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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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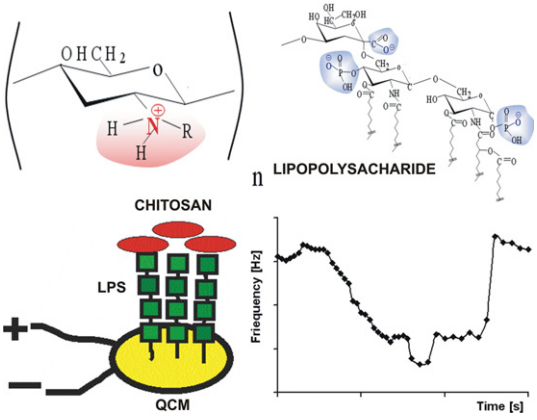
HIGHLIGHTS

- 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.