A New Nanomaterial for the Study of Protein Interactions

Alina VASILESCU[1], Iuliana MIHAI[1], Alis VEZEANU[1],
Veronica ANDREI[1], Georgian NEDELCU[1], Elena POPA[2],
Cristina PURCAREA[2], Palaniappan SUBRAMANIAN[3],
Yannick COFFINIER[3], Rabah BOUKHEROUB[3],
Sabine SZUNERITS[3]

[1]International Centre of Biodynamics, 1 B Intrarea Portocalelor, sector 6,
060101, Bucharest, Romania
[2]Institute of Biology of the Romanian Academy, 296 Splaiul Independentei,
060031 Bucharest, Romania
[3]Institut de Recherche Interdisciplinaire (IRI, USR CNRS 3078), Université Lille 1,
Parc de la Haute Borne, 50 Avenue de Halley,
BP 70478, 59658 Villeneuve d'Ascq, France

Abstract. Selective determination of amino acids such as tyrosine and tryptophan is highly relevant when studying protein interactions. We investigated the efficacy of *Boron-Doped Diamond NanoWires* (BDD NWs) for the sensitive detection of the two amino acids in binary mixtures, and in a protein, lysozyme. BDD NWs allowed differentiating the individual electrochemical signals due to tyrosine and tryptophan oxidation in binary mixtures, and were used for monitoring lysozyme aggregation by *Square Wave Voltammetry* (SWV) and *Electrochemical Impedance Spectroscopy* (EIS). BDD NWs represent thus an interesting alternative nanostructured electrode for the electrochemical detection of these amino acids from biological samples.

1. Introduction

Selective determination of amino acids is very important for several transformations suffered by peptides and proteins, including phosphorylation [1, 2], nitration, unfolding [3], and aggregation [4, 5], as well as for monitoring their interactions with various compounds [6] for drug screening purposes. Changes in the exposure of tyrosine and tryptophan residues during protein unfolding and aggregation were emphasized by several electrochemical studies, mainly concerning the direct electrochemical oxidation of these amino acid residues.

However, the ability to capture snapshots of structural changes in proteins by electrochemical methods depends not only on the nature of the protein, but also on the electrode material [7]. Electrochemical oxidation of tyrosine and tryptophan occurs at very similar potential values on a variety of materials. *Boron Doped Diamond* (BDD) films raisedhigh interest for applications in the field of electroanalytical chemistry, mostly due to their wide potential range for water stability as compared to other materials, their low background current and their biocompatibility[1, 8–14]. H-terminated BDD electrodes have been described as a hydrophobic, low-fouling substrate, ideal for electrochemical determinations in complex matrices. Oxidation of H-terminated BDD films by thermal, plasma, ozone treatment or electrochemical methods resulted in interfaces with applicative interest for electrochemistry, presenting different chemical reactivity and opening new avenues for functionalization [15]. BDD electrodes have been used for the detection of proteins [1], along with more classic carbon-based electrodes [7].

In the past decade, several reports have demonstrated the fabrication of nanostructured diamond interfaces [16–22]. The increase of the surface area of these interfaces resulted in higher sensitivities and selectivities when compared to planar BDD interfaces. Some of us have shown recently that BDD NWs can be prepared from polycrystalline BDD interfaces by reactive Ion Etching (RIE) with oxygen plasma in a maskless approach [18, 21–23]. These new interfaces allowed a sensitive detection of tryptophan with a detection limit of 5×10^{-7} M, representing a 20-fold improvement compared to planar electrodes from the same material [18].

These results prompted us to evaluate the utility of BDD NWs for the selective detection of tyrosine and tryptophan, both in binary mixtures and in a protein, represented by lysozyme, in a comparative study when using glassy carbon electrodes. The BDD NWs were used for monitoring the self-assembly of lysozyme in acidic media using *Square Wave Voltammetry* (SWV), and *Electrochemical Impedance Spectroscopy* (EIS). Fluorescence measurements using an amyloid indicator dye (Thioflavin T), *Atomic Force Microscopy* (AFM) and *Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis* (SDS-PAGE) were additionally performed in order to assess the degree of aggregation at various time points.

2. Material and Methods

Tryptophan was purchased from Fluka. Hen egg lysozyme,andtyrosinewere from Sigma Aldrich. Britton-Robinson buffer, pH 2–11, and 0.2 M acetate, pH 5, were used in the electrochemical tests. Glassy carbon electrodes were from BAS.

BDD NWs electrodes were prepared from polycrystalline highly boron-doped diamond electrodes (the boron concentration was $N_A= 8\times10^{19}$ B cm^{-2} determined by SIMS measurements) by Reactive Ion Etching with oxygen plasma, without using any mask or template [18]. *Reactive Ion Etching* (RIE) of BDD was performed

with oxygen plasma (Plasmalab 80 plus), with a radio frequency generator (13.56 MHz) for 10 min. Operating oxygen pressure, flow speed, and plasma power were: 150 mT, 20 sccm, and 300 W. The resulted BDD NWs were immersed for 15 min in an aqueous solution of HF (5% v/v) to dissolve the SiO_2 deposits. The dimensions of the obtained nanowires were 1.4 ± 0.1 μm long, with a tip and base radius of r_{tip} = 10 ± 5 nm and r_{base} = 40 ± 5 nm. Their density was (14 ± 3).10^8 nanowires cm $^{-2}$[9].

2.1. Forced Aggregation of Lysozyme

A solution of 10 mg/mL lysozyme in 0.015 M at pH 2, containing 5 mg/mL NaCl was filtered through a 0.22 μm Nylon membrane and incubated at 60ºC for up to 4 days. Aliquots were removed at various time intervals and diluted to 10-30 μg/mL with Britton-Robinson buffer pH 2 before analysis by electrochemical methods. The aggregation process was studied in detail by a variety of techniques as described elsewhere [24].

2.2. Electrochemical Studies

A VSP potentiostat/galvanostat from Bio-Logic S.A, France, equipped with EC-Lab software, was used for the electrochemical studies. Studies with BDD-NWs were performed in a flow-through cell of about 120 μL volume with BDD NWs as the working electrode, while the counter electrode was a platinum wire and the reference was a silver wire covered with silver chloride (Ag/AgCl). The geometric area of the BDD NWs exposed in the electrochemical cell was 4.1 mm^2.

The BDD NW electrodes were initially cleaned for 10 minutes in piranha (Hydrogen peroxide: sulphuric acid =1:3. *Warning: Piranha solution reacts violently with most organic materials and must be handled with extreme care*!) followed by a 15 minutes UV-ozone treatment (PSD-UV, NovaScan). After each measurement, the nanowires were cleaned in the presence of 0.2 M NaOH,by cycling the potential between -0.4 and 1.2 V *vs* Ag/AgCl, at 50 mV/s for 5-10 cycles.

In the case of glassy carbon electrodes, we used a classical setup, where the counter electrode was a Pt wire and the reference was Ag/AgCl, KCl (3M). The measurements were performed in 0.2 M acetate buffer pH 5, as described elsewhere [24]. The electrodes were cleaned by polishing with alumina slurry, followed by rinsing with water.

2.2.1. Tests with BDD NWs

Analysis of tyrosine and tryptophan

Solutions of 100 μg/mL tyrosine and 10 μg/mL tryptophan at pH ranging from 2 to 11 were prepared in Britton-Robinson buffer. For cyclic voltammetry

experiments, the potential was scanned at 50 mV/s, from 0 to 1.3 V, vs. Ag/AgCl. SWV analysis was optimized by analyzing the amino acid solutions of various concentrations at a frequency of 5 Hz, step height of 10 mV, and peak amplitude of 25 mV. The potential was swept directly from -0.25 to 1.2 V without any pre-adsorption.

Analysis of lysozyme by Square Wave Voltammetry (SWV)

The protein solution obtained in 20 mM Britton-Robinson buffer pH 2 was adsorbed on pseudo Ag/AgCl for 5 minutes at -0.25 V in the flow-through cell, and the potential was further increased to +1.5 V at 200 Hz, with a step height of 5 mV and a peak amplitude of 25 mV. In the case of the glassy carbon electrodes, the square wave voltammetry measurements (SWV) were performed in the presence of 0.2 M acetate buffer containing 0.1 M NaCl, pH 5, after first pre-treating the electrodes by polarizing them at +1.4 V vs. Ag/AgCl for 30 s [24].

Analysis of lysozyme by Electrochemical Impedance Spectroscopy (EIS)

The EIS spectra were recorded withina frequency range of 0.1–9.5 kHz, usingan equimolar mixture of 1 mM potassium ferricyanide and potassium ferrocyanide in PBS pH 7.4 buffer, at the formal potential of the ferri/ferro redox couple. A sinusoidal signal with 10mV amplitude was applied in addition of the formal potential. Data were fitted to an equivalent circuit (R1+Q2/(R2), using Zview software. The variation of the resistance to the ion transfer, before and after lysozyme adsorption, was determined as a function of lysozyme concentration (calibration) and incubation time (aggregation studies).

2.3. Study of the lysozyme aggregation by Thiflavin T fluorescence, SDS-PAGE and Atomic Force Microscopy

Thioflavin T fluorescence and Atomic Force Microscopy experiments were conducted as previously described [24], using a Glomax 20/20 Luminometer (Promega) with a Fluorescent Blue Module (excitation at 465–485 nm, emission at 515–575 nm). Samples of 10 µL protein solution were mixed with 1 mL of 28 mM thioflavin T in PBS pH 7.4 buffer,and the fluorescence was immediately measured. Results were expressed as the mean of five replicate measurements. AFM studies were carried on using a Nanowizard II AFM instrument (JPK, Germany) operating in intermittent contact in air, and the protein structure was visualized with an RTESP cantilever (resonant frequency of &300 kHz, force constant of ~40 N m^{-1}, (Veeco, USA). Aggregated lysozyme solutions(50 µg/mL)were adsorbed on mica and dried for 30 minutes at room temperature under vacuum, and the resulted mica samples were thoroughly washed with ultrapure water and dried under nitrogen flow. A SDS-PAGE analysis of aggregated lysozyme was performedusingan electrophoresis system with cooling (Biometra, Analytik Jena) to avoid

decomposition of aggregates at high temperature in SDS, and the protein oligomers were separated on 10% polyacrylamide gels at 20 mA and 40 mA/cm gel.Protein fragmentswere stained with Coomassie Blue for visualizing their migration position.

3. Results and Discussion

3.1. Detection of Tyrosine and Tryptophan in Simple Mixtures

A 10:1 mixture of tyrosine: tryptophan was analyzed by cyclic voltammetry in Britton-Robinson buffer with pH ranging from 2 to 11. The electrochemical oxidation signals for tyrosine and tryptophan were well separated at pH 2, while, at alkaline pH, the peaks completely overlapped (Fig. 1a). The redox peaks corresponding to the oxidation of tyrosine and tryptophan shifted towards more negative values as the pH increased, in accordance with electrode reactions involving transfer of protons. When glassy carbon electrodes were used, the two amino-acids were oxidized at very similar potential values, exhibiting fully overlapped peaks (Fig. 1b). The peak intensity also decreases upon cycling due to passivation of the electrode surface with adsorbed oxidation products.

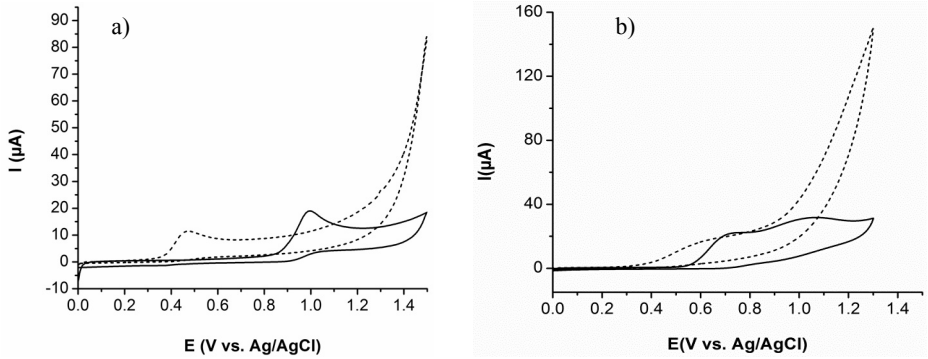

Fig. 1. Cyclic voltammograms of mixtures of tyrosine/tryptophan (100 µg/mL tyrosine and 10 µg/mL tryptophan recorded on (a)BDD NWs and (b) glassy carbon electrodes in Britton-Robinson buffer pH 2(full line) and pH 11 (dashed line) at a scan rate of 50 mV/s.

Our data, unraveling the ability of BDDNWs to discriminate between the electrochemical signature of tyrosine and tryptophan is a particularly important result, since very few materials that allow such separationhave been described so far.

We further explored the potential of BDD NWs for the sensitive detection of amino-acids using SWV. In SWV, the contribution from the charging capacitive current to the electrochemical signal is minimized, which can lead to improvement of the sensitivity of the detection compared to cyclic voltammetry.

Typical voltammograms recorded for tyrosine and tryptophan (Fig. 2) emphasize the difference in the magnitude of the oxidation peak for the two aminoacids at the same concentration. Depending on the ratio between tyrosine and

tryptophan, their accurate detection in binary mixtures would be hampered by the partial overlap of their electrochemical oxidation signals.

At pH 2, the tyrosine current intensity peak fromthe SWV profile showed a linear dependence with the amino-acid concentrations in the range 1–60 μM, according to: $I_{(\mu A)} = 0.0526 \times [\text{tyrosine}]_{(\mu M)} + 0.0779$($R^2 = 0.9972$), with a detection limit (S/N=3) of 0.86 μM. Under the same conditions, the current signal for tryptophan oxidation varied linearly with the protein concentration in the range 0.2–200 μM according to $I_{(\mu A)} = 0.0705 \times [\text{tryptophan}]_{(\mu M)} + 0.4557$ ($R^2 = 0.9947$) with a detection limit of 0.14 μM. When compared to similar experiments performed on screen-printed carbon electrodes, a lower detection limit of 10 nM and a linear range from 100 nM -200 μM were obtained for this aminoacid [2]. However, in this later case, the accumulation time on the surface of screen-printed electrodes prior to the voltammetric detectionwas of 10 minutes, while our tests with BDD NWs do not involve any pre-concentration of the electroactive species.

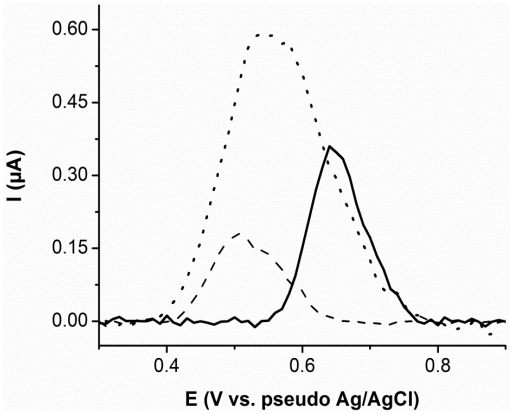

Fig. 2. SW voltammograms for 5 μM tyrosine (full line), 5 μM tryptophan (dotted line) and 0.5 μM tryptophan (dashed line) using BDD NWs in Britton Robinson buffer at pH 2.

The individual contributions of tyrosine and tryptophanto the electrochemical oxidation signal were previously studiedwhen using carbon electrodes for the analysis of binary aminoacid mixtures and small peptides[4]. In this case, a baseline separation was obtained between the oxidation signals of these two amino-acids fromthe 393 aminoacid cancer-relatedp 53 protein.

In the following we investigated the electrochemical detection of the aminoacids in lysozyme using BDDNWs electrodes. We further used the BDDNWs electrodes for the electrochemical monitoringof the aggregation process of this protein at pH 2.

3.2. Lysozyme Detection

Although lysozyme contains 3 tyrosine and 6 tryptophan residues, its electrochemical signal is determined by the exposed groups and influenced by the neighboring amino acids. SWV studies of lysozyme solutions using BDDNWs emphasized only one oxidation peak the signalsof tyrosine and tryptophan overlapping completely at pH 2–11 (Fig. 3). The current intensity peak value increased linearly with the lysozyme concentration in the range between 0.140 µM and 1.4 µM, with a detection limit (S/N = 3) of 70 nM (Fig, 3a and b) comparable to the analytical characteristics previously obtained with GC electrodes [24].

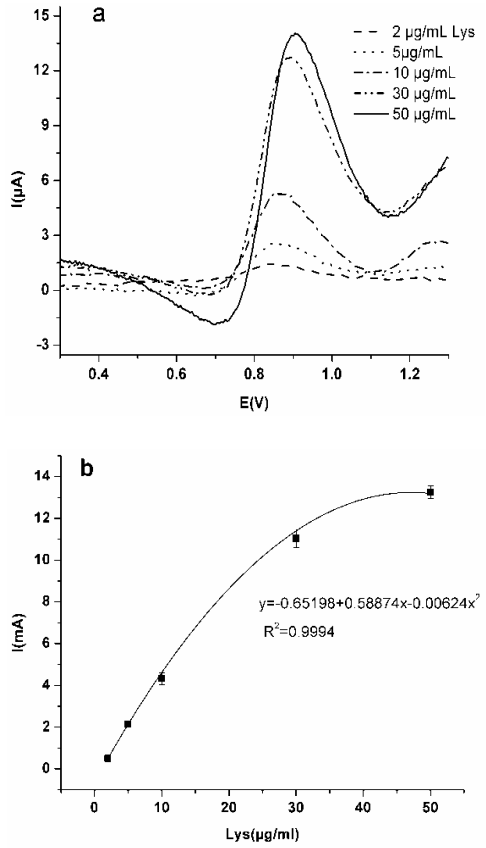

Fig. 3. SWV obtained on BDDNWs electrodes for different concentrations of lysozyme, after subtracting the base line (a), and the corresponding calibration curve (b).

When using EIS, similar results were obtained. The impedance spectra were fitted to a classic circuit (Fig. 4a and b), in order to determine the resistance to charge transfer (Rct). The charge transfer resistance showed a linear dependency

with the lysozyme concentration in the range of 0.140–1.4 μM (R^2 = 0.9901), with a detection limit of 70 nM.

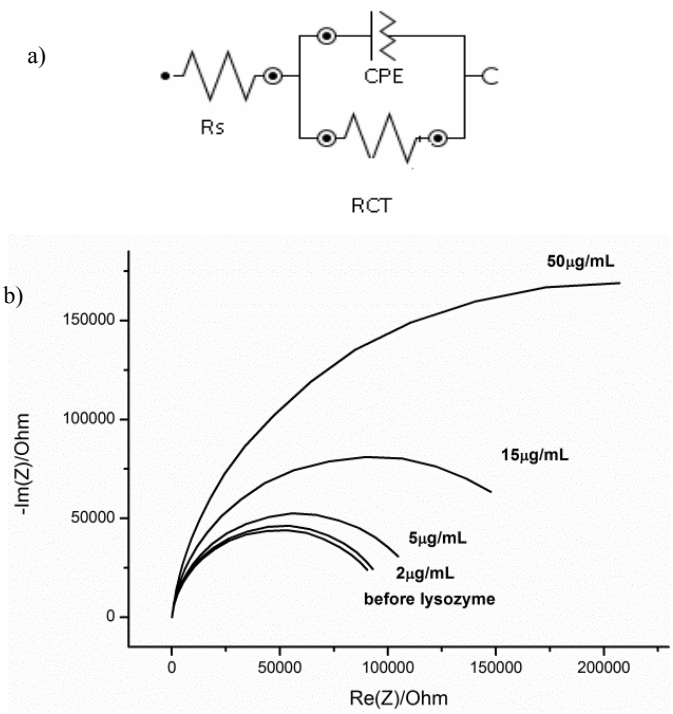

Fig. 4. Detection of lysozyme with BDD-NWs electrodes by EIS: Equivalent circuit used for fitting the data (a) and Nyquist plots for different concentrations of lysozyme (b).

3.3. Lysozyme Aggregation

The lysozyme aggregation process wasalso investigated by SWV and EIS. SWV showed an increase in the peak intensity during the initial 24 hours of the aggregation process, correlating with an increase in the charge transfer resistance to charge transfer using EIS. This was followed by a slow but continuous decrease of the signal therafter (Fig. 5a). A similar profile was previously observed for the first 24 hours of the same aggregation process monitored on glassy carbon electrodes [24] (Fig. 5b). However, both BDD NWs and glassy carbon electrodes did not allowed distinguishing between the individual contributions of tyrosine and tryptophan to the electrochemical signal recorded by SWV during the aggregation.

Our previous characterization of the lysozyme aggregation process using MALDI-TOF indicated that in the first 24 hours, the dominant processes were the lysozyme acid hydrolysis and formation of small soluble oligomers up to homopentameric quaternary structure [24].

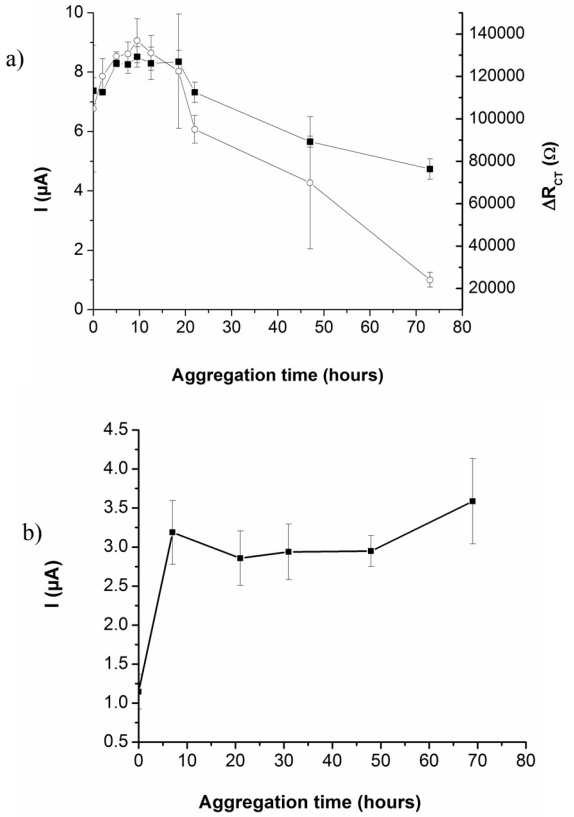

Fig. 5. Time course of lysozyme aggregation in the presence of (a) BDD –NWs using SWV (full squares) and EIS (open circles) and (b) in the presence of glassy carbon electrodes using SWV.

Moreover, the while oligomerization process from monomer to aggregates implies several probable pathways occurring in parallel [25], the population of protein species in solution at any given time is rather complex. In this context, the utilization of BDD NWs and electrochemical methods, such as SWV and EIS, allowed us to capture a snapshot of these transformations.

In order to correlate the electrochemical data with different stages of aggregation, we analyzed the aggregated lysozyme by not only by electrochemical methods but also by thioflavin T fluorescence, SDS-PAGE and AFM.Thioflavin T is widely used as an *in vitro* amyloid staining dye, based on the shift of its excitation maxima and enhanced fluorescence presented upon binding to β-sheets in protein fibrils. In our experiments, fluorescence measurements with thioflavin T were typical for aggregation studies leading to formation of amyloid fibrils and emphasized a lag period of about 24 hours, corresponding to a sharp increase in

fluorescence and a plateau after 3–4 days (Fig. 6). Within corroboration with the AFM results (Fig. 6, insets) and SDS-PAGE data (Fig. 7), this observation supports the SWV profiles obtained. In this case, the most extensiveshift in the electrochemical signal recorded with both BDD-NWs and glassy carbon electrodes occured in the first 24 hours, aspreviously observed[24]. These signalchanges are attributed to protein unfolding, exposing more electroactive tyrosine and tryptophan residues to the hydrophilic environment.

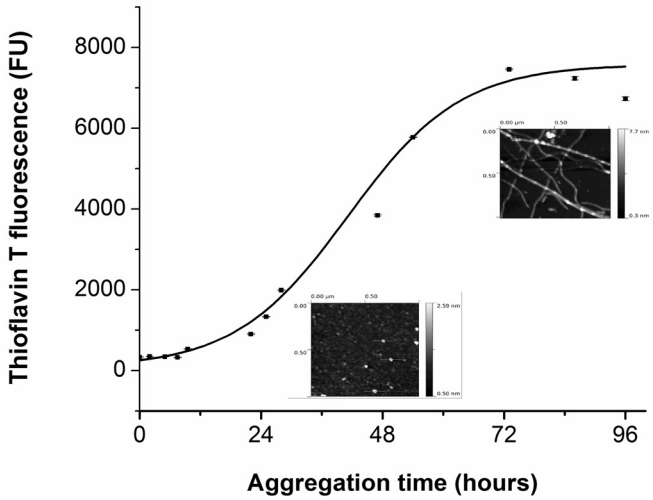

Fig. 6. Fluorescence of thioflavin T in solutions of lysozyme at different aggregation times. Insets: intermittent contact mode AFM images of fresh lysozyme (bottom inset; protein fibrils formed long, twisted protein fibrils (4 nm diameter) formed in lysozyme solutions kept for three days in the forced aggregation conditions (upper inset).

There was no increase in thioflavin T fluorescence in the first 24 hours,indicating that the fibrillation did not start after this period (Fig. 6). After 2–3 days of aggregation, the fibril formation determined by AFM (Fig. 6, insets) corresponded to the sharp increase in thioflavin T fluorescence (Fig. 6) due to its binding to amyloid fibrils [26].

Moreover, the fibrils formation leads to the decrease of the available electroactive amino-acid residues inducing an electrochemical signal decrease.

SDS-PAGE confirmed the results observed with the techniques detailed above. The concentration of monomers decreased after increasing aggregation time and in the presence of various NaCl concentrations, due to acidic hydrolysis,with the formation of increasing amounts of high molecular weight oligomers (around 80–120 kDa) (Fig. 7).

These results, together with data obtained with the complementary techniques presented above, substantiate our explanation of the trends observed with

electrochemical methods during aggregation, both using BDD NWs or glassy carbon electrodes.

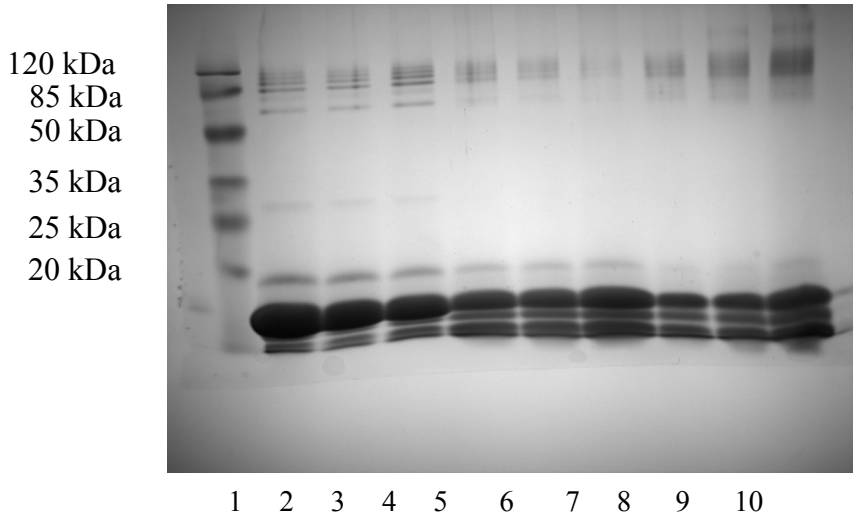

Fig. 7. SDS-PAGE analysis of lysozyme solutions. Samples of 10 mg/mL lysozyme solutions at pH 2 were incubated at 60°C for different periods of time and in the presence of various concentrations of NaCl: (1) MW Markers; (2) 2h, 3 mg/mL NaCl; (3) 2h, 5 mg/mL NaCl; (4) 4h, 5 mg/mL NaCl; (5) 18h, 5 mg/mL NaCl; (6) 20h, 5 mg/mL NaCl; (7) 24h, 5 mg/mL NaCl; (8) 28h, 5 mg/mL; (9) 41h, 3 mg/mL; (10); 41 h, 5 mg/mL NaCl.

Our results are in accordance with those obtained by the electrochemical monitoring of Alzheimer's disease (AD)amyloid beta peptides (Aβ-40, Aβ-42) aggregation process [5]. The present study also confirms that BDDNWs represent an adequate material for studying protein interactions, suggesting that it could be used as a general tool for studying proteins that are prone to aggregation.

4. Conclusions

Our results demonstrated that utilization of BDDNWs allowed not only the sensitive detection of tyrosine and tryptophan, but also partially resolved the individual electrochemical oxidation signals for simple mixtures analyzed at pH 2. This procedure provides a simple way to analyze aminoacids that could be explored for analyzing biological samples. However, under these conditions, it was not possible to resolve the contributions due to the tyrosine and tryptophan residues in lysozyme, not in the fresh protein or in aggregated samples. Future investigations are required to further improve the resolution between the electrochemical oxidation signals for the two aromatic amino-acids, by using approaches based on chemometrics.

Electrochemical detection of lysozyme with BDD-NWs was equally sensitive by using SWV and EIS, with a detection limit of 1 μg/mL. Moreover, the lysozyme adsorbed on the nanowires could be easily removed electrochemically after each analysis. Therefore, BDD-NWs could be useful in the flow injection analysis of lysozyme and, potentially, of other electroactive proteins. This is the first report of using BDDNWs for monitoring the aggregation of lysozyme using a forced degradation protocol leading to amyloid fibrils. The data obtained was further underlined by fluorescence measurements using Thioflavin T, SDS-PAGE and AFM, respectively. Moreover, the analytical performances and the trends observed with BDDNWs and glassy carbon electrodes when monitoring lysozyme aggregation were similar.

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