Anal. Chem. **2002,** *74,* 4788−4793

Label-Free Determination of Picogram Quantities of DNA by Stripping Voltammetry with Solid Copper Amalgam or Mercury Electrodes in the Presence of Copper

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Highly sensitive label-free techniques of DNA determination are particularly interesting in relation to the present development of the DNA sensors. We show that subnanomolar concentrations (related to monomer content) of unlabeled DNA can be determined using copper solid amalgam electrodes or hanging mercury drop electrodes in the presence of copper. DNA is first treated with acid (e.g., 0.5 M perchloric acid), and the acid-released purine bases are directly determined by the cathodic stripping voltammetry. Volumes of 5−3 μL of acid-treated DNA can easily be analyzed, thus making possible the determination of picogram and subpicogram amounts of DNA corresponding to attomole and subattomole quantities of 1000-base pair DNA. Application of this determination in DNA hybridization detection is demonstrated using surface H for the hybridization (superparamagnetic beads with covalently attached DNA probe) and the mercury electrodes only for the determination of DNA selectively captured at surface H.

More than 40 years ago it was shown by one of us (E.P.)[1,2] that the usual nucleic acid bases produced characteristic oscillopolarographic[3] signals in NaOH solutions; these signals were attributed to the formation of sparingly soluble compounds with the electrode mercury.[2] About 20 years later, we showed that the nucleic acid bases and some other purine and pyrimidine derivatives can be determined at nanomolar concentrations by cathodic stripping voltammetry (CSV).[4,5] Then Glodowski et al.[6] found that sparingly soluble compounds of copper(I) with adenine (A) and adenosine can be accumulated at the electrode surface either by reduction of copper(II) ions or by oxidation of the copper liquid amalgam electrode. The copper(I)/adenine deposit can be stripped either anodically or cathodically with detection limits 20 and 5 nM, respectively. Shiraishi and Takahashi[7] accumulated copper(I) compounds of adenine and guanine at carbon electrodes to determine the bases by anodic stripping voltammetry; linear calibrations of adenine and guanine were obtained in the range 0.5−8 μM at 5-min accumulation. Electrochemical methods were applied in studies of interactions of nucleic acid monomeric constituents with copper (ref 8 and references therein), but no attempt was made to utilize these interactions in electrochemical determination of DNA or RNA. Recently Farias et al.[9] used the hanging mercury drop electrode (HMDE) to determine low concentrations of adenine in the presence of copper. They used conditions different from those of Glodowski et al.[6] and reported a detection limit of adenine of 0.03 nM at 6-min accumulation time.[9] Using the same procedure they analyzed also calf thymus DNA, but they obtained sensitivity only at the micromolar level (related to the monomer content).

Until recently, mercury electrodes were not considered as detection electrodes suitable for DNA hybridization sensors because of difficulties with DNA hybridization at the hydrophobic mercury surface to which hydrophobic bases in single-stranded DNA were too strongly adsorbed,[10] thus not being available for the specific interaction with the DNA complementary strand. Recently we proposed a new method in which the hybridization is performed at one surface (surface H, which can be represented, e.g., by commercially available magnetic beads) and the electrochemical detection at another surface (detection electrode, DE).[11−13] In this method, the DE can be chosen only with regard

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10.1021/ac0200771 CCC: $22.00
Published on Web 08/16/2002

to the electrode process not considering its properties in relation to the DNA hybridization.

In this paper, we propose a new method of DNA determination using either HMDE in the presence of copper or a solid copper amalgam electrode. This method is almost by 3 orders of magnitude more sensitive than the DNA determination proposed by Farias et al.[9] Such a large increase in the sensitivity results from the acid treatment of DNA releasing purine bases from DNA; after this treatment, bases are electrochemically determined without separation from other products of the acid DNA degradation. Combining this highly sensitive determination of DNA with DNA hybridization at the magnetic beads, we propose a label-free method of highly specific and sensitive analysis of DNA sequences which can be used in an electrochemical DNA hybridization sensor.

EXPERIMENTAL SECTION

Apparatus and Electrochemical Procedures. Linear sweep voltammetry (LSV) measurements at HMDE were performed with an Autolab analyzer (EcoChemie) in connection with the VA-Stand 663 (Metrohm, Herisau, Switzerland). The standard cell with three electrodes was used. The working electrode was HMDE with the drop area of 0.41 mm^2, the reference electrode was Ag/AgCl/3M KCl electrode, and platinum wire was used as the auxiliary electrode. LSV experiments with HMDE were carried out at room temperature under following parameters: E_i −0.3 V; t_A 120 s, scan rate 0.5 V/s, and step potential 5 mV, if not stated otherwise. As background electrolytes, 0.005 M NaOH or 0.05 M Borax with 0.4 ppm Cu^{2+} were used.

The voltammetric measurements at the mercury meniscus-modified copper solid amalgam electrode (m-CuSAE) were carried out with the computer-controlled polarographic/voltammetric analyzer PC-ETP (Polaro-Sensors, Prague, Czech Republic). m-CuSAE served as the working electrode, a saturated calomel electrode (SCE) as reference, and a platinum wire (1 mm in diameter, length 7 mm) as auxiliary. Before starting the work, as well as after every pause longer than 1 h, the surface of m-CuSAE was electrochemically activated in 0.2 M KCl by applying −2.2 V for 300 s while stirring the solution. Moreover, before each measurement, the surface of the electrode was first regenerated automatically by 100 polarization cycles for a period of ∼10 s between −0.7 and −1.7 V and then kept for 30 s at −1.7 V. The differential pulse voltammetry (DPV) was applied with the pulse amplitude of 0.05 V, pulse width of 100 ms, and scan rate of 0.02 V/s. LSV measurements were carried out with t_A 300 s and scan rate 1 V/s. As inert gas, for deaerating solutions in the electrolytic cell, pure nitrogen was used. The measurements were done at room temperature in 0.005 M NaOH.

Chemicals. Adenine, guanine, and polyadenylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Calf thymus (CT) DNA isolation and thermal denaturation was carried out as previously described.[14] Apurinic acid (APA) was prepared by dialyzing the CT DNA sample against HCl (pH 1.6) for 26 h at 37 °C. Oligodeoxynucleotides (ODN) $(A)_{25}$ and $(A)_{25}(GAA)_4$ used in this study were synthesized by VBC-Genomics (Wien, Austria). Dynabeads Oligo$(dT)_{25}$ (DBT) and magnetic particle concentrator MPC-S were products of Dynal A.S. (Oslo, Norway). The nucleic acid concentration was determined spectrophotometrically using a HP 8452 spectrophotometer.

Other Procedures. *Hydrolysis of Oligodeoxynucleotides (ODN), DNA, and Poly(A).* Hydrolysis of ODN or DNA was performed by adding 20 μL of 1 M $HClO_4$ to a sample of the same volume of ODN or DNA at a concentration of 4 μM (related to the monomer) and heating for 30 min at 75 °C. The sample was cooled and neutralized with KOH, and aliquots were added to 1 mL of the background electrolyte and used for voltammetry measurements, if not stated otherwise.

Dynabeads Biomagnetic Separation Procedure. DNA and ODN samples for stripping voltammetry were treated using the following protocol. Aliquots (usually 20 μL) of DBT were washed twice in a 1:1 volume of 0.3 M NaCl, 50 mM phosphate buffer, pH 7.0. Then 20 μL of DNA or ODN solution at 20 μM concentration in the same buffer was added to the DBT. The samples were shaken for 30 min at 26 °C to allow hybridization between the DNA in solution and the oligo(dT) chains on the bead surface. After hybridization, the DBT were washed five times with 100 μL of 0.3 M $NaClO_4$. DNA was released from DBT by heating at 85 °C for 2 min in 20 μL of triple-distilled water. Then the samples were used for acid hydrolysis with perchloric acid.

RESULTS AND DISCUSSION

Earlier we showed[4] that low concentrations of adenine could be determined by CSV at HMDE in the presence of an excess of DNA. It has been known for a long time that as a result of acid treatment purine bases are released from DNA, producing a mixture of free adenine and guanine and APA (average MW of ∼15 000).[15,16] Here we attempted to determine purine bases released from DNA by acid treatment (without removal of APA) by means of voltammetric methods using either HMDE in the presence of copper or a solid copper amalgam electrode.

Hanging Mercury Drop Electrode. In the concentration range between 8 and 33 nM (related to monomer content), neither dialyzed APA (not containing any free purine bases) nor (acid-untreated) single-stranded (ss) calf thymus DNA produced any appreciable LSV signal at the accumulation time, t_A 120 s (Figure 1). The absence of any signal of 33 nM DNA is in agreement with the results of Farias et al.,[9] who showed a DNA response starting at a concentration ∼30 times higher. On the other hand, nanomolar concentrations of the same DNA treated with 0.5 M perchloric acid (30 min at 75 °C) produced well-developed cathodic LSV peaks at t_A 120 s (Figures 1A,B and 2). Under conditions used by Farias et al.,[9] i.e., LSV in 5 mM NaOH with 0.4 ppm Cu^{2+}, we obtained a linear calibration between 2 and 40 nM acid-treated DNA (slope −0.82 nA/1 nM, correlation coefficient 0.993) at t_A 120 s and −0.3 V deposition potential (ED) (scan rate 0.5 V/s). Peak potential (E_p) of the acid-treated DNA was ∼−0.64 V, which did not change with the DNA concentration in the given concentration range (Figure 1). At longer accumulation times, substantially lower DNA concentrations were determined (not shown).

Under air, the concentration of 5 mM NaOH and pH of the background electrolyte can undergo uncontrolled changes. We therefore tested other background electrolytes and we found that,

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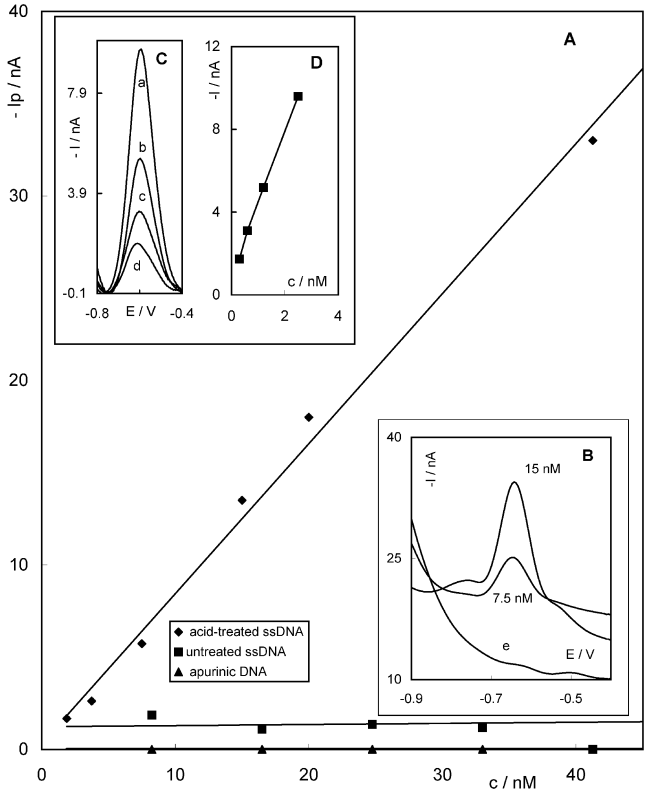

Figure 1. (A) Dependence of voltammetric stripping peak heights on concentration of acid-treated calf thymus DNA, ssDNA, and APA. (B) LS voltammograms of 15 nM (or 7.5 nM) acid-treated DNA. Background electrolyte (e) was 5 mM NaOH with 0.4 ppm Cu^{2+}, accumulation time t_A 120 s. (C) LS stripping voltammograms of acid-treated ODN $A_{25}(GAA)_4$ obtained at low ODN concentrations and at t_A 600 s. (a) 2.5, (b) 1.2, (c) 0.6, and (d) 0.3 nM. Background electrolyte 0.05 M Borax with 0.4 ppm Cu^{2+}. (D) Dependence of peak heights on acid-treated $A_{25}(GAA)_4$ concentration. In all cases, DNA (or APA) samples at a concentration of 2 μM (related to the monomer content) were treated with 0.5 M $HClO_4$ for 30 min, and after neutralization by NaOH, sample aliquots were added to the background electrolyte with 0.4 ppm Cu^{2+} and LSV curves were recorded. HMDE, scan rate 0.5 V/s, potential step 5 mV, starting potential −0.3 V, stirring 1450 revolutions/min, quiescent period 5 s; measurements were performed in a conventional voltammetric cell. All DNA concentration are related to the monomer content. The curves in (C) were corrected using linear baseline correction and background electrolyte was subtracted from the curves.

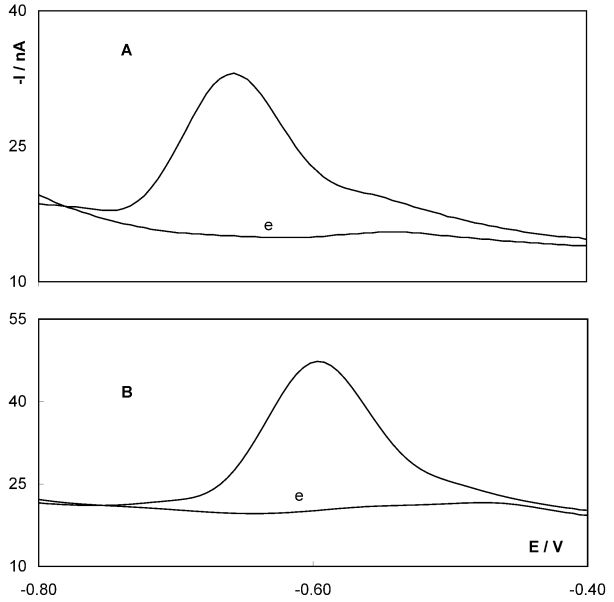

Figure 2. LS stripping voltammograms of 15 nM acid-treated DNA in (A) 0.005 M NaOH with 0.4 ppm Cu^{2+} or (B) 0.05 M Borax with 0.4 ppm Cu^{2+}. HMDE, scan rate 0.5 V/s, t_A 120 s, starting potential −0.3 V, and e background electrolyte. Other details as in Figure 1.

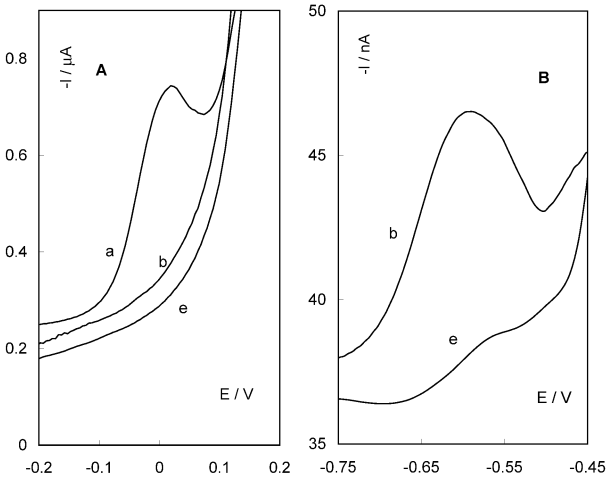

Figure 3. LS stripping voltammograms of acid-treated ssDNA (A) in the absence and (B) in the presence of copper. (a) 50 nM and (b) 2.5 nM acid-treated ssDNA, HMDE, scan rate 0.5 V/s, t_A 120 s, and e background electrolytes: (A) 0.05 M Borax (without copper); (B) 0.05 M Borax with 0.4 ppm Cu^{2+}. Other conditions as in Figure 1.

for example, in 50 mM Borax (pH 9.2), the 15 nM acid-treated DNA produced at t_A 120 s a well-developed LSV peak, comparable to that obtained in 5 mM NaOH (Figure 2). At longer accumulation times, subnanomolar concentrations of DNA were determined; for example, at t_A 10 min, we obtained a linear calibration between 0.3 and 2.5 nM (Figure 1C). A DNA concentration of 0.3 nM (related to monomer content) corresponds to ∼100 pg of DNA and ∼30 pg of adenine per milliliter. As we shall show further, the analysis of DNA in the presence of copper can easily be done in 5-μL volumes, thus allowing determination of femtogram amounts of purine bases or acid-treated DNA corresponding to attomole or subattomole amounts of 1000-base pair DNA. We compared the determination of acid-treated DNA in the presence and absence of copper. The CSV curve of 2.5 nM acid-treated DNA, obtained in the absence of copper, was almost identical with the background electrolyte (Figure 3A). The same concentration of DNA produced a well-developed peak in the presence of copper (Figure 3B) under otherwise the same conditions. Using baseline correction, a more symmetric peak was obtained (not shown) from the raw curve in Figure 3B, but it was impossible to generate any peak from the curve in Figure 3A produced in absence of copper (not shown). It follows from our results that the determination of acid-treated DNA in the presence of copper (Figures 1 and 2) is by ∼1 order of magnitude more sensitive than that in the absence of copper.[4,11] Increase in sensitivity in the presence of copper in anodic stripping of adenine and guanine was obtained also with carbon electrodes,[7] but the detection limit of guanine was ∼0.2 μM at 8-min accumulation, i.e., by 2−3 orders of magnitude higher as compared to the analysis with HMDE or m-CuSAE (see below).

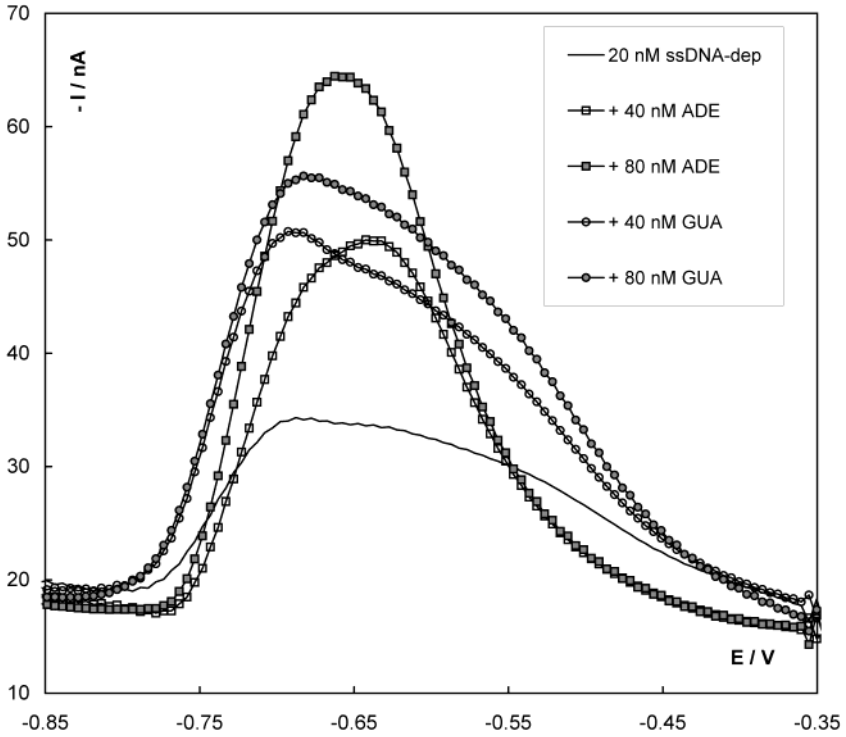

Figure 4. LS stripping voltammograms of 20 nM acid-treated DNA in 0.005 M NaOH with 0.4 ppm of Cu^{2+}; effect of addition of guanine or adenine. HMDE, scan rate 0.5 V/s, t_A 120 s, starting potential −0.3 V. Other conditions as in Figure 1.

In treating RNA with acid, we modified the conditions for the release of purine bases. We exposed polyriboadenylic acid [poly-(A)] to different conditions, using 1 or 2 M perchloric acid at 75 °C for 30 min, and measured the LSV responses of the released adenine bases in two different background electrolytes: 0.005 M NaOH and 0.05 M Borax. We found that poly(A) in 0.05 M Borax gave equally well-developed peaks as in 0.005 M NaOH and that poly(A) treatment with 2 M perchloric acid at 75 °C resulted in the highest signals. These results show that our method can be applied not only with 0.005 M NaOH but also with a more stable background electrolyte such as 50 mM Borax for the determination of both DNA and RNA. More details about the RNA analysis will be published elsewhere.

In contrast to poly(A), acid treatment of natural DNAs and RNAs will release not only adenine but also guanine. In the absence of copper, the optimum deposition potential ranges for the CSV determination of adenine and guanine were very narrow and sufficiently different,[4,17] making it possible to adjust conditions for adenine determination in the presence of guanine (and neglecting the small contribution of the latter). In the presence of copper, the region of optimum deposition potentials is wider and shifted to more negative potentials[9] and determining one type of purine base in the presence of the other one is more difficult. Thus, in the DNA determination (Figure 1), both adenine and guanine contributed to the measured signal. We measured 20 nM acid-treated DNA to which we added increasing concentrations of either adenine or guanine (Figure 4), observing increase in the height and width of the DNA peak. The response due to the addition of 80 nM guanine corresponded to 70% of that of 80 nM adenine. The analyte and the DNA used for calibration should therefore have the same adenine and guanine content.

Analysis of Microliter Volumes of DNA. Earlier we showed that DNA and RNA could be easily immobilized at the mercury[18,19] and carbon[18] electrodes by immersing the electrode for a short time in a 3−10-μL drop of the nucleic acid at an open current circuit. The electrode was then washed, the DNA- (or RNA-) modified electrode was immersed in the background electrolyte (not containing any nucleic acid), and the electrochemical measurements were performed. This medium exchange technique was called adsorptive transfer stripping voltammetry (AdTSV). Under suitable conditions, the electrochemical response obtained by AdTSV did not substantially differ from that observed with the electrode immersed in the nucleic acid solution during the measurement. We also showed that the TSV could be applied in the CSV determination of adenine.[20] In this case, however, it was necessary to carefully control the electrode potential during the deposition (because of the narrow optimum ED range), which appeared difficult in microliter adenine volumes. We found that in the analysis of acid-treated DNA in the presence of copper the accumulation step can be performed at an open current circuit (due to a different dependence of the peak height on the ED) without losing much of the sensitivity of the analysis. Using 3−5-μL drops of the 25−100 nM acid-treated DNA, we obtained well developed peaks at t_A 180 s (not shown).

Solid Amalgam Electrodes. For the determination of adenine, guanine, their mixtures, and acid-treated DNA, a pen-type

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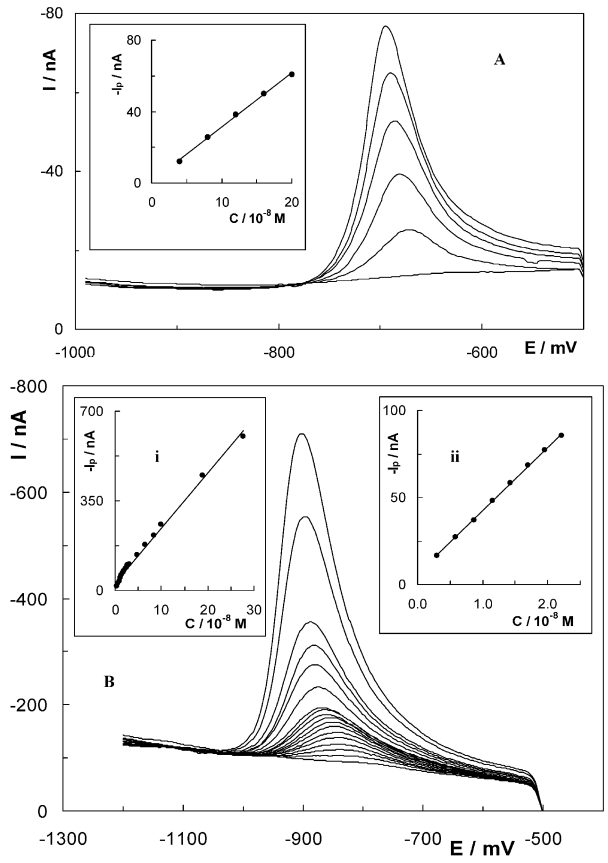

Figure 5. (A) DP and (B) LS voltammograms of acid-treated DNA samples at the m-CuSAE. In insets, the I_p dependence on concentration of acid-treated DNA is shown. LSV: scan rate 1 V/s, t_A 300 s. DPV: pulse amplitude 0.05 V, pulse width 100 ms, scan rate 0.02 V/s. Background electrolyte, 0.005 M NaOH.

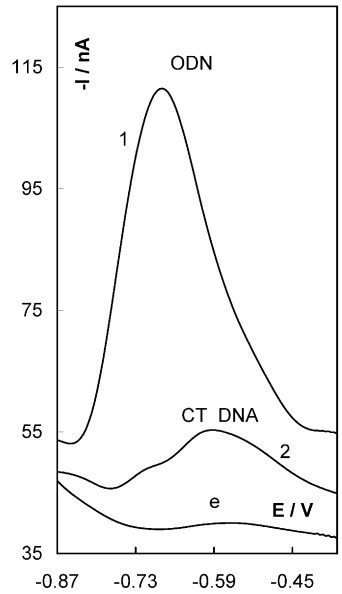

Figure 6. (A) Hybridization of DNA at magnetic beads with covalently attached DNA probe oligo(dT)$_{25}$ (DBT) followed by acid treatment of DNA released from DBT and DNA determination by stripping voltammetry in the presence of copper. The 25-mer (dA)$_{25}$ complementary to the DBT probe produced a well-developed LS stripping peak (1) as a result of selective capture of (dA)$_{25}$ at the beads; the peak of the noncomplementary calf thymus (CT) DNA (2) was much smaller. A small amount of CT DNA captured at the beads is probably connected with relatively the small content of (dA)$_n$ stretches which may be present in this chromosomal DNA.

copper solid amalgam electrode modified by a mercury meniscus[21] was used as an alternative to HMDE. The m-CuSAE shows values of hydrogen overvoltage and of background current comparable with those of HMDE; it requires no other surface regeneration than merely electrochemical, which has been in the present arrangement fully automated. This electrode can be considered as entirely nontoxic, as the solid copper amalgam is a component of dental amalgams.

After amalgamation of the solid electrode surface and activation of the surface of the formed meniscus, it can be considered that the meniscus consists of the saturated liquid amalgam of copper. The results of our experiments prove that the m-CuSAE behaves toward Ade and Gua in way analogous to a stationary electrode formed by liquid copper amalgam.[6,9] In contrast to the results obtained with HMDE (Figure 2), we have found that the best supporting electrolyte for the determination of adenine, guanine, their mixture, and acid-treated DNA by means of m-CuSAE is 5 mM NaOH. In that medium, the peaks are well defined and the sensitivity of determination is highest (the detection limit (3SD) = 4.4 nM of acid-treated DNA: LSV; scan rate 1 V/s; t_A 300 s; RSD = 3.7%; n = 11, at a DNA concentration of 40 nM). A comparison of results by DPV and LSV (Figure 5) shows the possibility of a successful application of both modes for DNA determination even though the LSV is more sensitive when applied at high rate of scan. Figure 5Bi displays almost linear dependence of the peak current on the DNA concentration; a careful inspection of this figure shows, however, that the dependence consists of two linear portions (Figure 5Bi and ii) with a small decline from linearity around 2.2×10^{-8} M. Similar nonlinear dependences were observed earlier;[22] they can be due to concentration-dependent changes in the structure of the adsorbed layer (connected with reorientation of the adsorbed molecules), formation of different types of adsorbed complexes, etc. Detailed information on comparison of various electrodes and methods for the adenine, guanine, and acid-treated DNA determination will be published elsewhere.[21] The simple and solid construction of m-CuSAE, its nontoxicity, high sensitivity, and good reproducibility of results are prerogatives for utilizing this electrode as the basic part of a DNA sensor.

DNA Hybridization. Mercury electrodes are not well suited for the DNA hybridization because of strong hydrophobic interactions of bases in ssDNA with the electrode surface.[10] DNA hybridization proceeds satisfactorily at other types of electrodes such as gold and carbon ones but working with long target DNAs and avoiding nonspecific DNA adsorption is difficult (reviewed in ref 23). To overcome these difficulties, we proposed a new method in which hybridization is performed at one surface (surface H) and electrochemical detection of the hybridization event at another surface using the mercury or solid electrodes as

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detection electrodes.[11−13] Here we use commercially available superparamagnetic Dynabeads Oligo$(dT)_{25}$ with covalently attached DNA probe $(dT)_{25}$ and HMDE as DE to determine purine bases released from captured DNA by acid treatment. We observed a well-developed peak of 37-mer ODN $(A)_{25}(GAA)_4$ or $(A)_{25}$ specifically captured by DBT, contrasting with the almost flat curve of the noncomplemetary $(GAA)_4$ (or $(CTT)_4$, not shown) or with that of chromosomal DNA differing little from the background electrolyte (Figure 6). Similar results were obtained with longer target DNAs and in the presence of a large excess of noncomplementary DNA (E. Palecek and F. Jelen, unpublished data). More details about the DNA hybridization at DBT with electrochemical detection have been published.[11−13]

CONCLUSIONS

Determination of the DNA sequences by DNA hybridization is gaining importance in connection with the attempts to develop fast methods for decentralized DNA diagnostics, for detection of bacteria and viruses (including biological warfare) in the field, etc. Such DNA (or RNA) sequencing can be performed by means of the DNA hybridization sensors with optical detection. Compared to optical detection, the electrochemical one offers important advantages, such as simple design, small dimensions, low cost, and low power requirements (reviewed in refs 23−25). This paper shows for the first time that a mercury-based nontoxic, renewable, solid amalgam electrode can be used for the determination of chromosomal DNA at the ppb level. Attomole or subattomole amounts of DNA can be determined at m-CuSAE or HMDE in several microliter volumes [only 1000-base pair DNA (MW ~6.6 × 10^5) was considered for calculation, but the length of real chromosomal DNA is usually orders of magnitude greater]. These electrodes can serve for a label-free DNA detection in a DNA hybridization sensor, if the new two-surface technique (hybridization at surface H and detection at DE) is used. Quite recently the two-surface technique has been successfully combined with other electrochemical detection methods, such as the enzyme-linked immunoassay at carbon electrodes,[12] the enzyme-linked sandwich assays involving avidin−biotin interactions,[26] or precipitation of silver on gold nanoparticles[27] in our laboratory[12,13] and in that of Wang.[26,27] The main advantages of the two-surface technique, using magnetic beads as surface H, are as follows: (a) a larger choice of DEs; our results suggest that in addition to carbon, gold, and tin oxide electrodes, usually used in DNA hybridization sensors,[23−25] other amalgam,[21] mercury film carbon, and other solid electrodes (E. Palecek, L. Trnkova, and F. Jelen, unpublished) can be used as DEs in the DNA hybridization sensors; (b) the detection of long target DNAs even in the presence of an excess of noncomplementary DNA; and (c) easy incorporation of this technique into microfluidic systems using pumping and magnetic field systems,[28] which can easily handle all the necessary steps, including washing and acid treatment.

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ACKNOWLEDGMENT

The authors are grateful to Dr. M. Heyrovsky for advice during the experimental work and for critical reading of the manuscript and to Mrs. Chroustova and Mrs. Rimankova for technical assistance. This work was supported by grant No. G203/02/0422 from the Grant Agency of the Czech Republic, by grants A1163201, S5004107, and Z5004920 from the Academy of Sciences of the Czech Republic and by november AG, Erlangen, Germany.

Received for review February 4, 2002. Accepted April 23, 2002.

AC0200771