

AFRL-ML-TY-TP-2007-4540

PREPRINT



ENTRAPMENT OF ENZYMES AND CARBON NANOTUBES IN BIOLOGICALLY SYNTHESIZED SILICA: GLUCOSE OXIDASE-CATALYZED DIRECT ELECTRON TRANSFER

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August 2007

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Submitted for publication in "Small" Journal.

**Air Force Research Laboratory
Materials and Manufacturing Directorate
Airbase Technologies Division
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REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

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| 1. REPORT DATE (DD-MM-YYYY) | | 2. REPORT TYPE | | 3. DATES COVERED (From - To) | |
| 4. TITLE AND SUBTITLE | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION/AVAILABILITY STATEMENT | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT | | | | | |
| 15. SUBJECT TERMS | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER (Include area code) |

Entrapment of Enzymes and Carbon Nanotubes in Biologically Synthesized Silica: Glucose Oxidase-Catalyzed Direct Electron Transfer

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[**] Acknowledgments: The UNM portion of this work was supported in part by a grant from DOD/AFOSR MURI Award Number: FA9550-06-1-0264, Fundamentals and Bioengineering of Enzymatic Fuel Cells and by the NSF/URC Center for Micro-Engineered Materials that operates the XPS with support from the Keck Foundation. AFRL research was funded by the US Air Force Research Laboratory, Materials Science Directorate. HRL is an employee of Universal Technology Corporation, 1270 N. Fairfield Drive, Dayton, Ohio.

Abstract

This work demonstrates a new approach for building bio-inorganic interfaces by integrating biologically-derived silica with single-walled carbon nanotubes to create a conductive matrix for immobilization of enzymes. Such a strategy not only allows simple integration into bio-devices but presents an opportunity to intimately interface an enzyme and manifest direct electron transfer features. Biologically-synthesized silica/carbon nanotube/enzyme composites were evaluated electrochemically and characterized by means of X-ray photoelectron spectroscopy. Voltammetry of the composites displayed stable oxidation and reduction peaks at an optimal potential close to that of the FAD/FADH₂ cofactor of immobilized glucose oxidase. The immobilized enzyme was stable for a period of one month and retained catalytic activity towards the oxidation of glucose. It was demonstrated that the resulting composite can be successfully integrated into functional bio-electrodes for biosensor and biofuel cell applications.

Keywords: Nano-composite electrodes, Glucose oxidase, Silica-immobilization, Direct electron transfer, X-ray photoelectron spectroscopy

1. Introduction

Direct bioelectrocatalysis of redox enzymes has attracted increasing attention for development of the next generation of electronic nano-scale biomaterials and devices for industrial, clinical, environmental, space exploration and defense applications.^[1,2,3,4,5] The fusion of electrocatalysis with biology, for example, facilitates the development and commercialization of disposable biochips with near perfect selectivity for a given target analyte.^[4,5] Efficient communication between enzyme and electrode can also aid the development of biofuel cells with high power-output.^[1,6,7] Direct electron transfer (DET) between an enzyme and an electrode provides the most potential for miniaturization and high-power output, as the requirement for complex electron mediators is negated. In addition, an enzyme-electrode based on DET theoretically functions at a potential range that is close to the redox potential of the enzyme itself. Immobilization of enzymes for DET, however, has proved to be problematic as accessibility of the enzyme redox center and hence efficient transfer of electrons to the electrode is limited.

Glucose oxidase (GOx) is a widely studied enzyme, particularly in respect to DET and as such, provides a suitable model system applicable to the development of biosensors and biofuel cells.^[8] GOx is a stable enzyme with high catalytically active and an inexpensive substrate source; utilizing glucose as a widely available fuel. In aqueous solution at physiological pH, the redox potential of FAD/FADH₂ at the enzyme active site is negative and therefore well suited to operation at the anode of a biofuel cell.^[7,9,10] The optimal redox potential of FAD/FADH₂, however, is only achieved under DET as redox mediators tend to shift the redox to a more positive potential. Several limitations therefore must be addressed for the successful application of GOx in direct bioelectrocatalysis. The FAD/FADH₂ redox center of GOx is located deeply in the apoenzyme (~13 Å) and hence the electron-transfer rate between the active site of glucose oxidase and the electrode surface is inherently slow.^[8,11,12] Over recent years, efforts have been made to reduce the electron tunneling distance between GOx and the electrode by

using different promoters.^[3,4,7,10,11,12,13] One strategy is to incorporate the enzyme into an electrically conductive matrix such as carbon nanotubes (CNT), which potentially reduces the distance from the redox-centre of GOx to the CNT; which then act as a microelectrode surface.^[14] We have previously demonstrated the ability to utilize multi-walled CNT as an efficient conductivity matrix for DET between the active site of GOx and a carbon electrode, indicating that CNT successfully orientates the enzyme active site and redox-active cofactor with respect to the electrode surface. The resulting electron transfer rate constant of $\sim 2.4 \text{ s}^{-1}$ indicated that the heterogeneous DET process was significantly greater than previously observed for unmodified electrodes. The three-dimensional network of electronically conductive CNT significantly increases the surface area for enzyme immobilization and provides an electronic circuit as a series of “nanowires” for the enzyme.^[3,9,11] The work reported herein now utilizes single-walled CNT in place of multi-walled CNT due to superior electron-transfer properties.

The crux of bioelectrocatalysis, however, is the development of enzyme immobilization techniques that provide continuous electron transfer from the enzyme to the electrode, whilst maintaining high catalytic activity and enzyme stability. The majority of methods for enzyme immobilization utilize an inert support that serves no further specific catalytic function. When the immobilization support is an electrode or transducer surface; highly integrated functional enzymatic systems can be realized. Recent studies have demonstrated the remarkable versatility of biosilicification as a means of enzyme immobilization.^[15,16] Biosilicification is a rapid ambient precipitation of silica mediated by a biological catalyst. A wide variety of peptides and proteins can catalyze the precipitation of silica and become encapsulated as the silica matrix forms.^[17] The reaction provides an efficient method for enzyme immobilization and provides significant mechanical stability to the resulting silica matrix. Lysozyme, for example, catalyzes the formation of silica particles when mixed with a silicic acid precursor. The process is a one-step procedure and additional enzymes added during the reaction become entrained and retain a high level of catalytic activity.^[18] Initial studies

demonstrated that the technique was successful for immobilizing enzymes directly at a surface with retention of catalytic activity, but extension of the application to bioelectrocatalysis has not yet been demonstrated.^[19] This work now describes an approach to create a bio-nano interface suitable for direct electrochemistry of enzymes. Direct bioelectrocatalysis of glucose oxidation is demonstrated by entrapping GOx in a silica/CNT composite obtained through lysozyme-catalyzed synthesis of silica.

2. Results and discussion

Biocatalytic precipitation of silica composites containing GOx, were prepared on Toray® carbon paper (TP) with and without, the addition of CNT. Lysozyme provides the scaffold for silica formation and binds to the carbon electrode surface by physical adsorption; negating any requirement for chemical modification or pre-treatment of the TP. The morphology of the resulting silica precipitate was investigated by scanning electron microscopy (SEM). The filaments of TP were clearly visible by SEM (Figure 1a) and following the biosilicification reaction, a surface coated layer of silica can be clearly differentiated from the uncoated fibers. The surface morphology of the TP appears more coarse, although still uniform, indicating that the silicification reaction occurred homogeneously on the surface (Figure 1b). The majority of the silica forms as a network of fused particles in agreement with previous studies (Figure 1c). The presence of CNT serving as a matrix of nanowires throughout the silica matrix is also evident (Figure 1d).^[15]

X-ray photoelectron spectroscopy (XPS) was used to analyze the chemistry of the silica composite. XPS is a powerful technique allowing estimation of the elemental and chemical composition of the upper 10 nm of a surface and has been demonstrated as an effective tool to quantify protein immobilized or adsorbed during enzyme immobilization.^[20] The surface elemental composition and bonding in respect to C1s, O1s, N1s and Si2p core level spectra were determined at a surface depth of ~10 and ~2 nm (Table 1). TP alone, with or without CNT exhibits the same elemental compositions

with ~2% oxygen, irrespective of sampling depth. Initially, the formation of silica resulting from reaction of lysozyme with hydrolyzed tetramethylorthosilicate (TMOS) was analyzed as a control sample and exhibited a significant increase in Si and N, as expected. The complete functional samples (i.e. silica on TP containing GOx and CNT) were composed of ~5% of N and ~15% of Si, confirming the presence of both enzyme and a silica matrix. In the absence of CNT, however, a lower N (0.6%) signal and relative higher Si and O signals are observed, suggesting either a lower degree of enzyme immobilization or a more complete coverage of GOx by silica that blocks the encapsulated enzyme from XPS analysis. No changes in composition with depth were observed for any of the samples.

XPS was also used to determine O/C and N/C ratios in an effort to obtain protein fingerprints for lysozyme and GOx. Analysis of soluble solutions of GOx and lysozyme, however, showed an O/C ratio of ~0.3 for both proteins, making distinction between the two difficult. In addition, the O/C ratios for composites containing silica were much higher due to the excess of silica (SiO_2). The N/C ratio may therefore be a more appropriate measure of the interaction of enzyme chemistry at the surface. The N/C ratio predicted by the polypeptide sequences is much larger for lysozyme (0.24) than for GOx (0.13). For samples containing both GOx and lysozyme, an N/C ratio of 0.12 is very close to that of GOx alone which may indicate that the majority of enzyme detected by XPS is GOx. By comparison, in a GOx-free control, the ratio is much larger (0.18) as would be expected for lysozyme alone.

High resolution C1s and O1s spectra of each sample were obtained and deconvoluted using conventional curve fitting (Figure 2) and quantitative results were calculated (Table 2). The C1s spectrum of GOx has three main peaks corresponding to the following bonds: aliphatic $\text{C}^*\text{H}-\text{CH}$ (284.8 eV) that may have originated from surface contamination, oxydrilic C^*-OH and amidic $\text{N}-\text{C}^*\text{H}-\text{CO}$ (286.6 eV) and $\text{N}-\text{CH}-\text{C}^*=\text{O}$ (288.3 eV), where the respective carbon species are marked by an asterisk. The analysis of the binding energies of the N1s (400.2 eV) and two peaks for O1s (532 and 533.2 eV) confirmed that these elements are attributable to the enzyme component. The C1s spectrum of lysozyme also has major peaks due to C-C and C-H carbon (284.8 eV), and higher binding energy

peaks at 285.8 and 288.2 eV that are attributed to the C*-N and -C*O- NH- or -C*OO- carbon, respectively. Smaller peaks at 284.2 and 286.6 eV are thought to be due to contamination during preparation but did not interfere with the chemical analysis.

High resolution C1s spectrum of the silica composite (containing lysozyme, GOx and CNT) contains features of both pure enzyme samples, i.e. a dominant peak at 286.6 eV which corresponds to the spectra for GOx and a secondary signal at 285.8 eV, which corresponds to the primary peak in the spectra of lysozyme. A background signal of Si-C was detected in all samples. High resolution O1s spectra for pure enzymes and the silica composite all exhibit spectra corresponding to the presence of immobilized biomolecules (533 eV). In addition, the composite exhibits a high binding energy which can be attributed to the presence of silica. Complete conversion of the TMOS precursor to silica is confirmed by the presence of a very small peak (O1s spectra) due to a Si-OCH₃ bond at ~532 eV. The position of a single Si2p peak (not shown) at 104 eV also confirmed that all of the silicon present occurs as silica (silicon dioxide). XPS analysis allowed for a detailed understanding of the overall chemical composition of the silica composite and confirmed the formation of silica with the incorporation of both enzymes and CNT.

The initial microscopy and spectroscopy demonstrated the formation of a heterogeneous matrix composed of silica particles that encapsulate GOx and CNT, attached to TP as a model electrode surface. The electrochemical characteristic of the GOx/CNT/TP composite was then investigated further by cyclic voltammetry. The key issue was to determine whether GOx can undergo DET when immobilized to a carbon electrode surface. Initially, the DET between the active sites of GOx and the electrode was investigated in the absence of glucose (i.e. no catalytic turnover). Two types of carbon electrodes (screen-printed and TP) showed similar behavior. The cyclic voltammograms of GOx/CNT modified carbon electrodes (Figure 3, curve 2) show that a pair of well defined redox peaks (reduction and oxidation) was observed. The formal redox potential is -406 mV at pH 6.2 vs. Ag/AgCl, which is close to the redox potential of the FAD/FADH₂ cofactor in the enzyme itself. The redox peaks can be

attributed to the redox reaction at the active site of GOx immobilized on the surface of the electrode. Control experiments show no redox peaks in the absence of the CNT; despite the presence of GOx (Figure 3, curve 1) suggesting that electrical contact between the redox center of GOx and the carbon electrode is provided by close proximity of the CNT incorporated into the silica film. This hypothesis was validated by XPS analysis, which confirmed the co-immobilization of GOx with CNT inside the silica matrix. The intensity of the anodic and cathodic peak currents increases with increasing scan rate (Figure 4a). In addition, there is a linear proportionality between the anodic and cathodic peak currents as the scan rate ranges from 40-260 mV/s (Figure 4b); a typical characteristic of thin layer electrochemical behavior. The electrochemical characteristics indicate that the reaction is not a diffusion-controlled process and the redox peaks are from the surface bound prosthetic group FAD of the GOx. The peak separations between 24-50 mV at a scan rate of 10 – 100 mV/s indicate that the heterogeneous pseudo reversible electron-transfer process was fast. The calculated electron transfer rate constant of 2.6 s^{-1} , is comparable to that previously reported for nanotube-modified electrodes and significantly greater than the magnitude reported for self-assembled monolayer electrodes.^[9,21]

The ability of glucose oxidase to simultaneously undergo DET with the electrode and retain its catalytic activity was confirmed by demonstrating the catalytic activity of the immobilized GOx/CNT in the presence and absence of glucose (Figure 5). It was further confirmed by a standard non-electrochemical activity assay during stability studies of the immobilized enzyme; through the manifestation of its catalytic activity over a period of time (see Figure 6). Figure 5 presents cyclic voltammetry observation of the redox transformations of FAD/FADH₂ associated with the active site of GOx immobilized in the CNT/silica matrix. In the presence of glucose, the anodic and cathodic peaks are being shifted significantly towards oxidative current values. This peak shift is as a direct result of enzyme activity and can be explained by an enzyme activity-induced decrease in oxygen at the surface of the GOx modified electrode.

The high efficiency of the biosilicification reaction for the simultaneous entrapment of CNT and GOx at an electrode surface is attributed to the mild immobilization conditions; which minimize enzyme denaturation. In addition, biosilicification provides a stabilization effect to the resulting composites. As such, we examined the stability of the silica-entrapped GOx/CNT composite in respect to catalytic activity. Enzyme activity of soluble GOx with glucose was comparable in the presence or absence of CNT (data not shown). Silica encapsulation of GOx provided an immobilization efficiency of ~18% (17.9 ± 1.9). The immobilization efficiency of GOx in the presence of CNT was slightly higher at ~25% (26.0 ± 0.55) and was attributed to non-specific binding of GOx to the CNT that provides a preliminary scaffold to stabilize the enzyme activity during subsequent silica encapsulation.^[22] The silica formation appears to cause some initial inactivation of the enzyme due to alkaline reaction conditions. Silica immobilization of GOx was not as high as has been observed for other enzymes and this is attributed to the loss of activity of GOx upon disparate changes in pH. Silica formation from lysozyme requires a $\text{pH} > 7$, whereas GOx has a pI at 4.2. Although, the silica formation reaction is rapid (<2 minutes), some loss of enzyme activity upon rapid changes in local pH may occur. The immobilized enzyme, however, retained its enzymatic activity and retains a pH activity profile comparable to the native GOx suggesting that no significant catalytic modification of the enzyme active site has occurred (Figure 6a). In addition, silica-immobilized GOx is stable when stored at 25°C for up to one month. Soluble GOx undergoes slow denaturation over time under the same conditions (Figure 6b). The enhanced stability provided by silica-encapsulation may therefore provide an opportunity to develop enzyme-based DET systems that can withstand continuous operation over a time frame that has not yet been realized.

3. Conclusions

In conclusion, we have demonstrated the potential to create bio-inorganic functional nano-materials that serve as a starting point for a variety of technology solutions. Potential applications include sensor systems, actuation devices and micro-power sources. The immobilization of GOx in a silica matrix, doped with CNT, was demonstrated and supported efficient electrical conductivity. The immobilization not only stabilized enzyme activity over a period of at least one month but also facilitates mediator-free DET coupled to the oxidation of glucose. The method of co-immobilization of enzyme and CNT based on a biological silicification reaction demonstrates a number of advantageous properties including; excellent film-forming ability, good adhesion, biocompatibility and bioelectrocatalytic properties. Enzyme immobilization with direct bioelectrocatalysis is widely applicable, for example, in mechanistic studies of enzyme reactions in biological systems. Primarily, however, the GOx electrode system demonstrated herein based on DET provides significant simplification and application to the design of an anode for biofuel cell applications. The inherent change in electrochemistry of the system in the presence and absence of glucose as substrate, however, also provides a further opportunity to develop the system for sensitive glucose detection and application in the field of reagent-free glucose sensors.

4. Experimental

Materials: Glucose oxidase (GOx) from *Aspergillus niger* (EC1.1.3.4), tetramethyl orthosilicate (TMOS), 99%, lysozyme from chicken egg white (EC3.2.1.17) and carboxylated single-walled carbon nanotubes were obtained from Sigma-Aldrich (St. Louis, MO). Toray® carbon paper (TP) TGPH-060, was obtained from E-TEK, New Jersey, US (now a division of BASF). Screen-printed electrodes were obtained from Alderon Biosciences Inc. All other reagents and chemicals were of analytical grade and

obtained from standard commercial sources. Enzyme stock solutions were prepared in 0.1 M phosphate buffer (pH 7.0). All other solutions were prepared with deionized water and filtered before use.

Preparation of the enzyme/CNT/silica composites: GOx and CNT were immobilized on a screen printed carbon electrode surface and on Toray® carbon paper (TP) by entrapment within a silica matrix using a modification of immobilization methods described previously.^[15,16] For screen-printed carbon electrodes, lysozyme was non-specifically adsorbed on the working carbon electrode surface by soaking the surface in a solution of lysozyme (0.5 mL, 25 mg mL⁻¹) and the excess removed by washing with phosphate buffer (0.1 M, pH 8). A homogeneous GOx/CNT suspension was prepared by sonicating CNT (2 mg) in phosphate buffer (1 mL, 0.1 M, pH 7) for 1 hour. An aliquot (0.1 mL) of the CNT suspension was then mixed with GOx (3 mg) and sonicated for a further 20 min. The silica precipitation reaction mixture consisted of phosphate buffer (0.7 mL, pH 8); TMOS (0.1 mL, 1M in 1 mM HCl) and GOx/CNT (0.1 mL in phosphate buffer, pH 7). The silica precipitation mix (20 µL) was dropped onto the lysozyme-modified surface and incubated for 30 min at room temperature to allow the silica to form. For the preparation of TP electrodes, the lysozyme modified TP (pre-washed by sonication) was placed into a mini-column (prepared from empty Qiagen spin columns modified to accommodate a circle of TP) and the precipitation mix was flowed through the mini-column four times under gravity. Finally, both screen-printed and TP modified with CNT, GOx, and silica were washed with water and dried before analysis.

Characterization of enzyme/CNT/silica composites: The surface morphology of the GOx/CNT/Silica modified carbon electrodes was visualized using a Hitachi (S-5200) SEM equipped with an Energy Dispersive Spectrometer. The microscope was operated at 10 kV for imaging. No conductive coatings or other treatment were performed on the samples prior to SEM observations. XPS spectra were acquired using a Kratos AXIS Ultra photoelectron spectrometer using a monochromatic Al K α source operating at 300W (Base pressure; 2×10^{-10} torr and operating pressure; 2×10^{-9} torr). Charge compensation was accomplished using low energy electrons. Standard operating conditions for good

charge compensation were -4.1 V bias voltage, -1.0 V filament voltage and a filament current of 2.1 A. The sample surface survey was completed initially, followed by determination of high-resolution spectra of C1s, O1s, Si2p and N1s for all samples. Take-off angles of 90° and 15° were selected for angle resolved studies, corresponding to ~ 8 - 10 and ~ 2 nm of the surface respectively. Data is presented as the average of 1-2 samples at 3-4 areas per sample. A linear background was used of C1s, N1s, O1s and Si2p spectra. Quantification utilized sensitivity factors provided by the manufacturer. All the spectra were charge referenced to the aliphatic carbon at 285 eV. Curve fitting was carried out using individual peaks of constrained width and shape. A 70% Gaussian/30% Lorentzian line shape was used for the curve-fits.

Electrochemical measurements: Electrochemical measurements were performed with a potentiostat/galvanostat (Princeton Applied Research, Model 263A) in a three-electrode cell with a 5 mL working volume and consisting of the silica/GOx/CNT working electrode, a carbon counter electrode and an Ag/AgCl reference electrode (Bioanalytical Systems Inc., MF-2052). The electrolyte solution comprised equal volumes of phosphate buffer (0.1 M, pH 6.2) and KCl (0.1 M). Electrochemical experiments were carried out at $20^\circ\text{C} \pm 0.5$. Cyclic voltammograms were used to calculate the electron transfer rate constant using the method of Laviron.^[23]

Determination of glucose oxidase activity

The enzymatic activity of GOx was determined according to the supplier's quality control test procedure (Sigma-Aldrich) with dextrose as substrate. For stability experiments, stock samples were incubated at 25°C with shaking for 1 month. Aliquots were removed periodically for analysis of GOx activity. The pH profile of GOx was determined using the same glucose oxidase assay adjusted to a range of pH values with 1M NaOH.

Table 1. XPS elemental quantitative results

Values shown are represented as a proportion of the total (taken as 100%). TP is Toray® Carbon Paper.

| Sample | Depth (nm) | C1s | O1s | Si2p | N1s | O/C | N/C | N/C |
|-------------------|-------------------|------------|------------|-------------|------------|-------------|-------------|-------------|
| | | (%) | (%) | (%) | (%) | | | |
| TP | 10 | 97.1 | 2.9 | | | | | |
| | 2 | 97.6 | 2.4 | | | | | |
| TP + CNT | 10 | 97.6 | 2.4 | | | | | |
| | 2 | 98.5 | 1.5 | | | | | |
| TP + Silica | 10 | 46.5 | 35.9 | 9.1 | 8.5 | 0.77 | 0.18 | 0.24 |
| | 2 | 45.7 | 35.5 | 10.3 | 8.5 | | | |
| TP/Silica/GOx | 10 | 36.9 | 48.5 | 14.0 | 0.6 | 1.31 | 0.02 | 0.01 |
| | 2 | 37.2 | 49.1 | 13.8 | 0.7 | | | |
| TP/Silica/GOx/CNT | 10 | 39.5 | 42.5 | 13.1 | 4.9 | 1.08 | 0.12 | 0.11 |
| | 2 | 35.4 | 45.6 | 14.0 | 5.1 | | | |
| GOx | | 68.1 | 23.1 | | 8.8 | 0.34 | 0.13 | 0.38 |
| Lysozyme | | 65.2 | 19.1 | | 15.7 | 0.29 | 0.24 | 0.82 |

Table 2. C1s and O1s deconvolution results of XPS analysis

Values shown are represented as a proportion of the total (taken as 100%)

| | C-Si, C=C | C-C, C-H | C-NH ₂ , CO-NH, C*-C-N, C*-C-O | N-C*H-C=O, C*-OH | CN, C*-O-C=O | N-CH-*C=O | O-C*=O | O-C | O=C | Si-O ₂ |
|-----------------------|-----------|----------|---|------------------|--------------|-----------|-------------|-------------|-------------|-------------------|
| <i>Binding Energy</i> | 284.2 | 284.8 | 285.8 | 286.6 | 287.2 | 288.2 | 531.9 | 533.2 | 534.1 | |
| GOx | | 27.1 | | 57.4 | | 15.5 | 23.8 | 76.2 | | |
| Lysozyme | 12.3 | 13.9 | 38.8 | | | 35.0 | 78.7 | 21.3 | | |
| TP + Silica | 5.1 | 13.9 | 32.6 | 8.3 | 20.6 | 19.4 | | 20.5 | 79.5 | |
| TP/Silica/Gox/CNT | 2.8 | 10.3 | 14.1 | 47.6 | 12.8 | 12.4 | 6.0 | 63.6 | 29.4 | |

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Figure Captions

Figure 1. SEM micrographs of carbon paper electrodes coated with lysozyme (a) and silica at low (b) and high magnifications (c-d)

Figure 2. XPS spectra of soluble GOx (a), soluble lysozyme (b) and silica composites with entrapped GOx + CNT (c)

Figure 3. Cyclic voltammogram of GOx modified electrodes

Line 1: GOx in silica; Line 2: GOx + CNT in silica. Electrolyte described in text. Scan rate is 20 mV s^{-1}

Figure 4. Cyclic voltammograms of GOx + CNT in silica showing effect of increasing scan rate from 40 to 250 mV s^{-1} (a) and the corresponding Laviron plot (b)

Figure 5. Cyclic voltammograms of the GOx + CNT in silica in the absence (Line 1) and presence (Line 2) of 40 mM glucose at a scan rate of 20 mV s^{-1}

Figure 6. Effect of silica immobilization upon the pH profile (a) and stability (b) of immobilized GOx

Key: soluble GOx (\diamond); soluble GOx + CNT (\blacklozenge); GOx immobilized in silica (\circ); GOx + CNT immobilized in silica (\bullet). Linear regression lines were calculated using GraphPad Prism (v3.03).

Figure 1

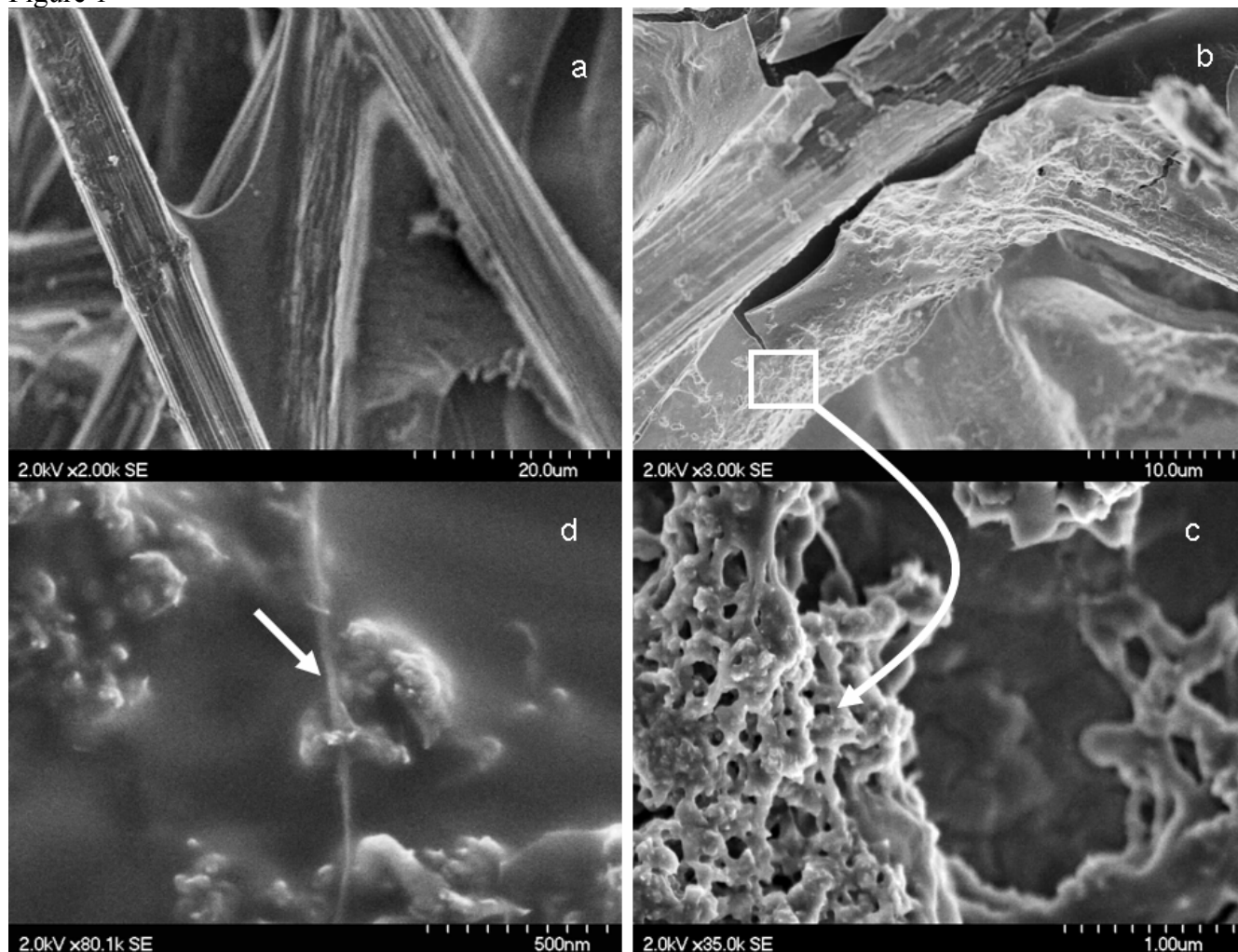


Figure 2

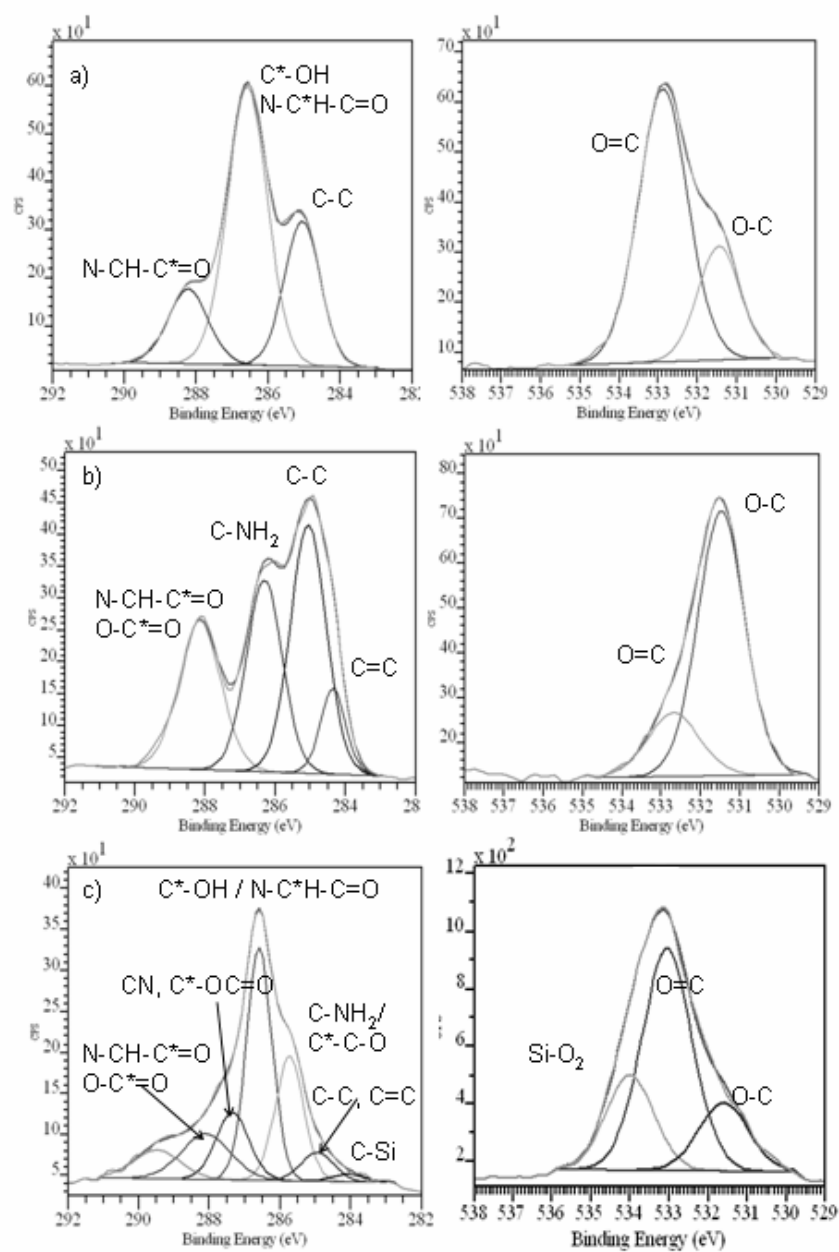


Figure 3

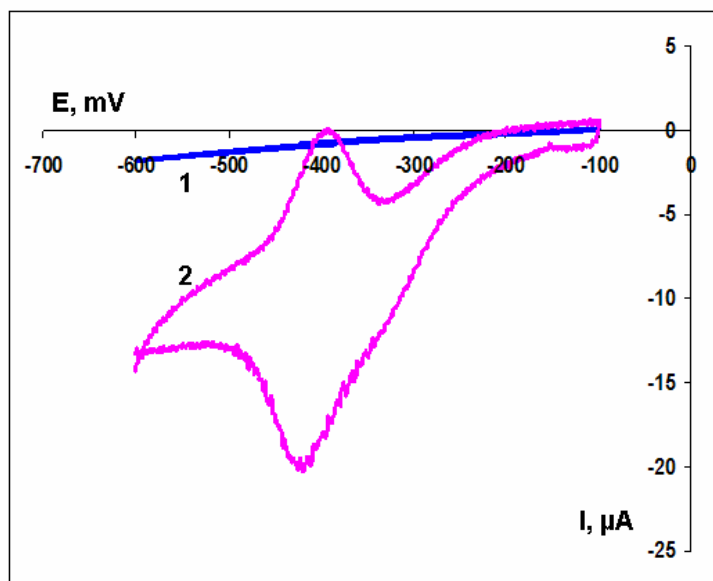


Figure 4

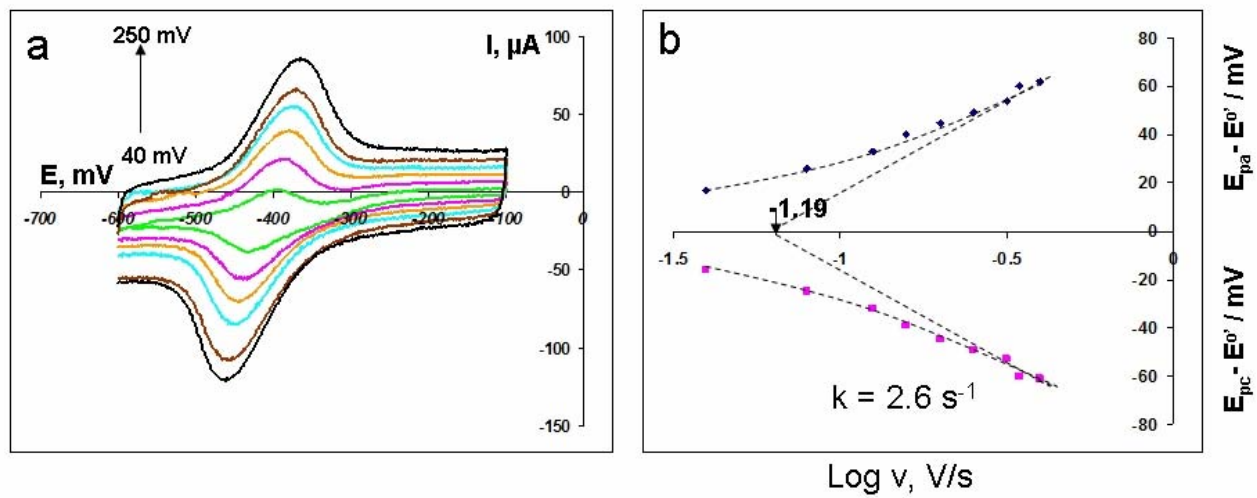


Figure 5

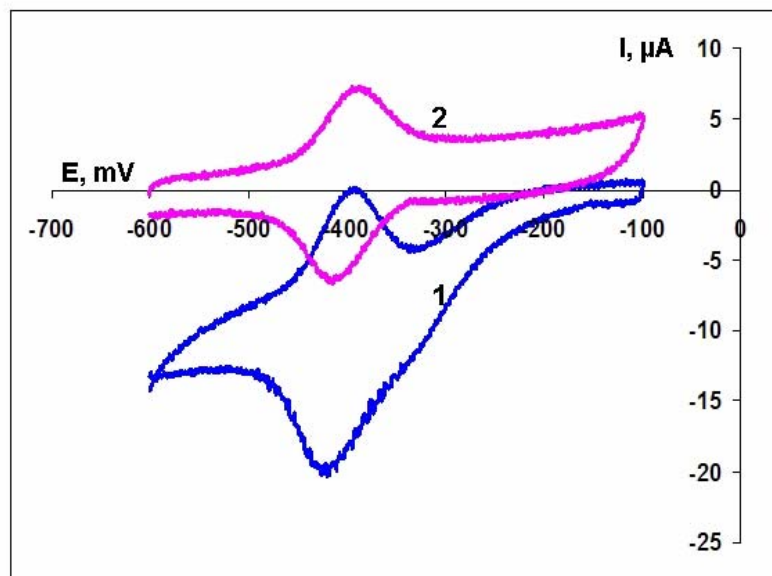


Figure 6

