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Computational, electrochemical, and spectroscopic, studies of acetycholinesterase covalently attached to carbon nanotubes

Murilo F. Cabral[a,*,1], Joseph D. Barrios[b,1], Erica M. Kataoka[a,1], Sergio A.S. Machado[a], Emanuel Carrilho[a,c], Carlos D. Garcia[b], Arturo A. Ayon[d]

[a] Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, CEP: 13560-970, São Carlos, SP, Brazil
[b] Department of Chemistry, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249, USA
[c] Instituto Nacional de Ciência e Tecnologia de Bioanalítica, Campinas, Brazil
[d] Department of Physics & Astronomy, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249, USA

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ABSTRACT

This manuscript describes results related to the characterization of electrodes modified with a composite of acetylcholinesterase covalently bound to carbon nanotubes (CNT). The characterization was performed by computational methods and complemented by cyclic voltammetry, infrared spectroscopy, and electrochemical impedance spectroscopy. *In-silico* simulations enabled the identification of the binding site and the calculation of the interaction energy. Besides complementing the computational studies, experimental results obtained by cyclic voltammetry showed that the addition of CNT to the surface of electrodes yielded significant increases in effective area and greatly facilitated the electron transfer reactions. These results are also in agreement with impedance spectroscopy data, which indicated a high apparent rate constant, even after the immobilization of the enzyme. These results lend new information about the physical and chemical properties of biointerfaces at the molecular level, specifically about the mechanism and consequences of the interaction of a model enzyme with CNT.

1. Introduction

The behavior of biomolecules attached to carbon-based nanomaterials has received increased attention in the past decade [1,2]. Among others, the remarkable mechanical, electrical, optical [3], and thermal [4] properties of carbon nanotubes (CNT) have enabled the development of new materials [5] and innovative applications in electronics [6], medicine [7,8], and environmental remediation [9]. Because they enable unique interactions with biological molecules, one of the key areas of research has been the development of CNT-based sensors [10–13], in which the functionalization of the CNT and subsequent attachment of the corresponding biorecognition element are critical to the performance of the sensor.

Several methods have been developed for the functionalization of CNT [14–16], being chemical oxidation amongst the preferred ones [17]. Besides removing organic contaminants and catalysts, this process typically results in the formation of short opened tubes with a high-density of oxygenated functions (carbonyl, carboxyl, hydroxyl, etc.) [18]. Although such modifications tend to affect the electroactivity and electrocatalytic properties of the CNT, they provide convenient ways to further modify the nanotubes through a variety of strategies [19,20] including adsorption or covalent binding, which accounts for approximately 77% of the papers published [14].

Several authors have reported that adsorption can provide a simple way to immobilize a variety of proteins to CNT [12,21–24]. However, one remaining concern is the long-term stability of such composites. Aiming to overcome this deficiency and taking advantage of the presence of carboxylic groups on the surface of CNT, the free amine groups of enzymes can be permanently attached to CNT using carbodiimide coupling chemistry. Such conjugates are stable, even at high temperatures, therefore providing a unique combination of useful attributes such as low mass transfer resistance, high activity and reusability [25]. Because such strategy has the potential to perturb the extended π-conjugation and the intrinsic electronic characteristic of the nanotubes [14] as well as the catalytic activity

* Corresponding author. Present address. Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083-970, Campinas, SP, Brazil.
Tel.: +55 1633738044.
E-mail addresses: mfcabral@gmail.com, mfcabral@lge.ibi.unicamp.br (M.F. Cabral).
[1] These authors contributed equally to this work.

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of the enzyme, a detailed understanding of the interaction is key aspect in the development of sensitive and stable biosensors.

Among other enzymes that can be used as a model to study interactions with carbon nanotubes, acetylcholinesterase (AChE) offers unique features. Besides its wide applicability to the detection and quantification of chemical warfare agents and pesticides [26,27], AChE is a very fast enzyme, functioning at a rate approaching that of a diffusion-controlled reaction [28]. Structurally, AChE is a polymorphic enzyme that appears to form amphiphilic and non-amphiphilic tetramers from a single variant that, when in solution, may present several conformational states of a flexible tetrameric arrangement of catalytic subunits [29]. Consequently, this enzyme has been used a model to investigate the interaction of AChE with a variety of inorganic and organic matrixes, commonly used in the development of biosensors [30–33].

Aiming to understand and characterize the properties of CNT, before and after the attachment of AChE through 1-ethyl-3-carbodiimide (EDC), this paper describes results obtained by a combination of electrochemical (cyclic voltammetry and electrochemical impedance spectroscopy) and spectroscopic techniques (Fourier-transform infra-red and UV–vis spectroscopy). Experimental results are complemented with results obtained by computational methods describing the docking site of AChE to CNT.

2. Experimental

2.1. Computational studies

The statistical analysis of the sequence of AChE (PDB ID: 1C2O, chain A) was performed using CLC's Protein workbench (http://www.clcbio.com/protein). Such analysis was used to calculate the number of residues with hydrophobic/hydrophilic behavior, as well as the relationship between protein charge and solution pH. To probe the interaction between AChE and the multi-walled carbon nanotubes (MWCNT) and to gain further insights about the potential docking sites, computational docking studies were also performed. For these calculations, the three-dimensional structure of selected nanotubes (exported as *.pdb files) was generated using a commercial software package (Nanotube Modeler; JCrystalSoft, 2011) by defining a diameter of 1.1 nm, and the chirality parameters $n = 14$ and $m = 0$. Nanotubes (defined as ligands) were then docked to the X-ray crystal structure of AChE (PDB ID: 1C2O, chain A), using the Lamarckian Genetic Algorithm provided by AutoDock 4.0 software [34]. The entire surface of the protein (which was considered as rigid body) was interrogated for possible interaction sites. The grid maps representing the protein were calculated using the AutoGrid option. A cubic box was built around the protein with $126 \times 126 \times 126$ points. A spacing of 0.41 Å between the grid points was used, placing the center of the protein at the center of the cube. The resulting top minimum-energy orientations with ≤ 2.0 Å r.m.s. deviation were subsequently clustered. The dockings were performed at least 10 times. AutoDock 4.0 software was run using distributed computing (www.pittgrid.pitt.edu).

2.2. Reagents

Acetylcholinesterase form *Electrophorus electricus* (EC#: 3.1.1.7) was purchased from Sigma–Aldrich (Saint Louis, MO) and used as received. Enzymatic activity was measured according a procedure described elsewhere [35]. Acetylthiocholine iodine, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma–Aldrich (Saint Louis, MO). All solutions were prepared using ultra-pure water (>18.2 MΩ cm^{-1}, Barnstead Nanopure, Dubuque, IA).

Multi-Wall CNT (purity > 90%, length 5–9 μm, OD 110–170 nm, prepared by chemical vapor deposition) were purchased from Sigma–Aldrich (Saint Louis, MO). In order to introduce carboxylic groups, MWCNT were suspended in a mixture of 3:1 98% H_2SO_4 and 70% HNO_3 and placed in an ultrasonic bath (GA/TA 08.25, Unique Group; SP, Brazil) at room temperature for 9 h. The MWCNT were then washed by suspension in DI water, centrifugation (15 min at 3000 rpm), and separation from the supernatant. This sequence was repeated until the pH of the dispersion was at least 6. The final product was dried in a convective oven at 60 °C overnight and kept in a vacuum desiccator until use.

2.3. Instrumentation

FT-IR spectra of bare and modified MWCNTs were performed at room temperature in a Bomem MB-102 (Hartmann & Braun-Michelson; Quebec, Canada) spectrophotometer by including the selected MWCNT in KBr pellets. In all cases, data was acquired in the range of 500–4000 cm^{-1}, using 20 scans. Background was corrected by the signal obtained using plain KBr pellets. Electrochemical measurements were performed using an Autolab potentiostat/galvanostat (PGSTAT40 Eco Chemie; Utrecht, Netherlands) coupled to a personal computer and controlled with GPES 4.9 software. A conventional three-electrode cell was used for all experiments herein described. In all cases, the corresponding modified electrodes were used as the working electrodes, a Ag/AgCl/KCl (3 mol L^{-1}) was used as the reference electrode, and a Pt plate (1 cm^{-2}) was used as the auxiliary electrode. The supporting electrolyte was 40 mmol L^{-1} Britton–Robinson buffer solution (pH 8), which was deoxygenated with high-purity nitrogen for 5 min before the electrochemical measurements. All the electrochemical measurements were performed at room temperature. Electrochemical impedance spectroscopy (EIS) data were obtained using a PC-controlled FRA2 (Eco Chemie, Utrecht, Netherlands) coupled to the aforementioned potentiostat, by scanning from 100 kHz to 40 mHz at a 10 mV amplitude, with 10 data points per frequency decade. The measurements were carried out in the presence of 5.0 mmol L^{-1} $K_4(Fe(CN)_6)$, using 40 mmol L^{-1} Britton–Robinson buffer (pH 8) as the supporting electrolyte. The impedance spectra were then analyzed with the simulation software Zview-Impedance®, version 2.4a. To determine the corresponding values for each electrical element, a Randles-type equivalent circuit (vide infra) was used.

2.4. Preparation of electrodes

Prior to all the experiments, pyrolytic carbon (PG) electrodes (Ø 3.0 mm, GoodFellow; Huntington, England) were polished with alumina (0.10 μm and 0.05 μm) and then ultrasonically cleaned in ethanol and water for 15 min each. The electrodes were then cycled in Britton–Robinson buffer 40 mmol L^{-1}, pH 8 (from −0.6 V to 0.8 V, 0.1 V s^{-1}) until a steady-state current was obtained, typically requiring ~20 cycles. These electrodes were then modified by dispensing 30 μL of an aqueous suspension containing MWCNT (1 mg mL^{-1} DI H_2O) on the surface of the PG electrode and letting the water evaporate at room temperature. The resulting electrodes will be referred to as PG/MWCNT.

In order to modify electrodes with AChE, the protein was covalently linked to MWCNT according to previous literature reports [36,37]. Briefly, the carboxylic groups present on the MWCNT walls were modified using EDC and N-hydroxysuccinimide (NHS) yielding the formation of active esters. Then, these esters were exposed to amino groups present in the AChE (in 40 mmol L^{-1} Britton–Robinson buffer, pH 8.0) leading to the MWCNT/AChE nanocomposite. PG electrodes were then modified by dispensing 30 μL of an aqueous suspension containing MWCNT/AChE