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Interfacial behavior of n-nitrosodiethylamine/bovine serum albumin complexes at the air-water and the chloroform-water interfaces by axisymmetric drop tensiometry

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Abstract. Interfacial properties of N-nitrosodiethylamine/Bovine Serum Albumin (NDA/BSA) complexes were investigated at the air-water interface. The interfacial behavior at the chloroform–water interface of the interaction of the phospholipid 1,2-dipalmitoyl-sn-Glycero-3-phosphocholine (DPPC), dissolved in the chloroform phase and the NDA/BSA molecules, in the aqueous phase, were also analyzed by using a drop tensiometer. The secondary structure changes of BSA with different NDA concentrations were monitored by circular dichroism spectroscopy at different pH and the NDA/BSA interaction was investigated by Fluorescence spectroscopy. Different NDA/BSA mixtures were formed to get 0, 7.5x10^{-5}, 2.2x10^{-4}, 3.7x10^{-4}, 5x10^{-4}, 1.6x10^{-3} and 3.1x10^{-3} M NDA concentrations in order to produce 0, 300/1, 900/1, 1500/1, 2000/1, 6000/1 and 12500/1 NDA/BSA molecular proportions, respectively in the aqueous solutions. α–helix content increments of BSA were obtained up to the 2000/1 NDA/BSA proportion, but for the 6000 and 12500 NDA/BSA proportions, the α–helix content practically disappeared. These BSA structure changes produced an enhancement of the surface pressure at the air-water interface, as the α–helix content increased with the NDA molecules. On the contrary, when α–helix content decreased, the surface pressure also appeared lower than the one obtained with pure BSA solutions. The interaction of DPPC with NDA/BSA molecules at the chloroform–water interface produced also a small, but measurable, pressure increment with the addition of NDA molecules. Dynamic Light scattering measurements of the NDA/BSA molecular sizes at pH 4.6, 7.1 and 8.4 indicated that the size of extended BSA molecules at pH 4.6 increased in a higher proportion with NDA molecules than for the other pH analyzed. Diffusion coefficients calculated from dynamic surface tension values, using a short-term solution of the general adsorption model of Ward and Tordai, also showed
differences with pH and the NDA concentration. Both, the storage and loss dilatational elastic modulus were obtained at the air-water and at the chloroform-water interfaces. The interaction of NDA/BSA with DPPC at the chloroform water produced a less rigid monolayer than the one obtained with pure DPPC (1x10^{-5} M), indicating a significant penetration of NDA/BSA at the interface. At short times and pH 4.6, the values of the storage elastic modulus were higher and more sensible to the NDA addition than the ones at pH 7.1 and 8.4, probably due to a gellike net formation at the air-water interface.

**Keywords:** BSA, interfaces, surface tension, elastic modulus, carcinogen
Introduction

Investigation of macromolecules (proteins and DNA) adducts has become in one of the most important research fields since time ago due to the direct or indirect connection with the carcinogenesis phenomenon. The protein and DNA adducts measurement is a form of chemical dosimetry which can be used to know the history of exposure to genotoxic substances\textsuperscript{1}. Albumin is one of the most investigated proteins and albumin adducts have been investigated long time ago, Bahl and Gutman in 1964 performed in vivo experiments to analyze the binding of rat serum albumin with N-2 fluorenylacacetamide\textsuperscript{2}. Due to the biological importance of serum albumin and the facility to obtain it, many investigations of serum albumin adducts have been performed\textsuperscript{3,4}. Recently Boysen and Hecht have reviewed the DNA and protein adducts of Benzo(a)pyrene\textsuperscript{5}. Protein interacting with water soluble carcinogens have also received attention since long time ago; Bemis et al. in 1966 investigated the effect of N-Nitrosodiethylamine and N-Nitrosomethyamine in the protein denaturing by using optical rotation, circular dichroism and light scattering techniques\textsuperscript{6}. N-Nitrosodiethylamine is present in foods, beverages, tobacco smoke, herbicides, pesticides, drinking water and industrial pollution\textsuperscript{7}. Gorsky and Hellenberg investigated the labeling of Albumin secreted from isolated rat hepatocytes during the metabolism of N-Nitrosodiethylamine (NDA)\textsuperscript{8}. However, most of the techniques used to investigate protein adducts are related to understand their biochemical and physicochemical properties in solution\textsuperscript{9,10,11}, leaving the surface and interfacial properties of these complexes. On the contrary, investigations of surface properties of pure proteins, like bovine serum albumin\textsuperscript{12}, $\alpha$-Lactalbumin and $\beta$-Lactoglobulin\textsuperscript{13}, Lysozime and others\textsuperscript{14} have received reasonable attention. For this reason, we think it would be
important to understand the influence of adducts on the surface properties of biomacromolecules and the interactions of protein adducts with different phospholipids at interfaces. In this paper we investigate the effect of the carcinogen NDA on the interfacial properties of bovine serum albumin (BSA) at the interface air-water and chloroform-water. For this purpose, we perform axial symmetric drop tensiometer measurements of the complexes NDA/BSA with different NDA concentrations. Due to the fact that BSA takes different conformations at different pH \(^{12}\), we analyze the short time behavior of the influence of NDA on the BSA adsorption at the air-water interface and measure the complex dilatational elastic modulus of the adsorbed complexes at the interface for different pH. Also, by dynamic light scattering (DLS), we compared the change of hydrodynamic radius of the NDA/BSA complexes at different pH. Circular dichroism of the NDA/BSA complexes was performed to investigate the change of BSA secondary structure in pure water and in presence of a buffer solution. The kind of the NDA/BSA bonding was estimated by using fluorescence measurements and the van't Hoff equation \(^{13}\). At times when both the surface tension and the elastic modulus were practically constant, we measured the effect of NDA on the BSA adsorption at the air-water interface and, at the chloroform-water interface we analyzed the interaction with the phospholipid 1,2-dipalmitoyl-sn-Glycero-3-phosphocholine (DPPC) dissolved in the chloroform phase. The characterization of the phospholipid/protein adsorption at the aqueous solution/chloroform interface was analyzed by Li et al. \(^{15}\) with different proteins and demonstrated the important role of the kind of the lipid head group and the lipid concentration, however they did not measure the elastic modulus of the interaction between lipids and the adsorbed proteins. In this work we used the Maxwell model \(^{16}\) and determined the relaxation times of the NDA/BSA complexes at both interfaces.
Experimental

Bovine serum albumin (BSA) was obtained from BD Biosciences (99 %, delipidized, and globulins free), 1,2-dipalmitoyl-sn-Glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids. Both were used without further treatment. N - Nitrosodyethylamine (NDA) was obtained from TCI America (99 %) and NaCl (reagent grade) was purchased from Sigma, U. S. A.). Chloroform (HPLC grade) and ethyl alcohol (reactive grade) were purchased from SIGMA-ALDRICH (U. S. A.). Water used through the experiments was filtered by an Easy pure/Barnstead instrument with a resistivity of 18.3 MΩ·cm.

Dynamic light scattering measurements. The size of BSA molecules and NDA/BSA complexes was estimated with the dynamic light scattering technique. Measurements were performed using an ALV-5000 digital correlator system (Langen-GmbH, Germany) fitted with a temperature control set at 25 ± 0.1 °C. The incident light was vertically polarized with a $\lambda_0 = 632 \text{nm}$ Argon laser (30 mW) and the scattered light was measured at 90°. The hydrodynamic radius, $R_h$, was obtained for diluted samples through the Stokes–Einstein relation $D_h = \frac{k_B T}{6\pi\eta R_h}$, where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ the viscosity of the solvent and $D_h$ the diffusion coefficient at infinite dilution.

Average hydrodynamic radius of BSA diluted solutions and two different NDA/BSA proportions were determined at pH 4.6, pH 7.1 and pH 8.4 in a 10 mM phosphate buffered solution. All measurements were done three times, each one in a time interval of 100 s and averaged with the software of the instrument (ALV 5000/E/WIN Software). The average size of the NDA/BSA molecules was measured about 30
minutes after the NDA solution was added to the BSA solution. All solutions were filtered with a 0.2 \( \mu \)m filter.

Surface tension and rheology measurements. Pendant bubble tensiometry was used to determine the dynamic interfacial tension and the interfacial dilatational rheology measurements at the air-water interface with the air bubble in the upward direction. The bubble was formed at the tip of a U shaped stainless steel needle (0.5 mm i. d.) immersed in the aqueous BSA or the NDA/BSA complexes solutions. The equipment used was a ‘Tracker’ tensiometer (I. T. Concept, France) capable of real time measurements. For the analysis of the chloroform water interface, a vertical stainless steel needle (0.5 mm i. d.) mounted on a syringe produces a pendant drop and allows to change the drop size by a mechanically controlled system. The needle is immersed into a quartz cuvette (103.051F-Og, 20-10, Hellma, Germany), filled with the BSA or the NDA/BSA complexes solutions, to produce the chloroform/water interface. The measurement of the interfacial tension and the rheological measurements is based on the digital profile of the drop image and the solution of the Gauss-Laplace equation. The software used for the axisymmetric drop shape analysis is the Win Drop software (I. T. concept, France).

BSA concentration was kept constant (2.5x10^{-7}M) in all performed experiments. Different NDA/BSA mixtures were formed to get 0, 7.5x10^{-5}, 2.2x10^{-4}, 3.7x10^{-4}, 5x10^{-4}, 1.6x10^{-3} and 3.1x10^{-3} M NDA concentrations in order to produce 0, 300/I, 900/I, 1500/I, 2000/I, 6000/I and 12500/I NDA/ BSA molecular proportions, respectively in the aqueous solutions.

For the analysis of the interfacial tension (\( \gamma \)), the dilatational elastic modulus (\( G' \)) and viscous modulus (\( G'' \)) at the air-water interface, we started the measurements after 1800 s. The used frequencies were 1, 0.5, 0.25, 0.1 0.05 and 0.033 s^{-1}. After this time,
minutes after the NDA solution was added to the BSA solution. All solutions were filtered with a 0.2 μm filter.

Surface tension and rheology measurements. Pendant bubble tensiometry was used to determine the dynamic interfacial tension and the interfacial dilatational rheology measurements at the air-water interface with the air bubble in the upward direction. The bubble was formed at the tip of a U shaped stainless steel needle (0.5 mm i. d.) immersed in the aqueous BSA or the NDA/BSA complexes solutions. The equipment used was a ‘Tracker’ tensiometer (I. T. Concept, France) capable of real time measurements. For the analysis of the chloroform water interface, a vertical stainless steel needle (0.5 mm i. d.) mounted on a syringe produces a pendant drop and allows to change the drop size by a mechanically controlled system. The needle is immersed into a quartz cuvette (103.051F-Og, 20-10, Hellma, Germany), filled with the BSA or the NDA/BSA complexes solutions, to produce the chloroform/water interface. The measurement of the interfacial tension and the rheological measurements is based on the digital profile of the drop image and the solution of the Gauss-Laplace equation. The software used for the axisymmetric drop shape analysis is the Win Drop software (I. T. Concept, France).

BSA concentration was kept constant (2.5x10^{-7}M) in all performed experiments. Different NDA/BSA mixtures were formed to get 0, 7.5x10^{-3}, 2.2x10^{-4}, 3.7x10^{-4}, 5x10^{-4}, 1.6x10^{-3} and 3.1x10^{-3} M NDA concentrations in order to produce 0, 300/1, 900/1, 1500/1, 2000/1, 6000/1 and 12500/1 NDA/ BSA molecular proportions, respectively in the aqueous solutions.

For the analysis of the interfacial tension (γ), the dilatational elastic modulus (G’) and viscous modulus (G’’’) at the air-water interface, we started the measurements after 1800 s. The used frequencies were 1, 0.5, 0.25, 0.1 0.05 and 0.033 s^{-1}. After this time,
all parameters were measured in a time interval of 400 s of the different oscillations with a 1 µL air bubble. All measurements were repeated three times. For the behavior of \( \gamma \) vs time and \( G' \) vs time with different pH, we averaged five measurements with an average error of 1 %.

For the measurements of \( \gamma \), \( G' \) and \( G'' \) at the chloroform water interface, we used a 1x10^-5 M DPPC dissolved in chloroform and the same aqueous BSA and NDA/BSA solutions mentioned above.

Spectroscopic methods.

Fluorescence spectra were performed with a Perkin Elmer Luminescence Spectrometer LS 50B. The emission spectra were recorded from 300 to 500 nm (excitation wavelength 280 nm) and the obtained intensity at 340 nm, for the different samples of BSA solutions (2.5x10^-7 M) and the different NDA /BSA complexes, was plotted for the different NDA /BSA proportions at different temperatures. The NDA concentrations were 7.5x10^-5, 1.5x10^-4, 2.2x10^-4 and 3.7x10^-4 M. All experiments were measured at four temperatures (293, 299, 305 y 310 K). The temperature sample was maintained by recycled water.

Circular dichroism (CD) measurements were performed in a JASCO J-810 spectropolarimeter at room temperature. A base line was obtained under the same experimental conditions as the samples and subtracted from the spectra of the samples. The BSA concentration was 0.1 mg/ml and the molecular proportion of NDA/BSA was the same as in the experiments at interfaces. The molecular weight of BSA used for the secondary structure calculations was 66500. Both sample and reference were run three times and averaged. The wavelength range used was 180-260 nm and the cell path length was 0.1 cm. The secondary structure was determined by using the DICHROWEB software with the program CDSSTR (http://public-lcryst.bbk.ac.uk/cdweb/html/)
Results.

CD and Fluorescence of NDA/BSA complexes.

In Fig. 1 we show the CD spectra of BSA and some NDA/BSA complexes in water without using any buffer. As can be noticed from the ellipticity values at 208 and 222 nm, the $\alpha$-helix content of BSA increases for the 1500 NDA/BSA molecular proportion and it is almost lost for the 6000 and 12500 NDA/BSA proportions. On the contrary, for these NDA concentrations, the $\beta$ sheet and the unordered structures increase. In Table 1 the secondary structure of BSA with all NDA concentrations used, is shown. We also show the secondary structure of BSA with different NDA concentrations for three different pH. The highest $\alpha$-helix content was obtained for pH 4.6 and also, the changes of BSA secondary structure were more sensitive to the NDA concentration than the ones observed for other pH. For the 1500 NDA/BSA molecular proportion, the $\alpha$-helix content decreased more than 20 %. The secondary structure did not show important variations at pH 7.1 and 8.4 for the same NDA concentrations. These differences could be due to the extended structure shown by the BSA molecule at pH 4.6, also found by other authors$^{14}$, producing more interaction sites with NDA molecules and, as we will show, a larger molecular size. We find important to compare the highest NDA concentration used by us (3.1x10^{-3} M) and the urea concentration used by Itri et al.$^{10}$ (3M) to diminish the $\alpha$-helix content from 66 % to 64 %. This would mean a high NDA/BSA interaction that induces a strong $\alpha$-helix content decrement.

Fluorescence of NDA/BSA complexes. The intensity of fluorescence decreased in an approximately regular form, named quenching, with the increasing of the NDA concentration (quencher). We analyzed the intensities with the Stern-Volmer equation$^{11}$.
\[ \frac{F_0}{F} = 1 + K_v [Q] \]  

(1)

Where, \( F_0 \) and \( F \) are the steady-state fluorescence intensities in absence and presence of the quencher, respectively. \( K_v \) is the Stern-Volmer constant and \([Q]\) is the concentration of the quencher (NDA). The results of \( K_v \) for the different temperatures used are shown in Table 2. Contrary to the tendency observed for \( K_v \) to grow for increasing temperatures by Hu et al., we obtained a decreasing effect, which is interpreted as a static quenching mechanism. Assuming that the enthalpy change (\( \Delta H^0 \)) does not vary significantly in the range of temperatures analyzed, we determined the entropy change (\( \Delta S^0 \)) with the Van’t Hoff equation

\[ \ln(K_v) = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \]  

(2)

Where \( K_v \) is the Stern-Volmer quenching constant at the corresponding temperature and \( R \) is the ideal constant gas. The data and the adjusted line with a 0.98 linear correlation are shown in Fig. 2. From the slope and the ordinate at the origin we obtained a negative enthalpy change and a positive entropy change. Finally, with the known thermodynamic relationship we get the free energy change (\( \Delta G^0 \))

\[ \Delta G^0 = \Delta H^0 - T\Delta S^0 \]  

(3)

As can be deduced from the results in Table 2, the negative sign of \( \Delta G^0 \) means that the NDA/BSA interaction is spontaneous, mainly entropic and the enthalpy is favorable. This probably would mean that not only the hydrophobic interactions are relevant, but also the hydrogen bond forces play an important role. As Kandagal et al. mention, electrostatic interactions are very small, so that we think the main interactions present in the NDA/BSA complexes are hydrogen bond and hydrophobic interactions.
Rheology of NDA/BSA complexes at the air-water and chloroform-water interface. For a given drop oscillation frequency $\omega$ produced by the tracker at every time $t$, we obtain a surface tension value $\gamma(t)$ and a drop area $A(t)$ which are used to calculate the complex elasticity modulus $G(\omega)$, defined as

$$G(\omega) = G'(\omega) + iG''(\omega)$$  \hspace{1cm} (4)

where $G'(\omega)$ and $G''(\omega)$ correspond respectively, to the real part and the imaginary part of the elasticity modulus. They are related with the change of $\gamma(t)$ and $A(t)$ in the interface by $G = -A \frac{d\gamma}{dA}$ and the phase angle $\phi$ between compression and expansion.

The real and imaginary parts are related with the complex modulus by $G' = G \cos(\phi)$ and $G'' = G \sin(\phi)$.

The imaginary part component is reflected in the phase difference between the stress $d\gamma$ and the strain $d \ln(A)$. If the phase difference approaches to zero, then the surfaces can be considered elastic, otherwise, the surface shows a viscoelastic behavior.

Assuming that the mechanical properties of the interface follows the Maxwell Model, the conservative part in eq. (4) is represented by

$$G'(\omega) = G'_0 + \Delta G' \frac{\omega^2 \tau^2}{1 + \omega^2 \tau^2}$$  \hspace{1cm} (5)

and the viscous part (loss part) is written

$$G''(\omega) = \Delta G'' \tau \frac{1}{1 + \omega^2 \tau^2}$$  \hspace{1cm} (6)
where $G_0'$ is the extrapolated value of the elastic modulus at the limit $\omega = 0$. $\Delta G'$ and $\Delta G''$ are values which are adjusted to the corresponding experimental data. $\tau$ is the relaxation time characteristic of the Maxwell model\textsuperscript{16}. The last three parameters were found by fitting the experimental values with the Origin 7.0 software adjusting the parameters given in equations (5) and (6).

The behavior of the real part of the complex elastic modulus vs time is shown in Fig. 3 for different pH and short times at the air-water interface. We also show the effect of the 1500 NDA/BSA concentration ($3.7 \times 10^{-4}$ M of NDA) for the three pH analyzed. We observe the influence of the extended BSA molecules at pH 4.6 on the elastic modulus at very short times (100 s), when the elastic modulus reaches almost 70 mN/m. On the contrary for pH 7.1 and 8.4, we observe very small elastic modulus values. This would mean that extended BSA molecules seem to be strongly attracted at the air-water interface and probably could build a protein net with large elastic modulus values. On the contrary for pH 7.1 and 8.4, BSA molecules remain less extended and almost independent at the air-water interface. It is also interesting to observe the influence of the NDA molecules on the elastic modulus. The elastic modulus remain almost insensible with the influence of the NDA molecules for pH 7.1 in comparison with the effect on the elastic modulus for pH 4.6 and 8.4, which means that for these cases, the protein membrane at the air-water interface is more flexible with the presence of NDA molecules attached to the BSA molecules.

The behavior of the modulus for BSA and NDA/BSA complexes at the air-water interface and the DPPC/BSA in the chloroform–water interface at long times is resumed in Table 3, where the parameters of the Maxwell model are given for different NDA concentrations. We observe that the relaxation times do not show appreciable changes with the NDA concentration, except for the highest NDA concentration used at
the air-water interface. The value 0 in columns 2 and 4 means that the behavior of the elastic modulus for BSA at the air water interface does not follow the Maxwell model when the proportion of NDA/BSA molecules is 12500. However, when using eq. (6) to fit the experimental data of $G''/\omega$, we found a relaxation time around 27 s. For this NDA concentration the $\alpha$-helix secondary structure of BSA is lost as was seen in Table 1. This could be one reason of the anomalous behavior of $G''$ for this NDA concentration.

The relaxation time of DPPC at the chloroform water interface is similar to the one found by Saulnier et al.\textsuperscript{16} by using a dichloromethane-water interface (10 s), however, they used a higher DPPC concentration (7.5x10\textsuperscript{-3} mg/ml), close to the DPPC c.m.c.. A slight increase of the relaxation time was observed due to the DPPC-NDA interaction for the highest NDA concentration used, varying from 11 s for DPPC alone to 13 s with the 3.1x10\textsuperscript{-3} M NDA concentration.

Notice that the presence of BSA in the water phase produces an increase of $r$, indicating a DPPC/BSA interaction at the chloroform-water interface. However, the presence of different NDA concentrations produces changes in the relaxation time which seems to be not monotonic.

The extrapolated values of the elastic modulus at large frequencies ($G'_0 + \Delta G'$) show differences when BSA is incorporated to the aqueous phase. This value decreases from approximately 30 mN/m, obtained with DPPC at the chloroform-water interface, to 14 mN/m, indicating a more rigid film when only DPPC are the adsorbed molecules at the interface. The variation of $G'_0$, considered as the as the conservative elastic response of molecules at the interface\textsuperscript{16}, shows a slightly decrement with the NDA concentration (around 2 mN/m for the 12500 NDA/BSA molecular proportion). On the contrary, $\Delta G'$,
characterized as the dissipative part of the rheological perturbation, shows the same slight average increment due to the NDA concentration. According to Freer et al., we could interpret $G'_0$ as the static response corresponding to a change in the surface pressure vs. interfacial area change and $\Delta G'$ as the dynamic contribution related to the rearrangement and reconfiguration of the protein molecules at the interface, therefore, a higher NDA concentration produces more changes in the BSA conformation, manifested also in important changes of the BSA secondary structure as mentioned before.

In Fig. 4 we show the behavior of the real and imaginary parts of the complex elasticity modulus at the air water and at the chloroform-water interface for different frequencies. The curves are the best approach to the experimental data using the Maxwell model equations (eq. 5 and eq. 6). The upper curve in Fig. 4a shows the behavior of $G'$ of BSA and one of the NDA/BSA complexes at the air water interface, the middle curve and experimental data correspond to DPPC at the chloroform–water interface and the lower curve corresponds to the behavior of $G'$ of the DPPC-BSA and DPPC/NDA/BSA interaction at the chloroform–water interface. The same sequence of the curves corresponds in Fig. 4b for $G'(\omega)/\omega$, which is interpreted as the viscosity at the corresponding interfaces. Notice that the Maxwell model fits better to the experimental data for low frequencies and at the chloroform–water interface, probably because of the instrument limitations for higher frequencies. The effect of NDA on the DPPC interaction at the chloroform-water interface does not seem to affect the elastic properties of the interface. However, for the concentration used of NDA, we observe a slight decrement of the $G'$ values at both, air-water and chloroform-water interface. In Fig. 4b we observe the effect of BSA and NDA/BSA molecules on the viscosity increment of the DPPC/BSA and DPPC/NDA/BSA films.
Surface tension measurements.

We investigated the air–water interfacial behavior of aqueous BSA solutions mixed with different NDA concentrations. The results for the BSA for different pH are shown in Fig 5. We observe that at short times, \( \gamma \) behaves approximately linear, but for pH 4.6, there is an important slope variation at about 25 s, interpreted as an increase of the magnitude of \( \frac{dy}{dh^{1/2}} \) and therefore, as an increment of the diffusion coefficient, according to the Ward–Tordai equation\(^{20,21}\).

The short time diffusion at the air-water interface can be approached by the asymptotic solution for \( t \rightarrow 0 \) of the diffusion coefficients, described by Miller and Fainerman\(^{22}\), which is given by

\[
D_{a-w} = \frac{\pi}{4} \left[ \frac{1}{RTC_0} \left( \frac{dy}{d\sqrt{t}} \right)_{t \rightarrow 0} \right]^2
\]  

(7)

where \( D_{a-w} \) is the diffusion coefficient near the air-water interface (m\(^2\)/s), \( R \) the universal gas constant (J mol\(^{-1}\)K\(^{-1}\)), \( T \) the temperature in K, \( t \) time in seconds, \( C_0 \) the bulk protein concentration (mol/m\(^3\)) and \( \gamma \) the surface tension in N/m.

The results for the three pH and two different NDA concentrations are shown in table 4, where also the bulk diffusion coefficients and the average hydrodynamic radius, obtained from the DLS experiments, are compared.

The results of the calculated short time diffusion constants are slightly dependent on the pH and, for pH 4.6 and 8.4, these are similar to the values obtained by Güzey et al.\(^{22}\) (0.5x10\(^{-8}\) m\(^2\)/s) for native BSA at the air-water interface. Also, our values of the bulk diffusion constants (\( D_b \)) for pH 4 and pH 8 are similar to the ones reported in the literature, 6x10\(^{-11}\) m\(^2\)/s\(^2\) \(^{23}\), and 6.7x10\(^{-11}\) m\(^2\)/s\(^2\) \(^{24}\). On the contrary, due to the increase of
the BSA hydrodynamic radius exhibited at pH 4.6, we obtained a lower bulk diffusion coefficient. The effect of the NDA/BSA interaction on the change of the bulk diffusion constant is interpreted as an increase of the average hydrodynamic radius. For pH 4.6, the increase of the NDA/BSA complex average radius for the 1500 NDA concentration was around 17% higher. On the contrary, for pH 7.1 and 8.4 the change was around 6%. Notice that for pH 4.6, the coefficient at the air-water interface increases with the NDA concentration. This behavior is probably due to the conformation changes of the BSA molecule and the different molecular environment at the air-water interface. A similar increase was obtained with denatured (ultrasonicated) BSA by Güzey et al.\textsuperscript{22} at the air-water interface.

The protein adsorption at the air-water interface for different pH at short time, \( t \), was determined with the Ward-Tordai equation\textsuperscript{21}

\[
\Gamma = 2C_0(D_0 / \pi)^{1/2} t^{1/2}
\]

The concentration of the BSA and NDA/BSA films at 25 s is shown in Table 4. We notice that the surface concentration is slightly dependent on the pH and the NDA concentration. At pH 4.6 the protein adsorption is about 10% lower in comparison with the adsorption at pH 7.1 and 8.6. The influence of the NDA molecules on the BSA adsorption is more evident at pH 4.6, where the bulk diffusion coefficient is smaller and BSA molecules are more extended. Similar results were found for human serum albumin (HSA) by Hansen and Myrvold\textsuperscript{11}, but the same surface concentration was reached at shorter times (< 1 s) by using a HSA concentration of 10\(^{-2}\) %.

The behavior of \( \Gamma \) for BSA aqueous solutions and NDA/BSA complexes at the air-water and the interaction of DPPC with NDA/BSA at the Chloroform-water interface were investigated at intermediate times. In fig. 6 we show the effect of the NDA concentration on the surface pressure of BSA solutions at different interfaces. Fig. 6a
shows the behavior of the surface pressure at the air-water interface as a function of time, \( \Pi(t) = \gamma_0 - \gamma(t) \), with \( \gamma_0 \) the surface tension for the corresponding NDA solutions and \( \gamma(t) \) is the surface tension at any time \( t \). We notice that a half an hour after the bubble formation, pressure increases with the NDA concentration and then, for the concentrations 6000 and 12500, the pressure decreases in comparison with the pressure for BSA alone. This could be explained as a consequence of the drastic conformation change of BSA molecules for the NDA/BSA molecular proportions 6000 and 12500. As observed in Table 1, the \( \alpha \)-helix content is almost eliminated in the BSA conformation and this would strongly change the hydrophobicity of BSA. The correlation between helix amphipathicity and surface activity has also been noticed by Suttriprasit et al.\(^{13}\), who has assumed the helices adsorption at the air-water interface. We can also distinguish the three regimes of tension lowering observed by other researchers for many proteins\(^{19}\). The induction zone, which is the time when negligible changes occur and molecules start migrating. In Fig. 6a we observe a small shift with the NDA concentration and reach almost 200 s for the 6000 NDA/BSA concentration. The monolayer saturation zone, which shows the largest surface pressure increment and also is shifted at larger times with NDA. The interfacial gelation zone, which is the time when molecules rearrange and build probably gel-like networks. Fig. 6b shows the behavior of the surface pressure vs time at the chloroform-water interface with DPPC dissolved in chloroform and NDA/BSA complexes dissolved in water. We noticed a clear pressure increment with the NDA concentration up to the 6000 NDA/BSA proportion and a pressure decrement for the highest NDA concentration used (not shown).

The behavior in the region time \( t \rightarrow \infty \) of the function \( \gamma \) vs \( t^{1/2} \) was obtained, assuming a diffusion controlled adsorption and is given by the Joos relationship\(^{25,26,27}\).
\[
\gamma - \gamma_\infty = \frac{RT}{2C_0} \left( \frac{\pi}{D_f} \right)^{1/2}
\]

(9)

Where \(\gamma_\infty\) is the extrapolated equilibrium interfacial tension at \(t \to \infty\), \(R\) is the ideal gas constant, \(T\) is the absolute temperature and \(\Gamma\) is the protein surface concentration at long times. The \(\gamma_\infty\) values for different NDA concentrations are obtained with the intersection of the ordinate of the extrapolated straight line. In all cases the linear correlation was larger than 0.99.

In order to evaluate the surface tension kinetics, we tried to find the best fit of the surface pressure vs time from the experimental values to get the relaxation modes through the relationship

\[
\frac{\Pi(t) - \Pi_{\infty}}{\Pi_0 - \Pi_{\infty}} = A_0 \exp(-t/\tau_1) + A_1 \exp(-t/\tau_2)
\]

(10)

Where \(\tau_1\) and \(\tau_2\) are the first and the second relaxation times, respectively, \(A_0\) and \(A_1\) are adjustable parameters, \(\Pi_{\infty} = \gamma_s - \gamma_\infty\), \(\Pi_0 = \gamma_s - \gamma_0\) and \(\Pi(t) = \gamma_s - \gamma(t)\), where \(\gamma_s\) is the solvent surface tension or the interfacial tension without BSA or NDA/BSA solutions.

Similar attempt was made by Suttiprasit et al.\textsuperscript{13} and Van der Vegt et al.\textsuperscript{14} with only one relaxation time. They used that equation to monitor penetration into the surface and configurational rearrangements of adsorbed protein molecules in a period of time beyond that of the time affected by diffusion. The results for the air- water interface are given in Table 5 for different NDA concentrations. We notice a decreasing effect of the first relaxation time for the NDA/BSA proportions 900, 1500 and 2000. It is also interesting that for these NDA concentrations, it appears a second relaxation time as a consequence of the protein rearrangement at the air water interface. Van der Vegt et al.\textsuperscript{14}, using the sessile droplet technique and 0.01 mg/ml BSA concentration, (our BSA concentration was 0.0165 mg/ml), found a relaxation time of BSA of 937 s. On the
other hand, they found for that BSA concentration, an induction time (period of time where negligible changes of surface tension occurs) around 240 s. In our case, we obtained an average initial induction time of 60 s. The largest relaxation time was found for the 6000 NDA/BSA concentration about 50% larger than the time without NDA molecules. The relaxation times of DPPC at the chloroform-water interface for different NDA concentrations are also shown in Table 5. Notice the relative lower values of the relaxation times for the NDA concentrations 300, 900 and 1500. In this case, by using eq. (10), we only obtained a unique relaxation time for each NDA concentration and practically no induction time. This is consistent with the results found by Li et al.\textsuperscript{26}. They demonstrated that DPPC is adsorbed at the chloroform/water interface by a diffusive mechanism as long as the DPPC concentration is lower than the c.m.c. concentration. Our DPPC concentration used was lower (1\times10^{-3} molar) but near the c.m.c. concentration and this could be a reason of the small variation of the relaxation times with the NDA concentration in the aqueous phase. However, for the 12500 NDA concentration, we found a relaxation time near 10% higher than the relaxation time without NDA molecules. Finally, in the same Table 5 we show the relaxation time for the chloroform/water interface (DPPC in the chloroform phase and BSA in the aqueous phase) and different NDA concentrations. We found two relaxation times for almost all NDA concentrations used, due to the BSA-DPPC rearrangement at the interface. The largest variation of the second relaxation time occurred at the 12500 NDA concentration (25% larger than the time obtained without NDA molecules) and the largest variation of the first relaxation time occurred for the 1500 NDA concentration (17% smaller than the time obtained without NDA molecules).
In Fig. 7 we resume the behavior of pressure of the NDA/BSA interaction for intermediate times (time when $|dy/dt| \approx 10^{-3}$) at the air/water and chloroform/water interfaces. For the air/water interface we observe a pressure increment up to the 2000 NDA/BSA concentration, and for higher NDA concentrations, the pressure decreases in almost 4 mN/m in comparison with the pressure of BSA alone. For the interface DPPC-chloroform/water we observe a small effect on the increment of pressure due to the increment of NDA concentration in the aqueous phase (near 3 mN/m for the 12500 NDA concentration). This would mean that NDA molecules could attract more DPPC molecular hydrophilic heads at the interface. The presence of NDA/BSA complexes in the aqueous phase produces a small increment of pressure (1-3 mN/m) indicating some interaction of NDA/BSA complexes with DPPC at the interface. Notice that for the 12500 NDA concentration, the pressure is lower in comparison with the pressure without BSA. This could mean an electrostatic repulsion with the interface due probably to the BSA abrupt configuration change.

Conclusions

In this paper we have investigated the effects of NDA on the interfacial properties of BSA at the air-water and at the DPPC/chloroform-water interface by axysymmetric drop tensiometer measurements. BSA molecules showed a larger size increase and larger changes in the BSA secondary structure with the NDA addition for pH 4.6 in comparison with the size increase and secondary structure changes shown at higher pH. It was possible to get almost destroyed the BSA $\alpha$-helix content for relative small NDA concentrations (1.6x10^{-3} M) in comparison with the concentrations needed with other compounds. The influence of relative small NDA concentrations on the diffusion and surface tension at short times were significantly larger for pH 4.6 obtaining also a larger
elastic modulus, due probably to the exposed $\alpha$-helix structures. The increase of the BSA $\alpha$-helix content at up to 1500/1 and 2000/1 NDA/BSA concentrations caused a pressure increment at the air-water and at the chloroform–water interfaces. The interaction of the NDA/BSA complexes with DPPC at the chloroform–water interface was enhanced with the NDA concentration. However, due to the DPPC concentration used, near the c.m.c., the changes of the interfacial pressure influenced by the NDA/BSA molecules were not as large as the ones observed at the air-water interface.

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References


CD secondary structure of BSA with different N – Nitrosodyethylamine/BSA molecular proportions. The analysis was performed using the DICHROWEB software with the program CDSSTR (http://public-1.cryst.bbk.ac.uk/cdweb/html/)

<table>
<thead>
<tr>
<th>NDA/BSA</th>
<th>α Helix</th>
<th>β sheets</th>
<th>Turns</th>
<th>Unordered</th>
</tr>
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<td>0.11</td>
<td>0.1</td>
</tr>
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<td>0.11</td>
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<tr>
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<td>0.1</td>
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<td>2000</td>
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<tr>
<td></td>
<td>1500</td>
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<td>pH 8.4</td>
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Table 2

Results of the Stern-Volmer constant (\( K_w \)) at different temperatures and the thermodynamic functions obtained from eqs. (2) and (3) in the text.

<table>
<thead>
<tr>
<th>T (K)</th>
<th>( K_w ) (l/mol)</th>
<th>( \Delta H_0 ) (kJ/mol)</th>
<th>( \Delta G_0 ) (kJ/mol)</th>
<th>( \Delta S_0 ) (J/mol)</th>
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Table 3

Relaxation times and elastic modulus at long times of different NDA/BSA concentrations at the air-water interface and the chloroform-water interface.

<table>
<thead>
<tr>
<th>NDA/BSA</th>
<th>Air/water $\tau \pm 1s$</th>
<th>DPPC/BSA Chloroform/water $\tau \pm 1s$</th>
<th>Air/water $G''_o + \Delta G''$ mN/m</th>
<th>DPPC/BSA Chloroform/Water $G''_o + \Delta G''$</th>
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<td>14</td>
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<td>18</td>
<td>16</td>
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<td>13</td>
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<tr>
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<td>0</td>
<td>15</td>
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</table>
Table 4

Diffusion coefficient near the air-water interface, $D_{a-w}$, and in bulk, $D_o$, hydrodynamic radius, $R_H$, and surface concentration, $\Gamma$, at 25 s at the air-water interface for different pH and NDA/BSA concentrations.

<table>
<thead>
<tr>
<th>pH</th>
<th>NDA/BSA</th>
<th>$D_o$ m$^2$/s x10$^{-11}$</th>
<th>$D_{a-w}$ m$^2$/s x10$^{-8}$</th>
<th>$R_H$ nm</th>
<th>$\Gamma$ (25 s) mg/m$^2$</th>
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<td>4.6</td>
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<td>7.1</td>
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<td>1.5</td>
<td>3.7</td>
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<tr>
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<td>6.4</td>
<td>0.5</td>
<td>3.8</td>
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<td>8.4</td>
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<td>0.5</td>
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<td>0.74</td>
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<tr>
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<td>4.0</td>
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Table 5

Relaxation times (in seconds) of the adsorption of NDA/BSA complexes at the air-water and the chloroform-water interface. The experimental data were adjusted to the eq. (10) in the text by using the Origin 7 software.

<table>
<thead>
<tr>
<th>NDA/BSA</th>
<th>BSA Air-water</th>
<th>DPPC Chloroform/water</th>
<th>DPPC/BSA Chloroform/water Relaxation time 1</th>
<th>DPPC/BSA Chloroform/water Relaxation time 2</th>
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<tr>
<td>0</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>582</td>
<td>28.4</td>
<td>10.8</td>
<td>148</td>
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</table>
Figure captions.

Fig. 1 CD spectra of BSA (0.1 mg/ml) mixed with different N-Nitrosodicyethylamine/BSA molecular proportions. The secondary structure analysis was performed using the DICHROWEB software with the program CDSSTR (http://public-1.cryst.bbk.ac.uk/cdweb/html/). Measurements were performed at room temperature. Numbers indicate the NDA/BSA proportions.

Fig. 2 Calculated $Ln(K_n)$ against $1/T$ ($T$, absolute temperature) and adjusted line for different temperatures. The enthalpy and entropy changes of the NDA/BSA interaction are obtained from the slope and the ordinate at the origin of the Van't Hoff equation (eq. (2)).

Fig. 3. Behavior of $G'$ vs time for the different pH used. BSA concentration was kept constant (2.5x10^{-7} M). Dark symbols correspond to BSA alone and open symbols to the 1500 NDA/BSA molecular proportion (3.75x10^{-4} M concentration of N-Nitrosodicyethylamine). Squares correspond to pH 4.6, triangles to pH 7.1 and circles to pH 8.4. measurements were performed at 25 ± 0.1°C.

Fig. 4. Behavior of $G'$ (4a) and the two dimensional viscosity $G''/\omega$ (4b) for different frequencies and different interfaces of aqueous BSA solutions. Filled symbols correspond to BSA solutions and open symbols to 3.7x10^{-4} NDA concentration solutions. Squares correspond to BSA at the air-water interface, circles to DPPC at the chloroform–water interface and triangles correspond to DPPC/BSA at the chloroform-water interface. BSA concentration was the same (2.5x10^{-7} M) and the concentration of DPPC dissolved in chloroform was 1x10^{-5} M.

Fig. 5. Surface tension of BSA aqueous solutions vs $t^{1/2}$ in phosphaed buffered solutions at different pH. BSA concentration was the same used. Experiments were carried on at 25 ± 0.1°C.
Fig. 6. Surface pressure vs time for different NDA/BSA molecular proportions in the aqueous phase. a) Air-water interface and b) chloroform-water interface with DPPC in chloroform 1x10^{-5} M. Temperature was kept at 25 ± 0.1°C. Numbers indicate the NDA/BSA proportions.

Fig. 7. $\Pi_{00}$ vs NDA concentration at different interfaces. Squares correspond to NDA/BSA complexes at the air-water interface, circles correspond to DPPC at the chloroform-water interface and triangles correspond to $\Pi_{00}$ due to the interaction DPPC with NDA/BSA at the chloroform-water interface.
Figures

![Graph showing wavelength vs. millidegree with different lines representing BSA and concentrations: 300, 1500, 6000, and 12500.](image)

Fig. 1
Fig. 2
Fig. 3
Fig. 4a
Fig. 6a
Fig. 6b