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ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2008

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Technical Notes

Femtomolar Electrochemical Detection of DNA Hybridization Using Hollow Polyelectrolyte Shells Bearing Silver Nanoparticles

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The preparation, and use as electrochemical labels, of polyelectrolyte shells bearing Ag nanoparticles is described. Their potential for highly sensitive detection is demonstrated. The shells are prepared by layer-by-layer self-assembly around templates (500 nm diameter) which are then dissolved. The shells can be opened and closed by adjustment of solution pH, and this process is utilized to encapsulate Ag nanoparticles, chiefly by adsorption to the inner walls of the capsules. Based on absorbance, TEM and voltammetric measurements, the highest loading achieved is approximately 78 Ag particles per capsule. The Ag capsules are used via biotin–avidin binding as labels for the detection of DNA hybridization, following acid dissolution and then detection of the Ag^+ by ASV. A 30-mer sequence specific to *Escherichia coli* is measured at DNA-modified screen-printed electrodes with a detection limit of ~25 fM, which corresponds to the detection of 4.6 fg (~3×10^5 molecules) in the 20 μL analyte sample. A 200 fM target containing a single mismatch gives a significantly (<74%) lower response than 200 fM of complementary target; 60 pM of noncomplementary target gives a negligible response.

The recognition of specific DNA sequences can be used for the diagnosis/quantification of viruses, pathogenic microorganisms and human genetic diseases. The usual method of detecting a ssDNA target is to use a labeled complementary ssDNA probe. Radioactivity,[1] fluorescence,[2] surface plasmon resonance[3] (SPR), and mass change detected by quartz crystal microbalance[4] (QCM) or microfabricated cantilevers[5] have been used for label detection. However, radioactive labels have inherent safety considerations, while fluorescence, SPR and microfabricated cantilevers require expensive instrumentation. QCM equipment is less expensive, but the QCM response in solution can sometimes be influenced/governed by nongravimetric effects.[6] These considerations have led to interest in electrochemical DNA sensors,[7] since electrochemical reactions produce an electrical signal without needing expensive transduction equipment. The direct electro-oxidation of guanine involves high background signals,[7] while the use of enzyme labels may involve deterioration of enzyme activity over time. Redox compounds which can intercalate with the probe−target duplex can provide a more stable signal[8] but do not always provide adequate sensitivity. This has led to interest in using metal nanoparticles as labels for electrochemical detection of DNA hybridization.[9–21] Typically, the nanoparticle is captured on the hybridized target and then quantified by anodic stripping voltammetry (ASV). The technique is popular because ASV allows

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10.1021/ac701867m CCC: $40.75
Published on Web 04/12/2008

highly sensitive detection due to the preconcentration step. Ag,[9–11] Au,[12–17] Cu/Au (core/shell),[18] CdS[19] and In nanoparticles[20] have been used as electrochemical labels. The field has been reviewed thoroughly by Willner and Katz.[21]

The achievable sensitivity of a nanoparticle−DNA detection scheme will depend on the following: electrical noise of the given hardware, to some extent the type of waveform used for stripping, and the amount of electrical charge provided by the nanoparticle. Therefore, the sensitivity of detection can be increased by increasing the nanoparticle mass attached to each oligonucleotide target. Methods of increasing this mass have included depositing Ag along the length of the dsDNA strand,[10] using a metal in the form of a microrod rather than a nanoparticle,[20] using a Au nanoparticle or a Au-coated polystyrene sphere as a label, on which further Au or Ag is deposited,[13,14,16] and using carbon nanotube labels bearing CdS nanoparticles.[19]

A method which has not so far been examined, but which is likely to be applicable to amplifying the mass of a wide range of nanoparticles, is encapsulation of the nanoparticles within hollow polyelectrolyte multilayer shells which are then used as labels. The shells are fabricated by the electrostatic layer-by-layer self-assembly method first described by Decher et al.[22] Films of typically several nanometers to tens of nanometers thickness are formed around colloidal template particles by the sequential deposition of oppositely charged polyelectrolyte layers,[23] following which the templates are dissolved to provide hollow shells.[24] Changes in solution pH[25] and ionic strength[26] can be used to alter the degree of ionization in the polyelectrolytes and thus weaken intermolecular binding, which results in an increased porosity to solutes in the bulk solution. Hence, reversals of pH can be used to entrap solute species within the microcapsules.[27] This procedure has been used to encapsulate enzymes[28] and dyes.[29] Au[30] and Ag[31] nanoparticles have been incorporated into the walls of such capsules, to provide light-assissted opening, but to the best of our knowledge such structures have not been used as electrochemical labels.

In this paper we wish to report proof-of-concept experiments in which we synthezise silver nanoparticles, encapsulate the nanoparticles within hollow shell polyelectrolyte capsules, and then use the resulting Ag capsules as labels for ssDNA targets by avidin–biotin binding. The Ag capsules are characterized by UV–vis spectrophotometry, voltammetry and transmission electron microscopy (TEM), and hybridization is monitored by ASV, following dissolution of the silver.

EXPERIMENTAL SECTION

Apparatus. Electrochemical experiments were performed using an Autolab PGSTAT10 computer-controlled potentiostat (Eco Chemie) with GPES software. The working electrode was either carbon paste (used with SCE reference and Pt coil counter) or a screen-printed carbon track (for DNA immobilization used with screen-printed Ag/AgCl combined reference and counter; for ASV used with Pt coil counter and Ag/AgCl (3 M NaCl) reference separated from the working solution by a salt bridge filled with 3 M KNO_3). UV–vis spectra were recorded using a Beckman model DU-7000 spectrophotometer. Transmission electron micrographs (TEMs) were obtained with a JEOL model JM-2100, operated at an accelerating voltage of 120 kV.

Electrode Preparation. The carbon paste electrode (4.8 mm diameter) contained graphite powder and paraffin oil (mass ratio = 70:30) in the cavity formed by platinum disk electrode recessed into a glass tube. The electrode surface was renewed and polished repeatedly on weighing paper prior to each measurement. Screen-printed electrodes (SPEs) were fabricated using a semiautomatic screen printer (model DEK248, DEK UK, U.K.). The carbon (type 145, MCA services, U.K.) and silver/silver chloride ink (type C2DR15, MCA services, U.K.) were printed onto PVC sheets (150 mm × 200 mm) through a patterned stencil to give a group of 24 SPEs (each consisting of a carbon working electrode and a Ag/AgCl combinded reference and counter electrode). Following each print, the electrodes were cured for 2 h at 55 °C and then allowed to cool at room temperature. A layer of insulating tape (Permacel model p221) was then placed over a portion of the conducting "lines", exposing a 1.5 mm × 4 mm working electrode, and a 2 mm × 4 mm reference/counter electrode.

Chemicals. Poly(sodium 4-styrenesulfonate) (PSS, MW ~70 000) and poly(allylamine hydrochloride) (PAA, MW ~70 000) were purchased from Aldrich. All reagents were of analytical grade and were used as received. The polystyrene-coacrylic acid (PSA) particles (diameter 500 nm) were prepared as described in the literature.[32] All solutions were prepared with deionized water. Target oligonucleotides were purchased from the Bioservice Unit (NSTDA, Thailand). A probe oligonucleotide with a biotin-modified 5′-position was acquired from BIONEER. The DNA strands had the following sequences: immobilized probe, biotinylated, biotin-5′CTT CCT GAG TAA TAA3′; target, 5′TAT TCA CTC AGG AAG TTA TTA CTC AGG AAG3′; single mismatch, 5′TAT TCA CTC AGG AAG TTA TTA CTC ACG AAG3′; noncomplementary strand, 5′CTT CCT GAG TCC TTC AAT AAT GAG TCC TTC3′.

The oligonucleotide stock solutions (1 mg mL^{-1}) were prepared with deionized water and kept frozen. Target oligonucleotide solution were diluted with 0.1 M phosphate buffer (pH 7). Capture probe solutions were diluted with hybridization buffer (5 × SSPE, pH 7.4, containing 2% SDS).

Preparation of Ag Nanoparticles. Ag nanoparticles were prepared by sodium borohydride reduction of $AgNO_3$ according to the literature[9] with some modifications. One volume of ice-cold 0.18×10^{-3} M $AgNO_3$ and an equal volume of 3×10^{-3} M $NaBH_4$ were mixed dropwise with stirring in an ice bath, and the mixture was then continuously stirred until the temperature

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naturally increased to room temperature. The solution was dialyzed against water for at least 24 h before use.

Preparation of Avidin-Coated Ag Capsules. An aliquot (200 μL of a 2.5 wt % dispersion) of PSA particles was incubated with 1 mL of PAA solution (1 mg mL^{-1} in 0.5 M NaCl) for 15 min, followed by three centrifugation/redispersion cycles, and was finally dispersed in water. A 1 mL aliquot of PSS solution (1 mg mL^{-1} in 0.5 M NaCl) was then added to the particle dispersion. After 15 min for adsorption, three centrifugation/redispersion cycles were performed again. These adsorption steps were repeated four times for each polyelectrolyte. Hollow polyelectrolyte capsules were then obtained by adding 600 μL of THF. Dissolution of the PSA template was noted as a change from a strong chalk-white appearance to turbid, and took approximately 1 h. The capsules were washed with THF, ethanol and deionized water (1 mL of each).

Ag-loaded capsules were prepared by adding the desired volume (10−200 μL of a 0.53 wt % dispersion) of microcapsules to a 2.5 mL dispersion of AgNPs. Sulfuric acid was added to the AgNPs/capsule mixture to give a pH of 4.0, and the mixture was kept at room temperature for a half-hour at this pH. The mixture pH was then adjusted to 8.0 using 0.1 M sodium hydroxide. After a half-hour at pH 8.0, three washing/centrifugations were carried out to remove residual unencapsulated AgNPs.

Avidin coated Ag capsules were prepared by mixing 10 μL of a Ag capsule solution with 12.5 μL of 3 mg mL^{-1} avidin in phosphate buffer, pH 7.0. After being left to stand for 2 h at room temperature, the particles were isolated by centrifugation for 5 min at 1.2×10^4 rpm. The absorbance of the supernatant was measured at 280 nm to confirm avidin coating on the capsules. The avidin-coated Ag capsule precipitate was washed with 0.1 M PBS. A 3-fold dilution of the solution in 0.1 M PBS was used as the work solution to perform the hybridization in this study.

Detection of DNA Hybridization Using Ag Capsules. Immobilization of target ssDNA on the SPE was performed by depositing 20 μL of target-solution in sterile phosphate buffer (pH 7) over both electrodes (carbon working electrode and Ag/AgCl combined reference and counter). A potential of 100 mV was then applied for 5 min. The electrode was rinsed three times with sterile phosphate buffer (approximately 250 μL each rinse) to remove nonadsorbed target, and the SPE left to dry at room temperature.

The hybridization reaction was carried out by dropping 10 μL of biotinylated probe (in 0.5% SDS phosphate buffer, pH 7) onto the DNA-modified SPE. After 20 min incubation at room temperature the electrode was washed three times with phosphate buffer to remove unhybridized probes and a 10 μL aliquot of avidin-coated Ag capsules deposited on the SPE and left 20 min for binding to occur. After being left to dry at room temperature, the electrode was rinsed three times with phosphate buffer and twice with deionized water.

The Ag capsules were dissolved by dropping 5 μL of 50% nitric acid solution on the SPE. After allowing 5 min for dissolution, the SPE was immersed in an electrochemical cell containing 0.8 mL of 10 mM HNO_3 + 10 mM KNO_3 as electrolyte.

Preparation of PAA/PSS-Coated Glass. Glass coverslips (22 mm × 22 mm, Menzel-Glaser) were dip-coated into the same PAA and PSS solutions used to modify the PSA particles, i.e., 1 mg mL^{-1} of polyelectrolyte in 0.5 M NaCl. Each coverslip was left in polyelectrolyte solution for 30 min, rinsed briefly with distilled water, left to air-dry at room temperature and then coated again if appropriate.

RESULTS AND DISCUSSION

As described in the Experimental Section, the silver nanoparticles were prepared by reduction with sodium borohydride and purified by dialysis against water. TEM images of the particles directly after synthesis, at a pH of approximately 6, gave an average diameter of 22.7 nm, based on a particle size histogram using >100 individual particles (Supporting Information, Figure S-1 (A)). Applying the pH change to be used for encapsulation, i.e., lowering the value to pH 4 (for 30 min) and then raising it to pH 8, produced particles with a mean diameter of 15.8 nm (Supporting Information, Figure S-1 (B)), which gives a mean mass of 2.2×10^{-17} g per particle based on the density of Ag (10.49 g cm^{-3} [33]). The zeta potential of Ag borohydride colloids has been found to vary in the range 6.5 mV to –56 mV from pH 2 to pH 11,[34] and hence the change in diameter here is likely to be due to the resulting change in aggregation with surrounding charge.

To determine the proportion of $AgNO_3$ converted to nanoparticles, an aliquot of Ag nanoparticles was dissolved using an equal volume of 50% HNO_3. An immediate change was observed from brownish yellow to colorless, confirming that dissolution was fast under these conditions. After waiting 10 min to ensure that dissolution was complete, the mixture was added to an electrolyte of 10 mM HNO_3 + 10 mM KNO_3 and the Ag^+ measured by ASV (E_{dep} = –0.2 V, t_{dep} = 400 s, scan rate = 50 mV s^{-1}, (see Supporting Information Figure S-2 for example of linear sweep voltammogram), using a carbon paste working electrode. A calibration curve of $AgNO_3$ plotted under the same conditions using the same electrode across the range 50 nM to 0.5 μM (Supporting Information, Figure S-3, gradient = 0.413 μA μM^{-1}, n = 18, r^2 = 0.9938) was then used to determine the concentration of Ag in the aliquot. From this value the Ag recovery was found to be 88%. Based on this recovery and the mean mass of the nanoparticles, the nanoparticle concentration was 7.86×10^{11} Ag particles mL^{-1}.

The templates for the multilayer capsules were polystyrene-coacrylic acid (PSA) particles with a mean diameter of 500 nm. After carboxylic groups were introduced onto the PSA surface, four layers of PSS/PAA were deposited. THF dissolution of the core produced hollow spheres of 482 nm diameter, as seen in the TEM image in Figure 1 (A). The shrinkage in size after dissolution is probably a result of drying the capsules for the TEM image. The shell thickness was approximately 37 nm, and hence the internal volume of each capsule was 3.55×10^{-14} cm^3. The mass of an empty capsule can be estimated as 2.6×10^{-14} g, assuming the density of the polyelectrolyte shell to be 1.1 g cm^{-3}.[35] Based on the mass of a 150 μL aliquot of capsules after evaporation to dryness, this gives a concentration of approximately 2.05×10^{11} capsules mL^{-1}. The incorporation procedure described in the Experimental Section was applied to hollow capsules in a solution containing Ag nanoparticles (see Scheme 1). A TEM of

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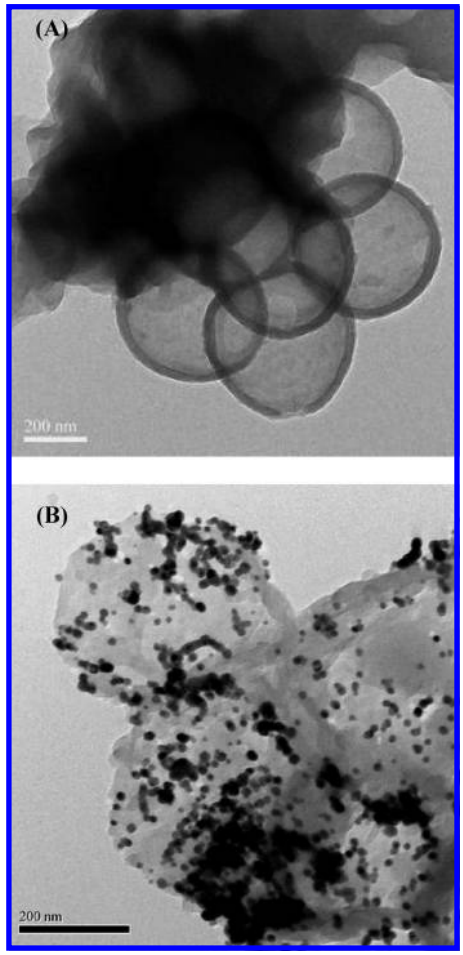


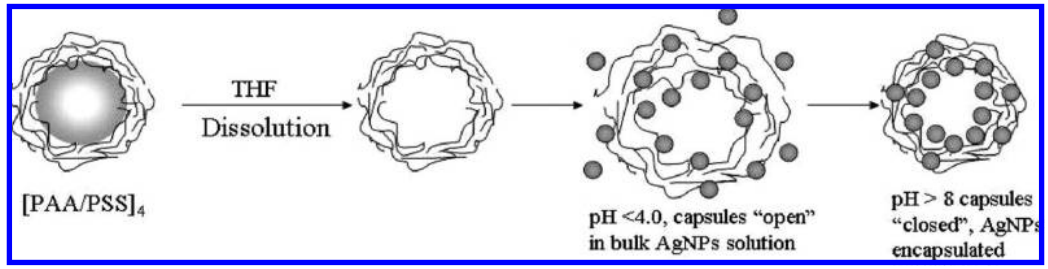
Figure 1. TEM images of hollow $[PAA/PSS]_4$ capsules obtained after core dissolution (A) and $[PAA/PSS]_4$ capsules loaded with Ag nanoparticles (B) (bar = 200 nm in both images).

the Ag-loaded capsules is shown in Figure 1 (B). The black spots represent the Ag nanoparticles. The distribution is nonuniform, which may partly be due to the effect of drying the sample for the TEM (see Supporting Information Figure S-4 for further TEM images of the loaded capsules). The quantity of Ag nanoparticles encapsulated was determined from the amount of unencapsulated nanoparticles remaining in the supernatant after centrifugation and removal, using UV absorbance at 406 nm. (See Supporting Information Figure S-5 for absorbance spectra of a Ag nanoparticle suspension and Figure S-6 for calibration curve plotted using stock Ag nanoparticle solutions, gradient = 29.86 g^{-1} L, $n = 18$, $r^2 = 0.998$.) Encapsulation experiments were performed using varying concentrations of hollow capsules with a fixed aliquot of silver nanoparticles. As shown in Figure 2 (A), the total amount of Ag encapsulated increased with the amount of capsules in the solution. Using the nanoparticle and capsule concentrations previously determined, the number of nanoparticles per capsule was estimated. Surprisingly, as shown in Figure 2 (B), there was a maximum in the achievable loading, at approximately 78 Ag nanoparticles per capsule. It is still unclear why this is, although we note that entry of nanoparticles into the capsules is driven by more than simply diffusion. Enzymes have been encapsulated within hollow polyelectrolyte shells at concentrations over 3 orders of magnitude higher than the concentration in the outer solution,[28] which means encapsulation *against* a concentration gradient. The explanation suggested for this was that the internal polyelectrolyte shell, or some residual template material, acted as a sorption material for the enzyme. The Ag nanoparticle intracapsule concentration here (78 particles in 3.6×10^{-14} $cm^3 = 2.2 \times 10^{15}$ particles mL^{-1}) was also greater than the outside concentration. To assess the role of sorption we deposited the capsule polyelectrolytes onto glass coverslips by dip-coating and then examined uptake of the Ag nanoparticles onto these layers. A nanoparticle suspension was synthesized, adjusted to pH 4 and the particle concentration determined as described previously. Two coverslips were then placed in the suspension for 30 min, followed by a further 30 min with the solution adjusted to pH 8 (i.e., the same conditions used for encapsulation). After removal of the coverslips, the quantity of Ag nanoparticles adsorbed onto the polyelectrolyte was calculated from the change in solution absorbance at 406 nm. Adsorption onto the glass surface itself was determined using uncoated coverslips, and the coverage of the polyelectrolytes adjusted for this value (9.0×10^8 particles cm^{-2}). A single PAA layer gave a Ag coverage of 1.13×10^{10} particles cm^{-2}, while a single PSS layer gave 0.19×10^{10} particles cm^{-2}. The greater degree of adsorption at the positive PAA is reasonable since the Ag nanoparticles have an isoelectric point of 2.7,[34] and therefore a negative zeta potential at these pH's. Layer-by-layer modification of coverslips by 3 PAA/PSS coatings, followed by a further PAA layer on top, gave a coverage of 1.40×10^{10} particles cm^{-2}. This increase, relative to 1 layer, suggests that as well as adsorption to the inner (PAA) and outer (PSS) capsule walls, Ag nanoparticles are also intercalated within the polyelectrolyte layers. From the capsule inner surface area (5.23×10^{-9} cm^2) the inner coverage corresponds to 59.1 particles per capsule; from the outer surface area (7.3×10^{-9} cm^2) the outer coverage corresponds to 13.9 particles per capsule; using the midpoint of the capsule membrane to approximate the surface area for intercalation (6.22×10^{-9} cm^2) the intercalated coverage is 11.2 particles per capsule. Hence, the sum of the coverages gives a loading of approximately 84 Ag nanoparticles per capsule, which is a good agreement with 78 nanoparticles per capsule calculated from the absorbance of the supernatant. The greater value from the coverslips possibly represents the greater accessibility of polyelectrolyte adsorption sites on the planar surface relative to the inner walls of the opened capsules. Given that the stock Ag nanoparticle concentration synthesized for the adsorption experiments was in fact slightly *lower* (6.84×10^{11} particles mL^{-1}) than the stock synthesized for the encapsulation experiments (7.86×10^{11} particles mL^{-1}), it seems likely that adsorption proccesses are responsible for all of the nanoparticle take-up. Hence, in terms of proportion we can estinmate that the Ag nanoparticle location is approximately 70% on the inner wall, 17% on the outer wall and 13% intercalated between.

The Ag content of the capsules could be dissolved using 50% HNO_3, since at this pH (≤ 4) the capsules would open. The inset to Figure 2 (B) shows the LSV stripping peak for the detection of the Ag^+ from the capsules indicated in the main figure (carbon paste working electrode, conditions as given earlier).

Use of the Ag capsules for detection of DNA hybridization followed five steps: (1) electrosorption of target oligonucleotide onto the carbon track of a screen-printed electrode, (2) hybridization with a biotinylated oligonucleotide probe, (3) binding of the

Scheme 1. Schematic Representation of Capsule Formation and Loading with Ag Nanoparticles by pH Control[a]



[a] Template for $[PAA/PSS]_4$ deposition is 500 nm diameter polystyrene-*co*-acrylic acid particle.

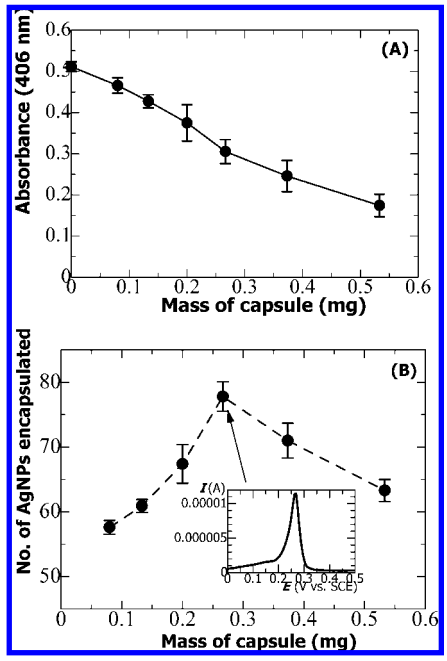


Figure 2. (A) Absorbance of unencapsulated Ag nanoparticles remaining in supernatant as a function of the mass of hollow capsules added to the nanoparticle suspension. (B) Number of Ag nanoparticles entrapped per capsule as determined from (A) using the nanoparticle and capsule concentrations calculated in the text. Inset: Linear sweep stripping voltammogram at carbon paste electrode from a capsule at the loading indicated (E_{dep} = –0.2 V, t_{dep} = 400 s, scan rate = 50 mV s^{-1}) following dissolution by 50% HNO_3. Absorbances were measured in triplicate. Error bars show ± 1 SD.

probe to an avidin-coated Ag capsule, (4) capsule opening and Ag dissolution by 50% HNO_3, (5) quantification of the Ag^+ ions at the screen-printed electrode by ASV (E_{dep} = –0.5 V, t_{dep} = 500 s, scan rate = 50 mV s^{-1}). The oligonucleotide used was a 30-mer sequence specific to *Escherichia coli*. As shown in Figure 3 (a) 200 fM of the complementary target produced a well-defined ASV stripping response, while 200 fM of target containing a single mismatch produced a significantly (<74%) lower response (Figure 3 (b)). A 300-fold higher (i.e., 60 pM) concentration of a non-complementary target produced a negligible response (Figure 3 (c)).

The accessible concentration range was examined by varying the concentration of oligonucleotide in the immobilization solution while keeping the concentration of biotinylated probe and Ag capsules constant. Three electrodes were used at each target concentration. As shown in Figure 4, the target was measured between 1 fM and 200 fM using a 20 min hybridization time. Given

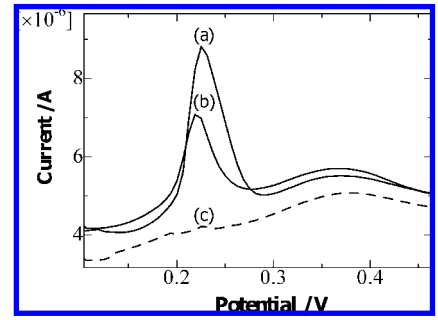


Figure 3. LSV recorded at carbon track screen-printed electrode after immobilization of target DNA, hybridization with biotinylated probe and binding of probe to Ag capsules, using 200 fM of target complementary to the probe (a), 200 fM of target containing a single mismatch (b) and 60 pM of a noncomplementary target (c). Capsules were prepared under conditions giving optimum Ag loading and were dissolved by 50% HNO_3. LSV: E_{dep} = –0.5 V, t_{dep} = 500 s, scan rate = 50 mV s^{-1}.

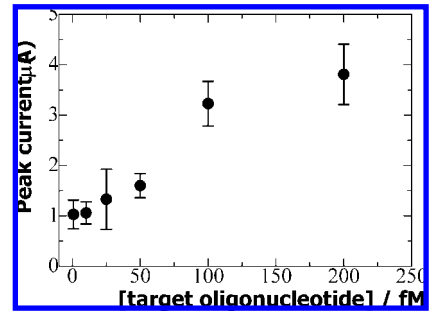


Figure 4. Calibration of target DNA at carbon track screen-printed electrodes by LSV following the detection sequence and LSV conditions given in Figure 3. Three electrodes were used at each concentration. Error bars show ± 1 SD.

the closeness of the response at 1 fM and 10 fM, the limit of detection was realistically ~25 fM (230 fg mL^{-1}). This corresponds to the detection of 4.6 fg (0.5 amol, i.e., ~3×10^5 molecules) of target in the 20 μL immobilization solution, which is a lower detection limit than other Ag nanoparticle-based hybridization assays reported recently,[9–11] as well as lower than many of the hybridization assays using other nanomaterials,[12–15,18–20] and approaches chemiluminescence (5 fM),[36] which is one of the most sensitive DNA detection methods using Ag nanoparticles.

Since capsule opening can also be affected by changes in ionic strength[26] and solvent composition (e.g., water:ethanol ratio[37]),

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there is scope for encapsulating a wide range of nanomaterials by this method. Thus it can provide a route to fabricating the simultaneous multianalyte assemblies previously reported,[38] where a single stripping voltammogram allows different DNA targets to be detected at different potentials. The Ag capsules should also be suitable for labeling antibodies using avidin–biotin binding. It is reasonable to suppose that replacement of LSV by a pulse method (e.g., differential pulse voltammetry) may improve the sensitivity.

ACKNOWLEDGMENT

TEM images were recorded at the Scientific and Technological Research Equipment Centre, Chulalongkorn University. Screen-printed electrodes were fabricated by Ms. Kritsanaporn Cheunrungsikul at PDTI. P.R. and M.S. are employees of BIOTEC.

SUPPORTING INFORMATION AVAILABLE

TEM images of Ag nanoparticles at pH 6 and pH 8. UV–vis absorbance spectra of Ag nanoparticle solution. Calibration curve for determining [Ag^+] by ASV using a carbon paste working electrode. Calibration curve for absorbance of Ag nanoparticle solutions at 406 nm. Further TEM images of Ag capsules. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review September 5, 2007. Accepted February 26, 2008.

AC701867M

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