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LABEL FREE IMMUNOSENSORS FOR THE DETECTION OF PROBIOTIC BACETRIA AND AFLATOXIN M_1

Helga Szalontai

PhD Dissertation Theses

Supervisor:
Nóra Adányi, DSc

Corvinus University of Budapest
Department of Food Science

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Name:	PhD School of Food Science
Field:	Food Science
Head:	**Prof. József Felföldi, PhD** Corvinus University of Budapest, Faculty of Food Science, Department of Physics and Automation
Supervisor:	**Nóra Adányi, DSc** National Agricultural Research and Innovation Centre, Food Science Research Institute (NARIC-FSRI), Department of Food Analysis

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Signature of Head of School	Signature of Supervisor

1. INTRODUCTION

Nowadays the biosensor applications have come to the front in the field of food analysis – mainly in the analysis of food safety related contaminants and microbes, and the food quality related components - besides the traditional and modern analytical methods and molecular biological techniques.

Various factors contribute to the dynamic development of biosensors. The sensitivity of the measurements is comparable with instrumental analytical techniques as high performance liquid chromatography (HPLC) or gas chromatography (GC). Usually simple sample preparation methods are required and the cost of the prime and the operation is lower.

There are many combinations existing considering the construction of biosensors thanks to the large variation of bio- or biomimetic receptors (antibody, antigen, oligonucleotide, tissue, whole cell, molecularly imprinted polymers etc.), the wide range of detection types (optical, electrochemical, mass etc.) and the dimension of the parts (micro, nano magnitude).

The antibody-based immunosensor applications have a prominent role among the bioaffinity sensors. The biosensor developers also utilize the positive features of antibodies that are also widely used in immunoanalytical techniques, like enzyme immunoassay (EIA) or enzyme linked immunosorbent assay (ELISA). The high affinity of antibodies for antigens, sensitivity and selectivity are the most common characteristics that should definitely be mentioned. Among immunosensors, label-free determination methods represent special, often simpler way to detect analytes, because the techniques allowing label-free detection do not need special labels such as enzymes or dyes to produce analytical signals. The main advantages are the reduced analysis time, the possibility of real-time determination and measurement of numerous samples and the cost-effective operation.

Taking all these benefits into consideration, the biosensor development laboratory of Egerfood Regional Knowledge Centre at the Eszterházy Károly College was established in 2006. Besides other techniques, we also started working with

sensing techniques that do not require any labels: quartz crystal microbalance (QCM) and optical waveguide lightmode spectroscopy (OWLS).

One of the most important goal was the bioanalytical determination and quantification of certain contaminants, bioactive components and microbes of milk and fermented milk products. The primary objective was to develop biosensors for monitoring these compounds with sufficient sensitivity (NTP-FUNKMILK OMFB-00386/2008 project).

Many biosensor articles were published related to bacterial contamination (primarily pathogenic bacteria) of milk and milk products due to the issue's importance concerning food safety. Probiotic bacteria represent another exciting field of research. Thanks to their beneficial physiological effects, probiotic bacteria are thoroughly examined by microbiologists and molecular biologists. The main research topics have been among others, the identification of certain strains, revealing and mapping the mechanisms in the digestive system. However, it can be noticed, that biosensor studies are rare in this field. Biosensor research can effectively contribute to food safety, e.g. by controlling the quality parameters indicated on the food packaging. For this reason the aim of this study was to develop antibody-based biosensors (QCM and OWLS) for the quantification of probiotic bacteria (*Lactobacillus acidophilus, Bifidobacterium bifidum*) widely used in dairy industry.

Aflatoxins, produced by mycotoxigenic moulds are the most significant health risk contaminants in milk besides veterinary residues and pesticides. During this work, an OWLS-based determination method for aflatoxin M_1 - the hydroxylated metabolite of aflatoxin B_1, secreted into milk - was elaborated as part of a food-safety related project (TÁMOP-4.2.2.A-11/1/KONV-2012-0008).

2. AIMS

The aim of the doctoral research were to develop QCM and OWLS based immunosensors for probiotic bacteria (*L. acidophilus* and *B. bifidum*) and to develop OWLS based immunosensor for the quantification of aflatoxin M_1.as follows:

1. Model examinations were carried out using BSA-antiBSA molecule pair in order to study the usability of the piezoelectric sensor in flow system for direct immunoanalytical measurements. Further aims were the comparison of antibody immobilization and measuring methods, the examination of the effect of the measuring parameters on the sensor sensitivity.
2. During the development of QCM and OWLS based immunosensors for probiotic bacteria, the aims were the optimization of measuring parameters, the calibration of the sensors with bacterium cells suspended in buffer solution and with bacterium cells suspended in milk, finally the determination of *B. bifidum* and *L. acidophilus* cell numbers in artificially contaminated fermented milk samples, furthermore the study of the antibodies selectivity.
3. During the development of OWLS-based aflatoxin M_1 biosensor the goals were the determination of the measuring parameters (temperature, optimal concentration of the aflatoxin M_1 antibody and the AFM_1-HRP conjugate to be immobilized), additionally the examination of the effects of different sample preparation methods on the recovery and on the sensor sensitivity. The final aim was to determine AFM_1 quantity in milk samples by the developed sensor.

3. MATERIALS AND METHODS

3.1 QCM and OWLS immunosensor developments for probiotic bacteria

The first step of the probiotic sensor development was the production of polyclonal antibodies raised against *L. acidophilus* and *B. bifidum* cell wall antigens by rabbit immunization. The selectivity of purified antisera was studied with indirect ELISA (NAIK-ÉKI).

The QCM (model 430A, CH Instruments, TX, USA) and OWLS (model 120, Microvacuum Ltd., Budapest) measuring systems were set-up for flow injection analysis, the buffer flow was supported by a peristaltic pump (Minipuls 3, Gilson, France) or syringe pump (Syringe Ne-1000, NY, USA), respectively. The sample injection was carried out using a Rheodyne injector (model 7725, CA, USA) fitted with a 500 or 200 µl sample loop.

The bioanalytical determination of bacteria was based on a direct immunorecognition method. The antibodies were immobilized on the QCM or OWLS sensor's surface with the adequate method. The bacterial cells were measured directly from the sample. During the biosensor investigations, the antibodies were bound to the sensors' surface covalently. Sulfo-LC-SPDP or MHDA cross-linking agents were used for QCM chips. As for OWLS, glutaraldehyde was utilized. In order to dissociate the immunocomplexes, thus regenerate the sensor surface, 1.2 mol/l NaOH (QCM) and 10 mmol/l HCl (OWLS) was applied.

Two types of flow through measuring methods were compared during the QCM immunosensor developments, the flow injection analysis and the flow injection analysis method with 10 minutes incubation time (stopped flow), whereas the examinations with OWLS were carried out using flow injection analysis.

The samples containing bacteria made for biosensor measurements were the following: (1.) bacterial cells (*L. acidophilus* or *B. bifidum*) suspended in buffer for the determination of the optimal measuring parameters, (2.) milk samples: bacterial cells (*L. acidophilus* or *B. bifidum*) suspended in milk, diluted 10× or 100×, (3.)

fermented milk samples: bifidus (*B. bifidum*) and acidophilus milk (*L. acidophilus*) diluted 10× or 100×.

Classical microbiological plate counting method was applied as for reference measurements. MRS and BSM bacterial growth media were used for *L. acidophilus* and *B. bifidum* bacteria.

3.2 OWLS immunosensor development for aflatoxin M_1

Indirect measuring method was used during the development of AFM_1 immunosensor, which is applicable for the quantification of low molecular weight compounds like AFM_1. For indirect measurements, AFM_1-HRP conjugate was covalently immobilized on the amino functionalized integrated optical waveguide sensor with the use of glutaraldehyde. AFM_1 antibodies were added in a slightly excess amount related to the amount of the antigen into the sample, and measured by the immobilized antigen. Applying this measuring method, only the antibodies remaining free in the sample mixture can bind to the antigens immobilized, so the amount of antibodies binding to the surface is inversely proportional to the quantity of the antigen in the sample. The immunocomplexes formed on the sensor surfaces were dissociated by 10 mmol/l HCl, thus regenerating the sensor.

The samples were prepared through AFM_1 standard addition, and three kinds of sample preparation methods were compared. First, milk was spiked with different concentrations of AFM_1, then (1.) simply filtered, (2.) centrifuged (3500 g, 10 min, 10°C) or (3.) size exclusion centrifuged (3K Pall macrosep filter, 4500 g, 30 min, 10°C). Finally, the samples were diluted appropriately (42 mmol/l Tris, pH 7.4). The AFM_1 antibody solution of appropriate concentration was mixed with the AFM_1 standard solutions immediately prior to measurement in 1:1 ratio. After 3 min incubation (24°C), the mixture was injected to the sensor surface.

Competitive ELISA format was used to control the results of the biosensor measurements.

4. RESULTS

The aim of this work was to develop antibody-based QCM and OWLS immunosenors for the determination of probiotic bacteria (*L. acidophilus* and *B. bifidum*) and OWLS immunosensor for aflatoxin M_1.

The applicability of the QCM measuring system for direct, antibody based determination was confirmed with model measurements. During the BSA-antiBSA model examinations, it was found that the self-assembled monolayer (SAM) constructed with sulfo-LC-SPDP cross linking agent was more effective in the process of antibody immobilization than the MHDA reagent. In the case of QCM immunosensor, the optimal flow rate was 0.1 ml/min, and the injected sample volume was 500 µl. The effect of the re-using of the same quartz crystal was examined. It was concluded, that the same sensor crystal could only be used four times with the applied surface cleaning method. No significant signal decrease was observed, when using the same crystal for four consecutive measurement sessions.

Specific polyclonal antibodies were raised against the target probiotic bacteria by rabbit immunization. Competitive ELISA test was carried out to determine the cross-reactivity and selectivity of the antibodies. During the development of the QCM and OWLS immunosenors, the optimal immobilized antibody concentration used on the quartz wafer and on the waveguide sensor was 50 µg/ml and 10 µg/ml, respectively. Further measuring parameters, flow rate and measuring temperature were also determined. The results of the ELISA confirmed the results of the selectivity investigation performed by the QCM and OWLS biosensors. The polyclonal antibodies showed cross-reactivity with the nonspecific bacteria from 1.0E+5 CFU/ml using QCM and from 1.0E+4 CFU/ml applying OWLS sensor. Further OWLS investigations regarding selectivity showed that the sensor was significantly more sensitive for specific heat treated cells than for untreated (native) cells. The dynamic measuring ranges for both bacteria suspended in buffer was 1.0E+4-1.0E+7 CFU/ml using QCM, while 1.0E+3-1.0E+7 CFU/ml

for *L. acidophilus* and 1.0E+3-1.0E+6 CFU/ml for *B. bifidum* in the case of OWLS. After the calibration of the sensors with the milk samples containing probiotic bacteria, the quantitative determination of *B. bifidum* and *L. acidophilus* in fermented milk samples were carried out. The cell numbers of fermented samples determined by the immunosenors and plate counting method were compared.

Experiments were carried out to explore the effect of the measuring method on the QCM's sensitivity. It can be stated that wider measuring range could be obtained using flow method for both bacteria (1.0E+3-5.0E+5 CFU/ml) in 100× diluted milk samples, than with stopped flow method including 10 min incubation (1.0E+3-1.0E+5 CFU/ml). As for the OWLS sensor, the dynamic measuring range of bacteria was 1.0E+3-5.0E+5 CFU/ml in milk samples, as in the case of QCM. The cell numbers of fermented milk samples determined by QCM and OWLS immunosensors were in good correlation (R^2 0.87-0.98) with the results of the plate counting method. There are many examples in the literature of biosensors developed to facilitate food microbiological examinations. As these developments target mainly pathogenic bacteria, the detection limits are too high (above 1.0E+3 CFU/ml, or even 1.0E+7 CFU/ml). These cell number values are unacceptable. In case a sample enrichment step is needed, the method will no longer be rapid. As for probiotic bacteria, high cell numbers are required in the product. This means, that after the application of a simple dilution step, an appropriate bacteria concentration can quickly be determined.

During the development of the OWLS-based determination method for AFM_1, indirect immunoassay format was applied. This method enabled the sensitive quantification of low molecular weight molecules, as AFM_1 (328.27 g/mol). The protein (HRP) - aflatoxin M_1 conjugate was immobilized on the sensor chip surface by glutaraldehyde (2.5%). The standards or samples were then mixed with the antibody solution of appropriate concentration in 1:1 ratio and incubated for 3 min, then finally injected. Applying this measuring method, only the antibodies remaining in free form in the sample mixture could bind to the antigens immobilized on the

sensor chip. We determined that the sensor showed the best sensitivity (0.001-100 ng/ml dynamic measuring range) using 3.0 µg/ml AFM_1-HRP conjugate and 21.25 µg/ml AFM_1 antibody at 24°C. These investigations were followed by the analysis of spiked milk samples. Three different methods were utilized for the preparation of spiked milk samples: filtration, centrifugation, size exclusion centrifugation. These samples were examined in 100 or 200 fold dilutions. Comparing these procedures and the dilution rates, it could be stated, that the best results were obtained when 100 fold diluted filtered or centrifuged samples were examined. The dynamic measuring ranges were 0.001-0.1 ng/ml (IC_{50}: 0.016 ± 0.002 ng/ml; LOD: 0.0005) and 0.0005-0.01 ng/ml (IC_{50} 0.0021±0.0004 ng/ml; LOD: 0.0001 ng/ml), respectively. Based on the results of the biosensor measurements and the reference ELISA test it could be concluded, that the developed label-free OWLS-based indirect immunoassay method was applicable for the quantification of AFM_1 in milk samples.

5. NEW SCIENTIFIC RESULTS

QCM- and OWLS-based flow through direct immunoanalytical methods were developed for *L. acidophilus* and *B. bifidum* bacteria and an OWLS-based indirect immunosensor was elaborated for aflatoxin M_1 quantification:

1. As the first step of the QCM biosensor development, the applicability of the QCM sensor for flow through measurements with direct determination method was confirmed with model examinations (BSA – anti-BSA molecule pair) by the examination of the basic operating parameters. It was stated, that the sulfo-LC-SPDP heterobifunctional crosslinker is more effective for antibody immobilization on the Au surface of the QCM sensor than the MHDA reagent. Comparing two measuring procedures– flow-through and stopped-flow – stopped-flow mode with 10 min incubation time using 500 µl sample loop proved to be effective based on the detected frequency (Hz). It was stated that the same sensor chip can be used up to 4 times, utilizing the pre-treatment and the surface modifications without any significant signal decrease.
2. Based on the model examinations and further optimization steps the QCM-based direct determination method for *L. acidophilus* and *B. bifidum* bacteria was elaborated. The dynamic measuring range for the bacteria suspended in buffer was found between 10^4-10^7 CFU/ml. It was stated that using the developed sensors, wider dynamic measuring range was observed for both bacteria in 100 fold diluted milk samples with the flow-through (10^3-5×10^5 CFU/ml) method than with stopped-flow method (10^3-10^5 CFU/ml). The applicability of the developed QCM sensors was confirmed with the measuring of 100 fold diluted fermented milk samples.
3. Direct, OWLS-based immunosensors were developed for *L. acidophilus* and *B. bifidum* bacteria. The dynamic measuring ranges when bacteria suspended in buffer were between 10^3-10^7 and 10^3-10^6 CFU/ml for *L.*

acidophilus and *B. bifidum*, respectively. The dynamic measuring range in 100 fold diluted milk samples for both bacteria using flow-through method was between 10^3-5×10^5 CFU/ml. The applicability of the developed OWLS sensors were confirmed with the measuring of 100 fold diluted fermented milk samples. The sensitivity and selectivity analyses of OWLS immunosensors showed that the antibodies bind to the heat treated cells with higher affinity, than to the untreated cells. However, the sensor gives selective, and significantly higher signals for the heat treated, specific cells than for untreated, native cells. The higher difference detected between signals of treated and untreated cells indicates the higher amount of specific bacteria.

4. An OWLS-based, indirect immunosensor was developed for the quantification of aflatoxin M_1. It was concluded, that the filtration and the centrifugation can be used effectively for the sample preparation. The dynamic measuring range of AFM_1 in 100 fold diluted milk samples was 0.001-0.1 ng/ml (LOD 0.0005 ng/ml) and 0.0005-0.01 ng/ml (LOD 0.0001 ng/ml) using filtration or centrifugation, respectively. The developed sensor can be used efficiently for the determination of AFM_1 contamination in 100 fold diluted milk samples.

6. PUBLICATIONS

Publication in journal

Articles with impact factor:

Szalontai, H., Adányi, N., Kiss, A. (2012): Development of piezoelectric immunosensor for the detection of probiotic bacteria. *Analytical Letters*, 45: 1214-1229. IF: 0,965

Szalontai, H., Adányi, N., Kiss, A. (2014): Comparative determination of two probiotics by QCM and OWLS-based immunosensors. *New Biotechnology*, 31: 395-401. IF: 2,106

Szalontai, H., Kiss, A., Adányi, N. (2014): Determination of aflatoxin M1 in milk samples by an OWLS-based immunosensor. *Acta Alimentaria*, 43: 148-155. IF: 0,427

Publication in conference proceeding

Proceedings in Hungarian:

Szalontai, H., Adányi, N., Kiss, A. (2013): Development of an OWLS-based immunosensor for the detection of Aflatoxin M1. Food Science Conference 2013 - With research for the success of Darányi Program, Budapest, Hungary. Book of proceedings (ISBN:978-963-503-550-2), p. 230-233 (oral presentation).

National conferences (abstracts):

Szalontai, H., Adányi, N., Kiss, A. (2011): QCM-alapú jelölésmentes immunszenzor alkalmazása probiotikus baktériumok kimutatására. MKE 1. Nemzeti Konferencia, Sopron, Magyarország. Program és előadás összefoglalók (ISBN:978-963-9970-11-3), p. 233 (poszter).

Szalontai, H., Adányi, N., Kiss, A. (2012): Probiotikumok csíraszámának meghatározása immunreakción alapuló bioszenzoros módszerekkel. Táplálkozástudományi Kutatások II., Kaposvár, Magyarország. Absztrakt CD (ISBN:978-963-9821-55-2), p. 25 (előadás).

International conferences (abstracts):

Szalontai, H., Adányi, N., Kiss, A. (2009): Quartz crystal microbalance (QCM) based immunosensor for food quality control. Lippay János - Ormos Imre - Vas Károly Scientific Conference, Budapest, Hungary. Book of abstracts (ISBN:978-963-503-397-3), p. 65 (oral presentation).

Szalontai, H., Adányi, N., Kiss, A. (2010): Development of piezoelectric biosensor for the detection of probiotic bacteria. 2nd Workshop on specific methods for food safety and quality, Belgrade, Serbia. Book of abstracts (ISBN:978-86-7306-113-9), p. 20 (oral presentation).

Szalontai, H., Adányi, N., Kiss, A. (2012): Label-free immunosensor for the detection of probiotics in fermented dairy products. 6th Central European Congress on Food, Novi Sad, Serbia. Book of abstracts (ISBN:978-86-7994-028-5), p. 284 (poster presentation).

Szalontai, H., Adányi, N., Naár, Z., Kiss, A. (2013): Development of OWLS-based biosensor for the detection of food contaminant Aflatoxin M1. 4th MoniQA International Conference, Budapest, Hungary. Book of abstracts (ISBN:978-3-9503336-1-9), p. 138 (poster presentation).

Szalontai, H., Adányi, N., Kiss, A. (2013): Quartz Crystal Microbalance and Optical Waveguide Lightmode Spectroscopy based immunosensors for probiotics detection. Advances in Biosensors and Biodetection, Barcelona, Spain (poster presentation.

Kiss, A., **Szalontai, H**., Adányi, N. (2013): Development of QCM-based direct, label-free immunosensor for rapid, cost-effective assessment of probiotic bacteria in

fermented dairy products. RME 2013, Food Feed Water Analysis innovations and breakthroughs, Noordwijkerhout, Hollandia. Book of abstracts, p. 115 (poster presentation).

Szalontai, **H.,** Csifáry, G., Adányi, N., Kiss, A. (2013): Development of two label-free immunosensors for the detection of probiotics. Sixth International Workshop on "Biosensors for Food Safety and Environmental Monitoring", Essaouira, Morocco. Book of abstracts, p. 54 (poster presentation).