oxidoreductase) class (Wilson et al., 1992). This enzyme catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone (δ-glucono-1,5-lactone) and H_2O_2 using molecular oxygen as an electron acceptor (Bankar et al., 2009). The holoenzyme contains 2 molecules of flavin adenine dinucleotide as a coenzyme non-covalently bound to the active site (Bankar et al., 2009). GOx can be extracted from many natural sources, in particular algae, plants, animals, insects, bacteria, and fungi (Vitolo, 2021). GOx from *Aspergillus niger* is most widely used in industry and laboratory research (Dubey et al., 2017). It has the following properties: molecular weight - 150-160 kDa, optimal operating temperature - 40-60 °C, and optimum pH 4.0-7.0 (Kornecki et al., 2020).

This enzyme is widely used in different processes of chemical technology and analytical chemistry (Tikhonov et al., 2019). However, the native form of GOx has a rather low resistance to inhibitory effects, in addition, it cannot be reused (Matos Trujillo et al., 2012). Immobilization of glucose oxidase on solid supports makes it possible to reuse the enzyme, increases its resistance to inhibitory effects, and strengthens the conformation of the protein component of the enzyme molecule (Ekinci et al., 2007). A wide range of solid supports can be used to immobilize of glucose oxidase (Tikhonov et al., 2019): coal, silicon and aluminum oxides, porous glass, natural and synthetic polymers, etc. Among the most promising carriers for enzyme immobilization,

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materials with magnetic properties (for example, magnetite – Fe_3O_4), which are easily separated from the reaction mixture using an external magnet, are currently of great interest (Sulman et al., 2023). For a more stable fixation of the enzyme on magnetite, it is advisable to modify its surface with various multifunctional reagents with target functional groups capable of forming a covalent bond with enzyme molecules (amide or peptide bond) (Podrepšek et al., 2020). Chitosan is one of these reagents.

Chitosan is a product of the deacetylation of chitin, a widespread component in the shells of crustaceans and insects, as well as the cell walls of many plants and fungi (Aranaz et al., 2021). In recent decades, the problem of the utilization of chitin-containing wastes has been an urgent task for researchers. The wastes are formed in large amounts in the seaside regions. The weight of the waste can reach up to 80 wt. % of the shellfish recovered (Wang et al., 2019). The storage of this waste leads to ecological problems due to the formation of toxins during the putrefaction and the foul smell. The utilization of chitin-containing waste via its processing can be a promising way to solve pollution problems by obtaining valuable chemicals. Chitosan has unique properties: it contains amino groups, forms a polycation in the dissolved state, and is capable of gelation and formation of thin films (Matveeva et al., 2023). In addition, this biopolymer is completely biodegradable (Cogollo-Herrera et al., 2018).

The methods of GOx immobilization on the magnetic nanoparticles modified with chitosan described recently in literature do not provide high activity and stability of the biocatalysts. This is due to the low active surface of the support and pure linkage of the enzyme with the modified MNPs, leading to fast enzyme loss.

In this regard, the purposes of this work were the synthesis of a biocatalyst based on GOx immobilized on chitosan-modified magnetic nanoparticles and the study of the main catalytic and physics-chemical properties of the synthesized biocatalyst. The novelty of the research presented in the paper is focused on the tailor-made synthesis of the mesoporous magnetite with the pore size, allowing the reaction to take place both on the support surface and in the pores. The last one can significantly increase the process contact surface and effectiveness of the immobilization. Besides, the application of the methods used allows chitosan to be cross-linked on the support surface with the ionic bonds using sodium tripolyphosphate, decreasing the loss of the biocatalyst components while its use and increasing the biocatalyst activity and operational stability.

2. Materials and methods

The following reagents and materials were used in this work: middle-viscous chitosan (CS, insoluble matter < 1 %, 400 KDa, «Biochimika», Japan); acetic acid (99.9 %, «Kupavnareactiv», Russia); carbodiimide (> 97.0 %, EDC, N-(3-dimethyl aminopropyl)-N'-ethyl carbodiimide hydrochloride, FLUKA, BioChemika, Japan); iron (II) chloride 4-hydrate ($FeCl_2 \cdot 4H_2O$, > 97.0 %, AppliChem, Panreac); iron (III) chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$, > 97.0 %, AppliChem, Panreac); N-hydroxysuccinimide (NHS, > 98.0 %, Acros Organics, China); sodium tripolyphosphate (TPP, > 99.0 %, Chimmedservis, Russia); D-glucose («Biochimika», > 98.0 %, Japan); soluble starch of Lyntner (ChimMedService, > 97.0 %, Russia), ammonium molybdate (Reachim, > 98.0 %, Russia), potassium iodide (Reachim, > 98.0 %, Russia), sodium hydroxide (Reachim, > 98.0 %, Russia), hydrogen peroxide (Rosbio, >98.0 %, Russia), phosphate-buffered saline (PBS, UralChimInvest, > 99.0 %, Russia) with different pH.

For the synthesis of magnetite nanoparticles (MNP), 15 mL of $FeCl_3 \cdot 6H_2O$ solution (2.16 g in 15 mL of distilled water) and 15 mL of $FeCl_2 \cdot 4H_2O$ solution (0.79 g in 15 mL of distilled water) were mixed in a glass reactor with a jacket equipped with a reflux condenser and a magnetic stirrer. The mixture was stirred for 15 min at room temperature, and then the temperature was increased up to 65 °C. The reaction mixture was stirred for 15 min. Then, 10 mL of 25 % aqueous ammonia solution was added dropwise. The mixture was maintained for 15 min at a constant stirring. A chitosan solution (0.1 g in 50 mL of 2 M acetic acid) was added to the mixture and stirred for 15 min. To crosslink chitosan on the surface of magnetic particles, the resulting sample was kept for 1 h in a solution of sodium tripolyphosphate (0.05 g in 50 mL of distilled water). Then, the particles were washed with distilled water several times, separated using an external magnet, and dried in air.

To immobilize glucose oxidase onto the synthesized magnetic particles, 0.25 g of dried particles were kept for 12 h in a solution containing 0.1 g EDC, 0.04 g NHS and 0.05 g GOx in 50 mL of distilled water.

Mixing GOx with EDC and NHS is necessary to activate the carboxyl groups of the enzyme to form a stable NHS ester, which then spontaneously reacts with the amino groups of the carrier to form an amide bond.

The synthesized biocatalyst was washed with distilled water several times and separated using a magnet. After the extraction, the biocatalyst was dried in air.

The scheme of the immobilized GOx preparation is shown in Figure 1.

To estimate the activity of the immobilized glucose oxidase, the biocatalysts obtained were mixed with 40 mL of D-glucose aqueous solution (2.2 mmol/L) in phosphate buffer (pH = 6.0) in a thermostatically controlled glass reactor with a magnetic stirrer. An activity index of GOx is a quantity of the hydrogen peroxide formed during the reaction (equimolar to the reacted D-glucose). The concentration of H_2O_2 was determined by the

iodometry through photometric measurements of the iodine-starch complex (Tikhonov et al., 2021). All kinetic experiments were repeated at least 3 times. The arithmetic mean was taken as the result.

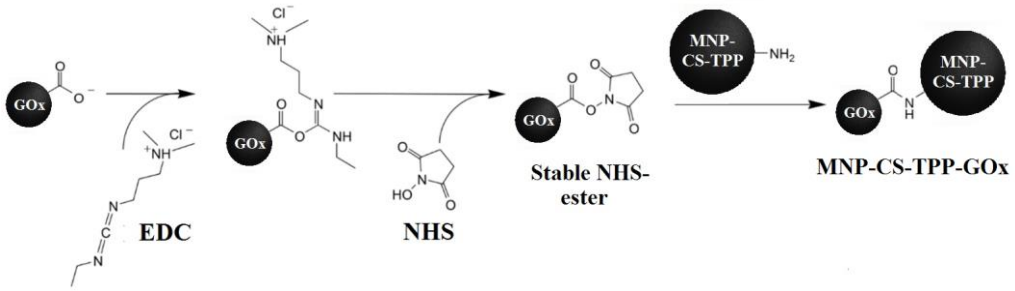

Figure 1: Scheme of synthesis of immobilized GOx

Particle size and zeta potential were determined by dynamic light scattering using a photon correlation spectroscopy spectrometer 90Plus (Brookhaven, USA). The FTIR spectra were recorded using the Fourier IR spectrophotometer IRPrestige-21 (Shimadzu, Japan) equipped with the diffuse reflection device. To estimate the porosity and surface properties of synthesized nanoparticles and biocatalysts, the specific surface area and porosity of synthesized samples of magnetic nanoparticles and biocatalysts were measured using low-temperature nitrogen physisorption on a Beckman Coulter™ SA 3100™ analyzer (Coulter Corporation, USA). Magnetization measurements of the samples were carried out using a developed vibrating sample magnetometer (VSM) with accuracy better than ± 0.01 emu/g, which allows measuring bulk and powder samples of 0.01-150 mg in the temperature range 80-1,000 K and magnetic field 0-2.5 T.

3. Results and discussion

The initial magnetite particles had good magnetic properties and were easily and completely separated from the reaction medium using a magnet. At the same time, the magnetization of particles after the application of chitosan, sodium tripolyphosphate, and glucose oxidase to magnetite decreased by no more than 15 % (for the initial magnetite – 65 Am^2/kg, for the biocatalyst - 56 Am^2/kg at 22 °C).

The results of kinetic experiments showed that the highest amount of hydrogen peroxide in the D-glucose oxidation is formed during the preparation of a biocatalyst using the following component contents: 0.1 g chitosan; 0.05 g sodium tripolyphosphate, 0.1 g EDC, 0.04 g NHS and 0.05 g GOx.

Figure 2 presents FTIR-spectra of the magnetic nanoparticles, chitosan, and magnetic nanoparticles modified with chitosan and sodium tripolyphosphate.

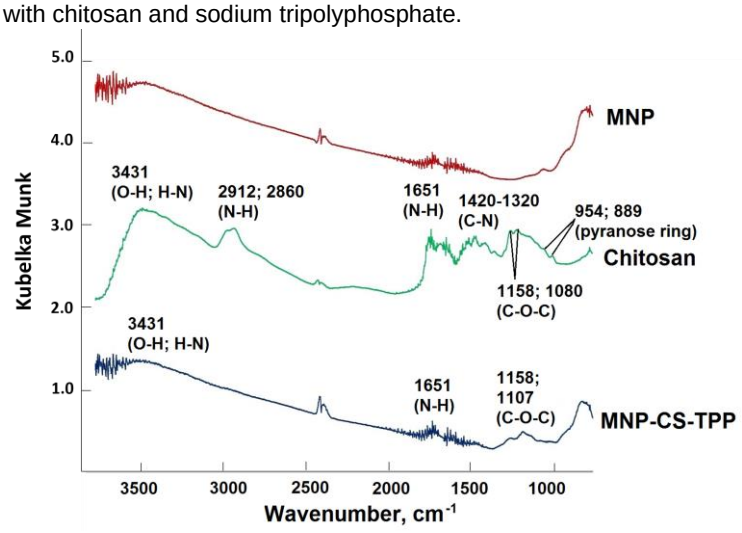

Figure 2: FTIR-spectra of the magnetic nanoparticles (MNP), chitosan (CS), and magnetic nanoparticles modified with chitosan and sodium tripolyphosphate (MNP-CS-TPP)

The chitosan spectrum contains characteristic absorption bands of pure chitosan (Guo et al., 2010): 3,431 (O-H and N-H stretching vibrations), 2,912, 2,860 (C-H stretching vibrations), 1,651 (N-H bending vibrations) and

1,158, 1,080 cm^{-1} (C-O-C stretching vibrations), as well as the peaks of the pyranose ring – deformational (889 cm^{-1}) and oscillatory (945 cm^{-1}). Chitosan absorption bands 1,420-1,320 cm^{-1} (C-N stretching vibrations) decrease sharply in the spectrum of magnetic nanoparticles coated with chitosan. It can be associated with the formation of the N-Fe complex (Pineda et al., 2014). At the same time, the bands at 3,431 (O-H and N-H stretching vibrations), 1651 (N-H bending vibrations), and 1,158, 1,107 cm^{-1} (C-O-C stretching vibrations), 945 and 889 cm^{-1} (pyranose ring) were observed in the spectrum of magnetic nanoparticles coated with chitosan, which confirms the presence of a chitosan layer on the surface of magnetic nanoparticles.

The studies of synthesized MNPs and biocatalysts by low-temperature nitrogen physisorption have shown that the application of chitosan, sodium tripolyphosphate, and glucose oxidase to the magnetite surface resulted in a slight increase in the surface area (MNPs – 98 m^2/g; MNP-CS-TPP-GOx – 102 m^2/g). This can be attributed to the distribution of these components in the large pores of the MNP, forming the cross-links with chitosan and sodium tripolyphosphate. Table 1 illustrates the pore size distribution for the studied samples (according to the BET model).

Table 1: The pore volume distribution of the studied samples (according to the BET model)

№	Sample	Pore diameter, nm							
		< 6	6 ÷ 8	8 ÷ 10	10 ÷ 12	12 ÷ 16	16 ÷ 20	20 ÷ 80	> 80
1	MNP	7.55	6.74	6.12	10.56	21.01	35.65	11.75	0.66
2	MNP-CS-TPP	9.18	9.99	9.49	16.53	35.7	13.56	5.09	0.46
3	MNP-CS-TPP-GOx	9.22	8.82	8.89	17.14	31.85	17.46	6.16	0.48

Based on Table 1, it can be observed that the application of organic components on the support surface resulted in a slight reduction in the average pore size. This confirms that chitosan and glucose oxidase are present not only on the surface of the support but also within its pores. More than 60 % of the pore volume of the samples is occupied by mesopores with a diameter of 10 ÷ 20 nm. However, the average pore diameter of the support is sufficient for the successful immobilization of glucose oxidase in the pores and for free access of the substrate (D-glucose) to the active sites of the immobilized enzyme without significant diffusion restrictions.

The effective diameter and zeta potential of the magnetic nanoparticles (MNP) and magnetic nanoparticles modified with chitosan and sodium tripolyphosphate (MNP-CS-TPP) were determined. Results are presented in Table 2.

Table 2: Effective diameter and zeta-potential of the magnetic nanoparticles (MNP) and magnetic nanoparticles modified with chitosan and sodium tripolyphosphate (MNP-CS-TPP)

№	Parameter	MNP	MNP-CS-TPP
1	Effective diameter, nm	207.8 ± 3.6	226.6 ± 2.9
2	Zeta-potential, mV	0 ± 0.5	56.9 ± 2.0

As can be seen from Table 2, the application of chitosan to magnetic nanoparticles led to an increase in the effective diameter of the particles by 5 %, while the zeta potential of the particles significantly shifted to the positive. This confirms the presence of a thin layer of chitosan polycation on the surface of nanoparticles which allows to counteract the adhesion of particles into larger clusters.

Experiments on the enzymatic activity were conducted, varying the initial concentration of D-glucose for the native and immobilized glucose oxidase. The kinetic curves for hydrogen peroxide formation in time are shown in Figures 3a and 3b. It can be seen from Figures 3a and 3b that GOx immobilized on magnetic particles has less activity compared to its native form. This is primarily due to the heterogenization of the process. However, enzyme immobilization on magnetic particles makes it easy to separate the biocatalyst from the reaction medium using a magnet and use it repeatedly. It compensates for the loss of activity of the immobilized enzyme. According to the results of the research, the kinetic parameters of the biocatalyst were calculated. Activity A, the maximum reaction rate, and the Michaelis K_M constant are shown in Table 3.

Table 3: Kinetic parameters of the native and immobilized GOx.

№	Parameter	GOx	MNP-CS-TPP-GOx
1	V_m, µmol/l·s	6.32	2.92
2	Activity, U/mg	0.038	0.017
3	K_M, µmol/l	7.688	3.083

Table 3 illustrates that the maximum rate for an immobilized enzyme is significantly lower than that for a native one. At the same time, immobilization led to a decrease in the Michaelis constant. These two parameters of the enzyme characterize different aspects of its activity. A decrease in the maximum reaction rate is primarily due to the diffusion restrictions when the process behaviors are at the solid-liquid interface. A decrease in the Michaelis constant indicates an increase in the enzyme's resistance to inhibitory effects and a fixation of its protein chain in a more efficient conformation than that for the native enzyme. The loss of enzyme activity is compensated by an increase in its stability and resistance to inhibitory effects.

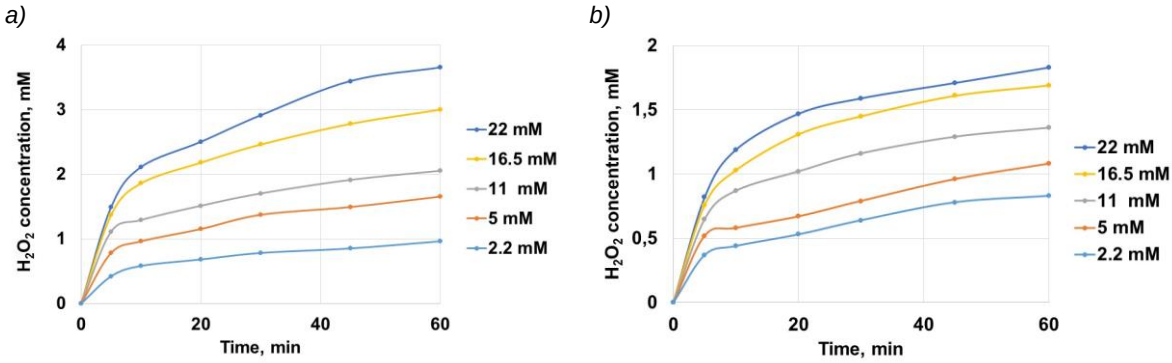

Figure 3: Kinetic curves of hydrogen peroxide formation for the (a) native and (b) immobilized forms of GOx varying the initial concentration of D-glucose

Active sites of GOx contain ionogenic groups that transform conformation and state depending on pH. Table 4 illustrates the dependence of the relative activity of the immobilized and native glucose oxidase on pH.

Table 4: Dependence of the activity of the native and immobilized GOx on the pH value of the reaction mixture

№	Sample	pH value						
		3	4	5	6	7	9	12
1	GOx	28.0	51.0	70.5	100	83.7	57.0	25.4
3	MNP-CS-TPP-GOx	40.9	60.3	89.3	100	90.9	61.4	42.9

The obtained data indicate that the relative activity of immobilized enzymes in the studied pH range is approximately 5-10 % greater than that of free enzymes.
Another factor that affects the enzyme efficiency is the reaction temperature. Table 5 illustrates the dependence of the relative activity of both native and immobilized enzymes on the reaction temperature.

Table 5: Dependence of the activity of the native and immobilized GOx on the temperature of the reaction mixture

№	Sample	Temperature of the reaction mixture, ºC												
		20	25	30	35	40	45	50	55	60	65	70	75	80
1	GOx	77.9	88.5	95.0	100	66.6	57.3	51.3	49.3	44.8	44.2	40.4	27.9	12.4
3	MNP-CS-TPP-GOx	85.4	91.7	99.5	100	88.8	81.6	66.0	58.3	52.9	51.0	46.6	31.1	21.4

As can be observed from Table 5, a reduction in temperature decreases the reaction rate, as the number of enzyme and substrate molecules with high energy decreases. An increase in temperature beyond optimal values initiates the denaturation of the enzyme. GOx immobilization allows for extending the temperature range, by stabilizing protein molecules in their effective conformation. The relative activity of the synthesized biocatalyst is 10-20 % greater at temperatures above 30 °C than that of the native enzyme.
The stability of the synthesized biocatalyst was investigated during its multiple reuses in the D-glucose oxidation reaction. The biocatalyst loses no more than 21 % of its activity in 10 consecutive cycles.

4. Conclusions

In the current study, the effectiveness of GOx immobilization on magnetic nanoparticles modified with chitosan and sodium tripolyphosphate has been proven. According to the experimental results, the optimal biocatalyst composition was determined as follows: 0.1 g of chitosan; 0.1 g of EDC, 0.04 g of NHS, and 0.05 g of GOx.

The presence of the chitosan layer on magnetic nanoparticles has been proven with FTIR and low-temperature nitrogen physisorption methods. Kinetic experiments have demonstrated that the immobilization of GOx on magnetic nanoparticles coated with chitosan and sodium tripolyphosphate allows for an expansion of the pH and temperature operating ranges compared to the native enzyme, as well as an increase in the resistance to inhibitory effects. The synthesized biocatalyst has the potential to be used in the industrial production of D-gluconic acid as well as for the analytical determination of D-glucose concentration in biological samples.

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