ROMA TRE
UNIVERSITÀ DEGLI STUDI

SCUOLA DOTTORALE IN BIOLOGIA

BIOLOGIA APPLICATA ALLA SALUTE DELL'UOMO

CICLO XXIV

Approach in Aptamer Based Biosensors For Human Health Applications

Studio di Biosensori Basati su Aptameri Applicati alla Salute dell'Uomo

Dr. Ilaria Lamberti

Tutor: prof. Antonio Antoccia

Coordinator: prof. Paolo Visca

Tutors: dr Sabrina Foglia (CNR)

dr Lucia Mosiello (ENEA Casaccia)

ROMA TRE
UNIVERSITÀ DEGLI STUDI

DOCTORAL SCHOOL IN BIOLOGY:

BIOLOGY APPLIED TO HUMAN HEALTH

COURSE XXIV

Approach in Aptamer Based Biosensors For Human Health Applications

Studio di Biosensori basati su aptameri Applicati alla Salute dell'Uomo

Dr. Ilaria Lamberti

December 2011

CONTENTS

Abstract

In recent decades the combination of different technologies and scientific disciplines like bioelectronics, genomics, materials science, biochemistry and computer science, has allowed the development and implementation of innovative analytical devices - biosensors - which allow to find new solutions in the field of diagnostic. In such devices the measurement of the target analytes is achieved by selective transduction of a parameter of the biomolecule-analyte reaction into a quantifiable signal, providing a selective identification of toxic chemical compounds at ultra trace levels in industrial products, chemical substances, environmental samples (e.g., air, soil, and water) or biological systems (e.g., bacteria, virus, or tissue components) for biomedical diagnosis. In life sciences, biosensors have offered new options for clinical diagnostic procedures. In the development of biosensors, nanotechnology is playing an increasingly important role, improving sensitivity and performance of their construction and allowing the capability of unambiguous identification and accurate quantification of big chemical constituents in complex systems.

Recently, biosensors based on the use of monoclonal or polyclonal antibodies have seen a great development in the field of small molecules analytical determination and specifically in the mycotoxins analyses for food safety application and in biomarkers detection for human health (i.e. caspases detection of apoptosis, playing a critical role in the development of therapeutics in many different fields, including cancer).

Affinity to specific molecular targets (such as nucleic acids, proteins, small compounds, or cells) of antibodies is overcome by aptamers, single stranded of DNA or RNA oligonucleotides of 15 to 60 base. In contrast to antibodies, aptamers are prepared by *in vitro* selection procedure called SELEX.

The purpose of this PhD thesis is to provide a study of a range of technologies and innovations in the field of the bio-sensible component of a biosensor, comparing different experimental approaches.

At the beginning, using as biological component monoclonal e polyclonal antibody, we performed an antibodies- based microarray biosensor for applications in proteome analysis, disease diagnostics and quantitative small molecules analysis. We discussed different microarray surfaces, immobilization techniques, detection systems and advantages and disadvantages of antibody microarrays.

During the second year, we approached a different bioreceptor: aptamers, that exhibit many advantages as recognition elements in biosensing, compared to traditional antibodies. The unique properties of aptamers and

the possibility of developing aptamers against different binding sites on the target analyte allowed high variability on the assay and a good use in biosensor field.
We used an acoustic sensor in a TSM format (Transverse Shear Mode methods) for studying protein/aptamer interaction. We detected subtle structural effects at the sensor surface connected with conformation changes of thrombin aptamers and ochratoxin induced by protein or low molecular mass ligand.
In the last year, an aptamer terminated sensing surface, was exploited for designing a nano-aptasensor for microfluidic device, coupled with a Surface Enhanced Raman Spectroscopy (SERS). In order to validate this strategy, the already experienced aptamer for thrombin is used.
During the PhD we moved in this direction, studying and defining different approaches to develop new biosensors for the most popular toxic and carcinogenic food contaminant class: micotoxins. We also focused our interest in thrombin and caspases, due to their importance as biomarker in several human pathologies, and in the development of the analytical devices.

1. General Background

The purpose of this PhD thesis is to provide a study of a range of technologies and innovations in the field of the bio-sensible component of a biosensor, comparing different experimental approaches. The availability of innovative tools for the detection of food contaminants and for the analysis of biomarkers of pathological conditions is closely related to the protection of human health. During my PhD, we moved in this direction, studying and defining different approaches to develop new biosensors.

1.1 What is a Biosensor?

A biosensor is a device which converts biological activity into a quantifiable signal, providing rapid analysis and real time detection [Schmid, 1987; Turner et al., 1987]. It consists of two main components: a bio-receptor and a transducer. The bioreceptor (or biological sensing

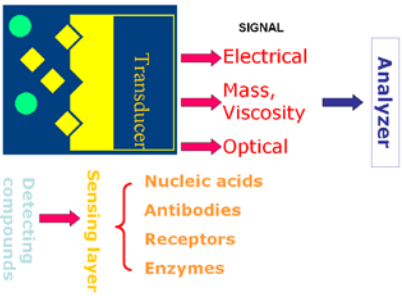


Fig. 1: Scheme of a Biosensor [Pacheco, 2011].

element or biological component) is a biomolecule, such as an antibodies, enzymes, cells or aptamers, in close contact with the transducer, which binds specifically to the analyte of interest. The transducer translates the binding event into detectable and measurable signal (fig.1). Interactions, via coupling of a biological recognition element to a transducer, are capable of providing qualitative or quantitative information [Malhotra et al. 2005]. The two most important properties of any proposed biosensor are the specificity and the sensitivity toward the target analyte. The specificity of a biosensor is entirely governed by the properties of biological component, because this is where the analyte interacts with the sensor. The sensitivity of the

integrated device is dependent on both the biological component and the transducer, because there must be a significant biomolecule-analyte interaction and high efficiency of subsequence detection of this reaction by the transducer. The range of potential biological components and the range of transducer technologies available for use in biosensors require a truly multi and interdisciplinary approach to research and development in this area. Much of the basic transducer technologies already exist, though it requires further development and optimization. A number of different techniques for measuring the response, as well as various types of transducer which are currently being evaluated for their suitability for a range of sensing applications, are listed in table 1. Biosensors based on DNA or RNA aptamers (aptasensors) represent new type of sensors that utilize unique properties of artificial receptor – aptamers. Aptasensors are of considerable interest, due to their application in detection of practically unlimited kinds of compound [Hianik and Wang, 2009].

Table 1

Biological applications	Tranducer system
Cofactors	Optical
Antibodies	Electrochemical
Receptors	Piezo-electric
Enzymes	Calorimetric
Membranes	Acoustic
Organelles	Mechanical
Cells	Densitometric
Aptamers	

The main advantages of biosensor technology, in comparison with traditional analytical methods, are fast detection (minutes) and response (seconds), high sensitivity (typically nM, improved sensitivity with nanoparticles pM and better), high selectivity, easy preparation and operation assay method. In addition, most of these devices are reusable and show low cost assay.

The methodology of surface chemistry is the basic know-how for obtaining reproducible results with biosensors and various strategies can be used [Gagliardi et al., 2007].

The key points to consider when selecting an appropriate surface and coating procedure are the low degree of unspecific binding sites and the uniform distribution of functional groups on the substrate surface.
For this reason, during biosensor development and testing, particular attention has to be focused on:

- Surface (on which sensing layer will be coated) characterisation
- Biological reagent (immunoglobulin, nucleic acid, ecc.) characterisation
- Uniformity of biological element
- Standard solution preparation
- Calibration and Standard Curve construction

1.2 Bioreceptor components

The variety of bioreceptors used in vast majority of biosensors, reported to date, have been based on antibodies, oligonucleotides, enzymes, whole cells, membrane and, more recently, aptamers. A brief overview of different types will be described next.

1.2.1 Immunosensors

Immunosensors use antibodies (monoclonal or polyclonal) as biological element and are defined affinity sensors. They are based on the immunological technique that can be applied to investigate and manipulate minute concentrations of complex molecules. Current immunological assays capable of detecting many biological agents utilize the sensitivity and specificity of polyclonal and monoclonal antibodies [Emanuel et al., 2000]. Polyclonal antibodies are obtained from animal serum and are produced by two type of blood cell, B lymphocytes and plasma cells, in response to a foreign substance. Monoclonal antibodies are produced *in vitro* by hibridoma technology. From the analytical point of view, the main difference between polyclonal and monoclonal antibodies is the fact that monoclonal recognizes on the molecule of antigen a single epitope only [Pohanka, et al., 2007]. Antibodies consist of four polypeptide sub-units comprising two identical large or heavy chains and two identical small and light chains which are held together by non covalent force and covalent inter chain disulphide bonds. The carbohydrate residues in antibodies are covalently bonded to the C-terminal half (Fc) of the molecule. The key portions of the antibody molecule that contain the antigen binding sites are called the Fab fragments. Each Fab fragment comprises an entire light chain and a segment of the heavy chain [Byfield and Abuknesha, 1994].
In biosensor based on antibodies the analyte is either the corresponding antigen of the antibody used. An antibody-antigen interaction is characterized by two major properties that may be exploited for sensing or detection purposes: a very high affinity constant (10^{-18} M of antigens can be detected) and a low cross-reactivity. The main important property of antibodies is their ability to bind to an extremely wide range of natural and man-made compounds [Vo-Dinh and Cullm, 2000].

1.2.2 Aptasensors

Aptamers are ligands with a high affinity for considerably differing molecules ranging from large targets as proteins over peptides, complex molecules to drugs and organic small molecules or even metal ions. They are widely used, including medical and pharmaceutical basic research, drug development, diagnosis, and therapy. Analytical and separation tools bearing aptamers as molecular recognition and binding elements are another big field of application.

Aptamers have become important tools for molecular diagnostics and therapeutics. In particular, the aptamers-based biosensors possess unprecedented benefits for their high specificity and affinity. In principle, they can be selected *in vitro* for each target, ranging from small molecules to large proteins, also cells. They can be synthesized with high

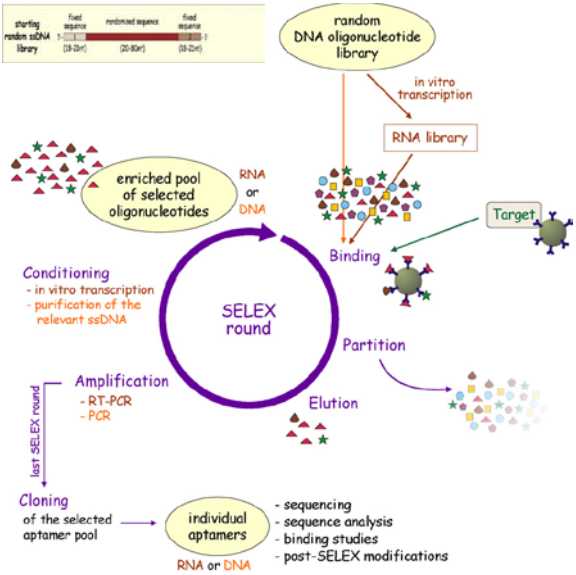


Fig. 2: Systematic Evolution of Ligands by Exponential enrichment - SELEX [Stoltenburg et al., 2000].

reproducibility and purity, DNA aptamers are usually highly chemically stable. The application of aptamers as biocomponents in biosensors offers a

multitude of advantages over the state of the art in affinity sensing [Song et al, 2008]. Aptamers are single-stranded RNA or DNA oligonucleotides 15 to 60 base in length that bind with high affinity to specific molecular targets such as nucleic acids, proteins, small compounds or cells. This oligonucleotides bind to their target with high selectivity and sensitivity due to their three-dimensional shape. Their specificity is comparable and in certain cases even higher than those of antibodies. In contrast to antibodies, aptamers are prepared by *in vitro* selection procedure: Systematic Evolution of Ligands by Exponential enrichment, also called SELEX, developed simultaneously in early 1990s by L. Gold and A. Ellington laboratories [Ellington and Szostak, 1990; Tuerk and Gold, 1990]; SELEX, aimed at the development of aptamers [Stoltenburg et al., 2000], involves three processes, namely: selection of ligand sequences that bind to a target; partitioning of aptamers from non-aptamers via affinity methods; amplification of bound aptamers (Fig.2) [Gopinath, 2007].

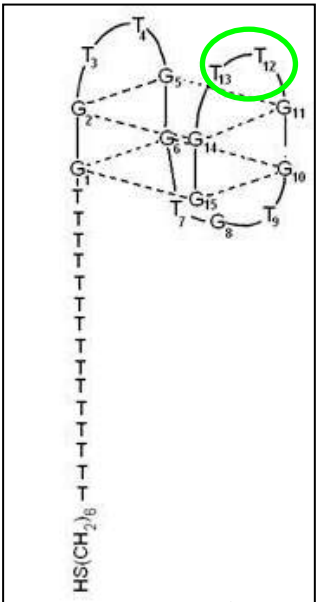


Fig. 3: Structures of DNA aptamers that selectively bind thrombind in its fibrionogen binding site [Hianik, 2009].

Aptamers have been selected to hundreds of small molecules and protein targets including, for example, ATP, GTP, B12, malachite green and caffeine, HIV-1 Rev peptide, MS2 coat protein, and thrombin. Several aptamer structures have been shown to be readily evolvable in terms of specificity. For example, three mutations within the binding loop of an *in vitro* selected l-citrulline aptamer were enough to change its specificity to l-arginine because the pattern of hydrogen bond donors and acceptors could be flipped in a simple way [Yang et al., 1996; Klussmann 2006].

Among aptamers, the thrombin aptamer was most extensively studied. The composition of the thrombin aptamer is as follows: d(T15GGTTGGTGTGGTTGG). A pair of thymines on the G- quandruplex of the aptamer characterizes thrombin binding site (fig. 3). The thrombin aptamer is the first example of ssDNA

oligonucleotides that bind a target protein with unknown specificity for nucleic acids and it is largely use in biosensor field [Bock et al., 1992].
Thrombin is a multifunctional serine protease, which plays an important role in pro-coagulant and anti-coagulant function. This protease converts soluble fibrinogen into insoluble strands of fibrin, which is responsible either for a physiological plug or pathological thrombus. Thrombin has two binding sites that are spatially separated and localized at opposite poles of thrombin molecules. These binding sites are sensitive to fibrinogen and heparin, respectively. Aptamers can be used as potential inhibitors of thrombin, therefore study of the mechanisms of interaction of thrombin with aptamers is of great importance for medicine [Coughlin, 2000].
Recently, also a DNA aptamer sensitive to ochratoxin A (OTA) has been developed [Cruz-Aguado and Penner, 2008]. OTA A is a fungal toxin, discovered as a metabolite of *aspergillus ochraceus* [Turner et al., 2009]. This aptamer is able to recognize OTA A with sensitivity in a ppb level and with high selectivity.
Mycotoxins, such are aflatoxins, fumonisine and ochratoxin, are toxical fungal metabolites that can occur in primary food products. This mycotoxin generally appears during storage of cereals, coffee, cocoa, dried fruit, pork etc. and occasionally in the field of grapes. It may also be present in blood and kidneys of animals that have been fed on contaminated feeds. Animal studies indicated that this toxin is carcinogenic [Turner et al., 2009]. Therefore, the European Commission has fixed maximum concentration of OTA in foodstuffs: 3 μg/kg (7.4 nM) for cereal products and 5 μg/kg (12.4 nM) for roasted coffee, respectively (Commission Regulation No. 1881/2006, 19 December 2006).
The establishment of an efficient method of this analyte detection is therefore of high importance. In addition to traditional, but expensive and time-consuming methods such as liquid chromatography, new trends consist in development of portable and easy to use biosensors [Tsai and Hsieh., 2007].

1.3 Types of Biosensor Trasducers

The availability of rapid and reliable methods for rapid determination of small molecules is an increasing need for human health. In order to monitoring small molecules proteins/marker related on Human Health, Gas Chromatographic (GC) High Pressure Liquid Chromatography (HPLC) methods and immunoassay are generally utilized, due to their high detection sensitivity and selectivity. However, GC and HPLC analyses are time consuming and needs sample pre-treatment or pre-concentration procedures [Sethi, 1994].

Immunoassays and biosensors are becoming a recognized alternative or complementary to conventional analytical techniques for the detection of different analytes. Recently, biosensors based on the use of monoclonal or polyclonal antibodies have seen a great development in the field of small molecules analytical determination. The absence of cross-reactivity obtained with most of these biosensor, the possibility of on-line measurement, the absence of sample pre-treatment, can really put it in competition with other conventional systems such as HPLC and ELISA [Pacheco, 2011].

In particular, we focused our attention on biosensors that utilize immunoglobulins or aptamer showing affinity for a correspondent analyte, associated to various transduction elements. Several biosensing platforms have been introduced for the Surface Enhanced Raman Spectroscopy (SERS) and for the Quartz Microbalance Crystals (QCM). Examples of microarray densitometric platform is been also presented.

Analytical methods used for small molecules determination for human health are mainly based on immunoassay as ELISA. Actually, biosensor and microsystem technologies are used for different applications including studies of human and veterinary diseases, drug discovery, genetic screening, clinical and food diagnostics. According to these approaches, the goal of many authors has been to transfer the methods of immunoassay from microtiter plates into a biosensor format to develop a fast, sensitive and inexpensive detection of protein markers for various diseases.

Microarray and biosensor technology enables the fast and parallel analysis of a multitude of biologically relevant parameters. Not only nucleic acid-based tests, but also peptide, enzyme antibody and aptamer assays using different formats of biosensor evolved within the last decade. Microarrays and biosensors are a powerful assay technology that can be used to generate rapid detection of analytes in complex samples which are also potentially useful for the generation of rapid tests for biomedicine and for food safety.

1.3.1 Densitometric

Densitometry is the quantitative measurement of optical density in light-sensitive materials. Densitometric analysis is the quantitative and qualitative study of color levels of the image obtained. It seems expecially suitable, since it allows rapid quantification. Densitometric evaluation is based on measurement of light reflected by a spot or of a native fluorescence. Today, modern computer-controlled densitometers allow quantitative determination of almost all chemicals, both colorless and colored which have UV-Vis absorbance or a capable of measuring fluorescence. The method is rapid and it offers possibility of analyzing simultaneously a large number of sample along with consecutive qualitative or semi-quantitative analysis. Several colorimetric and fluorometric methods are currently available for the measurement of proteins in biological extracts [Ghosh, 1988].

Enzyme linked immunosorbent assay (ELISA) and fluorescence immunoassay (FIA) are excellent survey tools for many analytical purposes because of their high-throughput, user friendliness, and field portability. Immunoassay is traditionally performed as individual test, however in many cases it is necessary to perform a panel of tests on each sample (detection of drug residues). To address this requirement, microarray-based immunoassay technologies have been developing utilizing microarray platform (multianalyte analysis) and classic immunoassay (multi-samples analysis).

In recent years, the antibody microarray technology has made significant progress, going from proof-of-concept designs to established high-performing technology platforms capable of targeting non-fractionated complex samples, as proteoma [Blohm and Guiseppi-Elie, 2001]. Microarrays provide a powerful analytical tool for the simultaneous detection of multiple analytes in a single experiment and consist of a biosensor *micro* o *nano* arrays.

Microarrays consist of immobilized biomolecules spatially addressed on planar surfaces, microchannels or microwells, or an array of beads immobilized with different biomolecules. Biomolecules commonly immobilized on microarrays include oligonucleotides, polymerase chain reaction (PCR) products, proteins, lipids, peptides and carbohydrates. Ideally, the immobilized biomolecules must retain activity, remain stable, and not desorb during reaction and washing steps. The immobilization procedure must ensure that the biomolecules are immobilized at optimal density to the microarray surface for efficient binding [Venkatasubbarao et al., 2004].

Some microarray applications are focused on current trends in the movement of this technology from being a purely research method to

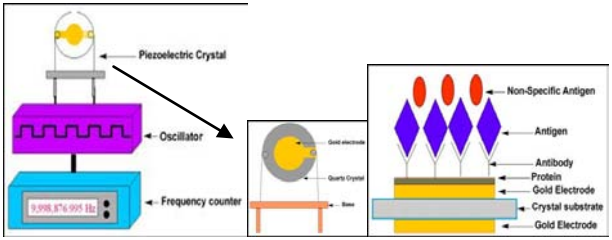
becoming an analytical instrument applicable in the clinic and as well as in human health [Koppal, 2004].

Research on microarrays as multianalyte biosystems has generated increased interest in the last decade. The main feature of the microarray technology is the ability to simultaneously detect multiple analytes in one sample by an affinity-binding event at a surface interface. In some cases immunoanalytical microarrays have the potential to replace conventional chromatographic techniques. They are applied if the number of samples is high or analysis by current methods is difficult and/or expensive. Therefore, microarray platforms have a great potential as monitoring systems for the rapid assessment of water or food samples. Antibody-based microarrays are a powerful tool for analytical purposes, also for aflatoxins and caspases detection application. Immunoanalytical microarrays are a quantitative analytical technique using antibodies as highly specific biological recognition elements.

In our two applications we developed a competitive immunoassay in a microarray format and observed, using the method described, different microarray patterns in samples containing aflatoxin-B1 or fumonisine at a ppb concentration range and either analytes in order to detect different caspases. The quality of the microarray data was comparable to data generated by a microplate-based immunoassay, but further investigations are needed in order to better characterize these methods.

1.3.2 Mass sensitive

Another form of transduction that has been used for biosensors is the measurement of small changes in mass. This has already been shown to capable of very sensitive measurements. The principle means of mass



a b c

Fig. 4 - a) and b): Mass piezoelectric transducer; **c):** A bioreceptor antibody-based on a Piezoelectric crystal transducer [Kumar, 2000].

analysis relies on the use of piezoelectric crystals. These crystals can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. The frequency of oscillation is therefore dependent on the electrical frequency applied to the crystal as well as the crystal's mass. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency of the crystal changes and the resulting change can be measured electrically and be used to determine the additional mass of the crystal [Vo-Dinh and Cullum, 2000].

Quartz crystal microbalances (QCM) are particularly suitable for the nucleic acids hybridization detection because no label is required to reveal the interaction between a strand immobilized on the sensor surface and the corresponding interacting molecule in solution. The limitation of this approach is non-specific adsorption of molecules present in real matrices. However, previous experiments with piezoelectric and DNA probes showed that non-specific binding can be avoided using an appropriate immobilisation chemistry [Tombelli et al., 2000]. Mass sensitive piezoelectric transducers are usually based on AT-cut quartz crystal covered by gold electrodes. The external alternating voltage induces oscillation of the quartz. The frequency of this oscillation depends on the transducer thickness (Fig. 4a and 4b).

In these biosensors the frequency value of the oscillation of the quartz is proportional to the mass of the crystal following the Sauerbrey law and decreases with increasing of the mass (Equation 1) [Sauerbrey, 1959].
However, exact relation between the changes of the resonant frequency and

$$\Delta f = -2.26 \times 10^{-6} f_0^2 (\Delta m / A)$$

Equation 1: Sauerbrey equation

mass is valid, according to Sauerbrey, only for dry crystal. In a solution, the contribution of viscoelasticity should be considered due to the possible friction between the biolayer at crystal surface and surrounding liquid. The analysis of viscoelastic contribution can be made by Thikness Shear Mode (TSM) method [Hianik et al., 2009]. This method is based on analysis of complex impedance spectra of quartz transducer. In addition to the resonant frequency also so called motional resistance (Rm), can be determined in this method. The Rm value is measure of viscoelastic contribution to the crystal oscillation [O'Sullivan and Guilbault 1990].

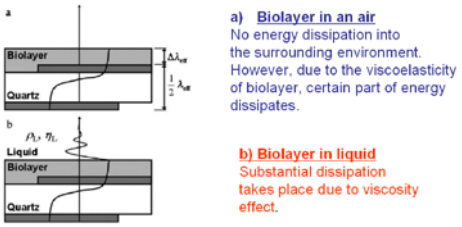


Fig. 5: Propagation of acoustic wave from the sensor surface [Pacheco, 2011].

TSM is certain analogy of QCM, however, in addition to mass, the TSM determines also the viscosity contribution arising from the friction between biolayer and the surrounding buffer (Fig. 5). This is important for detection of small molecules, such are mycotoxins for which the QCM detection is difficult due to small molecular weight of the analyte [Lamberti et al., 2011].

1.3.3 Optical

Among of all type of biosensors, optical biosensors are receiving considerable attentions because the advantages of using integrated optical or optoelectronic trasducer for measuring biological reactions have been realized. In such field the detection mode based on SERS (Surface Enhanced Raman Spectroscopy) opens a new way for bio-marker

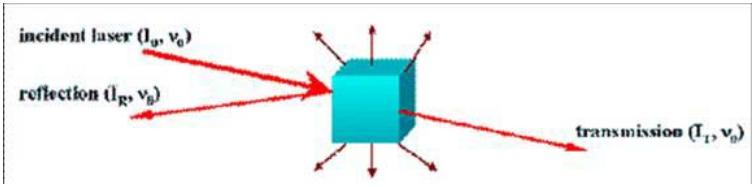


Fig 6: Raman spectroscopy: a photon of light interacts with a sample to produce scattered radiation of different wavelengths.

recognition [Wang et al., 2007].
In laser-based optical spectroscopy, Raman scattering generates a fingerprint-like vibrational spectrum for individual molecular species with features that are much narrower than fluorescence. Raman scattering can be generated using monochromatic far-red or near-IR light, photon energies too low to excite the inherent background fluorescence in biological samples (fig. 6). In addition, water is a very poor Raman scatterer, and combined with all the features described, makes Raman a useful tool in detecting molecular species in biological samples.
SERS takes advantage of strongly increased Raman scattering signals generated by local field enhancements near metallic nanostructure and can be applied for label free analyte detection, revealing molecular fingerprints. A Raman dye can be either fluorescent or non fluorescent, and a minor chemical modification of a dye molecule can lead to a new dye with a different Raman spectrum, even if the two dyes exhibit virtually indistinguishable fluorescence spectra [Sassolas et al., 2006]. A variety of metal structure (Au Ag and Cu) are used to induce SERS effect. These metal are used in different format as metal plates, colloids rods coating [Knauer et al., 2010].
The spectral specificity of a SERS scattering probe is excellent in comparison to that of the fluorescence method. For example, the spectral bandwidths of cresyl fast violet in UV adsorption and fluorescence are broad whereas the bandwidth of the SERS spectrum of the same dye is

narrower. The dye-labeled SERS active substrates can provide richer spectral information than fluorescence based signatures, which are often limited by spectral overlap of the chromophores and by background signals due to other components in the sample [Kneipp et al., 1997; Wang et al. 2007].

The SERS technique was reported as a tool for detecting specific nucleic acid sequences. A SERS aptasensor was described to detect thrombin. Thiolated thrombin-binding aptamers were immobilized onto a gold substrate. A sandwich structure was formed between the immobilized aptamer, the protein target and a secondary aptamer bound to AuNPs, which were labeled by a Raman reporter (R6G). Then, silver NPs aggregated on AuNPs yielding electromagnetic hot spots. Thus, the Raman signal of the R6G was greatly enhanced due to the large electromagnetic coupling effect produced by the hot spots between AgNPs and AuNPs. The detection limit of this SERS aptasensor was 5×10^{-10}M. A reagentless aptameric biosensor based on SERS spectroscopy was also developed to detect cocaine. Tetramethylrhodamine (TMR)-labeled aptamer was immobilized on a SERS substrate. In the absence of the target, the aptamer was partially unfolded. So the TMR moiety remained away from the sub-strate and yielded a weak SERS signal. In the presence of cocaine, the aptamer folded into stable three-way junction, in which the TMR moiety came in close proximity to the SERS substrate, generating an enhanced SERS signal. Detection limit was 10^{-6}M. SERS "aptatags" were used to develop a biosensor for thrombin detection. "Aptatags" were composed by AgNPs linked together using a small organic molecule bearing two thiol functionalities: biphenyl-4,4'-dithiol (DBDT). DBDT served as the linker and as the SERS reporters. Then, "aptatags" were functionalized with thiolated thrombin-binding aptamers. First, aptamers were immobilized onto a silver layer deposited over a silicon wafer. After interactions between thrombin and specific aptamers, the surface was treated with the SERS "aptatags". A sandwich complex was formed between immobilized aptamers, the target and aptamers on the SERS "aptatags". The SERS signature of the linker holding the nanoparticles together indicated the presence of thrombin. Detection limit was 10^{-10}M [Sassolas et al., 2006].

An optical aptamer-based detection system label free appears as highly efficient device with enormous potential. Unfortunately, such systems are still immature compared to immunoassays, reflecting the limited availability of aptamer types and the relatively poor knowledge of surface-immobilization technologies for aptamers [Song et al., 2008].

Reference

Byfield, M.P. and Abuknesha, R.A. (1994). Biochemical aspects of biosensors Biosensors and Bioelectronics 9 (4-5): 373-400.

Blohm, D.H., and Guiseppi-Elie, A. (2001). New developments in microarray technology. Current Opinion in Biotechnology, 12 (1): 41-47.

Bock, L.C., Griffin, L.C., Latham, J.A. Vermaas, E.H. and Toole J.J. (1992). Selection of single-stranded DNA molecules that bind and inhibit human thrombin Nature 355: 564-566.

Coughlin, S.R. (2000). Thrombin signalling and protease-activated receptors. Nature 407: 258–264.

Cruz-Aguado, J.A., and Penner, G. (2008). Determination of ochratoxin A with a DNA aptamer. Journal of Agricultural and Food Chemistry, 56 (22): 10456-10461.

Ellington, A.D., Szostak, J.W. (1990). In vitro selection of RNA molecules that bind specific ligands. Nature 346: 818–822.

Emanuel, P.A., Dang, J., Gebhardt, J.S., Aldrich, J., Garber, E.A.E., Henrieta, K., Stopa, P., Valdes, J.J., Schultz, A.D. (2000). Recombinant antibodies: a new reagent for biological agent detection. Biosens. Bioelectron., 14: 761-770.

Gagliardi, S., Rapone, B., Mosiello, L., Luciani, D., Gerardino, A., Morales, P. (2007). Laser- assisted fabrication of biomolecular sensing microarrays, IEEE Transactions on Nanobioscience, 6 (3): 242-248.

Ghosh, S, Gepsten, S., Heikkila, J.J., Dumbroff, E.B. (1988).Use of a Scanning Densitometer or an ELISA Plate Reader for Measurement of Nanogram Amounts of Protein in Crude Extracts from Biological Tissues Analytical Biochemistry 169. 377-233

Gopinath, S.C.B. (2007). Methods developed for SELEX Anal. Bioanal. Chem., 387:171–182.

Hianik, T., and Wang, J. (2009) Electrochemical Aptasensors – Recent Achievements and Perspectives. Electroanalysis, 21: (11) 1223-1235.

Hianik, T., Grman, I. and Karpisova, I. (2009). The effect of DNA aptamer configuration on the sensitivity of detection thrombin at surface by acoustic method. Chem. Commun., 7 (41): 6303–6305.

Klussmann, S. (2006). The Aptamers Handbook, Wiley-VCH.

Knauer, M., Ivleva, N.P., Liu, X., Niessner, R. and Haisch, C. (2010). Surface-Enhanced Raman Scattering-Based Label-Free Microarray Readout for the Detection of Microorganisms. Anal. Chem., 82 (7): 2766–2772.

Kneipp, K., Wang, Y., Kneipp, H, Perelman, L.T., Itzkan, I, Dasari, R.R, and Feld, M.S. (1997). Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS). Phys. Rev. Lett., 78: 1667–1670.

Koppal, T. (2004). Microarrays: migrating from discovery to diagnostics. Drug Discovery Development, 7: 30–34.

Kumar, A. (2000). Biosensors Based on Piezoelectric Crystal Detectors: Theory and Application JOM-e, 52 (10) on line.

Lamberti, I., Mosiello L., and Hianik, T. (2011). Development of thickness shear mode biosensor based on DNA aptamers for detection ochratoxin. Chemical Sensors, 1: 11-15.

Malhotra, B.D., Singhal, R., Chaubey, A., Sharma, S.K. and Kumar A. (2005). Recent Trends in Biosensors. Current Applied Physics, 5: 92-97.

O'Sullivan, C.K. and Guilbault, G.G. (1999). Commercial quartz crystal microbalances – theory and applications. Biosensor and Bioelectronics, 14: 663-670.

Pacheco I.T. (2011). Aflatoxin - Detection, Measurement and Control (from chapter Biosensors for Aflatoxin Detection by Mosiello L. and Lamberti I.). InTech.

Pohanka, M., Pavliš, O., and Skládal, P. (2007). Rapid Characterization of Monoclonal Antibodies using the Piezoelectric. Immunosensor Sensors, 7: 341-353.

Sassolas A, Blum L. J., Leca-Bouvier B. D. (2011). Optical detection systems using immobilized aptamers. Biosensors and Bioelectronics, 26: 3725–3736

Sauerbrey, G. (1959). The use of oscillator for weighing thin layers and for microweighing. Z. Phys., 155: 206–210.

Schmid, R.D. (1987). Biosensors, Nachr. Chem. Tech. Lab., 35: 910-914.

Sethi, R.S. (1994). Transducer aspects of biosensors Biosensors and Bioelectronics, 9 (3):243-264

Song, S.; Wang, L.; Li, J.; Fan, C.; Zhao, J. (2008) Aptamer-based biosensors. Trends in Analytical Chemistry, 27: 108-117.

Stoltenburg, R., Reinemann, C., Strehlitz, B. (2007). SELEX—A (r)evolutionary method to generate high-affinity nucleic acid ligands Biomolecular Engineering, 24: 381–403.

Tombelli, S., Mascini, M., Sacco, C., Turner, A.P.F. (2000). A DNA piezoelectric biosensor assay coupled with a polymerase chain reaction for bacterial toxicity determination in environmental samples. Analytica Chimica Acta, 418: 1–9.

Tsai, W.-C., Hsieh, C.-K. (2007). QCM-based immunosensor for the determination of ochratoxin A. Analytical Letters, 40:(10) 1979-1991.

Tuerk, C., Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science, 249: 505–510.

Turner, A.P.F., Karube, I. and Wilson, G.S. (1987). Biosensors - Fundamentals and Applications., Oxford University Press.

Turner, N.W., Subrahmanyam, S., Piletsky, S.A. (2009). Analytical methods for determination of mycotoxins: A review. Analytica Chimica Acta, 632 (2): 168-180.

Venkatasubbarao, S. (2004). Microarrays – status and prospects. Trends in Biotechnology, 22 (12): 630-637.

Vo-Dinh, T. and Cullum, B. (2000). Biosensors and biochips: advances in biological s in biological and medical diagnostics. Fresenius J Anal Chem, 366: 540–551.

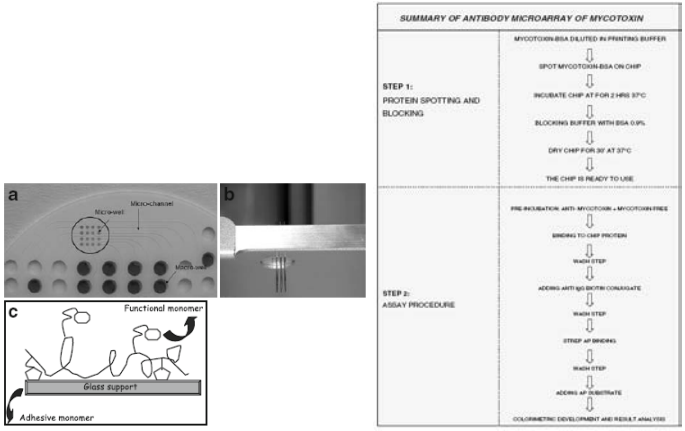
Wang, Y., Wei, H., Li, B., Ren, W., Guo, S., Dong, S., Wang, E. (2007). SERS opens a new way in aptasensor for protein recognition with high sensitivity and selectivity. Chem.Commun., 28 (48):5220-5222.

Yang, Y., Kochoyan, M., Burgstaller, P., Westhof, E., Famulok, M. (1996). Structural basis of ligand discrimination by two related RNA aptamers resolved by NMR spectroscopy. Science, 272 (5266): 1343-1347

Aims

Aim 1: Protein Microarray Applications

Research on microarrays as multi-analyte biosystems has generated increased interest in the last decade. According to this trend, we have tried to transfer the immunoassay method from microtiter plates into a microarray format in order to develop a multiparametric, rapid, sensitive and inexpensive method for the detection of mycotoxins for food safety application. To perform our test and check the feasibility of this format, we focused our studies on the most popular mycotoxins Aflatoxin B1 and Fumonisin B1 and developed a competitive immunoassay in a microarray format, using the Dr.Chip platform provided by Life Line Lab Co. (Pomezia, Italy) and used also for other applications.
Microarray platform is equipment to create microarrays and to read the final results, via densitometric detection, based on the enzymatic and colorimetric assay. In Fig. 7 A is reported a detail of the plastic probe tray for protein spotting and pins. In the same picture is also shown the scheme of the glass treated with functional protein linker.
As in other conventional competitive immunoassay, the color intensity and



A B

Fig. 7-A: Microarray spotting platform used for Aflatoxin B1 detection; **B:** Scheme of sensing assay for Aflatoxin B (Lamberti et al., 2009)

corrispondent grey values obtained from antigen microarrays BSA-Afla B1, prepared as described in this paper and used in our immunological tests, are in inverse proportion to antigen concentration in standard solutions. Assay method for Aflatoxin is described in Fig. 7 B.

The aim of our second application was to develop a new and rapid method for studying H460 cells that have been induced to apoptosis as a possible new tool for oncology studies. As main focus of this aim, we study the feasibility to transfer the methods of the immunological assay for caspases from classical western blot into a microarray format in order to develop a multiparametric, rapid immunoassay, sensitive and inexpensive assay.

Caspases are specific cytosolic proteases that are activated during apoptosis. Programmed Cell Death – PCD, or apoptosis, is a highly regulated process characterized by morphological and biochemical cellular changes. The selective induction of apoptosis in malignant cells may be an attractive mechanism to control neoplastic cell proliferation. Thus, factors that affect caspase activation and apoptosis might be important determinants for drug sensitivity. In addition, forms of cell death that are caspase dependent may also have a crucial role in the treatment response.

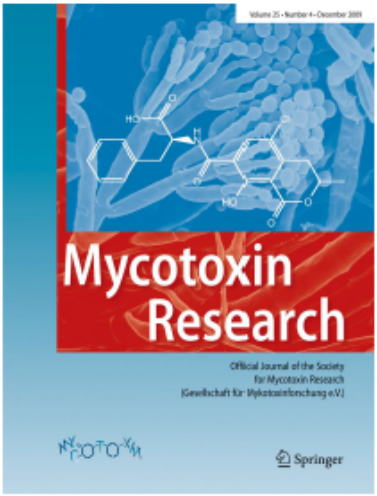
To test the feasibility of this format, we focused our studies on the three caspases that are critically involved in apoptosis: caspase-3 (both cleaved and uncleaved forms), caspase-8 and caspase-9. We developed different microarray formats using the commercial platform DR.Chip. Our results demonstrate that this platform is suitable for carrying out rapid immunological tests to identify cleaved and uncleaved caspase-3 as well as caspase-9. Simultaneous analyses for more of these proteases are mainly limited by the densitometric detection system associated with the microarray platform. In our opinion, the future use of fluorometric detection would offer great improvement for the microarray-based identification of caspases described in our work.

Our results demonstrate that this platform is suitable for carrying out rapid immunological tests to identify cleaved and uncleaved caspase-3 as well as caspase-9. Simultaneous analyses for more of these proteases are mainly limited by the densitometric detection system associated with the microarray platform. In our opinion, the future use of fluorometric detection would offer great improvement for the microarray-based identification of caspases described in our work.

An antibody-based microarray assay for the simultaneous detection of aflatoxin B1 and fumonisin B1 (2009) Lamberti, I., Tanzarella, C., Solinas, I., Padula, C., Mosiello, L. Mycotoxin Research, 25 (4), pp. 193-200. MYCOTOXIN RESEARCH ISSN: 01787888 DOI: 10.1007/s12550-009-0028-9

A Novel Based Protein Microarray for the Simultaneous Analysis of Activated Caspases (2010) Lamberti I, Mosiello L, Cenciarelli C, Antoccia A. and Tanzarella C. SENSORS AND MICROSYSTEMS, Lecture Notes in Electrical Engineering, 54 (4), pp. 323-326, DOI: 10.1007/978-90-481-3606-3_64

ISSN 0178-7888, Volume 25, Number 4



Mycotox Res (2009) 25:193–200
DOI 10.1007/s12550-009-0028-9

ORIGINAL PAPER

An antibody-based microarray assay for the simultaneous detection of aflatoxin B_1 and fumonisin B_1

Ilaria Lamberti · Caterina Tanzarella · Isabella Solinas · Cristiano Padula · Lucia Morello

Received: 4 September 2009 / Revised: 15 October 2009 / Accepted: 15 October 2009 / Published online: 10 November 2009

Abstract Advances in microsystem technology have enabled protein and nucleic acid-based microarrays to be used in various applications, including the study of diseases, drug discovery, genetic screening, and clinical and food diagnostics. Analytical methods for the detection of mycotoxins, however, remain largely based on thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), or enzyme-linked Immunosorbent assay (ELISA) . The aim of our work, therefore, was to transfer an immunological assay from microtitre plates into microarray format, in order to develop a multiparametric, rapid, sensitive and inexpensive method for the detection of mycotoxins for use in food safety applications. Microarray technology enables the fast and parallel analysis of a multitude of biologically relevant parameters. Not only nucleic acid-based tests but also peptide, antigen, and antibody assays, using different formats of microarrays, have evolved within the last decade. Antibody-based microarrays provide a powerful tool that can be used to generate rapid and detailed expression profiles of a defined set of analytes in complex samples and are potentially useful for generating rapid immunological assays of food contaminants. In this paper, we report a feasibility study of the application of antibody microarrays for the simultaneous (or independent) detection of two common mycotoxins, Aflatoxin B_1 and Fumonisin B_1. We present the development of microarray detection of aflatoxin B1 and fumonisin B1 in standard solutions with detection limits of 3 ng/ml of AFB1 and 43 ng/ml for FB1, and have developed a competitive immunoassay in microarray format for simultaneous analyses. The quality of the microarray data is comparable to data generated by microplate-based immunoassay (ELISA), but further investigations are needed in order to characterise our method more fully. We hope that these preliminary results might suggest that further research is warranted in order to develop hapten microarrays for the immunochemical simultaneous analysis of mycotoxins, as well as for other small molecules (e.g. bacterial toxins or biological warfare agents).

Keywords Mycotoxin · Microarray · Aflatoxin B_1 · Fumonisin B_1

I. Lamberti · L. Morello (✉)
ENEA, Italian National Agency for New Technologies, Energy and the Environment,
Via Anguillarese 301,
00060 Roma, Italy
e-mail: Lucia.morello@enea.it

I. Lamberti
e-mail: Ilaria.lamberti@enea.it

I. Solinas · C. Padula
Life Line Lab,
Via Castagnetta 7, Pomezia,
00040 Roma, Italy

C. Tanzarella
Department of Biology, Roma Tre University,
Vle G. Marconi 446,
00146 Roma, Italy

Introduction

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced mainly by three anamorphic species of the genus *Aspergillus*: *A. flavus*, *A. parasiticus* and *A. nomius* (Hurlich et al. 2003). They are the most potent, naturally occurring carcinogens known and have been linked to liver cancer and several other diseases in animals and humans (Otim et al. 2005; Turner et al. 2003; Valdivia

et al. 2008). Fumonisins are mycotoxins produced by *Fusarium moniliforme* or *verticillioides*, a prevalent fungus in the environment that infects corn and other cereal grains. Fumonisin B_1 (FB_1) is the most abundant mycotoxin produced by *F. verticillioides*, suggesting it may have toxicological significance. Ingestion of mouldy corn, infected by *F. verticillioides* or by closely related fungi, is linked to a higher incidence of primary liver cancer (Ueno et al. 1997) and oesophageal cancer in regions of South Africa and China (Marasas and Press 1982). Enzyme-linked immunosorbent assay (ELISA) and fluorescence immunoassay (FIA) are excellent analytical survey tools because of their ease of use and portability in the field, and because they can additionally be used in a high-throughput setting. These important characteristics make immunoassays attractive tools for, e.g., food testing to ensure food safety by regulatory agencies. An immunoassay is traditionally performed as an individual test; in many cases, however, a panel of tests is needed for each sample (e.g. in the detection of drug residues). To meet this need, microarray-based immunoassay technologies have been developed for multianalyte analysis as well as for classic immunoassay (multi-sample) analysis. In recent years, antibody microarray technology has made significant progress: from proof-of-concept design to establishing high-performance platforms capable of targeting non-fractionated complex samples, such as those arising from proteomic studies (Blohm and Guiseppi-Elie 2001). Microarrays consist of immobilised biomolecules located at specific spatial addresses on planar surfaces, microchannels or microwells, or as an array of beads immobilised with different biomolecules. Biomolecules commonly immobilised on microarrays include oligonucleotides, polymerase chain reaction (PCR) products, proteins, lipids, peptides and carbohydrates. Ideally, the immobilised biomolecules must retain activity, remain stable, and remain adsorbed to the surface during reaction and washing steps. The immobilisation procedure itself must ensure that the biomolecules are immobilised at optimal density to the microarray surface for efficient binding (Venkatasubbarao 2004). Some microarray applications are focused on the development of this technology from being a purely research method to becoming an analytical tool, applicable in the clinic as well as in other areas affecting human health, such as environmental testing and food safety (Koppal 2004).

In recent years, interest in rapid membrane-based immunoassay methods, such as flow-through immunoassays and lateral flow devices (LFDs), has strongly increased due to the need for rapid on-site (pre)-screening. A flow-through enzyme immunoassay was developed for the detection of ochratoxin A in roasted coffee (Sibanda et al. 2002). Requiring no sample preparation other than an extraction step, LFDs allow qualitative or semi-quantitative determination of mycotoxins in one-step strip tests within a few minutes. Such LFDs have been developed for selected mycotoxins, such as aflatoxin B1 (Delmulle et al. 2005) and fumonisin B1 (Wang et al. 2006). The strong interest is furthermore reflected in the increasing number of commercially available test kits for field use, based mostly on direct competitive assays (Krska and Janotta 2004; Janotta and Krska 2005; Krska et al. 2008;). In the past, array biosensors for detection of toxins have been developed (Ligler et al. 2003), and in particular an indirect competitive immunoassay using an array biosensor, offering rapid, sensitive detection and quantification of AFB1 in buffer, corn and nut products (Sapsford et al. 2006). With this in mind, we set out to transfer the immunoassay method from microtitre plates to a microarray format in order to develop a multiparametric, rapid, sensitive and inexpensive method for the detection of mycotoxins for use in food safety applications. To further develop and validate our test in a microarray format, we used the aflatoxin B1 (AFB1) and FB1 and developed a competitive immunoassay in microarray format.

Materials and methods

Chemicals and buffers

Anti-FB_1 polyclonal antibody produced in rabbit (catalogue number IPS0006-AB), FB_1-BSA conjugate (catalogue number IPS0006-C) and standard mycotoxin solutions of AFB_1 and FB_1 (within ppt or ppb range) were purchased from Geneaon (Modena, Italy). Polymer substrate, enzymatic and colourimetric reagents for developing the protein microarray, were provided by LifeLineLab (Pomezia, Italy). Anti-AFB_1 polyclonal antibody (produced in rabbit), AFB_1-BSA conjugate from *Aspergillus flavus*, anti-rabbit-IgG-biotin conjugate and all other chemicals were GR grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin-alkaline phosphatase (streptavidin-AP) conjugate (cat # CJ0100) and the AP substrate, nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP; catalogue number BUF0200) were purchased from LifeLineLab S.r.l. (Pomezia, Italy).

The solutions used for the antibody-based microarray development were as follows:

Printing Buffer 100 mM Na2HPO4.2H2O, 300 mM NaCl, 0.1% (w/v) Triton100, pH 7,2

Blocking Buffer 50 mM Na2HPO4.2H2O, 2% (w/v) BSA (Bovine Serum Albumine) 0.09% (w/v) sodium azide, pH 7.2

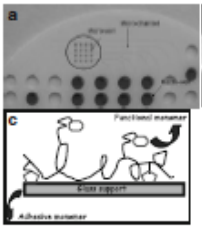


Fig. 1 a Plastic probe tray for protein spotting, b pin details, and c scheme of the glass treated with copolymer (N,Ndimethylacrylamide (DMA), N,N-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)-propyl] trimethoxysilyl (MAPS) used in protein spotting. MAPS represents functional linker to protein

Washing Buffer 500 mM Tris HCl, 2.5 M NaCl, 0.5% (v/v) Tween 20, pH 9.0

Binding Buffer 100 mM Tris HCl, 100 mM NaCl, 0.09% (w/v) sodium azide, 0.02% (w/v) Tween 20, 10 mM MgCl2, 100 mM ZnCl2, pH 7.2

Detection Buffer 100 mM Tris-HCl, 100 mM NaCl, pH 9.5 at 20°C

Microarray platform

The microarray platform used was DR. Fast Spot (DR. Chip Biotech, Chu-Nan, Taiwan) and was kindly donated by LifeLineLab. This spotting machine component produced a maximum spot density of 64 spots per 8×8 matrix yielding a spot diameter of 500–700 μm with 1,000 μm of distance between spots. For the microarray reactions, a high performance mini-incubator was used (DR. Mini Oven, DR. Chip Biotech) which included accurate temperature control and a vibration function to enable a linear rocking movement. For microarray reading, the optical scanner, DR. Aim (DR. Chip Biotech) was used. This had an optical resolution of 300–600 dpi, and was equipped with image and data analysis software so that the optical density of each spot could be measured and analysed.

Preparation of protein chips

Protein microarray plates or chips were prepared using the antigen conjugated AFB1-BSA and a plastic probe tray, fitted with spotting pins (Fig. 1) and mounted on the spotting device. In order to produce an antigen-microarray for the immunoassay (Fig. 2), different concentrations of AFB1-BSA, in a nano-volume range, were spotted onto a glass slide coated with a copolymer of N,N-dimethylacrylamide, N,N-acryloyloxysuccinimide and [3-(methacryloyl-oxy)-propyl] trimethoxysilyl (DMA-NAS-MAPS), according to the method described previously (Cretich et al. 2004). A BSA-alone microarray plate was produced in the same manner as a negative control. The polymeric surface, DMA-NAS-MAPS, has been demonstrated to be suitable as a covalent and stable coating for the slides for use with biological molecules in microarray experiments. Proteins immobilised on this surface have been shown to maintain an active conformation and are easily accessible by the test sample. It was

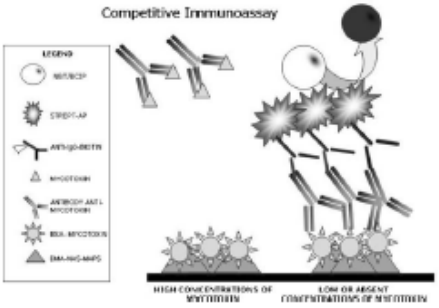


Fig. 2 Competitive immunoassay carried out on microarray for mycotoxins

previously demonstrated for this method that the ligands immobilised on the polymeric surface maintain an active conformation and are easily accessible, providing a detection limit of 54 amol/spot (Chiari, et al. 2005; Cretich et al. 2004).

Competitive binding assay for AFB_1

After spotting, the plates were blocked by washing with Blocking Buffer twice and finally incubated with the same buffer for 1 h at room temperature. After spotting, the plates

Table 1 Scheme of microarray antibody-based for mycotoxins

SUMMARY OF ANTIBODY MICROARRAY OF MYCOTOXIN	
STEP 1: PROTEIN SPOTTING AND BLOCKING	MYCOTOXIN-BSA DILUTED IN PRINTING BUFFER ⇩ SPOT MYCOTOXIN-BSA ON CHIP ⇩ INCUBATE CHIP AT FOR 2 HRS 37°C ⇩ BLOCKING BUFFER WITH BSA 0.5% ⇩ DRY CHIP FOR 30' AT 37°C ⇩ THE CHIP IS READY TO USE
STEP 2: ASSAY PROCEDURE	PRE-INCUBATION: ANTI- MYCOTOXIN + MYCOTOXIN FREE ⇩ BINDING TO CHIP PROTEIN ⇩ WASH STEP ⇩ ADDING ANTI IgG BIOTIN CONJUGATE ⇩ WASH STEP ⇩ STREP AP BINDING ⇩ WASH STEP ⇩ ADDING AP SUBSTRATE ⇩ COLORIMETRIC DEVELOPMENT AND RESULT ANALYSIS

Table 2 Samples containing mycotoxins applied on microarray for simultaneous detection

Solution A	Anti-AFB1	+	0 ppb di AFB1 (Binding Buffer)
Solution B	Anti-AFB1	+	50 ppb AFB1 free antigen
Solution C	Anti-FB1	+	0 ppb FB1 (Binding Buffer)
Solution D	Anti-FB1	+	50 ppb FB1 free antigen

were blocked by washing with Blocking Buffer. The antigen microarray plates and the control plates were incubated with solution of 50 µL of polyclonal antibody anti-AFB1 diluted at 1 µg/mL in Binding Buffer pre-mixed with the free antigen standard solutions of AFB1 at different concentrations ranging from 0 to 260 ng/mL in H_2O. Plates were incubated for 1 h at 37°C. The extent of antigen-antibody binding on the microarray plates was assayed by adding a secondary biotinylated antibody, 100 µL anti-rabbit-Fc specific (Sigma-Aldrich), diluted in binding buffer as described in the Sigma data sheet. After incubation, the plates were washed three times using washing buffer and incubated with 100 µL streptavidine-AP (1 mg/mL) diluted in binding buffer 1:10,000. Streptavidin-AP reacts with the chromogenic substrate NBT/BCIP, which is usually used for the sensitive detection of AP in immunoblotting and immunohistochemical assays. In our method, the antigen-antibody reaction gives rise to a dark blue colour in 5 min.

A flow chart for our method is described in Table 1.

Competitive binding assay for FB_1

The assay for FB_1 was performed following the same procedure as previously described for AFB_1, but using polyclonal antibody Anti-FB_1, premixed with the FB_1 standard solution at concentrations ranging from 0 to 300 ng/mL. Simultaneous binding assay for AFB_1 and FB_1. In order to demonstrate the applicability of the DR. Chip microarray platform for the simultaneous detection of the mycotoxins AFB1 and FB1, we performed the following experiment. AFB1-BSA and FB1-BSA conjugates were spotted at different concentration (ranging between 100 and 400 µg/mL) at different positions onto wells containing a glass slide coated with DMA-NAS-MAPS. The wells were blocked and then incubated for 30 min at room temperature using the following combinations of the mycotoxin solutions A–D, as shown in Table 2: (1) 50 µL solution A + 50 µL solution C, (2) 50 µL solution A + 50 µL solution D, (3) 50 µL solution B + 50 µL solution C, and (4) 50 µL solution B + 50 µL solution D. Antigen binding was assayed as previously described. BSA and anti-Rabbit IgG biotin conjugate were used as negative and positive controls respectively.

Data analysis using the microarray reader

The intensity of the dark blue spots on the microarray plates was determined using the microarray reader of the DR. Chip platform (DR. AiM Reader, DR. Chip Biotech). DR. ChiP platform incorporates a dedicate software (DR. Aim Soft, DR.Biotech) showing control of the scan conditions and scan area, reducing scan time (3 min/plate for 600 dpi; <3 min/plate for 300 dpi). This software automatically aligns feature-indicators with features; automatically associates individual microarray features with samples names and identities, automatically calculates and subtracts local

Fig. 3 AFB1 standard curve

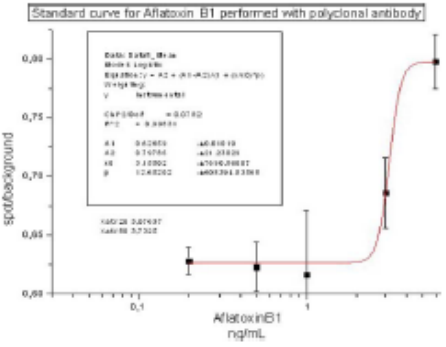
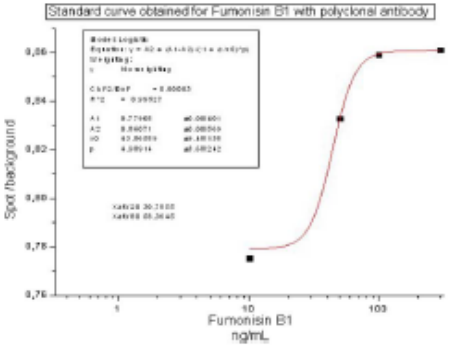


Fig. 4 FB_1 standard curve



background effects and finally presents the data in a spreadsheet for rapid sorting.

Relative gray-level statistics

The mean and standard deviation of the relative (i.e. 'difference') grey-level values of the positive spots and the negative spots, usually prove that they are more separable than that of absolute ones. In the program is also included an intelligent way of calculating the optimal 'relative' threshold on-the-fly (i.e. on a per image basis) automatically. Grey-scale relative values were collected for each spot and results were plotted in terms of ratio the "grey-scale value for each spot/grey-scale value for background". For background, we mean intensity of grey around each spot. Using this software, we read microarray spots, obtaining our densitometric results which were plotted for standard curves (SC) construction.

Results

Optimisation of conditions for microarray spotting and competitive binding assay

Different concentrations of the BSA-conjugated mycotoxins (AFB_1-BSA and FB_1-BSA) were tested in order to identify the best conditions for spotting the microarray plates. A concentration of the conjugated antigen at 100 μg/mL for AFB_1-BSA and 335 μg/mL for FB_1-BSA produced the best results. These concentrations were used for all subsequent competitive assays. Similarly, various dilutions of the polyclonal antibodies (anti-AFB_1 and anti-FB_1) were tested. Minimum antibody titres of 1:4,000 (from a 1 mg/mL solution) for anti-AFB_1, and 1:1000 (from a 1 mg/mL solution) for anti-FB_1, were established as suitable for the competitive immunoassay. These concentrations were used for the simultaneous binding assay.

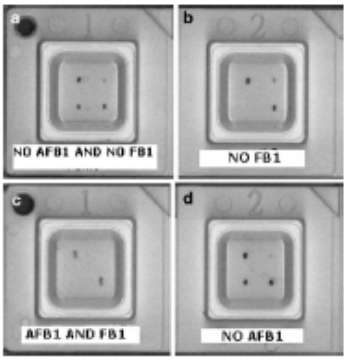


Fig. 5 Patterns for simultaneous AFB1 and FB1 microarray assay: concentrations of detectable of micotoxin ≥25 ppb. a Absence of AFB1 and FB1, b absence of FB1 and presence of AFB_1, c presence of AFB1 and FB_1, d absence of AFB1 and presence of FB1

Immunoassay sensitivity

Data collected from the competitive antibody microarray experiment, shown as relative grey scale intensities, was plotted in order to construct the SC for AFB_1 (Fig. 3). and with Origin® 7.0 software (OriginLab, Northampton, MA, USA). Spots obtained at different AFB_1 standard concentrations are show in the figure. SCs for the competitive assay were constructed using a 4-parametric fit. For the competitive assay, the dose detection limit was defined as the concentration of analyte that gives rise to 20% inhibition (IC20). The upper limit of the working range (at high concentrations of analyte) was defined as an inhibition of 80% (IC80), and the IC50 (corresponding to Xo values, reported in Figs. 3 and 4) value represents the concentration of analyte that results in a 50% decrease in the maximal corrected assay signal. From the SCs, detection limits of 3 ng/mL for AFB_1 and 43 ng/mL for FB_1 were obtained (Figs. 3 and 4 respectively). The optical density values obtained applying immunoassay in a microarray format previously described for FB_1 were also fitted by logistic function and the SC was obtained, as reported in Fig. 4. Detection limit for FB_1 obtained in our experiments corresponds to 43 ng/mL.

Microarray patterns arising from the immunoassay experiments

Standard solutions containing AFB_1 or FB_1, or both mycotoxins, were tested using the immunoassay microarray method described here. The resulting pattern of spots from the protein chip, corresponding to the presence or absence of mycotoxins in the samples, is shown in Fig. 5.

Discussion and conclusion

As in conventional competitive immunoassay experiments, the colour intensity (here measured as grey-scale values) obtained from the antigen-antibody microarray experiments described here are in inverse proportion to the antigen concentration of the standard solutions. For AFB1, the standard curve we reported in Fig. 3 the preliminary data obtained with microarray, while for FB1, we obtained standard deviations that were too large and consequently we decided not to report the values in Fig. 4. For this reason, our microarray method actually needs further improvements and standardisations. Our experiments were carried out using a microarray with a spots densitometric detection approach, with a poor discrimination between different experimental points, so for our next experiments we intend to add fluorescence. In any case, we would suggest that for the best fitting of these kinds of methods that the 4-parametric logistic function usually applied on most competitive immunoassays should be used. To our mind, it represents a useful preliminary insight in order to arrange further experiments for developing a rapid test or kits in an antibody-based microarray format for simultaneous analysis of mycotoxins. Our experiments show dose detection limits of 3 ng/mL for AFB_1 and 43 ng/mL for FB_1. This means that mycotoxin samples at higher concentrations would be detected by this method. For the simultaneous binding assay, we used standard mycotoxin solutions at concentrations in excess of these detection limits. When the combined total concentration of mycotoxin exceeded 50 ng/ml, we obtained clearly distinguishable microarray patterns. This concentration limit represents a critical cut-off value as at concentrations below this the microarray spots were not visible. For this reason, our method could be used as a semi-quantitative tool for rapid pre-screening for mycotoxins in potentially contaminated samples. Positive samples could subsequently be analysed by other conventional methods. Further investigation is needed in order to improve the reported microarray performance mainly in terms of sensitivity, if actually too high for the EU regulation, or in order to increase the range of mycotoxins that can be assayed. In any case, our method shows great potential for rapid simultaneous analysis. In order to do this, monoclonal and polyclonal antibodies are required for other mycotoxins, such as Ochratoxin A and Deoxyvalenol. Further investigation into the use of other microarray spotting methods is also needed, such as laser-assisted deposition. This method has been reported to be capable of forming microarrays of functionalised biological molecules with pattern definition on a nanometre scale (Gigliardi et al. 2007).

Acknowledgement This work was supported by the national project Me.di.T.A. funded for Applied Research by the Italian Ministry of Research. Ilaria Lamberti is a PhD student at Department of Biology, Roma Tre University.

References

Bioharn DH, Guiseppi-Elie A (2001) New developments in microarray technology. Curr Opin Biotechnol 12:41–47

Chiari M, Crotich M, Corti A, Damin F, Pirri G, Longhi R (2005) Peptide microarrays for the characterization of antigenic regions of human chromogranin. Proteomics 5:3600–3603

Crotich M, Pirri G, Damin F, Solinas I, Chiari M (2004) A new polymeric coating for protein microarrays. Anal Biochem 332:67–74

Delmulle BS, De Saeger SMDG, Sibanda L, Barna-Vetro I, Van Peteghem CH (2005) Development of an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. J Agric Food Chem 53:3364–3368

Ehrlich KC, Montalbano BG, Cotty PJ (2003) Sequence comparison of aflR from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. Fungal Genet Biol 38:63–74

Gagliardi S, Rapone B, Montillo L, Luciani D, Gerardino A, Morales P (2007) Laser- assisted fabrication of biomolecular sensing microarrays. IEEE Transactions on Nanobioscience 6:242–248

Janotta M, Krska R (2005) Entwicklung von Mykotoxin Screening Methoden mittels Protein Microarrays. Arbeitsgem Lebensmittel Veterinar Agrarwissen 2:42–45

Koppal T (2004) Microarrays: migrating from discovery to diagnostics. Drug Discov Devel 7:30–34

Krska R, Janotta M (2004) Technology and applications of protein microarrays. Anal Bioanal Chem 379:338–340

Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C (2008) Mycotoxin analysis: an update food additives and contaminants 25:152–163

Lipke PS, Taitt CR, Shriver-Lake LC, Sapsford KE, Shubin Y, Golden JP (2003) Array biosensor for detection of toxins. Anal Bioanal Chem 377:469–77

Marasas WFO (1982) Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. In: Cancer of the esophagus, vol I. CRC, Boca Raton, FL, pp 29–40

Otim MO, Mukiibi-Muka G, Christensen H, Bisgaard M (2005) Aflatoxicosis, infectious bursal disease and immune response to Newcastle disease vaccination in rural chickens. Avian Pathol 34:319–323

Sapsford KE, Taitt CR, Fertig S, Moore MH, Lassman ME, Maragos CM, Shriver-Lake LC (2006) Indirect competitive immunoassay for detection of aflatoxin B1 in corn and nut products using the array biosensor. Biosens Bioelectron 21 2:298–230

Sibanda L, De Saeger S, Barna-Vetro I, Van Peteghem C (2002) Development of a solid-phase cleanup and portable rapid flow-through enzyme immunoassay for the detection of ochratoxin A in roasted coffee. J Agric Food Chem 50:6964–967

Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP (2003) Modification of immune function through exposure to dietary aflatoxin in Gambian children. Environ Health Perspect 111:217–220

Ueno Y, Iijima K, Wang S-D, Sugiura Y, Sakijima M, Tanaka T, Chen C, Yu S-Z (1997) Fumonisin as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China by HPLC and ELISA. Food Chem Toxicol 35:1143–1150

Valdivia AG, Martinez A, Darnian FJ, Quezada T, Ortiz R, Maryinez C, Llamas J, Rodriguez ML, Yamamoto L, Jaramillo F, Loarca-Pina MG, Reyes JL (2001) Efficacy of N-acetylcysteine to reduce the effects of aflatoxin B1 intoxication in broiler chickens. Poult Sci 80:727–734

Venkatasubbarao S (2004) Microarrays - status and prospects. Trends Biotech 22:630–637

Wang S, Quan Y, Lee N, Kennedy IR (2006) Rapid determination of fumonisin B1 in food samples by enzyme-linked immunosorbent assay and colloidal gold immunoassay. J Agric Food Chem 54:2491–2495

A NOVEL BASED PROTEIN MICROARRAY FOR THE SIMULTANEOUS ANALYSIS OF ACTIVATED CASPASES

I. LAMBERTI, L. MOSIELLO

ENEA CR Casaccia, Dipartimento Biotecnologie e Protezione della Salute, Via anguillarese, 301, 00123 Roma

C.CENCIARELLI, A.ANTOCCIA AND C. TANZARELLA

Dipartimento di Biologia, Università "Roma Tre", V.le G. Marconi 446,00146 Roma

The aim of our work is to develop a new method to study caspases expression in apoptotic cells using an antibody-based protein microarray. Actually we performed the array for two different form of caspase-3 (cleaved and uncleaved), in the next future we plan to enlarge the microarray analysis using caspase-8 and caspase-9 antibodies in order to obtain a new analyticaltool to study the expression of these markers in apoptotic cells

1. Introduction

The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or cellular extracts. It uses gel electrophoresis to separate native or denatured proteins, then transferred to a nitrocellulose or PVDF membrane, and detected using antibodies specific to the target protein.

In recent years, the antibody microarray technology has made significant progress, going from proof-of-concept designs to established high-performing technology platforms capable of targeting non-fractionated complex samples, as proteoma. Antibody microarrays are an easy-to-use, cost-effective tool for high-throughput protein profiling using cell extracts, tissue lysates, and other biological samples. Apoptosis is the process of cell auto-destruction often called 'programmed cell death'. Apoptosis is a highly regulated process characterized by morphological and biochemical cellular changes, as the activation of specific cytolic proteases, called caspases. The aim of our work is to develop a new method to study caspases expression in cells induced to apoptosis using an antibody based protein microarray. According to this aim, we study the feasibility to transfer the methods of the immunological assay for caspases from classical western blot into a microarray format in order to develop a multiparametric, rapid, sensitive and inexpensive assay.

To check the feasibility of this format, we focused our studies on the most popular caspase (caspase-3, cleaved and un-cleaved forms) .

2. Experimental

2.1 Protein Chip platform

Our activities in microarray field was mainly devoted to develop protein microarray using the pulsed laser assisted deposition, PLD [4].
In this paper to perform microarray determination we used a commercial protein chip platform composed of a spotting machine (producing a maximum spot density of 64 spot for a 8x8 matrix and showing a spot diameter between 500-700 μm and a spot distance of 1 mm); for microarray reactions we used a high performance mini oven with accurate temperature control and vibration function with linear rocking movement. Finally for microarray reading we uses an optical scanner, with a 300-600 dpi resolution, equipped with image and data analysis software able to collected the results in terms of optical density for each spot. The protein chip platform is shown in Figure 1.

Figure 1. Protein chip platform

2.2 Apoptosis induction

To validate the protein chip, H460 lung tumor cells were induced to apoptosis by treating them for 4 hrs with 2μM staurosporine and performing Western blot with antibodies for activated caspase 3. In fact, the cleavage of inactive procaspase to active cleaved caspase is a recognized marker of apoptosis [5].

2.1. *Cell lysis and Immunoblot*

Western blot analysis (as control of microarrays) was carried out as described in Cenciarelli et al. [1] loading 35μg of whole cell lysate. Briefly, blotted filters were incubated with 1 μg/ml of specific antibodies recognizing caspase3-cleaved (Cell Signaling, MA, USA) and α-tubulin as loading control. Primary antibodies were detected using secondary anti-mouse antibody HRP conjugates and visualized using the enhanced chemioluminescence detection system.

2.4 Protein spotting and microarraying

In our method protein chip were prepared spotting crude and denaturated proteic lysate from cell H460, treated with staurosporine.The lysate was spotted at different concentrations onto a well containing a glass slide coated to copolymer of N,N-dimethylacrylamide (DMA), N,N-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)-propyl] trimethoxysilyl (MAPS) and in the same way proteins obtained from untreated cells were spotted, as negative control.
This polymeric surface is demonstrated to be suitable for covalent and stable coating of biological molecules and for protein microarray technology. The proteins immobilized on this polymeric surface maintain an active conformation and are easily accessible, providing a detection limit of 54 amol/spot.

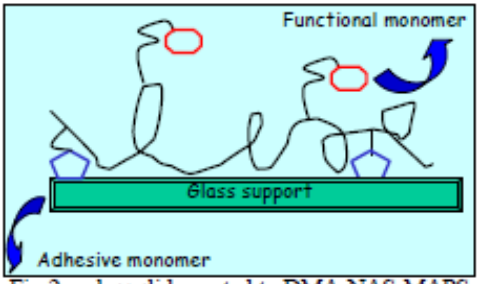


Fig 2 – glass slide coated to DMA-NAS-MAPS

2.5 Caspase-3 assay

After spotting and blocking with a solution of 2% (w/v) BSA in 0,05 M Na2HPO4.2H2O, the microarrays was incubated with a solution of a murine monoclonal antibodies anti-caspase-3 (cleaved and uncleaved forms) for 1 hr at 37°C. The antigen-antibody binding on the spotted microarrays was revealed adding a secondary biotinylated antibody anti-mouse-Fc specific and via the enzimatic reaction with a streptavidine-Alkaline-Phosphatase (AP) which reacts with a chromogenic substrate (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate toluidine salt, NBT/BCIP). This solution is usually used for the sensitive detection of alkaline phosphatase in immunoblotting and immunohistochemical assays and, in our method, gives a dark-blue color on the spot on which the reaction antigen-antibody is occurred. The scheme of glass slide copolymer treated, also provided by Life Line Lab (Pomezia, Italy) is reported in fig. 2.

2.6 Scan and data analysis

The dark color on the microarrays was finally determinated using the microarray reader and a grey scale relative values were collected for each spot. Because the

results are shown and plotted in term of ratio between "grey value for each spot/ grey value for background", the ratio decreased for spots more dark.

3. Results

In our results we observed a microarray dark spot only in sample derived from apoptotic cells and demonstrated the feasibility of microarray technology in order to characterize crude cell protein lysate for these or others tumor protein markers, comparable to the classical western blot analysis. In the Figure 3 are shown the microarray results for caspase- 3 (cleaved and uncleaved form) for the same samples spotted (crude proteins from apoptotic cells and from control cell culture). We plan in the next to enlarge our microarray analysis on others markers for apoptosis, as caspase-8 and -9 and to improve the presented method by application of a fluorescent reader for spot detection.

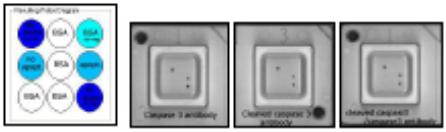


Fig.3 – microarray results for caspase 3 (cleaved and un-cleaved form)

4. Conclusions

We demonstrated that microarray platform Dr.CHIP, used in our experiments would be used in order to develop a microarray antibody for apoptosis markers, but it seem necessary to use the fluorescence as detection method. In our opinion the application of a fluorescent reader for spot detection, as a CCD camera, it would lead possible to enlarge microarray analysis on others markers for apopotosis, as caspase-8 and caspase-9 in order to obtain a multiparametric microarray assay and eliminate the need for multiple immunoprecipitation/ Western blot experiments.

Acknowledgments

We are particularly grateful to dr. I.Solinas and dr. C. Padula for assistance on analytic equipment (Dr. Chip platform) provided by Life Line Laboratory (Pomezia, Italy)

References

[1] Cenciarelli C, Tanzarella C, Vitale I, Pisano C, Crateri P, Meschini S, Arancia G, Antoccia A. (2008). Apoptosis. 13(5):659-69

[2] Chiari, M., Cretich, M., Corti, A., Damin, F., Pirri, G., Longhi, R. Peptide microarrays for the characterization of antigenic regions of human chromogranin A (2005) Proteomics, 5 (14), 3600-3603

[3] Cretich, M., Pirri, G., Damin, F., Solinas, I., Chiari, M. A new polymeric coating for protein microarrays (2004) Analytical Biochemistry, 332 (1), 67-74
[4] Gagliardi, S., Rapone, B., Mosiello, L., Luciani, D., Gerardino, A., Morales, P. (2007) IEEE Transactions on Nanobioscience, 6 (3), pp. 242-248
[5] Nuñez G, Benedict MA, Hu Y, Inohara N. (1998). Oncogene. 17(25):3237-45

Aim 2: Quartz Crystal Sensor

The aim of this work was to analyse the binding of human thrombin to the DNA aptamers that differ in structure of binding site. As a basic we used aptamer that selectively bind thrombin in its fibrinogen-binding site. A pair of thymines on the G- quandruplex of the aptamer characterize thrombin binding site. We analysed the aptamers in which AA replaced TT pair, (AA, AT or TA aptamers, respectively). The aptamers were purchased from Thermo Scientific (Ulm, Germany). We used acoustic method based on quartz crystal microbalance (QCM) and thickness shear mode (TSM) for monitoring the aptamer-thrombin interaction. We showed that substitution thymines by adenines in the thrombin-binding site of G-quadruplex substantially affect both thermodynamic and binding properties of the DNA aptamers immobilised on TSM transducer.

In a second application we used a DNA aptamer sensitive to ochratoxin A. The aptamers were immobilised at the gold layer of quartz crystal transducer.. We showed that TSM allowing detecting this mycotoxin with LOD 30 nM and with good selectivity. He also studied the stability of DNA aptamers depending on concentration of calcium ions, that are important for binding OTA to DNA aptamer.

A Piezoelectric Quartz Crystal Sensor Applied for Thrombin-Binding Aptamers (2011) Rakitka J., Hianik t. Lamberti I., Mosiello L., SENSORS AND MICROSYSTEMS 91 (5): 449-453, ISBN: 9789400713239, DOI: 10.1007/978-94-007-1324-6_73

Development of a Thickness Shear Mode Biosensor based on DNA Aptamers for detection Ochratoxin A (2011) Lamberti I Mosiello L Hianik T Chemical Sensors 1, 2011:11 ISSN 2231-6035

Chapter 73
A Piezoelectric Quartz Crystal Sensor Applied for Thrombin-Binding Aptamers

Ilaria Lamberti, Jan Rakitka, Tibor Hianik and Lucia Mosiello

Abstract The temperature-dependent UV spectroscopy and thickness shear mode acoustic method were applied to study of thermodynamics and binding properties of DNA aptamers sensitive to thrombin depending on the substitution of bases in G-quadruplex. The substitution of thymidines by adenines in TT and TGT loops of G-quadruplex resulted in destabilization of aptamers and in decrease of sensitivity to human thrombin.

73.1 Introduction

The DNA/RNA aptamers are single stranded in vitro selected oligonucleotides (typically 30–60 bases) that in certain conditions (ionic strength, pH) form in a solution three-dimensional structure with specific binding site to low or macromolecular ligands [1, 2]. The affinity of aptamers is similar or even higher than those of antibodies. However, the aptamers are more stable than antibodies and do not induce immune response. They can be chemically modified by various compounds, which increase their stability and allowing immobilisation at a surface. Increased interest in study of aptamers is connected with their high potential in cancer therapy, for the treatment of age related macular degeneration and for

I. Lamberti · J. Rakitka · T. Hianik (✉)
Department of Nuclear Physics and Biophysics, Comenius University, Mlynska dolina F1, 84248 Bratislava, Slovakia
e-mail: tibor.hianik@fmph.uniba.sk

I. Lamberti · L. Mosiello
ENEA, Italian National Agency for New Technologies, Energy and the Environment, Via Anguillarese 301, 00060 Rome, Italy

G. Neri et al. (eds.), *Sensors and Microsystems*,
Lecture Notes in Electrical Engineering 91, DOI: 10.1007/978-94-007-1324-6_73,

targeted drug delivery [2, 3]. The common binding motif of some aptamers including those of thrombin is guanine quadruplex (G-quadruplex) [4]. It is formed by guanine bases stabilized by Hoogsten hydrogen bonds [5]. The resulted planar G-quartets can be connected by intermediate sequences that form loops. The loops play key role in the aptamers stability and in their binding to the ligands. Among DNA aptamers those sensitive to fibrinogen binding site of thrombin is one of most studied [4]. According to NMR data [6], the eight guanine residues form G-tetrads that are connected at one end by two TT loops and at the other end by one TGT loop. This aptamer has been originally developed as inhibitor of coagulation effect of thrombin. However, later it has been used also for development various thrombin biosensors [7]. Among biosensors, those based on surface acoustic method are rather promising due to their sensitivity to surface modification. Recently it has been shown, that substitution of nucleotides in TT loop by U and substitution of G in TGT loops by A, C or T increased the stability of G-quadruplex [8]. We have studied how the substitution of thymidines by adenines in TT and TGT loops of G-quadruplex responsible for thrombin binding affects the thermodynamics and binding properties of the aptamers.

73.2 Materials and Methods

The HPLC purified DNA aptamers were purchased from Thermo Fisher Scientific (Ulm, Germany): TT: 5' GGT TGG TGT GGT TGG T_{15} 3'; AT: 5' GGT TGG TGT GG**A** TGG T_{15} 3'; AA: 5'GGT TGG TGT GG**A** **A**GG T_{15} 3'; AGA: 5' GGT TGG **A**G**A** GGT TGG T_{15} 3'. All aptamers were extended by 15-mer thymidine chains modified at 3' end by biotin, which was necessary for biosensor preparation. The TT is conventional aptamer sensitive to fibrinogen binding site at thrombin. Other aptamers: AT, AA and AGA differ from TT aptamer by substitution of thymidines by adenines in the position marked by underlined bold. The thermodynamic properties of aptamers have been studied by UV absorption spectroscopy. The melting temperature at wavelength 297 nm has been determined using thermoelectrically controlled UV–VIS spetrometer Shimadzu 1700 (Japan) with a scan rate 0.5°C/min. The melting of aptamers at this wavelength is characterized by larger hypochromic effect in comparison with hyperchromic effect at 260 nm [8]. The melting temperature T_m and Van Hoff enthalpy changes, ΔH_{VH}, have been determined by fitting the normalized plot of absorbance versus temperature using Eq. (73.1).

$$A = \frac{A_F + A_U e^{(\Delta H_{vH}/R)*(1/T-1/T_m)}}{1 + e^{(\Delta H_{vH}/R)*(1/T-1/T_m)}} \tag{73.1}$$

where A, A_F and A_U are total, folded and unfolded aptamer normalized absorbance, respectively. R is gas constant and T is absolute temperature. The binding of human thrombin (Sigma–Aldrich, USA) to the aptamers was analyzed by

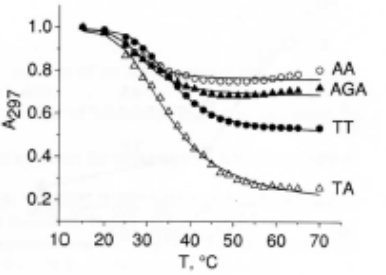


Fig. 73.1 Normalized plot of absorbance at wavelength 297 nm versus temperature for all aptamers studied. The full lines are fits according to Eq. (73.1)

Table 73.1 The thermodynamics parameters of aptamers studied: T_m—melting temperature, ΔH_{VH}—Van Hoff enthalpy changes, $\Delta G^0_{(20)}$—changes of standard Gibbs energy at 20°C, ΔS—entropy change ($\Delta G^0_T = \Delta H_{cal}\ (1-T/T_m) = \Delta H_{VH}\ (1-T/T_m) = \Delta H_{VH} - T\Delta S$, where ΔH_{cal} is calorimetric enthalpy. $\Delta H_{cal}/\Delta H_{VH} = 1$ for two-state transition [8]). Results are mean ± S.D. (n = 3)

Aptamer	T_m (°C)	ΔH_{VH} (kcal/mol)	$\Delta G^0_{(20)}$(kcal/mol)	$T\Delta S$ (kcal/mol)
TT	35.3 ± 0.5	−40.4 ± 0.4	−2.00 ± 0.02	−38.40 ± 0.40
TA	34.2 ± 0.4	−31.6 ± 0.6	−1.46 ± 0.03	−30.14 ± 0.60
AA	26.8 ± 1.0	−45.6 ± 5.9	−1.03 ± 0.13	−44.57 ± 5.90
AGA	29.6 ± 0.8	−40.5 ± 2.8	−1.28 ± 0.09	−39.22 ± 2.80

thickness shear mode (TSM) acoustic method. The aptamers have been immobilised at clean gold surface of TSM transducer (fundamental frequency 8 MHz) by neutravidin-biotin technology [9]. The TSM method is based on determination of series resonant frequency, f_S, and motional resistance, R_m, from impedance spectra of quartz transducer. f_S and R_m reflect energy storage and dissipation, respectively [9, 10]. Thermodynamic and binding experiments were performed in following buffer: 140 mM NaCl + 5 mM KCl + 1 mM $CaCl_2$ + 1 mM $MgCl_2$ + 20 mM Tris–HCl (pH 7.4). TSM experiments were performed at T = 20°C.

73.3 Results and Discussion

The plot of absorbance versus temperature for all aptamers studied is presented in Fig. 73.1. It is seen that the absorbance has sigmoid shape typical for two-state transition. The fit of experimental results using Eq. 73.1 allowing determination of thermodynamics properties of aptamers (Table 73.1). It is seen from Table 73.1, that substitution of nucleotides resulted in decrease of T_m and in less favorable Gibbs energy changes in comparison with TT aptamers. Most remarkable instability took place for AA aptamers. The favorable changes in enthalpy are in this

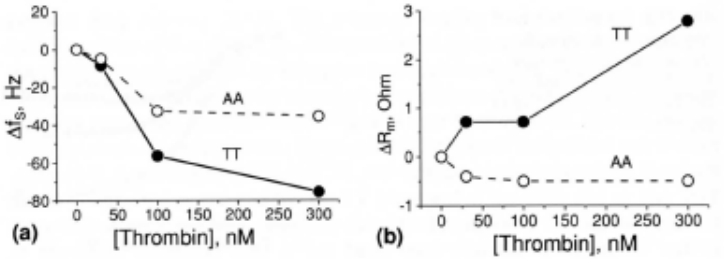


Fig. 73.2 Representative plots of Δf_S (**a**), and ΔR_m (**b**), as a function of thrombin concentration of TSM sensor with immobilised TT or AA aptamers. S.D. did not surpass 15%

case compensated by entropy contribution. The changes in entropy can be due to uptake of ions or water into the G-quadruplex [8]. The lower values of T_m in comparison with those reported in [8] are due to less KCl concentration used, presence of NaCl and due to thymidine spacer.

The interaction of thrombin with aptamers immobilised at TSM transducer correlates with thermodynamic properties of aptamers. Most remarkable differences in the acoustic parameters following addition of thrombin were observed for TT and AA aptamers. The resonant frequency for both aptamers decreased with increasing thrombin concentration. However, for TT aptamers these changes were substantially larger, which suggest higher sensitivity to the thrombin. Substantial differences were observed in changes of motional resistance for these aptamers. While for TT aptamers the motional resistance increased with increasing the thrombin concentration, opposite changes in R_m took place for AA aptamers (Fig. 73.2). This suggests different conformation/surface properties of the thrombin-aptamer complexes for these aptamers and as a result changes in coupling of the surface and liquid-higher coupling for TT (increase in R_m), decoupling for AA (decrease in R_m) [10]. AGA and AT have revealed comparable sensitivity with those of AA in respect of frequency changes, but R_m slightly increased for these aptamers.

73.4 Conclusions

We showed that substitution thymidines by adenines in the thrombin-binding site of G-quadruplex substantially affect both thermodynamic and binding properties of the DNA aptamers immobilised on TSM transducer.

Acknowledgments We thank to Slovak National Scholarship Program and to Agency for Promotion Research and Development (project APVV-0362-07) for financial support.

References

1. Ellington AD, Szostak JW (1990) Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA. Nature 344:467–469
2. Jayasena SD (1999) Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem 45:1628–1650
3. Vorhies JS, Nemunaitis JJ (2007) Nucleic acid aptamers for targeting of shRNA-based cancer therapeutics. Biol: Targets Ther 4:367–376
4. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ (1992) Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 355:564–567
5. Hardin CC, Perry AG, White K (2001) Thermodynamic and kinetic characterization of the dissociation and assembly of quadruplex nucleic acids. Biopolymers 56:147–194
6. Schultze P, Macaya RF, Feigon J (1994) Three-dimensional solution structure of the thrombin-binding DNA aptamer d(GGTTGGTGTGGTTGG). J Mol Biol 235:1532–1547
7. Hianik T, Wang J (2009) Electrochemical aptasensors—recent achievements and perspectives. Electroanalysis 21:1223–1235
8. Ohsen CM, Lee H-T, Marky LA. Unfolding thermodynamics of intramolecular G-quadruplex: base sequence contribution of the loops. J Phys Chem B 113: 2587–2595
9. Hianik T, Grman I, Karpisova I (2009) The effect of DNA aptamer configuration on the sensitivity of detection thrombin at surface by acoustic method. Chem Commun pp 6303–6305
10. Ellis JS, Thompson M (2004) Acoustic coupling at multiple interfaces and the liquid phase response of the thickness shear-mode acoustic wave sensor. Chem Commun pp 1310–1311

Development of thickness shear mode biosensor based on DNA aptamers for detection of ochratoxin A

I. Lamberti[a], L. Moriello[b], T. Hianik[c,*]

[a] University of RomaTre Department of Biology Viale Marconi 446, 00149 Roma, Italy
[b] ENEA, Italian National Agency for New Technologies,Energy and the Environment Via Anguillarese 301, 00060 Roma, Italy
[c] Department of Nuclear Physics and Biophysics, Comenius University, Mlynska dolina F1, 842 48 Bratislava, Slovakia

*Author for correspondence: T. Hianik, email: tibor.hianik@fmph.uniba.sk
Received 21 Feb 2011; Accepted 28 Mar 2011

Abstract

We developed aptamer based biosensor for label-free detection of ochratoxin A (OTA) using thickness shear mode acoustic method (TSM). This method is sensitive to mass adsorbed at the sensor surface, measured by changes of resonant frequency, f_s, and to the viscoelastic contribution, measured by motional resistance, R_m. Addition of OTA to a sensor surface with immobilised biotinylated DNA aptamers resulted in decrease of f_s and increase of R_m. We were able to detect OTA with limit of detection of 30 nM and determined the equilibrium dissociation constant K_D=43.9±30 nM. The OTA interacted with aptamer only in the presence of calcium ions. Therefore binding studies were performed in the presence of 20 mM Ca^{2+}. No significant changes of f_s and R_m were observed without calcium. We analyzed also changes of acoustic parameters in the presence of possible interference N-acetyl-L-phenylalanine (NAP). Addition of NAP in a concentration range 25-740 nM resulted similar frequency changes like that induced by OTA, however significant but much lower changes of motional resistance were observed only at the highest NAP concentration analyzed (740 nM).

Keywords: Ochratoxin A; DNA aptamers; Thickness Shear Mode acoustic method; Biosensor

1. Introduction

The mycotoxins, such are aflatoxin or ochratoxin A are toxical fungal metabolites that can occur in primary food products. Ochratoxin A (OTA) was discovered as a metabolite of *Aspergillus Ochraceus* [1]. OTA generally appears during storage of cereals, coffee, cocoa, dried fruit, pork etc. and occasionally in the field of grapes. It may also be present in blood and kidneys of animals that have been fed on contaminated feeds. Animal studies indicated that OTA is carcinogenic [2]. Therefore, the European Commission has fixed maximum concentration of OTA in foodstuffs: 3 µg/kg (7.4 nM) for cereal products and 5 µg/kg (12.4 nM) for roasted coffee, respectively (Commission Regulation No. 1881/2006, 19 December 2006). The establishment of efficient method of detection of OTA is therefore of high importance. In addition to traditional, but expensive and time-consuming methods such as liquid chromatography [2], new trends consist in development of portable and easy to use biosensors [3]. Most of the sensors for detection OTA developed so far were based on electrochemical detections such as oxidation of OTA at glassy carbon electrode (limit of detection (LOD) 0.26 µM) [4], or reduction of horseradish peroxidase (LOD 0.25 nM) [5]. Immunosensors using antibodies immobilised on various substrates for example carbon nanotubes (LOD 6.2 pM) [6], chitosan/polyaniline layers (LOD 2.5 nM) [7], and that utilizing competitive immunoassay (LOD 20 pM) [8], were reported. Surface plasmon resonance (SPR) immunosensor was also used for detection of OTA in corn (LOD 1.2 nM) [9]. Immunosensor based on quartz crystal microbalance (QCM) was recently reported as well [10]. In this sensor anti-OTA antibodies were immobilised on the surface of 16-mercaptohexadecanoic acid. The detection based on the competitive binding between free OTA and that conjugated with BSA provided LOD of 40 nM. Recently a DNA aptamer sensitive to OTA has been developed [11]. This aptamer was able to recognize OTA with sensitivity in a ppb level and with high selectivity. The electrochemiluminiscence biosensor using aptamers as receptors was recently developed (LOD 17 pM) [12]. This sensor utilizes DNA aptamer as a receptor (DNA 1) and complementary DNA strand modified by electrochemiluminiscence probe (DNA 2). The binding of OTA to DNA 1

induced dissociation of the DNA 2 and resulted in decrease of luminescence that served as the analytical signal.

Thus most of the biosensors for OTA reported were based on indirect detection methods. Would be, however, rather useful to develop biosensor based on direct method that does not require additional modification of receptor or complicated multi stage assay. In this work we made therefore an attempt to develop biosensor for OTA based on thickness shear mode acoustic method (TSM) using biotinylated DNA aptamers immobilised on the surface of quartz crystal transducer covered by neutravidin. TSM is certain analogy of QCM, however, in addition to mass, the TSM determines also the viscosity contribution arising from the friction between biolayer and the surrounding buffer. This is important for detection of small molecules, such as OTA for which the QCM detection is difficult due to small molecular weight of the analyte. We showed that TSM allowing the detection of OTA with LOD 30 nM and with good selectivity. We also studied the stability of DNA aptamers depending on concentration of calcium ions, that are important for binding OTA to DNA aptamer.

2. Experimental

The HPLC purified DNA aptamers were purchased from Thermo Fisher Scientific (Ulm, Germany). The nucleotide composition of aptamer was identical with that published in Ref. [11], (aptamers 1.12:2), but containing at 3' end the biotinylated dT_{15} spacer: 5' GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA - dT_{15}- 3'- Biotin. The aptamers were immobilised at the gold layer of quartz crystal transducer of the area 0.2 cm^2 (CH Instruments, USA). For this purpose the transducer was carefully cleaned (see [13], for details) and mounted between two silicon O-rings in the flow-through cell [14]. The analyte was introduced into the cell by means of a Genie Plus syringe pump (Kent Scientific, USA) with a flow rate 50 µl/min. Each solution was applied until the resonance frequency stabilized. The transducer surface was first washed with Millipore water, and then 0.2 mg/ml neutravidin (Pierce, USA) in water was applied. This was followed by washing with water in order to remove non-chemisorbed neutravidin molecules. Then 1 µM aptamer solution in a binding buffer was applied and subsequently the crystal was rinsed with this buffer again. As a binding buffer we used 10 mM HEPES + 120 mM NaCl + 5 mM KCl + 20 mM $CaCl_2$ (pH = 7.4). The OTA was added to the sensor surface in the binding buffer.

The binding of OTA (Sigma-Aldrich, USA) to the aptamers was analyzed by TSM method. This method is based on determination of series resonant frequency, f_s, and motional resistance, R_m, from impedance spectra of quartz transducer determined by vector network analyzer 8712 ES (Agilent Technologies, USA). For details of experimental setup see Ref. [16]. f_s and R_m reflect energy storage and dissipation, respectively [15-17]. The TSM experiments were performed at T=20 °C.

Cruz-Aguado and Penner [11], showed, that Ca^{2+} ions are necessary for binding OTA to aptamers. No binding was observed without calcium and at increased calcium concentration (5 to 20 mM) the affinity of OTA to aptamers increased. We therefore studied also the thermodynamic properties of the aptamers depending on the concentration of Ca^{2+}. For this purpose we used UV absorption spectroscopy and determined the melting temperature of aptamers at wavelength 297 nm. For this purpose we used thermoelectrically controlled UV-VIS spectrometer Shimadzu 1700 (Japan) and applied a scan rate 0.5 °C/min. The melting of aptamers at this wavelength is characterized by larger hypochromic effect in comparison with hyperchromic effect at 260 nm [18]. The melting temperature T_m and Van Hoff enthalpy changes, ΔH_{VH}, have been determined by fitting the normalized plot of absorbance vs. temperature using Equation [19]:

$$A = \frac{A_F + A_U e^{(\Delta H_{VH}/R)(1/T - 1/T_m)}}{1 + e^{(\Delta H_{VH}/R)(1/T - 1/T_m)}} \quad (1)$$

where A_t, A_F and A_U are total, folded and unfolded aptamer normalized absorbance, respectively. R is gas constant and T is absolute temperature.

3. Results and Discussion

3.1. Thermodynamic properties of the aptamer

The plot of absorbance vs. temperature for aptamers without and with various concentrations of Ca^{2+} ions is presented in Figure 1. It is seen that the absorbance has sigmoid shape typical for two-state transition. The fit of experimental results using Equation (1) allows the determination of thermodynamic properties of aptamers (Table 1). It is seen from Table 1, that with increasing of the Ca^{2+} concentration the melting temperature increases which suggests increase in the aptamers stability. This is confirmed also by changes of

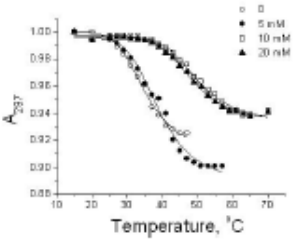


Figure 1. Normalized plot of absorbance at wavelength 297 nm vs. temperature for DNA aptamers in the presence of various concentrations of Ca^{2+} in the buffer. The full lines are fit according to Equation (1).

Table 1. The thermodynamics parameters of aptamers in the presence of various concentrations of Ca^{2+} ions. Enthalpy changes, $\Delta G^0_{(20)}$ – changes of standard Gibbs energy at 20 °C, ΔS - entropy change ($\Delta G^0_T = \Delta H_{vH}(1-T/T_m) = \Delta H_{VH}(1-T/T_m) = \Delta H_{VH}-T\Delta S$, where ΔH_{vH} is calorimetric enthalpy. $\Delta H_{vH}/\Delta H_{VH}=1$ for two-state transition [19]). Results are means± SD (n=3).

$[Ca^{2+}]$, mM	T_m (°C)	ΔH_{VH} (kJ/mol)	$\Delta G^0_{(20)}$ (kJ/mol)	$T\Delta S$ (kJ/mol)
0	35.1±0.7	-201.9 ±0.2	-9.89±0.01	-192.0±0.2
5	39.2±0.8	-208.0±0.3	-12.83±0.02	-195.2±0.3
10	49.9±0.6	-176.8±0.1	-16.36±0.01	-160.4±0.1
20	48.3±0.5	-180.8±0.1	-15.92±0.01	-164.9±0.1

Gibbs energy which is significantly larger for aptamers in the presence of relatively high Ca^{2+} concentrations (10-20 mM) in comparison with that without calcium or at relatively low calcium concentration (5 mM). Less favorable changes in enthalpy are, however compensated by entropy contribution. It is likely that calcium is important for providing aptamers stability and possibly also the proper folding into 3D structure allowing formation of binding site for OTA. This conclusion follows from the fact that without Ca^{2+}, no binding of OTA to aptamers was observed [11]. Therefore in binding experiments we used the buffer containing 20 mM Ca^{2+}. At this condition the dissociation constant was minimal K_D = 49±3 nM, which corresponds to maximal affinity of OTA to the aptamer [11].

3.2. Detection of OTA by aptamers biosensor

The TSM method allows checking all steps of sensor preparation and to study the binding of OTA to the aptamers. The representative plot of the changes of series resonant frequency, f_s, and motional resistance, R_m, following the modification of TSM transducer by neutravidin, aptamers and by addition of the OTA in the concentration range 25-740 nM is presented on Figure 2. It can be seen that addition of neutravidin (NA) resulted in substantial decrease of the frequency by 120 Hz, which suggests strong chemisorption to the gold surface. Washing with deionised water slightly increased the resonant frequency due to desorption of weakly adsorbed NA molecules. The changes of R_m were rather small suggesting that NA form rather rigid layer. The obtained results are in good agreement with previously reported data [14,17]. The changes of frequency allowing estimation of surface concentration of NA using Sauerbrey equation that describes the relationship between the changes of resonant frequency (Δf_s) and the adsorbed mass (Δm) [20]:

$$\Delta f_s = -2f_0^2 \rho_q (\mu_q \rho_q)^{-1/2} = -2.26\times10^{-6} f_0^2 (\Delta m/A) \quad (2)$$

(f_0 is the fundamental frequency of the quartz, ρ_q is the mass density, μ_q =2.95x10^{10} Pa is shear stiffness of quartz, ρ_q = 2650 kg m^{-3} is the quartz density and A is the sensor area). In equation (2) the surface mass loading ($\Delta m/A$) is in g.cm^{-2} [21]. This equation is valid for dry crystal in vacuum. The oscillations of quartz in a liquid are, however, affected by viscosity of surrounding liquid, which cause additional

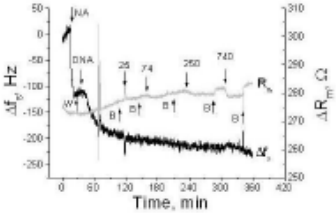


Figure 2. The representative plot of the kinetics of the changes in the series resonance frequency (Δf_s, black) and in the motional resistance (ΔR_m, gray) following various modifications of the TSM transducer surface. The surface was modified consecutively with neutravidin (NA), DNA aptamers (DNA) and OTA in different concentrations (nM). The additions are shown by arrows. W and B are the washing steps in deionised water or buffer, respectively.

frequency changes [22]. However, for analytical purposes even Equation (2) can be used assuming that a correction factor of approx. 2 could be applied to correct changes of frequency affected by viscoelasticity [23], i.e. viscoelasticity causes approx. 2 times larger decrease of the frequency change in comparison with mass effect only. However, exact correction of frequency changes have to be determined in special experiment using impedance analysis of crystal oscillations by means of vector network analyzer [16]. Thus, the values of frequency change Δf_s =120 Hz correspond according to Equation (2) to a change of mass loading $\Delta m/A=8.3\times10^{-7}$ g.cm^{-2} and the surface concentration of NA molecules can be calculated as $(\Delta m/A)x(N_A/M_w)$ = 8.3 x10^{-12} cm^{-2}, where N_A=6.02x10^{23} mol^{-1} is Avogadros number and M_w = 60 kDa is molecular weight of NA. Due to negligible changes of R_m, it is not necessary to apply the correction factor.

Addition of DNA aptamers resulted also in decrease of resonant frequency (by approx. 86 Hz), which is evidence of adsorption of the biotinylated aptamers to the NA layer due to high affinity of biotin to the neutravidin. However decrease of the frequency is accompanied by increase of the motional resistance by 4.9 Ω. This increase is evidence of existence of the friction between the surface of TSM transducer covered by brushes of randomly oriented DNA aptamers and the buffer. In analogy with above estimations the obtained frequency changes Δf_s = 86 Hz following the addition of aptamers correspond to mass loading $\Delta m/A=5.9\times10^{-7}$ g.cm^{-2} and the surface concentration of aptamers will be $(\Delta m/A)x(N_A/M_w)$ = 3.0 x10^{13} cm^{-2}, where M_w = 11.9 kDa is molecular weight of aptamer. Considering the correction factor of 2, finally the surface concentration of aptamers can be estimated as 1.5 x10^{13} cm^{-2}. Comparing the surface concentrations of NA and aptamers one can obtain approx. 1.8 times more aptamers in comparison with chemisorbed NA molecules. This ratio is in good agreement with the structural peculiarities of NA. It is known that NA contains 4 binding sites for biotin. However, due to chemisorption probably 2 binding sites at each NA molecule are faced to a gold layer and thus are out of the access of DNA aptamers.

Addition of OTA to the TSM sensor surface resulted in increase of the R_m and decrease in resonant frequency. This is better seen on Figure 3 where the plot of the changes of f_s and R_m vs. OTA concentration is presented. The shape of the frequency and resistance changes is typical for Langmuir isotherm. This means that the OTA binds to the aptamer binding sites independently on the other OTA molecules. The binding of OTA to the aptamers can be quantitatively characterized by Langmuir equation [24], For example for changes of R_m one can write:

$$\Delta R_m=(\Delta R_m)_{max}[c/(K_D+c)] \quad (3)$$

where $(\Delta R_m)_{max}$ is the maximal changes of the motional resistance. The K_D value is a measure of the affinity of OTA to the aptamers at the sensor surface. Using Equation (3) and the least square method the K_D value has been determined as K_D=43.9±30 nM. This value is in good agreement with that reported in Ref [11], for free aptamers in the presence of 20 nM Ca^{2+} (fluorescence detection of OTA, K_D=49±3 nM).

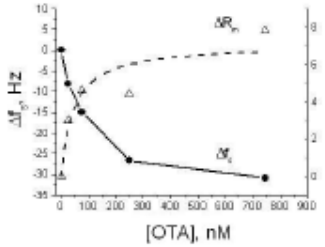


Figure 3. The representative plot of the dependence of the changes of resonant frequency, Δf_n, and motional resistance, ΔR_m, as a function of OTA for TSM sensor. Dashed line is the fit according to Langmuir isotherm (Equation (2).

The limit of detection (LOD) for TSM sensor was 30 nM. It has been determined using common criteria of significant analyte determination at the level corresponding to signal to noise ratio, S/N=3. The obtained LOD is comparable with QCM sensor based on indirect competitive detection method [10]. This LOD is above the requirements of OTA contamination according to European Commission rules. However, amplification of detection is possible using for example nanoparticles conjugated with antibodies. We studied also interaction of OTA with the TSM surface when no calcium was present in a buffer. In this case we did not observe significant changes in frequency and motional resistance in the concentration range of OTA 25-740 nM. These results agree well with the Ref. [11], in which no interaction of OTA was observed without calcium ions.

In order to check specificity of the aptamers we analyzed also interaction of potential interference compound *N*-acetyl-L-phenylalanine (NAP). According to Ref. [11], NAP in 100 fold excess does not significantly bind to the aptamer. Addition of NAP to the TSM sensor surface resulted in the decrease of the resonant frequency similar to that caused by OTA, however, we did not observe significant changes in motional resistance. Only at rather high NAP concentration (740 nM) the increase of R_m by 1.15 Ω was observed. This is approx. 7.8 times less in comparison with the effect induced by OTA. The decrease of the frequency could be due to various reasons. This can be due to non-specific binding of NAP to DNA, or to the neutravidin layer not covered by aptamers. It is likely that certain optimization of the immobilisation conditions would be necessary for development of the biosensor for detection of OTA in real samples. These experiments as well as selection of the amplification conditions for improvement the sensor sensitivity are in progress.

4. Conclusion:

We showed that TSM acoustic method is perspective tool for label-free detection of low molecular toxicant – OTA using DNA aptamers as specific receptors. The detection of OTA with aptamers requires the presence of calcium ions that are most probably responsible for stability of aptamer and for formation of 3D structure of specific binding site for OTA. The analysis of thermodynamic properties of aptamers certainly indicates the increased melting temperature of aptamers, and hence increased stability, with increased concentration of calcium in a range 0-20 mM. The limit of detection of the biosensor (30 nM) was comparable with QCM based acoustic sensor, but utilizing indirect, competitive assay. In contrast with Ref. [11], we observed certain interference of aptamers sensor with NAP that affects significantly the frequency, but not the motional resistance. It is possible that NAP interact non-specifically with DNA or with neutravidin layer. Therefore further optimization of TSM sensor will be necessary.

Acknowledgements:

This work was supported by Agency for Promotion Research and Development under the contracts No. APVV-0362-07, by Slovak Grant Agency (Project No. 1/0794/10), MNT-ERA.NET project IntelliTip (to T.H.) and by scholarship program of SAIA (to I.L.).

References

1. Van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, D.B. Theron, J.J., Nature 205 (1965) 1112.
2. Turner, N.W., Subrahmanyam, S., Piletsky, S.A., Anal. Chim. Acta 632 (2009) 168.
3. Sionteros, C.G., Nikolelis, D.P., Miernik, A., Krull, U.J., Electrochim. Acta 43 (1998) 3611.
4. Oliveira, S.C.B., Diculescu, V.C., Palleschi, G., Compagnone, D., Oliveira-Brett, A.M., Anal. Chim. Acta 588 (2007) 283.
5. Alonso-Lomillo, M.A., Domínguez-Renedo O., Ferreira-Goncalves, L., Arcos-Martínez, M.J., Biosens. Bioelectron. 25 (2010) 1333.
6. Ajeet Kaushik, A., Solanki, P.R., Pandey, M.K., Kaneto, K., Ahmad, S. Malhotra, B.D., Thin Solid Films 519 (2010) 1160.
7. Khan, R., Dhayal, M., Biosens. Bioelectron. 24 (2009) 1700.
8. Liu, X-P., Deng, Y-J, Jin, X-Y., Chen, L-G., Jiang, J-H., Shen, G-L., Yu, R-Q., Anal. Biochem. 389 (2009) 63.
9. Yuan, J., Deng, D., Lauren, D.R., Aguilar, M-I., Wu, Y., Anal. Chim. Acta 656 (2009) 63.
10. Tsai, W.C., Hsieh, C.K., Anal. Lett. 40 (2007) 1979.
11. Cruz-Aguado, J.A., Penner, G., J. Agric. Food Chem. 56 (2008) 10456.
12. Wang, Z.P., Duan, N., Hua, X., Wu, S.J., Anal. Bioanal.Chem. 398 (2010) 2125.
13. Hianik, T., Ostatná, V., Sonlajtnerova, M., Grman, I., Bioelectrochemistry 70 (2007) 127.
14. Tassew, N., Thompson, M., Biophys. Chem. 106 (2003) 241.
15. Ellis, J.S., Thompson, M., Chem. Commun. (2004) 1310.
16. Snejdarkova, M., Svobodova, L., Polohova, V., Hianik, T., Anal. Bioanal.Chem. 390 (2008) 1087.
17. Hianik, T, Grman, I., Karpisova, I., Chem. Commun. (2009) 6303.
18. Kankia, B.I., Marky, L.A., J. Am. Chem. Soc. 123 (2001) 10799.
19. L.A. Marky, K.J. Breslauer, Biopolymers 26 (1987) 1601.
20. Sauerbrey, G., Z. Phys. 155 (1959) 206.
21. Hayward, G.L., Chu, G.Z., Anal. Chim. Acta 288 (1994) 179.
22. Kanazava, K.K., Gordon, J.G., Anal. Chim. Acta 175 (1985) 99.
23. Zhou, X.C., Huang, L.Q., Li, S.F.Y., Biosens. Bioelectron. 16 (2001) 85.
24. J. Wang, Analytical Electrochemistry, Wiley-VCH, New York (2006).

Cite this article as:

T. Hianik *et al.*: **Development of thickness shear mode biosensor based on DNA aptamers for detection of ochratoxin A.** *Chemical Sensors* 2011, 1: 11

Aim 3: Sensing Surface for SERS Application

We present the preparation of an aptamer terminated sensing surface, allowing a fast and cheap system for assay of analytes unlimited by size and tossicity. The designed platform is suitable for an aptamer-based microfluidic device, coupled with a Surface Enhanced Raman Spectroscopy (SERS). To accomplish this, a multi-step deposition sequence was performed: as a start, a mixed self assembled monolayer (SAM) containing a binary mixture of biotinylated alkylthiol (BAT) [1]. with the capacity to graft neutravidin proteins and diluent methyl-terminated alkylthiol, was prepared on a gold thin film. The chemical and electronic structure of the mixed SAMs was investigated by X-ray Photoelectron Spectroscopy and IRRAS (Infrared Spectroscopy in Reflection Mode) [2].
In a second step, the bioconjugation with an avidin-gold sol is performed. Monodispersed 5 nm gold particles were prepared in aqueous medium and covered with neutravidin, overcoming possible non-specific bindings, taking usually place at the isolectric point (~ 6) of neutravidin [3, 4].
The sample morphologies were observed by atom force microscopy (AFM), the size was determined by Dynamic Light Scattering and the concentration of gold species in the colloid was monitored by UV-vis spectra [5, 6].
In the ending step, a sensing aptamer (i. e. thrombin-binding aptamer) is bond to the surface through the avidin-biotin linkage. The resulting SERS changes involved was observed when the target molecule (i.e. thrombin) interacts with its own aptamer [7].
This aptamer terminated sensing surface is fitted for affinity based microfluidic devices, providing controlled fluid transport, rapid affinity assay and cost saving advantages over conventional methods for biological and medical applications. Through such design, the sensing surface overcomes the sandwich structure, formed between the immobilized aptamer, the protein target and a secondary aptamer bound to the Raman probe (i.e. gold nanoparticles), usually realized in SERS aptasensors, limited by consuming and inconvenient handling step [8].

References

[1] Ptrats-Alfonso E., Garcia-Martin F.,Bayo N., Cruz L.J., Pla-Roca M., Samitier J., Errachid A., Albericio F., (2006) Tetrahedron, 62, 6876-6881.

[2] Nelson K.E., Gamble L., Jung L S., Boeckl M S., Naeemi E., Golledge S L., Sasaki T, Castner D.G., Campbell C.T., and Stayton P.S. (2001)

Surface Characterization of Mixed Self-Assembled Monolayers Designed for Streptavidin Immobilization Langmuir. 17: 2807–2816.

[3] Grabar, K.C., Freeman R.G., Hommer M.B., and Natan M.J. (1995) Preparation and Characterization of Au colloid Monolayers, Anal. Chem., 67: 735-743.

[4] Morris R.E. and Saelinger C.B., (1984) Visualization of intracellular trafficking: use of biotinylated ligands in conjunction with avidin-gold colloids. J.Histochemistry and Cytochemistry, 32, 124-128.

[5] Kimling J, Maier M, Okenve B, Kotaidis V, Ballot H, Plech A. (2006) Turkevich method for gold nanoparticle synthesis revisited. J Phys Chem B., 110 (32):15700-15707.

[6] Ji X, Song X, Li J, Bai Y, Yang W, Peng X.,. (2007) Size control of gold nanocrystals in citrate reduction: the third role of citrate. J.Am.Chem.Soc. 129: 13939-13948.

[7] Nie S.; and Emory S. R., (1997) Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering Science 275: 1102,.

[8]. Sassolas A, Blum L. J., Leca-Bouvier B. D., (2011) Optical detection systems using immobilized aptamers Biosensors and Bioelectronics 26: 3725–3736

Aptamer based SERS sensing surface for direct detection of proteins.

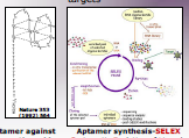
*Lamberti I., * Antoccia A., **Battocchio C. *** Quagliano L.G, * Tanzarella C., ** Iucci G., and ***Foglia S.

*Dipartimento di Biologia Università RomaTre Viale Marconi,446 00146 Rome Italy**Dipartimento di Fisica Università RomaTre Via della Vasca Navale,84 00146 Rome Italy, ***IFN-CNR, via Cineto Romano,42 00156 Rome Italy

sabrina.foglia@ifn.cnr.it

APTAMERS

single-stranded RNA or DNA oligonucleotides that bind with high affinity to specific molecular targets

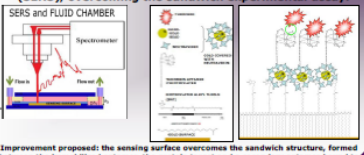


Aptamer against human thrombin

Aptamer synthesis-SELEX Systematic Evolution of Ligands by EXponential enrichment

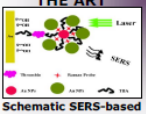
OBJECTIVE:

Design a nanoaptasensor sensing surface for microfluidic device, coupled with Surface Enhanced Raman Spectroscopy (SERS), overcoming the sandwich experimental assay.



Improvement proposed: the sensing surface overcomes the sandwich structure, formed between the immobilized aptamer, the protein target and a secondary aptamer bound to the Raman probe (i.e. gold nanoparticles), usually realized in SERS aptasensors, limited by consuming and inconvenient handling steps

STATE OF THE ART



Schematic SERS-based sandwich assay.

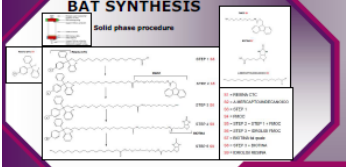
Biosensors and Bioelectronics 26(9), 2011

Sensing surface assembling Pathway

STEP	PREPARATIONS	CHARACTERIZATIONS	
1: Surface fuctionalization	BAT Chemical Syntesis Macromolecules surface deposition	IR, XPS, AFM	HERE REPORTED
2: Conjugation of Gold nanoparticle	Gold nanoparticle syntesis Bioconjugation avidin-gold sol	TEM, XPS, UV-vis ...	
3: Binding nanoparticles-surface	Deposition of gold-neutravidin conjugates on macromolecules surface	Raman Spectroscopy	IN PROGRESS
4: Binding Aptamer-nanoparticles	Catch of thrombin aptamer biothinylated with gold-neutravidin conjugates	Raman Spectroscopy	
5: Binding Thrombi-Aptamer	Thrombin-Aptamer detection	SERS Analysis	

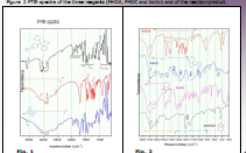
BAT SYNTHESIS

Solid phase procedure

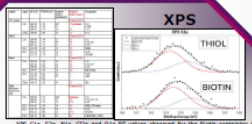


Tetrahedron 62(2006) 6876-6881

FT-IR

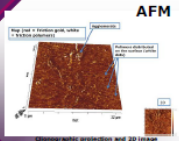


XPS



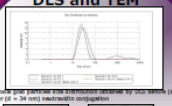
XPS C1s, S2p, N1s, C2p and O1s BE values observed for the Biotin-containing thiol are fully consistent with the values observed for the reference samples Biotin, CTC and thiac. Semiquantitative XPS analysis was also carried out, and the evaluated intensity ratios (Nature'Y' / Nature 'selective') confirm the proposed chemical structure. In the figure, S2p1/S spectra measured on the pristine Biotin sample and on the freshly synthesized biotin-containing thiol are shown. S2p3/2 BE values are indicative for an alkylthiol-like sulfur atom, as well as for a sulfur atom inserted in the biotin ring.

AFM

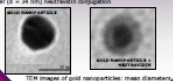


Chronographic projection and 2D image of a self-assembled monolayer of long-chain alkylthiolates on gold surface. (Langmuir 2001, 17, 2807)

DLS and TEM



The monomodal gold particles size distribution obtained by DLS before (d = 26 nm)and after (d = 34 nm) neutravidin conjugation



TEM images of gold nanoparticles: mean diameters confirm DLS measurements

Acknowledgements:

we thank M. Papi and F. Porcaro for their helpful collaboration

6 WORLD CONGRESS on Biomimetics, Artificial Muscles and Nano-Bio

Publications

Impedimetric DNA Aptasensor for Sensitive Detection of Ochratoxin A in a Food. Castillo G., Lamberti I., Mosiello L., Hianik T. ELECTROANALYSIS (in press)

Biosensors for Aflatoxin Detection Mosiello L., and Lamberti I., Chapter Title from *Aflatoxin - Detection, Measurement and Control* by Irineo Torres Pacheco. ISBN:978 953 307 711 6

High Sensitive Impedimetric Aptasensor for Detection Ochratoxin A In Food Castillo G., Lamberti I., Mosiello L., Hianik T. SENSORS AND MICROSYSTEMS World Scientific Publishing co. ISBN:978114609342 in press

***A Piezoelectric Quartz Crystal Sensor Applied for Thrombin-Binding Aptamers** (2011) Rakitka J., Hianik T., Lamberti I., Mosiello L., SENSORS AND MICROSYSTEMS 91 (5): 449-453, ISBN: 9789400713239, DOI: 10.1007/978-94-007-1324-6_73

***An antibody-based microarray assay for the simultaneous detection of aflatoxin B1 and fumonisin B1** (2009) Lamberti I., Tanzarella C., Solinas I., Padula C., Mosiello L. Mycotoxin Research, 25 (4): 193-200. MYCOTOXIN RESEARCH ISSN: 01787888 DOI: 10.1007/s12550-009-0028-9

***Development of a thickness shear mode biosensor based on DNA aptamers for detection ochratoxin A** (2011) Lamberti I., Mosiello L., Hianik T., Chemical Sensors 1: 11-15 ISSN 2231-6035.

***A Novel Based Protein Microarray for the Simultaneous Analysis of Activated Caspases** (2010) Lamberti I, Mosiello L, Cenciarelli C, Antoccia A. and Tanzarella C. SENSORS AND MICROSYSTEMS, Lecture Notes in Electrical Engineering, 54 (4): 323-326, DOI: 10.1007/978-90-481-3606-3_64.

Development of a Protein Microarray System Antibody based for Mycotoxins Determination Mosiello L, Lamberti I, Vitali F, Misiti S And

Di Giorgio G, SENSORS AND MICROSYSTEMS World Scientific Publishing co. ISBN: 10 981-283-597-0.

Toxicity of toner nanoparticles on RT112 cell cultures Mosiello, L., Zappa, G., Zoani, C., Lamberti, I., Gatti, R., Pilloni, L.(2009) Proceedings Of The 9th IEEE Conference on Nanotechnology, IEEE NANO 2009, art. no. 5394752, pp. 616-618.

Conference

Integrated approach to a sensitive platform for SERS aptamer biosensors, suitable for a microfluidic device. I. Lamberti, A. Antoccia, F. Duconge, G. Iucci, M. Papi, L.G. Quagliano and S. Foglia CONVEGNO NAZIONALE SENSORI (2012) (submitted)

Overcoming of gold nanoparticles sandwich for sensitive platforms in microfluidic devices, based on surface-enhanced Raman scattering Foglia S., Lamberti I.,Quagliano L.G., Papi M., Antonini G., Polzonetti G., and Tanzarella C. NANOTECHITALY 2011 NOVEMBER 23rd, 24th November

Aptamer based SERS sensing surface for direct detection of proteins. Lamberti I., Antoccia A., Battocchio C. Quagliano L, Tanzarella C, Iucci G., and Foglia S. Proceedings Of The 6th Mondial Conference on Biomimetics, Artificial Muscles and Nano-Bio Cergy-Pontoise, France 25-26-27 October 2011.

Development of thickness shear mode biosensor based on DNA aptamers for detection ochratoxin A Lamberti I., Mosiello L., Hianik T. Sensors And Microsystems, Proceedings Of The 16th Italian Conference, Rome, Italy 7- 9 February 2011 ENEA –C.R. Casaccia - Via Anguillarese, 301 - 00123 Roma; Edited By C Di Natale, A D'amico (University Of Rome "Tor Vergata" and Cnr-Imm, Italy)

A Piezoelectric Quartz Crystal Sensor Applied for Thrombin-Binding Aptamers Jano Rakitka, Tibor Hianik Ilaria Lamberti, Lucia Mosiello, Proceedings Of The 15th Italian Conference, Messina, Italy 8- 10 February 2010 Edited By Signorino Galvagno, Giovanni Neri, Nicola Donato

(Università degli Studi di Messina, Italy) A D'amico (University Of Rome "Tor Vergata" and Cnr-Imm, Italy)

A Novel Based Protein Microarray for the Simultaneous Analysis of Activated Caspases Lamberti I., Mosiello L., Cenciarelli C., Antoccia A., Tanzarella C. Sensors And Microsystems, Proceedings Of The 14th Italian Conference, Pavia, Italy 24-26 February 2009 Edited By Andrea Baschirotto, Piero Malcovati e Arnaldo D'Amico (University Of Rome "Tor Vergata" and Cnr-Imm, Italy)

Development of a Protein Microarray System Antibody based for Mycotoxins Determination Mosiello L, Lamberti I, Vitali F, Misiti S And Di Giorgio G, Sensors And Microsystems, Proceedings Of The 13th Italian Conference, Rome, Italy 19- 21 February 2008 Edited By C Di Natale, A D'amico (University Of Rome "Tor Vergata" and Cnr-Imm, Italy)

Toxicity of toner nanoparticles on RT112 Cell Cultures Lucia Mosiello, Giovanna Zappa, Claudia Zoani, Ilaria Lamberti, Rosanna Gatti, Luciano Pilloni Proceedings Of The 9th Nanotechnology Conference IEEE NANO 2009 Genoa July 26-30 2009

Materiali di Riferimento per la determinazione di micotossine e la valutazione di contaminazioni fungine in prodotti alimentari Rosanna Gatti, Ilaria Lamberti, Lucia Mosiello, Giovanna Zappa, Claudia Zoani ENEA – Dipartimento Biotecnologie Agroindustria e protezione della Salute C.R. Casaccia - Via Anguillarese, 301 - 00123 Roma; giovanna.zappa@.enea.it III Congresso Nazionale: Le Micotossine nella Filiera Agro-Alimentare e Zootecnica 28-29-30 settembre 2009 Roma By Istituto superiore di Sanità Dipartimento Sanità Pubblica Veterinaria e Sicurezza Alimentare Reparto OGM e Xenobiotici di Origine Fungina

Conclusions

The main results obtained during the PhD work consist in the characterization of the best approach for the development of biosensors in order to detect concentrations and ligand affinity parameters of free unlabeled molecules in real time.

In recent years, the antibody microarray technology has made significant progress, going from proof-of-concept design to established high-performing technology platforms capable of targeting non-fractionated complex samples, as proteoma.

We have studied microarray detection in standard solutions with a low detection limits and developed a competitive immunoassay in microarray format for simultaneous analysis. The quality of the microarray data is comparable to data generated by microplate based immunoassay, and for this reason it represents just a useful insight in order to arrange further experiments to develop a rapid test or kits. Our method could be used as a semi quantitative tool for rapid pre-screening and further investigations are needed in order to improve the reported microarray performance mainly in term of sensitivity.

We moved from antibodies to aptamers because the use of functional aptamers as bioreceptors has become a new interdisciplinary field that aims at providing new hybrid sensing systems for specific and high sensitive molecular recognitions. This novel integration has yield various types of sensor for selective and sensitive detection of a wide range of analytes. Then, we focused on QCM aptasensor. We used, as model systems, two different analytes, thrombin and ochratoxin, in association with specific their aptamers as immobilized ligands. Our experiments prove that aptamers are equivalent to antibodies in terms of specificity and sensitivity and are one of the best approach for affinity biosensors construction.

We showed that acoustic methods (such as QCM) are perspective tool for label-free detection of low molecular toxins and protein markers. The detection of aptamers requires the present of ions that are probably responsible for their stability and formation of 3-D structure of the specific binding site.

We tried to develop a biosensor, based on direct method that do not require additional modification of receptor or complicated multi stage assay, with the aim to overcome the indirect detection methods in published literature.

Another evidence is that aptamers can mimic antibodies for application on affine layers for biosensors or chips.

In comparison to antibodies as capture molecules in biosensors, small aptamer receptors have a number of advantages. Because of their small size, denser receptor layers can be generated, meaning for a given receptor affinity, the sensitivity of these layers can be increased. They are produced *in vitro*; indeed no animals are needed.
The improvement of sensitivity, produced by changing the selective element (from antibodies to aptamers), has been assisted by a sensitility enhancement, applying a different trasducer (from acustic to optical methods).
We have approached the development of an affinity based detection system for analytes unlimited by size and tossicity, designing a nanoaptasensor sensing surface for microfluidic device, coupled with Surface Enhanced Raman Spectroscopy (SERS). Among the optical detection methods, SERS distinguishes itself with several advantages: the spectrum change brought by a single molecule, is induced by the specific interaction between aptamers and their own proteins and the spectral probe specificity is excellent in comparison to the fluorescence methods.
A thrombin SERS aptasensor is usually realized by a sandwich structure, formed between the immobilized aptamer, the protein target and a secondary aptamer bound to a Raman probe (i.e. gold nanoparticles), which requires a consuming and inconvenient handling step.
In attempt to overcome this drawback we designed an aptamer terminated sensing surface, allowing a faster and cheaper system and suitable for an analytical label free measurements, in order to perform a direct analysis of biological samples.
We also developed a multi-step depositional sequence where the last stage is the assay itself of the target molecules (i.e. thrombin), instead of the SERS probe deposition (i.e. gold nanoparticle), as planned to a classic sandwich structure. Such advantage allows to make improvements to costly and inconvenient handling steps.
In conclusion, we have investigated different experimental approaches of biosensible components of biosensors. Firstly, we developed a multi-parametric, rapid, sensitive and inexpensive method for the detection of mycotoxins for food safety and caspases for human health applications (immunoassay in a microarray format). Then, we moved to a different bioreceptor, choosing aptamers as selective element, improving the method sensitivity (QCM). Finally we designed a novel sensitive surface for a different detection mode (i.e. optical method, SERS) with higher sensitivity compared to the acoustic methods. This final improvement allows the possibility of submitting a patent application.

Acknowledgement

I would like to express my gratitude to Prof.ssa Caterina Tanzarella and Prof. Antonio Antoccia, my super advisors at the biologist department, University of RomaTre, my tutors Dr. Lucia Mosiello, (ENEA) and Dr Sabrina Foglia (CNR) for their great support.

I also would like to thank for their helpful collaboration:
Prof. Tibor Hianik, head of the Laboratory of Biophysics at Department of Nuclear Physics and Biophysics, Faculty of Mathematics, Physics and Computer Sciences, Comenius University.
Prof Giovanna Iucci, Dr Chiara Battocchio and Prof G. Polzonetti, CISDiC University of RomaTre and M. Papi, Physics departement, Catholic University S.C. of Rome.