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Determination of the parameters of binding between lipopolysaccharide and chitosan and its N-acetylated derivative using a gravimetric piezoquartz biosensor

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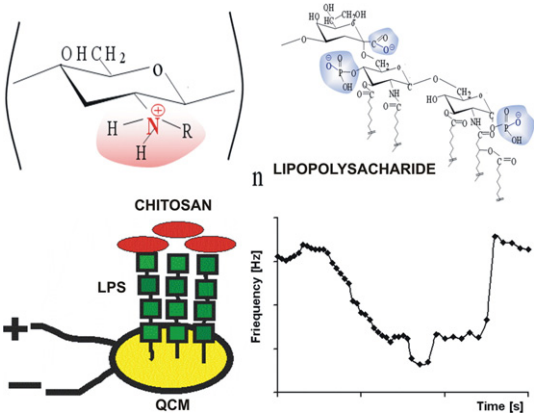
HIGHLIGHTS

- Interaction of lipopolysaccharide with chitosan and its N-acylated derivative was examined.
- Association, dissociation rate constants and affinity for LPS binding with chitosans are determined.
- Affinity of binding N-acylated chitosan with LPS was higher, than the parent chitosan.

GRAPHICAL ABSTRACT



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ABSTRACT

The interaction of endotoxin (lipopolysaccharide — LPS) with low molecular weight chitosan (5.5 kDa), its N-acylated derivative and chitoliposomes was studied using a gravimetric piezoelectric quartz crystal microbalance biosensor. The optimal conditions for the formation of a biolayer based on immobilized LPS on the resonator surface and its regeneration were elaborated. The association and dissociation rate constants for LPS binding to chitosans were determined and the affinity constants (K_{af}) were calculated based on the data on changes in the oscillation frequency of the quartz crystal resonator. The K_{af} values correlated with the ones obtained using other methods. The affinity of N-acylated chitosan binding to LPS was higher than that of the parent chitosan binding to LPS. Based on the results obtained, we suggest that water-soluble N-acylated derivatives of chitosan with low degree of substitution of amino groups could be useful compounds for endotoxin binding and neutralization.

Abbreviations: LPS, lipopolysaccharide; Ch-LM, low molecular weight chitosan; Ac-Ch-LM, N-3-hydroxytetradecanoil chitosan; *E. coli*, *Escherichia coli*; APTES, γ-Aminopropyltriethoxysilane; GA, glutaraldehyde; FIA, flow injection analysis.

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1. Introduction

Lipopolysaccharide (LPS, endotoxin), one of the main components of the outer membrane of Gram-negative bacteria, plays a key role in the emergence of sepsis, the complex clinical syndrome. An LPS molecule is composed of the hydrophobic (lipid A) and hydrophilic (O-specific polysaccharide, core oligosaccharide) parts.

Glycolipid fragment as lipid A is the toxic center of LPS; its structure is similar for many Gram-negative bacteria [1]. Lipid A consists of a diphosphorylated β-D-1,6-linked D-glucosamine disaccharide acylated with varied amounts of ester- and amide-linked fatty acids. The inner part of the core oligosaccharide of LPS also contains a sufficiently large amount of anionic groups (phosphate, pyrophosphate and carboxyl moieties). The negative charge and the amphiphilic nature of LPS make it capable of binding to the positively charged and amphiphilic ligands with high affinity.

A series of cationic proteins, peptides, and lipopolyamines that are capable of binding to LPS and modifying its endotoxic properties have currently been synthesized or isolated from a variety of natural sources [2–5]. Fluorescently labeled ligands have been widely used to estimate the LPS-binding capacity of polycations and to study their interaction with endotoxins [6]. Parameters of LPS binding to various proteins and peptides were also determined using the methods based on optical biosensors [7]. The application of piezoelectric sensors with the microbalance operating principle opens new opportunities for determining the parameters of binding of a ligand to the receptor [8,9]. The analytic signal of a gravimetric piezoelectric sensor is the change in the resonator oscillation frequency (Δf) caused by increasing or decreasing mass of the bio-receptor layer as the ligand–receptor complex is either formed or destroyed [10]. This method does not require any labels for real-time registration of biochemical interaction and allows one to determine the mass of a ligand linked to the receptor at the micro- and nanogram levels. The highly sensitive quartz crystal microbalance (QCM) method opens new possibilities when used to study complementary biochemical interactions in vitro [11–13]. The method has been developed to monitor the interaction between an LPS and LPS-binding peptides using a piezoelectric quartz crystal [14].

As indicated earlier, the cationic polyelectrolyte chitosan, which is a linear polysaccharide composed of β-1,4-linked glucosamine residues, reacts with LPS to form stable complexes with various compositions [15,16]. It has been determined that water-soluble hydrophobic chitosan derivatives are promising for high-affinity binding of LPS [17]. This work continues the previous research in this field. The purpose of this study was to investigate the interaction of endotoxins with low molecular weight chitosan and its mono-N-acylated derivative. The receptor layer of the sensor consists of immobilized LPS that can connect with a polycation—chitosan and its derivative—in a solution to form a stable complex. The quartz crystal microbalance method has made it possible to determine the association (k_a), the dissociation (k_d), and the affinity (K_{af}) constants of LPS binding to chitosan.

2. Experimental

2.1. Chemicals

We used LPS 055: B5 of *Escherichia coli*, γ-aminopropyltriethoxysilane (APTES); glutaraldehyde (GA) chloride, hydrophosphate and sodium azide; chloride, dihydrophosphate and potassium thiocyanate, hydrochloric acid (Sigma, USA), and an ultrafiltration membrane (Millipore, USA). All other reagents were of a reagent grade (Reachim, Russia) and were used without additional purification.

2.2. Low molecular weight chitosan (Ch-LM) and N-3-hydroxytetradecanoil chitosan (Ac-Ch-LM)

Low molecular weight chitosan (Ch-LM) and N-3-hydroxytetradecanoil chitosan (Ac-Ch-LM) were prepared as described previously [17].

2.3. Immobilization of LPS on the surface of gold and silver electrodes of piezoelectric resonators

AT-cut piezoelectric resonators (10 MHz ± 2 Hz) with gold and silver electrodes (8 mm in diameter) obtained by magnetron sputtering of silver and gold (OAO Quantum, ZAO Etna, Russia) were used. The biolayer was formed on the sensor after the electrode surface of the piezoelectric resonator was activated with an APTES solution by applying an aqueous LPS solution (0.1%) on the sensor electrode surface and keeping it for 16 h at 25 °C [11]. The excess of unlinked LPS molecules was removed by washing the sensor with a phosphate buffer solution to stabilize the frequency of the device.

The mass gain of receptor coating on the electrode was calculated according to the Sauerbrey equation (taking into account the corresponding analytic signal of the sensor) [10]:

$$\Delta f = -2,3 \cdot {f_0}^2 10^{-6} \Delta m/A, \quad (1)$$

where Δf is the intrinsic crystal oscillation frequency, MHz; Δm is the change in mass of the receptor film formed on the electrode surface, g; and A is the squared surface of sensor electrode, cm^2.

2.4. Regeneration of biolayer

Regeneration of biolayer after binding to chitosan was performed using 0.1 M Tris–HCl buffer (pH 9).

2.5. The LPS-binding activity of C-LM and its acetylated derivative

The LPS-binding activity of C-LM and its acetylated derivative was determined by passing chitosan solutions of (2.5–12.5 μg/ml) through the microcell with the sensor. The difference in oscillation frequency (Δf) between the stages of chitosan injection and washing of the quartz crystal resonator by the buffer from unbound ligand was recorded as an analytic signal. The control measurement was performed using ovalbumin, which cannot interact specifically with LPS.

2.6. The association (k_a) and dissociation (k_d) rate constants of reactions

The association (k_a) and dissociation (k_d) rate constants of reactions on the surface of the LPS-bioreceptor layer of the sensor were determined according to the common approach previously described in [8,9]. The kinetic rate was described through the frequency characteristics using the following equation:

$$-\Delta f/\Delta t = (k_a * C + k_d) f - k_a * f_{sen} * C, \quad (2)$$

where $\Delta f/\Delta t$ is the rate of change in frequency of the piezoelectric sensor; C is the ligand concentration; k_a and k_d are the kinetic rate constants of direct and reverse reactions (association and dissociation) of the complex, respectively; f is the experimental frequency of the sensor; and f_{sen} is the intrinsic frequency of the sensor with immobilized LPS.

We used the kinetic binding curves to plot the graphs showing the rate of changes in the frequency of the sensor with immobilized LPS after binding to Ch-LM and Ac-Ch-LM ($\Delta f/\Delta t$ versus f). The equilibrium or affinity (K_{af}) constants were calculated as the ratio between the rate constants of the direct (k_a) and reverse (k_d) reactions.

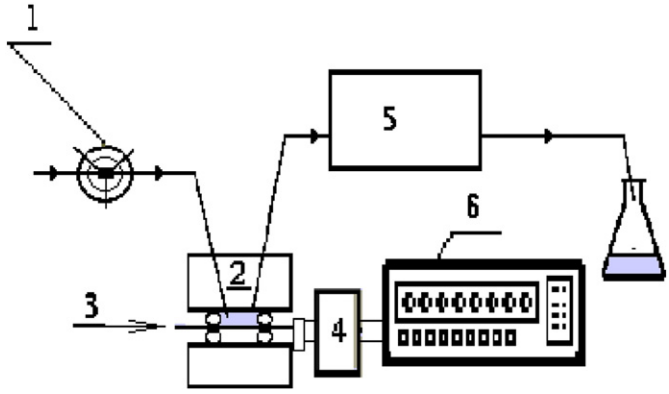

Fig. 1. Scheme of the flow-injection analysis: 1—dosator for injecting the sample (buffer or regenerating solution); 2—micro-cell with QCM-sensor (3); 4—oscillator of frequency; 5—peristaltic pump; and 6—computer for registration of the change of sensor's signal. The 0.05 M phosphate buffer (pH 6) was used as a carrier solution. The flow rate of liquid was 30 μl/min.

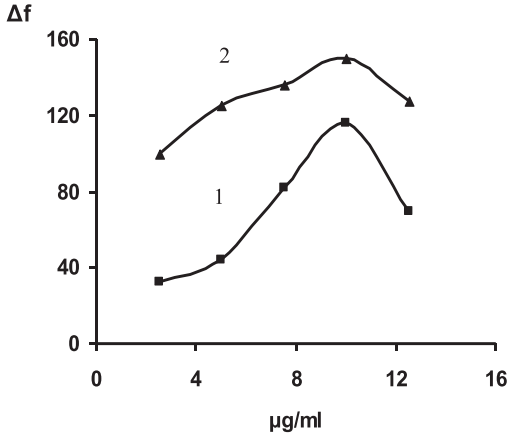

Fig. 3. Effect of C-LM (1) and Ac-C-LM (2) concentrations on analytic signal of the sensor.

3. Results and discussion

3.1. The flow injection analysis (FIA) setup

The flow injection analysis (FIA) setup consisted of a peristaltic pump, a dosing device, and a 15–20 μl microcell with the LPS-sensor fixed inside the cell. The sensors contacted with the liquid phase only on one side (Fig. 1).

3.2. Formation of bioreceptor layer on the sensor surface

We have shown previously that chitosan reacted with the positively charged LPS fragments (core oligosaccharide and lipid A) and lipid A bound to chitosan with a higher affinity [17]. The orientation and availability of active sites of the immobilized LPS molecules for interaction with ligands on the electrode surface are the most important parameters for the binding activity of these molecules. The reproducibility of the results and duration of the biosensor performance depend on the stability of the bioreceptor layer. APTES, which forms a strong and dense cover, was used to obtain the siloxane film on the sensor surface. It was assumed that a hydrophilic modifier (APTES) provides greater accessibility of the inner parts of LPS for contacting with the chitosan solutions under analysis [11]. LPS was immobilized on the APTES-activated sensor surface using several methods. The first method was based on the formation of ionic bonds between the amino groups of the substrate (APTES) and the negatively charged LPS and involved keeping the aminated sensor with a drop of an LPS solution in humid atmosphere. The second method (covalent cross-linking with a bifunctional reagent GA) ensures a more durable covalent attachment of LPS to the sensor electrode surface. Two ways of action of the cross-linking agent were also tested: incubation of the sensor with a drop of the LPS solution in GA vapor and sequential application of aqueous GA and LPS solutions on the APTES-activated electrode.

It is assumed that in the former case the bifunctional reagent interacts with LPS located on the surface of the liquid phase. This step should contribute to preserving the native conformation of the LPS. In the

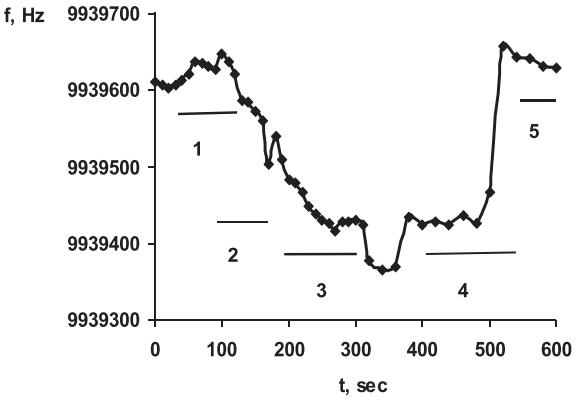

Fig. 2. Analytic signal of the sensor during one measurement cycle. Description of the graph is given in the text.

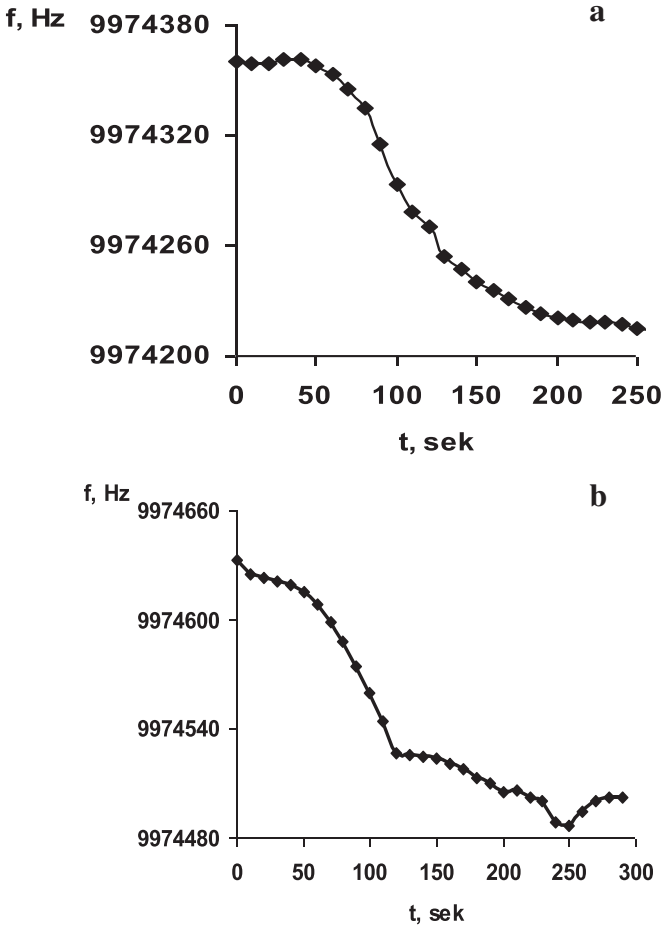

Fig. 4. Kinetic curves for binding of immobilized LPS to Ch-LM (a) and Ac-Ch-LM (b), concentration is 7.5 μg/ml.

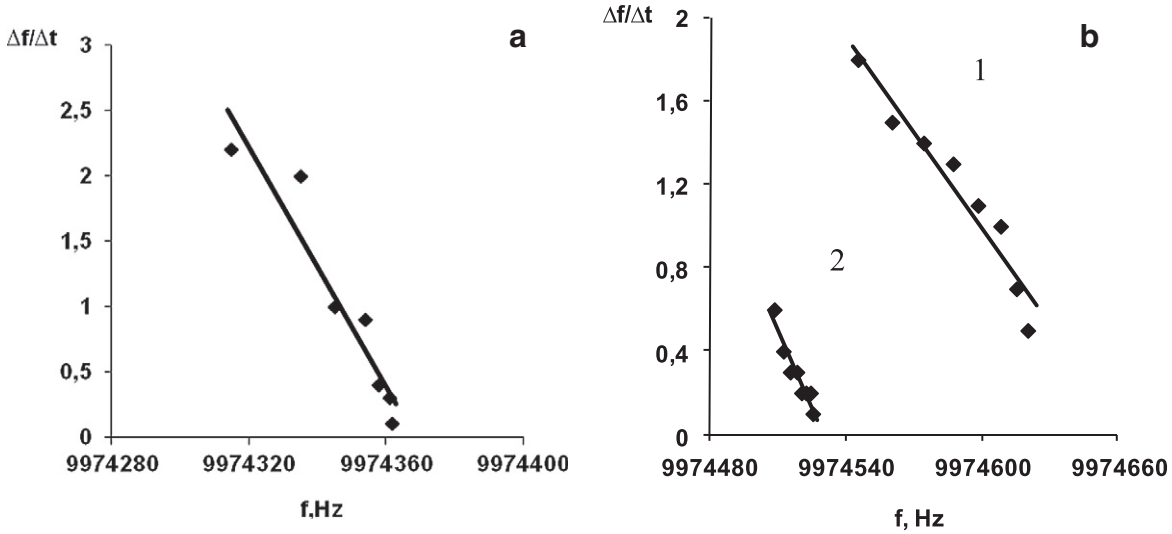

Fig. 5. Dependence of the rate of sensor oscillation frequency on its frequency during binging of LPS to Ch-LM (a) and Ac-Ch-LM (b, 1—the fast and 2—the slow phases).

second way of immobilization, a large number of the LPS molecules interact with the APTES via GA methylene groups, thus increasing the stability of the receptor layer [11].

The LPS sensors obtained were used as detectors in FIA to study the interaction of LPS with Ch-LM and its N-acylated derivatives. A graph showing the changes in sensor response during one measurement cycle is given in Fig. 2.

The cycle comprises the following steps: 1—passing the phosphate buffer through the cell to stabilize the LPS sensor signal; 2—introducing a chitosan sample to the flow; 3—passing the buffer solution to remove unbound chitosan; 4—feeding the regenerating solution for dissociation of the LPS–chitosan complex that is formed on the surface of the sensor electrodes; and 5—passing the phosphate buffer to restore the original sensor frequency. The difference in oscillation frequencies of the quartz crystal resonator (Δf) between stages 1 and 3 (Fig. 2) was recorded as an analytic signal, and this part of the curve was used to calculate the kinetic parameters of binding.

Stability of the sensor after introduction of chitosan was determined based on the difference between the initial oscillation frequency of the resonator and its frequency after washing with all buffers including the regeneration phase. It was demonstrated that immobilized LPS does not give a stable receptor layer due to formation of ionic bonds. The increase in sensor frequency to 30–60 Hz after each rinsing step indicates that chitosan-bound LPS is removed. The sensor designed using covalent immobilization of LPS retains its oscillation frequency during measurements. However, the sensitivities of the two sensors differ significantly. The frequency of the sensor with LPS immobilized in GA vapors was insignificant ($\Delta f = 22$ Hz) after interaction with chitosan. It constituted 15% of the analytic signal of the second sensor ($\Delta f = 150$ Hz) with LPS immobilized on the activated APTES coating. Therefore, the second sensor was used in the following work.

3.3. Choice of regeneration methods

An important feature of the flow injection analysis is that the receptor layer can be regenerated due to the dissociation of the ligand–receptor complex, while maintaining the initial receptor layer. The following regeneration solutions were used for this purpose: 1.5 M potassium thiocyanate, distilled water, 0.1 M acetate buffer (pH 3), and 0.1 M Tris–HCl buffer (pH 9). The first three solutions did not restore the initial frequency of the sensor. The regeneration with alkali solution allows one to achieve the initial oscillation frequency and does not reduce sensor sensitivity. Therefore, Tris–HCl buffer (pH 9) was used for further regeneration of the biolayer providing about 15 measurement cycles per sensor. Ionization of the chitosan amino groups is apparently suppressed under alkaline conditions and ionic bonds with LPS are ruptured, leading to the dissociation of the LPS–chitosan complex.

3.4. Interaction between chitosan and surface-immobilized LPS

The effect of concentration (2.5–12 μg/ml) of low molecular chitosan and its N-acylated derivative on binding with LPS was investigated.

Fig. 3 demonstrates that the binding activity of acylated chitosan (Fig. 3, curve 2) is higher than that of chitosan. The amount of bound chitosan decreases with its concentration increasing above 10 μg/ml (Fig. 3), which apparently results from self-association of surface-bound chitosan molecules and the changes in the visco-elasticity properties of the bio-receptor layer [18,19].

The experimentally observed deviation from the linear dependence of QCM resonant frequency on mass deposition (Fig. 3) hinders the determination of the saturation concentration of the binding reaction between LPS and acyl-chitosan and the use of Scatchard plots to calculate the equilibrium binding constant [19]. Therefore, the affinity constants (K_{af}) were calculated as the ratios between the rate constants of the association and dissociation reactions. As seen from Fig. 4, the kinetic curves for the interaction between Ch-LM and Ac-Ch-LM (ligand concentration 7.5 μg/ml) and LPS are different. The kinetic curve of Ac-Ch-LM consists of two independent fast and slow phases, which may be due to the fact that the ligand binds to the sites on the LPS molecule with different affinities.

A large body of the previously obtained data support this assumption. It was shown that polymyxin B recognizes LPS in a biphasic manner [20]. As mentioned earlier [4], the amphiphilic acylated polyamines with distantly located hydrophobic and hydrophilic regions inside the same molecule, which are structurally similar to Ac-Ch-LM, interact with the core and lipid A fragments of LPSs. These interactions play a crucial role in neutralization of the toxic activity of LPS. Molecular modeling of the structure of the LPS–chitosan complexes has demonstrated that both the core and lipid A moieties of LPS were involved in interaction with acyl chitosan [17].

The rate constants of the association (k_a) and dissociation (k_d) reactions of the LPS binding to Ch-LM (Fig. 5a) and Ac-Ch-LM (Fig. 5b) were determined using plots of $\Delta f/\Delta dt$ vs. f, which subsequently provided the kinetic constants from Eq. (2) to calculate K_{af} values (Table 1).

Table 1 demonstrates that Ac-Ch-LM binds to two types of sites on the LPS molecules that differ in their affinities. The order of magnitudes

Table. 1
Kinetic parameters of interaction of LPS with low molecular weight chitosan and its N-acylated derivatives.

Sample	Rate constants of the direct reaction (association) (k_a) · 10^4 M^{-1} s^{-1}	Rate constants of reversed reaction (dissociation) (k_d) · 10^{-2} s^{-1}	Affinity constants $K_{af} = k_a / k_d$ (K_{af}) · 10^5 M^{-1}
Ch-LM	1.72	0.055	2.13
Ac-Ch-LM	1.56	0.044	3.54
	3.16	0.075	4.21

of the binding constants ($10^5 \cdot M^{-1}$, Table 1) coincides with that of the values obtained earlier using the solution-based methods [15,16]. The affinity constants of Ac-Ch-LM and Ch-LM binding to LPS differ 1.5-fold on average. We have previously shown using the direct binding methods and computer modeling techniques that the introduction of a fatty acid residue to a chitosan molecule gives rise to additional binding sites [17]. The study of the binding activity of LPS to acylated chitosan compared to that of the unsubstituted chitosan supports the recent results showing that the introduction of an acyl substituent increases chitosan affinity for binding to LPS [17].

Thus, this study is one of the first examples of determining the separate kinetic parameters of LPS binding to polycation on the surface of the quartz crystal biosensor. The elaborated technique of using flow injection analysis to determine the LPS-binding activity using a piezo sensor can be used to study and characterize the LPS-binding and antimicrobial compounds.

Acknowledgments

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References

[1] K. Brandenburg, A. Wiese, Endotoxins: relationships between structure, function, and activity, Curr. Top. Med. Chem. 4 (2004) 1127–1146.
[2] D.M. Bowdish, R.E. Hancock, Anti-endotoxin properties of cationic host defence peptides and proteins, J. Endotoxin Res. 11 (2005) 230–236.
[3] K. Brandenburg, J. Andra, P. Garidel, T. Gutsmann, Peptide-based treatment of sepsis, Microbiol. Biotechnol. 90 (2011) 799–808.
[4] S.A. David, Towards a rational development of anti-endotoxin agents: novel approaches to sequestration of bacterial endotoxins with small molecules, J. Mol. Recognit. 14 (2001) 370–387.
[5] M. Zorko, A. Majerle, D. Sarlah, M.M. Keber, B. Mohar., R. Jerala, Combination of antimicrobial and endotoxin—neutralizing activities of novel oleoylamines, Antimicrob. Agents Chemother. 49 (2005) 2307–2313.
[6] S.J. Wood, K.A. Miller, S.A. David, Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for high-throughput identification of lipopolysaccharide-binding agents, Comb. Chem. High Throughput Screen. 7 (2004) 239–249.
[7] J.T. Celestine, N. Surolia, A. Surolia, Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B, J. Biol. Chem. 274 (1999) 29624–29627.
[8] P.J. Skladal, Piezoelectric quartz crystal sensors applied for bioanalytical assays and characterization of affinity interactions, J. Braz. Chem. Soc. 14 (2003) 491–495.
[9] T.N. Ermolaeva, E.N. Kalmykova, Piezoelectric immunosensors. Analytical potential and prospects, Uspechi Chimii (Mosc.) 5 (2006) 445–459.
[10] G.Z. Sauerbrey, Use of quartz vibrator for weighing thin films on a microbalance, Z. Phys. 155 (1959) 206.
[11] T.N. Ermolaeva, E.N. Kalmykova, Capabilities of piezoelectric immunosensors for detecting infections and early clinical diagnostics (Monograph. The Chapter 5), in: Norman H.L. Chiu, Theodore K. Cristopoulos (Eds.), Advances in Immunoassay, InTech, ISBN: 978-953-51-0440-7, 2012, pp. 81–108 (Chapter 5).
[12] E.N. Kalmykova, E.S. Dergunova, T.N. Ermolaeva, R.P. Gorshkova, N.A. Komandrova, Piezoquartz immunosensors for assessing the interactions between *Yersinia enterocolitica* lipopolysaccharides and antibodies to them, Appl. Biochem. Microbiol. 44 (4) (2007) 372–377.
[13] B. Godber, M. Frogley, M. Rehak, A. Sleptsov, K.S.J. Thompson, Y. Uludag, M.A. Cooper, Profiling of molecular interactions in real time using acoustic detection, Biosens. Bioelectron. 22 (9-10) (2007) 2382–2386.
[14] H.C. Chang, C.C. Yang, T.M. Yeh, Detection of lipopolysaccharide binding peptides by the use of a lipopolysaccharide-coated piezoelectric crystal biosensor, Anal. Chim. Acta. 340 (1997) 49–54.
[15] V.N. Davidova, G.A. Naberezhnykh, I.M. Yermak, V.I. Gorbach, T.F. Solov'eva, Determination of the binding constants of different structure lipopolysaccharides with chitosan, Biochemistry (Mosc) 71 (2006) 332–339.
[16] I.M. Yermak, V.N. Davidova, V.I. Gorbach, P.A. Luk'yanov, T.F. Solov'eva, A.J. Ulmer, B.U. Beckmann, E.T. Rietschel, Yu.S. Ovodov, Forming and immunological properties of some lipopolysaccharide–chitosan complexes, Biochimie 88 (2006) 23–30.
[17] G.A. Naberezhnykh, V.I. Gorbach, G.N. Likhatskaya, V.N. Davidova, T.F. Solov'eva, Interaction of chitosans and their N-acylated derivatives with lipopolysaccharide of Gram-negative bacteria, Biochemistry (Mosc) 73 (2008) 432–441.
[18] K. Lebed, A.J. Kulik, Llo Forro, M. Lekka, Lectin–carbohydrate affinity measured using a quartz crystal microbalance, J. Colloid Interface Sci. 299 (2006) 41–48.
[19] M.V. Voinova, M. Jonson, B. Kasemo, ‘Missing mass’ effect in biosensor's QCM applications, Biosens. Bioelectron. 17 (2002) 835–841.
[20] C.J. Thomas, N. Surolia, A. Surolia, Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B, J. Biol. Chem. 274 (1999) 29624–29627.