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BIOSENSORS

Determination of the Fluoroquinolones Levofloxacin and Ciprofloxacin by a Piezoelectric Immunosensor Modified with Multiwalled Carbon Nanotubes (MWCNTs)

Evgenia I. Shinko[a], Olga V. Farafonova[a], Il'ja A. Shanin[b], Sergei A. Eremin[b], and Tatyana N. Ermolaeva[a]

[a]Department of Chemistry, Lipetsk State Technical University, Lipetsk, Russia; [b]Department of chemical enzymology, M.V. Lomonosov Moscow State University, Moscow, Russia

ABSTRACT

Methods for the high-sensitivity determination of fluoroquinolones using a piezoelectric immunosensor based on multi-walled carbon nanotubes (MWCNTs) are developed. The use of MWCNTs in the formation of a stable piezoelectric sensor detection layer increases the active specific surface area which is necessary for receptor molecule binding. The concentrations of hapten-protein conjugates (35/65 serial concentration) and polyclonal antibodies are determined in the direct (15/85 for levofloxacin, 18/82 for ciprofloxacin) and in the competitive immunoassay formats (14/86 for levofloxacin, 10/90 for ciprofloxacin). Conditions for the analysis in the flow-injection mode were studied. The carrier flow rate was 30 and 50 μl/min depending on the detection layer formation method. The characteristics of MWCNTs-based piezoelectric immunosensors were characterized for the determination of fluoroquinolones in the static and in the flow-injection modes. The direct and competitive immunoassay formats (detection limit, linear range of target concentrations, reproducibility) were determined. The detection limits were 9 and 8 ng/ml for levofloxacin and ciprofloxacin in the competitive format and 25 and 21 ng/ml in the direct assay. The piezoelectric immunosensors were employed for the analysis of milk.

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affinity interactions; ciprofloxacin; levofloxacin; multi-walled carbon nanotubes (MWCNTs); piezoelectric immunosensor

Introduction

Fluoroquinolones (FQs) are a large group of highly effective antimicrobial drugs that are widely used in veterinary practice (Wang et al. 2021; Dinh et al. 2020; Egunova et al. 2020; Cheng et al. 2020). Inside the body, FQs are poorly metabolized and accumulate (Cao et al. 2020; Rusch et al. 2019; Feng et al. 2019), which is why their residual contents are found in animal products such as milk and meat. The consumption of these products results both in direct toxic effects of antibiotics on the human body and in the development of resistance to these substances (Sazykin et al. 2021; Yu et al. 2020). The World Health Organization (WHO) lists antibiotic resistance among the

CONTACT Evgenia I. Shinko shukshina_evg@mail.ru Department of Chemistry, Lipetsk State Technical University, 30 Moskovskaya Street, Lipetsk 398055 Russia.

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most important problems of modern medicine, since this resistance is spreading at an incredible rate thus posing a threat to human health (Skepper et al. 2020; Pascucci et al. 2021; Karadag et al. 2021; Yu et al. 2021; Nji et al. 2021; Yin et al. 2021).

The widespread use of these drugs necessitates the development of sensitive, rapid and easy-to-perform methods for determining the residual content of fluoroquinolone antibiotics. The most commonly used method today is HPLC (Asu et al. 2021; Hu et al. 2021; Pang et al. 2019; Li et al. 2019; Wu et al. 2019; Moudgil et al. 2019). A fluorimetric detector allows the determination fluoroquinolone from 0.14 to 1.1 ng/l (Pang et al. 2019), while the level of a mass spectrometric detector is 0.0014 to 0.023 μg/l (Li et al. 2021). However, these methods require expensive equipment and highly qualified personnel, as well as rather complicated sample preparation, which limits their use for routine analysis.

An alternative to chromatographic methods in the determination of fluoroquinolone is immunochemical methods with their relative ease of implementation and high selectivity. Among the immunochemical methods, it is important to note the enzyme-linked immunosorbent assay (ELISA) (Acaroz et al. 2020) and the fluorescence polarization immunoassay (FPIA) (El Kojok et al. 2020; Shen et al. 2019).

Considerable attention is paid to the development of immunosensors to determine fluoroquinolones with minimal sample preparation. Most common in this respect are electrochemical immunosensors (Cardoso et al. 2021; Rudnicki et al. 2020) that require the use of special labels (e.g., enzyme labels). Label-free impedimetric immunosensors for the determination of ciprofloxacin at the pg/ml level are also described (Lamarca et al. 2020). In addition, to perform highly sensitive determination of fluoroquinolones without any special labels, it is suggested to use sensors based on surface plasmon resonance (Pan et al. 2017; Sari et al. 2018).

Piezoelectric immunosensors are rarely used in determining fluoroquinolones. In a piezoelectric sensor, the analytical signal is the change in the frequency of its oscillations during the formation of the antigen/antibody affinity complex (Yun et al. 2019). In terms of sensitivity, piezoelectric sensors are comparable to and in some cases even surpass widely used optical, spectrophotometric, fluorescent, and electrochemical sensors (Medyantseva et al. 2021; Cervera-Chiner et al. 2020). Therefore, it is expedient to use them for the determination of trace concentrations of drugs in food products and biological media.

To enhance the analytical signal of a piezoelectric gravimetric immunosensor, two approaches are reported: (i) an increase in the number of detection sites on the surface of its electrode by using multi-walled carbon nanotubes and (ii) the use of gold nanoparticles or secondary antibodies for the analyte (Kwak & Lee 2019).

The purpose of this study is to develop a highly sensitive method for the determination of fluoroquinolones using a piezoelectric immunosensor based upon multi-walled carbon nanotubes.

Materials and methods

Reagents and immunoreagents

The objects of this study are fluoroquinolones: ciprofloxacin (cip) and levofloxacin (lev) (Sigma-Aldrich corporation, St. Louis, Missouri, USA). The immunochemical determination used the corresponding polyclonal antibodies (ab:lev; ab:cip).

The following reagents were used: ethanol, acetone (Quimica, Barcelona, Spain), hydrochloric acid, potassium thiocyanate, ammonium sulfate (chemically pure, Reachem, Moscow, Russia), dimethylformamide (DMF), (chemically pure, Merck, Darmstadt, Germany), 2-amino-3-mercaptopropionic acid (cysteine), glutaraldehyde (GA), N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma-Aldrich, St. Louis, Missouri, USA), and bovine serum albumin (BSA) (PanEco, Moscow, Russia).

Multi-walled carbon nanotubes (MWCNTs) were obtained from the Institute of Microelectronics Technology and High Purity Materials of the Russian Academy of Sciences (Chernogolovka, Russia) during catalytic pyrolysis of ethanol vapors from 400 to 550 °C. The precatalyst was nickel nitrate which was thermally decomposed to metal immediately before the nanotube deposition. During the catalytic decomposition of ethanol vapors, the nanotubes were deposited onto the surface of the catalyst (nickel) which was washed with acids after the synthesis. After washing the catalyst, the samples were washed twice with deionized water, dried, and sieved (Grazhulene, Red'kin, and Telegin 2012).

The multi-walled carbon nanotubes were activated as follows: 800 µl of 1:3 HNO_3 and H_2SO_4 were added to 1 mg of MWCNTs and treated with ultrasound for 3 hours at 50 °C in an ultrasonic bath (PSB-Galas, Moscow, Russia).

A phosphate physiological buffer solution (PBS) (pH = 7.2) was prepared by dissolving 8.0145 g of NaCl, 0.2012 g of KCl, 2.864 g of $NaH_2PO_4{\cdot}12H_2O$, and 0.204 g of KN_2PO_4 in 1 dm^3 of bidistilled water.

Hapten-protein conjugates (Lev-BSA, Cip-BSA) were synthesized by carbodiimide condensation. 2 mg of ciprofloxacin (6 μmol) or 2.2 mg of levofloxacin (6 μmol) and 30 mg of EDAC (156 μmol) were added to a solution of 5 mg of BSA (0.07 μmol) in 2.5 cm^3 of distilled water. The reaction mass was kept for 4 hours at room temperature and for 16 hours at 4 °C. Purification was carried out by dialysis against 0.2% aqueous NaCl for 4 days; the solution was changed periodically.

Instrumentation

AT-cut piezoelectric resonators with gold electrodes (4 mm in diameter) and a natural oscillation frequency of 10 MHz ± 1 Hz (Etna JSC, Moscow, Russia) were used as the sensor's physical transducer. The resonators were obtained by magnetron gold sputtering. The sensor's analytical signal was recorded in the static mode on a CPNA-330 device (ETNA JSC, Moscow, Russia) and in the flow-injection mode on a unit consisting of a 15 to 20 µl flow cell providing contact with the sample on one only side of the sensor, a peristaltic pump (Knauer, Berlin, Germany), a DiSkop digital module (Bafika, Moscow, Russia), and a personal computer.

The operational principles for piezoelectric immunosensors involve microweighting. The analytical signal is the piezoelectric resonator frequency change at an increase in the receptor layer mass due to the formation of an immunocomplex (Dergunova et al. 2008; Don et al. 2016).

The quartz resonator frequency is related to the mass of the coating applied to its surface via the Sauerbrey equation:

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\mu\ \rho_q}}$$

where Δf is the piezoelectric resonator frequency change (Hz), f_0 is its natural frequency, A is the surface area of the electrodes, ρ_q is quartz density (2.65 g/cm^3), μ is the quartz shear modulus (2.95×10^{11} dyne/cm^2) and Δm is the resonator mass change during surfacing. Thus, the biolayer mass (ng) is equal to:

$$\Delta m = 1.23 \cdot \Delta f$$

Methods of forming the sensor detection layer

Method 1. The resonator electrode surface was defatted with ethanol, and 2 µl of cysteine ethanol solution (1 µM) were introduced with a microsyringe, maintained at room temperature for 90 minutes, and 5 µl of 5% GA solution were added. After 15 to 20 minutes, the sensor was washed with PBS and 5 µl of 0.05% hapten-protein conjugate solution were applied. The immunosensor was then placed in a humid chamber for 10 to 12 hours at 4 °C (Karaseva & Ermolaeva 2012).

To form a MWCNTs-based detection layer, the electrode surface was modified with cysteine. 2 µl of a MWCNTs colloidal solution (100 mg/dm^3) were dosed onto the cysteine substrate and maintained for 24 hours at 4 °C. Carboxyl groups were activated on the MWCNT surface using EDAC and NHS solutions (2 µl were applied of the mixture of 5 mg EDAC and 5 mg NHS in 200 µl DMF) for 90 minutes, after which the hapten-protein conjugates (***Method 2***) or antibodies to fluoroquinolones (***Method 3***) were immobilized (Farafonova et al. 2018).

Determination of fluoroquinolones

In the static mode, 5 µl of the pre-prepared test solution were dosed onto the sensor surface containing either immobilized hapten-protein conjugates (Method 1, Method 2) or antibodies to fluoroquinolones (Method 3). After 5 minutes, the sensor surface was washed with unbound PBS reagents (pH 7.2), dried to a constant weight, and the analytical signal was measured in air.

The flow-injection determination of fluoroquinolones was performed in a competitive assay. A decrease in the sensor frequency (Δf) was recorded while an immunocomplex was formed between the hapten-protein conjugate immobilized on the electrode surface (Method 2) and antibodies to fluoroquinolones that were unbound to the determined compound in the sample.

The analytical measurement was followed by the biolayer regeneration when a regenerating solution washed the sensor surface or when a 200 µl 0.003 M KCNS solution which promotes dissociation of the surface immunocomplex was dosed onto the sensor surface (Dergunova et al. 2008).

Standard FQ solutions (2 to 400 ng/cm^3) for plotting a calibration graph were obtained by dissolving the analytes in bidistilled water.

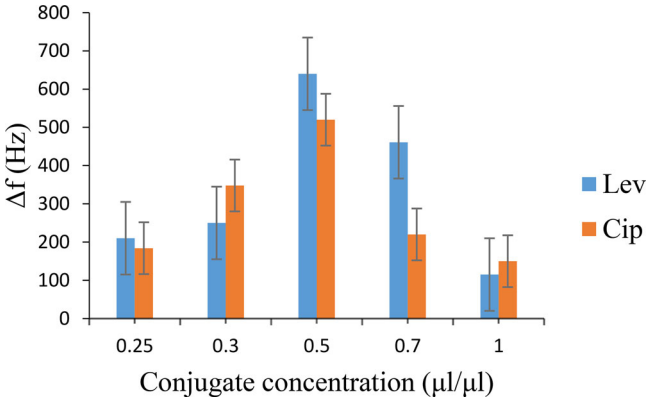

Figure 1. Dependence of levofloxacin-protein (Lev) and ciprofloxacin-protein conjugates (Cip) on the analytical signal (Δf). Conditions: modifier: cysteine, MWCNTs (100 mg/l): 2 µl, 100 ng/ml levofloxacin, and 100 ng/ml ciprofloxacin.

sample preparation

10 cm^3 of the sample (milk diluted 3-fold) were added to 5 cm^3 of ethanol for fat hydrolysis and 2 cm^3 of ammonium sulfate were added. The sediment was separated by centrifugation (3 min, 7000 rpm) (MPW centrifuge, Warszawa, Poland). The supernatant was used for the analysis.

Results and discussion

When using a piezoelectric immunosensor to determine low-molecular compounds, a competitive immunoassay is typically employed to indirectly determine the analyte by the number of antibodies bound to the hapten-protein conjugate immobilized on the sensor surface (Karaseva & Ermolaeva 2012). The direct immunoassay format to determine low-molecular compounds is rarely used. However, the increase in the specific surface area of the sensor when using multi-walled carbon nanotubes due to the appearance of a three-dimensional high-porosity boundary layer that allows the determination of low-molecular compounds in the direct assay format.

The possibility was considered to use a piezoelectric affinity sensor to determine ciprofloxacin and levofloxacin in the competitive and direct assay formats in the static and flow-injection modes.

Formation of the sensor detection layer

Figure S1 shows the conditions for forming the sensor detection layer. 2-Amino-3-mercaptopropionic acid (cysteine), which is highly adhesive to the gold electrode surface and forms a thin film with terminal amino groups that bind to biomolecules via a glutaraldehyde molecule, was chosen to be the modifier of the sensor surface (Figure S1, method 1). Carbon nanotubes are covalently bound to the modifier and biomolecules due to the interaction of MWCNTs carboxyl groups with amino groups (Figure S1, method 2-3).

Table 1. Effect of immobilization methods on piezoelectric immunosensor characteristics under static conditions.

Method	Fluoroquinolone	Layer mass Δm_{pl} (ng)	Concentration sensitivity S_c ($Hz \cdot cm^3 \cdot \mu g^{-1}$)	Number of detection cycles N
1	Levofloxacin	33.50	670.0	26
	Ciprofloxacin	32.98	659.6	26
2	Levofloxacin	64.63	1313.4	28
	Ciprofloxacin	64.35	1307.7	27
3	Levofloxacin	62.15	1263.0	28
	Ciprofloxacin	61.94	1258.7	27

To assess the layer quality, the piezoelectric microweighting method was used which uses the equation $\Delta f = k\Delta m$ to determine the layer mass ($\Delta m_{пл}$, ng) and to calculate the concentration sensitivity of the sensor (S_c, $Hz \cdot cm^3 \cdot \mu g^{-1}$) and the stability of the bio-layer (N). The former characterizes the efficiency of the affine reaction on the electrode surface and the latter shows the number of detection cycles without a significant change in the analytical signal (Table 1). The bio-layer stability largely depends on the surface modifier used and on the strength of the bonds formed during its formation. Experimental studies have shown a similar stability of the detection layer formed by different methods (26 to 28 cycles) which is caused by the application of cysteine providing high adhesion to the gold electrode surface.

Investigation of conditions for the determination of fluoroquinolones

To obtain comparable characteristics of sensors with a detection layer formed according to Method 1, Method 2 and Method 3, the concentrations of hapten-protein conjugates and antibodies to fluoroquinolones used at the immobilization stage or in the competitive assay format were preselected experimentally.

The basis for the optimal concentration of hapten-protein conjugate was the dependence of the analytical signal of the sensor on the concentration of conjugate in the range from 0.25 to 1.00. The analytical signal of the sensor for both Lev and Cip reaches its maximum when a 35/65-concentration conjugate solution is used (Figure 1).

The basis for the optimal concentration of antibodies to fluoroquinolones in the competitive and direct assay format was the dependence of the analytical signal upon the degree of concentration of polyclonal antibodies (Figures 2 and 3). The concentration of antibodies in the competitive format corresponds to 50% binding, which achieves the optimal ratio of active sites on the sensor surface and the number of antibody molecules that did not bind to a homogeneous affinity complex with fluoroquinolones (concentration degree of antibodies is 14/86 and 10/90 for levofloxacin and ciprofloxacin, respectively) (Figure 2) (Dergunova et al. 2008; Karaseva & Ermolaeva 2012). In the direct assay format, the optimal concentration of antibodies corresponds to the graph maximum (Figure 3) indicating the saturation of the detection layer (15/85 for levofloxacin and 18/82 for ciprofloxacin).

To assess the selectivity of determining fluoroquinolone, the cross-reactivity coefficients (CR, %) of polyclonal antibodies to other compounds together with levofloxacin and ciprofloxacin were calculated. The results show that polyclonal antibodies to levofloxacin and ciprofloxacin are highly specific (Table 2).

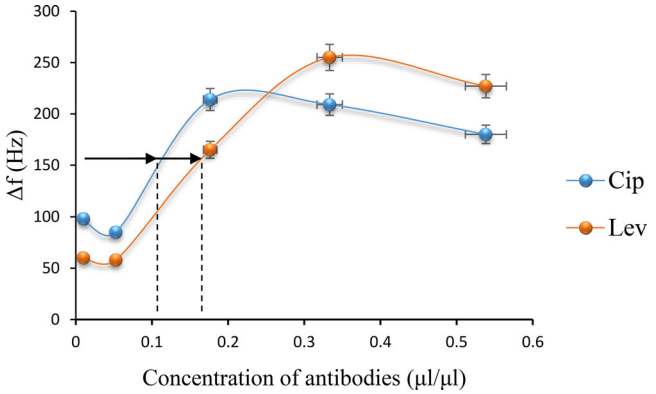

Figure 2. Determination of the polyclonal antibodies to levofloxacin (Lev) and ciprofloxacin (Cip) corresponding to 50% binding based upon the analytical signal (Δf) The arrows show selected values. Conditions: modifier: cysteine, MWCNTs (100 mg/l): 2 μl, 100 ng/ml levofloxacin, and 100 ng/ml ciprofloxacin.

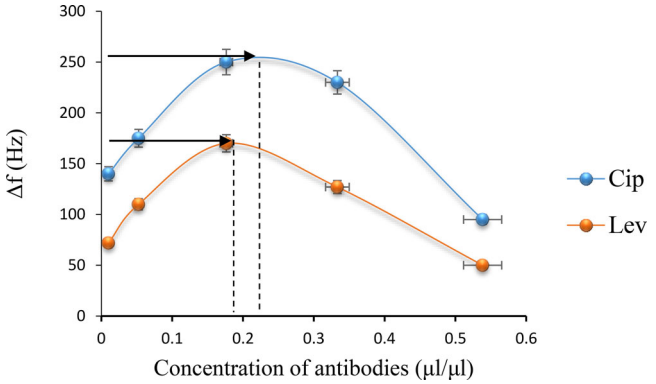

Figure 3. Determination of concentrations of polyclonal antibodies to levofloxacin (Lev) and ciprofloxacin (Cip) for the direct format depending on the analytical signal (Δf). The arrows show selected values. Conditions: modifier: cysteine, MWCNTs (100 mg/l): 2 μl, 100 ng/ml levofloxacin, 100 ng/ml ciprofloxacin.

When the Δm_{pl} and S_c values for sensors using Method 1 and Method 2 were compared, the application of carbon nanotubes at the immobilization stage increases the added mass and the concentration sensitivity of the sensor and consequently the efficiency of affine interactions for both fluoroquinolones. The application of carbon nanotubes in the formation of an antibody-based detection layer (Table 1) results in higher S_c values compared to the coating obtained according to Method 2, which demonstrates the use of a piezoelectric sensor in the direct immunoassay format to determine fluoroquinolone. The sorption layers formed according to Method 2 and Method 3 demonstrate almost equal stability and a similar bio-layer mass.

The value of the analytical signal of a piezoelectric sensor depends on the measurement method. For instance, under static conditions, the value depends on the time the sensor contacts the sample. This time was set in advance to 20 minutes. At the same

Table 2. Cross-reactivity coefficients for polyclonal antibodies.

Antibiotic	Antibodies, cross-reactivity (CR) % Antibodies: Levofloxacin	Antibodies: Ciprofloxacin
Levofloxacin	100	9
Ciprofloxacin	12	100
Tetracycline	1	1
Polymyxin	2	1

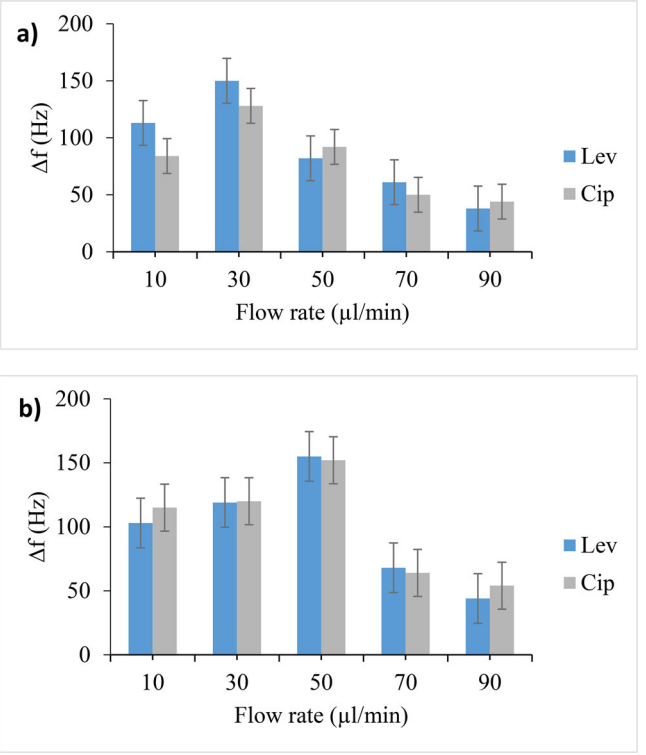

Figure 4. Influence of flow rate on the analytical signal (Δf): (a) Method 1 [conditions: modifier: cysteine, hapten-protein conjugate (35/65): 5 µl, 100 ng/ml levofloxacin (Lev), and 100 ng/ml ciprofloxacin (Cip)] and (b) Method 2 [conditions: modifier: cysteine, MWCNTs (100 mg/l): 2 µl, 100 ng/ml levofloxacin (Lev), and 100 ng/ml ciprofloxacin (Cip)].

time, when measuring in the flow-injection mode which reduces the duration of the analysis, it is important to choose the optimal flow rate of the PBS carrier solution.

When characterizing the effect of the carrier solution flow rate (1 to 120 µl/min) on response of the sensor, the maximum Δf value for fluoroquinolones in the epy competitive immunoassay format is achieved at 30 µl/min by Method 1 and Method 2 (Figure 4). The possibility of measurement at a higher flow rate of the carrier solution using a MWCNTs bio-layer indicates both a higher concentration of surface detection sites and the steric availability of immobilized hapten-protein conjugates for interaction with antibodies. The optimal flow rate of the carrier solution is equal for levofloxacin and ciprofloxacin and does not depend upon the fluoroquinolone structure.

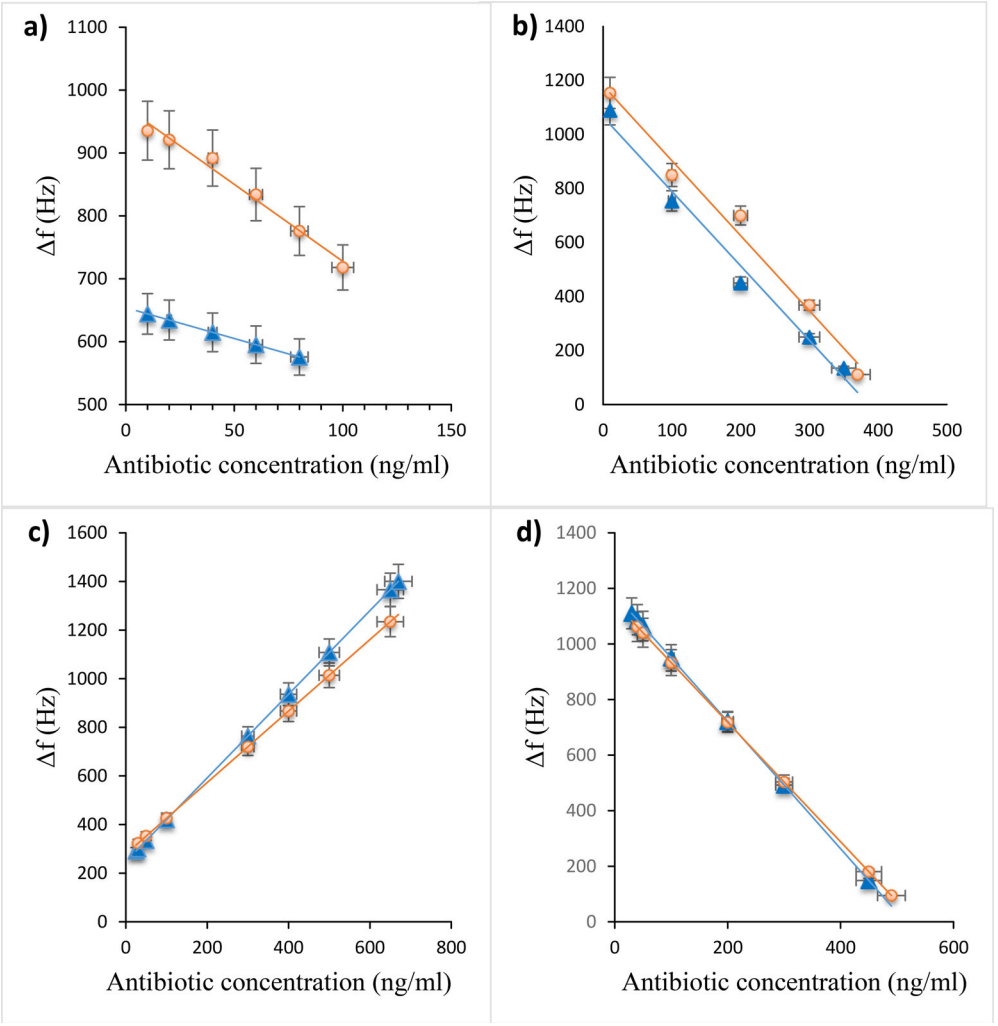

Figure 5. Calibration relationships for the determination of fluoroquinolones where the blue triangles represent ciprofloxacin and red circles levofloxacin: (a) method 1, (b) method 2 in static mode, (c) method 3, and (d) method 2 in flow-injection mode.

Tracing the calibration function

A methodology for determining fluoroquinolones in the flow-injection and static modes using various detection layers was developed (Figure 5). The metrological characteristics in Table 3 show that the minimum value of the C_{min} detection limit is achieved using a sensor modified with only cysteine. However, MWCNT application contributes to the expansion of the range of determined levofloxacin and ciprofloxacin concentrations both in the competitive and direct assay formats. The developed methods allow the determination in a range covering considerable areas exceeding those presented previously (Pinacho et al. 2014; Lavaee et al. 2017; Abnous et al. 2017; Hu et al. 2018), which makes the methodology applicable for various products.

Table 3. Metrological characteristics for fluoroquinolone determination.

Method	Fluoro-quinolone	Detection limit LOD (ng/cm^3)	Range of target concentrations (ng/cm^3)	Calibration relationship	Coefficient of determination R^2
		Static mode			
Method 1	Levofloxacin	3	10-100	y=-0.98x + 654	0.95
	Ciprofloxacin	4	5-80	y=-2.9x + 950	0.95
Method 2	Levofloxacin	9	10-350	y=-2.76x + 1065	0.96
	Ciprofloxacin	8	10-370	y=-2.78x + 1181	0.96
Method 3	Levofloxacin	25	30-650	y = 1.47x + 279	0.97
	Ciprofloxacin	21	25-670	y = 1.72x + 248	0.98
		Flow-injection mode			
Method 2	Levofloxacin	9	30 – 450	y=- 2.29x + 1179	0.94
	Ciprofloxacin	9	40 – 490	y=- 2.15x + 1148	0.93
		Alternative methods			
Pinacho et al. 2014	Ciprofloxacin	0.009	0.043-7.38		
Lavaee et al. 2017	Ciprofloxacin	1.06	1.3-165.7		
Hu et al. 2018	Ciprofloxacin	0.5	0.5-64		
Abnous et al. 2017	Ciprofloxacin	0.009	0.3-132		

Table 4. Results of fluoroquinolone determination in milk by the spike-and-recovery method (P = 0.95, n = 3).

Fluoroquinolone	Spiked (ng/cm^3)	Recovered (ng/cm^3)	Recovery (%)	Reproducibility RSD
		Competitive analysis format		
Levofloxacin	20.0	19.5 ± 6.8	97.5	0.08
	50.0	51.8 ± 7.0	103.6	0.05
	70.0	71.1 ± 8.6	101.6	0.03
Ciprofloxacin	20.0	19.1 ± 3.0	95.5	0.06
	50.0	51.6 ± 5.7	103.2	0.05
	70.0	70.2 ± 9.2	100.3	0.03
		Direct assay format		
Levofloxacin	50.0	49.3 ± 5.5	98.6	0.03
	100	99.0 ± 3.7	99.0	0.04
	300	294 ± 14	98.0	0.03
Ciprofloxacin	50.0	49.2 ± 6.9	98.4	0.04
	100	98.0 ± 6.1	98.0	0.05
	300	297 ± 17	99.0	0.04

Determination of fluoroquinolones in real samples

The accuracy of the determination of levofloxacin and ciprofloxacin was assessed by spike-and-recovery measurements of milk. Comparison of Student's coefficients given in the table and calculated during the measurements did not reveal any significant differences between the spiked and recovered concentrations of fluoroquinolones (Table 4).

The relative standard deviation s_r values show favorable reproducibility of the results (s_r does not exceed 0.08).

Conclusion

Conditions for creating high-capacity detection layers based on multi-walled carbon nanotubes with the use of piezoelectric quartz microweighting have been studied. The formation of the sensor layer was controlled layer-by-layer: a substrate with high

adhesion to the gold electrode surface was obtained and antibodies or hapten-protein conjugates were covalently immobilized. The conditions for the determination of fluoroquinolone using piezoelectric immunosensors modified with carbon nanotubes were investigated. The concentrations of immunoreagents were selected and the cross-reaction coefficients (CR, %) of polyclonal antibodies were determined to assess the antibiotic selectivity. The application of carbon nanotubes promotes a wider range of levofloxacin concentrations (10 to 100 ng/cm^3 without MWCNTs and 30 to 650 ng/cm^3 with MWCNTs) and ciprofloxacin (5 to 80 ng/cm^3 without MWCNTs and 25 to 60 ng/cm^3 with MWCNTs). The developed methodology provides sensitive, rapid, and selective determination of fluoroquinolones in the competitive and direct assay formats. The sensors were tested for the determination of these antibiotics in milk.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not involve human participants or animals.

Author contributions

All authors contributed to the study conception, and design; to the preparation of materials, data collection, and analysis; and read and approved the final manuscript.

Informed consent

Informed consent is not applicable to this study.

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ORCID

Evgenia I. Shinko http://orcid.org/0000-0001-7554-7308
Olga V. Farafonova http://orcid.org/0000-0002-2234-9321
Tatyana N. Ermolaeva http://orcid.org/0000-0003-4137-3420

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