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Screening and selection of synthetic peptides for a novel and optimized endotoxin detection method

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ABSTRACT

The current validated endotoxin detection methods, in spite of being highly sensitive, present several drawbacks in terms of reproducibility, handling and cost. Therefore novel approaches are being carried out in the scientific community to overcome these difficulties. Remarkable efforts are focused on the development of endotoxin-specific biosensors. The key feature of these solutions relies on the proper definition of the capture protocol, especially of the bio-receptor or ligand. The aim of the presented work is the screening and selection of a synthetic peptide specifically designed for LPS detection, as well as the optimization of a procedure for its immobilization onto gold substrates for further application to biosensors.

1. Introduction

In the Intensive Care Units all over the world sepsis constitutes one of most severe and dreaded medical conditions (Rubulotta et al., 2009). In fact, the rate of death ascribable to sepsis is comparable to that related to heart attack. This entails about 130,000 annual sepsis episodes in Spain with a mortality rate as high as 13% (Esteban et al., 2007), whereas in the United States only between 55% and 65% of the 750,000 yearly patients survive to the episode (Wang et al., 2010).

Sepsis involves a general affection induced by the presence of pathogens or their toxins in blood. This pathology is the consequence of a disproportionate response of the organism to PAMPs (pathogen-associated molecular patterns) and can lead to severe sepsis and septic shock, two complications that are frequently lethal (Annane et al., 2005). Probably the most potent and widespread PAMP is the lipopolysaccharide (LPS) from Gram-negative bacteria, which can be released from the bacteria cell wall during their growth or by the action of antibiotics. Those compounds provoke an overwhelming immune system response that leads to a release of cytokines and inflammatory molecules which can cause death (Alexander and Rietschel, 2001).

Therefore the main regulation agencies such as the Food and Drug Administration (FDA) or the European and other international Pharmacopoeias have set highly demanding endotoxin limits for medicines, drugs and other products for medical or clinical (FDA 1987).

At present the Gold Standard of the methods approved and validated by international Pharmacopoeias for the analytical determination of pyrogens is the *Lymulus* Amoebocyte Lysate test (LAL test) (FDA 1987). Based on an enzymatic coagulation cascade of the lysate obtained from the blood of horseshoe crabs, this test is extremely sensitive to the presence of endotoxins, however it lacks significantly of repeatability, robustness and it is also strongly sensitive to interferences such as pH, presence of divalent cations, anticoagulants, chelators, proteases or serum among others. Moreover, this assay presents other drawbacks such as its laboriousness, batch-to-batch variability and its reliance in the user skills. Bearing all these issues in mind, the identification of several improvable features related to the LAL based tests has led the users to demand new endotoxin detection strategies to avoid those limitations. In fact there are several approaches in the literature of the last years showing that the future of these assays could rely on biosensors due to their versatile performance, cost efficiency, robustness, sensitivity and ease for use. Table 1 summarizes, for the main references, the detection principle, the capture molecules used and the sensitivity achieved.

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Table 1
Different techniques for LPS detection.

	Sensing principle	Capture molecule	Sensitivity (EU/ml)
Hreniak et al. (2004)	Luminiscence	TEOS/APTES + $-NH_2$ + Concanavalin-A + FITC	2000
Ding et al. (2007)	Electrochemical impedance spectrosc.	4,4-Dithiodibutyric acid + Polymyxin B	2–8
Kato et al. (2007)	Cyclic voltammetry	BSA + Diaphorase + Ferrocenylboronic acid	500
Bouafsoun et al. (2008)	Impedance spectroscopy	Polystyrene + Fibronectin	5000
Inoue et al. (2010)	Differential pulse voltammetry	Recombinant factor C zymogen	1–5
Kim et al. (2012)	Electrochemical impedance spectrosc.	Single-stranded DNA aptamers	0.1–10
Su et al. (2012)	Cyclic voltammetry and electrochemical impedance spectroscopy	LPS specific single stranded DNA (ssDNA) aptamer	0.01–10

Based on the information above presented it can be stated that the key feature that makes the difference among these approaches stems from on the capture protocol rather than on the sensing principle itself. The highest sensitivity is provided by those molecules with specific affinity for LPS. There are many molecules that can act as LPS receptors, namely compounds similar to lipid A (like lipid X) (Lam et al., 1987), LPS-binding protein (Wright et al., 1990), anti-LPS antibodies (Dunn et al., 1985) or antibiotics such as Polymyxin B (Bannatyne et al., 1977). Nevertheless the most relevant ones are, together with anti-LPS antibodies, the Polymyxin B (PmB) and those molecules designed specifically for LPS neutralization, namely aptamers and peptides.

The novel LPS detection method optimized in this work aims at improving the present biosensor biofunctionalization protocols. Therefore it makes use of a series of synthetic anti-LPS peptides that have already been proved to bind highly specifically to LPS (Brandenburg, 2009). These peptides are based on proteins such as lactoferrin (Brandenburg et al., 2001), NK-lysine (Andrä et al., 2004a), *Limulus* anti-LPS factor or LALF (Andrä et al., 2004b; Liu et al., 2011; Mora et al., 2008) and shrimp anti-LPS factor or SALF (De la Vega et al., 2008; Lin et al., 2010). The pursued goal is the design and optimization of a biofunctionalization protocol for gold surfaces (one of the main materials for biosensor electrodes) that interacts with high sensitivity and specificity with LPS molecules present in a sample. The strong LPS neutralization ability of these peptides, based on the binding of the peptide to the lipid A portion of the LPS (Brandenburg, 2009), makes them remarkably suitable for this aim. ELISA and mass measurements performed by Quartz-Crystal Microbalance with Dissipation (QCM-D) have been the main techniques used for the proposed work.

2. Materials and methods

2.1. Reagents

Polymyxin B sulfate salt (PmB), biotin, sodium periodate, Tween 80, ABTS, 11-mercaptoundecanoic acid (11-MUA), H_2O_2, pyrogen-free water, sodium acetate, orthophosphoric acid, sodium dihydrogen phosphate anhydrous (NaH_2PO_4), citric acid monohydrate, sodium hydroxide and chloridric acid were purchased from Sigma Aldrich, whereas 3-mercaptopropanoic acid (MPA) was supplied by Fluka. 1-Ethyl-3-(3-dimethylaminopropyl) (EDC) carbodiimide HCl and N-hydroxysuccinimide (NHS) were provided by Thermo Scientific and HEPES by Fischer. Sodium acetate, glycerol, DMSO, ethanol 99.5% pure and NH_3 were supplied by Panreac, while Hellmanex II was supplied by Hellma, glycylglycine by from Scharlab and H_2O_2 at 30% by Merck. The barbital sodium was provided by the Department of Microbiology of the University of Navarra.

2.2. Synthetic anti-LPS peptides (SALP)

The synthetic peptides used in this study involved small cationic peptides and lipopeptides derived from the LPS-binding domain from human lactoferricin (LFcin) (Sánchez-Gómez et al., 2011) provided by NeoMPS and *Limulus* anti-LPS factor (LALF) (Brandenburg, 2009) kindly provided by the Biophysics Division of Forschungszentrum Borstel Leibniz-Zentrum für Medizin und Biowissenschaften in Germany. The sequences of the peptides are summarized in Table 2. Four peptides out of 40 derived from human lactoferricin, whereas most of them are based on different series derived from LALF factor. These are peptides of linear, non-cyclic structure comprising 17 to 23 amino acids wherein the amino acids in positions 1 to 23, counted from the N-terminus, are as follows (1) G, S or lacking; (2) C or lacking; (3) K or R; (4) K or R; (5) Y, W or F; (6) K or R; (7) K or R; (8) F, W or L; (9) K or R; (10) K or L or lacking; (11) W, L or F; (12) K or R; (13) F, Y or C; (14) K or R; (15) G or Q; (16) K or R; (17) F, L or W; (18) F or W; (19) F, L or W; (20) W or F; (21) C or lacking; (22) F or G or lacking (23) G or lacking.

2.3. Lipopolysaccharide (LPS) and outer membrane vesicles (OMVs) extraction and characterization

Antigens for LPS detection were obtained from the following strains: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* PAO1, *Brucella abortus*, *Salmonella minnesota* R595, *Serratia marcescens* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Burkholderia cepacia* (clinical isolate) and *Ralstonia picketti* (clinical isolate).

All the strains were grown until their stationary phase at 37 °C. Then, bacteria were killed with phenol at 0.5%. Cells were removed by centrifugation (10,000 × *g*, 10 min) and used for LPS extraction. OMVs or blebs were obtained from the supernatant by a concentration through a 100 kDa tangential filtration unit (Millipore). The retentant was frozen in order to induce larger blebs. Then, they were centrifuged at 47,000 × *g*, 2 h. OMVs samples were resuspended in distilled water, dialyzed and lyophilized.

LPS of *E. coli* ATCC 35218 was obtained from the aqueous phase of a water–phenol extract according to a published procedure (Leong et al., 1970). LPS extracts were dialyzed, lyophilized and purified following published protocols to remove traces of nucleic acids or proteins that could interfere with endotoxin detection (Hirschfeld et al., 2000). Specifically, LPS was treated with proteinase K, DNAse, RNAse and phenol–deoxycholate and then dialyzed and lyophilized. By standard analytical methods (Daniels

Table 2
Peptides and corresponding amino acid sequences.

Peptides	Amino acid sequence
LFcin derived peptide	
P2-29	FWRIWRWR
P3-24	PFFWRIRIRR
LP3-55	Octanoyl-FWRIRIRR
LP3-58	6-Methyl-octanoyl-FWRIRIRR
LALF derived peptide series	
Pep19-2	GCKKYRRFRWKFKGKFWFWCG
Pep19-3	GCRRYKKFKWRYRGRFWFWCG
Pep19-7	GKKYRRFWKFKGKWFFWG
Pep19-8	GRRYKKFRWKFKGRWFWFG
Pep19-9	GCRRFKKFRWKYKGKFWFWCG
Pep19-10	GRRYKKFKWRFRGRFWFWG
Pep19-12	GCRRFKKFKKWRYRGRFWFWCFG