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The power of electron paramagnetic resonance spectroscopy in pharmaceutical analysis

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Introduction

Electron Paramagnetic Resonance (EPR) spectroscopy, also know as Electron Spin Resonance (ESR), is arguably the most powerful technique for studying unpaired electrons. Since its discovery in 1944 it has been applied to study a wide range of organic free radicals and paramagnetic transition metal species. In the case of organic free radicals EPR may be used, in a manner analogous to Nuclear Magnetic Resonance (NMR), to identify an unknown radical but may also provide information on the structure, reactivity and environment surrounding such a radical.

The basic theory of EPR[1] is essentially similar to NMR, with some changes to allow for the larger magnetic moment of the electron compared to the proton. An unpaired electron may exist in one of two possible spin states (M_S=+½ or −½) and under normal circumstances these two spin states are degenerate. When placed in an external magnetic field, however, the degeneracy between the two states is lifted, producing Zeeman energy levels such that the M_S=−½ state is lower in energy than the M_S=+½ state. This may be thought of in the lower energy case as a bar magnet placed in a large magnetic field with its south pole pointing towards the north pole of the external magnetic field and *vice versa* in the higher energy case. The separation between these two states is proportional to the magnitude of the applied magnetic field, see Figure 1, and given by the expression

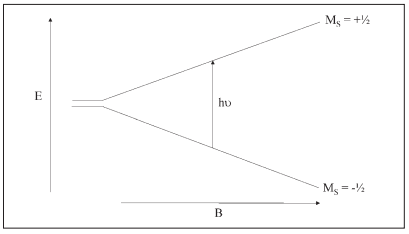

Figure 1. The Zeeman energy levels and the EPR experiment.

$$\Delta E = h\nu = g\mu_B B$$

where h is Planck's constant, ν is the frequency of the electromagnetic radiation, g is a dimensionless constant called the g factor (for an unpaired electron in free space g=2.0023), μ_B is the Bohr magneton and *B* is the applied magnetic field. The SI units of magnetic field strength, or more correctly magnetic flux density, are Tesla (T); however, for historical reasons the older units of Gauss (G) are still in common use (often quoted in the literature as 1 mT=10 G).

The above expression shows that if electromagnetic radiation of the correct energy is applied to the sample in a magnetic field then resonance transitions may be induced between the two energy levels. Hence for an unpaired electron in free space (so called *free spin*) a magnetic field of 0.35T would require radiation of a frequency of 9.8 GHz (the microwave X-band region of the electromagnetic spectrum) or a magnetic field of 1.25T would require radiation of a frequency of 35 GHz (the microwave Q-band region).

The g factor

The g factor in EPR behaves in a manner analogous to the chemical shift in NMR and gives information about the type of radical giving rise to the EPR spectrum. However, organic radicals have g values close to free spin (variations are typically in the third or fourth decimal place) and thus their accurate determination requires either careful measurement of the magnetic field and microwave frequency or a reliable standard. For the analysis of organic free radicals the hyperfine splittings are therefore more important and informative.

Hyperfine splittings

Just as a magnetic nucleus may interact with other magnetic nuclei in NMR to produce splittings in the spectrum, so too may the unpaired electron interact with magnetic nuclei to produce splittings in the EPR spectrum; such splittings are called *hyperfine splittings* (symbol *a*). As in NMR, the number of lines and their relative intensities caused by the hyperfine splitting may be determined by reference to Pascal's triangle. For example an unpaired electron coupled to a single proton will split the single EPR line into two lines of equal intensity, separated by the hyperfine splitting constant. Similarly in the methyl radical, see Figure 2, where the unpaired electron is coupled to three equivalent protons, the EPR spectrum is split into four lines (i.e. $2nI+1$=number of lines, where I=½ for a proton and n is the number of equivalent nuclei) of inten-

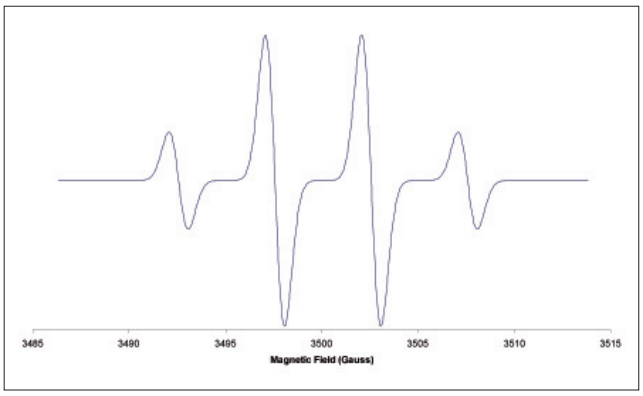

Figure 2. Simulated EPR spectrum of the methyl radical.

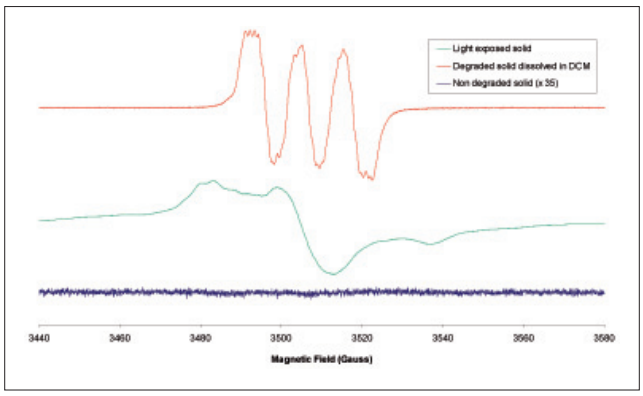

Figure 3. X-band EPR spectra of photo-degraded Nifedipine. Bottom, non-degraded solid ×35; middle, degraded solid dissolved in DCM; top, solid after exposure to light.

sity 1:3:3:1; again separation between the four lines of this quartet corresponds to the hyperfine splitting constant. More complex hyperfine patterns occur when multiple sets of inequivalent nuclei contribute to the spectrum.

Pharmaceutical analysis

Within pharmaceutical analysis EPR spectroscopy has a wide range of applications. Since it can be used to study and characterise organic free radical species, one area of application is in the understanding of radical driven degradation mechanisms, such as photo and oxidative degradation. This can include mechanistic or kinetic studies of the degradation of an active pharmaceutical ingredient (API) or formulation and quantitative measurements to determine the extent of degradation of a sample. Free radical formation or decay can also occur when an API is mixed with an excipient used in a formulation and therefore EPR can also be used to monitor these interactions. Terminal sterilisation, such as autoclaving or γ-irradiation, is often used to produce sterile pharmaceutical products and can result in free radical formation that can be characterised by this technique. EPR spectroscopy can also be used to study paramagnetic transition metal species. Within pharmaceutical analysis this provides not only a means of detecting trace level impurities in APIs or excipients, but also the ability to monitor redox processes or study APIs that are themselves paramagnetic transition metal complexes. Some of the applications described above will now be explored in more depth.

Photodegradation

Current regulations require that the photostability of all new drug substances and products be evaluated to demonstrate that light exposure does not result in an unacceptable change.[2] Samples after exposure to a defined amount of ultra violet and visible light are normally analysed by High Performance Liquid Chromatography (HPLC) to determine any change in product strength or levels of degradation products. HPLC-Mass Spectrometry and Nuclear Magnetic Resonance (NMR) are then used to identify any degradation products. By 1998 there had been more than 100 reports of light-induced decomposition of drug molecules in the literature and the European Pharmacopoeia described light protection for more than 250 drugs.[3]

EPR spectroscopy can be used to monitor free radical formation during photodegradation, identify the radical intermediates and help determine the reaction mechanism. Nifedipine is a widely studied photo-labile drug, used in the treatment of hypertension. When exposed to light in the solid state high concentrations of stable free radical species are formed, producing a solid state EPR pattern as shown in Figure 3 (the EPR spectrum of the non-degraded sample is also shown but has been expanded ×35 to show the absence of any free radical species before exposure to light). When dissolved in dichloromethane (DCM) some of the radicals present in the solid are still stable and so can be detected by EPR, producing a simpler isotropic spectrum. Interpretation of the spectral profile and hyperfine splitting confirms that a nitrogen-based free radical is involved in the photodegradation of Nifedipine.

The extent of free radical formation on exposure of an API in the solid state to a defined amount of light can be used to determine the photostability characteristics of the sample. Samples can be exposed to light directly in the EPR sample cavity and the extent of free radical formation monitored, as shown in Figure 4. In this way kinetic information about the photodegradation of samples can also be determined.

Oxidative degradation

Oxidation is one of the major causes of drug instability,[4,5] so the ability to probe and understand oxidative degradation mechanisms is extremely important. When an oxidation reaction follows a free radical pathway, EPR spectroscopy can be utilised to identify the radical intermediates, determine the oxidation mechanism and study reaction kinetics. In the food industry EPR has successfully been used to predict the oxidative stability of food products[6] and this application could also be applied in the pharmaceutical industry.

For example, stability test samples of an API stored at both 25°C/60% relative humidity (RH) and 40°C/75% RH for up to 12 months were analysed by EPR at each time point to determine the free radical content and identify the radi-

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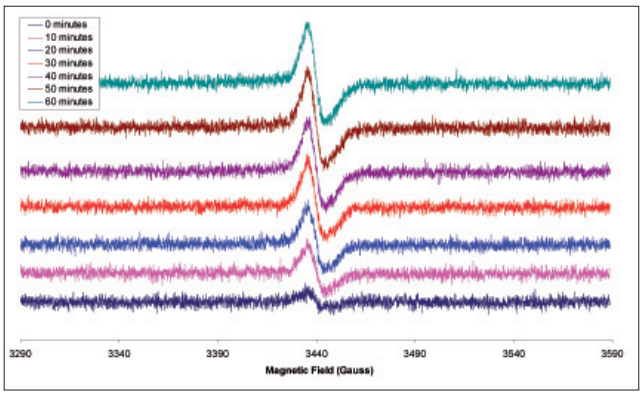

Figure 4. X-band EPR spectra of an API sample exposed to light *in situ* in the spectrometer and monitored over time. Bottom to top: 0, 10, 20, 30, 40, 50, 60 minutes.

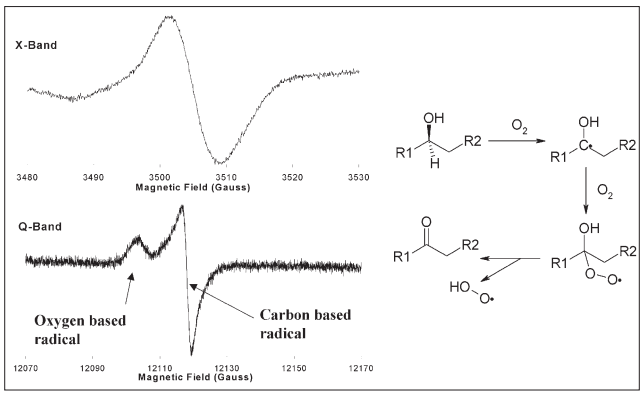

Figure 5. Variable frequency EPR spectra (298 K) and proposed oxidative degradation mechanism of an API. Reprinted with permission from *Analytical Chemistry* **78(2),** 604–608 (2006). Copyright 2006 American Chemical Society.

cal intermediates.[7] EPR analysis at two different frequencies, X-band (9 GHZ) and Q-band (35 GHz), confirmed that two different radical species (a carbon-based and an oxygen-based free radical) were involved in the oxidation mechanism, see Figure 5.

Excipient interactions

Excipient compatibility studies form an important part of the development of a pharmaceutical formulation. Interactions between an API and an excipient can both stabilise or destabilise a formulation, so choosing appropriate excipients for a formulation is paramount. In certain cases the interaction between an API and excipient can involve the formation or reaction of free radical species, so EPR can be used to investigate these interactions. Paramagnetic transition metals present at trace levels in an excipient also have the potential to catalyse the degradation of an API and this effect can again be studied by EPR spectroscopy, see the Paramagnetic transition metal analysis section.

One example involves the enhanced oxidative degradation of an otherwise stable API when formulated into tablets and even during the manufacture of the tablets. EPR spectroscopy was used to show that the degraded tablets contained at least two structurally similar free radical species and analysis of each excipient confirmed that the radicals originated from lactose monohydrate, used as a filler in the tablets, as shown in Figure 6.

EPR studies of binary mixes of the lactose and API confirmed that the radical content of the lactose decreased in the presence of the API, suggesting that the lactose radicals were reacting with the API to form diamagnetic products. Subsequently stability testing showed that enhanced degradation of the API was seen in the presence of lactose.

Effects of sterilisation techniques

Terminal sterilisation is an important step in manufacturing sterile pharmaceutical products. For polymers used in controlled release formulations, such as poly(lactide-co-glycolide) (PLG) copolymer, γ-irradiation is the preferred method of sterilisation. Free radical formation occurs in PLG samples during γ-irradiation leading to polymer degradation. The free radicals formed can interact with any drug molecules present and so potentially also result in degradation of the API.

In recent studies[8] using both EPR and the related technique of ENDOR (Electron Nuclear DOuble Resonance) the radicals formed in PLG by γ-irradiation have been identified as –•CHO–,

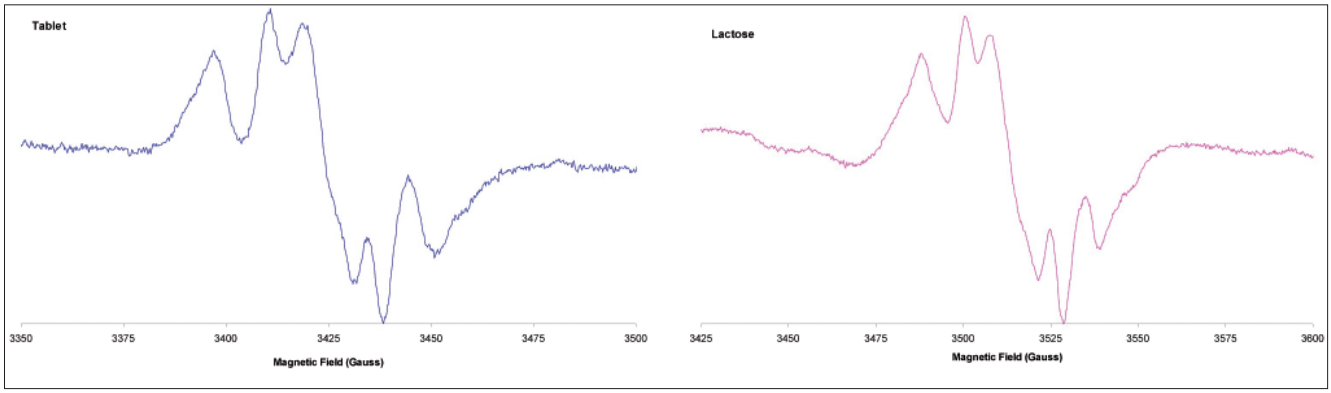

Figure 6. X-band EPR spectra of degraded tablet and lactose excipient.

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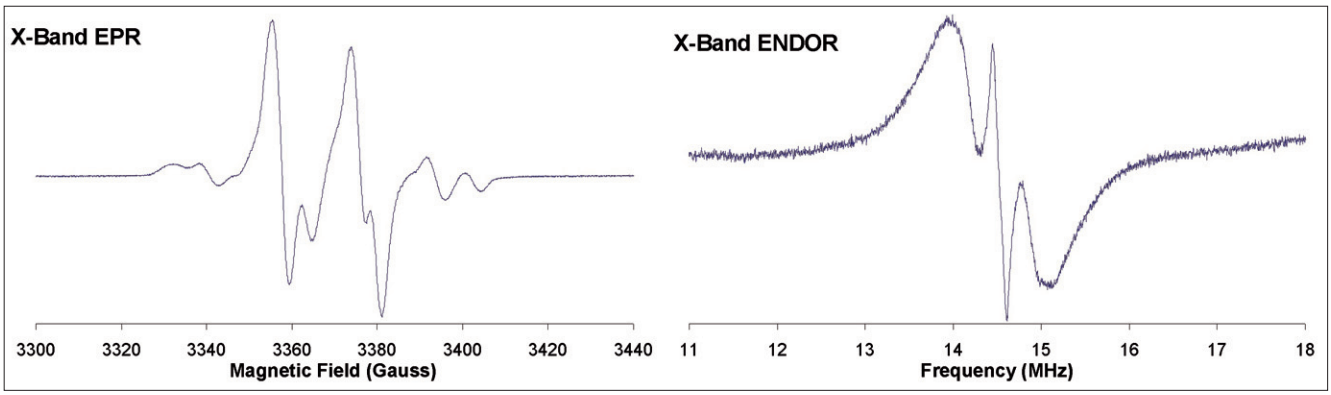

Figure 7. EPR and ENDOR spectra of γ-irradiated PLGA co-polymer.

–•C(CH$_3$)O– and –•C(CH$_3$)OR (example spectra shown in Figure 7) and the relative distribution of the radicals present was found to depend on the lactide : glycolide ratio.

Paramagnetic transition metal analysis

EPR spectroscopy can be used to detect paramagnetic transition metals down to levels of parts per million. Trace metals are often present in excipient or even API samples and EPR can be used to identify and quantify any paramagnetic transition metals. Such species have the potential to catalyse degradation of an API or excipient species.[9] For example, manganese (II) present at trace levels in the excipient calcium hydrogen phosphate, that is commonly used as a filler in tablets, can very easily be detected and quantified by EPR as shown in Figure 8.

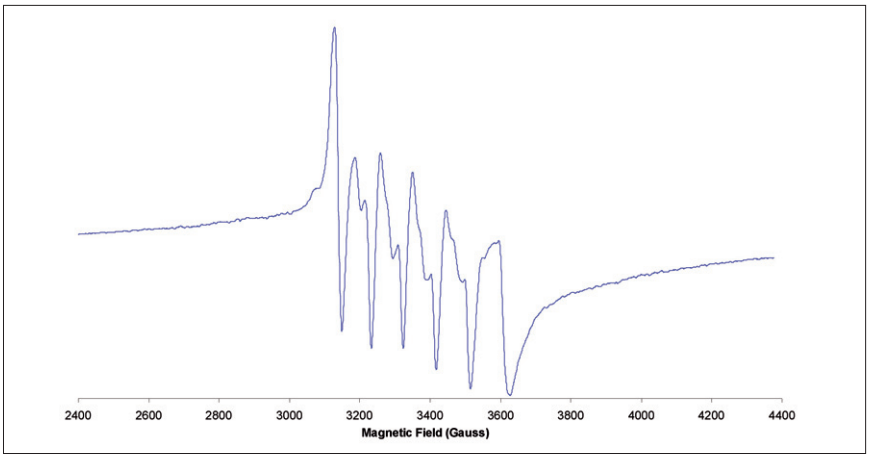

Figure 8. EPR spectrum of Mn(II) in calcium hydrogen phosphate.

Conclusions

EPR is a powerful tool within pharmaceutical analysis and has a wide range of application areas. It is a non-destructive and very sensitive technique that requires only a small sample size and it can be applied to a wide range of samples (solids, liquids, suspensions, solutions, whole tablets etc.). It can be performed over a wide temperature range to gain further information about any radical species present or to monitor thermal reactions. Samples can be photolysed *in situ* in the spectrometer. In solution reactants can even be mixed *in situ* to allow direct detection of less stable radical species. All these features make EPR a very flexible technique over a wide range of applications. It should be noted, however, that the pharmaceutical analysis applications discussed here are not meant to be exhaustive.

Acknowledgements

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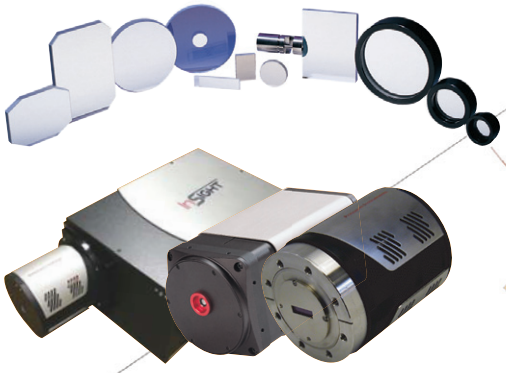

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