Neutron scattering provides unique insights for drug R&D

Drug discovery and development is a long and expensive process. Techniques, such as computer modelling, that make the search for promising candidates easier are usually taken up enthusiastically. Neutron scattering, an emerging technique in this field, can provide drug developers with crucial information not available through any other means about atomic and molecular structure and interactions. The Science and Technology Facilities Council's ISIS Neutron Source at the Rutherford Appleton Laboratory in Oxfordshire is a UK research centre open to all.

By Dr Martyn Bull

Many, if not most, organisations that undertake drug research and development have access to x-rays, nuclear magnetic resonance (NMR) and other core crystallography and spectroscopy techniques. Neutron sources are in short supply, which largely explains the limited use to date of neutrons in the drug discovery and development process.

ISIS uses neutrons to provide unique insights into the arrangement and behaviour of atoms and molecules in a material. It explores the properties of matter by measuring the locations of atoms and the forces between them. Neutrons can be used for research into physics, chemistry, engineering, materials science, environmental and geological sciences, and increasingly the life sciences. In terms of drug discovery and development, neutrons can investigate: advanced materials such as catalysts; molecular materials such as pharmaceuticals; proteins, DNA and cell membranes; complex biomedical materials and disordered materials and liquids.

Last year ISIS opened a second target station (TS2), widening its capability for research into soft matter and bioscience. It now has more than 30 instruments with more in design. Each instrument is optimised for different types of experiment, providing diverse and complementary information on samples. Instruments include:

- **Diffractometers** designed to analyse atomic-level structures.
- **Reflectometers** for studies of surfaces and interfaces.
- **Spectrometers** measuring the energies of scattered neutrons provide information about atomic motions and magnetic and electronic behaviour.

Neutrons have several desirable characteristics for drug discovery and development:

- Studies can range from the distances between atoms (0.1nm) to those associated with the structures of large molecular arrays (over 500nm) (see **Figure 1**).
- Neutrons can penetrate deep inside a sample.
- Neutrons are very sensitive to light atoms such as hydrogen (which are almost invisible to x-rays).
- Different isotopes scatter the neutrons differently, so can be used to identify the location and orientation of a particular element or component.

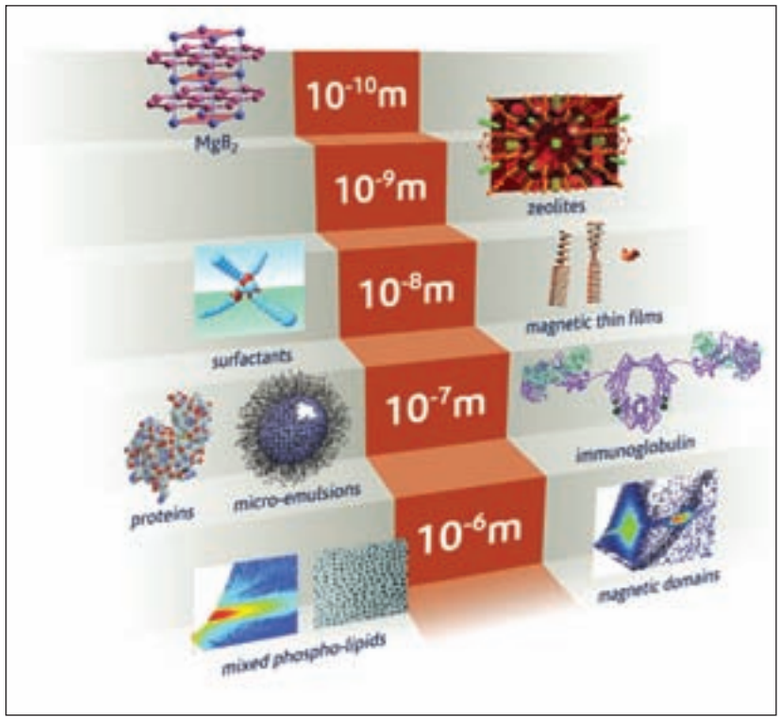


Figure 1

- Surface and interface structure can be revealed by neutron reflectometry.
- It is possible to scan samples under real conditions to monitor interactions in real time.
- Neutrons are non-destructive so can be used on delicate biological samples.
- Biological samples can be examined *in vitro*, they do not need to be frozen or chemically fixed.
- Neutrons can provide both low and high resolution information about biological interactions:
  - Low resolution information about large features such as lipids surrounding a macromolecule.
  - High resolution information about water solvent structure, hydrogen binding and precise active site geometry.

How ISIS works

Neutrons are released from a small tungsten target (see 3 in **Figure 2**) when a high energy proton beam (see 1) is fired at it. The target is housed in a concrete structure at the centre of the experimental hall (see 2). Bursts of neutrons are released 50 times a second.

Once the neutrons have been released from the target, they travel down beam pipes (see 4) leading to instruments (see 5) and are used in experiments to characterise samples. There are more than 30 separate instruments at ISIS spread across two experimental halls. Each instrument can be operated independently of the others.

Examples of ISIS research with impact for drug discovery and development

Crystallography and spectroscopy

Neutrons are a complement to core crystallography and spectroscopy techniques such as x-rays and NMR because neutrons interact with matter according to a different set of principles. Unlike x-rays, which are scattered by the electrons in an atom, neutrons are scattered by the nucleus through the strong nuclear force, meaning that the strength of scattering of elements is not systematically dependent on atomic number. This means that even atoms with low atomic weight such as hydrogen – virtually invisible to x-rays – are easily perceived with neutrons. Indeed, according to ISIS user Professor Chick Wilson, Regius Professor of Chemistry at the University of Glasgow: "Neutron scattering is the most powerful technique available for pinpointing hydrogen atoms."

Neutrons are excellent for determining molecular structure and precise atomic arrangement, but are especially useful in understanding how and why the complex tertiary structure of a molecule forms. The ability to identify the location and orientation of hydrogen atoms means that neutrons are particularly effective at allowing the determination of surface charge distribution (since exterior molecule surfaces often have a hydrogen 'coat' or tail) and electrostatic interactions, which are crucial for molecule binding and affinity.

Prof Wilson and his team have used ISIS to investigate polymorphism[1,2] – where the same compound has more than one crystalline form (see **Figure 3**). They have used neutron diffraction to provide information about the intermolecular forces holding the different crystal structures together. These forces often involve hydrogen bonds, and are thus ideally investigated by neutron diffraction.

Polymorphism is important for pharmacology and drug development as variants can have dramatically different physical properties, such as solubility, which can affect their suitability as a drug candidate. The adoption of different polymorphs can be governed by very small differences in the solid-state equilibrium energy. Computational methods indicate that the energy landscape of potential stable configurations accessible to a solid-state molecule are often clustered at the bottom of an energy spread – typically with eight to 10 variants spread over just 3-5kJ/mole. Prof Wilson's team have shown that neutrons can measure the 'energy cost' of an intramolecular shift of single hydrogen atom (found to be around 1kJ/mole) [3], and have related this to accurate experimental and computational studies of

polymorphs with energy differences of a few 1kJ/mole [2].

Professor Wilson's group works closely with computational chemists. "A combined experimental and computational approach is essential in this area, for example in our UCL-led CPOSS (Control and Prediction of the Organic Solid State) Basic Technology collaboration, which tackles the predictability of polymorphism," says Professor Wilson. "Neutrons are an essential part of our experimental process. Investigating the subtle effects controlling polymorphism requires the most accurate experimental methods and we couldn't get that data through any other means. As we extend this work, the new capability of the Second Target Station at ISIS is of enormous interest to us."

ISIS instruments used by Prof Wilson's team included: Sxd (single crystal diffractometer) which uses the time-of-flight Laue technique to access large 3D volumes of reciprocal space in a single measurement; Gem (general materials powder diffraction) which can be used to perform high intensity, high resolution experiments to study the structure of disordered materials and crystalline powders; and Hrpd (high resolution powder diffraction) which is the highest resolution neutron powder diffractometer of its type in the world.

Organic molecule arrangements – aromatic π-π interactions

In April 2010, a team of scientists led by UCL and ISIS Neutron Source published exciting new experimental data that demonstrated how aromatic molecules such as benzene arrange themselves in liquids[4]. This new knowledge should lead to more efficient drug design by the more accurate selection of the lead drug molecule, reducing the time and cost of developing new drugs to market.

Understanding the aromatic π-π interactions of benzene-like chemical groups is extremely important for developing models of their biochemical interactions. These interactions play a role in the stereochemistry of organic reactions, organic host-guest chemistry and crystal packing, protein

Figure 2

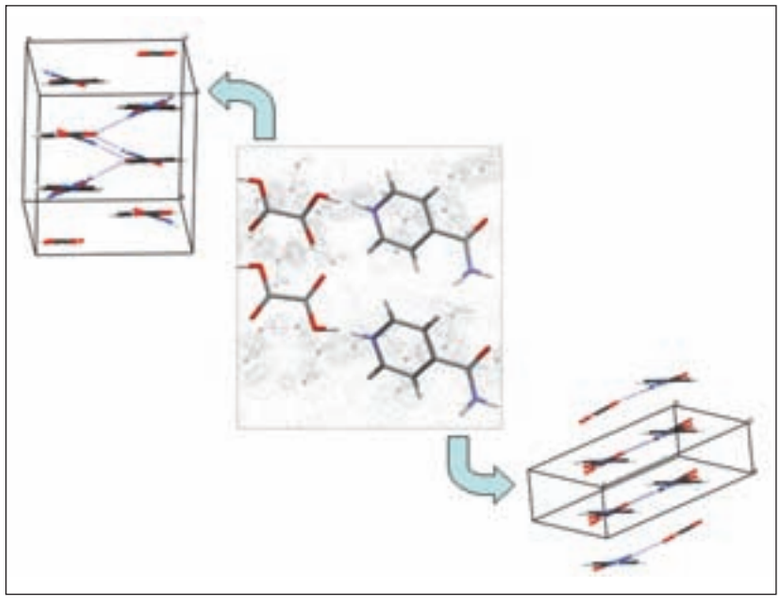


Figure 3
A change in conformation leads to dramatic changes in crystal packing in this molecular complex. Neutron studies at ISIS, along with x-ray diffraction and computational chemistry, showed, however, that the energy difference between the two polymorphs is only around 3kJ/mol. Accessing the small energy differences between polymorphic forms is vital in the understanding and potential control of this phenomenon in pharmaceutical materials

folding and structure, DNA and RNA base stacking, protein-nucleic acid recognition, and drug design and development.

Many classes of drug molecule contain a benzene-type functional unit within their structure. For example, pain-killers such as aspirin, ibuprofen and paracetamol all contain such a unit, despite their otherwise relatively simple molecular form. In many cases the presence of the benzene-type functional unit in the drug molecule is essential for its biological activity, because one of the mechanisms by which the drug interacts with the target biological molecule (usually an enzyme) will be through aromatic π-π interactions. Better understanding the nature of such interactions will improve our understanding of the mechanisms by which drugs exert their activity.

The team investigated benzene (C_6H_6) as it is the archetypal aromatic liquid and the simplest molecule – a carbon ring with six hydrogen atoms – with which to attempt to understand structures resulting from intermolecular π-orbital interactions. Aromatic rings of six carbons are found in many biological molecules and pharmaceuticals. Aromatic liquids are also important non-polar organic solvents, used in a wide range of laboratory and industrial processes. For example, benzene is used as a solvent in the pharmaceutical industry, where many materials require a non-polar environment to solubilise them for chemical processing. Benzene derivatives are also important reactants used in the manufacture of drugs, eg isobutyl benzene is used in the production of ibuprofen.

A combination of high-resolution neutron diffraction and isotopic substitution of hydrogen for deuterium was used to determine the detailed structure of liquid benzene. This showed that the theoretical model underpinning many biological calculations may need significant rethinking. Current theoretical calculations are limited as they are predominantly based on how two molecules interact, rather than on many molecules packing together as occurs in liquids.

The structure of liquids is complex, but scientists typically visualise benzene via four 'motifs' (molecule arrangements) known as sandwich (S), parallel displaced (PD), T-shaped (T), and Y-shaped (Y) (see **Figure 4**).

Theory found that T and PD had the lowest energies, and so predicted that they would be the most common. Data analysis resulted in a full six-dimensional spatial and orientational picture of the liquids (see **Figure 4**), which showed that PD and Y-shaped are in fact the most common (although all four motifs are present). This makes sense as they are more efficient at 'stacking' when there are lots of molecules together.

Whether the molecules assume a PD or Y formation depends on the distance between the pair. The experiment found that the nearest neighbour co-ordination shells contain approximately 12 molecules. When viewed as a whole these shells are orientationally isotropic, but detailed analysis reveals that the favoured molecule arrangements is PD at the smaller separations (<5 Å) and Y-shaped at the larger separations (see **Figure 5**).

"It's like a zipping up action – as the molecules get closer they flip from Y to PD. This is important information for understanding the crystallisation of biological molecules and proteins," said Dr Daniel Bowron, the ISIS research scientist who worked on the project.

The team found the Sandals (small angle neutron diffractometer for liquids and amorphous samples)

ISIS stats and facts

- ISIS uses neutrons to provide unique insights into the arrangement and behaviour of atoms and molecules in materials.
- ISIS can investigate a wide range of length scales, from 10^{-6} to 10^{-10} metres.
- Access to ISIS beam time is allocated by peer review and is free to UK researchers; there are two proposal deadlines a year – April and October.
- ISIS has over 30 instruments that run 24/7 to maximise experiment time, with ISIS staff on a 24-hour call-out rota to provide support.
- ISIS instrument scientists are paired with visiting teams to help them to get the best out of their experiments.
- ISIS has five laboratories for preparing samples and characterising samples using standard lab techniques.

instrument at ISIS particularly useful because it enables isotope labelling, providing information on the distance and orientation of atoms. This enabled investigation of the angle of neighbouring molecules to see how they were orientated in the liquid. Normal hydrogen (H) was substituted with deuterium ('heavy hydrogen'). Because deuterium has a proton as well as a neutron the benzene structure diffracts the neutron beam differently, indicating molecule orientation.

"This added clarity to the structure like adding focus and colour to a fuzzy black and white picture," said Professor Neal Skipper, Professor of Physics in the Condensed Matter and Materials Physics at UCL. "The difference we found between theory and experiment is very important. The benzene molecule is six-fold symmetric and there's a rotation of one whole order of symmetry between the simulation and our experiment. If you have a side group on the benzene ring then there is a significant difference in the calculated position and what is actually found – this is a problem if you're using models to determine the likely nature of the interactions between the putative drug and biological molecule of interest."

The group is now researching molecule interactions and positioning when functional groups are added to the benzene ring.

Biological molecule interactions

Neutrons have several advantages when it comes to investigating biological molecule interactions. It is possible to scan samples under real conditions to monitor interactions in real time, neutrons are non-destructive so can be used on delicate biological samples, and biological samples can be examined **in vitro** – they do not need to be frozen or chemically fixed. Both low and high resolution information about biological interactions can be determined from low resolution information about large features (eg lipids surrounding a macromolecule) to high resolution information about water solvent structure, hydrogen binding and precise active site geometry.

Neutron scattering was of great use for Dr David Barlow, a principal investigator at Kings College London's Pharmaceutical Biophysics Group, who

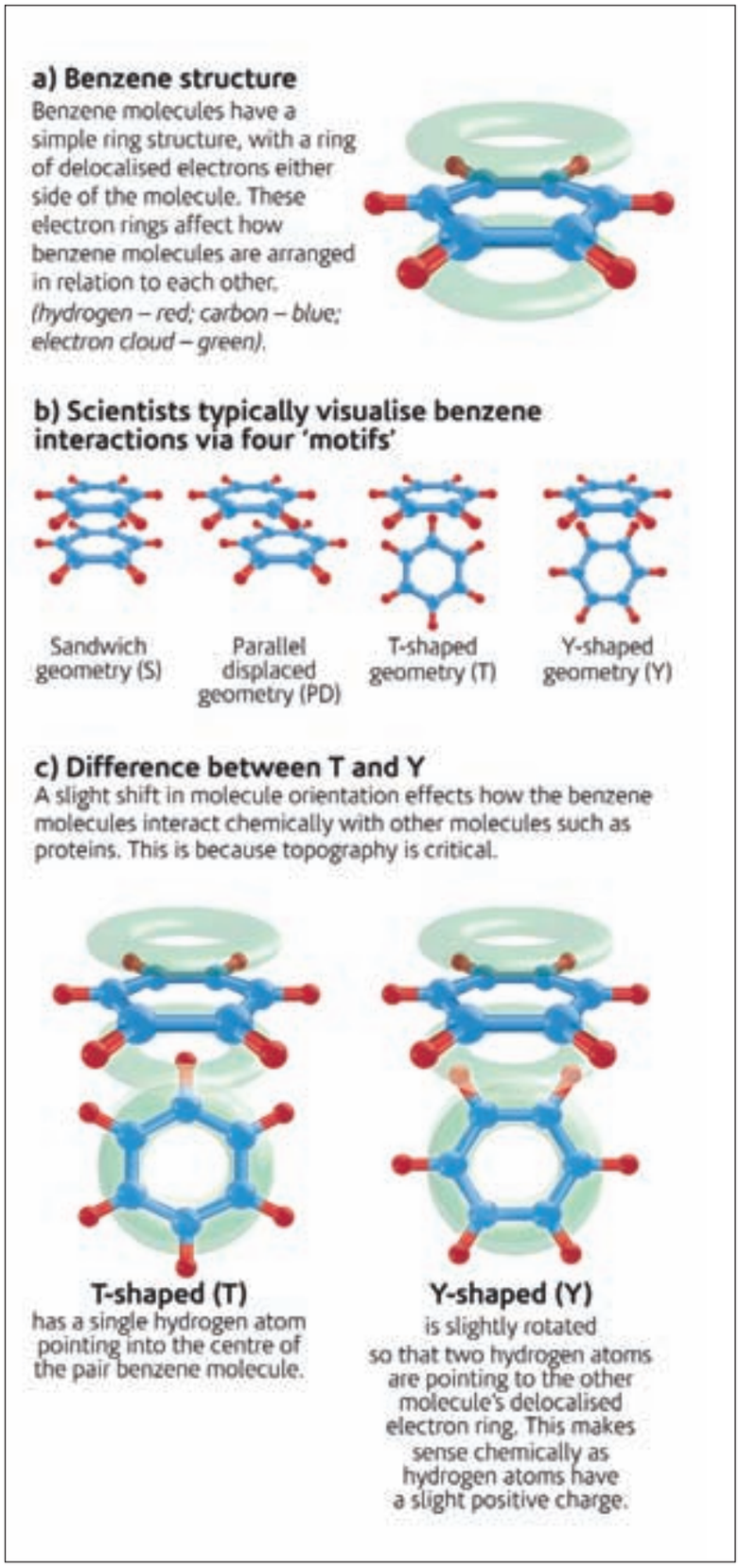
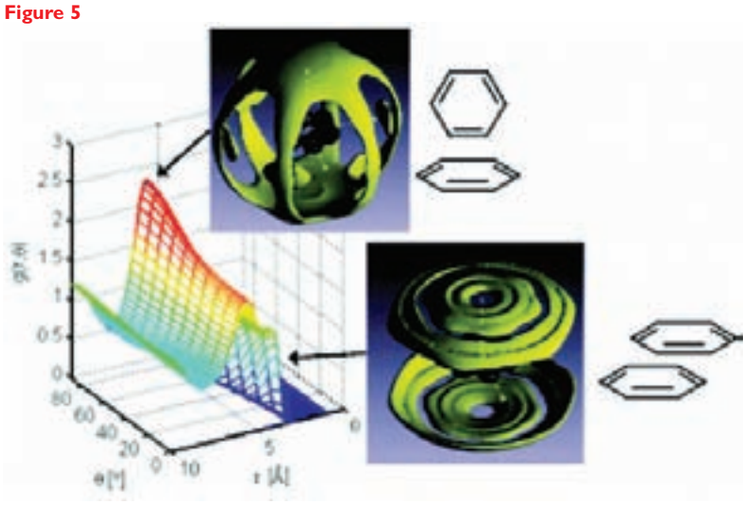


Figure 4

The structure of π-π interactions in liquid benzene, showing the predominance of Y-shaped (top) and parallel displaced (bottom) arrangements. The former manifests itself as a six-fold symmetric Chinese lantern, which reflects the underlying symmetry of the benzene molecules

Figure 5



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is conducting retrospective drug discovery research into the mode of action of amphotericin B[5].

Amphotericin B has been the first line of defence against fungal infections since the mid-1950s, but unfortunately resistance is beginning to emerge. This is of grave concern because immunosuppressed people such as AIDS and chemotherapy patients often get fungal infections, which become a problem if they spread to the lungs or circulatory system. Reported mortality rates of chemotherapy, immunosuppressed or AIDS patients associated with invasive Candida infections are as high as 49%, while invasive aspergillosis (IA) has emerged as a leading cause of morbidity and mortality in immunocompromised patients with an associated mortality rate of 65%.

The normal route to replacement drugs would be to examine compounds that have a similar mechanism of action. The problem is that amphotericin B's mechanism of action is not properly understood, meaning a return to first principles of drug discovery and development is necessary.

The effects of amphotericin B are well documented – it punches holes in the fungal cell walls, and the leaky cells then die. This could potentially happen to a patient's cells but the drug has a much higher selectivity for fungal cells, so at normal doses side-effects are minimal and tolerable. However, the raised doses necessary to overcome resistance mean the side-effects can become problematic.

Quite how amphotericin forms holes in the cell walls is not entirely clear, but one idea concerns one of the key components of cell walls, sterol. Fungal cell membranes contain ergosterol rather than cholesterol. It is thought to be the preferential interaction of amphotericin B with the fungal sterol that confers the drug selectivity.

Dr Barlow's research aims to find out precisely why this difference between cholesterol-containing and ergosterol-containing cell membranes is critical, and why, therefore, amphotericin B is so damaging to fungi and not to humans. The research uses liposomes, prepared using different mixtures of fats and either cholesterol, ergosterol or some other kind of steroid, so that they mimic fungal or human cells.

The structures of the membranes surrounding these liposomes, and how they were altered when saturated with amphotericin B, were studied using the Loq instrument at ISIS, the most successful time-of-flight SANS (small angle neutron scattering) instrument in the world.

"Interestingly, we found that they were equally affected. However, this experiment only investigated the end point as the drug was delivered directly to the membrane. We're now looking at the preceding steps to see how the drug would normally get taken up by the membrane – this may be where the difference in fungal and human cells lies," said Dr Barlow.

Conclusion

Neutron research conducted at ISIS is of great benefit and provides many insights on different aspects of the drug discovery and development process. It can help identify and develop the best drug candidates by improving our understanding of chemical and protein structure, elucidating a drug's mode of action, or investigating how biological interactions occur.

Those interested in finding out more about what neutron scattering can do should visit www.isis.stfc.ac.uk. DDW

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