Current Chemical Biology Centre Doctoral Training Centre Projects

Projects 2008/09

Project Title

1. N-myristoyl transferase in apoptosis and disease: a novel target for cancer therapy
2. Single molecule studies on the allosteric behaviour of a protein folding nanomachine
3. An integrated theoretical and experimental platform to probing Protein-Protein Interactions (PPIs)
4. Modelling mutant receptor-ligand analogue pairs to predict unique phosphoinositide-protein interactions
5. FRET analysis of interactions of the macromolecular complex AMPK, a regulator of cellular metabolism
6. Biophysical Requirements of Zipper-Like Phagocytosis
7. Fragment tethering for the development of small molecule inhibitors of protein/protein interactions
8. How does alpha toxin break down the membrane?
9. Single molecule measurements of the catalytic events in proteolysis
10. Substrate-directed synthesis of chemical receptors for phosphatidylinositol phosphates to moderate lipid-protein interaction
11. Bridging the In-vivo In-Vitro Divide in Non-Specific Binding

Projects 2007/08

Project Title

1. Lipidation in retinal disease: applying chemistry to proteomics
2. Chemical Genetic Dissection of Histone Deacetylase function
3. Probing Cellular Membrane Dynamics using Dielectrophoretic Trapping
4. ‘Smart’ Supramolecular Polymers for Gene Delivery
5. ‘Smart’ molecular networks to unravel cell signalling
6. AAA Proteins: Using Chemistry to Understand a Universal Biomolecular Machine
8. Flipping DAG
9. Model Studies of an S-Acylated protein with cellular membranes

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Projects commencing 2008/09

1. N-myristoyl transferase in apoptosis and disease: a novel target for cancer therapy

Supervisors: Ed Tate, David Mann & Stuart Haslam

In contrast to other protein modifications, the factors that regulate protein acylation with long-chain acyl groups such as myristate (a C\textsubscript{14} unsaturated fatty acid) are not well understood. Cells make use of N-terminal protein myristoylation to anchor proteins to membranes in a reversible manner, and N-myristoyl
transfersases (NMTs), the enzymes that catalyse myristoylation in living organisms, play an important role in protein transport and signalling events at the membrane. In addition to a key role in programmed cell death (or ‘apoptosis’), NMT is widely recognised as an important but as yet poorly understood target for potential therapeutic intervention. Preliminary studies in mammalian disease models have demonstrated that NMT is a valid drug target in both cancer and infection (fungal and parasitic).

Despite the importance of myristoylation in health and disease, until very recently there has been no effective method available to analyse the protein targets of NMT in living cells. In common with other post-translational modifications, myristoylation has proven very difficult to study using traditional proteomic techniques, and yet this is critical for understanding the role NMT plays both in apoptosis and in the initiation and progression of cancer. This project will combine a novel chemical tagging technique with cutting edge post-translational proteomics to enable us to analyse global myristoylation in normal and cancerous cells for the first time. This approach will then be applied to probe the effect of novel chemical NMT inhibitors to understand how these potential drugs affect myristoylation in living cells, with the ultimate aim of designing new anti-cancer chemotherapeutics.

2. Single molecule studies on the allosteric behaviour of a protein folding nanomachine

*Supervisors: Keith Willison & David Klug*

It is becoming increasingly evident that proteins carry out their functions in cells as components of protein complexes which contain many other proteins; so-called protein nanomachines. The cytosolic chaperonin (CCT) is an ATP-dependent nanomachine which folds cytoskeletal proteins and regulates cell cycle progression in cells. CCT has 16 protein subunits, a mass of 1MDa, and is barrel-shaped with dimensions 15x16 nm. We have developed a pure system from yeast for studying CCT-folding of actin in ensemble and at single molecule level. This project will use fluorescent probes to label CCT and actin in order to analyse folding cycles at single molecule level using FRET in a TIRF microscope. Mutants of CCT and yeast actin will be used to unpick transitions and verify models derived from kinetic analysis of the wild type system. The student will measure distances and angular distributions between landmark probes in the protein assembly and their time-dependent appearance and decay during the folding process which takes a few minutes to complete. The aim is to determine the pathway in which CCT captures an actin folding intermediate and conveys it to the native state to elucidate the biophysical and energetic consequences of this process for the function of native actin in its filamentous form.

3. An integrated theoretical and experimental platform to probing Protein-Protein Interactions (PPIs)

*Supervisors: Ian Gould, Robin Leatherbarrow & Ed Tate*

Protein-protein interactions (PPIs) mediate all cellular processes, and are a central focus of post-genomic biology. A rich variety of PPIs in both humans and pathogenic organisms have been validated as candidates for chemotherapy; however, the large interacting surface areas involved (>1600Å²) have proved extremely difficult to target using traditional small molecule approaches. It has been estimated that there are about 40,000 therapeutically important PPIs in humans alone and a universal chemical method for blocking and modulating these interactions would have a dramatic and wide-ranging impact in both basic research and drug discovery.

This highly multidisciplinary project will bring together innovations in molecular modelling, microprotein synthesis and *in silico* and *in vitro* directed evolution to develop highly structured macrocyclic probes engineered to make multiple specific contacts at a protein-protein interface. Cyclotides, naturally-occurring head-to-tail cyclic proteins containing a cysteine knot motif, have emerged as an ideal experimental platform to develop such probes: they possess a highly constrained and well-defined structure that is well-suited to biomolecular modelling and structural studies, but are small enough (ca. 30 residues) for chemical synthesis; they also possess extraordinary bio-stability and a wide range of known biological activities. This
potentially general platform for interrupting PPIs in a highly selective manner will be applied to target PPIs involved in real-world problems in basic biology and disease processes.

4. Modelling mutant receptor-ligand analogue pairs to predict unique phosphoinositide-protein interactions

Supervisors: Ian Gould, Piers Gaffney, & David Mann

The proper working of multi-cellular organisms depends on signalling between and within cells to orchestrate life’s processes. A major player in this symphony is a class of lipids, the phosphoinositides (PIs), which relay the signals underlying, for example, insulin action and growth factor stimulation. These external hormones’ information is initially transmitted from receptors to the inner surface of the cell membrane where PIs create docking sites that downstream proteins recognise. Malfunctions here can be responsible for diseases such as diabetes and cancer, but understanding the signalling networks so that a rational site for medicinal intervention can be identified is fraught with difficulty. It is particularly hard in this case because there are many PIs classes of PI binding proteins, and apparently separate pathways often disentangle the outputs from each other. We have been pursuing a program of receptor-ligand engineering to disentangle these pathways. For this purpose analogues of PI lipid head-groups have been prepared with unnatural polar or bulky protrusions that prevent them fitting into their native PI binding proteins. The aim of this project is to identify mutants of PI binding domains that complement the modified head-groups, re-establishing specific recognition, but now of only one protein with one unique lipid. We plan to short-cut laborious screening in vitro by docking modified PI head-groups to mutant binding domains in silico using state of the art molecular modelling techniques. Not only will this allow us to test a far greater number of ligands on a far wider range of proteins than is practical in vitro, it will also allows us to optimise the fit and propose second generation synthetic targets and engineered proteins. Best fits will then be reality-checked in the wet lab studying the strengths of binding between our PI analogues and mutant PI binding domains. Ultimately a successful mutant PI binding domain will be re-engineered into full-length signalling proteins and expressed in cells. It is anticipated that when the corresponding modified PI is added, only the signalling system directly downstream of this unnatural receptor-ligand pair will be activated, and so the components of this system should be much easier to characterise.

5. FRET analysis of interactions of the macromolecular complex AMPK, a regulator of cellular metabolism

Supervisors: Alessandro Sardini, David Carling, Paul French, Mark Neil & Chris Dunsby

AMP-activated protein kinase (AMPK) is a macromolecular complex constituted of three protein subunits (α, β and γ). Recently we (CSC) reported (Nature; doi:10.1038/nature06161) the crystal structure of the regulatory fragment of the mammalian AMPK and determined the basis of AMP and ATP binding to the complex. This has suggested that AMP binding triggers the formation of inter-subunit interactions that are not possible when ATP is bound. Although binding of nucleotides to the γ subunit of the complex has been extensively studied, the fundamental issue of the consequent activation, upon binding of AMP, of the kinase domain, which resides in the α subunit, has remained elusive. Changes in the quaternary AMPK complex structure, upon binding of AMP, will be investigated by FLIM analysis of FRET signals of appropriately positioned fluorophores genetically codified. The acceptor-donor fluorophore couple will be inserted in the AMPK subunits in such a way to report their reciprocal movements. Initial FRET experiments will be performed on proteins in solution, by purification of the individual subunits of AMPK and by reconstitution of the complex, using a novel hyperspectral lifetime (HSL) fluorimeter developed in Physics. Subsequently we will image single cells transfected with the individual subunits using a unique new high-speed automated optically-sectioning FLIM-FRET microscope system, currently under development in Physics. Known human AMPK sequence mutations responsible for changes in enzymatic activity will be also tested as potential modifiers of subunit interactions. AMPK activity is also regulated by the phosphorylation of a critical residue (T172) within the catalytic domain by the upstream kinases LKB-1 and CamKK β. The
kinetics of such regulation and its cellular location will be followed in live cells by FRET analysis. Factors that influence phosphorylation, such as pH, Ca$^{2+}$, growth factors and pharmacological agents, will be investigated. Multiplexed FRET experiments will also developed to synchronously measure the AMPK FRET signal with Ca$^{2+}$, pH or NADH signals, using extrinsic probes and/or intrinsic autofluorescence as read outs to study influence of activation of AMPK on cellular metabolism.

6. Biophysical Requirements of Zipper-Like Phagocytosis

*Supervisors:* Emmanuelle Caron & Robert Endres

Phagocytosis allows cells to uptake particles (bacteria and organic material above 0.5 μm in diameter), and is predominantly performed by specialized cells of the immune system. Importantly, a successful uptake can only be completed if interactions between cell surface receptors and ligands on the particle occur circumferentially around the whole of the particle (Zipper model). However, almost nothing is known of the biophysical requirements of the recognition and uptake process, such as the ligand density, spatial organization of receptors and ligands, and to what degree the process is cooperative. To address these questions the student will develop new experimental and theoretical approaches.

7. Fragment tethering for the development of small molecule inhibitors of protein/protein interactions

*Supervisors:* Alan Armstrong, David Mann & Caroline Low

Members of the cdc25 family of protein phosphatases (enzymes which remove phosphate groups from protein substrates) are key regulators of the eukaryotic cell division cycle and so are important potential targets for anti-cancer agents. To date, drug development studies have targeted the catalytic domain of the cdc25 phosphatases, but the relatively shallow nature of this binding site has made it difficult to generate selective small-molecule inhibitors. This project aims to develop small molecules which effect phosphatase inhibition in a different way: by targeting a binding "hotspot" between the phosphatase and its substrate protein which is remote from the catalytic domain. The high potential therapeutic value (anti-cancer) of successful inhibitors, as well as the novel demonstration of the possibility of development of small molecule inhibitors of a protein-protein interaction at a remote interaction “hotspot”, means that successful results would be of very high profile and of exceptional interest to the pharmaceutical industry. The project will involve novel synthetic chemistry (small molecule synthesis, library preparation), molecular biology and molecular modelling. In order to find new small molecule inhibitors which bind at the remote binding site, we will use a technique known as "fragment tethering" which involves engineering a cysteine residue close to the target site. The resulting thiol unit will then be exposed to a focused library of small molecules which will can attach to the cysteine thiol by reversible formation of disulfide or thioester bonds. The members of the library which bind most strongly to the target site should selectively "stick" to the engineered thiol. Identification of these fragments, in conjunction with molecular modelling studies to help understand how they bind to the target site, should then allow us to design and synthesise novel phosphatase inhibitors.

8. How does alpha toxin break down the membrane?

*Supervisors:* Katherine A. Brown, Richard Templer, Neil Fairweather

Bacterial phospholipases play the key role in enabling bacterial pathogens to cross the cell membrane barrier, but the processes by which they destroy the membrane are poorly understood. This project will probe the processes guiding molecular recognition, phospholipid hydrolysis and membrane destruction by the Clostridial phospholipase, alpha toxin. We are particularly interested in how changes in membrane stress and interfacial curvature, caused by hydrolysis products, affect the phospholipase’s activity and the membrane’s porosity. The student will study the effects of systematic changes to both enzyme and model lipid membrane. Recombinant forms of the enzymes, including truncated domains and mutants which alter substrate
recognition and hydrolysis, will be generated and their behaviour measured as a function of the stored elastic energy and interfacial charge state of the lipid membrane. Using physical techniques developed in the Brown and Templer groups will enable the student to measure enzyme partition coefficient, lipid degradation products rates, some changes in enzyme structure and changes in membrane structure and internal membrane stress during binding and hydrolysis.

9. Single molecule measurements of the catalytic events in proteolysis

*Supervisors:* Robin Leatherbarrow & Liming Ying

We wish to characterise the molecular interaction between a protease enzyme and a target polypeptide using a single molecule approach. These measurements will encompass both the molecular encounter and the catalytic processes, giving rise to a full kinetic description of the enzyme-catalyzed events. Unlike conventional protein chemistry, this will not be determined for an ensemble but for individual molecules. The system to be studied will involve the 3C protease from Foot and Mouth Disease Virus, which is a highly specific enzyme involved in the viral life cycle of this pathogen— inhibitors of 3C are currently being developed as potential disease control agents. This enzyme is available in recombinant form, has recently had its structure solved and has well-defined substrate specificity (Leatherbarrow and S. Curry). Making use of techniques developed by a current CBC student, we are able to add fluorescent tags to the N-terminus of a protein in a highly selective manner; chemical synthesis of polypeptide substrates will be used to introduce further fluorescent tags either side of the scissile bond. This will provide 3 specific loci that will be used to measure either the enzyme-substrate interaction or the catalytic turnover by making use of single pair fluorescence resonance energy transfer (sp-FRET). We will apply high order correlation analysis to the fluorescence trajectory during enzyme turnover to explore possible dynamic disorder associated with the enzyme kinetics.

10. Substrate-directed synthesis of chemical receptors for phosphatidylinositol phosphates to moderate lipid-protein interaction

*Supervisors:* Ramon Vilar & Rudiger Woscholski

Phosphoinositide signalling is an important research area for our understanding of many diseases and human health in general. For example, diabetes, cancer and neurodegenerative diseases are strongly linked to defects in these pathways. Elucidating these signalling pathways was greatly facilitated by the advent of more or less specific inhibitors to the enzymes involved in the turnover of these inositol lipids. In particular, pathways that are governed by the phosphoinositide 3-kinase, which is responsible for the generation of the second messenger PtdInsP3, have mapped with the help of chemical intervention (Wortmannin and LY294002). However, these inhibitors are not very specific for a particular lipid. They will, when applied to cells, take out all 3-phosphorylated inositol lipids and will also affect several related protein kinase (eg. DNA-PK, ATM, mTOR). Thus, there is a considerable void in lipid specific inhibitors, which would have a much-improved specificity as compared to ATP-competitive kinase inhibitors. We therefore propose to generate chemical receptors that are capable of specifically binding to the second messenger PIP3, in order to provide a novel tool to disturb cellular PIP3 levels without affecting any other lipid or metabolite directly. The development of these chemical receptors will be based on a target-guided synthetic approach in which several small building blocks will be joined together using PIP3’s head group as a template. Our previous work on chemical receptors indicates that this goal can be realistically achieved within a PhD project and would build on this experience to generate this important chemical intervention tool.

11. Bridging the In-vivo In-Vitro Divide in Non-Specific Binding

*Supervisors:* Tony Gee, Richard Templer, Nick Long & Oscar Ces

Recent research at Imperial has uncovered significant evidence that CAD (Cationic Amphiphilic Drug) molecules may be transported across biological membranes by a novel phospholipid hydrolysis mechanism. This may be an important mechanism which if understood and controlled 1) may enable the efficient design of in-vivo imaging agents (eg. PET probes) by tuning their non-specific binding (NSB) properties, 2) enable
the more efficient design and selection of drugs acting at target proteins by providing engineering rules and novel tools that predict their NSB profile and 3) may generate an understanding of toxicological processes such as phospholipidosis. The aim of this project is to investigate the structure-activity relationship for CAD catalysis of phospholipid membranes by synthesising a suite of structurally modified compounds (Long). The modifications will include the effect of structural modifications to protonated amine functionalities that are implicated in the ester hydrolysis phenomenon. The membrane binding affinities and NSB properties of this novel suite of molecules will be predicted and tested using a multidisciplinary closed feedback loop that will incorporate: (a) in-silico ab-initio platforms (preliminary studies have already demonstrated correlations between NSB behavioural patterns and the interaction energy between a single phospholipid molecule and a given test CAD compound), (b) the measurements of in-vitro membrane hydrolysis rates using assays developed by Templer and Ces using model membrane systems, (c) SSNMR to determine atomic interactions and spatial alignment in membrane constructs (in collaboration with R.Law) and (d) in-vivo validation by labelling with positron emitting radionucleides and determination of non-specific binding in authentic tissue using tissue section autoradiography and PET imaging (Gee).

NB: All the projects listed below were selected to fit the original DTC theme “Multidisciplinary molecular studies at or near the membrane”. The DTC theme changed in 2008/09 to “Protein-protein and protein-lipid interactions as targets for biological intervention” – see projects listed above.

Projects 2007/08

1. Lipidation in retinal disease: applying chemistry to proteomics
Supervisors: Ed Tate & Miguel Seabra
Student: Alex Berry

Post-translational geranylgeranylation of Rab proteins is known to play a central role in intracellular organelle biogenesis, motility and communication between them via vesicular transport. Rab geranylgeranyl transferase (RGGT), the enzyme that performs this post-translational modification, plays a key role in trafficking proteins between the membrane-delimited compartments of the cell. Importantly, defects in this fundamental process have serious medical implications. For example, defective geranylgeranylation of Rab27 has been shown to contribute to degenerative retinal disease in Choroideremia, a form of inherited blindness. A generic method for the identification of the targets of GGTs would enable and accelerate investigations into the functional biology of protein lipidation, but current techniques lack the requisite selectivity for proteins bearing a specific prenyl modification. Tagging-by-substrate (TBS) is a powerful emerging technology that overcomes many of the problems commonly encountered in proteomics of post-translationally modified proteins. In this approach a synthetic transferase substrate bearing a small and biologically inert chemical tag is fed to cells and incorporated into modified proteins metabolically in vivo. Tagged proteins are then captured using a highly selective bio-orthogonal reaction, and by incorporating a dye and/or affinity label into the capture reagent the modified proteome can be visualised or enriched, greatly enhancing identification. The MRes/PhD project will develop TBS for the enrichment, visualisation and identification of geranylgeranylated proteins and, by applying TBS to cell and mouse disease models, identify mechanisms by which defective lipidation of Rab proteins in retinal cells may contribute to degenerative retinal disease.

Techniques that the student will learn during the project include organic synthesis, molecular biology (expression of recombinant proteins), biophysical assays (membrane-binding affinity of lipidated proteins), proteomics (2DGE, protein mass spectrometry) and cell biology (cell culture, microscopy).

2. Chemical Genetic Dissection of Histone Deacetyrase function
Supervisors: Eric Lam, Alan Armstrong & David Mann
Student: Robert Felstead

Breast cancer is one of the most commonly diagnosed malignancies and one of the main causes of cancer death among women. It has become apparent that imbalances of histone acetylation play a significant role
in cancer development and progression, and HDACs (histone deacetylases) have an important role in tumourogenesis. We aim to generate inhibitors specific for particular HDACs to understand their roles and to restore normal cell control in breast cancer

3. Probing Cellular Membrane Dynamics using Dielectrophoretic Trapping

Supervisors: Joshua Edel, Andrew de Mello & Tony Cass
Student: Fabrice Gielen

This research project will focus on the development of novel microfluidic methods for electrical trapping of cells or small particles (1-20μm) over extended periods of times. This will facilitate probing of single molecule membrane dynamics using total internal reflection spectroscopy on timescales on the order of minutes. The microfluidic platform will enable the variables such as buffer composition to be continuously varied while probing changes in membrane dynamics in real time. The programme can be broken down into four distinct phases. 1) Design, fabrication and testing of microfluidic structures with ‘built in’ electrodes having dimensions on the order of 20-100 μm. 2) Creation of optical detection technology and dielectrophoretic trapping electronics. 3) Development of algorithms to trap and track single molecule membrane activity. Single molecule tracking will be performed via imaging fluorescence emission using an electron multiplying CCD camera. Micrometer sized fluorescently labelled particles will be used as proof of principle to test phases 1-3. 4) Further experiments will involve to delivery of rat basophilic leukaemia cells containing fluorophore – labelled C18 in their cell membrane into the dielectrophoretic trap. Monitoring of C18 diffusing along the membrane will be performed under varying buffer and temperature compositions. Such experiments open the door to developing novel technologies for monitoring membrane dynamics of extended periods of time with single molecule resolution.

4. ‘Smart’ Supramolecular Polymers for Gene Delivery

Supervisors: Joachim Steinke, Maya Thanou & Sunil Shaunak
Student: Bryn Monnery

Polycations are used widely in drug and gene delivery. They condense and protect nucleic acids or proteins and by doing so, allow for successful delivery into cells. Nevertheless the issue of their safety appeared recently to be controversial as well as the function of the polycations for endocytosis. This project aims at designing, preparing and characterising a series of structurally well-defined polycations for further investigations of their association with cell membranes. Therefore, in this project a structure-activity relationship will be investigated. Libraries of polycations varying in structure and charge density will be prepared and investigated in a number of cell lines for their effect on the cell membrane using semi high-throughput techniques.

This is the first time that polymer architecture, size and charge will be studied in a systematic manner. The student will perform investigations to answer the following questions:
Effect of chemical functionality and local concentration of the polycation on the cell membrane
Effect of architecture on cell membrane perturbation: linear versus branched (dendritic)
Effect of size (hydrodynamic radius and molecular weight) of both linear and branched polymers on the cell membrane integrity
Effect of charge on cell membrane association (pH dependent versus quaternised cations)

5. ‘Smart’ molecular networks to unravel cell signalling

Supervisors: Joachim Steinke, David Mann & Ramon Vilà
Student: Chirag Patel

Reversible protein phosphorylation is a universal means of regulating many cellular processes including cell division, glucose metabolism, development and differentiation. Aberrant phosphorylation often causes or exasperates disease progression (e.g. cancer). While significant progress has been made in the
identification and biochemical characterisation of the protein kinases and phosphatases themselves, it has proven significantly more problematical to identify their substrates. In vitro consensus phosphorylation sites have been defined for many kinases. Unfortunately, these tend to be of little predictive value in substrate identification; they are generally common motifs of a few amino acids that occur stochastically with high frequency in the proteome. In this project we propose to utilise molecularly imprinted polymers (MIPs) to physically isolate phosphopeptides from complex peptide mixtures via an affinity separation process. MIPs are synthetic solid phase receptors which can be prepared to possess specific selectivity towards a particular target structure; in our case a particular epitope of a kinase substrate. The generation of a phosphopeptide-binding matrix would enable purification of phosphorylated proteins/peptides and their subsequent identification by mass spectrometry, considerably advancing our ability to analyse and interpret cell signalling processes

6. AAA Proteins: Using Chemistry to Understand a Universal Biomolecular Machine

Supervisors: Martin Buck, Ed Tate & Xiaodong Zhang

Student: Lucy Rush

The AAA (ATPase Associated Activity) proteins are a large family of mechanoenzymes that have evolved unique ways of using a fundamentally similar conformational change in many different biological settings. A conserved ATP-dependent conformational switch applies tension to bound substrates and thereby allows AAA proteins to unfold polypeptides, open double-stranded DNA, dissociate protein-protein interactions, or generate unidirectional motion along a track, and thus AAA proteins play key roles in myriad cellular functions in both prokaryotic and eukaryotic cells. Studies on these important proteins has been limited by drawbacks in current techniques: the use of genetics to study the role of AAA protein complexes is compromised by the pleiotropic and/or lethal effects of AAA mutants, and targeting the ATP binding site using small molecules lacks specificity towards key protein-protein interactions. This project aims to develop a physical technique, termed structure-guided chemical genetics, to modulate specific protein-protein interactions in wild-type AAA complexes in a manner that overcomes the problems inherent in existing methods.

The initial target of this work will be PspF, an archetypal AAA protein from E. coli for which we have extensive structural data and well-established in vitro and in vivo functional assays. PspF represents an ideal tractable model system in which to study the conserved ATP-dependent conformational switch, and work on this protein will enable us to obtain wide-ranging mechanistic insights that apply generally to AAA proteins, including those from eukaryotic organisms. PspF is a transcriptional activator that uses the energy released during ATP-hydrolysis to remodel the o54-RNA polymerase holoenzyme, causing its isomerisation. It forms a stable complex with sigma factor o54 at the point of ATP hydrolysis, and recent work in our labs has shown that binding occurs via two mobile surface loops that transfer energy from PspF to RNAP via o54. The student will leverage this structural data to design and synthesise constrained peptide mimics of these loops that can inhibit specific PspF-PspF and PspF-o54 interactions. Established transcriptional activation assays will be used to assess the efficacy of the inhibitors in vitro and their potency will be optimised by one-bead one-peptide library techniques; binding of the strongest inhibitors will be characterised by physical techniques (ITC, NMR/crystallography). In the later stages of the PhD the student will develop methods for delivering the peptide tools into live cells, and the exquisite selectivity of these inhibitors for different functional forms of the PspF-holoenzyme complex will be exploited as a tool to illuminate AAA mechanism in vivo. A further important outcome of this work will be tools and technology that will enable parallel research on eukaryotic AAA proteins (e.g. p97, an AAA protein involved in membrane biogenesis) using structure-guided chemical genetics. Techniques the student will learn during the project include synthetic chemistry, library generation and screening, molecular biology (expression of recombinant AAA proteins), biophysical assays (measurement of binding affinity), structural biology (NMR, crystallography) and in vitro and cell-based assays.


Supervisors: Ramon Vilar & Rudiger Woscholski
Student: Verity Stafford

The symptoms of patients suffering from the inborn error “oculocerebrorenal syndrome of Lowe” (OCRL) are due to the loss of the phosphatidylinositol 4,5-bisphosphate (PIP2) 5-phosphatase activity encoded by the OCRL1 gene, which is missing or truncated in OCRL patients. Consequently, high levels of PIP2 are an indication of the disease, which together with reduced phosphatase levels form the basis for the currently employed biochemical diagnosis of OCRL. However, the biochemical OCRL diagnosis is cumbersome and restricted to a few places worldwide, which is hindering any progress in diagnosis and clinical research. Thus, it would be extremely useful to have an easy, robust and sensitive method to detect the amount of PIP2 in the cell and/or tissue sample. The aim of this project is to develop a synthetic PIP2 receptor capable of selectively binding to the head-group of this substrate (inositol trisphosphate; IP3). Such a receptor (when properly coupled to a luminophore or chromophore) would be able to detect nanomol amounts of PIP2 and/or IP3; hence, it could be used as a diagnostic tool for detecting OCRL

8. Flipping DAG
Supervisors: Tony Magee, John Seddon & Rob Law
Student: Arwen Tyler

How cells interact or “talk” with each other is vital to understand the process of disease. The signalling protein molecules exist at the boundary of cells in a bilayer called the plasma membrane, which has inner and outer layers. This membrane is a two-dimensional fluid made up of amphiphilic or detergent type molecules called phospholipids. Previously, it has been thought that the signalling proteins which span across or bind to the plasma membrane were not influenced by it, however, recently this premise has been questioned. It is now believed that cholesterol plays a vital part in changing how the phospholipids behave and consequently how the signalling proteins aggregate in the plasma membrane. The cholesterol rich microdomains thought to be responsible for the modulation of membrane protein behaviour are called “lipid rafts” and reside mainly of the outer layer of the plasma membrane where conditions are better suited for their formation. An important cell signally pathway involves two special lipids, phosphatidylinositol 4,5-biphospahte (PIP2) and diacylglycerol (DAG). It is now thought that DAG rapidly flips from inner side of the bilayer to the oily or hydrophobic lipid rafts areas on the outside, where it temporarily resides. The outer lipid rafts now act a subtle moderator on the rate of the translocation back to the inner membrane where it can reactivate its signalling pathway. The focus of this project is the examination of the lipid rafts on the rate of DAG flipping

9. Model Studies of an S-Acylated protein with cellular membranes
Supervisors: John Seddon, Rob Law & Tony Magee
Student: Anna Markiewicz

Lipid modifications of proteins play key roles in localising them to cellular membranes, and hence affect function. In eukaryotic cells a major lipid modification is S-acylation with long chain fatty acids. One major function of S-acylation is targeting to membrane microdomains such as “lipid rafts” (LRs), which are formed by lateral segregation of lipids and cholesterol within the plane of cellular membranes due to differential lipid-lipid packing. We have shown that LRs are hotspots for both protein and lipid signalling pathways in immune cells. The developing view of LRs is of a highly dynamic situation where ordered lipid domains are transient and very small (<50nm across) but can be stabilised by subtle changes in membrane organisation, e.g. by promoting protein-protein interactions. Biophysical studies suggest that defined mixtures of saturated and unsaturated glycerophospholipids and cholesterol form a system that can separate into disordered (La-like) and ordered (Lo-like) phases under physiological conditions. However, the interactions of lipid-modified proteins with such lipid bilayers have until now not been studied. Here we plan to study the interaction of a recombinant S-acylated protein with lipid membranes of defined composition and phase, using a range of cell biological and biophysical techniques such as fluorescence microscopy,
differential scanning calorimetry, pressure perturbation calorimetry, solid-state NMR and X-ray diffraction. This will greatly enhance our understanding of the formation of LRs and their role in vivo.

Projects 2006/07

1. PhD Studentship: V2D: Virtual 2-Dimensional Capillary Electrophoresis for Protein Separation & Identification
   
   **Supervisors:** John Hassard, Andrew de Mello & Tony Cass
   
   **Student:** Andrea Laine

   Increasing attention has been directed towards proteins and their roles in diseases and their utility in diagnosis. For protein investigation, gel separation over 2 different attributes of a protein is a powerful tool, generally considered to be the cornerstone of wide sectors of biomedicine, drug discovery, diagnosis and the elucidation of disease. While conventional techniques such as 2-dimensional SDS polyacrylamide gel electrophoresis allow separation, identification and capture of proteins, they have significant limitations including complexity, speed, resolution, accuracy, dynamic range, quantification and reproducibility. In the proposed project, a system will be developed where proteins in complex mixtures are identified by controlled separation with a microfluidic device. Conventional separation over two attributes in two dimensions will be replaced by a ‘virtual’ 2-dimensional system in which proteins are separated by capillary electrophoresis, resulting in 2 independent measures of the protein for identification purposes and an accurate measure of how much protein is present. This will enable the system to be more powerful, accurate, cheaper, faster and far easier to use than existing technologies. The project will encompass the development and testing of a pH gradient switch within a microfluidic chip, the integration with an optical detection system, advanced information processing and validation of the system against key biological samples.

2. The investigation of sub-millisecond biological reaction kinetics using continuous flow microfluidics
   
   **Supervisors:** Andrew de Mello, Paul French, Geoff Baldwin, Mark Neil, John Eccleston
   
   **Student:** Tom Robinson

   This project aims to develop a new generation of ultra-fast microfluidic mixers for probing reaction which occur on timescales significantly less than 1ms. Such timescales are an order of magnitude faster than current stopped-flow systems, which have dead times of 1.5ms and will therefore allow the investigation of biochemical reaction mechanisms that are currently beyond the scope of modern technologies. Using our approach, we expect to achieve a temporal resolution below 50μs, whilst only consuming nanolitres of fluid per second and will use multidimensional fluorescence imaging (MDFI) to enhance the information content. The first phase of the project will involve the design, fabrication and testing of the microfluidic mixers using model reaction systems and MDFI to provide 3D images of fluid dynamics. We will use this new microfluidic technology to probe biochemical reaction mechanisms on ultra-short timescales. We aim to investigate the kinetics of cell signalling processes using the Ras GDP complex, which is membrane bound and the activating nucleotide exchange factor SOS. This interaction has been difficult to follow using conventional rapid kinetic methods. In addition, the interaction of proteins and DNA is of fundamental importance and is ultimately the final stage in many signalling cascades. Understanding how proteins locate their target site on DNA has been the subject of much study, but has been hampered by the limitations of conventional kinetic techniques. We plan to use uracil DNA glycosylase (UDG) as a model system. We already have significant information for this system, which will make it an ideal test bed for the new technology, whilst the ability to resolve faster events will enable us to make a critical advancement in our understanding of DNA-protein interactions.

3. Studies of the non-specific/specific binding of drug molecules to biological membranes.
   
   **Supervisors:** Oscar Ces, Nick Long, Antony D Gee
Student: Duncan Casey

We have recently discovered a novel translocation mechanism for the CAD class of drugs: a catalytic reaction that degrades the phospholipid fabric of the cell membrane. This work points to a previously unreported mechanism for membrane translocation in which the resulting membranous fragments carry the drug across and away from the membrane barrier. We have also discovered that there is a direct correlation between the rate of this reaction and the ability of a drug to bind specifically/non specifically in-vivo. Being able to predict the specific/non-specific binding properties of drug molecules a priori is one of the most intense areas of research in the pharmaceutical industry. In collaboration with GSK we aim to study the mechanism of this reaction by synthesising a number of systematically modified CAD molecules that have predicted differences in the degree of non-specific binding, based on this emerging hypothesis. Compounds with chemical fragments of these CAD’s such as amide and fluorinated benzoyl species will also be utilised and their behaviour monitored at the membrane in order to test the hypothesis and evaluate whether any sites on the CADs are particularly important. This will allow us to develop a predictive model which can be tested both in-vitro and then in-vivo using PET labelled 11C and 18F analogues. The chemical effect of CADs on model membranes and its link to specific/non-specific binding will be studied using transmission electron microscopy (TM), fluorescence microscopy (FM), nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS) and small angle X-ray scattering (SAXS). Whilst our initial focus will be on PET-labelled species, in the longer term we envisage that the systematically-modified CAD molecules will also be tagged by simple organic fluorophores or quantum dots, resulting in the formation of novel dual imaging (PET/optical) agents.

4. Protein folding in regulation of the cell cycle
Supervisors: Keith Willison, Robin Leatherbarrow, Steve Matthews
Student: Sarah Todman

The anaphase-promoting complex (APC) is an E3 ubiquitin ligase which targets cell cycle proteins for degradation. APC is a complex protein nanomachine of 1MDa mass composed of more than 12 subunits. Its specificity towards its protein substrates is determined by two co-activators, CDH1 and CDC20, which function at different phases of the cell cycle. Both these proteins have a 7-bladed WD40 repeat propeller encoded at their C-terminus. Each WD40 blade is constructed from 4 β-strands (ABCD) which fold up into a wedge. Each blade is encoded in this order except for the seventh; strand 7D is encoded at the N-terminus of the first repeating unit and strands 7ABC at the C-terminus after the sixth unit. This causes a protein folding problem because 7D requires insertion at the final stage of ring closure. This folding step performed while the WD40 protein is bound to the cytosolic chaperonin CCT, itself a 1MDa protein nanomachine.

The project involves synthesising and/or expressing single blades and sets of blades for analysis using a combination of biophysical techniques and structural biology. The various components of the system will be analysed using NMR in order to define the structural organisation of the propeller architecture. Hybrid and segmental labelling schemes will facilitate simplification of NMR spectra and the assignment process and we will use TROSY NMR spectroscopy to monitor chemical shift and relaxation information in large fragments of the CCT/CDH1 complex. TROSY NMR spectroscopy results in a dramatic decrease in resonance line-widths that enables spectra to be obtained for proteins in excess of 100kDa. Model systems will also be constructed that incorporate just the 7-bladed propeller along with additional reporter groups that will allow us to study the ring closure events that occur during CCT-mediated folding using FRET and this will allow direct analysis of the ring closing kinetics. Such information will be related to the structural and dynamics features of CCT/CDH1 in its various functional states.

5. Identifying protein kinase substrates using chemical biology
Supervisors: David Mann, Alan Armstrong, Peter Parker
Student: Karen McCague
Protein phosphorylation is a universal means of regulating protein function. These phosphorylation events are carried out by the protein kinase family of enzymes. Mammalian cells possess many hundreds of different protein kinases all targeting a distinct set of substrates. Since many of these different kinases are active simultaneously within a cell, it is extremely difficult to determine which kinase phosphorylates which substrate protein. Small molecule inhibitors have been useful in dissecting some functions of protein kinases but, since all protein kinases share a similar catalytic architecture, many small molecules will inhibit a large subset of the cellular kinases. This project aims to circumvent this problem by using a chemical genetic approach to the identification of protein kinase substrates. The student will make mutations in one specific protein kinase catalytic site so that the enzymatic function is not compromised but which provide space for accepting a more bulky version of a known protein kinase inhibitor; this inhibitor analogue will not bind to wild type protein kinases. This approach will allow the small molecule to target one specific protein kinase. This approach will be extended so that the inhibitor molecule includes a suitably positioned azide group which, when appropriately irradiated, will cross-link to nearby proteins. It is envisaged that this cross-linking unit will covalently attach to the substrates of the mutant kinase, providing a means of substrate isolation and hence identification.

6. Probing Signalling in membrane nanotubes using multidimensional fluorescence imaging.  
Supervisors: Dan Davis, Paul French, Mark Neil  
Student: Stephane Oddos

Broadly, how cell-cell interactions over a few minutes lead to the decision whether or not a cell is diseased if far from understood. Using a combination of novel and established physical technologies as well as molecular and cellular biology methods we aim to help understand how the spatial and temporal organisation of molecules controls intercellular communication in the immune system. We have recently identified that long membrane tethers between cells, or membrane nanotubes, may facilitate as yet unknown modes of intercellular communication. We now wish to investigate the possibility that nanotubes can facilitate novel functional signalling between cells over large distances. This will be undertaken building upon the unique multidimensional fluorescence imaging developed in the Photonics Group at Imperial College, first using FRET techniques to establish whether intracellular signals can propagate down a membrane nanotube and then developing multi-parameter fluorescence imaging techniques such as simultaneous FRET and calcium imaging applied to live cells.

7. Phosphatidylinositol transfer proteins: Does the topology and the stored curvature elastic stress of lipid bilayers regulate membrane-association and lipid abstraction?  
Supervisors: Shamshad Cockcroft, Oscar Ces  
Student: Natalia Goehring

Phosphatidylinositol transfer proteins α and β (PITPα and PITPβ) are soluble proteins that have the property of associating with membranes transiently to selectively extract and thus promotes exchange of phosphatidylinositol (PI) and phosphatidylcholine (PC) between lipid bilayers. In higher eukaryotes PITPα is required for cellular functions such as phospholipase C-mediated signalling, regulated exocytosis, and secretory vesicle formation. Despite their central role in lipid metabolism, signal transduction and membrane trafficking processes, the molecular mechanisms that regulate the rate and specific targeting of each PITP to a specific location within the cell remain unexplored. Since PITPα interactions with the membrane are dependent on cell stimulation, we hypothesise that the curvature of the membrane and the membrane composition-dependent local micro-mechanical stresses stored therein may be responsible for regulating PITP membrane association. We plan to express and purify recombinant PITPα and PITPβ (and mutants that have increased or decreased membrane–association properties) and examine the conditions that influence the association with membranes with respect to the micro-mechanics of the membrane and the topology of the bilayer using a Biomechanical microscope. The PITP proteins and the selected mutants will be analysed for lipid transfer and binding in a biological setting using native membranes and for their ability to interact with specific membranes in cells by microscopy. We will resolve what properties of
membranes determines the specific interactions of PITPα with the plasma membrane compared to PITPβ with Golgi membranes. Membrane association studies will be mimicked in vitro by developing in parallel a high-throughput approach for such studies based upon supported bilayer composition arrays within microfluidic devices.


*Supervisors: Matilda Katan, Edward Morris, Piers Gaffney*

*Student: Alessia Arduin*

Being able to observe the atomic structure of key signalling proteins that regulate physiological processes and that can be subverted in cancer, is important for understanding their regulatory mechanisms and for later development of specific inhibitors as potential therapeutic agents. Many of these regulatory events take place at membranes but this has historically been a very difficult environment in which to determine structures. Recent successful studies for investigating large proteins associated with lipid membranes have employed cryo-electron microscopy. In this project novel technologies, including both lipid synthesis and protein modification, will be explored to obtain protein arrays on a lipid monolayer. These arrays will then be used to obtain 3D protein structures in the membrane environment by cryo-electron microscopy.

9. Selective imaging of Nitric Oxide on the cell's membrane

*Supervisors: Ramon Vilar, Nick Long, Rudiger Woscholski*

*Student: Neil Wilson*

Nitric Oxide (NO) is a well-known atmospheric pollutant. However, to the surprise of the scientific community, studies carried out in the past decade have unveiled the extremely important role of NO as a messenger molecule in the cardiovascular, nervous and immune systems. Although NO is a relatively stable free radical it readily reacts with free radicals and metal centres in proteins, exerting physiological and in some cases pathological effects. Consequently, it is essential to develop efficient methods to detect and image this molecule in living organisms.

In this project we propose to develop a series of new chemical receptors for the detection of NO. In particular, we propose to synthesise imaging probes that can be easily anchored in the cell’s membrane and consequently can be used to image the traffic of this small messenger molecule in the boundaries of the membrane. The project will involve the synthesis of new metal-containing species with the capability of selectively binding NO. Upon binding it, a physical response will be triggered that can then be easily detected (initially we will concentrate on optical probes but other signals such as MR can be explored). In order to anchor the probes in the cell’s membrane, they will contain lipid substituents. Once their imaging behaviour has been confirmed, their role in a biochemical context will be studied. In particular, their ability to disrupt NO dependent signalling (membrane anchored probe) as well as the ability to sense NO generated by cells to various stimuli will be tested. There are currently colorimetric/fluorogenic detection methods, but their sensitivity is not as good and they are also subject to interference by other radicals such as reactive oxygen species, including lipid peroxides.

10. Development of microfluidic and supported bilayer technologies for the study of drug-membrane interactions

*Supervisors: Andrew de Mello, Oscar Ces, Antony D Gee*

*Student: Claire Stanley*

We have recently discovered that cationic amphiphilic drugs (CADs), one of the largest and most important class of drug molecules, catalyze the ester hydrolysis of phospholipids which in turn leads to the degradation of biological membranes. This degradation mechanism has now been linked directly to the
ability of drug molecules to bind specifically or non-specifically in-vivo and also forms the basis of a novel drug translocation mechanism. In collaboration with GSK we aim to study the mechanism of this reaction so as to develop a predictive model which can be tested both in-vitro and then in-vivo using PET labelled 11C and 18F analogues of the drug molecules under consideration. The successful development of such a model will have a considerable impact upon rational drug design. We will use patterned lipid bilayers and tailored lipid composition arrays in microfluidic devices for these studies. As it is possible to deposit patterned bilayers using vesicles into which membrane proteins have been re-constituted these drugs studies will be expanded to investigate the competition between binding to the membrane (as a function of composition) and binding to an e.g. target receptor(s) such as D2. In addition we also aim to develop a microfluidic device in which hydrodynamically focused laminar streams are used to facilitate spatially specific removal as a function of time of supported bilayer material from the surface of patterned bilayer systems to which a CAD has been applied for subsequent chemical analysis. Such a device would provide the capability for being able to monitor the rate of acid-catalyzed ester hydrolysis in real time. Label free high-sensitivity analysis of the stripped material will be performed downstream using mass spectrometry, HPLC and or 2DIR fingerprinting.

11. Application of microfluidics to study membrane polarity and vesicular transport in neurons

Supervisors: Giampietro Schiavo, Joao Cabral, John Seddon
Student: Guillermo Garcia Menendez

The maintenance of neuronal morphology is necessary for neuronal functions and survival. The preservation of the identity of the axonal and somatodendritic membranes and their homeostasis require a complex network of trafficking pathways, which allows efficient communication over long distances. Axonal transport constitutes the backbone of this long-distance crosstalk in neurons and is crucial in cells with extended axons such as motor and sensory neurons. Impairment of this process is at the basis of several neurodegenerative and incurable disorders, such as motor neuron disease. Despite its importance, the biochemical and biophysical analysis of axonal transport has been hindered by the lack of amenable experimental systems allowing the polarised growth of neurons in a chemically-controlled microenvironment. We are now proposing to fill this gap by optimising microfluidic devices to grow differentiated neurons in a polarised fashion by means of stable growth factor gradients. High-resolution confocal and multiphoton microscopy will be used to decipher the signals responsible for long-range communication along the axon coupling distal synapses with the neuronal soma. This approach will allow us to further our understanding into the molecular mechanisms regulating membrane dynamics in neurons in health and disease.

12. Molecular mechanisms of membrane association of prenylated GTPases

Supervisors: Miguel Seabra, Oscar Ces
Student: Marie Kirsten

The remarkable degree of specificity with which Rab GTPases recognise distinct subsets of intracellular membranes forms the basis of their ability to act as key cellular regulators, determining the recruitment of downstream effectors to the right membrane at the right time. Given the importance of Rabs in cellular physiology and their involvement in a variety of genetic and acquired diseases it is important to understand the molecular mechanisms, which will till now have proved elusive, controlling Rab localisation. It is becoming increasingly apparent that multiple factors contribute to the specificity of Rab localisation and the close coordination of membrane targeting with Rab activation. We believe that a combination of protein and lipid factors are important for membrane recruitment of Rabs. The objective of this study will be to determine whether the lipid composition of membranes influences Rab-membrane association. Given the highly specific lipid compositions that are maintained across a variety of membrane compartments within the cell this could provide a mechanism that could account for the specific targeting of Rab proteins. Geranylgeranylated Rabs will be modified in vitro and isolated in complex with its solubilising factor, REP. REP:Rab-GG complexes will be incubated with artificial liposomes of defined composition and the kinetics
of Rab association with liposomes will be measured. Fluorescence microscopy using a combination of a novel Biomechanical Microscope that allows us manipulate the micro-mechanics of model membrane vesicles and labelled Rabs will also allow us to correlate the function of Rab proteins with a variety of membrane properties. These include the stored curvature elastic stress, average lipid geometry, headgroup chemistry and average interfacial charge

13. Analysis of the binding site for fibroblast growth factor receptor on neural cell adhesion molecule

Supervisors: Robin Leatherbarrow, Jane Saffell
Student: Kate Bowman

This project is to recreate this binding site using the constrained synthetic peptides (10-20 aa) that aim to reproduce these sites, and test the degree to which they retain the FGFR target binding affinity and bioactivity of NACM. Linear and cyclised peptides corresponding to the binding loops in Fn I and II will be synthesised and optimal sequence and structure determined using NMR; it is also hoped to express the whole Fn domains for structural studies.

The project is highly multidisciplinary and will involve a wide range of different methodologies. These include synthetic chemistry (peptide synthesis), molecular biology (expression of recombinant Fn I and II domains, site directed mutagenesis), biophysical assays (measurement of binding affinity) structural biology (NMR analysis) and cell-based assays (signalling, survival, proliferation, axon outgrowth).


Supervisors: Richard Templer and Oscar Ces
Student: Joe Kaplinsky

The analysis and measurement of all the thousand of protein types in an individual cell is one of the key challenges in post-genomic science. This is the subject of proteomics, and it underpins a vast range of modern life-sciences research from personalised medicine to the engineering of more efficient plants. Despite the importance of proteomics in many areas of research, one of the technological bottle-necks remains the ability to selectively disrupt and digest single cells. This project involves the development of novel physical, chemical and biological approaches to selectively digest the plasma membrane of single cells within microfluidic devices.

We aim to measure the protein composition of the plasma membrane of selected cells as this structure is the subject of many disease states and a target for numerous therapeutic interventions. The plasma membrane is not only the entry and exit route for all cellular components but also transduces a large number of signalling pathways and is the means by which cells interact and communicate with their environment.

This project is a component of the £5 million multidisciplinary Single Cell Proteomics Project funded by the Engineering and Physical Sciences Research Council (EPSRC) to create an unrivalled suite of technologies for the study of single cells using proteomic approaches. The project consortium includes teams from the Imperial College Departments of Chemistry and Physics, the Institute of Cancer Research and the London Research Institute of Cancer Research UK and is conducted under the auspices of the Chemical Biology Centre (CBC). This is a programme of research.

15. Multidimensional Fluorescence Imaging for Single Cell Proteomics

Supervisors: Paul French, Mark Neil
Student: Ali Salehi-Reyhani
The analysis and measurement of all the thousand of protein types in an individual cell is one of the key challenges in post-genomic science. This is the subject of proteomics, and it underpins a vast range of modern life-sciences research from personalised medicine to the engineering of more efficient plants. Despite the importance of proteomics in many areas of research, the existing analysis tools methods are relatively slow and cumbersome. This project aims to develop novel multidimensional fluorescence imaging (MDFI) technology for application to the monitoring and analysis of single cells held in an optical trap. These trapped cells will be selectively digested and novel microfluidic technology will be employed to separate different components for downstream proteomic analysis using novel vibrational spectroscopic techniques. The MDFI technology will involve advanced microscopy techniques including spectrally resolved fluorescence lifetime imaging (FLIM) and will also be applied to monitoring and optimising the microfluidic systems to molecular analysis of the separated components.

This project is a component of the £5 million multidisciplinary Single Cell Proteomics Project funded by the Engineering and Physical Sciences Research Council (EPSRC) to create an unrivalled suite of technologies for the study of single cells using proteomic approaches. The project consortium includes teams from the Imperial College Departments of Chemistry and Physics, the Institute of Cancer Research and the London Research Institute of Cancer Research UK and is conducted under the auspices of the Chemical Biology Centre (CBC). This is a programme of research.

16. Femtosecond/Picosecond Infra-Red Methods for the analysis of Protein Composition and Structure
Supervisors: David Klug, Keith Willison
Student: Christian Loeffeld

The analysis and measurement of all the thousand protein types in an individual cell is one of the key challenges in post-genomic science. This is the subject of proteomics, and it underpins a vast range of modern life-sciences research from personalised medicine to the engineering of more efficient plants. Despite the importance of proteomics in many areas of research, the existing analysis tools methods are relatively slow and cumbersome. Ultra-short infra-red laser pulses can be used to study protein composition and structure in a manner analogous to Two-Dimensional Nuclear Magnetic Resonance, but with much higher sensitivity. This project involves the construction and application of new Femtosecond/Picosecond infra-red laser instruments to study protein composition and structure.

This project is a component of the £5 million multidisciplinary Single Cell Proteomics Project funded by the Engineering and Physical Science Research Council (EPSRC) to create a suite of technologies for the study of single cells, using proteomic approaches. The project consortium includes teams from the Imperial College Departments of Chemistry and Physics, the Institute of Cancer Research and the London Research Institute of Cancer Research UK and is conducted under the auspices of the Chemical Biology Centre (CBC). This is a programme of research.

Projects 2005/06

1. Synthesis and Evaluation of Novel PI 3-Kinase Activators and Inhibitors
Supervisors: Helen Hailes, Rudiger Woscholski, Herbert Waldman
Student: Richard Gunn

Our overall aim is to utilise synthetic chemistry and biochemistry to identify novel phosphoinositide (PI) 3-kinase activators and inhibitors to facilitate research into the role and interactions of PI kinases, an area of utmost importance for cellular function and human diseases such as diabetes or cancer.

Using the PI 3-kinase dependent phosphorylation of protein kinase B (PKB), on Serine 473 as a monitor, we will synthesise and screen small monomeric molecules, building upon our preliminary SAR data, that are capable of either activating or inhibiting PI 3-kinase dependent signalling pathways. Having identified key
compound leads we will enhance activities by synthesising short novel oligomers constructed from the monomeric compound via the development of new synthetic methodology. We will then attempt to unravel the identity of the unknown S473 kinase target by generating tools, through the synthesis of derivatives of active compounds that are capable of cross-linking beads, dyes or proteins. These will facilitate the identification of the target proteins for our compounds, and enable us to characterise the mechanism controlling these novel controlling elements of PI 3-kinase dependent signalling.

2. New reagents for protein localisation
Supervisors: Robin Leatherbarrow, Rudiger Woscholski
Student: Gillian Busch

The metal coordinating properties of poly histidine tags have been widely exploited for the purification of proteins by affinity chromatography. This project aims to refine both the ligand and the protein site to allow greater specificity and affinity than existing methodologies. A variety of approaches will be taken, including novel metal binding ligands, optimised peptide sequences and multiple interaction sites. Our improvements will allow designer ligands to localise specifically tagged proteins in solution or within cellular systems, providing the tools for delivery of probes for a variety of purposes including fluorescent localisation. The project involves synthesis of metal complexing agents, synthesis of peptide tags, measurement of binding constants and optimisation of ligand-metal-peptide interactions. Finally, the probes will be tested in biological systems.

3. Phosphoester affinity purification: a novel tool for the “omics”
Supervisors: Joachim Steinke, Rudiger Woscholski, Rainer Cramer
Student: Sally Ewen

For more information on this project please contact Joachim Steinke: j.steinke@imperial.ac.uk

4. Inositol Phospholipids: Do Structural and Energetic Properties Affect Phosphatase Activity?
Supervisors: Piers Gaffney, Rudiger Woscholski, Richard Templer, Oscar Ces
Student: Sam Furse

Phosphoinositides (Pis) are essential components of intracellular signalling systems. PI lipids perform this function by acting as protein docking sites at biological membranes. At its simplest level, these potent signals (for cell survival, proliferation, glucose metabolism etc.) are mediated by the degree and position of phosphorylation of the inositol head-group. The kinases and phosphatases that switch these signals are therefore tightly controlled. There is some evidence that micro-mechanical stresses in the membrane modulate these enzymes’ activity and this effect may indeed be an integral part of PI signalling. We plan to synthesise a series of PI lipids varying in both degree of head-group phosphorylation and tail unsaturation. The effects that these graduated molecular alterations have on the micro-mechanical stress stored in a membrane will be studied by observing the changes in the lyotropic liquid crystalline phase behaviour of these synthetic molecules. This will be done using polarising microscopy, DSC and small angle X-ray diffraction. Subsequently phosphatase processing of PI lipids within vesicles having controlled micro-mechanical stress will be examined for correlations of enzyme rate with membrane state.

5. The effect of local composition on the mechanics of lipid bilayer membranes and membrane fusion processes
Supervisors: Kim Parker, Rudiger Woscholski, Richard Templer, Oscar Ces
Student: Jessica Knott
The budding of vesicles in cell membranes plays a crucial role in a number of biological processes such as endocytosis, the formation of vesicles in endothelial cells and the release and scavenging of neurotransmitters. There is an increasing body of evidence in the literature suggesting that local micro-mechanical stresses stored in the membrane can be induced by chemical modifications to the headgroups of lipids within the membrane. We plan to develop an axisymmetric, continuum model of lipid bilayer membranes to study the relationship between the local composition of the membrane and how these local material properties can in turn alter the overall configuration of the vesicle assembly and drive membrane fusion processes. This novel computational framework will be used to simulate a vital but as yet poorly understood class of lipids, inositol phospholipids (PI) and their roles in endocytosis and membrane invagination processes. In tandem with the development of the computational model the student will experimentally determine the effect of PI lipid composition in mixed-lipid vesicle systems and their metabolising enzymes upon in-vitro and in-vivo fusogenic processes. By then determining the local material properties of these mixed-phosphoinositol lipid systems (e.g. shear and bending moduli) the student will be able to use the computational model to explain and simulate the observations made in these fusogenic assays.

6. Expression of cross-linking collagens
*Supervisors:* Paul French, Yoshi Itoh
*Student:* Hugh Manning

Collagen is one of the most predominant proteins in the body and is essential for the structural integrity and mechanical properties of the tissues in which it is present. The proposed research will exploit the intrinsic molecular contrast afforded by the autofluorescence of collagen cross-links and will employ wide field fluorescence life imaging and hyperspectral imaging to characterise the aggregation of collagen during expression from cells and extracellular fibril formation and its degradation in the presence of matrix metallo-proteinases. Studies will be conducted in normal physiological environments and in various conditions which are expected to occur in damaged or diseased tissues, in which shifts are observed in the type of collagen that is expressed. Coupling these studies with observations in whole tissues will lead to improved understanding of the role of altered collagen expression and degradation in many pathological processes.

7. Enhancing the binding of the lantibiotic nisin to lipid II
*Supervisors:* Alethea Tabor, Piers Gaffney, Paul Driscoll
*Student:* Daniel Nicolau

The lantibiotic nisin is a complex polycyclic peptide with multiple thioether side-chain bridges and a unique mode of antibiotic activity. This involves specific binding to Lipid II, an essential subunit with a vital role in cell wall biosynthesis. Recent NMR results indicate that the C-terminal 12 residues of nisin, which include rings A and B, are key to the binding of the pyrophosphate moiety of lipid II, via a highly conserved cage structure. Progress in understanding the mode of action of nisin has so far focussed on the use of recombinant nisin: however, truncated nisin structures cannot be expressed and non-natural analogues cannot be easily biosynthesised. We aim to use our recently reported methods for the solid-phase synthesis of peptide containing single and multiple thioether bridges to prepare analogues of nisin(1-12) (rings A+B), to screen these for tighter and more selective binding to lipid II, and to investigate the role of the different nisin residues in binding to the pyrophosphate of lipid I.

Projects 2004/05

1. FRET of Molecular Interactions
*Supervisors:* Matilda Katan, Mark Neil, Paul French
*Student:* David Grant
This project involves the development and application of FRET ( Förster Resonant Energy Transfer) techniques – including instrumentation and biological constructs – to a number of applications including the elucidation of signalling processes via small GTP-ase Ras. In particular, we will develop FRET based on spectrally-resolved fluorescence lifetime imaging (FLIM), which will be implemented in wide-field and confocal-multi-photon scanning microscopes. While this project will not be exclusive to one sample or experimental programme, the main emphasis of the PhD student’s work will be to study signalling processes via small GTP-ase Ras. Signalling via Ras, which in its mutated/activated form appears at a high rate in several cancer types, is crucial for insights into regulation of many biological processes and deregulation in cancer. One of the downstream components stimulated upon Ras activation is recently discovered phospholipase C epsilon (PLC). Although our evidence supports activation of PLC through Ras, their direct interaction and molecular mechanism of PLC activation are not clarified. This will be addressed using FRET with the two components (Ras and PLC) containing compatible fluorescent tags. After transfection with fluorescent constructs, cells will be stimulated with epidermal growth factor known to stimulate PLC via activation of Ras. This will not only dissect the molecular nature of the interaction between the signalling molecules but also reveal spatial and temporal aspects of this interaction in the cellular context. It is our goal to ultimately develop systems to image this FRET in real-time, although we will begin our studies using our scanning microscope system.

2. The Study of Protein-Inositol Phospholipids Interactions Using Synthetic Fluorescent Ligand Analogues

**Supervisors:** Piers Gaffney, Banafshe Larijani
**Student:** Trung Huynh

Nuclear envelope (NE) disassembly and reassembly are essential processes during mitosis. We can mimic this process in sperm nuclei post-fertilisation using a sea urchin cell-free system. Upon fertilisation, precursor membrane-vesicles (MV) isolated from sea urchin egg extracts (S10) bind to demembranated sperm nuclei through an ATP-dependant manner; fusion of these MVs occurs via a GTP-induced step forming a NE surrounding the chromatin. The initial population of MVs which bind to chromatin are enriched with the specific steryl (18:0), arachidonyl (20:4) phosphoinositol (PI). This PI is reduced upon fusion of MVs. Addition of exogenous phospholipase C (PLCγ) or bacterial PI-PLC triggers fusion of MVs. It is suggested DAG produced from PLC hydrolysis is vital to promote fusion of chromatin bound MVs by increasing membrane curvature stress. However, details of this association are difficult to probe. We plan to synthesise fluorescent PIs as a unique tool to investigate PI hydrolysis. Alkylation of the inositol 6-O (which does not usually participate in protein recognition); with a hydrophilic spacer between the fluorophore and lipid head-group will minimise disruption of the lipid-protein interaction. We aim to form giant unilamellar vesicles from the synthetic PI and upon addition of bacterial PI-PLC, monitor the critical distribution and dynamics of the probes by fluorescence.

3. Short Pulse laser enhancements of MALDI-TOF

**Supervisors:** David Klug, Keith Willison
**Student:** Elizabeth Gardner

MALDI-TOF is a powerful method for the analysis of the protein content of biological samples, but many problems with the method remain. In particular, it is difficult to achieve reliable peptide fragmentation, ablation and ionisation. These can all be controlled and enhanced by the application of femto and picosecond laser methods. The project involves the coupling of tunable short pulse laser systems to a MALDI-TOF spectrometer to develop novel and controlled ablation, ionisation and fragmentation of protein samples.

Particular goals of the project include enhancing the ability of MALDI-TOF to identify chemical modifications to the polypeptide and to improve the method’s ability to identify and determine the chemical condition of membrane proteins.
4. Development and application of multi-parameter fluorescence imaging technology  
Supervisors: Paul French, Mark Neil, Dan Davis  
Student: Sunil Kumar

This project concerns the development and application of multi-parameter fluorescence imaging technology, particularly exploiting multi-spectral and fluorescence lifetime imaging for application to cell biology, including the study of protein interactions and the immune system.

5. Imaging Lipid Rafts in Living cells  
Supervisors: Tony Magee, Mark Neil, Paul French  
Student: Dylan Owen

Eukaryotic cells contain cell surface “lipid raft” microdomains that are involved in signalling processes, concentrating specific proteins and excluding others. Receptor engagement can promote aggregation of lipid rafts, which facilitates co-localisation of signalling proteins while excluding negative regulators, thereby potentiating signalling (Janes et al., 2003). We propose to image these domains using living cells and coverslip-bound bilayers composed of lipid mixtures capable of forming lipid raft domains and incorporating proteins that will partition between raft and non-raft regions. The fluorescence lifetime of enhanced Green Fluorescent Protein (EGFP) and other probes may be a sensitive reporter of the environment of the fluorophore displaying variation between different cellular locations (Suhling et al., 2002). The student will use fusion proteins in artificial lipid raft and non-raft membranes to see if this could be cells expressing similar constructs and manipulated their lipid raft content by altering cholesterol and/or glycosphingolipid levels. We will also use the well-characterised outer leaflet raft marker cholera toxin B subunit in similar studies. The rotational mobility of lipid raft and non-raft proteins tagged with EGFP as well as lipid probes will also be tested by fluorescence anisotropy measurements. These studies will help to elucidate the structure and dynamics of lipid rafts in living cells. They will provide the student with a cross-disciplinary training in molecular cell biology and state-of-the-art imaging methods.

6. Probing the PKB/Akt Activation Mechanism by Chemical Genetics and Phosphoinositides  
Supervisors: Piers Gaffney, David Mann  
Student: Sam Cooper

Protein Kinase B (PKB) is a critical signalling kinase that controls the diverse cellular functions which are important both physiologically (e.g. insulin signalling) and pathologically (e.g. cancer). PKB is activated at the plasma membrane by the Inositol phospholipids PIP3 in combination with a second kinase PDK1. Unfortunately PIP3 is recognised by many other proteins, including PDK1, making this activation mechanism very hard to dissect. We propose to break this linkage by mutating PKB to accept an unnatural lipid using reverse chemical genetics – the “bump and hole” approach. Accordingly an unnatural substituent is attached to PIP3 by chemical synthesis, preventing lipid recognition by the PH-domain of the PKB. Mutation of PKB is used to create an additional pocket to complement the modified lipid; a suitable site for mutation of PKB and a corresponding modification of PIP3 have already been identified. Initial studies will be conducted on the PH-domain alone and will include lipid analogue dot blots, observing the protein’s interaction with vesicles containing modified lipid, and ITC measurement of head-group/protein affinity. Protein crystallography of successful protein/lipid pairs will provide insight for the rational design of second generation protein/lipid pairs. When a successful bump-hole pair has been identified, full length mutant PKB will be expressed in vivo when it is anticipated that the addition of modified lipid will highlight purely PKB dependent down-stream events.

7. Structure of the Membrane Pore Formed by Alpha-latrotoxin
Supervisors: Yuri Ushkaryov, Ardan Patwardan  
Students: Roberto Abbondati

Tetramerisation of alpha-latotoxin (LTX), normally a soluble protein, causes conformational changes that allow the toxin to insert itself into the membrane and form transmembrane pores. In neurones, this action is mediated by specific receptors and results in extensive transmitter secretion. Cryo-electron microscopy was used to determine the 3D structure of LTX tetramers at 14 Å resolution; this method also enables us to visualise toxin molecules inserted into a lipid bilayer. The proposed project will seek to improve the resolution of cryo-EM images of membrane-embedded LTX and to understand the molecular basis for synchronous opening and closing of toxin pores in native membranes. To achieve this, we will reconstitute liposomes with LTX receptors and thus create favourable conditions for multiple pore formation. The presence of receptor on liposomes will also allow the removal of unincorporated LTX molecules and creation of a larger dataset, including both side and end views. We have found that under certain conditions, LTX tetramers spontaneously form flat 2D crystals. The project will, therefore, also aim at finding and analysing crystalline regions in receptor-containing liposomes and 2D lipid sheets. Results of these studies may help to explain the synchronous behaviour of the native LTX pores and will provide important insights into the process of pore formation by different toxins and the mechanism of LTX-evoked transmitter release.

8. The Dynamic Membrane – How Membrane Chemistry and Membrane Stress Couple  
Supervisors: Richard Templer, Oscar Ces, Piers Gaffney  
Student: Emma Clay

Cell membranes are the site of intense chemical activity during signalling events. In many cases the chemical activity makes major alterations to the lipids that are the fabric of the membrane wall. We know that this will often result in significant changes in the mechanical stresses in the membrane. We wish to study how these stresses may build up in the membrane and affect signalling behaviour. To this end we are developing instrumentation capable of holding single cells whilst chemically active species are accurately delivered to specific locations. Using dipyrenylphosphatidylcholine molecules we will monitor spatial and temporal changes in mechanical stress and via micromechanical manipulation we will measure average variations in membrane rigidity.

9. The Molecular and Physical Basis of SNARE-Mediated Vesicle Membrane Fusion  
Supervisors: Paul Freemont, Oscar Ces, Richard Templer  
Student: Christina Turner

SNARE proteins are part of the basic machinery needed to make membranes fuse. By self-assembling into a ring-like complex, anchored to the membrane, they bring together and define the site of membrane fusion. However, the SNARE complex is very difficult to disassemble and yet it must do so to catalyse further fusion events. It has been shown that a protein called NSF is a necessary component in the ATPase mediated disassembly of the SNARE complex, but this aside, very little is known about the dynamics disassembly or indeed assembly of these complexes. In this project we will be elucidating the dynamics of these processes. To do this we will develop techniques to directly visualize and physically characterise SNARE mediated fusion using fluorescence imaging of SNARE proteins in vesicles whose mechanical and compositional state can be manipulated. The development of techniques to micromanipulate vesicles under the microscope will be done in collaboration with other students working in the Doctoral Training Centre.

10. Structure and Dynamics of Lipid Rafts  
Supervisors: Rob Law, Tony Magee and John Seddon  
Student: Alex Trevenen
Lipid rafts have reported to be micro-domains present with the plasma membrane of a cell. They are potentially a revolution in understanding of cell-cell signalling interactions. Lipid rafts have been attributed to many different cell signalling processes and may be responsible for the aggregation or recruitment of membrane proteins. The structure of lipid rafts, however, is very poorly understood. It has been suggested that they are cholesterol rich micro-domains which preferentially separate from the fluid liquid crystalline phase of the plasma membrane. This project will involve both cell biology and a variety of biophysical techniques including solid state nuclear magnetic spectroscopy, x-ray scattering. This will be carried out on labelled cell line extracts and model membranes to elucidate the structure, role and dynamics of lipid rafts.