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Abstract

We demonstrate substitution of the custom-synthesized alkaline phosphatase (AP) substrate, *p*-aminophenyl phosphate (pAPP), with the commercially available 1-naphthyl phosphate (1-NP) as applied in the enzyme-linked immunomagnetic electrochemical (ELIME) detection of the pathogenic bacterium, *Escherichia coli* O157:H7. ELIME entails 'sandwiching' bacterial analyte between antibody-coated magnetic beads and an AP-conjugated antibody. The beads (with or without bound bacteria) were localized onto the surface of magnetized graphite ink electrodes in a multi-well plate format. Enzyme substrate (pAPP or 1-NP) was added and conversion to an electroactive product was measured using Osteryoung square wave voltammetry. Using this technique, quantitative detection of *E. coli* O157:H7 bacterial cells was achieved with a minimum detectable level of $\leq 4.7 \times 10^3$ cells ml^{-1} in buffer or porcine carcass wash water within ca. 80 min. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Bacteria; Biosensors; Detection; *Escherichia coli*; Immunomagnetic beads; Osteryoung square wave voltammetry

1. Introduction

Many rapid methods for detecting bacteria approach the selectivity and sensitivity, yet circumvent the relatively long analysis time of conventional culture techniques [1–3]. Some of these methods have employed the selectivity of immunoassays along with the rapidity and sensitivity of electrochemistry for the detection of molecular analytes or bacteria [4–6]. In addition, several methods have combined the selectivity and capture capability of μm-sized superparamagnetic antibody-coated particles (immunomagnetic beads or IMB) with electrochemistry for the specific detection of analytes [7–10]. The utility of IMB is particularly evident in the detection of analytes contained in complex sample matrices (e.g. heterogeneous food mixtures) which may exhibit either poor mass transport in immunoassays that utilize planar surfaces for binding/interaction or physical blockage of surfaces as with the clogging of filtration membranes.

In this study, we have applied enzyme-linked immunomagnetic electrochemistry (ELIME; Fig. 1) to the rapid detection of *Escherichia coli* O157:H7 cells in either buffer or porcine carcass wash water. Our choice of this particular pathogenic bacterium stems from its notorious association in food poisoning cases; acute renal failure in infants and young children is commonly caused by the development of hemolytic uremic syn-

Abbreviations: AP, alkaline phosphatase; IMB, immunomagnetic beads; ELIME, enzyme-linked immunomagnetic electrochemistry; 1-NP, 1-naphthyl phosphate; pAPP, *p*-aminophenyl phosphate; TBS, Tris-buffered saline.

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

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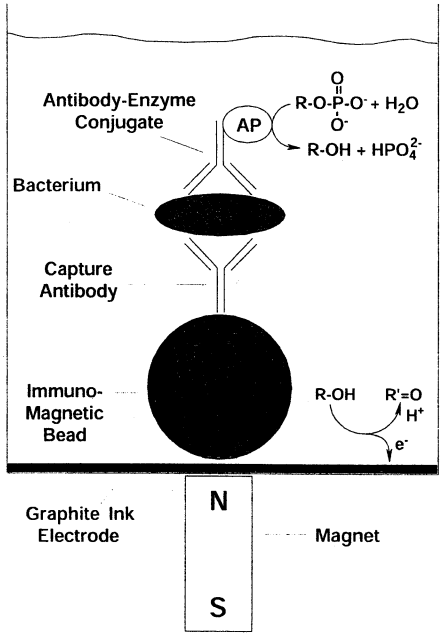

Fig. 1. Schematic representation of the enzyme-linked immunomagnetic electrochemical (ELIME) assay. Multiply immunogenic analyte (bacteria, for example) is sandwiched between antibody-coated superparamagnetic beads (immunomagnetic beads or IMB) and antibody–enzyme conjugate. IMB are trapped magnetically onto the electrode surface, exposed to substrate, and electroactive product is detected electrochemically. Abbreviations are as follows: AP, alkaline phosphatase.

drome upon infection with *E. coli* O157:H7 [11]. The ELIME methodology involved 'sandwiching' of *E. coli* O157:H7 cells between IMB and an alkaline phosphatase (AP)-conjugated antibody. The IMB (with or without bound bacteria) were localized onto graphite ink strip electrodes with the aid of permanent magnets. Enzyme substrate was then added and conversion to an electroactive product was quantified using Osteryoung square wave voltammetry [12,13]. The custom synthesized substrate for AP, *p*-aminophenyl phosphate (pAPP), used in our previous study with *Salmonella typhimurium* [7], was demonstrated to be replaced effectively by the commercially available reagent 1-naphthyl phosphate (1-NP). The substitution was deemed additionally favorable since *p*-aminophenol, the product of cleavage of pAPP by AP, is unstable under the alkaline measurement conditions that were employed [14].

2. Experimental

2.1. Materials

Materials used in this research included: AP-conjugated goat anti-*Escherichia coli* O157:H7 (anti-*E. coli* O157:H7 conjugate), AP-conjugated goat anti-*Salmonella* (anti-*Salmonella* conjugate), and heat-killed *Salmonella typhimurium* cells (Kirkegaard & Perry, Gaithersburg, MD), *E. coli* O157:H7 93-933 (Centers for Disease Control, Atlanta, GA), goat anti-*E. coli* O157 M-280 immunomagnetic beads (IMB), goat anti-*Salmonella* M-280 IMB, and magnetic particle concentrator (MPC-M; Dynal, Lake Success, NY), graphite ink silk screen printed on Mylar (1 cm wide graphite ink strips separated by 3 mm on 31×38 cm^2 Mylar sheets; Motson, Flourtown, PA), Alnico magnets (cylindrical 4.8 mm diameter $\times$ 25.4 mm; Edmund Scientific, Barrington, NJ), 1-NP (disodium salt, 98%) and platinum wire (0.25 mm diameter, 99.99%; Aldrich, Milwaukee, WI), 1% Blocker™ casein (Pierce, Rockford, IL), tryptic soy broth and Butterfield's buffer (Fisher Scientific, Pittsburgh, PA), and double coated plastic Tuck carpet installation tape (Tesa tape, Charlotte, NC). The synthesis of pAPP was performed as described previously [7]. Other chemicals used were of reagent grade. Porcine carcass wash water, obtained from a local abattoir, was collected from a single carcass during the second wash immediately following evisceration; carcass wash water was used for experimentation the same day that it was collected unless otherwise noted.

2.2. Apparatus

All reactions with shaking were performed on a Vortex-Genie 2™ (Scientific Industries, Bohemia, NY). Bacteria samples were enumerated with a Petroff–Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, NJ). Electrochemical analyses of samples were performed in a custom-built multi-well electrode/magnet assembly constructed from polymethyl methacrylate blocks, Alnico magnets, double-sided tape, and graphite ink strip electrodes [7] and quantified with a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN) and BAS 100W software (version 2.0). An Ag | AgCl | 3 M KCl reference electrode ($0.6 \times \sim 7$ cm^2, Vycor™ tipped, Bioanalytical Systems), wrapped with a platinum wire that served as a counter electrode, was positioned in the test solutions during electrochemical measurements.

2.3. Growth of E. coli O157:H7

A loopful of *E. coli* O157:H7 cells, collected from a slant, 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 by centrifugation at $1725 \times g$ for 30 min. The pelleted cells were suspended in 100 ml of sterile Butterfield's buffer and centrifuged (as above). The cells were resuspended in 10 ml Butterfield's buffer and a 1:100 diluted aliquot was

enumerated (ten fields) with the bacterial counting chamber.

2.4. Irradiation of E. coli O157:H7

Irradiation of *E. coli* O157:H7 cells was performed in a manner consistent with a previously reported method [15]. Briefly, cells were diluted to 1×10^{10} cells ml^{-1} in Butterfield's buffer and exposed to 12 kGy, at a rate of 0.097 kGy min^{-1}, of gamma radiation from a self-contained ^{137}Cs source. The samples were maintained at 5 ± 1°C during the irradiation process. The irradiated cells, stored at −70°C for up to 3 years, 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 6 months.

2.5. AP assay

The determination of Michaelis–Menten constants for anti-*Salmonella* conjugate was investigated with pAPP and 1-NP as substrates for AP. Substrate serial dilutions (0–3 mM, 0.1 M Tris, 40 mM KCl [pH 9.6]), were utilized for generating kinetic activity profiles of the anti-*Salmonella* conjugate. AP activity was ascertained by monitoring the release of inorganic phosphate using a colorimetric assay based on the formation of a green ($\lambda_{max} = 660$ nm) phosphate–molybdate–malachite complex [16].

2.6. ELIME detection of bacteria

Using reaction volumes suggested by Dynal, 20 μl of IMB were placed in 1.5 ml polypropylene microcentrifuge tubes, 1 ml of bacteria (in either TBS [25 mM Tris, 150 mM NaCl, pH 7.6] or 0.9 ml porcine carcass wash water diluted with 0.1 ml 10X TBS) was added, and the mixture was incubated with shaking (agitation just sufficient to prevent settling of the IMB) for 30 min. The tubes were placed into the magnetic particle concentrator for 3 min in order to trap the IMB against the walls of the tubes and the liquid was removed by aspiration. During IMB concentration, the particle concentrator was inverted gently several times to dislodge any IMB located in the cap of the tubes and to focus the IMB into a single spot. The IMB were resuspended by gentle vortexing with 1 ml of either anti-*E. coli* O157:H7 or anti-*Salmonella* conjugate (diluted 1:500 in 1% Blocker™ casein) for 30 min. The IMB were again separated using the magnetic particle concentrator for 3 min and the liquid was removed. The IMB were then washed/resuspended twice with 1 ml TBS followed by magnetic separation (as above). Finally, the IMB were resuspended with 200 μl TBS.

For electrochemical analysis, 200 μl of the IMB suspension was added to the solution holding block of a multi-well electrode/magnet. The beads were magnetically trapped against the electrodes for 2 min and the liquid was removed by aspiration. With the magnetic field applied and the counter/reference electrode inserted into the sample well, 200 μl of pAPP or 1-NP (2.7 mM in 0.2 M Tris, pH 9.6) was added to the well and allowed to react for 5 min. The production of electroactive *p*-aminophenol or 1-naphthol was measured using Osteryoung square wave voltammetry (−300 to 300 mV for pAPP or 50 to 500 mV for 1-NP, 25 mV sweep width amplitude, 5 Hz frequency, 83.4 ms delay time, 16.6 ms sampling time, 4 mV step potential, 10^{-6} A/V sensitivity); the peak current was determined by drawing a tangent line across the base of the peak using the BAS 100W software. The electrochemical detection of immunomagnetically captured bacteria is represented schematically in Fig. 1.

Table 1
Comparison of Michaelis–Menten constants of two substrates for alkaline phosphatase

Substrate	K_m/μM	V_{max}/μmol mg^{-1} min^{-1}
p-Aminophenyl phosphate	480 ± 20	470
1-Naphthyl phosphate	650 ± 30	850

3. Results and discussion

Previous investigations in this laboratory employed the custom-synthesized AP substrate, pAPP, for the ELIME detection of *S. typhimurium*. The requirement to synthesize pAPP and the instability of *p*-aminophenol, the enzymatic product of AP acting upon pAPP, at alkaline pH [14] prompted the study of commercially available 1-NP as an alternative substrate for AP. The Michaelis–Menten kinetic constants of the two substrates with antibody-conjugated AP were compared. In addition, ELIME standard curves for *S. typhimurium* with 1-NP were compared with data obtained previously with pAPP.

The enzymatic activity of anti-*Salmonella* antibody conjugated AP was monitored through colorimetric determination of released inorganic phosphate upon reaction with either of the AP substrates, pAPP or 1-NP. Apparent Michaelis–Menten constants for the substrates (Table 1) were derived from double reciprocal plots of AP activity at different substrate concentrations.

The AP substrates were also compared as candidates for use in ELIME detection of *S. typhimurium*. Essentially, the substrates were tested for reactivity with AP and electrochemical activity of their enzymatic products in a comparison of two individual experiments. The resultant current versus bacteria concentration profile

obtained with 1-NP was related to the previous results that utilized pAPP [7]. For this investigation, heat-killed *S. typhimurium* cells again were sandwiched between anti-*Salmonella* coated IMB and anti-*Salmonella* conjugate. However, instead of pAPP, 1-NP was substituted as a substrate for AP prior to electrochemical detection. Table 2 displays current versus bacteria concentration for quadruplicate measurements utilizing 1-NP as compared with pAPP.

Additional experiments were performed to challenge our ELIME method with the detection of bacteria contained in a relatively complex sample matrix, porcine carcass wash water. The experimental results represent the ELIME detection of *E. coli* O157:H7 cells in buffer (TBS; Figs. 2(A and B)) or carcass wash water (Table 3 and Fig. 3). To obtain the results, irradiated *E. coli* O157:H7 cells were diluted serially in TBS or TBS-buffered carcass wash water followed by isolation with IMB. The IMB were magnetically separated, incubated with anti-*E. coli* O157:H7 conjugate (or anti-*Salmonella* conjugate as a control) and the IMB were then washed to remove unreacted conjugate. Thus, the bacteria were captured with IMB and labeled with AP in a sandwich immunoassay format (Fig. 1). The sandwiched bacteria were then subjected to electrochemical analysis. Figs. 2(B) and 3 (and Table 3) display the electrochemical response versus the number of *E. coli* O157:H7 bacteria/ml tested. The data in Table 3 demonstrate the variability of ELIME responses achieved with different lots of carcass wash water samples. Fig. 3 displays three selected wash water data sets, representing the scatter in regression slopes for the data in Table 3. The ELIME data for the bacteria in TBS only, excluding those for the controls, was obtained in an experiment that was repeated three times on separate days using quadruplicate measurements per datum point. Results for the controls in either Figs. 2(B) or 3 were obtained in one experiment with quadruplicate measurements per datum point.

Table 2
Comparison of substrates in the ELIME detection of heat-killed *Salmonella typhimurium*

Cell concentration/ # bacteria ml^{-1}	*p*-Aminophenyl phosphate (−current/nA) [a]	1-Naphthyl phosphate (−current/nA) [b]
0	16.92 ± 4.54	98.03 ± 5.40
1000	7.688 ± 3.19	nd [c]
2500	nd	112.8 ± 4.68
5000	51.60 ± 21.2	118.9 ± 4.09
10 000	86.88 ± 42.8	152.6 ± 7.78
50 000	427.1 ± 136.4	389.0 ± 57.3
100 000	659.2 ± 158.3	nd
500 000	3591 ± 598	nd
Line of regression	$y = 7.1 \times 10^{-3}x + 12$ $r^2 = 0.999$	$y = 5.9 \times 10^{-3}x + 95$ $r^2 = 0.999$

[a] $n = 3$.
[b] $n = 4$.
[c] nd = Not determined.

4. Conclusions

Previous research has shown the AP substrate, 1-NP, to be an effective substrate for uses in electrochemical immunoassays that incorporated AP as a labeling enzyme [14,17]. This investigation has revealed that 1-NP was suitable for ELIME that utilized the same enzyme label, AP, as demonstrated by similarities to pAPP in enzyme activity and electrochemical response to 1-naphthol (Tables 1 and 2). Although the oxidation of 1-naphthol appeared to foul the graphite ink strip electrodes utilized in ELIME, the intent of incorporating graphite ink strip electrodes into ELIME analysis was to take advantage of the disposable nature of the electrodes which would circumvent any problems associated with either such electrode deterioration or contamination due to carryover.

The background current observed in the absence of bacteria was significantly higher for 1-NP than for pAPP. We attribute this to impurities or direct oxidation of the parent 1-NP in the commercial 1-NP, and experiments with recrystallized 1-NP showed much lower background (data not shown). However, this observation was not pursued in this work since the variance in the background was similar for pAPP and 1-NP and variance is the primary factor influencing the detection limit and reproducibility (see below).

Many researchers often specify the limit of detection (LOD) as 2 or 3 × the standard deviation of the mean of a system 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. Since ELIME does not demonstrate constant variance in the replication of response over the range of *E. coli* O157:H7 cell concentrations measured, an alternate protocol was used. The LOD was determined by generating a calibration curve, produced by replicated analyses of standards containing 0, 1.0×10^3, 2.5×10^3, 5.0×10^3, and 1.0×10^4 cells ml^{-1} (actual data used were adapted from Figs. 2(B) and 3 although a conservative assumption of quadruplicate measurements was used for the former), fitting a line by regression of the data, and adapting the following formula [18]:

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

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

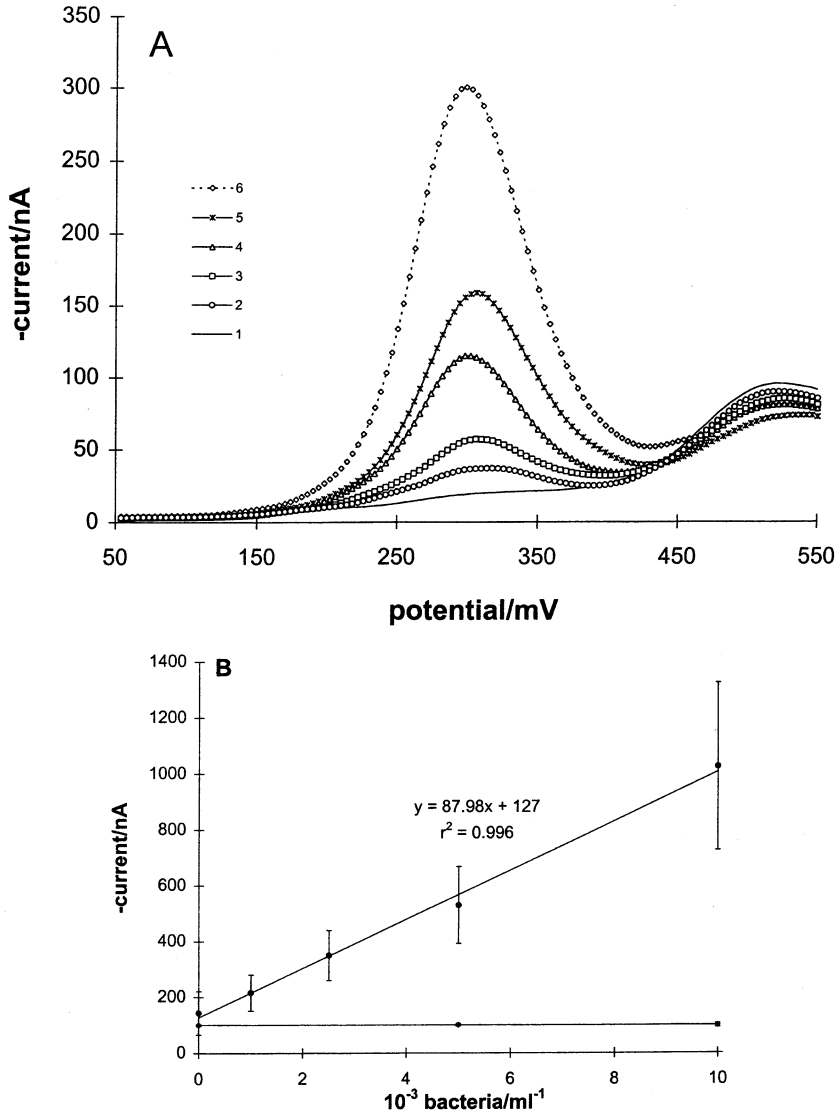

Fig. 2. ELIME detection of gamma irradiated *E. coli* O157:H7 cells. (A) Typical ELIME responses as represented by raw Osteryoung square wave voltammograms. ELIME was performed on 1 ml samples of *E. coli* O157:H7, diluted serially to the following concentrations per ml: (1) 0; (2) 6.2×10^3; (3) 1.25×10^4; (4) 2.5×10^4; (5) 5.0×10^4; and (6) 1.0×10^5 in Tris-buffered saline (TBS), by immunomagnetic capture with anti-*E. coli* O157:H7 IMB, reaction with either AP-labeled anti-*E. coli* O157:H7 conjugate (alkaline phosphatase [AP] labeled antibody), and electrochemical detection as described using 1-NP as an enzymatic substrate. (B) *E. coli* O157:H7 cells (bacteria) were diluted serially in TBS and aliquots (1 ml) were analyzed using ELIME that incorporated either anti-*E. coli* O157:H7 conjugate (circles) or anti-*Salmonella* conjugate (control; diamonds). The above plot displays the electrochemical (current) responses for the varying concentrations of bacteria that were tested.

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 2.5×10^3 cells ml^{-1} standard as an estimate of the variance for S_D we calculated a LOD of 4.7×10^3, 1.2×10^3 (Lot 2), 3.2×10^2 (Lot 4) and 2.9×10^3 cells ml^{-1} (Lot 5B) for *E. coli* O157:H7 in TBS and the three porcine carcass wash water samples represented in Figs. 2B and 3, respectively. The total assay time, based on a single sample, was ~ 80 min for ELIME. However, since the samples are reacted concurrently in a multi-well format, only ~ 5 min is required for the analysis of additional samples. The additional 5 min corresponds to the time needed for substrate reaction and electrochemical detection which may have been avoided if a multiplex electrochemical arrangement, analogous to that of a previously described system [19], that allows for simultaneous electrochemical detection at multiple working electrodes was employed. Such a multiplex arrangement for ELIME in a multi-well format would necessitate a common reference and counter electrode, perhaps in the form of a strip of silver and platinum, respective, running the length of the multiplate wells on the Mylar plastic that contains the silk screen printed graphite ink strips.

Table 3
ELIME detection of *E. coli* O157:H7 in fresh (unless otherwise noted) porcine carcass rinse, electrochemical responses (using 1-naphthyl phosphate as an alkaline phosphatase substrate) listed are current (−nA)

Cells ml^{-1}	$-I$/nA							
	Lot 1	Lot 1A [a]	Lot 2 [b]	Lot 3	Lot 4	Lot 5 [c]	Lot 5A [c]	Lot 5B [c]
0	60.77	146.6	**95.90**	89.90	**159.5**	145.0	144.0	**129.4**
	62.83	109.3	**113.3**	89.95	**167.0**	117.4	114.4	**102.4**
	81.66	171.7	**114.9**	93.56	**173.9**	146.4	162.7	**113.0**
	–	165.0	**119.8**	98.46	**185.4**	129.0	153.6	**160.9**
1000	–	297.4	**297.7**	–	**252.1**	246.0	213.4	**250.2**
	–	191.6	**308.8**	–	**284.4**	165.1	180.7	**163.0**
	–	321.3	**335.9**	–	**294.4**	277.9	248.3	**245.5**
	–	228.1	**337.5**	–	**315.6**	276.0	185.3	**200.1**
2500	–	527.4	**539.0**	–	**455.1**	446.4	420.0	**394.7**
	–	339.8	**671.6**	–	**460.2**	413.8	307.6	**260.1**
	–	532.4	**713.9**	–	**465.0**	467.9	441.0	**428.8**
	–	545.0	**741.2**	–	**465.3**	510.6	356.0	**343.0**
5000	584.5	603.8	**941.1**	554.1	**652.2**	561.6	612.6	**769.1**
	648.8	563.0	**1052**	559.7	**694.9**	604.2	748.9	**536.0**
	676.5	862.7	**1075**	610.5	**732.0**	764.0	665.0	**729.7**
	–	546.7	**1370.**	624.7	**845.3**	517.0	593.6	**431.1**
10 000	–	998.8	**1964**	923.2	**1353**	1173	1208	**957.1**
	–	987.2	**2021**	1039	**1371**	1129	1415	**963.0**
	–	1711	**2076**	1323	**1428**	1349	1293.	**1040.**
	–	1288	**2441**	1328	–	920.1	830.0	**938.9**

[a] Lot 1 carcass wash stored for 7.5 months frozen (−10°C) until thawed and warmed to room temperature immediately prior to use.
[b] Values in bold-face are represented in Fig. 3.
[c] Carcass wash stored at 4°C for 1, 2, and 4 days (Lot 5, 5A, and 5B, respective) until a portion was removed and warmed to room temperature immediately prior to use.

The blank values for the ELIME detection of *E. coli* O157:H7 in porcine carcass wash water samples, relative to detection in TBS, were observed to be very consistent from day to day. Furthermore, the response was linear but the slope varied considerably. Several experiments were conducted to identify the source of this variation. The carcass wash water samples were rosé colored, due to hemolysis of red blood cells, and turbid, due to the presence of suspended fat globules. The varying lots of carcass wash water tested were buffered with TBS so that ionic strength and pH were similar to TBS and remained consistent between the lots as confirmed by conductivity and pH measurements (data not shown). The Bradford assay [20] was used to test protein content in selected lots of wash water that were found to contain a range of 0.25–0.5 mg ml^{-1} protein. Varying lots of wash water were determined consistently, through non-selective plate culturing, to contain natural flora at approximately 10^2 total aerobic bacteria per ml. The negligible ELIME response of the negative controls in Figs. 2(B) and 3 demonstrates the lack of cross-reaction between *E. coli* O157:H7 cells and AP-labeled antibody directed against other bacteria (*Salmonella*). The negligible response also indicates a lack of non-specific binding between the antibody–enzyme conjugate to either the IMB or carcass wash matrix that may be potentially bound to the bacterial cells and/or the IMB. Plans to elucidate the varied ELIME response for carcass wash water samples through investigation of the possibility of non-specific binding of a sample matrix constituent to the IMB as it effects either IMB/bacteria binding efficiency or electrochemical activity are under consideration.

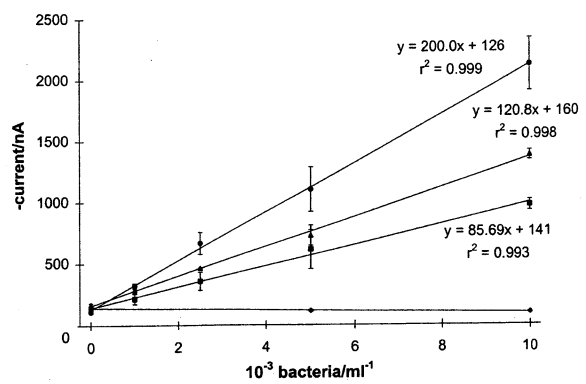

Fig. 3. ELIME detection of gamma irradiated *E. coli* O157:H7 cells in porcine carcass wash. Irradiated *E. coli* O157:H7 cells (bacteria) were diluted serially in porcine carcass wash water, aliquots (1 ml) were captured immunomagnetically with anti-*E. coli* O157:H7 antibody-coated magnetic beads, reacted with either anti-*E. coli* O157:H7 antibody conjugate (circles, triangles, and squares represent three different sets of data) or anti-*Salmonella* antibody conjugate (control; diamonds), and detected electrochemically as described. The above plot displays the electrochemical (current) responses for the varying concentrations of bacteria that were tested.

Our application of ELIME resulted in a minimum detectable level (MDL) of $\leq 4.3 \times 10^3$ *E. coli* O157:H7 cells/ml in buffer or porcine carcass wash water within ~ 80 min. This result compares favorably with current rapid methods developed by other researchers for the specific detection of *E. coli* O157:H7. Such current methods include: filtration capture/immunoelectrochemical detection (MDL of $\sim 5 \times 10^3$ cells/ml in an assay time of 25 min) [21], immunoligand assay/light-addressable potentiometry ($\sim 7.1 \times 10^2$ cells ml^{-1}, 45 min) [22], immunomagnetic-electrochemiluminescence (10^2–10^3 *E. coli* O157 cells ml^{-1}, < 1 h) [8], immunomagnetic separation/flow cytometry ($< 10^3$ colony forming units or CFU ml^{-1}, ~ 1 h) [23], immunomagnetic separation/flow injection analysis/mediated amperometric detection ($\sim 10^5$ CFU ml^{-1}, ~ 2 h) [24], and multiplex polymerase chain reaction/agarose gel electrophoresis (1.3×10^4 CFU ml^{-1}, 2.75 h) [25].

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