Microspot Enzyme Assays with Scanning Electrochemical Microscopy

C. Ajith Wijayawardhana,1 Niina J. Ronkainen-Matsuno,1 Svetlana M. Farrell,1 Gunther Wittstock,2 H. Brian Halsall,1,1 and William R. Heineman1,1

† University of Cincinnati, Department of Chemistry, P. O. Box 210172, Cincinnati, OH 45221-0172, USA.
(E-mail: heinemwer@mail.uc.edu, Brian.Halsall@uc.edu)

2 University of Leipzig, Wilhelm Ostwald Institute of Physical and Theoretical Chemistry, Linnestrasse 2, D-04103 Leipzig, Germany

A procedure is described to deposit “microspots” of paramagnetic beads on surfaces for immunoaassay applications. The shape and size of the domains are characterized by scanning electron microscopy (SEM) and the immunochromological activity by scanning electrochemical microscopy (SECM). This approach is first demonstrated with beads modified with anti-mouse IgG and reacted with an excess of alkaline phosphatase (ALP) labeled mouse IgG. Signal is acquired in the generation-collection SECM mode by oxidizing 4-aminophenol formed in the ALP-catalyzed hydrolysis of 4-aminophenyl phosphate. The feedback SECM mode is used next to image streptavidin-coated beads that have been labeled with biotinylated glucose oxidase. Finally, the study is extended to a miniaturized sandwich assay for mouse IgG and a 1.5 x 10^{-15} moles of mouse-IgG is obtained. A micro-volume electrochemical cell is also suggested as an alternative to SECM.

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For over a decade our group has been exploring different ways to miniaturize electrochemical immunoassays (ECIA). Miniaturized immunoassays are essential for testing small samples such as in the health care of neonates or the critically injured.1 Miniaturized immunoassays are also pursued for their promise of high-throughput, multi-analyte assays.2 With respect to enzyme ECIA in particular, miniaturization can play a key role in enhancing the assay sensitivity by virtue of reducing the dilution of enzyme product.3

In heterogeneous immunoassays, miniaturization is often achieved by forming closely spaced microscopic Ab sites, often in an array format with each site targeting a different analyte.7 A single exposure of such a surface to a small amount of sample can, therefore, be adequate to assay for a number of analytes. The technologies currently used for patterning biochemically active microsurfaces include ink-jet technology,7 screen printing,5 microcontact printing,6 local photochemical activation of pre-immobilized coupling agents,8 and local electropolymerization of polymers for subsequent enzyme coupling.9

For such multi-analyte immunoassays, it is necessary to be able to address each Ab site individually in the final detection step. Although using an electrode directly patterned with Abs seems attractive because it combines sampling and detection on a single surface, its implementation is made difficult by a number of factors. Many enzymes and antibodies tend to denature at surface, its implementation is made difficult by a number of factors. Many enzymes and antibodies tend to denature at surface, its implementation is made difficult by a number of factors. Many enzymes and antibodies tend to denature at surface, its implementation is made difficult by a number of factors. Many enzymes and antibodies tend to denature at surface, its implementation is made difficult by a number of factors. Many enzymes and antibodies tend to denature at surface, its implementation is made difficult by a number of factors. 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layer can be expected which simplifies analytical determinations. However, the spatial resolution of the GC mode is inherently poor in comparison to the feedback mode because there is no mediator in GC to confine the signal to a narrow region above the enzyme region. In ECIA, SECM has been used to detect immobilized Ab¹³ and for detecting the same analyte in different samples,¹⁴ and for multiple analytes in the same sample.²⁵

This paper describes our recent efforts at applying GC and feedback modes of SECM to miniaturized heterogeneous enzyme immunoassays. The heterogeneous surfaces are mound-shaped microspots consisting of a pre-determined number of paramagnetic immunobeads. For the GC mode assays, the enzyme-substrate (E-S) pair alkaline phosphatase (ALP) – 4-aminophenyl phosphate (PAPP) was used to generate the electroactive enzyme product 4-aminophenol (PAP). For the feedback mode assays, the E-S pair glucose oxidase (GOx) – glucose is used with the electroactive mediator dimethylaminomethyl ferrocene (DMAMFc). Since SECM calls for fairly sophisticated instrumentation and an SECM instrument is typically not available in a lab involved in ECIA, we show that it is possible to use a simple instrumental mimic of SECM that works surprisingly well.

**Experimental**

**Reagents**

Biotinylated sheep anti-mouse IgG F(ab’)² (515-065-072), Chrompure mouse IgG (015-000-003), Chrompure mouse IgG conjugated to alkaline phosphatase (mouse-IgG-ALP), and rat anti-mouse IgG (H+L) conjugated to alkaline phosphatase (415-055-100) were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Biotinylated glucose oxidase (GOx) was from Vector Laboratories (Burlingame, CA, USA), glucose from Fluka (Buchs, Switzerland), and dimethylaminomethyl ferrocene (DMAMFc) from ACR GmbH (Karlsruhe, Germany). Streptavidin-coated paramagnetic beads were either M-280 Dynabeads (2.8 µm diameter beads in a 6.7×10⁵ beads mL⁻¹ suspension) from Dynal Inc., (Great Neck, NY, USA) or 1 µm diameter beads from Bangs Laboratories (Fishers, IN, U.S.A). Anti-mouse IgG coated immunobeads with physical specifications identical to the M-280 beads above were from Dynal, Bovine serum albumin (BSA) fraction V powder 98+% was from Boehringer Mannheim (Indianapolis, IN, USA), tris(hydroxymethyl)aminomethane (Tris), K₂HPO₄, NaH₂PO₄, and NaN₃, were from Fluka (Deisenhofen, Germany), and Tween 20 from Sigma (Deisenhofen, Germany). 4-Aminophenyl phosphate (PAPP) was synthesized from 4-nitrophenyl phosphate (Aldrich, Milwaukee, WI, USA) as reported.¹⁶

The following two aqueous buffer solutions were used. PBS/BSA buffer: 0.044 M NaH₂PO₄, 0.056 M K₂HPO₄, 0.15 M NaCl, 0.01 % (w/v) NaN₃, 0.5 % (v/v) Tween 20, 1 % (w/v) BSA, pH 7.4. Tris buffer: 0.1 M Tris-HCl, 1 g L⁻¹ MgCl₂, 0.1 M KCl, 0.02 % (w/v) NaN₃.

**Preparation of beads for SECM imaging and microdrop assay**

**Beads coated with GOx for SECM.** Using polystyrene tubes, 10 µL of a suspension of streptavidin-coated Bangs beads were allowed to react for 45 min with 1000 µL of a solution of 0.005 mg mL⁻¹ biotinylated GOx in 0.05 M acetate buffer (pH 4.5). The beads were then rinsed with Tris buffer containing 0.25 % Tween 20 using a rare earth magnet to hold the beads against the tube for discarding supernatant. GOx was detected using a deoxygenated solution of 50 mM glucose and 1 mM dimethylamino-methyl ferrocene (DMAMFc) in phosphate buffer (pH 6.9).

**Anti-mouse IgG beads saturated with mouse-IgG-ALP for SECM.** Twenty µL of undiluted anti-mouse coated Dynal beads were first incubated for 45 min with 3 µL of 11.4 mg mL⁻¹ mouse-IgG-ALP in Tris buffer (pH 9). The beads were rinsed with Tris buffer containing 0.25% Tween 20 prior to testing.

**Beads for microspot SECM immunoassay.** The beads were modified in PBS/BSA buffer unless stated otherwise. In the first step, 40 µL of streptavidin-coated Dynabeads were mixed with 40 µL of biotinylated sheep anti-mouse-IgG diluted to 40 µg mL⁻¹. The mixture was agitated gently on a plate-shaker for 30 min. Excess Ab was removed in three rinses with 120 µL buffer. After re-suspending the beads in 90 µL of buffer, 22 µL aliquots were transferred to three fresh tubes. Samples of 22 µL of Ag (analyte mouse IgG) of 1150, 115, and 11.5 ng mL⁻¹ were added to the tubes and shaken for 30 min. After washing the beads as before, they were re-suspended in 48 µL of buffer, and 15 µL of enzyme-labeled antibody (7 µg mL⁻¹) added. Following 20 min on the shaker, the beads were washed three times with 150 µL of buffer. Finally, the buffer was changed to Tris by washing 3 times with 150 µL of the buffer and re-suspending the beads in each tube with 150 µL. The final concentration of beads in the test tube was 4×10⁷ beads mL⁻¹.

**Beads for microdrop ALP assays.** Biotinylated ALP in concentrations of 0.5, 1, 2, 4, and 6 µg/mL were reacted with an excess of streptavidin-coated beads in PBS buffer. The beads were washed three times with PBS buffer, five times with Tris buffer, and re-suspended in Tris buffer prior to use.

**Microspotting beads for SECM**

One µL of a bead suspension was taken up in a variable volume micropipette that was mounted on the translation stage of the SECM (see next section for details of the SECM). Next, the pipette tip was lowered to within 500 µm from a hydrophobic slide (with a rare earth magnet glued to the lower surface) and a 0.3 µL drop extruded as shown in Fig. 2. After allowing 30 s for the magnet to pull all the beads to the bottom of the microdrop, the translation stage was lowered at 8 µm s⁻¹ to bring the drop in contact with the hydrophobic surface. Finally, the pipette was retracted, and because of the hydrophobicity of the surface, the drop adhered to the retracting tip leaving behind a mound of beads.

**Imaging the beads with SECM**

The SECM images were taken with a home-built SECM instrument described elsewhere.¹⁷ The three-electrode system of the SECM consisted of a 10 µm Pt UME (Cypress Inc., Lawrence, KS, USA), a Pt wire auxiliary, and a silver wire quasi-reference electrode. The UME was held at +290 mV to detect 4-aminophenol (PAP) in GC experiments and at +400 mV to detect DMAMFc in feedback experiments. The horizontal translation rate was 8 µm s⁻¹ in both cases. For GC experiments, the UME was positioned above the surface by applying a -0.6 V to the UME and moving it perpendicularly towards the insulating hydrophobic surface (0.7 µm s⁻¹) and retracting it 45 µm after the O₂ reduction current had reached half its limiting current in bulk (iₜ). Similarly for feedback experiments the electrode was retracted 25 µm from the surface after the DMAMFc oxidation current had reached half its iₜ. But it was important to carry out
the vertical placement of the UME several hundred micrometers away from the microspot so as to avoid the feedback effects of GOx. Scanning electron micrographs were acquired in a DSM 940 (Zeiss, Oberkochen, Germany) after sputter-coating the polymer beads with gold.

**Micro-volume ALP assays**

The microvolume ALP assays were carried out in a 40 µL drop sandwiched between a hydrophobic slide and a 20 µm diameter carbon fiber electrode. A Pt wire and a silver wire served as the auxiliary and the reference electrode respectively. The 40 µL drop contained 5 µL of beads, 10 µL of 16 mM PAPP, and 25 µL of Tris buffer. The beads were added last to the drop and a rare earth magnet placed below attracted the beads to an approximately 1 mm diameter circle. The current-time plot was taken 30 s after the addition of beads.

**Results and discussion**

**Monitoring microspot enzyme activity with SECM**

The deposition procedure led to well-defined microspots of beads as shown in Fig 3. The size of the microspot is determined primarily by the number of beads in the bead suspension drawn into the pipette. We have shown elsewhere that the microspot shape too depends on the number of beads; when the number of beads is only a several hundred or less the beads form a monolayer instead of a mound. Thus the size and shape of the microspot can be controlled by the concentration of the bead suspension used for deposition. Furthermore, a micro-positioning system for the pipette such as that provided by the SECM translation stage gives free choice in the placement of microspots. The microspots are stable after drying and remain adhered to the support even when the magnet is removed. The biochemical activity of the microspot can be restored in a suitable buffer.

Fig. 3 shows the SECM GC-mode image obtained by oxidizing PAP produced at a microspot of beads (bottom) saturated with mouse-IgG alkaline phosphatase and exposed to the enzyme substrate 4-aminophenyl phosphate.

Fig. 4 Feedback images of a microspot of beads saturated with glucose oxidase (above) with its cross-section showing the signal width (below).

The SECM GC-image (top) obtained by oxidizing PAP produced at a microspot of beads (bottom) saturated with mouse-IgG alkaline phosphatase and exposed to the enzyme substrate 4-aminophenyl phosphate.

layer of enzyme product PAP is expected in analogy with diffusion profiles at a disk-shaped microelectrode. Indeed, the general shape of the SECM image reflects this, and the significantly larger lateral width of the signal compared to the microspot diameter (>10) is in agreement with the GC mode imaging of enzyme product as illustrated in Fig. 1. The relatively large working distance (d = 45 µm) of the SECM tip and the high PAPP concentration prevented the UME from shielding the diffusion of PAPP to the beads. If the latter case, the \( i_T \) would have first increased with the approaching UME but decrease sharply after reaching a maximum (typically several tenths of microns above the surface). For very low detection limits, a smaller \( d \) would be beneficial but this would limit the working range at high mouse IgG-ALP concentrations because of hindered PAPP diffusion.

As illustrated in Fig. 1 the GC mode is very sensitive but offers poor spatial resolution for multi-analyte array formats. Hence it sets a fundamental limit on how close two neighboring microspots can be placed before the diffusion profiles of the two overlap and complicate the spatial addressing of individual microspots. In contrast, the signal of an enzyme-mediated feedback mode in principle confines the signal to the region right above the microspot by virtue of the mediator shuttling between the UME and the enzyme. This was tested with the GOx coupled beads. The ferrocinium form of DMAMFc produced at the electrode surface is a highly efficient electron acceptor for GOx. Naturally, the ensuing feedback effect is confined to regions of GOx activity. Fig. 4 shows the feedback SECM image obtained over a microspot of approximately 30 µm in diameter. The high resolving power of the feedback mode is evident in the sharp peak and the full width at half maximum of 25 µm is evidence of the excellent spatial resolving power of feedback mode. These results illustrate that both feedback and GC modes of SECM are appropriate for applying to microspot immunoassay. Because virtually all our immunoassays are based on the ALP-PAPP E-S pair, we chose the GC mode for the first microspot assay.
Microspot immunoassay with SECM detection

The immunoassay work was done on microspots of approximately 100 µm radius. The substrate PAPP was added in excess to ensure that the ALP is saturated with PAPP. As above, the SECM images show the quasi-stationary concentration of PAP above the microspot. The steady-state concentration gradients of PAP enable different assay points to be compared by taking the peak height against the baseline as the analytical signal.

As shown in Fig. 5, this signal increases in going from

![Fig. 5 SECM profiles of bead agglomerates prepared from suspension after sequential reaction of the Ab-coated beads with mouse IgG and ALP-Ab conjugate. Concentration of mouse-IgG in ng mL⁻¹: a) 1150, b) 115, c) 11.5.](image)

11.5 to 1150 ng mL⁻¹ mouse IgG concentration as expected from a sandwich immunoassay. The lowest concentration of mouse IgG detected corresponds to a molar concentration of 6.4 × 10⁻¹⁵ moles of mouse IgG.

Assay in the micro-volume electrochemical cell

The results so far have described the use of an SECM as a detector for microspot assays. However, since very few labs are equipped with an SECM instrument, we tested the feasibility of substituting the SECM by applying a drop of highly concentrated PAPP solution to a microspot of beads where the small solution volume would take the place of the SECM tip in limiting enzyme product dilution. The current-time (I-t) plot from the microelectrode would then give the enzyme progression curve. Under substrate saturation conditions, the initial slope of the I-t plot (approximated by the slope between 90 to 120 s) would be linearly proportional to the enzyme concentration. Fig. 6 is the calibration curve for ALP thus obtained. The plot is linear up to about 1 µg/mL. The signal leveling off at higher concentrations is attributed to the loss of substrate saturation conditions. Therefore, the cell volume, the substrate concentration, and perhaps the concentration of beads need to be chosen such that the signal falls within the dynamic range. For example, if it is known that only a trace amount of the antigen is present, then a small cell volume and/or a high concentration of beads would be necessary.

Conclusion

Microspots of beads can be assembled on hydrophobic surfaces for immunoassays with SECM detection, or when an SECM instrument is unavailable, with a simple micro-volume electrochemical cell. The size and shape of the microspot can be varied easily by controlling the number of beads in the microspot. The assays described do not require lengthy incubations because a rapid steady-state concentration of enzyme product is established over the microspot.

The results also suggest that a surface patterned with an array of microspots, each targeting a different analyte, can be coupled easily to an SECM or similar apparatus to yield a simple multi-analyte electrochemical immunoassay.

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