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# Immunochemical Assays for Bacteria: Use of Epifluorescence Microscopy and Rapid-Scan Electrochemical Techniques in Development of an Assay for *Salmonella*

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Immunochemical sensors in which the sensor surface functions as both analyte capture phase and electrochemical detector have recently been developed for bacteria analysis. The speed and sensitivity of these devices make them very attractive for applications such as the detection of pathogenic microorganisms in food and water. However, the development and optimization of assays utilizing these sensors can be complicated by undesired interactions between the capture and detection functions. Modification of the sensor to achieve improvements in one function can have deleterious effects on the other function, and such effects can be difficult to diagnose and correct. In the course of investigations on immunochemical detection of *Salmonella*, we developed a rapid, nondestructive epifluorescence microscopy method to determine bacteria capture efficiency. This method enabled us to study capture and detection functions independently and efficiently identify performance-limiting factors. Rapid-scan electrochemical methods were used to optimize detection sensitivity and to provide diagnostic information on detection performance.

Foodborne illnesses caused by pathogenic microorganisms pose a serious threat to public health. Over 5 million cases of foodborne bacterial disease, resulting in hundreds of deaths and costing nearly 7 billion dollars, were estimated to occur annually in the United States during the years 1981–1986.<sup>1,2</sup> Current practices for preventing microbial contamination of foods rely upon careful control of all aspects of food handling (e.g., heat treatment, equipment sterilization) in order to ensure product safety. Testing of food products to verify the absence of contamination could significantly reduce the incidence of foodborne illness, but effective testing requires methods of analysis that meet a number of very challenging criteria. Speed of analysis is critical, since modern processing and distribution systems operate very rapidly. Very low detection limits are required, since an infectious dose may, in principle, be as little as one organism. Because pathogenic bacteria may comprise a very small fraction of an otherwise benign population of microorganisms, extremely selective detection methodology is required. Existing methods are unable to meet

these requirements,<sup>3</sup> and considerable effort is now directed toward the development of new methods that can rapidly detect low levels of pathogens in foods, water, and clinical samples.

Approaches currently under investigation include immunoassay,<sup>4,5</sup> immunofluorescence microscopy,<sup>6</sup> DNA-based tests,<sup>7–9</sup> and optical<sup>10</sup> and piezoelectric sensors.<sup>11</sup> A very promising approach was recently reported<sup>12,13</sup> which used an enzyme-linked immunochemical (IEC) method to detect fewer than 100 cells/mL *Staphylococcus aureus* and *Escherichia coli* in a 20 min analysis. IEC assays were initially developed ~15 years ago<sup>14,15</sup> in order to exploit the high sensitivity of electrochemical detection methods as a replacement for radioactive detection. Virtually all the immunoassay formats that have been developed for optical or radioactive detection now have IEC analogs.<sup>16</sup> Initial applications used electroactive antigens or antibodies/antigens with electroactive labels. The enzymes commonly used in enzyme-linked immunosorbent assays (ELISA)<sup>17</sup> can also produce electrochemically detectable products, and the chemical amplification possible with enzyme-linked assays has led to the dominance of this approach for both electrochemical and optical immunoassays. Most applications of IEC assays have been in clinical pharmaceutical analysis, with relatively few studies aimed at microbial analysis. Kroll's group<sup>18–22</sup> utilized electrochemical detection in conjunction

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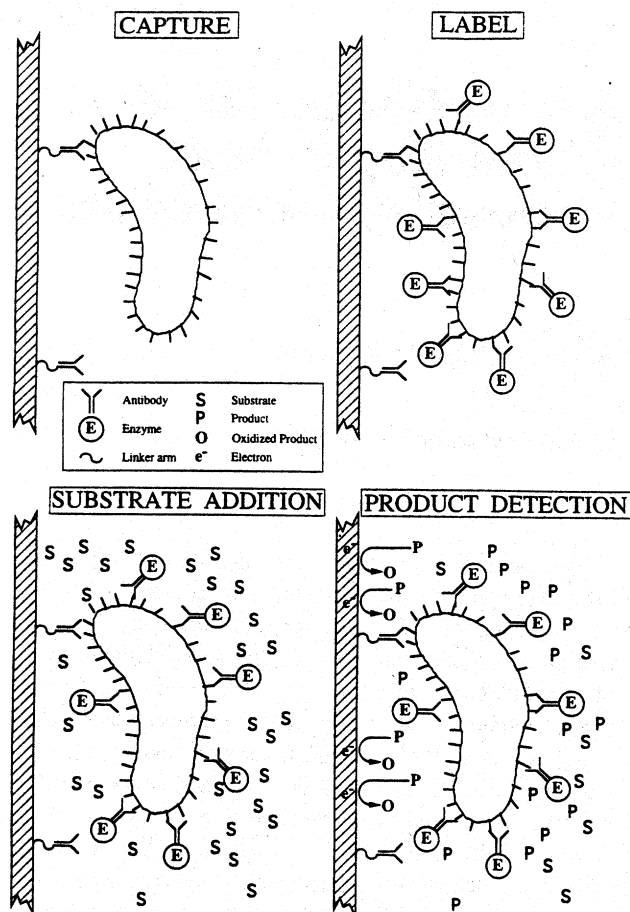
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**Figure 1.** Outline of the surface-capture immunoelectrochemical IEC technique. No linker arm was used in this work (capture antibody was adsorbed directly to the electrode).

with conventional ELISA methods. Bacteria were captured by antibody adsorbed to the walls of polystyrene microwell plates, labeled with antibody-enzyme conjugate, and incubated with substrate. The product formed at the surface of the plate diffused into the bulk solution, where it was detected by potentiometric or amperometric measurement. This approach gave detection limits of  $\sim 10^4$  cells/mL for *Salmonella*,  $\sim 1$  order of magnitude lower than the same assay using optical absorbance detection. In the approach used by Rishpon's group,<sup>12,13</sup> the surface of the electrode itself was used as the solid phase.<sup>23,24</sup> The basis of this surface-capture IEC technique is outlined in Figure 1. Antibodies to the target organism were immobilized at the surface of an electrode, which was then exposed to the sample solution. The target organism was captured on the surface, and the remaining sample constituents were washed off. After incubation with an enzyme-labeled antibody and washing, the electrode was placed in a solution containing an electrochemically inactive substrate, *p*-aminophenyl phosphate (*p*-APP). The electroactive product formed by the enzyme *p*-aminophenol (*p*-AP), was oxidized at the electrode surface, producing a measurable current. The layer of

bound enzyme very close to the surface produced a high local concentration of product, which diffused slowly into the bulk solution. Because the electrochemical response is proportional to the surface concentration of product, while absorbance and fluorescence respond to the average concentration throughout the solution, the surface-capture IEC assay can be orders of magnitude more sensitive than conventional ELISA using optical detection. The use of porous, high-surface area electrodes and efficient agitation of solutions, as well as the rapid generation of signal at the surface, provided very short assay times.

The electrode is a particularly critical component in surface-capture IEC, since it is required to function as both a platform for immobilization of the capture antibody and as a detector. These functions are not independent and may, in fact, be antagonistic. For example, high antibody loading and large electrode area can enhance capture efficiency—but may reduce overall performance by inhibiting electron transfer and/or increasing background and charging currents. Our initial efforts at detection of *Salmonella* using porous carbon felt electrodes with chronoamperometric detection gave highly variable results and relatively low sensitivity. It was difficult to determine whether the poor performance was due to inefficient capture of bacteria or to problems with electron transfer, since only the overall current could be measured. After limited success with trial-and-error approaches to optimization, we sought some means of independently monitoring the capture and detection functions in order to identify the performance-limiting factor(s) and rationally develop improvements. We found fluorescence microscopy and rapid-scan electrochemical techniques to be very effective for monitoring bacteria capture and electron transfer, respectively. A description of these techniques and their use in improving a surface-capture IEC assay being developed for *Salmonella* is presented here.

## EXPERIMENTAL SECTION

**Materials.** Water was deionized in-house with a Nanopure water treatment system (Barnstead, Dubuque, IA). The enzyme substrate *p*-APP was synthesized as described below. Affinity-purified goat antibody to *Salmonella* Common Structural Antigens and alkaline phosphatase (AP)-conjugated goat antibody to *Salmonella* Common Structural Antigens, and heat-killed *Salmonella typhimurium* cells were from Kirkegaard & Perry Laboratories, Inc., (Gaithersburg, MD). Affinity-purified AP conjugate rabbit anti-goat IgG, Sigma-FAST pNPP substrate tablets (containing *p*-nitrophenyl phosphate and Tris buffer), bovine serum albumin (BSA) fraction V, and Tween-20 were from Sigma (St. Louis, MO). Disodium *p*-nitrophenyl phosphate (*p*-NPP) was from Pierce (Rockford, IL). Ten percent palladium on charcoal was from Baker & Co. Catalysts Inc. (Newark, NJ). Flat-bottomed polystyrene microwell plates were from Fisher Scientific (Pittsburgh, PA). Glassy carbon disk electrodes consisting of a 3 mm diameter disk embedded in the end of a 6 mm diameter Kel-F cylinder, Ag/AgCl reference electrodes, and polishing materials were from Bioanalytical Systems, Inc. (West Lafayette, IN). Abrasive paper was obtained from a local store. All other chemicals were of reagent grade.

**Apparatus.** A BAS 100B/W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN) equipped with a C-2 cell stand and RDE-1 rotating disk electrode module was used for all electrochemical measurements. An EL 311s microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by a Macintosh Plus computer (Apple Computer Inc., Cupertino, CA)

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running  $\Delta$ Soft software (BioMetallics Inc., Princeton, NJ) was used to read absorbance of microwell plate samples at 405 nm. Epifluorescence microscopy was conducted with a Leitz Orthoplan microscope equipped with a 200 W high-pressure mercury lamp, and H3 and M2 filter sets. Digital microscope images were acquired with a Nikon Diaphot TMD epifluorescence microscope (Nikon, Inc., Melville, NY) equipped with a 200 W high-pressure mercury lamp, a STAR I cooled CCD camera (Photometrics, Inc., Tucson, AZ), controlled by a Macintosh Power PC computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health).

**Synthesis of *p*-APP.** *p*-Aminophenyl phosphate was prepared by catalytic hydrogenation of *p*-NPP using the procedure of Boyland and Manson<sup>25</sup> with the following changes. In a 100 mL glass hydrogenation vessel, 2.503 g of *p*-NPP 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 19 psi. The mixture was filtered on a Buchner funnel to remove the catalyst and the volume of the filtrate reduced to ~10 mL using a rotary evaporator. The oily residue was adjusted to 20 mL with water and filtered. Cold ethanol (4 °C, 20 mL) was added to the filtrate, and the precipitated product was recovered by filtration, dried under vacuum, and stored at -10 °C. The purity was >95% by NMR and no *p*-AP was detectable electrochemically.

**Solutions.** Carbonate buffer, 0.2 M sodium carbonate, pH 9.6; TBS, 25 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM sodium chloride, pH 7.6; TTBS, 25 mM tris(hydroxymethyl)aminomethane, 150 mM sodium chloride, 0.05% Tween-20, pH 7.6; *Fe(II)*, 1 mM  $K_4Fe(CN)_6$  in carbonate buffer; *p*-NPP, Sigma-FAST *p*-NPP and buffer tablets dissolved in water following the manufacturer's directions; *blocking*, 0.1% BSA in TBS; *substrate*, 5 mM *p*-APP in carbonate buffer prepared shortly before use; *Acridine Orange*, 0.02% acridine orange in water; *capture antibody*, 5  $\mu$ g/mL goat anti-*Salmonella* in TBS; *reporter antibody*, 0.5  $\mu$ g/mL alkaline phosphatase conjugated goat anti-*Salmonella* in TBS; *anti-goat antibody conjugate*, alkaline phosphatase conjugated rabbit anti-goat antibody (6250 AP units/mL) diluted in TTBS; *bacteria*, lyophilized cells reconstituted in 50% glycerol to yield stock solution ( $5 \times 10^9$  cells/mL), which was aliquoted and frozen at -10 °C; aliquots thawed and diluted in TBS to the desired concentration shortly before use.

**Electrochemical Measurements.** Electrodes were placed in a small glass vessel containing 1.5 mL of *Fe(II)* or substrate solution, a Ag/AgCl reference electrode, a Pt wire auxiliary electrode, and a magnetic stir bar. For testing electrode performance and reproducibility cyclic voltammograms of *Fe(II)* were recorded from 0 to 500 mV at 25 mV/s without stirring. For assays, Osteryoung square wave voltammetry<sup>26</sup> was conducted under the following conditions: initial potential, -200 mV; final potential, 200 mV, square wave amplitude, 25 mV; frequency, 6 Hz; step potential, 4 mV; quiet time, 2 s; sensitivity:  $10^{-6}$  A/V. The electrode was placed in stirred substrate solution and equilibrated for at least 30 s. The stirrer was then stopped and the first scan initiated. After 180 s a second scan was initiated. The measurement cycle (stirring followed by two scans without stirring) was repeated two to three times. The current from the first scan in each cycle was subtracted from the second scan, and

the net peak current for each cycle was recorded. The conditions used in other experiments are given below.

**Colorimetric Measurement.** Electrodes were placed in 1.5 mL polypropylene centrifuge tubes containing 500  $\mu$ L of *p*-NPP solution. After incubation for 60–120 min at room temperature the electrode was removed and two 200  $\mu$ L aliquots of the liquid were transferred to microplate wells. The amount of *p*-nitrophenol produced was determined by measuring the absorbance at 405 nm.

**Electrode Preparation.** Electrodes as received from the manufacturer were wet-sanded for ~15 s using 320 grit silicon carbide abrasive paper (roughened electrodes). The electrodes were rinsed thoroughly in a stream of methanol and then a stream of water and sonicated for 5 min in water before further use. The electrode performance and reproducibility was tested as above using *Fe(II)* solution, followed by thorough washing in water. Electrodes that exhibited anomalous currents, peak widths, or peak separations were reroughened. All incubations with solutions were conducted at room temperature (21–24 °C) in a small chamber saturated with water to prevent evaporation. The electrodes were held vertically (active surface up) and solution was pipetted onto the surface. The electrode was more readily wetted than the hydrophobic sheath, so that small volumes (<50  $\mu$ L) of liquid formed a rounded droplet several millimeters high, which covered only the electrode surface. Somewhat larger volumes of liquid (75–90  $\mu$ L) covered the end of the electrode and sheath but did not flow onto the even more hydrophobic sides of the sheath cylinder. Electrodes were washed by pipetting 2 mL of buffer over the surface in a gentle stream. In some experiments, electrodes were incubated with bacteria by immersion in a polypropylene microcentrifuge tube containing 1 mL of solution and a small magnetic stir bar.

**Capture Antibody Loading Measurement.** The electrode was incubated for 60 min with 50  $\mu$ L of capture antibody solution. It was then washed (TBS), incubated for 30 min with 75  $\mu$ L of blocking solution, and washed (TTBS) again. A 50  $\mu$ L aliquot of a 1:2000 dilution of anti-goat antibody conjugate solution was carefully pipetted so that the solution covered only the electrode surface and it was incubated for 30 min. The electrode was then washed with TTBS and carbonate buffer. The electrodes were kept in 12  $\times$  75 mm glass test tubes containing 0.5 mL of carbonate buffer until assayed colorimetrically. The response obtained was compared to a calibration curve obtained from known amounts of anti-goat antibody conjugate in solution in order to determine the amount of anti-goat antibody conjugate immobilized on the electrode.

**Anti-Goat Assay.** The electrode was incubated for 60 min with 50  $\mu$ L of capture antibody solution. It was then washed (TBS), incubated for 30 min with 75  $\mu$ L of blocking solution, washed (TTBS), incubated 30 min with 50  $\mu$ L of anti-goat antibody conjugate solution (various dilutions), and washed with TTBS and carbonate buffer. The electrodes were kept in 12  $\times$  75 mm glass test tubes containing 0.5 mL of carbonate buffer until assayed by Osteryoung square wave voltammetry.

**Bacteria Assay.** Capture antibody solution (75  $\mu$ L) was pipetted onto the electrode and incubated for 1 h. The electrode was then washed (TBS), incubated for 30 min with 75  $\mu$ L blocking solution, and washed (TBS) again. Following incubation for 60 min with 75  $\mu$ L of bacteria sample solution, the electrode was washed (TBS) and incubated 60 min with reporter antibody

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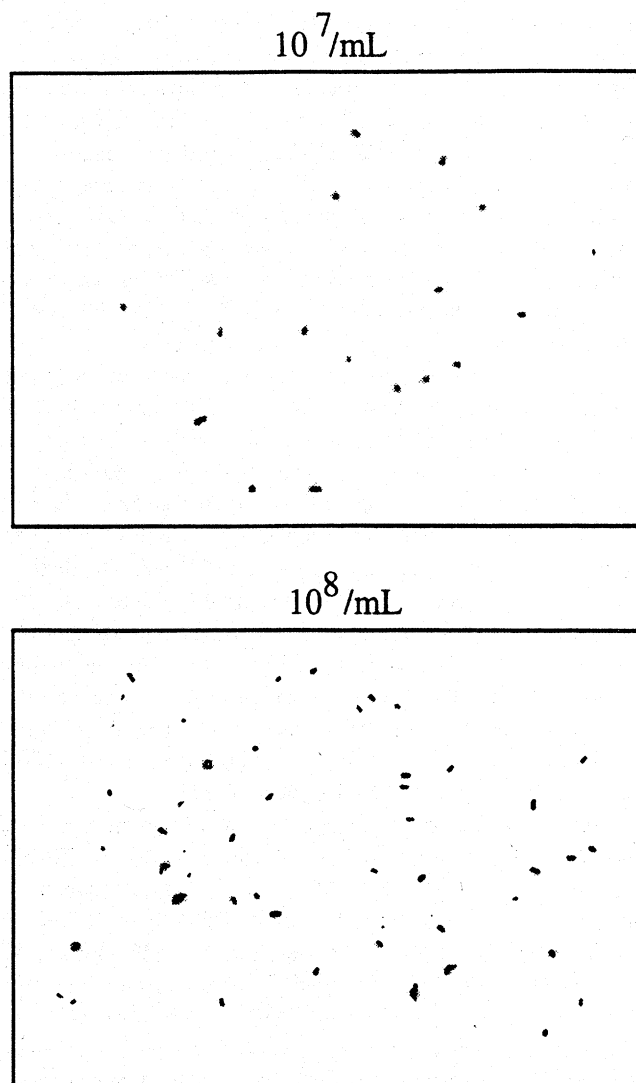
solution. The electrodes were then washed with TBS and covered with 75  $\mu$ L of TBS until assayed.

**Bacteria Counting.** Electrodes with bound bacteria were held vertically (active surface up), and 75  $\mu$ L of Acridine Orange solution was pipetted onto the surface. After 2 min the electrode was washed with 2 mL of water and allowed to dry, and the number of bacteria in a series of randomly selected fields was counted at 40 $\times$  magnification ( $\sim$ 450  $\mu$ m diameter field). The depth of field was greater than 2  $\mu$ m under these conditions, and all the bacteria ( $\sim$ 1  $\mu$ m long) in a field could be counted without changing the focus. The average number was multiplied by 45 (ratio of the electrode area to image area) to determine the total number of captured bacteria.

**ELISA.** All procedures were performed at room temperature. Individual wells of polystyrene microwell plates were incubated for 60 min with 200  $\mu$ L of antibody solution (or TBS for determining nonspecific binding), emptied, incubated for 30 min with 300  $\mu$ L of BSA blocking solution, and washed twice with  $\sim$ 200  $\mu$ L of TTBS using a plastic wash bottle. Sample or standard (200  $\mu$ L) was added (four replicates for each concentration) and incubated for 30 min. For determining nonspecific binding, 200  $\mu$ L of  $10^8$  cells/mL standard was used. Wells were emptied, washed twice with  $\sim$ 200  $\mu$ L of TTBS, and incubated for 30 min with antibody AP conjugate. Wells were washed four times with  $\sim$ 200  $\mu$ L of TTBS, allowing the last wash to remain for 5 min. *p*-NPP substrate solution (200  $\mu$ L) was added to each well, and absorbance at 405 nm was measured at 0, 15, and 30 min. Bacteria concentration was determined from a calibration curve generated from the standards.

## RESULTS AND DISCUSSION

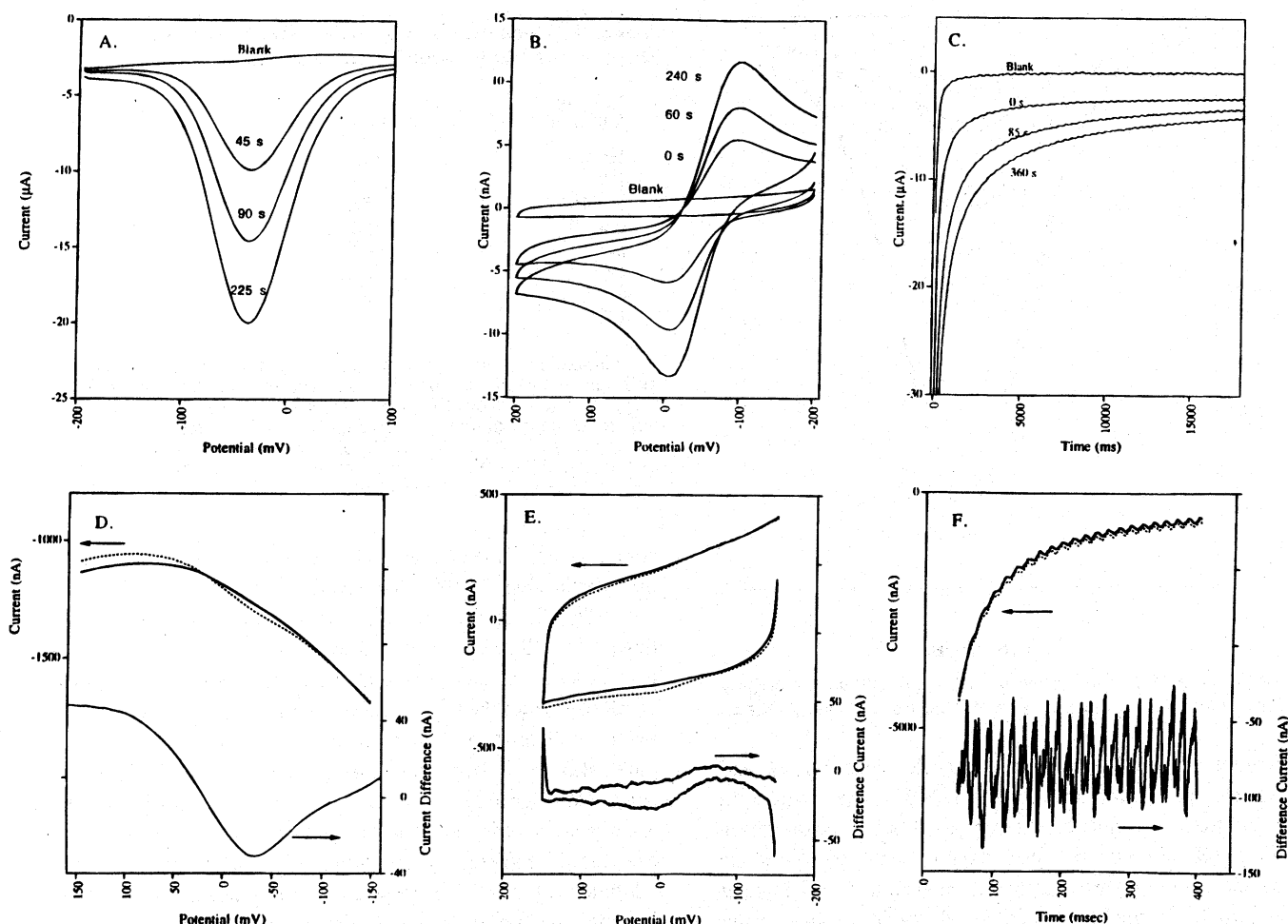
**Selection of Electrodes and Staining Procedures.** In order to utilize microscopy for monitoring bacteria capture, planar electrodes of relatively small area were required. Glassy carbon was selected as a commercially available planar electrode material similar to carbon felt, although the two materials have significant differences in surface chemistry and area. To achieve high sensitivity and avoid interference from particulates, epifluorescence microscopy was used. A number of stains and staining protocols were tested in efforts to find a procedure that would give quick, reproducible fluorescent staining of bacteria bound to electrodes, good contrast between bacteria and the electrode, and minimal loss of captured cells. Staining with Acridine Orange in water for 2 min proved to be the simplest and gentlest procedure tested and was quite effective. The fluorescence of stained cells faded with a half-life of  $\sim$ 20 min, but cells could be restrained without difficulty. High-contrast images were obtained, and bacteria could be detected at 40 $\times$  without oil or water immersion lenses. Bacteria were typically distributed uniformly over the surface as individual cells (see Figure 2). It was possible to reanalyze electrodes electrochemically after staining and measure currents that were within 10% of the initial currents. This result indicated that the staining did not cause significant loss of bacteria or enzyme label and did not substantially alter the electron transfer function of the electrode. To allow completion of an assay with a set of six to eight electrodes (including bacteria counting) in 1 day, it was necessary to limit the number of fields observed on each electrode to approximately five. Estimates of bacteria coverage made from such observations were only approximate, but the level of precision proved to be adequate for studying assay performance. The brightness of the fluorescence made it unlikely



**Figure 2.** Negative epifluorescence image of *S. typhimurium* captured on antibody-covered glassy carbon electrodes at 40 $\times$ . Initial bacteria concentrations as shown.

that significant numbers of bacteria were not observed, and similar bacteria counts were obtained consistently when capture conditions were replicated. More sophisticated measurements such as digital image capture and automated counting could clearly be used to improve precision, if required.

**Selection of Assay Conditions.** An ELISA was developed for *S. typhimurium* using the same cells, antibody, antibody conjugate, and blocking reagents as used in the immunoelectrochemical assay. Detection limit for the assay was  $\sim 5 \times 10^5$  cells/mL, comparable to other reported methods.<sup>5</sup> The ELISA results served to guide selection of antibody concentrations, incubation times, etc. Adsorption of antibody proved to be a simple, reproducible method for producing stable antibody-coated electrodes. The amount of adsorbed capture antibody, estimated by reaction with AP conjugated anti-goat antibody and colorimetric determination of the bound AP, was  $\sim 0.2$  ng/electrode. This calculation assumed that the enzymatic activity of the bound anti-goat antibody conjugate was equal to the free conjugate. Although this assumption and any assumed correspondence between binding of anti-goat conjugate and bacterial cells is questionable, the results indicated that functional capture antibody was present at reasonably high and reproducible levels. As this coverage was



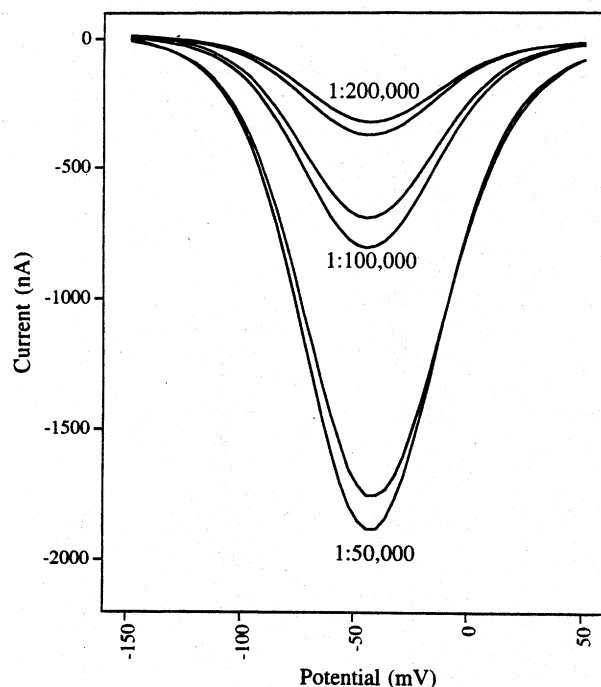
**Figure 3.** Comparison of electrochemical techniques. All potentials vs Ag/AgCl reference. (A, D) Osteryoung square wave voltammetry: initial potential,  $-200$  mV; final potential,  $200$  mV, square wave amplitude,  $25$  mV; frequency,  $6$  Hz; step potential,  $4$  mV; quiet time,  $2$  s; sensitivity,  $10^{-6}$  A/V. (B, E) Cyclic voltammetry: initial potential,  $-200$ ; final potential,  $200$ ; scan rate,  $25$  mV/s; sensitivity,  $10^{-6}$  A/V. (C, F) Chronoamperometry: initial potential,  $-200$  mV; step potential,  $200$  mV; final potential,  $-200$  mV; sensitivity,  $10^{-6}$  A/V. For panels A–C, electrode treated following capture antibody loading protocol. For panels D–F, electrode treated following bacteria assay protocol without sample.

adequate for capture of test analytes and bacteria, no further optimization of antibody loading was performed in this study. In order to conserve reagents and prevent adsorption of samples or reagents to the sheath material, which could result in high colorimetric or electrochemical background, we adopted the practice of applying droplets of liquids to the electrode tip rather than immersing electrodes in reagent solutions.

**Evaluation of Electrochemical Techniques.** Most amperometric electroanalytical methods are based on changing the working electrode potential from a value at which the analyte is unreactive to a potential at which it undergoes transport-limited electron transfer. The potential change may be a step function (chronoamperometry), a ramp (cyclic voltammetry), or a more complex series of steps (e.g., square wave voltammetry). Current flow arises from both charge transfer processes (faradaic current) and from charging the capacitance of the electrode–solution interface (charging current). The charging current is proportional to electrode surface area and the rate at which the potential is changed and decays exponentially following a potential step. The faradaic current is proportional to surface area, analyte concentration, and a fractional power of the rate of potential change and decays with a  $t^{-1/2}$  dependence following a potential step. The slower decay of the faradaic current after a potential step can be used to discriminate the faradaic signal from the charging current

background in chronoamperometry. More sophisticated techniques that take the difference in current between steps in two directions can almost completely eliminate charging current contributions. However, there are important instrumental limitations to use of such techniques with high surface area electrodes. First, the currents and voltages required to rapidly change the potential of the electrode can exceed the capabilities of many electroanalytical instruments. Second, the charging current may be so large that the faradaic current is near or below the resolution of the signal-processing system and becomes lost in the instrumental noise.

Published reports on detection of bacteria with surface-capture IEC<sup>12,13</sup> used high surface area electrodes and a custom-made signal-averaging chronoamperometric detection system.<sup>26,27</sup> Using commercially available instrumentation, we were unable to obtain results with electrochemical methods other than chronoamperometry with these electrodes except at very low scan rates. This failure was attributed to the high surface area ( $\sim 10$  cm<sup>2</sup>) and resulting high charging current of the felt. With the low surface area ( $0.071$  cm<sup>2</sup>) planar glassy carbon electrodes it was possible to explore other methods to determine whether they could provide greater diagnostic capability and/or greater sensitivity than



**Figure 4.** Detection of anti-goat alkaline phosphatase conjugate by OSWV. Conjugate diluted as shown. See text for assay details.

chronoamperometry. Two methods, cyclic voltammetry and Osteryoung square wave voltammetry (OSWV), were studied in depth, and their performance was compared to chronoamperometry (Figure 3). Studies were performed under assay conditions with an electrode prepared using the capture antibody loading protocol (high enzyme loading) and an electrode prepared using the bacteria assay protocol (low enzyme loading). In the latter case, no bacteria were applied to the electrode, so that only the background level of antibody conjugate was present. Sequential runs by all three methods on an electrode with high enzyme loading (Figure 3A–C) showed large current increases as the enzymatic reaction proceeded. The performance at low enzyme loading (Figure 3D–F) was quite different. In these experiments the analytical signal was recovered from the background by making two scans 180 s apart and subtracting the initial scan (in which the signal was dominated by background processes) from the second scan. Chronoamperometry (Figure 3F) gave virtually no change in response as product was formed. Measurements by cyclic voltammetry (Figure 3E) were noisy, though useful signal could be extracted. OSWV provided the best rejection of background currents and highest signal-to-noise ratio. OSWV peak shapes and peak potentials were sensitive indicators of the status of the sensor surface and permitted rapid diagnosis of problems such as fouling or contamination. Such information was difficult to extract from chronocoulometric or chronoamperometric data. The reproducibility in response for a set of electrodes prepared at the same time was good with “small” analytes such as anti-goat AP conjugate. As shown in Figure 4, replicate electrodes exposed to the same dilution of the conjugate gave peak currents that agreed within 15%. Replicate analyses on the same electrode gave peak currents with relative standard deviations of less than 5% over a current range of 10–200 nA.

In early experiments with planar electrodes, the bacteria capture step was performed by immersing electrodes in 0.5–1 mL volumes of sample solution with agitation (the protocol followed with carbon felt electrodes), with detection by cyclic

**Table 1. Surface-Capture IEC Detection from Stirred Solutions of *Salmonella typhimurium*<sup>a</sup>**

<i>Salmonella</i> (cells/mL)	average current (nA)	SD, <i>n</i> = 2 (nA)
10 <sup>6</sup>	84.5	1.94
10 <sup>6</sup>	112	16.3
5 × 10 <sup>5</sup>	98.0	10.73
5 × 10 <sup>5</sup>	48.2	0.61
10 <sup>5</sup>	38.9	1.87
10 <sup>5</sup>	33.4	6.48
0	nd <sup>b</sup>	nd
10 <sup>6</sup> <sup>c</sup>	31.5	8.90

<sup>a</sup> Conditions: assay conditions as given in the Experimental Section with the exception of the bacteria capture and detection method. Capture was performed by placing electrodes in 1 mL of magnetically stirred sample solution. Detection by cyclic voltammetry. Initial potential, −200 mV; final potential, 200 mV, scan rate, 25 mV/s; sensitivity, 10<sup>−6</sup> A/V. <sup>b</sup> nd, not determined. <sup>c</sup> No capture antibody.

voltammetry. Detection limits in these studies were >10<sup>6</sup> cells/mL. Application of OSWV reduced the detection limit to less than 10<sup>5</sup> cells/mL, as shown in Table 1. However, the currents observed in this experiment were much lower than predicted based on the number of cells in the sample, estimated numbers of AP molecules/cell from ELISA assays (data not shown), and the current observed when the immobilized surface antibody molecules were saturated or nearly saturated with anti-goat AP conjugate (Figure 3A). It could not be determined whether this discrepancy was due to inefficient capture of bacteria, to inefficient labeling of bacteria by anti-*Salmonella* AP conjugate, or to inhibition of electrode response after exposure to bacteria. Experiments were performed (before the microscopic method was fully developed) to determine the number of captured bacteria by incubation of the electrodes in *p*-NPP solution followed by colorimetric detection of *p*-NP product. To prevent interference from bacteria and anti-*Salmonella* AP conjugate adsorbed to the electrode sheath, which could not be distinguished from bacteria on the electrode, reagents and samples were applied in small volumes (50 μL) directly to the electrode surface, where they formed droplets due to the hydrophobicity of the sheath material. It was expected that smaller numbers of bacteria would be captured, since the immersed electrodes were exposed to 20 times more cells and transport of cells to the electrode surface was far more efficient in stirred solutions. However, experiments with bacteria captured from droplets consistently gave currents more than 10 times higher than with immersion. The explanation which appeared most credible was that the electrodes were being fouled by a sample constituent and that fouling was more pronounced for the immersed electrodes since they were exposed to a larger mass of solution/unit area and agitated more efficiently than the droplet-treated electrodes. Further experiments were then conducted in which antibody-coated, BSA-blocked electrodes were exposed to high concentrations of bacteria in four ways: by immersion in unstirred solution, by immersion in stirred solution, and by placing 25 or 75 μL droplets directly on the electrode surface. After incubation for 1 h, the electrodes were washed, stained, and examined microscopically. The results of this experiment, shown in Table 2, showed that far more bacteria were captured on the droplet treated electrodes than on the immersed electrodes and that the capture efficiency was very small in both cases. With microscopy we were able to quickly and unequivocally confirm that the low currents observed in the original



**Table 2. Effect of Incubation Conditions on Capture of  $10^7$ /mL *Salmonella* on Roughened Glassy Carbon Electrodes**

incubation method	agitation	vol of solution (mL)	no. of cells applied	no. of cells captured	% captured
droplet	no	25	$2.5 \times 10^5$	1125	0.45
droplet	no	25	$2.5 \times 10^5$	900	0.36
droplet	no	75	$7.5 \times 10^5$	2475	0.33
droplet	no	75	$7.5 \times 10^5$	2250	0.30
immersion	no	1000	$10^7$	<225	<0.01
immersion	no	1000	$10^7$	<225	<0.01
immersion	yes	1000	$10^7$	315	<0.01
immersion	yes	1000	$10^7$	<225	<0.01

**Table 3. Surface-Capture IEC Detection and Epifluorescence Counting of *Salmonella* on Roughened Glassy Carbon Electrodes**

capture antibody	<i>Salmonella</i> (cells/mL)	av peak current (nA)	no. of cells captured
yes	$10^7$	3354	2025–4000
yes	$10^7$	5968	2025–4000
yes	$10^7$	2755	2250–4500
yes	$10^6$	1744	1350–1800
yes	$10^6$	739	1125–1575
yes	0	261	0
no	$10^6$	252	<225
no	$10^7$	262	450–900

experiment (Table 1) were due to inefficient capture and not to changes in electrochemical performance upon exposure to large sample volumes. This task would have been far more difficult if only current measurements were available. Although the reason for the 10-fold increase in binding efficiency for sample solutions applied to the electrode in droplets remains to be determined, the droplet protocol was used in subsequent work.

Results from a typical *Salmonella* assay using liquid droplets are shown in Table 3. In contrast to the reproducibility obtained in model assays with anti-goat antibody conjugate (Figure 3), replicate electrodes gave currents that deviated by as much as 50% from the mean. The detection limit was  $\sim 5 \times 10^4$  bacteria/mL, only moderately better than that obtained in our ELISA ( $5 \times 10^5$  bacteria/mL). Comparison of these data to the results obtained with anti-goat antibody conjugate (Figure 3) indicated that variability between electrodes cannot be attributed to irreproducibility in capture antibody loading, in reaction with the

reporter antibody, or in the detection step, since all these sources of error were also present in the anti-goat antibody conjugate study. This high variability must be attributed to the bacteria capture process and clearly indicates that the capture step requires further optimization. It was possible to estimate an intrinsic detection limit (assuming 100% capture efficiency) for this system using the data on peak current and number of cells captured. There was a good correlation between these numbers at both  $10^7$  and  $10^6$  cells/mL, and the same figure of  $\sim 1$  nA/cell was typically observed in these assays (data not shown). The intrinsic detection limit was therefore governed by the smallest current that could be reliably detected by OSWV, estimated as  $< 10$  nA (Figure 3D). Detection of  $< 100$  bacteria, as reported by Rishpon et al.,<sup>12</sup> can therefore be achieved with the system described here provided that bacteria capture efficiency on the order of 10% can be obtained.

## CONCLUSIONS

Surface-capture IEC sensors achieve very high speed and sensitivity by combining analyte capture and detection functions on a single surface. This tight coupling of the two functions can make development and optimization of assays very challenging. By using microscopy, it was possible to decouple these functions and independently and unequivocally assess the capture efficiency of a bacteria sensor. The rapid, sensitive, nondestructive epifluorescence microscopy method reported here was invaluable in resolving problems such as the unusual dependence of assay response on sample volume. In conjunction with high-sensitivity electrochemical detection using OSWV, microscopic evaluation of capture efficiency has permitted us to estimate an intrinsic detection limit for the assay and to identify the performance-limiting factor in the current method as bacteria capture. Future efforts will focus on improving capture efficiency, with the expectation that detection limits for the assay can be reduced from the current level of  $\sim 5 \times 10^4$  cells/mL to less than 100 cells/mL.

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