How neutron science has enabled innovation in drug design and delivery

Meeting global health challenges, from cancer to viral infections, requires a profound understanding of living processes – not just at the level of cells but right down at the molecular level. And designing the most efficient therapeutics requires an in-depth understanding of the disease. Most diseases result from biomolecular processes going wrong. Viruses invade cells and take over their molecular machinery, eventually killing the cells and bacteria may release toxins that block a vital molecular interaction. The ambitious aim of biologists today is thus to observe the structure and behaviour of biological molecules at the atomic scale, as they function in their natural, physiological environment. Understanding the complex biological processes that regulate our bodies requires insight into structure and dynamics at the molecular scale. And for that we need particularly potent analytical weapons. While x-rays are well-known to most, the use of neutrons in health-related research is not common knowledge. The Institut Laue-Langevin (ILL), the world's flagship centre for neutron science, is using its vast array of instruments to piece together the huge biological puzzle of life.

By Dr Giovanna Fragneto

This article will provide some background information on each neutron technique, and will explain why neutrons are invaluable in the investigation of therapeutics. The collection of studies that follow stems from recent results obtained on ILL instruments – employing neutrons directly generated in the Institute's reactor. Each has been carefully selected to provide an overview of the use of neutrons on drug investigations. They demonstrate that understanding biological processes at the atomic level helps to rationalise the effect of disease on our bodies and yields insights which directly impact drug innovation and lead to novel or better-adapted treatments.

The molecules of life

40,000 to 100,000 different proteins are specified by the human genome. Each protein structure consists of a chain of molecular units, amino acids, folded into a distinctive 3D shape that allows them to do a particular job. Very often, proteins do not act alone but as components of complex interactive molecular assemblies. One significant example of an assembly is a cell membrane. Biologists are becoming more and more aware of the importance of exploring in detail the structure and behaviour of membranes: how the lipids and embedded proteins interact to mediate the membrane's complex functions.

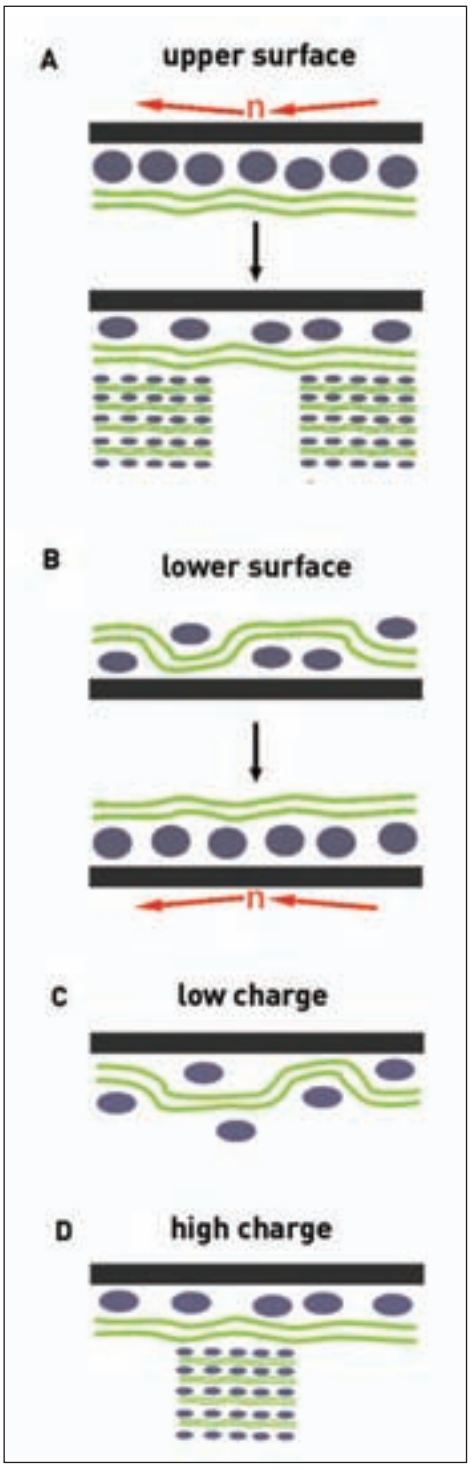

Figure 1
The interactions of a lipid-dendrimer particle suspension (molecules in green and purple respectively) with model silicon supports, positioned (A) above and (B) below the sample, and with supported lipid bilayers of (C) low and (D) high electrostatic charge

Another major subject of study is how proteins fold in cells. Subtle changes in the local physiological environment can trigger misfolding to create structures associated with diseases such as Alzheimer's and Parkinson's. So for drug design and delivery methods, developing an understanding of protein structure and function is essential.

In recent years, neutron scattering has played an increasingly important role in observations of macromolecules such as proteins. The various neutron techniques now available can reveal aspects of structure and dynamical behaviour not easily accessed by other methods.

Advantages of neutrons

Neutrons are found in the atomic nucleus, and are produced or released by either energetically breaking up nuclei (fission) or by knocking them directly out of nuclei (spallation). The ILL's nuclear reactor employs fission to produce the most intense beams of neutrons in the world. Neutrons provide an invaluable analytical tool for studying the properties of matter at the atomic and molecular scales. Because neutrons are quantum particles, they have a characteristic wavelength directly related to their energy. A neutron's wavelength is at least 1,000 times shorter than that of visible light, so, like x-rays, they can 'see' matter at scales between tenths of a nanometre and micrometres. Neutrons can also be 'tuned' to penetrate a structure and be scattered by the nuclei of the target material's atoms.

Neutron beams are particularly suitable for biological research. They can be generated with energies and wavelengths appropriate for probing a range of biological structures, from small molecules such as peptides, to larger molecules and molecular assemblies, including viruses. Being electrically neutral, neutrons can travel deep into materials without being deflected, and are non-destructive.

One of their most important advantages is that they interact quite strongly with hydrogen nuclei, so can identify the hydrogen atoms in a structure, including those trapped in water molecules. This is in contrast to x-rays, which are unable to image hydrogen atoms. Determining the precise locations and orientations of the hydrogens in a molecule is crucial to understanding its biological behaviour.

The only way is up – neutrons highlight the potential of advanced drug delivery systems

A key goal for drug designers is to find ways of delivering therapeutic agents effectively to sites of disease in the body. Ideal carriers must be able to bind selectively with target cells, and must mediate the passage of an optimised concentration of drug molecules across the cell membrane in a sustained and controlled way. An exciting new approach is to attach 'reservoirs' of the drug to the membrane in the form of liquid-crystalline particles. These particles form spontaneously from long-chain

lipids and branched tree-like molecules called dendrimers. They are ideal candidates for transporting large amounts of small drug molecules for diffusion across cell membranes.

Delivery systems using similar principles are already being tested in clinical studies. However, the development of commercial products would benefit from a more detailed understanding of the interactions of the drug reservoirs with membranes. The composition of the particles will affect how they interact with the cell membrane – an important concern in controlling the dosage over time. In addition, given that cancer cells differ electrically from healthy ones, knowledge of how the charge on the membrane affects the binding process will influence the effectiveness of selective targeting.

Interactions with model cell membranes

Neutrons have another property useful in biological research, and that is they can be reflected at grazing angles from surfaces – this is known as neutron reflectometry. The analysis of the signal after the interaction with the interface can reveal the 2D organisation of a surface layer. Neutron reflectivity offers an ideal tool for studying layered structures such as the cell membrane. Recent advances show that it may be possible to prepare model membrane systems in aqueous solutions that mimic the physiological environment of a real membrane – that is, with a typically fluctuating complex composition involving different lipids and proteins.

Recently, experiments were carried out, in which the interactions of a suspension of lipid-dendrimer particles with model surfaces were investigated. The surfaces were located both above and below the lipid-dendrimer particles solution[1]. Over time, as a result of their lower density, the particles separate out into a concentrated phase floating on a more dilute phase. The reflectivities of the layers that formed on the model surfaces were then regularly measured, determining their evolving structures over a period of 30 hours.

These measurements were possible thanks to the unique capability of the ILL's FIGARO instrument to take measurements from both above and below a horizontal sample in kinetic mode, revealing the membrane structure, and that of any attached material. This ability proved to be essential in this study.

It was found that when the suspension interacted with a model surface positioned below, only a single lipid-dendrimer layer formed on it. However, when the model surface was above the lipid layer, the liquid-crystalline particles in the concentrated phase attached themselves to it, creating the kind of reservoir-surface configuration that had been sought. The dramatic differences in the surface interactions provided an important message to formulation scientists: gravity can have a substantial effect, so the orientation of the surface during trials of new products can be critical to their success.

Similar experiments were then carried out using silicon supports positioned above the suspension, which were coated with one of three model membranes, each a lipid bilayer with a different electrostatic charge. The liquid-crystalline particles quickly attached to the two bilayers with the greatest negative charge, and the dendrimer molecules diffused across to the other side. However, the particles did not attach to the supported bilayer with the lowest charge, and the dendrimer diffusion was much slower. This result demonstrates the potential for tuning the charge within new lipid-dendrimer formulations to deliver drugs for targeting specific cell types.

Key questions remain on the mechanisms that control the diffusion of the dendrimer molecules across the membrane, and the attachment of the reservoirs. Future research is planned which includes encapsulating small drug molecules in the particles for controlled slow release.

New vistas in drug design – neutron crystallography is providing breakthroughs in developing improved antiviral drugs

Most drugs work by binding to a specific enzyme involved in the proliferation of a disease, so that its function is inhibited. Modern drug design focuses on analysing and optimising the interactions between the drug and its target, and x-ray crystallography has been the method of choice to unravel these structural details. However, given that these interactions are often mediated by hydrogen bonds, x-rays often only give incomplete, or sometimes even misleading, answers.

Neutrons, however, can locate hydrogen, and so they provide a powerful tool for analysing drug-binding interactions. In a crystal, the atoms are arranged in a regular array called a lattice. As the neutron waves hit the crystal, they interfere with each other (a process called diffraction) – like ripples meeting on the surface of a pool. This produces a scattering pattern that is characteristic of the atomic arrangement. Modern molecular biology emerged through the application of x-ray crystallography of key biological structures such as

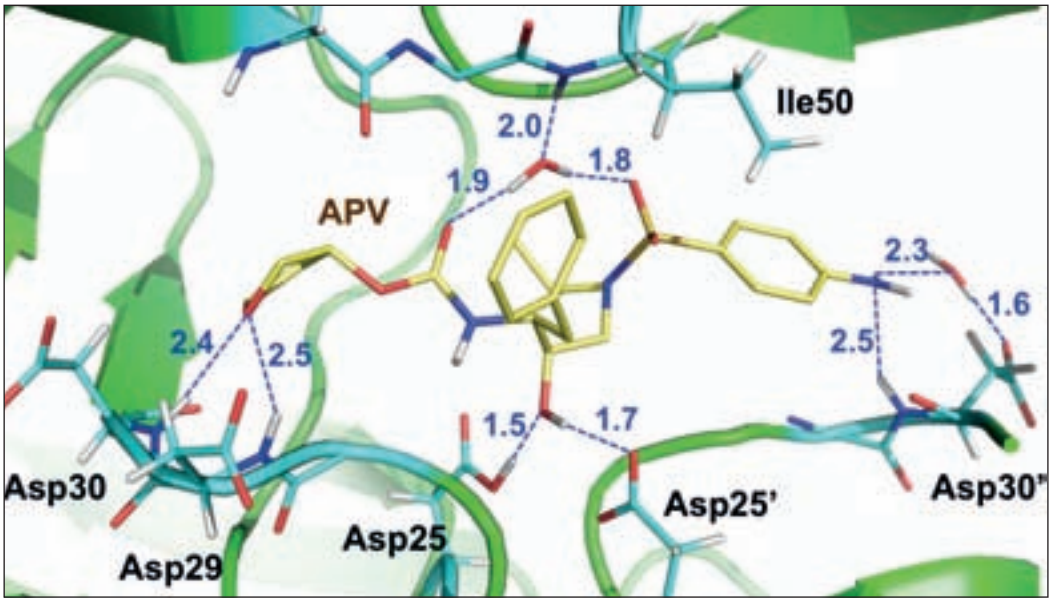

Figure 2
The active site in HIV-1 protease

DNA and proteins. This remains the principal and routine method for solving the structure of large biomolecules at atomic resolution.

Until recently, neutron crystallography was hampered by the fact that large crystals were required and data took a long time to collect. However, thanks to advances in instrumentation and sample preparation at the ILL, crystals can now be much smaller, only 0.05mm^3, opening up new avenues for structure-guided drug design using neutrons.

The potential has been acutely demonstrated in a recent study of the binding between an anti-retroviral HIV drug (amprenavir) and its target enzyme, HIV-1 protease[2]. This is a key enzyme in the HIV lifecycle, breaking down viral polypeptides to create the proteins needed for the maturation and the production of new infectious virus particles.

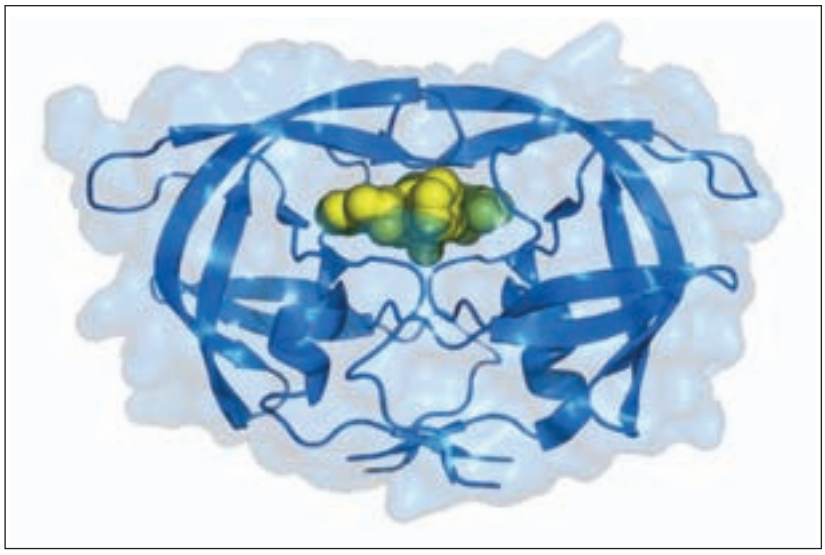

Figure 3: Amprenavir bound to the enzyme HIV-1 protease

Using a small crystal of HIV-1 protease bound to amprenavir, neutron diffraction data were collected at a resolution down to 0.2 nanometres. These data allowed the research team to locate the positions of the hydrogen atoms in the enzyme-drug complex, and, critically, to identify those participating in hydrogen bonding between the drug and the enzyme.

Previous x-ray studies had speculated that several hydrogen-bond interactions were important in the binding; however, the neutron study showed that, in fact, only two strong, direct hydrogen bonds exist between the drug and the enzyme. This finding offers drug designers new ways to strengthen the binding through subtle modifications of the drug's molecular structure, thereby increasing the effectiveness of the drug and reducing the necessary dosage. For example, the two strong hydrogen bonds can be made yet stronger by creating so-called low-barrier hydrogen bonds, via the introduction of a reactive atom such as fluorine. Alternatively, the weaker water-mediated hydrogen bonds could be replaced by stronger, direct hydrogen-bond interactions, by incorporating larger groups of atoms in the structure that would expel water molecules currently found in the binding site.

Overcoming drug resistance

Another important issue in combatting HIV infection is drug resistance. The evolution of the virus over time produces enzyme variants with weakened

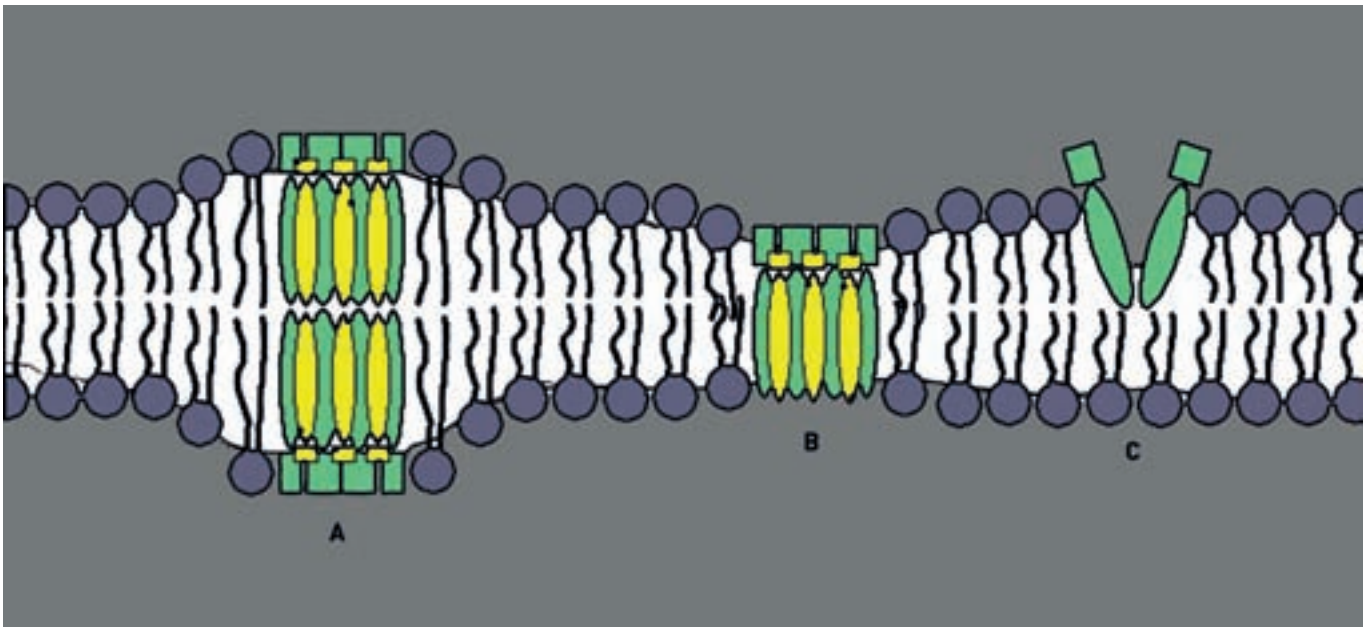

Figure 4
The various structures proposed for the pores formed by amphotericin in lipid bilayers: aligned half-pores/ion channels (A), half-width pores (B) and half-pores (C). Amphotericin molecules are shown in green, sterols, yellow and phospholipids, red

binding affinity – a process that is actually sped up by the introduction of the drugs themselves. One way around this would be to improve the binding of the drug with the atoms in the enzyme's main-chain rather than side-chain atoms, as the main-chain atoms cannot mutate. Before this latest study, researchers thought that the potential for advances in this area were limited, because the hydrogen bonds with the main-chain atoms were already strong. However, this has now been shown not to be the case, creating a new avenue for the development of HIV pharmaceuticals much less affected by virus evolution and resistance.

Improved antifungal drugs – neutron diffraction of Amphotericin B provides the first step in antibiotic-resistant drug development

Amphotericin B (AmB) is a drug widely used to treat life-threatening fungal infections that may arise in severely immuno-compromised patients, such as those who have undergone chemotherapy or contracted AIDS. Recently, however, fungal pathogens resistant to AmB have emerged. This has led to higher doses of amphotericin being prescribed, which can result in kidney damage. There is therefore an urgent need to find new drugs that work in the same way as AmB, but with a sufficiently-different molecular structure to stop the fungi overcoming its toxic effects.

Despite the fact that AmB has been in clinical use for more than 50 years, we still do not know exactly how it works at the molecular level. The accepted idea is that the molecule alters the structure of the cell's lipid membrane – a double layer of phospholipids embedded with other fatty molecules such as sterols. It is known that fungal cell membranes are slightly different from human ones in that they contain the sterol, ergosterol, instead of cholesterol. AmB is thought to interact more strongly with ergosterol than with cholesterol – but exactly how remains unclear. We looked to neutrons to find some answers.

Pinpointing the differences

Several ideas for the mechanism have been proposed, and the most classically accepted one is that the drug molecules insert themselves across the membrane so as to form pores. These facilitate the indiscriminate passage of ions in and out of the cell so that it can no longer function correctly and eventually dies. Recent evidence, however, has suggested that this description is too simplistic. The aim of the neutron studies described below was to find out the precise difference between the interactions of AmB and cell membranes containing cholesterol and those with ergosterol. In this way, it was hoped to pinpoint the critical chemical features needed in an effective anti-fungal agent. Neutron diffraction provided the methodology to do this.

In diffraction studies, the angle of scattering is inversely proportional to the scale of the structure producing the diffraction pattern. Beams of neutrons, deflected by an angle of only a degree or so, thus provide structural information at scales

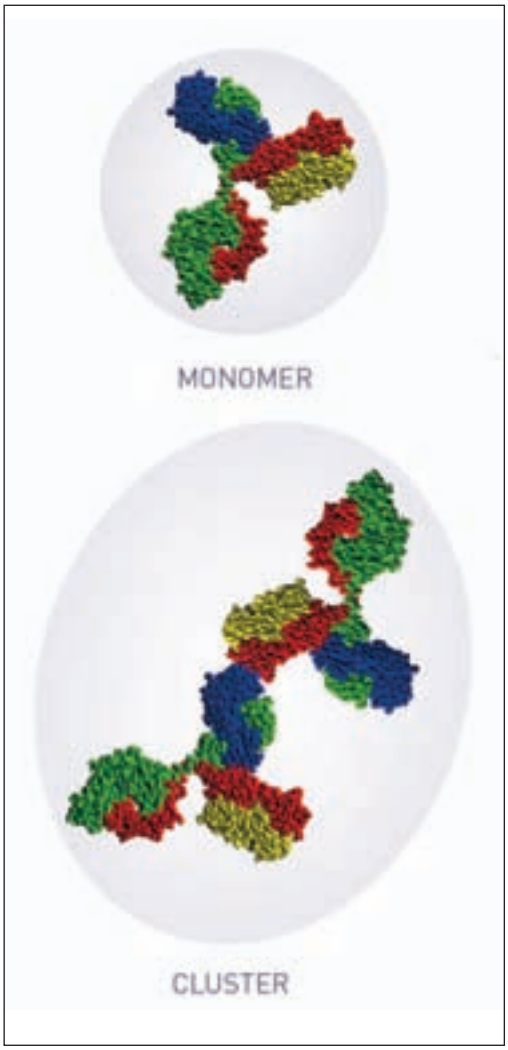

Figure 5
Monoclonal antibody proteins form clusters in solution which thickens it, making them difficult to inject

between one and several hundred nanometres, depending on the wavelengths used. Small-angle neutron scattering (SANS) is becoming ever more popular for probing larger cellular and other biological structures.

Experiments were carried out using model lipid membranes supported on silica, containing either cholesterol or ergosterol, with and without AmB[3]. The lipid layers were partially deuterated so that the position and orientation of AmB within them could be readily distinguished in the scattering patterns.

The results showed that AmB combined with both sterols to form pores in the lipid bilayer. However, the ergosterol molecules were more extended across both lipid layers, and that may help to stabilise the formation of continuous channels right across the membrane. The cholesterol-AmB combination tended to form separate 'half-channels' for each lipid layer, which were less likely to be aligned. The insertion of AmB also caused the sterols to become tilted with respect to the membrane, with cholesterol tilting more than ergosterol. It may be that AmB is able to react more readily with the latter.

A further study investigating the role of membrane phospholipid composition was conducted using neutron reflection, making use of the large difference in neutron scattering between hydrogen and deuterium isotopes to achieve contrast inside the complex membrane system[4]. It was found, for the first time ever, that the structure of fungal membranes depends on the degree of lipid polyunsaturation, which has an impact on the structural consequences of AmB activity. AmB inserts in membranes even in the absence of ergosterol; in ergosterol-containing membranes, AmB insertion is accompanied by ergosterol extraction into this layer. The resulting hydrophobic mismatch is likely to interfere with a much broader range of membrane protein functions than those directly involving ergosterol, and suggests that polyunsaturated lipids could boost the efficiency of AmB. Furthermore, a low degree of lipid polyunsaturation leads to least AmB insertion and may protect host cells against the toxic effects of AmB.

These experiments have provided a clear view of how AmB interacts with fungal and human cell membranes. These results provide a new framework based on lipid composition and membrane structure through which we can understand its antifungal action and develop better treatments. We now plan more detailed studies in which we hope to uncover the exact chemical differences between them and use that to design new anti-fungal drugs which lack AmB's damaging side-effects.

Towards self- injectable targeted drugs – neutron beams reveal how antibodies cluster in solution

Targeted treatment using monoclonal antibodies (mAbs) is an important tool in modern pharmacology; these large proteins provide the basis for a growing number of successful drugs for treating cancer, and also autoimmune disorders such as arthritis and multiple sclerosis. As agents for targeted therapy with a good safety profile, they are an alternative to harsher, traditional chemotherapy treatments.

The mAbs work by attaching themselves to specific protein targets (an antigen), for example, on cancerous cells or in a known biochemical pathway responsible for a disease. These treatments usually require high doses, which are fed through a series of intravenous drips in the clinic. Recently, there has

References

1 Campbell et al. ACS Macro Lett. 2014, 3, 121.
2 Weber et al. J. Med. Chem. 2013, 56, 5631.
3 Foglia, F et al. Sci. Rep. 2, 778; DOI:10.1038/srep00778 (2012).
4 de Ghellinck et al. Biochim Biophys Acta, Volume 1848, Issue 10, Part A, October 2015, Pages 2317–2325, DOI:10.1016/j.bbamem.2015.06.006.

been considerable interest in moving to a more convenient subcutaneous delivery (that is, via a shallow injection just below the skin, as routinely self-administered by diabetics). However, progress has been hampered by the fact that solutions containing large amounts of protein are very viscous for some mAbs. This makes them not only difficult to administer, but also presents considerable challenges to their large-scale production and purification. As a result, researchers have been trying to understand the root cause of this thickening so that less-viscous, mAbs-loaded systems can be devised.

To investigate the structure and dynamics of the clustering at the molecular length-scale, an international collaboration was lead by the US NIST Center of Neutron Research (NCNR) and included researchers from the biotechnology company Genentech, the University of Delaware and the ILL. By combining two neutron techniques – SANS and neutron spin-echo (NSE) – they studied two types of antibody: one known to increase the viscosity of the solution and one that does not, so as to compare their behaviour at the microscopic scale.

Probing cluster formation

Neutrons can be scattered in different ways. Instead of elastically bouncing off an atom like a tennis ball hitting a wall, they may lose or gain energy to or from the atom, such that the atom itself changes its motion. We can use this property to examine how a molecule moves and determine if a particular part of it is rigid or flexible. The way in which the molecule functions will often depend on this flexibility and the ability to adapt to the other molecules around it. Inelastic scattering techniques, including both spin-echo and quasi-elastic scattering, allows us to explore motions over periods from picoseconds to hundreds of nanoseconds, and so probe a wide range of dynamics, from the fast motions of small groups of atoms in proteins and other macromolecules to slower collective changes in large molecular assemblies and cellular structures. Dynamics studies are expected to become increasingly essential to understanding biological behaviour, and neutron-scattering experiments will be a crucial tool here.

NSE is able to track the individual movement of protein structures, as in the case of cluster formation of large biological molecules like mAbs. The unrivalled high resolution and neutron intensity provided by the ILL's NSE instrument, IN15, enabled many different mAb samples to be explored.

Previous light-scattering studies from mAb solutions had suggested that the high viscosity could be due to the formation of protein clusters. The results from the neutron-scattering experiment revealed that the mAb solutions with high viscosity are dominated by small mAb clusters. Once formed, the size of these clusters is almost constant over a wide range of concentrations.

This understanding provides the basis for designing an optimal device system for delivering injectable biopharmaceuticals at very high concentrations. In addition, the subject of protein clustering is an extremely interesting area in its own right. Many well-known phenomena, such as the cataracts in our eyes, or Alzheimer's disease, are the results of proteins clustering in our bodies. Neutron techniques provide a unique, high-resolution tool to investigate these complex interactions. This result also provides new physics insights into the phenomenon of protein clustering.

The future

The future role for neutron experiments in understanding biological processes is thus set to expand. The ILL will continue developing the necessary techniques and instruments, and providing experimental support and knowledge, to researchers in the life sciences who wish to exploit the huge analytical potential of neutrons. Neutron-based research will have a special role in not only uncovering the extraordinary subtleties of living processes but also in contributing to the research of treatments that enable us to live long and healthy lives. DDW

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