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Electrochemical sensor for the detection of serine β-lactamase catalytic activity

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Abstract

A new approach is proposed based on the use of electrodes modified with carbon nanomaterials to determine enzymatic activity and screening for inhibitors of serine β-lactamases such as extended spectrum β-lactamases (ESBLs). These enzymes are responsible for the development of antibiotic resistance of pathogenic bacteria to β-lactam antibiotics. Electrochemical oxidation of cephalosporin antibiotic cefotaxime was effectively registered at a potential *E* from +596 to +625 mV (relative to Ag/AgCl). This property makes it possible to determine the change in cefotaxime concentration in solution upon interaction with serine β-lactamases. By analyzing the electrochemical characteristics of the cefotaxime oxidation reaction, the kinetic parameters of its hydrolysis catalyzed by the serine β-lactamase CTX-M-116 were determined. The Michaelis constant was K_M = 50 μM and the maximum rate of the catalytic reaction was $1.67 \cdot 10^{-6}$ M/min. A comparative analysis of the electrochemical parameters of the enzyme/substrate cefotaxime and enzyme/substrate cefotaxime/inhibitor sulbactam (SBT) systems was carried out. Inhibition of β-lactamase by sulbactam was characterized by an IC_{50} value of 2.5 μM. The proposed approach can be used for screening new substrates and inhibitors of β-lactamases.

Keywords

electroanalysis
nanostructured screen-printed electrodes
β-lactamase
cefotaxime
sulbactam
bioelectrochemistry

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Key findings

- Oxidation of antibiotic cefotaxime was effectively registered on nanostructured screen-printed electrodes at a potential of *E* from +596 to +625 mV.
- Enzymatic activity of β-lactamase was determined by means of electrochemical technique based on registration of cefotaxime inactivation.
- Sulbactam efficiently inhibits β-lactamase catalytic activity.

1. Introduction

The rise in resistance to antibiotics (antibacterial drugs) has become a major concern worldwide in recent years. According to World Health Organization, in Europe alone, about 25,000 patients die annually from infections caused by antibiotic resistance [1–3]. Among all antibacterial drugs, β-lactam antibiotics are the most efficient and frequently used ones [4]. The main processes associated with the development of bacterial resistance to antibiotics are determined by β-lactamases. These enzymes, the substrates of which are β-lactam antibiotics, appeared evolutionarily in bacteria to inactivate them by hydrolysis of the C–N bond of the β-lactam ring [5–7]. β-Lactamases form a superfamily of enzymes divided into serine hydrolases and metalloenzymes [8]. In serine β-lactamases, at the first stage, the side chain of the catalytic serine residue is acylated with the formation of an acyl-enzyme complex, which is then hydrolyzed with the participation of a water molecule [9]. Analysis of the catalytic activity of serine β-lactamases is of interest for identifying new potential substrates of this en-

zyme with antibacterial activity. The search for new inhibitors of these enzymes to suppress antibiotic resistance of bacterial pathogens and increase the efficiency of existing β-lactam antibiotics is a very promising area of pharmacology and medicine [3, 9].

Cefotaxime (7-[a-aminothiazol-4-yl)-a-(z)-methoxy-iminoacetamido]-3-(1-methyl acetate)-methyl-3-cephem-4-carboxylate) is a third generation antibiotic belonging to the class of cephalosporins, usually recommended for the clinical treatment of various bacterial infections caused by gram-negative bacteria belonging to the family Enterobacteriaceae [10]. It is also used in the food industry and canning [10].

Cefotaxime is the main substrate of CTX-M-type serine β-lactamases, which are the most common extended spectrum β-lactamases (ESBLs) of pathogenic infectious agents [11]. The main reason for bacterial resistance to β-lactam antibiotics is the hydrolysis of these drugs by β-lactamases [12].

This paper proposes a new efficient approach for determining the catalytic activity of serine β-lactamases based on registration the electrochemical parameters of the oxidation of cefotaxime. Antibiotic before and after β-lactamase impact was immobilized on screen-printed graphite electrodes, modified with carbon nanotubes. The recombinant β-lactamase CTX-M-116, which belongs to the enzymes of the most common subcluster CTX-M-1, was chosen as a model serine ESBL [13, 14].

The resistance of pathogenic bacteria to β-lactam antibiotics can be evaluated by registering the activity of β-lactamase. There are different approaches for the determination of catalytic activity of β-lactamases enzyme superfamily [5]. Phenotypic detection as a traditional approach is a golden principle, but it is time-consuming and expensive methods [5, 15]. Methods for the registration of β-lactamases based on hydrolysis of antibiotics are also used with detection of substrate or hydrolyzed products by acidity method, iodometry, and spectrophotometry. Hydrolysis-based β-lactamase detection methods also include mass-spectrometry-based methods, lateral flow immunoassays and fluorescent assays [5, 7, 15].

The novelty of the approach proposed in this work is the development of a platform of nanostructured screen-printed graphite electrodes with an immobilized electroactive substrate for studying the catalytic activity of β-lactamase CTX-M-116 and the effect of inhibitors on the hydrolytic properties of the enzyme.

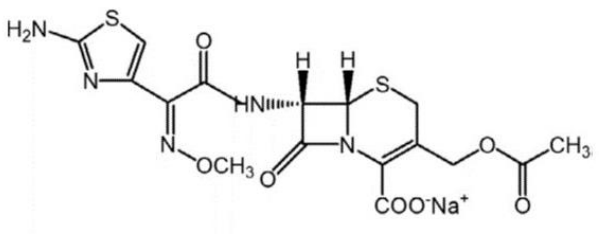

Figure 1 Chemical structure of Cefotaxime (7-[a-aminothiazol-4-yl)-a-(z)-methoxy-iminoacetamido]-3-(1-methyl acetate)-methyl-3-cephem-4-carboxylate).

2. Materials and Methods

2.1. Reagents and Materials

Water dispersion of 0.4% single-wall carbon nanotubes (SWCNT, diameter 1.6±0.4 nm, length > 5 μM, surface area 1000 m^2/g) TUBALL™ BATT H2O stabilized by carboxymethylcellulose was obtained from OCSIAL Ltd. (https://ocsial.com, Oksial Additives NSK LLC, Novosibirsk, Russia). Cefotaxime sodium salt (CFT) and sulbactam (SBT) were obtained from Sigma–Aldrich (c7039 and s9701, correspondingly). Stock solutions of CFT and SBT were prepared as 10 mM in 0.1 MM potassium phosphate with 50 mM NaCl at pH 7.4 (PBS). Recombinant β-lactamase CTX-M-116 was expressed in Escherichia coli as described in [16]. The cells were grown under vigorous shaking at 30 °C in 300 mL LB medium containing 50 μg/mL kanamycin. When the optical density (OD600) of the culture medium reached the value of 0.8, the expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were further grown for 5 h and then harvested by centrifugation (3,000 g, 15 min, 4 °C). The periplasmic protein fraction was isolated using osmotic shock, by re-suspending the cell pellet in 10 mM Tris/HCl pH 8.0, containing 1 mM EDTA and 20% (w/v) sucrose for 15 min at room temperature. After removing the spheroplasts by centrifugation (10,000 g, 10 min, 4 °C), the supernatant containing the periplasmic fraction was dialyzed against 50 mM Na-acetate buffer pH 5.5. The crude extract was applied to S-Sepharose cation exchange column (Amersham-Pharmacia-Biotech). The recombinant enzyme was eluted by a linear salt gradient of 0–0.3 M NaCl at a concentration of 100–150 мM NaCl. Fractions containing β-lactamase CTX-M-116 were pooled and dialyzed against 10 mM Tris/HCl pH 8.0.

2.2. Electrochemical measurements

Cyclic voltammetry (CV) and square wave voltammetry (SWV) measurements were performed using an Autolab PGSTAT12 potentiostat/galvanostat (Metrohm Autolab, the Netherlands) equipped with GPES software (version 4.9.7). All electrochemical experiments were carried out at room temperature in 0.1 M potassium phosphate with 50 mM NaCl at pH 7.4. CV experiments were carried out in a 1 mL electrochemical cell by potential sweeping from an initial potential of 0 V to an end-point potential of +1 V at different scan rates in a range of 10–100 mV/s.

2.3. Square wave voltammetry (SWV) measurements

The SWV settings were as follows: potential range of 0–1.2 V, pulse amplitude of 0.005 V, potential step of 0.005 V, and frequency 10 Hz. SWV experiments were carried out in a 60 μL drop applied onto the electrode to cover all three electrodes in a horizontal arrangement.

Electrochemical studies were performed in PBS. For preparation of the modified electrodes, 2 μL of SWCNT in

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carboxymethylcellulose diluted with water as 1:4 was dropped onto the working area of the SPE (denoted as SPE/SWCNT) and incubated for 15 min. All potentials were referenced to the Ag/AgCl electrode. The data are presented as average values ± standard deviations.

Modified SPE/SWCNTs were utilized for a single measurement to avoid surface fouling, cross-contamination and blockage of the electrode surface by oxidation products of protein. Each sample was tested with three independent measurements. The relative standard deviation in all cases did not exceed 10%. In the figures, data points represent the mean values of the detected peak currents (I_p) or potential maxima (E_{max}), with the error bars showing the confidence intervals. Each voltammogram was processed with Savitzky-Golay level 3 smoothing and baseline corrected to a peak width of 0.003 V using the GPES moving average baseline correction tool. Oxidation peak current and peak potential values were then recorded for each measurement.

2.4. Preparation of modified electrode

For incorporation of CFT onto the surface of modified SPE (SPE SPE/SWCNT), 60 μL of the CFT solution at specified concentrations prepared in PBS, pH 7.4, was dropped onto the surface of modified electrode (SPE/ SPE/SWCNT) and incubated for 30 min before measurements. Horizontal arrangement of the electrodes was used for all experiments.

The electrochemical active surface area on SPE/SPE/SWCNT was determined from the cyclic voltammetry of 5 mM $K_4[Fe(CN)_6]$ in 0.1 M KCl solution according to the Randles-Shevchik Equation [17, 18] and corresponded to 0.1258 cm^2 [19].

2.5. Catalytic activity and inhibition analysis of β-lactamase CTX-M-116

Experiments to determine catalytic activity and inhibition analysis of β-lactamase CTX-M-116 were performed in Eppendorf tubes with 100 μM CFT in the presence of 17 nM β-lactamase CTX-M-116 in PBS solution for 5–50 min [8–10]. Then the enzyme-substrate mixture in a volume of 60 μL was applied to the SPE/SWCNT electrode and incubated for 10 min. Inhibition assay was performed with 50 μM CFT in the presence of 17 nM β-lactamase CTX-M-116 in PBS solution and SBT in the concentration range of 2–50 μM in in Eppendorf tubes. Then the enzyme-substrate-inhibitor mixture in a volume of 60 μL was applied to the SPE/SWCNT electrode and incubated for 10 min before SWV measurements.

3. Result and Discussion

Enzymes possess unique catalytic activities and catalyze extremely selective chemical reactions in living systems. Electroanalysis of enzyme catalytic activity is based on the two main approaches. The first one is enzyme immobilization with a biocatalytic layer on an electrode transducer [18–22]. The functional electrode contains immobilized enzymes that retain their catalytic activities. The second approach is immobilization of substrate on the electrode surface [23–25] and monitoring of chemical reactions catalyzed by active enzyme with subsequent measuring of product formation or substrate depletion.

The key point of electroanalysis is the informed choice of both the type of electrodes and methods for modifying their working surfaces to enhance the useful signal and increase the signal-to-noise ratio [26–28]. Screen-printed electrodes are widely used as sensing elements in bielectrochemical area [28, 29]. Cyclic voltammetry measurements in 5 mM $K_4[Fe(CN)_6]$ in 0.1 M KCl as external electrolyte were used to investigate the electroactive surface area of the modified SPE/SWCNTs [18, 19]. Carbon nanotubes enhanced conductivity and electron transfer properties of electrodes. Modification of the working surface of screen-printed electrodes with dispersion of carbon nanotubes significantly improves the analytical characteristics of sensor elements [30–33].

In our investigation, for the determination of catalytic activity of β-lactamase CTX-M-116 we used immobilization of substrate cefotaxime. The decrease of its concentration was monitored after enzymatic reaction catalyzed by β-lactamase CTX-M-116.

Cefotaxime was analyzed by cyclic voltammetry (Figure 2A) and square wave voltammetry (Figure 3A). At potential E from +596 to +625 mV, a peak was recorded corresponding to the electrochemical oxidation of the amino group of the thiaazole ring [33] (Figure 2A). In the range of 2–200 μM, a linear, directly proportional relationship is observed between the maximum amplitude of the electrooxidation current of cefotaxime adsorbed on the surface of the nanostructured electrode and its concentration (Figure 3B). The sensitivity of the method and the limit of detectable concentrations correspond to 0.0426 μA/μM and 0.67 μM (Table 1).

Oxidation peak potential (E_{pa}) of CFT cyclic voltammetry shifted towards the positive direction with the increase in the scan rate. This is a characteristic feature of an irreversible electrode reaction (Figure 2A) [35–37]. A linear relationships between the oxidation peak current and the scan rate ν and between the oxidation peak current and square root of scan rate $\nu_{1/2}$ for SPE/SWCNT were registered (Figure 2B, C). The explanation of these experimental dependences indicated that the CFT electrochemical oxidation process possesses mixing mechanism, controlled by both adsorption and diffusion [37–39]. The slope of the $\log I_{pa}$ against $\log\nu$ is 0.17 (Figure 2D); the equation's slope confirmed that the electrochemical oxidation process of CFT is characterized by a mixed mechanism controlled by a mixed diffusion-controlled electrochemical reaction [35–38].

Since cefotaxime exhibits substrate properties towards β-lactamase CTX-M-116, a quantitative electroanalysis of the antibiotic was carried out before and after the enzymatic reaction. 100 μM CFT was incubated in the presence of 17 nM β-lactamase CTX-M-116 in PBS solution for 5–50 min [8–10].

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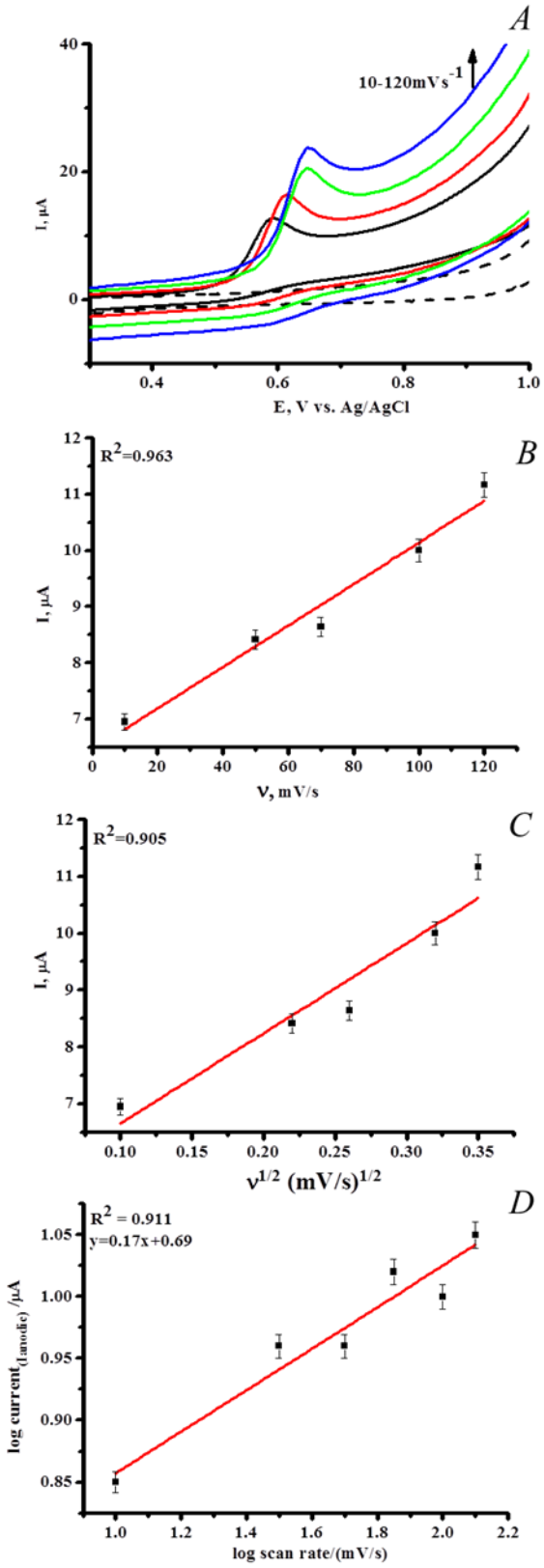

Figure 2 Cyclic voltammograms of 500 μM cefotaxime on SPE/SWCNT at scan rates from 10 to 300 mV/s (A); the dependence of the oxidation peaks current vs the scan rate (B); the linear plot of the oxidation peaks current of CFT vs. the square root of the scan rate (C); log(I_p) dependence on log (ν) (D).

Then the enzyme-substrate mixture in a volume of 60 μL was applied to the SPE/SWCNT electrode and incubated for 10 min.

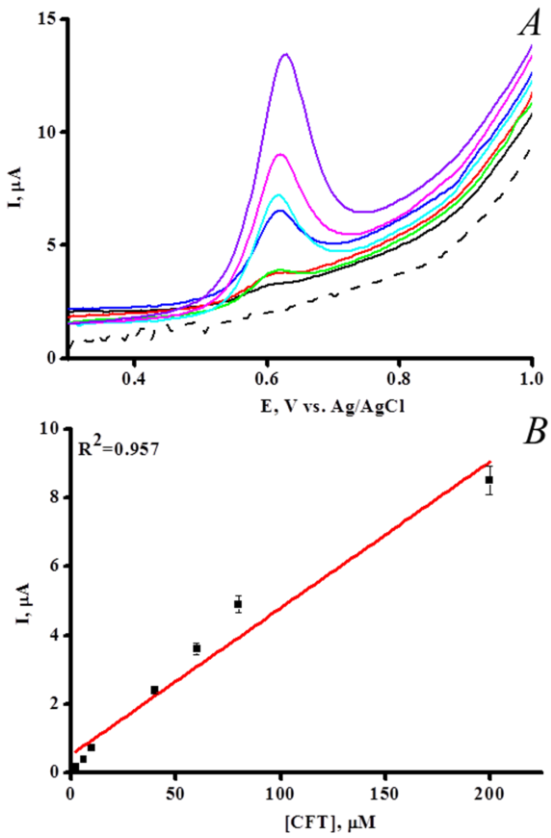

Figure 3 Square wave voltammetry of SPE/SWCNT with increasing cefotaxime (CFT) concentration in the range of 2–200 μM (-) (A); concentration dependence of SWV peak current of CFT for SPE/SWCNT (B).

Table 1 Electroanalytical parameters of SWV for cefotaxime determination by SPE/SWCNT.

Parameters	SPE/SWCNT/CFT
E_{ox}, V	0.63±0.05
Sensitivity, μA/μM (Slope)	0.043
Linear range, μM	2–200
Limit of detection LOD, μM	0.67
Equation for linear regression*	I = 0.043±0.004c(CFT)+0.5±0.3
Determination coefficient, R^2	0.957

Square-wave voltammograms of cefotaxime before and after the β-lactamase-catalyzed enzymatic reaction clearly demonstrate the decline of peak current as a result of enzyme activity (Figure 4A). A decrease in the intensity of the peak corresponding to cefotaxime is recorded depending on the time of incubation with the enzyme due to an enzymatic reaction leading to the destruction of the antibiotic (Figure 4B).

To combat β-lactamase-mediated resistance, β-lactamase inhibitors were used that restore the therapeutic activity of β-lactam drugs. The development of effective β-lactamase inhibitors is an important direction for increasing the efficiency of β-lactam antibiotics in the treatment of bacterial infections caused by antibiotic-resistant pathogenic bacteria [3, 39].

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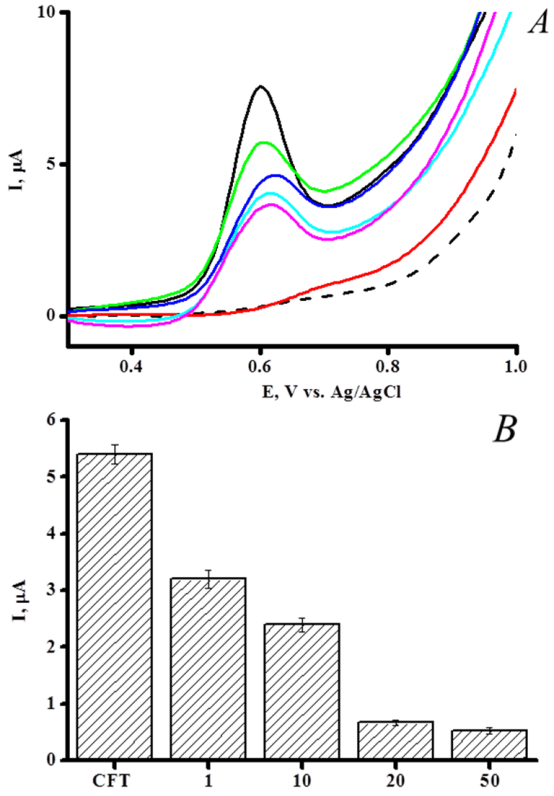

Figure 4 (A) Square-wave voltammograms of SPE/SWCNT /cefotaxime electrodes before (-) and after (colored lines) hydrolysis of 100 μM cefotaxime in the presence of 17 nM β-lactamase CTX-M-116 depending on the incubation time in solution (in Eppendorf tube) (0– 50 min). (-) SPE/SWCNT/cefotaxime 100 μM without enzyme degradation, (-) SPE/SWCNT/cefotaxime, after 1 min incubation in the presence of 17 nM β-lactamase CTX-M-116, (-) SPE/SWCNT/cefotaxime, after 10 min incubation in the presence of 17 nM β-lactamase CTX-M-116, (-) SPE/SWCNT/cefotaxime, after 20 min incubation in the presence of 17 nM β-lactamase CTX-M-116, (-) SPE/SWCNT/cefotaxime, after 50 min incubation in the presence of 17 nM β-lactamase CTX-M-116. The electrode SPE/SWCNT is represented as (---), SPE/SWCNT/β-lactamase is represented as (-). (B) Histogram of oxidation peak current of substrate CFT concentration on SPE/ SWCNT before and after incubation with 17 nM β-lactamase CTX-M-116. Incubation time in solution for the enzymatic reaction to occur is 1 min, 10 min, 20 min, 50 min.

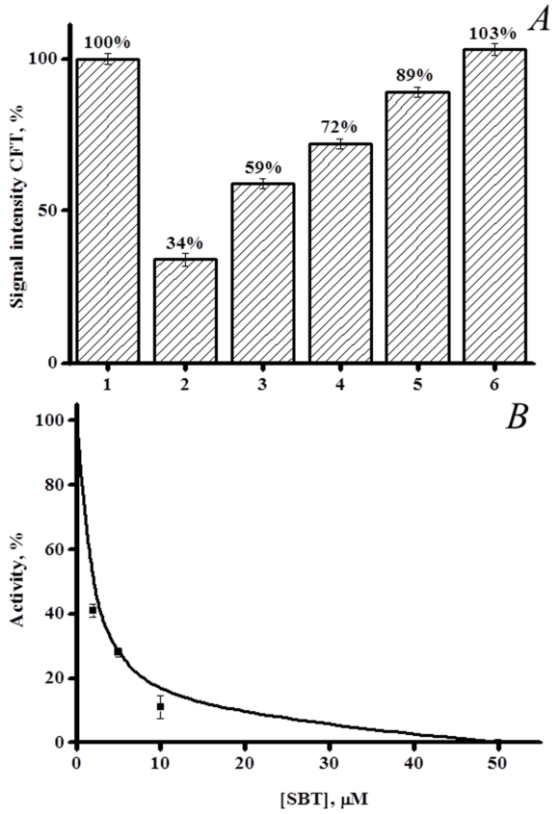

Figure 5 (A) Signal intensity of inhibition of β-lactamase CTX-M-116 by sulbactam in the concentration range of 2-50 μM, (1) 50 μM cefotaxime, (2) incubation of 50 μM cefotaxime with 17 μM β-lactamase CTX-M-116, (3) incubation of 50 μM cefotaxime with 17 μM β-lactamase CTX-M-116 and 2 μM inhibitor sulbactam, (4) incubation of 50 μM cefotaxime with 17 μM β-lactamase CTX-M-116 and 5 μM inhibitor sulbactam, (5) incubation of 50 μM cefotaxime with 17 μM β-lactamase CTX-M-116 and 10 μM inhibitor sulbactam, (6) incubation of 50 μM cefotaxime with 17 μM β-lactamase CTX-M-116 and 50 μM inhibitor sulbactam. (B) Effect of the inhibitor sulbactam on the catalytic activity of β-lactamase CTX-M-116 (IC_{50} = 2.5 μM).

In our work, the effect of the inhibitor sulbactam on the enzymatic reaction of cefotaxime hydrolysis catalyzed by β-lactamase CTX-M-116 was investigated. SBT forms a kinetically stable acyl-enzyme intermediate, inhibiting serine β-lactamase [39, 40]. Incubation of the enzyme and substrate mixture in the presence of the inhibitor leads to inhibition of β-lactamase catalytic activity. Effective action of inhibitor SBT corresponds to the registration of the oxidation current of cefotaxime with the intensity corresponding to the oxidation current of the substrate not subjected to the catalytic action of the enzyme (Figure 5A, B). As follows from the presented experimental data, sulbactam completely inhibits the enzyme activity at a concentration of 50 μM, with IC_{50} = 2.5 μM.

4. Limitation

There are no special limitations in our study.

5. Conclusions

Antibiotic resistance of microorganisms is becoming one of the most serious health problems worldwide. The solution to the problem consists of two things. One direction is the chemical synthesis or biotechnological production of new drugs with antibacterial activity. The second one is the synthesis of new inhibitors of β-lactamases for the concerted action of the drug and the inhibitor.

In our investigation, we have developed a new approach for screening antibacterial compounds as substrates and inhibitors of bacterial serine β-lactamases using nanostructured screen-printed electrodes. The electrode registers the current of substrate electrochemical oxidation, which is proportional to its concentration in the medium. The comparative analysis of electrochemical parameters of the enzyme/substrate or enzyme/substrate/inhibitor systems is an efficient approach to the analysis of catalytic activity of

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β-lactamases. The registering of antibiotic cefotaxime electroanalytical parameters before and after β-lactamase CTX-M-116 catalysis permits detecting enzymatic catalysis and its suppression in the presence of the inhibitor sulbactam. The developed principle of determining the electrochemical oxidation of antibiotics on electrodes can be used to create efficient sensor platforms for screening new antibacterial compounds and inhibitors of enzymes responsible for the development of bacterial resistance to antibiotics.

• Supplementary materials

No supplementary materials are available.

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• Author contributions

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Formal Analysis: V.S., M.R., G.P.
Funding acquisition: V.S., M.R.
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Methodology: V.S., M.R.
Project administration: V.S.
Resources: V.S., M.R., V.G.
Software: G.P.
Supervision: V.S., M.R.
Validation: M.R., G.P.
Visualization: T.B., G.P.
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• Conflict of interest

The authors declare no conflict of interest.

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