New insights into bioprotective effectiveness of disaccharides: an FTIR study of human haemoglobin aqueous solutions exposed to static magnetic fields

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Abstract The aim of this study was the investigation of static magnetic field effects on haemoglobin secondary structure and the bioprotective effectiveness of two disaccharides, sucrose and trehalose. Samples of haemoglobin aqueous solutions, in the absence and in the presence of sucrose and trehalose, were exposed to a uniform magnetic field at 200 mT, which is the exposure limit established by the ICNIRP recommendation for occupational exposure. Spectral analysis by FTIR spectroscopy after 3 and 7 h of exposure revealed a decrease in the amide A vibration band for haemoglobin in bi-distilled water solution. Analogue exposures did not produce any appreciable change of amide A for haemoglobin in sucrose and trehalose solutions. Otherwise, no relative increase of β -sheet contents in amide I and II regions was detected for haemoglobin aqueous solutions, leading us to exclude the hypothesis that static magnetic fields can induce the formation of aggregates in the protein. In addition, a decrease in CH_3 stretching linkages occurred for haemoglobin in bi-distilled water solution after exposure, which was not observed for haemoglobin in sucrose and trehalose aqueous solutions, providing further evidence of a bioprotective compensatory mechanism of such disaccharides.

Keywords Haemoglobin · Static magnetic field · Electromagnetic field · Infrared spectroscopy · Disaccharides · Trehalose

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1 Introduction

The main objective of this study was to investigate the influence of bioprotectants on the stability of haemoglobin perturbed by a static magnetic field. The hypotheses suggested for understanding bioprotection mechanisms are generally suitable for narrow temperature and hydration ranges [1–4]. Recent investigations carried out on lysozyme [5–7] and bovine serum albumin [8] thermal denaturations have pointed to a common mechanism of bioprotection by disaccharides.

Disaccharides are cryptobiotic activating substances represented by the chemical formula $C_{12}H_{22}O_{11}$ and the same number of hydroxyl groups, with different bioprotectant effectiveness. Previous research carried out up to now on protein thermal denaturations has confirmed a bioprotection mechanism by disaccharides [5, 8]. In particular, trehalose is a disaccharide that has been largely recognized as capable of protecting many organisms in extreme life conditions and preserving proteins from several stress agents [9–11].

Crowe et al. [12] formulated the so-called water replacement hypothesis to explain why trehalose is particularly effective. Such hypothesis is supported by the simulation of [13], who infer that the structure of trehalose is perfectly adaptable to the tetrahedral coordination of pure water, whose structural and dynamical properties are not significantly affected. Furthermore, inelastic neutron spectra revealed a more marked downshift of the OH stretching mode and a narrower bandwidth for the trehalose/ H_2O mixture, indicative of a disaccharide–water interaction of a higher order [14]. In addition, OH stretching Raman spectra of disaccharide aqueous solutions showed that the area of the tetrahedric contribution is smaller in the case of trehalose, indicating that a more marked destructuring effect occurs. This result, which was confirmed in all the investigated concentration range by the trend of the integrated intensity of the low density contribution to be smaller for trehalose with respect to its homologous sucrose and maltose, constituted a conclusive proof of the fact that trehalose frustrates ice formation better than the other disaccharides [15].

Experiments on trehalose/ H_2O , maltose/ H_2O and sucrose/ H_2O mixtures, through the intensity profile of the bending modes identified by a density functional simulation, appeared more structured, indicating that, in combination with water molecules, trehalose forms entities which show a more “cryptocrystalline” behaviour: the macroscopically glassy protective shell hides a local crystalline character [11, 16, 17].

The aim of this work was to study bioprotective mechanisms of disaccharides, trehalose in particular, to defend organic systems against environmental stress, represented by exposure to a static magnetic field. Uniform intensity magnetic fields are, indeed, environmental sources normally not suspected of being harmful because we always live with the Earth’s magnetic field, which ranges from 30 to 70 μT , depending on location. This is the reason why researchers have not given various health effects from this kind of field nearly as much attention as fields produced by alternating current. While a few studies have examined possible long-term effects of exposure, particularly in relation to cancer, the results are in general inconclusive.

A static magnetic field (SMF) is generated wherever direct current (DC) is used, such as in electric trains or industrial processes such as aluminium production. These can be more than 1,000 times stronger than the Earth’s natural magnetic field and some technological innovations have led to the use of magnetic fields up to 100,000 times stronger than the Earth’s magnetic field, such as magnetic resonance imaging.

Studies on work exposure and health effects have been carried out almost exclusively on workers exposed to moderate SMF by equipment using large DC currents, such as welders, aluminium smelters or workers in various industrial plants using large electrolytic cells in chemical separation processes.

Transport systems often use DC traction motors, giving rise to appreciable values of SMF [18, 19]. Other sources of SMF in transportation systems can be represented by smoothing and line filter inductors, and inverters, used when DC is for power supply [20]. SMF under DC transmission lines are about 0.02 mT. Artificial sources of static fields are common, such as audio speaker components, battery-operated motors and refrigerator magnets. These small magnets can produce fields of 1–10 mT within a few centimetres from their magnetic poles.

Measurements by the National Institute for Occupational Safety and Health in various industries, relative to the production of SMF, found that, in aluminum production, magnetic fields can reach 330 μ T, electrogalvanizing processes can produce up to 460 μ T and telecommunications switching rooms can produce up to 130 μ T [21]. The highest SMF exposures of the general public are from magnetic resonance imaging, where the fields range from 150–2,000 mT. Furthermore, magnetically levitated trains do not suffer the rolling resistance of conventional railways trains, but strong magnetic fields of around 1 T are required to achieve levitation, and flux density of up to 1.33 mT inside passenger cabins has been measured in magnetic levitation systems [22].

For SMF, short-term exposure to very strong fields does seem to induce a number of measurable effects in the body. Computer simulations suggest possible effects to the heart of electric currents induced by blood flowing through a strong magnetic field, although this has not been experimentally verified. The use of increasingly stronger fields makes interactions with the body more likely and further research is needed to investigate possible health effects of strong fields and long-term exposure. However, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) recommends exposure limits for SMF. The exposure limit is 200 mT averaged over the working day, the maximum being 2 T, whereas for the general public the ICNIRP recommends an exposure limit of 40 mT [23].

Fourier transform infrared (FTIR) spectroscopy provides accurate information on the secondary structure of proteins, in H₂O-based structure or in deuterated form, as largely demonstrated up to now [24–27]. In particular, previous research studied the effects on human and in vitro red blood cells induced by microwaves and radiofrequencies, such as diminution of sedimentation speed or erythrocyte haemolysis [28–30].

We use this FTIR spectroscopy to investigate SMF effects on the infrared vibration bands of haemoglobin around the limits recommended by the ICNIRP for occupational exposure, focusing our attention on eventual bioprotective effectiveness of disaccharides such as sucrose and trehalose.

2 Materials and methods

2.1 Haemoglobin samples

Twelve healthy human subjects (aged 20–30 years, six males and six females) living in southern Italy were recruited for this study, and written authorizations were collected.

Blood samples were obtained by venipuncture and anticoagulated by using ethylenediamine-tetraacetic acid bi-sodium salt (Na_2EDTA) 0.5 M (10 nanomol/ml blood sample). After centrifugation at 1,500 rpm for 5 min, each sample was immediately washed five times with a physiological solution (NaCl 0.9%), whereas the white blood cell pellet was removed by using a water pump. The erythrocyte pellet obtained was treated with an equal volume of bi-distilled water and lysed by vigorously shaking and freezing/thawing. The haemoglobin solution was centrifuged at 6,000 rpm (mod. MR 23, Thermo Electron Corporation, USA) at 4°C in order to precipitate the insoluble stroma. The quantity of each haemoglobin solution was acquired by spectrophotometric analysis (mod. T70 UV/VIS, Full Tech Instruments, USA) at 420 nm against a calibrator. For each sample of 250 μl , three haemoglobin solutions were prepared at 150 mg/ml concentration in bi-distilled water, sucrose 50 mg/ml and trehalose 50 mg/ml, respectively, and immediately subjected to the following assays.

2.2 Experimental design

The exposure system consisted of a couple of Helmholtz coils, with round parallel polar faces, to produce a uniform magnetic field at the centre of the coil distance. An SMF was generated by coils powered by a DC generator, producing a uniform magnetic field intensity of 200 mT, corresponding to the exposure limit for SMF recommended by [17] for occupational exposure. Samples were placed at the centre of the generated uniform field area between the coils.

The magnetic field intensity was continuously monitored by a magnetic field probe (GM07 Gaussmeter, HIRST Magnetic Instruments Ltd., UK).

2.3 Infrared spectroscopy

FTIR absorption spectra were recorded at room temperature by a Vertex 80 v spectrometer (Bruker Optics). The attenuated total reflection (ATR) method was chosen for spectrum collection. Transmission of FTIR requires the placement of proteins between two calcium fluoride windows with thin path length spacers, with a potential for solvent interference. ATR is the desired method to overcome solvent masking since the penetration depth of infrared light is inherently limited to a fraction of the wavelength estimated to be $\lambda/10$, permitting rapid secondary structure analysis on small volumes of protein in H_2O solution [31]. Therefore, the effective path length for ATR-FTIR is sufficiently short to enable the analysis of protein structure in H_2O solution.

Haemoglobin solution (150 mg/ml) samples of 250 μl , in the absence or presence of sucrose and trehalose, were placed between a pair of CaF_2 windows separated with a 25- μm Teflon spacer. For each spectrum, 128 interferograms were collected and co-added by Fourier transform employing a Happ-Genzel apodization function to generate a spectrum with a spectral resolution of 4 cm^{-1} in the range from 7,500 to 350 cm^{-1} . IR spectra of water solution were subtracted from the spectra of haemoglobin at the corresponding temperature. Each measurement was performed under vacuum conditions to eliminate minor spectral contributions due to residual water vapor. Additionally, a smoothing correction for atmospheric water background was performed. IR spectra were baseline corrected and area normalized for exposed and unexposed samples.

Vector normalization was used, calculating the average value of the spectrum and subtracting it from the spectrum in order to decrease the mid-spectrum. The sum of the

squares of all values was calculated, and the spectrum was divided by the square root of this sum. The automatic baseline scattering correction function was used to subtract baselines from spectra, which allows us to obtain spectra with band edges of up to the theoretical baseline.

Fig. 1 **a** Representative spectra of haemoglobin in bi-distilled water solution after 3 h of exposure to SMF at 200 mT (the *dashed line* refers to exposed sample). The *circled part* of the spectrum corresponds to amide I and II regions analysed by second derivative spectra as represented in (**b**). The *dashed line*, representing the exposed sample spectrum, is almost overlapped by the *solid line*, representing the control sample, confirming that no appreciable alteration in β -sheet, turns or random coil contents occurred in amides I and II after the exposure to SMF

Interactive baseline concave rubberband correction was used for the acquired spectra, to confirm the decrease in amide A intensity. This method also uses a rubberband that is stretched from one spectrum end to the other, and the band is pressed onto the spectrum from the bottom up with varying intensity. It performs iteratively, depending on the number of iterations in the algorithm and the baseline as a frequency polygon consisting of n baseline points.

ATR spectra were smoothed by the Loess algorithm and the deconvolved spectra were fitted with Gaussian band profiles. Initial values for the peak heights and widths were estimated from the deconvolved spectra.

In order to enhance the fine spectral structure, second-derivative analysis of infrared spectra was performed. In the second-derivative spectrum, the intrinsic shape of an infrared absorbance is approximated by a Lorentzian function [26], and the peak frequency is practically identical with the original peak frequency, but the half-bandwidth is reduced. The height of a second-derivative peak is proportional to the original peak height with an opposite sign, and the half-bandwidth of the second-derivative peak is proportional to the original half-bandwidth [32].

3 Results and discussion

The secondary structure of a protein is very sensitive to the environment in which it is embedded. It has been largely demonstrated that several stress agents such as temperature, pH or chemical agents have a serious impact upon protein secondary structure. Hence, structural changes of vibration bands of human haemoglobin induced by exposure to SMF, in the absence or presence of sucrose and trehalose, were studied by FTIR.

The magnetic field intensity of 200 mT selected for this study corresponds to the maximum level suggested by the ICNIRP for SMF for occupational exposure [23].

Haemoglobin in bi-distilled water, sucrose and trehalose aqueous solutions, prepared as described in the preceding section, were exposed for 3 and 7 h to a uniform electromagnetic field of 200 mT, at the room temperature of 20°C. Analogue unexposed samples were used as a control.

Representative exposed and unexposed ATR-FTIR spectra obtained after 3 h of exposure for haemoglobin aqueous solution are shown in Fig. 1a. The assignment of the main vibration bands, based on previous spectroscopic studies, showed an intense amide I band centred at approximately $1,654\text{ cm}^{-1}$, corresponding to α -helix structure content due to C=O stretching vibration, a low-intensity amide II at $1,542\text{ cm}^{-1}$, originating from the N-H bending and C-N stretching modes, and a strong amide A band, centred at $3,293\text{ cm}^{-1}$ [33–36].

In order to estimate quantitatively further structural changes, IR spectra of haemoglobin solutions were differentiated twice, revealing three bands centred at $1,654$, $1,633$ and $1,685\text{ cm}^{-1}$ in the amide I region. The band at $1,654\text{ cm}^{-1}$ that was due to the α -helix content of the secondary structure is an appropriate indicator of the intactness of the protein

Fig. 2 Representative infrared spectra of amide A region for haemoglobin in bi-distilled water (a), sucrose (b) and trehalose (c) solutions after 3 h of exposure to SMF at 200 mT (*dashed lines* represent exposed sample spectra). The decrease in amide A vibration band appears clearly for exposed samples of haemoglobin in bi-distilled water solution

structures and at around 1,635 and 1,685 cm^{-1} can be associated with β -sheet features [37, 38].

No increase of β -sheet bands relative to α -helix content was found in amide I and II regions for samples in haemoglobin aqueous solutions after exposure (Fig. 1b). This circumstance led us to exclude the possibility that SMF can induce the formation of aggregates in haemoglobin.

In contrast, the spectral region from 3,500 to 3,000 cm^{-1} exhibited a clear change after exposure, as the amide A band close to 2,995 cm^{-1} decreased strongly for haemoglobin in bi-distilled water solution after 3 h of exposure.

Analogue ATR-FTIR spectral analyses were conducted for haemoglobin samples in sucrose and trehalose aqueous solutions after 3 h of exposure, providing the result that no appreciable change occurred in that region, as shown in Fig. 2, suggesting the hypothesis of a bioprotective effectiveness against SMF exposure. Also, such bioprotective effects were evidenced for haemoglobin aqueous solutions exposed to a 50-Hz electromagnetic field [39].

The amide A vibration band is one of the most prominent spectral features of peptides, polypeptides and proteins, due mostly to the peptide linkage N–H stretching mode, and it may be considered a useful indicator of secondary protein structure, which often appears as a doublet band arising from two different structures, the stronger of the two components being associated with the standard α -helical structure in the chain [40, 41].

Previous studies showed that local environments and hydrogen bonding configurations can play a role in determining the line shape of amide A vibration [42–44].

It is widely accepted that it is derived from an overtone of amide II (NH in-plane angle bend) or a combination of amides I and II that interacts with the strong NH fundamental through a cubic anharmonic potential, acquiring enough intensity to be observable through a Fermi resonance and resulting in two bands (amides A and B) whose separation is proportional to the interaction strength [45, 46]. Nevertheless, the amide A band might be considered too strong to be only an overtone or a combination of lower frequency bands [45, 46]. Such explanation is in agreement with our results that the decrease in amide A band due to the exposures to EMF exposure is not necessarily accompanied by an equal decrease in amides I and II modes.

Further ATR-FTIR analyses were conducted after 7 h of exposure. A decrease in amide A intensity was confirmed for haemoglobin in bi-distilled water solution, as represented in Fig. 3a. No appreciable change was observed for haemoglobin in sucrose and trehalose aqueous solutions (Fig. 3b–c).

A computation of the ratio between the amide A integrated area from 3,380 to 3,260 cm^{-1} with respect to the integrated area from 3,800 to 2,800 cm^{-1} of exposed samples and that relative to unexposed samples was conducted to quantify the evolution of the amide A features for haemoglobin in bi-distilled water, sucrose and trehalose aqueous solutions after exposure, providing the following values (this ratio must be 1 before exposure, obviously). This ratio decreased to 0.76 ± 0.09 and 0.74 ± 0.11 after 3 and 7 h of exposure, respectively, for haemoglobin in bi-distilled water solution. In contrast, the values of 1.03 ± 0.12 and 0.99 ± 0.04 were obtained after 3 and 7 h of exposure, respectively, for

Fig. 3 Representative infrared spectra of amide A region for haemoglobin in bi-distilled water (a), sucrose (b) and trehalose (c) solutions after 7 h of exposure to SMF at 200 mT (*dashed lines* represent exposed sample spectra). The decrease in amide A band intensity is still evident for exposed haemoglobin in bi-distilled water solution

Fig. 4 Amide A integrated area ratio between exposed and control samples for haemoglobin in bi-distilled water, sucrose and trehalose aqueous solutions, after 3 and 7 h of exposure to SMF at 200 mT. Its value is close to 1 after exposures for haemoglobin in sucrose and trehalose aqueous solutions

haemoglobin in sucrose aqueous solution. Finally, analogue computation for haemoglobin in trehalose aqueous solution provided the values of 1.03 ± 0.08 and 1.02 ± 0.06 after 3 and 7 h of exposures, respectively, proving that no significant effects occurred after exposure when compared to controls. These levels of amide A integrated area ratio are represented in Fig. 4. The computations were performed for 12 spectra for every exposure, providing levels significantly different in comparison to controls ($p < 0.05$).

These results led us to consider the possibility of a loss of NH linkage in the secondary structure of haemoglobin due to SMF exposure, suggesting the hypothesis that disaccharides such as sucrose and trehalose can preserve proteins from eventual structural changes due to an exposure to SMF. Furthermore, accurate qualitative and quantitative analysis by curve fitting to the second derivative spectra around the region of $3,000 \text{ cm}^{-1}$ revealed changes in the features of the aqueous haemoglobin solution. Between $2,850$ and $3,000 \text{ cm}^{-1}$,

Fig. 5 Second derivative spectra between $3,000$ and $2,800 \text{ cm}^{-1}$ for haemoglobin in bi-distilled water (a), sucrose (b) and trehalose aqueous solutions (c) after 7 h of exposure (dashed lines represent exposed sample spectra). The decrease in CH_3 symmetric and asymmetric stretching vibrations around $2,960$ and $2,870 \text{ cm}^{-1}$ is evident for haemoglobin in bi-distilled water solution after the exposure, but not for haemoglobin in sucrose and trehalose solution

symmetric and asymmetric CH_2 and CH_3 stretching vibrations of protein side chains are generally observed, assigned to methylene and methyl groups. In particular, the bands close to 2,960 and 2,870 cm^{-1} originate, respectively, from the asymmetric stretching $\nu_{\text{as}}\text{CH}_3$ and the symmetric stretching $\nu_{\text{s}}\text{CH}_3$ of CH_3 methyl groups [47, 48]. Decreases of such vibration bands in CH_3 chains were clearly evident for haemoglobin in bi-distilled water solution after 7 h of exposure to SMF, as shown in Fig. 5a.

No appreciable change of these vibration bands was observed for haemoglobin in aqueous sucrose and trehalose solutions after SMF exposure (Fig. 5b–c), giving another proof of the bioprotective effectiveness of such disaccharides.

4 Conclusion

Effects of static magnetic fields at 200 mT on different haemoglobin aqueous solutions, in the absence and in the presence of sucrose and trehalose, were studied by means of FTIR techniques. A decrease in the amide A vibration band was observed after 3 and 7 h of exposures for haemoglobin in bi-distilled water solution. The addition of disaccharides in aqueous haemoglobin solution inhibited such alteration, in agreement with our previous studies relative to the effects of extremely low frequency electromagnetic fields. These results led us to conclude that static magnetic fields can affect the N–H stretching vibrations of peptide linkages, suggesting compensatory mechanisms of environmental biochemical agents, such as a protective effectiveness of trehalose and sucrose.

No alteration of the ratio α -helix/ β -sheet contents was observed in the secondary structure, which can be considered as a primary indicator of the intactness of a protein, proving that exposure to static magnetic fields is not denaturing for haemoglobin. Furthermore, a decrease in CH_3 symmetric and asymmetric stretching vibrations of protein side chains was observed for haemoglobin in bi-distilled water solution after the exposure, but not for haemoglobin in sucrose and trehalose aqueous solutions.

Further investigations on other proteins could confirm such bioprotection properties of trehalose and sucrose relative to static magnetic field exposures.

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