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Carborane- or Metallacarborane-Linked Nucleotides for Redox Labeling. Orthogonal Multipotential Coding of all Four DNA Bases for Electrochemical Analysis and Sequencing

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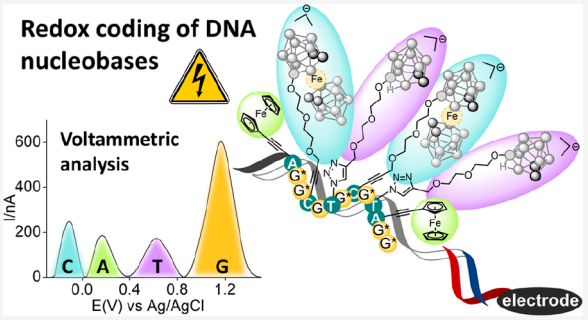
ABSTRACT: We report a series of 2′-deoxyribonucleoside triphosphates bearing dicarba-*nido*-undecaborate ($[C_2B_9H_{11}]^{1-}$), [3,3′-iron-bis(1,2-dicarbollide)]$^-$ (FESAN, $[Fe(C_2B_9H_{11})_2]^{2-}$) or [3,3′-cobalt-bis(1,2-dicarbollide)]$^-$ (COSAN, $[Co(C_2B_9H_{11})_2]^{2-}$) groups prepared either through the Sonogashira cross-coupling or the CuAAC click reaction. The modified **dN^{X}TPs** were substrates for KOD XL DNA polymerase in enzymatic synthesis of modified DNA through primer extension (PEX). The *nido*-carborane- and FESAN-modified nucleotides gave analytically useful oxidation signals in square-wave voltammetry and were used for redox labeling of DNA. The redox-modified DNA probes were prepared by PEX using tailed primers and were hybridized to electrode (gold or glassy carbon) containing capture oligonucleotides. The combination of *nido*-carborane- and FESAN-linked nucleotides with 7-ferrocenylethynyl-7-deaza-dATP and 7-deaza-dGTP allowed polymerase synthesis of DNA fully modified at all four nucleobases, and each of the redox labels gave four differentiable and ratiometric signals in voltammetry. Thus, the combination of these four redox labels constitutes the first fully orthogonal redox coding of all four canonical nucleobases, which can be used for determination of nucleobase composition of short DNA stretches in one simple PEX experiment with electrochemical readout.



INTRODUCTION

Electrochemical analysis of nucleic acids[1] is an attractive and cost-effective alternative to fluorescence-based techniques, which are routinely used for sequencing of DNA[2] or detection of particular DNA or RNA sequences.[3] For most electrochemical bioanalytical or diagnostic applications, redox labeling of DNA bases[4] is achieved through an attachment of some oxidizable or reducible molecules or functional groups with characteristic redox potentials,[5] which need to be within a potential window compatible with the type of electrode used. The redox label can be attached at position 5 of pyrimidine or at position 7 of 7-deazapurine 2′-deoxyribonucleoside triphosphate (dNTP) and such modified dNTPs are typically very good substrates for DNA polymerases in primer extension (PEX) or polymerase chair reaction (PCR) synthesis of redox-labeled DNA for subsequent electrochemical detection. Recently, we have developed the enzymatic synthesis of redox-labeled DNA probes, which are flanked by tails complementary to capture oligonucleotides covalently attached to gold electrodes and have exploited these in the construction of electrochemical genosensors.[6] A combination of several orthogonal redox labels with differing redox potentials might in principle be applicable in redox coding of DNA bases[7] for applications in sequencing or in the PEX-based electrochemical determination of the nucleobase composition of an unknown sequence. A number of redox-active groups have already been reported for the labeling of DNA. Anthraquinone,[7,8] nitrophenyl,[9] benzofurazane,[10] or azidophenyl[11] were studied as reducible groups, whereas aminophenyl,[9] methylene blue,[7,12] Nile blue,[12,13] and phenothiazines,[7] as well as ferrocene[14−16] or substituted ferrocene labels[17] can be oxidized on various types of electrodes. Previously, we attempted to combine some of them, but only a combination of two reducible groups, i.e. nitrophenyl with either benzofurazane or azidophenyl,[10] or a combination of two oxidizable groups (ferrocene and ferrocenecarboxamide)[17] were truly orthogonal and ratiometric and thus useful for redox coding of two

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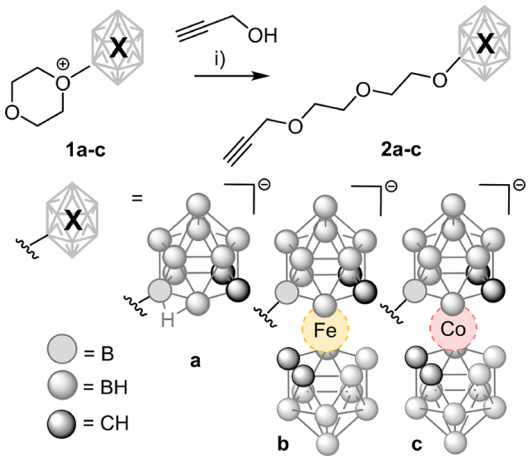
nucleobases. For a full set four redox labels for encoding of all four nucleobases, it would be practical to have either all four oxidizable or all four reducible so that all of them can be simultaneously detected on one electrode using monodirectional and highly sensitive voltammetric detection techniques including square-wave and differential pulse voltammetry. It is, however, very challenging to achieve an adequate resolution of differentiable peaks, due to the limited potential windows available on specific types of electrodes. Mercury electrodes, for example, are excellent for the detection of signals due to electrochemical reductions but poorly useful for oxidations due to the anodic dissolution of mercury at potentials ≥+0.2 V (Ag|AgCl|3 M KCl),[18] while the use of gold electrodes in the presence of chlorine ions becomes unreliable at potentials higher than +0.9 V vs Ag/AgCl, but this potential window can be extended to +1.2 to +1.3 V vs Ag/AgCl if a chloride-free electrolyte such as strontium nitrate ($Sr(NO_3)_2$) is used.[19] On the other hand, carbon electrodes usually offer the widest positive potential window (≥+1.2 V), making them the electrodes of choice for the detect oxidation reactions.[20] An additional problem is the limitation of polymerase-mediated enzymatic incorporation of high-density modified nucleotides. However, recently we have shown that DNA polymerase can be used even for synthesis of long sequences containing all four modified bases using either PEX or asymmetric PCR (aPCR).[21]

Carborane and metallacarborane clusters are highly hydrophobic sphere-shaped molecules that have been used in medicinal chemistry,[22] either as potential boron neutron-capture-therapy (BNCT) agents[23] or as components of enzyme inhibitors,[24] forming strong interactions with hydrophobic pockets of target proteins. They have also been attached to nucleosides and some of them incorporated into oligonucleotides[25] by chemical synthesis on solid supports. We have recently reported[26] the polymerase incorporation of *p*-carborane-linked dNTPs into DNA. Many carborane and metallacarborane derivatives are also redox active.[27−31] In particular, 7,8-dicarba-*nido*-undekaborate anion,[29] as well as [3,3′-cobalt bis(1,2-dicarbollide)]$^-$ (COSAN)[30] and [3,3′-iron-bis(1,2-dicarbollide)]$^-$ (FESAN)[31] clusters have been used as oxidizable redox labels in the chemical synthesis of labeled nucleic acids and their redox potentials are distinctly different, presenting them as potential candidates for orthogonal labeling. Therefore, we decided to explore these carborane and metallacarborane clusters as potential oxidizable redox labels in our quest for the development of a fully orthogonal set for the redox coding of all four DNA nucleobases.

RESULTS AND DISCUSSION

Synthesis. The attachment of the bulky (metalla)carborane labels was envisaged through a flexible linker that would allow the polymerase to incorporate the corresponding nucleotide into DNA.[32] We thus designed the diethylene glycol linked (metalla)carborane terminal alkynes **2a−c** suitable to be used in either the Sonogashira reaction[33,34] or Cu-catalyzed alkyne azide cycloaddition (CuAAC).[35] They were prepared (Scheme 1) through dioxane ring-opening from their dioxane-carborane precursors **1a−c**[36−38] by treatment with *in situ* generated sodium propargylalcoholate similar to a procedure previously published[36] for 8-(5-propargyl-3-oxa-pentoxy)-3,3′-cobalt bis-(1,2-dicarbollide) (**1c**).

Scheme 1. Synthesis of Alkyne-Linked Carboranes[a]



[a]Reagents and conditions: (i) NaH, toluene (dry), reflux, 4 h or 22 °C, 48 h.

The modified cytosine or 7-deazaadenine nucleoside triphosphates (**dC^{X}TP** and **dA^{X}TP**) were prepared through a direct one-step aqueous Sonogashira cross-coupling reaction[17,33,34] of halogenated nucleoside triphosphates (**dC^{I}TP** or **dA^{I}TP**) with alkyne-linked carboranes **2a−c** (Scheme 2). The cross-coupling reactions were performed in the presence of $Pd(OAc)_2$, TPPTS (triphenylphosphine-3,3′,3″-trisulfonate) ligand, CuI and Hünig's base in water/acetonitrile (2:1). The desired modified triphosphates were isolated as trisodium salts following HPLC purification and ion exchange, achieving moderate to good yields (26−62%). The yields were lowered due to the partial hydrolysis of triphosphates and difficulty in isolation.

In the case of modified thymidine triphosphates (**dT^{X}TP**), we selected an alternative synthetic route using CuAAC click reaction of known **dU^{MeN3}TP**[39] with the terminal alkynes **2a−c** (Scheme 2). This approach prevented possible undesired cyclization of 5-alkynyluracils to furopyrimidines which often occurs during the Sonogashira coupling of 5-iodouracil derivatives with alkynes.[15] The CuAAC reactions catalyzed by $CuSO_4$ in the presence of sodium ascorbate proceeded smoothly giving the desired modified **dT^{X}TP**s in good yields (45−66%). In all cases modified **dN^{X}TP**s were prepared in sufficient amounts for the subsequent biochemical and electrochemical experiments.

Enzymatic Incorporation Study of Modified dN^{X}TPs. Modified nucleotides were tested in primer extension experiments (PEX) using 19-mer (temp^A, temp^C, temp^T) or 31-mer (temp^{rnd16}) templates (encoding for one or four modified nucleotides) and 15-mer (prim^{rnd}) primer (for sequences of all oligonucleotides see Table S1 in the Supporting Information). KOD XL DNA polymerase was selected as this enzyme has previously been reported to efficiently incorporate multiple modifications.[17,21] Primarily, we tested incorporation of just one type of modified nucleotide (in the presence of the other 3 natural dNTPs). Figure 1 shows the PAGE analysis of the PEX products. In all cases, it confirmed formation of full-length products, where bands of modified products are typically shifted slightly upward in comparison to the natural ones, due to their bulkiness and higher molecular weight. In some cases of multiply modified **31DNA_4N^x**, we observed some smearing, suggesting possible aggregation of modified single

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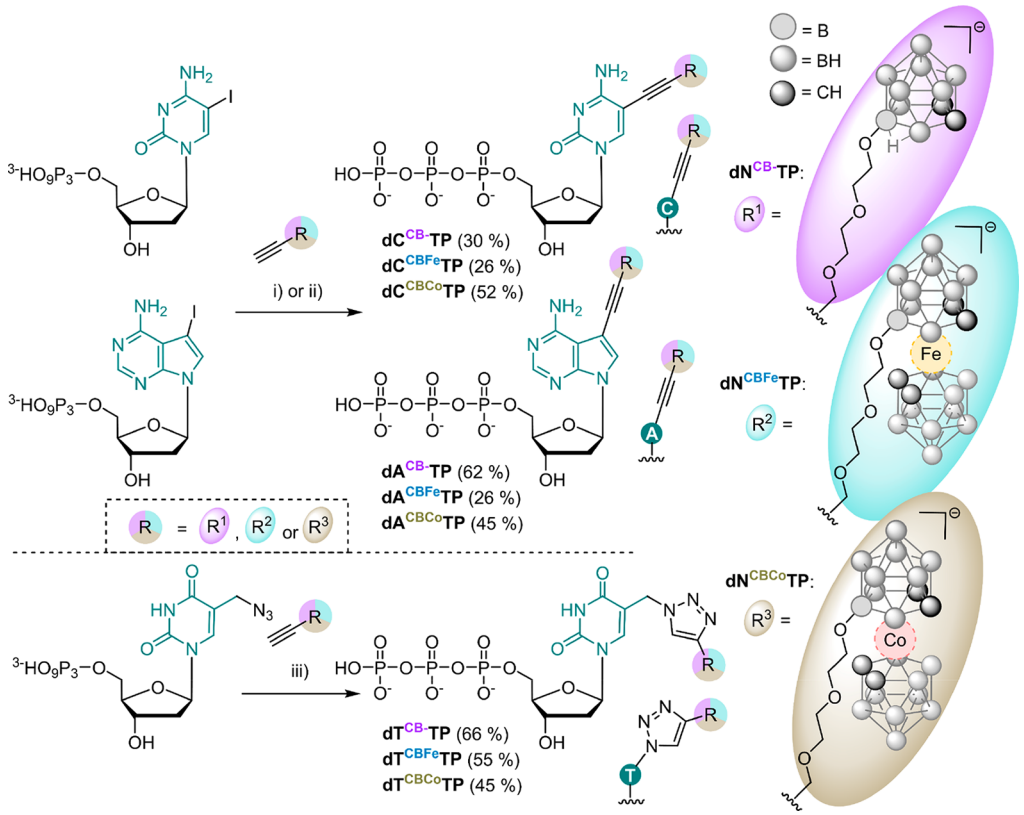
Scheme 2. Synthesis of Modified dN^{X}TPs[a]



[a]Reagents and conditions: (i) $Pd(OAc)_2$, CuI, TPPTS, DIPEA H_2O/CH_3CN (2:1), 75 °C, 30 min; (ii) $Pd(PPh_3)_2(Cl)_2$, CuI, PPh_3, Et_3N, H_2O/CH_3CN (2:1), 75 °C, 1 h; (iii) alkyne, sodium ascorbate, $CuSO_4 \cdot 5H_2O$, H_2O/t-BuOH (1:1), 22 °C, 16 h.

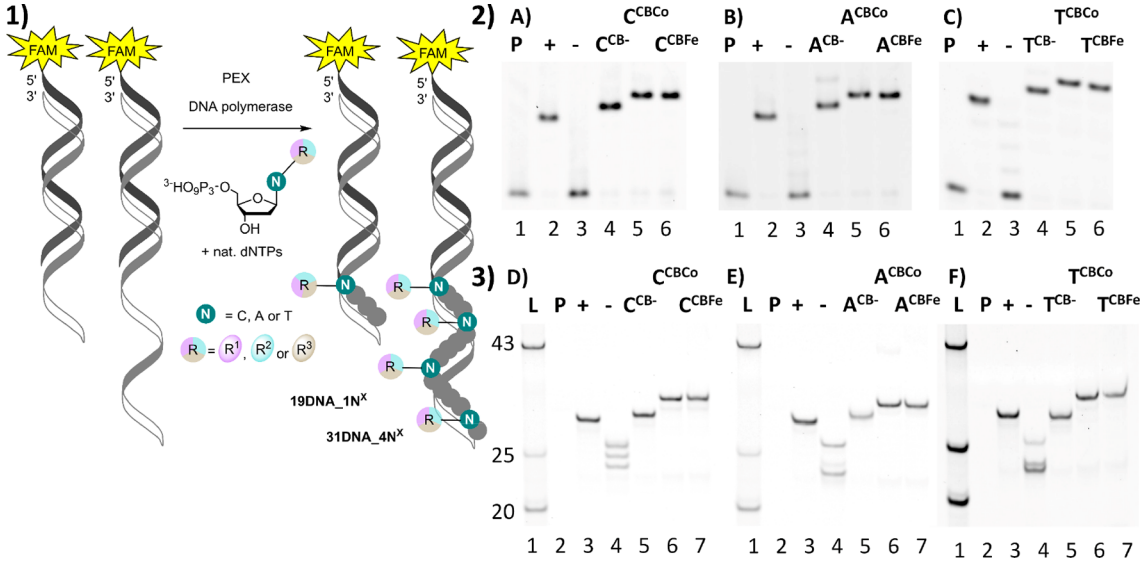


Figure 1. (1) PEX with KOD XL DNA polymerase using a single modified **dN^{X}TPs**. (2) Denaturating PAGE analysis of PEX using 5′-(6-FAM) labeled prim^{rnd} and (A) temp^C, **dC^{X}TP**; (B) temp^A, **dA^{X}TP**; (C) temp^T, **dT^{X}TP** [lanes (1) primer; (2) positive control, natural dNTPs; (3) negative control, without studied dNTP; (4) reactions containing **dN^{CB-}TP**; (5) **dN^{CBCo}TP**; (6) **dN^{CBFe}TP**]. (3) Native PAGE analysis of PEX using 5′-(6-FAM) labeled primer^{rnd}, temp^{rnd16}, and (D) **dC^{X}TP**; (E) **dA^{X}TP**; (F) **dT^{X}TP** [lanes (1) dsDNA ladder with denoted length; (2) primer; (3) positive control, natural dNTPs; (4) negative control, without studied dNTP; (5) reactions containing **dN^{CB-}TP**; (6) **dN^{CBCo}TP**; (7) **dN^{CBFe}TP**].

stranded ONs (ssONs) (data not shown). This could be resolved by analysis on native PAGE (Figures S2−S4). The identity of the products was also confirmed using MALDI-TOF analysis (Table S2 in the Supporting Information) of ONs after a larger scale PEX with a biotinylated template followed by magnetoseparation purification.[16] The successful PEX incorporations of the modified **dN^{X}TPs** shows that they are reasonably good substrates for the KOD XL polymerase,

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and hence, they have potential for enzymatic incorporation of carborane-labeled dNTPs.

We also attempted the polymerase chain reaction (PCR) with the carborane modified **dT^{X}TPs** under different conditions, but no amplified product was observed. (Figure S5) We assume that the bulky lipophilic nature of the modified nucleotides in the template hinders the ability of the polymerase to incorporate these bases, thus preventing effective elongation.

Electrochemistry of Modified Nucleotides on Pyrolytic Graphite Electrode. Electrochemical experiments studying carborane-linked **dN^{X}TPs** were performed using square-wave voltammetry (SWV) on a pyrolytic graphite electrode (PGE) in acetate buffer (pH = 5.0). In the *nido*-carborane-linked nucleotides **dN^{CB-}TPs**, we observed a signal at +0.6 V (vs Ag/AgCl) corresponding to irreversible oxidation of the boron cluster. In FESAN-derivatives **dN^{CBFe}TPs**, electrochemical peaks were detected at −0.4 V due to the reversible oxidation of iron and at +1.2 V due to irreversible boron cluster oxidation. For the COSAN nucleotides **dN^{CBCo}TPs**, the oxidation peak due to reversible oxidation of Co was observed at very low potential −1.4 V. In the 7-deazaadenine nucleotides **dA^{X}TPs**, an additional peak at ca. +1.2 V was also observed due to the oxidation of the pyrrolopyrimidine moiety. The electrochemical signal of the COSAN nucleotides (at −1.4 V) is already out of the potential range for practical analytical applications, whereas the oxidation signals of the *nido*-carborane and FESAN are within the range of gold or carbon electrodes and they significantly differ from one another, suggesting their applicability for combinations in orthogonal redox coding. We thus have further studied *nido*-carborane- and FESAN-linked nucleotides as building blocks for the redox labeling of DNA (Figure 2).

Synthesis of Redox-Labeled DNA Probes and Analysis on Different Electrodes. In order to investigate the electrochemical behavior of carborane-labeled DNA, the modified DNA containing one (**19DNA_1N^{X}**) or four (**31DNA_4N^{X}**) modifications were synthesized using a PEX reaction followed by purification using the QIAquick Nucleotide Removal Kit. These modified ONs were then studied using SWV on the PGE (analogously to our previous works[9,16]). Unfortunately, the observed signals were very weak (Figure S9), which can probably be attributed to the low amounts of the modified DNA adsorbed on the electrode surface. Moreover, appearance of parasitic peaks at ca. +0.2 V due to oxygenous surface groups at the PGE prevents the combination with ferrocene-linked nucleotides.[17]

We subsequently turned our attention to our recently reported approach[6,17] based on the synthesis of tailed-PEX products and their hybridization with electrode-tethered oligonucleotides (Figure 3). We designed three 37-mer ON templates flanked on its 5′-end with a 20-mer ssON which were separated by a C3 spacer (−(CH$_2$)$_3$−). The templates were designed to contain a range of mixtures (4:4; 2:6; 6:2) of dT and dG, thus encoding for different ratios of modified **dA^{X}** and **dC^{X}** nucleotides. In the first instance, we tested combinations of either the *nido*-carborane- or FESAN-linked **dC^{CB-}TP** or **dC^{CBFe}TP** with our previously reported 7-ferrocenylethynyl-7-deaza-dATP (**dA^{EFc}TP**).[16] The PEX reaction was then used to generate 37-mer DNA bearing a corresponding number of modifications and a 20-mer single stranded tail. The C3 spacer prevented further polymerase mediated extension, thus maintaining the single-stranded tail

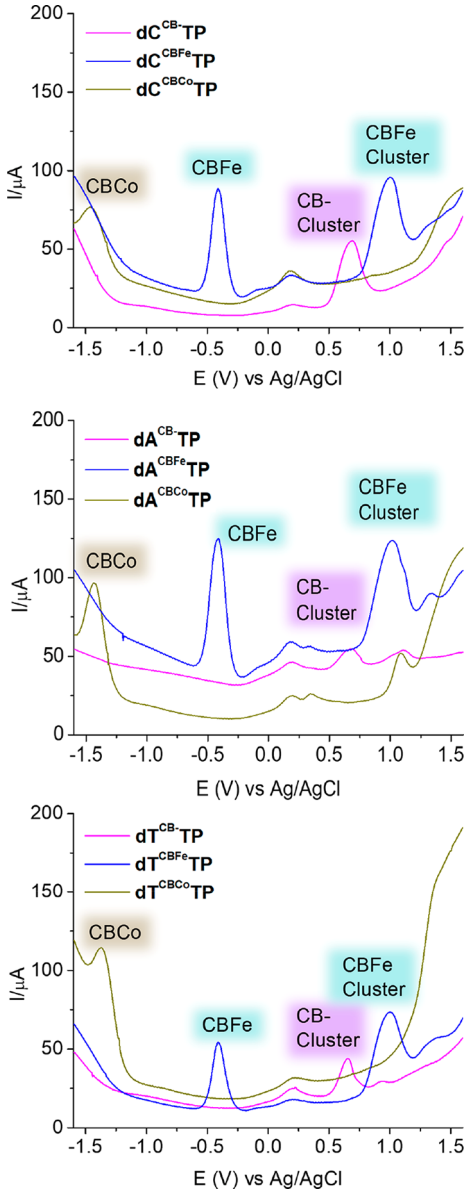

Figure 2. Square-wave voltammograms of modified **dN^{X}TPs** (40 μM) measured at pyrolytic graphite electrode in 0.2 M acetate buffer (pH = 5.0).

ON, which was complementary to a capture ON immobilized on the electrode surface. In parallel, we have conducted a series of analytical solution-based PEX experiments using 5′-(6-FAM) labeled primer to demonstrate that full length PEX products were formed, as expected. The presence of these expected PEX products was indeed confirmed by PAGE and MALDI-TOF analysis. (Figures S3−S4 and Table S2 in the Supporting Information).

The capture ON was linked to the electrode either through immobilization of 3′-thiol-linked ON on the surface of a gold electrode[6] or *via* the addition of thiolated probes to maleimide-modified glassy carbon electrodes (GCEs).[40] The tailed PEX product was then hybridized with the modified electrode, followed by thorough washing, thus removing any non-hybridized amplicons and facilitating direct SWV measurements without the need for any postextension purification step of the PEX product. A 0.1 M Sr(NO$_3$)$_2$ solution was used as an electrolyte solution containing divalent cations, due to the

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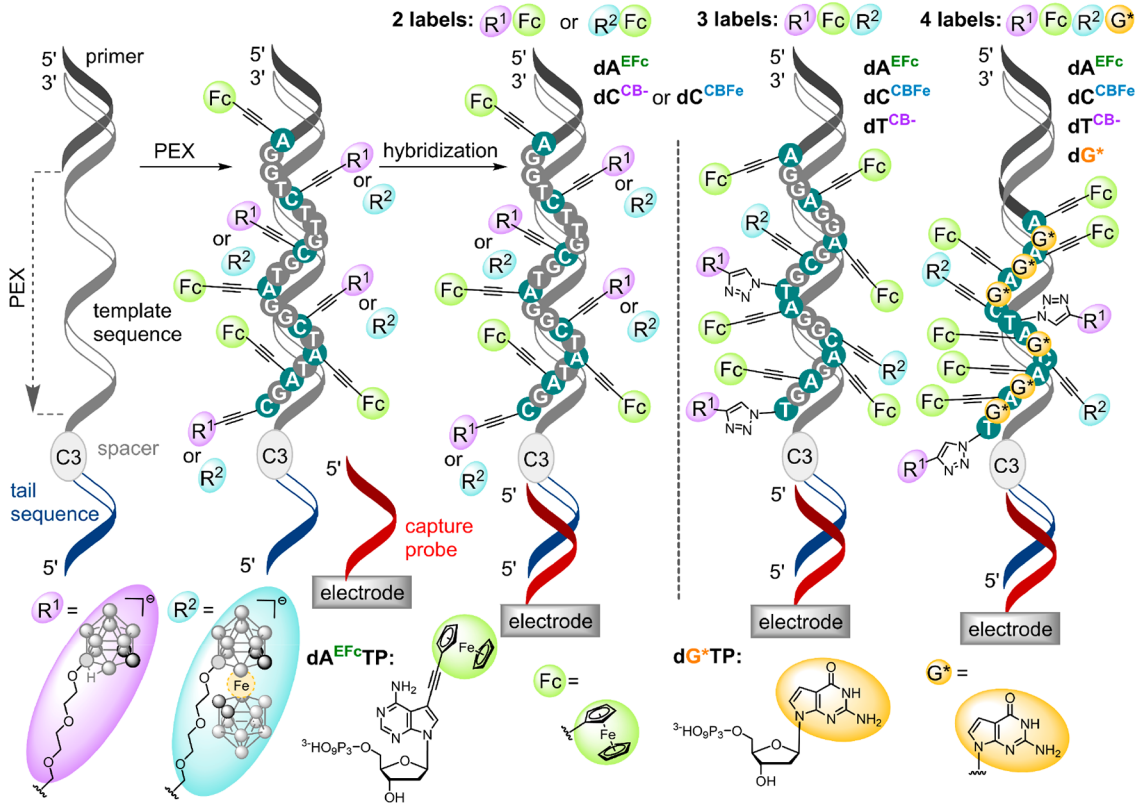

Figure 3. PEX synthesis of the modified DNA probes (bearing 2, 3, or 4 modified nucleotides) and their capture on the electrode.

ability of these cations to shrink and compact DNA,[41] allowing maximal hybridization between immobilized probes and their complementary sequences.[42]

Figure 4 (part 1) shows the PAGE analyses of the different PEX reactions using different templates and combinations of **dC^{CB-}TP** or **dC^{CBFe}TP** with **dA^{EFc}TP**. In all cases, we observed formation of the full-length modified DNA products. The voltammograms on glassy carbon electrode (Figure 4, part 2) reveal clearly distinguishable peaks corresponding to the oxidation FESAN at −0.1 V or *nido*-carborane at +0.6 V and ferrocene at +0.2 V (all vs Ag/AgCl).

Since the first report[43] of the use of the ratiometric responses of electrochemical redox labels in electrochemical sensors in 2014, there have been multiple approaches devised, with the vast majority of these using square wave (SWV) or differential pulse voltammetric (DPV) measurement.[44] While cyclic voltammetry can provide useful information regarding reaction mechanisms, kinetics, and electron transfer rates, SWV/DPV provides better developed signals and enhanced sensitivity of detection of both reversible and nonreversible redox labels, including those used in this study. Metallacarboranes give (quasi)reversible redox electrochemistry due to their metal components, while the carborane cages are known to undergo irreversible electrochemical oxidation[28] and indeed cyclic voltametric (CV) evaluation of the carborane modified nucleotides demonstrated this expected behavior (Figure S7). In addition, 7-deaza-dGTP, which was used for redox coding of G was evaluated using CV and observed to undergo irreversible oxidation (Figure S8), in agreement with previous reports.[45] SWV was thus selected as the preferred method for the voltammetric measurement of the redox labels used throughout this work.

As each of the redox labels will transfer electrons at different rates, with ferrocene transferring most rapidly, followed by the ferrocarborane and then the metal-free nidocarborane, the effect of frequency on the SWV response was evaluated in order to obtain a reproducible and ratiometric signal. As can be seen in Figure S10, the use of a 10 Hz frequency resulted in the best ratiometric signal, which was also observed to provide high intersynthesis batch (Figure S11) and interelectrode reproducibility (Figure S12). As can be seen, in Figure S12, all SWV were automatically background corrected. It should be highlighted that while ratiometric responses are reproducibly obtained for each DNA sequence under interrogation, the signal intensities obtained are not absolute and cannot be used as "calibrator values". Thus, the signal intensities used to measure the ratiometric ratios of redox labels in one ON sequence cannot be extrapolated to another ON, but each individual experiment reliably determines the ratios in the ON being evaluated, as exemplified in Figure S13.

Remarkably, we were able to observe a proportional correlation between the ratio of signal intensities with the known ratio of incorporated labeled nucleotides. The SWV observed with the same amplicons hybridized to probes surface-tethered on gold electrodes also showed equivalent differentiable and ratiometric signals (Figures S14).

Encouraged by the successful combinations of two redox-labeled nucleotides, we advanced to testing a combination of three orthogonally labeled nucleotides. We used a combination of the above-mentioned **dC^{CBFe}TP** and **dA^{EFc}TP** with *nido*-carborane modified thymidine **dT^{CB-}TP**. To this end, we designed a 36-mer template which was used for the PEX synthesis of a tailed DNA product containing 2 **dC^{CBFe}**, 6 **dA^{EFc}**, and 2 **dT^{CB-}** nucleotides. Figure 5 shows the PAGE analysis of the successful PEX reaction and SWV voltammo-

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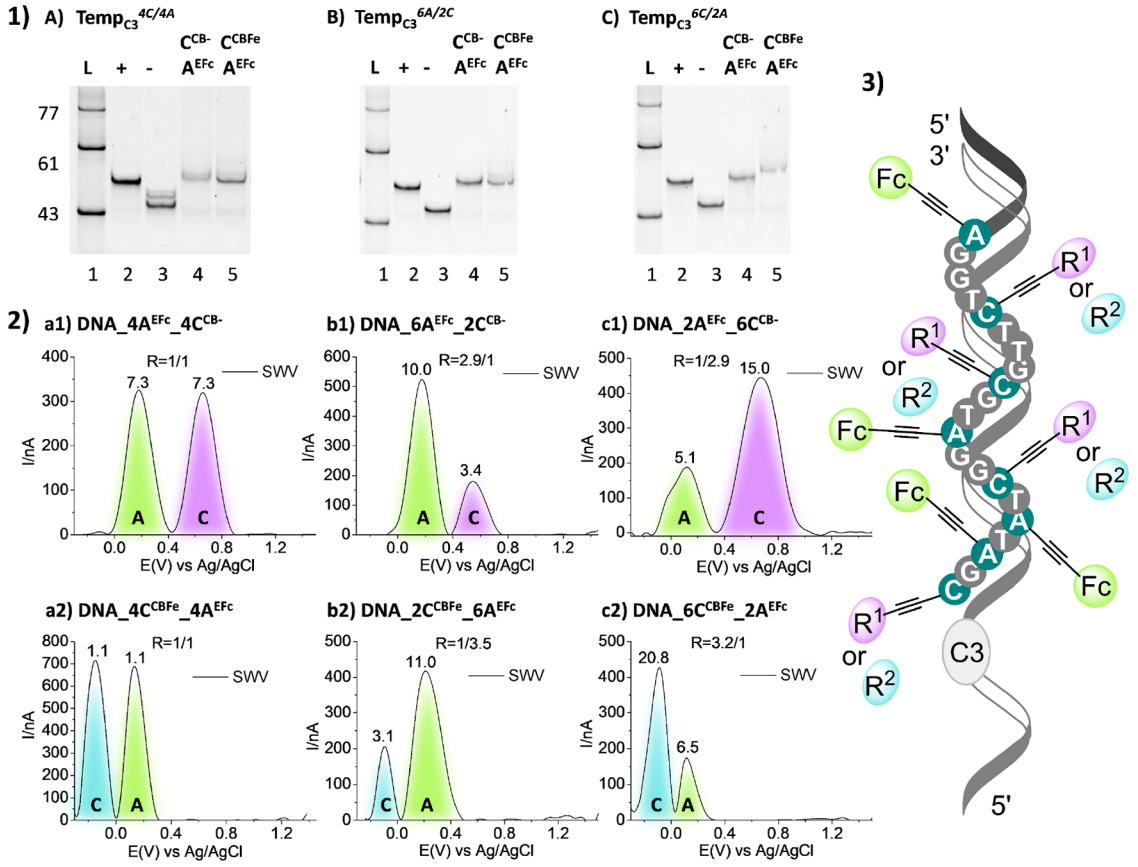

Figure 4. (1) Native PAGE analysis of PEX with KOD XL DNA polymerase using combination of modified **dC^{X}TP**, **dA^{EFc}TP**, 5′-(6-FAM) labeled prim^{C3}, and (A) temp$_{C3}^{4C/4A}$; (B) temp$_{C3}^{6A/2C}$; (C) temp$_{C3}^{6C/2A}$ [lanes (1) dsDNA ladder with denoted length; (2) positive control, natural dNTPs; (3) negative control, without studied dNTPs; (4) reactions containing **dA^{EFc}TP**, **dC^{CB-}TP**; (5) **dA^{EFc}TP**, **dC^{CBFe}TP**]. (2) SWV of modified DNA hybridized with complementary capture probe immobilized on GCE measured in 0.1 M Sr(NO$_3$)$_2$. Cyan, green, and violet designation corresponds to **dC^{CBFe}**, **dA^{EFc}**, and **dC^{CB-}**, respectively. The black traces correspond to the experimental SWV signals of **dA^{EFc}/dC^{CB-}** or **dA^{EFc}/dC^{CBFe}** ratios equal to (a1−2) 4/4; (b1−2) 6/2; (c1−2) 2/6, respectively. (3) Illustrative example of analyzed PEX products.

gram (GCE) confirming the response of the electrochemical analysis correctly corresponding to the ratio of modified nucleotides (observed ratio C/A/T 1/3.1/1.1). This ratiometric sensitivity was also observed using gold electrodes (Figure S15).

The final most challenging step was the extension to a combination of four different redox-labeled nucleotides covering the whole genetic alphabet. An empty "window" in an applicable range of electrochemical spectra in the potential window between 0.8 and 1.4 V vs Ag/AgCl led us to the selection of previously published[45] and commercially available 2-deoxy-7-deazaguanosine triphosphate (**dG*TP**). 7-Deazaguanine in DNA is known[45] to show an intrinsic oxidation peak at ca. 1.1 V which is significantly different from the signal of guanine. As gold electrodes are not highly reliable at potentials >+0.9 V vs Ag/AgCl when using chloride-containing electrolytes, the experiments with the full set of all four modified nucleotides could only be performed with the glassy carbon electrodes. We thus performed the PEX reactions with a combination of **dC^{CBFe}TP**, **dT^{CB-}TP**, **dA^{EFc}TP**, and **dG*TP** using three different templates encoding for different ratios of the four nucleotides in the extended DNA products. Figure 6 shows the PAGE analyses of the successful PEX reactions showing the full-length products in all cases. The voltammograms on glassy carbon electrodes in this figure also reveal four clearly differentiated oxidation signals for each of the four redox labels. Integration of the signal intensities corresponded very well with the known ratio of redox labels and hence with the relative nucleobase composition of the DNA.

CONCLUSIONS

We have designed and synthesized a series of new dNTPs bearing dicarba-*nido*-undecaborate, [3,3′-cobalt-bis(1,2-dicarbollide)]$^-$ or [3,3′-iron-bis(1,2-dicarbollide)]$^-$ groups tethered to nucleobases *via* a flexible diethyleneglycol linker either through the Sonogashira cross-coupling or the CuAAC click reaction. Despite the very bulky and lipophilic substituents, the modified **dN^{X}TP**s were efficient substrates for KOD XL DNA polymerase and were used in enzymatic synthesis of modified DNA *via* the PEX reaction. Electrochemical study using SWV revealed that each of these (metalla)carborane groups are oxidized at distinctly different potentials. The cobaltacarborane cluster showed oxidation at a very low potential, while the other two clusters gave analytically useful signals at −0.4 V (FESAN) and +0.6 V (*nido*-carborane) vs Ag/AgCl, respectively.

In order to develop a multipotential redox coding of DNA nucleobases, we primarily combined either the FESAN or *nido*-carborane with our previously reported ethynyl ferrocene modification. A combination of two modified **dN^{X}TP**s incorporated *via* PEX using a tailed template, followed by hybridization with a capture ON immobilized on either gold or

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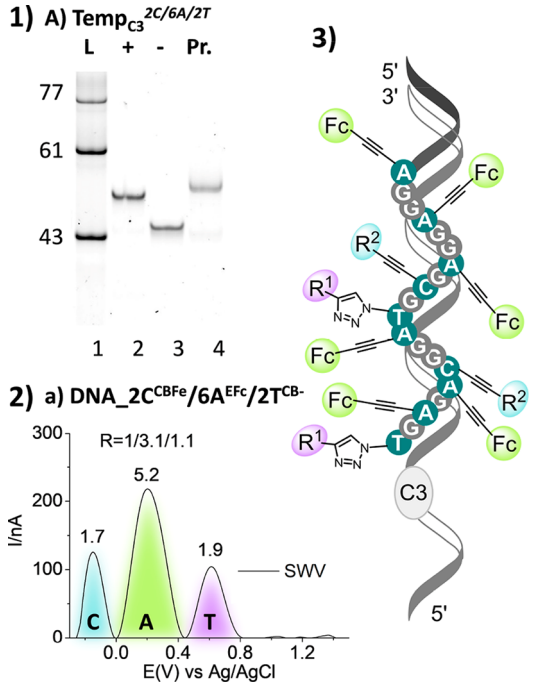

Figure 5. (1) Native PAGE analysis of PEX with KOD XL DNA polymerase using (A) combination of **dC^{CBFe}TP**, **dA^{EFc}TP**, **dT^{CB-}TP**, 5′-(6-FAM) labeled prim^{C3} and temp$_{C3}^{2C/6A/2T}$ [lanes (1) dsDNA ladder with denoted length; (2) positive control, natural dNTPs; (3) negative control, without studied dNTPs; (4) reaction containing **dA^{EFc}TP**, **dC^{CBFe}TP**, **dT^{CB-}TP**; (2) SWV of modified DNA hybridized with complementary capture probe immobilized on GCE measured in 0.1 M $Sr(NO_3)_2$. Cyan, green, and violet designation corresponds to **dC^{CBFe}**, **dA^{EFc}**, **dT^{CB-}**, respectively. The black trace corresponds to the experimental SWV signals of **dC^{CBFe}/dA^{EFc}/dC^{CB-}**, ratio equals to (a) 2/6/2, respectively. (3) Illustrative example of analyzed PEX product.

glassy carbon electrodes enabled facile electrochemical measurements. The SWV showed that the signals of (metalla)-carborane and ferrocene oxidations appeared at distinctly different potentials and were ratiometric, corresponding to the ratio of labeled nucleobases in the newly synthesized sequence. Subsequently, a combination of three different redox labels (FESAN, *nido*-carborane, and ferrocene) at each of three different dNTPs (C, T, and A) were successfully and efficiently incorporated into elongating DNA, and the subsequent SWV readout clearly showed three distinctly different oxidation signals, which were ratiometric with respect to the modified nucleobases of each type. The final and most challenging goal was the development of a full set of four orthogonal redox labels for encoding the four canonical nucleobases. Here, we chose 7-deazaguanine as the fourth redox label and we succeeded in the PEX synthesis of DNA using all four modified **dN^{X}TP**s and the SWV analysis of the fully modified DNA on glassy carbon electrode revealed four fully distinguished, and ratiometric, oxidation peaks. The use of carbon electrodes facilitated a wide enough potential window to detect all four redox labels, while gold electrodes, although slightly easier to functionalize with the thiolated surface-tethered probes, can only be used for the simultaneous detection of three orthogonal labels.

In previous works, either a combination of several labeled dNTPs was used but only one modification at a time (to verify which single nucleotide has been incorporated)[7,9] or a combination of a maximum of two different reducible[10,11] or two oxidizable[17] labeled nucleotides at the same time (to observe the ratio of two nucleobases), was observed to be feasible. Here, we present a fully orthogonal combination of four redox labels encoding all four canonical nucleobases, which are fully ratiometric and facilitate the readout of the proportion of each of the four nucleobases in a newly synthesized DNA sequence using a single modified electrode. While in this initial work the use of four different modifications is limited by the maximum length of the newly synthesized fully modified sequence to ca. 16 nucleotides, we anticipate that further optimization and other combinations will allow synthesis of longer stretches of hypermodified DNA. Incorporation of a known labeled sequence for signal normalization (e.g., via the primer tail) may be used to facilitate determination of the absolute number/mol of each label present in unknown sequences if needed for a specific application.

While optical detection technologies such as fluorescence are prone to background interference and require relatively complex instrumentation, not typically compatible with portable, or point-of-need application, there are increasing numbers of examples of optical detection enabled smartphone technologies.[46] However, multiplexing still remains a grand challenge due to the complex optics and multiple excitation and emission wavelengths as well as the broad emission spectra of fluorescent dyes, requiring either spatial separation of sensors and detection using CCD as exploited in sequencing-by-synthesis approaches,[2,47] or use of individual detectors, such as the zero-mode waveguide nanostructure arrays employed by Pacific Biosciences,[48] and indeed both of these approaches have finally resulted in elegant, high-throughput, massively parallelized, sequencing platforms. Surface-enhanced resonance scattering (SERS) can overcome the problems due to the overlapping, broad fluorescent spectra as the peaks obtained in Raman spectra have narrow spectral widths, allowing for ease of spectral separation and multiplexed detection,[49] but despite instrumental advances it still requires a complex optical setup with an inherent cost impact.

Electrochemical detection, such as that detailed in this work, on the other hand, is highly sensitive, simple, inexpensive, compatible with miniaturization and integration with microfluidics, and can exploit advanced semiconductor technologies, facilitating rapid uptake and mass production, as evidenced by the Ion-Torrent[50] and Oxford Nanopore sequencing[51] technologies. Ion-torrent sequencing remains a laboratory-based instrument, requiring preamplification, and is highly prone to homopolymer read errors. Nanopore's sophisticated MinION addresses portability and does not require preamplification, but the platform still faces issues of high error rates and stability of the protein nanopore-lipid bilayer membrane.[52]

While the work reported here is in its infancy, being just at the proof-of-concept phase, the potential achievable via upscaling and parallelization on electrode arrays is immense. The ratiometry achievable could address problems encountered with homopolymers, the cost of the redox labeled dNTPs is relatively low, massively multiplexed electrode arrays and accompanying microfluidics can be facilely produced using established microfabrication technologies. In addition, solid-phase amplification could facilitate sequencing through synthesis akin to the Pacific Biosciences approach.

Furthermore, the approach reported can find niche applications where more focused sequencing is required, and

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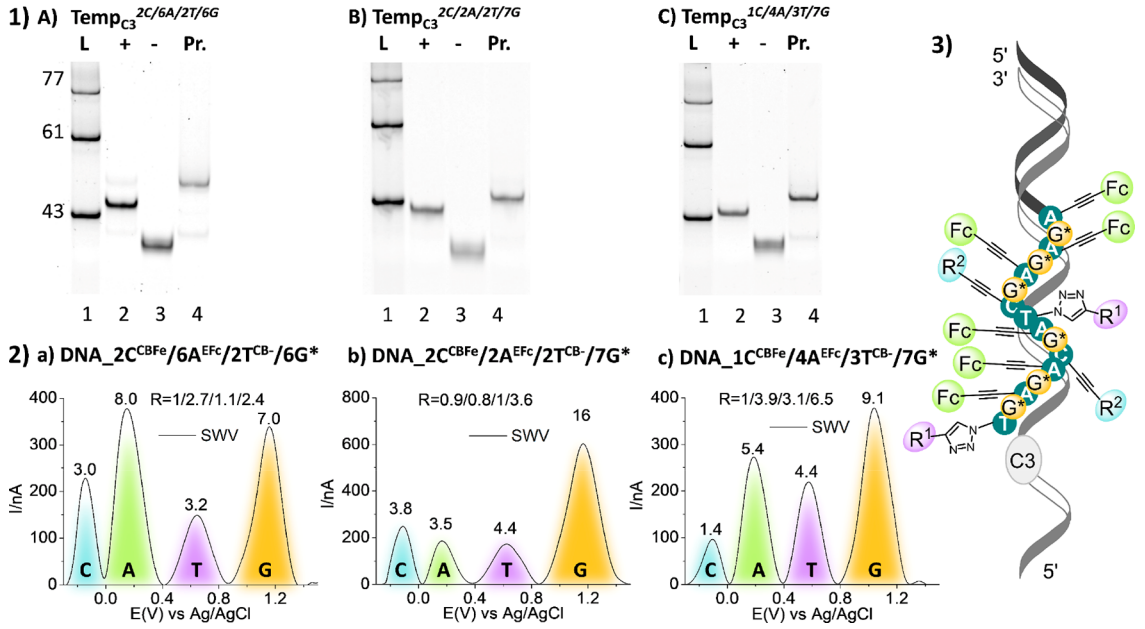

Figure 6. (1) Native PAGE analysis of PEX with KOD XL DNA polymerase using combination of **dC^{CBFe}TP**, **dA^{EFc}TP**, **dT^{CB-}TP**, **dG*TP**, 5′-(6-FAM)-prim^{C3}, and (A) temp$_{C3}^{2C/6A/2T/6G}$; (B) temp$_{C3}^{2C/2A/2T/7G}$; (C) temp$_{C3}^{1C/4A/3T/7G}$ [lanes (1) dsDNA ladder with denoted length; (2) positive control, natural dNTPs; (3) negative control, without studied dNTPs; (4) reactions containing **dA^{EFc}TP**, **dC^{CBFe}TP**, **dT^{CB-}TP**, **dG*TP**]. (2) SWV of modified DNA hybridized with complementary capture probe immobilized on GCE measured in 0.1 M $Sr(NO_3)_2$. Cyan, green, violet and orange designation corresponds to **dC^{CBFe}dA^{EFc}**, **dT^{CB-}**, **dG*** respectively. The black traces correspond to the experimental SWV signals of **dC^{CBFe}/dA^{EFc}/dC^{CB-}/dG*** ratios equal to (a) 2/6/2/6; (b) 2/2/2/7; (c) 1/4/3/7, respectively. (3) Illustrative example of analyzed PEX products.

one can envisage a number of diverse applications including minisequencing of short DNA stretches,[53] detection of specific sets of single-nucleotide polymorphisms,[54] the detection of multiple mutations in consecutive codons, or even the use of the redox coding with electrochemical readout DNA-encoded libraries.[55] Furthermore, (metalla)carborane-linked nucleotides may be used in synthesis of hypermodified DNA-based polymers,[21] synthesis of boron-rich oligonucleotides for BNCT,[23] and in SELEX to generate novel modified aptamers[56] or other functional DNA constructs. All these applications are currently being explored in our laboratories.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02222.

Full experimental details, methods, and procedures and full characterization of all compounds, tables of sequences of oligonucleotides, additional and full uncut gels, additional electrochemical measurements, and copies of MALDI and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Paleček, E.; Bartošík, M. Electrochemistry of nucleic acids. *Chem. Rev.* **2012**, *112*, 3427−3481.
(2) Shendure, J.; Balasubramanian, S.; Church, G. M.; Gilbert, W.; Rogers, J.; Schloss, J. A.; Waterston, R. H. DNA sequencing at 40: past, present and future. *Nature* **2017**, *550*, 345−353.
(3) (a) Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. Isothermal amplification of nucleic acids. *Chem. Rev.* **2015**, *115*, 12491−12545. (b) Fozooni, T.; Ravan, H.; Sasan, H. Signal Amplification Technologies for the Detection of Nucleic Acids: from Cell-Free Analysis to Live-Cell Imaging. *Appl. Biochem. Biotechnol.* **2017**, *183*, 1224−1253.
(4) Hocek, M.; Fojta, M. Nucleobase modification as redox DNA labelling for electrochemical detection. *Chem. Soc. Rev.* **2011**, *40*, 5802−5814.
(5) Kang, D.; Ricci, F.; White, R. J.; Plaxco, K. W. Survey of Redox-Active Moieties for Application in Multiplexed Electrochemical Biosensors. *Anal. Chem.* **2016**, *88*, 10452−10458.
(6) Magriñá, I.; Toldrà, A.; Campàs, M.; Ortiz, M.; Simonova, A.; Katakis, I.; Hocek, M.; O'Sullivan, C. K. Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP. *Biosens. Bioelectron.* **2019**, *134*, 76−82.
(7) Debela, A. M.; Thorimbert, S.; Hasenknopf, B.; O'Sullivan, C. K.; Ortiz, M. Electrochemical primer extension for the detection of single nucleotide polymorphisms in the cardiomyopathy associated MYH7 gene. *Chem. Commun.* **2016**, *52*, 757−759.
(8) (a) Gorodetsky, A. A.; Green, O.; Yavin, E.; Barton, J. K. Coupling into the base pair stack is necessary for DNA-mediated electrochemistry. *Bioconjugate Chem.* **2007**, *18*, 1434−1441. (b) Balintová, J.; Pohl, R.; Horáková, P.; Vidláková, P.; Havran, L.; Fojta, M.; Hocek, M. Anthraquinone as a redox label for DNA: synthesis, enzymatic incorporation, and electrochemistry of anthraquinone-modified nucleosides, nucleotides, and DNA. *Chem. - Eur. J.* **2011**, *17*, 14063−14073. (c) Li, H.; Arroyo-Currás, N.; Kang, D.; Ricci, F.; Plaxco, K. W. Dual-Reporter Drift Correction To Enhance the Performance of Electrochemical Aptamer-Based Sensors in Whole Blood. *J. Am. Chem. Soc.* **2016**, *138*, 15809−15812.
(9) Cahová, H.; Havran, L.; Brázdilová, P.; Pivonková, H.; Pohl, R.; Fojta, M.; Hocek, M. Aminophenyl- and nitrophenyl-labeled nucleoside triphosphates: synthesis, enzymatic incorporation, and electrochemical detection. *Angew. Chem., Int. Ed.* **2008**, *47*, 2059−2062.
(10) Balintová, J.; Plucnara, M.; Vidláková, P.; Pohl, R.; Havran, L.; Fojta, M.; Hocek, M. Benzofurazane as a new redox label for electrochemical detection of DNA: towards multipotential redox coding of DNA bases. *Chem. - Eur. J.* **2013**, *19*, 12720−12731.
(11) Balintová, J.; Špaček, J.; Pohl, R.; Brázdová, M.; Havran, L.; Fojta, M.; Hocek, M. Azidophenyl as a click-transformable redox label of DNA suitable for electrochemical detection of DNA−protein interactions. *Chem. Sci.* **2015**, *6*, 575−587.
(12) (a) Dauphin-Ducharme, P.; Arroyo-Curras, N.; Plaxco, K. W. High-precision electrochemical measurements of the guanine-, mismatch- and length-dependence of electron transfer from electrode-bound DNA are consistent with a contact-mediated mechanism. *J. Am. Chem. Soc.* **2019**, *141*, 1304−1311. (b) Mahshid, S. S.; Camiré, S.; Ricci, F.; Vallée-Bélisle, A. A Highly Selective Electrochemical DNA-Based Sensor That Employs Steric Hindrance Effects to Detect Proteins Directly in Whole Blood. *J. Am. Chem. Soc.* **2015**, *137*, 15596−15599. (c) Pheeney, C. G.; Barton, J. K. Intraduplex DNA-mediated electrochemistry of covalently tethered redox-active reporters. *J. Am. Chem. Soc.* **2013**, *135*, 14944−14947. (d) Pheeney, C. G.; Guerra, L. F.; Barton, J. K. DNA sensing by electrocatalysis with hemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 11528−11533. (e) Cash, K. J.; Ricci, F.; Plaxco, K. W. An electrochemical sensor for the detection of protein-small molecule interactions directly in serum and other complex matrices. *J. Am. Chem. Soc.* **2009**, *131*, 6955−6957.
(13) (a) Zwang, T. J.; Hürlimann, S.; Hill, M. G.; Barton, J. K. Helix-Dependent Spin Filtering through the DNA Duplex. *J. Am. Chem. Soc.* **2016**, *138*, 15551−15554. (b) Gorodetsky, A. A.; Hammond, W. J.; Hill, M. G.; Slowinski, K.; Barton, J. K. Scanning electrochemical microscopy of DNA monolayers modified with Nile Blue. *Langmuir* **2008**, *24*, 14282−14288.
(14) (a) Anne, A.; Demaille, C. Electron transport by molecular motion of redox-DNA strands: unexpectedly slow rotational dynamics of 20-mer ds-DNA chains end-grafted onto surfaces *via* C6 linkers. *J. Am. Chem. Soc.* **2008**, *130*, 9812−9823. (b) Di Giusto, D. A.; Wlassoff, W. A.; Giesebrecht, S.; Gooding, J. J.; King, G. C. Multipotential electrochemical detection of primer extension reactions on DNA self-assembled monolayers. *J. Am. Chem. Soc.* **2004**, *126*, 4120−4121. (c) Di Giusto, D. A.; Wlassoff, W. A.; Giesebrecht, S.; Gooding, J. J.; King, G. C. Enzymatic synthesis of redox-labeled RNA and dual-potential detection at DNA-modified electrodes. *Angew. Chem., Int. Ed.* **2004**, *43*, 2809−2812. (d) Wlassoff, W. A.; King, G. C. Ferrocene conjugates of dUTP for enzymatic redox labelling of DNA. *Nucleic Acids Res.* **2002**, *30*, No. 58e. (e) Yeung, S. S. W.; Lee, T. M. H.; Hsing, I.-M. Electrochemical real-time polymerase chain reaction. *J. Am. Chem. Soc.* **2006**, *128*, 13374−13375. (f) Patolsky, F.; Weizmann, Y.; Willner, I. Redox-active nucleic-acid replica for the amplified bioelectrocatalytic detection of viral DNA. *J. Am. Chem. Soc.* **2002**, *124*, 770−772.
(15) Yu, C. J.; Yowanto, H.; Wan, Y.; Meade, T. J.; Chong, Y.; Strong, M.; Donilon, L. H.; Kayyem, J. F.; Gozin, M.; Blackburn, G. F. Uridine-Conjugated Ferrocene DNA Oligonucleotides: Unexpected Cyclization Reaction of the Uridine Base. *J. Am. Chem. Soc.* **2000**, *122*, 6767−6768.
(16) Brázdilová, P.; Vrábel, M.; Pohl, R.; Pivonková, H.; Havran, L.; Hocek, M.; Fojta, M. Ferrocenylethynyl derivatives of nucleoside triphosphates: synthesis, incorporation, electrochemistry, and bioanalytical applications. *Chem. - Eur. J.* **2007**, *13*, 9527−9533.
(17) Simonova, A.; Magriñá, I.; Sýkorová, V.; Pohl, R.; Ortiz, M.; Havran, L.; Fojta, M.; O'Sullivan, C. K.; Hocek, M. Tuning of oxidation potential of ferrocene for ratiometric redox labeling and coding of nucleotides and DNA. *Chem. - Eur. J.* **2020**, *26*, 1286−1291.
(18) Fojta, M. Mercury electrodes in nucleic acid electrochemistry: sensitive analytical tools and probes of DNA structure. A review. *Collect. Czech. Chem. Commun.* **2004**, *69*, 715−747.
(19) Ferapontova, E. E. Electrochemistry of guanine and 8-oxoguanine at gold electrodes. *Electrochim. Acta* **2004**, *49* (11), 1751−1759.
(20) Dhanjai; Sinha, A.; Lu, X.; Wu, L.; Tan, D.; Li, Y.; Chen, J.; Jain, R. Voltammetric sensing of biomolecules at carbon based electrode interfaces: A review. *TrAC, Trends Anal. Chem.* **2018**, *98*, 174−189.
(21) Ondruš, M.; Sýkorová, V.; Bednárová, L.; Pohl, R.; Hocek, M. Enzymatic synthesis of hypermodified DNA polymers for sequence-

https://doi.org/10.1021/jacs.1c02222
J. Am. Chem. Soc. 2021, 143, 7124−7134

specific display of four different hydrophobic groups. *Nucleic Acids Res.* **2020**, *48*, 11982−11993.
(22) (a) Lesnikowski, Z. J. Challenges and opportunities for the application of boron clusters in drug design. *J. Med. Chem.* **2016**, *59*, 7738−7758. (b) Sivaev, I. B.; Bregadze, V. V. Polyhedral boranes for medical applications: current status and perspectives. *Eur. J. Inorg. Chem.* **2009**, *2009*, 1433−1450.
(23) (a) Hawthorne, M. F. The role of chemistry in the development of boron neutron capture therapy of cancer. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 950−984. (b) Suzuki, M. Boron neutron capture therapy (BNCT): a unique role in radiotherapy with a view to entering the accelerator-based BNCT era. *Int. J. Clin. Oncol.* **2020**, *25*, 43−50.
(24) (a) Scholz, M.; Hey-Hawkins, E. Carbaboranes as pharmacophores: properties, synthesis, and application strategies. *Chem. Rev.* **2011**, *111*, 7035−7062. (b) Issa, F.; Kassiou, M.; Rendina, L. M. Boron in drug discovery: carboranes as unique pharmacophores in biologically active compounds. *Chem. Rev.* **2011**, *111*, 5701−5722. (c) Cígler, P.; Kozísek, M.; Rezácová, P.; Brynda, J.; Otwinowski, Z.; Pokorná, J.; Plesek, J.; Grüner, B.; Dolecková-Maresová, L.; Másа, M.; Sedlácek, J.; Bodem, J.; Kräusslich, H.-G.; Král, V.; Konvalinka, J. From nonpeptide toward noncarbon protease inhibitors: metallacarboranes as specific and potent inhibitors of HIV protease. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 15394−15399. (d) Brynda, J.; Mader, P.; Šícha, V.; Fábry, M.; Poncová, K.; Bakardiev, M.; Grüner, B.; Cígler, P.; Řezáčová, P. Carborane-based carbonic anhydrase inhibitors. *Angew. Chem., Int. Ed.* **2013**, *52*, 13760−13763.
(25) (a) Lesnikowski, Z. Boron Clusters− A New Entity for DNA-Oligonucleotide Modification. *Eur. J. Org. Chem.* **2003**, *2003*, 4489−4500. (b) Druzina, A. A.; Bregadze, V. I.; Mironov, A. F.; Semioshkin, A. A. Synthesis of conjugates of polyhedral boron hydrides with nucleosides. *Russ. Chem. Rev.* **2016**, *85*, 1229−1254.
(26) Balintová, J.; Simonova, A.; Białek-Pietras, M.; Olejniczak, A.; Lesnikowski, Z. J.; Hocek, M. Carborane-linked 2′-deoxyuridine 5′-O-triphosphate as building block for polymerase synthesis of carborane-modified DNA. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4786−4788.
(27) Núñez, R.; Tarrés, M.; Ferrer-Ugalde, A.; de Biani, F. F.; Teixidor, F. Electrochemistry and photoluminescence of icosahedral carboranes, boranes, metallacarboranes, and their derivatives. *Chem. Rev.* **2016**, *116*, 14307−14378.
(28) (a) Fojt, L.; Fojta, M.; Holub, J.; Grüner, B.; Vespalec, R. Electrochemistry of different boranes, carbaboranes and their exo -skeletal hydroxy derivatives at the graphite carbon electrode in aqueous phosphate buffers. *Electrochim. Acta* **2016**, *205*, 8−14. (b) Fojt, L.; Grüner, B.; Šícha, V.; Nekvinda, J.; Vespalec, R.; Fojta, M. Electrochemistry of icosahedral cobalt bis(dicarbollide) ions and their carbon and boron substituted derivatives in aqueous phosphate buffers. *Electrochim. Acta* **2020**, *342*, 136112.
(29) Jelen, F.; Olejniczak, A. B.; Kourilova, A.; Lesnikowski, Z. J.; Palecek, E. Electrochemical DNA detection based on the polyhedral boron cluster label. *Anal. Chem.* **2009**, *81*, 840−844.
(30) Olejniczak, A. B.; Corsini, M.; Fedi, S.; Zanello, P.; Lesnikowski, Z. J. Nucleoside−metallacarborane conjugates for multipotential electrochemical coding of DNA. *Electrochem. Commun.* **2007**, *9*, 1007−1011.
(31) Grabowska, I.; Stachyra, A.; Góra-Sochacka, A.; Sirko, A.; Olejniczak, A. B.; Leśnikowski, Z. J.; Radecki, J.; Radecka, H. DNA probe modified with 3-iron bis(dicarbollide) for electrochemical determination of DNA sequence of Avian Influenza Virus H5N1. *Biosens. Bioelectron.* **2014**, *51*, 170−176.
(32) Hottin, A.; Marx, A. Structural Insights into the Processing of Nucleobase-Modified Nucleotides by DNA Polymerases. *Acc. Chem. Res.* **2016**, *49*, 418−427.
(33) Cho, J. H.; Prickett, C. D.; Shaughnessy, K. H. Efficient Sonogashira Coupling of Unprotected Halonucleosides in Aqueous Solvents Using Water-Soluble Palladium Catalysts. *Eur. J. Org. Chem.* **2010**, *2010*, 3678−3683.
(34) Shaughnessy, K. H. Palladium-catalyzed modification of unprotected nucleosides, nucleotides, and oligonucleotides. *Molecules* **2015**, *20*, 9419−9454.
(35) Amblard, F.; Cho, J. H.; Schinazi, R. F. Cu(I)-catalyzed Huisgen azide-alkyne 1,3-dipolar cycloaddition reaction in nucleoside, nucleotide, and oligonucleotide chemistry. *Chem. Rev.* **2009**, *109*, 4207−20.
(36) Wojtczak, B. A.; Andrysiak, A.; Grüner, B.; Lesnikowski, Z. J. Chemical ligation: a versatile method for nucleoside modification with boron clusters. *Chem. - Eur. J.* **2008**, *14*, 10675−10682.
(37) Řezáčová, P.; Pokorná, J.; Brynda, J.; Kožíšek, M.; Cígler, P.; Lepšík, M.; Fanfrlík, J.; Řezáč, J.; Grantz-Šašková, K.; Sieglová, I.; Plešek, J.; Šícha, V.; Grüner, B.; Oberwinkler, H.; Sedláček, J.; Kräusslich, H.-G.; Hobza, P.; Král, V.; Konvalinka, J. Design of Potent HIV Protease Inhibitors Based on Inorganic Polyhedral Metallacarboranes. *J. Med. Chem.* **2009**, *52*, 7132−7141.
(38) Plešek, J.; Grüner, B.; Macháček, J.; Císařová, I.; Čáslavský, J. 8-Dioxane ferra(III) bis(dicarbollide): A paramagnetic functional molecule as versatile building block for introduction of a Fe(III) centre into organic molecules. *J. Organomet. Chem.* **2007**, *692*, 4801−4804.
(39) Ren, X.; El-Sagheer, A. H.; Brown, T. Azide and trans-cyclooctene dUTPs: incorporation into DNA probes and fluorescent click-labelling. *Analyst* **2015**, *140* (8), 2671−2678.
(40) Bartolome, J. P.; Echegoyen, L.; Fragoso, A. Reactive Carbon Nano-Onion Modified Glassy Carbon Surfaces as DNA Sensors for Human Papillomavirus Oncogene Detection with Enhanced Sensitivity. *Anal. Chem.* **2015**, *87* (13), 6744−6751.
(41) Magriñá, I.; Ortiz, M.; Simonova, A.; Hocek, M.; O' Sullivan, C. K.; Forster, R. J. Ferrocene-Containing DNA Monolayers: Influence of Electrostatics on the Electron Transfer Dynamics. *Langmuir* **2021**, *37*, 3359−3369.
(42) Špringer, T.; Šípová, H.; Vaisocherová, H.; Štěpánek, J.; Homola, J. Shielding effect of monovalent and divalent cations on solid-phase DNA hybridization: surface plasmon resonance biosensor study. *Nucleic Acids Res.* **2010**, *38* (20), 7343−7351.
(43) Du, Y.; Lim, B. J.; Li, B. L.; Jiang, Y. S.; Sessler, J. L.; Ellington, A. D. Reagentless, ratio- metric electrochemical DNA sensors with improved robustness and reproducibility. *Anal. Chem.* **2014**, *86*, 8010−8016.
(44) Yang, T.; Yu, R.; Yan, Y.; Zeng, H.; Luo, S.; Liu, N.; Morrin, A.; Luo, X.; Li, W. A review of ratiometric electrochemical sensors: From design schemes to future prospects. *Sens. Actuators, B* **2018**, *274*, 501−516.
(45) Pivoňková, H.; Horáková, P.; Fojtová, M.; Fojta, M. Direct Voltammetric Analysis of DNA Modified with Enzymatically Incorporated 7-Deazapurines. *Anal. Chem.* **2010**, *82* (16), 6807−6813.
(46) Shrivastava, S.; Trung, T. Q.; Lee, N.-E. Recent progress, challenges, and prospects of fully integrated mobile and wearable point-of-care testing systems for self-testing. *Chem. Soc. Rev.* **2020**, *49*, 1812−1866.
(47) (a) Bentley, D.; Balasubramanian, S.; Swerdlow, H.; et al. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* **2008**, *456*, 53−59. (b) Shendure, J.; Porreca, G. J.; Reppas, N. B.; Lin, X.; McCutcheon, J. P.; Rosenbaum, A. M.; Wang, M. D.; Zhang, K.; Mitra, R. D.; Church, G. M. Molecular biology: Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* **2005**, *309*, 1728−1732.
(48) Eid, J.; Fehr, A.; Gray, J.; Luong, K.; Lyle, J.; Otto, G.; Peluso, P.; Rank, D.; Baybayan, P.; Bettman, B.; Bibillo, A.; Bjornson, K.; Chaudhuri, B.; Christians, F.; Cicero, R.; Clark, S.; Dalal, R.; Dewinter, A.; Dixon, J.; Foquet, M.; Gaertner, A.; Hardenbol, P.; Heiner, C.; Hester, K.; Holden, D.; Kearns, G.; Kong, X.; Kuse, R.; Lacroix, Y.; Lin, S.; Lundquist, P.; Ma, C.; Marks, P.; Maxham, M.; Murphy, D.; Park, I.; Pham, T.; Phillips, M.; Roy, J.; Sebra, R.; Shen, G.; Sorenson, J.; Tomaney, A.; Travers, K.; Trulson, M.; Vieceli, J.; Wegener, J.; Wu, D.; Yang, A.; Zaccarin, D.; Zhao, P.; Zhong, F.;

https://doi.org/10.1021/jacs.1c02222
J. Am. Chem. Soc. 2021, 143, 7124−7134

Korlach, J.; Turner, S. Real-time DNA sequencing from single polymerase molecules. *Science* **2009**, *323*, 133−138.
(49) Laing, S.; Gracie, K.; Faulds, K. Multiplex in vitro detection using SERS. *Chem. Soc. Rev.* **2016**, *45*, 1901−1918.
(50) Rothberg, J. M.; Hinz, W.; Rearick, T. M.; Schultz, J.; Mileski, W.; Davey, M.; Leamon, J. H.; Johnson, K.; Milgrew, M. J.; Edwards, M.; Hoon, J.; Simons, J. F.; Marran, D.; Myers, J. W.; Davidson, J. F.; Branting, A.; Nobile, J. R.; Puc, B. P.; Light, D.; Clark, T. A.; Huber, M.; Branciforte, J. T.; Stoner, I. B.; Cawley, S. E.; Lyons, M.; Fu, Y.; Homer, N.; Sedova, M.; Miao, X.; Reed, B.; Sabina, J.; Feierstein, E.; Schorn, M.; Alanjary, M.; Dimalanta, E.; Dressman, D.; Kasinskas, R.; Sokolsky, T.; Fidanza, J. A.; Namsaraev, E.; McKernan, K. J.; Williams, A.; Roth, G. T.; Bustillo, J. An integrated semiconductor device enabling non-optical genome sequencing. *Nature* **2011**, *475*, 348−352.
(51) Branton, D.; Deamer, D. W.; Marziali, A.; Bayley, H.; Benner, S. A.; Butler, T.; Di Ventra, M.; Garaj, S.; Hibbs, A.; Huang, X.; Jovanovich, S. B.; Krstic, P. S.; Lindsay, S.; Ling, X. S.; Mastrangelo, C. H.; Meller, A.; Oliver, J. S.; Pershin, Y. V.; Ramsey, J. M.; Riehn, R.; Soni, G. V.; Tabard-Cossa, V.; Wanunu, M.; Wiggin, M.; Schloss, J. A. The potential and challenges of nanopore sequencing. *Nat. Biotechnol.* **2008**, *26*, 1146−1153.
(52) Dohm, J. C.; Peters, P.; Stralis-Pavese, N.; Himmelbauer, H. Benchmarking of long-read correction methods. *NAR Genom. Bioinform.* **2020**, *2*, lqaa037.
(53) Pastinen, T.; Kurg, A.; Metspalu, A.; Peltonen, L.; Syvänen, A. C. Minisequencing: a specific tool for DNA analysis and diagnostics on oligonucleotide arrays. *Genome Res.* **1997**, *7*, 606−614.
(54) Shen, W.; Tian, Y.; Ran, T.; Gao, Z. Genotyping and quantification techniques for single-nucleotide polymorphisms. *TrAC, Trends Anal. Chem.* **2015**, *69*, 1−13.
(55) (a) Dickson, P.; Kodadek, T. Chemical composition of DNA-encoded libraries, past present and future. *Org. Biomol. Chem.* **2019**, *17*, 4676−4688. (b) Liszczak, G.; Muir, T. W. Nucleic Acid-Barcoding Technologies: Converting DNA Sequencing into a Broad-Spectrum Molecular Counter. *Angew. Chem., Int. Ed.* **2019**, *58*, 4144−4162. (c) Goodnow, R. A.; Dumelin, C. E.; Keefe, A. D. DNA-encoded chemistry: enabling the deeper sampling of chemical space. *Nat. Rev. Drug Discovery* **2017**, *16*, 131−147.
(56) (a) Diafa, S.; Hollenstein, M. Generation of Aptamers with an Expanded Chemical Repertoire. *Molecules* **2015**, *20*, 16643−16671. (b) Gawande, B. N.; Rohloff, J. C.; Carter, J. D.; von Carlowitz, I.; Zhang, C.; Schneider, D. J.; Janjic, N. Selection of DNA aptamers with two modified bases. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 2898−2903. (c) Cheung, Y.-W.; Röthlisberger, P.; Mechaly, A. E.; Weber, P.; Levi-Acobas, F.; Lo, Y.; Wong, A. W. C.; Kinghorn, A. B.; Haouz, A.; Savage, G. P.; Hollenstein, M.; Tanner, J. A. Evolution of abiotic cubane chemistries in a nucleic acid aptamer allows selective recognition of a malaria biomarker. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 16790−16798.

https://doi.org/10.1021/jacs.1c02222
J. Am. Chem. Soc. 2021, 143, 7124−7134