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Tissue-Inspired Engineering

By Cleo Choong, Timothy Tan and Andrew Tan

Nature's Design Principles To Develop Bio-Inspired Technologies

The phrases "nature-inspired" and "biomimetic approach" have often been used to describe research that is motivated by natural systems. Tissue-inspired engineering (TIE) draws its inspiration from the ways cells as the consummate engineers of the body's architecture and organisation integrate biochemical, physical and environmental cues to communicate between different tissues and organs. Distinct from classical tissue engineering that aims to restore, replace or improve biological functions using a combination of cells, scaffolds/biomaterials and biological signals, TIE is about understanding tissue function, structure and properties using engineering techniques and principles. Engineered biomimetic 3D environments at micro- and nano-levels allow the study of cell-cell and cell-material interactions and aid in the repopulation of specific cell types and thus in tissue healing processes. These engineered tissues are of direct clinical relevance in regenerative medicine, drug efficacy testing, understanding of disease progression and treatment of diseases such as cancer, diabetes and obesity as well as in patient-specific studies.

TIE is highly multidisciplinary (Fig 1), requiring close collaborations between biologists, material scientists and nanotechnologists. While the biologists' role is to understand cell-cell and cell-matrix interactions within a tissue and the intracellular molecular circuitries, the material scientists create the appropriate microenvironment for the cells enabling them to perform their engineering duties, and the nanotechnologists develop materials on a nanoscale level that are instructive (i.e. influence cell behaviour) and interactive (i.e. responsive to cellular cues).

In collaboration with Tan Tock Seng Hospital, National University Hospital, KK Women's and Children's Hospital, Singapore General Hospital, National Skin Centre and National Cancer Centre Singapore, the team aims at developing TIE tools and solutions to face real-life clinical problems and to advance personalised healthcare.

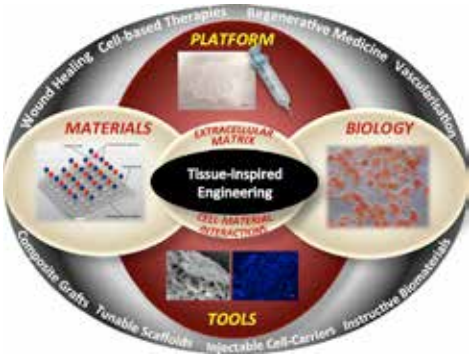

Fig 1: A decoupage of TIE illustrating various engineering platforms and tools used to study the interactions between cells and materials aimed at providing solutions for various biomedical problems such as wound healing and vascularisation.

Next Generation Skin Substitutes: A Multi-Modal Solution For Diabetic Wound Healing

Normal wound healing proceeds via a continuum of events, including acute inflammation, proliferation and maturation phases, which are altered in the diabetic state. Diabetic ulcers are characterised by an accumulation of devitalised tissue, increased/prolonged inflammation, poor angiogenesis and deficiencies in the extracellular matrix (ECM) components. Chronic diabetic wounds show elevated levels of proteolytic activities culminating in a corrupt ECM that cannot support healing. Thus, wound-healing strategies targeted at replacing the dysfunctional ECM would be highly beneficial.

The TIE team designs ECM-based scaffolds that closely mimic the components of normal ECM and the anisotropic properties of skin. Antimicrobial and enhanced wound healing properties support the growth of the various cell types in the different tissue layers of the skin (Fig 2). Additional features such as pH sensitive materials that release drugs in response to pH changes in infected wounds allow for interactions with the microenvironment.

The projects are led jointly by Asst Prof Cleo Choong Swee Neo from the School of Materials Science and Engineering, Asst Prof Timothy Tan Thatt Yang from the School of Chemical and Biomedical Engineering, and Asst Prof Andrew Tan Nguan Soon, recipient of NTU's Nanyang Award in Research Excellence 2011, from the School of Biological Sciences.

Presentations on the projects won three awards at the 2nd International Symposium of Materials on Regenerative Medicine 2012 in Taipei, Taiwan. Parts of this research were published in Cell Death Differ *(2011), 18: 1120-1129;* J Biol Chem *(2010), 285(43): 32999-33009 (highlighted in* www.bioportfolio.com *and* Vascular Biology Publications Alert*); and* J Cell Biol *(2009), 184: 817-831 (highlighted in* J Cell Biol's "In Focus" *(2009), 184: 767, and on the cover page). Funding support came from the National Medical Research Council, the NTU-National Healthcare Group Innovation Seed and collaborations with Johnson & Johnson and Procter & Gamble*

Dynamic Scaffolds: Injectable Porous Scaffolds For Post-Implantation Bone Regeneration

Bony defects requiring bone replacements pose major challenges to reconstructive surgeons. An alternative to bone autografts and allografts are synthetic materials such as metals and ceramics that provide strong mechanical support. However, metal implants poorly integrate with neighbouring tissues and can fail due to infection or fatigue loading. Hence, tremendous efforts have been devoted to the development of tissue-compatible and biodegradable scaffolds. To successfully regenerate tissue, scaffold degradation rates that depend on scaffold composition and prevailing physical, chemical and biological conditions should match tissue growth rates. However, tissue growth rates can vary significantly among tissue types and individuals due to differences in age, diet and lifestyle-related factors.

The TIE team is designing porous scaffold systems that enable post-implantation alteration of the pore structure *in vivo* through minimally invasive means (e.g. via injection) or external stimulation (e.g. light-triggered mechanisms), resulting in structures with larger pores and higher porosity that enhance new tissue and vasculature formation (Fig 3). TIE developed strategies that allow adjustments of the post-implantation scaffold structure to the tissue's growth rate or the patient's recovery rate open up huge potentials in personalised tissue engineering.

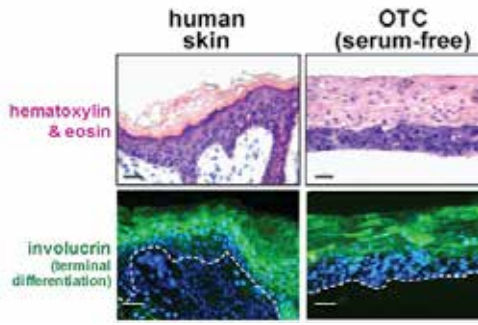

Fig 2: Improved stratification of epidermis in 3D organotypic skin culture (OTC) maintained in serum-free defined medium. Immuno-fluorescence staining of involucrin (green), a protein marker for terminal differentiation of epidermis, of a 2-week old OTC. Human skin biopsy was used for comparison. Dotted white lines show the epidermal-dermal junction.

Bone Regeneration Through 3D Architecture-Directed Stem Cell Differentiation And Electrical Stimulation

Directed differentiation of stem cells into ECM has traditionally employed cocktails of ECM proteins. However, due to the short half-life of the proteins, either multiple injections or a controlled release system are required to achieve full stem cell differentiation. On the other hand, electrical stimulation of cells has been shown to increase cell proliferation, induce angiogenic and osteogenic factors and improve tissue healing.

Fig 3: Bone regeneration. A 3D hydrogel, encompassing mesoporous particles loaded with cell differentiation-stimulating ECM proteins, can be injected into implanted scaffolds at bone defect sites.

To integrate electrical properties, the TIE team is coupling electrically conductive materials such as graphene-polymer composites with biochemical and mechano-biological approaches in injectable bioactive 3D constructs that aid in bone stem cell differentiation and regeneration.

Green Processing And Waste-To-Resource Strategies

Resource resilient materials such as biological waste products and environment-friendly processing methods are used to create scaffolds for tissue regeneration and other useful materials for various biomedical applications. Cells and ECM material are isolated from clinical waste in form of both lipoaspirate and fat grafts (Fig 4). Collagen gained from fish-scales, a waste product from the fish processing industry, is used to fabricate collagen-based wound dressings. Abundantly available natural materials such as ovalbumin (gained from chicken eggs) and alginate (from seaweed) are used to create injectable microcarriers and as encapsulation material. The material properties of these biocompatible matrices can be further tuned to improve entry of nutrients and oxygen and disposal of toxic metabolites and carbon dioxide, and to enhance shielding against the patients' immune defence systems.

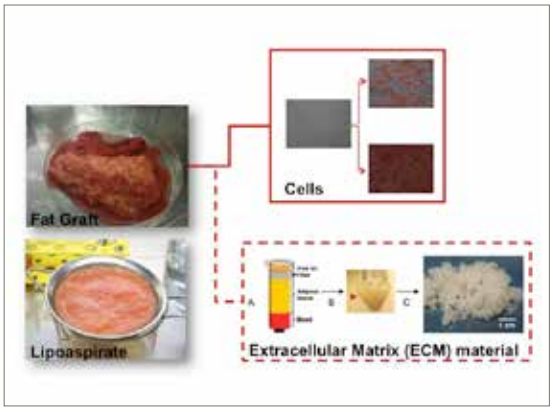

Fig 4: Waste-to-resource strategies: Isolation of stem cells and ECM from clinical waste materials such as fat graft and lipoaspirate.

Enemy At The Gate

By Richard Sugrue

Making Sense Of Pathogen-Host Interactions During Virus Maturation

Assoc Prof Richard Sugrue is the Head of the Molecular Genetics & Cell Biology Division at NTU's School of Biological Sciences.
The described projects are collaborations with Agri-Food and Veterinary Authority of Singapore (AVA), DSO National Laboratories, KK Children's and Women's Hospital (KKH), and the Singapore-MIT Alliance for Research and Technology Interdisciplinary Research Group (SMART-IRG) on Infectious Diseases. Funding support came from the National Medical Research Council, Defence Science and Technology Agency (DSTA), NTU and the National Research Foundation.

Every year a large proportion of the world's population succumbs to virus infections of the respiratory tract. The outcomes of these infections are largely determined by molecular interactions between the virus and host that manifest in a range of phenomena, from virus transmission to virus pathogenicity. My research team focuses on the molecular interactions between virus and host cell during the process of virus maturation. In the past, this important stage of virus replication has been the target of several successful antivirus strategies such as retrovirus protease inhibitors and influenza virus neuraminidase inhibitors.

Human Respiratory Syncytial Virus (RSV): Significant Health Concerns Locally And Globally

Human respiratory syncytial virus (RSV) is responsible for globally approximately 64 million infections and 160,000 deaths each year and the predominant cause of lower respiratory tract (LRT) virus infection in young children. Human metapneumovirus (HMPV), a virus closely related to RSV and only discovered in 2002, typically infects older children, leading to generally less severe disease symptoms. In Singapore, RSV and HMPV infections pose important health concerns. A study, led by our team in collaboration with Singapore's KK Women's and Children's Hospital (KKH) and DSO National Laboratories, estimated that both RSV and HMPV infections together account for approximately 17 % of children hospitalised at KKH with virus related respiratory tract infections. Other high-risk groups for severe RSV and HMPV infections include the elderly and adults with impaired immune systems. The lack of effective vaccines and the limited availability of cost-effective therapeutics aggravate this clinical scenario.

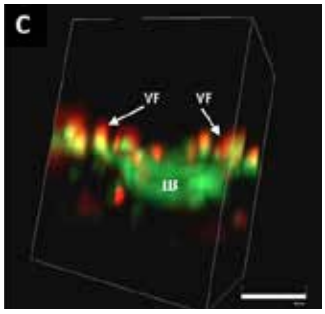

Fig 1. The association of virus filaments and inclusion bodies in RSV infected cells. A 3D projection of an image obtained by confocal microscopy showing an inclusion body (IB) and associated virus filaments (VF).

RSV Infection, Particle Formation And Transmission To Other Cells

During RSV infection, two distinct virus structures – inclusion bodies and virus filaments (Fig 1) – are formed. Inclusion bodies, made up of aggregates of proteins, are associated with virus gene replication and transcription and thus reproduction of virus particle components. Virus filaments, forming on the surface of infected cells, are the sites where mature virus particles are assembled and subsequently transmitted to other cells. Our current studies on HMPV maturation indicate that the mechanisms of virus particle maturation are similar to those of RSV and thus conserved in these viruses.

The main focus of our studies is towards a better understanding of virus filaments formation and RSV particle assembly. Several host cell proteins that are usually associated with cell membranes or scaffolding, e.g. caveolin-1 and actin (Fig 2), were detected in the envelopes of purified RSV particles. Further research showed that virus assembly takes place at complex cell membrane structures composed of specialised lipid membrane structures (lipid-raft microdomains) that are stabilised by actin. In addition, activation of several small GTPases, enzymes that play a role in regulating actin structure, is also required for RSV filament formation. Current work is in progress to characterise the interactions between the virus and cell proteins and to elucidate the cell signal networks that lead to virus assembly and facilitate intercellular transmission of RSV particles.

Infected Macrophages Do Not Produce Virus Particles But Induce Inflammation

Lung macrophages are proposed to play an important role in the early clinical response to RSV infection. In contrast to cell types that are permissive to RSV infection, RSV infection of macrophages results in a phenomenon known as 'abortive infection'. Although virus infection of macrophages leads to the expression of several virus proteins and formation of inclusion bodies (Fig 4), infectious virus particles are

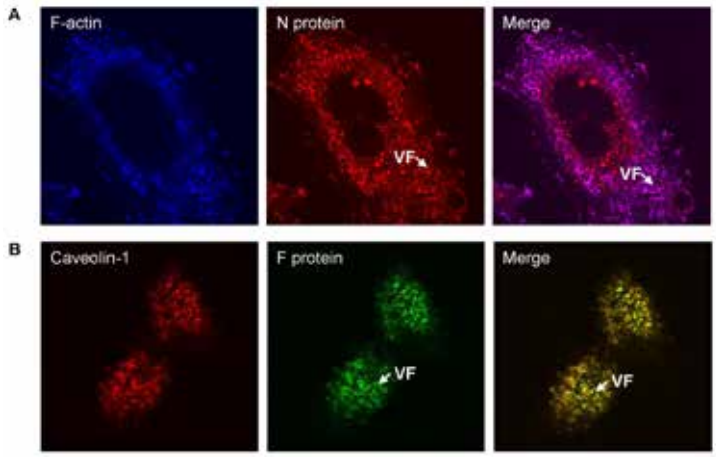

Fig 2. Immunofluorescence confocal microscopy showing the association of F-actin and Caveolin-1 with virus structures in RSV-infected cells.

(A) Left: cellular protein F-actin stained in blue; middle: viral N protein stained in red; right: merging of both images: overlap of the staining of cellular F-actin and viral N protein (purple) indicates that both types of proteins are found within the virus filament (VF).

(B) The cellular membrane protein Caveolin-1 (red) and the viral F protein (green) are both located within the virus filament (VF) in the merged image.

not produced. However, infection with RSV induces strong and sustained activation of cytokines, a category of signalling molecules, which promote systemic inflammation.

The research team aims to understand the molecular mechanisms that impair virus particle formation in these cells and to decipher the processes that lead to inflammation. Since RSV is harmful to cells of the immune system, a greater understanding of these processes will advance the development of therapeutics to mitigate the strong inflammation that is associated with severe RSV infections.

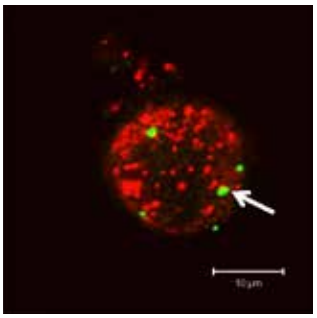

Fig 3. RSV-infected lung macrophages were stained for RSV (green) and a cell protein (red) and visualised by fluorescence scanning confocal microscopy at magnification x100. The white arrow highlights the presence of virus inclusion bodies.

Avian And Human Influenza Virus

According to estimates of the World Health Organization (http://www.who.int/), seasonal influenza accounts for approximately 0.2 billion infections and 500,000 deaths each year. Although seasonal influenza virus infects primarily humans and is typically transmitted from person to person via aerosols, influenza viruses can also be transmitted as zoonotic infections from avian species or swine. The research team aims to understand the molecular mechanisms that allow avian viruses to become adapted to mammalian hosts. Adaptation has been the driver in all past influenza pandemics, and will most likely be the major factor in future influenza pandemics.

The team uses model viruses of human and avian origin such as low pathogenic avian influenza (LPAI) viruses of H5N2 and H9N2 subtype, isolated from live broiler ducks that were detected by the Agri-Food and Veterinary Authority of Singapore when imported into Singapore, and pH1N1 viruses that were isolated from patients in Singapore during the influenza pandemic in 2009. These viruses have been completely characterised at the genetic level and their biological properties were established in several cell types.

Infection of human airway cells with the LPAI virus isolates (H5N2 and H9N2) leads to inflammatory responses not seen with viruses adapted to laboratories such as the H1N1/WSN and H1N1/PR8 isolates that are commonly used to examine influenza virus replication. In addition, the LPAI viruses were not able to reproduce, assemble and transmit virus particles. Further examination of the biological properties of LPAI viruses and human influenza virus isolates will provide a clearer understanding of species adaptation.

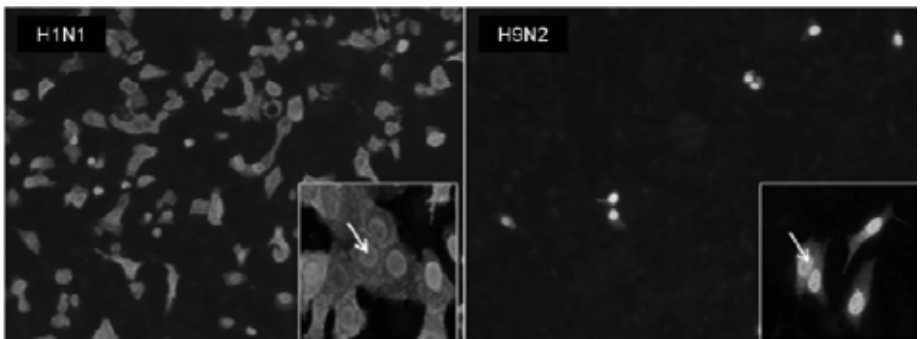

Fig 4. Virus assembly in H9N2 virus-infected cell cultures is impaired. Cell cultures infected with either H1N1/WSN lab-adapted viruses or H9N2 virus isolates were stained for viral N protein and viewed using a fluorescence microscope. The nuclei are highlighted (white arrows). Staining of most cells infected with lab-adapted virus H1N1/WSN show generalised cell staining indicating virus assembly has taken place. In contrast, cells infected with the H9N2 virus show only brightly stained nuclei, indicating that the virus N protein is not transported from the cell nucleus to the sites of virus assembly at the cell surface. Insets show the staining pattern at higher magnification.

Bio-Inspired Materials Science

Translating Biological Design Into Eco-Friendly Materials

By Paul A. Guerette, Ondrej Zvarec, Vitali Lipik and Ali Miserez

Asst Prof Ali Miserez is a Nanyang Assistant Professor at the Schools of Materials Science and Engineering (MSE) and Biological Sciences (SBS) and Head of the Biological and Biomimetic Materials Laboratory (BBML). Dr Paul A. Guerette and Dr Vitali Lipik are Senior Research Fellows and Dr Ondrej Zvarec is a Research Fellow, all at MSE.

Bio-inspired engineering has attracted considerable research interest in the past decade, though nature as a source of inspiration for scientists is not new. For instance, in the mid 20th century, zoologists exhibited a keen interest in understanding topics such as insect flight physiology that eventually led to identification and in-lab replication of the key material (called resilin) for insect flight. In the past two decades, materials scientists and chemists have extensively studied a wide range of biological materials, from soft to hard tissues, and this growing interest has led to remarkable progress in linking the intriguing properties of biological materials to their structure and chemistry. In addition, tremendous advances in molecular and genetic engineering technologies and instrumentation now allow affordable high-throughput genetic sequencing of a vast and diverse array of biological systems and materials.

The research team at NTU's Biological and Biomimetic Materials Laboratory (BBML) aims at elucidating the molecular, structural and functional relationships of unique natural materials in a synergistic approach that combines techniques such as protein and genetic sequencing, structure-property relationships at the nano-scale, recombinant protein expression and self-assembly, as well as chemical functionalisation and polymer synthesis (Fig 1). Many of our model systems are marine organisms from Singapore's intertidal zone, which contains hundreds of intriguing species. Conveniently accessible, these organisms include mussels, marine snails, cephalopods and stomatopods (Fig 2).

Core research focuses on understanding how robust multi-functional materials are being fabricated under benign environmental conditions, i.e. without the need for high temperatures, high pressures or harsh chemicals. The biological tissues of the study organisms – produced from natural chemicals in the

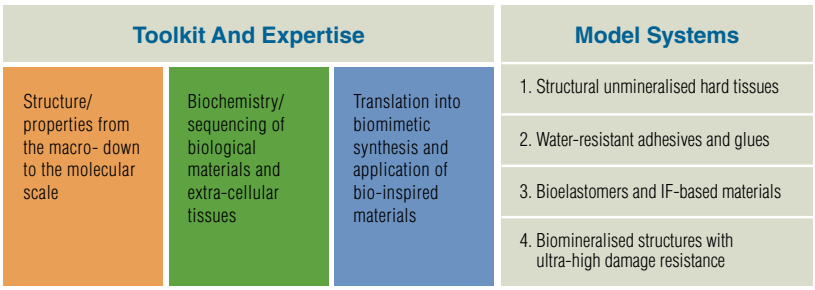

Fig 1. Four types of model systems are studied at BBML: (1) wear-resistant tissues and materials that *do not* contain a hard mineral phase; (2) water-resistant natural glues secreted by mussels and other marine molluscs; (3) bioelastomeric materials made of intermediate-filament (IF) protein types; (4) ultra-high damage resistant biomineralised structures.

These biological materials are studied by combining expertise in Materials Science (materials structure-properties relationships) and Life Sciences (biochemistry / sequencing of protein-based materials). Translation into synthesis of biomimetic materials is carried out by genetic engineering as well as polymer chemistry approaches.

aqueous milieus of tropical oceans under conditions of low temperatures and pressures and thus without much energetic input – can provide invaluable lessons in sustainability and "green chemistry". Ultimately, the aim of the BBML researchers is to mimic such chemistry, following two approaches: One approach attempts to completely reproduce model materials using genetic engineering strategies. The second approach aims at replicating the key physico-chemical interactions discovered in model systems using polymer chemistry and functionalisation approaches.

Molecular Biomimetics: From Genotype To Phenotype

A complete approach to biomimetics requires detailed knowledge of primary protein sequence design and processing conditions. One of the cornerstones of the BBML involves the identification and sequencing of genes that encode key structural proteins of high-performance biomaterials. Examples include (1) squid sucker teeth, which are robust materials found in the tentacles of squid that perform grappling functions (Fig 2A, Fig 3); (2) squid beaks, which exhibit extreme hardness and impressive wear resistance

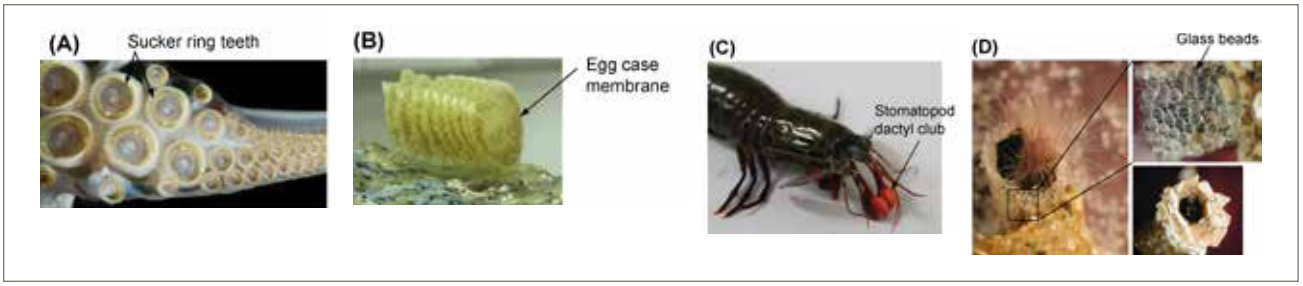

Fig 2. Model systems. (A) Sucker ring teeth are fully proteinaceous yet robust materials that line the tentacles of squid, and are self-assembled only through weak interactions between the constitutive proteins. (B) The ability of marine snails' elastomeric egg case membranes to absorb mechanical shock is unmatched in synthetic elastomers. (C) Stomatopods have evolved remarkable "dactyl clubs", which they use to shatter the shells of molluscs with impact forces up to 500 N (500 kg·m/s^2). (D) Sandcastle worms construct a tubular protective casing using water-resistant glue. This highly versatile polypeptidic glue can stick together diverse solid materials (here glass beads and egg shell fragments). BBML scientists are synthesising polypeptides that closely resemble the native glue to produce adhesives for biomedical applications.

(see Fig 3); (3) marine snail egg cases, which feature elastomeric materials with unique shock absorbing properties (Fig 2B); and (4) mussel byssal materials, which are made of self-healing fibers and water-resistant adhesives.

In collaboration with scientists from the Molecular Engineering Lab at Singapore's Agency for Science, Technology and Research (A*STAR), the research team uses state-of-the-art next-generation sequencing technology combined with proteomics tools to reveal the primary sequences, designs and processing mechanisms of these diverse materials. With the genes and protein sequences in hand, novel proteins can be designed and engineered in high amounts using recombinant protein expression tools. These recombinant proteins are then purified, assembled and/or processed further into high-performance fibres, films, adhesives and bulk materials that mimic the structural as well as biophysical and mechanical properties of these remarkable native materials (Fig 3).

Materials Synthesis: Utilising Key Physical-Chemical Principles

In parallel to genetic and protein-engineering strategies, the team is employing powerful reductionist approaches in the biomimicry of model systems. Based on a fundamental understanding of the key biological and chemical processes and principles that are responsible for the unique characteristics of natural materials, chemists at the BBML are replicating these principles by tailoring the physico-chemical behaviour of natural polymers through highly controlled chemical modification processes.

Fig 3. Advanced bioinspired materials. In the "Biological approach" genes encoding proteins of model systems provide templates for novel protein designs. Recombinant proteins are assembled into high-performance fibres, films, adhesives and bulk materials. In the "Synthetic approach" polymer scaffolds are chemically modified to incorporate additional functionality and chemically cross-linked with peptides or inorganic particles to mimic chemical interactions observed in model materials in order to yield designed biomimetic materials (e.g. biocompatible hydrogel films or adhesives).

The major goal is to combine biomimetic principles gathered from various model systems into one material. As an example, squids employ controlled dehydration in order to gradually strengthen the tissue of their beak from soft to hard by diffusing hydrophobic proteins and chemicals into a hydrophilic polysaccharide network. This controlled desolvation can be utilised to create strong hydrogel materials that are chemically resistant, biocompatible, and orders of magnitude stiffer than currently available hydrogels. In parallel, mimetic peptidic domains are synthesised based on sequences discovered in the generated genetic databases and incorporated into chemically modified polymer matrices. This approach aims at the complete mimicry of the structural phenomena observed in the model systems. In the near future, this multifaceted approach will enable the engineering of materials that are truly self-healing and multi-functional for possible applications in regenerative medicine.