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Specific carriers and bioconjugates in modern analytical methods

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Abstract:

This thesis deals with the biofunctionalization of carriers and bioconjugation of metallic-based nanoparticles and their subsequent use in modern bioanalytical methods. Two types of magnetic immunosorbents were prepared by biofunctionalization of magnetic microparticles. Carriers were used to isolate and detect bacterial cells in dairy products or to detect specific antibodies in the serum of Alzheimer's patients. Gold nanoparticles-based bioconjugates were prepared for electrochemical detection of bacterial cells. It was experimentally verified that the conjugated bioactive components reacted specifically with the bacterial cell surface and allowed their detection and quantification in the analyzed sample.

Abstrakt:

Předložená disertační práce se zabývá biofunkcionalizací nosičů a biokonjugací nanočástic a jejich následným využitím v moderních bioanalytických metodách. Biofunkcionalizací magnetických mikročástic byly připraveny dva druhy magnetických imunosorbentů. Tyto nosiče byly použity pro izolaci a průkaz bakteriálních buněk v mléčných výrobcích nebo pro průkaz specifických protilátek v séru pacientů s Alzheimerovou chorobou. Pro elektrochemický průkaz bakteriálních buněk byly připraveny biokojugáty na bázi zlatých nanočástic. Bylo experimentálně ověřeno, že konjugované bioaktivní složky reagovali specificky s povrchem bakteriálních buněk a umožnili jejich průkaz i kvantifikaci v analyzovaném vzorku.

Key words:

Biofunctionalization, bioconjugates, immunosorbents, immunomagnetic separation, rapid detection of food-borne bacteria

Klíčová slova:

Biofinkcionalizace, biokonjugáty, immunosorbenty, imunomagnetická separace, rychlý průkaz bakterií v potravinách

Table of Contents

1 Introduction

1.1 Bioconjugation and biofunctionalization of modern materials

The terms bioconjugation and biofunctionalization generally describes processes in which two or more molecules are coupeled together and at least one of the bound molecules is biomolecule [1]. The most commonly used biologically active molecules include peptides [2,3], proteins [4,5] (enzymes [6]), nucleic acids [7], etc. These can be conjugated to a wide range of other molecules or materials. The most common are nano- and microparticles [8-10], synthetic polymers [11], fluorescent labels [12] or drugs [13]. It could be said that these processes are a certain connection between biology, chemistry and physics, because the (bio) chemical or physical properties of one molecule and at the same time the biological properties of the other molecule are used here.

The complexes created in this way can then be used mainly for the following purposes:

• Capture, remove, purify of a wide range of analytes [14-16]

• Determination and quantification of a wide range of analytes [17-19]

• Monitoring and imaging (eg. *in vivo* and *in vitro* tissues) [20-22]

• Therapy, *in vivo* diagnostics [23-25]

Due to the great popularity of the fields of materials engineering and nanotechnology, the preparation and use of metal-based particles, which began to be used in many applications from diagnostics to therapy is in the center of attention [26-29]. The main advantages of working with metal-based materials are the diversity of their physicochemical properties [30]. If we consider using these systems in diagnostics or therapy, it is possible to choose a material that is non-toxic and is so-called biocompatible. An indisputable advantage of using micro- and nanoparticles is also a large specific surface, to which a large number of different molecules can be attached. More precisely biomolecules, that brings to particles a very diverse use. For example, gold [31] and silver nanoparticles [32], quantum dots [29] and magnetic micro- or nanoparticles [33] are most widely used in biomedicine.

The introduction of functional groups on the surface of magnetic particles is called surface modification and the subsequent binding of biomolecule is denoted as biofunctionalization. The magnetic particle thus becomes a magnetic carrier.

Generally, nanoparticles are usually defined as particles with diameter between the range 1-100 nm. [34]. Thanks to the great advances in the synthesis, modification and subsequent bioconjugation of metal nanoparticles, a very wide range of applications is offered, from separation [16], targeted drug delivery [35], gene therapy [36], radiotherapy [21], *in vivo* imaging techniques and detection [22]. Thus, when we talk about a

bioconjugate, we mean biomolecules as the antibody, peptides, enzymes or nucleic acids) covalently bound with nanoparticles. The biological molecule ensures the contact with the environment and the nanoparticles are able to provide a measurable signal that is detected due to their specific physical and chemical properties. Figure 1 describes the processes of biofunctionalization and bioconjugation as presented in this dissertation.

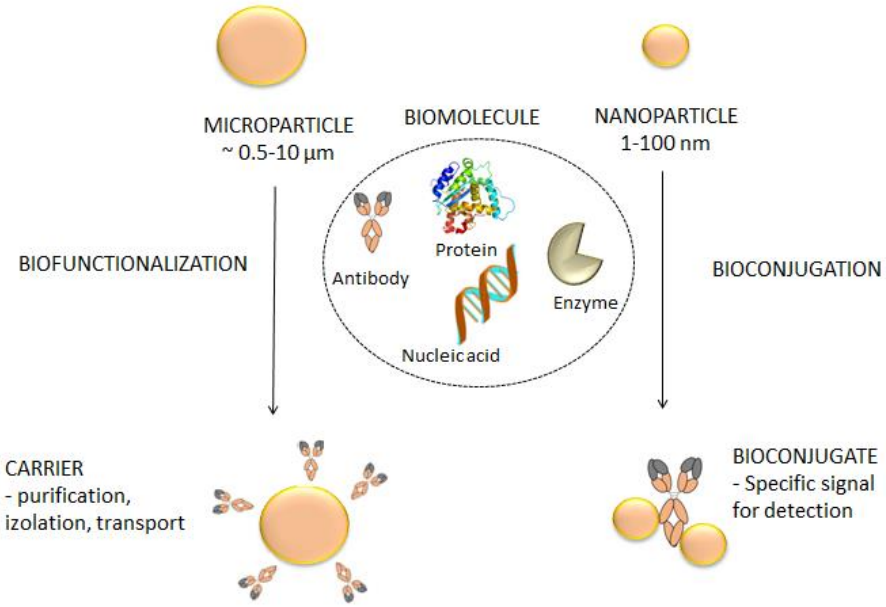

Figure 1: Binding of biological molecules to micro- and nanoparticles.

1.2 Magnetic carriers in modern analytical methods

Due to their physical and chemical properties, magnetic particles have great benefits. Mainly they are superparamagnetic particles, so they exhibit a magnetic moment only in the presence of an external magnetic field. Thus, in the absence of a magnetic field, the particles should not interact with each other to form unwanted aggregates. An important advantage is that due to the superparamagnetic properties, the particles can be resuspended if the magnetic field stops acting on them. Iron oxides, magnetite Fe_2O_3 or maghemite (γ-Fe_2O_3) are most often used to prepare superparamagnetic particles. Great advantage is their biocompatibility and biodegradability [37]. An integral part of the particles is a non-magnetic component, which can be various organic or inorganic molecules, enveloping the magnetic core ensuring colloidal stability and solubility [38]. Magnetic particles with sizes of 50 nm to 10 µm are mostly used in bioapplications [39]. According to the size, these particles can be divided into micro- and nano-. This division is then related to their use in various areas. Magnetic nanoparticles exert have great potential especially in *in vivo* medical applications, namely in the fields of diagnostics [40] and therapy [41]. In contrast, magnetic microparticles in sizes ranging from hundreds of nanometers to units of micrometers are used mainly in *in vitro* diagnostics, for example in the immunomagnetic separation or diagnosis of cells [42], proteins [43] and pathogenic microorganisms [44].

1.2.1 Magnetic separation as a tool for purification and isolation of a specific substance from a sample

The process of purification and isolation of specific molecules is used in almost all biotechnological branches. Chromatographic [45,46] and electrophoretic methods [46], ultrafiltration [47], centrifugation [48], precipitation and others are most commonly used for this proces. The above-mentioned techniques are commonly used in commercial laboratories for the purification of synthesized peptides, antibodies, enzymes, oligonucleotides, but also drugs.

In addition to these methods, magnetic separation (MS) has built up its position in modern bioanalytical methods in recent years. MS combines the advantages of affinity chromatography with the properties of magnetic particles and is therefore specific, selective, fast and it can be integrated into chips, flow cells and automated [49,50]. After biofunctionalization of the surface of a magnetic particle, we obtain a magnetic carrier (sorbent) enabling specific and selective isolation of target molecules [51]. Magnetic carriers are easily resuspended in the whole sample volume and thus readily accessible for binding with target molecule. After capture of such targer molecul the particles can be concentrated with the help of an external magnetic field and separated from the rest of the sample containing unbound (undesired) parts. Thanks to this easy and fast handling, the time for preanalytical phase is significantly reduced [16].

1.3 Bioconjugates based on gold nanoparticles

Gold nanoparticles are defined as a stable colloidal solution of clusters of individual gold atoms in the size range from 1 to 100 nm [52]. Due to these very small dimensions, they have different physical and chemical properties compared to gold in their elemental form [31]. Gold nanoparticles have become widely used in biomedicine and biotechnology mainly due to their unique physicochemical properties such as their size, shape, optical and electronic properties, large specific surface area, excellent biocompatibility, chemical stability and minimal toxicity. [55,54]. In the field of biomedicine, their applications can be divided into several main areas. They are routinely used in microscopic imaging methods [55], another area is their use in therapy [56-58], where they are used as drug carriers in drug delivery system. They are also used in hyperthermic therapy to kill targeted (malignant) cells due to their ability to heat up strongly after interacting with strong light pulses. [59,60]. They are also used in diagnostics, where they are preferably used as electrochemically or optically active labels for the detection of nucleic acids [61,62], proteins [63,64], tumor cells [65], but also, for example, bacterial cells [66].

1.4 Magnetic carriers and bioconjugates as a part of Lab-on-a-chip platforms

Thanks to the rapid development in the field of microfluidics and nanotechnologies, the miniaturization and automation of analytical methods has become a major trend in recent decades. Knowledge in the field of microfluidics has become the basis for the creation of miniature systems, where the flow of small amounts of liquid (from micro- to picoliters)

inside microchannels is precisely controlled and monitored by electrokinetic or compressive forces [67]. The result is systems that include all steps of analysis from sample introduction to detection on a single platform of a few millimeters or centimeters. These highly integrated miniature platforms include micro-valves, pumps, mixers and electrodes, which together form a system in which processes such as enrichment, separation, mixing and final sample detection take place [68].

These systems are referred to as Lab-on-a-chip (LOC) and enjoy great interest in scientific laboratories. The main advantages are the speed of sample analysis, easy operation and analysis, low consumption of sample and reagents related to the size of the platform and small volumes of channels, which of course lead to a reduction in analysis costs. Last but not least, it is the possibility of parallel analyzes and the possibility of automation. In addition, automation, miniaturization, and integration on a single platform provide higher sensitivity and reproducibility of assays [69,70].

1.4.1 Use of magnetic carriers and bioconjugates in the detection of bacterial cells within Lab-on-a-chip platforms

Magnetic particles inside microfluidic chips are generally used for several processes. These are: fluid mixing [71], selective capture of specific analytes by the immunomagnetic separation (IMS) process, and the associated transport of analytes within the chip. Since the mixing of fluids inside the chip channels is usually performed passively, using different channel geometries [72], the most important is the use of magnetic particles to separate the analyte from the sample. The integration of biofunctionalized magnetic particles into a microfluidic device significantly increases the specific surface area for capturing the analyte within the chip. This ensures a high capture efficiency [73].

Various analytical methods can be used to detect and identify microorganisms within microfluidic chips. According to the need to use specific tags, we can generally divide them into two groups. The first one are methods which, do not require any specific labeling necessary for subsequent detection. These are molecular biology techniques [74] or, for example, mass spectrometry [75]. While the second group includes methods that still require specific labeling of isolated microorganisms using a conjugate. This includes fluorescence spectrometry methods [76] and electrochemical techniques.

If the polymerase chain reaction (PCR) method is used as a detection technique inside the chip, careful control of all reaction conditions, such as accurate temperature, introduction of primers, but also other reagents, must be ensured. For example, Ganesh et al. in their study developed an integrated system for the detection of *E. coli*. After a short multiplication with simultaneous IMS (25 minutes), the sample was integrated into a chip, where magnetic separation of the particles with the captured bacteria took place. Bacteria were subsequently detected by PCR. Using this relatively simple arrangement, they were able to detect 10^3 CFU / ml in less than two hours [73]. These promising results show that by combining and miniaturizing IMS and PCR, an integrated system for easy and rapid detect bacterial cells

detection can be obtained. Like the PCR method, the Loop-mediated isothermal amplification (LAMP) method can be used. Compared to PCR, LAMP is a more modern, faster, but also more sensitive method [77]. Sun et al. developed an eight-chamber chip for the simultaneous detection of *Salmonella ssp*. in eight food samples. Again, a combination with IMS was used to concentrate the sample. Using this system, 10 CFU / μl were detected in less than 40 minutes [74].

If electrodes are integrated into the microfluidic chip, a system with very sensitive electrochemical detection is created. Chen et al. in their work describe the development of a platform for fast and sensitive *Listeria* detection. Here they combined IMS with impedimetric detection. Using such a combined microfluidic device, they reached a detection limit of 10^2 CFU / ml in one hour [78].

Very interesting is the work that describes the construction of a microfluidic device for the detection of *Salmonella typhimurium* on the principle of magnetoimmunoassay. Bacteria are separated from the sample by magnetic carriers by IMS, then labeled with antibodies conjugated with AuNPs and the whole complexes are integrated into a microfluidic device, where they are captured on the surface of a screen printed carbon electrode (SPCE). In this device, parallel detection of eight samples was possible. The whole analysis lasted 1.2 hours with LOD 7.7 cells / ml [79].

In the platform described above is interconnected the use of magnetic carriers and bioconjugates of gold nanoparticles and their subsequent integration into the chip. The advantages of immunosorbents and bioconjugates can be in practice summarized on the example of this platform. The magnetic immunosorbent serves to concentrate and separate the bacterial cells from the sample. Antibodies bound to a magnetic particle provide selectivity and specificity, and thanks to the superparamagnetic properties of the particles, the trapped bacteria can be very easily separated from the other contaminants of the solution. The bioconjugate of gold nanoparticles serves here as an electrochemically active label, where antibodies bound to AuNPs provide selective labeling of bacterial cells and the gold nanoparticle itself is responsible for the sensitive signal. In the context of the protection of human health, the development of such LOC platforms that are easy, fast, portable and sensitive is very important for comprehensive and rapid testing of a wide range of samples with different analytes. In order to build such a functional, highly integrated system, it is necessary to optimize all partial parts of the platform, as well as individual methodological procedures. The key is the development of carriers, bioconjugates and the production of microfluidic equipment. The culmination is their purposeful interconnection.

2 Experimantal part

2.1 Aims and objectives of Ph.D. study

The research tasks realized within the doctoral study were focused on biofunctionalization / bioconjugation of metal micro and nanoparticles for subsequent application in modern bioanalytical methods. Specifically, biofunctionalization of magnetic microparticles and bioconjugation of gold nanoparticles was carried out with the help of specific antibodies, synthetic peptides and oligonucleotides as biologically active ligands. A schematic representation of the three partial aims of the work is described in Figure 2.

The main goal of this work was the preparation and use of magnetic immunosorbents, which were developed to separate bacterial cells from dairy products. These experiments were performed in two consecutive European projects, LOVE-FOOD, Project No. 317742, and LOVE-FOOD2MARKET, Project No. 687681. Appropriate commercially available magnetic particles and antibodies were selected and conjugation conditions were optimized to prepare the most appropriate immunosorbents. Subsequently, their binding efficiency, colloidal, operational, storage stability and cytotoxicity were tested. The prepared magnetic carriers were used for immunomagnetic separation of bacteria from milk. With the prepared immunosorbents, the bacteria capture was optimized both in batch and microfluidic arrangements from different sample volumes from 50 µl up to 25 ml. The resultof all these sub-steps was the final detection of bacteria isolated from the sample using the above mentioned immunosorbents on two detection devices: I) on a LOC platform with a sensor operating on the surface acoustic wave II) on a printed electrode working on the anodic dissolution voltammetry. At the same time, the successful integration of the developed magnetic immunosorbent into the microfluidic magnetic bed took place.

In addition to the preparation of magnetic carriers with antibodies, the partial task was to optimize the procedures for the preparation of magnetic particles with synthetic peptides. These immunosorbents have been prepared to isolate specific antibodies from the serum of patients with non-degenerative diseases.

The last task was the preparation of bioconjugates of gold nanoparticles. Procedures for binding on the surface of functionalized gold nanoparticles and particles without functional groups have been optimized. Specific antibodies and synthetic oligonucleotides were used as biologically active ligands. Bioconjugates of gold nanoparticles and specific antibodies were subsequently used as labels for the electrochemical detection of *Listeria monocytogenes.*

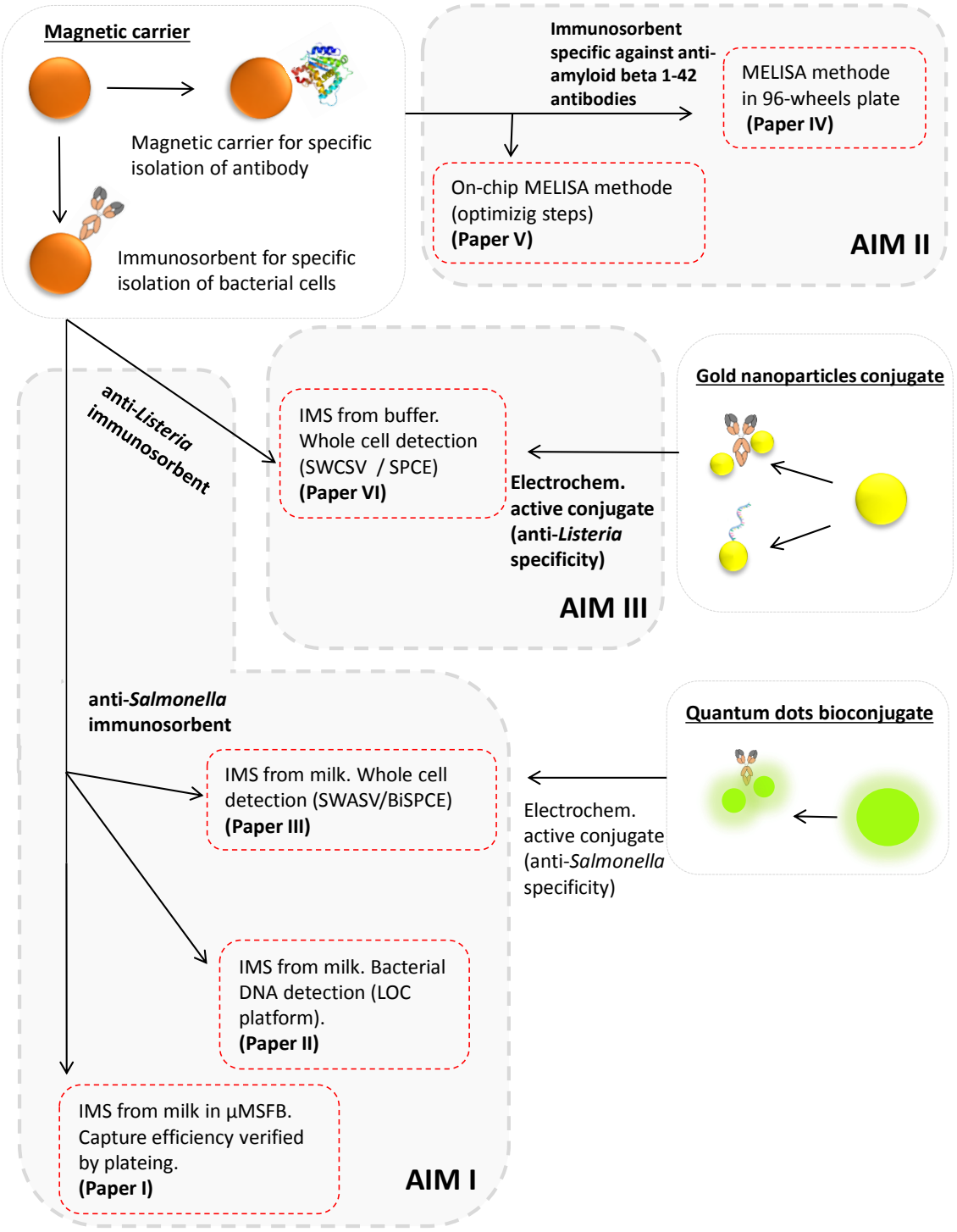

Figure 2: Schematic representation of the objectives of the dissertation.

2.2 Results

2.2.1 Paper I and II

Main part of the dissertation includes experiments that were performed in two consecutive European projects LOVE-FOOD (2012-2016) and LOVE-FOOD2MARKET (2016-2019). The output of these projects was to develop a highly integrated, portable and user-friendly platform for the detection of bacteria in dairy products. The end product should be able to detect only one bacterial cell in a 25 ml sample in less than 4 hours. All optimization steps were performed with *Salmonella ssp*. and the optimized procedures were subsequently applied to other bacteria (*Listeria monocytogenes, Escherichia coli* and *Bacillus cereus*). Our task within the projects was to prepare the magnetic immunosorbent for specific isolation of bacteria from the sample that will be compatible with the whole platform. During optimizations steps, several commercially available magnetic carriers and commercially synthesized antibodies were tested in order to develop the most stable and effective carrier. The long-term experience of our research team with magnetic beads and their *in vitro* application were taken into account when considering the selection and subsequent modification of magnetic particles. The selection of suitable antibodies was guided not only by the quality but also by the price and availability on the market.Immunosorbents were tested for their colloidal, operating and storage stability, cytotoxicity, binding activity, specificity, selectivity and other necessary parameters. After proper characterization of the carrier, we got 4 specific and effective immunosorbents for 4 different bacteria. Only the most interesting and innovative results were published.

Paper I: Advanced immunocapture of milk-borne *Salmonella* by microfluidic magnetically stabilized fluidized bed

At the beginning of the first aforementioned project, a new type of microfluidic chip working on the principle of a magnetically stabilized fluidized bed was developed and later patented at a partner workplace, the Curie Institute in Paris. This chip was developed to capture bacterial cells in a microfluidic arrangement and provide all benefits associated with the microfluidics .

The goal of this study was to develop an efficient magnetic immunosorbent with specificity for *Salmonella (S. typhimurium)* a representative of common foodborne pathogens. Attention was paid to the choice of superparamagnetic microparticles and the anti-Salmonella Ab. We were focused on microspheres in the size range between 1 and 3 μm, suitable for in vitro laboratory diagnostics and at the same time suitable for microfluidics. Particles with terminal carboxyl functional groups which are suitable for covalent biofunctionalization using carbodiimide chemistry were preferred. Five different commercial microspheres from different suppliers were compared and binding capacity of the microparticles was evaluated. The highest binding efficiencies (82%) were repeatedly achieved with Promag microparticles from Bangs Laboratories, Inc. (Fishers, IN, USA). Then the behaviour of Promag particles in a microfluidic PDMS chip was examined and found to be excellent.The most important parameters which considered during the selection

of the anti-Salmonella Ab were high immunoreactivity, purity, and integrity of the Ab. Goat anti-Salmonella sp. provided by KPL (Gaithersburg, MD, USA) were chosen as the best due to highest capture efficiency of bacteria that did not fall below 89.5 ± 3.3% after IMS from10^2 CFU/ml.

The CE of *S.* typhimurium using Promag-KPL immunosorbent was evaluated directly in whole UHT milk in batch and in microfluidic chip. Milk samples were spiked with an increasing concentration of *S.* typhimurium (from 10^1 to 10^5 CFU per reaction volume, i.e. 50 µL, which corresponds to 2×10^2–2×10^6 CFU/mL), followed by IMS (see Fig. 3). In the case of microfluidic chip, excellent capture rates were achieved even in low-concentrated samples (92.5 ± 5.4%), and they did not fall under 90% (with the exception of the highest tested bacterial concentration).

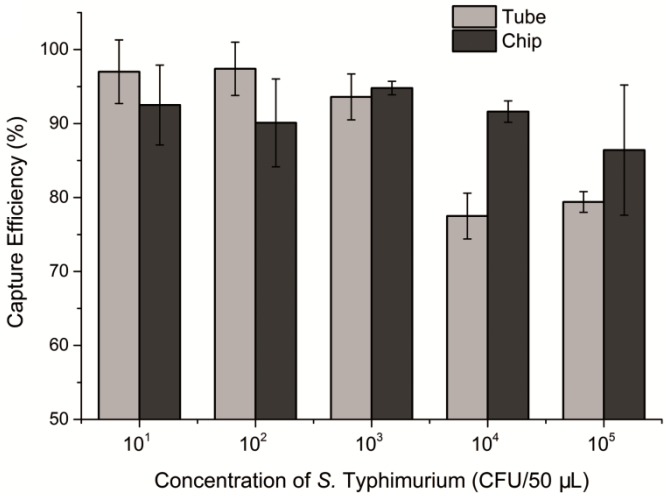

Figure 3: The effect of increasing concentration of S. typhimurium on capture efficiencies from UHT whole milk using Promag-KPL (reaction volume: 50 µL, amount of immunosorbent: 50µg).

After that, an aliquot of milk was artificially spiked with a mixed culture of *S.* typhimurium and *E. coli* in two different ratios (1:1 and 1:100) to confirm the selectivity of the Promag-KPL immunosorbent in the microfluidic chip (Figure 4). Both achieved CE exceeded our expectations; the surplus (100:1) of competing bacteria increased the CE of *S.* typhimurium to as high as 99.3 ± 0.5%. The nonspecific capture of *E. coli* was negligible and only reached 2.1 ± 0.8%.

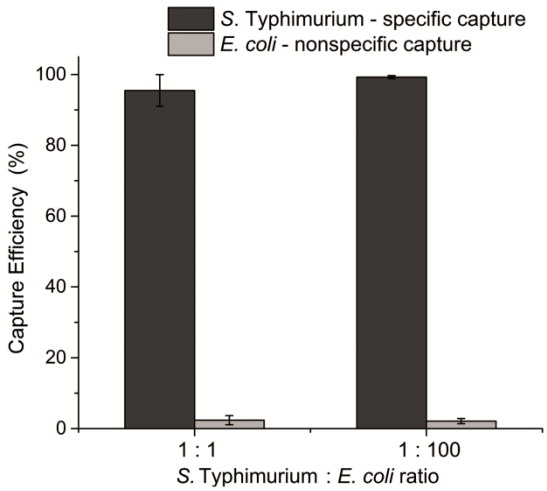

Figure 4: The effect of presence of competing bacteria (*E. coli*) on capture efficiency of S. *typhimurium* in chip. Mixed culture of S. *typhimurium* and *E. coli* in two different ratios (1:1 and 1:100), reaction volume: 50 μL, amount of immunosorbent: 50μg.

Author's contribution: In this publication, the author participated in all experiments related to the optimization of IMS in the chip, which were the content of her internship at the Institute Curie in Paris. She modified the method for preparing a sample for electron microscope analysis an and participated in the writing of the manuscript.

Paper II: Micro-nano-bio acoustic system for the detection of foodborne pathogens in real samples

During the second project in a partner laboratory of the Institute of Molecular Biology and Biotechnology – FORTH, Heraklion, Crete, an integrated LOC platform for the isothermal amplification of target-DNA in a microfluidic chamber followed by detection with a surface acoustic wave (SAW) sensor was developed. The goal of this study was to take advantage of IMS and connected with LOC platform to get a micro-nani-bio acoustic system for detection od bacteria cells provides the means for ultra-fast, sensitive and cost-effective analysis, always within the existing legislation framework.

Since the standardized sample according to ISO standards is 25 ml of milk, it was not easy to conect the concentration of the sample with the rest of the platform.
Our initial task was to assess the minimum time needed for preenrichment of sample to have enough bacteria for subsequent LOC amplification and detection. Starting from 1 to 25 cells in 25 ml of milk, we performed different pre-enrichment times varying from 3 to 10 h at 37 °C without adding any growth medium (selective or non-selective). It was observed that around 500 CFU were reached after 3 h of culturing and approximately twice as many within 4.5 h.

In a following step, magnetic beads functionalized with polyclonal anti-Salmonella *typhymurium* antibodies (Promag-KPL immunosorbent) were added in milk cultures spiked with *Salmonella* cells. In search of the optimum conditions for bacteria cells capturing, the following parameters were taken into account: amount of particles (1.0, 0.5and 0.2 mg) added in 25 ml of milk, time at which beads were added in the sample (at the beginning or end of the culture period) and crossreactivity with other cells. It was repeatedly confirmed that bacteria cells were able to grow and divide even when they were captured on the surface of the beads. For this reason, samples containing immunobeads from the start of incubation were cultured for 2.5, 3.0 and 3.5 h and then put into contact with a magnet for 20 min. Resuspended beads were plated and colonies enumeration showed an average of 85% capturing efficiency for all growth times tested (Figure 5).

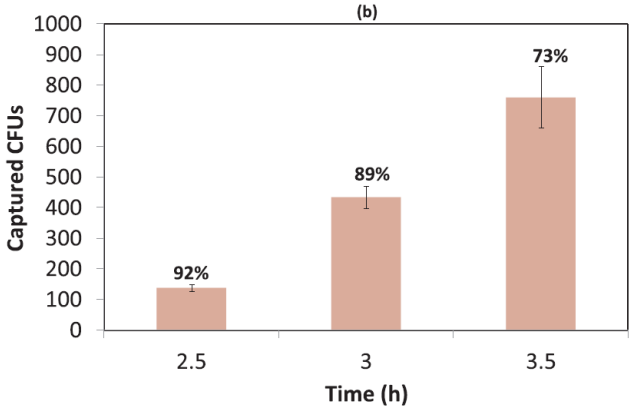

Figure 5: Plating results showing the absolute number of *Salmonella* bacteria extracted with the magnetic immunobeads as a function of the culture time starting from ~1 cell in 25 ml of milk; the capturing efficiency corresponds to Nc / N0 x 100, where Nc is an average total number of cells captured with the immunosorbent (CFU/ml) and N0 is an average total number of cells counted after plating of the original cell suspension (growing without beads) (CFU/ml). In all cases, immunobeads were added at the beginning of the enrichment step.

Such optimized IMS was connected with LOC platform. The complete procedure for the detection of food pathogens comprised the following steps: (1) 25 ml of milk were mixed with 1 mg of magnetic particles functionalized with antibodies and incubated at 37 °C for 3 h; (2) Enriched bacterial cells were captured by an external magnet, washed with buffer and resuspended in 25 µl of a solution containing Triton-X 100 for lysis and the LAMP ingredients and (4) The 25 µl is injected in the LOC where isothermal DNA amplification is performed at ~65 °C for 30 min followed by rapid acoustic detection (~60 s) using a SAW sensor. Using our methodology and integrated platform, we have achieved the detection of 1 cell in 25 ml of milk.

Author's contribution: In this publication, the author optimized the procedures for IMS from a volume of 25 ml. During an internship at the Institute Pasteur in Paris, she participated in experiments to optimize the preenrichment step and immunomagnetic separation of bacterial

cells from a 25 ml milk sample. And during an internship in the laboratory of the Institute of Molecular Biology and Biotechnology - FORTH, Heraklion, Crete, she participated in the connection of optimized procedures for the preenrichment and IMS of bacteria from a sample with the LOC platform. She also participated in writing the corresponding chapters of the manuscript.

2.2.2 Paper III

Paper III: Direct culture-free electrochemical detection of Salmonella cells in milk based on quantum dots-modified nanostructured dendrons

Rapid, timesaving and sensitive method plays a key role in the detection of bacterial cells contamination. Classical cultivation methods, are still among the most widely used but they are relatively time consuming, especially due to the time required for enrichment in nutrient media and growth of colonies on plates. For this reason, a new method which could potentially replace classical methods has been optimized in our laboratory.

Our goal was to create a unique combination of a very specific and selective magnetic carrier with a highly sensitive electrochemically active conjugate. The principle of our developed system involved the magnetoimmunocapturing (with ProMag-KPL immunosorbent as mentioned in paper I a II) of target bacterial cells together with specific labelling using an electrochemically active probe. After the incubation phase the anti-*Salmonella* IgG – *Salmonella* – anti-*Salmonella* IgGDs-CdTeQDs immunocomplexes were magnetically separated and finally analyzed by square-wave anodic stripping voltammetry. For the preparation of sensitive electrochemically active probes, polymeric dendrons modified with quantum dots were exploited. Commercially available bifunctional dendrons synthesized of polyester bis-MPA with defined number of functional groups (1 amino/8 carboxyl) were combined with CdTe/COOH QDs (cadmium content 0.6 mg/mL, verified by ICPOES). Bifunctionality of dendrons allowed their functionalization with QDs on its N-side, whereas the opposite C-side with eight carboxylic groups ensured their stable, and presumably, multi-site attachment onto antibodies. This unique combination has not yet been used for any application. Moreover, the insertion of dendrons improved the elimination of non-specific sorption of the labelled antibodies (anti-Salmonella IgGDs-CdTeQDs) leading to high current responses of negative controls. Using this immunosensor, even 4 *Salmonella* cells were detected in 1 ml of milk (Figure 6).

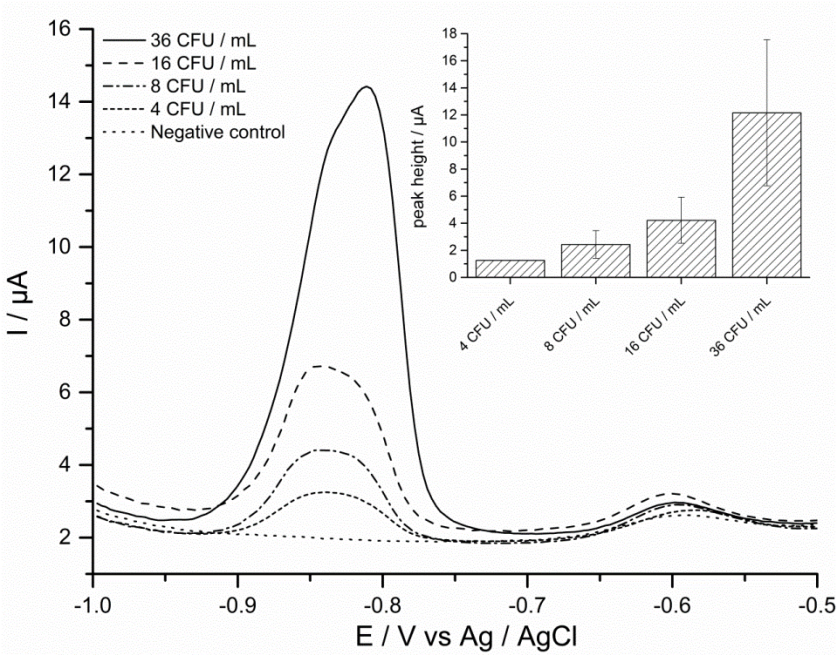

Figure 6: Square-wave voltammograms and current responses (inset graph) of the detection of Salmonella cells from milk using our electrochemical immunosensor. Concentrations of Salmonella artificially added to the samples: 36, 16, 8, and 4 CFU/mL. Measurements were performed with BiSPCE using electrochemical detection conditions specified in the experimental section (n= 3).

Author's contribution: The author contributed for the most parts of experiments as well as in the writing of manuscript. The author prepared a magnetic carrier, participated in the preparation of the conjugate, performed all separations steps, was presented during the electrochemical measurement and wrote a manuscript.

2.2.3 Paper IV and V

Rapid and early stage diagnosis is a key step in initiating treatment and suppressing of pathophysiological proccesses in almost all diseases. Detection of anti-Aβ1-42 antibodies (anti-Aβ1-42 IgG / IgM) as potential biomarkers of Alzheimer's disease is now performed by solid phase enzyme-linked immunosorbent assay (ELISA). In order to increase the sensitivity of detection of anti Aβ 1-42 antibodies, magnetic microparticles were used instead of the surface of the wells of the microtiter plate for antigen fixation (Aβ1-42). The aim was to increase the specific surface for the capture of possible antibodies and thus achieve a higher sensitivity of their detection. Protocols of MELISA have been optimized for batch-wise arrangement using a microtiter plate (Paper IV) and for chip-based application (Paper V).

Paper IV: Development of the magnetic bioaffinity carrier for the anti-amyloid *beta*1-42 antibodies detection

The main task was to prepare magnetic particles functionalized with synthetic Aβ1-42 peptide for the antibody detection in serum of AD patients. Due to the fact that serum is a very complex biological material, great attention was focused on the prevent of non-specific interaction of magnetic carrier with undesirable components of serum. Moreover due to the small size of Aβ1-42 peptide, it was necessary to ensure their steric availability for the binding sites of captured antibodies. Ligand binding via spacer has proven to be a suitable solution. Bifunctional polyethylene glycol was used as a spacer. It was experimentally confirmed that the spacer increased the binding capacity of the magnetic carrier. In the Figure 7 you can see the higher OD value in the case of bioaffinity carrier (particles with spacer) which corresponds to the higher amount of antibodies captured with such carrier. Data suggest a significantely positive effect of the bisamino-polyethylene glycol spacer.

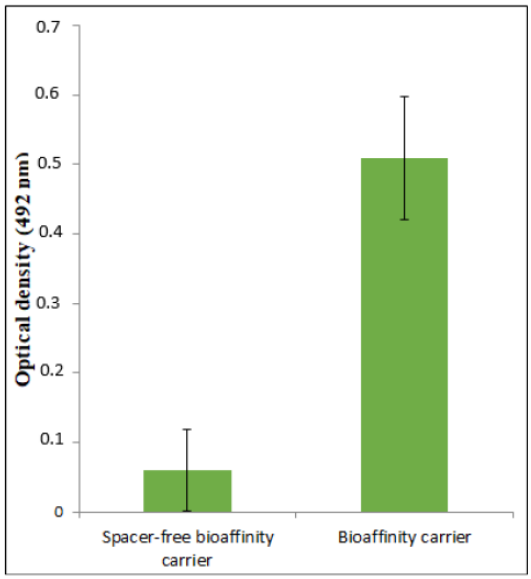

Figure 7: Influence of Ab 1-42 peptide magnetic bioaffinity carrier preparation method to acquired values anti-Ab Abs detected in control serum. OD values are acquired from three repetitions.

The optimized protocol was then used to analyze a series of samples from patients with established Alzheimer's disease. The aim of this pilot experiment was to observe different levels of specific antiodies between patients and helthy controls. Results are summarized in the Figure 8. The antibody levels measured in the control sera group showed a large dispersion. Similarly, large dispersion of data was evident for sera from patients with AD. A single group of MKP patients data gave homogenous results. The developer magnetic bioaffinity carrier used for the anti-Aβ Abs detection was able of detectin anti-Aβ Abs with some differences between the groups of sera. But these data are only pilot one and did not revealed statistically significant differences between the control samples and those obtained from patients with AD. From this reason another properly study of the role of anti-Aβ antibodies in the body is neccessary.

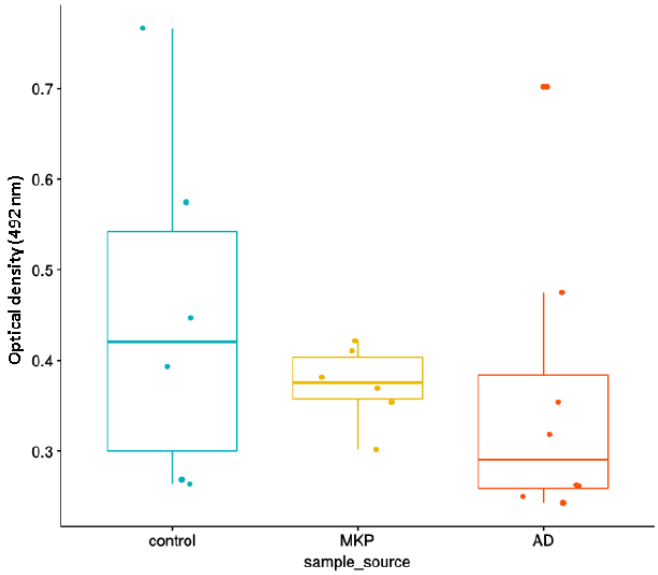

Figure 8: Box plot of acquired values anti-Aβ Abs detected in control and patients sera using the Aβ 1-42 peptide magnetic bioaffinity carrier. MKP-mixed cognitive impairment, AD-Alzheimer disease. Values are acquired from three repetitions.

Author's contribution: In this work, the author participated in the preparation of a magnetic carrier and optimization of the magnetic ELISA method on magnetic particles. During writing the publication author participated in the methodological part and discussion of the results.

Paper V: On-chip ELISA on magnetic particles: isolation and detection of specific antibodies from serum

The aim of this work was to optimize the MELISA protocol for on-chip antibodies detection. Simple commercial microfluidic set from microfluidic ChipShop was employed. This device provides easy liquid application, automatic mixing of the MPs and high ratio of used MPs vs. chamber volume. Therefore, this setup enables one to capture specific antibodies on the surface of the magnetic biofunctionalized microparticles that can be (i) eluted and subsequently detected and/or (ii) directly detected by MELISA protocol upon the application requirements. Both MELISA arrangements (in microtitration plate and/or in microfluidic chip) were performed simultaneously under the same conditions and the results were subsequently compared. The preliminary results of on-chip MELISA and MELISA in microtitration plate for detection/isolation of anti-chymotrypsin antibodies are presented in this paper. The same protocol for determination of anti-chymotrypsin antibodies in hyperimmune porcine serum was repeated in three subsequent days on always fresh aliquot of the bioactive carrier with immobilized chymotrypsin. The data from the three measurements in microtitration plate and their trend lines are shown in Figure 9. The coefficient of determination (R2) were for day 1 (0.986), for day 2 (0.906) and for day 3

(0.845). The standard deviation between the three measurements in microtitration plate for each serum dilutions were: 1:30,000 (0.057); 1:40,000 (0.120); 1:60,000 (0.081); 1:80,000 (0.065); 1:120,000 (0.029). The results from the on-chip application, for serum dilution 1:30,000, are also presented in Figure 9 (circled one) and their values went along the trend of the results from the microtitration plate. The standard deviation among the three subsequent measurements in microfluidic setup was 0.183. Such variability between the measurements were probably due to the technique of the final sample volume uptake from the outlet of the device. For the future it is neccessary to develop a robust technique which would enable us to reach the more reproducible results.

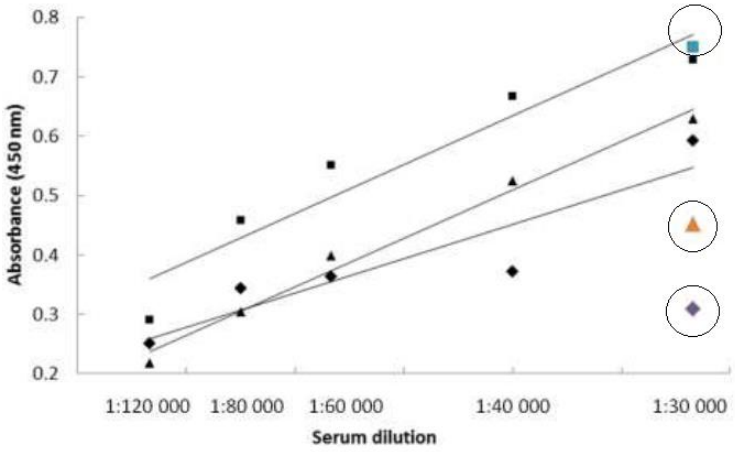

Figure 9: The results from MELISA determination of anti-chymotrypsin specific antibodies in hyperimmuneporcine serum (high titer of specific antibodies to chymotrypsin). The measurement of 5 different serumdilutions was repeated in 3 subsequent days. The black in the lines symbols correspond to results from MELISA on microtitration plate and the circled symbols correspond to on-chip MELISA (only for dilution 1:30,000). The day one (triangel), the day two (square), the day three (rhombus)

Author's contribution: In this work, the author performed experiments related to the magnetic ELISA method in a microtiter plate. While writing, she participated in chapters related to the above-mentioned experiments.

2.2.4 Paper VI

Paper VI: Different approaches for preparation of AuNPs-based conjugates as a signal-generating unit for electrochemical biosensors

The last part of this dissertation was the effort to develop gold nanoparticles bioconjugates. The main goal of this work was to optimize the binding strategy for modification of AuNPs with thiolated oligonucleotides or specific antibodies. Two different types of AuNPs, two different bonds and two different types of biomolecules were selected for preparation of conjugates potentially usable as a signal generating molecule for electrochemical biosensors.

Surface-unmodified AuNPs were used to bind synthetically prepared DNA oligonucleotides with a reactive thiol group. Under standard conditions, the DNA molecules are highly negatively charged and being repelled by AuNPs. To screen the negative charge of AuNPs, a solution with high concentration of NaCl should be added although this addition of highly concentrated salt may lead to the irreversible aggregation of particles. This problem can be solved by the process called "salt aging", when such a salt is gradually added together with the molecules of DNA. While DNA is still at an excess against the salt, the aggregation of particles is then prevented. In this work, four various protocols working with different buffers and different processes using the salt aging method were performed and compared; see Figure 10.

	Gold nanoparticles		Oligonucleotides	Binding
	Size [nm]	Concentration of AuNP in reaction [nmol]	Concentration of oligonucleotides in reaction [nmol]	Protocol
1	10	230	5	Incubation for 4 h under 37 °C; in **water**; salt stabilization 0.1M NaCl, overnight 38 °C
2				**10 mM citrate-HCl buffer pH 3**; lab. temp. incubation 10 min.; salt stabilization 0.3 M NaCl; lab. temp. incubation 20 min
3				**5 mM HEPES buffer pH 9**; 16 h incubation at lab. temp.; than salt stabilization 0.1 M NaCl.
4				**10mM PBS buffer pH 7.4** , 16 h incubation at lab. temp.; add **0.01% SDS**, salt stabilization 1M NaCl, 30 min incubation at lab. temp.

Figure 10: Overview of binding protocols / procedures

For optimizing steps, the DNA molecules labelled with fluorescent tag Cy3 were used for easier determination of capture efficiency, when the intensity of fluorescence correlated with the concentration of bound DNA oligonucleotides. The capture efficiency expressed by numbers of DNA chains bound on the surface of AuNPs was calculated from calibration curve. Because there exists a mathematical relationship for predicting suitable saturation of the nanoparticles surface with oligonucleotides, we could compare our results with theoretical assumption. Namely, one should bound around 68 ± 10 chains per one unit of AuNP with diameter 10 nm. We were not far from this prediction, so there was no need to change the protocol to increase the capture efficiency. Except the binding in water (63 chains of DNA per one AuNP), there were no significant differences among the individual protocols; see Figure 11. The most effective was the procedure with 10mM PBS containing 0.01% SDS used as the detergent avoiding undesirable aggregation of AuNPs. We could estimate that, by this protocol, 154 of DNA oligonucleotide chains were bound on the surface of one nanoparticle. For future application, protocol with citrate buffer was chosen. As we have estimated, around 134 of DNA oligonucleotide chains were bound on the surface of one nanoparticle. Moreover, from the sufficient binding efficiency, this protocol was not time-consuming and the conjugates could be prepared in 1.5 hours.

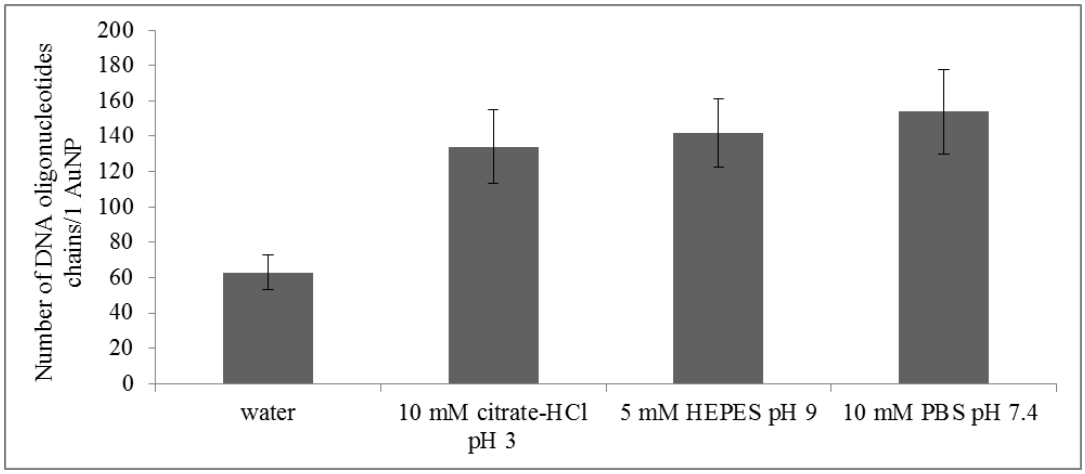

Figure 11: Immobilization of reduced oligonucleotides on AuNPs in four different buffers

The second part of this work dealt with the modification of AuNPs by antibodies via crosslinker. On the surface of AuNPs containing functional groups, the specific anti-Listeria antibodies were bound. One- or two-step carbodiimide methods were tested. In addition, the different ratios of AuNPs and antibodies (1:1 or 2:1, resp.) were optimized. The functionality and usability of these conjugates were then verified by model samples with *Listeria* cells. For preliminary experiments, two conjugates were used (prepared by one- or two steps method with initial ratio of AuNPs:Ab 2:1). Conjugates were mixed together with anti-*Listeria* magnetic immunosorbent and sample spiked with *Listeria* cells. During 60 min. incubation, *Listeria* cells should be captured by magnetic immunosorbent and so caught cells were labelled with the conjugates at the same time. After incubation, these complexes were captured from the samples (due to magnetic immunosorbent), washed, and the signal generated from electrochemically active conjugate measured on SPCE. In Figure 12, a voltammogram with positive and negative control is drawn clearly showing the differences. The signals of both negative controls were nearly 0 μA and for positive samples provided signals –0.157 μA and 0.165 μA, resp. These results indicate that the prepared conjugates give rise to an electrochemically measurable signal. Using these advanced conjugates we were able to detect 10^4 CFU in 1ml of milk.

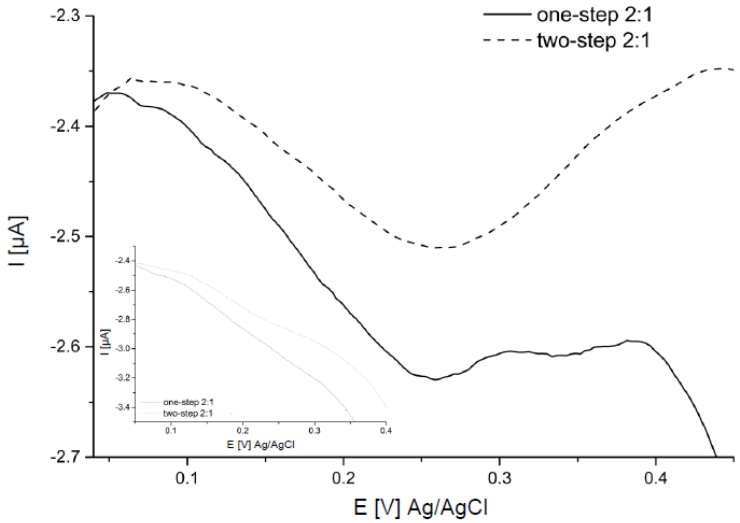

Figure 12: Square-wave voltammogram of Listeria cells detection with the help of prepared conjugates as a part of biosensor. Measurements were performed with SPCE upon conditions of the electrochemical detection specified in the experimental

Our electrochemical experiments have confirmed that AuNPs provide lower electrochemical responses (in the order of tenths of µA units) compared to, for example, conjugates with quantum dots. Thus, for next experiemnts we have to prepare conjugate with higher amount of AuNPs per 1 molecule of IgG. This is the only way to achieve the required sensitivity.

Author's contribution: The author contributed in this work as well as in writing the publication is majority. With the exception of electrochemical measurements, she performed all the experiments.

Conclusions

The presented dissertation dealt with the biofunctionalization of magnetic microparticles and the conjugation of gold nanoparticles with a bioactive ligand. The resulting carriers and bioconjugates were developed as part of analytical methods, which combined the physical and chemical properties of metal particles with the biological properties of ligands bound on their surface. The binding of specific antibodies or peptides on the surface of magnetic particles resulted in so-called sorbents, which were used for example, to specifically isolate and purify bacterial cells from milk samples or selected dairy products or to isolate clinically important antibodies from human serum. Gold nanoparticles and their electrochemical properties were used for the production of bioconjugates as one part of an electrochemical sensor for direct detection of pathogenic bacteria in milk.

The largest part of the work was devoted to the preparation of immunosrobents based on magnetic microparticles and their use for the isolation of bacterial cells from milk samples. This was a combination of a short-term pre-enrichment phase and immunomagnetic separation. The aim was to eliminate the time-consuming pre-enrichment phase (tens of hours) commonly used in practice. With the help of by us developed immunosorbent, very good results of binding efficiency were achieved. The conditions of effective isolation from standardized 25 ml of the initial sample, in our case cow's milk or homogenized Ricotta cheese, were optimized. Furthermore, the immunosorbent showed excellent selectivity even in the presence of another bacterial culture, which was in multiple excess. Furthermore, it was experimentally confirmed that this carrier can also be used as an integral part of a microfluidic device. Detection of pathogenic bacterial cells was performed in the LOC platform together with integrated bacterial cell lysis, DNA amplification and SAW detection. In accordance with the legislation, this method provided sufficient sensitivity for the detection of bacterial cells, when the total analysis time did not exceed 4 hours. It could therefore be a suitable variant for field testing of food and clinical samples, where it is not possible to use classical confirmation methods. In addition, with the great benefit of time saving and without the need for complex instrumentation.

In addition to modern biosensors and LOC platforms, magnetic carriers can also be advantageously used in traditional methods, such as ELISA. Here, magnetic carriers can be used as a solid phase in order to increase the sensitivity of the method and to shorten the incubation times in the individual steps of the method. In our case, we used this method to detect anti-Aβ 1-42 antibodies from the sera of patients with Alzheimer's disease. The key step was the preparation of the magnetic carrier. The magnetic ELISA assembled by us proved to be a suitable alternative, but it is still necessary to work more on the surface modification of the magnetic carrier, especially in terms of non-specific sorption of undesirable proteins and ballast components from serum, which is very disruptive in the assay.

In the next phase of the research, we decided to experimentally verify whether it is possible to create a biosensor for the detection of bacterial cells occurring in small concentrations in

complex and highly heterogeneous material. Our strategy was based on the combination of a very specific and selective magnetic carrier (see above) with an electrochemically active conjugate. The conjugate was an anti-salmonella antibody labeled with CdTe quantum dots, whereas to increase the signal intensity, a multifunctional dendron was incorporated into the conjugate structure. It turned out that the combination of quantum dots and dendrons created a sensitive electrochemical label, the use of which has not yet been published. In combination with effective isolation using a magnetic carrier, a very sensitive immunosensor was created with the possibility of use for fast and cheap detection of bacterial contamination. In addition, AuNPs-based conjugates have been tested as potential electrochemical labels for the detection of *Listeria monocytogenes* contamination. However, our results so far do not provide the necessary sensitivity.

In conclusion, it can be stated that one of the most important components of biosensors, which clearly decides about its analytical parameters, is the conjugate carrying the signal generating label. Another advantage is the combination of an electrochemical sensor with immunomagnetic separation, where we obtain an analyte in sufficient concentration and quality. The ability to integrate the entire system into the LOC platform is another benefit related to this technique.

This can create integrated LOC-based detection platforms for fast and accurate sample testing, which plays today a key role in human health protecting.

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