Reagentless and non-reagentless nicotinamide adenine dinucleotide (NAD\(^+\))-dependent glucose dehydrogenase (GDH) bioanodes integrating multiwalled nanotubes (MWNTs)-bucky papers are introduced in this study. The NAD\(^+\) was tethered by pyrene butanoic acid succinimidyl ester (PBSE) to the wall of the carbonic MWNTs composing the bucky paper by π-π stacking interactions. The designs have been evaluated electrochemically and by X-ray spectroscopy. The electrodes have been assembled to a quasi-2D microfluidic system with capillary driven flow. Michaelis-Menten analysis on CMN-MG-PBSE-NAD\(^+\)-GDH, reagentless bioanode, in an electrolytic cell shows a limiting current and constant of \(I_{\text{Lim}} = 3.252 \pm 0.134 \text{ mA cm}^{-2}\) and \(K_M = 48.3 \pm 4.6 \text{ mM at } 4^\circ \text{C}\), and \(I_{\text{Max}} = 2.568 \pm 0.008 \text{ mA cm}^{-2}\) and \(K_M = 13.6 \pm 1.8 \text{ mM at } 25^\circ \text{C}\) for 0.1 M glucose. Results demonstrate the enzymatic system, in non-reagentless and reagentless bioanode, has an extraordinary current density generation. In the reagentless bioanode design, the enzyme and its cofactor are highly catalytically active, and the design can be feasible integrated into biofuel cell assembly and later used as a power source for small portable devices.

© The Author(s) 2014. Published by ECS. This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 License (CC BY, http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse of the work in any medium, provided the original work is properly cited. [DOI: 10.1149/2.0051413jes] All rights reserved.

The development and implementation of alternative energy systems is highly desired and envisioned for overcoming environmental issues. Furthermore, designing environmentally friendly power devices is needed because current systems such as conventional batteries have shown to contain toxic compounds and produce toxic residues.\(^1\) Harvesting energy from glucose, for example, would make possible the utilization of fruits as well as commercial drinks as source of biofuel and the products of their oxidation are naturally biodegradable. Enzymatic biofuel cells are devices engineered for that purpose.\(^2-4\) These biofuel cells are envisioned to power small portable devices and devices for biomedical applications.\(^3-17\)

Increasing the efficiency of the biofuel cell systems requires decreasing limitations on the kinetics of the catalytic layer, resistivity losses of the electrode materials and mass transport losses. In previous work, we reported the integration of bunny papers\(^5\) (multiwalled carbon nanotube (MWNTs)-based papers) to bioelectrode designs to reduce kinetic and ohmic limitations. The employment of a quasi-2D microfluidic system (paper-based fan) was employed to decrease mass transport limitations of fuel to the catalytic layer. The enzymatic systems were immobilized within CNTs network-Chitosan 3D-matrix in order to improve electron transfer and enzyme lifetime. Herein, the improvement of the NAD\(^+\)-dependent glucose dehydrogenase (GDH)-based bioanode is presented.

Harvesting energy from glucose by enzymatic biofuel cells could be performed by using oxidoreductase enzymes such as glucose dehydrogenase (GDH) on the anode\(^6\) and analogous enzymatic systems on the cathode for reduction of oxygen from air.\(^6,21\) At the anode, GDH shows a biocatalytic activity when coupled to its NAD\(^+\)/NADH-cofactor.\(^13,22-25\) The oxidized form of the cofactor (NAD\(^+\)) is reduced to (NADH) while glucose is oxidized to gluconolactone. The reoxidation of NADH is needed for the GDH enzymatic activity to continue. Since NADH oxidation occurs at high overpotential, mediators such as methylene green (MG) are employed to overcome this energetic limitation.\(^6,26-28\) NAD\(^+\) is externally fed into the system with the biofuel solution. MG is polymerized on the electrode’s surface. Then, engineering of a reagentless bioanode where NAD\(^+\)/NADH-cofactor is immobilized on the bioanode’s surface allows for no-external feeding of the cofactor. This is highly desired for improved cofactor-enzyme-electrode interaction and improved friendly-user design of the biofuel cell.

The development of reagentless bioanodes employing NAD\(^+\)/NADH-dependent dehydrogenases has been the area of study of researchers designing anodes for biosensor applications primarily. According to literature, several approaches considered the integration of the cofactor to polymeric structures such as dextran,\(^29\) alginate,\(^30\) polyethylene glycol,\(^31\) chitosan,\(^32\) polyethyleneimine,\(^33,34\) and entrapment in sol-gel using glycidoxypropyltrimethoxysilane\(^35,36\) as linker. Then, the improvement of the dehydrogenases-based anode designs involve the tethering of the NAD\(^+\)/NADH-cofactor in proximity to the enzyme to enhance electron transfer in order to create an efficient reagentless enzymatic biofuel cell.

In our research, the tether selected is pyrene butanoic acid succinimidyl ester (PBSE) which interacts with the surface of carbonaceous MWNTs without disrupting its conductivity. The use of an adsorbed crosslinking agent on the MWNTs is desired in order to maintain the benzene-ring like structure and the quasi-metallic electronic conductivity of their walls. Chen et al reported the successful use of PBSE as noncovalent functionalization agent for protein immobilization on single walled nanotubes (SWNTs) by π-stacking.\(^37\) The pyrenyl group of the compound was reported to interact with the graphite plane and the SWNTs surface. However, due to the planar structure of the pyrenyl ring, this moiety has better interaction with carbonic MWNTs. These have a larger diameter than SWNTs and wall circumference which is ‘seen’ by the pyrenyl rings as approximately planar structure which improves π-π interaction. At the opposite side of the tether chain, at the N-hydroxy succinimide side of the PBSE agent, an amine group of the enzyme is attached covalently to the tether by forming an amine bond. The immobilization of enzymatic structures on MWNTs-based materials for biofuel cell applications was successful.\(^38\) Similarly, due to the presence of an amine moiety in the molecular structure of NAD\(^+\)/NADH, the formation of a covalent amide bond with the N-hydroxy succinimide side of PBSE is expected. The NAD\(^+\)/NADH-PBSE structure would be absorbed by π-π interaction on the surface of the MWNTs integrating the bioanode material.

Herein, we present two non-reagentless and reagentless bioanode designs. The latter is characterized for having the NAD\(^+\)/NADH-tethered on the MWNTs-based bunny paper PBSE by π-π interaction while the GDH enzyme is entrapped within a CNTs/Chitosan

---

\(^*\)Electrochemical Society Student Member.

\(^+\)Electrochemical Society Active Member.

\(^\text{E-mail: plamen@unm.edu}\)
3D-matrix. Additionally, we have integrated a new bucky paper, CMN-grade, which has demonstrated to increase the current output performance in the GDH-bioanode design. The objective of this research is to minimize the inherent limitations of the bioanodic system and improve the bioanode design to a reagentless GDH-based anode for biofuel cell applications.

**Experimental**

**Apparatus.**—The electrochemical experiments were performed in a three-electrode cell by conventional potentiostat: Gamry Reference 600 Potentiostat/Galvanostat. All potentials are reported vs Ag/AgCl. The material characterization was performed with a scanning electronic microscope Hitachi (S-5200) equipped with an energy dispersive spectrometer (EDS) and Kratos Axis Ultra DLD X-ray photoelectron spectrometer.

**Chemicals.**—Buckeye Paper (BEP) of thickness of 15–25 μm and purity of ~100% MWNTs and CMN-grade-bucky paper of 15–25 μm and a purity of ~100% were obtained from Buckeye Composites Inc. (Kettering, OH). Isopropanol (purity >99%), monobasic and dibasic sodium phosphates were obtained from EMD Chemicals Inc. and used to prepare pH 7 and pH 7.5 phosphate buffer stock solutions. Methylene Green (MG) Zinc Chloride double salt (purity Inc. and used to prepare pH 7 and pH 7.5 phosphate buffer stock solutions. Methylene Green (MG) Zinc Chloride double salt (purity >99%, from Fluka Cat. 66870) and KNO3, from EMD Chemicals Inc. were used to make MG growing solution in phosphate buffer of pH7. NAD+ (purity >98%, Fluka Cat. 43407). D(-)-Glucose (purity >99.5%), Lyophilized Glucose Dehydrogenase (GDH-2) from Pseudomonas sp. (272 U/mg activity, Cat. 19359) from Amano Enzyme USA Co., Ltd. (Elgin, IL). Koptec 200 Proof Ethanol from VWR (King of Prussia, PA). Pyrene butyric acid succinimidyl ester (PBSE) (Cat. 457078) from Sigma–Aldrich (St. Louis, MO).

**Anode design procedure.**—BEP and CMN-based bioanode designs and their respective control electrodes were developed, evaluated and compared in this study. Three set of each electrode were analyzed for reproducibility and reported herein. Each electrode material was washed with IPA initially, and DI water later. The polymerization of MG proceeded as described in previous work by cyclic voltammetry. For electrodes with no cofactor immobilized, the deposition of the GDH was performed after PMG formation. For electrodes employing NAD+–tethered on the electrode surface, NAD+ and PBSE dissolved, mixed and deposited on the bucky paper’s surface after PMG formation. PBSE tether was dissolved in DMSO and the NAD+ cofactor in phosphate buffer (PB) 0.1 M at pH 7.5. The amount of PBSE and NAD+ used maintained the 1:1 molar ratio. For a 3.7 cm² of geometrical surface area of bucky paper, 5 mg NAD+ is dissolved in 100 μl PB 0.1M, pH 7.5 and 2.9 mg PBSE in 100 μl DMSO. After the electrode was dried for an hour at room temperature (25 ± 1°C), the immobilization of the GDH is performed by mixing the enzyme within 150 μl of 95/5 v/v Chitosan/CNTs mixture, deposited on the electrode and left to dry overnight at 4°C. The electrodes fabricated were BEP-MG-GDH and CMN-MG-GDH (with no tethered NAD+), and BEP-MG-NAD+–GDH and CMN-MG-NAD+–GDH (with tethered NAD+). Control electrodes, CMN and CMN-MG electrodes, were evaluated in similar conditions.

**X-Ray photoelectron spectroscopy.**—XPS measurements were performed with a Kratos Axis Ultra DLD X-ray photoelectron spectrometer using a monochromatic Al Kα source operating at 225W. The data obtained are average from 3 separate areas per sample. High resolution spectra of C 1s, O 1s, N 1s and S 2p were acquired after the survey was taken. No charge compensation was necessary. The samples MG, GDH-Chitosan, NAD+, PBSE, plain BEP, plain CMN, BEP-MG, CMN-MG, BEP-MG-PBSE-NAD+ CMN-MG-PBSE-NAD+, BEP-MG-PBSE-NAD+–GDH (BEP full set) and CMN-MG-PBSE–NAD+–GDH (CMN full set) were analyzed. Additionally, the analysis of samples with NAD+ tethered by PBSE was done. After the bioanode was electrochemically tested, we have analyzed it with respect to time in continuous operating conditions. The sampling times were at 0, 1, 3, 6, 9, 12, 15, 18 and 24 hours.

Data analysis and quantification were performed with the CasaXPS software. A linear background was used for C 1s, N 1s, O 1s, S 2p and P 2p spectra. Quantification utilized sensitivity factors that were provided by the manufacturer. A 70% Gaussian / 30% Lorentzian (GL (30)) line shape was used for the curve-fits. Full width at half maximums (FWHMs) used for curve fits of N 1s and S 2p spectra were constrained to 1.1 ± 0.2 eV for all spectra acquired. These are based upon analysis of reference materials. Curve fitting analysis was completed first on the high resolution spectra of reference compounds using the above parameters and then similar constraints were applied to mixture spectra and new peaks were added (if necessary) to obtain an optimal curve fit. Identification of peaks is based on Scienta ESCA300 database[14] and NIST Standard Reference Database[15].

**Chronoamperometry.**—The BEP and CMN-grade electrodes were tested by chronoamperometry, the current generated was monitored while step potentials were applied. Each step was maintained for 300 seconds and incremented by 50 mV with respect to the previous; the potential range started from the OCP up to 350 mV. The electrochemical test was performed in a three electrode-electrolytic cell as well as in the 270° paper-based fan cell at room temperature as well as 4°C. The results were used to generate the potentiostatic polarization curves (potential vs. current density). Later, similar procedure was used to monitor the electrode behavior with respect to time, at this time every stepping potential was applied during an hour. The overall test lasted 20 hours.

**Chronopotentiometry.**—The electrodes were evaluated by chronopotentiometry, the steady-state current obtained by chronoamperometry (described above) was applied to the electrodes, and the resulting voltage was monitored. Each step was maintained for 500 seconds. These tests were performed also on both electrolytic and fan cell-setup at room temperature. The resulting potentials were used to generate the galvanostatic polarization (potential vs. current density).

**Michaelis–Menten analysis.**—The kinetic activity of the enzyme on the anodic surface was monitored as a function of substrate concentration and temperature by chronoamperometry. At 50 mV of constant applied potential, current generated was monitored when the aliquots of substrate were added to the electrolytic cell every 400 seconds. The results obtained were used to develop the Michaelis–Menten curve (current vs. concentration) and to find the respective parameters.

**Stability study.**—In previous research, we analyzed the stability of the catalytic layer of the BEP-MG-GDH bioanode in storage in ‘dry’ conditions at 4°C for 30 days. In the current study, the electrode integrating CMN-grade bucky paper was evaluated similarly. Additionally, the stability study at continuous working conditions was performed on the reagentless bioanode design. Also, XPS analysis was performed for the test in continuous operation of the anode.

**Results**

**Bioanode design.**—The bioanodes developed integrated BEP and CMN-grade bucky papers, and the NAD+/NADH-cofactor and GDH-enzyme immobilized on the bioanode surface. SEM images show the nanoarchitecture of the plain BEP and CMN surface differ from each other in the diameter range of MWNTs-integrating the material. The surface of BEP-paper is constituted by MWNTs of more uniform size distribution, diameters ranged between ~15 nm to 40 nm (Figure 1a). The CMN-nanoarchitecture shows to be constituted by MWNTs of a wider range in diameter, from ~10 nm to 200 nm (Figure 1b). Due to the wide diameter/size differences of the MWNTs, the CMN-paper has a wider range of porous structures as well (Figure 1c). When the cofactor and enzyme in Chitosan/CNTs mixture is deposited on the electrode surface, the mixture is capable of penetrating the small and wide porous structures and form a 3D-CNTs network of unique...
conformation, architecturally distinct from BEP at the nanoscale (Figure 1d). The CMN-nanostructure demonstrates to enhance electron transport and current generation shown below.

**XPS analysis.**—In a recent minireview, we have discussed that through (1) careful selection of reference materials, which constitute part of the multicomponent nanoarchitectures and individual relevant mixtures, and (2) constraining curve fits using information obtained from the references, it is possible to differentiate between chemically similar biological compounds in the matrix of organic materials and to establish interaction between them.39 As was discussed in the Experimental section, we have analyzed all reference materials, such as CMN and BEP paper, and powders of MG, PBSE, chitosan, GDH and NAD$^+$.

In addition, we have analyzed not only full sets but also mixtures such as BEP-MG, CMN-MG, BEP-MG-PBSE-NAD$^+$, CMN-MG-PBSE-NAD$^+$, BEP-MG-PBSE-Chitosan-GDH, CMN-MG-PBSE-Chitosan-GDH. Table I shows XPS elemental composition and Figure 2 shows a subset of high resolution spectra.

Plain paper has only carbon and very small amount of oxygen detected. All other components of bioanode have various amounts of N, while MG has also $\sim$3% of S and NAD$^+$ has $\sim$4% of P. The presence of N, S and P is XPS spectra confirm the expected qualitative composition of all mixtures. Based on N/C ratio for pure MG sample, MG constitutes 29% (CMN) and 23% (BEP) for MG-paper sets. N/S for both MG-paper samples is very close to that from pure MG reference. Based on N/S ratio for MG, in CMN-MG-NAD$^+$ 50% of N signal comes from MG and 50% from NAD$^+$. For BEP-MG-NAD$^+$ set, 40% of N signal comes from MG and 60% from NAD$^+$. This is also confirmed by P/N ratio for reference NAD$^+$. Significant increase in O and N is observed for both full sets due to the contribution from all constituents – MG, chitosan, GDH, PBSE and NAD$^+$. Based on amounts of sulfur and phosphorous detected we confirm that for CMN based full set, total N consists of 31% from MG, 49% from NAD$^+$ and 20% from the PBSE/chitosan/GDH mixture. For BEP based full set, total N consists of 18% from MG, 45% from NAD$^+$ and 38% from the PBSE/chitosan/GDH mixture.

Figure 2 shows high resolution N 1s and S 2p spectra from reference pure constituents, i.e. MG, PBSE, NAD$^+$, and a full set on CMN paper. Pure MG has a main peak at 399.5 eV due to amine, peak at 401.3 eV due to N-C=O and peaks due to NOx species between 404 and 408 eV. Pure NAD$^+$ has a unique peak at 400.1 eV due to amide group in addition to peaks due to amine and N$^+$. GDH and Chitosan have single peak due to amine at 399.5 eV (not shown). Main peak of PBSE is due to amide at 401.3 eV. From the reference spectra, we can conclude that MG has a unique peak at high BE between 404 and 408 eV due to NOx. NAD$^+$ has a unique peak at 401.3 eV due to amide, while PBSE has a large peak at 401.3 eV, but both MG and NAD$^+$ also contribute in the same BE region due to similar N-C=O types of species or some charged N$^+$. This information can be used to study the composition of partial and full sets on paper. Results of

| Sample Identifier | C 1s% | O 1s% | N 1s% | S 2p% | P 2p% |
|-------------------|------|------|------|------|------|
| MG reference      | 73.4 | 11.3 | 12.5 | 2.8  |
| Chitosan reference| 67.1 | 28.7 | 4.3  |      |
| GDH reference     | 64.9 | 22.6 | 12.3 |      |
| NAD reference     | 56.2 | 29.7 | 10.6 | 3.5  |
| PBSE reference    | 79.3 | 18.8 | 1.9  |      |
| CMN               | 99.3 | 0.7  | 0.0  | 0.0  |
| CMN MG            | 89.0 | 5.7  | 4.4  | 1.0  |
| CMN MG GDH Chit CNT| 72.8 | 19.8 | 7.1  | 0.3  |
| CMN MG NAD        | 82.7 | 11.1 | 4.9  | 0.5  | 0.9 |
| CMN full set      | 66.0 | 23.4 | 8.6  | 0.6  | 1.4 |
| BEP               | 99.2 | 0.7  | 0.1  | 0.0  |
| BEP MG            | 92.4 | 4.1  | 3.0  | 0.6  |
| BEP MG GDH Chit CNT| 73.9 | 18.5 | 7.4  | 0.2  |
| BEP MG NAD        | 87.6 | 7.7  | 3.6  | 0.4  | 0.8 |
| BEP full set      | 67.2 | 21.4 | 10.1 | 0.4  | 0.9 |
N fit for all samples are shown in Table 1S. All composite samples with MG have peak at 407 eV detected due to MG and its relative contribution decreases with the addition of other constituents. In a full set, this peak is 7.7% for CMN and 5.6% for BEP representing, based on the ratio of this peak to the total N amount, 39% of total nitrogen detected from CMN and 28% from BEP, respectively. In samples where PBSE is present, there is an evident increase in imide peak at 401.3 eV. In partial set with NAD+, PBSE and MG on both papers, peak due to NAD+ at 399.7 is present. This peak, however, is absent in a full set. It may be explained by either attenuation of the NAD+ signal with the added layer of GDH-Chitosan or change in the chemical environment of NAD+. GDH and Chitosan have a single peak at 399.5 eV (Table 1S). Total amount of N increases from paper-MG sample to both MG-GDH-Chitosan and a full set (Table 1); but the peak at 399.5, where Chitosan and GDH should contribute, the amount of N decreases significantly. This fact may be due to change in a chemical environment of amines present in Chitosan and GDH. Moreover, MG-GDH-Chitosan and a full set have large peak at 400.6 eV (Figure 2d) that is not present exclusively in either of the pure constituents. This suggests a shift in BE due to the interaction between constituents. At this BE, multiple possible species may contribute, including secondary amines, protonated amines as well as N-C-O species. Change in a chemical environment of NAD+ (amide), Chitosan and GDH (primary amines) and appearance of a new peak due to secondary amines point to the interaction between constituents of a nanocomposite.

S is only present in MG itself. In addition to the majority of S present as S-C, there is some amount present as S-O (Figure 2e).
When GDH/Chitosan is added to the system, the ratio of S-C to S-O changes to having more S-O (Table 1S). When MG mixed with NAD\(^+\), in addition to larger relative% of S-O, new peak at 168.2 eV due to sulfoxides \(R_2S=O\) or some charged sulfonic \(S=O^+\) appears (Table 1S). In a full set, the relative% of S-OX is largest among all the samples, especially for CMN sample (Figure 2f).

The stability of the full set nanostructures was studied by XPS. The CMN-based bioanode was electrochemically tested at 0, 1, 3, 6, 9, 12, 15, 18 and 24 hours, and these bioanodes were then analyzed by XPS. Table 2S shows full results while Figure 3 shows changes in the composition as a function of time for a subset of species. S/N ratio stays the same throughout the series demonstrating overall stability of MG in the matrix. There is, however, a decrease on P/N ratio with electrochemical testing pointing toward loss of NAD\(^+\) in the nanostructure. The peak that we have identified as an interaction component between constituents of composite due to the formation of secondary amines by participating amides and primary amines, increases during electrochemical testing that is accompanied by loss of oxidized types of nitrogen. Sulfur environment also undergoes changes in which oxygenated S species decrease while S-C increases.

**Polarization curves for BEP-MG-GDH/chitosan/CNTs and CMN-MG-GDH/chitosan/CNTs anodes.**—The potentiostatic and galvanostatic polarization curves were developed by chronoamperometry and potentiometry, respectively, in order to evaluate the bioanodes performance in both electrolytic and paper-based fan shape setups. The potentiostatic polarization curves started from the OCP up to 0.35 V. Control electrodes, systems without enzyme, show negligible current generation demonstrating the material has not significant catalytic properties for glucose oxidation (Figure 4A and 4D) as demonstrated in previous study as well.\(^{19}\) For BEP-MG-GDH/Chitosan/CNTs the OCP was \(-0.18\) V\(^{19}\) and for CMN-MG-GDH/Chitosan/CNTs the OCP \(-0.225\) V (Figure not shown). The potentiostatic polarization curves of BEP-MG-GDH/Chitosan/CNTs (-\(\bullet\), Figure 4A) in the electrolytic cell with the NAD\(^+\)-cofactor in solution generates \(2200\ \mu\)A.cm\(^{-2}\) at 50 mV and 2250 \(\mu\)A.cm\(^{-2}\) at 0.1 V \((2.5 \text{ mAA.cm}^{-2} \text{ at } 0.35 \text{ V})\).\(^{19}\) Similar procedure on the CMN-MG-GDH/Chitosan/CNTs bioanode (-■, Figure 4A) produces larger current densities, with observations of \(3100 \mu\)A.cm\(^{-2}\) (3.1 mA.cm\(^{-2}\)) at 50 mV and 3600 \(\mu\)A.cm\(^{-2}\) at 0.1 V (4.5 mA.cm\(^{-2}\) at 0.35 V). From comparison of the polarization curves, it is possible to recognize that at low current densities, the kinetic activity of the enzymatic system on the CMN-paper is slightly slower than on the BEP-paper. At larger current densities (>2000 \(\mu\)A.cm\(^{-2}\)), it is possible to observe the transport limitation of the substrate to the catalytic layer on the BEP-paper is much greater than on CMN-paper. Both bioanodes were prepared to use the same procedure of MG deposition and enzyme immobilization (in Chitosan/CNTs) and both materials present comparable ohmic resistivity. The aforementioned results allow us to infer that the porosity of the CMN-bucky paper conferred by the large range of MWNs-size/diameter provides the CMN-structure with extraordinary nanoarchitecture demonstrating improved electron transfer and mass transfer of substrate at the catalytic layer due to an improved integration of the GDH-CNTs-Chitosan mixture within the electrode material compared to the architecture of BEP surface.

Furthermore, potentiostatic and galvanostatic curves were performed to analyze the behavior of the bioanodes in simulated real-life working conditions. The galvanostatic polarization curves for both bioanodes in the electrolytic cell (Figure 4B) show that at applied current densities higher than 2000 \(\mu\)A.cm\(^{-2}\) (>0 V) the potential recorded increased drastically compared to the potentiostatic curve (comparing right upper quadrant of Figure 4A and 4B). At applied currents below 2000 \(\mu\)A.cm\(^{-2}\), the behavior of the curves were similar (comparing left bottom quadrant of Figure 4A and 4B). At current densities below 2000 \(\mu\)A.cm\(^{-2}\) and 0 V, the electrochemical processes of the enzymatic catalytic layer of both bioanodes are approaching steady-state behavior, current densities and measured potentials are similar in magnitude. When potentials are increased from 0 V to 0.35 V in potentiostatic mode, the polarization curve shows that the currents generated range from 2,000 \(\mu\)A.cm\(^{-2}\) to 2,500 \(\mu\)A.cm\(^{-2}\) and 2,000 \(\mu\)A.cm\(^{-2}\) to 4,250 \(\mu\)A.cm\(^{-2}\) for BEP and CMN-based anodes, respectively. On the other hand, when applying those currents to the electrodes to generate galvanostatic polarization curves, the potentials generated are observed to range from 0 V to \(\sim 1.2\) V and 0 V to \(\sim 1.3\) V for BEP and CMN-based anodes, respectively (Figure 4A and 4B). The potentiostatic and galvanostatic polarization curves have different behavior, the systems abandoned the steady-state at potentials >0 V and current densities >2,000 \(\mu\)A.cm\(^{-2}\). At high current densities and potentials (>2000 \(\mu\)A.cm\(^{-2}\), >0 V), the contribution of processes involving the material’s performance may be affecting the system inducing a transient or unsteady state.

In order to minimize mass transport limitations, the bioanodes were assembled to the quasi-2D microfluidic system, and their performance was electrochemically evaluated. The OCPs were obtained and employed in chronoamperometric tests. The BEP-based anode presented an OCP \(-0.225\) V and the CMN-based anode’s OCP was \(-0.275\) V. In order to evaluate the catalytic layer utilizing the capillary driven microfluidic system to diffuse the biofuel, potentiostatic polarization curves were generated by chronoamperometry from the OCP up to 0.35 V. Using this 270° paper-based fan setup, the polarization curve of the enzymatic system on the BEP-based anode exhibits a slower kinetic activity represented by the lower current density generated compared to the CMN-based anode, approximately 200 \(\mu\)A.cm\(^{-2}\) for BEP (-\(\bullet\)) and 1100 \(\mu\)A.cm\(^{-2}\) for CMN (-■) at 0.35 V (Figure 4C). Both enzymatic systems exhibit shift to decreased current densities on the polarization curves at potentials higher than -50 mV for BEP and -150 mV (--0.15 V) for CMN. Above 0.1 V, the curve of the CMN-based bioanode starts recovering its performance by increasing the current densities generated. At 0.35 V, the CMN-based anode gives around 900 \(\mu\)A.cm\(^{-2}\). The BEP-based anode displays a
Figure 4. A) Potentiostatic and B) galvanostatic polarization curves performed on BEP-MG-GDH/Chitosan/CNTs and CMN-MG-GDH/Chitosan/CNTs in electrolytic cells. C) Potentiostatic and D) galvanostatic polarization curves performed in 270° paper-based fan cell. Experiments were performed in 1 mM NAD\(^+\), 0.1 M glucose in 0.1 M PB and 0.1 M KCl at pH 7.5 at room temperature (25 °C).

Decreasing polarization performance above ~50 mV and does not recover but maintains overall constant 620 \(\mu\)A.cm\(^{-2}\) of current density at potentials higher than 0.2 V. These results indicate the catalytic layer of the BEP-based paper presents a nanostructural architecture of smaller porosity and active site exposed to the biofuel mass transport. This observation reflects limitation of flow of the substrate to feed the catalytic layer; therefore, a decrease of current density is observed. The potentiostatic polarization curve of the CMN-based anode demonstrates decreasing electron transfer or current density due to minimized mass limitation. Although the current output decreases when the anode is assembled into the paper-fan cell when compared to the static electrolytic cell, the design is energetically convenient since no external energy is applied to drive the flow of fuel to the anode. This design could be feasible employed in biofuel cell applications that employ the glucose as biofuel.

Storage stability study on CMN-MG-GDH anode.— The stability study on the bioanodes was performed in a time range of 30 days. Potentiostatic polarization curves were developed to analyze the current output on the enzymatic systems on the electrodes at different days of storage (inset Figure 5A). The experimental results, current as a function of time, were analyzed at 0.35 V of applied voltage (Figure 5A). The CMN-bioanode system maintains approximately 73% of its initial performance up to 25 days and ~65% up to 30 days of storage in ‘dry’ conditions at 4 °C. The enzymatic system is stabilized within the Chitosan/CNTs 3D-structure and is capable of generating an average of 1,800 \(\mu\)A.cm\(^{-2}\) at 25 days in the electrolytic cell setup.

Michaelis-menten analysis on GDH/tethered NAD\(^+\)/CMN catalytic surfaces.— In order to improve the design of the bioanode to a reagentless NAD\(^+\)/NADH-dependent GDH anode, the immobilization of the cofactor was performed. PBSE tethering NAD\(^+\)/NADH-cofactor has a pyrenyl moiety that acts as footprint interacting with the MWCNTs surface by cloud overlap of \(sp^2\) orbitals. The Michaelis-Menten parameters of the enzymatic system having the cofactor tethered to the electrode surface were found from the curve of current generated as a function of substrate concentration at 0.35 V by chronoamperometry. The Michaelis-Menten curve shows that at 4 °C and 0.1 M glucose a limiting current of approximately \(I_{\text{Max}} = 3,252.5 \pm 133.9 \mu\)A.cm\(^{-2}\) is observed (-□-, Figure 5B and Table II). At 25 °C, the limiting current is \(I_{\text{Max}} = 2,568.1 \pm 85.0 \mu\)A.cm\(^{-2}\) (-■-, Figure 5B and Table II). The Michaelis-Menten constant at 4 °C is \(K_M = 48.3 \pm 4.6 \text{ mM}\) and \(K_M = 13.6 \pm 1.8 \text{ mM}\) at 25 °C (Table II). The resulting information corroborated the catalytic activity of the GDH coupled
to the tethered NAD$^+$-cofactor for glucose oxidation on the bioanode surface. The kinetic of the reaction is faster at 25°C showing the smaller Michaelis-Menten constant, indicating $I_{\text{Max}}$ is reached faster at 25°C than at 4°C. However, the limiting current is higher for the process at 4°C which is reflected by the $I_{\text{Max}}$ (4°C) > $I_{\text{Max}}$ (25°C). This may be explained from a kinetic point of view, slower movement of the cofactor-PBSE structure that allows an increased number of interactions by affinity between the substrate-active site-cofactor-electrode at 4°C when compared to 25°C.

### Table II. CMN-MG-PBSE-NAD$^+$-GDH/Chitosan/CNTs at 4°C and 25°C, Michaelis-Menten parameters obtained by chronoamperometry at 50 mV.

| Temperature (°C) | $I_{\text{Max}}$ ($\mu$A.cm$^{-2}$) | $K_M$ (mM) |
|------------------|---------------------------------|----------|
| 25°C             | 2568.15 ± 85.05                  | 13.6 ± 1.8 |
| 4°C              | 3252.5 ± 133.9                   | 48.3 ± 4.6 |

Figure 5. A) Stability study for CMN-MG-GDH/Chitosan/CNTs bioanode showing the current density as a function of time at 0.35 V obtained from the potentiostatic polarization curves at 0.35 V (inset). B) Michaelis-Menten curves for CMN-MG-PBSE-NAD$^+$-GDH/Chitosan/CNTs bioanode at 4°C and 25°C where the current generated is analyzed as a function of glucose concentration.

Figure 6. A) Open circuit potential of CMN-MG-PBSE-NAD$^+$-GDH/Chitosan/CNTs in absence (light gray line) and presence of substrate (gray line) at day 1 and in the presence of substrate at day 2 in an electrolytic cell. B) Potentiostatic polarization curves performed on CMN-MG-PBSE-NAD$^+$-GDH/Chitosan/CNTs in an electrolytic cell in the absence (●) and presence of substrate (0.1 M glucose, 0.1 KCl in 0.1 M PB) at day 1: measurements taken at 300 seconds intervals (●) and at 1 hour interval after OCP, potentiostatic polarization by chronoamperometry and depolarization (●, starting after 7 hours of working conditions) at day 2: intervals of 600 seconds (∙).
current density had decreased from 1,750 μA.cm⁻² (−0.225 V) to 1,250 μA.cm⁻² (−0.225 V) at 0.35 V in 20 hours of continuous test (−40% of initial activity was preserved). The polarization curve obtained at day 2 shows the electrode is generating ∼100's of μA.cm⁻² (−0.35 V). Later in day 2, after renewing the electrolytic solution (1 mM NAD⁺, 0.1 M glucose in 0.1 M PB and 0.1 M KCl at pH 7.5), the electrode began generating current densities close to the ones obtained in day 1 (plot not shown). These results indicate that continuous interaction of the tethered NAD⁺ with GDH-enzyme and PMG (to produce NADH and regenerate NAD⁺, respectively) induces the release of the tethered cofactor to the solution. This phenomenon is also corroborated by ultra-violet light spectroscopy (not shown) and XPS analysis (Table 2S). The structure of the enzyme however is preserved on the electrode surface, and it maintains its catalytic activity.

The nanoarchitecture of the entrapment matrix has adopted exceptional characteristics that increased enzyme loading and fuel mass transport. The chitosan/CNTs 3D-structure on the CMN-bucky paper forms a CNTs-3D network that helps to maintain the active 3D-enzyme conformation and enhances electron transfer as observed on the previous study on BEP-based GDH-bioanodes.14 The increased current density demonstrated by the CMN-based anode compared to BEP-bioanodes may be due to the increase in the porosity range that allows the GDH/Chitosan/CNTs mixture to reach inner depths on the CMN-material. The large range of pore size allows further minimization of substrate mass transport limitations.

Conclusions

This study proposes various advances in GDH and bucky paper-anode designs looking to minimize kinetic, ohmic and mass transport limitations for feasible application into fuel cell designs. The improvement in current density generation has been possible by increasing the surface to volume ratio and porosity of the electrode material that increases enzyme loading and fuel mass transport to the catalytic layer. The stability of the enzymatic system has been preserved in a Chitosan/CNTs-matrix that posses a unique CNTs-3D network that induces improvement in substrate-enzymatic active site-electrode surface interaction enhancing electron transfer and current output.

The first improvement in design was presented in the BEP-MG-GDH anode which showed OCP of −0.18 V, and current generation of approximately 2.2 mA.cm⁻² at 50 mV and 2.5 mA.cm⁻² at 0.35 V.15 The later improvement consisted in modifying the afore-mentioned nanostructural architecture of the enzymatic catalytic layer introducing a new material, the CMN-bucky paper. The current generated by the CMN-MG-GDH bioanode has extraordinary performance approaching −0.225 V of OCP, and a current generated of ∼3.1 mA.cm⁻² at 50 mV and 2.45 mA.cm⁻² at 0.35 V in the electrolytic cell.

Next, the use of a paper-based quasi-2D microfluidic system improved the biofuel feeding process with non-external energy supplied because it consisted of a capillary driven flow maintained by liquid-vapor equilibrium. The integration of such system to the half-cell anode, was successfully achieved. This assembly presents an OCP of −0.225 V and −0.275 V for BEP and CMN-based anodes, respectively and displays 200 μA.cm⁻² and 1.1 mA.cm⁻² at −0.15 V for BEP- and CMN, respectively. This bioanode satisfactorily exceeds the performance of recent MWNTs-based designs.16

The bioanode was redesigned to a reagentless NAD⁺-dependent GDH anode. The NAD⁺-cofactor was tethered to the surface of the MWNTs-wall by employing a linker capable of adsorbing on the CNT-wall by π–π stacking interaction from the pyrenyl end of the molecule. At the alkyl-ester end, the molecule forms a covalent amide bond with the amine moiety of the NAD⁺ molecule. These bonds make feasible the immobilization of the mentioned cofactor which can be successfully coupled to the glucose oxidation by the enzymatic process and can be regenerated by action of the MG mediator. This design has a working duration time of approximately 16 hours which shows an improvement with respect to previous research.17

The reagentless and non-reagentless NAD⁺-dependent GDH-based bioanode integrating CMN-bucky paper presented in this research has demonstrated to be reproducible and feasible to employ in half-electrolytic and paper-based fan cells. These designs could be employed in the real fuel cell applications to power small devices as well. Further studies have to be performed to prolong the continuous working conditions of the reagentless bioanode and storage life as well in order to meet manufacturability standards and user needs. However, to the author’s knowledge, the bioanodes introduced in this research have shown the best performance among previous NAD⁺-dependent GDH-based bioanodes or other enzymatic system used for glucose oxidation.

Acknowledgment

This work was supported at UNM by AFOSR Bioenergy Program and NSF-CBET grant number 1158596.

References

1. K. Fisher, E. Wallén, P. P. Laenen, and M. Collins, Battery Waste Management Life Cycle Assessment; Environmental Resources Management, DEFRA: 2006.
2. S. Calabrese Barton and P. Atanassov, Preprint Papers - Am.Chem.Soc., Div. Fuel Chem., 49(2), 476 (2004).
3. P. Atanassov, C. Apblert, S. Banta, S. Brozk, S. C. Barton, M. J. Cooney, B. Y. Liaw, S. Mukerjee, and S. Minteer, Electrochemical Society Interface, 16, 25 (2007).
4. R. Arechaderra and S. D. Minteer, Electrochimica Acta, 53, 6698 (2008).
5. A. Heller, Physical Chemistry Chemical Physics, 6, 209 (2004).
6. S. Calabrese Barton, J. Galloway, and P. Atanassov, Chemical Reviews, 104, 4867 (2004).
7. F. von Stetten, S. Kerzenmacher, R. Sumbarajau, R. Zengerle, and J. Ducrè, Proc. Euroensors. XX, 222 (2006).
8. F. Davis and S. P. J. Higson, Biosensors and Bioelectronics, 22, 224 (2007).
9. S. D. Minteer, B. Y. Liaw, and M. J. Cooney, Current Opinions in Biotechnology, 18, 228 (2007).
10. M. Togo, Y. Yatagawa, M. Okie, H. Kaji, T. Abe, and M. Nishizawa, Proceedings of PowerMEMS 2008+ microEMS 2008, 2008, Sendai, Japan (November 9-12).
11. I. Willner, Y. M. Yan, B. Willner, and R. Tel-Vered, Fuel Cells, 9(1), 7 (2009).
12. L. Coman, R. Ludwig, V. Garretier, D. Hallisch, L. Gorton, T. Razgas, and S. Shleev, Fuel Cells, 10(1), 9 (2010).
13. P. K. Addo, R. L. Arechaderra, and S. D. Minteer, Electroanalysis, 22, 807 (2010).
14. T. Miyake, K. Haneda, N. Nagai, Y. Yatagawa, H. Oumuri, S. Yoshihito, T. Abe, and M. Nishizawa, Energy and Environmental Science, 4, 5008 (2011).
15. D. Bhattacharj, S. Xu, C. Fischer, R. L. Arechaderra, and S. D. Minteer, Physical Chemistry Chemical Physical, 13, 86 (2011).
16. P. Atanassov, C. Lau, C. W. Narvárez Villarrubia, G. Cincagino, S. O. Garcia, R. Rincon, S. Sibbett, T. Pisev, S. Minteer, M. Moehlenbrock, R. Arechaderra, S. Banta, Y. H. Kim, E. Campbell, H. Luckariff, and G. Johnson, 21st ECS Meeting, The Electrochemical Society 2012, Abstract #1452.
17. M. Falk, C. W. Narvárez Villarrubia, S. Babanova, P. Atanassov, and S. Shleev, Chemphyschem 2013.
18. R. L. D. Whitby, T. Fukuda, T. Maekawa, L. S. James, and S. V. Mikhailovsky, Carbon, 46, 949 (2008).
19. C. W. Narvárez Villarrubia, S. O. Garcia, C. Lau, and P. Atanassov, ECS J. Sol. State Sci. and Tech., 2(10), M3156 (2013).
20. G. Gupta, C. Lau, V. Rajendran, F. Colon, B. Branch, D. Ivntski, and P. Atanassov, Electrochemistry Communications, 13, 247 (2011).
21. G. P. M. K. Cinagino, C. Lau, A. Cochrane, S. S. Sibbett, E. R. Gonzalez, and P. Atanassov, Electrochimica Acta, 82, 208 (2012).
22. L. Gorton, P. D. Hale, B. Persson, L. L. Boguslavsky, H. I. Karan, H. S. Lee, A. T. Skotheim, H. L. Lan, and Y. Okamoto, ACS Symp. Ser., 487, 56 (1992).
23. M. J. Cooney, W. Windmesser, C. Lau, B. Y. Liaw, T. Klotzbach, and S. Minteer, J.Mater Chem. 6, 667 (2008).
24. I. Katakis and E. Dominguez, Microchimica Acta, 126(1–2), 11 (1997).
25. L. Gorton and P. N. Bartlett, (2008), NAD(P)-Based Biosensors. In P. N. Bartlett(Ed.), Bioelectrochemistry: fundamentals, experimental techniques, and applications (157–198). West Sussex: John Wiley & Sons, Inc.
26. D. Zhou, H.-Q. Fang, H.-Y. Chen, H.-D. Zhou, H.-Q. Fang, H.-Y. Chen, H.-X. Ju, and Y. Wang, Analitica Chimica Acta, 329(1–2), 41 (1996).
27. V. Svoboda, C. Rippolz, M. J. Cooney, and B. Y. Liaw, The Journal of Electrochemical Society, 154(3), D113 (2007).
28. C. W. Narvárez Villarrubia, R. A. Rincon, V. K. Radhakrishnan, V. Davis, and P. Atanassov, ACS Applied Materials and Interfaces, 7(6), 2402 (2011).
29. M. Montagné and J.-L. Marty, Analyt. Chem. Acta, 315(3), 297 (1995).
30. S. L. Syve, H. Zheng, H. Okada, and T. Hori, Sens. Actuat B-Chem., 108(1–2), 671 (2005).
31. K. K. W. Maka, U. Wollenberger, F. W. Scheller, and R. Renneberg, *Biosen. and Bioelect.*, **18**(9), 1095 (2003).
32. M. Zhang, C. Mullens, and W. Gierski, *Anal. Chem.*, **79**(6), 2446 (2007).
33. H. Zheng, J. Zhou, J. Zhang, R. Huang, H. Jia, and S.-I. Suye, *Microchim Acta*, **165**, 109 (2009).
34. B. L. Hassler, N. Kohli, J. G. Zeikus, I. Lee, and R. M. Worden, *Langmuir*, **23**, 7127 (2007).
35. Z. Wang, F. Quiòès, G.-W. Kohring, and A. Walcarius, *Biosens. and Bioelect.*, **32**(1), 111 (2012).
36. Z. Wang, M. Etienne, V. Urbanova, G.-W. Kohring, and A. Walcarius, *Anal. Bioanal. Chem.*, **405**, 3899 (2013).
37. R. J. Chen, Y. Zhang, D. Wang, and H. Dai, *Journal of the American Chemical Society*, **128**(16), 3838 (2001).
38. R. P. Ramasamy, H. R. Luckarift, D. M. Ivnitski, P. B. Atanassov, and G. R. Johnson, *Chemical Communications*, **46**, 6045 (2010).
39. K. Artjushkova and P. Atanassov, *Chemphyschem*, **14**(10), 2071 (2013).
40. R. C. Reid, F. Giroud, S. D. Minteer, and B. K. Galea, *J. Electrochem. Soc.*, **160**(9), H612 (2013).