Electrochemical magneto-immunosensing of Salmonella based on nano and micro-sized magnetic particles

D Brandão1, S Liébana1, S Campoy2, P Cortés3, S Alegret1, M I Pividori1,3
1Grup de Sensors i Biosensors, Unitat de Química Analítica, Universitat Autònoma de Barcelona, 08193, Bellaterra, Barcelona.
2Grup de Microbiologia Molecular, Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Spain
E-mail: isabel.pividori@uab.cat

Abstract. A very simple and rapid method for the detection of S. enterica is reported. In this approach, the bacteria were captured and preconcentrated with magnetic particles through an immunological reaction. A second polyclonal antibody labeled with peroxidase was used for the electrochemical immunosensing based on a magneto-electrode. Different nano and micro-sized magnetic particles were evaluated in this approach. The ‘IMS/m-GEC electrochemical immunosensing’ system shows a limit of detection of $5 \times 10^4$ and $1 \times 10^4$ CFU mL$^{-1}$ in BHI culturing media when micro and nanoparticles are used respectively. These LOD were achieved in a total assay time of 1 h without any previous culturing preenrichment step. Moreover, this system was able to clearly distinguish between food pathogenic bacteria such as S. enterica and E. coli. The features of this approach were discussed and compared with conventional culture methods.

1. Introduction
The increasing incidence of food-borne illnesses is becoming an important public health concern for consumers worldwide [1]. Conventional methods for food pathogen detection depend upon microbiological culturing techniques combined with standard biochemical identifications. These methods are time-consuming, laborious and might introduce sampling and enumeration errors due to the extremely low concentration of pathogenic bacteria in food samples able to produce disease [2].

In this context, electrochemical immunosensors presents a good advantage due to their high sensitivity, rapidity, low cost and possibility of being a hand-held platform for field applications [3].

Magnetic particles with sizes ranging from a few nanometres up to micrometer dimensions have been widely used for detection, concentration, separation, purification and identification of specific targeting ligands [4]. Their easily surface modification with functional organic molecular groups, such as amine, hydroxyl, carboxyl, aldehyde or thiol allows the immobilization of biomolecules (enzymes, antibodies, oligonucleotides or other biologically active compounds). Moreover, the possibility of being manipulated by an external magnetic field gradient provides a selective capture of target cells, offering an attractive technology for the design of biosensors systems, as well as microfluidic devices [5-7]. Recently in our group, a rapid strategy for the detection of S. enterica in milk combining immunomagnetic separation (IMS) and electrochemical magneto-immunosensing was developed,

3 To whom any correspondence should be addressed.
using commercial anti-Salmonella particles [3]. In the present work, an electrochemical immunosensor for Salmonella detection with magnetic nano and micro-sized particles is reported. The strategy is based on the detection of the whole bacteria by a double immunological recognition. The effect of the size particle was evaluated by comparing nano and micro-sized magnetic particles modified with antibodies for the immunomagnetic separation (IMS) of the bacteria. Then, the captured bacteria were detected using a polyclonal anti-Salmonella-HRP antibody. The modified magnetic particles were easily captured by a magneto-electrode based on graphite-epoxy composite (m-GEC), which was also used as the transducer for the electrochemical detection. The features of this approach were discussed and compared with conventional culture methods.

2. Materials and methods

2.1. Chemicals and Biochemicals

Dynabeads M-280 Tosylactivated (tosyl-MMP, Product No. 142.04) and Anti-Salmonella magnetic beads (Product No. 710.02) were purchased from Dynal Biotech ASA (Oslo, Norway). Carboxyl-Adembeads (carboxyl-MNP, Product No. 0213) were supplied by Ademtech (Pessac, France). Mouse monoclonal Anti-Salmonella (Product No. ab 8274), rabbit polyclonal Anti-Salmonella (HRP) (Product No. ab 20771) and goat polyclonal secondary antibody to mouse IgG (HRP) (Product No. ab 20043) were purchased from Abcam (Cambridge, UK). The graphite-epoxy composite was prepared with graphite thin powder (1.04206.2500, Merck, Darmstadt, Germany) and Epo-Tek H77 (epoxy resin and hardener both from Epoxy Technology, USA). Hydrogen Peroxide and TMB (3,3’,5,5’-tetramethylbenzidine) solutions used for optical measurements (TMB Substrate Kit, Reference no. 4834021) were purchased from Pierce (Rockford, USA).

Activation and storage buffers used for carboxyl-MNP were supplied from Ademtech (Pessac, France). All other buffer solutions were prepared with milli-Q water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions was: MES buffer (0.1 mol L⁻¹ [2-(N-(morpholino)ethanesulfonic acid)], 0.9 % (w/v) sodium chloride, pH 4.7); borate buffer (0.1 mol L⁻¹ H₃BO₃, pH 8.5); ammonium sulphate buffer (3 mol L⁻¹ [(NH₄)₂SO₄] in borate buffer); phosphate blocking buffer (10 mmol L⁻¹ sodium phosphate, 0.5% w/v BSA, pH 7.4); Ademtech blocking buffer (activation buffer, 0.5% w/v BSA); phosphate storage buffer (10 mmol L⁻¹ sodium phosphate, 0.1% w/v BSA, 0.02 % (w/v) sodium azide, pH 7.4); PBS (10 mmol L⁻¹ , pH 7.4); PBS 3% BSA (0.01 mol L⁻¹ , pH 7.4, 3% w/v BSA); sulphuric acid solution (2 mol L⁻¹ H₂SO₄); PBST (10 mmol L⁻¹ sodium phosphate, 0.8% w/v NaCl, pH 7.4, 0.05% v/v of Tween 20), PBST 2% BSA (10 mmol L⁻¹ sodium phosphate, 0.8 % w/v NaCl, pH 7.4, 0.05 % v/v of Tween 20, 2% w/v BSA) and PBSE (0.1 mol L⁻¹ sodium phosphate, 0.1 mol L⁻¹ KCl, pH 7.0).

The bacterial strains used in this work were S. enterica serovar Typhimurium LT2 and E. coli DH5a. Both strains were grown at 37°C in Luria-Bertani (LB) agar plates. Bacterial suspensions were performed in Brain Heart Infusion (BHI) from freshly grown plates of the appropriated bacteria and its turbidity was adjusted at OD₆₀₀ of 0.2. The exact concentration of each bacterial suspension was quantified by plating serial dilutions onto LB plates.

2.2. Instrumentation

All the incubations and washing steps were performed on microtiter plates under shaking conditions using a MiniShaker MS1 (IKA, Germany). Temperature-controlled incubations with Eppendorf tubes were performed in an Eppendorf Thermomixer compact. Covalent immobilization of Anti-Salmonella antibody on the magnetic particles was performed using a rotor for test tubes (Micro Bio Tec-TTR 79). Magnetic separation during the washing steps was performed using a magnetic separator Dynal MPC-S (Product No. 120.20D, Dynal Biotech ASA, Norway). Polystyrene MaxiSorp microplates were purchased from Nunc (Catalogue no. 442404, Roskilde, DK). Optical measurements were performed on a TECAN Sunrise microplate reader with a Magellan v4.0 software. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc, USA). A three electrode setup was used comprising a platinum auxiliary electrode (Crison 52-67 1), a double
junction Ag/AgCl reference electrode (Orion 900200) with 0.1 mol L⁻¹ KCl as the external reference solution and a working electrode (the magneto electrode, m-GEC). The detailed preparation of the m-GEC electrodes has been extensively described by Pividori et al. [8, 9].

2.3. Covalent immobilization of anti-Salmonella antibody on magnetic particles and coupling efficiency

Anti-Salmonella antibody was covalently coupled on both magnetic particles, tosyl-modified magnetic microparticles (tosyl-MMP, 2.8 μm, 30 mg mL⁻¹, 2 x 10⁸ MP mL⁻¹) and activated carboxyl-modified magnetic nanoparticles (activated carboxyl-MNP, 300 nm, 30 mg mL⁻¹, 1 x 10¹² MP mL⁻¹). Before antibody immobilization, carboxyl-MNP were activated as follows, 140 μL of carboxyl-MNP were resuspended and washed twice in 1 mL of activation buffer then, 4 mg mL⁻¹ of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECD) and 8 mg mL⁻¹ of N-hydroxy-sulfosuccinimide (sulfo-NHS) were added at a final volum of 320 μL in MES buffer and incubated under shaking at room temperature during 10 h.

For the covalent immobilization, a volume of 140 μL of both tosyl-MMP and activated carboxyl-MNP were washed twice with 1 mL of borate buffer and activation buffer respectively, avoiding foaming. Afterwards, tosyl-MMP were resuspended in 560 μL of borate buffer and 400 μL of ammonium sulphate buffer, while activated carboxyl-MNP were resuspended in 960 μL of borate buffer. Then, 40 μL of the Anti-Salmonella antibody was added, reaching a concentration of 83.3 μg mL⁻¹ in the immobilization solution. The MPs were incubated for a total reaction time of 13 h, 8 h at 37°C and 4 h at room temperature, with slow tilt rotation, using a rotor for the test tubes. After incubation, the supernatant was collected to perform the quantification of the remaining protein by ELISA immunoassay. Then, 1 mL of phosphate and Ademtech blocking buffers were added to tosyl-MMP and activated carboxyl-MNP respectively and incubated under shaking for 2 h at 37 °C, in order to inactivate the remaining tosyl and carboxyl molecular groups. Finally, the modified antibody-MPs were washed and resuspended in phosphate storage buffer (tosyl-MMP) or storage buffer (activated carboxyl-MNP) to reach a concentration of 5.0 mg mL⁻¹ and stored at 4°C. Before each assay, MPs were washed twice and resuspended in PBST buffer.

In order to evaluate the antibody coupling efficiency for each magnetic particle, the concentration of antibody in the supernatant before and after the immobilization step was determined by ELISA immunoassay. In this context, serial dilutions of the supernatant collected during the covalent immobilization, were adsorbed on a 96-well microplate overnight at 4 °C. Two calibration curves were prepared with serial dilutions of anti-Salmonella antibody ranging from 0 to 0.15 μg mL⁻¹ in 0.31 mol L⁻¹ ammonium sulphate dissolved in 10 mmol L⁻¹ PBS for tosyl-MMP and 24 mmol L⁻¹ borate buffer for activated carboxyl-MNP. After two washing steps with 200 μL of phosphate buffer were performed with shaking at room temperature for 5 min, plate blocking was performed under shaking with 200 μL of PBS 3% BSA, at room temperature for 2 h. Then, 3 washing steps with 200 μL of phosphate buffer were performed with shaking at room temperature for 5 min. The immobilized antibody was revealed with 100 μL of secondary antibody labeled with the enzyme HRP, diluted 1/3000 in PBST 2% BSA for 1 h, under shaking at room temperature. Afterwards, according to the kit manufacturer, a mixture of TMB/H₂O₂ in the proportion of 1:1 was reacted with the secondary peroxidase antibody, at room temperature with shaking for 30 min. The enzymatic reaction was stopped by adding 100 μL of H₂SO₄ (2 mol L⁻¹) Finally, the absorbance was measured at 450 nm after a short shaking and the standard curve was obtained plotting Abs vs. concentration (μg mL⁻¹) (Figure 2), for the quantification of anti-Salmonella attached on the magnetic particles.

2.4. Immunomagnetic separation. Evaluation by conventional culture methods

The schematic procedure for the immunomagnetic separation (IMS) of S. enterica is outlined in Figure 1, B. A volume of 10 μL (0.05 mg) of both anti-Salmonella modified magnetic particles (nano and micro-sized) and Dynabeads® anti-Salmonella was added to 500 μL of several serial dilutions of a S. enterica suspension ranged from a concentration of 4.42 x 10⁵ to 4.42 x 10⁷ CFU mL⁻¹ in BHI broth.
An incubation step was performed for 30 min with slight agitation. After that, the bacteria attached to the particles were separated with a magnet and the supernatant was collected for plating. Then, MPs were washed with PBST (x5) shaking for 1 min at room temperature. Finally, the collected modified MPs were resuspended in 110 μL of PBST. Both, MPs with the attached bacteria and supernant were plated on LB agar and grown for 18-24 h at 37 °C.

2.5. Electrochemical magneto-immunosensing

The IMS/m-GEC electrochemical immunosensing procedure is schematically outlined in Figure 1, A, and it is based on the following steps: (i) immunomagnetic separation (IMS); (ii) immunological reaction with the anti-Salmonella-HRP antibody; (iii) electrochemical detection. The IMS step was performed as described in Section 2.4. For each concentration of bacteria ranged from 0 to 3.70 x 10⁵ CFU mL⁻¹ for carboxyl-MNP and from 0 to 5.48 x 10⁵ CFU mL⁻¹ for tosyl-MMP in BHI broth. After discarding the supernatant, the collected MPs were further reacted with 140 μL of Anti-Salmonella HRP antibody (diluted 1/1000 in PBST 2% BSA) for 30 min at room temperature. Finally, the magnetic particles were washed twice and resuspended in 140 μL of PBST. After the final washing step, the modified magnetic beads were captured by dipping the magneto electrode (m-GEC) inside the reaction tube. The modified m-GEC electrode was immersed into the electrochemical cell containing 20 mL of PBSE buffer with 1.81 mmol L⁻¹ hydroquinone, and under continuous magnetic stirring, a potential of -0.100 V vs Ag/AgCl was applied. When a stable baseline was reached, 500 μL of H₂O₂ was added into the electrochemical cell to a final concentration of 4.90 mmol L⁻¹ (which corresponds to the H₂O₂ concentration capable to saturate the whole enzyme amount employed in the labeling procedure), and the current was measured until the steady state current was reached. This steady-state current was used for Figures 3 and 4.

![Figure 1](image)

**Figure 1.** Schematic representation of electrochemical immunosensing procedure for *S. enterica* (up panel), as well as for the evaluation for the immunomagnetic separation (IMS) by conventional culture methods (down panel).

2.6. Specificity study

In order to verify the specificity of this approach, the above procedure was also performed using carboxyl-MNP with 5.90 x 10⁵ CFU mL⁻¹ of *E. coli*, 1.85 x 10⁵ CFU mL⁻¹ of *S. enterica* and, finally, a
sample containing both bacterial species (5.90 x 10^4 and 1.85 x 10^5 CFU mL\(^{-1}\), respectively for \textit{E. coli} and \textit{S. enterica}) artificially inoculated in BHI. The specificity was studied for tosyl-MMP as well, testing 4.90 x 10^4 CFU mL\(^{-1}\) of \textit{E. coli}, 2.74 x 10^5 CFU mL\(^{-1}\) of \textit{S. enterica} and, finally, a sample containing both bacterial species (4.90 x 10^4 and 2.74 x 10^5 CFU mL\(^{-1}\), respectively for \textit{E. coli} and \textit{S. enterica}) artificially inoculated in BHI. Two negative controls were also performed for each type of magnetic particle.

3. Results and Discussion

Covalent immobilization efficiency of anti-\textit{Salmonella} antibody on magnetic particles was performed by quantifying the amount of antibody present in the supernatant before and after the immobilization step through an ELISA immunoassay. Figure 2 shows the calibration curves for both types of magnetic particles, presenting a coupling efficiency of 90 and 99% of antibody immobilized on the magnetic particles, for nanoparticles (carboxyl-MNP) and microparticles (tosyl-MMP), respectively.

The immunomagnetic separation step (IMS) was evaluated by conventional culture methods for the modified magnetic particles, as well as for the commercial anti-\textit{Salmonella} particles. Table 1 shows the bacteria capture efficiency obtained by culturing MPs after the IMS. Similar capture efficiencies were obtained for all magnetic particles, showing promising capability for the further electrochemical immunosensing.

Table 1. Immunomagnetic separation of \textit{Salmonella} with Dynabeads® anti-\textit{Salmonella} and nano and micro-sized magnetic particles.

| \textbf{S. enterica concentration/ CFU mL\(^{-1}\)} | \textbf{Immunomagnetic separation (IMS)/ %} |
|-------------------------------------------------|------------------------------------------|
| \textbf{Dynabeads® anti-Salmonella} | \textbf{Carboxyl-MNP} | \textbf{Tosyl-MMP} |
| 10^5 | 92 | 85 | 90 |
| 10^4 | 77 | 73 | 85 |
| 10^3 | 90 | 95 | 83 |
| 10^2 | 82 | 95 | 95 |
| 10^1 | 100 | 100 | 100 |
The IMS/m-GEC electrochemical immunosensing procedure was developed for both magnetic particles (tosyl-MMP and carboxyl-MNP). The amperometric signal corresponding to the LOD was estimated by processing the negative control samples of 0 CFU mL\(^{-1}\) in BHI and performing three different single inter-day assays, using ten different magneto electrode devices, obtaining a mean value of 0.32 µA with a standard deviation of 0.07 µA for the tosyl-MMP and a mean value of 0.51 µA with a standard deviation of 0.08 µA for the carboxyl-MNP. The amperometric signal corresponding to the LOD value was then extracted with a one-tailed t-test at a 99 % confidence level, giving a value of 0.53 and 0.73 µA respectively for tosyl-MMP and carboxyl-MNP (shown in Figure 3 as the dotted and solid horizontal lines, respectively). The results obtained show that IMS/m-GEC electrochemical immunosensing approach was able to detect 5 × 10\(^4\) CFU mL\(^{-1}\) (tosyl-MMP) and 1 × 10\(^4\) CFU mL\(^{-1}\) (carboxyl-MNP) of \(S.\) enterica in BHI broth, in 1 h.

**Figure 3.** ‘IMS/m-GEC electrochemical immunosensing’ approach for the detection from 10\(^3\) to 10\(^5\) CFU mL\(^{-1}\) of \(S.\) enterica cells artificially inoculated in BHI broth. The error bars show the standard deviation for \(n = 2\), except for the negative control (\(n = 9\) for Carboxyl-MNP and \(n = 7\) for Tosyl-MMP in BHI broth). The LOD values for tosyl-MMP (dotted line) and carboxy-MNP (solid line) are also displayed.

Figure 4 shows the results obtained for the specificity study. As expected, the electrochemical signal obtained for \(E.\) coli was similar to the value obtained when processing the negative control, while the mix of both pathogens (\(E.\) coli and \(S.\) enterica) gave a similar signal than the sample spiked just with \(S.\) enterica for both particles nano and micro-sized. These results confirm the specificity of the ‘IMS/m-GEC electrochemical immunosensing’ approach.

**Figure 4.** Specificity study for the electrochemical immunosensing approach. The bars show the electrochemical signal for BHI broth with: 0 CFUmL\(^{-1}\) (negative control); 5.9×10\(^4\) CFU mL\(^{-1}\) of \(E.\) coli; 1.85×10\(^3\) CFU mL\(^{-1}\) of \(S.\) enterica; and a mix solution containing 5.9×10\(^4\) CFU mL\(^{-1}\) of \(E.\) coli and 1.85×10\(^3\) CFU mL\(^{-1}\) of \(S.\) enterica. The error bars show the standard deviation for \(n=2\).
4. Conclusions
A specific and sensitive electrochemical magneto immunosensor for \textit{S. enterica} detection was developed. Evaluation of different magnetic particles (nano and micro-sized) functionalized with different molecular groups showed to be very efficient on the IMS of \textit{S. enterica}, which capture was achieved as low as 10 CFU mL\(^{-1}\) by conventional culturing methods. Furthermore, the use of nanoparticles improved the IMS/m-GEC electrochemical immunosensing approach being able to detect as low as 1 x 10\(^4\) CFU mL\(^{-1}\) in BHI broth, in 1 h without any culturing enrichment. Magnetic nanoparticles offer a good advantage for food pathogenic bacteria detection, presenting a rapid alternative to the laborious and time consuming culturing detection methodologies. The high sensitivity of the approach conferred by the m-GEC electrochemical immunosensing coupled with magnetic separation using magnetic nanoparticles, results in an extremely specific, rapid, robust and sensitive procedure, all of them promising features for being implemented as microfluidic system mainly for food industry applications. Future work will focus on further validation of this assay in artificially inoculated as well as in naturally contaminated meats, poultry, dairy products, and environmental samples.

Acknowledgments
Financial support from BioMaX: Novel diagnostic bio-assays based on magnetic particles Marie Curie Initial Training Networks (ITN) Call: FP7-PEOPLE-2010-ITN, Ministry of Science and Innovation (MEC), Madrid (Project BIO2010-17566) and from Generalitat de Catalunya (Projects SGR 323 and SGR2009-1106) are acknowledged. Ademtech (Pessac, France) is also acknowledged for providing Carboxyl-Adembeads magnetic nanoparticles.

5. References
[1] Suo B, He Y, Paoli G, Gehring A, Tu S, Shi X 2010 \textit{Mol. Cell. Probes} 24 77–86
[2] Liébana S, Lermo A, Campoy S, Barbé J, Alegret S, Pividori M I 2009 \textit{Anal. Chem.} 81 5812–5820
[3] Liébana S, Lermo A, Campoy S, Barbé J, Alegret S, Pividori M I 2009 \textit{Biosens. Bioelectron.} 25 510-513
[4] Roque A C A, Bispo S, Pinheiro A R N, Antunes J M A, Gonçalves D, Ferreira H A 2009 \textit{J. Mol. Recognit.} 22 77–82
[5] Hsing I-Ming, Xu Y, Zhao W 2007 \textit{Electroanalysis} 19 755 – 768
[6] Yang H, Li H, Jiang X 2008 \textit{Microfluid. Nanofluidics} 5 571–583
[7] Den Dulk R C, Schmidt K A, Sabatté G, Liébana S, Prins M W J, 2013 \textit{Lab Chip} 13 106-118
[8] Pividori M I, Alegret S, 2005 \textit{Anal. Lett} 38 2541-2565
[9] Pividori M I, Lermo A, Campoy S, Barbe J, Alegret S 2007 \textit{Electrochemical Sensor Analysis} Elsevier Amsterdam 221-226