Comparative study of the iron cores in human liver ferritin, its pharmaceutical models and ferritin in chicken liver and spleen tissues using Mössbauer spectroscopy with a high velocity resolution

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ABSTRACT

Application of Mössbauer spectroscopy with a high velocity resolution (4096 channels) for comparative analysis of iron cores in a human liver ferritin and its pharmaceutically important models Imferon, Maltofer® and Ferrum Lek as well as in iron storage proteins in chicken liver and spleen tissues allowed to reveal small variations in the ⁵⁷Fe hyperfine parameters related to differences in the iron core structure. Moreover, it was shown that the best fit of Mössbauer spectra of these samples required different number of components. The latter may indicate that the real iron core structure is more complex than that following from a simple core–shell model. The effect of different living conditions and age on the iron core in chicken liver was also considered.

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1. Introduction

Iron, essential element for all forms of life, is stored by both plants and animals mainly in a form of ferritin. The ferritin is a soluble iron-storage protein that contains nanosized hydrous ferric oxide core of about 8 nm diameter in the form of ferrihydrite (FeOOH)₈(FeO:OPO₃H₂) surrounded with a protein shell consisting of 24 subunits. The iron core represents a polynuclear system with up to 4500 iron atoms [1]. It was shown that ferritin from different living systems as well as from different organs and tissues within one living system may be different [2]. Moreover, the complicated process of the iron core formation may cause variations in the iron cores. Disturbances of iron metabolism in a body may be a result of iron overload diseases and/or an iron deficiency anemia. In the latter case some medicines with a high amount of iron are commonly used or being developed in order to treat this pathology. Among them there are ones that can be considered as ferritin models. They also contain nanosized hydrous ferric oxide cores in the form of β-FeOOH or other structural modifications of the hydrous ferric oxides coated with a polysaccharide shell instead of a protein

one. It is plausible that the iron core structure in the pharmaceutical models of ferritin may be related to the effect of these medicines. Therefore, a comparative study of natural iron storage proteins and its pharmaceutically important model seems to be of interest.

Previous Mössbauer spectroscopy studies have shown that physical properties of the iron cores in ferritin and iron–dextran complexes were similar, however, the average composition of the iron cores was found to be different, namely (5Fe₂O₃·9H₂O) and β-FeOOH, respectively [3,4]. The core–shell model based on relaxometry and magnetometry data was proposed for the explanation of the iron core complicated structure [5]. In this model the ferritin iron core is a system composed of an inner antiferromagnetic region whose the Néel temperature, $T_N > 310$ K, and an amorphous surface layer with a very low value of T_N where exchange forces are not strong enough to maintain an antiferromagnetic order. Consequently, the ferritin iron core has different internal and surface magnetizations, and it represents two-phase spin system of interior and surface spins. This magnetic structure becomes more evident at a low iron-loading when the surface-to-volume ratio is enhanced. This model is still in use for interpretation of the ferric hydrous oxides Mössbauer spectra measured with a low velocity resolution [6–8]. According to the core–shell model the Mössbauer spectra of ferritin were fitted using two paramagnetic doublets at 295 K and two magnetic sextets at 4.2 K. For Imferon, one of the ferritin

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models, the proposed structure of the iron core consisted of two nonequivalent positions of ferric ions, therefore an approach with two components was also applied to fit the Mössbauer spectra of Imferon [9]. However, some authors think that it is insufficient to use only two components to fit such complicated spectra [8,10–12]. In particular, an application of the Mössbauer spectroscopy with a high velocity resolution showed that the best fit of the room temperature Mössbauer spectra of ferritin and Imferon was obtained using a superposition of four and three quadrupole doublets, respectively [11,13,14]. Therefore, this technique was used for a further comparative study of the iron cores in a human liver ferritin, chicken liver and spleen as well as in pharmaceutically important ferritin models like Imferon, Maltofer®, Ferrum Lek.

2. Materials and methods

Lyophilized human liver ferritin was prepared at the Russian State Medical University (the process of ferritin preparation was described earlier [15]), and used as a powder with a sample's weight of 100 mg. Samples of chicken liver and spleen were obtained from two different poultry farms (1 and 2), then washed from the blood and, finally, lyophilized. The samples' powder weight in the latter case was between 1200 and 1600 mg. Commercially available pharmaceuticals such as Imferon (Fisons, UK), Maltofer® (Vifor Inc., Switzerland) and Ferrum Lek (Lek, Slovenia) were used in this study. A sample of Imferon solution, the iron–dextran complex, was used in a lyophilized form for a room temperature measurement, and in a frozen solution for a 90 K measurement. Two iron–polymaltose complexes Maltofer® and Ferrum Lek were prepared for measurements by powdering 1/3 of a tablet. The effective thicknesses of the samples for Imferon, Maltofer® and Ferrum Lek were 5, 10 and 10 mg Fe/cm², respectively.

The Mössbauer spectra were measured using an automated precision Mössbauer spectrometric system with a high velocity resolution. Two sources of the gamma-rays, each of about 1.8×10^9 Bq ⁵⁷Co in chromium and rhodium matrixes, were used at room temperature. A more detailed description of the system was given elsewhere [16,17]. The spectra were measured at 295 K and 90 K in a transmission geometry with moving absorber placed in a liquid nitrogen cryostat and registered in 4096 channels. For a further analysis the spectra of ferritin, Imferon and Maltofer® were converted into 2048 channels by the consequent summation of two neighboring channels, the spectra of chicken tissues with a very low iron content were converted into 1024 channels by the consequent summation of four neighboring channels, while the spectra of Ferrum Lek were analyzed as measured. The number of channels for a spectra presentation was determined by the number of counts per one channel and a signal-to-noise ratio. A statistical counting rate for the measured spectra was in the range from 0.6×10^6 to 5.0×10^6 counts per channel, and the signal-to-noise ratio was in the range between 12 and 133. The spectra were computer fitted using UNIVEM-MS program with a least-squares procedure using the Lorentzian line shape. It should be noted that there was no thickness effect in the spectra of ferritin and its models with a large amount of iron. Therefore, there was no need for a transmission integral method to be used for the spectra fitting. Spectral parameters determined for the measured spectra were isomer shift, δ , quadrupole splitting, ΔE_Q , line width, Γ , relative subspectrum area, S , and statistical quality, χ^2 . An instrumental (systematic) error for a velocity scale was ± 0.5 channel for each spectrum point, the systematic error for the hyperfine parameters was ± 1 channel while that for a line width was ± 2 channels. It should be noted that these spectrometer characteristics determined an integral velocity error (total mechanical and electronics systematic and random errors) which was several times less than

a half of a channel value expressed in mm/s during the spectra measurements using 4096 channels [16]. A relative error for S did not exceed 10%. If an error calculated with the fitting procedure (fitting error) for the parameters exceeded the instrumental (systematic) error, we used the larger error. A velocity resolution in the spectra was about 0.001 mm/s per channel for Ferrum Lek, 0.002–0.003 mm/s per channel for ferritin, Imferon and Maltofer®, and about 0.005 mm/s per channel for the chicken liver and spleen tissues. Values of the isomer shift are given relative to that of the α -Fe at 295 K.

3. Results and discussion

The Mössbauer spectra of ferritin demonstrated non-Lorentzian line shape [13,14] like those of other hydrous ferric oxides [18,19]. They can be better fitted using a superposition of individual paramagnetic and/or magnetic components (quadrupole doublets and/or magnetic sextets), or, alternatively, applying a distribution of a quadrupole splitting and/or a hyperfine field. Such an approach to a spectra analysis corresponds to the heterogeneous iron core model i.e. the one with a complex structure. We are of opinion that a more reliable picture can be obtained in the frame of the heterogeneous iron core model using the superposition of several independent spectral components rather than by applying the distribution method. In addition, we also analyze the spectra using one component only which corresponds to a homogeneous iron core model. By doing so we want to visualize and compare small differences between the hyperfine parameters obtained for some samples using this rough approximation. This approach for the spectra analysis can be well demonstrated using the example of the Mössbauer spectrum of Ferrum Lek measured at 295 K in 4096 channels (Fig. 1). It is clearly seen that the χ^2 -values (standard deviation $\sigma = 0.022$) and difference spectrum significantly changed if the number of doublets included into the analysis was varied from one up to five, while adding a sixth doublet did not changed either χ^2 -value or the difference spectrum in comparison with the fit obtained using five doublets. Therefore, the Mössbauer spectra recorded for all studied samples were fitted in above mentioned way to reach the best fit.

3.1. Ferritin and its pharmaceutically important models

The Mössbauer spectra of the human liver ferritin and its models Imferon, Maltofer® and Ferrum Lek measured at 295 K are shown in Fig. 2. These spectra represent similar paramagnetic doublets but with a different absorption effect. Variations in the absorption effect were related to a different iron content in the samples, while in the case of Ferrum Lek this effect was caused by using another registration tract for γ -rays which resulted in a better resonance efficiency. The best fits of these spectra were obtained using four doublets for the ferritin, three doublets for Imferon and five doublets for both iron–polymaltose complexes. The Mössbauer hyperfine parameters which were obtained using the rough approximation appeared to be different within the error (Fig. 2e). It should be emphasized that thanks to the improved velocity resolution we were able to distinguish the hyperfine parameters for the two iron–polymaltose complexes Maltofer® and Ferrum Lek. These results indicated that Fe(III) in iron cores of the human liver ferritin and its models had some small stereochemical variations which may be related, for instance, to differences in oxygen and iron atoms packing.

It was interesting to see approximately the same differences of the isomer shift and the quadrupole splitting for the ferritin, on one hand, and those for Maltofer® and Ferrum Lek, on the other hand, as found from the spectra measured at 90 K (see Fig. 3). Such

Fig. 1. Room temperature Mössbauer spectra measured in 4096 channels on the Ferrum Lek as fitted with a different number of doublets. Difference spectrum is shown below each spectrum.

Fig. 2. Room temperature Mössbauer spectra of: (a) human liver ferritin, (b) lyophilized Imferon, (c) Maltofer®, (d) Ferrum Lek, (a)–(c) are presented in 2048 channels, (d) is presented in 4096 channels, difference spectrum is shown below each spectrum, and (e) a plot of the Mössbauer hyperfine parameters obtained within the homogeneous model for the human liver ferritin (◆), Imferon (■), Maltofer® (▲) and Ferrum Lek (●).

Fig. 3. Mössbauer spectra recorded at 90 K on: (a) human liver ferritin, (b) Maltofer®, (c) Ferrum Lek; (a), (b) are presented in 2048 channels, (c) is presented in 4096 channels; difference spectrum is shown below each spectrum; and (d) plot of the Mössbauer hyperfine parameters obtained within the homogeneous model for the human liver ferritin (●), Maltofer® (▲) and Ferrum Lek (●).

differences of the hyperfine parameters found for the ferritin and two iron–polymaltose complexes both at 295 and 90 K may indicate some variations in the structure of the iron cores of these samples. An increase in the quadrupole splitting from the ferritin to Imferon, Ferrum Lek and Maltofer® at both temperatures means an increase in the electric field gradient on ^{57}Fe nuclei resulting from a local environment and a stereochemical distortion of iron ions, impurities, etc. The same number of the quadrupole doublets needed to obtain the best fit of the corresponding spectra measured at 90 and 295 K can be regarded as a justification for the chosen model of spectra analysis. On the other hand, a different number of the doublets needed to obtain the best fit of the Mössbauer spectra recorded on the ferritin and its models may be interpreted as an indication of differences existing in the complicated structure of the iron cores of the investigated samples. For instance, four doublets necessary to obtain the best fit of the Mössbauer spectra of the ferritin may indicate a complicated polycrystalline structure of its core, or an effect of the surface and that of several internal layers, etc. The actual iron core structure may result from processes involved in the core formation.

As far as the ferritin pharmaceutical models are concerned, their iron cores are usually prepared using FeCl_3 . Therefore, $\beta\text{-FeOOH}$ structure of the iron cores formed in the presence of chlorine (and, likely, other impurities), and in different conditions, may be responsible for differences in the iron cores. The different number of the quadrupole doublets necessary to obtain the best fit, on one hand, and differences in the Mössbauer spectral parameters for the ferritin and its pharmaceutical models, on the other hand, may, therefore, be reflecting variations in the iron core structures.

Although the Mössbauer spectra measured at 295 and 90 K of the both iron–polymaltose complexes viz. Maltofer® and Ferrum Lek were fitted with the same number of the quadrupole doublets, the values of the hyperfine parameters appeared to be different for the spectral components with similar relative areas. Therefore, we conclude that the iron cores in these models (iron–polymaltose complexes) are different possibly due to a manufacturing process. The relative areas of corresponding components obtained at 295 K appeared to be equal within the error limit in case of the first, the second and the fourth components (Fig. 4a). This fact may indicate that regions of the iron cores corresponding to these components were similar in the size and ^{57}Fe content. However, small variations of the hyperfine parameters related to the corresponding regions in two iron–polymaltose complexes may indicate different degree of crystallinity, density of atoms package, etc. in these regions. Relative areas of the third and the fifth components were different in the spectra of Maltofer® and Ferrum Lek. This fact may indicate different sizes of the corresponding regions in the iron cores of both complexes. At 90 K relative areas of all corresponding components in the Mössbauer spectra of Maltofer® and Ferrum Lek were different within the error limit except the third component.

3.2. Chicken tissues

The Mössbauer spectra of chicken livers and spleen measured at 295 K are shown in Fig. 5. The spectra are in form of doublets with slightly different values of the absorption effect. They were fitted using two models: (a) homogeneous and (b) heterogeneous iron core. In the first case some small differences in the hyperfine

Fig. 4. Comparison of relative areas of spectral components obtained from the best fit of the Mössbauer spectra of: Maltofer® (□) and Ferrum Lek (■) measured at (a) 295 K and (b) 90 K.

parameters were revealed, and are shown in the plot of the quadrupole splitting versus the isomer shift (Fig. 5e). Using this data we were able to distinguish between the human liver ferritin and chicken tissues containing ferritin, between the chicken liver and spleen, and between livers from different chickens. It was interesting to observe small variations in the Mössbauer hyperfine

parameters for the chicken liver as obtained from the poultry farms 1 and 2. The latter may be a result of a different chicken feeding used in these farms. Differences observed among chicken with different age (148 and 200 days) indicate that the iron cores structure might vary with the age. Moreover, the absorption effect for a younger chicken was slightly smaller than that for an older one which may be related to iron content and a degree of crystallization of the iron cores. The above discussed results showed that the iron core formation in ferritin depends on various factors and, therefore, to obtain a correlation between the Mössbauer hyperfine parameters, iron core structure and various factors such as feeding, age, etc. one has to carry out studies thoroughly. Within the heterogeneous model the best fits of the Mössbauer spectra of the chicken tissues measured earlier [11] and in this work, were obtained using the superposition of only two quadrupole doublets instead of four doublets as in the case of the extracted human liver ferritin with 10 times larger resonance effect. It is very likely that this is the result of a poor signal-to-noise ratio observed in the spectra of the chicken tissues. It is possible that an increase in statistical counts rate may improve this fit. However, it would take a significantly longer measurement time. Nevertheless, the Mössbauer spectral parameters obtained for the chicken tissues appeared to be different for all samples. A comparison for the chicken livers obtained from the poultry farms 1 and 2 are shown in the plots of the quadrupole splitting and isomer shift for doublets 1 and 2 (Fig. 6). The ^{57}Fe hyperfine parameters differ for the chicken liver obtained from the poultry farms 1 and 2 for the both quadrupole doublets. In contrast, the values of the parameters are the same within the error limit for doublet 1 in the case of the chicken liver from chicken with the age of 148 and 200 days, while those for doublet 2 appeared to be slightly different. However, the absorption areas of doublets 1 and 2 are different for the chicken livers from the chicken with different age. This fact may reflect structural differences of the iron cores in the chicken liver ferritin as a result of a different iron core formation process possibly due to a different feeding of chicken in the two poultry farms, and a different chicken age.

Fig. 5. Room temperature Mössbauer spectra presented in 1024 channels of (a) normal chicken liver and (b) spleen from the poultry farm 1, chicken liver from chicken of the age of: (c) 148 days and (d) 200 days from poultry farm 2 (indicated components are the results of the best fit), difference spectrum is shown below each spectrum; and (e) plot of the Mössbauer hyperfine parameters obtained within the homogeneous iron core model for: human liver ferritin (◆), normal chicken liver (■) and spleen (▲) from poultry farm 1, preliminary results for the normal liver from chicken of the age of 148 days (□) and 200 days (■) from poultry farm 2.

Fig. 6. Plot of the Mössbauer hyperfine parameters obtained within the heterogeneous model from the room temperature spectra for the fresh chicken liver (■) from poultry farm 1, normal chicken liver from chicken of the age of 148 days (□) and 200 days (◐) from poultry farm 2: (a) quadrupole doublet 1, (b) quadrupole doublet 2; 1024 channels, $T = 295$ K.

4. Summary

We have demonstrated that the Mössbauer spectra recorded with the high velocity resolution equipment and having a high statistical quality needed to be fitted with more-than-two quadrupole doublets. This fact reflects, in our opinion, a complex structure of the iron core in the studied samples of the ferritin and its pharmaceutical models. Each doublet may be related to a characteristic feature of the iron core such as: its surface, internal layers with different size, degree of crystallization, density of iron and oxygen atoms packing and complicated multi-domain structure. In the case of the chicken tissues, the corresponding spectra were analyzed in terms of only two doublets, but such fitting procedure was due to the low signal-to-noise ratio observed in these samples. A very low concentration of iron in the natural samples did not allow to record spectra with a high statistical quality within a reasonable span of time. Contrary to previous Mössbauer studies of ferritin and its models performed with a low velocity resolution equipment, our present investigation has shown that in order to obtain good quality fits of the Mössbauer spectra of the human liver ferritin and Imferon it was necessary to take into account four and three doublets, respectively. Five doublets were needed to get good fits in the case of the two iron–polymaltose complexes. Based on these results it seems reasonable to assume that the iron core structure in the ferritin and its models discussed in this paper is more complicated than the one proposed by the core–shell model [5]. Differences in the values of the spectral parameters observed in the samples taken from chicken of different origin and age gave a further support to the heterogeneous structure of the core as well as to a possible effect of various factors such as feeding, age, etc. on the iron core formation. However, further investigations are required to better evidence our interpretation.

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