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PROTEINS IN AQUEOUS SOLUTION UNDER HIGH FREQUENCY ELECTROMAGNETIC FIELD: VISCOELASTIC APPROXIMATION OF A COMPLEX SYSTEM

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ABSTRACT. Proteins in aqueous solution are complex systems that are made of many interacting and non-interacting elements whose behavior can be predictable upon the application of non-linear models. The aim of this study was to show that this complex system behaves like a viscoelastic system under exposure to high frequency electromagnetics (HF-EMFs). To this aim, typical proteins in water solution were exposed for 3 h to a high frequency electromagnetic field at the power density of 1 W/m^2. Fourier Transform Infrared (FTIR) spectroscopy was used to study the response of proteins to exposure to HF-EMFs. Proteins α-helices aligned along the direction of the field and the integrated areas of proteins β-sheet content increased linearly as a function of proteins dipole moment. This result can be explained assuming that proteins in aqueous solution under HF-EMF behave like a viscoelastic system.

1. Introduction

A complex system can be defined as a system which is composed by many components that mutually interact each other and with environment. Studying complex systems is a new field of science and the great variety of complex systems has led us to use different types of techniques and methodologies to study their structure and behavior (Magazù *et al.* 1997; Magazù 2000; Magazù *et al.* 2005; Marwan *et al.* 2007; Bonaccorsi *et al.* 2009; Bertin 2012; Kwapien and Drozdz 2012; Christakis *et al.* 2013; Kiselev *et al.* 2013; Holovatch *et al.* 2017; Kiselev and Lombardo 2017; Marchese *et al.* 2017; Cannuli *et al.* 2018; Caccamo and Cannuli 2019) and to investigate their response to external stress agents (Chrousos and Gold 1992; Eisler *et al.* 2008; Calabrò and Magazù 2012; Calabrò *et al.* 2013; Caccamo *et al.* 2016; Koorehdavoudi and Bogdan 2016; Magazù *et al.* 2016; Calabrò and Magazú 2017; Calabrò and Magazù 2017, 2018a,b,c).

Proteins in biological environment are subjected to a large amount of different forces such as intermolecular and electrostatic interactions, van der Waals forces, hydrogen bonding, hydrophobic interactions and hydration, ion dispersion forces. As a result, protein's response to external stresses is unpredictable but it may be often predictable upon the application of statistical methods. Previous work stated that proteins are the systems in which the laws of

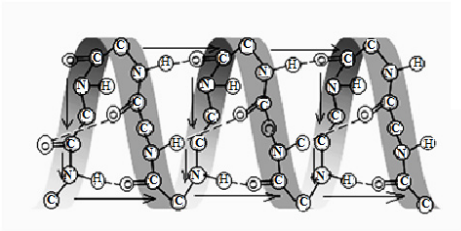

FIGURE 1. Scheme of the *alpha*-helix structure in a typical protein.

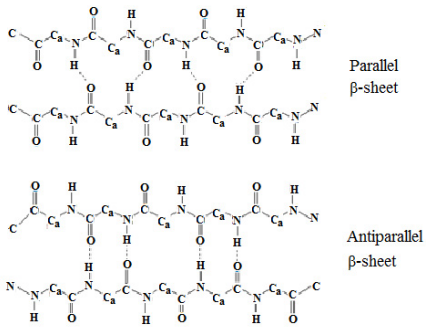

FIGURE 2. Scheme of parallel and antiparallel β-sheet components in a typical protein.

complexity can be studied better than anywhere else (Frauenfelder and McMahon 2001) Indeed, proteins show a rich phase behavior and can assume a large number of different structures. These systems have a free energy landscape and their fluctuations are crucial for the behavior of biological systems. A protein is a complex system which is composed by interconnected parts that as a whole exhibit more properties "hard to predict" from the properties of the individual parts (Frauenfelder 2010; Magazù and Frauenfelder 2013). The most representative element of these interconnected parts of a protein is the α-helix structure, a righthand-spiral conformation in which every backbone N-H group donates a hydrogen bond to the backbone C-O group of the amino-acid located three or four residues earlier along the protein sequence (see Figure 1).

The use of Fourier Transform Infrared (FTIR) spectroscopy can be successfully used to study the secondary structure of a protein. In particular, FTIR spectra of typical proteins show C=O stretching and N-H bending vibrations that give rise to the Amide I band and are oriented along the α-helix axis. In contrast, C-N stretching and N-H bending vibrations give rise to the Amide II band, that are perpendicular to the α-helix axis (Parker 1971; Stuart n.d.) such as schematized in Fig2. Another important component of proteins secondary structure of a protein is the β-sheet feature, which consists of pleated β-strands formed by polypeptide chains connected laterally by hydrogen bonds, forming parallel or antiparallel conformations, such as represented in Fig2.

Despite of these two main structures give stability to proteins folding, the behavior of a protein in aqueous solution under an external stress is not easily predictable. The aim of this study was to show that the application of a HF-EMF to a typical protein gives rise to a predictable behavior so that a protein under a HF-EMF can be considered a viscoelastic system. In order to study proteins response to HF-EMFs, the following typical proteins were used: hemoglobin (HB), myoglobin (MB), bovine serum albumin (BSA) and lysozyme (LYS). HB is a tetrameric protein, which is present in erythrocytes, whose main function is the binding and transport of oxygen to cells and tissues. MB is a protein which has the fundamental function to transport the oxygen in muscle cells. BSA is the most abundant protein in the blood plasma, whose the most important property is the ability to transport various endogenous and exogenous substances in blood. LYS is a protein that is present in secretions such as mucus, saliva and leukocytes. These proteins have been often used as a model for many biophysical and biochemical studies because proteins represent the basis of vital functions of life bodies. FTIR spectroscopy analysis has been already used to test the effects of MW radiations on the secondary structure of proteins. Previous studies have already shown that exposure to EMFs can induce unfolding of BSA, MB, LYS, HB (Magazù *et al.* 2010; Magazù and Calabrò 2011; Calabrò *et al.* 2012; Magazù *et al.* 2012; Calabrò and Magazù 2014a,b, 2015a). Furthermore, it was demonstrated that HF-EMFs can orient molecular chains of organic compounds (Calabrò and Magazù 2013a; Calabrò and Magazù 2013b; Calabrò and Magazù 2015b; Calabrò 2016; Calabrò and Magazù 2016). In this study we have shown that the response of typical proteins to HF-EMFs exposure is linear and easy predictable, so that we can affirm that proteins under HF-EMFs exposure behave like a viscoelastic system unlike a protein is a complex system.

2. Material and Methods

2.1. Proteins samples. HB was obtained by blood samples following the procedure accurately described in Magazù *et al.* (2010) and Calabrò and Magazù (2012). BSA, MB, and LYS samples were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in D2O at 20°C at the concentration of 100 mg/ml. After these treatments, proteins solutions were placed in sterile microcentrifuge tubes, polypropylene, 1.5 ml capacity (BR780400, Sigma–Aldrich, Milan, Italy) and submitted to the following assays.

2.2. Experimental design. The exposure system consisted of three operational mobile phones (Nokia 1208, LG model U8330, Motorola V635) that were used to irradiate the samples of proteins by MWs at 1800 MHz frequency at the average power density of 1 W/m2 for three hours. The power density of the EMF produced by the exposure system was continuously monitored by a SRM-3000 device of Narda Safety Test Solutions. Narda SRM 3000 frequency range can vary from 100 kHz to 3 GHz. It was linked through a cable to a Narda three axis antenna covering the frequency range from 75 MHz to 3 GHz, determining the three spatial components of the EMF being measured. MWs frequencies were detected by the SRM-3000 device using the spectrum analysis mode as a preliminary analysis with a resolution bandwidth RBW= 6 MHz and measurements averaged over a number of 16 replications. Then, time analysis mode was chosen to monitor the intensity of the power density during exposure. Specific Absorption Rate (SAR) of proteins solution was calculated using the expression $SAR = \sigma E^2/\rho$ where E, ρ and σ are the amplitude of the electric

field, the mass density and the electrical conductivity of the exposed sample, respectively. The electrical conductivity of proteins solutions was measured using a conductivity meter (Digimeter L21, Conductivity Meter, Mach wiss-Techn, D812 Walhuim, Germany), and the electric field amplitude was measured using electrodes spaced by 0.5 cm and dipped in the proteins solutions, connected to an oscilloscope (MS-5100A, Iwatsu Electric, Tokyo, Japan). Such measurements were used to calculate proteins SAR using the appropriate equation, providing values much lesser than the limits of 1.6 and 2 W/kg recommended in the USA and Europe, respectively. Samples of 200 μL of each protein solution were introduced into 1.5 mL sterile polypropylene microcentrifuge tubes (BRAND, 780400 Aldrich, Milan, Italy) and were exposed to MWs at a distance of 4 cm from the operational mobile phone. Analogue unexposed samples were used as un-exposed controls.

2.3. Infrared Spectroscopy. FTIR spectroscopy analysis was carried out using a spectrometer Vertex 80v (Bruker Optics Inc.). Proteins samples in bidistilled water solution at 150 mg/ml concentration were placed between two CaF_2 windows separated with a 25 μm Teflon spacer. FTIR spectra were obtained by 128 interferograms that were acquired for each spectrum with a spectral resolution of 4 cm^{-1}. The IR spectrum of bidistilled water was subtracted from the spectra of each protein in water solution at the corresponding temperature and smoothing correction for atmospheric water background was performed. In addition, baseline correction was performed for exposed and un-exposed protein samples. Further analysis was also performed using vector normalization. Moreover, automatic baseline scattering correction was preliminary applied to the acquired spectra in order to subtract baselines from spectra getting spectra with band edges of up to the theoretical baseline. Further correction was applied to the acquired spectra using interactive baseline rubber-band correction. This method uses a rubber-band which is stretched from one spectrum end to the other and the band is pressed onto the spectrum from the bottom up with varying intensity, depending on the number of iterations and baseline points in the algorithm, so that the resultant spectrum will be the original spectrum less the baselines points manually set and a concave rubber-band correction. In this study the values of 50 baseline points and 60 iterations were used. Differences were considered to be significant when the probability values were lower than 0.05.

3. Results

Representative exposed and unexposed spectra in the IR region from 1700 to 1500 cm^{-1} of Hb, Bsa, Lys and Mb in bidistilled water solutions are represented in Figure 3 (A-D), in which exposed samples spectra are represented in red color. In these spectra, the intense vibration bands at 1654 cm^{-1} and 1545 cm^{-1} can be observed, that correspond to the Amide I and Amide II vibration bands, respectively. The Amide I is produced by proteins α-helix structure, that is due to the spectral contribution of the C=O stretching vibration and N-H bending mode, the Amide II is due to the N-H bending and C-N stretching modes (Wada 1976; Hol *et al.* 1981; Surewicz *et al.* 1993). A significant result which was observed after exposure to HF-EMF, was the increasing in intensity of the Amide I band in the exposed samples spectra.

In order to highlight another possible correlation between the HF-EMF and some physicochemical property of the exposed proteins, the integrated area of the β-sheet content of

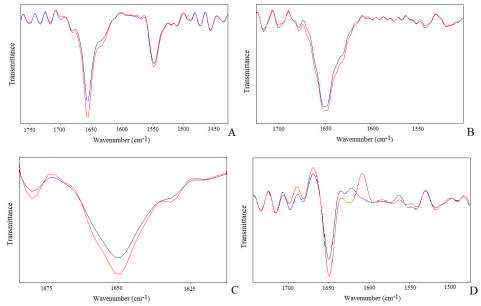

FIGURE 3. Representative exposed and unexposed spectra of Hb (A), Bsa (B), Lys (C) and Mb (D) samples in bidistilled water solutions in the range 1700-1500 cm^{-1} after 4 h exposure to 1750 MHz microwaves at the average power density of 1 W/m^2 (red line refers to exposed sample). Significant increase in intensity of the Amide I vibration band was observed after exposure to MWs ($p < 0.05$).

each protein was calculated in the Amide I region and was plotted as a function of proteins dipole moment. The observed results indicated that proteins β-sheet structure increases parallel to an increase of the dipole moment of the protein (Calabrò and Magazù 2016).

4. Discussion

A reasonable explanation of this result is that an alignment of proteins α-helices occurred in the direction of the applied field (Calabrò and Magazù 2017) Proteins in aqueous solution represent a complex system because proteins movement can be represented by Brownian motion, due to thermal molecular agitation, that is unpredictable. Otherwise, a HF-EMF generates a torque on a protein, due to the dipole moment of the α-helix, so that the response of a protein in aqueous solution to the external stress can be predictable, depending on some physical variable of the protein. Indeed, proteins α-helices form a macrodipole which will place itself at an average position along the direction of the field, that is a predictable behavior similar to macroparticles subjected to an applied EMF, a phenomenon that is named "pearl chain", that has been studied for many years (Sher 1968; Schwan 1969; Griffin and Ferris 1970; Hu and Barnes 1975; Takashima and Schwan 1985). Contrary to this experimental result, Adair indicated that the levels of exposure that were generally used in previous studies to test the effects of EMFs seem too small to overcome thermal agitation that the molecules are subjected to at room temperature (Adair 2000). In its simulation Adair considered the impulse from a EMF with amplitude E = 100 kV/m , integrated over an interval t =1 ns (the Sandia electric pulse) and performed a simulation using Hb. The angular impulse from EMF is $E * \mu$, which have to be compared with the mean angular momentum from thermal agitation $\sqrt{2I_h kT}$ in which Ih is the moment of inertia of Hb. The mean angular momentum from thermal agitation resulted significantly larger than the impulse from the EMF despite of the large amplitude E = 100 kV/m which was used. Nevertheless, in the simulation of Adair, the value of impulse t = 1 ns that was taken into account is the time integrated over a period of t = 1 ns, but we should take into account the time $\Delta t = 3h$

corresponding to the exposure used in our study, given that the protein cannot follow the oscillation of the field due to its inertia and to the viscosity of the medium in which it is embedded. In this scenario, the impulse from the EMF will overhead the mean angular momentum from thermal agitation.

4.1. Applying the viscoelastic model to a protein in aqueous solution. A protein in aqueous solution can be described by a viscoelastic model in which the spring, damper and mass elements are the representative parameters of this system, supposed to be under exposure to an applied EMF. For simplicity, we may consider an electric field acting on the system, but this study can be easily extended to an applied magnetic field or HF-EMF. The equation of motion of this viscoelastic model is given by the following equation:

$$m\frac{d^2x}{dt^2} = qE - m\omega_s^2 x - mv\frac{dx}{dt} \tag{1}$$

where qE is an applied electric field, m is the mass of the system, v is the damper coefficient of the medium and ω_s is a fundamental frequency of the system. In addition, $\frac{d^2x}{dt^2}$ and $\frac{dx}{dt}$ are, respectively, the acceleration, velocity and complex displacement of a particle which is subjected to the applied electric field.

The external force acting on the system can be represented by an exponential function

$$\mathbf{E} = E_0 e^{j\omega t} \tag{2}$$

where E_0 is the amplitude of the field and ω is its frequency.

The generic solution of Eq. (1) is an exponential function, as well,

$$x = x_0 e^{j\omega t - \phi} \tag{3}$$

where x_0 and ϕ are the amplitude of displacement and the phase angle, respectively. Let we consider that the oscillation of the system begin with the beginning of exposure to the external field, so that it is $\phi = 0$. Hence, we may rewrite Eq.(3) as follows:

$$x = x_0 e^{jwt} \tag{4}$$

The corresponding velocity and acceleration of the charged particle are, respectively,

$$\frac{dx}{dt} = j\omega x_0 e^{jwt} \tag{5}$$

$$\frac{d^2x}{dt^2} = -\omega^2 x_0 e^{jwt} \tag{6}$$

Solving Eq.(1) for the displacement, putting $x_0 = x$ for simplicity, we get:

$$x = \frac{\frac{q}{m}E}{\omega_s^2 - \omega^2 + j\omega v} \tag{7}$$

A resonance phenomenon occurs when the frequency of the applied field is close to a fundamental frequency of the system $\omega = \omega_s$, giving the maximum value of displacement x which is provided by Eq. (7), that is the behavior of a viscoelastic system. Eq. (7) can be written as follows

$$x = D/md * f(E, \omega, \omega_s, v) \tag{8}$$

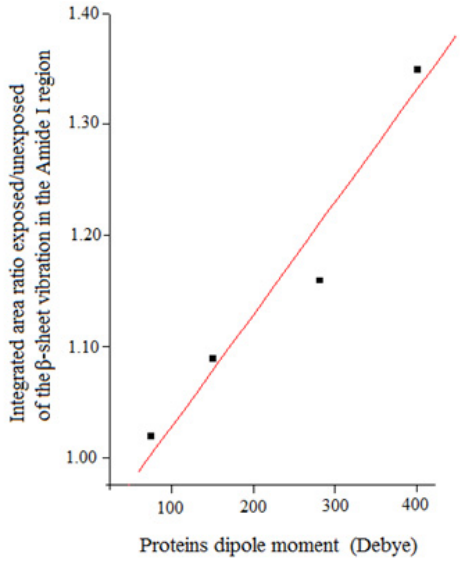

FIGURE 4. Integrated area ratio of β-sheet content in the Amide I region as a function of dipole moment of four typical proteins: hemoglobin, bovine serum albumin, lysozyme and myoglobin. The red line represents a linear regression with a correlation coefficient r= 0.969.

where D is the dipole moment of the protein, d is the distance between the positive and negative charges of the dipole that represent the protein and $f(E, \omega, \omega_s, \nu)$ is a function of module and frequency of the applied field, of the fundamental frequency of the system and of the damper coefficient of the medium.

Finally, the viscoelastic model implies that the displacement of a representative point of a protein embedded in aqueous solution under a HF-EMF is a linear function of proteins dipole moment. This model is in agreement with previous results in literature, in particular with the linear correlation between proteins β-sheet feature and proteins dipole moment which resulted after exposure to HF-EMF (Calabrò and Magazù, 2016), as represented in Figure 4.

5. Conclusion

A protein in aqueous solution is a complex system which can be described by Brownian motion due to thermal molecular agitation, whose behavior is unpredictable. Nevertheless, it was found that 3 hours exposure of typical proteins to a high frequency electromagnetic field induces that the impulse from the electromagnetic field can overhead the mean angular momentum from thermal agitation, so that the behavior of this system can be described by a viscoelastic model. This scenario is in agreement with experimental results that were found in previous literature.

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