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Use of biocompatible redox-active polymers based on carbon nanotubes and modified organic matrices for development of a highly sensitive BOD biosensor

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ABSTRACT

This work investigated the use of redox-active polymers based on bovine serum albumin and chitosan, covalently bound to mediators neutral red and ferrocene and containing carbon nanotubes, for immobilization of *Paracoccus yeei* VKM B-3302 bacteria. The structures of produced polymers were studied by IR spectroscopy and scanning electron microscopy. Cyclic voltammetry and impedance spectroscopy found the electrochemical characteristics of the investigated systems: the heterogeneous electron transfer rate constant, the constant of the rate of interaction with *P. yeei* bacteria and the impedance. The systems containing carbon nanotubes and ferrocene-based redox-active polymer proved to be the most promising. Biosensors formed using the hybrid polymers had a high sensitivity with the lower boundary of 0.1 mg/dm^3 of the detected BOD_5 concentrations and a high correlation ($R = 0.9916$) with the standard BOD assay of surface water samples.

1. Introduction

Rapid assessment of environmental contamination, in particular, detection of organic impurities in surface, ground and effluent waters, is a topical task. An important characteristic of water contamination by organic substances is the biochemical oxygen demand (BOD) index. The standard BOD assay is at least 5 days long (BOD_5), during which time contaminants can get into natural water bodies, cause their eutrofication and death of useful hydrobionts [1]. There are several techniques providing for the high accuracy of BOD measurements; however, they are time-consuming and require expensive specialized equipment and qualified personnel [2].

Alternatives to the standard method are microbial biosensors [3–5]. Mediator biosensors are the most promising as their analytical signal does not depend on the concentration of dissolved oxygen, thus enabling the analysis under anaerobic conditions. Besides, the working potential can be changed, thereby decreasing noise related to redox reactions of oxidants or reductants occurring in the sample [6]. A modern and efficient approach to the development of mediator biosensors is the use of redox-active polymers produced by crosslinking the mediator with polymers used to immobilize microorganisms [7]. Interest in such developments is caused by advantages of these materials in forming biosensors on their basis. Redox-active polymers are rather efficient, provide for biosensor stability and reagent-free assay, no mediator wash-out during the procedure and can be used for creating biosensors based on microbial cells [8].

At present, redox-active polymers come into use in mediator biosensors the most frequently for immobilization of enzymes [9–11] and affinity bioreceptors [12,13]. The complexity of using these systems for immobilization of microorganisms is due to the necessity for covalently bound mediator to penetrate into the cell via its membrane to couple the biochemical reactions with the electrochemical converter. Thus, a mediator biosensor developed in [14] for measurements of BOD at low concentrations is based on *Bacillus subtilis* cells immobilized in a three-dimensional porous graphene–polypyrrole composite produced by a hydrothermal method followed by electropolymerization. The range of assayed BOD_5 values is 4–60 mg/dm^3. A mediator-type bioelectrochemical biosensor developed in [15] using polypyrrole with

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immobilized mediator potassium hexacyanoferrate (III) and *Pseudomonas* cells is used for rapid determination of BOD. This biosensor enables a stable signal (at a relative standard deviation of 2%) within a wide BOD range of 5–100 mg/dm^3. A mediator microsensor for BOD assessment proposed in [16] is based on poly(neutral red) and *Pseudomonas aeruginosa* bacteria immobilized in a polypyrrole matrix on the surface of an ultramicroelectrode. The microsensor enables a stable signal within the BOD range of 5–100 mg/dm^3; however, each measurement takes about 20 min. The synthesis of bovine serum albumin (BSA) covalently bound to ferrocene is reported in [17]. At the first stage of crosslinking, Schiff bases are formed; then double bonds are reduced using sodium hydroborate. The BOD biosensor based on this gel has a high operational stability (5%); the range of assayed concentrations is 10–180 mg O_2/l.

Despite numerous advantages of synthesized hydrogels, they have some serious drawbacks. The major of them are low sensitivity due to the complexity of coupling biomaterial with the electrode, large duration of measurements due to the poor permeability of matrices and low long-time stability determined by the poor biocompatibility of conducting systems used. The aim of this work was to investigate the possibility of developing bioelectrodes based on *P. yeei* bacteria and redox-active polymers based on bovine serum albumin and chitosan, modified by electron transfer mediators and carbon nanotubes, for creating a highly sensitive BOD biosensor.

2. Experimental

2.1. Reagents and materials

Ferrocenecarboxaldehyde (FC) (Sigma-Aldrich, Germany) and neutral red (NR) (Diaem, Russia) were used as electron transfer mediators. Non-conducting matrices based on low-molecular weight chitosan of an average molecular weight 50–190 kDa (Sigma-Aldrich, Germany) and bovine serum albumin (Sigma-Aldrich, Germany) were used as the basis of redox-active polymer. A working carbon paste electrode was formed using graphite of particle size 75 microns and high purity 99.997 % (Fluka, Germany), paraffin oil (Fluka, Germany) and dialysis membrane with an MWCO of 14 kDa (Roth, Germany). Biosensor measurements were carried out in a sodium–potassium buffer solution, pH 6.8 (33 mM KH_2PO_4 + 33 mM Na_2HPO_4, Diaem, Russia).

2.2. Cultivation of microbial cells

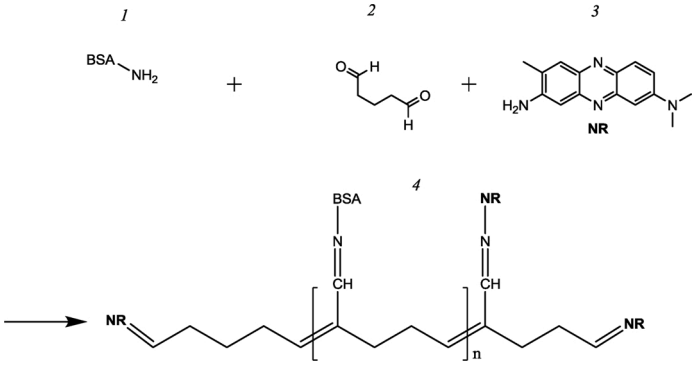
Cells of *Paracoccus yeei* VKM B-3302 isolated from activated sludge [18] were grown on a rich Luria–Bertani mineral medium. The cell growth medium was sterilized by autoclaving at a pressure of 1.15 atm for 45 min. Cells were grown aerobically for 18–20 min in 750-cm^3 shaken flasks at a temperature of 29 °C. Biomass produced was centrifuged at room temperature for 10 min (8000 rpm). Further on, the centrifugate was washed for 20 min with a phosphate buffer, pH 6.8. Sedimented cells were transferred into fresh portions of buffer, distributed portion-wise and sedimented on a centrifuge (Eppendorf, Germany) for 5 min at 8000 rpm. Washed biomass was weighed and stored in microtubes at a temperature of −25 °C.

2.3. Formation of working electrodes based on BSA redox-active polymers

2.3.1. Synthesis of redox-active polymer based on BSA and mediator neutral red; formation of a working electrode (BSA–NR)

A 5-μl amount of a 0.6-M solution of neutral red and 50 μl of phosphate buffer, pH 6.8, were added to 3.5 mg of BSA. The mix was shaken for 5 min. Then 7.5 μl of glutaraldehyde (GA) was poured into the solution, and this mix was shaken for 30 s. The formed polymer was applied in the amount of 10 μl onto a ground carbon-paste electrode fabricated according to the following method [19]: 100 mg of graphite powder was mixed with 40 μl of paraffin oil; a plastic tube of the working surface area 6.3 mm^2 was filled with the produced paste.

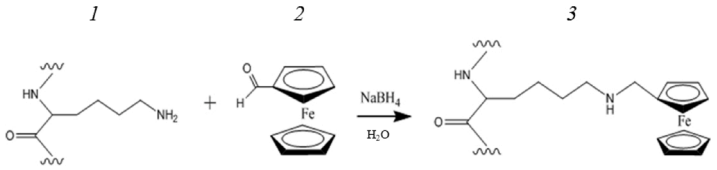
Taking into account the various forms of glutaraldehyde in water solution [20], one of the possible structures of neutral red- and BSA-based redox-active polymer can be as follows.



Scheme of the synthesis of BSA–NR redox-active polymer: 1, BSA; 2, glutaraldehyde; 3, neutral red mediator; 4, BSA–NR.

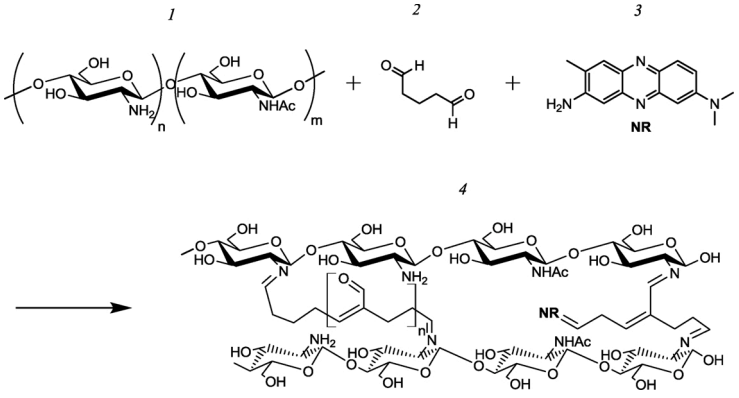
2.3.2. Synthesis of a redox-active polymer based on BSA and mediator ferrocenecarboxaldehyde; formation of a working electrode (BSA–FC)

To form a conducting matrix, 0.5 *g* of BSA was dissolved in a 5-ml buffer solution. Then 0.05 *g* of ferrocenecarboxaldehyde was dissolved in 5 mL of acetone; this mix was poured into the BSA solution. The produced solution was adjusted to pH 9.3 by adding 5% K_2CO_3. The reaction was continued for 1 h at a temperature of 30 °C. After that, 0.01 *g* of $NaBH_4$ was added to the mix. The produced mix was stirred using a magnetic stirrer for 10 min and kept at room temperature for 6 h. The pH value of the mix was adjusted to pH 6.5 for the remaining $NaBH_4$ to decompose. Then the pH of the mix was adjusted to pH 8.5 by adding a 0.1-M NaOH solution by 20-μl portions. Further on, the mix was centrifuged at 3000 rpm for 20 min. The supernatant produced was dialyzed by a phosphate buffer solution for two days at a temperature of 4 °C to separate the nonreacting ferrocenecarboxaldehyde. The produced redox-active polymer was dried to a constant weight at a temperature of 60 °C.



Scheme of the synthesis of a BSA–FC redox-active polymer: 1, BSA; 2, ferrocenecarboxaldehyde mediator; 3, BSA–FC.

To form a measuring electrode, the redox-active polymer of a 0.0035-g weight was dissolved in 50 μl of phosphate buffer, pH 6.8; then, 7.5 μl of glutaraldehyde was added. The produced mix in the amount of 10 μl was applied onto a carbon paste electrode and left for complete dry-out (s 1–4).



Scheme of the synthesis of CHIT–NR redox-active polymer: 1, CHIT; 2, glutaraldehyde; 3, neutral red mediator; 4, CHIT–NR.

2.4. Formation of working electrodes based on redox-active polymers of chitosan

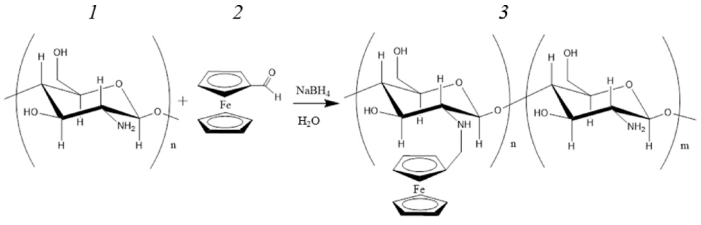
2.4.1. Synthesis of redox-active polymer based on chitosan (CHIT) and mediator neutral red; formation of a working electrode (CHIT–NR)

A 5-μl amount of a 0.6-M neutral red solution, 50 μl of phosphate buffer (pH 6.8), 7.5 μl of glutaraldehyde were added to 500 μl of a 1 % chitosan solution in 1% acetic acid. The formed redox-active polymer was applied onto the ground carbon paste electrode in the amount of 10 μl and left for complete dry-out. Taking into account the various forms of glutaraldehyde in the water solution [21] one of the possible structures of neutral red- and chitosan-based redox-active polymer can be as follows.

2.4.2. Synthesis of redox-active polymer based on chitosan and mediator ferrocenecarboxaldehyde; formation of a working electrode (CHIT–FC)

To form a conducting matrix, 0.1 g of chitosan was dissolved in 10 mL of 3 % acetic acid, mixed with a solution of 0.01-g ferrocenecarboxaldehyde in 7 mL of acetone. Further on, the mix was stirred for 24 h. Then 53.0 mg of $NaBH_4$ was added under stirring. The reaction mix was stirred for 24 h. The modified polymer was precipitated by a solution of 5.0-mM NaOH to pH 10.0.

The precipitated polymer was centrifuged and dialyzed, after which it was dried for 2 h in a drying chamber.



Scheme of the synthesis of CHIT–FC redox-active polymer: 1, CHIT; 2, ferrocenecarboxaldehyde mediator; 3, CHIT–FC.

To form the electrode, 2.5 mg of produced polymer was dissolved in 250 μl of 1% acetic acid. Glutaraldehyde in the amount of 7.5 μl was added to 50 μl of the produced solution; 10 μl of the formed polymer was applied onto the surface of the carbon paste electrode and left for complete dry-out.

2.5. Modification of redox-active polymers by carbon nanotubes

For modification of redox-active polymers, 100 μl of deionized water was added to 0.5 mg of a suspension (0.5 wt%) of single-walled carbon nanotubes (Carbon Chg, Chernogolovka, Moscow Region, Russia) and left in an ultrasonic bath for 15 min. The produced suspension in the amount of 10 μl was added to redox-active polymer at the preparation of working electrodes.

In this way, the following electrodes were formed: BSA–NR–CNT, BSA–FC–CNT, CHIT–NR–CNT, CHIT–FC–CNT.

2.6. Immobilization of microorganisms into conducting matrices and formation of biosensors

To form a biosensor, 20 μl of a suspension contaning *P. yeei* bacteria (200 mg wet weight/mL) was applied onto a redox-active polymer-modified electrode and left for complete dry-out. Further on, one more layer of conducting redox-active polymer was applied. After drying, the electrode was coated with a dialysis membrane.

2.7. Biosensor measurements

Electrochemical measurements were conducted using an IPC-Micro galvanopotentiostat (OOO NTF Volta, St. Petersburg, Russia) integrated with a personal computer. The range of registered currents was from 5 up to 20 μA. The potential measurement error was no more than 0.1 mV for the interval of ±5 mV. The working potential was −380 mV vs Ag/AgCl for gels based on neutral red and +250 mV vs Ag/AgCl for gels based on ferrocene. Measurements were conducted at a temperature of 20 °C; the volume of the measuring cell was 5 cm^3. The measurements proceeded at constant stirring of the solution by a magnetic stirrer (300 rpm). After a stable level of current was settled on, a required amount of an investigated compound was introduced into the cell. The cell was washed with a potassium–sodium–phosphate buffer, pH 6.8, after each measurement. The measured parameter of the sensor response was the current amplitude determined as a difference between the final and initial values of current before and after adding analytes into the measuring cuvette.

2.8. Current–voltage characteristics

Voltammograms were registered by a three-electrode system using an Ekotest-VA voltammetric analyzer (OOO Ekoniks-Ekspert, Russia). A carbon paste electrode with an applied polymer composite was used as a working electrode; a platinum electrode was auxiliary. A saturated Ag/AgCl electrode was used as a reference electrode. Cyclic voltammograms were recorded at a scan rate of 20–100 mV/s in a potassium–sodium–phosphate buffer, pH 6.8.

2.9. Determination of the content of iron in specimens by atomic absorption spectroscopy

Produced specimens of conducting matrices were dried; a 1.2-mg amount of the dry substance was dissolved in 10 mL of deionized water with addition of 3-ml concentrated sulphuric acid and 2.5-ml hydrogen peroxide. The produced solution was boiled down for 2 h to a volume of ~2–3 mL and then diluted with 50 mL of deionized water. For the assay, the solution was diluted 1000-fold; 30 μl of the diluted solution was introduced into the measuring cuvette. Atomic absorption spectra were registered by an MGA-915 M spectrophotometer (Lumeks, Russia).

2.10. Determination of the content of NR in redox-active polymer by spectrophotometry

2.10.1. Determination of the NR content in a CHIT–NR redox-active polymer

A 5-μl amount of a 0.6-M neutral red solution, 50 μl of phosphate buffer (pH 6.8), 7.5 μl of glutaraldehyde were added to 500 μl of a 1% chitosan solution in 1% acetic acid. Unreacted NR was separated by dialysis and determined by an SF-104 spectrophotometer (Akvilon, Russia).

2.10.2. Determination of the NR content in a BSA–NR redox-active polymer

A 5-μl amount of a 0.6-M solution of neutral red and 50 μl of phosphate buffer, pH 6.8, were added to 3.5 mg of BSA. The mix was shaken for 5 min. Then 7.5 μl of glutaraldehyde (GA) was poured into the solution. Unreacted NR was separated by dialysis and determined by an SF-104 spectrophotometer (Akvilon, Russia).

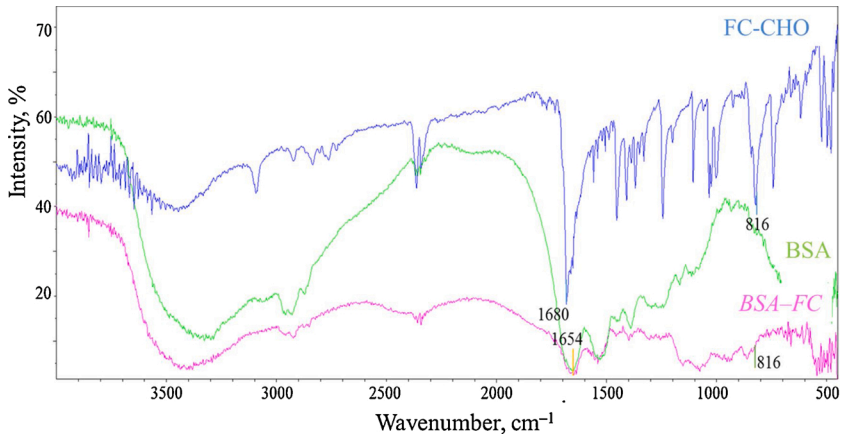


Fig. 1. IR spectra of the conducting matrix from FC-modified BSA (BSA–FC), of BSA and ferrocenecarboxaldehyde (FC-CHO).

2.11. Impedance spectroscopy

Impedance characteristics were measured on a VersaSTAT 4 galvanopotentiostat (Ametek Inc., USA). The spectra were obtained using a three-electrode measurement scheme, where a carbon paste electrode was the working electrode and a platinum plate of 30 mm^2 area served as an auxiliary electrode. An Ag/AgCl electrode was used as a reference electrode. Impedance measurements were conducted at a voltage of +350 mV (vs Ag/AgCl) within the frequency range of 40 kHz down to 0.02 Hz at a voltage modulation amplitude of 10 mV. The measurements were carried out in a 25-mM potassium–phosphate buffer solution, pH 6.8, containing 10 mM of sodium chloride.

2.12. Electron microscopy

Thin layers of a platinum–carbon mix were sputtered onto specimens of matrices in a JEE-4X vacuum evaporator (JEOL, Japan). Electron microscopy of the specimens was carried out using a JSM-6510 LV scanning electron microscope (JEOL, Japan) in a high vacuum mode at the registration of secondary electron images.

2.13. IR spectroscopy

IR spectra of the initial substances and reaction products were registered using an FMS infra-red Fourier spectrometer (OOO Monitoring, Russia). To measure the spectra of solid substances, KBr disks were used: 1–3 mg of a solid was thoroughly mixed in a mortar with spectroscopically pure potassium bromide (150–200 mg); the mix was pressed at $(7.5–10)\times10^3$ kgf/cm^2 for 2 min. The spectrum of the produced specimen was taken relative to the air.

2.14. Determination of BOD by the dilution method

The dilution technique was used as a reference method for determining BOD_5. The analysis was carried out in accordance with the method in [1]. The dissolved oxygen content was determined using an Ekspert-001-4.0.1 BOD thermo-oximeter (OOO Ekoniks-Ekspert, Russia).

3. Results and discussion

3.1. Production of biocompatible redox-active hydrogels based on polymers covalently bound to mediators

Neutral red and ferrocenecarboxaldehyde were used as mediators to produce conducting systems due to the presence of the required functional groups in them and their mediator properties. The choice of bovine serum albumin and chitosan for synthesis of redox-active polymers was determined by their high biocompatibility and non-toxicity. Immobilized microorganisms in the biocompatible base of the polymer provide a favourable microenvironment for the biocatalyst, increasing the time of functioning of the receptor element [22]. In addition, both polymers contain free amino groups required for targeted modification with redox-active compounds. These properties are required when using the developed redox-active polymers as matrices to immobilize microorganisms at the development of biosensors. As shown in [18], *Paracoccus yeei* VKM B-3302 bacteria are capable of oxidizing a wide range of organic compounds; for this purpose, they were chosen in this study as the basis of a bioreceptor element. To facilitate the transfer of electrons between mediator and electrode, single-walled carbon nanotubes were introduced into the system.

The synthesis of polymer matrices proceeded by nucleophilic addition, where glutaraldehyde played the role of a crosslinking reagent. The structures of produced matrices modified by the mediator were studied using IR spectroscopy.

After modification of BSA and chitosan with ferrocenecarbaldehyde, a new $-CH_2-NH-$ bond is formed, and similar phenomena are observed in the IR spectra (Fig. 1). Formation of a new bond is evidenced by a weak frequency band at 1654 cm^{-1} (bending vibrations of the secondary amino group δ –NH). Bending vibrations of the primary amino group at 1607 cm^{-1} are decreased in modified polymers against BSA and chitosan before modification, which indicates the bonding of part of the amino groups with mediator (the other part of the amino groups must remain for crosslinking). The absorption band of C═O group valent vibrations (wavenumber, 1680 cm^{-1}) of ferrocenecarboxaldehyde vanished in the modified matrix. This is due to the fact that the interaction of the aldehyde with the amino group is accompanied by the breaking of the —C═O bond. The presence of ferrocenyls in the formed film was

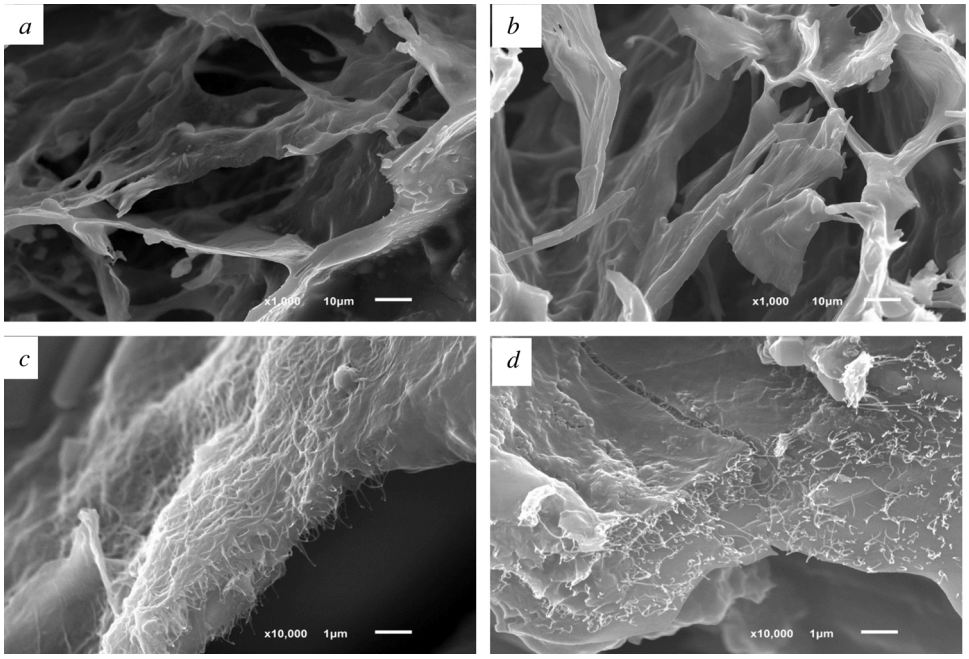


Fig. 2. Scanning electron micrographs of redox-active polymers: a, BSA–FC; b, CHIT–FC; c, BSA–FC–CNT; d, CHIT–FC–CNT.

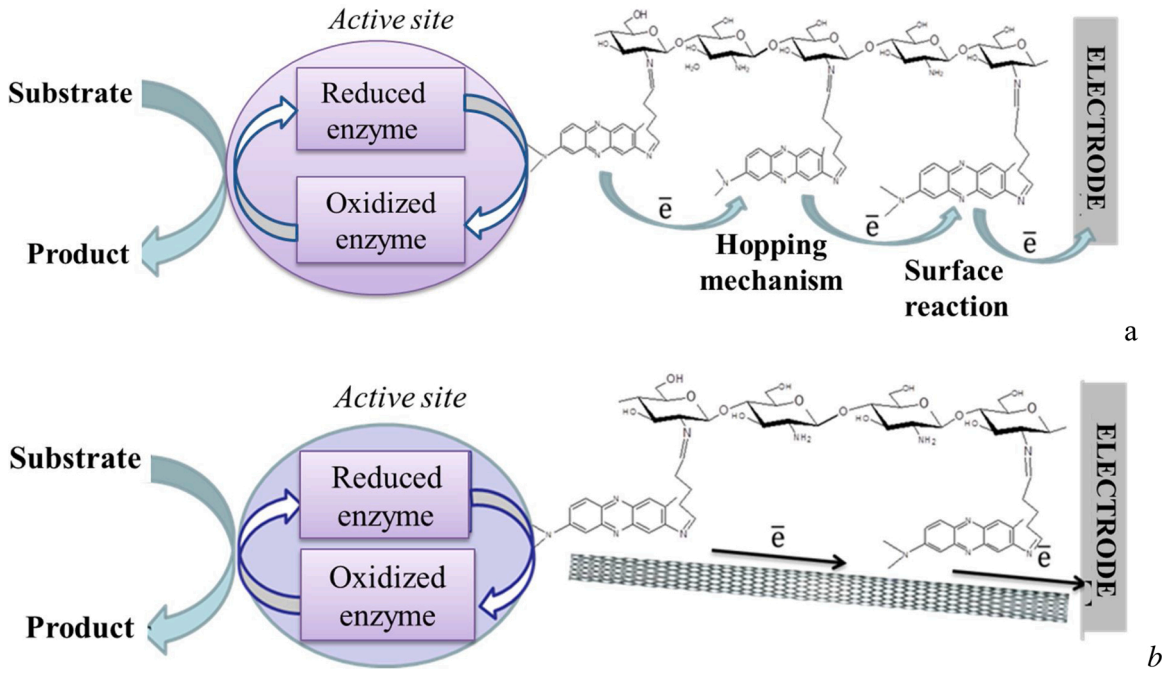


Fig. 3. Transfer of electrons to the electrode in a CHIT–NR redox polymer: a) in the absence of CNT; b) in the presence of CNT.

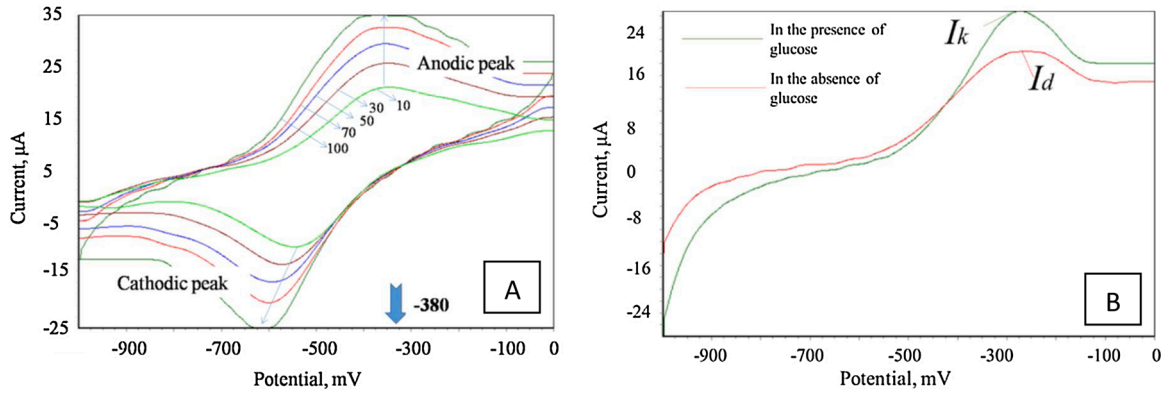


Fig. 4. Typical current–voltage dependences for studies of the electrochemical properties of formed matrices: a) a voltammogram of a BSA–NR redox-active polymer at an increase of the scan rate from 10 up to 100 mV/s; b) a change of the limiting anodic current as the result of the oxidation of glucose by P. yeei bacteria immobilized in a BSA–NR polymer at a scan rate of 10 mV/s.

determined by the occurrence of the absorption band of C—H deformation vibrations at a wavenumber of 820 cm^{-1}. In the formed film this absorption band is shifted to emerge at a wavenumber of 850 cm^{-1}; the results are consistent with [11], which had investigated the IR spectrum of a ferrocene-modified chitosan redox-active polymer.

Neutral red was crosslinked with chitosan and BSA without reducing the imide bond of the Schiff base; for this reason, a new absorption band corresponding to C═N valent vibrations of the imine bond of the Schiff base emerged in all spectra for a wavenumber of 1633 cm^{-1}. This absorption band appears in both redox-active polymers based on neutral red. Indirect evidence of the formation of a redox-active polymer was determined by the emergence of an absorption band at a wavenumber of 1500 cm^{-1}, characteristic of C = C–benzene ring valent vibrations. This band is absent in the initial chitosan matrix, which confirms the crosslinking of neutral red to the matrix. An increase was noted in the intensity of the band at a wavenumber of 2919 cm^{-1} in modified chitosan; this band corresponds to symmetric valent vibrations of CH_3 groups, which can also be explained by the introduction of methyl groups of neutral red into the structure of chitosan.

Determination of iron content in the ferrocene-based synthesized matrixes by atomic absorption spectroscopy showed the mass fraction of ferrocene in the BSA–FC matrix to be 14.1 %; in the CHIT–FC matrix, 6.2 %. Spectrophotometric determination of the NR content in the NR-based synthesized matrices showed the mass fraction of NR in the BSA–NR matrix to be 23 %; in the CHIT–NR matrix, 3%. Thus, it is more likely that NR–NR–BSA, BSA–NR dimeric fragments are formed after redox-active polymer synthesis, and an insignificant amount of NR-NR dimers, which are eliminated during the dialysis. The opposite situation is observed with neutral red-modified chitosan: formation of a larger number of NR–NR fragments, which are washed out by dialysis. Thus, despite a lower content of free amino acids in the BSA protein. BSA was subjected to modification more efficiently than chitosan. It is most likely due to the fact that the BSA is crosslinked with ferrocene or neutral red in an alkaline medium, which significantly facilitates the reaction of nucleophilic addition. Crosslinking of chitosan in an alkaline medium is difficult to perform due to its low solubility.

The structures of conducting hydrogels were studied by scanning electron microscopy. Their images are shown in Fig. 2.

Table 1
The heterogeneous electron transfer rate constants for investigated matrices and the constants of the rate of interaction of *P. yeei* bacteria with covalently bound mediators.

Redox-active polymer	Limiting stage of electron transfer	Heterogeneous transfer constant, cm/s	Constant of the rate of interaction of *P. yeei* bacteria with covalently bound mediators, $dm^3/(mg \cdot s)$
BSA-NR	SR[a]	0.0119 ± 0.0006	36 ± 2
BSA-NR-CNT	SR	0.041 ± 0.001	73 ± 3
CHIT-NR	HM[b]	0.054 ± 0.003	not detected
CHIT-NR-CNT	SR	0.14 ± 0.01	51 ± 2
BSA-FC	SR	0.45 ± 0.01	51 ± 4
BSA-FC-CNT	SR	0.55 ± 0.01	55 ± 1
CHIT-FC	SR	0.44 ± 0.02	52 ± 3
CHIT-FC-CNT	SR	0.55 ± 0.03	57 ± 2

[a] surface reaction.
[b] hopping mechanism.

Fig. 2a,b shows the porous structures of redox-active polymers. The produced hydrogels have pore sizes of about 10 μm. Thus, application of the produced gels onto the electrodes significantly increases the working surface area, the porous structure enables incorporation of *P. yeei* bacteria into the polymer. Carbon nanotubes (Fig. 2 c,d) contribute to their uniform distribution in the structure of the formed matrices. New chemical bonds are not formed between carbon nanotubes and the redox-active polymer; the incorporation into the structure of the redox-active polymer occurs due to the physical entrapment into the polymer backbone.

Determination of the electrochemical properties of produced redox-active polymers

Two stages can be singled out in the investigated electrochemical system in the absence of carbon nanotubes: the hopping mechanism (HM), the transfer of electrons between covalently bound electrons of the mediator, and the surface reaction (SR), the transfer of electrons from the mediator to the electrode (Fig. 3a).

When the distance between covalently bound mediators is large (Fig. 3a), the rate of the electron-transfer hopping component may decrease. Incorporation of carbon nanotubes into the polymer structure decreases the effective distance between mediator molecules (Fig. 3b). At a low level of modification of the polymers with redox-active compounds, when electroactive particles are not enough for the rapid transfer of electrons to the electrode, addition of nanomaterials into the system removes kinetic barriers due to, first, an increase in the effective surface area of the electrode and, second, due to the support of the conductivity between the covalently bound redox particles in the polymer, between which electron transfer does not occur without nanotubes. Addition of nanoparticles into the composition of a redox-active polymer makes it possible to "connect" more redox-active centres of polymer, and, consequently, to increase the recorded analytical signal, and, thus, to increase the measurement sensitivity.

The electrochemical properties of the produced conducting gels were investigated using cyclic voltammetry. In the case of an immobilized mediator process the most common case is the limitation by the surface reaction on the electrode and the current is proportional to the scan rate [23]. Another situation is realised when bonded redox species are mobile in the matrices and the peak current is primarily generated by a diffusion control redox hopping, it should obey the Randles–Sevcik equation [24,25]. Blauch and Saveant developed a model to describe electron motion in polymer films using percolation concepts. When the physical motion of the segments is either nonexistent or is very slow, the charge transfer takes place primarily via percolation through interconnected clusters of redox centres [26]. Typical cyclic dependences are shown in Fig. 4a.

The limiting stages of the electrochemical process for the formed matrices are given in Table 1. The finding of the limiting stage made it possible to use the Laviron model (eq. (1)) [23] in cases with the difference between the anodic and the cathodic peak larger than 200/n mV when the process is limited by the surface reaction on the electrode for determining the heterogeneous electron transfer constant. The model is applicable both for reversible and irreversible systems [27]:

$$\log(k_s) = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\left(\frac{RT}{nFv}\right) - \frac{\alpha(1-\alpha)nF\Delta E}{2.3RT} \quad (1)$$

where k_s is the heterogeneous constant of the rate of the electrochemical system (s^{-1} cm; n, the number of electrons involved; F, the Faraday constant (C/mol); ν, the potential scan rate (V/s); R, the universal gas constant (J molK); T, temperature (K); α, the cathodic transfer coefficient; $(1-\alpha)$, the anodic transfer coefficient; ΔE, the potential difference between the anodic and cathodic peaks (V).

To investigate the use of redox-active polymers for immobilizing microorganisms, the constants of interaction of microorganisms with covalently bound electron transfer mediators were founded, using Nicholson–Shain modelling [28] widely employed for the analysis of the constants of the rate of interaction of mediators, including modified

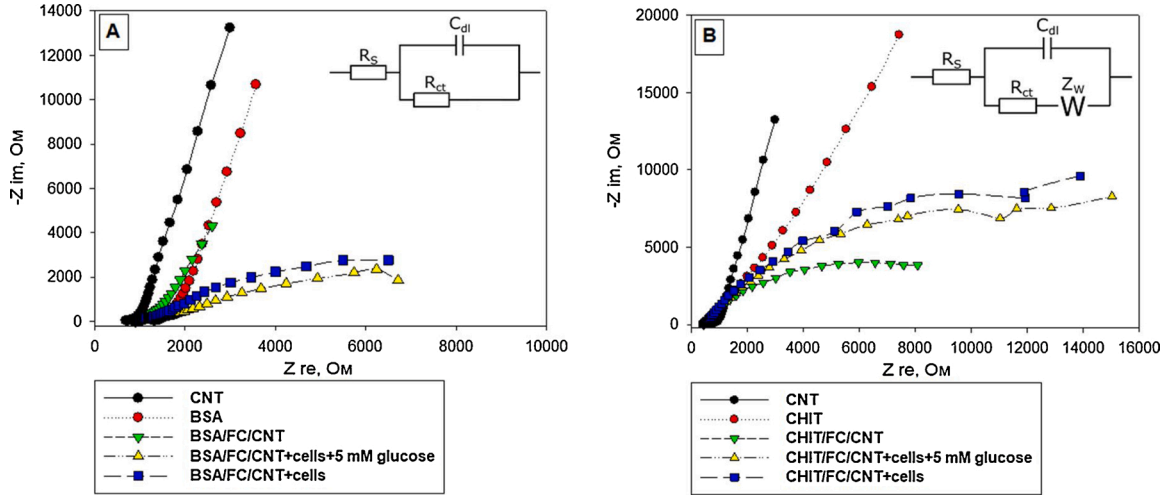


Fig. 5. Impedance spectroscopy of investigated matrices. A, plots corresponding to a BSA matrix-modified electrode; B, plots corresponding to a chitosan matrix-modified electrode.

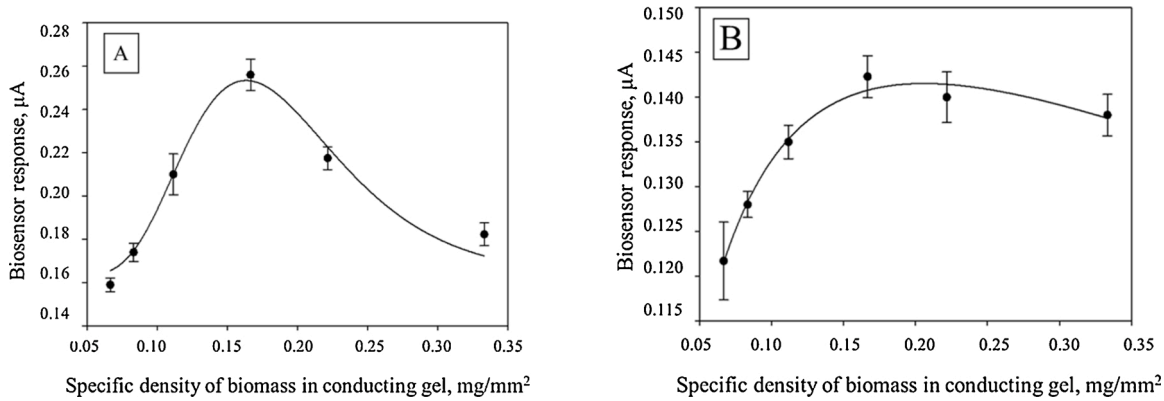


Fig. 6. Effect of the amount of biomaterial on the value of the generated biosensor signal. Conducting systems are based on BSA–FC–CNT (A) and CHIT–FC–CNT (B).

ones, with various enzymes [29][30]. Our previous research had shown the possibility of using this model with microbial cells [19]. The model considers a change of the limiting anodic current before and after the introduction of an oxidized substrate into the system. At an excess concentration of substrate and a low concentration of mediator, the rate of the biochemical stage of mediator–to–biomaterial interaction is of a pseudo first order, and the interaction constant is related to the change of limiting anodic current by the Nicholson–Shain equation (eq. 3). Typical curves are shown in Fig. 4b, and the values of the calculated constants are given in Table 1.

$$\frac{I_k}{I_d} = \sqrt{\frac{k_{\text{interact}}[E]RT}{nFv}} \tag{3}$$

where I_k is the limiting current in the presence of a substrate, A; I_d, the limiting current in the absence of a substrate, A; k_{interact}, the mediator–biomaterial interaction rate constant ($dm^3/(mg \cdot s)$); R, the universal gar constant, J/(mol K); T, temperature, K; $[E]$, the titre of cells, mg/l); ν, the scan rate, V/s; n, the number of transferred electrons; F, the Faraday constant, C/mol.

The largest rate of electrode exchange was achieved using matrices based on BSA modified by ferrocene. It should be noted that modification of matrices by nanomaterials assumes an increase of the rate of electron transfer to the electrode and, therefore, an increase of the heterogeneous electron transfer rate, which is supported by the obtained results. Presumably, this is due to an increase of the total conductivity of the gel; in particular, the increasing efficiency of the electron-transfer hopping component. The best rate interaction constants were also achieved upon addition of carbon nanotubes, which supports the prospects of their introduction into the matrix. On the whole, the constants of the interaction of covalently bound mediators with *P. yeei* bacteria are higher than using non-modified mediators [19]. It should be noted that the largest interaction rate constant is achieved in a BSA–NR–CNT system; however, the same system featured the lowest heterogeneous electron transfer rate constants, which eventually may affect negatively the sensitivity of the biosensor. Thus, polymers based on the covalently bound mediator ferrocene are more preferable both in respect with the heterogeneous electron transfer constant and the constant of the rate of interaction with *P. yeei* bacteria.

Features of the electrochemical properties of ferrocene-based redox-active polymers were investigated by impedance spectroscopy. Measurements of the impedance characteristics of the systems yielded frequency dependences of the real and imaginary components of impedance presented as Nyquist plots (Fig. 5).

It should be noted that a modification of a carbon-paste electrode by a CHIT–FC–CNT matrix considerably decreases the charge transfer resistance, whereas at a modification of the electrode by a composite the charge transfer resistance of the electrode decreases insignificantly. This can be due to the fact that chitosan-based matrix interacts more efficiently with the electrode and carbon nanotubes, creating a larger contact area and providing for an easier passage of electric current along the conducting channels. At the same time, it should be noted that at a modification of the electrode with the CHIT–FC–CNT matrix by bacterial cells a noticeable increase of the electrode impedance is observed. For electrodes with a BSA–FC–CNT matrix, the opposite effect is observed. It could be assumed that the BSA–FC–CNT matrix interacts better with bacterial cells and provides for a more reliable electron transfer from cells to the electrode surface due to the presence of a larger amount of crosslinked ferrocene groups (in accordance with the results of atomic absorption spectroscopy, the content of iron in modified BSA is almost

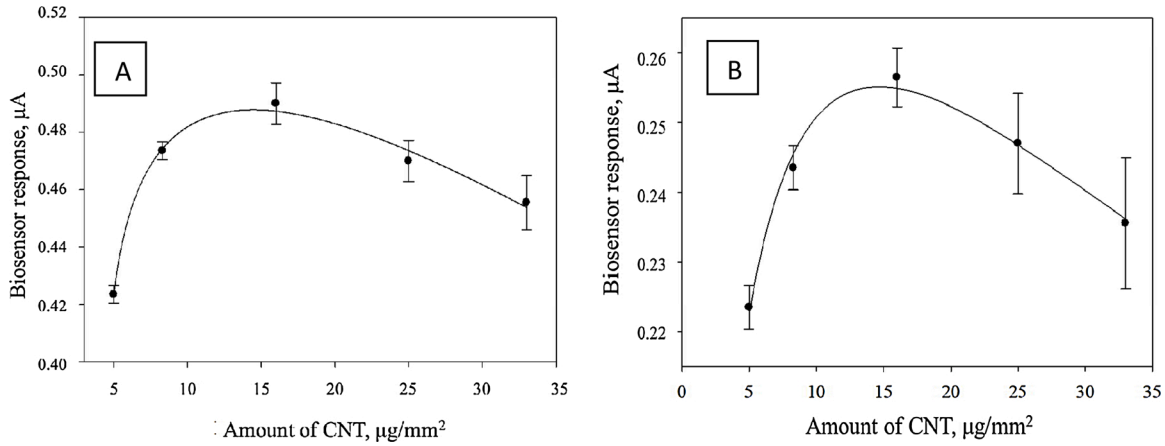


Fig. 7. Effect of the amount of CNT on the value of the generated biosensor signal. Conducting systems are based on BSA–FC–CNT (A) and CHIT–FC–CNT (B).

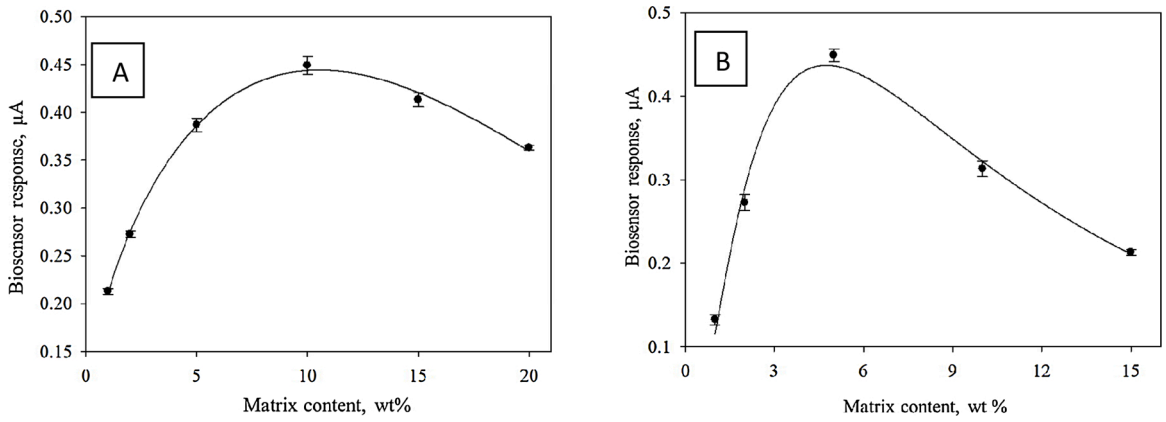


Fig. 8. Effect of the content of a matrix on the value of the generated biosensor signal. Conducting systems are based on BSA–FC–CNT (A) and CHIT–FC–CNT (B).

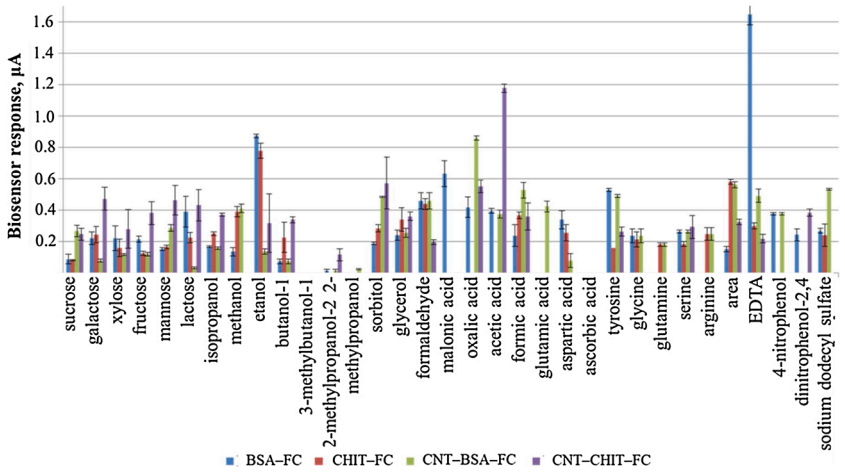


Fig. 9. Substrate specificity of P. yeei cells immobilized in redox-active polymers.

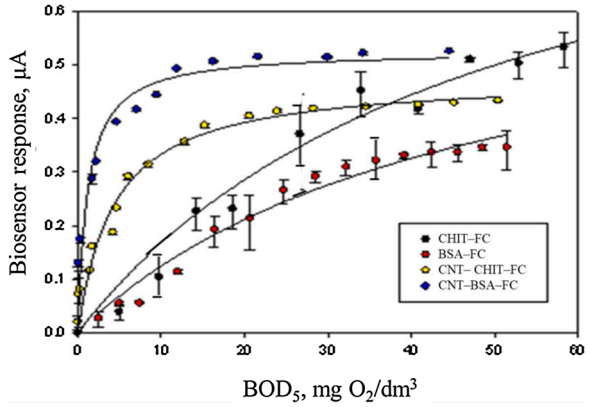


Fig. 10. Calibration dependences of the response of a biosensor based on P. yeei bacteria, immobilized in redox-active polymers, on BOD_5 concentration.

twice as high as in modified chitosan). It should be noted that upon addition of substrate into the cell, the impedance of electrodes based on both types of matrices was observed to decrease (from 7520 down to 6950 ohms in the case of using BSA–FC–CNT and from 14,825 down to 13,923 ohms in the case of CHIT–FC–CNT redox-active polymer). Also, using a modified CHIT–FC–CNT polymer, a bend in the Nyquist plots was observed within the region of high frequencies, which can be related to the occurrence of a Warburg element. This implies insignificant diffusion limitations in the system, which are absent almost totally when using a BSA–FC–CNT matrix.

Thus, it can be concluded from the results of cyclic voltammetry and impedance spectroscopy of the formed polymers that a better conductivity is achieved using CHIT–FC–CNT and BSA–FC–CNT systems. This determined the selection of these matrices for forming BOD biosensors.

3.2. *Formation of a biosensor based on produced redox-active polymers*

When creating biosensors, it is important to determine the working parameters at which the current generated by the biosensor would be maximal. The magnitude of current generated by the biosensor in the presence of a substrate may change depending on conditions under which the measurements are carried out (concentrations of mediators, weights of biomaterial on the electrode, pH of the medium).

The dependence of biosensor responses on pH of the medium can be determined by both the properties of biomaterial used and the nature of mediator. Proceeding from this, dependences of biosensor responses on pH of the medium were studied within the pH range from 5.6–8.0. To maintain the required pH, use was made of a potassium–sodium phosphate buffer solution with various ratios of the buffer system components (30 mM potassium dihydrophosphate and 30 mM sodium hydrophosphate). The experimentally largest responses for both biosensors were registered at pH 6.8, which was taken as the working value.

The amount of biomaterial on the electrode has a direct effect on the parameters of the developed biosensor; however, an increase of the biomaterial layer may lead to diffusion limitations both for the used substrate and for mediator.

The values of biosensor responses, as it is shown in Fig. 6, rise

Table 2
Comparative analytical and metrological characteristics of mediator biosensors.

Biomaterial/Conducting system	Long-time stability, days	Relative standard deviation, % (n = 15)	BOD_5 linear range, mg/dm^3	Assay time, min	Ref.
P. yeei/BSA–FC	38	5.7	2.5–47	5	This work
P. yeei/BSA–FC–CNT	25	5.9	0.1–2	5	This work
P. yeei/CHIT–FC	49	6.6	2.5–18.9	5	This work
P. yeei/CHIT–FC–CNT	50	5	0.1–4.5	5	This work
B. subtilis/PHCF–GF–PPy	60	1.8	4–60	15	[14]
P. aeruginosa/PHCF–PPy	5	2	5–100	10	[15]
Activated sludge/MB–CHIT–GF	65	2.9	1–100	9	[32]
P. aeruginosa/pNR–PPy	10	3	5–100	20	[16]
C. violaceum/PHCF	10	15.4	20–225	30	[33]

PHCF, potassium hexacyanoferrate (III); GF, graphene; PPy, polypyrrole; MB, methylene blue; pNR, poly(neutral red).

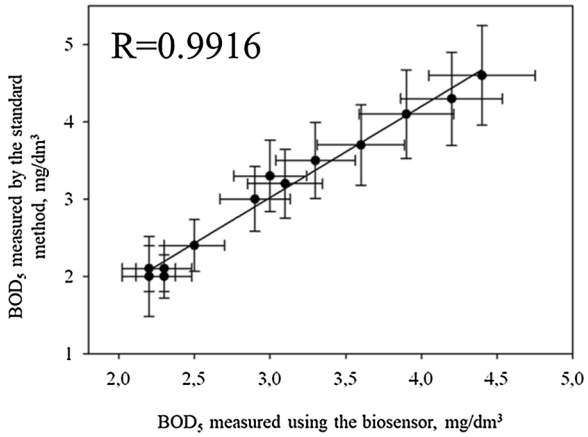


Fig. 11. The linear dependences of the results of a BOD_5 assay by the standard method and using the developed biosensor.

linearly at an increase of the amount of biomaterial in the redox-active polymer, owing to the fact that the generated current is proportional to the concentration of the enzyme per unit area of the electrode surface. At an increase of the number of bacteria on the electrode surface up to values exceeding 0.16 mg/mm^2 the biosensor responses go down, which is due to the emerging diffusion limitations.

A high concentration of CNT increases the generated currents (Fig. 7) and, therefore, the analytical signal but, at the same time, may cause a toxic action on microbial cells [31]. Mediator biosensors based on redox-active polymers possess a maximal analytical signal at a CNT content on the electrode of an order of 16 μg/mm^2.

To form the receptor system, a mass concentration of redox-active polymer based on a covalently bound mediator was chosen: the higher the initial concentration of the modified matrix, the higher the concentration of mediator in the system was. To find an optimal concentration of the matrix, 1–20 wt % of the initial redox-active polymers was taken based on the mediator per the same content of glutaraldehyde (Fig. 8).

It is seen from the data of Fig. 8 that the biosensor response decreased with the increase of the content of the matrix above certain values. Based on these data, the optimal concentrations of the matrices were chosen to be 10 % for BSA–FC–CNT and 5% for CHIT–FC–CNT. Further studies were conducted at these parameters.

3.3. Determination of the analytical and metrological characteristics of biosensors based on produced redox-active polymers

For a BOD_5 assay to be conducted, it is required that the developed biosensor register biochemical oxidation of a wide range of organic substrates. Substrate specificity of biosensors based on the developed conducting systems was investigated. Fig. 9 presents the obtained results.

Analysis of the substrate specificity enables a conclusion that the character of substrate oxidation changes depending on the matrix used. In the presence of assayed compounds, the sensor will generate a signal corresponding to the joint oxidation of the substrates by microorganisms. The sensor is not suitable for selective detection of assayed compounds; however, for joint pollution measurements, when each substrate increases the BOD value, such an analytical system becomes appropriate. On the whole, all developed biosensors make it possible to register the oxidation of a sufficiently wide range of organic compounds by *P. yeei* bacteria, which ensures the accuracy of BOD_5 assay results.

For assaying the content of analyzed substances in a sample, the hyperbolic dependences of biosensor responses on BOD_5 were investigated (Fig. 10); the dependences were approximated by the Michaelis–Menten equation:

$$\upsilon = \frac{\upsilon_{max}[S]}{K_M + [S]}, \quad (4)$$

where υ is the rate of the enzymatic reaction; υ_{max} is the maximal rate of the enzymatic reaction; K_M, the apparent Michaelis constant; [S], the initial concentration of the substrate.

For the analysis of the stability and invariability of the sensor response at a long-time use, the long-time and operational stabilities were investigated. Table 2 presents all characteristics of the developed mediator biosensors based on *P. yeei* bacteria and of analogues.

Comparing the metrological characteristics of the developed systems and their analogues, it can be seen that the lower limit of the determined BOD_5 concentrations of the developed biosensors based on redox-active BSA-FC and Chit-FC polymers is about 2.5 mg O_2/ dm^3, which is better than many known analogues with their lower limits of 1–10 mg O_2/dm^3 [14–16,32,33]. CNTs enable increasing the efficiency of electron transfer to the electrode and provide the high sensitivity of the biosensor (quantification limit, 0.1 mg O_2/dm^3), making possible the monitoring of natural waters, where the BOD index is within the range of admissible concentrations (up to 2 mg O_2/dm^3). Proceeding from the obtained results, by its selectivity, long-time stability and range of assayed concentrations the biosensor based on *P. yeei* bacteria and CHIT–FC–CNT conducting system is promising for developing a BOD biosensor.

3.4. Appraisal of the developed biosensor

The biosensor based on *P. yeei* bacteria and a CHIT–FC–CNT conducting system was appraised on 14 samples of surface waters (Fig. 11). Sample taking and BOD_5 determination by the standard method were conducted according to the effective regulatory documents.

The statistical treatment (using a modified Student's t-test) of the produced results showed that the data obtained by both methods differed insignificantly. The developed biosensor can be used as an alternative to the standard assay.

4. Conclusion

This work developed redox-active polymers based on bovine serum

albumin and chitosan modified by neutral red and ferrocenecarboxaldehyde. IR spectroscopy of produced matrices confirmed the formation of mediator-to-polymer crosslinking. Redox-active polymers based on neutral red featured an absorption band at 1633 cm^{-1}, corresponding to C═N valent vibrations of the imine bond of the Schiff base; in ferrocene-based polymers a weak absorption band appeared at a frequency of 1654 cm^{-1} (deformation vibrations of the secondary amino group δ –NH). Electron microscopy showed the matrices to have porous structures and the size of pores pore sizes of about 10 μm to enable immobilization of the used microorganisms. Atomic absorption spectroscopy determined the mass fractions of ferrocene to be 14.1 % for the BSA–FC matrix and 6.2 % for the CHIT–FC matrix. The mass fractions of neutral red were determined spectrophotometrically to be 23 % for the BSA–NR matrix and 3 % for the CHIT–NR matrix.

Cyclic voltammetry determined the electrochemical parameters of the formed matrices, such as the constant of the rate of interaction of *P. yeei* bacteria with covalently bound mediators and the heterogeneous electron transfer rate constant. These data enable a conclusion that the rate constants of the electrochemical processes increase at the introduction of carbon nanotubes. The most promising are the redox-active polymers BSA–FC–CNT and CHIT–FC–CNT, which have the largest heterogeneous electron transfer rate constants (0.55 ± 0.01 and 0.55 ± 0.03 cm/s, respectively) and the constants of the rate of interaction with *P. yeei* bacteria (55 ± 1 and 57 ± 2 $dm^3/(mg \cdot s)$), as well as the lowest impedance.

Biosensors based on *P. yeei* bacteria and the developed matrices were formed. Biosensor characteristics, such as the long-time and operational stabilities, substrate specificity, the lower limit of the determined content for each matrix, were found. The obtained data showed the redox-active polymers based on BSA and chitosan covalently bound to ferrocene and modified by carbon nanotubes to have the highest specificity (quantification limit, 0.1 mg O_2/dm^3).

The biosensor based on the matrix of chitosan modified by ferrocene and CNT was used to carry out a surface water assay. The obtained data differed insignificantly from the results of the standard method (high correlation coefficient (R = 0.9916)), which is indicative of the prospects of using the developed biosensor as an alternative to the standard analysis.

CRediT authorship contribution statement

V.A. Arlyapov: Data curation, Formal analysis, Validation, Writing. **A.S. Kharkova:** Writing, Visualization, Investigation. **S.K. Kurbanaliyeva:** Writing, Visualization, Investigation. **L.S. Kuznetsova:** Writing, Visualization, Investigation. **A.V. Machulin:** Software, Data curation, Investigation. **S.E. Tarasov:** Methodology, Visualization. **P.V. Melnikov:** Data curation, Formal analysis. **O.N. Ponamoreva:** Validation, Formal analysis. **V.A. Alferov:** Resources, Validation. **A.N. Reshetilov:** Writing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

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