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# Mössbauer Spectroscopy as a Valuable Analysis Technique in Biomedical Research

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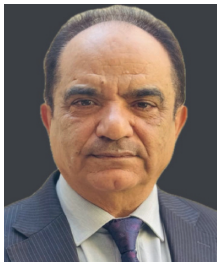
*Abstract*: Mössbauer spectroscopy is an effective technique used to examine the iron atom electronic environments in both biomolecular molecules and whole animal studies. Because of its sensitivity to nuclear hyperfine interactions, this technique yields incredibly accurate data regarding the electronic and magnetic states of nuclei, chemical bonds, and the local electronic environment structure around iron atoms. This review demonstrates how Mössbauer spectroscopy contributes to biomedical sciences. The use of Mössbauer spectroscopy in the fields of general biology is discussed, as well as studies that included bacterial analyses, studies related to protein materials, and pharmaceutical studies. In addition, although beyond the scope of this review, the use of Mössbauer spectroscopy to study model compounds to aid in understanding the iron proteins is briefly referred to.

**Keywords**: Bacteria · Biological science · Hyperfine interaction · Mössbauer spectroscopy · Pharmaceutical studies · Protein



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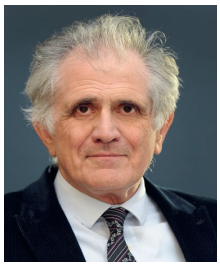

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## 1. Introduction

Mössbauer spectroscopy is not considered as a common technique in the fields of biology and medicine. However, this technique is sensitive enough to detect species at concentrations between 10 and 20 μM for iron containing species.[1] While Mössbauer spectroscopy is effective for biological systems containing iron, it is not practicable for transition metals such as copper, manganese, or zinc, due to a lack of suitable isotopes and low recoil-free fractions.[1,2] The importance of coordination compounds with an iron metal center in inorganic and bio-inorganic chemistry as models for biologically important proteins cannot be overstated. Iron has a biological role that no other metal can match. Nearly every lineage of life contains enzymes that have heme units as their active centers. Of particular note in this regard are the hydrogenase enzymes, which show promise as materials for hydrogen storage.[3]

Mössbauer spectroscopy was shown to be very effective for investigating proteins and enzymes having iron active sites, as well as Fe/S clusters. It can be utilized to obtain kinetic and mechanistic information about processes involving Fe-containing proteins.[4,5]

The technique has also proved useful for studying numerous iron-containing medicaments, vitamins, and dietary supplements that have been used or created for the treatment and prevention of iron deficiency anemia, a condition which is extremely dangerous for humans and can lead to a variety of illnesses.[6,7]

The main aim of this review is to provide an overview of how Mössbauer spectroscopy has/can be used in the field of biomedicine to acquire a deeper understanding of the characteristics, roles, and activity of iron-containing proteins/compounds within biological systems.

We will illustrate some of the many breakthroughs and new understandings that the use of Mössbauer spectroscopy has facilitated in the general field of biomedical and associated animal, bacterial and pharmaceutical studies.

We shall briefly review the Mössbauer parameters that can be derived from the Mossbauer spectra. These include the isomer shift, quadrupole splitting, and magnetic hyperfine field, all of

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which can provide important insights on the valence state and spin state of iron as well as the electronic environment of iron.[8] A new Mössbauer spectrometer system has been developed. The system has high velocity resolution and a temperature variable liquid nitrogen cryostat with a moving absorber. The method reduces experimental error in determining $^{57}$Fe hyperfine parameters. This has led to more accurate analysis of small variations in the iron electronic structure and reliable fitting of complex spectra.[9]

## 2. Mössbauer Spectroscopy

The Mössbauer effect is the recoilless emission and resonant reabsorption of nuclear gamma-rays between the ground and normally the first excited state of identical nuclei of the source and the sample.[10] The Mössbauer effect has been observed in 72 isotopes of 42 different elements for a total of nearly 90 γ-ray transitions.[11] Resonance absorption can only be observed when there is sufficient overlap between the emission and absorption lines. The emission or absorption of γ *quanta* with energy $E_\gamma$ in a freely moving atom or molecule (gas, liquid) results in a recoil effect with energy $E_R = E^2_\gamma / 2mc^2$, which is several orders of magnitude greater than the natural line width Γ. There is no possibility of resonance between free atoms or molecules. As a result, the Mössbauer effect cannot be observed for freely moving atoms or molecules, *i.e.* in a gaseous or liquid state. In the solid state, recoilless emission and absorption of γ *quanta* is possible, and the essentially unshifted transition lines can (at least partially) overlap and nuclear resonance absorption can be observed. This was demonstrated experimentally by cooling the source and absorber to temperatures close to liquid nitrogen, where atoms are tightly bound in the lattice and the recoil effect is greatly reduced.[12] While it can also be used to describe many nuclei, its application to iron compound characterization is by far more common.[13]

The radioactive $^{57}$Co isotope with a half-life of 271.8 days, which decays by electron capture to the nuclear isomer $^{57m}$Fe, is a common source for excited state iron nuclei (Fig. 1).[14] The sample (absorber) is then probed using the γ-ray that is released during the relaxation of the spin $I = 3/2$ to the nuclear ground $I = 1/2$ state of the iron nucleus.[10]

The resonant absorption of γ-rays can be observed by fixing the nucleus in a crystal lattice and lowering the temperature of the source and the absorber. The recoil energy of the nucleus can be transferred to the lattice.

The Mössbauer spectrum is obtained by plotting the percentage transmission (or the number of counts registered by the detector) against Doppler velocity. Fig. 2 shows the Mössbauer spectrum for an identical source and absorber.[15,16]

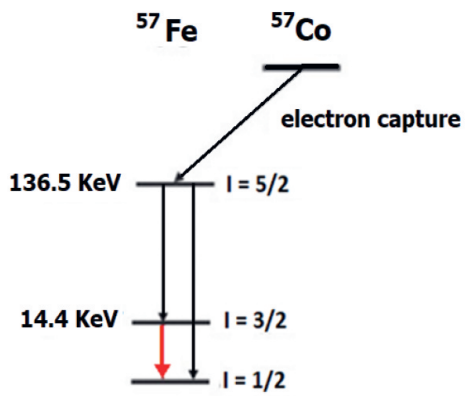


Fig. 1. Decay scheme of $^{57}$Co to $^{57}$Fe and the respective $^{57}$Fe nuclear transitions.

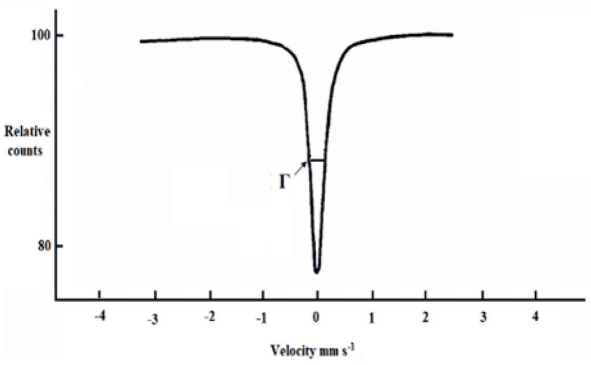


Fig. 2. Mössbauer spectrum.

### 2.1 *Measurable Parameters of Mössbauer Spectroscopy*

There are three parameters that are the most important in the Mössbauer technique; they are the isomer shift, the quadrupole splitting, and magnetic hyperfine splitting.

#### *Chemical Isomer Shift (δ)*

A shift of the center of the resonance line occurs when the source and absorber are in different environments. This shift, referred to as isomer shift, arises due to the difference in *s*-electron density between the source and the absorber (Fig. 3).

Only *s*-electrons have a finite probability of penetrating the nucleus, and any change in *s*-electron density at the nucleus results in a change in chemical isomer shift. The presence of *p*, *d*, and *f* electrons can influence the electron density of the *s*-electrons through shielding effects.

The isomer shift can be expressed as:

$$\delta = \tfrac{4}{5}\pi\, Ze^2\, r^2\, (\Delta r/r)(|\Psi_a(0)|^2 - |\Psi_s(0)|^2) \qquad (1)$$

where z is the nuclear charge, e is the electron charge, $\Delta r = \gamma_e - \gamma_g$ is the difference in the nuclear radii of the excited and the ground states, $|\Psi_a(0)|^2$ and $|\Psi_s(0)|^2$ are the total *s*-electron density at the nuclei of the absorber and the source respectively. This equation contains two terms; the first term $\frac{4}{5}\pi\, Ze^2\, r^2\, (\Delta r/r)$, contains the nuclear parameter, in particular the mean difference in the radius between the ground and the excited states. The second term, $(|\Psi_a(0)|^2 - |\Psi_s(0)|^2)$, represent the change in *s*-electron density.[15,16]

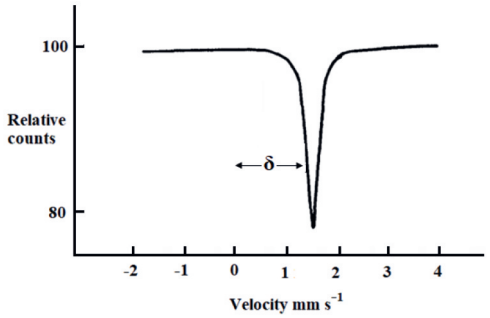


Fig. 3. The chemical isomer shift.

#### *Quadrupole Splitting (Δ)*

The derivation of the expression for isomer shift assumed a spherical nucleus with a uniform charge density. If the nucleus is non-spherical it will possess a nuclear quadrupole moment. Nu-

clei with spin quantum number $I > ½$ possess an electric quadrupole moment. In a non-cubic symmetry, the interaction between the electric field gradient at the nucleus and the electric quadrupole moment Q causes the quadrupole splitting. The electric quadrupole moment is a measure of the deviation from spherical symmetry of the nuclear charge. The sign of Q is positive for an elongated (prolate) and negative for a flattened (oblate) nuclei. Nuclei with $I = ½$ or 0 are spherically symmetrical and do not possess quadrupole moments.

The quadrupole splitting is the result of the interaction of an electric quadrupole moment and the electric field gradient (Fig. 4). The Hamiltonian, which describes this quadrupole interaction, is:

$$H = Q \,.\, VE$$

where Q is the nuclear quadrupole moment, VE is the electric field gradient tensor.

The interaction of an electric quadrupole moment with an electric field gradient results in a splitting of the energy levels, which are given by:

$$EQ = \frac{e^2\, qQ}{4I\,(2I-1)}\,[3M_I^2 - I\,(I+1)]\,(1+\frac{\eta^2}{3})^{1/2} \qquad (2)$$

where eQ is the nuclear quadrupole moment, eq is the electrostatic field gradient, $M_I$ is the magnetic quantum number, I is the spin quantum number, η is the symmetry parameter which is related to the magnitude of three components of the electrostatic field gradient by:

$$\eta = (V_{xx} - V_{yy} / V_{zz})$$

where $0 < \eta < 1$

For $^{57}$Fe, the two allowed values for $M_I$ for the first excited states are ½ and ⅔. When these atoms occupy a lattice site where the electric field gradient tensor is non-vanishing (*i.e.* $q \neq 0$), the ⅔ state is split into two levels, one of which is raised in energy by an amount:

$$EQ\,(M_I = 3/2) = \frac{e^2\, qQ}{4}\,(1+\frac{\eta^2}{3})^{1/2} \qquad (3)$$

and the other level is lowered by an identical amount. As there is no quadrupole moment associated with the ½ state, the quadrupole splitting may be represented by this energy separation:[15,16]

$$\Delta = \pm\, 1/2\; e^2\, qQ\,(1+\frac{\eta^2}{3})^{1/2} \qquad (4)$$

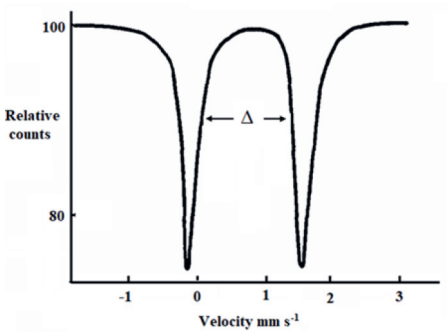


Fig. 4. The quadrupole splitting.

*Magnetic Hyperfine Splitting*

The nucleus has a magnetic moment μ, when the spin quantum number *I* is greater than zero. The magnetic moment will interact with a magnetic field at the nucleus and the interaction can be expressed by the Hamiltonian:

$$H = -\,\mu.B = -\,g\,\mu_N\, I.B$$

where B is the magnetic flux density, g is the nuclear factor, $\mu_N$ is the nuclear magneton, I is the angular momentum. The solution of this equation gives the energy levels as:

$$E_m = -\,g\,\mu_N\, Bm_z$$

where $m_z$ can take values I, I-1, ……. -I. The magnetic field will split each energy level into 2I+1 separate equally spaced energy levels. Splitting of the energy levels arises either from an externally applied field or from the sample itself (the latter originates as an internal field) which can produce a sufficient field to cause observable splitting. The magnetic field removes degeneracy from nuclear energy levels, allowing for transitions that obey the selection rule $\Delta m = 0, \pm 1$ to occur. Fig. 5 shows the six lines, which are the allowed transitions in the case of $^{57}$Fe. The lines are not of equal intensity, but a 3:2:1:1:2:3 ratio is often found.[15,16]

To sum up, the chemical or isomer shift gives information about the *s* electron density at the nucleus, and *p* and *d* electron shielding. It is often studied by comparing differences in relation to a range of similar compounds.

The quadrupole splitting gives information of the shape of the electric field around the Mössbauer nucleus, the greater the quadrupole spitting the more distorted the electric field.

The magnetic hyperfine splitting yields information as to the sign of the field gradient.

Thus, a Mössbauer nucleus such as $^{57}$Fe can be used to probe the chemical environment around the iron atom.

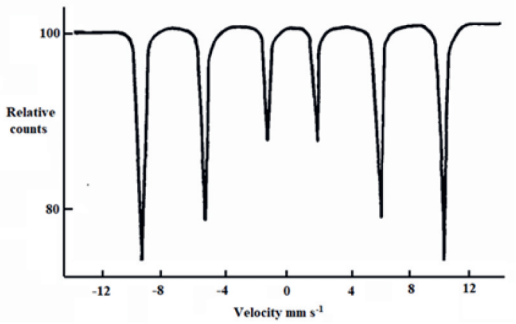


Fig. 5. Origin of magnetic hyperfine splitting and spectrum of natural iron-used for calibration of the spectrometer.

## 3. General Biomedical Studies

In biomedical studies, Mössbauer spectroscopy has two applications in studying the electronic state of atoms in molecules and identifying chemicals. Since many biological molecules have a transition metal atom at the active center, studying the electronic state of an atom can be very powerful in giving chemical information about the active center. Magnetic measurements can provide information on the chemical state of the atom as well as the electron transfer that occurs during a biochemical reaction.[17]

Mössbauer spectroscopy offers physical parameters and information about the electronic structure of iron in biomolecules, model compounds, and pharmaceutical samples. It also measures

qualitative and quantitative changes in iron-containing biomolecules during pathological processes or environmental factors.[18] Measuring Mössbauer spectra is crucial in biomedical applications, particularly when comparing normal and pathological subjects.[19]

### 3.1 *Studies on Dynamic Processes and Iron-Sulfur Proteins*

The use of Mössbauer spectroscopy can also provide insights into the dynamic behavior of iron centers in biomolecules, in addition to their structural and electrical features,[20] and can serve as a biophysical probe of iron metabolism in cells and organelles.[1] Mössbauer spectroscopy was employed to analyze Co complexes with several small biomolecules, such as 4-*n*-hexylresorcinol, homoserine lactone, and a few amino acids. The spectra were obtained for rapidly frozen dilute aqueous solutions or for samples dried at 80 K.[21] The identification of the electronic states of polynuclear iron sulfur centers was one of the major successes of Mössbauer spectroscopic applications in biological chemistry. It was formerly utilized to look into how an iron-sulfur core functions in a unique biological process,[22] and also to identify an unusual 4Fe-4S-center in the Lytb-protein, known as IspH. In many bacteria and the malaria parasite *Plasmodium falciparum*, this protein is required for the biosynthesis of isoprenoids.[23] The electronic structures of the 'well-established' $[2Fe\text{-}2S]^{2+/+}$, $[3Fe\text{-}4S]^{1+/0}$, and $[4Fe\text{-}4S]^{3+/2+/1+/0}$ clusters were extensively characterized using Mössbauer spectroscopy, as were novel Fe/S clusters, such as the [4Fe-3S] cluster of the $O_2$-tolerant hydrogenase from *Aquifex aeolicus*.[5] Iron sites in Fe/S clusters are generally tetrahedral with four sulfur-coordinating atoms. The Mössbauer technique was used to identify the $[2Fe\text{-}2S]^{+}$ cluster of the IscR promoter as containing three cysteines and one histidine. The histidine is bound to the ferrous center. IscR promotes Fe/S cluster biosynthesis through the Isc pathway in aerobic conditions.[24]

### 3.2 *Studies on Direct Medical Applications in Human or Animal Subjects*

Mössbauer spectroscopy has been used to compare healthy donor spleen and liver tissues to those from patients with non-Hodgkin B-cell lymphomas, acute myeloid leukemia, and primary myelo fibrosis. The study found minor changes in the ferritin iron core structure in spleen and liver tissues between healthy donors and patients with hematological malignancies.[25] Mössbauer spectroscopy was employed to assess three samples with known amounts of natural iron (400, 800, and 1600 g), as well as a sample of lyophilized human brain tissue from globus pallidus.[26] It has also proved useful in examining the oxidative effects of acetylphenylhydrazine in red blood cells from healthy donors and breast cancer patients.[27] The technique has been utilized to investigate important issues such as iron deposits in biological tissue of Basal Ganglia,[28] analyzing data for Co complexes with several small biomolecules, such as 4-*n*-hexylresorcinol, homoserine lactone, and a few amino acids,[21] and understanding ferrate thermal decomposition, heavy metal encapsulation, and thiol oxidation pathways.[29]

The Mössbauer technique offers structural and quantitative insights into the coordination microenvironment, chemical state, and transformations of Mössbauer nuclides in metal-containing proteins, supramolecular structures, and microbial cells/tissues.[30] The anti-tumor antibiotic bleomycin was the subject of several Mössbauer spectroscopic studies in the 1980's.[31–33] Iron-bleomycin in various oxidation states and in complexes with dioxygen or carbon monoxide was examined. Ferrous bleomycin is a high spin ferrous complex. It was found that oxygenated bleomycin is diamagnetic.[31] It has also been used to aid other techniques in the characterization of the magnetic nanoparticles prepared and coated with poly(L-lysine), poly(*N*,*N*-dimethylacrylamide-co-acrylic acid), L-ascorbic acid, D-galactose, D-mannose, and sucrose. The nanoparticles are a possible platform for colon cancer theranostics.[34] A recent study has suggested the use of the Mössbauer effect as a potential low-dose technique for treating glioblastoma. The Mössbauer effect was found to impair cellular viability in both 2D and 3D models of U87 GBM cells.[35] The magnetic characteristics of the iron-containing cores of the biological iron storage material haemosiderin formed under normal and various pathological situations have been shown to be different.[36] Mössbauer spectroscopy has also been used to study iron distribution in rat organs, specifically in liver subcellular fractions. Throughout a 6-day period, 0.5 ml doses of a Fe-sucrose complex solution were injected into the tail veins of animals. The spectra were obtained in the spleen, blood, liver, and liver subcellular fractions.[37] The technique was utilized to analyze iron content, redox state, and binding sites in the *substantia nigra* of Parkinson's and control brains. Measurements on fresh-frozen, formalin-fixed, and lyophilized materials revealed only ferric iron. Ferrous iron may only account for 5% of the total iron content.[38] The possible role of iron in the degeneration of nervous cells in Parkinson's disease was also investigated. The spectra were found to be very similar to ferritin spectra. Small differences in the spectra obtained from Parkinson's disease and control parkinsonian *substantia nigra* were detected, which could be due to a slight difference in the composition of the ferritin-like iron cores or the presence of about 8% non-ferritin-like iron in parkinsonian *substantia nigra*.[39]

## 4. Studies Involving Bacteria

Many Mössbauer studies involving bacteria have appeared in the literature, and it is not the aim of this review to cover them all, rather we wish to provide the reader with a taste of what has been achieved. Early attempts were made to use Mössbauer spectroscopy to detect bound iron in bacterial cells of *Desulfovibrio vulgaris* in the wet state.[40] *Pseudomonas aeruginosa* samples were examined. The samples included entire cells, membranes, and soluble fractions from cells cultured with ferric chloride, ferric citrate, or incubated ferripyoverdine.[41] A high velocity resolution study was used to compare two biomass samples of the rhizobacterium *Azospirillum brasilense* (strain Sp245) generated under various conditions to human liver ferritin at room temperature. This study found that the bacterial spectra contained ferritin-like iron, similar to the ferritin spectrum.[42] Fd 11, a tetrameric ferredoxin form of *Desulfovibrio gigas*, facilitates electron transport between cytochrome C3 and sulfite reductase. The two stable oxidation states of this protein were examined using Mössbauer spectroscopy.[43] It was also used in the identification of iron(II) enterobactin and its possible role in *Escherichia coli* iron transport.[44]

Under aerobic conditions of iron deficiency, the ferric enterobactin $[Fe(ent)^{3-}]$ siderophore complex has a highly stable role in mediating iron uptake by *Escherichia coli*. Following the uptake of $^{57}Fe(ent)$ by the cells, the destiny of the iron was monitored using Mössbauer spectroscopy.[45] Mössbauer spectroscopic studies on entire cells of *Pseudomonas aeruginosa* cultured under various conditions show that the main type of iron in the cells varies dramatically. These distinctions are viewed as changes in the nature of the iron cores of the bacterial ferritin, which emerge from differing growing conditions.[46] The technique was utilized to investigate the binding and transformation of $^{57}Co(II)$ traces in living and dead (hydrothermally treated) cells of the rhizobacterium *Azospirillum brasilense* (strain Sp7) at 80 K in frozen aqueous suspensions and dried residues.[47,48] Indigenous iron-oxidizing bacteria isolated from Baiyin copper mine stope, China, were studied using a modified selective 9KFe medium.[49] Mössbauer spectroscopy was used to study the Mo-Fe protein and the Fe protein that together constitute the nitrogenase of *Klebsiella pneumonia*.[50] The bio-oxidation of Fe(II) in reduced nontronite coupled with nitrate reduction by *Pseudogulbenkiania sp.*

strain 2002 was studied using Mössbauer spectroscopy.[51] Using the Mössbauer spectroscopic $^{57}$Co emission, the interaction of cobalt(II) at micromolar concentrations with living cells of the plant-growth-promoting rhizobacterium *Azospirillum brasilense* (strain Sp245) and further transformations of the metal cation were monitored.[52] The disassembly of iron-sulfur clusters in *Escherichia coli* FNR protein by O: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity was analyzed.[53] Additionally, investigations have been conducted on the ribonucleotide reductase of *Escherichia coli*, which consists of two distinct subunits, namely Protein Bl and Protein B2. The results indicate that Protein B2 contains a binuclear complex with two non-identical high spin Fe(III) ions. This complex exhibits antiferromagnetic coupling and shares resemblances with both oxyhemerythrin and methydroxohemerythrin.[54] Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) from *Pseudomonas aeruginosa* was studied using Mössbauer spectroscopy.[55] The acquisition of iron by *Pseudomonas aeruginosa* cells after incubation with ferripyoverdine for 20, 40, 60, 120, or 360 minutes was also studied.[56] The 88-kDa corrinoid protein from *Clostridium thermoaceticum*, which serves as a methyl carrier in the production of acetyl-coA was also investigated using this technique.[57] To study metabolic iron accumulation, Mössbauer spectroscopic analyses of dry biomass samples of the cyanobacterium *Arthrospira platensis* (formerly known as *Spirulina platensis*) was conducted.[58] The β-monomers of *Escherichia coli* NADPH-sulfite reductase have been studied. The Mössbauer spectroscopic data revealed that the siroheme and $Fe_4S_4$ cluster are tightly exchange-coupled.[59] After purification of a Strep-tagged nonactivated benzylsuccinate synthase from recombinant *Escherichia coli*, Mössbauer spectroscopy revealed the presence of FeS clusters as additional cofactors.[60] The method is sensitive for detecting metal binding and transformation in live bacterial cells. A comparison of Mössbauer parameters between live and dead bacterial cells shows that the primary fast adsorption of cobalt(II) by living cells is chemically similar to its interaction with dead bacteria (hydrothermally destroyed).[47] Mössbauer spectroscopy was used as a tool for the study of activation/inactivation of the transcription regulator fumarate nitrate reduction in whole cells of *Escherichia coli*.[61] Mössbauer spectroscopy was also found to be suitable to analyze the MoFe-protein of *Azotobacter vinelandii*, isolated under dithionite and taken at temperatures ranging from 125 K to 175 K.[62] The two-iron ferredoxins found in spinach, parsley, *Azotobacter vinelandii*, *Closlridium pasteurianum*, and pig adrenal cortex were investigated.[63] Mössbauer spectroscopy was used to investigate Fe-enriched ribonucleotide reductase subunit B2 from *Escherichia coli* strain N6405/pSPS2 in both its native diferric state and in a new diferrous form.[64] The technique was also utilized to study ferritin cores in human spleen, limpet (*Patella vulgata*) haemolymph, and bacterial (*Pseudomonasaeruginosa*) cells. It was found that the typical superparamagnetic blocking temperature of limpet ferritin cores is approximately 25 K, whereas that of human ferritin cores is approximately 40 K.[65] The method was used with other techniques to evaluate surface-modified magnetic nanoparticles as a platform for colon cancer cell theranostics.[66]

Before leaving the Mössbauer spectroscopic studies on bacteria it is important to note that in the last three decades such studies on aerobic bacteria including *porphyromonas gingivalis*[67-73] and other oral anaerobes[74,75] found evidence for the presence of iron-protoporphyrinIX (Fe(PPIX)) species on the surface of the bacteria; these Fe(PPIX) moieties were identified as being collected and stacked on the bacterial surfaces. It was later established that a new group of enzymes (hemophores) haem transport proteins (used by bacteria and fungi to scavenge and transport haems from mainly animal sources) were the molecules responsible for the scavenging and possibly stacking of the Fe(PPIX) species on the surfaces of the bacteria. Indeed, it was these Mössbauer spectroscopic studies that provided the evidence for the existence of the hemophores and for their characterization.[76]

## 5. Studies Involving Proteins

Mössbauer spectroscopy has made substantial contributions to the study of iron-containing proteins. In natural enzymes iron(II) protoporphyrin IX, [Fe(PPIX)] also known as haem b, has been found to be widespread in nature.[77–81] [Fe(PPIX)] and related haems (other iron porphyrin macrocycles), for example haem c and haem a,[81] have been shown to be the active centers in a wide range of biological molecules, each crucial for living organisms. The haem groups are utilized to perform a diversity of roles including oxygen transport (haemoglobin) and storage (myoglobin), electron transport (the cytochromes) and in the elimination of toxic and unwanted compounds (cytochrome $P_{450}$).[77–81] This is in addition to their role in the group of enzymes known as the (hemophores) haem transport proteins (used by bacteria and fungi to scavenge and transport haems from mainly animal sources) mentioned in the previous section of this review. Early uses of Mössbauer spectroscopy resulted in thorough electronic characterizations of hemeproteins, which improved our understanding of their chemical characteristics.[82,83] There are various advantages that can be used in the study of heme proteins. In contrast to electron paramagnetic resonance (EPR), Mössbauer spectroscopy is applicable regardless of the iron atom's charge or spin state.[71–84] Fe-enriched horse hemoglobin and sperm whale myoglobin were investigated at temperatures ranging from 80 K to 260 K.[85] The technique was used to analyze Red Blood Cells (RBC) samples from healthy volunteers and patients with β-thalassaemia major and β-thalassaemia intermedia. The spectra from thalassaemia patients' RBCs revealed large quantities of ferritin-like iron, particularly in 82% of β-thalassaemia intermedia cases.[86] It was also used to investigate the temperature dependency of the mean square displacement of the iron atom in reduced and oxidized cytochrome c. Protein flexibility labeled by the modes coupling to the iron, is diminished upon reduction.[87] Human normal, adult, and fetal oxyhemoglobins as well as those from leukemic patients were analyzed. Quadrupole splitting and isomer shift estimates enabled differentiation of leukemic, fetal, and adult oxyhemoglobins. Analysis was done on the variations between the active site molecular structure and $Fe^{2+}$ electronic structure of leukemic and fetal oxyhemoglobins.[88] Mössbauer spectroscopic studies have shown the presence of two major inequivalent heme species in the reduced and oxidized states of the tryptophan 2,3-dioxygenase enzyme.[89] The Mössbauer spectroscopy spectra of Fe in human and rabbit transferrin were obtained under different magnetic fields and temperatures. The measured spectra in an applied field were interpreted using a high spin ferric spin Hamiltonian in a rhombic setting.[90] High velocity resolution Mössbauer spectroscopy was utilized to investigate small variations in $^{57}$Fe hyperfine parameters of iron-containing proteins from human and rabbit oxyhemoglobins.[91] Mössbauer spectra of 12 normal human spleen and 12 normal human liver tissues (post mortem) from Australia and Thailand were measured at 78 K. The spectra reveal the presence of iron in the form of ferrihydrite, as well as some deoxyhemoglobin and methemoglobin in certain samples.[92] The iron cores in various human and animal hemosiderin's were also studied using Mössbauer spectroscopy.[93] The high velocity resolution version of Mössbauer spectroscopy was used to study normal oxyhemoglobins in frozen red blood cell solutions of humans, rabbits, and pigs. The observed variances in Mössbauer hyperfine parameters were attributed to structural differences in human, rabbit, and pig oxyhemoglobins, as well as changes in binding affinities across these proteins.[94] A comparison between the oxyhemoglobins of pigs, rabbits, normal humans, and patients with blood system malignancies was

conducted.[95] At different temperatures, zero-field Mössbauer spectra of a hemoglobin model molecule that can undergo reversible oxygenation were recorded.[96] Iron deposits in patients with abnormal iron metabolism was examined using Mössbauer spectroscopy.[97] Mössbauer spectroscopic investigations were undertaken on human recombinant ferritins and horse spleen, in order to determine the path iron takes from the time it is absorbed by the protein from an $Fe_2SO_4$ solution to the building of the iron core of ferritin.[98] The technique has been found to be a powerful investigatory probe into spin relaxation processes of nano size magnetic systems of ferritin core constitutes.[99] Mössbauer spectra of ferritin from three different tissues, pancreas, liver and spleen,[100–105] have revealed interesting results. It was revealed that ferritin contains iron cores based on the ferrihydrite ($5Fe_2O_3.9H_2O$) structure.[100] Ferritin and hemosiderin separated from iron-overloaded human spleens were studied at temperatures ranging from 1.3 to 200 K.[101] Additionally, ferrum lek, an iron-polymaltose complex that is believed to be an analog of ferritin and is utilized as an antianemia medication, and human liver ferritin were studied.[102,103] A high velocity resolution was used to compare iron cores in human liver ferritin, its pharmaceutically significant models Imferon, Maltofer®, and Ferrum Lek, as well as iron storage proteins in chicken liver and spleen tissues.[104] Human liver ferritin and spleen tissues from healthy individuals and patients with primary myelofibrosis were also analyzed.[105]

Mössbauer spectroscopy was used to study the active-site models of [Fe] hydrogenase, yielding structural information about the species present.[106]

## 6. Pharmaceutical Studies

Mössbauer spectroscopy has long been used to investigate medicines including both organic and inorganic iron-based chemicals. The advantages the technique offers includes:- the identification of the type of metal compound present, the ability to detect impurities, and also yield information on the coordination polyhedron, valence, and spin states of iron and other isotopes in these materials.[107] Using Mössbauer spectroscopy, the iron content of several commercial vitamin and nutritional supplement products including ferrous fumarate, ferrous bisglycinate chelate (Ferrochel), and ferrous sulfate were examined. Numerous ferrous and ferric contaminants were discovered.[108] Mössbauer spectroscopy has been used to investigate the iron state in selected outdated drugs. Drugs containing ferrous sulfate and ferrous fumarate exhibit the appearance of a minor content of ferric compounds and some instability of ferrous sulfate and fumarate with the formation of ferrous compounds. Drugs containing iron chelates, on the other hand, exhibit considerable iron chelate instability, with the formation of ferric compounds even in fresh samples, and their amount increases with age.[109] Several commonly available drugs containing ferrous fumarate ($FeC_4H_2O_4$) and ferrous sulfate ($FeSO_4$) as sources of ferrous iron were investigated utilizing high velocity resolution. Minor variances were found in the primary components of both medications, indicating discrepancies in ferrous fumarates and ferrous sulfates.[110] A comparative investigation of ferrous gluconate, as well as fresh and expired Ascofer® tablets, was carried out using the technique with a high velocity resolution. The results showed that all investigated samples including three ferrous and one ferric component, manifested impurities which might be attributed to ferrous gluconate molecules and ferric contamination, and/or aging effects.[111] Mössbauer spectroscopy was used to investigate the state of several industrial samples of vitamins and nutritional supplements containing iron ions that are used to treat anemia. The results showed that the determination of the iron status ($Fe^{2+}$ or $Fe^{3+}$) in pharmaceuticals is critical for evaluating their quality.[112] The iron status of iron-containing vitamins and dietary supplements was investigated with high velocity resolution.[113] The effect of $NO^-$ and $PO^{3-}$ on ciprofloxacin adsorption on humic acid/ferrihydrite composite, synthetic ferrihydrite, and humic acid was evaluated at controlled pH 7, ionic strength (0.1 M), and temperature (25 °C). The study characterized both the composite and iron oxide.[114] The technique was used to evaluate the cytotoxicity of the curcumin-Fe(III) complex, which was produced by refluxing a slightly basic methanolic solution of curcumin and $Fe(NO_3)_3{\cdot}9H_2O$ precursor.[115] The iron state in commercial pharmaceutical products that contained ferrous fumarate ($FeC_4H_2O_4$), ferrous sulfate ($FeSO_4$), ferrous bisglycinate chelate (Ferrochel®), and ferrous iron (hydrolyzed protein chelate) were analyzed.[116] Mössbauer spectroscopy and other tools were used to study the iron(III)-polymaltose pharmaceutical ferritin analogue Ferrifol® to get novel information about the structural arrangement of the iron core.[117] The iron content and oxidation state ($Fe^{2+}$ or $Fe^{3+}$) in commercial drugs containing ferrous fumarate $FeC_4H_2O_4$ were determined.[118] The iron state of commercial drugs comprising ferric and ferrous iron compounds that are used to treat iron insufficiency was examined. Small differences in the FeOOH cores of injectable iron-dextran complexes were discovered.[119] The effect of iron concentration on the oxidation state and iron microenvironments in iron-polygalacturonate compounds produced from pectin was investigated.[120] Mössbauer spectroscopy was used to compare horse spleen ferritin to pharmaceutically significant model complexes of hydrous ferric oxide produced from $FeCl_3$ and dextran (Imferon) or chondroitin sulfate (Blutal).[121] The hyperfine interactions in the iron cores of pharmaceutically relevant industrial and elaborated iron-dextran complexes (ferritin models), as well as human ferritin were reported.[122] The technique was used to evaluate four distinct iron compounds used in oral, intravenous, and intramuscular therapy.[123] Mössbauer spectra for human liver ferritin and its pharmacological analogs Ferrum Lek and Maltofer® were measured at temperatures ranging from 295 to 83 K.[124] Mössbauer spectroscopy was used to investigate an antianemia drug called Aronamin C Plus, which contains simple iron-bearing components.[125] Mössbauer spectroscopy was also used to analyze antianemia medication Ascofer® and its main iron component, ferrous gluconate. Room temperature spectra revealed the presence of two iron phases: ferrous ($Fe^{2+}$) (about 85±5%) and ferric ($Fe^{3+}$) (15±5%).[126] The physiological behavior of iron-pectin beads using ionic gelation was investigated to assess their potential use in the food industry.[127] Mössbauer spectroscopy has been used to examine two fresh commercial pharmaceutical drugs, Tardyferon® and Fenules®, that contain ferrous sulfate. The result showed some variations in the iron local microenvironments.[128] The oral hematinic marketed as Niferex, whose active component is a polysaccharide-iron complex was recharacterized using Mössbauer spectroscopy.[129] The antianemia drug ferrous gluconate, ferrous fumarate, and a Dynabi tablet containing a basic iron-bearing component were studied.[130] The injectable iron-dextran complex used as a hematinic was also investigated.[131] The technique was used to examine iron containing biomolecules, pharmaceutical products, meteorite samples and nanoparticles using high velocity resolution.[19] The physicochemical properties of iron nanoparticle drug products, specifically brand and generic sodium ferric gluconate were evaluated using Mössbauer spectroscopy.[118–132] It has also been used to quantify the iron content of numerous powdered drugs.[119–133] Iron Maltol (*mer*-tris (3-hydroxy-2-methyl-3-hydroxypyran-4-onato) iron(III), a potential iron pharmaceutical composition for the treatment of iron deficiency anemia) was investigated by Mössbauer spectroscopy and X-ray diffraction.[7] Mössbauer spectroscopic studies have been reported on haem-antimalarial complexes of pharmacological interest.[134,135]

## 7. Other Studies Related to Iron Oxides Nanoparticles

The common limpet *Patella vulgata*'s radula teeth were examined for iron oxide biomineralization using Mössbauer spectroscopy.[136] The technique was used in studies on the synthesis of magnetite nanoparticles and their functionalization with glycine (MNPGly), β-alanine (MNPAla), L-phenylalanine (MNPPhAla), and D-(-)-α-phenylglycine (MNPPhGly) amino acids.[137] Additionally, the technique was employed to study low toxic maghemite nanoparticles for theragnostic applications.[138]

## 8. Studies on Small Molecules Used to Model the Iron Active Centers of Iron Containing Proteins and other Small Molecules Found in Living Organisms

All the Mössbauer papers on both natural and synthetic heme complexes are really beyond the scope of this work. However, it is worth pointing out to the reader that such studies have been very useful in providing insights into how the heme proteins discussed above carry out their roles.

Similarly, Mössbauer spectroscopic studies on model compounds aimed at furthering the understanding of the *modus operandi* of for example, iron siderophores have been carried out.

In view of the possibility the reader may want to know more about the Mössbauer spectra of model compounds for the heme proteins we are providing 9 references so that they can follow up the field for themselves as these references contain many references to other work in the field.[139–147] Similarly, we are providing follow-up references for iron siderofores models.[148–154]

## 9. Other Studies

It is also worth noting that Mössbauer spectroscopy has been used to develop materials used in medical diagnostics equipment that image the body. Again, this is off the main theme of this work, but an example is given here for reference. $Eu^{2+}$-doped fluorochlorozirconate glasses and glass ceramics for medicinal and photovoltaic applications were analyzed.[155] Mössbauer spectroscopy was used in the optimization of storage phosphors for radiography.

## 10. Conclusions

The study of physiological and pathological processes in living systems requires the application of physical techniques that can yield molecular-level information. In this review, we have shown that Mössbauer spectroscopy has contributed to the advancement of biological and medical research. The technique has proved to be successful in providing useful information on proteins and enzymes that have iron active sites. It has also long been used to study drugs that contain both organic and inorganic iron-based compounds and some of this work has been reviewed herein. Mössbauer spectroscopy has been used to assist in identifying the type of metal compound, detecting impurities, and determining the coordination polyhedron, valence, and spin states of iron and other isotopes in these biological materials. In addition, it has been used to study model compounds of many of the iron proteins allowing further insight into both the model compounds and the proteins.

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