Biomacromol. J. Vol. 1, No. 2, 187-195, December 2015.

Biomacromolecular Journal
www.bmmj.org

Change in Oxygen Absorption of Human Adult and Fetal Hemoglobin Due to 940 MHz Electromagnetic Field Radiation Exposure

A. Banaei[a,b], H. Ghourchian[a,]*, R. Faraji Dana[c], A.A. Moosavi-Movahedi[a], H. Naghavi[c], S.M. Saviz[c], R. Amjadi[a] and R. Hosseinzadeh[a]

[a]*Institute of Biochemistry & Biophysics, University of Tehran, Tehran, Iran*

[b]*Research Institute of Applied Science, Academic Center of Education, Culture and Research (ACECR), Shahid Beheshti University, Tehran, Iran*

[c]*School of Electrical and Computer Engineering, University of Tehran, Tehran, Iran*

(Received 26 August 2015, Accepted 29 January 2016)

ABSTRACT

The effects of electromagnetic fields (EMFs) radiation at the frequency of 940 MHz on the structure and function of human adult and fetal hemoglobin (HbA and HbF) were studied. After extraction and purification of HbA and HbF, the oxygen absorption values for exposed and unexposed HbA and HbF to EMF were compared. The slope of oxygen absorption curve for exposed HbA was increased while that for HbF was decreased compare to those before EMF exposing. Furthermore, the oxygen absorption saturation values were changed from 3.4-5.1 and from 5.1-3.1 mg l^{-1}, respectively for HbA and HbF after exposing to EMF. The UV-Vis, circular dichroism and fluorescence spectroscopy confirmed the quaternary structural changes of both proteins after EMF exposure. So that, the structural transition of HbA from tense to relaxed state caused an increasing in oxygen absorption; whilst in HbF, transition from relaxed to tense state was occurred and therefore oxygen absorption was decreased.

Keywords: Electromagnetic fields radiation, Fetal hemoglobin, Conformational changes, Structural transition

INTRODUCTION

Currently, scientists are involving the interaction of electromagnetic fields (EMFs) and various life processes. Attention has been focused on EMFs in different frequency ranges, of which microwave radiation ranging in frequency from 300 MHz to 300 GHz forms an important part [1], Some of these reports focused on different biological effects depending upon frequencies, wave forms, modulation and duration of exposure [2,3]. Due to an increased level of reactive oxygen species because of EMF radiation, the biological effects such as enzyme induction and toxicological effects including genotoxicity and carcinogenicity, testicular cancer and reproductive outcomes are observed [4]. According to the literature, two types of effects can be occurred to EMF radiation, *i.e.* thermal and non-thermal [5,6]. The thermal effects are attributed to the heat generated *via* absorption of EMF energy radiation by the water medium or by organic complex systems. For example, Tahvanainen *et al.* reported the exposure brings about a temperature rise in the ear canals [7]. On the other hand, many non-thermal effects following exposure of biosystems to EMF have been reported in recent years; among them some enzyme systems such as lymphocyte protein kinases [8], hepatoma cell ornithine decarboxylase [9] and acid phosphatase [10] respond to microwave radiation. Moreover, a significant inhibition of red cell Na^+/K^+ ATPase, perhaps related to conformational changes of the protein, has been reported [11,12]. Lai and Singh first reported DNA strand breaks from microwave radiation [13]. Recently, it was reported that 940 MHz EMF can alter the structure of DNA and the displacement of electrons in DNA by EMFs may lead to conformational changes of DNA and DNA disaggregation [14]. The described mechanisms for EMF non-thermal effect were either causing ions to accelerate and collide with other molecules or dipoles to rotate and array rapidly with alternating electric field [1].

*Corresponding author. E-mail: ghourchian@ut.ac.ir

Many factors, including EMFs affect on the structure and function of hemoglobin. In a recently published work, Mousavy *et al.* showed that EMF alters the oxygen affinity and tertiary structure of adult hemoglobin (HbA) [15]. Since HbA and HbF have different structures, it is expected that EMF has different effects on HbF and HbA. Although these proteins are essentially similar in spectroscopic characteristics and in molecular weight, they exhibit dissimilarity in structure and function [16-19]. HbF possesses the same α subunit as HbA, but its β subunit is replaced by similar, but not identical, γ subunits [20]. The homology of the primary structure between β and γ subunits is 73%. Interactions among the subunits in hemoglobin caused conformational changes that change the protein oxygen affinity [21,22]. Normal hemoglobin is an essential component of the circulatory system of vertebrates. Its chief physiological function is to transport oxygen from the lungs to the tissues [23]. Oxygen binds to the heme component of the protein hemoglobin in red blood cells. The oxygen then travels through the blood stream to be dropped off at cells. For mothers to deliver oxygen to their fetus, it is necessary for the HbF to extract oxygen from the maternal oxygenated hemoglobin across the placenta. This requires the HbF to have a higher oxygen affinity than that of the HbA [24].

In the present report a waveguide channel was designed for evaluation of the effects raised from HbA and HbF exposure to EMF. The possible changes of EMF radiation at the frequency of 940 MHz on the oxygen absorption and structure of HbA and HbF were estimated and compared. The changes were interpreted in terms of the differences between β- and γ-chains amino acids.

MATERIALS & METHODS

Reagents

All chemicals were of analytical grade and purchased from Merck (Germany).

Preparation of Hemoglobin

HbA and HbF were extracted from human red blood cells of healthy donors and umbilical cord blood of newborn from Royan Institute (Tehran, Iran) respectively, according to the Riggs method [25]. At birth, HbF constitutes approximately 60-80% of the umbilical cord blood [26].

HbF was purified based on the different resistances of fetal and adult hemoglobin against denaturation at high pH [27]. The solution was brought to pH 13 by gradual addition of 3 M NaOH. The denaturation of HbA reached to completion after 15 min at pH 13. Then, HbF was separated by centrifuging at 3000 rpm for 15 min followed by passing the solution through filter paper and dialyzed against 50 mM phosphate buffer solution at pH 7.4 (PBS) three times. Additionally, the prepared Hbs were analyzed by conventional native gel electrophoresis for purity control [28]. Finally, the purified HbA and HbF aliquots were frozen in liquid nitrogen and stored at -70 °C. Protein concentrations of HbA and HbF were determined using a UV-Vis absorption spectrophotometer (100 Bio-model, USA), with the extinction coefficient of 131936 M^{-1} cm^{-1} (monomer basis) at 280 nm for oxyhemoglobin [29]. All protein samples were prepared at 3 μM in PBS.

Exposure Setup

Figures 1a and b represents the experimental setup for exposure of Hbs to EMF. It was including a signal generator, an amplifier, a thermo-stated waveguide (170 × 170 × 600 mm) with the horizontal and vertical polarization ports and a spectrum analyzer. 7 ml of sample solution in a glass tube was fixed in the sample holder and placed in the waveguide. The sample was exposed to radiation at frequency of 940 MHz produced by a signal generator (model SML03, Rohde and Schwarz, Germany). The signal was amplified and fed through the coaxial cables (model RG223, Draka Comteq, Finland) into the sample holder. An Attenuator (model 40024, 20 dB, Narda, USA) and a level control panel on signal generator were used for regulating the incident power. Radio frequency power incident on the sample-holder cavity and the transmitted power, were measured using Spectrum Analyzer (model R3131, Advantest, Japan). The sample characteristics were obtained by Dielectric Probe Kit (model 85070E, Agilent, USA). Temperature was controlled within ±0.1 °C by an incubator (Perssojen, Iran). The unexposed sample, as control, was kept in the same experimental conditions, except that it was isolated in a 20 mm thickness aluminum holder. To have a uniform temperature in incubator space, a set of fans was used for air circulation. The fluctuation of temperature inside the tube upon interaction with EMF was controlled

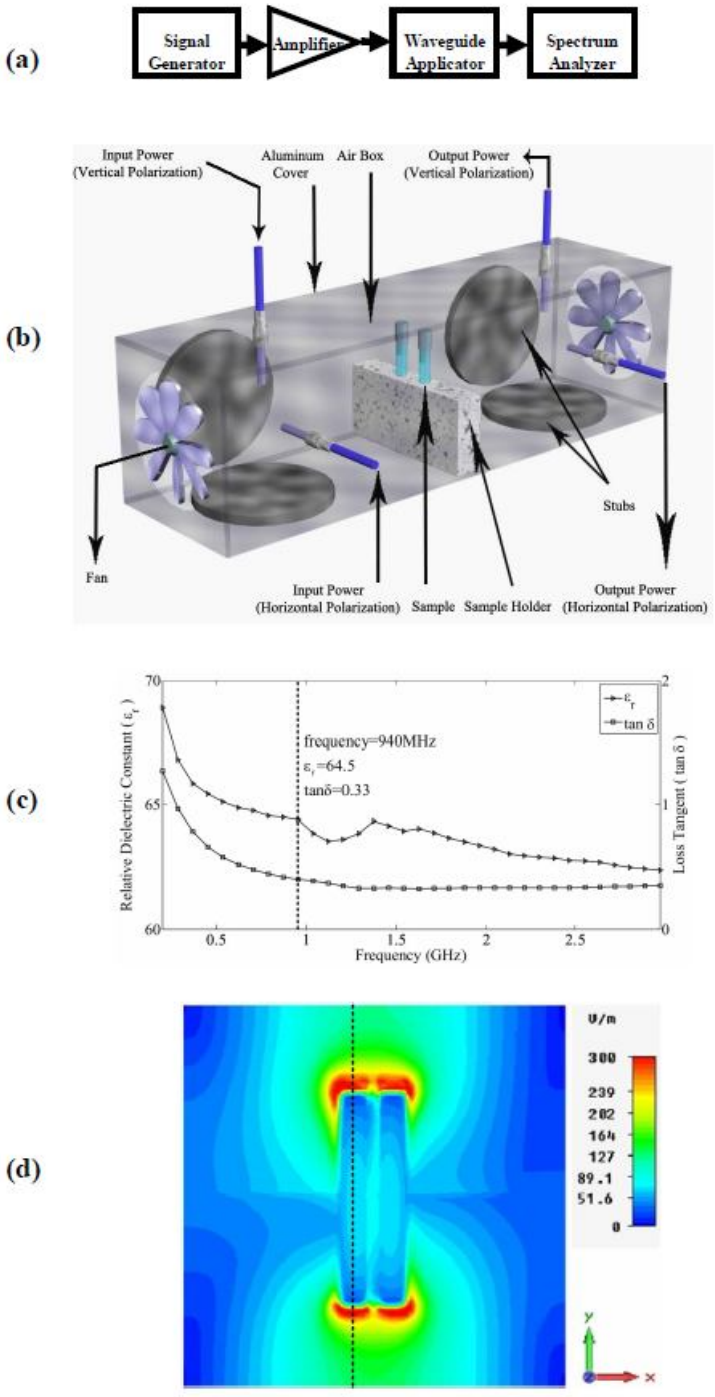

Fig. 1. (a) Diagram for illustrating the exposure of HbA and HbF to EMF radiation, (b) components of waveguide applicator including sample holder, (c) variation of dielectric constant (ε_r) and tan δ against frequency (vertical dashed line shows the values of frequency, ε_r and tan δ at 940 MHz) and (d) vertical profile of the simulated electric field intensity in and around the samples (color bar, on the right, shows the magnitude of electric field in each point).

which was negligible. The uniformity of electric field was studied using CST microwave studio simulator (CST MWS, a special tool for the 3D EM simulation of high frequency components). In microwave studio simulation process, adaptive mesh refinement was enabled to accurately illustrate the distribution of electric field inside the sample.

Oxygen Absorption Measurements

The oxygen absorption of Hb samples was determined *via* measuring the amount of dissolved oxygen in 50 mM phosphate buffer solution at pH 7.4 ($O_{2,PBS}$) and in phosphate buffer solution containing Hb ($O_{2,PBS+Hb}$). The absorbed oxygen by Hb samples ($O_{2,Hb}$) was calculated by Eq. (1):

$$O_{2,Hb} = O_{2,PBS} - O_{2,PBS+Hb} \quad (1)$$

The amount of dissolved oxygen in the solutions was determined using oxygen measuring instrument WTW Handheld meter (Oxi 330i, Germany). Prior to measurements, the solutions (either PBS or PBS containing Hb) were purged with highly pure nitrogen gas for at least 10 min to remove the dissolved oxygen. Then, the deoxygenated solution in a sealed cell was kept under nitrogen atmosphere throughout the experiments. Thereafter, the highly pure oxygen was injected into both solutions under the constant flow rate of 1 ml min^{-1} for a certain time (1.5, 2, 2.5,… , 8 min). The oxygen purging flow rate was also controlled by a volumetric flow meter (Dwyer, rma150ssv model, USA). In all measurements, solutions were stirred on a magnetic stirrer to obtain a uniform distribution of oxygen.

UV-Vis Spectroscopy

The UV-Vis absorption of Hbs was measured using spectrophotometer (100 Bio-model, USA) in the wavelength range from 200-700 nm. For all measurements the proteins concentration was 3 μM in phosphate buffer (50 mM, pH 7.4).

Far UV Circular Dichroism Spectroscopy

The secondary structure change of Hbs was studied using circular dichroism (CD) spectropolarimeter (model Aviv 215, Lakewood, NJ, USA) equipped with a temperature controller. Changes in the secondary structures were monitored in the far-UV region (190-260 nm) using 1-mm path length. Protein secondary structure was analyzed by CDNN software, version 2.1.0.223. All measurements were performed at 37 °C.

Fluorescence Spectroscopy

A Hitachi spectrofluorimeter (MPF-4 model, Japan) equipped with a temperature controller bath was used to measure the fluorescence emission of the Hb samples. The excitation wavelength was adjusted to 285 nm, which was specific for the excitation of tryptophan (Trp) residues. Emission spectra were recorded in the wavelength range between 290 and 400 nm at the bandwidth of 10 nm. Hemoglobin samples were 3 μM in a phosphate buffer (50 mM, pH 7.4). All experiments were conducted using a 1 cm path length fluorescence cuvette and 10 nm slit size.

RESULTS AND DISCUSSION

By exposing the Hbs to EMF variation of the permittivity (relative dielectric constant, ε_r) and tan (δ) against frequency was recorded at 940 MHz for 40 min (Fig. 1c). Moreover, the distribution of electric field in every point of waveguide channel was obtained using CST Microwave Studio Simulator. As shown in Fig. 1d, the distribution of electric field inside the sample was uniform and the effective electric field on sample was approximately constant.

Oxygen Absorption Study

After exposing HbA and HbF to the radiation at the frequency of 940 MHz for 40 min, the trend in oxygen absorption by either exposed or unexposed Hbs were recorded and illustrated in Fig. 2. As seen, oxygen absorption by both exposed and unexposed HbA (Fig. 2a) and HbF (Fig. 2b) increased as a function of oxygen purging time. But, the oxygen absorption slope of exposed HbA shifted to higher values while as for HbF it was shifted to lower rates, relative to their native states. Moreover, in the presence of EMF, the oxygen absorption saturation value for HbA was increased from 3.4-5.1 mg l^{-1} while this value for HbF was decreased from 5.1-3.1 mg l^{-1}.

According to Perutz [30] deoxy-Hb and oxy-Hb have

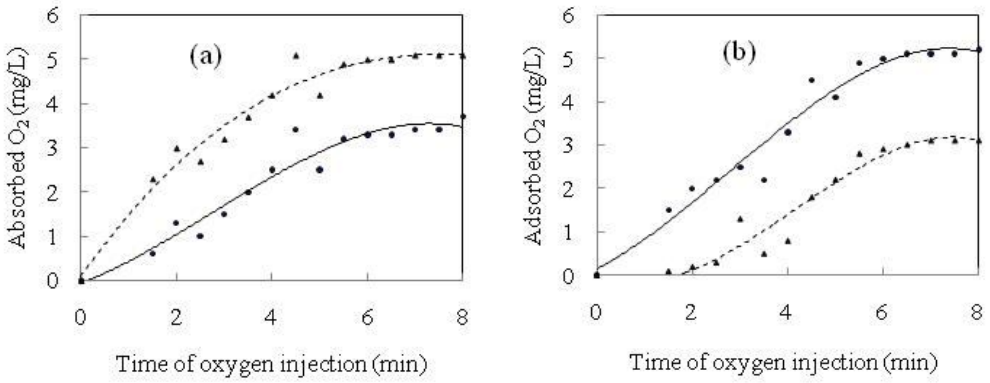

Fig. 2. The absorbed oxygen *vs.* time of oxygen injection with 1 ml min^{-1} flow rate for (a) HbA and (b) HbF. Solid lines and dashed lines represent the fitted curves of the unexposed (●) and exposed (▲) proteins, respectively. The protein concentration was 3 µM in PBS (50 mM, pH 7.4).

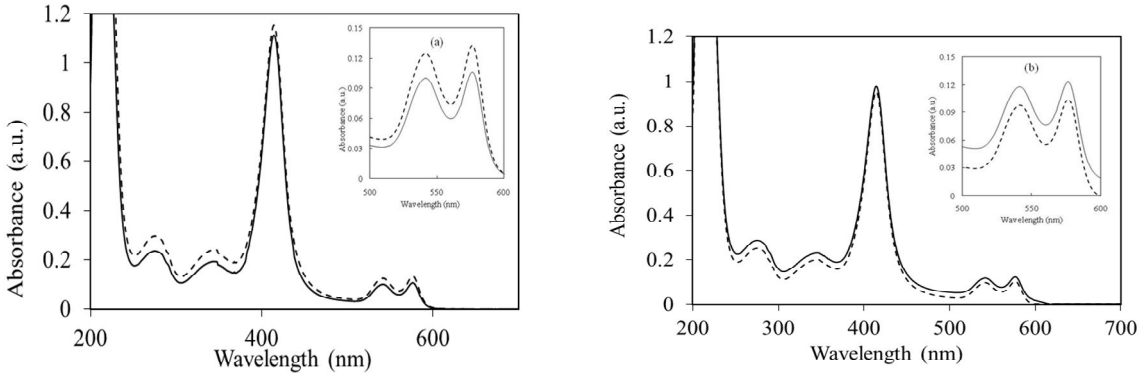

Fig. 3. UV-Vis absorption spectra of (a) HbA and (b) HbF. Solid lines and dashed lines represent the spectra of the unexposed and exposed proteins, respectively. The EMF was applied at frequency of 940 MHz for 40 min. The insets show the magnified spectra at the wavelength from 500-600 nm. The conditions are the same as Fig. 2, but at 37 °C.

two unique quaternary structures (T- and R-quaternary) having low and high O_2-affinities, respectively. Since then, the following assumptions [29] has been accepted as unquestionable principles of the structure-function correlation in the allosteric mechanism of Hb and widely used as the base for almost all the subsequent studies of allosteric mechanism of Hb [31]:

[Deoxy-state] = [T-quaternary structure] = [low O_2-affinity state]

[Oxy-state] = [R quaternary structure] = [high O_2-affinity state]

The results obtained by oxygen absorption study suggested that the absorbed oxygen in HbA solution increased by applying EMFs radiation while it was decreased in HbF. Therefore by these results together with the above relations, one may come to this conclusion that EMF caused the transition of T to R-quaternary structure in HbA and that of R to T-quaternary structure in HbF. Since

quaternary structure changes in proteins are usually occurred via changes in secondary and tertiary structures, therefore the structural changes of HbA and HbF were also compared in the presence and absence of EMF using UV-Vis, CD and fluorescence spectroscopy.

Oxy-Hemoglobin Spectroscopy

Hb, in addition to the specific absorbance at 280 nm due to Trp and 410 nm due to porphyrin [32], shows two other bands (Q-band) including α-band at 575 nm and β-band at 540 nm [33], related to the $\pi \rightarrow \pi^*$ transition of porphyrin. O_2 binding to Hb alters the spectral properties in this range. Therefore, studies on the spectrophotometric properties of Hb, is widely used. Figure 3 shows the absorption spectra of HbA and HbF before and after exposure to EMF in the spectral range from 200-700 nm. Figure 3a revealed that the absorption of exposed HbA at 541 nm compared to unexposed HbA has increased from 0.100-0.124 (24%) and at 576 nm from 0.104-0.130 (25%). This could be a reason for increasing the amount of oxy-Hb. In contrast, as shown in Fig. 3b, the absorption of exposed HbF at 541 nm compared to the unexposed HbF has decreased from 0.118-0.097 nm (22%) and at 577 nm from 0.123-0.103 (22%) which indicates a decreasing in the amount of oxy-Hb. Decreasing in absorption at these two wavelengths indicates reducing the amount of oxy-hemoglobin. This intensity change of Q-bands could be an indication that the Hb oxidation process could be affected due to EMF exposure.

Effect of EMF on Hbs Secondary Structure

CD spectroscopy is a quantitative technique to investigate the conformation of proteins in aqueous solution. To investigate the effect of EMF on the secondary structure of Hbs, the far-UV CD spectra of 3 μM Hbs in PBS buffer was recorded under the same conditions as Fig. 3. In Fig. 4, the far-UV CD spectra of HbA and HbF before and after exposure to EMF have been compared. The intensive positive peak at about 195 nm and prominent negative bands at about 209 and 222 nm are the characteristics of α-helical content. Analyzing the CD spectra by CDNN software (Table 1) indicated an increasing of about 9% in HbA alpha-helix after EMF exposure (Fig. 4a) perhaps due to HbA compactness. This implicates EMF exposure may causes a transition from T- to R-quaternary structure. While 22% decrease in HbF α-helix (Fig. 4b) indicates that EMF causes HbF to be partially unfolded and perhaps a transition from the R- to T-quaternary structure was occurred. Oxygen absorption by Fe^{2+}, located in porphyrin plane, causes the histidine (His) residue at the fifth coordination site of Fe^{2+} to move [34]. Since His is part of α-helix, this rearrangement causes a shift in secondary structure of HbA and HbF and changed the α-helical content [35].

Effect of EMF on Hbs Tertiary Structure

The intrinsic fluorescence of Hb especially around Trp residues (α-14, β-15 and β-37), is sensitive to its microenvironments and could be exploited for estimation of conformational changes in Hb [36]. Figure 5a shows that by exposing the protein to EMF, the fluorescence emission of HbA was quenched from 153-117. This suggested that the chemical environment of Trp was changed slightly into hydrophobic. This supposition is in agreement with the idea that Trp sense the hydrophobic pocket of heme group. The intrinsic fluorescence of HbA, which primarily originates from the β-37 Trp, is responsible for the fluorescence signal of HbA and is sensitive to the R- to T-quaternary structure transition [37]. In R structure, the iron atom is in the plane of porphyrin system and in this situation because of the overlap of UV-Vis absorbance spectrum of heme and the fluorescence spectrum of the β-37 Trp, there is efficient energy transfer from the β-37 Trp to the porphyrin system [38]. Due to exposure of EMF, protein structure even become more compact and therefore the β-37 Trp would be closer to the porphyrin system. This could be the reason for fluorescence quenching in HbA. Indeed, reducing of fluorescence intensity suggests T- to R-quaternary structure transition occurred in HbA and so oxygen absorption increased.

However, in HbF, α-14, γ-15, γ-37 and γ-130 Trp are the main fluorophores, among them γ-37 and γ-130 Trp are located in heme neighborhood [39]. As discussed previously, a partial unfolding was happened by exposing HbF to EMF which results a slight increase in distance between Trp (γ-37 and γ-130) and hydrophobic heme pocket. Therefore, the fluorescence emission of HbF was increased from 78-97 (Fig. 5b). This changes Trp micro-environment into hydrophilic and causes red shift in

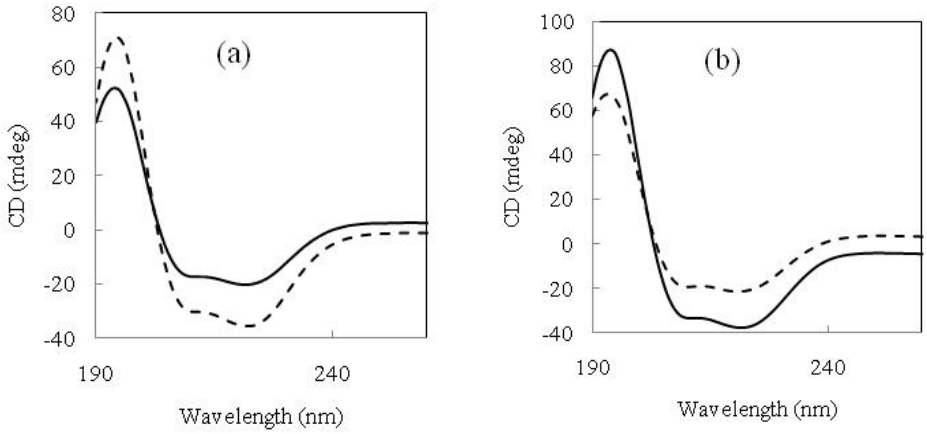

Fig. 4. The far-UV CD spectra of (a) HbA and (b) HbF. Solid lines and dashed lines represent the spectra of the unexposed and exposed proteins, respectively. The conditions are the same as Fig. 3.

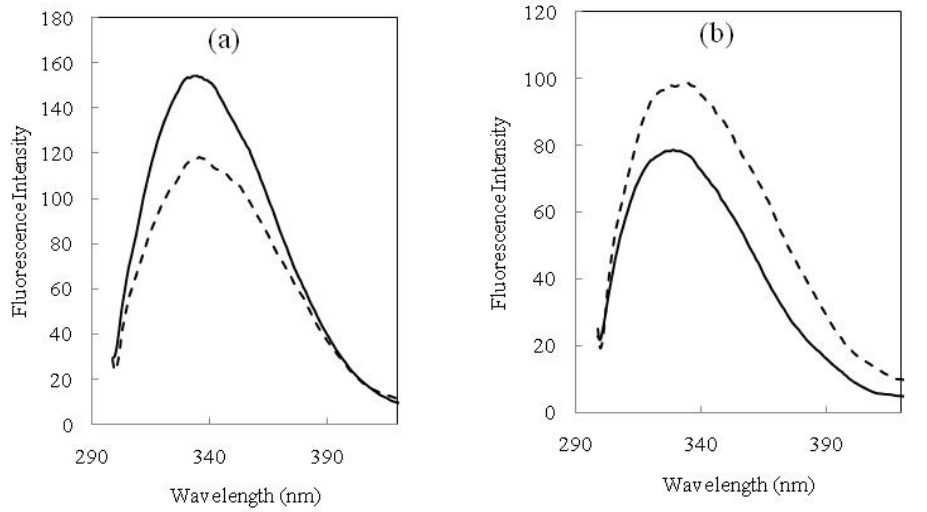

Fig. 5. Intrinsic fluorescence spectra of (a) HbA and (b) HbF. Solid lines and dashed lines represent the spectra of the unexposed and exposed proteins, respectively. The conditions are the same as Fig. 3.

Table 1. The Percentage of Secondary Structure Components of Exposed and Unexposed Hbs. Data were Extracted from Fig. 4 Using CDNN Software

Secondary structure	HbA unexposed	HbA exposed	HbF unexposed	HbF exposed
Alpha helix	59	68	66	44
Beta sheet	5	4	5	11
Beta turn	11	12	11	15
Random coil	25	16	18	30

fluorescence peak from 326-329 nm. Also getting Trp (γ-37 and γ-130) farer from heme, prevent the energy dissipation due to overlapping of Trp excitation and heme absorption. This could be another reason for increasing the fluorescence intensity at 326 nm. In fact, increasing of fluorescence intensity leads to R- to T-quaternary structure transition in HbF and so decrease in oxygen absorption.

CONCLUSIONS

Comparison of the absorption spectra of HbA and HbF at 541 and 576 nm before and after EMF exposure indicated that the amount of oxy-Hb for HbA was increased after exposing to EMF at 940 MHz, while that for HbF was decreased. Deep insight into the structure changes of Hbs revealed that EMF caused HbA to become more compact due to increase in α-helical content while HbF was faced with a partially unfolding due to decrease in α-helix and consequently it caused the transition of T to R-quaternary structure in HbA and that of R to T-quaternary structure in HbF. This was confirmed with the intrinsic fluorescence results, too. In summary, EMF at 940 MHz caused a transition in HbA to R-quaternary structure, which is a high oxygen-affinity state, while exposing to this radiation changed the HbF in to T-quaternary structure which is a low oxygen-affinity state.

ACKNOWLEDGEMENTS

The support provided by Research Council of the University of Tehran is gratefully appreciated. The authors are also grateful to Jalil Badraghi and Yashar Mayamay of Research Institute of Applied Science (ACEAR) and Iman Tavakolnia of University of Tehran for their useful helps.

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