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Enzymatic Redox 3′-End-Labeling of DNA Oligonucleotide Monolayers on Gold Surfaces Using Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Single Base Extension

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DNA oligonucleotide monolayers end-tethered to gold surfaces are of considerable interdisciplinary importance. They are actively investigated for their applications, either singly or in an array format, in a variety of DNA-related biotechnologies ranging from biosensing[1−4] to bio-nanomanufacturing[5−7] but also for the general interest in understanding the behavior of short nucleic acids at interfaces.[8] In this vein, alkanethiol-linked (self-assembled, SAMs) monolayers of single-stranded (ss) DNA that incorporate a discrete redox-active label at their free end, commonly a ferrocene (Fc), have recently proven, with the power of existing electrochemical methods, to constitute a new class of invaluable tools for characterizing and exploiting differences in DNA physical properties.[9−16] More specifically, studies on some well-defined Fc-DNA layer systems, typically of a few tens of nanometers in thickness, have remarkably evidenced that the magnitude of the faradaic current response, reflecting the electron transport efficiency of the DNA borne-Fc head to and away from the electrode surface, is not only a function of the through-space distance between the redox label and the electrode, that is, of the conformation of the DNA layer, but is also intimately related to the dynamics of the DNA chain.[11,16]

Many new methods for manipulating surface-tethered DNAs that exploit the utility and fidelity of nucleic-acid modifying enzymes have recently grown in importance.[5−7,17−19] A real major advance has been the introduction of nucleotide triphosphates NTPs for surface-enzymatic extension of DNA oligonucleotide monolayers on gold by polymerases[17d,19] or terminal transferases.[7] Notably, ferrocene-labeled NTPs, have rapidly evolved as promising tools to enhance the redox properties of surface-immobilized DNA.[18−19] However, all the reported surface-incorporation redox strategies, even in single-base extended DNA assays, are polymerase-template DNA dependent, precluding their use as universal labeling methods for detection of nucleic acids.

We introduced earlier the first synthesis of a Fc-labeled dideoxynucleotide (chain terminator), FcddUTP, and its efficient DNA terminal deoxynucleotidyl transferase enzyme (TdT) template-independent catalyzed incorporation into the 3′-OH of ssDNA oligonucleotides in solution.[20] In this paper, as an extension of our previous work, we report a novel TdT-mediated method for direct, facile, and efficient redox end-labeling of ssDNA oligonucleotide monolayers on gold. Cyclic voltammetry (CV) is used with the complementary combined atomic force electrochemical microscopy (AFM−SECM) technique to finely characterize the structural properties of the DNA layer before and after the single-base extension labeling event (see Scheme 1).

A molecular monolayer of homopolymeric $(dT)_{19}$ strands was 5′-thiol end-grafted onto a (polycrystalline or flat template-stripped) gold surface via a C6-alkyl linker as previously described.[11,16] As seen in Figure 1A (green curve), the CV of the thus thiolated-$(dT)_{19}$ electrode recorded in a 1 M $NaClO_4$ aqueous solution exhibits an almost ideally shaped capacitive current response, confirming for this system the absence of interfering electroactive and foreign species in the potential window useful for Fc electrochemistry.

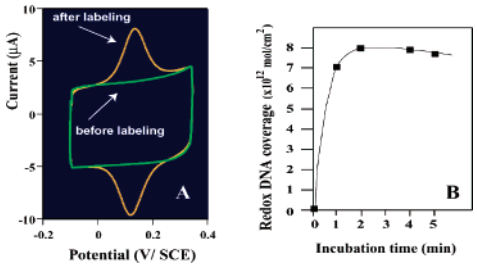

Figure 1. Cyclic voltammetric detection of 3′-redox FcddU-end-labeling event of a $(dT)_{19}$ ssDNA monolayer 5′-thiol-grafted on a gold surface electrode. (A) Raw voltametric data before (green trace) and after 2 min incubation time in TdT reaction mixture (orange trace). (B) Kinetics of TdT-catalyzed FcddU incorporation measured by CV. Scan rate $\nu = 100$ V/s; $T = 21$ °C. The supporting electrolyte is 1 M $NaClO_4$ + 25 mM sodium phosphate buffer pH 7. Each reaction was repeated four times, and representative curves are shown. TdT labeling reaction: TdT recombinant (Roche Diagnostics) 60 U/μL, 60 mM FcddUTP, 5 mM Co^{2+}, $T = 37$ °C.

Scheme 1. TdT-Mediated 3′-Redox Single Base Extension of a Au-ssDNA Oligonucleotide Surface with Ferrocene-Labeled (Fc)ddUTP

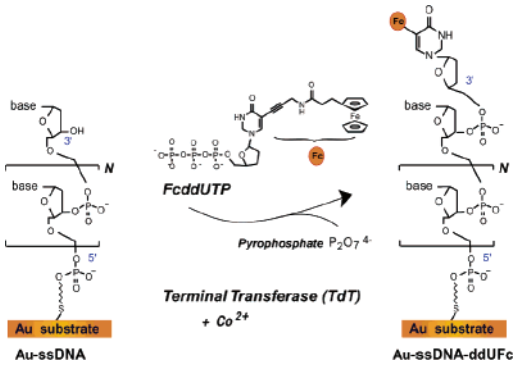

Figure 2 shows the deflection and current versus distance approach-curves of the thiolated-$(dT)_{19}$ gold flat surface, acquired by in-situ AFM-SECM utilizing a tip-probe acting simultaneously as a cantilever and a working electrode.[21] Compared to bare gold, a notable increase in the deflection curve is observed from a tip−substrate separation of ∼12 nm, demonstrating that the tip detects the oligonucleotide layer and starts to compress it within a distance consistent with the contour length of a 19-bases C6-linked ssDNA.[16,22i] From this result, it may be inferred that the 5′-anchored $(dT)_{19}$ strands are not lying flat on the gold surface, leaving the bases and the free 3′-end unobstructed for biochemical event. As shown in Figure 2B, compression of the redox-unlabeled ssDNA layer did not result in any current flow through the tip, the

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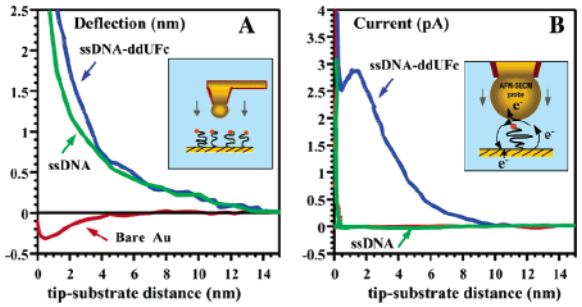

Figure 2. AFM−SECM: Simultaneously recorded tip-deflection (A) and tip-current (B) approach curves versus tip-to-substrate distance for a $(dT)_{19}$ layer 5′-end grafted on a gold-substrate before (green line) and after a 2 min redox FcddU-labeling accomplished with TdT (blue line). The insets show the schematics of a combined AFM-SECM probe approaching a gold substrate-grafted ssDNA layer (in A) and of the tip-to-substrate redox cycling of the Fc head (in B). Conditions are as follows: supporting electrolyte, 1 M $NaClO_4$ solution; (DNA) surface coverage, $\sim 6 \times 10^{-12}$ mol/cm^{-2}; gold substrate potential, −0.1 V/SCE; tip bias, +0.4 V/SCE; approach rate, 10 nm/s; estimated accuracy on the tip−substrate distance, approximately ±1 nm; averaging, ∼10 curves.

sudden current jump observed in the current-approach curve down to 1 nm, also recorded for a bare surface, being attributed to tunneling.[21]

Surface enzymatic FcddU redox-labeling was performed by incubating the $(dT)_{19}$-reactant gold surface with TdT recombinant (Roche Diagnostics) at 60 U/μL, in a Tris-HCl buffer solution (Roche Diagnostics) containing 5 mM Co^{2+} to activate the enzyme and 60 μM FcddUTP nucleotide at 37 °C for 2 min. The electrode was subsequently rinsed with cold deoinized water and 1 M $NaClO_4$ to deactivate the enzyme and then analyzed.[22ii]

As shown in Figure 1A, the CV of the resulting FcddU-3′-extended $(dT)_{19}$ electrode displays a remarkable reversible faradaic signal, whose features are as expected for an ideal Nernstian surface bound ssDNA-Fc:[11,16] the peak heights are proportional to the scan rate υ, and the peak-to-peak separation is less than 10 mV at high υ = 500 V/s, indicating that charge-transfer kinetics does not interfere. The standard potential of the Fc head as determined from the common value of the anodic and cathodic peak potentials, $E°$ = 130 mV/SCE is the same as the one previously reported for FcddU−ssDNA freely diffusing in aqueous solutions, showing that the environment experienced by the Fc heads borne by the strands is similar in both cases.[23] In addition, the peak width at midpeak height is ca. 95 mV, which is close to the predicted value of 90 mV for identical, non-interacting tethered redox centers, located outside the double layer. Hence, integration of the background-subtracted peak currents provided, for complete FcddU-labeling of grafted $(dT)_{19}$ strands, a coverage value of $(6 - 8) \times 10^{-12}$ mol/cm^2 (per effective area) (see Figure 1B). This result, which is in agreement with packing densities reported by others for well-defined ssDNA oligonucleotide monolayers on gold,[1,8] suggests that all the chains are labeled.

The ability to efficiently label the $(dT)_{19}$ monolayer in a 2 min incubation time, without loss of surface-linked ssDNA strands nor changes in the morphology of the layer, was further confirmed by AFM-SECM studies. As shown in Figure 2A, the deflection-approach curves, before and after the extension labeling event, are quite similar, evidencing that the tip compresses in both cases a DNA layer with an average height of ∼12 nm and addresses the same number of anchored ssDNA chains. As shown in Figure 2B, following the labeling event, the redox head is electrochemically detected from a tip−substrate separation of ∼10 nm, confirming that the Fc-unit is, as expected, capping the free 3′-end of the ssDNA strand. As the tip−substrate distance is made smaller, the recorded current increases since the time required for the tip-to-substrate travel of the Fc-head becomes concomitantly shorter (see inset in Figure 2B).[22iii,iv] Importantly, the intensity of the current-approach curves recorded at various locations along the gold-ssDNA-Fc substrate differed by less than 10%, ascertaining that the TdT enzymatic FcddU-labeling reaction proceeded homogeneously all over the surface.

In summary, we have demonstrated the ability of TdT to incorporate efficiently and rapidly a Fc-labeled chain terminator at the free 3′-end of a ss-DNA monolayer. This unprecedented surface redox postlabeling method has the advantage of simplicity over existing chemical labeling strategies, which are not always practical before achieving DNA immobilization. Finally, the major strength of our technology is its universality, making it suitable for labeling any 5′-tethered ssDNA whatever its length, base composition, sequence, or nature.

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(22) (i) Force curves recorded at different locations on the surface differed by <20%, demonstrating the homogeneous distribution of grafted DNA. (ii) No faradaic signal was observed in the absence of TdT nor in the presence of deactivated TdT. (iii) The specificity of the electrochemical detection of the Fc head was proved by the S-shaped variation of the tip current versus tip potential, characterized by a half-wave potential of ∼135 mV/SCE, a value close to the $E°$ of the Fc-head. (iv) The peak observed in the current approach curve, is due to lateral surface displacement and/or lateral elongation of the overcompressed chains (ref 21b).
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