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Subfemtomolar Electrochemical Detection of Target DNA by Catalytic Enlargement of the Hybridized Gold Nanoparticle Labels

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After showing the failure of conventional gold-enhancement procedures to amplify the gold nanoparticle-based electrochemical transduction of DNA hybridization in polystyrene microwells, a new efficient protocol was developed and evaluated for the sensitive quantification of a 35 base-pair human cytomegalovirus nucleic acid target (tDNA). In this assay, the hybridization of the target adsorbed on the bottom of microwells with an oligonucleotide-modified Au nanoparticle detection probe (pDNA-Au) was monitored by the anodic stripping detection of the chemically oxidized gold label at a screen-printed microband electrode (SPMBE). Thanks to the combination of the sensitive Au^{III} determination at a SPMBE with the large number of Au^{III} released from each pDNA-Au, picomolar detection limits of tDNA can be achieved. Further enhancement of the hybridization signal based on the autocatalytic reductive deposition of ionic gold (Au^{III}) on the surface of the gold nanoparticle labels anchored on the hybrids was first envisaged by incubating the commonly used mixture of Au^{III} and hydroxylamine (NH₂OH). However, due to a considerable nonspecific current response of poor reproducibility it was not possible to significantly improve the analytical performances of the method under these conditions. Complementary transmission electronic microscopy experiments indicated the loss of most of the grown gold labels during the post-enlargement rinsing step. To circumvent this drawback, a polymeric solute containing polyethyleneglycol and sodium chloride was introduced in the growth media to act as aggregating agent during the catalytic process and thus retain the enlarged labels on the bottom of the microwell. This strategy, which led to an efficient increase of the hybridization response, allowed detection of tDNA concentrations as low as 600 aM (i.e., 10⁴ lower than without amplification) and thus offers great promise for ultrasensitive detection of other hybridization events.

Introduction

The rapid, inexpensive, sensitive and specific detection of nucleic acids is of central importance for the diagnosis of infections, identification of genetic mutations and forensic analysis. Currently, one of the most conventional methods for sequence-specific DNA analysis is polymerase chain reaction (PCR) followed by hybridization of the target PCR-amplified product with a single-stranded oligonucleotide-labeled probe. Because of their exceptionally high sensitivity and specificity, oligonucleotide labelled by metal nanoparticles probes have shown great promise for sequence specific DNA testing over the past 10 years.¹ Although various metal and semiconductor nanoparticle tags (gold, silver, zinc sulfide, lead sulfide...) have been described as suitable biodetection agents, gold nanoparticle labels have by far witnessed the most tremendous interest owing to their unique physical and chemical properties. The first indication of their potential in

45 biodiagnostic screening of nucleic acids was reported by Mirkin's group with the observation that oligonucleotide-modified gold nanoparticles in the presence of complementary target DNA yield aggregate assemblies that can be easily monitored by a change of solution color from red to blue.² Since then, gold nanoparticle labels have been involved in a wide variety of DNA hybridization assays in which absorbance,³ light scattering,⁴ optical,⁵ fluorescence,⁶ surface plasmon resonance,⁷ quartz cristal microbalance,⁸ conductimetric⁹ and electrochemical¹⁰ techniques were employed to monitor the hybridization events.

Among these transduction methods, the electrochemical detection is particularly well suited for the quantitative determination of the colloidal gold label because of its sensitivity, cost and time-effectiveness, portability, minimal power requirement and compatibility with microfabrication technology. Basically, two main electroanalytical schemes have been proposed so far: either the direct oxidative measurement of the electroactive gold nanoparticle anchored in the hybrids immobilized onto the electrode surface or the indirect anodic stripping analysis of the amount of gold (III) ions released from each gold label after an oxidative treatment. This latter approach which was first demonstrated in our group for a noncompetitive heterogeneous immunoassay¹¹ and was further applied to the sensitive quantification of a PCR amplified 406-base pair human

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cytomegalovirus (HCMV) DNA sequence. The combination of the large number of Au^{III} ions released from each gold particle anchored in the hybrid using an acidic bromine-bromide solution (e.g., 2.3×10^5 gold atoms are theoretically contained in a 20-nm spherical gold particle) with the sensitive measurement of gold (III) by anodic stripping voltammetry (ASV) at a sandwich type carbon screen-printed microband electrode (SPMBE) indeed allowed the determination of amplified HCMV DNA at picomolar levels.^{10a} Similar sensitive potentiometric stripping measurements of the dissolved tags were reported at the same time by Wang and co-workers for the detection of nucleic acid segments related to the breast cancer BRCA1 gene in a metallogenomagnetic assay.^{10b} Alternatively, the direct oxidation of the gold nanoparticle label contained in the hybrid attached onto a graphite pencil electrode surface was explored by Ozsoz *et al.* and they were able to determine factor V Leiden mutation from PCR amplified samples in the picomolar range.^{10c} Yet, despite the good sensitivity of all the above hybridization tests for PCR-amplified samples, further improvement is required to detect as low as hundreds copies of target DNA per few tenths of microliters of sample, avoiding the need for target pre-amplification schemes such as polymerase chain reaction.

Since the measured electrochemical signal is proportional to the size of the colloidal gold label, a significant increase in the gold response, and consequently a higher sensitivity for DNA sequence analysis, should be expected when using larger Au nanoparticles. However, large gold colloidal particles with diameter larger than 20 nm were seldom employed to prepare the oligonucleotide-modified gold probes for several reasons including a wider size distribution,¹² a poor stability in solution of the resulting bioconjugates and a lower hybridization rate.¹³ Alternatively, once hybridized, the gold label can be enlarged by forming shells of gold or silver surrounding the gold core through an autometallographic process based on the colloidal Au-surface catalyzed reduction of gold or silver ions, respectively. The silver amplification of the hybridization events,¹⁴ followed by the stripping analysis of the dissolved silver ions¹⁵ or the direct oxidation of the silver deposits¹⁶ have been especially investigated. While all these approaches provided an increase in sensitivity, the achieved detection limits were still restricted to the picomolar range because of a significant background noise arising from the nonspecific silver deposition both on the hybridization solid-phase supports (magnetic beads or electrodes) and/or on the negatively charged DNA, which can act as the binding and nucleation sites of silver ions.¹⁷ An interesting issue consists in using gold ions instead of silver ones, as recently demonstrated by Hainsfeld *et al.* in electron microscopy experiments on the *in situ* detection of nucleic acids.¹⁸ In their work, the authors have shown that while providing an effective gold signal amplification, the gold autometallographic process offered cleaner backgrounds (mainly resulting from minimal autonucleation and less interactions between the anionic AuCl₄⁻ gold complexes and the negatively charged DNA). To our knowledge, Wang *et al.*, have been so far the only ones who associated the gold autometallographic technique with the use of colloidal gold

nanoparticle labels in an electrochemical DNA hybridization test.^{10b,d} Despite the dramatic increase in the gold stripping response reported for such an amplification route, a non-negligible background signal seriously hampered the subpicomolar DNA determinations. The minimization of the gold noise signal – mainly ascribed to the nonspecific deposition of the Au^{III} ions on the magnetic beads – was envisaged by the authors; however, it has not been demonstrated yet.

In this context, the main task of the work described below was to provide a gold enlargement procedure which not only enhances the electrochemical signal generated by the colloidal gold label but also allows the detection of subpicomolar HCMV DNA sequences. The overall DNA hybridization assay was done in polystyrene microwells. It involves the four main steps depicted in the upper part of Figure 1, i.e., (1) passive adsorption of target DNA on the bottom of the microwell, (2) hybridization with an oligonucleotide probe labelled by a gold nanoparticle, (3) oxidative dissolution of the gold metal atoms anchored on the hybrids and (4) ASV detection of the released Au^{III} ions at a SPMBE. The gold autometallographic amplification stage sketched at the bottom of Figure 1 was first investigated by incubating standard [Au^{III}/NH₂OH] aqueous growth mixtures, as previously reported in the literature.¹⁹ However, the resulting electrochemical responses complemented by transmission electronic microscopy (TEM) had established the absence of significant improvement of the ASV gold label response under such experimental conditions. As it will be shown, this is only by a careful control of the growth media composition that the sensitivity of the hybridization assay will be improved.

Experimental

Reagents and solutions

Chloroauric acid (HAuCl₄, 3H₂O), trisodium citrate, concentrated bromine (CAUTION: bromine is a toxic and harmful reagent), 3-phenoxypropionic acid, cetyltrimethylammonium bromide (CTAB), 3-(amidinothio)-1-propanesulfonic acid, sodium salts of bromide, chloride and PEG 35000 MW were obtained from Aldrich as well as the gold atomic absorption standard solution (1000 µg.mL⁻¹) used to check all the concentrations of our home-made Au^{III} solutions. Hydroxylamine hydrochloride (NH₂OH) was purchased from Fluka. Hydrobromic acid (47 %) was supplied by Merck as Suprapur grade reagents and its residual amount of bromine was eliminated as previously described.¹¹ All of the oligonucleotides were synthesized by MWG Biotech France and they had the following sequences: *tDNA*: 5'-(T)₁₀GGA TCC GCA TGG CAT TCA CGT ATG T^{3'}, *pDNA*: ^{5'}HS-(CH₂)₆-ACA TAC GTG AAT GCC ATG CGG ATC C^{3'}. The Hybridowell™ kit – which denaturation solutions, coating, hybridization and washing buffers were employed in the HCMV DNA hybridization assay – was provided by Argene-Biosoft (<http://www.argenebiosoft.com>). Phosphate-buffered saline (PBS: 4.3 mM NaH₂PO₄, 15.1 mM NaHPO₄, and 50

185 mM NaCl, pH 7.4) and all of the solutions were prepared with MilliQ 18 M Ω water (Millipore purification system). Other chemicals used were of reagent grade.

Electrodes and instrumentation

SPMBEs were prepared as previously^{10a} (four per array, 190 0.0085 mm² for the geometric working area) with a manual screen-printer (Circuit Imprimé Français, Bagneux, France) in association with a screen stencil of 120 threads.cm⁻¹ and using a conductive carbon-based ink (Electrodag PF 407A, Acheson Colloids Co) and high-impact polystyrene (Séricol, France). 195 The electrochemical measurements were performed at room temperature by immersing a SPMBE in a 60- μ L droplet electrochemical cell as well as a small-sized home-made saturated Ag/AgBr reference with a double frit separation and a platinum wire counter electrode. An Autolab potentiostat (EcoChemie) interfaced to a PC system with GPES version 200 4.9 software was used for ASV measurements under the following instrumental conditions: 5-min electrodeposition step at -0.3 V immediately followed by a positive potential scan ($v = 50$ mV.s⁻¹). The integration of the peak current (Q_p) 205 located at $\sim +0.95$ V was chosen as the analytical response. Before each measurement, the microband electrode was regenerated by cutting a small slice of its extremity with a scalpel. Absorption spectra of the oligonucleotides and nanoparticles solutions were recorded using a SAFAS DES 210 spectrophotometer. Transmission electron microscopy (TEM) images of DNA functionalized Au nanoparticles were obtained on a Hitachi H600 transmission electron microscope. Samples were prepared by pipetting a 5- μ L sample onto a copper grid and allowing these latter to dry at room 215 temperature for two days.

Synthesis of colloidal gold

Colloidal gold nanoparticles were prepared by citrate reduction of chloroauric acid according to protocols described by Mirkin and co-workers.²⁰ The gold nanoparticles used in 220 this study have an average diameter of 12.3 ± 0.3 nm as measured by TEM and were referred as Au nanoparticles in this work. Assuming spherical nanoparticles, their molar concentration was calculated by comparing the number of gold atoms in a particle of known diameter to the total number 225 of atoms in solution. The extinction coefficient at 520 nm for 12.3 nm diameter particles was determined to be $\epsilon_{520} = 2.3 \times 10^8$ M⁻¹.cm⁻¹.

Preparation of gold nanoparticle-labeled oligonucleotide

Gold nanoparticles were functionalized by derivatizing 0.5 230 mL of the aqueous Au colloid (15 nM) with 36 μ L of an aqueous solution of pDNA (46 μ M) as previously described.^{10a} Briefly, after standing for 20 h at room temperature, the solution was aged in a 73 mM NaCl, 10 mM phosphate (pH 7.4) for three days. The oligonucleotide-colloidal gold conjugate (pDNA-Au) was then isolated by 235 centrifugation (14000 rpm, 1h) to remove excess reagents, washed with PBS and centrifugated again (14000, 1h). Following removal of the supernatant, the gold nanoparticle probes were redispersed in an equivalent volume of glycerol 240 and stored at -20°C. The concentration of this stock solution

was estimated by using its absorbance value at 520 nm in conjunction with the extinction coefficient ϵ_{520} . A freshly diluted probe solution was prepared prior to each hybridization step.

245 Gold nanoparticle-based hybridization assay

The electrochemical HCMV DNA sequence detection involving a colloidal gold label was performed as reported in a previous work,^{10a} according to a four step procedure (Figure 1) which was an adaptation of the commercially-available 250 Hybridowell™ kit protocol. In short, the tDNA sample was diluted with the coating solution and 100 μ L of this solution was incubated in polystyrene microwells (Maxisorp, Nunc, France) overnight. After removing the solution, 100 μ L of pDNA-Au diluted in the hybridization buffer (200 pM) were 255 added and incubated into the wells at 37°C for 30 min. Following this hybridization step, the microwells were drained and subjected to a washing cycle consisting of five washes for 1 min with 300 μ L of washing buffer (1 X) followed by two rinses for 1 min with 300 μ L of PBS. After 260 carefully removing the rinsing solution, the microwells were filled with 100 μ L of an acidic bromine-bromide solution (0.1 M HBr containing 10^{-4} M Br₂), and after waiting for 10 min, 13 μ L of a fresh solution of 3-phenoxypropionic acid (4×10^{-3} M in 0.1 M HBr) was added and mixed into the wells.¹¹ 265 Finally, a 60- μ L droplet of the solution was transferred onto the bottom of a reversed microwell and the released gold (III) ions were quantified at a SPMBE by ASV under the instrumental conditions described above. All of the experiments were carried out at room temperature, unless 270 otherwise stated.

Gold enhancement procedure

The gold-based autometallographic enlargement of the gold labels anchored onto the hybrids was an additional step in the hybridization protocol which was carried out prior to the gold 275 metal chemical dissolution. The gold enhancement solution (G_S^*) was prepared just before use by successively mixing freshly prepared 0.8 mM Au^{III} and 1 mM NH₂OH aqueous solutions in a 15 % PEG/0.3 M NaCl aqueous media containing 0.1 mM CTAB. The growth method consisted in 280 treating the microwells with 100 μ L of G_S^* for 30 min followed by a rinsing step with a 0.5 M (HCl / NaCl) washing solution (W_S , 5×300 μ L, 1 min for each).

Results and discussion

Autocatalytic growth of gold nanoparticle labels using 285 Au³⁺/NH₂OH aqueous mixtures

The enlargement of the gold nanoparticles anchored in the immobilized hybrids was first achieved by adapting the hydroxylamine seeding method reported by Natan and co-workers,¹⁹ i.e.; by incubating 100 μ L of a 250 μ M HAuCl₄ + 290 40 mM NH₂OH aqueous solution in the polystyrene microwell for 10 min followed by two rinses with H₂O for 1 min each. In agreement with the results previously reported by Wang and coll.,^{10b,d} this enlargement process provided a significant increase of the ASV response (factor of ~ 100) but also led to a 295 huge background current response of very poor

reproducibility (relative standard deviation of 40 %). Such a variable nonspecific response is obviously highly deleterious to the improvement of the hybridization assay detection limit. Contrary to optical or gravimetric detections which rely on a threshold amount of gold metal particles for visualization¹⁸ or microgravimetric⁸ measurements, the nonspecific adsorptions of Au^{III} on the walls of the polystyrene microwells during enlargement process was here of major concern because of trace detection of gold (III) by ASV at a SPMBE (Au^{III} detection limit at nanomolar levels). In order to minimize this undesirable Au^{III} background contribution, the influence of the Au^{III} concentration in the growth mixture and the efficiency of several post-enlargement washing solutions were studied. A growth mixture (G_S) containing 3 μM Au^{III}, 30 μM NH₂OH and 0.1 mM CTAB,²¹ was selected for all of the further experiments as well as a cycle of five 1-min post-enlargement washings with the acidic solution (W_S) to remove the excess of Au^{III}. Under these conditions, the autometallographic amplification with the G_S solution was then investigated and the resulting series of ASV curves is shown in Figure 2. As expected, the background signal recorded in the absence of DNA target was negligible (curves a, a') whereas the comparison of curves b and b' obtained for a 30 nM tDNA concentration only just indicates a 3-fold ASV current increase after the enlargement process. The gold ions concentration contained in the hybridized gold nanoparticle probes ([Au^{III}]₀) was determined from the integration of the stripping peak current obtained without gold enhancement (curve b in Figure 2) and the ASV calibration plot of AuBr₄⁻ recorded at a SPMBE^{10a}. A value of [Au^{III}]₀ = 47 nM was found here which corresponds to a hybrid surface coverage of 89 amol.cm⁻². Since at such a low surface coverage, the immobilized gold nanoparticles grow individually, in analogy to the process in solution,²² the efficiency of the proposed enlargement protocol was assessed by TEM analysis after incubating in a microcentrifuge tube 93 amol of pDNA-Au (53 nM of Au^{III}) with 100 μL of G_S for 30 min. As shown by the images in Figure 3, the small pDNA-Au (Figure 3a) were enlarged into gold spheres and nanorods (Figure 3b) which definitely provides support for the notion that growth occurs in our experiments. Therefore, all of these results strongly suggest that the small amplification observed by ASV (compare again curves b and b') is mainly due to the loss of most of the enlarged gold nanoparticle labels during the growth and subsequent rinsing steps. The validity of this hypothesis was evidenced by the TEM observations of 5 μL of G_S removed from the microwell just after the autometallographic stage and placed on the copper grids. In the absence of tDNA, i.e., hybridized pDNA-Au, the TEM micrograph (Figure 4a) shows dispersed small nanoparticles, suggesting the occurrence of gold autonucleation events in the polystyrene microwell.²³ In contrast, for a 30 nM tDNA sample (Figure 4b), large aggregates with a wide size dispersion are observed in addition to the small seeds thus confirming the above assumption. So, the question we address now is why most of the enlarged gold nanoparticle labels are released from the walls of the microwells during the growth and subsequent rinsing steps. The physical desorption of the gold-labeled hybrid from the polystyrene surfaces was

proposed as first explanation. Hence, a few experiments were carried out by following the protocol depicted in Figure 1, except for the step 1 in which the passive adsorption of the tDNA was replaced by the covalent binding of the 5'-phosphorylated tDNA target on aminated polystyrene microwells.²⁴ Despite this stronger fixation of the target, the gold enhancement still led to the same poor amplification of the ASV signal (data not shown). Several control experiments were then carried out to assess the stability of the gold-labeled hybrids over each G_S's reagents and W_S, but none of them were found to dissociate the gold nanoparticle-labeled hybrid. Finally, the engulfing of the hybridized pDNA-Au by the gold metal layer deposited during the autocatalytic process can be evoked, with no further experimental evidence at the moment, as an explanation for the uncoupling of the grown gold label from the microwell, and thus for the poor enhancement of the gold ASV response after the amplification step with G_S.

Use of PEG-NaCl in the enlargement mixture

To effectively detect all of the enlarged gold nanoparticle labels by ASV at the end of the hybridization assay, each pDNA-Au must be irreversibly immobilized on the walls of the polystyrene microwell during the amplification stage. To achieve this goal, a macromolecular crowding polymeric solute, which has both the properties to aggregate colloidal gold labeled oligonucleotides in the absence of complementary DNA and to increase the DNA duplex stability,²⁵ was added to the G_S solution. The addition of 0.3 M NaCl and 15 % PEG (PEG/NaCl) to the G_S solution was initially adopted. Under these selected conditions (Entry 1 in Table 1), a 150-fold enhancement of the ASV response was reached for a 30 pM tDNA concentration. After correction from the nonspecific Au^{III} ASV blank signal recorded in the absence of tDNA, it corresponds to an amplification factor of 100. This is roughly 11 times higher than when the growth stage occurred in the absence of PEG/NaCl (Entry 2).²⁶ It clearly indicates that the addition PEG/NaCl in G_S solution prevents the release of the enlarged labels during the growth stage. With the aim to assess the respective role of PEG and NaCl during the autocatalytic process, the same experiment was repeated by introducing either 15 % PEG or 0.3 M NaCl in the G_S mixture. Surprisingly, poor amplification rates were obtained in both cases (Entries 3-4 in Table 1). The addition of the PEG polymer alone tends to increase the Au nonspecific signal without improving the growth response, whereas the addition of NaCl tends to reduce both the specific and nonspecific but without improving the amplification factor.²⁷ The influence of the NaCl concentration in the G_S-PEG solution on the ASV hybridization signal of 0, 3 and 3000 fM tDNA was also examined. As shown in Figure 5, the stripping current was clearly impacted by the amount of salt introduced in the polymeric growth mixture and an optimal ASV response was obtained for a 0.3 M NaCl concentration, allowing to detect the specific response provided by a 3 fM tDNA concentration (■ symbol in Figure 5). At a lower NaCl concentration, the observed smaller signal is consistent with the fact that the stability of the DNA-Au aggregates decreases when reducing the salt concentration in the polymeric solution.²⁵ On the other hand, the autometallographic process

tends to be less and less efficient when increasing the values of NaCl concentrations above 0.3 M. Though this latter observation is not fully understood, all these results show that it is very important to carefully balance the NaCl concentration in the polymeric G_S solution to effectively enhance the electrochemical DNA hybridization signal. A G_S mixture containing 15 % PEG and 0.3 M NaCl (G_S^*) was used for all of the further studies. With the aim to better understanding the origin of the high nonspecific signal recorded in the absence of tDNA, the entire assay was carried out for a 3 pM tDNA concentration without incubating pDNA-Au during the hybridization step. An average stripping charge of 7 ± 0.3 nC was obtained. This high value, which roughly contributes to 70 % of the ASV blank signal, can be explained by the spontaneous formation of gold nanoparticles through reactions of Au^{III} complexes with PEG in aqueous solutions at ambient temperature.²⁸ Other likely reasons for this high blank signal may be the nonspecific gold deposition onto other components of the DNA hybridization assay in the microwells and the occurrence of a low nonspecific binding of pDNA-Au. Despite this background signal, it should be noted that the enlargement procedure with G_S^* provides consistent results with an average stripping charge $Q_{G,0}$ of 10.7 ± 0.86 nC (relative standard deviation < 8 % for 2 separate series of 4 assays different from that which yielded the results of Table 1).

Characterization of the analytical performance

The sensitivity of the amplified gold-based electrochemical detection of DNA hybridization was investigated by varying the tDNA concentration over the 300 aM–30 nM range. The corresponding calibration plot i.e.; the stripping charge versus tDNA concentration in log scale is shown in Figure 6 (curve A). With a dynamic range extended between 3 fM and 300 pM, this is 2 orders of magnitude larger than without gold enlargement (compare curves A and B in Figure 6). In both cases, the upper concentration limit is around the nanomolar level due to the saturation of the tDNA capturing sites on the surface of microwells. A detection limit of 600 aM (~ 1.4 zmol or 840 tDNA sequences per microwell) can be estimated using a signal to noise ratio of 3. The sensitivity of this assay is 10^4 lower than that observed without gold amplification (5 pM) and compares favorably to values reported for other electrochemical, gravimetric gold-enhanced DNA analysis.^{8,10b,d} Moreover, the tDNA detection limit obtained in the present work is competitive with lowest values recently reported for electrochemical hybridization assays based on electroactive polystyrene microbead labels (100 aM or 3100 copies),²⁹ enzyme-amplified amperometric detection on microelectrodes (500 aM or 1000 copies),³⁰ carbon nanotube-derived amplification (5.4 aM or 80 copies),³¹ and biometallization process (100 aM or 6000 copies).³² Finally, our approach allows to envision hybridization assays devoid of any target amplification. It is important to note that in our experiments the tDNA was systematically immobilized on the walls of the polystyrene microwell in the presence of a high amount of unrelated long DNA.³³ The results in Figure 6 clearly show that the presence of unrelated DNA does not affect gold deposition and moreover, without tDNA, lead to a

low nonspecific gold response (i.e., a charge value of 11.30 ± 0.76 nC). By extrapolation, we can assume that background components contained in real samples should thus not modify the gold deposition and consequently the sensitivity of the method.

Conclusions

In this work, we have first shown that classical gold-enhancement procedures cannot provide an effective amplification of the gold nanoparticle-based electrochemical transduction of DNA hybridization in polystyrene microwells. The addition of a carefully selected amount of PEG/NaCl in the growth mixture has proved to be decisive in the amplification factor of the Au^{III} ASV response. This approach, which competes favourably with the most sensitive methods involving enzyme labels, is a promising alternative for gene detection and other biological assays since the gold tags have the advantages over enzymes of being stable and the labeling procedure is very simple. However, the sensitivity of the method may be improved by replacing the PEG/NaCl with other aggregating agents which yield less background signal and/or using three-dimensional DNA-linked aggregate hybridization formats. Some of these studies are currently underway to envision the ultrasensitive detection of DNA without any pre-amplification of the targets.

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