Engineered (Lys)$_6$-Tagged Recombinant Sulfide-Reactive Hemoglobin I for Covalent Immobilization at Multiwalled Carbon Nanotubes

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Supporting Information

ABSTRACT: The recombinant HbI was fused with a poly-Lys tag ((Lys)$_6$-tagged rHbI) for specific-site covalent immobilization on two carbon nanotube transducer surfaces, i.e., powder and vertically aligned carbon nanotubes. The immobilization was achieved by following two steps: (1) generation of an amine-reactive ester from the carboxylic acid groups of the surfaces and (2) coupling these groups with the amine groups of the Lys-tag. We analyzed the immobilization process using different conditions and techniques to differentiate protein covalent attachment from physical adsorption. Fourier transform infrared microspectroscopy data showed a 14 cm$^{-1}$ displacement of the protein’s amide I and amide II peaks to lower the frequency after immobilization. This result indicates a covalent attachment of the protein to the surface. Differences in the morphology of the carbon substrate with and without (Lys)$_6$-tagged rHbI confirmed protein immobilization, as observed by transmission electron microscopy. The electrochemical studies, which were performed to evaluate the redox center of the immobilized protein, show a confinement suitable for an efficient electron transfer system. More importantly, the electrochemical studies allowed determination of a redox potential for the new (Lys)$_6$-tagged rHbI. The data show that the protein is electrochemically active and retains its biological activity toward H$_2$S.

1. INTRODUCTION

The immobilization of proteins on different surfaces has been an extremely active field of study in which a great variety of strategies have been described for further applications. For the immobilization of proteins onto supports, several factors need to be considered depending on the application, including the assembly of biomaterials, the strength of the immobilization, the influence of the surface electrode in the final assembly, and more importantly, the retention of the biomaterial properties after the process. Therefore, the type of immobilization (covalent or noncovalent), as well as the control of a homogeneous orientation of the protein, becomes increasingly important.

Recent studies explore the use of endogenous functional groups present in the side chains of the amino acids to immobilize proteins at different surfaces by covalent bonds. However, due to multiple copies of the same amino acids in a protein, random orientations of the protein are possible. Fusing the proteins with a tag added at the C or N terminus can overcome the random orientation, and moreover it can contribute to the purification process.

Another technique for the immobilization of proteins is the use of fusion proteins with an extension of charged amino acids, such as polyarginine, polyhistidine, polyaspartic, and polylysine tails. The polyhistidine tail (His-tag) is the most commonly used method to purify and immobilize proteins on numerous surfaces but through a noncovalent, reversible immobilization. On the other hand, tags of repeated polar amino acids, such as aspartic acid, lysine, and arginine, are suitable for covalent immobilization. For example, Allard et al. and Ladavie et al. successfully immobilized a polylysine tagged protein by a covalent bond. Our recombinant hemoglobin I (HbI) from Lucina pectinata was engineered to contain a polylysine tag, which allowed it to attach covalently on conductive and biocompatible multiwalled carbon nanotubes (CNTs). This will be a viable approach to further develop a prototype detection system for hydrogen sulfide because HbI binds H$_2$S with high affinity. For this future application, it is imperative to determine if the protein is electrochemically and biologically active after immobilization and to prove the capability of an electron transfer process between the protein and the electrode.
The overall goal of this work is to immobilize a Lys tag recombinant HbI on a conductive surface via the formation of covalent bonds with the retention of the protein’s biological properties. We previously reported the construction, expression, and purification of this new (Lys)₆-tagged rHbI together with its structural and functional properties. The results showed that the (Lys)₆-tagged rHbI is structurally analogous to the native HbI and to the (His)₆-tagged rHbI protein that binds H₂S. In addition, the results indicated that the lysine residues located in the tag are more solvent exposed (>60%) than the other three native lysine residues (<60%). Thus, the polylysine tag is most likely responsible for the interaction between the (Lys)₆-tagged rHbI and biocompatible surfaces. Indeed, the results show that the Lys₆-tag offers an alternative for the proper immobilization of the protein on reactive surfaces due to the high abundance of lysine residues exposed to the solvent in the C-terminal. In general, these residues are certainly suitable to anchor the protein on different surfaces. For example, using a coupling agent, the (Lys)₆-tagged rHbI was immobilized via an amide bond to the carbonyl-oxidized surfaces of carbon nanotubes. Carbon nanotubes (CNTs) not only have an excellent transfer rate due to their high reaction area and their ultrasmall size, but they can be very useful materials to make electrodes.

Nanotubes have unique electrical properties that allow direct electron transfer between the redox active center of the protein and the electrode, which makes them suitable for this application. One disadvantage of the CNTs is their chemical inertness and high hydrophobicity that generally allows them to form insoluble aggregates. Many efforts have led to the development of versatile chemical modification methodologies for the surface functionalization of CNTs to solve the insolubility and inertness problem. One of the most reported paths for functionalization of CNTs is the formation of carboxylic acids groups (COOH) by the oxidation of the CNTs with strong acids. Here, CNTs were oxidized to create a series of defects on the carbon surfaces of carbonyl groups to generate a reactive surface. For protein immobilization, both oxidized multiwalled CNTs (MWCNTs-COOH) and single-walled CNTs (SWCNTs-COOH) have been demonstrated to immobilize many types of proteins. Although SWCNTs are attractive because of their higher surface for proteins interactions, MWCNTs are desirable for their easy dispersibility and lower cost. Several techniques are used to immobilize proteins on CNTs (using either MWCNTs or SWCNTs) ranging from reversible physical adsorption to irreversible stable covalent bonds.

Although in most cases, noncovalent absorption immobilization preserves the protein’s and the CNTs’ chemical and physical properties, the major drawback of this approach is the eventual leakage of the protein from the CNTs. In addition, in many occasions, the physical adsorption of the proteins requires the use of other substances like polymers and/or surfactants, which add complexity to the immobilization process. In contrast, covalent conjugation not only provides a direct link between the protein and the CNTs, preventing the protein to leak from the active surfaces, but it can also maintain the properties of the protein. To covalently attach the protein on CNTs, surface functionalization of CNTs is required. Among the various surface functionalization techniques, the most studied are the amidation reaction of the oxidized carbon nanotubes. Nonetheless, as explained by Fleming et al., the individual protein molecules can adopt many different orientations on an electrode. These aspects, as well as the difficulty for these macro-biomolecules to achieve a direct electron exchange at any electrode surface, are important features to evaluate. They usually have large and complex structures, where the redox centers are deeply immersed in the protein body. For this reaction, several coupling agents are used to activate the surface of oxidized CNTs.

Our data demonstrate that the (Lys)₆-tag is suitable to attach this new rHbI on transducer surfaces like MWCNTs. Important techniques like Fourier transform infrared (FTIR), transmission electron microscopy (TEM), scanning electron microscopy/energy-dispersive spectroscopy (SEM/EDS), and atomic force microscopy (AFM), as well as electrochemical analysis were used to show that (Lys)₆-tagged rHbI was immobilized by covalent attachment to the carbonyl oxide surface of MWCNTs. Electrochemical analysis also demonstrated protein activity after the immobilization of the (Lys)₆-tagged rHbI at the oxidized vertically aligned multiwalled carbon nanotubes (VACNTs-COOH) electrode. (Lys)₆-tagged rHbI/VACNTs response toward H₂S was evaluated. The results suggest the potential applicability of this electrode to detect H₂S.

2. RESULTS AND DISCUSSION

2.1. Immobilization Reaction of (Lys)₆-Tagged rHbI at MWCNTs-COOH. UV–vis studies and Bradford assay showed that the immobilization reactions of (Lys)₆-tagged rHbI at MWCNTs-COOH were successful. Bradford differential analysis, as shown in Table 1, suggests that approximately 27% of the protein treated with coupling agents (EDC and N-hydroxysuccinimide (NHS)) bound to the MWCNTs-COOH, whereas only less than 7% were bound to the MWCNTs-COOH when the system was not treated with coupling agents. This was established by monitoring the UV–vis concentration of the protein remaining in the supernatant after immobilization process and subtracted from the initial concentration of the (Lys)₆-tagged rHbI as explained in Section 4.1. As predicted, the solution containing coupling agents bound more protein than the solution lacking coupling agents.

Validating the covalent conjugation of the (Lys)₆-tagged rHbI onto the carboxylated MWCNTs using EDC/NHS required rigorous scrutiny to differentiate a covalent immobilization from protein adsorption. Therefore, we monitored the amide bands of the (Lys)₆-tagged rHbI before and after immobilization. The FTIR spectra of the controls (Lys)₆-tagged rHbI without coupling agents and the (His)₆-tagged rHbI with agents) and the oxidized MWCNTs without (Lys)₆-tagged rHbI were also evaluated. The first control, corresponding to the immobilized (Lys)₆-tagged rHbI without the use of...
coupling agents, provides information of the contribution or existence of noncovalent interaction, for example, irreversibly bound intercalated, entrapped, or hydrophobically attached. It is known that the coupling agents are necessary to promote the covalent immobilization by forming amide linkage between primary amine groups of the lysine tag and the COOH of the MWCNTs-COOH. Thus, the presence of (Lys)$_6$-tagged rHbI at the MWCNTs-COOH after several washing cycles is caused by irreversible interactions different from covalent attachment. The second control is (His)$_6$-tagged rHbI with EDC/NHS. It is known that the lateral chain of His$_6$-tag is an imidazole group where its secondary amine is not capable of forming an amide bond, whereas the primary amino groups of each residue were already forming peptide bonds to rHbI. Then, this control provides information of the possible immobilization of the protein via amine groups of other lysines or residues at the surfaces of the protein or other interactions.

It should be noted that IR microspectroscopy is a quantitative technique that can be used to determine the absolute protein concentration given the thickness of the sample and using Beer’s Law. In this work, the absolute protein concentration was not determined; however, the peak height (intensity) of the amide I was used to establish a relative concentration and to monitor changes after several washes. Figure 1 shows the amide bands of the (Lys)$_6$-tagged rHbI after the immobilization and washing processes (black line) and the controls (without EDC/NHS and the (His)$_6$-tagged rHbI, blue and red lines, respectively). As shown in Figure 1, the intensity of the amide bands of the treated (Lys)$_6$-tagged rHbI/MWCNTs-COOH are remarkably higher than the controls, implying a higher concentration of the protein on MWCNTs-COOH. The band at 1682 cm$^{-1}$, which has been assigned to the C-OH stretch of traces of water, the immobilization of (Lys)$_6$-tagged rHbI/MWCNTs-COOH using the coupling agents EDC/NHS. When the immobilization process was conducted without EDC and NHS, the amide I and amide II bands decrease dramatically (~69%) after various washes (blue line). However, it is observed that the control with (His)$_6$-tagged rHbI in the presence of EDC and NHS kept a noticeable, although not strong, intensity of the amides bands. This control suggests a strong interaction with the MWCNTs-COOH, which could be attributed to the formation of covalent bonds with some of the exposed lysine residue on the surface of the protein. In the absence of high density of lysine in certain areas, like the tag, the immobilization onto MWCNTs-COOH would take place at random on any of the three lysine residues available on the surface. This strongly supports the notion of a strong covalent bond between the lysine tag of the (Lys)$_6$-tagged rHbI and the MWCNTs-COOH.

The (His)$_6$-tagged rHbI has comparable structural and functional properties to the native HbI and the (Lys)$_6$-tagged rHbI.$^{12}$ The main difference between these two rHbI mutants is the histidine tag at the N-terminus instead of a lysine tag at the C-terminus. This (His)$_6$-tagged rHbI has the same quantity of lysine residues, except for the presence of the (Lys)$_6$-tagged at the C-terminus. Thus, this strongly suggests that the lysine residues within the tag are the reactive species, and the other Lys residues in the protein do not contribute to the covalent attachment of the protein. This is in accordance with the results previously published by this group in which the (Lys)$_6$-tag was used to interact electrostatically with a cation exchange resin for purification purposes.$^{12}$ There it was demonstrated that due to the high abundance of lysine exposed to the solvent in the C-terminal (polylysine tag), it is reasonable to suggest that these residues are more likely responsible for the coupling reaction between the (Lys)$_6$-tagged rHbI and the activated carboxylic groups of the MWCNTs-COOH. Other authors that use polylysine tag employed similar rationalization.$^{11,17}$

Additionally, displacement of the (Lys)$_6$-tagged rHbI amide bands supports the covalent immobilization of the protein on the MWCNTs-COOH. For example, Figure 2 shows the FTIR spectra of (a) (Lys)$_6$-tagged rHbI, (b) MWCNTs-COOH, and (c) (Lys)$_6$-tagged rHbI/MWCNTs-COOH. Figure 2a shows the presence of the vibrational normal mode of amide I at 1657 cm$^{-1}$, whereas amide II appeared at 1548 cm$^{-1}$. This is in agreement with the expected amide I and II absorption bands for proteins with predominantly α-helix structures, which are usually found in the spectral range of 1652–1657 cm$^{-1}$ and 1545–1551 cm$^{-1}$, respectively.$^{26}$ The FTIR spectrum of MWCNTs-COOH, Figure 2b, displayed a band at 1734 cm$^{-1}$, which has been assigned to the C=O stretch mode of carboxylic acid. The band at 1682 cm$^{-1}$ has also been assigned to the stretch mode of traces of water. The immobilization of (Lys)$_6$-tagged rHbI to MWCNTs-COOH can be demonstrated by the changes in the FTIR spectra of these species, as observed in Figure 2c of the resulting (Lys)$_6$-tagged rHbI/MWCNTs-COOH conjugation. The disappearance of the band at 1734 cm$^{-1}$, attributed to the C=O bonds of MWCNTs-COOH,
suggests that the carboxylic groups of MWCNTs-COOH react almost completely with the amino groups of the (Lys)$_6$-tagged rHbI. The vibration of $-\text{NH}_3^+$ also appears in the spectrum of the (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite, which indicates the existence of native amine groups in the (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite region and (black star) solvent region. All of the spectra represent an average of two or more regions in the sample.

Interestingly, the spectra showed a displacement to lower frequencies in comparison to the same bands of the (Lys)$_6$-tagged rHbI alone (Figure 2c,a, respectively). This displacement supports the notion of a covalent attachment (strong interactions) between the protein and the MWCNTs-COOH. The adsorption frequency of the amide I and amide II bands provides information about the secondary structure of the proteins. Slight changes in the global structure of the protein should cause variations in the length and directions of the hydrogen bonds that are part of the amide bonds buried inside the protein. This promotes variations in the strength of the hydrogen bonds between the secondary structures, which results in the displacement of the amide I frequency. Thus, the 14 cm$^{-1}$ displacement of the amide I of the (Lys)$_6$-tagged rHbI toward lower frequencies after its conjugation at the MWCNTs-COOH suggests changes in the global structure of the (Lys)$_6$-tagged rHbI. These changes are expected because the protein was attached through its polylysine tag, causing the hydrogen bond of the secondary structure to be slightly shorter (stronger), displacing in turn the amide I to lower frequencies. Amide II vibration equally shifts toward lower frequencies and shows a broadening, which is related to the protein’s interactions with the solvent. This is attributed to the presence of water, considering that then (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite was more difficult to dry out completely when preparing the samples for the FTIR analysis.

Similarly, TEM analyses were done to further support the immobilization process. Figure 3a shows the MWCNTs-COOH without the (Lys)$_6$-tagged rHbI, whereas Figure 3b illustrates the (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite system where a spiderweb on the sidewalls of the MWCNTs can be easily observed. In addition, there are differences in the diameters of the MWCNTs-COOH with (15 ± 5 nm) and without the protein (10 ± 5 nm). The former shows an increment in diameter attributed to the immobilization of the protein at the MWCNTs-COOH sidewall. The fact this spiderweb appears only in some regions of the MWCNTs-COOH surface supports the suggestion of a covalent interaction, given that the protein is capable of only binding to the oxidized part of the surface. As mentioned in Section 4.1, the (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite was rinsed with deionized water several times with the intention of eliminating any weak interactions (physical adsorption) between the MWCNTs-COOH and the protein. Therefore, the only interactions that are expected are strong covalent

![Figure 2](image.png)

Figure 2. FTIR microspectroscopy of the (a) (Lys)$_6$-tagged rHbI, (b) MWCNTs-COOH, and (c) (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite. The inset show a micrograph of the sample (circled white star) (Lys)$_6$-tagged rHbI/MWCNTs-COOH biocomposite region and (black star) solvent region. All of the spectra represent an average of two or more regions in the sample.

![Figure 3](image.png)

Figure 3. TEM images of MWCNTs-COOH (a) without (Lys)$_6$-tagged rHbI and (b) with (Lys)$_6$-tagged rHbI. The arrow indicates the typical feature of the attached (Lys)$_6$-tagged rHbI.
bonds between the oxidized part of MWCNTs-COOH and the (Lys)_6-tagged rHbI. Because the oxidation on the MWCNTs sidewalls is unevenly distributed, it is expected that the conjugation of the (Lys)_6-tagged rHbI on the surface of MWCNTs-COOH will also be uneven. Therefore, it is not surprising that TEM images of the biocomposite are not equally distributed or smooth in appearance. Nonetheless, the differences in the morphology on the sidewall of MWCNTs-COOH with/without (Lys)_6-tagged rHbI observed by TEM agree with the FTIR results, which strongly support the argument that the protein is covalently attached to the MWCNTs-COOH.

Recently, some research groups reported excellent results of hemeprotein immobilization on multiwalled carbon nanotubes using a different strategy. They modified the MWCNTs-COOH surfaces with L-lysine by electrodeposition followed by a second step involving the catalase immobilization onto the poly-L-lysine-MWCNTs. However, the present work is entirely different because the polylysine tag was added to the C-terminal codons of a recombinant hemoglobin I expression construct. Thus, this new (Lys)_6-tagged rHbI was immobilized onto the functionalized MWCNTs-COOH by EDC/NHS. The FTIR and TEM results demonstrated chemical attachment instead of (Lys)_6-tagged rHbI/MWCNTs biocomposite protein adsorption. Thus, this is an alternative that can be used to attach a biocomponent.

2.2. Surface Analysis of (Lys)_6-Tagged rHbI/VACNTs-COOH Biocomposite. MWCNTs-COOH powder was first used as a model to determine the effectiveness of the immobilization. We then moved to an electrode of oxidized VACNTs-COOH, which also have carbonyl sites where the protein can be attached, but the main advantage of this electrode is that it is highly sensitive due to the high surface area, which improves the detection limits and the signal-to-noise ratio. We therefore used the VACNTs-COOH electrode for SEM and electrochemical analyses. Figure 4a,b shows impressive SEM images of VACNTs-COOH and (Lys)_6-tagged rHbI/VACNTs-COOH. The images demonstrate that the VACNTs-COOH and the (Lys)_6-tagged rHbI/VACNTs-COOH biocomposite have the appearance of bonfires. The differences in the appearance of both bonfires are due to the immobilization of (Lys)_6-tagged rHbI. Although this bundle formation (Figure 4a) can be initially due to the exposure of the VACNTs-COOH to the solution and the drying process, we suggest that the differences in Figure 4a,b are due to the immobilization of (Lys)_6-tagged rHbI. As shown in Figure 4b, the (Lys)_6-tagged rHbI/VACNTs-COOH electrode is wider on top and on the base of the bonfire as compared to the VACNTs-COOH bunches (Figure 4a). Similarly, the three-dimensional (3D) AFM images show a slight increase in height, as well as width on the top and the base, of the bonfires arrangements. These results are in accordance with the results obtained by the TEM images (Figure 3b) where it can be easily observed how the (Lys)_6-tagged rHbI attach to the MWCNTs-COOH sidewall, causing an increment in its thickness. The height differences can be assessed by the increment in the root mean square roughness (Rq), which varies from 963 to 1168 nm, whereas the width increases from 457 ± 60 to 781 ± 101 nm. This represents an increment of approximately 58%, similar to the one observed with TEM analysis of modified and unmodified MWCNTs-COOH. EDS spectra corroborate the...
presence of the protein, taking into consideration the absence of nitrogen in the bare VACNTs-COOH versus the presence of nitrogen (1–2 atom %) when the (Lys)$_6$-tagged rHbI was immobilized on the surface of VACNTs. Overall, the SEM and AFM results demonstrated that the (Lys)$_6$-tagged rHbI was successfully attached to the new VACNTs-COOH electrode.

2.3. Electrochemical Characterization of the (Lys)$_6$-Tagged rHbI/Carbon Substrate Biocomposite and the Interaction with H$_2$S.

To further evaluate the covalent immobilization of the (Lys)$_6$-tagged rHbI on the oxidized VACNTs electrode and the electrochemical response of this biocomposite, cyclic (CV) and square-wave voltammetry (SWV) were employed.

2.3.1. Immobilization of the (Lys)$_6$-Tagged rHbI at VACNTs-COOH by CV. The measurement of the electrochemical response of the electrode to K$_3$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ by CV is a valuable tool for validating the immobilization on the VACNTs. For example, an increase in the current implies an efficient electron transfer process between the electroactive species in the solution and the electrode surface, whereas a decrease in the current indicates that there are molecules attached on the electrode surface that reduce to some extent the electron transfer process. Changes in the capacitance while keeping the same surface with the same dielectric constant also provide information about the area, in this case, about the electrode surface area. Consequently, a decrease in the capacitance implies a decrease in the surface area of the VACNTs-COOH due to the blocking effects of the molecules modifying the surfaces of the nanotubes. A high capacitance indicates a large surface area, which is the characteristic of oxidized empty VACNTs.

Figure 5 shows the CV of VACNTs-COOH before (solid black) and after (dashed red) the immobilization of (Lys)$_6$-tagged rHbI in the presence of 1.0 mM K$_3$Fe(CN)$_6$ (0.1 M PBS, pH 7.0), with a scan rate of 50 mV s$^{-1}$.

![Image](https://example.com/image)

**Figure 5.** Cyclic voltammograms of the oxidized VACNTs-COOH before (solid black) and after (dashed red) the immobilization of (Lys)$_6$-tagged rHbI in the presence of 1.0 mM K$_3$Fe(CN)$_6$ (0.1 M PBS, pH 7.0), with a scan rate of 50 mV s$^{-1}$.

Figure 5, a decrease in the non-Faradaic current after immobilization of the (Lys)$_6$-tagged rHbI is observed. This decrease in the capacitance is attributed to the (Lys)$_6$-tagged rHbI being on the VACNTs-COOH surface or between them, diminishing the access of the electrolyte solution to the interior of the VACNTs-COOH film. In others words, the immobilized protein at the sidewall and/or top of the VACNTs-COOH decreases its surface area, reducing to some extent the accessibility of the K$_3$Fe(CN)$_6$ solution. In conclusion, the decrease in capacitance after protein immobilization suggests that the (Lys)$_6$-tagged rHbI not only attaches to the VACNTs-COOH but also allows the transfer of electrons to the electrode. To prove this suggestion, we use the square-wave voltammetry (SWV), which is the most appropriate technique to overcome the larger double charging in the VACNTs-COOH due to the Faradaic current because it suppressed the non-Faradaic background currents much more effectively than CV. SWV measures the Faradaic current at a suitable time after the potential pulse occurs in which the non-Faradaic current decays to the point of becoming negligible.

2.3.2. Characterization of the (Lys)$_6$-Tagged rHbI/VACNTs-COOH by Square-Wave Voltammetry (SWV). SWV is a powerful technique for electroanalysis with the advantages of speed and very low detection limits. SWV is widely known for its better signal-to-noise ratio and resolution when compared to CV due to the minimization of current capacity. It allows one to effectively discriminate non-Faradaic current from Faradaic current, which enables very low detection limits. Faradaic current yields information about the electrochemical behavior of the oxidized VACNTs with and without (Lys)$_6$-tagged rHbI. Figure 6a shows the SWV for the VACNTs-COOH before and after the immobilization of (Lys)$_6$-tagged rHbI. There is a remarkable displacement toward more negative potentials and a decrease in current upon conjugation with the protein. This shift in the peak potential ($E_p$) from 0.029 V (solid black line, empty VACNTs-COOH) to $E_p$ at $-0.170$ V (dashed red line) after immobilization implies that the protein is indeed attached to the VACNTs-COOH. Therefore, this $E_p$ at $-0.170$ V represents the redox potential of the protein. This was confirmed by analyzing the forward (oxidation) and reverse (reduction) process.

Figure 6b shows the forward (dashed red) and reverse (dashed black) scan of SWV for the (Lys)$_6$-tagged rHbI/VACNTs-COOH at pH 6.0. The data clearly suggest that the redox process is reversible. To determine the net current, we subtracted the forward from the reverse process, which is shown in the inset. The observation that the net current is positive confirms that the redox process is reversible. In both cases, the forward and reverse peaks were broad, suggesting an heterogeneous VACNTs-COOH surface, which is in accordance with the SEM and AFM data. As shown in the inset of Figure 6b, a large net current is produced because the forward and reverse currents contribute almost equally to it. A similar phenomenon is seen in films with reversible electron transfer that have restricted internal diffuse mass transport.

It also important to note the broad SWV exhibited by the (Lys)$_6$-tagged rHbI/VACNTs-COOH biocomposite in Figure 6b. Numerous aspects can contribute to the broadening of the SWV peak, which suggests possible thermodynamic or kinetic dispersions. Some of the reasons for this behavior can be attributed to the repulsion between immobilized (Lys)$_6$-tagged...
rHbI, differences in the spatial distribution of its redox centers, and dispersion of peak potentials ($E_p$). In this case, the repulsion between the immobilized (Lys)$_6$-tagged rHbI is attributed to the protein distribution and its volume, whereas the dispersion of peak potentials is related to its redox center (mixture of Fe(III)/Fe(II)). This (Lys)$_6$-tagged rHbI/VACNTs-COOH biocomposite behaves similar to those models of confined redox molecules, such as Cytochrome c oxidase, which strongly adsorbs in a stable functional state at near-monolayer coverage on carboxylic acid terminated self-assembled monolayer. Thus, SWV not only showed that (Lys)$_6$-tagged rHbI is electrochemically active but that it also exhibited a remarkable displacement in the $E_p$ that clearly distinguished the VACNTs-COOH alone from the (Lys)$_6$-tagged rHbI/VACNTs-COOH electrode compared to VACNTs-COOH without protein that under the same conditions exhibited an average $E_p$ of (0.039 ± 0.005) V vs Ag/AgCl. The $I_p$ has a higher standard deviation $I_p$ = (407 ± 99) μA, which is not surprising considering that the nonhomogeneous surface of CNTs does not necessarily generate a uniform protein distribution as explained in Section 2.2. Because TEM images indicate that MWCNTs are not modified evenly, we can assume an uneven modification in the VACNTs as well. Thus, there will be slight differences between the modified electrodes that affect the $I_p$ and $E_p$ measurements.

2.3.3. Electrochemical Response toward H$_2$S. To evaluate the activity of (Lys)$_6$-tagged rHbI immobilized on VACNTs-COOH, the SWV of (Lys)$_6$-tagged rHbI/VACNTs-COOH biocomposite in the presence of different concentrations of H$_2$S was analyzed. This study helps to provide information about the protein function retention after immobilization. The electrochemical behavior of (Lys)$_6$-tagged rHbI toward H$_2$S was evaluated at pH 6.0. H$_2$S is a weak acid with a first pKa value between 6.97 and 7.06 at 25 °C. This is very important to control because according to the Le Chatelier’s Principle, at this pH, the equilibrium will continually shift to the left, generating H$_2$S(aq).

$$H_2S(g) \leftrightarrow H_2S(aq) \leftrightarrow HS^-(aq) + H^+(aq) \leftrightarrow S^{2-}(s) + 2H^+(aq)$$

It is well established that at pH = 7.4, the ratio is ~30% as H$_2$S(aq) and 70% HS$^-$(aq). Thus, at pH 6, the (Lys)$_6$-tag rHbI Fe(III)–H$_2$O complex was present and, after addition of H$_2$S, the following equilibrium is suggested.

$$\text{(Lys)$_6$-tag rHbI Fe(III)} \leftrightarrow \text{rHbI Fe(III)} \leftrightarrow \text{H}_2\text{S} + \text{H}_2\text{O}$$

Before adding H$_2$S, several scans were conducted to assure the electrochemical oxidation of Lys-tag rHbI Fe(II) to (Lys)$_6$-tag rHbI Fe(III). The strategy was to monitor the current until no changes were observed, which confirmed that all of the protein was in the Lys$_6$-tag rHbI Fe(III) oxidation state. Before the successive scanning from −0.400 to 0.400 V, the potential was held at −0.400 V for 60 s. During this holding period, H$_2$S was added. After the addition of H$_2$S, formation of the Fe(III)–H$_2$S derivative (see reaction 2) is expected, considering the large association rate constant $1.4 \times 10^6$ M$^{-1}$ s$^{-1}$ for the formation of the (Lys)$_6$-tag rHbI Fe(III)–H$_2$S at neutral pH.

Figure 7 shows the electrochemical response of the (Lys)$_6$-tag rHbI/VACNTs in three replicate electrodes after the addition of H$_2$S. As shown in Figure 7, small changes in the (Lys)$_6$-tag rHbI Fe(II)/(III) oxidation peak current of (Lys)$_6$-tag rHbI/VACNTs-COOH were observed upon H$_2$S addition. For each electrode and before any H$_2$S addition, a forward scan was done to determine the $E_p$. Then, for each subsequent H$_2$S addition, changes in the current peak ($I_p$) were monitored. As previously discussed, several reasons are responsible for the variation in $E_p$ but on average, we report an $E_p$ of (−0.18 ± 0.04) V. Although the objective of the work presented here is to show that the (Lys)$_6$-tagged rHbI/MWCNTs biocomposite is a robust electrode construction and an electrochemically active system capable of binding a heme ligand, using hydrogen sulfide as an example, the present bioelectrode serves as a proof of concept, capable of binding and detecting H$_2$S. Although the system is not optimized as a quantitative sensor for hydrogen...
In all of the cases, the oxidation peak current for the (Lys)_6-tagged rHbl/VACNTs-COOH increases with an increase in H_2S concentration. This suggests that as soon as Fe(II) is oxidized to Fe(III) in an H_2S environment, the formation of the (Lys)_6-tagged rHbl molecules are available to form (Lys)_6-tagged rHbl (Fe(III)−H_2S) and a plateau is finally reached. As shown in Figure 7, changes in the (Lys)_6-tagged rHbl oxidation peak current of the (Lys)_6-tagged rHbl/VACNTs were observed initially until a plateau was reached. In general, the observed increased in current is attributed to a change in Hbl environment, indicating that reaction 2 is taking place. Once the reaction is completed, the Hbl binding sites are occupied by H_2S, the reaction reached an equilibrium and a plateau is observed. It is possible to observe that at low concentrations, the current is directly proportional to the (Lys)_6-tagged rHbl/VACNTs-COOH concentrations, whereas at higher concentrations (greater than 150.0 μM), where the number of vacancies (Fe(III)) on the (Lys)_6-tagged rHbl/VACNTs will be limited, the current begins to reach a plateau. This observed behavior of the interaction, chemisorption of H_2S at Fe(III)−(Lys)_6-tagged rHbl/VACNTs looks like a Langmuir–Freundlich isotherm, where Fe(III)−(Lys)_6-tagged rHbl/VACNTs would represent the adsorbent, whereas H_2S would be like an adsorbate. Because the data fit very well for both sorption isotherms, we do not consider the random sequential adsorption model in this analogy. However, the authors understand that an additional analysis is necessary before establishing a model and to avoid a misleading approach.

Overall, all of the replicate electrodes showed changes in current and a small displacement toward more positive potentials (30–40 mV) with each H_2S addition. This suggests that the electronic transfer between the protein and electrode is slower. Moreover, we observed that the coverage does not affect the E_p. The results not only showed the electrochemical response of the (Lys)_6-tagged rHbl/VACNTs-COOH and toward H_2S, but it also demonstrates that the behavior is reproducible.

3. CONCLUSIONS

The results demonstrated that the Lys tag interacts strongly with the MWCNTs-COOH as well as with VACNTs-COOH. This suggests that the tag can also be used to covalently anchor the protein on conductive supports like VACNTs-COOH arrays for a potential diverse application including a future development of a bioelectrode for H_2S detection. Overall, the results established that the location of the poly-Lys tag facilitates the site-selective and covalent immobilization of the (Lys)_6-tagged rHbl. We propose that amidation between the carboxylic groups of MWCNTs-COOH and the amine groups of the lateral chain of the lysine tag was successfully achieved. Declaring this type of conjugation results is difficult when it is known that CNTs have a natural affinity for diverse proteins through hydrophobic and electrostatic interactions. However, some considerations were taken to differentiate between covalent attachments and protein adsorption. First, the immobilization reaction was performed in two steps, where an amine reactive NHS ester was promoted by the addition of coupling agents and then the (Lys)_6-tagged rHbl was added. Second, the resulted (Lys)_6-tagged rHbl/MWCNTs bicomposite was washed several times with deionized water to remove noncovalent adsorbed (Lys)_6-tagged rHbl. The FTIR,
TEM, SEM, and electrochemical analysis support this suggestion. For example, FTIR microspectroscopy results showed a displacement in the peaks corresponding to amide I and amide II, 1657 and 1548 cm⁻¹, respectively, toward 1643 and 1535 cm⁻¹, when the hemeprotein was immobilized on the oxidized MWCNTs surface. Differences in morphology on the sidewall of MWCNTs before and after (Lys)₆-tagged rHbI were observed by TEM, SEM, and AFM, supporting the FTIR findings. The electrochemical (CV and SWV) studies showed the clear evidence of the presence of immobilized (Lys)₆-tagged rHbI at VACNTs.

The (Lys)₆-tagged rHbI/VACNTs showed good electrochemical activity, suggesting that other carbon substrates can also be used to improve this behavior. In addition, SWV demonstrated to be an effective technique to estimate the potential for the (Lys)₆-tagged rHbI Fe(III)/Fe(II) redox couple. More importantly, the (Lys)₆-tagged rHbI/VACNTs biocomposite showed good electrocatalytic activity with various H₂S concentrations. The use of the (Lys)₆-tagged rHbI as a bioelectrode for H₂S detection system will be the subject of further studies.

4. EXPERIMENTAL SECTION

MWCNTs-COOH, in powder form, was first used as a model to understand and to evaluate the immobilization process. We then used VACNTs-COOH because they exhibit a high electrocatalytic activity and fast electron transfer, which can be used for highly sensitive detection of redox species. In addition, vertical nanotube arrays are suitable for fabricating various electronic devices and sensors.

4.1. Immobilization Reaction of (Lys)₆-Tagged rHbI at MWCNTs-COOH. The immobilization reaction was conducted with the (Lys)₆-tagged rHbI in the presence and absence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) and with the (His)₆-tagged rHbI in the presence of EDC/NHS. EDC and NHS were employed to transform the hydroxyl group of the carboxylic group into a good leaving group (amine-reactive NHS ester), which favors the amide bond formation with the protein. This amide bond formation occurs by a nucleophilic attack of the amine side chain of the lysine. Thus, in the absence of EDC/NHS, no covalent modification is expected. Equally, no covalent immobilization is expected for the (His)₆-tagged rHbI through the His tag. As stated in the Introduction, His tagged proteins are mainly attached to the surfaces via noncovalent immobilization such as adsorption. These two reactions were therefore used as controls. It is important to mention that unoxidized MWCNTs were not evaluated here because previous studies have demonstrated that the amino acids with aromatic side chains in (Lys)₆-tagged rHbI, which have π electrons available to interact with the π electrons on the surface of CNTs, are buried residues and most likely would not react with the CNTs. In addition, many of the literature reviewed suggest a more efficient electron transfer between proteins and electrodes when exist a direct and strong attachment among them.

The reaction of the (Lys)₆-tagged rHbI in the presence of coupling agents was conducted by sonicking the MWCNTs-COOH with MES buffer pH 6.0, containing 0.2 M EDC and 0.1 M NHS for 1 h to ensure homogeneous dispersion, as well as the generation of a semistable reactive NHS ester intermediate. Purified (Lys)₆-tagged rHbI (5–20 mg in 0.1 M PBS, pH 7) was then added, and the reaction was stirred for 16–18 h at 4 °C. Nonconjugated adsorbed hemeprotein was removed by thoroughly washing the nanotubes with water until no (Lys)₆-tagged rHbI UV–vis signal was detected in the washings (about eight washing cycle). (Lys)₆-tagged rHbI shows a strong Soret band at 407 nm, with the corresponding Q bands at 502 and 633 nm. The absence of these bands indicates the removal of protein in the suspension. The amount of immobilized protein was evaluated by measuring the concentration of the hemeprotein remaining in the supernatant after the immobilization process using the Bradford method. In the Bradford method, the absorbance was measured at 595 nm and related with protein concentrations. The same procedure was carried out without coupling agents and with the (His)₆-tagged rHbI as controls. In both cases, once the reaction was completed, the collected supernatant in each washing step was analyzed by UV–vis and Bradford assay to determine the amount of protein bound to the MWCNTs-COOH using differential analysis. Table 1 shows an example of the mathematical analysis to determine the concentration of immobilized protein.

4.2. Immobilization Analysis of (Lys)₆-Tagged rHbI/MWCNTs-COOH Biocomposite. FTIR microspectroscopy and TEM were used to assess the conjugation of the (Lys)₆-tagged rHbI at oxidized MWCNTs. For the FTIR analysis, 1 μL of the sample was pipetted on the CaF₂ window and dried under N₂ atmosphere. IR microspectra were recorded using a Perkin Elmer Spectrum Spotlight microscope equipped with a 16-element quasi-linear, photoconductive mercury cadmium telluride detector array. Spectra were collected in transmission mode from 4000 to 700 cm⁻¹, with 20 scans per point and an aperture of 50 × 50 μm². For background correction, one measurement of an empty site with only the window present was used. The same procedure was used for the controls ((Lys)₆-tagged rHbI without coupling agent and (His)₆-tagged rHbI with coupling agent). To correct for scattering effect, a baseline correction with a piecewise liner approach was performed on each spectrum. TEM were recorded on a JEOL JEM-1400 TEM operating at 120 kV with a charge-coupled device camera. A drop of sample dispersed in water was placed on a copper grid and dried. TEM analysis was conducted at the Center of Functional Nanomaterials at Brookhaven National Laboratory.

4.3. Immobilization Analysis of (Lys)₆-Tagged rHbI on a VACNTs-COOH. The immobilization procedure on this substrate was conducted following the same procedure described in Section 2.1, with minor modifications. For example, the surface was immersed for at least 30 min on 50 mM MES pH 6.0 to guarantee the carboxylate groups. The carboxylic acid groups were activated by adding 200 μL of 400 mM EDC/100 mM NHS in 100 mM MES at pH 6.0 to the electrode surface for 1 h, and then the (Lys)₆-tagged rHbI was immobilized at the oxidized carbon surface. The immobilization reaction was conducted by the addition of 100 μL of the (Lys)₆-tagged rHbI (1–5 mg/mL) for 16–18 h at 4 °C. The nonimmobilized protein was removed by washing with 0.1 M MES pH 6 as explained before.

The aligned carbon nanotube array used was grown by plasma-enhanced chemical vapor deposition (PEVCD) on 50 nm chromium-coated Si substrate, with a nanotube diameter ~100 nm and target length 5–10 μm. Nanotubes site density is ~5 × 10⁶ CNTs/cm² and attaching COOH groups to PEVCD carbon nanotubes were part of the properties of this VACNTs.
They were purchased from NanoLab, Inc. with the above-described characteristics.

4.4. Surface Analysis on VACNTs-COOH. The atomic force microscopy experiments were done on a Nanoscope Va from Digital Instrument using a silicon tip at ambient atmosphere and temperature. AFM images were obtained in tapping mode. Scan rates were varied from 1 to 2 Hz. The images presented here were repeatedly obtained in several spots of the sample. All of the images (256 × 256 pixels) were essentially unfiltered and analyzed with NanoScope 8.0 software. Scanning electron microscopy (SEM) was carried out using a JEOL JSM-6480 LV, and energy-dispersive spectroscopy (EDS) was done using an EDAX Genesis 2000 at 20 kV.

4.5. Electrochemical Immobilization Analysis of (Lys)₆-Tagged rHbI on a VACNTs-COOH. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were performed using an EC-Lab v10.44 workstation (BioLogic Science Instrument). A conventional three-electrode cell was used with a silver chloride (Ag/AgCl, NaCl 3 M) reference electrode, a platinum wire counter electrode, and with VACNTs-COOH (carbon substrate) as working electrode. The cyclic voltammetry experiments were done in a solution of 1.0 mM of K₂Fe(CN)₆ in 0.1 M PBS at pH 7.0 from −0.400 V to 0.200 V vs Ag/AgCl, NaCl 3 M with a scan rate of 50 mV s⁻¹. Cyclic voltammetry on electrodes before and after immobilization of (Lys)₆-tagged rHbI was done in buffer solutions without the protein. Buffers were purged with highly purified nitrogen for at least 10 min before a series of experiments. The cell was closed during the experiment to protect the solution from oxygen. In the experiments with H₂S, experiments. The cell was closed during the experiment to refrigerate at 2°C. Subsequently with a rubber stopper, kept in the dark, and immobilization of (Lys)₆-tagged rHbI was done in buffer solutions with VACNTs-COOH (carbon substrate) as working electrode. All of the experiments were done at ambient temperature (20.0 ± 0.5°C).

For the characterization with H₂S, a solution 4 mM in H₂S was used. The H₂S stock solution was prepared by placing 20 mL of 0.1 M succinic acid, at pH 6.0, in a vial and purged vigorously with nitrogen gas for 15 min before adding 9.60 mg of sodium sulfide (Na₂S·9H₂O). The vial was sealed subsequently with a rubber stopper, kept in the dark, and refrigerated at 2–8°C until used after no more than 6 h of storage.

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**REFERENCES**

(1) Steen Redeker, E.; Ta, D. T.; Cortens, D.; Billen, B.; Guedens, W.; Adriaensens, P. Protein engineering for directed immobilization. *Bioconjug. Chem.* 2013, 24, 1761–77.

(2) Johnson, C. P.; Jensen, I. E.; Prakash, A.; Vijayendran, R.; Leckband, D. Engineered protein a for the orientational control of immobilized proteins. *Bioconjug. Chem.* 2003, 14, 974–8.

(3) Kweon, D. H.; Kim, S.-G.; Han, N.-S.; Lee, J.-H.; Chung, K. M.; Seo, J.-H. Immobilization of Bacillus macerans cyclodextrin glycosyltransferase fused with poly-lysine using cation exchanger. *Enzyme Microb. Technol.* 2005, 36, 571–578.

(4) Bello-Gil, D.; Maestro, B.; Fonseca, J.; Fellu, J. M.; Climent, V.; Sanz, J. M. Specific and reversible immobilization of proteins tagged to the affinity polypeptide C-LyTA on functionalized graphite electrodes. *PLOS One* 2014, 9, No. e87995.

(5) Kweon, D. H.; Lee, D. H.; Han, N. S.; Rha, C. S.; Seo, J. H. Characterization of polycationic amino acids fusion systems for ion-exchange purification of cyclodextrin glycosyltransferase from recombinant *Escherichia coli*. *Biotechnol. Prog.* 2002, 18, 303–8.

(6) Brewer, S. J.; Sassenfeld, J. M. The purification of recombinant proteins using C-terminal polyarginine fusions. *Trends Biotechnol.* 1985, 3, 119–122.

(7) Nock, S.; Spudich, J. A.; Wagner, P. Reversible, site-specific immobilization of polyarginine-tagged fusion proteins on mica surfaces. *FEBS Lett.* 1997, 414, 233–8.

(8) Pessela, B. C.; Mateo, C.; Carrascosa, A. V.; Vian, A.; Garcia, J. L.; Rivas, G.; Alfonso, C.; Guisan, J. M.; Fernandez-Lafuente, R. One-step purification, covalent immobilization, and additional stabilization of a thermophilic poly-His-tagged beta-galactosidase from Thermus sp. strain T2 by using novel heterofunctional chelate-epoxy Sepabeads. *Biomacromolecules* 2003, 4, 107–13.

(9) Ley, C.; Holtmann, D.; Mangold, K. M.; Schrader, J. Immobilization of histidine-tagged proteins on electrodes. *Colloids Surf., B* 2011, 88, 539–51.

(10) Allard, L.; Cheynet, V.; Oriol, G.; Mandrand, B.; Delair, T.; Mallet, F. Versatile method for production and controlled polymer-immobilization of biologically active recombinant proteins. *Biotechnol. Bioeng.* 2002, 80, 341–8.

(11) Ladavie, C.; Delair, T.; Domard, A.; Novelli-Rousseau, A.; Mandrand, B.; Mallet, F. Covalent immobilization of proteins onto (Methane anhydride-alt-methyl vinyl ether) copolymers: enhanced immobilization of recombinant proteins. *Bioconjug. Chem.* 1998, 9, 655–61.

(12) Díaz-Ayala, R.; Moya-Rodriguez, A.; Pietri, R.; Cadilla, C. L.; Lopez-Garriga, J. Molecular Cloning and Characterization of a (Lys)₆-

**ASSOCIATED CONTENT**

Supporting Information

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Current responses exhibited by the (Lys)₆-tagged rHbI/MWCNTs biocomposite toward different H₂S concentration (PDF)

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Notes

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Tagged Sulfide-Reactive Hemoglobin I from Lucina pectinata. Mol. Biotechnol. 2015, 57, 1050–62.

(13) Kraus, D. W.; Wittenberg, J. B. Hemoglobins of the Lucina pectinata/bacteria symbiosis. I. Molecular properties, kinetics and equilibria of reactions with ligands. J. Biol. Chem. 1990, 265, 16043–53.

(14) Kraus, D. W.; Wittenberg, J. B.; Lu, J. F.; Peisach, J. Hemoglobins of the Lucina pectinata/bacteria symbiosis. II. An electron paramagnetic resonance and optical spectral study of the ferric proteins. J. Biol. Chem. 1990, 265, 16054–9.

(15) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. Direct Electrochemistry of Redox Proteins. Acc. Chem. Res. 1988, 21, 407–413.

(16) Baffert, C.; Sybina, K.; Ezzano, P.; Lautier, T.; Hajj, V.; Meynial-Salles, I.; Soucaille, P.; Bottin, H.; Leger, C. Covalent attachment of FeFe hydrogenases to carbon electrodes for direct electron transfer. Anal. Chem. 2012, 84, 7999–8005.

(17) Lao, H.; Zhao, H.; Chang, Y.; Wang, Q.; Yu, H.; Shen, Z. Oriented immobilization and characterization of a poly-lysine-tagged cephalosporin C acylase on glyoxyl agarose support. J. Chem. Rev. 2010, 110, 5366–5397.

(18) Karousis, N.; Tasis, D.; et al. Current Progress on the Chemical Modification of Carbon Nanotubes. Chem. Rev. 2010, 110, 5366–5397.

(19) Chen, R. J.; Drouvalakis, K. A.; Shi Kam, N. W.; Shim, M.; Li, Y.; Kim, W.; Utz, P. J.; Dai, H.; et al. Noncovalent functionalization of carbon nanotubes for high specific Electronic biosensors. Proc. Natl. Acad. Sci. U.S.A. 2003, 100, 4984–4989.

(20) Wu, X. C.; Zhang, W. J.; Sammynaiken, R.; Meng, Q. H.; Yang, Q. Q.; Zhan, E.; Liu, Q.; Yang, W.; Wang, R. Non-functionalized carbon nanotube binding with hemoglobin. Colloids Surf., B 2008, 65, 146–9.

(21) Safiuddin, N.; Raziah, A. Z.; Junizah, A. R. Carbon Nanotubes: A Review on Structure and Their Interaction with Proteins. J. Chem. 2013, 2013, 1–18.

(22) Vardharajula, S.; Ali, S. Z.; Tiwari, P. M.; Erogul, E.; Vig, K.; Dennis, V. A.; Singh, S. R. Functionalized carbon nanotubes: biomedical applications. Int. J. Nanomed. 2012, 7, 5361–74.

(23) Fleming, B. D.; Zhang, J.; Bond, A. M.; Armstrong, F. A.; et al. Application of Power Spectra Patterns in Fourier Transform Square Wave Voltammetry To Evaluate Electrode Kinetics of Surface-Confined Proteins. Anal. Chem. 2006, 78, 2948–2956.

(24) Verma, M. L.; Naebu, M.; Barrow, C. J.; Puri, M. Enzyme immobilisation on amino-functionalised multi-walled carbon nanotubes: structural and biocatalytic characterisation. PLoS One 2013, 8, No. e73642.

(25) Susi, H.; Byler, D. M. Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. Biochem. Biophys. Res. Commun. 1983, 115, 391–7.

(26) Jackson, M.; Mantsch, H. H. The use and misuse of FTIR spectroscopy in the determination of protein structure. Crit. Rev. Biochen. Mol. Biol. 1995, 30, 95–120.

(27) Gao, Y.; Kyrtatzis, I. Covalent immobilization of proteins on carbon nanotubes using the cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide—a critical assessment. Bioconjug. Chem. 2008, 19, 1945–50.

(28) Liu, Y.-T.; Zhao, W.; Huang, Z.-H.; Gao, Y.-F.; Ye, X.-Y.; et al. Noncovalent surface modification of carbon nanotubes for solubility in organic solvents. Carbon 2006, 44, 1613–1676.

(29) Ezhil Vilian, A. T.; Chen, S. M.; Lou, B. S. A simple strategy for the immobilization of catalase on multi-walled carbon nanotube/poly (L-lysine) biocomposite for the detection of H2O2 and iodate. Biosens. Bioelectron. 2014, 61, 639–47.

(30) Li, J. S. R.; Deltzeit, L.; Ng, H. T.; Cassell, A.; Han, J.; Meyyappan, M.; et al. Electronic properties of multiwalled carbon nanotubes in an embedded vertical array. Appl. Phys. Lett. 2002, 81, 910–912.

(31) Madduri, N. Electrochemical Capacitance Measurements to Study Molecular Surface Interactions; Clemson University, 2012.
(55) Li, J.; Cassell, A.; Tan, W.; Chen, H.; Yi, Q.; Koehne, J.; Han, J.; Meyyappan, M.; et al. Carbon Nanotube Nanoelectrode Array for Ultrasensitive DNA Detection. *Nano Lett.* 2003, 3, 597–602.

(56) Gooding, J. J.; Wibowo, R.; Liu, J.; Yang, W.; Losic, D.; Orbons, S.; Mearns, F. J.; Shapter, J. G.; Hibbert, D. B. Protein electrochemistry using aligned carbon nanotube arrays. *J. Am. Chem. Soc.* 2003, 125, 9006–7.

(57) Barker, P. D.; Di Gleria, K.; Hill, H. A.; Lowe, V. J. Electron transfer reactions of metalloproteins at peptide-modified gold electrodes. *Eur. J. Biochem.* 1990, 190, 171–5.

(58) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248–54.

(59) Baker, M. J.; Trevisan, J.; Bassan, P.; Bhargava, R.; Butler, H. J.; Dorling, K. M.; Fielden, P. R.; Fogarty, S. W.; Fullwood, N. J.; Heys, K. A.; Hughes, C.; Lasch, P.; Martin-Hirsch, P. L.; Obinaju, B.; Sockalingum, G. D.; Sule-Suso, J.; Strong, R. J.; Walsh, M. J.; Wood, B. R.; Gardner, P.; Martin, F. L. Using Fourier transform IR spectroscopy to analyze biological materials. *Nat. Protoc.* 2014, 9, 1771–91.