SCHOOL OF BIOLOGICAL SCIENCES

COLLEGE OF SCIENCE, NTU, SINGAPORE

A Research Institute Dedicated to Research Excellence in Life Science
When founded in 2002, the School of Biological Sciences (SBS) had a mission to make a strong contribution to biological- and biomedical sciences. Since then, many talented individuals from around the world and Singapore have joined SBS—scientific leaders, researchers, PhD- and Master students, working in the two divisions of Structural Biology and Biochemistry as well as Molecular Genetics and Cell Biology. In this publication we celebrate recent achievements of our scientists in diverse areas such as Peptide Chemistry, Drug Discovery, Genetics & Disease, Infectious Diseases, Protein Engineering and Environmental Biotechnology. We hope that you enjoy this first print edition of SBS Research and that through it you can gain a better picture of the variety of cutting-edge research being done at SBS.

However, the magazine you are holding is only part of the story. Updated every six months, SBS Research brings you the very latest research from SBS across a broad spectrum in life sciences.

We hope all of you will enjoy reading SBS Research and that, over time, this publication will help to share the entire span of research activities among ourselves, students and partners in research and in industry.

Prof. M. Featherstone
Chair of SBS

Prof. G. Grüber
Associate Chair (Research) of SBS
Peptide nucleic acid (PNA) is a DNA mimic with a polyamide backbone. The high hybridizing affinity and specificity of PNA has made it an attractive agent for antigene and antisense research. But poor cellular uptake is a major disadvantage that has severely limited its use in functional genomics and molecular therapy. The laboratory of A/Prof Chuan-Fa Liu (SBS, NTU) has designed a new type of PNA analogue that has built-in cell-permeability. In this design, structural moieties with inherent membrane translocation ability are built onto PNA’s polyamide backbone without affecting its hybridization activity. This is achieved by introducing a guanidine-bearing peptoid-like side chain onto the γ-N of the PNA backbone. Thus, this novel class of nucleic acid mimics possesses all the desired properties expected for an ideal antigene or antisense agent: base-pairing ability, cell-permeability, in vivo stability and ease of preparation due to the simplified pseudo-peptide backbone. The new PNA analogue will be a useful gene-knockdown agent both for basic biomedical research and therapeutic development.

Bioactive peptides would be excellent drug candidates if not for their poor pharmacokinetic profiles like very short half-lives *in vivo*. A major recent breakthrough is the advent of the so-called “peptibody” platform technology. In a peptibody a bioactive peptide of interest is fused to the Fc domain of human IgG at either the N- or C-terminus. A fusion protein so-created has been shown to have a dramatically increased half-life that may last for weeks in an animal’s body. Such peptide-Fc fusion constructs are therefore very useful formats for designing peptide-based drugs, as validated by the successful development of the first peptide drug – Nplate® (romiplostim) – which was discovered by *A/Prof Liu Chuan-Fa* (SBS, NTU) and colleagues while he was at Amgen.

PEPTIDE DENDRIMERS AS NOVEL PLATFORM FOR VACCINE DEVELOPMENT

The laboratory of Prof James P. Tam (SBS, NTU) invented a new technological platform known as multiple antigen peptides (MAP) or peptide dendrimers for development of safe and effective vaccines. In the MAP system, multiple copies of antigenic peptides are bound to a non-immunogenic Lys-based dendritic scaffold. The MAP molecules acquire proteolytic stability, enhanced molecular recognition by immune cells, and induction of stronger immune responses compared with monomeric peptides. This structure permits controlled structure-activity relationship studies, and is applicable for oral or nasal delivery to elicit mucosal immune responses. The MAP-based vaccines also eliminate the need for the inclusion of adjuvants found to be toxic to humans, and avoid the adverse effects associated with conventional vaccines (i.e., live-attenuated, killed or inactivated pathogens), carrier proteins and cytotoxic adjuvants.

Since the introduction of MAPs by Tam’s research group, over 200 applications of MAPs have been documented in the literature. It has become a popular method with many vaccines currently being studied in clinical trials in the treatment for malaria, influenza, tuberculosis and HIV. Commercial companies specializing in peptide reagents now offer either reagents for synthesizing MAPs in the laboratory or by custom synthesis services.

2. WO Patent no. 011778: Dendritic Polymer of Multiple Antigen Peptide System Useful as Anti-Malarial Vaccine.
5. U.S. Patent no. 5,229,490: Multiple antigen peptide system.
Genetic manipulation of genomes is an important component of many applications in biotechnology and molecular medicine. Prominent examples include generation of genetically modified cells for the production of protein therapeutics/diagnostics, drug testing/development and gene therapy. However, existing gene insertion technologies often lack desired target sequence specificity and are mostly irreversible. These features can result in potentially harmful genomic mutations and/or unpredictable complications. The laboratory of A/Prof Peter Dröge (SBS) possesses a patented, site-specific genome insertion technology which has found commercial application. It enables users to express any transgenic DNA construct of interest from a pre-determined, single genomic docking site. Examples of potential applications of this technology, in particular in conjunction with our novel “in-house”-developed embryonic stem cell pluripotency reporter system, include GMO production, gene therapy, and drug screening.


Sequence-specific gene silencing by short hairpin (sh) RNAs has recently emerged as an indispensable tool for understanding gene function and a promising avenue for disease therapies. However, a wider biomedical use of this approach is hindered by the lack of straightforward methods for achieving uniform expression of shRNAs in mammalian cell cultures. To this end, the group of Asst Prof Eugene Makeyev (SBS, NTU) has developed a high-efficiency and low-background (HILO) recombination-mediated cassette exchange (RMCE) technology that yields virtually homogeneous cell pools containing doxycycline-inducible shRNA elements in a matter of days and with minimal efforts (1, 2). The utility of this approach has been shown for a number of commonly used human and mouse cell lines. Because of its technical simplicity and cost efficiency, the technology should facilitate both low- and high-throughput shRNA-based RNA interference projects. Moreover, a recent study by the Makeyev lab demonstrates (3) that HILO-RMCE will be useful for a wider range of molecular and cell biology applications by allowing one to rapidly engineer cell populations expressing essentially any transgene of interest.

The cyclic dinucleotides c-di-GMP and c-di-AMP are newly discovered bacterial messenger molecules with potent immunostimulatory properties. With their potential to be used as immunologic or vaccine adjuvants, efficient synthesis of the dinucleotides in large quantity is essential for research and clinical trial purposes. Currently, c-di-GMP and c-di-AMP can be obtained by multi-step chemical synthesis methods that are not suitable for large scale production. Enzymatic synthesis of c-di-GMP using mesophilic enzymes suffers from low production yield due to protein instability and strong product inhibition. The laboratory of A/Prof Zhao-Xun Liang (SBS, NTU) lab has cloned and engineered two hyperthermophilic enzymes: a diguanylate cyclase (tDGC) and a diadenylate cyclase (tDAC), for the enzymatic synthesis of c-di-GMP and c-di-AMP. The product inhibition that previously limited production yield of c-di-GMP was significantly alleviated by protein engineering in the putative regulatory inhibition site. With the engineered enzymes, the lab demonstrated that grams of c-di-GMP/AMP can be readily prepared by using the procedures optimized for enzymatic reaction and product purification. The thermophilic enzymes are not only highly valuable for large-scale c-di-GMP/AMP production but also suitable for the preparation of radioisotope-labeled c-di-GMP/AMP derivatives.

Proteolytic peptides as biomarkers for hemoglobinopathies and other diseases

Protein biomarker discovery efforts are usually hampered by the fact that most of the candidate markers identified result from the body’s response to diseases, rather than disease-causing proteins themselves. This often raises serious questions about the specificity of these candidate markers. On the other hand, disease-causing protein mutations, if known and easily detected, could serve as ultimate biomarkers. However, proteins carrying such mutations are rare and often in low abundance, making them difficult to be detected. The laboratory of Asst. Prof Kai Tang (SBS, NTU) hypothesized that these proteins are degraded by endogenous proteases and the proteolytic peptides carrying the mutation could be more readily detected and serve as potential biomarkers for diseases. They tested this hypothesis in α-thalassemia patients with stop-codon mutations and identified a number of proteolytic peptides. The sequence of these peptides matched with the C-terminal fragments of the elongated α-globin. They have been identified in peripheral blood carrying the Hb CS (Hb Constant Spring) or PS (Pakse) allele, including homozygotes and heterozygotes.

The researchers also found that in samples carrying HbE allele, a common Hb variant in Southeast Asia due to mutation in beta globin chain, proteolytic peptides carrying the HbE mutation could also be found in peripheral blood of both homozygous and heterozygous carriers. Furthermore, proteolytic peptides from sickle cell samples carrying specific mutations HbS and HbC can also be found in peripheral blood. When validated, the method can be used for high-throughput screening of hemoglobinopathies or other diseases using tandem mass spectrometry.

Invasion by the malaria merozoite depends on recognition of specific erythrocyte surface receptors by parasite ligands. *Plasmodium falciparum* uses multiple ligands, including the reticulocyte binding protein homologues (RHs). RH are conserved in all species of *Plasmodium* and play an important role in host cell sensing and recognition as well as virulence. The group of Prof Peter Preiser (SBS, NTU) has identified the functional region of RH proteins that mediates the initial binding of the protein to a specific receptor on the erythrocyte surface. Antibodies against this domain are able to block invasion of the merozoite efficiently. The relatively small (334 amino acid) region of RH that encodes the binding domain is ideal for recombinant protein expression and can serve as a potential component of an invasion blocking antibody.

The concept of *Bioaugmentation* in Environmental Biotechnology is not new – this involves adding microbial strains with specialized ability, e.g. degradation of toxic pollutants, to the site where the specific function is needed. However, applying a single strain has often proven ineffective. Asst Prof Sze Chun Chau’s laboratory (SBS, NTU) explores and exploits bacterial interplay in order to achieve a more sophisticated level of Bioaugmentation. Interactions between various bacterial species bring about differing outcomes. A compatible combination of bacterial species, with complementary and synergistic metabolic capabilities, can result in a potent co-culture suitable for handling applications not possible with pure cultures. One of the greatest advantages of bacterial co-cultures over pure cultures is that co-cultures are often more resistant to environmental disturbances and fluctuation, and hence, more self-sustaining. This work has benefitted our industry partners in the *Environmental Biotechnology sector*, e.g. an air-purification device for removing Volatile Organic Compound (VOC) will be making use of the bacterial co-culture formulated by Asst Prof Sze’s laboratory, which can remove VOC more than 400% faster, compared to their original version. Licensing terms are being negotiated currently.
The laboratory of A/Prof Jaume Torres (SBS, NTU) developed together with Aquaporin A/S (Denmark) a method to create giant protein vesicles (GPVs). Fluorescence microscopy is used to characterize GPV morphology and protein–lipid hydrophobic interactions. Specifically, they employed generalized polarization imaging to monitor the polarity around the protein transmembrane region of aquaporins labeled with the polarity-sensitive probe Badan.

Their recently published work (1,2), describes how giant protein vesicles (GPVs) of ≥10 μm can be created by solvent-driven fusion of large vesicles (0.1-0.2 μm) with reconstituted membrane proteins. The researchers found that formation of GPVs proceeded from rotational mixing of protein-reconstituted large unilamellar vesicles (LUVs) with a lipid-containing solvent phase. GPVs can be made by using n-decane and squalene as solvents, and applied generalized polarization (GP) imaging to monitor the polarity around the protein transmembrane region of aquaporins labeled with the polarity-sensitive probe Badan. Specifically, they created GPVs of spinach SoPIP2;1 and E. coli AqpZ aquaporins. There findings show that hydrophobic interactions within the bilayer of formed GPVs are influenced not only by the solvent partitioning propensity, but also by lipid composition and membrane protein isoform.

Together with the NS5 polymerase, the NS3 helicase has a pivotal function in flavivirus RNA replication and constitutes an important drug target. The laboratory of Prof Julien Lescar (SBS, NTU) and the Novartis Institute for Tropical Diseases (Singapore) captured the dengue virus NS3 helicase at several stages along the catalytic pathway including the forms bound to single-stranded (ss) RNA, to an ATP analogue, to a transition-state analogue and to ATP hydrolysis products. RNA recognition appears largely sequence independent in a way remarkably similar to eukaryotic DEAD box proteins Vasa and eIF4AIII. On ssRNA binding, the NS3 enzyme switches to a catalytic-competent state imparted by an inward movement of the P-loop, interdomain closure and a change in the divalent metal coordination shell, providing a structural basis for RNA-stimulated ATP hydrolysis. These structures demonstrate for the first time large quaternary changes in the flaviviridae helicase, identify the catalytic water molecule and point to a β-hairpin that protrudes from subdomain 2, as a critical element for dsRNA unwinding. They also suggest how NS3 could exert an effect as an RNA-ancho ring device and thus participate both in flavivirus RNA replication and assembly.

FUNCTIONAL ANALYSIS OF TWO CAVITIES IN FLAVIVIRUS NS5 POLYMERASE

Flavivirus NS5 protein encodes methyltransferase and RNA-dependent RNA polymerase (RdRp) activities. Structural analysis of flavivirus RdRp domains uncovered two conserved cavities (A and B). Both cavities are located in the thumb subdomains and represent potential targets for development of allosteric inhibitors. The laboratory of A/Prof Susana Geifman Shochat (SBS, NTU) analyzed in collaboration with the Novartis Institute for Tropical Diseases (Singapore) the function of the two RdRp cavities. Amino acids from both cavities were subjected to mutagenesis analysis in the context of genome-length RNA and recombinant NS5 protein; residues critical for viral replication were subjected to revertant analysis. For cavity A, they found that only one (Lys-756) of the seven selected amino acids is critical for viral replication. Alanine substitution of Lys-756 did not affect the RdRp activity, suggesting that this residue functions through a nonenzymatic mechanism. For cavity B, all four selected amino acids (Leu-328, Lys-330, Trp-859, and Ile-863) are critical for viral replication. Biochemical and revertant analyses showed that three of the four mutated residues (Leu-328, Trp-859, and Ile-863) function at the step of initiation of RNA synthesis, whereas the fourth residue (Lys-330) functions by interacting with the viral NS3 helicase domain. Collectively, their results have provided direct evidence for the hypothesis that cavity B, but not cavity A, from dengue virus NS5 polymerase could be a target for rational drug design.

The laboratory of A/Prof Richard Sugrue (SBS, NTU) and researchers of JEOL U.S.A. Inc. (Massachusetts) identified the presence of virus-associated cellular proteins that may play a role in respiratory syncytial virus (RSV) maturation. Fluorescence microscopy of virus-infected cells revealed the presence of virus-induced cytoplasmic inclusion bodies and mature virus particles, the latter appearing as virus filaments. In situ electron tomography suggested that the virus filaments were complex structures that were able to package multiple copies of the virus genome. The virus particles were purified, and the protein content was analyzed by one-dimensional nano-LC MS/MS. In addition to all the major virus structural proteins, 25 cellular proteins were also detected, including proteins associated with the cortical actin network, energy pathways, and heat shock proteins (HSP70, HSC70, and HSP90).

Immunofluorescence microscopy of infected cells stained with antibodies against relevant virus and cellular proteins confirmed the presence of these cellular proteins in the virus filaments and inclusion bodies. The relevance of HSP90 to virus infection was examined using the specific inhibitors 17-N-Allylamino-17-demethoxygeldanamycin. Although virus protein expression was largely unaffected by these drugs, we noted that the formation of virus particles was inhibited, and virus transmission was impaired, suggesting an important role for HSP90 in virus maturation. This study highlights the utility of proteomics in facilitating both our understanding of the role that cellular proteins play during RSV maturation and, by extrapolation, the identification of new potential targets for antiviral therapy.

Crystal structure analysis of Flavivirus methyltransferases uncovered a flavivirus-conserved cavity located next to the binding site for its cofactor, S-adenosyl-methionine (SAM). Chemical derivatization of S-adenosyl-homocysteine (SAH), the product inhibitor of the methylation reaction, with substituents that extend into the identified cavity, generated inhibitors that showed improved and selective activity against dengue virus methyltransferase (MTase), but not related human enzymes. The crystal structure of dengue virus MTase with a bound SAH derivative, solved by the laboratory of A/Prof Julien Lescar (SBS, NTU) and the Novartis Institute for Tropical Diseases (Singapore), revealed that its N6-substituent bound in this cavity and induced conformation changes in residues lining the pocket. These findings demonstrate that one of the major hurdles for the development of methyltransferase-based therapeutics, namely selectivity for disease-related methyltransferases, can be overcome.

A NOVEL MECHANISM OF INHIBITION OF THE TB DRUG TMC207

With one-third of mankind infected subclinically, and an incidence of nine million new cases of active tuberculosis disease (TB) and two million deaths per year, *Mycobacterium tuberculosis* remains the most important bacterial pathogens in the world. TMC207 was identified in a phenotypic whole cell screen and described to interfere with the *M. tuberculosis* F-ATP synthase.

Using NMR-, SAXS, fluorescence spectroscopy and mutagenesis, the laboratory of Prof Gerhard Grüber (SBS, NTU) in collaboration with the Novartis Institute for Tropical Diseases (Singapore) demonstrated that the new TB drug candidate TMC207, proposed to bind to the proton translocating c-ring, also bind to the coupling subunit ε of the *M. tuberculosis* F-ATP synthase. The data demonstrate that TMC207 forms a wedge between the two rotating subunits c and ε by interacting with the residues W15 and F50 of ε and the c-ring, respectively. T19 and R37 of ε provide the necessary polar interactions with the drug molecule. This new mechanism of TMC207 binding provides the basis for the design of antimycobacterials, targeting the F-ATP synthase in *M. tuberculosis*.

BACTERICIDAL BOOMERANGS

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is perhaps best recognized as a potent inducer of the innate immune system. However, LPS also acts as a barrier to exogenous compounds and a chaperone to aid in the folding of outer membrane proteins. Therefore, molecular interactions with LPS should be considered when designing antimicrobial drugs. Dr. Surajit Bhattacharjya and colleagues (SBS, NTU) do just that, describing structure activity correlations for a series of 12-residue peptides (based on chemokines and neutrophil bactericides) in LPS. They observed that long range aromatic-aromatic packing between residues located at positions 4 and 9 (forming a boomerang-like conformation) was crucial for both neutralizing LPS and antimicrobial activity; the ability of these active peptides to neutralize LPS appeared to correlate with their ability to perturb LPS micelles. This atomic level knowledge should be useful in providing the building blocks for designing novel peptides for bacterial outer membranes.