EPR spectroscopy of free radicals and proteins: Effect of solvents on EPR spectra.

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Summary:

Abstract

Electron Paramagnetic resonance or electron spin resonance is a versatile and analytical technique that allows us to study different types of samples: inorganic, biological, free radicals, etc., in different states (solid, frozen solution,etc.), at different temperatures and gives us information about each sample such as the identification of the metal, the effect of solvents on biological samples and the presence of hyperfine structure to characterize paramagnetic centers in bioinorganic, organic, and inorganic materials and molecules. These measurements are enabled with the help of paramagnetic species, such as organic free radicals and ions, electronically excited states, and metal-ligand compounds, some of which with biological importance. The EPR spectral analysis of DPPH (at room temperature), dry myoglobin, myoglobin in H_2O & glycerol (at 77K), and $MnCl_2$ / H_2O were recorded at Professor Doros Petasis's Lab at Allegheny College. The effects of solvents on the EPR absorption spectrum have been discussed and analyzed by comparing the spectrum of myoglobin in varying solvent.

Introduction

EPR mainly deals with the interaction of microwave radiation with spin magnetic moment of unpaired electrons in atoms- paramagnetic ions. It is an ideal technique for detecting the magnetic properties of individual atoms and the interactions between individual magnetic moments. EPR along with the magnetic susceptibility experiments can yield a wealth of information about the magnetic properties of samples. The experiments performed will be focused on analysing the EPR spectrum of the following samples:

DPPH is an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-coloured crystalline powder. Myoglobin is a metalloprotein composed of a polypeptide globin and a heme residue with iron ion. The present spin states of the iron ion in the heme structure greatly influences the EPR spectrum for myoglobin. Manganese(II) ions have a nuclear spin of I = 5/2 and their EPR spectrum shows hyperfine splitting.

The main purpose of the experiments are to identify features about the samples by analysing the spectrum and getting the g-values. The experiments would also be focused on identifying the reasons behind the variation in EPR results of myoglobin due to different solvents. There is limited research performed on analysing the effect of solvents on X-band EPR spectra of myoglobin and further analysis is needed. Furthermore, the EPR spectrum will be used to identify the: hyperfine splitting, the hyperfine constant, the effect of temperature on EPR spectrum, the electron spin relaxation time and the spin states of iron ion in myoglobin.

Background Information

EPR Theory and Zeeman Effect:

The EPR technique is based on the Zeeman effect, which is the interaction of an external magnetic field B with the magnetic moments μ of unpaired electrons. The electronic magnetic moment is due to the spin angular momentum S of the electron. The interaction of the external magnetic field and the magnetic moment of an electron is described by the Zeeman Hamiltonian (Abragam & Bleaney, 1970)

$$\mathcal{H}_Z = -\mu \cdot B = g\beta S \cdot B \quad (1)$$

where g is the spectroscopic g-factor, β is the Bohr magneton (9.2741024 J/T). The magnetic field B defines an axis of quantization (typically the z-axis) with S_z the projection of S onto B that allows the dot product to be expressed as

$$S \cdot B = S_z B = m_s B \quad (2)$$

Where m_s is the spin magnetic quantum number which has the values $m_s = \pm 1/2$. This leads to electronic energy states given by $\pm 1/2\, g\beta B$ that produces a linear splitting of the degenerate m_s spin energy levels as a function of magnetic field as shown in Fig.1A. An electron on the lower energy level (spin down) can absorb microwave radiation and make a transition to the upper state (spin up) giving rise to an EPR signal. An electronic transition is possible only when the resonance condition is satisfied:

$$h\nu = \delta E = g\beta B \quad (3)$$

Where h is Planck's constant (6.626×10^{34}Js) and ν is the frequency of the electromagnetic radiation. The frequencies of the electromagnetic radiation are in the GHz region (microwave radiation) with magnetic fields between 0 and 1 T. In a typical EPR experiment, a microwave source produces radiation at a constant frequency, while the magnetic field is swept through the desired range. Microwave energy is absorbed when the magnetic field goes through a value that satisfies the resonance condition. This absorption is called an "EPR resonance line" and appears as a Gaussian or Lorentzian curve in the microwave power spectrum (Fig. 2B). EPR spectrometers employ modulation of the magnetic field with phase-sensitive detection to significantly increase the signal-to-noise ratio, which results in the first derivative of this line as shown in Fig.1C. The area under the curve in Fig. 1B is proportional to the number of spins that contribute to the EPR signal. Integration of this line allows determination of the species concentration of EPR samples. The resonance condition can be written in a form that makes it easy to convert magnetic fields into g-values. With the frequency in GHz and the magnetic field in kG, the constants h and β can be combined into one numerical constant that allows the resonance condition to be expressed in the form (D.T.Petasis & M.P.Hendrich, 2015):

$$g = 0.71449\, \frac{\nu\ (\mathrm{GHz})}{B\ (\mathrm{kG})} \quad (4)$$

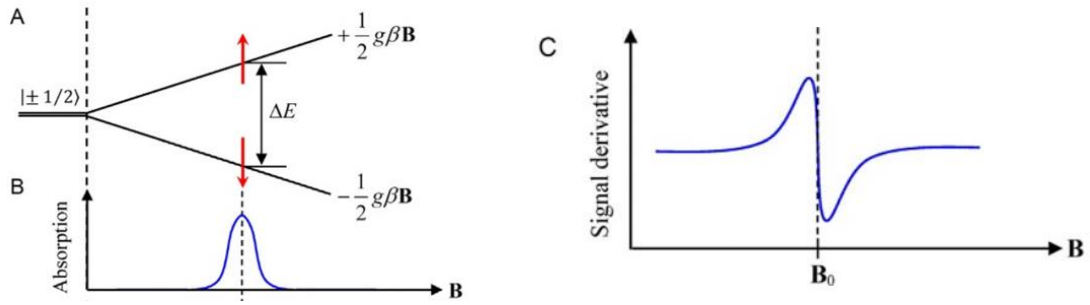

Figure .1. (A) Zeeman splitting of the degenerate electronic spin states for an S= $1/2$ system. (B) An electron can absorb energy to make a transition to the excited state resulting in an EPR absorption line. (C) Typical experimental EPR resonance line. The point where the line crosses the baseline determines the resonance magnetic field, B_0, which allows the determination of the g-factor of the system. (D.T.Petasis & M. P.Hendrich, 2015).

Hyperfine Structure:

Many transition series ions have nuclei with spin that interact with unpaired spins and produce splitting in the EPR spectra. These electron-nuclear interactions give rise to hyperfine structure. A nucleus with spin I interacting with an electron with spin S produces splitting's in the m_s electronic levels. The multiplicity of these levels is given by(2I+1) and each level is labelled by the quantum number m_I as shown in Figure 2. Transitions are now allowed between the m_I levels obeying the selection rules: $\Delta m_s = \pm 1$ and $\Delta m_i = 0$. These transitions give rise to (2I+1) equally spaced EPR lines of equal intensity. The magnetic field separation of the hyperfine lines is called the hyperfine constant A and it is characteristic of the particular ion. Typical transition series ions with nuclear spin are $Cu^{2+}(I = 3/2), Mn^{2+}(I = 5/2)$, and $Co^{2+}(I = 7/2)$. (D. T. Petasis & M. P. Hendrich, 2015)

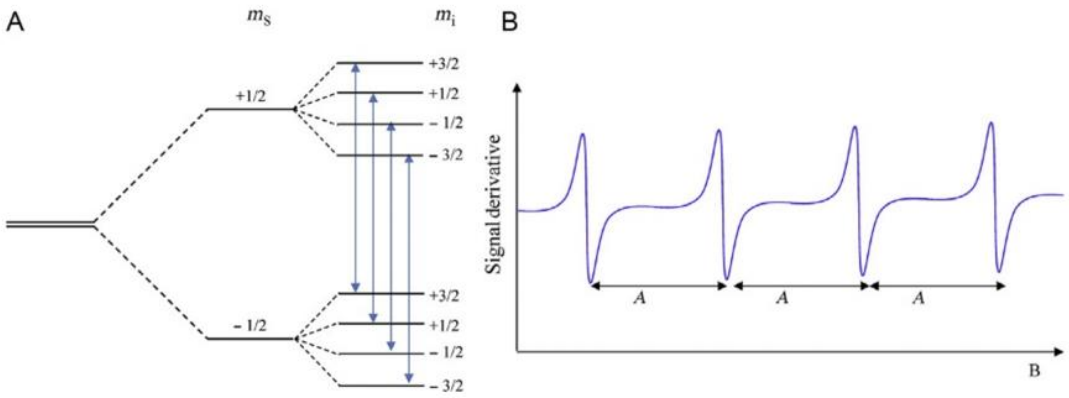

Figure 2 (A) Hyperfine splitting's in a system with S = $^1/_2$ and I = $^3/_2$. Allowed transitions between the nuclear levels obey the selection rule $\Delta m_s = \pm 1$ and $\Delta m_i = 0$ and are indicated by the arrows. (B) Ideal EPR hyperfine spectrum for a species with I = $^3/_2$. The g-factor is determined from the field position of the centre of the spectrum. (D. T. Petasis and M. P. Hendrich, 2015)

Experimental Procedures

The EPR measurements were carried out with a Varian E-3 X-band spectrometer that has a 100kHz field modulation and a nitrogen cryostat that allows the spectrometer to conduct experiments at temperatures as low as 100K. The spectrometer operates at frequencies ranging from 8.5-12GHz. Before starting with the experiments the start-up procedures for the instrument were carried out: water coolant hose was opened, intensity of the oscilloscope was adjusted and finally the frequency channel was set to channel three for accurate readings before putting in the sample. The temperature controller was setup before taking EPR measurements of samples at low-temperatures. Multiple EPR test runs were done before setting the conditions for the EPR run of each sample.

For DPPH a small amount of vacuum grease was placed, on the inside of a clean EPR tube at the location correlating to the cavity region, and the sample of DPPH was placed on the grease spot. After inserting the sample into the cavity, the oscilloscope was read and the dip was placed a little to the right from the centre. The central field was set to 3.3kG and the sweep range was set at ±500 G. The microwave power = 1.25mW, modulation field = 20G, scan time was 4 min and gain = 5×10^2. The microwave frequency was recorded as 9.139 GHz for DPPH. The EPR spectroscopy was carried out at room temperature (approximately 298K).

The dry myoglobin was placed in a vacuum EPR tube, in a powdered state. The settings of the Varian E-3 X-band were changed to the following to obtain a satisfactory spectrum. The microwave power was set to 5mW and the centre field to 3kG with a sweep range of ±2500G. The modulation field, sweep time and modulation frequency were kept at 20G, 4min and 100kHz respectively. The Microwave frequency, υ, was read to be 9.146 GHz. The EPR spectroscopy was carried out at room temperature (approximately 298K).

The EPR tube was filled with myoglobin suspended in water and the tube was placed in a dewar flask filled with liquid Nitrogen to freeze the sample. The purpose of this was to reduce the kinetic energy of the molecules, slowing them down, so to get a better EPR spectrum without a lot of interference. The temperatures were lowered carefully to ensure the glass tube doesn't crack due to expansion of the water, during the flash freezing process. The sample was left in the Dewar flask and before using the sample the condensed oxygen was wiped off with a kimwipe. The cavity was prepared by first running nitrogen gas through, at a pressure of about 25 to 30 psi (Varian E-3 X-Band Manual), to remove any oxygen present, this is necessary as oxygen is highly paramagnetic and will affect the EPR results. The dewar was then slowly filled with liquid nitrogen to cool down the nitrogen gas flowing into the cavity. The process was carried out slowly so that the parts got time to cool down and there were no sudden drops in the temperature that could damage the equipment. The cavity was cooled down from an ambient temperature of 297.95K to 117K. The microwave power was set to 25mW and the centre field to 3kG with a sweep range of ±2500G. The modulation field, sweep time and modulation frequency were kept at 40G, 2 min and 100 kHz respectively. The Microwave frequency, υ, was recorded as 9.120 GHz.

A sample of frozen myoglobin suspended in glycerol was placed in the cryostat and the conditions of the Varian E-3 X-band were varied to the following. The microwave power was set to 25mW and the centre field to 3kG with a sweep range of ±2500G. The modulation field, sweep time and modulation frequency were kept at 40G, 2min and 100kHz respectively. The Microwave frequency, υ, was read to be 9.122 GHz. The EPR spectrum was recorded at a temperature of 110K.

The manganese(II)chloride solution was carefully flash-frozen using liquid nitrogen in a dewar and it was, later, placed in the cavity. The Microwave frequency was recorded as 9.144 GHz. The settings of the Varian E-3 X-band were tweaked after running a few test runs in order to get a satisfactory EPR spectrum for the sample. The final settings were as follows: the microwave power was set to 5mW and the centre field to 3kG with a sweep range of ±2500G. The modulation field, sweep time and modulation frequency were kept at 40G, 2min and 100kHz respectively. The EPR spectrum was recorded at a temperature of 104K: the lowest temperature the instrument reached during experimentation.

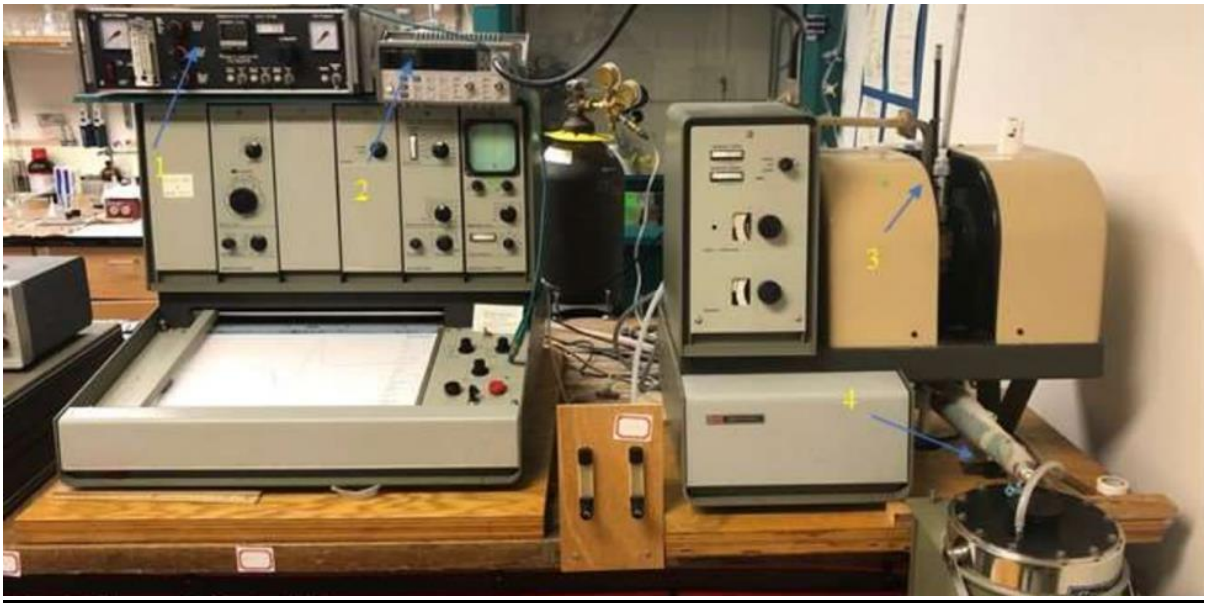

Figure 3. Instrument Setup Varian E-3 X-band Spectrometer. D.Petasis Lab, Allegheny College. 1- Temperature controller, allows us to monitor the temperature and control Nitrogen flow during low temperature run. 2- Universal counter: displays the frequency, υ, throughout the run. 3- Cavity: where samples are placed. 4- Dewar: stores liquid nitrogen that is supplied to the cavity for cooling it down during low temperature EPR runs. (D. T. Petasis and M. P. Hendrich, 2015)

Results and Discussion

The EPR spectrum obtained for DPPH suggests it is a free radical with a g factor equal to 2.017 at 298K (Figure 4) . The signal at 3236.8G depicts a very narrow linewidth, i.e. a small ΔB value, a result of the very highly concentrated dried polycrystalline used. The g value for a free radical is calculated to be 2.003; the value obtained from the EPR spectrum is closely in agreement to this, with a percentage difference of only 0.7%. The EPR line in Figure 4 shows an isotropic spectrum, suggesting the symmetry produced by all the other atoms in the lattice at the location of the paramagnetic ion belongs to the cubic system.

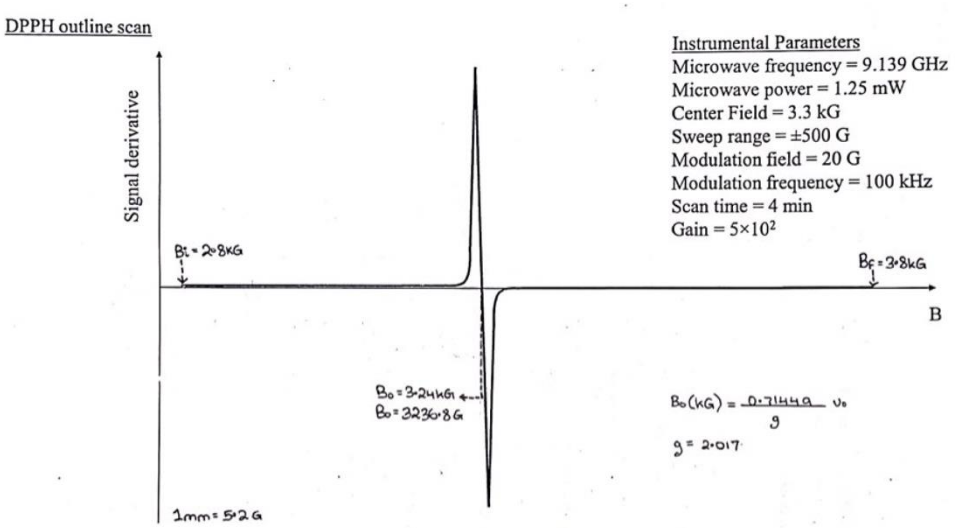

Figure 4. EPR absorption spectrum of DPPH. X-band EPR spectrum of DPPH recorded at 298K. The Central field =3kG, $B_i = 2.8$ kG and $B_f = 3.8$ kG. Here, $B_0 = 3236.8$ G. $h \times \beta_B = 0.71449$ and g factor $= 2.017$. The spectrum has a scale of 1mm = 5.2G. (Refer to **Experiment Procedure** for the detailed conditions of the EPR measurement.)

The EPR spectrum obtained for dry-powdered myoglobin suggests that it has a g factor of 1.941 at $B_0 = 3366.72$G (298K) (Figure 5) . The signal at $B_1 = 2951.8$G depicts a moderate linewidth due to one of the many factors: spin-lattice relaxation, spin-spin relaxation or unresolved hyperfine structures. From the EPR signatures we can analyse that the Iron in the heme has a low-spin ($S = 1/2$), $g_x = 2.69, g_y = 2.2138, g_z = 1.83$. Suggesting it has an axial spectrum (hexagonal symmetry) ,that is, one of the axis of the molecule is unique (usually the z-axis) and the other two orientations are equivalent.

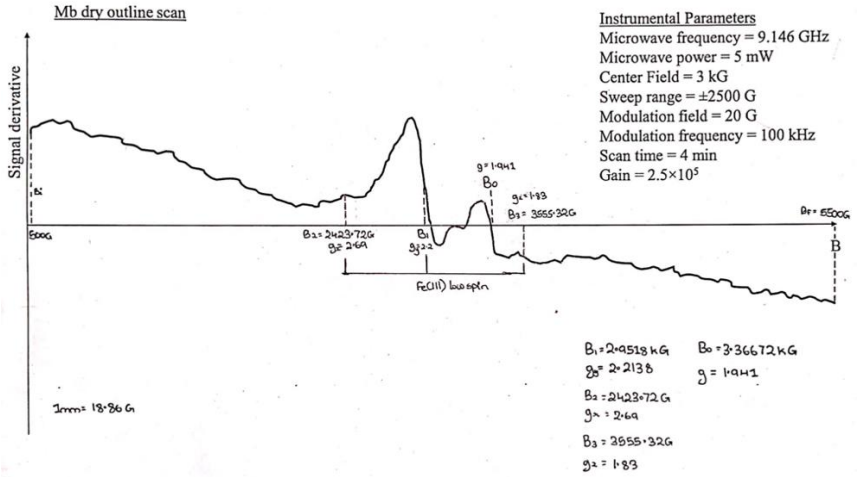

Figure 5. EPR absorption spectrum of Dry myoglobin. The scale is 1mm = 18.86 G. $B_1 = 2951.8$ G, $B_2 = 2423.72$ G and $B_3 = 3555.32$ G. The g values for the spectra are $g_x = 2.69, g_y = 2.2138, g_z = 1.83$, suggesting the Iron in heme has a low spin state.

The EPR spectrum obtained for myoglobin in water suggests that it has a g-factor = 2.0078 at $B_0 = 3245.36$G (117K) (Figure 6). Comparing the g factors with the EPR signatures we see that the Fe(III) ion has a low spin(S = 1/2): $g_x = 2.74, g_y = 2.5, g_z = 1.855$, and a high spin (S = 5/2): $g_{\parallel} = 2.0078, g_{\perp} = 6.03$, and an axial spectrum, meaning that one of the axis of the molecule is unique (usually the z-axis) and the other two orientations are equivalent. At $B_4 = 1593.68$ we get a junk Fe(III) reading with g = 4. The EPR line is further analysed to help draw conclusions on how a solvent affects the EPR absorption spectrum(look at conclusion).

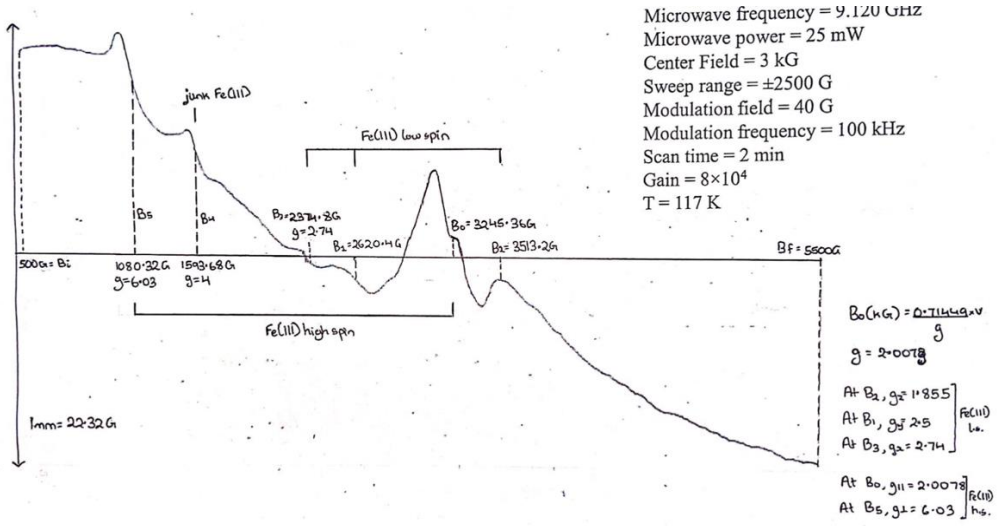

Figure 6. EPR absorption spectrum for myoglobin in water. The X-band EPR spectrum of frozen myoglobin in water recorded at 117K. The Central field = 3kG, $B_i = 0.5$kG and $B_f = 5.5$ kG. Here,$B_0 = 3245.36$ G, $B_1 = 2620.4$ G, $B_2 = 3513.2$ G, $B_3 = 2374.8$ G, $B_4 = 1593.68$G and $B_5 = 1080.32$. The spectrum has a scale of 1mm = 22.32G.

The EPR spectrum obtained for myoglobin in glycerol suggests that it is a free radical with g-factor=2.0099 at $B_0 = 3242.6$G (110K) (Figure 7). The signal at B_0 has a broad linewidth, $\Delta B = 1217.4$ G. The myoglobin suspended in glycerol causes the EPR line to change drastically as we see the noise disappears and we get one significant signal.

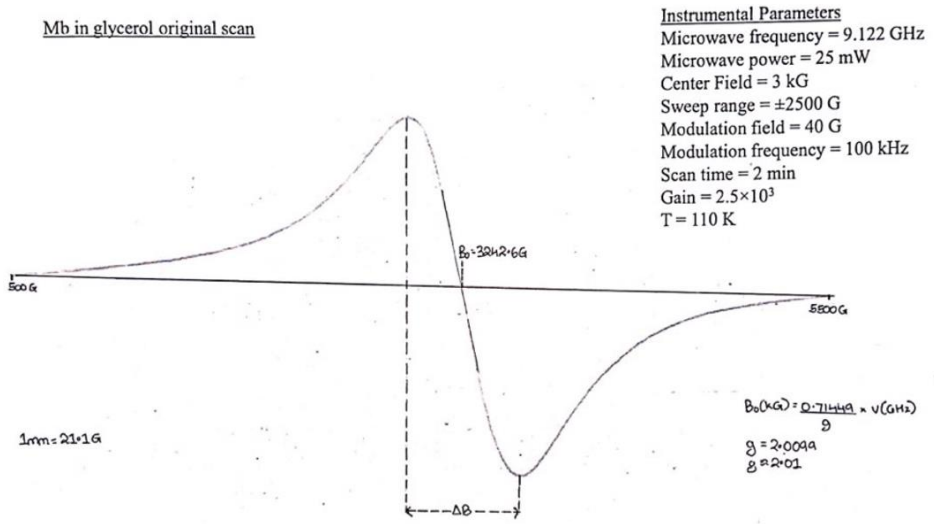

Figure 7. EPR absorption spectrum for myoglobin in glycerol. The X-band EPR spectrum of frozen myoglobin in glycerol recorded at 110K. The Central field = 3kG, $B_i = 0.5$ kG and $B_f = 5.5$ kG. Here, $B_0 = 3242.6$ G and $\Delta B = 1217.4$ G. The spectrum has a scale of 1mm = 21.1G. The g-factor value is $g = 2.0099$.

The EPR spectrum of frozen $MnCl_2/H_2O$ (Figure 8) appears as six hyperfine lines due to coupling of the S = 1/2 electron spin with the I = 5/2 nuclear spin of the Mn^{2+}ion. The hyperfine coupling constant *A* is taken to be 109.65G (5mm x 21.93G). The g-factor at B_0=2715G is 2.4; the intensities of all the signals are approximately equal and the variations in the g-factor, hyperfine coupling constant(*A*), and intensities may be caused due to the solvent used while carrying out the experiments. Furthermore, the electron spin relaxation time of the Mn(II), as reflected in the widths of the EPR lines, is a sensitive indicator of molecular motion in the vicinity of the ion. The collisions with the solvent affect this parameter.

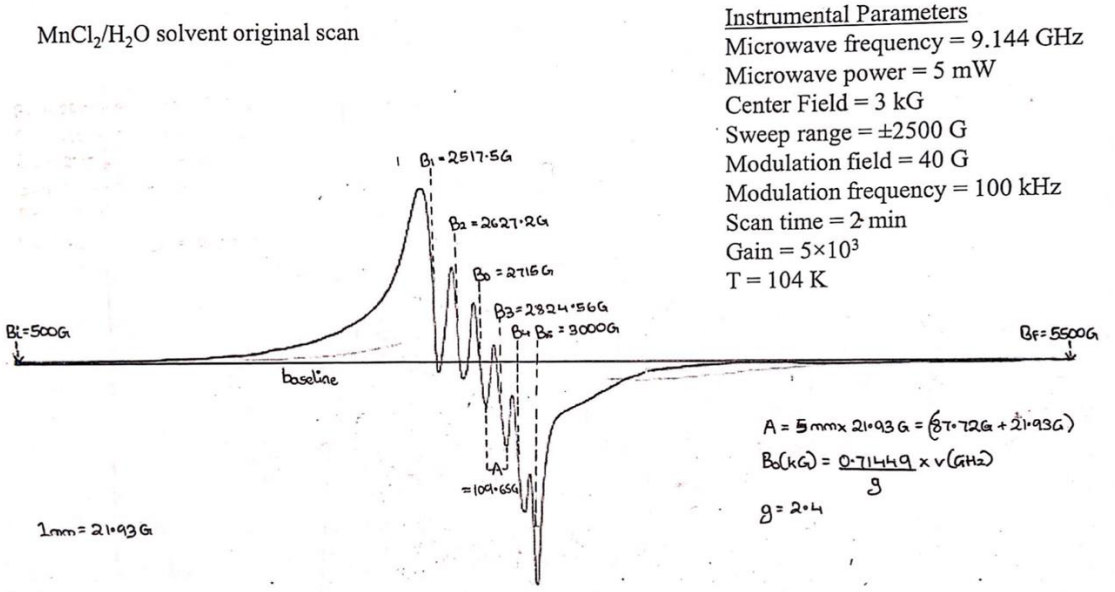

Figure 8. EPR absorption spectrum for $MnCl_2$ in water. The X-band EPR spectrum of frozen $MnCl_2$ in water recorded at 104K. The Central field = 3kG, $B_i = 0.5$ kG and $B_f = 5.5$ kG. Here, $B_0 = 2715$ G, $B_1 = 2517.5$G, $B_2 = 2627.2$G, $B_3 = 2824.56$G, $B_4 = 2890.37$G,= and $B_5 = 3000$G. The spectrum has a scale of 1mm = 21.93G. The g-factor = 2.4.

Conclusion

The results determined for all 5 samples were in agreement with the existing theories and research in EPR. The EPR spectra conducted for myoglobin in water at low-temperature and the dry myoglobin at room-temperature showed severe temperature drifts. A source of error may arise from this as the baseline was taken to be horizontal, however, it is negligible. The use of glycerol as a solvent for myoglobin helps the EPR run as on freezing it has a glassy structure allowing for better readings. However the glycerol has an impact on the results as it diminishes the axial and rhombic signals. The feature $g \approx 6$ of the axial species gets broadened and shifts to lower magnetic fields. The glycerol also reduced but did not eliminate the low spin signal intensities. This explains the significant reduction in noise for the EPR spectrum of myoglobin in glycerol when compared to myoglobin in water or dry myoglobin. Furthermore, the polarity of solvents like water and glycerol may impact the EPR absorption spectra. The g-values may decrease and there might be an increase in the hyperfine coupling constant *A*(as seen in the EPR spectra of $MnCl_2$). Another impact of the solvents are on the linewidth,ΔB, of the single derivative and the most prominent affect is seen in myoglobin dissolved in glycerol where the linewidth is 1217.4 G. The g factors for all the samples were analysed using the EPR signatures. The g-factor is the ratio of the electron magnetic dipole moment to its angular momentum, for free radicals g has a constant value of 2.0023 and the results are in agreement with this value. The g factor also helps in identifying samples and a summary of the results are given(Table 1).

Sample	Spectrum Details
DPPH	g = 2.017
Dry Mb	l.s axial: $g_x = 2.69, g_y = 2.2138, g_z = 1.83$
Mb in water	h.s axial: $g_{\parallel} = 2.0078, g_{\perp} = 6.03$ and l.s : $g_x = 2.74, g_y = 2.5, g_z = 1.855$
Mb in glycerol	$g = 2.0099, \Delta B = 1217.4$ G
$MnCl_2$ in water.	h.s: 6 splittings spaced at, *A*=109.65 G, centred on g=2.4

Table 1. Summary of obtained EPR results. All spectroscopic measurements of samples were recorded on a Varian E-3 X-band EPR spectrometer equipped with liquid nitrogen cryostat. High spin and low spin have been abbreviated to h.s and l.s respectively. *A* represents the hyperfine coupling constant measured for Mn(II) ion.

References

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