Video Article

Synthetic Methodology for Asymmetric Ferrocene Derived Bio-conjugate Systems via Solid Phase Resin-based Methodology

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Abstract

Early detection is a key to successful treatment of most diseases, and is particularly imperative for the diagnosis and treatment of many types of cancer. The most common techniques utilized are imaging modalities such as Magnetic Resonance Imaging (MRI), Positron Emission Topography (PET), and Computed Topography (CT) and are optimal for understanding the physical structure of the disease but can only be performed once every four to six weeks due to the use of imaging agents and overall cost. With this in mind, the development of “point of care” techniques, such as biosensors, which evaluate the stage of disease and/or efficacy of treatment in the clinician’s office and do so in a timely manner, would revolutionize treatment protocols.[1] As a means to exploring ferrocene based biosensors for the detection of biologically relevant molecules[2], methods were developed to produce ferrocene-biotin bio-conjugates described herein. This report will focus on a biotin-ferrocene-cysteine system that can be immobilized on a gold surface.

Video Link

The video component of this article can be found at https://www.jove.com/video/52399/

Introduction

Biosensors are small devices that employ biomolecular recognition technology as the platform for selective analysis and are utilized for their specificity, speed, and low-cost. Electrochemical biosensors for the detection of biomolecules are at the forefront of this field due to their simplicity, cost effectiveness, and high sensitivity.[1,3] The general anatomy of these sensors is an electrode equipped with a recognition molecule specific for the biological marker of interest. Binding of the biomarker by the recognition molecule results in a local change of potential or current that can be detected by simple measurement. To date the recognition moiety can range from enzymes,[4-8] antibodies,[9-12] whole cells,13-16 receptors,[17-20] peptides[21-23] and DNA[24] and have largely focused on larger, biological molecules.[25-28] Research efforts in this arena have concentrated mainly on immunosensors where an immunoglobulin is immobilized with a redox active core (such as ferrocene) and used to detect an antibody of interest. These studies have been excluded from clinical applications due to poor precision and time consumption stemming from the complications arising from use of antigen/antibodies.[1,3] Growing attention has focused on the detection of small molecules (less than 1 kg/mol) of biomedical, food and environmental interest in addition to national security.[29] The best known examples of biosensor devices are self-test glucose monitors, which have screen printed enzyme electrodes coupled to a pocket-size amperometric meter. These systems typically utilize a coulometric method where the total amount of charge generated by the glucose oxidation reaction is measured over a period of time. Marketable devices must be portable, robust and hand-held to make use facile for the population at large.

Redox tags such as ferrocene are necessary to provide the electrochemical detection of biomarkers or small molecules in solution as most biomarkers are not intrinsically electrochemically active.[30-38] Ferrocene is an organometallic molecule that is a gold standard for electrochemistry, which makes it an excellent choice for integration into electrochemical biosensors. Ferrocene-based redox active species have already garnered considerable attention due to their small size, good stability, convenient synthetic access, easy chemical modification, relative lipophilicity, and ease of redox tuning.[3,30-42] Small molecules based on the ferrocene core have been used extensively as detectors of metal ions and small molecules.[32-38,43] Systems targeting larger species such as biomolecules have utilized the attachment of large antibodies or immunoglobulins to ferrocene derivatives that have been embedded onto an electrochemical surface.[1,3,39,44] In each case, the potential and current intensity of the Fe^{III}/Fe^{II} redox couple was altered upon molecular coupling, thus producing a new spectroscopic handle indicating the presence of the analyte molecule. This change arises from the extensive overlap that occurs between the pi-system of the cyclopentadienyl rings and the iron d-orbitals. If the pi-system is modified, *i.e.,* derivatized or reacted, then the orbital interaction will, in turn, change. This will affect the Fe core and can be observed as a shift in the potential of the Fe^{III}/Fe^{II} couple.[40,45,46] These properties make such a system attractive for use as a quantifying agent in an electrochemical immunoassay or biosensor.

In order to produce ferrocene containing systems specific for biosensor capacities it is optimal to modify one Cp ring with the bio-receptor specific for a target molecule and utilize the other Cp ring as a molecular tether to the electrochemical readout or electrode (**Figure 1**). Synthesis of these asymmetric ferrocene derivatives is challenged by side reactions and the formation of dimeric and polymeric species formed upon intermolecular cross-linking.[47] However, coupling chemistry producing an amide bond is the most direct route to provide simple derivatives of

ferrocene involving biological components such as peptides and their metabolites. Therefore, solid phase techniques first developed in the 1950s by Merrifield for peptide synthesis can be applied to organometallic compounds containing ferrocene. Through the use of the orthogonally substituted 1'-Fmoc-amino-ferrocene-1-carboxylic acid molecule, a ferrocene system that can contain a receptor moiety (biotin), electrochemical readout (ferrocene), and immobilizing-linker component (cysteine) has been constructed and detailed herein. The synthesis of this bio-conjugate is discussed as well as evidence for immobilization on a gold surface. This work represents the first presentation of a system composed of biotin, ferrocene and an amino acid for immobilization on a gold surface.

Protocol

1. Synthesis of Biotin-Fc-cysteine (1)

1. Solid phase methods to produce resin-bound **1**.
    1. Place biotin loaded resin (250 mg, 0.145 mmol) into a fritted syringe and swell the resin by drawing up dimethylformamide (5 ml) and shaking the syringe on a lab shaker for 20 min. Expel the solution and repeat dimethylformamide swelling one more time.
    2. Remove the Fmoc protecting group by adding 4-6 ml of 20% piperidine in dimethylformamide to the syringe followed by 10-15 min of shaking. Repeat the deprotection process with another 4-6 ml of piperidine. Wash the resin with a sequence of 3x dimethylformamide, 3x dimethylformamide:methanol (1:1), 3x methanol:dichloromethane (1:1), 3x dichloromethane, ~5 ml each. Do a ninhydrin test (+) on a small sampling (~10) of the beads to confirm successful deprotection by the presence of blue upon heating.
    3. Mix a solution containing 1'-Fmoc-amino-ferrocene-1-carboxylic acid (203.3 mg, 0.4350 mmol), 1-hydroxybenzotriazole hydrate (58.8 mg, 0.413 mmol), diisopropyl carbodiimide (0.0673 ml, 0.435 mmol), diisopropyl ethyl amine (0.0757 ml, 0.435 mmol), and a 4:1 mixture of dichloromethane and dimethylformamide. Draw this into the fritted syringe and gently shake on a lab shaker for 6 hr. Then expel the solution from the syringe and wash as previously described.
    4. Perform the ninhydrin test (-) as described above to confirm coupling. The ninhydrin test can still be useful in confirming coupling despite the orange color of the bead derived from the attachment of the iron containing moiety.
    5. Then remove the Fmoc group by the addition of 20% piperidine in dimethylformamide and washed as described above. The ninhydrin test (+) should be used to confirm Fmoc removal.
    6. Prepare a solution composed of Fmoc-Cys(Trt)-OH (254.8 mg, 0.4350 mmol), 1-hydroxybenzotriazole hydrate (58.8 mg, 0.4125 mmol), diisopropyl carbodiimide (0.0673 ml, 0.4350 mmol), diisopropyl ethyl amine (0.0757 ml, 0.4350 mmol), and a 4:1 mixture of dichloromethane and dimethylformamide. Add this cysteine coupling cocktail the fritted syringe and gently shake for 6 hr. Wash using the protocol described previously.
    7. Confirm coupling using the ninhydrin test (-), followed by removal of the Fmoc component with 20% piperidine and washing. Verify the free terminal amine using the ninhydrin test (+).

2. Cleavage of **1** from the resin.
    1. Make a solution of TFA (9.45 ml), water (0.25 ml), 1,2-ethanedithiol (0.25 ml), and triisopropyl silane (0.1 ml), add it to the syringe and shake gently for 4 hr.
    2. Collect the resulting red-brown solution in an Eppendorf tube and evaporate the TFA slowly using a stream of air.
    3. Add cold diethyl ether (~15 ml) to the Eppendorf tube to precipitate **1**, which will form gentle agitation. Isolate the product via centrifugation (1 g, 5 min). Then repeat cycles of diethyl ether washings (~60 ml total) and centrifuge to obtain **1** as a red/brown solid.

2. Characterization and Analysis of 1

1. Confirm that the identity matches the connectivity and composition shown in **Figure 2** using ^{1}H (16 scans) and ^{13}C NMR (512 scans) in deuterated methanol (300 µl) and ESI-MS analysis.
   Expect the following results:
   ^{1}H NMR spectrum (CD_3OD) δ/ppm: 1.407-1.684 (m, 6H), 2.245 (t, 2H), 2.665-3.150 (m, 12H), 4.015 (t, 1H), 4.104 (d, 2H), 4.274 (q, 1H), 4.426 (d, 2H), 4.479 (q, 1H), 4.595 (t, 1H) and ^{13}C NMR spectrum (CD_3OD) δ/ppm: 24.644 (CH_2), 25.472 (CH_2), 28.051(CH_2), 28.300 (CH_2), 35.474 (CH_2), 38.698 (CH_2), 39.241 (CH_2), 39.717 (CH_2), 55.340/55.538 (Cp-ring), 60.286 (CH), 61.964 (CH), 62.521/62.821 (Cp-ring), 66.038/66.170 (Cp-ring), 69.153/69.328 (Cp-ring), 71.468/71.593 (Cp-ring), 76.466 (CH), 171.770 (C=O), 175.361 (C=O).
   ESI-MS (m/z): Found: 639.00 [**1**+Na]$^{+}$, Theoretical: 639.1 [**1**+Na]$^{+}$ and HR-MS (m/z): Found: 617.2049 [**1**+H]$^{+}$, Theoretical: 617.1622 [**1**+H]$^{+}$.
2. Perform HPLC, elemental analysis to confirm the composition of isolated **1**.
   Carry out HPLC chromatograms using a C8 reversed phase column with 100% MeOH at a flow rate of 0.5 ml/min. Note: HPLC retention times were: 3.198-4.674 min.

3. Immobilization of 1 on a Gold Surface

1. Cut polymer backed gold slides into squares of ~0.25 in^2.
2. Fill a 50 ml beaker with a DI water solution of **1** (~1 mM).
3. Add the gold slide to the beaker and cover with a watch glass. Allow the glass slide to incubate O/N at RT with no agitation.
4. Remove the gold slide from the solution and allow it dry in air.
5. Obtain scanning electron microscopy images using a scanning electron microscope (or equivalent) to observe immobilized **1**.

Representative Results

The resin bound form of **1** is shown in **Figure 2**. The covalent attachment of the ferrocene component gives rise to an orange tint to the resin beads that is persistent with continuous washing and indicative of an immobilized iron containing complex as opposed to iron absorption by the PEG component of the resin bead. The resin-free form of **1** is identical in color to the resin beads. Following removal of the compound from the resin-beads, the purity and yield (68%) resulting from the methods is far superior to typical solution methodology. Elemental analysis of the product showed that **1** was isolated as the TFA salt: Calcd (Found) for $C_{26}H_{36}FeN_6O_4S_2$4TFA: C, 38.07 (38.90); H, 3.76 (4.20); N, 7.83 (7.70). The resulting yield (105.1 mg, 68%) from a typical reaction is based on the elemental analysis results. NMR analysis in deuterated methanol provided ^{1}H NMR spectrum (CD_3OD) δ/ppm: 1.407-1.684 (m, 6H), 2.245 (t, 2H), 2.665-3.150 (m, 12H), 4.015 (t, 1H), 4.104 (d, 2H), 4.274 (q, 1H), 4.426 (d, 2H), 4.479 (q, 1H), 4.595 (t, 1H) and ^{13}C NMR spectrum (CD_3OD) δ/ppm: 24.644 (CH_2), 25.472 (CH_2), 28.051(CH_2), 28.300 (CH_2), 35.474 (CH_2), 38.698 (CH_2), 39.241 (CH_2), 39.717 (CH_2), 55.340/55.538 (Cp-ring), 60.286 (CH), 61.964 (CH), 62.521/62.821 (Cp-ring), 66.038/66.170 (Cp-ring), 69.153/69.328 (Cp-ring), 71.468/71.593 (Cp-ring), 76.466 (CH), 171.770 (C=O), 175.361 (C=O). HPLC retention times were: 3.198-4.674 min. The multiple peaks observed in HPLC analysis were confirmed to be TFA salts of **1** as described above using elemental analysis. Mass spectrometry correlated to the structure shown in **Figure 2**: ESI-MS (m/z): Found: 639.00 [**1**+Na]$^+$, Theoretical: 639.1 [**1**+Na]$^+$ and HR-MS (m/z): Found: 617.2049 [**1**+H]$^+$, Theoretical: 617.1622 [**1**+H]$^+$. Electronic absorption spectra were obtained in water and showed λ_{max} (ε, M^{-1} cm^{-1}) 268 (4,779.8), 434 (324.26).

The methods used to incubate a small gold slide in a solution of bioconjugate **1** are represented in the schematic drawing in **Figure 3**. A thin layer of gold back with polymeric material was added to a solution of **1** and allowed to incubate O/N. The gold slide was then washed with DI water and allowed to dry. Concomitantly, a gold slide was co-incubated in DI water and washed in the same manner. SEM images of the two samples, shown in **Figure 4**, showed that the surface of the gold slide co-incubated with **1** was modified. This indicates that the thiolate interactions of **1** provide an anchor of attachment to the gold surface.

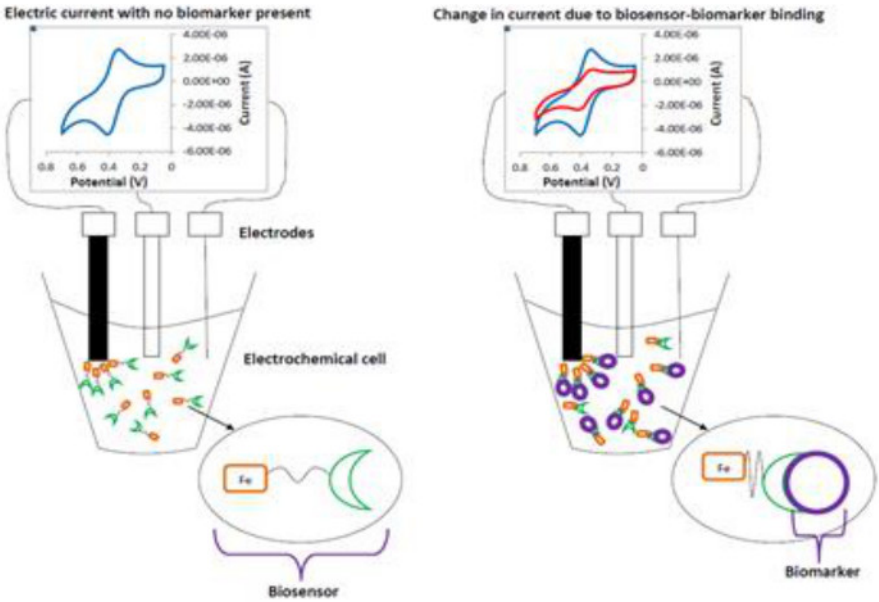

Figure 1. Basics of a biosensor. A specific example of an electrochemical biosensor to directly detect a target in solution.

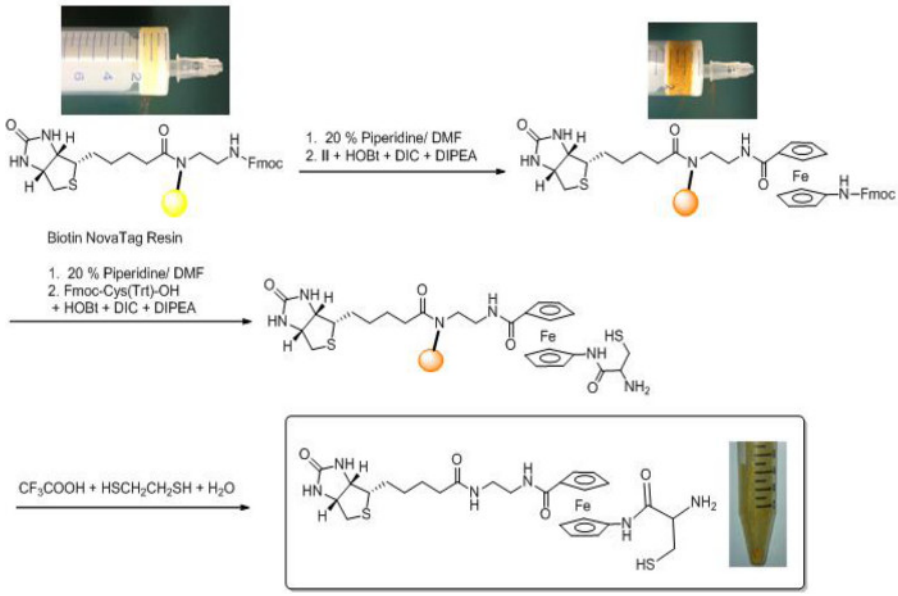

Figure 2. Synthetic methods used to produce 1. The methods used to incubate a small gold slide in a solution of bioconjugate **1** are represented in the schematic drawing in **Figure 3**. A thin layer of gold back with polymeric material was added to a solution of **1** and allowed to incubate O/N . The gold slide was then washed with DI water and allowed to dry. Concomitantly, a gold slide was co-incubated in DI water and washed in the same manner. SEM images of the two samples, shown in **Figure 4**, showed that the surface of the gold slide co-incubated with **1** was modified. This indicates that the thiolate interactions of **1** provide an anchor of attachment to the gold surface.

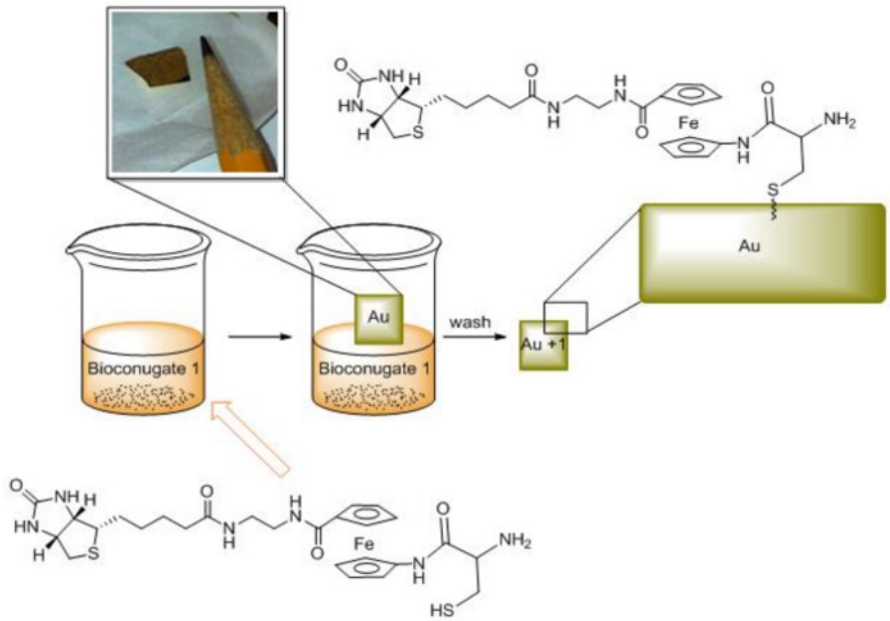

Figure 3. Schematic drawing representing the immobilization of 1 on to a gold slide. The process involves dissolving the bioconjugate in water and adding the gold slide. Incubation O/N is followed by washing of the slide. Analysis for successful immobilization is carried out using scanning electron microscopy shown in **Figure 4**.

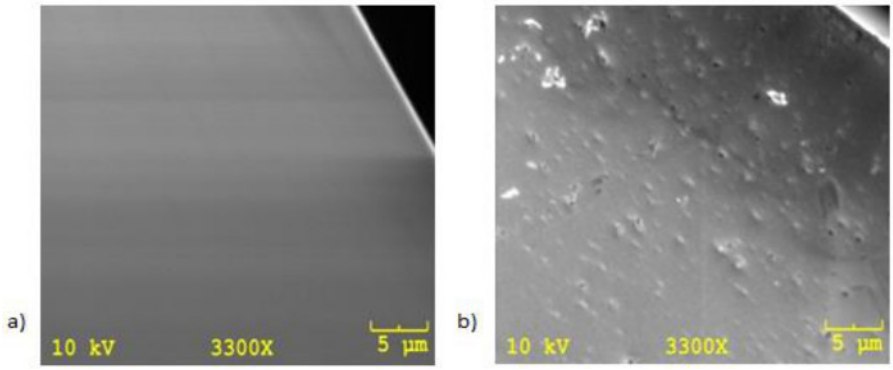

Figure 4. SEM images of gold layered onto a plastic-polymer film. (**A**) incubation *sans* **1** and (**B**) following incubation with **1** in water O/N and rinsing with water.

Discussion

The synthesis of asymmetric ferrocene derivatives is challenging in solution. For example, attempts to produce **1** in solution resulted in low yields of the desired product (less than 20%). Likewise, reactions utilizing 1'-amino-ferrocene carboxylic acid (*sans* Fmoc) and resin bound biotin resulted in insoluble product consistent with the polymerized product reported by Baristic *et al*. and minimal product.[47] This is further complicated by ferrocene and its derivatives being light sensitive and that the amino congeners are prone to dimerize in solution. These issues make extensive reactions and workups challenging. However this reactivity can be circumvented using solid phase methods first developed by Merrifield for synthesis of peptides. The synthesis of ferrocene-peptide systems has garnered attention by several groups and has led to a library of asymmetric peptide-ferrocene systems.[46,48-52] The work described herein details the synthesis of the first asymmetric ferrocene bio-conjugates containing biotin-ferrocene-cysteine. This compound serves as a model through which other small molecule receptors could be considered for detection of biologically relevant molecules. In this work was utilized as an immobilization tether to a gold surface.

The first phase of synthesis was to obtain an immobilized biotin core onto a solid-state resin using an amine-based linker. The *N*-Biotin-*N'*-Fmoc-ethylenediamine resin can be purchased commercially as the Biotin NovaTag Resin and was used as the foundation of bio-conjugate **1**. The Fmoc-amino protecting group was removed using a 20% solution of piperidine in dimethylformamide and a positive (blue) ninhydrin test confirmed successful deprotection. The free amine of the resin-bound biotin was then used to anchor ferrocene using a coupling cocktail composed of diisopropyl ethyl amine, diisopropyl carbodiimide, and 1-hydroxybenzotriazole hydrate.[40,46] A variety of ferrocene precursors are available for such purposes and include ferrocene carboxylic acid and 1'-Fmoc-amino-ferrocene carboxylic acid. Use of the former system yields mono substituted bio-conjugates having only one of the cyclopentadienyl rings modified with a biotin appendage. The latter ferrocene derivative is comprised of orthogonal substitution of the cyclopentadienyl rings with a carboxylic acid and an Fmoc-protected amino group. Such substitution allows for extended asymmetric modification of the ferrocene core allowing for separate modification of the cyclopentadienyl rings to produce bio-conjugates such as **1**. The light yellow beads changed into a bright orange hue upon successful coupling of the ferrocene to the biotin-resin core as shown in **Figure 2**.

In the next phase of synthesis a variety of linkers could be attached to the free amine of the resin-bound ferrocene congener. For the purposes of this system, cysteine was utilized as it is a thiolate containing amino acid. The thiolate component allows for attachment of the bio-conjugate to a gold surface, as discussed below. Modification with cysteine proceeded by reaction of the resin bound system with Fmoc-Cys(Mmt)-OH. Removal of the Fmoc group with 20% piperidine in dimethylformamide yields the resin bound forms of **1**. The resin-bound bio-conjugate was removed from the solid support by cleavage of the bio-organometallic systems using a solution of trifluoroacetic acid (TFA), water and triisopropyl silane. Evaporation of the acid solution and addition of cold diethyl ether yielded bio-conjugate **1** as a red-orange solid determined to be the TFA salt of **1** as confirmed by the elemental and NMR analysis. Purity was confirmed by HPLC analysis.

To show proof of principle for the cysteine component providing an attachment tether for bio-conjugate **1**, deposition of **1** on a gold surface was explored. The well-known Au-S affinity allows for facile immobilization of **1** on a polymer backed gold surface. In this experiment, the gold surface was cleaned and gently polished. The slide was then dipped into a ~1 mM water solution of **1** and allowed to set O/N . The Au surface was then washed with distilled water and dried with a Kimwipe. The gold slides were then evaluated for modification using SEM. The images shown in **Figure 4** are representative of a monolayer of **1** having formed onto the gold surface. The imperfections in the surface are postulated to be a result of 'holes' in the monolayer of **1** and are under further exploration by our group.

Overall, the synthetic methods to produce ferrocene bioconjugates that can be immobilized on a gold surface are reported. The work is novel in that synthesis of asymmetric ferrocene compounds is challenged by an array of side reactions leading to low yields and purity. As ferrocene-bioconjugates similar to **1** have applications as potential biosensors, overcoming these synthetic difficulties is paramount. Using solid-phase methods akin to solid-phase peptide synthesis, a well characterized bioconjugate **1** that contains biotin-ferrocene-cysteine has been produced. Furthermore, SEM was used to show that this system is able to adhere to a gold surface thanks to the thiolate moiety of the cysteine component. Overall, The simple synthetic methodology provided herein can easily be modified for peptide sequences and bio-conjugate systems for an array of applications.

Disclosures

The authors have nothing to disclose.

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