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Phagomagnetic Separation and Electrochemical Magneto-Genosensing of Pathogenic Bacteria

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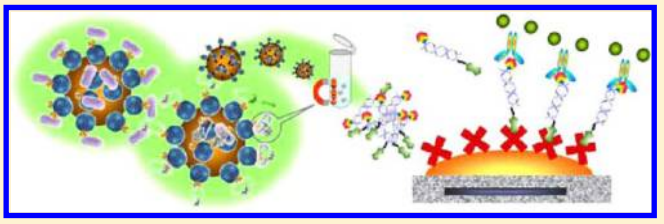
Supporting Information

ABSTRACT: This paper addresses the use of bacteriophages immobilized on magnetic particles for the biorecognition of the pathogenic bacteria, followed by electrochemical magneto-genosensing of the bacteria. The P22 bacteriophage specific to *Salmonella* (serotypes A, B, and D_1) is used as a model. The bacteria are captured and preconcentrated by the bacteriophage-modified magnetic particles through the host interaction with high specificity and efficiency. DNA amplification of the captured bacteria is then performed by double-tagging polymerase chain reaction (PCR). Further detection of the double-tagged amplicon is achieved by electrochemical magneto-genosensing. The strategy is able to detect in 4 h as low as 3 CFU mL^{-1} of *Salmonella* in Luria−Bertani (LB) media. This approach is compared with conventional culture methods and PCR-based assay, as well as with immunological screening assays for bacteria detection, highlighting the outstanding stability and cost-efficient and animal-free production of bacteriophages as biorecognition element in biosensing devices.



Bacteriophages (or phages) are natural host-specific, self-reproducing, and self-assembling nanostructured particles, with both structure and function encrypted in the genomic DNA. Bacteriophages bind to specific receptors on the bacterial surface in order to inject the genetic material inside the bacteria, using the host's own replication machinery for multiplication. The replicated virions are eventually released, killing the bacteria and allowing the infection of other host cells. Beside the promising features of phage therapy,[1] bacteriophage-based diagnostic is attracting much interest[2] due to the high specificity of phages, which makes them ideal agents not only for the detection of bacteria, but also for the detection of almost all kinds of targets, ranging from small molecules to proteins and even cells, by using the phage display technique.[3] As phages have the ability to display peptides or proteins on their surface, those showing a very high affinity and specificity for a target can be selected out of a library. Unfortunately, as the use of phages as biorecognition elements is in its infancy, the range of commercially available bacteriophages is still limited. Another important advantage is the fast, cheap, and animal-friendly phage production, which is achieved by just infecting the host bacteria.[3] Moreover, phages are stable in a range of harsh conditions including pH and temperature.[3] Phages can even be used in the presence of nucleases or proteolytic enzymes, without degradation. The high stability of phages in a variety of environmental conditions makes them suitable for in situ monitoring of food and environmental contaminants. These naturally occurring nanoparticles have other interesting properties in comparison with synthetic nanoparticles: all bacteriophages are nearly identical, being monodisperse in shape and size, a fact difficult to achieve by laboratory synthesis. On the contrary, these nanoparticles are self-synthesized in their specific host, by producing a large amount of viral coat proteins with a large surface for further chemical modification.

The reported methods for bacteria detection using bacteriophages include (i) expression of bacteriophage-encoded bioluminescent genes which produce visible products within the specific target cells (lux-bacteriophage strategy),[4] (ii) fluorescence-labeled phage, which can be combined with immunomagnetic separation (labeled phage strategy),[5] (iii) detection of bacteria by the intracellular replication of specific bacteriophages (named "phage amplification" strategy),[6,7] and the (iv) detection of the phage-mediated bacterial lysis and release of host enzymes (e.g., adenylate kinase) or ATP (termed "lysin-release ATP bioluminescence strategy").[8]

Bacteriophages recognize the bacterial receptors through their tail spike proteins. This biorecognition is highly specific and has been employed for the typing of bacteria. This level of specificity and selectivity opens avenues for the development of specific pathogen detection technologies and for the creation of biosensing platforms. Biosensing approaches based on quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) as transduction platform were reported.[9−11] These early reports relied on physical adsorption of the bacteriophage on the sensor surface. Single-point, oriented, covalent attachment of the bacteriophages on different surfaces and transducers was

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also reported in order to yield better coverage and to improve the performance of these devices. Streptavidin-mediated attachment of bacteriophages that were genetically modified to directly express biotin on their capsid was reported.[12,13] Covalent immobilization of bacteriophages on gold surface,[14] screen-printed carbon electrode,[15] and glass substrates[16] for biosensor application was also reported.

This paper addresses the use of bacteriophage nanoparticles as a highly specific biorecognition element for the capture and preconcentration of pathogenic bacteria by using "phagomagnetic separation" (PMS), followed by electrochemical magneto-genosensing detection. The main advantage of using bacteriophages relies on cost-efficient and animal-free production, as well as their outstanding stability, overcoming thus the main challenges of the biorecognition elements in biosensing devices. The icosahedral-shaped bacteriophage (P22) specific to the pathogenic bacteria *Salmonella* was studied as a model.[17,18] The immobilization of the native, non-modified, P22 phage nanoparticles on tosylated magnetic particles was achieved throughout the amine moieties of the lysine residues in the main capsid monomeric protein (gp5)[19] by covalent amine linkage. After preconcentration of the bacteria on the magnetic particles by PMS, the bacteria were easily detected by double-tagging polymerase chain reaction (PCR) amplification of the DNA of the captured bacteria followed by electrochemical magneto-genosensing (PMS/double-tagging PCR/m-GEC genosensing).[20]

The main features of the PMS/double-tagging PCR/m-GEC electrochemical genosensing approach are compared with conventional culture methods and PCR-based assay.

EXPERIMENTAL SECTION

Instrumentation. Temperature-controlled incubations were performed in an Eppendorf Thermomixer compact. The magnetic separation during the washing steps was performed using a magnetic separator Dynal MPC-S (product no. 120.20D, Dynal Biotech ASA, Norway). The PCR reaction was carried out in an Eppendorf Mastercycler personal thermocycler. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc., U.S.A.). 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 and co-workers[21,22] (Figure i, Supporting Information). The scanning electron microscopy (SEM) images were taken with the scanning electron microscope Hitachi LTD S-570 (Hitachi LTD, Tokyo, Japan).

Chemicals, Biochemicals, and Materials. Tosylactivated magnetic particles (MP-Tosyl) (Dynabeads M-280, product no. 142.03) as well as the streptavidin magnetic particles (Dynabeads M-280 Streptavidin, product no. 112.05) were purchased from Life Technologies, Invitrogen Dynal AS (Oslo, Norway). AntiDig−HRP (antidigoxigenin−POD, product no. 11.207.733.910) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Bradford assay was performed with the Coomasie Bradford protein assay kit, ref. 23200, Pierce, U.S.A.

The Expand High Fidelity PCR System kit (Roche Molecular Biochemicals) was used for performing the PCR. The primers for the double-tagging PCR amplification in the genosensing strategy were obtained from TIB-MOLBIOL (Berlin, Germany). These primers were selected for the specific amplification of the *IS200* insertion sequence[23] related to *Salmonella* spp. The primer sequences were biotin-*IS200* up: 5′ BIO-ATG GGG GAC GAA AAG AGC TTA GC 3′, digoxigenin-*IS200* down: 5′ DIG-CTC CAG AAG CAT GTG AAT ATG 3′.

The buffer solutions were prepared with Milli-Q water. All other reagents were analytical reagent grade supplied from Sigma and Merck. The composition of the solutions is detailed in the Supporting Information.

Bacterial Strains and P22 Bacteriophage. The P22 bacteriophage (ATCC 19585-B1), *Salmonella enterica* serovar Typhimurium LT2, and *Escherichia coli* K12 strains were used in this work. The bacteriophage lysate was obtained by infecting exponential cultures of *Salmonella* Typhimurium LT2 (10^8 CFU mL^{-1}) with the P22 bacteriophage, and by further purification with cesium chloride gradient,[24] as detailed in the Supporting Information. The bacteriophage titer was determined by plating them using double agar layered conventional method (Figure iv, part A, Supporting Information). The phage stock solutions were maintained in $MgSO_4$ 10 mM in Milli-Q water solution at 4 °C retaining a constant titer for several months. When specified, the P22 bacteriophages were inactivated by exposure to a UV-C (254 nm) germicidal lamp to avoid the lytic cycle.

Covalent Immobilization of P22 Bacteriophage on Magnetic Particles and Coupling Efficiency Study. The native P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by the reaction of aminated aminoacidic lysine moieties of the main capsid monomeric protein (gp5)[18] (as schematically outlined in Figure ii, Supporting Information), by an amine linkage, in order to obtain the P22 phage-modified magnetic particle conjugate (P22-MP). The binding was performed using purified P22 phage stock solution (200 μL) at a concentration level of 2×10^{12} PFU mL^{-1}, reaching a concentration in the immobilization solution of 4×10^{11} PFU mL^{-1} as explained in detail in the Supporting Information.

The coupling efficiency was evaluated by the Coomassie Bradford protein assay,[25] analyzing the protein concentration of the P22 phage capsid in the supernatant after the covalent attachment, and performing the calibration curve with the purified P22 phage solution from 2×10^{10} to 5×10^{11} PFU mL^{-1}, as described in the Supporting Information, Figures ii and iii.

A similar approach was performed by the double agar layered conventional method for counting active phages. In this approach, 10-fold dilutions of the supernatant after the covalent attachment were plated through the double agar layered method as described in the Supporting Information (Figure iv).

Evaluation of the Immobilized P22 Bacteriophage on Magnetic Particles by SEM and Conventional Culture Methods. The evaluation of the immobilized P22 bacteriophages was performed by microscopic techniques (SEM) as well as by conventional culture methods. For the microscopic evaluation by SEM, 10 μL of the P22 phage-modified magnetic particles (P22-MPs) in 5 mL of Milli-Q water (1924 PFU/MP) was filtered through a Nucleopore membrane (25 mm Ø, 0.2 μm pore size). The filters were then fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydratated with ethanol, and dried by CO_2 critical point before gold metallization and observation.[26]

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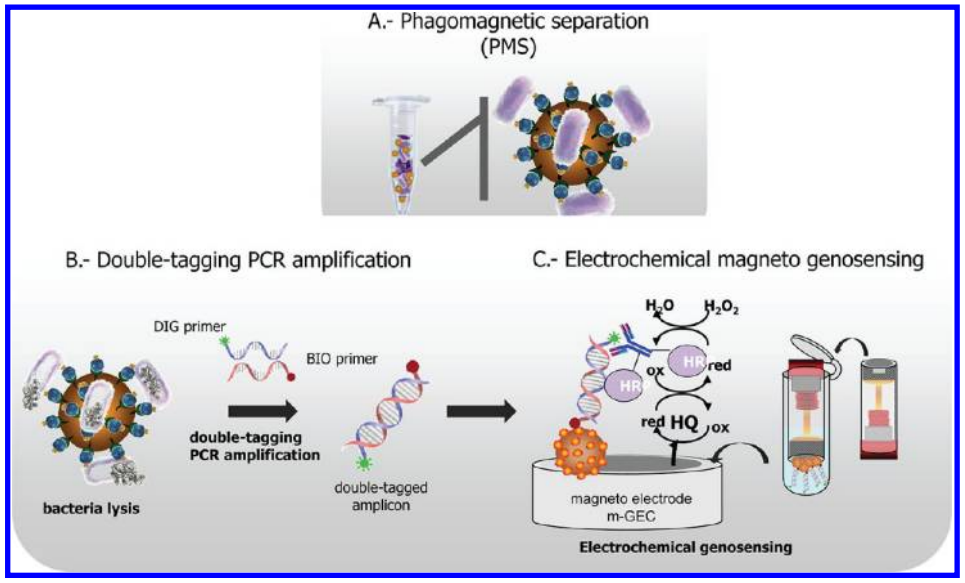

Figure 1. Schematic representation of the phagomagnetic separation (PMS) (A) of the bacteria followed by the double-tagging PCR (B) and the electrochemical magneto-genosensing (C) of the attached bacteria (PMS/double-tagging PCR/m-GEC electrochemical genosensing).

As previously addressed, the orientation of the bacteriophages on the solid support is an important issue to be considered. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22-MPs, since oriented phages immobilized on magnetic particles will produce bacteria attachment and further infection of viable bacteria, producing the plaques (Figure v, Supporting Information). A 10-fold dilution of the P22-MPs was plated through the double agar layered conventional method as previously explained (Figure iv, Supporting Information).

Phagomagnetic Separation of *Salmonella*. Evaluation by SEM and Conventional Culture Methods. The procedure for the PMS of the bacteria is schematically outlined in Figure 1A. Inactivated bacteriophages by UV radiation were used for the phagomagmetic separation to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured, preconcentrated, and cultured since both SEM and culturing require non-infected bacteria.

Bacterial solutions that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} in Luria–Bertani (LB) broth were performed for the PMS of *Salmonella* Typhimurium LT2. A negative control of LB broth was also processed. The culture in LB (500 μL) was mixed with 50 μL of P22-MPs (Figure 1A). An incubation step was performed for 30 min at 37 °C without agitation. After that, the magnetic particles with the attached bacteria were separated with a magnet and then washed with PBST for 5 min (3×) at room temperature. Finally, the modified magnetic particles were resuspended in 80 μL of Milli-Q water.

The evaluation of the PMS (Figure 1A) was performed by SEM and conventional culture methods. For the SEM study, the PMS was performed with a concentration of bacteria of 2.9×10^7 CFU mL^{-1}, and the filters were treated as above-described. In order to study the efficiency of the PMS step by conventional culture method, 10 μL of modified magnetic particles of each solution that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} in LB broth including LB broth as negative control was plated in LB agar and grown for 18–24 h at 37 °C.

Phagomagnetic Separation, Double-Tagging PCR Amplification, and Electrochemical Magneto-Genosensing. The procedure for the PMS of the bacteria followed by the double-tagging PCR and the electrochemical magneto-genosensing of the attached bacteria (PMS/double-tagging PCR/m-GEC electrochemical genosensing) is schematically outlined in Figure 1. In this case, UV-inactivated P22 phage nanoparticles were also used for the phagomagmetic separation to avoid the lytic cycle and the release of the genomic DNA of the bacteria while being captured and preconcentrated, since DNA is required for the double-tagging PCR amplification. For each concentration of bacteria in LB (from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}), the lysis of the bacteria attached on the inactivated P22-MPs was performed at 99 °C for 20 min in order to break the cells and to achieve the releasing of the genomic DNA and the cellular debris to the solution for the PCR amplification (Figure 1B). The amplification of the specific *IS200* insertion sequence related to *Salmonella* spp. was thus performed (Figure 1B) by a double-tagging PCR using two labeled primers with biotin and digoxigenin[27] (Figure vii, expanded version of Figure 1B, Supporting Information). During the PCR, not only the amplification of pathogenic bacteria genome was achieved, but also the double tagging of the amplicon ending with (i) the biotinylated capture primer to achieve the immobilization on streptavidin-modified magnetic particles and (ii) the digoxigenin signaling primer to achieve the enzymatic detection through antiDig–HRP reporter.

All of these amplifications included not only a positive control, but also a blank as a negative control, which contained LB broth without *Salmonella* spp. template. The double-tagged amplicon was analyzed by electrochemical genosensing with the m-GEC electrodes as well as with the conventional gel electrophoresis.

The electrochemical genosensing strategy of the double-tagged amplicon (Figure 1C) comprises the following steps, as outlined in the Supporting Information and the Figure viii, expanded version of Figure 1C: (a) immobilization of the double-tagged amplicon in which the 5′ biotin end was immobilized on the streptavidin magnetic particles; (b) enzymatic labeling with the antibody antiDig–HRP able to bond the 5′ digoxigenin end of the ds-DNA amplicon; (c) magnetic capture of the modified magnetic particles by the m-GEC electrode; (d) amperometric determination.

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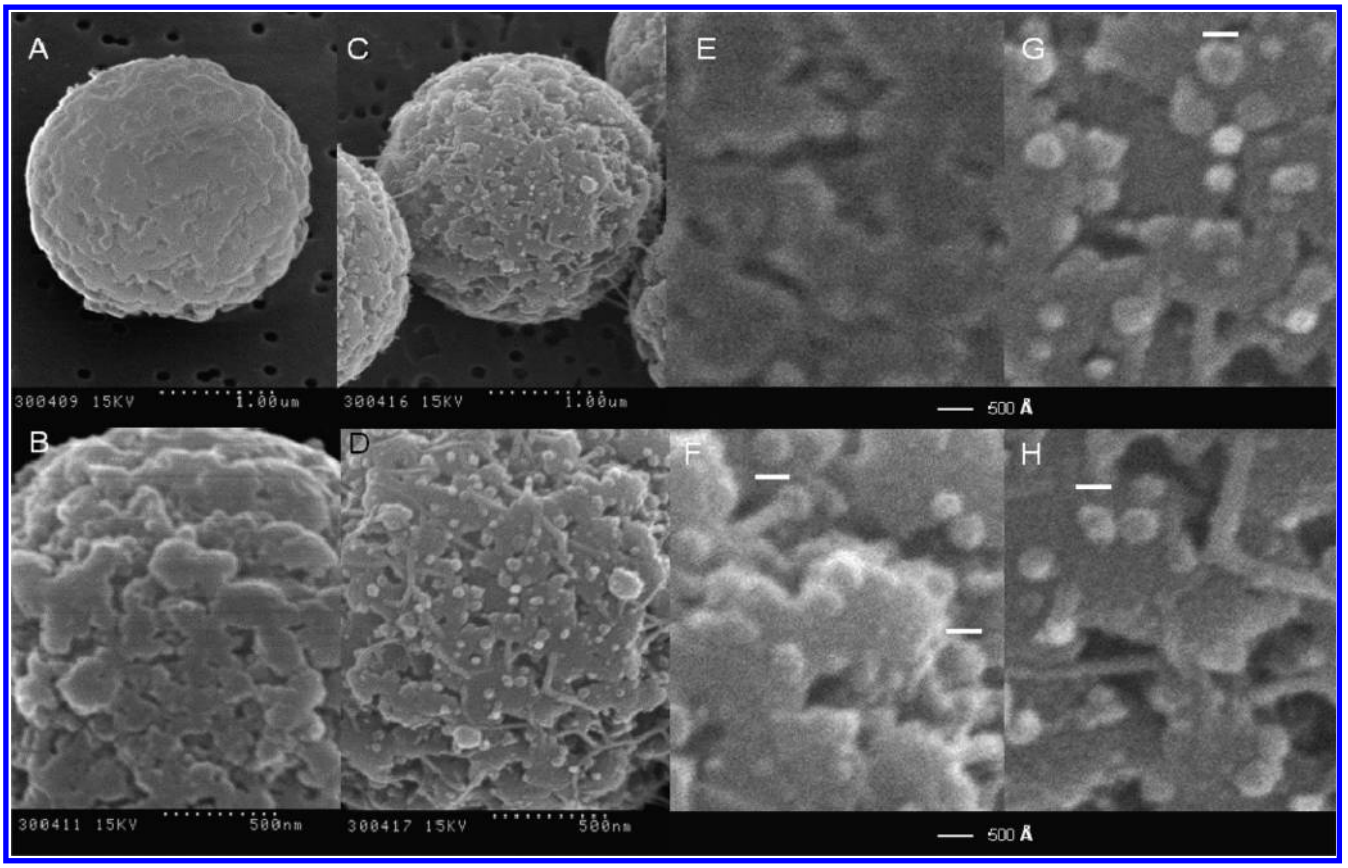

Figure 2. Evaluation of the immobilized P22 bacteriophage on magnetic particles by SEM (1924 PFU/MP). Images C, D, and F–H show, at different resolution levels, the P22 bacteriophages attached to the magnetic particles. Panels A, B, and E show the magnetic particle without modification as a negative control. In all cases, identical acceleration voltage (15 kV) was used.

Specificity Study of the "PMS/Double-Tagging PCR/m-GEC Electrochemical Genosensing" Approach. In order to verify the specificity of this approach, the above procedure was also performed with 4.5×10^5 CFU mL^{-1} of *E. coli*, *Salmonella*, and finally, a sample containing both bacterial species (4.3×10^5 CFU mL^{-1} of each bacterial specie) artificially inoculated in LB, as well as a negative control.

Safety Considerations. All the procedures involving the manipulation of potentially infectious materials or cultures were performed following the guidelines for safe handling and containment of infectious microorganism.[28] Strict compliance with BSL-2 practices was followed in all experiments involving *Salmonella* Typhimurium LT2, *E. coli* K12, and active P22 bacteriophage, and proper containment equipment and facilities were used. The ultimate disposal was performed according to local regulations.

RESULTS AND DISCUSSION

Covalent Immobilization of P22 Bacteriophage on Magnetic Particles and Coupling Efficiency Study. The native P22 phage nanoparticles were covalently coupled for the first time to tosyl-activated magnetic particles by the reaction of aminated aminoacidic moieties of the main capsid monomeric protein (gp5).[18] The amount of viral protein present in the supernatant before and after the immobilization step was determined by the Bradford test to quantify the coupling efficieny of the capsid protein to the magnetic particle. As shown in Figure iii, Supporting Information, a good calibration curve was obtained with the Bradford method of the P22 phage nanoparticles showing good reproducibility at each concentration level ($n = 3$) and a linear range from 2×10^{10} to 5×10^{11} PFU mL^{-1} ($r = 0.968$). By comparing the phage concentration before and after immobilization, the coupling efficiency of nonmodified P22 bacteriophages (4×10^{11} PFU mL^{-1}) on tosyl-activated magnetic particles on both 7×10^8 and 7×10^7 magnetic particle units was found to be 100.4% and 23.6%, respectively, with ratios of 626 and 1924 P22 phage nanoparticles (PFU) immobilized per magnetic particle, respectively. Moreover, the immobilization of an increased amount of P22 phage nanoparticles (1×10^{12} PFU) on the same amount of magnetic particles (7×10^7) showed a similar coupling efficiency (25.6%), with a ratio of 2163 P22 phage nanoparticles (PFU) per each magnetic particle, indicating a plateau in the immobilization efficiency in approximately 2000 PFU/MP. A similar approach for coupling efficiency study was performed by quantifying the plaque forming units (PFU) in the supernatant before and after the immobilization step by the double agar layered conventional method for counting active phages (as explained in detail in the Supporting Information, Figure iv). After comparing the bacteriophage counting (PFU) before and after the immobilization, the coupling efficiency for 1.44×10^{11} PFU on 7×10^7 magnetic particle units was found to be 37%, with a ratio of 757 phage nanoparticles (PFU) immobilized in each magnetic particle. The results are comparable to those obtained by the Bradford method, considering that, in this last case, the starting amount of phage for immobilization (1.44×10^{11} PFU) was around 35% of the amount used for Bradford (4×10^{11} PFU, the saturating

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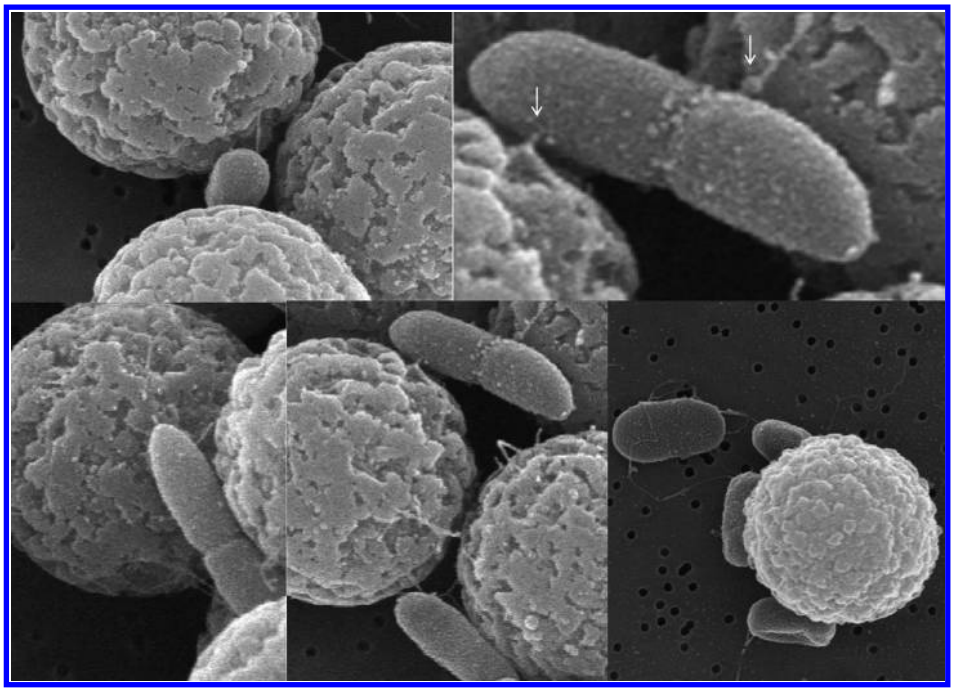

Figure 3. Evaluation of the PMS by SEM at a *Salmonella* concentration of 2.9×10^7 CFU mL^{-1}. The images show the *Salmonella* cells attached to the magnetic particles through the tail spikes. In all cases, identical acceleration voltage (15 kV) was used.

phage concentration), the immobilized phages on 7×10^7 MP being thus also approximately 35% (757 PFU per MP) of the saturated value (2000 PFU phage nanoparticles per magnetic particle) obtained by Bradford. The Bradford method showed thus good performance as a rapid alternative for the time-consuming microbiological methods in order to estimate the coupling efficiency of phage nanoparticles, not only on magnetic particles, but also in other supports. Finally, the optimum ratio to achieve the higher covering of P22 bacteriophages on 7×10^7 magnetic particles was found to be 4×10^{11} PFU mL^{-1}, reaching a coupling efficiency of around 25% with approximately 2000 PFU per MPs.

Evaluation of the Immobilized P22 Bacteriophage on Magnetic Particles by SEM and Conventional Culture Methods. Figure 2 shows the microscopic characterization by SEM of non-modified (Figure 2, parts A, B, and E) and modified (Figure 2, parts C, D, and F–H) magnetic particles with P22 phages nanoparticles. Figure 2 shows the spherical structures of P22 bacteriophages (~600–700 Å in diameter[19]) (Figure 2F–H) uniformly distributed on the surface of the magnetic particles (Figure 2, parts C and D).

Although the successful in the immobilization of P22 phage nanoparticles on magnetic particles was demonstrated by different methodologies (Bradford, phage counting on the supernatant by the double agar layered conventional method, and SEM), none of these methods can ensure the orientation of the tail spikes away from the solid support. This orientation was studied by the double agar layered method and enumeration of plaques by culturing the P22 phage-modified magnetic particles (P22-MPs). The quantification of the number of bacteriophages per magnetic particle is not possible by plating the P22-MPs conjugates, due to the fact that all the bacteriophages attached on the same magnetic particle will produce a unique plaque, as explained in Figure v (Supporting Information). However, the phage counting for the immobilization of 1.44×10^{11} PFU on 7×10^7 magnetic particle units was found to be 5.3×10^7, which demonstrated lytic activity in at least 75% of the magnetic particles and, as such, confirmation of the oriented immobilization of the phages on the magnetic particles.

Phagomagnetic Separation of *Salmonella*. Evaluation by SEM and Conventional Culture Methods. The microscopic characterization by SEM was also performed for the evaluation of the PMS, i.e., the bacteria attachment to the magnetic particles throughout the interaction between the tail spikes and the O-antigen polysaccharide receptor on the bacteria.[17] In this case, instead of active P22 phages, UV-inactivated P22 phage nanoparticles were used for the phagomagnetic separation to avoid the lytic cycle in order to keep the attached bacteria as a whole cell while being captured. The procedure for the PMS of the bacteria is schematically outlined in Figure 1A.

Figure 3 shows that the binding was achieved with more than one specific binding site of the bacteria to the magnetic particle. Single-point attachment of the bacteria to the magnetic particle was mostly observed. Moreover, a unique magnetic particle was able to attach more than one bacterium. Finally, some aggregates were observed due to the binding of two or more different magnetic particles by a unique bacterium cell.

Conventional culture method was also performed by growing the bacteria attached on magnetic particle for 18–24 h at 37 °C, as schematically outlined in Figure vi, part A (Supporting Information). Colony counting was clearly decreasing from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}. The corresponding plates are also shown, displaying the characteristic colony features of *Salmonella* in LB media. However, an underestimation of the expected amount of bacteria was observed in all the concentration range. The counted colony number was found to be in all cases under 10% of the expected amount, perhaps due to the formation of the aggregates observed by SEM, formed by several bacterium cells but growing at a unique colony point in the agar plate or, for instance, due to infection

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of remaining active bacteriophages and, thus, under growing of the attached bacteria.

Phagomagnetic Separation, Double-Tagging PCR Amplification, and Electrochemical Magneto-Genosensing. The second step in the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is the double-tagging PCR for the amplification of the *Salmonella* spp. genome for the final genosensing detection.[20,27] The chosen set of primers amplified exclusively the *IS200* insertion sequence, producing only the expected 201 bp fragments, according to the agarose gel electrophoresis shown in Figure 4, for the

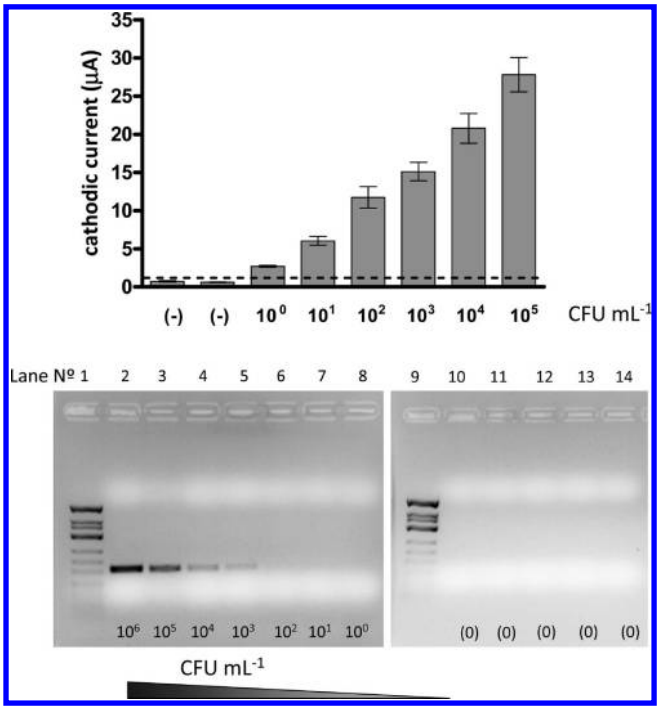

Figure 4. Top: Electrochemical signals for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach. Gray bars show the signal by increasing the amount of *Salmonella* ranged from 3.2×10^0 to 3.2×10^5 CFU mL^{-1} artificially inoculated in LB broth. Two negative controls (0 CFU mL^{-1} and PCR negative control) are also shown, respectively. In all cases, $n = 4$, except for the 0 CFU mL^{-1} negative control ($n = 8$). Bottom: Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "PMS/double-tagging PCR/electrophoresis" approach. Lanes 2−8 are 10-fold dilutions that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1}. Lanes 10−13 are 0 CFU mL^{-1} negative controls, while lane 14 is the PCR negative control. Lanes 1 and 9 are the molecular weight marker (ΦX174-Hinf I genome).

concentration that ranged from 3.2×10^6 to 3.2×10^0 CFU mL^{-1} (lanes 2−8) in LB broth artificially inoculated with *Salmonella*. As shown in Figure 4, the limit of detection (LOD) for the PMS/double-tagging PCR/electrophoresis was found to be 3.2×10^3 CFU mL^{-1} (lane 5 in the gel electrophoresis). No bands were observed for the negative controls (0 CFU mL^{-1}) performed in LB broth (Figure 4, lanes 10−13). In order to increase the sensitivity of the assay, instead of the "PMS/double-tagging PCR/electrophoresis" approach, the proposed methodology is based on the "PMS/double-tagging PCR/m-GEC electrochemical genosensing", by replacing the electrophoresis detection for the electrochemical magneto-genosensing of the double-tagged amplicon[20,27] (Figure viii, expanded version of Figure 1C, Supporting Information). The amperometric response of the doubly labeled product was evaluated for artificially inoculated bacteria in LB (Figure 4). The amperometric signal corresponding to the LOD was estimated by processing the negative control samples of 0 CFU mL^{-1} in LB and performing three different single interday assays, using six magneto-electrode devices from different batches, obtaining a mean value of 0.75 μA with a standard deviation of 0.20 μA. 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 1.33 μA, respectively (shown in Figure 4 as the dotted horizontal line).

As shown in Figure 4, the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is able to give a clear positive signal (15.1 μA with a standard deviation of 2.08 μA) and a signal-to-background ratio value of 20 for 3.2×10^3 CFU mL^{-1}, while the electrophoresis for the same concentration shows a weak positive band (Figure 4, lane 5). On the other hand, as low as 3 CFU mL^{-1} was clearly detected with a total assay time of 4 h for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach, with an amperometric signal of 2.7 μA, a standard deviation of 0.20 μA, and a signal-to-background ratio value of 3.6. Compared with other biosensing methodologies for detecting pathogenic bacteria (ref 27 and references therein, Table A, Supporting Information), excellent detection limits were achieved with this procedure. In addition, this method is more rapid and sensitive than other rapid antibody-based and nucleic acid-based PCR methods that have been previously reported (ref 27 and references therein, Table A, Supporting Information). Moreover, the procedure is able to detect at least 3 CFU mL^{-1} in 4 h without the use of any culturing pre-enrichment or selective plating enrichment steps, with higher sensitivity than PCR followed by electrophoresis or plating by conventional culture method. Other rapid approaches based on immunological recognition coupled with electrochemical impedance spectroscopy or fluorescence detection are able to detect the bacteria faster (ranging from 6 min to 2.5 h) but with significantly higher LODs (from 10^2 to 10^5 CFU mL^{-1}) (Table A, Supporting Information). Regarding other rapid approaches based on genetic recognition, most of them are demonstrated with synthetic oligonucleotides, and only few procedures are based on inoculated bacteria detection obtaining LODs ranged from 10 to 10^4 CFU mL^{-1} (Table A, Supporting Information). To the best of our knowledge, only detection techniques based on fluorescence are able to obtain similar features to the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach.

Comparing the procedure for the bacteriophage-based and the immunological magnetic separation[27] coupled with double-tagging PCR/m-GEC electrochemical genosensing, the PMS approach gave significantly lower background values for the negative control (0.75 vs 2.2 μA, respectively), better standard deviation values (0.2 $n = 8$ vs 0.65 $n = 35$), and thus lower amperometric signal corresponding to the LOD value (1.33 vs 3.78 μA) allowing better discrimination at lower concentration levels. It should be also pointed out that remarkably improved LOD was also achieved with the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach compared with LODs reported for other biosensing approaches using bacteriophages (Table A, Supporting Information). This fact can be ascribed to the combined used of the magnetic separation and the sensitivity of the amplicon detection with the m-GEC electrochemical genosensing strategy.

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Specificity Study of the "PMS/Double-Tagging PCR/m-GEC Electrochemical Genosensing" Approach. Figure 5A shows the results of the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach for LB artificially inoculated, in all cases, with 4×10^5 CFU mL^{-1} of *E. coli*, *Salmonella*, and both *E. coli* and *Salmonella*, as well as a negative control. Figure 5B shows the corresponding electrophoresis images of the double-tagged amplicon ("PMS/double-tagging PCR/electrophoresis" approach). As expected, the electrochemical signal obtained for *E. coli* is similar to the negative control signal, while the solution of both pathogens gave a similar signal to that of the sample spiked just with *Salmonella*. Similarly, no electrophoresis band was observed for *E. coli* (Figure 5B, lane 4), while the mix of both pathogens (Figure 5B, lane 2) and the *Salmonella* (Figure 5B, lane 5) gave a unique positive electrophoresis band producing only the expected 201 bp fragments, corresponding to the amplification of the *IS200* insertion sequence specific for *Salmonella*, as confirmed for the positive PCR control (lane 3). The same results were obtained by plating the bacteria attached to the magnetic particles in LB agar for 18−24 h at 37 °C. No growing was observed for *E. coli*, while typical colony features of *Salmonella* were observed for the mix of both pathogens as well as for just *Salmonella*. These results confirm that the specificity of the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach is coming mainly from the PMS step, due to the P22 bacteriophage specific to *Salmonella* which coated the magnetic particles whose tail-spike proteins specifically recognize the repetitive O-antigen part present in the lipopolysaccharides (LPS) of *Salmonella* serotypes A, B, and D_1 outer membrane.[29] The selection of the *IS200* specific set of primers for *Salmonella* spp.[20,23,27] in the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach provides an additional source of specificity, coming from the double-tagging PCR. This fact could be especially useful in other applications when bacteriophages with low specificity are involved in the PMS step.

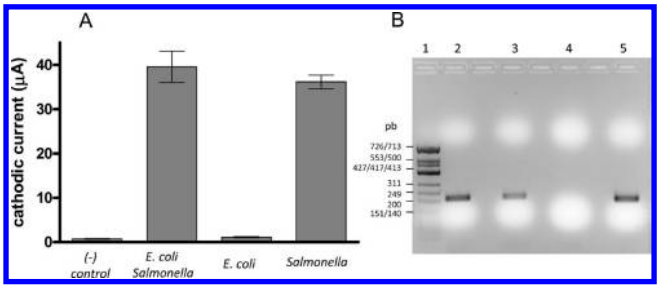

Figure 5. (A) Specificity study for the "PMS/double-tagging PCR/m-GEC electrochemical genosensing" approach. Gray bars show the electrochemical signal for LB artificially inoculated, respectively, with 0 CFU mL^{-1} (negative control, $n = 4$), 4.3×10^5 CFU mL^{-1} *E. coli* and *Salmonella* spp. ($n = 4$), 4.5×10^5 CFU mL^{-1} *E. coli* ($n = 4$), and 4.5×10^5 CFU mL^{-1} of *Salmonella* ($n = 5$). (B) Agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "PMS/double-tagging PCR/electrophoresis" approach: lane 2, 4.3×10^5 CFU mL^{-1} *E. coli* and *Salmonella* spp.; lane 4, 4.5×10^5 CFU mL^{-1} *E. coli*; lane 5, 4.5×10^5 CFU mL^{-1} of *Salmonella*. Lane 1 is the molecular weight marker (ΦX174-Hinf I genome) while lane 3 is a PCR positive control.

CONCLUSIONS

A rapid and sensitive assay combining PMS, double-tagging PCR, and electrochemical magneto-genosensing of the double-tagged amplicon for *Salmonella* is presented. This is the first time that native, whole bacteriophages are used as a biorecognition element for magnetic separation and bacteria preconcentration. The main advantages of using phagomagnetic instead of the immunomagnetic separation rely on the use of the bacteriophages for biorecognition. Contrary to antibody generation, phages are animal-free, cost-efficiently produced by bacteria infection, taking only few hours. Another feature which makes them suitable as a biorecognition element is their outstanding stability. The specificity is mainly conferred by the P22 bacteriophage specific to serotypes A, B, and D_1 during the PMS, being thus an extremely useful tool to trace the source of outbreaks by phage typing. A phage cocktail can be employed by using the same strategy to increase the host range of this assay or for multiplexing the bacteria detection toward others food-borne pathogens, such as *Listeria* or *E. coli*.

This strategy is able to detect in 4 h as low as 3 CFU mL^{-1} of bacteria in LB media. As in the case of other rapid methods, such as PCR and immunological assays, the primary use of this approach is focused on screening out negative samples. As such, positive test results should be always considered presumptive and must be confirmed by an approved culture method. The high sensitivity of the approach conferred by the m-GEC electrochemical genosensing coupled with magnetic separation results in an extremely specific, rapid, robust, and sensitive procedure, all of them promising features for being implemented as a 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 by assaying in parallel with standard plating techniques. Moreover, and in order to reach the LODs according to the legislation (absence of *Salmonella* in 25 g, sampled in five portions of 5 g each in different points, Real Decreto 1679/1994, BOE 24-09-94), a pre-enrichment step of the sample in LB will be implemented. Other approaches based on PMS followed by electrochemical immunosensing, as well as the use of phage as tags to increase the sensitivity of the detection, are currently being studied.

ASSOCIATED CONTENT

Supporting Information

Figures i−viii, expanded version of Figure 1, parts B and C, and Table A. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Pirisi, A. *Lancet* **2000**, *356*, 1418.
(2) Smartt, A. E.; Ripp, S. *Anal. Bioanal. Chem.* **2011**, *400*, 991−1007.
(3) Van Dorst, B.; Mehta, J.; Bekaert, K.; Rouah-Martin, E.; De Coen, W.; Dubruel, P.; Blust, R.; Robbens, J. *Biosens. Bioelectron.* **2010**, *26*, 1178−1194.
(4) Loessner, M. J.; Rees, C. E.; Steward, G. S.; Scherer, S. *Appl. Environ. Microbiol.* **1996**, *62*, 1133−1140.
(5) Goodridge, L.; Chen, J.; Griffiths, M. *Int. J. Food Microbiol.* **1999**, *47*, 43−50.
(6) Stewart, G. S. A. B.; Jassim, S. A. A.; Denyer, S. P.; Newby, P.; Linley, K.; Dhir, V. K. *J. Appl. Microbiol.* **1998**, *84*, 777−783.
(7) Favrin, S. J.; Jassim, S. A.; Griffiths, M. W. *Appl. Environ. Microbiol.* **2001**, *67*, 217−224.
(8) Blasco, R.; Murphy, M. J.; Sanders, M. F.; Squirrell, D. J. *J. Appl. Microbiol.* **1998**, *84*, 661−666.
(9) Olsen, E. V.; Sorokulova, I. B.; Petrenko, V. A.; Chen, I.; Barbaree, J. M.; Vodyanoy, V. J. *Biosens. Bioelectron.* **2006**, *21*, 1434−1442.
(10) Balasubramanian, S.; Sorokulova, I.; Vodyanoy, V. I.; Simonian, A. L. *Biosens. Bioelectron.* **2007**, *22*, 948−955.
(11) Nanduri, V.; Sorokulova, I. B.; Samoylov, A. M.; Simonian, A. L.; Petrenko, V. A.; Vodyanoy, V. *Biosens. Bioelectron.* **2007**, *22*, 986−992.
(12) Gervais, L.; Gel, M.; Allain, B.; Tolba, M.; Brovko, L.; Zourob, M.; Mandeville, R.; Griffiths, M.; Evoy, S. *Sens. Actuators, B* **2007**, *125*, 615−621.
(13) Tolba, M.; Minikh, O.; Brovko, L. Y.; Evoy, S.; Griffiths, M. W. *Appl. Environ. Microbiol.* **2010**, *76*, 528−535.
(14) Singh, A.; Glass, N.; Tolba, M.; Brovko, L.; Griffiths, M.; Evoy, S. *Biosens. Bioelectron.* **2009**, *24*, 3645−3651.
(15) Shabani, A.; Zourob, M.; Allain, B.; Marquette, C. A.; Lawrence, M. F.; Mandeville, R. *Anal. Chem.* **2008**, *80*, 9475−9482.
(16) Handa, H.; Gurczynski, S.; Jackson, M. P.; Auner, G.; Walker, J.; Mao, G. *Surf. Sci.* **2008**, *602*, 1392−1400.
(17) Lander, G. C.; Tang, L.; Casjens, S. R.; Gilcrease, E. B.; Prevelige, P.; Poliakov, A.; Potter, C. S.; Carragher, B.; Johnson, J. E. *Science* **2006**, *312*, 1791−1795.
(18) Kang, S.; Hawkridge, A. M.; Johnson, K. L.; Muddiman, D. C.; Prevelige, P. E. *J. Proteome Res.* **2006**, *5*, 370−377.
(19) Jiang, W.; Li, Z.; Zhang, Z.; Baker, M. L.; Prevelige, P. E.; Chiu, W. *Nat. Struct. Biol.* **2003**, *10*, 131−135.
(20) Lermo, A.; Campoy, S.; Barbé, J.; Hernández, S.; Alegret, S.; Pividori, M. I. *Biosens. Bioelectron.* **2007**, *22*, 2010−2017.
(21) Zacco, E.; Pividori, M. I.; Alegret, S.; Galve, R.; Marco, M. P. *Anal. Chem.* **2006**, *78*, 1780−1788.
(22) Pividori, M. I.; Alegret, S. In *Electrochemical DNA Biosensors*; Ozsoz, M., Ed.; Pan Stanford Publishing: Singapore, 2012; pp 57−93.
(23) Gibert, I.; Carroll, K.; Hillyard, D. R.; Barbé, J.; Casadesus, J. *Nucleic Acids Res.* **1991**, *19*, 1343−1344.
(24) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; pp 2.73−2.76.
(25) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248−254.
(26) Van Harreveld, A.; Khattab, F. I. *J. Cell Sci.* **1968**, *3*, 579−594.
(27) Liébana, S.; Lermo, A.; Campoy, S.; Barbe, J.; Alegret, S.; Pividori, M. I. *Anal. Chem.* **2009**, *81*, 5812−5820.
(28) U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health. *Biosafety in Microbiological and Biomedical Laboratories*; Chosewood, L. C., Wilson, D. E., Eds.; U. S. Government Printing Office: Washington, DC, 2007; pp 44−49. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm.
(29) Thouand, G.; Vachon, P.; Liu, S.; Dayre, M.; Griffiths, M. W. *J. Food Prot.* **2008**, *71*, 380−385.