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A piezoelectric immunosensor for chloramphenicol detection in food

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ABSTRACT

A piezoelectric immunosensor on the basis of electrogenerated polymer for detecting trace quantities of chloramphenicol, a drug which is used in veterinary for treatment and prevention of infectious diseases, was developed. The detection was conducted in a competitive format with the use of hapten–protein conjugate and monoclonal antibodies. The limit of detection is 0.2 ng ml^{-1}, the calibration curve is linear in the range of concentrations 0.5–100.0 ng ml^{-1}. Chloramphenicol was detected in food (meat, milk, egg, honey).

1. Introduction

Chloramphenicol (CAP) is widely used for treating and preventing infectious diseases of cattle and poultry; it has a broad spectrum against gram-positive and gram-negative microorganisms. The trace quantities of the antibiotics accumulate in meat and milk, which may cause allergic reactions, resistance to microorganisms and general immunity lowering. MRL of chloramphenicol in food is 0.3 μg kg^{-1} [1].

Microbiology and chromatography methods for chloramphenicol detection are characterized by duration, labour content and multistageness [2,3]. At present immunochemical test-methods and the methods of immunoenzyme analysis are used as screening methods [4,5]. They are based on the specific fixation of the detected compound by corresponding antibodies, on conditions that a label is introduced into one of the initial components of the reaction system; the label is easily detected with a corresponding highly sensitive physical and chemical method.

The methods where sensors are applied are becoming widely used. Electrochemical, gravimetrical and optical sensors, the latter including surface plasmon resonance sensors, have been developed to detect chloramphenicol [6–14].

The application of gravimetrical sensors allows detecting drugs in composite matrixes at quite a low level. An important stage is to obtain a sensor electrode coating which is characterized by a minimum mass and stability both in liquid media and at regeneration. The detection limit, range of detection, accuracy and reproducibility depend on the properties of this coating.

Stable coatings of gravimetrical immunosensors are obtained by the method of covalent immobilization of biomolecules. The support is most often formed by the method of self-assembled monolayers.

In [13] anti-CAP antibodies were covalently linked to the pre-formed monolayers on the siloxane base by an activation procedure using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysulfosuccinimide. Low-molecular chloramphenicol was detected in a direct format of analysis at the level of 10 μg ml^{-1}.

The creation of a receptor coating on the electrogenerated polymeric layer is quite promising. While electrogenerating the polymer on the surface of the gold electrode, it is possible to control the mass and thickness of coating in the process of its obtaining by changing the potential and the scan rate [14]. This procedure provides a strong bond with the gold electrode and the possibility of forming a sufficient number of functional groups for the connection with biomolecules. It enhances the biolayer stability at regeneration, which in its turn allows multiple usage of one receptor layer for analysis in a flow-injection mode.

The aim of this research is to develop a piezoelectric sensor on the base of an electrosynthesized platform to detect chloramphenicol in food.

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Table 1
The choice of the concentration of hapten–protein conjugate.

Concentration of the hapten–protein conjugate ($\mu g\,ml^{-1}$)	Concentration of antibodies ($\mu g\,ml^{-1}$)	Signal, Δf (Hz)
25.0	10.0	24
	20.0	36
10.0	10.0	22
	20.0	44
5.0	10.0	25
	20.0	50
1.0	10.0	20
	20.0	38

2. The experiment

2.1. Chemicals and immunoreagents

Hydrochloric acid, sulfuric acid, nitric acid, perchloric acid ("Vecton", Russia), ethanol (Russia), acetone (Russia).

2.1.1. Reagents for immobilization

(3-Aminopropyl) triethoxysilane – APTES ("Reanal", Hungary), glutaraldehyde solution – GA ("Reanal", Hungary), pyrrole ("Vecton", Russia).

2.1.2. Immunoreagents

Monoclonal antibodies to chloramphenicol MoAb/CAP, protein (soybean tripsin inhibitor) – chloramphenicol conjugate (CAP-STI) (supplied by A.N. Bach Institute of Biochemistry of RAS, Russia).

Phosphate buffer, pH 7.2, was prepared containing 0.137 M NaCl, 0.0027 M KCl, 0.008 M $Na_2HPO_4{\cdot}12H_2O$, 0.0015 M KH_2PO_4 in double distilled water.

2.2. Immobilization

2.2.1. Cleaning the electrode surface

Before measuring, the electrode surface was cleaned by nitric acid and defatted by acetone and then dried in air at room temperature.

2.2.2. Immobilization on the surface of the sensor's gold electrode

The formation of the receptor layer included monomer electropolymerization followed by its activation by glutaraldehyde across amino- and carbonyl groups and covalent binding of chloramphenicol conjugate on the surface of the polymer. This allowed to conduct accurate localization of biomolecules preserving the recognition properties and specified orientation.

A polypyrrole coating is obtained in cyclic voltamperometry using a potentiostat (PI-50-1) in the range between −0.2 and 0.8 V versus the silver chloride reference electrode at the scan rate of $10\,mV\,s^{-1}$ in the sodium chloride medium. The newly prepared polymer, dried for 30 min, was placed into a camera with the glutaraldehyde vapours for 24 h, then 10 μl of CAP-STI conjugate was applied onto one side of the gold electrode and stored in the damp camera for 24 h (Fig. 1). Before measuring, the sensor surface was washed by the sodium phosphate buffer to remove non-bound reagents. Covalent binding facilitated the formation of a linker bridge of optimal length, which provided spatial availability of active recognition centres.

2.3. Immunoassay format

A competitive format of analysis, which included the injection of fixed antibody quantities into standard analyte samples and the passage of this solution over the surface of the sensor electrode, was used. The sensor's frequency change is explained by the antibody concentrations, which were not bound with the analyte in the sample. Standard chloramphenicol solutions were prepared by dilution in a buffer solution (pH 7.2) with the addition of ethanol and Tween 20. The standard solutions were mixed with a fixed MoAb/CAP antibody concentration ($17\,\mu g\,ml^{-1}$). The solutions were kept for 5 min and then injected into the cell. The value of resonance frequency was measured on-line. The regeneration of the biolayer was accomplished by 0.04 mM KCNS.

2.4. QCM immunosensor

The AT-cut 10 MHz resonator with gold electrodes obtained by magnetron deposition was purchased from ELECTRONIC FIRM ETNA COMPANY (Moscow, Russia).

A piezoelectric sensor with one immobilized side was placed into the flow detection cell. Only one side of the sensor was allowed to be in contact with the solution during the analysis. The flow cell was part of a general system, including a hopper for injecting the sample, a peristaltic pump providing a continuous stream of liquid, a frequency meter and a personal computer for recording the frequency measurement.

The samples were introduced into the flow cell at a rate of $60\,\mu l\,min^{-1}$. The flow-injection immunoassay included:

- passing the buffer solution through the cell to fix the constant value of the signal;
- introducing the sample containing the working antibody concentration and various chloramphenicol concentrations into the cell;

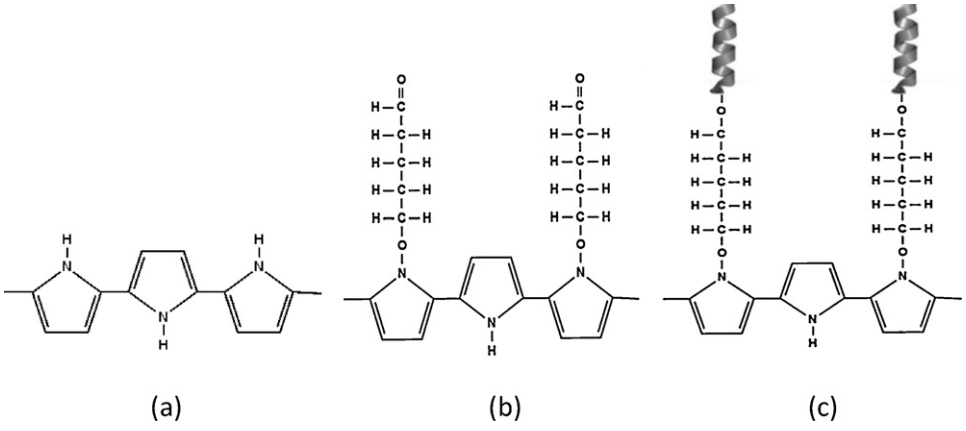

Fig. 1. The scheme of immobilization (a) electrosynthesis of polypyrrole coating, (b) activation by glutaraldehyde, and (c) covalent binding of chloramphericol conjugate.

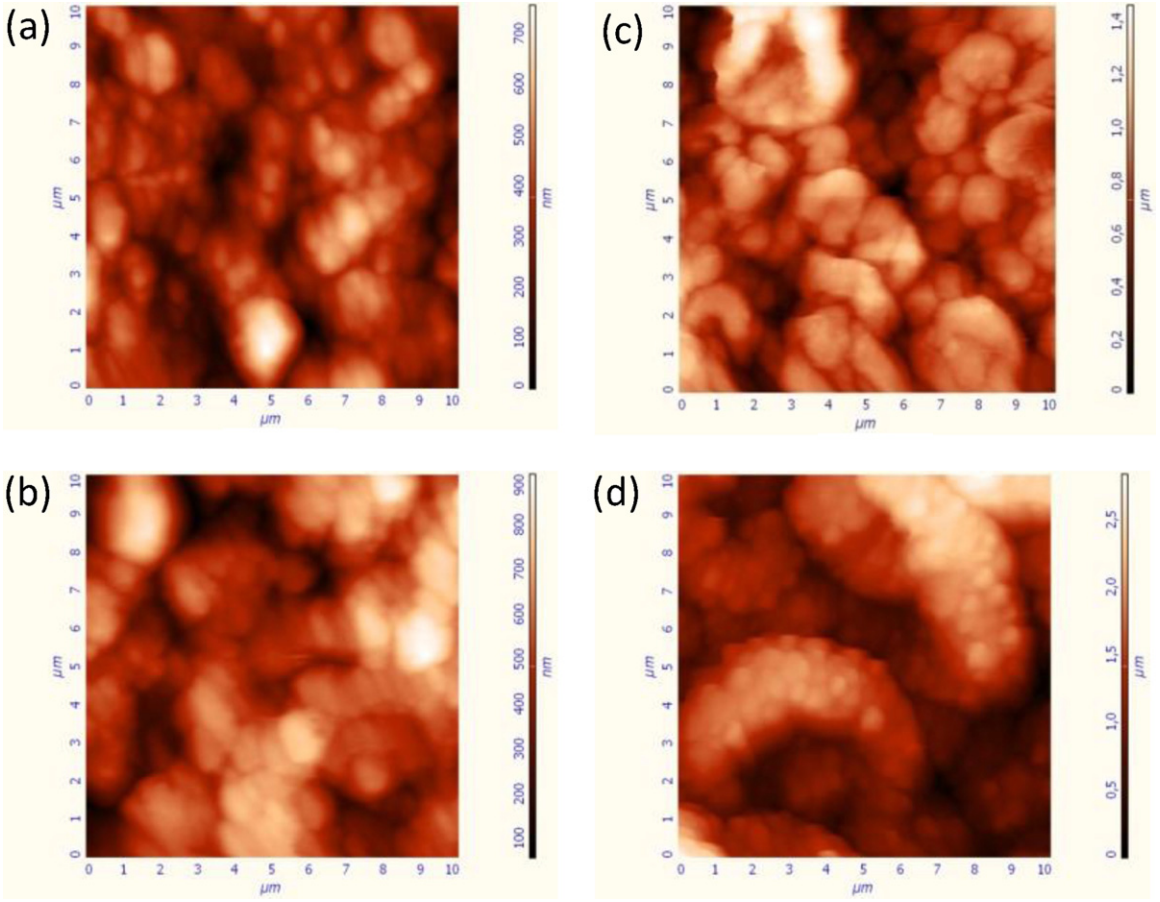

Fig. 2. AFM pictures of piezoelectric sensors' surfaces with applied polypyrrole coating in solutions (a) 0.1 M HCl, (b) 0.1 M $HClO_4$, (c) 0.1 M NaCl, and (d) 0.1 M $NaClO_4$.

- passing the buffer solution to remove non-bound antibodies;
- injecting the regenerator solution of potassium thiocyanate;
- passing the buffer solution to fix the constant value of the signal.

The full cycle, including the biolayer regeneration, lasted about 20–30 min. After the analysis the surface of the sensor was washed with the buffer solution and placed into the damp camera at 4 °C.

2.5. The AFM procedure

The changes in the morphology and the structure of the surface were controlled by the method of atomic force microscopy (the scan microscope Solver P47-PRO, produced by Nanotechnology-MDT, Russia) in the semi-contact mode in air with the scan rate of 120–180 kHz.

2.6. The calculation of kinetic characteristics and affinity constants

Solutions with different concentrations of antiserum were used to assess the binding of low-molecular compounds, immobilized as hapten–protein conjugates on the surface of the sensor electrode, with corresponding antibodies.

The constants of the destruction rate and of the immunocomplex formation rate were calculated by the Scatchard method. Experimental and calculated values were presented as graphs of the kinetic dependence of the sensor's frequency modulation change and the dependence of the reaction rate on the sensor's frequency change.

The values of the angular coefficient of equation a and of the segment on ordinate axis b, allowed to calculate the rate constants k_o, k_p.

$$y = ax + b$$

$$-\frac{df}{dt} = (k_o \cdot c + k_p) \cdot f - k_o \cdot f_m \cdot c$$

The affinity constant was calculated as the ratio of the constant of the destruction rate to the constant of the formation rate of the antigen–antibody complex.

2.7. Sample pretreatment

For the pretreatment of chicken meat, a piece (1 g) of muscular tissue cut from the middle part of the sample was chopped by scissors with the addition of 2 ml of phosphate buffer solution (pH 7.2), carefully stirred and placed into a centrifuge tube. The extraction of antibiotic was carried out for 90 min in a thermostat at (37 ± 1) °C. Then the samples were heated for 30 min on the water bath at (65 ± 1) °C and centrifuged at 3000 rpm for 20 min. The supernatant was separated and used in the analysis.

The pretreatment of milk was carried out in the following way: 1 ml of saturated solution of ammonium sulphate was added to 1 ml of the sample, stirred, centrifuged for 3–5 min at 3000 rpm.

The sample of eggs was homogenized, then 2.0 ml of this mixture was taken, 2.0 ml of ammonium sulphate was added, stirred, centrifuged for 5 min at 8000 rpm. The supernatant was taken and analyzed.

The following method of pretreatment was used for honey: 5 g was dissolved in 10 ml of the phosphate buffer; 12 ml of ethyl acetate was added and stirred for 30 min. After centrifugation 8 ml of the organic layer was removed and dried. The residue was dissolved in the buffer and centrifuged for 10 min.

3. Results and discussion

3.1. Optimization of the QCM method

Low-molecular compounds, such as chloramphenicol, are detected in a competitive format. The receptor layer is hapten–protein conjugate of chloramphenicol and soybean tripsin inhibitor.

The important stage in the formation of a biosensitive coating on the surface of sensor electrodes is the formation of the support, the latter providing a strong bond with the surface of the gold electrode and forming a sufficient number of equally distributed functional groups on its surface. The polypyrrole coating obtained in the cyclic regime from −0.2 to 0.8 V vs. AgCl electrode at the scan rate of 10 mV s^{-1} was used as the support. Solutions of acids and salts (NaCl, HCl, $NaClO_4$, $HClO_4$) were used as supporting electrolytes. Anions and cations are incorporated into the structure of the polymer to establish the electrical neutrality of the film during the electropolymerization process.

It was found by the method of atomic force microscopy that the character of ions influences the morphology and mass of the coating: in hydrochloric acid and sodium chloride solutions the polypyrrole coating demonstrates a ragged granular structure, in solutions with perchlorate ions – a granular porous structure (Fig. 2). The concentration of electrolyte influences the coating mass (the rate of film formation increases linearly with the concentration increase).

In choosing the concentration of hapten–protein conjugate it was considered possible to obtain a large number of active centres on the surface of the immunosensor electrode. The dependence of the sensor's value Δf on the concentration of hapten–protein conjugate (1–25 μg ml^{-1}) at direct interaction with specific antibodies in the analyzed sample were investigated (Table 1). The largest analytical signal was obtained in the application of conjugate with the concentration of 5 μg ml^{-1}. In the range of lower and higher concentrations of conjugate the signal of the sensor lowers as a result of the insufficient specific concentration of active centres or their steric inaccessibility.

3.2. The detection of the concentration corresponding to 50%-inhibition of antibodies

Graphic dependences of the value of the analytical signal on the concentration of antibodies were obtained experimentally to choose the concentration of specific antibodies (Fig. 3). The concentration of specific antibodies corresponding to 50%-inhibition of active centres of the bioreceptor coating of the piezoelectric immunosensor was detected in the range of the linear dependence of these parameters. The application in a competitive format of the concentration of antibodies equal to half the linear section of the graph makes it possible to reach the optimal correlation between the number of active centres on the surface of the immunosensor and the number of the molecules of the antibodies which remained unbound on the stage of the homogeneous immunochemical reaction.

It is stated that the working concentration of monoclonal antibodies to chloramphenicol equals 17 μg ml^{-1}.

3.3. The calculation of kinetic characteristics and affinity constants of a reversible immunochemical reaction

The affinity constant of antibodies and the constants of the formation rate and the immunocomplex dissociation rate were calculated by the Scatchard method. The affinity constant of the immunocomplex formation with hapten–protein conjugate immobilized on the coating on the base of polypyrrole is 3.4×10^{10} M^{-1}. The high rate of the immunocomplex formation (7.14×10^{6} M^{-1} c^{-1}) confirms the conformation availability of hapten–protein conjugate functional groups for binding.

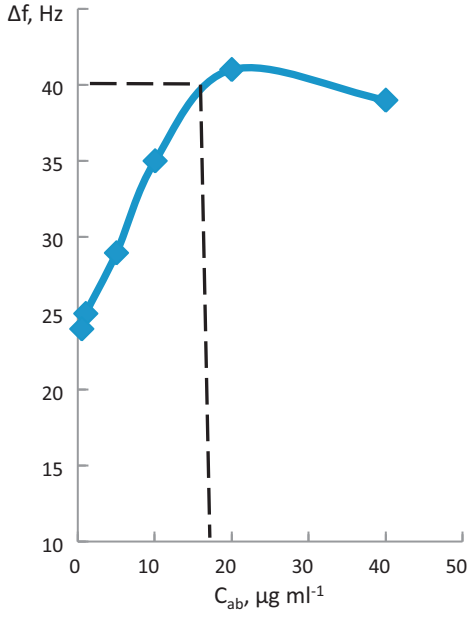

Fig. 3. The choice of the concentration of specific antibodies corresponding to 50%-inhibition.

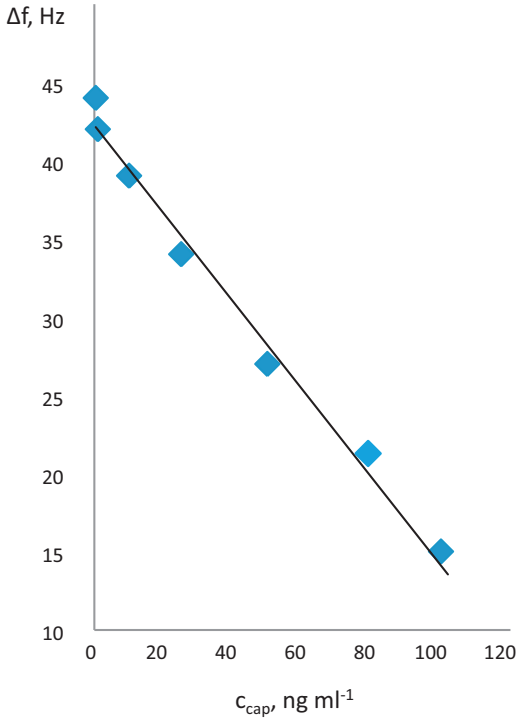

Fig. 4. The calibration curve for chloramphenicol detection.

Table 2
Chloramphenicol detection in food by the "added-fount" method.

Sample	Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Sr
Chicken meat			
1	5.0	5 ± 1	0.05
2	10.0	10 ± 1	0.04
Milk			
1	5.0	4 ± 1	0.07
2	10.0	9 ± 1	0.05
Egg			
1	5.0	5 ± 1	0.06
2	10.0	10 ± 1	0.05
Honey			
1	5.0	5 ± 1	0.06
2	10.0	9 ± 1	0.05

3.4. Drawing a calibration curve of the dependence between the sensor's signal and the analyte's concentration

0.04 mM KCNS solution was used for regeneration. The introduction of the potassium thiocyanate solution provided for the biolayer's complete regeneration without its destruction, which allowed to conduct the measurement on one coating for 12 cycles. The biolayer was removed and immobilization was repeated after the mass had decreased by more than 5%.

The calibration curve for the detection of trace quantities of chloramphenicol (Fig. 4) is linear in the range of concentrations 0.5–100.0 ng ml^{-1} ($y = -5.1733x + 59.31$) (linear working range, in which analyte concentrations produced signals between 80 and 20% of the maximum), the limit of detection, calculated as the chloramphenicol concentration that provided 90% of the maximum signal, was 0.2 ng ml^{-1} [15].

3.5. Analysis of food

Chloramphenicol was detected in samples of meat, milk, eggs and honey (the pretreatment method was described above). The concentration of the antibiotic was detected in the supernatant obtained after extracting the drug from the products tested (Table 2).

The studied samples did not reveal exceeding chloramphenicol MRL [2].

4. Conclusion

Immunosensors are widely used for detecting different types of compounds in a direct format of analysis. In detecting low-molecular compounds, such as antibiotics or pesticides, immunosensors are used competitively with immobilized hapten–protein conjugate bound with antibodies which did not form a complex with analyte. This method allows to reach highly sensitive and selective detection.

A piezoelectric sensor for the detection of trace quantities of chloramphenicol in food was developed. The ways of forming an immunoaffinity coating of the sensor on the base of an electrogeneration polypyrrole coating were suggested.

The detection method was developed; the limit of detection corresponds to 0.2 ng ml^{-1}. The piezoelectric immunosensor was tested in analyzing samples of meat, milk, eggs and honey. At present we are investigating the possibility of applying this method to the detection of beta-lactam group antibiotics.

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