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Label-Free Sequence-Specific DNA Sensing Using Copper-Enhanced Anodic Stripping of Purine Bases at Boron-Doped Diamond Electrodes

Stanislav Hasoň,* Hana Pivoňková, Vladimír Vetterl, and Miroslav Fojta*

Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i., Královopolská 135, CZ-612 65 Brno, Czech Republic

Stripping voltammetric determination of purine bases in the presence of copper ions at mercury, amalgam, or carbon-based electrodes has recently been utilized in analysis of DNA or synthetic oligodeoxynucleotides (ODNs). Here we report on copper-enhanced label-free anodic stripping detection of guanine and adenine bases in acid-hydrolyzed DNA at anodically oxidized boron-doped diamond electrode (AO-BDDE). The AO-BDDE was successfully applied in a three-electrode microcell in which an ∼50 μL drop of the analyte solution can be efficiently stirred during the accumulation step by streaming of an inert gas. Accelerated mass transport due to the solution motion in the presence of copper resulted in enhancement of the guanine oxidation signal by about 2 orders of magnitude (compared to accumulation of the analyte from still solution not containing copper), allowing an easy detection of ∼25 fmol of the ODNs. The proposed technique is shown to be suitable for a determination of purine (particularly guanine) content in DNA samples. Applications of the technique in magnetic bead-based DNA assays (such as hybridization with DNA sequences exhibiting asymmetrical distribution of purine/pyrimidine nucleotides between the complementary strands or monitoring of amplification of specific DNA fragments in a duplex polymerase chain reaction) are demonstrated.

Recent progress in electrochemical nucleic acids sensing has made the electrochemical detection platform a powerful alternative to the optical detection principles used in most of the current commercially available gene analyzers.[1−4] Besides techniques using a variety of nucleic acids labels (e.g., transition metal complexes,[5−7] nanoparticles, "quantum dots",[8,9] or enzymes[10−12]) developed to improve sensitivity and specificity of the DNA electrochemical analysis, label-free approaches utilizing intrinsic electrochemical properties of nucleic acid constituents are in focus of the researchers due to their inherent simplicity and inexpensiveness. Particularly, approaches based on DNA depurination upon acid hydrolysis followed by determination of the released purines by means of stripping voltammetry[13−17] belong to techniques exhibiting a considerable sensitivity (allowing easy determination of the nucleobases at nanomolar levels). Stripping detection of purines in the DNA hydrolysates involves formation of sparingly soluble complexes with mercury or copper ions that are efficiently accumulated at surfaces of mercury,[15,18] amalgam,[16,17,19] as well as carbon[14,20] electrodes. Hg or Cu(I) ions have been generated via anodic oxidation of the electrode material (hanging mercury drop electrode (HMDE) or copper amalgam electrode) or via reduction of Cu(II) from solution. The stripping voltammetric detection involves either cathodic reduction of the metal component of the accumulated complex (at mercury or amalgam electrodes) or its anodic oxidation (at carbon electrodes). Moreover, it has been shown[14,20,21] that accumulation of the purine−copper complexes at the carbon electrodes results in considerable enhancement of the guanine and adenine electrooxidation signals. The stripping voltammetric determination of purines in acid-hydrolyzed DNA has been applied[16,18,21] in the

* To whom correspondence should be addressed. E-mail: fojta@ibp.cz (M.F.), hasons@ibp.cz (S.H.).

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"double-surface" DNA hybridization assays involving magnetic microparticles.[2]

Miniaturization of the electrochemical measuring systems together with application of advanced electrode materials belongs to the key steps in development of devices applicable in practical analyses such as genetic assays, DNA diagnostics, DNA damage monitoring, etc.[1,3,22] In our previous work[14] we proposed a concept of an inverted drop electrochemical microcell allowing reduction of the sample volume to ~50 μL while maintaining critical parameters of the analysis. Three-electrode setup allowed precise control of the working electrode potential, and efficient stirring of the analyte solution was attained via streaming of an inert gas.

Boron-doped diamond (BDD) is one of most promising advanced electrode materials having recently found application in electroanalysis of diverse analytes.[23−27] BDD possesses unique characteristics, including chemical inertness, high electrical conductivity, extraordinarily low catalytic activity for both hydrogen and oxygen evolution, and very low background current within a wide working potential range.[24,25] Several groups have demonstrated the possibility of unmodified nucleic acids detection with the BDD electrode (BDDE) at micromolar levels via oxidation signals of guanine and adenine residues.[28−30] It was also shown[29] that a hydrogen-terminated diamond surface (as-deposited diamond film) exhibited better sensitivity of DNA determination than oxygen-terminated diamond (anodically oxidized surface) due to a lower electrostatic repulsion of negatively charged DNA molecules from the hydrogen-terminated BDDE surface (possessing a certain positive polar charge) in contrast to the oxygen-terminated surface (with a considerable negative polar charge). For free purine and pyrimidine bases no significant influence of the BDD surface modification was observed, in agreement with the neutral or slightly positively charged character of these molecules under the conditions used.[23] The higher overpotential of oxygen evolution on the anodically oxidized BDD makes it possible to observe well-developed anodic peaks of purine and even pyrimidine bases[23,31] that are better separated from background discharge, compared to signals of nucleic acids constituents measured at other types of carbon-based electrodes.[32,33]

In this paper we utilize anodic stripping of the electrochemically accumulated complexes of Cu(I) with purine bases at the anodically oxidized BDDE (AO-BDDE) in electrochemical sensing of synthetic ODNs or polymerase chain reaction (PCR)-amplified DNA fragments. We show that the purines can easily be determined at picomolar levels in acid-hydrolyzed DNA (ODN) samples. We show that the proposed technique is suitable for a reliable determination of purine (particularly guanine) content in a DNA sample and, in connection with magnetic beads technology, for sensing DNA hybridization or monitoring PCR amplification of specific DNA fragments.

EXPERIMENTAL SECTION

Materials. Synthetic oligodeoxynucleotides (ODNs; for nucleotide sequences, see Table 1) were purchased from Thermo Electron (Ulm, Germany), except for the PCR primers which were supplied by VBC Biotech (Vienna, Austria). Plasmid DNAs pT77[34] and $pBSK_{(-)}$[35] were isolated and purified from *E. coli* cells and linearized using *Eco* RI restrictase (Takara, Japan). The ODN and DNA concentrations were determined spectrophotometrically using a Libra S22 spectrophotometer. *Pfu* DNA polymerase was obtained from Promega (U.K.), and deoxynucleotide triphosphates (dNTP: dATP, dTTP, dCTP, and dGTP) were from Sigma (U.S.A.). Magnetic beads Dynabeads oligo$(dT)_{25}$ (DBT) and Dynabeads streptavidin M-280 (DB_{Stv}) were supplied by Dynal A.S. (Norway). Other chemicals were of analytical grade.

Acid Hydrolysis of Oligodeoxynucleotides. Hydrolysis of the ODNs was performed by mixing of the ODN sample with the same volume of 1.0 M $HClO_4$ followed by incubation at 75 °C for 30 min. After that, the samples were cooled down, neutralized with an equal volume of 0.5 M NaOH, and diluted by the background electrolyte solution (see below) so that the desired final concentration was reached. The same procedure was used in the magnetic bead-based assays (see below).

DNA Hybridization at Magnetic Beads. The magnetic "double-surface" DNA hybridization procedure was performed as described.[5,10,16,18] Briefly, aliquots (usually 40 μL) of the stock DBT suspension were washed twice in a 1:1 volume of 0.3 M NaCl + 50 mM phosphate buffer, pH 7.0 (buffer H). Then 40 μL of 0.125 μM target ODN solution in the buffer H was shaken with the DBT in a Thermomixer Comfort (Eppendorf, Germany) for 30 min at 25 °C to capture the target ODN on the beads surface. After washing (twice by 50 μL of buffer H), the beads were incubated in the same way with 40 μL of 0.85 μM *4GAA* probe in buffer H (the 6.8-fold excess of the probe was used to ensure saturation of all binding sites in the target strands). Then, the DBT was washed thrice with 100 μL of 0.3 M $NaClO_4$ and the hybridized ODNs were released into 40 μL of triple-distilled water by heating at 85 °C for 2 min. In all steps, the beads were separated from the supernatant using the magnetic concentrator and resuspended in new medium by short vortexing. Finally, the output samples were subjected to the acid hydrolysis (see above) and mixed with the background electrolyte containing copper ions (this procedure resulted in 8-fold dilution of the output sample solution).

PCR and Magnetic Separation of End-Biotinylated DNA Fragments. Fragments of the pT77 and/or $pBSK_{(-)}$ plasmids were amplified using a mixture of primers *t77-f*, *t77-r*, *bsk-f*, and *bsk-r* (with various combinations of biotinylated or nonbiotinylated

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Table 1. Nucleotide Sequences of ODNs Used in This Work

acronym	length (nucleotides)	sequence	note
A30	30	5′AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA3′	
G30	30	5′GGGGGGGGGGGGGGGGGGGGGGGGGGGGGG3′	
A20Py10	30	5′ATAACAATCAATAATACAATATCAAAAAAA3′	
G20Py10	30	5′GTGGCGGTCGGTGGTGCGGTGTCGGGGGGG3′	
G4A4Py22	30	5′TTCAGTCCTGGCTTTTCCTTTCTCCCAGAA3′	ODNs with different G and/or A contents
G8A8Py14	30	5′AAGAGTCCTGGCTTTTCCTTTGAAGGAGAA3′	
G15A15	30	5′AAGGGAAAAGGGAAGGGGAAAGAAGGGGAA3′	
Py30	30	5′TTCCCCTTCTTTCCCCTTCCCTTTTCCCTT3′	
A25Py24	49	5′ $(TTC)_8(A)_{25}$3′	
G4A25Py20	49	5′ $(TTC)_4TGCGTCTGCGTC(A)_{25}$3′	
G8A25Py16	49	5′GTCGTCTGCGTCTGCGTCTGCGTC$(A)_{25}$3′	
G16A25Py8	49	5′GTGGGCTGGGTGGGCGTGGGCGTG$(A)_{25}$3′	
G24A25	49	5′GGGGGGGGGGGGGGGGGGGGGGGG$(A)_{25}$3′	
12TTC	61	5′$(TTC)_{12}(A)_{25}$3′	target strands
12Gnt	71	5′TGGGTGTTGTCTGCGTTTTGTGTTTCTCTTGTTTTTTCTGTTTTTG$(A)_{25}$3′	
24TTC	97	5′ $(TTC)_{24}(A)_{25}$3′	
12G12TTC	97	5′GCGCGCGGCGCCGCCCCCGGCTGGCTTTTCTTTGTT$(TTC)_{12}(A)_{25}$3′	
4GAA	12	5′ $(GAA)_4$3′	hybridization probe
t77-f	20	5′GAGGTTGTGAGGCGCTGCCC3′	PCR primers[a]
t77-r	20	5′TCCTCTGTGCGCCGGTCTCT3′	
bsk-f	20	5′CTGGCGAAAGGGGGATGTGCTGCA3′	
bsk-r	20	5′CTGGCACGACAGGTTTCCCGACTGG5′	

[a]The *t77-f* and *bsh-f* primers were used either unmodified or biotinylated at their 5′-end.

t77-f and *bsk-f*), *Pfu* DNA polymerase, and a mix of dNTPs; primer concentrations were 1 μM of each, and dNTP concentrations were 150 μM of each. The PCR involved 30 cycles (denaturation 94 °C/90 s, annealing 60 °C/120 s, polymerization 72 °C/180 s). After the PCR, 10 μL of the reaction mixture was diluted by 40 μL of deionized water and the sample was added to DB_{Stv} (20 μL of the stock suspension, washed by buffer H) and incubated for 30 min at 20 °C under mild shaking. During this procedure, DNA fragments synthesized with biotinylated primers were captured at the DB_{Stv}. Then the beads were subsequently washed thrice by 100 μL of PBS (0.14 M NaCl, 3 mM KCl, 4 mM Na_2PO_4 pH 7.4) containing 0.05% of Tween 20 (Sigma, U.S.A.) and thrice by 100 μL of 0.3 M $NaClO_4$. Release of the nonbiotinylated DNA strand from the beads and the acid hydrolysis were performed as above.

Voltammetric Measurements. Voltammetric measurements were performed using an Autolab PGStat12 potentiostat (Eco-Chemie, The Netherlands) connected to a three-electrode system involving a 3 mm diameter AO-BDDE sealed in an inert plastic body (Windsor Scientific, U.K.), a Ag|AgCl|3 M KCl reference electrode, and a platinum wire (1 mm diameter) auxiliary electrode. All measurements were carried out at room temperature either in the classical electrochemical cell (usually in 1 mL volume) or in a homemade inverted drop microcell (usually 50 μL volume) derived from the previously reported concept[14] (Figure 1). The surface of the BDDE, which is commercially available in a polished form, was electrochemically activated by cycling the potential in vigorously stirred aqueous 1 M HNO_3 between 0 and +5 V until a stable signal was detected. In this way, the AO-BDDE was prepared according to Marken et al.[26] After this initial activation, the AO-BDDE surface was mechanically polished on cloth with 1.0 μm abrasion particles before each next measurement.

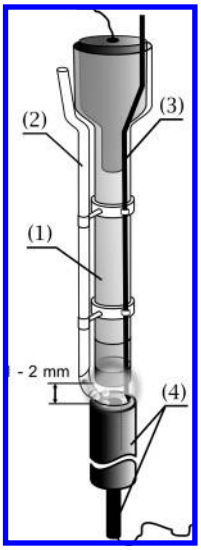

Figure 1. Scheme of the inverted drop microcell: (1) a glass tube (2 mm diameter) filled by background electrolyte and terminated by Vycor resin serving as a bridge of the Ag|AgCl|3 M KCl reference electrode; (2) plastic tube (diameter 0.3 mm) feeding the inert gas into the analyzed ODN droplet; (3) platinum wire (diameter 1 mm) serving as a counter electrode; (4) plastic body of the boron-doped diamond working electrode (diameter 3.0 mm). A 50 μL aliquot of the analyte solution in background electrolyte is placed on the anodically oxidized boron-doped diamond electrode (AO-BDDE) surface. The reference electrode, auxiliary electrode, and the argon inlet form the upper removable part of the microcell, which touches the analyte drop.

The oxidation peaks of the guanine (G^{ox}) and adenine (A^{ox}) were measured by means of anodic stripping differential pulse voltammetry (DPV) in 0.2 M acetate buffer (pH 5) containing 0.5 mM Cu(II), with the following settings: pulse amplitude of 25 mV; pulse width 50 ms; scan rate 15 mV s^{-1}; potential of accumulation, E_A = 0.008 V in the usual voltammetric cell or

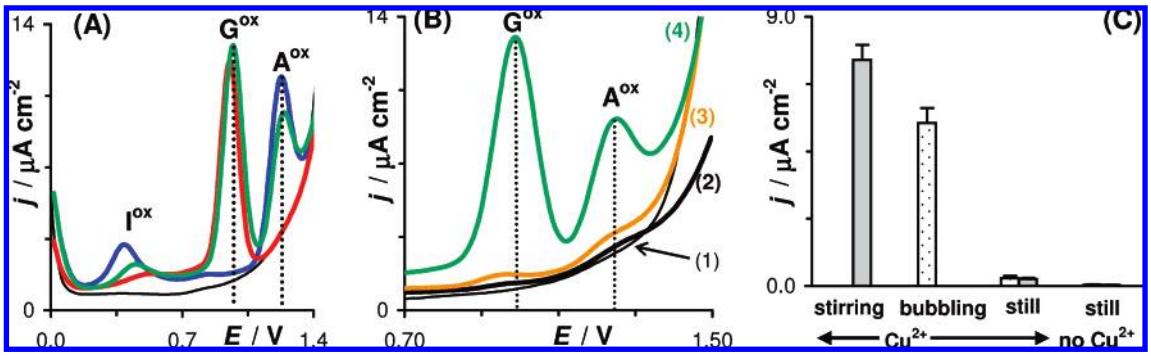

Figure 2. (A) Differential pulse voltammograms of 20 nM acid-hydrolyzed ODNs: (blue) *A30*, (red) *G30*, and (green) a mixture of both in 0.2 M acetate buffer (pH 5.1) containing 0.5 mM Cu(II) at the anodically oxidized boron-doped diamond electrode (AO-BDDE). The thin black line represents the blank response (0.5 mM Cu(II) alone). (B and C) Enhancement effects of copper and/or solution stirring during accumulation on the purine oxidation signals. (B) DPV responses measured for (curve 1) 0.5 mM Cu(II), no ahODN, no stirring; (curve 2) a mixture of *A30* and *G30* (20 nM of each) in the absence of Cu(II), no stirring; (curve 3) the *A30* + *G30* mixture in the presence of 0.5 mM Cu(II), no stirring; (curve 4) same as curve 3 but stirred during accumulation. (C) Current densities of the peak G^{ox} produced by the *A30* + *G30* ahODN mixture (20 nM of each) measured in the presence or absence of copper in the usual voltammetric cell (gray columns) or in the inverted 50 μL droplet microcell (dotted columns). Accumulation conditions (stirred or still solution) are given in the caption below the panel. Measurements in (A) and (B) were performed in a usual voltammetric cell (1 mL volume); accumulation conditions: $E_A = +0.008$ V (1 mL cell) and/or +0.068 V (inverted 50 μL drop microcell; $t_A = 8$ min; rate of stirring $\omega = 3000$ min^{-1}; solution in the inverted drop microcell was bubbled by argon at a constant pressure of 0.04 bar.

+0.068 V in the inverted drop microcell (see the Supporting Information, Figure S1); accumulation time $t_{AC} = 8$ min; rate of stirring of the bulk solution was $\omega = 3000$ min^{-1}; solution in the inverted drop microcell was bubbled by argon at a constant pressure of 0.04 bar. All voltammograms were smoothed using a Savicky−Golay algorithm and eventually baseline-corrected by the moving average method (peak width of 1 mV), using the GPES software (EcoChemie, The Netherlands).

RESULTS AND DISCUSSION

Sensing of Acid-Hydrolyzed Oligodeoxynucleotides at the BDD in the Presence of Copper Ions. Synthetic ODNs *A30* or *G30* were subjected to the hydrolysis procedure as described previously,[14−16] and anodic stripping DPV responses of the ODN hydrolysates (ahODN) were measured in 0.2 M acetate buffer containing 0.5 mM Cu(II). Voltammograms shown in Figure 2, parts A and B, were recorded in a usual voltammetric cell containing 1 mL of the analyte solution. The potential-controlled accumulation of Cu(I)−purine complexes was performed at the potential of oxidation peak of copper in the same medium[14] (for 0.5 mM Cu(II) at $E_A = +0.008$ V; see Figure S1 in the Supporting Information) for accumulation time of $t_A = 8$ min while stirring the solution (3000 rpm). In absence of the ahODN, no voltammetric peak was detected (black curve in Figure 2A). When homoadenine *A30* ahODN sample was added (concentration in solution, 20 nM), two well-developed peaks appeared at the potentials of $E_I = +0.41$ V (peak I^{ox}) and $E_{Ade} = +1.27$ V (peak A^{ox}) (Figure 2A, blue curve). The peak I^{ox} resulted from anodic oxidation of copper in the accumulated Cu(I)−adenine complex.[14,20,36] The more positive peak A^{ox} corresponded to oxidation of adenine residues.[37] When homoguanine *G30* ahODN sample was added to the Cu(II)-containing background electrolyte instead of the *A30* ahODN (Figure 2A, red curve), the peak I^{ox} due to the Cu(I) oxidation was much less well developed and shifted by about 120 mV to more positive potentials, compared to the analogous peak obtained for the *A30*. In addition to this signal, a well-developed, symmetrical peak G^{ox} appeared at +0.97 V, i.e., by about 300 mV less positive than the peak A^{ox} of *A30*. The peak G^{ox} was attributed to electrochemical oxidation of the accumulated guanine bases.[37] Under the given conditions (i.e., 20 nM *A30* or *G30*), the peak G^{ox} was about 1.7-fold higher than peak A^{ox}. Adenine or guanine bases (purchased as chemical individuals, i.e., not released from the ODNs) added to the copper-containing background electrolyte exhibited qualitatively the same behavior as the *A30* or *G30* ahODNs, respectively (see the Supporting Information, Figure S2), thus verifying the above ascription of the voltammetric signals.

We further prepared a mixture of the *A30* and *G30* ODNs (20 nM each) and performed the same hydrolysis and measuring procedures. The resulting voltammogram (Figure 2A, green curve) showed the peak I^{ox} (the potential and intensity of which were intermediate compared to the same signal obtained for *A30* or *G30* alone) and both base-specific signals (peaks G^{ox} and A^{ox}, Figure 2A). Interestingly, although the height of peak G^{ox} was only slightly influenced by the presence of the *A30* ODN, the height of peak A^{ox} obtained for the ahODN mixture was significantly lower than that obtained for *A30* alone (thus increasing the signal intensity ratio G^{ox}/A^{ox} to 2.8). Measurements with *A30* and *G30* mixed in different ratios revealed the peak A^{ox} to be remarkably affected by increasing concentrations of *G30*, but there were only negligible effects of *A30* on the peak G^{ox} (for more details, see the Supporting Information, Figure S3). Again, analogous results were obtained for mixture of pure nucleobases (see the Supporting Information, Figure S2).

Signal Enhancement Effects and Measurements with the Inverted Drop Microcell. Results shown in Figure 2, parts B and C, demonstrate strong effects of accelerated transport of the analyte (purine nucleobases in the ahODN) to the electrode surface,

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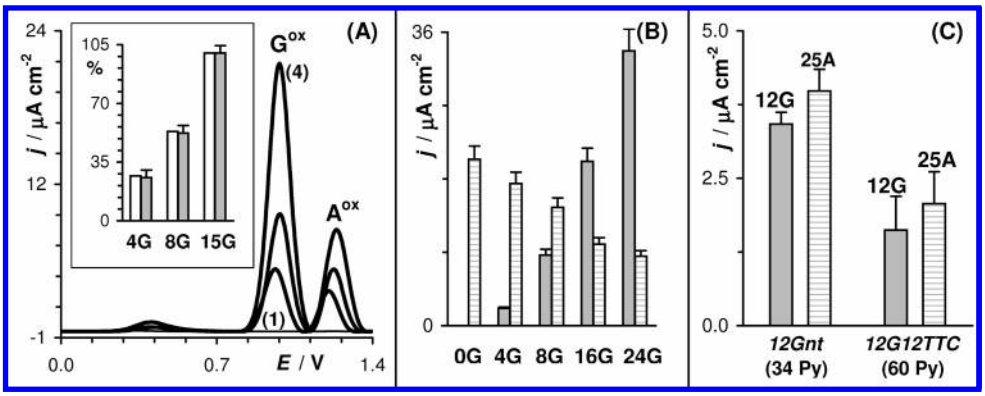

Figure 3. (A and B) Effects of the proportion of guanine and/or adenine residues in synthetic ODNs on the purine oxidation signals. (A) Sections of baseline-corrected DPVs measured for 100 nM 30-mer ahODNs: (1) *Py30*, (2) *G4A4Py22*, (3) *G8A8Py14*, and (4) *G15A15*. Inset: correlation between the proportion of guanine units within the 30-mer ODNs (white columns) and the intensity of the oxidation peak G^{ox} (gray columns; the current densities are means ± standard deviations of 10 experiments). The number of guanine units within *G15A15* and peak G^{ox} intensity obtained for the same ahODN was taken as 100%. (B) Current densities of peak G^{ox} (gray columns) or peak A^{ox} (striped columns) for a set of five different 100 nM 49-mer ahODNs (*A25Py24*, *G4A25Py20*, *G8A25Py16*, *G16A25Py8*, and *G24A25*). Each of them contains a fixed $(A)_{25}$ tail and varying number of guanine units (given below the panel). (C) Effect of excess apurinic fraction of the ahODN on the intensities of peak G^{ox} (gray columns) or peak A^{ox} (striped columns). The two ODNs, *12Gnt* and *12G12TTC*, contained a fixed number of guanine and adenine residues (12 or 25, respectively) and varying number of pyrimidines (34 or 60, respectively). The values are means ± standard deviations of five experiments. Other conditions were the same as in Figure 2.

together with formation of the Cu(I)−purine complexes, on the intensities of the guanine and adenine oxidation peaks. A mixture of *G30* and *A30* ahODN (20 nM each), unstirred during an 8 min accumulation step at +8 mV, yielded in the absence of copper only negligible purine oxidation peaks (Figure 2B, thick black curve 2), regardless of whether the measurements were performed in a classical voltammetric cell containing 1 mL of the analyte solution or in the inverted drop microcell containing 50 µL of the solution. The addition of copper to the still ahODN mixture (i.e., the Cu(I)−purine complexes were generated and accumulated without rotation of the analyzed solution) led to about 6-fold or 3-fold increase of the heights of the peak G^{ox} or A^{ox}, respectively (Figure 2B, yellow curve 3). Stirring of the ahODN mixture in the presence of copper during the accumulation step (in the usual voltammetric cell) resulted in about 30-fold enhancement of the peak G^{ox} height, when compared to the unstirred copper-containing ahODN mixture. The combined effect of stirring and formation of the sparingly soluble copper−guanine complexes thus resulted in about 200-fold signal enhancement (Figure 2C). Motion (rotation) of the solution in the three-electrode inverted drop microcell during the accumulation step was attained by inert gas streaming (bubbling) through the sample drop.[14] In this way, about 115-fold or 20-fold enhancement of the peak G^{ox} intensity was reached, when compared to the "still" conditions of accumulation in the absence or presence of copper, respectively (spotted columns in Figure 2C; voltammograms are shown in the Supporting Information, Figure S4). The height of the peak G^{ox} obtained for measurements in the microcell was about 28% lower compared to the same signal intensity obtained in the usual electrolysis cell after accumulation from a stirred solution (see above). On the other hand, application of the microcell allowed working with at least 20-times-reduced sample volumes and hence with substantially smaller analyte amounts.

After accumulation from stirred (streamed) solution containing copper, the *G30* could easily be detected down to 0.5 nM in 1:1 mixture with the *A30* on the AO-BDDE (for more details, see the Supporting Information, Figure S5). For a 50 µL sample analyzed in the inverted drop microcell, 0.5 nM corresponds to the amount of 25 fmol of the *G30* ODN. Measurements of the peak G^{ox} close to the detection limit exhibited a reasonable reproducibility (relative standard deviations for 0.5, 2.0, or 20.0 nM *G30* were ~11%, ~5%, or 2.7%, respectively). Such low detection limits could be attained thanks to low noise levels and a "clean" background response (not exhibiting any significant current signals in the absence of the analyte) at the AO-BDDE. With other carbon-based (such as glassy carbon, pyrolytic graphite, or carbon paste) electrodes, the lowest detectable concentration was at least 1 order of magnitude higher due to considerable background current perturbations that were affecting ahODN responses (for more details, see the Supporting Information, Figure S6). Determination of ahODN using either stirred bulk or argon-bubbled 50 µL sample volumes was about 2 orders of magnitude more sensitive in the presence of copper than that in its absence.

Effects of ODN Nucleobase Composition on the Enhanced Purine Oxidation Signals. The above results suggest that (1) measurements of the enhanced guanine- or adenine-specific signals at the AO-BDDE may be useful for determination (estimation) of the purine base content in acid-hydrolyzed DNA and (2) particularly changes in the guanine content may be easily and reliably detectable due to a strong enhancement of the peak G^{ox} in stirred (argon-streamed) copper-containing medium and a weak interference of adenine. To prove these assumptions, we analyzed a set of acid-hydrolyzed random-sequence 30-mer ODNs differing in relative content of the purines (for nucleotide sequences see Table 1). The homopyrimidine *Py30* ahODN did not produce any voltammetric signal in agreement with absence of the purine bases (Figure 3A, curve 1). The height of the peak G^{ox} increased almost linearly with increasing number of guanine units within a set of the ahODNs *G4A4Py22*, *G8A8Py14*, and *G15A15* (Table 1). If we took the height of the G^{ox} peak of*G15A15* as 100%, then the signal intensities obtained for *G8A8Py14* or *G4A4Py22* were 52% ± 3% or 26% ± 3%, respectively. These values were in a good agreement

with the percentage variations of the guanines units within the ODN set (inset in Figure 3A). In comparison to the peak G^{ox}, the peak A^{ox} did not display such a good correlation between the percentage of adenine residues within the 30-mers and the signal intensity. The peak A^{ox} height also increased with increasing number of the adenine residues, but the relative increments were significantly lower than those corresponding to the adenine portion increments (Figure 3A). Such behavior was in accordance with the above-mentioned interference of increasing amounts of guanine.

We further tested the effect of varying number of guanine units (from 0 to 24) within a set of five 49-mers containing a constant number of adenine residues (25) on the guanine and adenine signal intensities. Similarly as observed for the *A30* + *G30* mixture (see above), heights of the peak A^{ox} were decreasing significantly with increasing number of guanine residues per ODN. For *G24A25* the height of the peak A^{ox} was about 58% lower compared to the guanine-lacking *A25Py24* (Figure 3B). The intensity of the peak G^{ox} increased with the number of guanine residues per ODN molecule, varying proportionally to the variations in number of guanines per a 49-mer for *G24A25*, *G16A25Py8*, and *G8A25Py16* (Table 1). For *G4A25Py20* the intensity of peak G^{ox} was lower than expected on the basis of guanine proportion (representing 24% of the peak height observed for the *G8A25Py16*, Figure 3B), but the signal was still well developed and reproducibly measurable.

Real DNA samples (as well as the ODNs used above) contain not only purine but also pyrimidine nucleotides. The apurinic fraction, albeit not yielding any anodic stripping signal in the presence of copper (curve 1 in Figure 3A), can influence intensities of the peak G^{ox} and A^{ox} due to concurrent adsorption at the AO-BDDE and/or interactions with copper ions in the sample solution or at the electrode surface. To test interferences of the apurinic component, we measured responses of two ahODN samples, *12Gnt* and *12G12TTC* (Table 1), containing a fixed number of purine (12 guanine + 25 adenine) and different number of pyrimidine (34 or 60 Py in sum, respectively) units. Figure 3C shows that, for the same molar concentration of the whole ODNs (and thus of the adenine and guanine residues), increasing the proportion of the apurinic fraction about 2-fold caused the intensities of both purine signals (peak G^{ox} and peak A^{ox}) to decrease by about 50% (Figure 3C). Despite the signal intensities reduction, the ratio of the purine signals remained practically unaffected. This phenomenon may have caused the supraproportional decrease of the peak G^{ox} intensity upon lowering the number of guanine residues from 8 to 4 in the 49-mer ODN series where the guanines were replaced by pyrimidine units (Figure 3B).

Analytical Applications. The above results demonstrate that the enhanced stripping voltammetric detection of purine bases in the presence of copper at the AO-BDDE, especially in connection with the inverted drop microcell application, is suitable for DNA microanalysis. Detection of guanine residues via the peak G^{ox} appears to be analytically useful due to an excellent correlation between the signal intensity and guanine content within the acid-hydrolyzed DNA (ODN) sample. Besides a simple determination of DNAs with average nucleotide composition (more or less "balanced" A + T/G + C content), the possibility of selective detection of the guanine (and/or adenine) residues suggest particular applicability of the presented electrochemical technique in analysis of DNA samples with asymmetric purine−pyrimidine distribution (involving, for example, some repetitive genomic DNA elements whose replication instability is closely related to pathogenesis of severe hereditary disorders[10,38−40]). The excess of purine (guanine and/or adenine) residues within one of the complementary strand can be exploited in label-free electrochemical sensing of DNA hybridization with such DNA elements.

DNA Hybridization with Target/Probe Pairs Asymmetrical in Their Purine Contents. For the DNA hybridization experiments we used the double-surface (DS) technique[2,8−10,12,18] involving magnetic Dynabeads oligo(dT)$_{25}$ (DBT). We designed a set of target ODNs with an A_{25} adaptor allowing their capture at the DBT (Figure 4A). The targets involved a $(CTT)_n$ (n = 0, 12, or 24) triplet repeat segment and/or a random-sequence stretch containing 12 guanine residues (see Table 1). The target ODNs were captured at the DBT, followed by hybridization with a probe *4GAA* (see the scheme in Figure 4A). The number of the probe molecules hybridized with the target strand depended on the length of the repetitive $(TTC)_n$ segment,[10,39] being in average equal to $n/4$ for the *4GGA* probe. After magnetoseparation and washing, the hybrid ODN "sandwiches" anchored at the beads were heat-denatured and the released ODNs subjected to the acid hydrolysis procedure and stripping voltammetric detection in the presence of copper at the AO-BDDE, using the inverted drop microcell.

Figure 4B summarizes results of the DNA hybridization experiments. When capture of any target strand was omitted, no signal was detected regardless of whether the second hybridization step (with the *4GAA* probe) was performed or not. This was in agreement with absence of any DNA in the output samples after the DBT magnetoseparation procedure (the *4GAA* probe alone could not be captured at the beads due to lack of sequence complementarity). When target ODNs *12TTC* and *24TTC* (containing only the triplet repeat segments coupled to the A_{25} adaptor) were captured at the DBT but no *4GAA* was hybridized in the second step, only peak A^{ox} (due to the A_{25} stretches of the target ODNs) was detected (not shown). The absence of peak G^{ox} was in accordance with the lack of guanine residues in these two target strands. After hybridization with the *4GAA* probe, well-developed signals appeared due to guanine residues originating from the probe. The peak G^{ox} obtained for the *24TTC* target was about twice as intense as the same signal obtained for the *12TTC* target, thus exhibiting a good correlation with the length of the repetitive stretch or the expected stoichiometry of the probe/target hybridization (each four TTC triplets in the target can accommodate one probe molecule).

Another couple of target ODNs possessed a random-sequence stretch involving 12 guanine residues (*12Gnt* and *12G12TTC*). When these ODNs were captured at the DBT and the *4GAA* hybridization was omitted, peak G^{ox} due to these guanines was detected (Figure 4B). The intensity of this signal was similar as

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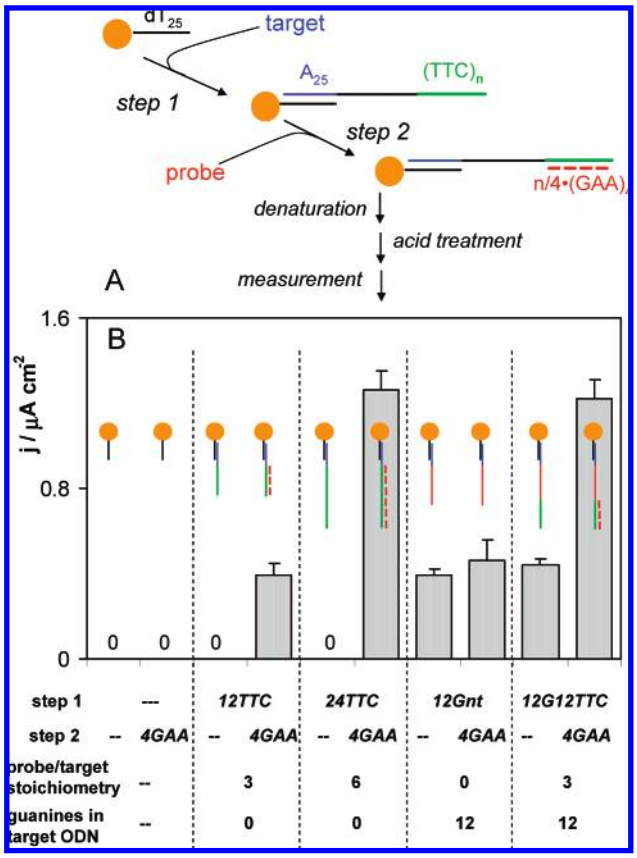

Figure 4. Hybridization of target/probe DNAs with asymmetrically distributed guanine nucleotides. (A) Scheme of a "double-surface" DNA hybridization experiment. A target DNA strand with a homopyrimidine stretch [a $(TTC)_n$ triplet repeat] is captured at the magnetic Dynabeads oligo$(dT)_{25}$ (DBT) via an A_{25} adaptor. Then, a homopurine probe [$(GAA)_4$ spanning four TTC triplets] is hybridized with the target. The number of the probe molecules hybridized, and thus the number of guanine residues collected, per target ODN molecule depends on the triplet repeat length. After separation, the hybridized ODNs are subsequently detached from the DBT by thermal denaturation and acid-hydrolyzed and analyzed using the enhanced anodic stripping technique. (B) Current densities of peak G^{ox} resulting from the DNA hybridization experiments. The concentration of target ODNs in the "input" samples was 125 nM, and the probe concentration was 850 nM; both hybridization steps were conducted at 20 °C for 30 min. Schemes of ODN "sandwiched" formed at the DBT are shown as insets for each sample (green color denotes the $(TTC)_n$ stretch, red symbolizes guanine-containing stretches; for more details see the caption below the graph and text).

detected after the *12TTC·4GAA* hybridization, in agreement with the same number of guanine residues collected per target ODN strand (12 in all three cases). Hybridization of the *4GAA* probe with the *12Gnt* ODN lacking the TTC triplet repeat did not result in any significant change in the peak G^{ox} intensity (compared to the above experiment in which the DBT-captured *12Gnt* was in the second step incubated in hybridization medium not containing the *4GAA* probe), in agreement with the inability of the *4GAA* probe to hybridize with *12Gnt*. The same experiment performed with the *12G12TTC* target (involving 12 triplet repeat units in addition to the random-sequence stretch with the 12 guanines) showed a considerable increase of the peak G^{ox} intensity after hybridization with the *4GAA* probe. Notably, the height of the peak G^{ox} resulting from the latter experiment (corresponding to a sum of 24 guanines per hybrid duplex) was very similar to the peak height observed for the *24TTC·4GAA* hybrid (also 24 guanines per target).

Data shown in Figure 4 were obtained for 125 nM target ODNs (first step) and 850 nM *4GAA* probe (second step) in 40 μL samples incubated with the magnetic beads. Analogous hybridization experiments were performed using different concentrations of the target ODNs (keeping the probe/target ratio constant). For the *12G12TTC* target, well-developed signals reliably responding to hybridization with the *4GAA* probe were detected down to ~60 nM (i.e., ~2.5 pmol in 40 μL of sample incubated with the beads). Upon increasing the tDNA concentration, the signal intensities increased accordingly. On the other hand, the correlation between number of guanines collected per hybridization event and the peak G^{ox} height was changed for the worse when the tDNA concentration was increased to 8 μM (probably due to the above-mentioned interferences of the ODN apurinic component; data not shown).

Electrochemical Monitoring of Polymerase Chain Reactions. The enhanced purine oxidation signals can be utilized for sequence-specific DNA sensing even for DNA samples not exhibiting any asymmetry in purine−pyrimidine distribution between their complementary strands. In such instances, the desirable sequence specificity can be attained by, for example, PCR amplification of a specific DNA fragment using specifically designed primers. Figure 5A shows a procedure we used for monitoring simultaneous amplification of two DNA fragments using two primer pairs. Specificity of detection of a particular amplicon was attained by using 5′-end-biotinylated primers. After the PCR, the DNA fragment amplified using the biotinylated primer was captured at magnetic beads coated with streptavidin (DB_{Stv}). After separation from the reaction mixture, the DNA duplexes anchored at the DB_{Stv} were heat-denatured and the released (nonbiotinylated) DNA strand was subjected to the acid hydrolysis, followed by the copper-enhanced anodic stripping measurements in the inverted drop microcell.

Figure 5B shows results of the PCR experiments. All reaction mixtures contained two pairs of primers, *t77-f* + *t77-r* (to amplify a fragment of human tumor suppressor gene *p53* cDNA cloned into pT77 plasmid[34]) and *bsk-f* + *bsk-r* (to amplify a fragment of the $pBSK_{(-)}$ plasmid vector[35]). The *t77-f* or *bsk-f* primers were used as either 5′-biotinylated or unlabeled. Agarose gel electrophoresis showed that in the presence of the pT77 (but not pBSK) template, only one product was formed (inset in Figure 5B, panel i, lanes a and b). When the biotinylated *t77-f* primer was used, an intense peak G^{ox} was detected after the magnetoseparation and hydrolysis procedure (Figure 5B, sample a). On the contrary, when *bsk-f* was biotinylated and *t77-f* nonbiotinylated, and the sole pT77 template was used again, no electrochemical signal was detected (sample b). Control agarose electrophoresis performed with PCR mixtures after incubation with the DB_{Stv} (panel ii in Figure 5B, inset) showed disappearance of the biotinylated (lane a) but not the nonbiotinylated (lane b) pT77 amplicon. The electrophoresis data were in accord with the electrochemical ones: the DNA fragment withdrawn from the PCR mixture by means of the DB_{Stv} (band disappeared) was detected, after the separation procedure, by the stripping voltammetric technique (peak G^{ox} appeared). In further experiments, both pT77 and pBSK templates were mixed and various combinations of biotinylated and nonbiotinylated primers

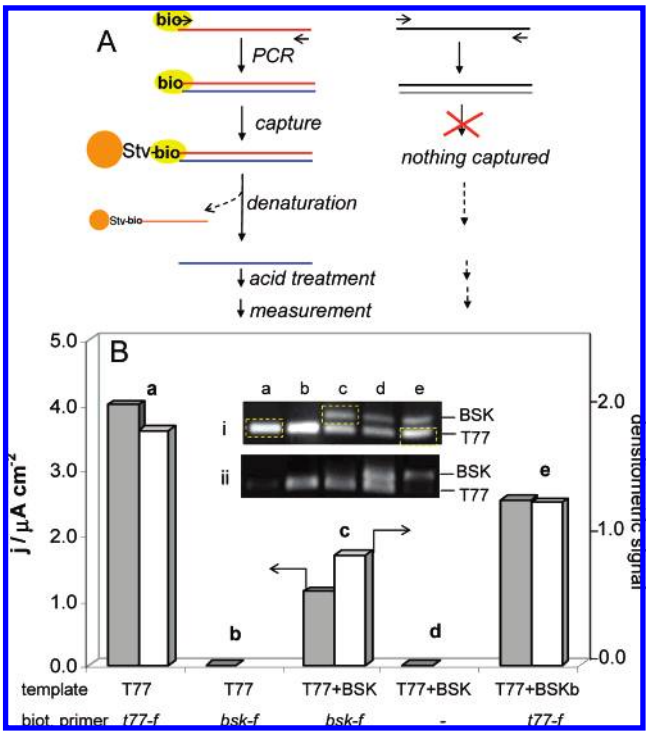

Figure 5. Monitoring of specific DNA fragment amplification by polymerase chain reaction. (A) Scheme of the experiment. Left: using a primer biotinylated at its 5′-end, biotin-tagged DNA fragments are PCR-amplified. Such fragments are captured at streptavidin (Stv)-coated magnetic beads. After separation from the reaction mixture and washing, the nonbiotinylated strand of the amplicon is released via thermal denaturation and subjected to acid hydrolysis, followed by voltammetric detection of released purines. Right: when both PCR primers are nonbiotinylated, the resulting amplicon is not captured at the beads and thus not extracted from the PCR mixture. (B) Heights of peak G^{ox} (gray columns) and ethidium-stained band intensities (white columns) resulting from analysis of amplicons obtained for different combinations of templates, biotinylated, and nonbiotinylated primers (in all mixtures, all *t77-f*, *t77-r*, *bsk-f*, and *bsk-r* primers were present; the caption below the panel shows which primer was biotinylated and what template(s) were present). Inset: agarose gel electrophoresis of the PCR products (i) prior to or (ii) after incubation of the samples with DB_{Stv}. Marking of lanes in the gels corresponds to marking of samples in the column graph; bands in dashed yellow boxes are due to the biotin-labeled DNA fragments. The biotin-tagged amplicons were captured at the DB_{Stv} from 50 μL of 5-times-diluted PCR mixtures for 30 min at 20 °C; other conditions were as in Figure 4.

were added. Under these conditions, two products were detected in the gel (Figure 5B; the lengths of the T77 and BSK amplicons were 348 or 448 bp, respectively). Electrochemical responses obtained after the DB_{Stv} magnetoseparation procedure revealed amplification of both pT77 and pBSK fragments: the peak G^{ox} was observed when the biotinylated primer was *t77-f* (sample c in Figure 5B), as well as when the *bsk-f* was biotinylated (sample e). When none of the primers was biotin-labeled, no electrochemical response was detected (sample d). Again, the control electrophoresis of the PCR mixtures after incubation with the DB_{Stv} revealed extraction of the biotinylated (but not nonbiotinylated) fragments (Figure 5B, inset, panel ii). Relative intensities of the peak G^{ox} obtained for different PCR reactions correlated with relative intensities of the ethidium-stained bands corresponding to the biotinylated amplicons (Figure 5B). Hence, the presented electrochemical technique can be used as a powerful alternative method not only for qualitative detection but also for reliable semiquantitative determination of individual amplicons in multiplex PCR. Under the given conditions, both techniques exhibited comparable sensitivities (for more details, see the Supporting Information, Figure S7). Advantages of the magnetoseparation step[2] preceding the electrochemical detection can be exploited not only in manually conducted experiments (as in this work) but also in automated formats involving microfluidics, magnetically operated microreactors, etc.[41−46]

CONCLUSION

Boron-doped diamond electrode, in connection with an inverted drop three-electrode microcell, was successfully applied for the anodic stripping determination of purine bases in acid-hydrolyzed DNA involving electrochemically controlled accumulation of purine−Cu(I) complexes. The enhanced anodic stripping voltammetric peaks of the purines reflected changes in their relative content in the DNA (ODNs). Particularly, peak G^{ox} due to the guanine oxidation exhibited a good correlation with percentage of guanine in synthetic oligonucleotides and a lack of interferences of excess adenine, allowing detection of several tens-of-femtomole amounts of the ODNs.

We applied measurements at the AO-BDDE in the inverted drop microcell, in connection with a magnetic bead-based microseparation technology, in sandwich DNA hybridization or duplex PCR assays. Hybridization between target $(CTT)_n$ repeats and a $(GAA)_n$ probe at the magnetic DBT was detected via measurements of the enhanced peak G^{ox}. Changes in the homopyrimidine triplet repeat length were reflected in changes of the peak G^{ox} intensity in agreement with accommodation of more guanine-containing probe molecules by the longer repeat. In addition, the hybridization events were easily detectable even when the $(CTT)_n$ stretch was present in an ODN containing additional guanine residues in another part of the molecule. Specific detection of biotin-tagged DNA fragments amplified in a duplex PCR (involving simultaneous amplification of two nucleotide sequences) was achieved via their capture at streptavidin-coated magnetic beads followed by measurements of the enhanced peak G^{ox} in the DNA hydrolysate. Our results suggest applicability of the label-free electrochemical detection, particularly in connection with the magnetoseparation techniques (that can be automated, multiplexed, and/or integrated in microfluidic and/or lab-on-a-chip devices[41−47]) in practical DNA assays.

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SUPPORTING INFORMATION AVAILABLE

The way of accumulation potentials choice (E_A), control and supplementary experiments focused on evaluation of the analytical performance of the technique, comparative measurements of purines in the presence of copper with different carbon electrodes, and comparison of detection of a DNA fragment using ethidium-stained agarose gel or using the presented electrochemical technique. This material is available free of charge via the Internet at http://pubs.acs.org.

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