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# An Alternative Spectroscopy Technique for Biopharmaceutical Applications

**Spectroscopy using light is a familiar technique and the electromagnetic spectrum from UV to IR is routinely employed. This article looks at the advantages and applications of spectroscopy using sound waves, with special reference to pharmaceutical research.**

Spectroscopic techniques are widely used in the analysis of bio/pharmaceutical materials. They enable the non-destructive characterization of samples by measuring the parameters of signals passed through them. Such signals are composed of a combination of waves. However, only one wave dominates the field of material analysis — the electromagnetic wave, which represents the oscillations of electrical and magnetic fields. This wave probes the electromagnetic properties of materials (such as electron transitions) and is employed in optical spectroscopy and its related derivations, such as FTIR (Fourier transform infrared), fluorescence and NMR (nuclear magnetic resonance). In addition, there is a second, alternative wave that propagates through materials, the acoustical wave. When this wave penetrates a material, oscillating pressure (stress) causes fluctuations in the compression properties (mechanical deformation) of a material, so it is defined as a rheological wave.

## High-resolution ultrasonic spectroscopy

High-resolution ultrasonic spectroscopy is an alternative technique for material analysis employing high-frequency acoustical (ultrasonic) waves, that is, waves with a frequency greater than 100 KHz.

The application of ultrasound is well known; in medicine, ultrasound is used to visualize the internal organs of a patient's body. It is also used by submarines for underwater navigation. To explain further, ultrasound waves 'probe' intermolecular forces, providing information regarding the interior of a sample. Alternating compression (and decompression) forces in ultrasonic waves cause oscillation of the molecular structures in a sample, which respond by demonstrating an intermolecular attraction or repulsion. The amplitude of the deformations caused by analytical ultrasonic waves is extremely small, thus making it a non-destructive technique. Another important feature of an ultrasonic wave is its ability to propagate through a broad range of samples, including opaque materials.

In the past, the limited resolution of the measurements and the large sample volumes required prevented the use of this technique in analytical or research and development laboratories. Recently, however, a range of commercial ultrasonic instruments, such as the HR-US 101 and HR-US 102 high-resolution spectrometers (Ultrasonic Scientific Ltd, Dublin, Ireland), has overcome these limitations, allowing a wide variety of analytical tasks to be performed.

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## Perfluorocarbon liquids are well known for their high capacity to solubilize gases such as oxygen and carbon dioxide, and therefore have applications as synthetic blood substitutes.

### Requirements and applications

These instruments require small sample volumes, typically 0.03–1 mL (custom made) and can perform analyses in various regimes with superior resolution. They can be used for composition analysis and measurements of concentration; analysis of aggregation in suspensions and emulsions; particle sizing; formation of particle and polymer gels; micellization; adsorption on particle

surfaces; sedimentation; analysis of enzymatic reactions; conformational transitions in polymers; biopolymer–ligand binding; and antigen–antibody interactions.

A high-resolution ultrasonic spectrometer measures two parameters, *ultrasonic attenuation* and *velocity*. The two factors are physically independent, allowing the different levels of molecular organization of the samples to be characterized.

Attenuation is mainly determined by the scattering of ultrasonic waves in non-homogeneous samples (emulsions and dispersions) and fast chemical relaxation. Ultrasonic velocity is determined by the density and the elasticity of the medium.

### Heat stability of pharmaceutical emulsions

Figure 1 shows the application of ultrasonic spectroscopy to analyse the stability of pharmaceutical emulsions. The stability of an emulsion (including its composition and microstructure) is a key element in determining the lifetime and temperature conditions for the storage and use of the product. Ultrasonic measurements allow for a very simple procedure when evaluating the stability of emulsions. In this experiment, ultrasonic analysis of the thermal stability of a pharmaceutical water/oil emulsion (0.5  $\mu\text{m}$ ) was performed during gradual heating of the sample. The changes in the ultrasonic attenuation and relative ultrasonic velocity were measured.

The arrows in Figure 1 indicate the temperature corresponding to the destabilization of the sample. (Temperature measurements were taken at various frequencies in the range 2–15 MHz.) The rise in the attenuation at 44  $^{\circ}\text{C}$  provides clear evidence of the destabilization of the emulsion. The increase in attenuation can be attributed to the flocculation of dispersed aqueous droplets induced by heating. As seen in Figure 1, the change in ultrasonic velocity deviates from the baseline at the

same temperature that the ultrasonic attenuation begins to rise. This demonstrates the sensitivity of both ultrasonic parameters (attenuation and velocity) for accurate characterization of an emulsion.

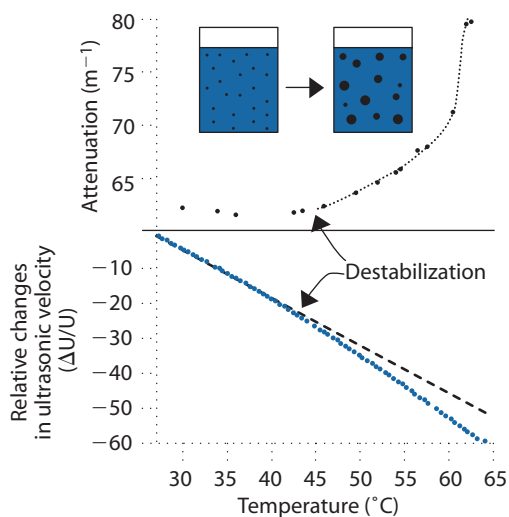
Another advantage of the ultrasonic technique is the ability to make measurements directly, in the original emulsion, without having to make dilutions. The preparation of serial dilutions is commonly required when using optical techniques.

### Sedimentation in emulsions and suspensions

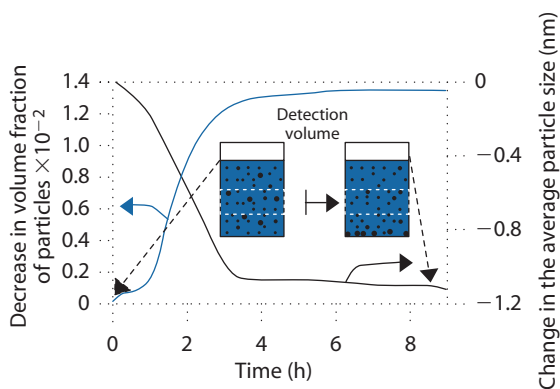
Figure 2 shows the ultrasonic monitoring of sedimentation in a perfluorocarbon emulsion. Perfluorocarbon liquids are well known for their high capacity to solubilize gases such as oxygen and carbon dioxide, and therefore have applications as synthetic blood substitutes. Commercially available blood substitutes are typically supplied as concentrated perfluorocarbon emulsions in water. The functional properties of the emulsion are determined by the stability of the perfluorocarbon droplets. Perfectly suspended emulsions generally consist of small particles (approximately 100 nm). The droplet size of these emulsions increases with storage time. In particular, the fast precipitation of perfluorocarbon droplets is related to the growth in droplet size, because of the higher density of the perfluorocarbons compared with water. This indicates a breakdown of the functional properties in the emulsion. However, ultrasonic spectrometers enable the direct detection of the changes in the structure of the emulsion, allowing the efficacy or usefulness of such a preparation to be assessed.

To demonstrate, 1 mL of a commercial emulsion of perfluorocarbons in water (10% v/v) was agitated and loaded into an ultrasonic cell. The precipitation of large particles resulted in a shift of particle distribution across the cell, which was monitored by measuring ultrasonic parameters in the middle of the cell (that is, the area of the ultrasonic beam). The measurements were performed simultaneously at a number of frequencies (2–15 MHz).

**Figure 1** Application of ultrasonic spectroscopy to analyse the stability of pharmaceutical emulsions.



**Figure 2** Monitoring the precipitation of a perfluorocarbon emulsion used as a synthetic blood substitute (25  $^{\circ}\text{C}$ , 2–15 MHz).



The ultrasonic velocities were used to calculate the emulsion concentration in the beam (volume fraction) and the ultrasonic attenuation was used to calculate the average particle size of perfluorocarbon droplets. The results, shown in Figure 2, enable the ageing of the emulsion to be directly evaluated.

### Unfolding and aggregation of proteins

Figure 3 shows the ultrasonic monitoring of the aggregation of carbonic anhydrase in solution, induced by thermal denaturation. Carbonic anhydrase metalloenzymes are involved in critical physiological processes related to the respiration and transport of  $\text{CO}_2/\text{HCO}_3^-$  between metabolizing tissues and the lungs, pH homeostasis and various biosynthetic reactions such as gluconeogenesis and lipogenesis. There are a number of different forms of carbonic anhydrase isoenzymes that can be obtained from living organisms or by genetic engineering. One of the most important characteristics of the enzymes is thermal stability, which is determined by the chemical structure and conformation of protein molecules.

To examine this concept, 1 mL of freshly prepared enzyme solution (1% w/v) in Tris-buffer (pH 7.4) was loaded into an ultrasonic cell, and a reference cell was filled with just the buffer. The difference in ultrasonic velocities between the two solutions and the change in attenuation in carbonic anhydrase solution upon heating the samples at a rate of  $0.1\text{ }^\circ\text{C}/\text{min}$  was measured. As seen in Figure 3, the difference in ultrasonic velocity (solution–buffer) decreases gradually within the temperature range of  $30\text{--}58\text{ }^\circ\text{C}$ . The decrease in ultrasonic velocity is attributed to the different temperature dependencies of ultrasonic velocity (such as density and compressibility) in the hydration shell of protein compared with bulk water.

As a result of heating, the beginning of protein aggregation is indicated clearly by the sharp growth in ultrasonic attenuation above  $50\text{ }^\circ\text{C}$ . The formation of protein particles and the scattering of ultrasonic waves increase the attenuation, which at higher frequencies is more

sensitive to the formation of small particles.

The main heat-induced transition (protein denaturation) is observed (protein denaturation) is observed between  $58\text{--}64\text{ }^\circ\text{C}$  and is indicated by

## In the past, the limited resolution of the measurements and the large sample volumes required prevented the use of this technique in analytical or research and development laboratories.

sharp changes in both ultrasonic parameters (increase in attenuation/decrease in velocity). The decrease in velocity demonstrates the formation of a highly compressible hydrophobic core of protein aggregates in which hydrophobic amino acid residues stick to each other. This ‘extra’ compressibility of the core decreases the ultrasonic velocity, similar to what is generally observed in hydrophobic aggregation during the formation of surfactant micelles. Figure 3 also shows that whereas the main transition occurs within a narrow temperature range, the increase in attenuation and the decrease in velocity at temperatures greater than  $64\text{ }^\circ\text{C}$  shows that aggregation continues at higher temperatures.

The insert shows the ultrasonic temperature profiles in the carbonic anhydrase solution measured after several weeks of storage at  $4\text{ }^\circ\text{C}$ ; the transition temperature is almost the same as that of the fresh sample. However, the effect of transition on the ultrasonic parameters is different. The ultrasonic attenuation is nearly twice as high in the aged sample compared with the fresh sample, and the changes in ultrasonic velocity are the opposite. This indicates that the ageing of the enzyme solution during storage can be analysed by high-resolution ultrasonic spectroscopy.

### Enzymatic catalase

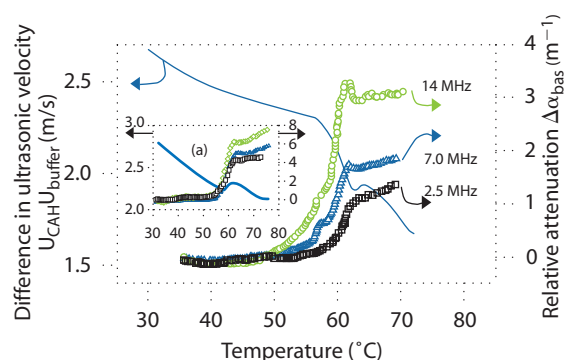
Figure 4 shows the ultrasonic determination of the enzymatic activity of catalase. Catalase is an enzyme that is present in almost all animal cells and organs, and in aerobic microorganisms. Using  $\text{H}_2\text{O}_2$  as a substrate to produce  $\text{H}_2\text{O}$  and  $\text{O}_2$ , catalase is of commercial interest wherever hydrogen peroxide is used as a germicide. It has applications in the

food industry, for example, for disposing of  $\text{H}_2\text{O}_2$  used in pasteurizing milk prior to cheese-making, as well as for micro-encapsulation. The most common technique for

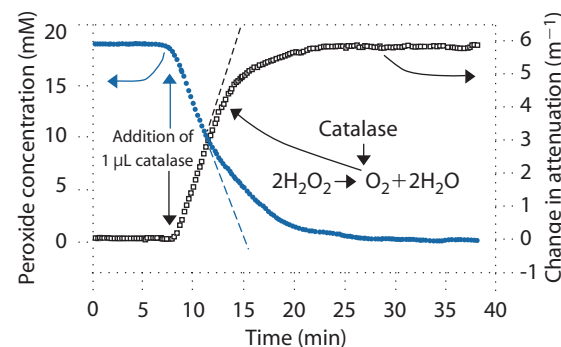
detecting enzymatic reactions is based on standard UV/visible spectrophotometric assays. However, in many cases this requires special markers, making the analysis more complicated. Ultrasonic measurements allow the direct detection of an enzymatic reaction by monitoring the change in concentration of both the substrate and products, as highlighted below.

In this experiment (Figure 4), an aqueous peroxide solution (19 mM)

**Figure 3** Ultrasonic monitoring of heat-induced aggregation in freshly prepared 1% carbonic anhydrase solution in Tris-buffer (pH 7.4).



**Figure 4** Change in peroxide concentration and ultrasonic attenuation after the addition of 1 mL of catalase solution.



in 0.05 M phosphate buffer (pH 7) was loaded into ultrasonic cells at 25 °C. An aliquot (1 mL) of 40 mg/mL catalase solution in the same buffer was then added to the cell. The enzymatic reaction was monitored continuously by measuring both the ultrasonic velocity and attenuation in the samples. The change in ultrasonic velocity was directly related to the change in chemical composition of the solutions (for example, peroxide concentration and products of the reaction) allowing the concentration of peroxide during the enzymatic reaction to be calculated. The increase in the ultrasonic attenuation was attributed to the scattering of ultrasonic waves by oxygen-gas bubbles, which are formed upon decomposition of peroxide. The saturation of the enzymatic reaction after 20 min corresponds to the point at which the ultrasonic velocity and attenuation stabilize. The data show the activity of the enzyme to be 3000 units/mg. The advantage of the ultrasonic technique is that it allows the direct measurement of enzymatic kinetics without any markers and it can perform the analysis in opaque systems (such as blood).

### Summary

These four examples show the potential of ultrasonic spectrometry in the monitoring of reactions. Its ability to monitor both structural and biochemical processes in real-time in the same machine make it a simple yet powerful analytical technique.

The theory of acoustic analysis has been known for many years, but practical applications are recent and affordable laboratory-scale instruments more recent still.

The arrival of such instruments opens up new possibilities in difficult to analyse samples. The specification of ultrasonic spectrometers has improved dramatically during the past few years and is likely to have a profound impact on analytical techniques.

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