

Enzyme-linked immunomagnetic electrochemical detection of *Salmonella typhimurium*¹

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Abstract

There is a need for rapid methods to detect pathogenic bacteria in food products as alternatives to the current laborious and time-consuming culture procedures. We report a microbial detection technique that combines the selectivity of antibody-coated superparamagnetic beads with the rapidity and sensitivity of electrochemical detection in a format termed enzyme-linked immunomagnetic electrochemistry. In it, *Salmonella typhimurium* were sandwiched between antibody-coated magnetic beads and an enzyme-conjugated antibody. With the aid of a magnet, the beads (with or without bound bacteria) were localized onto the surface of disposable graphite ink electrodes in a multi-well plate format. Enzyme substrate was added and conversion of substrate to an electroactive product was measured using electrochemical detection. The electrochemical response was directly proportional to the number of captured bacteria. Using this technique, a minimum detectable level of 8×10^3 cells/ml of *Salmonella typhimurium* in buffer was achieved in ca. 80 min.

Keywords: Magnetic bead; Electrochemistry; Immunomagnetic; *Salmonella*; Bacterium; Detection

1. Introduction

Conventional culture methods achieve highly sensitive and selective bacterial detection, but typically require days to weeks to complete. In recent years, researchers have strived to develop 'rapid' methods

that can replace arduous culture techniques (Feng, 1992; Hartman et al., 1992). Rapid methods require assay times on the order of minutes to days and include ATP bioluminescence (Griffiths, 1993), the antibody-direct epifluorescent filter technique (Tortorello and Stewart, 1994), enzyme immunoassays (Ibrahim, 1986; Park et al., 1994), impedance (Connolly et al., 1993), the polymerase chain reaction (Bej et al., 1994), the resonant mirror biosensor (Watts et al., 1994), and the quartz crystal microbalance biosensor (Minunni et al., 1995).

Electrochemical methods typically have the advantage of being highly sensitive, rapid, and inexpensive. While immunoassays using electrochemical detection were developed about 15 years ago (Heine-

Abbreviations: AP, alkaline phosphatase; IMB, immunomagnetic beads; ELIMC, enzyme-linked immunomagnetic colorimetry; ELIME, enzyme-linked immunomagnetic electrochemistry; pAPP, *p*-aminophenylphosphate; TBS, Tris-buffered saline

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¹ Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

mann et al., 1979), application of this combination for the rapid detection of whole bacterial cells has only recently been reported (Brooks et al., 1992; Hadas et al., 1992). In a typical ELISA assay, an enzyme-conjugated antibody or antigen is immobilized on the walls of tubes or microwells via an immunological interaction. During incubation with substrate, (typically 30–120 min) the product is formed at relatively high concentration at the wall surface, and then diffuses from the walls into the bulk solution. Following incubation, the concentration of product in the (now) homogeneous solution is measured by appropriate means. This homogeneous detection approach was used by Brooks et al. (1992), who employed a substrate/enzyme system which produced an electroactive product that could be quantitated amperometrically using electrodes inserted sequentially into separate microwells.

Hadas et al. (1992) utilized a heterogeneous detection approach, in which capture antibody was immobilized directly on a carbon felt electrode surface. An ensuing sandwich immunoassay resulted in localization of an antibody-enzyme conjugate at the surface and formation of a relatively high local concentration of product at the electrode after addition of enzyme substrate. Because electrochemical response is proportional to the surface concentration of analyte, the heterogeneous detection method can be orders of magnitude faster and more sensitive than homogeneous methods. Using the heterogeneous method, a limit of detection of ~ 10 cells/ml of *Staphylococcus aureus* and *Escherichia coli* bacteria with ~ 25 min total assay time has been reported (Rishpon et al., 1992).

The procedure of Hadas et al. (1992) employed a rotating disk electrode with high surface area to promote rapid and efficient antibody-mediated capture of bacteria. However, the electrode was exposed to both sample and conjugate solutions, and interference due to adsorption of sample and/or conjugate constituents cannot be ignored. The high capacitance of the felt electrodes can also result in slow rise times and high charging currents which effectively prevent the use of sensitive pulse electrochemical techniques. To circumvent these problems, an alternative approach can be used that retains a high surface area solid phase for efficient antibody-mediated analyte capture, while minimizing the size of the

electrode and eliminating contact between the electrode and the sample and/or conjugate solutions. Immunomagnetic beads (IMB) have been successfully applied to rapidly and conveniently separate cells from complex matrices by Molday et al. (1977). Recently, IMB have also been utilized in several rapid methods for the capture of bacteria prior to analysis (Fratamico et al., 1992; Olsvik et al., 1994). IMB have also been applied in immunoelectrochemical assays for the detection of choriogonadotropin (Robinson et al., 1985) and immunoglobulin G (Weetall and Hotaling, 1987) in cell-free systems. These latter methods, which for convenience, we shall refer to as ELIME (enzyme-linked immunomagnetic electrochemistry), combined the selectivity of antibody, the convenience of separation with magnetic particles, and the sensitivity of electrochemical detection.

In this study, we combined a sandwich immunoassay and immunomagnetic separation that included anti-*Salmonella* antibody-coated IMB, the blocking reagent casein, and alkaline phosphatase-conjugated anti-*Salmonella* antibody with Osteryoung square wave voltammetry performed at a magnetized graphite ink strip electrode surface for the analysis of *Salmonella typhimurium*. The results demonstrated that this modified form of ELIME shows promise for the rapid detection of whole bacterial cells.

2. Material and methods

2.1. Materials

Materials used in this research included: *Salmonella typhimurium* ATCC 14028 (Difco Laboratories, Detroit, MI), heat-killed *Salmonella typhimurium* cells (reconstituted in 50% glycerol and stored at -10°C until use) and alkaline phosphatase (AP)-conjugated goat anti-*Salmonella* antibody (anti-*Salmonella* conjugate; Kirkegaard & Perry Laboratories, Gaithersburg, MD), goat anti-*Salmonella* M-280 Dynabeads (immunomagnetic beads or IMB; $\sim 6.5 \times 10^8$ /ml, Dynal, Lake Success, NY), 10% palladium on charcoal (Baker & Co. Catalysts, Newark, NJ), tryptic soy broth, Butterfield's buffer (Fisher Scientific, Pittsburgh, PA), AP-conjugated

rabbit anti-goat antibody (anti-goat conjugate; product #A4187), gelatin (300 bloom, porcine skin), and Tween 20 (Sigma, St. Louis, MO), flat bottomed polystyrene microwell plates (Nunc, Denmark), graphite ink silk screen printed on Mylar (1 cm wide graphite ink strips separated by 3 mm on 31 cm \times 38 cm Mylar sheets; The Motson Co., Flourtown, PA), Alnico magnets (cylindrical, 4.8 mm diameter \times 25.4 mm; Edmund Scientific Co., Barrington, NJ), double coated plastic Tuck Carpet Installation Tape (Tesa Tape, Charlotte, NC), platinum wire (0.25 mm diameter, 99.99%; Aldrich, Milwaukee, WI), and disodium *p*-nitrophenyl phosphate (pNPP) along with the following blocking buffers in Tris-buffered saline: Blocker Blotto, 1% Blocker casein, 10% bovine serum albumin (BSA), and 1% SuperBlock blocking buffer (Pierce, Rockford, IL). Other chemicals used were of reagent grade.

2.2. Apparatus

All reactions with shaking were performed on a Vortex-Genie 2 (Scientific Industries, Bohemia, NY) at 'shake' setting #1. All reactions with rotating were performed on a Labquake Shaker (Labin-dustries, Berkeley, CA). A magnetic tube holder that incorporated fixed Alnico magnets with their poles

directed towards the sloping lower sides of micro-centrifuge tubes was custom-built in our laboratory. Bacteria samples were counted on a Petroff-Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, NJ). All colorimetric measurements were obtained using a Bio-Tek EL 311s microplate autoreader (Bio-Tek Instruments, Winooski, VT) in the dual wavelength mode ($\lambda_1 = 405$ nm, $\lambda_2 = 630$ nm). All electrochemical measurements were obtained with a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN) and accompanying BAS 100W software (version 1.0). An Ag/AgCl reference electrode (0.6 cm \times \sim 7 cm, Vycor tipped, Bioanalytical Systems), wrapped with a platinum wire that served as a counter electrode, was inserted into the tested solutions during electrochemical measurements.

2.3. Multi-well electrode / magnet assembly

A locally constructed 12-well electrode/magnet (see Fig. 1) was fashioned from two polymethyl methacrylate blocks, cylindrical Alnico magnets, graphite ink electrode strips on Mylar, and double coated tape. A solution holding block was constructed by boring cylindrical holes (6.4 mm diameters on 12.7 mm centers) through a polymethyl

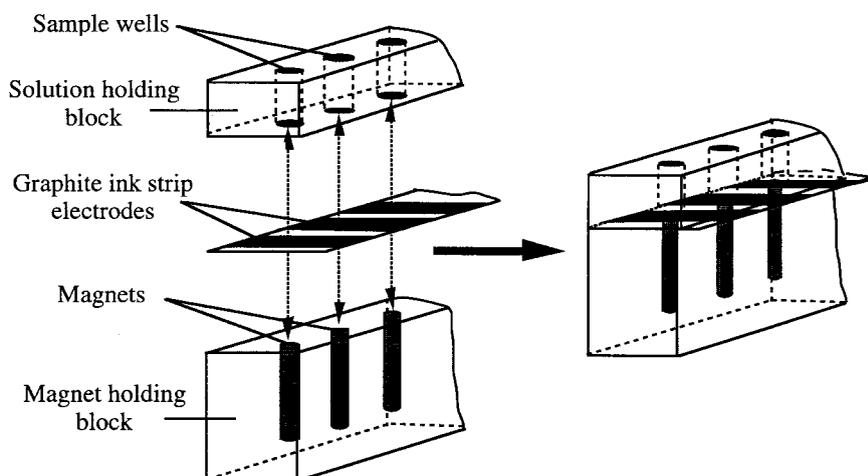


Fig. 1. Multi-well electrode/magnet assembly. The double-headed arrows indicate positioning of the solution and magnet holding blocks thus precisely aligning each magnet underneath separate sample wells. The individual strip electrodes were effectively sandwiched between a magnet and a sample well with the graphite ink exposed to and forming the bottom of each sample well. Only three of the 12 sample wells are shown.

methacrylate block with the dimensions 9.5 mm (h) \times 2.54 cm (w) \times 16.5 cm (l). The electrode strips (1 cm in width) were cut to \sim 2.5 cm in length and fixed to the bottom of the solution holding block with the double-sided tape so that a few millimeters protruded from the edge of the block to provide an electrical contact point. Holes were cut in the tape at the bottom of the solution wells using a punch (6.4 mm outside diameter) prior to adhering the electrode strips. Finally, a magnet holding block was constructed by boring holes (4.8 mm \times 25.4 mm depth on 12.7 mm centers) into the top side of a polymethyl methacrylate block that had the dimensions 3.2 cm (h) \times 2.54 cm (w) \times 16.5 cm (l) and inserting the Alnico magnets into each hole. The magnet holding block could be positioned directly beneath the solution holding block so that the magnets were precisely aligned and in close contact with the electrode strips which formed the bottoms of the solution wells. Prior to use, the electrode strips were pre-treated by addition of 0.6 M NaOH solution to the solution wells followed by three cyclic voltammetry sweeps from 0.3 to 1.2 V at 250 mV/s. The electrode strips then were rinsed extensively with nanopure water to remove any residual NaOH.

2.4. Synthesis of pAPP

Synthesis of pAPP by catalytic hydrogenation of pNPP was performed using the procedure of Boyland and Manson (1957) with the following modifications. In a 100 ml glass hydrogenation vessel, 2.503 g of pNPP was dissolved in 30 ml of 50% ethanol containing 0.109 g of 10% palladium on charcoal catalyst. The hydrogenation reaction was conducted overnight at room temperature at an initial pressure of 1.3 atm. The resultant mixture was filtered on a Büchner funnel to remove the catalyst and the volume of solvent was reduced to \sim 10 ml using a rotary evaporator. The oily residue was diluted to 20 ml with distilled, de-ionized water and clarified by filtration. Cold ethanol (20 ml, 4°C) was added to the filtrate and the precipitated product was recovered by filtration, dried under vacuum, and stored at -10°C . The pAPP product was greater than 95% pure as determined by NMR and electrochemical methods.

2.5. Growth of *Salmonella typhimurium*

A single Bactrol disk containing *Salmonella typhimurium* ATCC 14028 was inoculated into 10 ml of tryptic soy broth and incubated at 37°C for 4.5 h. The inoculant (1 ml) was transferred to a 500 ml baffled shake flask containing 100 ml of tryptic soy broth and shaken (150 rpm) overnight at 37°C. The cells were pelleted through centrifugation at $1725 \times g$ for 30 min and the supernate was discarded. The pelleted cells were resuspended in 100 ml of sterile Butterfield's buffer, centrifuged (as above), and the supernate was discarded. The cells again were resuspended in 10 ml Butterfield's buffer and a 1/100 diluted aliquot was counted (ten fields) on a bacteria counting chamber.

2.6. Irradiation of *Salmonella typhimurium*

Irradiation of *Salmonella typhimurium* ATCC 14028 cells was performed in a manner consistent with the method reported by Thayer and Boyd (1993). Cells were diluted to 1×10^{10} cells/ml in Butterfield's buffer and exposed to 12 kGy of gamma irradiation from a self-contained ^{137}Cs source. The samples were maintained at $5 \pm 1^\circ\text{C}$ during the irradiation process. The irradiated cells were further diluted 1:10 in phosphate-buffered saline (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) and aliquots were stored at -10°C for up to 3 months.

2.7. Comparison of blocking buffers

All of the following reactions in this and the remaining Materials and methods subsections were performed at room temperature. IMB (20 μl) were reacted by shaking for 30 min with either TBS (25 mM Tris, 150 mM NaCl, pH 7.6) or irradiated *Salmonella typhimurium* (diluted to 1×10^6 /ml in TBS) in 1.5 ml polypropylene microcentrifuge tubes. The tubes were placed into the magnetic tube holder for 3 min in order to trap the IMB (a portion containing bound bacteria when present) against the walls of the tubes and the liquid was removed by aspiration. Anti-*Salmonella* conjugate solutions were prepared by dilution of anti-*Salmonella* conjugate 1/500 in either TBS, 1% gelatin (in TBS), 2% Tween 20 (in TBS), 1% BSA (diluted from the 10% stock with TBS), or the 'ready-to-use' solutions of Blocker Blotto, 1% Blocker casein, or 1% Su-

perBlock. The IMB were resuspended in 1 ml of anti-*Salmonella* conjugate solution by gentle vortexing. The mixtures again were reacted by shaking for 30 min followed by separation of the IMB with the magnetic tube holder for 3 min. The IMB were washed twice with TBS to facilitate the removal of unreacted anti-*Salmonella* conjugate. Each wash consisted of resuspension of the IMB in 1 ml of TBS followed by separation with the magnetic tube holder for 3 min and removal of the liquid. The IMB then were resuspended in 0.2 ml TBS, transferred to the solution holding block of the multi-well electrode/magnet (Fig. 1), the magnet holding block was applied for 2 min to trap the IMB against the electrodes, and the liquid was removed by aspiration. After removing the magnets, the IMB were resuspended by gentle vortexing in 0.22 ml pNPP (2.7 mM in 0.2 M Tris, pH 9.6) and allowed to react for 90 min. During the last 2 min of the pNPP reaction, the magnet holding block was reapplied for 2 min to trap the magnetic beads at the electrode surface. The liquid (0.2 ml) was immediately transferred to polystyrene microwell plates and absorbance was measured at 405 nm. All absorbance values were pNPP blank subtracted to compensate for any spontaneously formed *p*-nitrophenol present in the pNPP.

2.8. Preparation of alkaline phosphatase-associated IMB (AP-IMB)

IMB were mixed with anti-goat conjugate in a 1.5 ml polypropylene microcentrifuge tube and diluted with TBS to yield a final concentration of $\sim 1.3 \times 10^7$ IMB/ml and a 1/280 anti-goat conjugate dilution. The tube was rotated for 30 min to allow the anti-goat conjugate to react with the IMB. To facilitate the removal of unreacted anti-goat conjugate, the mixture was centrifuged ($2000 \times g$ for 2 min) followed by removal of the supernate by aspiration. The pellet was washed twice by resuspension through gentle vortexing in TBS containing 0.05% Tween 20, centrifugation ($2000 \times g$ for 2 min), and removal of the supernate. The pellet then was washed by resuspension in TBS, centrifugation ($2000 \times g$ for 2 min), and removal of the supernate. The resultant AP-associated IMB (AP-IMB) were resuspended in TBS to a concentration of $\sim 1.3 \times 10^7$ AP-IMB/ml. Serially diluted AP-IMB, covering a range from 8.1×10^2 to 2.6×10^5 AP-IMB/ml, were mixed

with unmodified IMB so that the total bead concentration remained at 1.3×10^7 beads/ml. This gave bead suspensions having varying numbers of AP-IMB yet a constant total amount of beads.

2.9. Colorimetric and electrochemical detection of AP-IMB

Colorimetric analyses were conducted by placing 0.2 ml aliquots of the serially diluted AP-IMB in 0.6 ml polypropylene microcentrifuge tubes and pelleting them by centrifugation at $2000 \times g$ for 5 min. Only 180 μ l of the supernates were removed by aspiration, 0.2 ml of pNPP (2.7 mM in 0.2 M Tris, pH 9.6) was mixed by gentle vortexing into each tube, and colorimetric development was allowed to proceed for 30 min without agitation. The reaction was stopped by the addition of 25 μ l of 6 M NaOH and the tubes were again centrifuged at $2000 \times g$ for 5 min. Aliquots (200 μ l) of each supernate were transferred to a polystyrene microwell plate and absorbance was measured at 405 nm. All absorbance values were pNPP blank subtracted.

For electrochemical analysis, 200 μ l of the serially diluted AP-IMB were added to the solution holding block of the multi-well electrode/magnet (Fig. 1). The beads were magnetically trapped against the electrodes by application of the magnet holding block for 2 min and the liquid was removed by aspiration. With the magnetic field applied, 200 μ l of pAPP (2.7 mM in 0.2 M Tris, pH 9.6) was added to the well and allowed to react for 3 min. Production of electroactive *p*-aminophenol was measured using Osteryoung square wave voltammetry (-300 – 300 mV, 25 mV sweep width amplitude, 5 Hz frequency, 4 mV step potential, 10^{-6} A/V sensitivity) and the peak current was determined by drawing a tangent line across the base of the peak using the BAS 100W software.

2.10. Enzyme-linked immunomagnetic colorimetric (ELIMC) and ELIME detection of *Salmonella*

Using reaction volumes suggested by Dynal, 20 μ l of IMB were placed in 1.5 ml polypropylene microcentrifuge tubes, 1 ml of heat-killed *Salmonella typhimurium* (in TBS) was added, and the mixture was incubated by shaking for 30 min. The tubes were placed into the magnetic tube holder for 3 min in order to trap the IMB (a portion containing bound

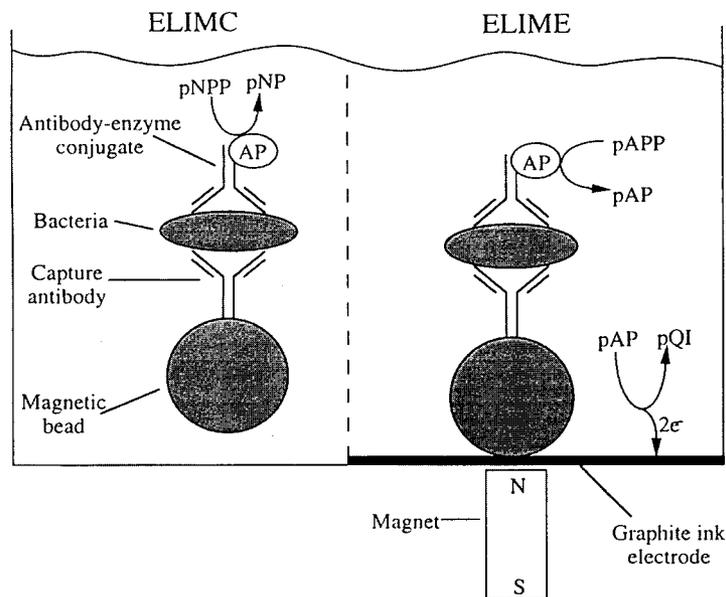


Fig. 2. Schematic representations of enzyme-linked immunomagnetic colorimetric (ELIMC) and electrochemical (ELIME) assays. Analyte is sandwiched between antibody-coated magnetic beads (immunomagnetic beads or IMB) and antibody-enzyme conjugate followed by addition of substrate. For ELIMC, suspended IMB are mixed with pNPP and product formation is measured colorimetrically. For ELIME, IMB are magnetically trapped onto the electrode surface, exposed to pAPP, and the product, pAP, is electrochemically detected. Abbreviations are as follows: AP, alkaline phosphatase; pNPP, *p*-nitrophenyl phosphate; pNP, *p*-nitrophenol; pAPP, *p*-aminophenyl phosphate; pAP, *p*-aminophenol; and pQI, *p*-quinone imine.

Table 1

Comparison of blocking agents for the control of non-specific binding of AP-labeled goat anti-*Salmonella* antibody conjugate to immunomagnetic beads (IMB)

Sample	Blocking agent	Absorbance at 405 nm (absorbance units)	Signal-to-background ratio
Blank	None	2.142 ± 0.185	
10 ⁶ bacteria	None	2.513 ± 0.231	1.2
Blank	Blotto	0.011 ± 0.019	
10 ⁶ bacteria	Blotto	0.005 ± 0.002	0.4
Blank	1% BSA	0.140 ± 0.067	
10 ⁶ bacteria	1% BSA	0.277 ± 0.111	2.0
Blank	1% casein	0.002 ± 0.004	
10 ⁶ bacteria	1% casein	0.185 ± 0.094	90
Blank	1% gelatin	0.189 ± 0.061	
10 ⁶ bacteria	1% gelatin	0.345 ± 0.202	1.8
Blank	1% SuperBlock	0.012 ± 0.006	
10 ⁶ bacteria	1% SuperBlock	0.111 ± 0.051	9.2
Blank	2% Tween 20	0.047 ± 0.002	
10 ⁶ bacteria	2% Tween 20	0.259 ± 0.016	5.5

IMB, incubated with either 0 (blank or background) or 10⁶ (signal) irradiated *Salmonella typhimurium*, were further reacted with AP-labeled goat anti-*Salmonella* antibody conjugate in the presence of the blocking agents listed above. After washing to remove unbound conjugate, any AP associated with the IMB was detected colorimetrically and signal-to-background ratios are indicated above.

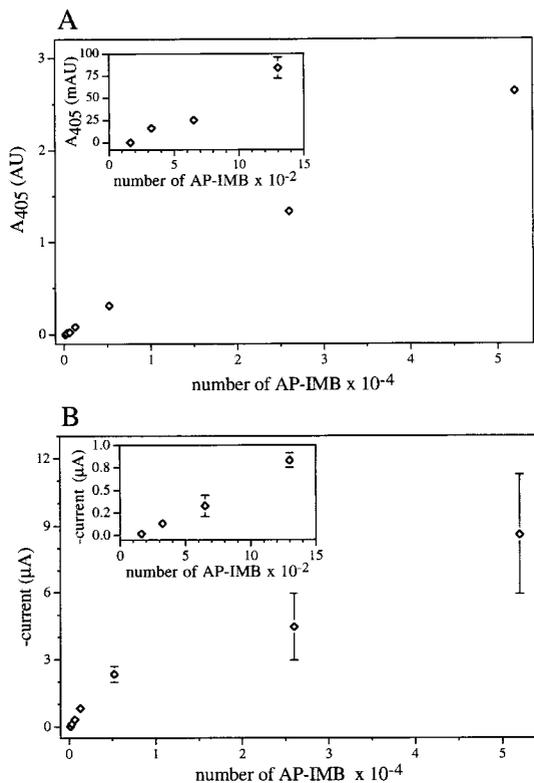


Fig. 3. Colorimetric vs. electrochemical detection of AP-associated immunomagnetic beads (AP-IMB). IMB were reacted with AP-labeled rabbit anti-goat antibody conjugate, washed to remove unbound conjugate, serially diluted, and associated AP was detected either colorimetrically or electrochemically, as previously described. The above curves show either the colorimetric (A: absorbance at 405 nm) or the electrochemical (B: current) responses for the serially diluted AP-IMB. The insets display an expanded portion of the low concentration region near the origin of each curve.

bacteria when present) against the walls of the tubes and the liquid was removed by aspiration. The IMB were resuspended by gentle vortexing and reacted by shaking with 1 ml of anti-*Salmonella* conjugate (diluted 1/500 in 1% Blocker casein) for 30 min. The IMB again were separated using the magnetic tube holder for 3 min and the liquid was removed. The IMB then were washed/resuspended twice with 1 ml TBS followed by magnetic separation for 3 min in the magnetic tube holder and removal of the liquid after each wash. Finally, the IMB were resuspended with 0.2 ml TBS.

For colorimetric analysis, the 0.2 ml IMB samples were transferred to 0.6 ml microcentrifuge tubes,

centrifuged at $2000 \times g$ for 5 min, and the supernates were removed by aspiration. The pellets then were reacted with 0.2 ml of pNPP for 30 min in the tubes. The reaction was stopped by the addition of 25 μl of 6 M NaOH and the contents of the tubes were again centrifuged at $2000 \times g$ for 5 min. Aliquots (200 μl) of the supernates were transferred to polystyrene microwell plates and absorbance was measured at 405 nm. All absorbance values were pNPP blank subtracted. The colorimetric detection of immunomagnetically captured bacteria is schematically represented in Fig. 2.

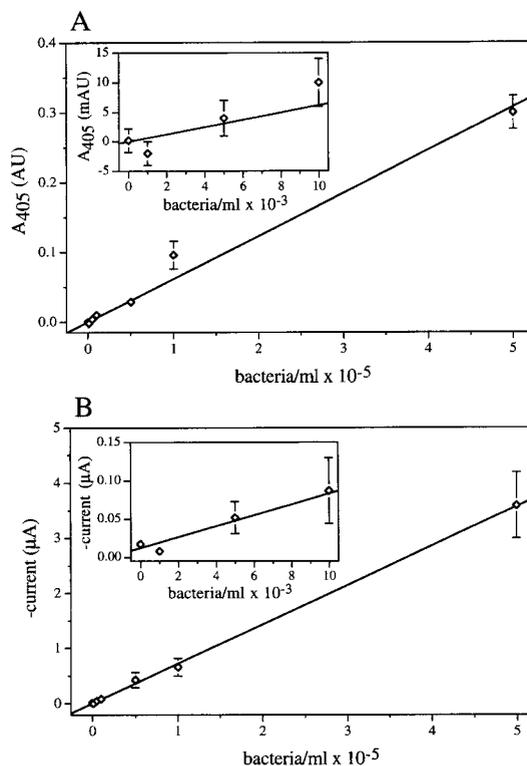


Fig. 4. ELIMC vs. ELIME detection of *Salmonella typhimurium* (bacteria). Bacteria samples (1 ml) were immunomagnetically captured with anti-*Salmonella* antibody-coated magnetic beads, labeled with AP-labeled goat anti-*Salmonella* antibody conjugate, and detected either colorimetrically or electrochemically as previously described. The above plots show either the colorimetric (A: absorbance at 405 nm) or the electrochemical (B: current) responses for the serially diluted bacteria. The insets display an expanded portion of the low concentration region near the origin of each curve.

For electrochemical analysis, the 0.2 ml IMB samples were transferred to the multi-well electrode/magnet (Fig. 1). After 2 min of magnetic separation by application of the magnet holding block, the liquid was removed by aspiration and the samples were assayed (as above for the electrochemical detection of AP-IMB). The electrochemical detection of immunomagnetically captured bacteria is schematically represented in Fig. 2.

3. Results

3.1. Control of the non-specific binding of anti-*Salmonella* conjugate

The background response generated from the non-specific binding of anti-*Salmonella* conjugate to IMB was determined colorimetrically. Table 1 shows the effectiveness of selected, commercially available, blocking reagents in preventing the conjugate from binding to IMB colorimetric results are displayed for the blank (background) and for the 10^6 cells/ml of *Salmonella typhimurium* bacteria (signal) along with the signal-to-background ratio. The first row of data in Table 1 demonstrates the high level of non-specific binding of anti-*Salmonella* conjugate to IMB (approximately 1.3×10^7 IMB are represented) in the absence of blocking reagent. The absorbance values in Table 1 represent the mean of at least three replicate measurements plus or minus the standard deviation from the mean.

3.2. Detection of AP-IMB

In order to test the multi-well electrode/magnet (Fig. 1) and the ELIME method (Fig. 2), various concentrations of AP-associated IMB were assayed both colorimetrically and electrochemically and the results are compared in Fig. 3. IMB were incubated with anti-goat conjugate to effectively immobilize AP onto the beads. The resultant beads, termed AP-IMB, were serially diluted in the presence of IMB that were not reacted with conjugate so that a constant total number of particles was maintained. AP-IMB were magnetically separated and then analyzed both colorimetrically and electrochemically. Fig. 3 displays the colorimetric (A) or electrochemical (B) response versus the number of AP-IMB tested. Samples corresponding to the four highest

concentrations were prepared and analyzed on the first day. On the second day, samples corresponding to the three lowest concentrations and to two of the higher concentrations were prepared and analyzed. Means and standard deviations were calculated from at least three replicates and include data from both days, where available. Data from the colorimetric plot was blank (substrate only) subtracted.

3.3. Detection of immunomagnetically isolated *Salmonella*

A comparison of ELIMC versus ELIME detection of *Salmonella typhimurium* is shown in Fig. 4. Bacteria were isolated from solution using IMB. The IMB (a portion containing bound bacteria when present) were magnetically separated, incubated with anti-*Salmonella* conjugate (in 1% Blocker casein), and then the IMB were washed to remove unreacted anti-*Salmonella* conjugate. Essentially, the bacteria were captured with IMB and AP-labeled in a sandwich assay format (Fig. 2). The sandwiched bacteria were then subjected to either colorimetric or electrochemical analysis. Fig. 4 displays the colorimetric (A) and electrochemical (B) response versus the number of *Salmonella typhimurium* bacteria/ml tested. All of the ELIMC or ELIME data points were fitted using error weighted linear regression. The ELIMC data was obtained in one experiment whereas the ELIME plot was constructed from overlapping measurements obtained during two separate experiments performed on different days. ELIME measurements were obtained for the even bacteria concentrations on one day and the odd bacteria concentrations were measured on the other. Data points from both plots represent the mean of three replicate measurements plus or minus the standard deviation from the mean.

4. Discussion

As with many immunological assays, the limit of detection (LOD) is proportional to the background response that arises from the non-specific binding of reporter antibody to the analyte capture phase. Therefore, we tested several blocking reagents for their ability to minimize background response in our system (i.e. electrochemical signal originating from electroactive analytes produced by enzyme-antibody

conjugate non-specifically bound to IMB). It should be noted that background response due to non-specific binding is distinct from background current that may originate from charging current and/or solvent oxidation during electrochemical processes. Since ELIME employs a small electrode surface area (relative to carbon felt immunoelectrodes), background current is expected to be considerably reduced with this system. As shown in Table 1, the use of Blocker casein was found to elicit the highest signal-to-background response ratio and hence the most effective at blocking the non-specific binding of anti-*Salmonella* conjugate to IMB.

The suspended flocculent solids in Blocker Blotto appeared to interfere with the magnetic isolation of the IMB by the custom-built magnetic tube holder. The subsequent poor isolation efficiency (observed visually) of the IMB in the presence of Blotto probably accounted for the low signal displayed (Table 1). Poor isolation of the IMB was also observed when gelatin was used, however the relatively high colorimetric signals reported may reflect an overall poor blocking ability for gelatin. Low isolation efficiencies for beads in glycerol solutions by the magnetic tube holder (data not shown) suggest that high viscosity contributed to the poor IMB isolation in the gelatin solutions. The use of either stronger magnets or longer magnetic isolation times with the magnet tube holder is expected to increase bead isolation efficiency. Research groups that have used magnetic devices, containing rare earth metals, have reported that complex sample matrices, including food, did not adversely affect the separation of bacteria with IMB (Skjerve et al., 1990; Luk and Lindberg, 1991; Skjerve and Olsvik, 1991).

For the detection of AP-IMB (Fig. 3), both the colorimetric and electrochemical techniques exhibited similar LOD. However, the electrochemical detection of AP-IMB was observed to become nonlinear for amounts of AP-IMB that corresponded to current responses of $\sim 2\text{--}3\ \mu\text{A}$. Although the nonlinear electrochemical response was a reproducible phenomenon, it was not further investigated since relatively high analyte (enzyme-labeled bacteria) concentrations that produced current responses greater than or equal to this were not of interest. Variance in the electrochemical responses was observed to increase with the concentration of analyte,

but this phenomenon also was not further investigated for the same reason.

In Fig. 4, the results for both the ELIMC and ELIME detection of *Salmonella typhimurium* are shown. As for the detection of AP-IMB, both techniques produced similar responses and LOD. Many researchers often specify the LOD as two or three times the standard deviation of the mean of a systems blank response added to the blank response. However, this method for defining LOD presumes a constant variance for the responses over the range of analyte concentration measured. ELIME, and to a lesser extent ELIMC, response was observed to display higher variance with increasing concentration of bacteria. We therefore determined the LOD for the two techniques using an alternative protocol. Calibration curves that included data from Fig. 4 were produced by running replicated analyses of standards containing $0, 5 \times 10^3, 1 \times 10^4,$ and 5×10^4 cells/ml and fitting a straight line by weighted linear regression of the data. The LOD was determined using the following formula (adapted from Currie, 1968):

$$\text{LOD} = \left[t(n_B - 1, \alpha) S_B n_B^{-1/2} + t(n_D - 1, \beta) S_D n_D^{-1/2} \right] m^{-1}$$

where n is the number of replicates, $t(k, P)$ is the abscissa of the t (Student's) distribution for k degrees of freedom at the probability level $1 - P$, S is the standard deviation of the replicates, and m is the slope of the calibration line. The subscripts B and D refer to the blank and the LOD, respectively. Using $n_B = n_D = 3$, $\alpha = \beta = 0.05$ (95% confidence limit), and using the standard deviation for the 5×10^3 cells/ml standard as an estimate of the variance for S_D we calculated a LOD of 2×10^4 cells/ml for ELIMC and 8×10^3 cells/ml for ELIME. The total assay times, based on a single sample, were ~ 110 min for ELIMC and ~ 80 min for ELIME. The rapidity of the ELIME assay relative to ELIMC is attributed to the ten times shorter substrate reaction time and ultimately the highly sensitive nature of electrochemical detection.

Future work will address reducing both the LOD and assay time for ELIME detection of bacteria. The LOD may be lowered by increasing the bead/bacteria capture efficiency. Recovery of *Salmonella livingstone* in phosphate-buffered saline

has been reported to be $51.0 \pm 7.8\%$ for 10^4 cells/ml reacted for 60 min with 10^7 antibody-coated Dynabeads (Vermunt et al., 1992). Increased bead/bacteria incubation temperature may also improve capture efficiency and/or analysis speed. The use of protein A in immobilizing antibodies to beads in a 'proper' orientation has been demonstrated to enhance the recovery of *Salmonella* (Widjoatmodjo et al., 1993). Lectins (Payne et al., 1993) or carbohydrates (Nilsson and Mandenius, 1994) added to or replacing the antibodies on the beads may also enhance recovery.

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