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A Comparison of Five Bioconjugatable Ferrocenes for Labeling of Biomolecules

*** Supplementary Information ***

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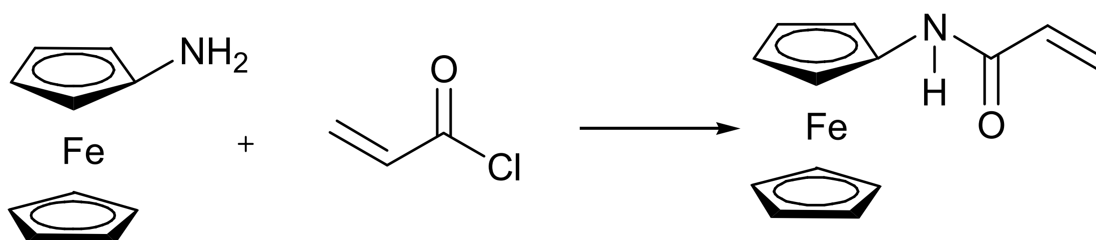
S.1. Materials. Aminoferrocene, 96 % purity, was from TCI America (Portland, OR, USA). Other chemicals and organic solvents were reagent grade from Sigma-Aldrich or Fluka. All chemicals were used without further purification. Aqueous solutions were prepared using deionized (18.2 MΩ cm) water. DNA oligodeoxyribonucleotides (Integrated DNA Technologies, Coralville, IA, USA) were purified by reverse phase high-performance liquid chromatography (HPLC) by the provider. A 20mer DNA "probe", of the sequence 5'-TTT TAA ATT CTG CAA GTG AT-$(CH_2)_3$-SS-$(CH_2)_3$-OH-3', was immobilized via the disulfide terminus to gold electrode supports for hybridization assays (see below). 18mer oligodeoxyribonucleotides, of sequence 5'-CAC TTG CAG AAT TTA AAA-3' complementary to the first 18 bases on the probe solution side, were purchased with either a primary amine (H_2N-$(CH_2)_6$-DNA) or disulfide ($HO(CH_2)_6$-S-S-$(CH_2)_6$-DNA) at the 5' terminus for modification with amine- or thiol-reactive ferrocene tags, respectively. The labeled strands were used as analyte "target" molecules in hybridization assays for testing tag stability.

S.2. Synthesis of *N*-ferrocenylacrylamide (FA0). FA0 was prepared using a one-step Schotten-Baumann synthesis, Scheme 1. Aminoferrocene (0.1 g, 0.48 mmol) and acryloyl chloride (0.065 g, 0.7 mmol) were dissolved in dry CH_2Cl_2 (20 mL) to which 3 mol L^{-1} aqueous NaOH was added dropwise to neutralize HCl generated by the reaction. The mixture was stirred in an ice-water bath for 8 h. After reaction, the bottom organic layer was collected, washed with water (10 mL, 3 times), and dried over $MgSO_4$ after removal of the upper aqueous layer. The CH_2Cl_2 solvent was removed under reduced pressure, leaving a crude orange product. The product was eluted on a silica gel column using 90:10 CH_2Cl_2:MeOH. The purified fraction was

collected and the solvent was evaporated off to leave an orange product with a melting point of 158-159 °C. Yield: 0.11 g (87 %). ^{1}H NMR (δ in ppm, *d*-DMSO; Figure S1): 9.57 (1H, s, -N*H*CO-), 6.26 (1H, t, -C*H*=CH_2), 6.20 (1H, d, -CH=CH*H*, -NHCO- cis), 5.68 (1H, d, -CH=C*H*H, -NHCO- trans), 4.66 (2H, s, H ortho to amide on Cp ring), 4.12 (5H, s, H of unsubstituted Cp ring), 4.01 (2H, s, H meta to amide on Cp ring); ^{13}C NMR (δ in ppm, *d*-DMSO; Figure S2): 162.87 (1C, -NH*C*O-), 131.56 (1C, -*C*H=CH_2), 125.58 (1C, -CH=*C*H_2), 94.96 (1C, substituted C on Cp ring), 68.83 (5C, C of unsubstituted Cp ring), 64.04 (2C, C meta to amide on Cp ring), 60.90 (2C, C ortho to amide on Cp ring). MS (ESI): 254.94 (M^+), calculated: 255.08.



Scheme 1

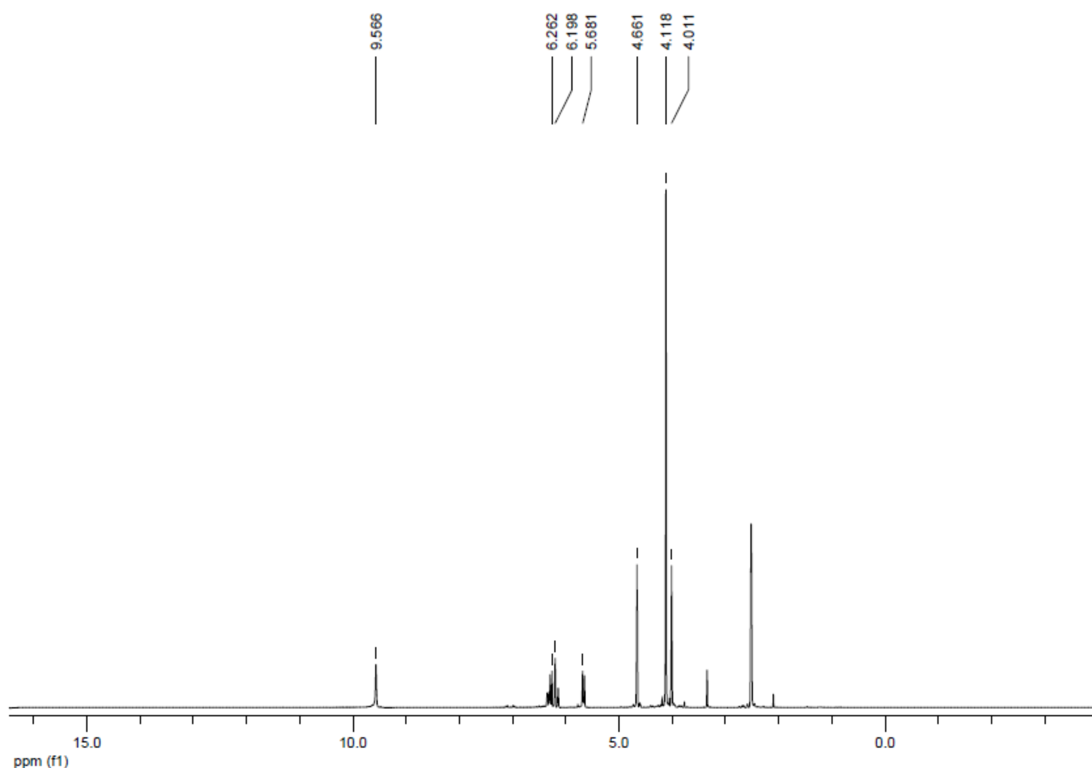


Figure S1. ^{1}H NMR spectrum of FA0.

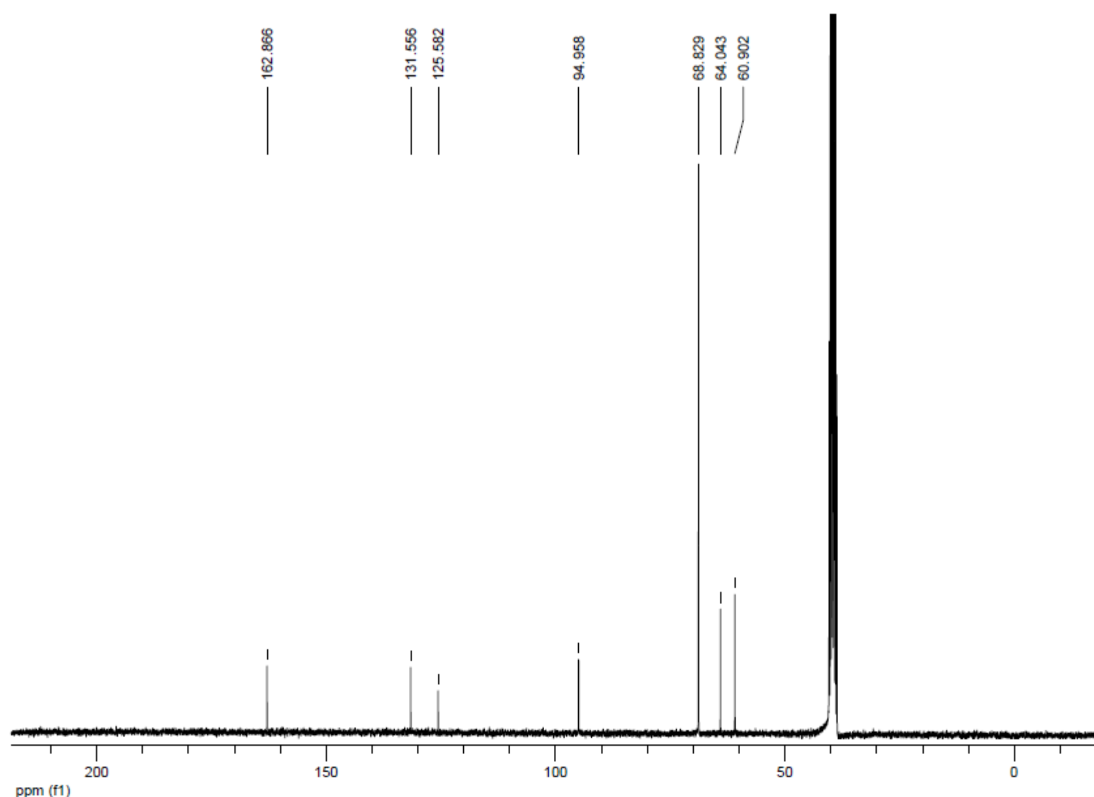
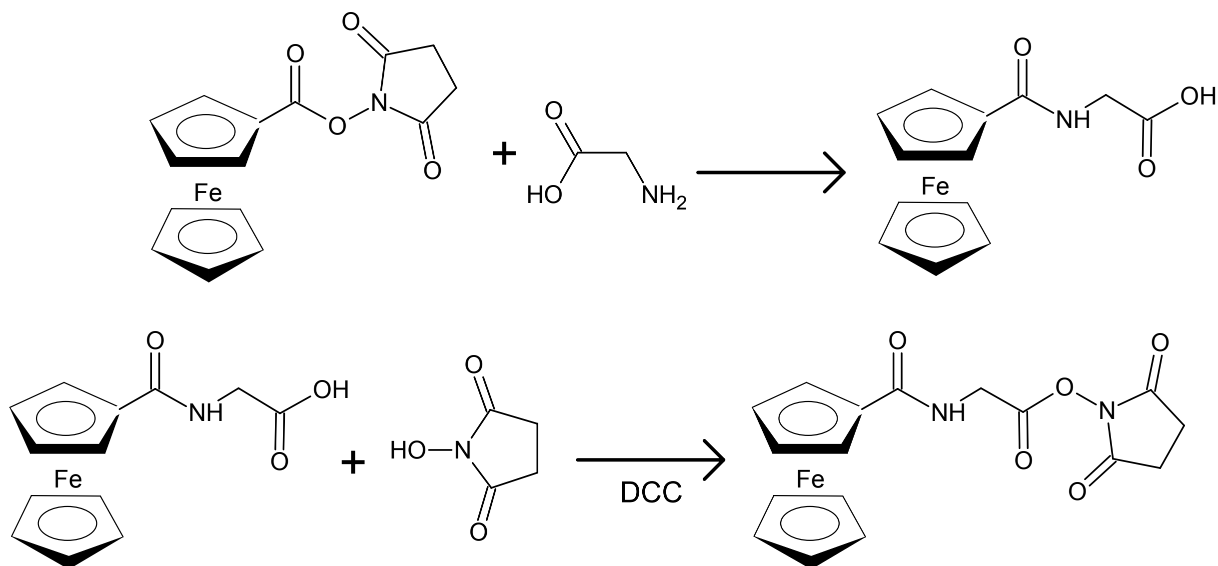


Figure S2. ^{13}C NMR spectrum of FA0.

S.3. Synthesis of *N*-hydroxysuccinimide Ester of *N*-ferrocenylformylglycine (FcFG-NHS). FcFG-NHS was prepared from FcCA-NHS, Scheme 2. FcCA-NHS (4.1 g, 12 mmol) was dissolved in dioxane (150 mL) and, while stirring, glycine (2.73 g, 36 mmol) dissolved in 0.5 mol L^{-1} pH 8.0 $NaHCO_3/Na_2CO_3$ buffer (45 mL) was added. The reaction was allowed to proceed, under stirring and at room temperature, for 24 h. The mixture was next evaporated to dryness, and the residue was redissolved in deionized water (150 mL). Sufficient hydrochloric acid (1 mol L^{-1}) was added to adjust the pH down to 5.0. The formed precipitates from the acidified solution were filtered off and the collected filtrate was dried under reduced pressure. The crude solid was chromatographed on a silica gel column using 98:2 CH_2Cl_2:MeOH as eluent to recover purified *N*-ferrocenylformylglycine (FcFG) as a light yellow solid.

The conversion from FcFG to FcFG-NHS ester followed standard methods. FcFG (0.66 g, 2.2 mmol) and *N*-hydroxysuccinimide (NHS) (0.29 g, 2.5 mmol) in dioxane (20 mL) was combined, while stirring, with *N,N'*-dicyclohexylcarbodiimide (DCC) (0.52 g, 2.5 mmol) in

dioxane (5 mL) at room temperature for 24 h. The precipitate (dicyclohexylurea) was filtered off using a fritted funnel, and the filtrate was evaporated to dryness. The solid product (FcFG-NHS) was washed rapidly with cold absolute methanol (-20 °C) on a fritted funnel and dried under reduced pressure. The FcFG-NHS ester was obtained as an orange powder with a melting point of 139-140 °C. Yield: 0.4 g (45 %). ^{1}H NMR (δ in ppm, *d*-DMSO; Figure S3): 8.46 (1H, t, -CON*H*CH$_2$COO-), 4.81 (2H, s, H ortho to carboxy group on Cp ring), 4.39 (2H, s, H meta to carboxy group on Cp ring), 4.34 (2H, d, -CONHC*H*$_2$COO-), 4.22 (5H, s, H of unsubstituted Cp ring), 2.83 (4H, s, H of succinimide); ^{13}C NMR (δ in ppm, *d*-DMSO; Figure S4): 170.04 (2C, C of carboxy group of succinimide), 169.80 (1C, -CONHCH$_2$*C*OO-), 166.68 (1C, -*C*ONHCH$_2$COO-), 75.00 (2C, C ortho to carboxy group on Cp ring), 70.33 (2C, C meta to carboxy group on Cp ring), 69.47 (5C, C of unsubstituted Cp ring), 68.26 (1C, substituted C on Cp ring), 33.34 (1C, -CONH*C*H$_2$COO-), 25.46 (2C, aliphatic C of succinimide). MS (ESI): 383.98 (M^{+}), calculated 384.15.



Scheme 2

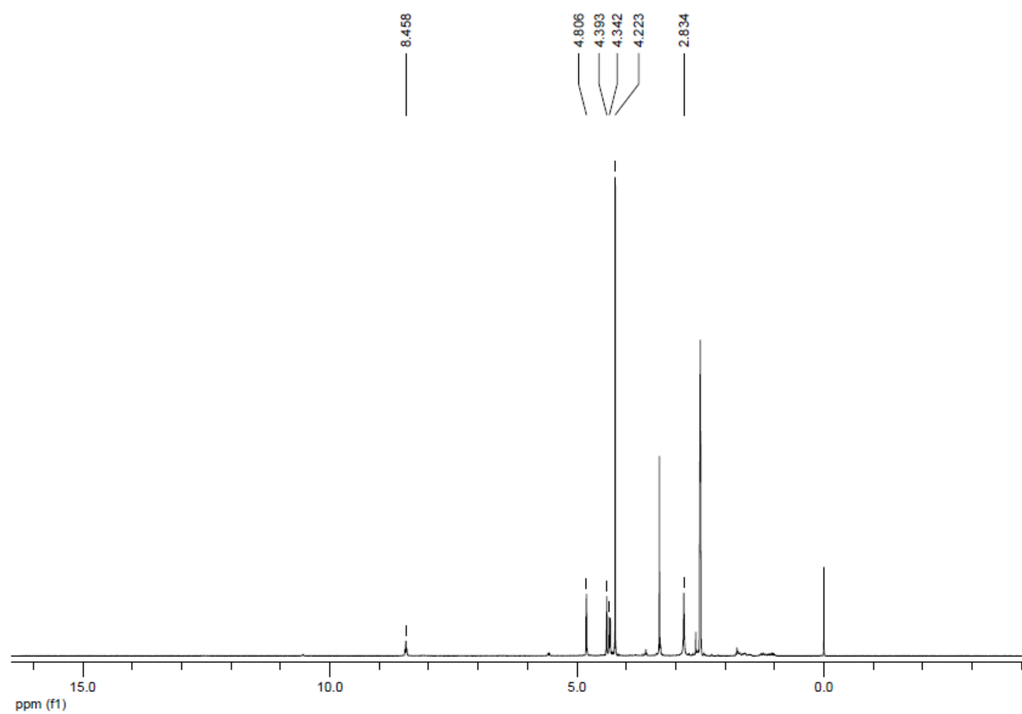


Figure S3. ^{1}H NMR spectrum of FcFG-NHS.

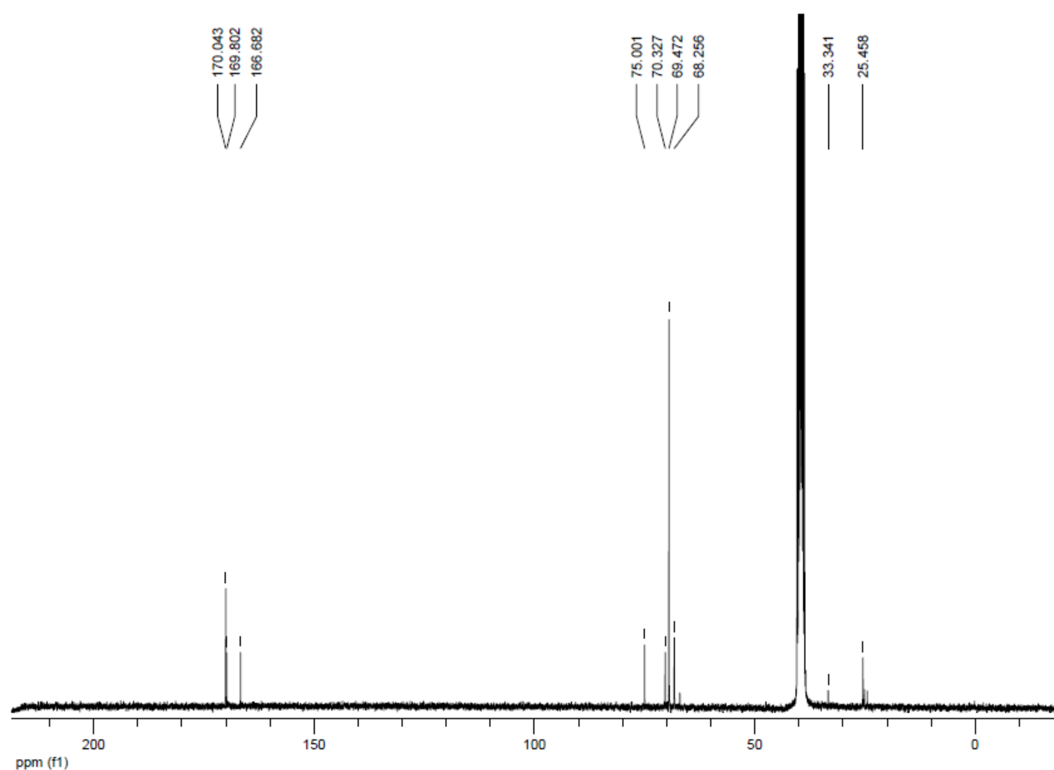


Figure S4. ^{13}C NMR spectrum of FcFG-NHS.

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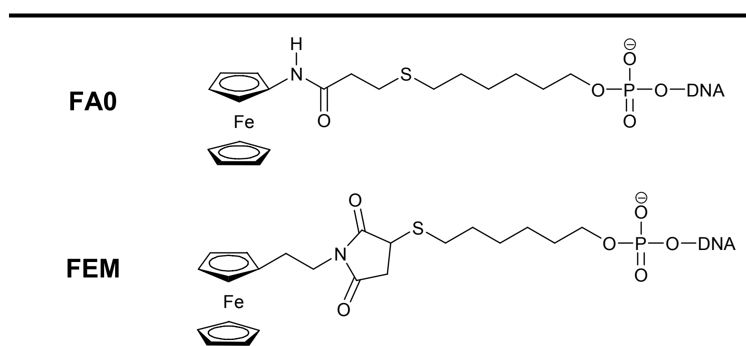
S.4. Bioconjugation with DNA. NHS-ester tags were reacted directly with 5' aminohexyl (H_2N-$(CH_2)_6$-DNA) modified target sequences by combining 0.1 mmol L^{-1} oligonucleotide in 0.5 mol L^{-1} pH 9.0 sodium carbonate buffer with a 100-fold excess of the tag, at room temperature for 24 h. FA0 and FEM tags were conjugated to targets bearing a 5' C6 disulfide $CH_3(CH_2)_5$-SS-$(CH_2)_6$-DNA. The terminal thiol was deprotected with dithiothreitol (DTT) using 10 mmol L^{-1} DTT in 10 mmol L^{-1} TRIS, 1 mmol L^{-1} EDTA, pH 8.0, for 2.5 hrs. After removal of excess DTT on a NAP-10 column (GE Healthcare, Waukesha, WI, USA), the ~ 100 µmol L^{-1} solution of oligonucleotide was reacted with a 100-fold excess of FA0 or FEM in 200 mmol L^{-1} pH 7.3 SPB overnight. Tag-DNA conjugates were purified by passage through two separate NAP-10 columns to remove most of the excess tag and salts, followed by reverse-phase HPLC (Beckman Coulter Gold® 125; Clarity 3 µm Oligo-RP column from Phenomenex®). HPLC conditions were 25 °C, 0.5 ml min^{-1}, and a linear gradient of 12 to 60 % methanol in hexafluoroisopropanol / triethylamine buffer (HFIP-TEA; 100 mmol L^{-1} HFIP, 4.5 mmol L^{-1} TEA, pH 8.0) over 22 min. Table S1 shows the molecular structures of the tag-DNA conjugates.

Table S1. Molecular structures of ferrocene-DNA conjugates.

Tag	Molecular Structure
FcFG-NHS	
FcAA-NHS	
FcCA-NHS	



S.5. Electrochemical Setup. CV voltammograms were recorded on a CHI 660C electrochemical workstation. The three electrode setup used a 1.6 mm diameter polycrystalline gold disc as the working electrode, an Ag/AgCl/3 mol L^{-1} NaCl reference electrode (Bioanalytical Sciences; 0.209 V vs NHE at 25 °C), and a Pt wire counter electrode. Temperature control was provided by a recirculating water bath connected to a 10 mL jacketed electrochemical cell, to an accuracy of ± 1 °C. To improve dissolution of unconjugated tags, tags were first dissolved in 100 μL of acetonitrile followed by addition to 10 mL of SPB.

S.6. Preparation of DNA Monolayers. Working electrodes were mechanically polished with 1-μm diamond slurry, rinsed with copious amounts of methanol and deionized water in sequence, and immediately electrochemically polished by cycling 30 times between -0.20 V and 1.75 V (vs. Ag/AgCl/3 mol L^{-1} NaCl) in 0.5 mol L^{-1} H_2SO_4 at 100 mV s^{-1}. After washing with water, cleaned electrodes were immediately immersed, while still wetted, in 1 μmol L^{-1} solution of the probe DNA sequence 5'-TTT TAA ATT CTG CAA GTG AT-$(CH_2)_3$-SS-$(CH_2)_3$-OH-3' in 1 mol L^{-1} $MgCl_2$ for 20 min. After immobilization of the probe, electrodes were blocked with 1 mmol L^{-1} 3-mercapto-1-propanol (MCP) in deionized water for 90 min. The above procedure resulted in a coverage of ~ 5×10^{12} strands cm^{-2} as determined by repeating the same immobilization protocol with FcFG-labeled probe strands and integrating the redox peaks to extract strand coverages.[1]

S.7. Hybridization Assays for Tag Degradation. Degradation of ferrocene moieties in tag-DNA conjugates was investigated in deoxygenated, nitrogen-blanketed SPB (O_2 concentration of 10 μmol L^{-1}) at 65 °C. 10 μmol L^{-1} solutions of tag-DNA conjugates were kept at 65 °C for 0

min, 60 min, 120 min, or 240 min prior to hybridization, with 0 min corresponding to the time when solution temperature first reached 65 °C. After each designated period, samples were rapidly cooled by 100-fold dilution with room temperature SPB to yield 10 mL of 0.1 μmol L^{-1} conjugate target solution which was then hybridized, while stirring, to probe DNA monolayers until the ferrocene peak no longer increased (~ 40 min). Hybridizations were performed at room temperature, and the same monolayer was used for all hybridizations of a given tag (~ 6 h total duration) so as to maintain a comparable coverage of probes. Monolayers were regenerated between hybridizations by a 15 s immersion in deionized (18.2 MΩ cm) water; efficiency of regeneration was confirmed by noting disappearance of the ferrocene signals.

S.8. Control Experiments for Ferrocene-Mediated DNA Scission. FcAA-DNA and FA0-DNA conjugates were heated at 65 °C for 60 min (FcAA-DNA) and 240 min (FA0-DNA) in SPB following the experimental procedures described in section S.7. MALDI-TOF mass spectra were obtained from the conjugates before and after degradation. Each matrix solution was prepared by mixing 10 μL of a saturated solution of 6-aza-2-thiothymine (ATT) in acetonitrile with 10 μL of 0.1 mol L^{-1} aqueous solution of diammonium hydrogen citrate. 20 μL of an aqueous (deionized water) solution of the conjugate (10-20 μmol L^{-1} of oligonucleotide) were added and the mixture was briefly vortexed. 1 μL of this mixture was applied onto a metallic substrate and the solvent was removed in a vacuum desiccator. Spectra were recorded on an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc., Germany). The instrument was operated in a linear mode and the desorbed positive ions were accelerated by a static electric potential of +20 kV. All spectra were obtained as abundance intensities vs mass-to-charge ratio (*m/z*) by overlaying at least 200 laser shots. The analyzer used a pulsed ion extraction with a delay time of 450 ns. Calibration was performed with adrenocorticotropic hormone (residues 7-38; MW = 3656.9 daltons) and a DNA sequence $dA_8T_5C_3G_2$ (MW = 5490.7 daltons) as external standards.

The heat treatment at 65 °C caused degradation of the tags as evidenced by decreases in *m/z*, Figs. S5 and S6. In all cases the spectra show a single dominant size range whose width was comparable for the before- and after-degradation samples. Moreover, no features indicative of strand scission appeared, which would be expected to occur at the sugar positions[2] and lead to

peaks at lower *m/z*. These results show that under conditions of the study, degradation of ferrocene tags did not lead to significant DNA scission.

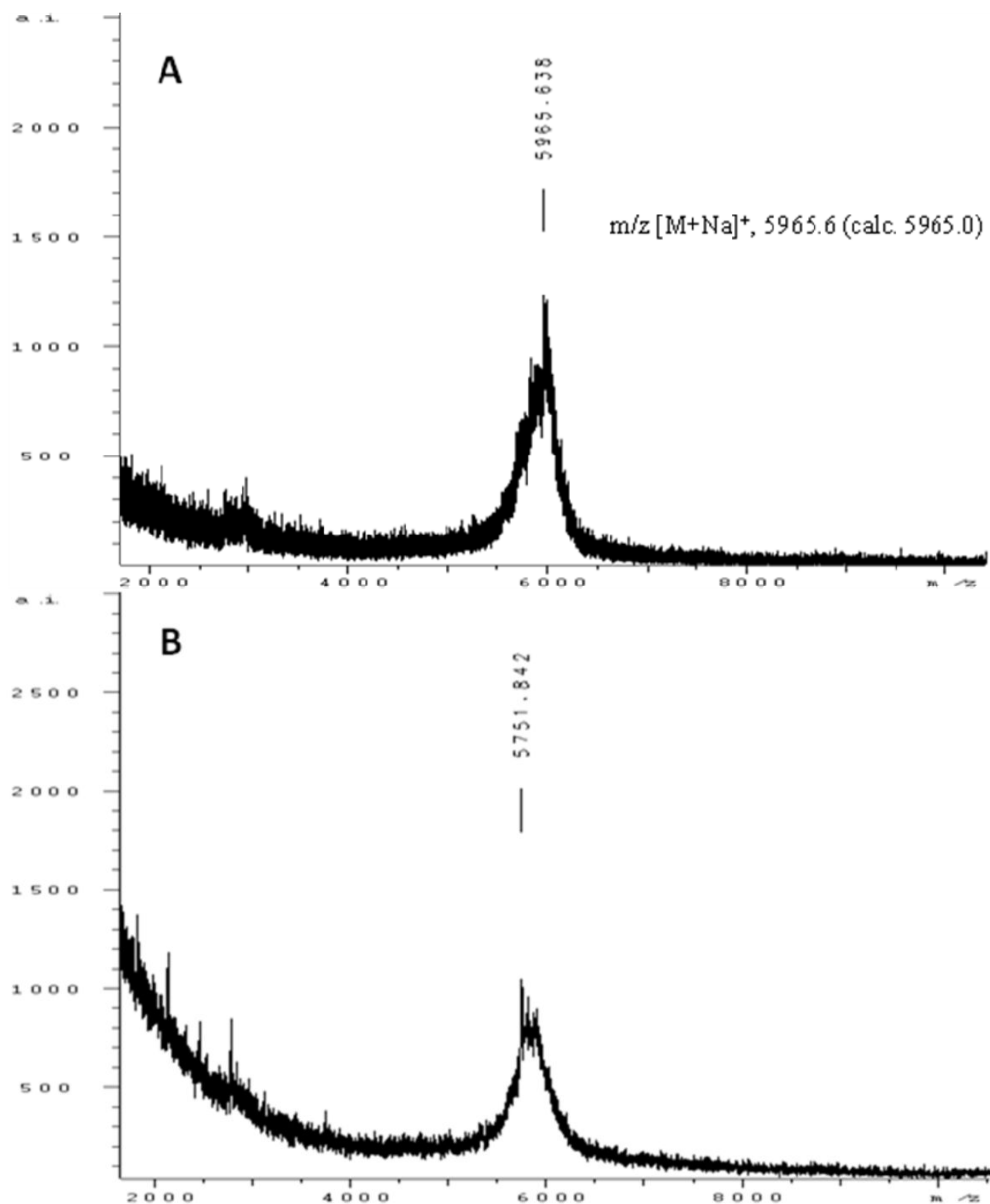


Figure S5. Comparison of MALDI-TOF mass spectra (abundance intensity vs *m/z*) of FA0-DNA before (A) and after (B) treatment in SPB at 65 °C for 4 h.

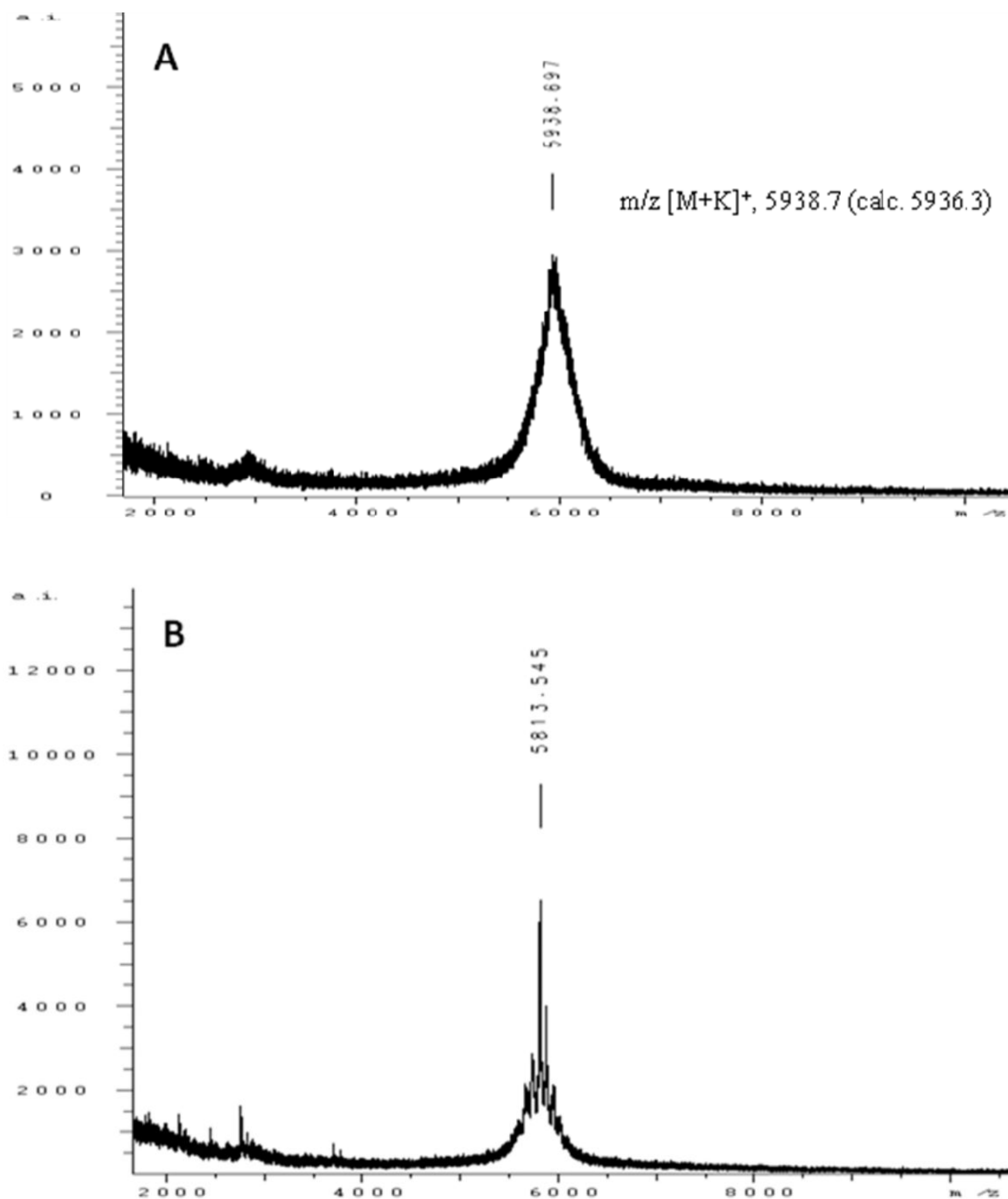


Figure S6. Comparison of MALDI-TOF mass spectra (abundance intensity vs *m/z*) of FcAA-DNA before (A) and after (B) treatment in SPB at 65 °C for 1 h.

References for Supplementary Information

1. N. Tercero, K. Wang, P. Gong and R. Levicky, *J. Am. Chem. Soc.*, 2009, **131**, 4953-4961.
2. R. P. Hertzberg and P. B. Dervan, *Biochemistry*, 1984, **23**, 3934-3945.