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The entire editorial board of TSSR would like to take a moment to convey our profound gratitude for the diligence, patience, and hard work of the Faculty of the Engineering, Mathematics and Science in Trinity College Dublin. Without their support, Volume IV would not have been possible.

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Contents

Life Sciences

Contents

Contents

Natural Sciences

Contents

Chemistry

Contents

Physics

Welcome

"So you believe in science only?"

"I have already explained to you that I don't believe in anything; and what is science—science in the abstract? There are sciences, as there are trades and professions, but abstract science just doesn't exist."

- Ivan Turgenev, *Fathers and Sons*

"We are grateful also to those who made our work possible, those who made our lives possible, and those who make our lives worth living. To all of you and all of them we say, 'Thank you.'"

- William C. Campbell, Nobel Laureate; Trinity alumnus

In our often-vulgar world, science is regularly held up as the antithesis to all that is violent, oppressive, and wrong. This is done without necessarily considering the discipline's inherent flaws and biases, its politics, and without considering the question of whom science serves.

Here at Trinity Student Scientific Review, we do not believe in abstract science.

We do not believe in impenetrable theories with little application to our everyday lives. We do not believe in scientific dogma that does little but preserve our established order. We do not believe in research that doesn't fulfil a simple criterion: whether it makes our world a better place. To paraphrase Nobel Laureate William C. Campbell, science that helps make our lives worth living.

It is with immense pride that we bring to you volume four of the Trinity Student Scientific Review, a journal that, we hope, is characterised not by the arcane, but of simple-yet-powerful suggestions to improve our world.

The publication of this review would not have been possible without the unswerving support of Dean Vinny Cahill and all those in the Faculty of Engineering, Maths and Science, to whom we owe an enormous debt of gratitude. I would also like to thank the team behind the publication of last year's review, in particular Sarah Jennings.

And, of course, we would not have this review without this year's incredible team: Conor Rossi, Andrew Connolly, Eleanor Windle, Isabel Jorgensen, Alva Casey, Áine McCabe, Eleanor Mullen and Rachel Hanger. I thank each of you for your tireless work—for making this review a reality.

We hope you find it incisive and insightful, low on the abstract and heavy on the tangible.

Gemma Mortell
General Manager, Vol IV.

Life Sciences

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Letter from the Editor

The Life Sciences section comprises reviews on the study of microorganisms, plants, and animals as well as related concerns like bioethics. Research of life sciences is vital in improving human living standards. Advances in the life sciences provide a foundation for advances in pharmaceuticals and medicine, and may hold solutions to global issues in agriculture and climate change. As the Trinity Student Scientific Review journal enters its fourth year, Life Sciences once again proved to be the most popular section for submission. The moderatorship of experimental science was first taught in Trinity College Dublin in 1851. At inception, the subjects taught were physics, chemistry and mineralogy. Since then, the

range of moderatorships has diversified to fifteen distinct fields and students in Trinity embrace the biological sciences more than any other. This enthusiasm is reflected in the amount of submissions to Life Sciences this year.

The Editorial Board wished to select publications that reflect the current diversity of subjects in the life sciences. As such, you will find that the selected reviews encompass research fields of neuroscience, immunology, genetics, microbiology and more. The selected reviews have captured the cutting edge development of current scientific thinking. Further, they successively display a comprehension of subject that is indicative of critical scrutiny of the current literature. The difficulty of producing such a high-quality piece is testament to the passion and dedication of the authors and they should be commended for it.

The editorial process was directed by the comments and advice of our peer reviewers. We would like to express our sincere gratitude to the team of PhD reviewers who validated the work of the authors. Notably, a previous editor of the TSSR is counted amongst our PhD reviewers this year. It is heartening to recognise that the TSSR has already become a link for alumni even as a relatively new endeavor. We would also like to commend every student who took the time to research and prepare a submission for publication. The task of evaluating the breadth of research is not an easy one. The commitment

from those who submitted is a positive reflection of the academic drive that Trinity encourages.

It has been a privilege for us to act as Editorial Board for the Life Sciences section this year. The ability of researchers to effectively communicate their science is as important as it ever has been. It has been encouraging to see the enthusiasm that both students and academic advisers have for the TSSR. We have no doubt that every author, published or not, has improved their scientific-writing and communication skills during the writing of these reviews. These are skills that will serve the authors well in their academic futures. For now, we hope you enjoy these selected reviews as much as we have.

Conor Rossi, Áine McCabe, and Alva Casey
Life Sciences Editors
Trinty Student Scientific Review 2018

Circadian Regulation and its Role in the Development of Alzheimer's Disease

Natalie Ness
Junior Sophister
Genetics

Rhythmic changes in behaviour and physiology have been observed in all living organisms, and arise from molecular circadian rhythms.[1] Disruption and dysregulation of circadian rhythms have tremendous consequences on human health and play a major role in the development and progression of numerous diseases, such as Alzheimer's disease, which is the most prevalent age-related neurodegenerative disease. This review aims to explore the genetic regulation of circadian rhythms in Drosophila and mammals, and its relevance in human health and disease by exploring its role in the development of Alzheimer's disease.

Introduction

The 2017 Nobel Prize in Physiology or Medicine was awarded jointly to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for their research on the *period (per)* gene in *Drosophila melanogaster*, which led to significant advances in the understanding of the molecular mechanisms that regulate the circadian rhythm. They carried out genetic screens on *per* mutants and isolated several mutants that displayed extended or shortened circadian rhythms as well as arrhythmic mutants.[2] Rhythmicity could be restored by insertion of the wildtype per locus.[3] Subsequent immunohistochemical staining of the *per* gene product PER revealed that the protein is distributed in a variety of tissues and that its protein and mRNA concentrations in these tissues oscillate over the circadian cycle with a peak in the middle of the night. The fact that PER concentration is highest within nuclei led to the conclusion that oscillations in PER could be due to *per* mRNA oscillation and indicated the presence of a feedback loop, in which PER causes cycling of its own mRNA.[4–6] Another gene was associated with the circadian clock when *timeless (tim)* null mutants were discovered to be arrhythmic. Further investigation suggested that TIM may be involved in nuclear localization of PER, thereby establishing nuclear PER cycling.[7] These discoveries demonstrate how the circadian rhythm

is regulated on various levels through transcriptional and translational feedback loops.

As a circadian cycle typically lasts for approximately 24 hours[8], it may seem evident that the circadian rhythm is governed by the earth's rotation around its axis and thus arises as a response to external cues. However, the circadian rhythm is under genetic control and rhythmicity is endogenous and persists even in the absence of external cues.[9] However, although circadian rhythms are self-sustained, they can be entrained by external cues, referred to as *zeitgebers*, in order for the organism to adjust to its external environment. Furthermore, a lack of synchrony of the internal circadian rhythm with the external environment is a major cause of health problems, such as impaired cognitive and hormonal function arising from shift work and sleep deprivation.

Entrainment of the Circadian Clock

The circadian rhythm is hypothesized to be established by a series of oscillators, which drive rhythmic expression of genes. In mammals, the primary circadian clock was found to be located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus and coordinates peripheral oscillators to establish a coherent circadian rhythm throughout the body.[10,11] A widely accepted model outlining the network of oscillators found in *Drosophila* hypothesizes that pigment-dispersing factor-expressing lateral neurons serve as master pacemaker cells.[12]

Light is the most influential *zeitgeber* as it resets the circadian clock at the beginning of the day and thereby induces all hormonal, metabolic and locomotive activity required when the organism is awake. The primary photoreceptor in mammalian systems are intrinsically photosensitive retinal ganglion cells (ipRGCs), which are depolarized in response to light even in the absence of synaptic input from retinal rod or cone cells.[13] The action spectrum of ipRGCs suggests that melanopsin is the major photopigment.[14] The ipRGCs innervate the SCN, thereby ultimately mediating phosphorylation of Ca^{2+}/cAMP-response element binding (CREB) proteins, which bind to CREs found in the promoters of several core clock genes, thereby affecting transcription.[15,16] Two such light-inducible core clock genes are *mPer1* and *mPer2*.[17] While there is an increase in the level of PER1 and PER2 at the start of the day in the endogenous circadian rhythm, light-induced signals further enhance the increase in PER1 and PER2, thereby shifting the circadian phase to more closely align with local time.[18] Therefore, photic entrainment of the circadian clock is achieved by light-induced transcriptional control of clock genes.

The main photopigment in *Drosophila* is cryptochrome (CRY).[19] Photic entrainment of *Drosophila* is mainly mediated by enhancing the degradation of TIM via an ubiquitin-proteasome mechanism, which also takes place under free-running conditions.[20,21] Light accelerates degradation by increasing TIM phosphorylation and/or proteolytic activity. Organisms may adapt to the light intensities in

their environment by expressing a specific isoform of TIM with a suitable photosensitivity.[22] In fact, regional differences in circadian photosensitivity are likely to be naturally selected for by selection for the most suitable *tim* polymorphism.[23]

Other *zeitgeber*s include temperature, oxygen availability, physical exercise and several neurotransmitters and hormonal signals.[24–27]

Regulation by Transcriptional Feedback Loops

The circadian oscillators drive a network of transcriptional-translational feedback loops that establish rhythmic expression and translation patterns of the core clock components. *Zhang et al.* found that about 43% of the mammalian protein-coding transcriptome is under circadian control.[28] In mammals, the primary autoregulatory transcriptional feedback loop involves the major clock components CLOCK and BMAL1. The equivalent transcription factors in *Drosophila* are CLOCK (CLK) and CYCLE (CYC). The CLOCK and BMAL1 proteins form a heterodimer that initiates the transcription of a number of clock genes by binding to E-box *cis*-regulatory enhancer sequences, as shown in Figure 1. Mammalian genes whose transcription is activated by CLOCK and BMAL1 include *mPer1*, *mPer2* and *mPer3*, as well as the *cryptochrome* genes *mCry1* and *mCry2*.[29,30] Similarly, CLK and CYC initiate the transcription of PERIOD (PER) and TIMELESS (TIM) in *Drosophila*[31], as shown in Figure 2. In mice, *Clock* and *Bmal1* transcription takes place at daytime and transcription of their target genes is initiated in the afternoon. This causes accumulation of PER and CRY in the evening.[32]

Negative feedback is mediated by CRY and PER accumulation, dimerization and translocation into the nucleus to directly interact with the CLOCK-BMAL1 heterodimer, which inhibits transcription of its target genes, including *Cry* and *Per* themselves.[33] As this occurs in response to accumulation of CRY and PER, translocation into the nucleus and inhibition occur at night time. In *Drosophila*, a complex of PER and TIM is translocated back into the nucleus, where it represses its own transcription by binding to CLK in a similar manner.[34]

One of the major interlocked feedback loops in mammals involves the ROR and the REV-ERB α and β genes, whose transcription is activated by the CLOCK-BMAL1 heterodimer. ROR and the REV-ERB proteins compete for ROR response elements in the *Bmal1* promoter. ROR activates transcription, whereas the REV-ERBs repress transcription of *Bmal1*.[35] This affects the period length and phase-shifting properties of the clock, thereby stabilizing core clock function.[36] An analogous stabilising feedback loop also exists in *Drosophila* and involves the transcription factors vrille (vri) and Par Domain Protein 1 (Pdp1).[36] VRI levels were found to peak 3-6 hours before PDP1 levels, which allows for precise circadian regulation of dCLK as VRI

represses and PDP1 activates dClk transcription.[37]

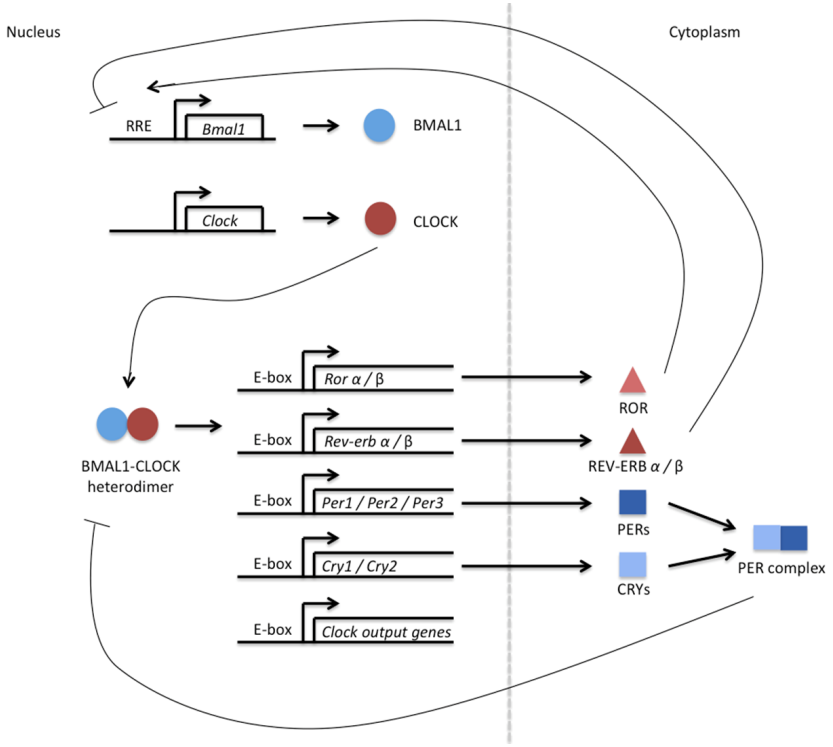

Figure 1: Schematic representation of the main mammalian circadian clock transcriptional feedback loops. The regulatory relationships involved in both the main autoregulatory feedback loop involving BMAL1, CLOCK and the PER complex, as well as the major interlocking feedback loop involving ROR and REV-ERBs are illustrated.

Cis-regulatory elements, such as E-boxes and REV-ERB and ROR binding elements (RRE) are found in many genes and display a repressor-precedes-activator regulatory pattern. This results in delayed transcriptional activity when repressors are present, and causes the genes to be highly transcriptionally active or inactive in each phase.[38]

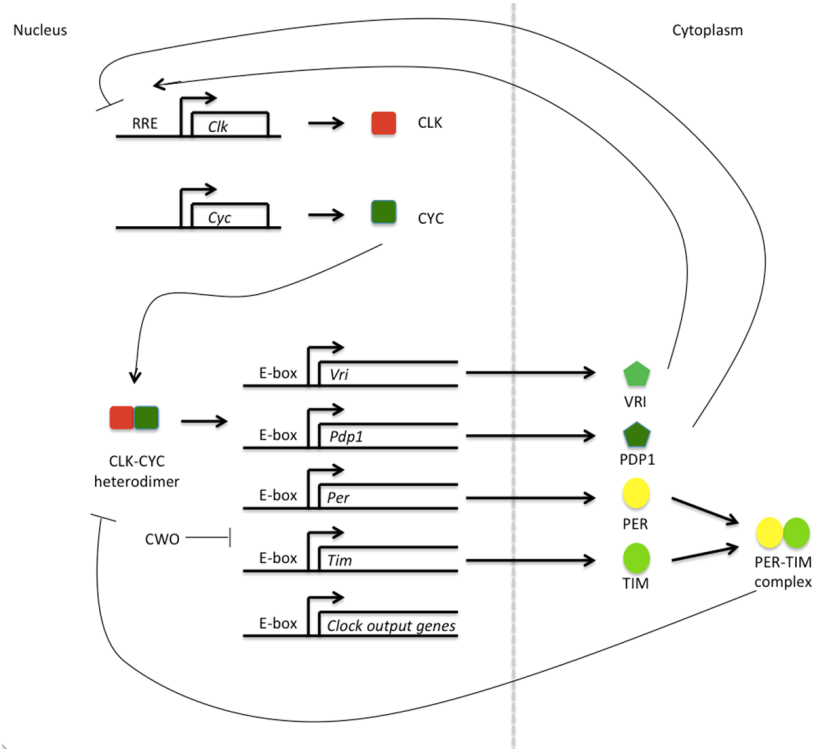

Figure 2: Schematic representation of the Drosophila core circadian clock transcriptional feedback loops. The major autoregulatory feedback loop involving CLK, CYC, PER and TIM, as well as the stabilising feedback loop driven by temporal expression of VRI and PDP1 are shown.

Transcriptional feedback loops are widely accepted to be the main drivers of circadian rhythm, however this clock model has been challenged. For instance, *Fujimoto et al.* found that mice, in which *mPer2* is constitutively expressed, still display rhythmic PER2 activity, which suggests that post-translational modification of PER2 is sufficient for oscillation.[39] Post-translational regulation is required for proper clock function as it allows for a delay in transcription of many genes and plays a vital role in targeting proteins for proteasome-mediated proteolysis.[40] Furthermore, genome-wide temporal RNA polymerase II DNA occupancy and histone modification profiles have shown that RNA polymerase II recruitment is highly regulated by dynamic epigenetic changes, and transcriptionally active chromatin conformations coincide with clock gene transcription. This has led to the hypothesis that epigenetic control mechanisms contribute to transcriptional regulation of clock genes in mice. [41]

Role of Circadian Regulation in Human Physiology and Behavior

As chronic stress, sleep disruptions and irregular eating times are very common and easily arise from shift work and stress, it is important to understand how this affects the circadian clock in order to minimize consequences. For example, the mood stabilizer lithium normally inhibits GSK-3β, however, chronic stress was found to elevate GSK-3β activity by phosphorylation in mice. This affects *Per2* transcription and abnormally high activity may diminish PER2 rhythmicity.[42] The effect of chronic stress is especially severe in peripheral clocks as entrainment occurs through the actions of stress-induced corticosterone and adrenaline/noradrenaline, and unlike peripheral oscillators, the SCN lacks glucocorticoid receptors.[43] Thus, chronic stress causes phase delays and dysregulation of peripheral circadian rhythms, and thereby leads to desynchronization from the SCN.[44] Internal desynchronization is referred to as jet lag and renders the circadian system dysfunctional.[45]

An important consideration in disease treatment is chronopharmacology, in which timing of drug administration is tailored to the circadian clock to maximize absorption and desired effects, while minimizing toxicity.[46,47] For instance, aminoglycosides are often used as part of antimicrobial therapy but can be toxic for the kidney. The extent of their nephrotoxicity has been found to vary greatly depending on the time of administration, as renal toxicity is higher during rest.[48,49]

Circadian regulation is involved in the development and progression of numerous diseases, such as cancer, neurodegenerative diseases and metabolic diseases.[50–52]

Role of circadian regulation in age-related neurodegenerative disease

Aging is commonly associated with a decrease in quality and duration of nocturnal sleep as well as an increase in daytime fatigue.[53,54] Experimental studies on *Drosophila* and mice suggest that this may be due to an age-related decline in the efficacy of communication between clock neurons among each other and with target cells, despite normal molecular oscillations in the clock neurons.[55,56] Chronic sleep deprivation is associated with brain cell damage, blood-brain barrier (BBB) breakdown and impairments in cognitive function by inhibiting hippocampal neurogenesis.[57,58] As mice with *Bmal1* knockout mutations exhibit disruptions in the regulation of reactive oxygen species homeostasis, *Ali et al.* proposed that the age-dependent decline in hippocampal neurogenesis may be a consequence of increased oxidative stress.[59] *Kondratov et al.* found that administration of antioxidants to *Bmal1*-deficient mice significantly extended lifespan but did not reduce the occurrence of age-related pathologies such as joint ossification and thus proposed that BMAL1 is involved in the response to oxidative stress by expression

of major antioxidant enzymes and that loss of this activity is responsible for some age-related symptoms.[60]

BBB integrity reduction occurs age-dependently and is involved in the development of multiple neurological diseases such as Alzheimer's disease (AD).[61] BMAL1 deficiency has been associated with BBB hyperpermeability due to loss of pericyte coverage of blood vessels in the brain.[62] BBB breakdown causes capillary leakages, which facilitate entry of β-amyloid into the brain, thereby accelerating accumulation.[61] Pericytes are also involved in controlling β-amyloid precursor processing and thus loss of pericytes accelerates β-amyloid plaque buildup.[63] The accumulation of β-amyloid contributes to the progression of AD.

AD is mainly characterized by sleep problems and circadian rhythm dysfunction.[64] Common circadian symptoms of AD are a phase delay in body temperature rhythms[65] and "sundowning", which refers to exacerbation of behavioural symptoms of AD late in the day.[66] As mentioned previously, ipRGCs mediate light entrainment of the SCN, and the loss of ipRGCs has been identified to be a major contributor to circadian rhythm dysfunction in AD.[67–69] ipRGC loss is likely to be caused by the symptoms of AD. For instance, AD is characterized by the buildup of β-amyloid plaques and tau containing neurofibrillary tangles, which cause cellular damage and have been found to accumulate in retinas of AD patients. Detection in the retina often precedes detection in the brain, suggesting that the consequences of ipRGC impairment may play a large role in disease progression at early stages.[70–72] Studies in mice have shown that the level of β-amyloid accumulation in the brain interstitial fluid (ISF) (equivalent to accumulation in the cerebrospinal fluid in humans) is directly related to the amount of sleep obtained. While the molecular mechanism of β-amyloid regulation is not fully understood, *Cirrito et al.* found that inhibiting the Extracellular Regulated Kinase (ERK), an enzyme involved in the orexin receptor signaling pathway, increases β-amyloid levels by 50%.[73] This suggests that the orexin pathway, which is regulated by input from the SCN, mediates β-amyloid levels during sleep and wakefulness.[74]

β-amyloid plaques may alter circadian regulation by inducing post-translational degradation of the circadian clock regulator CBP, which is involved in epigenetic regulation of genes under the transcriptional control of BMAL1, as well as BMAL1 itself.[75] Thus, circadian rhythm disruption promotes the development of neurodegenerative disease, which in turn alters circadian regulation. The dynamic relationship between circadian disruption and AD is illustrated in Figure 3.

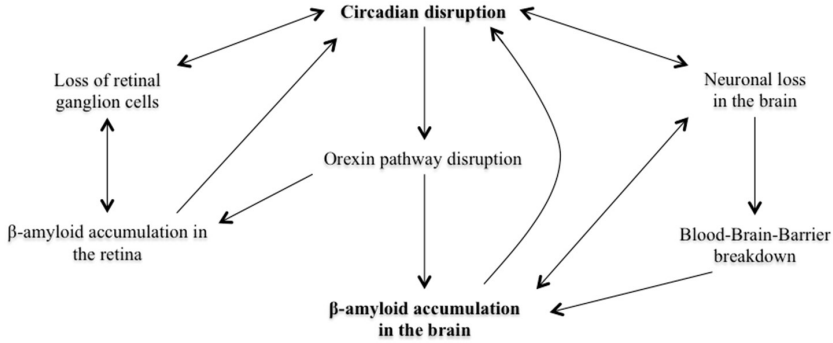

Figure 3: Diagram illustrating the relationship between circadian disruption and AD. The relationships between symptoms reflect the disease's self-propagating nature.

As the circadian rhythm plays a major role in the progression of AD, the circadian system may serve as a target for treatment. Light therapy has been successfully used to stimulate entrainment of the circadian clock and thereby improve sleep quality and reduce depression and agitation.[76]

Oral administration of melatonin is considered a promising pharmacological treatment for AD and its potential is currently being tested in clinical trials.[77] Melatonin acts as a *zeitgeber* on the circadian clock and has been shown to facilitate the breakdown of β-amyloid plaques by inhibiting β-amyloid precursor processing and stimulating the expression of α-secretases in hippocampal neurons, and alleviate cognitive deficit in mice.[78–80] There are concerns over the short half-life of melatonin as it is rapidly metabolized and thus selective melatonin receptor agonists such as ramelteon and tasimelteon are also being considered.[81] However, long-term safety studies of their metabolites are lacking.[46]

Circadian disruption also occurs in neurodegenerative diseases that are not strongly associated with age. Cluster headache and bipolar disorder for example are both recurrent in nature and often preceded by sleep-wake disturbances. Genetic predisposition to circadian dysregulation plays a major role in the development of these diseases.[82]

Conclusion

Circadian rhythms in physiology and behaviour are driven by endogenous molecular oscillations regulated by the circadian clock, which is entrained by environmental cues. The most commonly accepted circadian clock model proposes transcriptional feedback loops to be the main driver of molecular oscillations. However, this model has been challenged by a body of evidence that emphasizes

the importance of post-translational and epigenetic regulation of circadian gene expression. Integration of these various layers of regulation gives a highly dynamic and complex system with high levels of redundancy that enable the generation of robust rhythmic mRNA and protein profiles. There is still much research to be done to fully elucidate circadian regulation, however, the recently awarded Nobel Prize in this area will hopefully encourage further research and clearly indicates its importance in the scientific community.

Circadian disruption and dysregulation have been implicated in the development of numerous diseases, such as Alzheimer's disease, and chronopharmacological approaches have proven to be highly effective in maximizing the effect of drugs. Understanding the highly complex dynamics of the circadian clock is thus key to finding new, innovative approaches to diagnosis and treatment of diseases.

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Copper and Gram-Negative Pathogenic Bacteria: A Toxic Relationship

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Bacteria have had a relationship with copper for billions of years, those unable to cope, succumbing to the toxic effects of this heavy metal. Although toxic to life at certain concentrations, bacteria have evolved to take advantage of copper for their benefit, similar to their utilisation of the human body as a source of nutrients. As copper is used as an antimicrobial agent, it is important that we do not underestimate the ability of microorganisms to adapt in response. To predict or target these adaptations, we first need to understand how bacteria can and are responding to copper and how this contributes to pathogenesis. This review will focus on key features of copper homeostasis, discussing how gram-negative bacterial pathogens maintain a fine balance between what is toxic and what is useful.

Introduction

Microorganisms have been replicating in the presence of heavy metals long before humans walked on Earth. Heavy metals include elements such as iron, cobalt, nickel, zinc and copper and are commonly found in most environments, at varying concentrations. As human populations rose and disease became a more common part of life, the use of naturally occurring substances for health grew in popularity. One such substance was copper, a heavy metal first described as medically useful in the Smith Papyrus, an Egyptian text dated at 2600-2200 B.C.E.[1] Over four thousand years later, copper is still manipulated for its antimicrobial capabilities. We are entering an era in which antibiotics are becoming ineffective against many life-threating bacteria and to combat this, previously used antimicrobial agents, like copper, are being reconsidered for use. In order to prevent microbial contamination, copper has been suggested as a coating for hospital hand rails, doors and bathroom surfaces, due to its antimicrobial action.[1] While adding copper to object exteriors could vastly reduce the occurrence of nosocomial infections, it has been shown

that certain bacteria are less susceptible to copper-coated surfaces. This has been ascribed to copper-balancing or homeostatic abilities.[2,3] Thus, our understanding of copper homeostasis mechanisms in pathogenic bacteria will be an important aspect of evaluating adoption of copper as a commonly used surface component. The use of copper as an antimicrobial substance is not limited to hospitals, with places with high contact levels, such as airports, trains and gyms also being considered.[4] To examine the interaction of copper and bacteria we need look no further than our own immune system. Upon activation by inflammatory agents, production of copper transport protein 1 (Ctr1) is increased in macrophages.[5] Ctr1 is located at the macrophage plasma membrane and along with copper pump ATP7A, can provide copper with a means of translocating the membranes (Figure 1).[6,7] The partial localisation of ATP7A to phagolysosomes, specialised bacteria-killing compartments in macrophages, suggests copper plays a role in destroying bacteria taken up by the macrophage.[7]

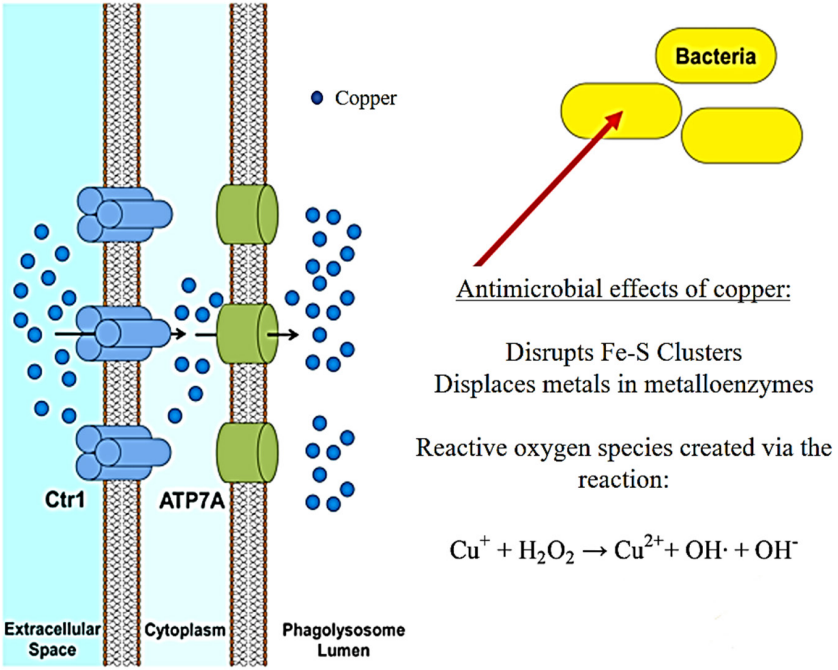

Figure 1: The use of copper by macrophages in the phagolysosomal compartment. Ctr1 imports copper through the macrophage membrane. Antioxidant 1 (Atox1) copper chaperone transfers the copper to ATP7A. ATP7A transfers it across the phagolysosome membrane. Copper then aids in the destruction of the bacterium within the phagolysosome. *Adapted from Fiesta & Thiele, 2012.*[7]

Copper can have several toxic effects, including the production of reactive

oxygen species, displacement of other metals from their relative enzymes and disruption of iron-sulfur clusters (Figure 1).[8] However, bacterial infections still arise, suggesting these bacteria have means of controlling environmental copper. This review will discuss these mechanisms in detail, in relation to relevant human pathogens, predominantly discussing gram-negative organisms and *Mycobacterium tuberculosis*. This review will highlight not only what is known but what is unknown in the dynamics of copper homeostasis.

Copper Binding Proteins

Based on the antimicrobial effects of copper listed in Figure 1, it would be easy to assume bacteria purely act to remove copper from their environment. However, copper plays an essential role in bacteria. Due to its reactive nature, natural abundance and ability to form stable molecular interactions, copper is ideal for biological reactions.[7,9] As such, copper is often integrated into structures to form functional proteins which, without copper, would be inactive. These copper-containing proteins (cuproproteins) are generally found within the bacterial cell envelope.[10] Notable functions of cuproproteins include cytochrome oxidases, NADH dehydrogenases, both used in the electron transfer chain, polysaccharide oxygenase and superoxide dismutases (SODs).[10] Cuproproteins are central to copper homeostasis as bacteria need to ensure there are enough copper ions present for these protein structures, while preventing free molecules from harming the cell.[11] Cuproproteins can effectively act as copper stores, although this is generally not their primary function. A protein which has been suggested to be a 'copper sink', is the *Mycobacterium* metallothionein (MymT) of *M. tuberculosis*.[12] Metallothioneins are small, cysteine rich proteins which have one or more metal ions bound.[13] They are rarely found in bacteria, potentially due to difficulty in their discovery, because of their small size and varying sequence. MymT has a high affinity for copper, holding up to seven ions, and is required for *M. tuberculosis* copper tolerance.[12]

Different heavy metal homeostatic mechanisms can overlap with one another, particularly where proteins have affinity for more than one specific substrate. One example of this is yersiniabactin (Ybt), an iron chelator, once primarily associated with iron homeostasis.[14] Ybt in *Escherichia coli* can also bind Cu^{2+} and convert reactive oxygen species (ROS) to hydrogen peroxide and oxygen.[15] It effectively sequesters Cu^{2+} in the extracellular space, preventing it from entering the cell. In uropathogenic *E. coli* (UPEC), Ybt has been shown to aid survival in the phagosome, presenting once again an association between heavy metal homeostasis and pathogenesis.[16]

Dynamics of Copper Uptake

Bacteria, as already discussed, need copper for various cellular functions. So how

do bacteria acquire the copper necessary for such functions? The mechanism of copper entry into gram-negative bacteria remains largely uncharacterised. It has been suggested that copper might simply diffuse across the membrane of gram-negative bacteria, however, this mechanism remains undefined and it seems unlikely given the low permeability of lipid membranes to copper.[12,17] Furthermore, cuprous copper (Cu^{+}) is considered to have greater cytoplasmic membrane-translocating ability than cupric copper (Cu^{2+}) and is considered the most toxic biologically.[18,19] Certain pathogens have mechanisms which block or inhibit uptake of copper ions by alteration of the ions' charge. Found in *S. typhimurium*, *Acinetobacter baumanaii* and *E. coli* is a protein called CueO, which has cuprous oxidase activity within the periplasm.[20,21] As its name suggests, CueO can convert the ion of copper, Cu^{+} to less harmful Cu^{2+}.[22] Other examples of such multicopper oxidases are the plasmid encoded *pcoA*, found in *K. pneumoniae* and *E. coli*, and MmcO protein of *M. tuberculosis*.[23,24]

CueO is found within the regulon of transcription regulator CueR.[25] CueR is capable of sensing zeptomolar concentrations of copper in the cytoplasm.[17] Importantly, CueR is an easy target for proteolysis in *E. coli* and its stability is not dependent on copper concentrations.[26] This allows the bacterium to be prepared for changing environments, which is significant for a pathogen experiencing the range of harsh conditions met in the human body. While CueO helps inhibit Cu^{+} passing through to the cytoplasm, another *E. coli* protein, ComC helps prevent passage of copper into the periplasm.[27] ComC, a protein bound at the outer membrane, makes the bacterial membrane less permeable to copper.[27] The expression of ComC is repressed by ComR, which does so in a copper concentration-dependent manner.[27]

Specific copper uptake systems are rarely found in bacteria. No importers have been described in *E. coli*, which is one of the most studied organisms in terms of copper homeostasis.[12] A suggested copper uptake protein HmtA has been described in *P. aeruginosa*. Overexpression of HmtA results in copper hypersensitivity and increased intracellular concentrations of copper.[28] The lack of known importer systems in human pathogens could possibly be because they haven't yet been discovered or alternatively, because gram-negative bacteria are under more pressure to detoxify themselves of copper than to acquire it.

Copper Efflux

Efflux pump systems are a ubiquitous method of removing toxic substances from inside the cell, literally by pumping them from the intracellular environment. Due to the current antibiotic resistance crisis, efflux pumps are receiving a lot of attention, often implicated in drug resistance and pathogenesis.[29] Exposure to copper can co-select for antibiotic resistance, as well as copper tolerance and this co-selection is mainly attributed to these efflux mechanisms.[30] With this and the role of copper in innate immunity, understanding copper efflux mechanisms in

pathogenic bacteria is of paramount importance.

Cus Copper Efflux System

Found in *K. pneumoniae* and highly pathogenic *E. coli* strains, the Cus efflux system can detoxify bacteria of both silver and copper.[31,32] The pump itself is a resistance-nodulation-cell division (RND) type pump which requires influx of protons to efflux copper ions.[33] It is composed of three proteins; CusA, CusB and CusC, arranged as shown in Figure 2.[33] It is made of six subunits of CusB with three subunits of both CusA and CusC.[33] The Cus system functions to remove copper, specifically Cu^{+}, from the cytoplasm.[34] It can also remove Cu^{+} from the periplasm, with the aid of CusF. CusF can then transfer copper to CusB, causing a conformational change in the latter, allowing transport of periplasmic copper out.[34] As is commonly found, a two-component system, CusRS, regulates expression of this efflux pump.[35] *cusCFBA* are encoded on one operon with a secondary operon containing *cusRS*. In the CusRS two component regulatory system, CusS is a histidine kinase and CusR is a response regulator.[35] CusS spans the inner membrane, detects copper in the periplasm and thus phosphorylates CusR, which affects gene transcription.[35,36] In *K. pneumoniae*, expression of both *cusRS* and *cusCFBA* operons increases in the presence of high quantities of copper, in both anaerobic and aerobic conditions.[31] In comparison, in *E. coli*, the Cus system only becomes necessary in anaerobic conditions.[35] CusCFBA's role in copper homeostasis is suggested to be only significant when copper levels overwhelm the other copper detoxification systems.[35]

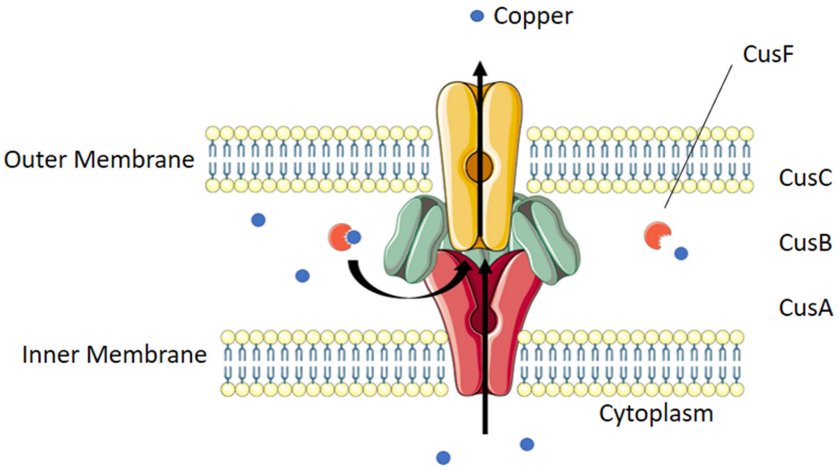

Figure 2: The Cus efflux pump system of *Escherichia coli*. The Cus efflux pump spans both the inner and outer membranes. It is composed of CusA (pink), the periplasmic adaptor CusB (green/grey) and CusC (yellow). Copper is transported via chaperone CusF. *Based on structure from Delmar et al., 2013.*[33]

Cue Copper Efflux System

While Cus plays an important role in anaerobic copper homeostasis in *E. coli*, the Cue system is considered to be more important in aerobic conditions.[35] In *E. coli*, the Cue system is composed of CopA, CueO and CueR. *copA* is encoded alongside *cueO* and is regulated by CueR.[22] The *copA* gene encodes a P_{1B}-type ATPase located at the cytoplasmic membrane which can transport copper out of the cytoplasm.[37] This CopA type pump is found in several pathogens including, but not limited to, *S. typhimurium*, *L. pneumophila* subspecies *pneumophila*, as well as gram positive pathogens such as *Staphylococcus aureus*.[38-40] As an ATPase type pump CopA relies on ATP hydrolysis to exclude copper from the cytoplasm.[41] The crystal structure of CopA shows eight transmembrane domains and three cytoplasmic domains.[41] The components of this structure are shown in Figure 3.

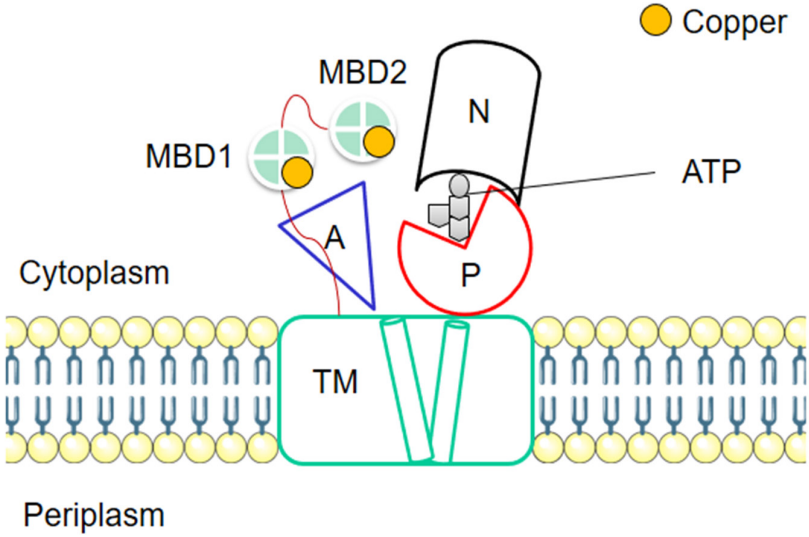

Figure 3: Structure of CopA. The transmembrane (TM) section is composed of eight helices, two shown here prepared to accept copper at the inner membrane. Cytoplasmic actuator (A), phosphorylation (P) and nucleotide-binding (N) domains are shown with ATP bound. Metal binding domains (MBD) 1 and 2 facilitate transport of copper. *Based on Drees et al., 2015.*[42]

In some bacteria encoding CopA, for example *Listeria monocytogenes*, copper is transported by an associated chaperone CopZ.[43] Chaperones and transcription regulators are key to the control of any efflux system's activity. The amount of metallochaperone present in the cell can determine how efficient the efflux system is, depending on the concentration of copper. It has been discovered in *E. coli*, that the CopA(Z) chaperone is produced from the first 69 amino acids of *copA* and can be produced in a greater ratio to the pump itself via a programmed ribosomal frameshift.[44] Interestingly, interaction between CopA and the chaperone from the Cus system, CusF, has also been observed.[45] *S. typhimurium*, which lacks a Cus system, has been found to have an additional chaperone, CueP, which is not found in *E. coli*.[46]

Pco/Sil Efflux Systems

The plasmid-borne copper resistance (Pco) system includes *pcoABCDRSE*, whose expression is stimulated in the presence of copper ions.[47] PcoA has multicopper oxidase activity, similar to CueO. PcoRS form a two-component system to activate the response to copper, similar to those found in the efflux systems previously discussed.

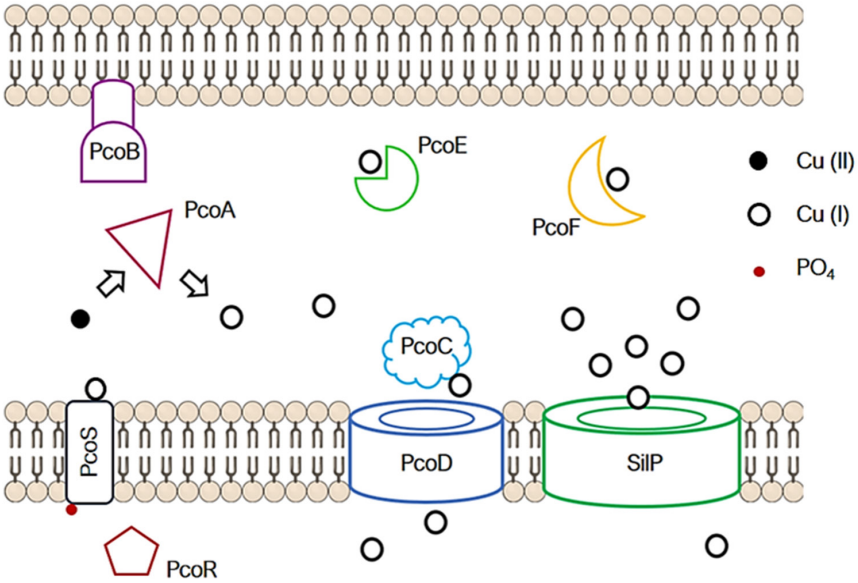

Figure 4: Model of Pco and Sil copper detoxification systems. Shown are the predicted contributions of the pco genes A-F to copper efflux alone with their regulatory system PcoRS. SilP, suggested inner membrane silver/copper transporter is also shown. *Based on Staehlin et al., 2016.*[3]

PcoB encodes an outer membrane protein, shown in Figure 4, which binds copper and PcoC is a periplasmic chaperone.[48] These components are also found in *P. aeruginosa*.[49] The role of PcoD is largely unknown but it is suggested to have a role in copper transport across the inner membrane.[47] The role of PcoE is likewise, undefined, but considered to be a potential chaperone in the periplasm.[11] Unusually, it is regulated separately by the CusRS system.[50] Some homologous proteins of this Pco system are found in the silver-resistance system named Sil. This was discovered in *S. typhimurium* and itself shares functional and sequence homology with the Cus system.[36] *silCBA* form a transmembrane pump and *silP* encodes a P_{1B}-type ATPase pump in the inner membrane.[51] Although these genes are associated with silver resistance, Cu^{+} transport is suggested to be carried out by the SilP efflux pump, as shown in Figure 4, although this has not been demonstrated experimentally.[36,52]

Mycobacterium tuberculosis Efflux Systems

M. tuberculosis is a highly adapted intracellular human pathogen, one of the oldest we are aware of, and it currently kills about two million people worldwide every year.[53] Unfortunately, there are many aspects of this pathogen and its genome that remain unknown. Many of its copper homeostasis mechanisms, in particular efflux systems, are still only at the theoretical stage of discovery. *M. tuberculosis'* encoded

cation transporting protein V (CtpV), is predicted to be a P-type ATPase copper transporter, with eight transmembrane helices and three domains in the cytoplasm.[54] Its expression is induced in the presence of copper, and CtpV has similarities to the amino acid sequence of CopA in *E. coli*.[54] Unusually, CtpV has no known copper binding domain, although it has many typical P-type ATPase motifs.[12] Its function is currently predicted, with no direct transport of copper by CtpV yet shown.[12] CtpV was originally discovered after the operon on which it is encoded was found to be relevant for *M. tuberculosis* pathogenesis in mice.[55] This operon, the copper sensitive operon (cso), encodes four genes (Figure 6). Two are of unknown function, *rv0968* and *rv0970*, with *ctpV* encoded in between. The other gene was found to encode the operon regulator, CsoR.[56] This regulator binds to *cso*, preventing transcription and is only released when copper is bound.[56] CsoR has been shown to only regulate its own operon, dedicated to regulation of expression of just four genes.[57]

Mycobacterial copper transport protein B (MctB/Rv1698) is a protein of unknown structure and specific function. It has been described as an outer membrane protein analogous in function to gram-negative porins.[58] More recent studies indicate that MctB is actually most likely located at the inner membrane of *M. tuberculosis* (Figure 6).[59] Deletion of *mctB* leads to increased intracellular copper and the same occurs with deletion of its homologue in the pathogen *Mycobacterium smegmatis*.[60] The exact role of this protein in copper homeostasis has yet to be elucidated.

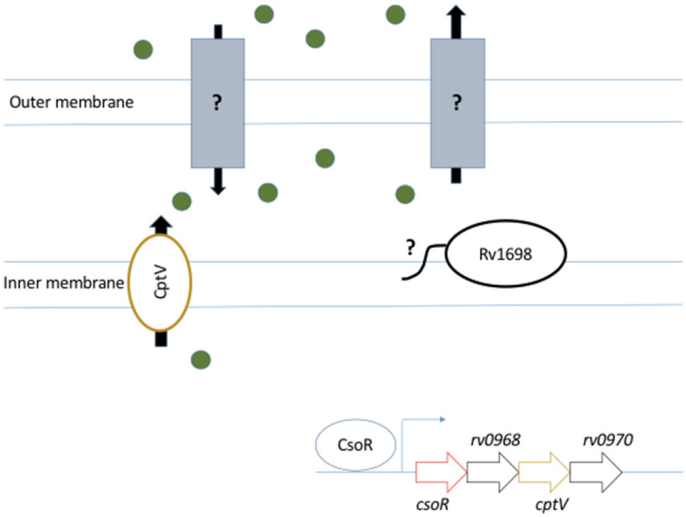

Figure 5: *cso* operon and CtpV efflux model in *Mycobacterium tuberculosis*. Shown are the genes encoded in the *cso* operon, regulated by CsoR. CtpV, encoded by the operon, is a suggested transporter of copper (yellow) across the inner membrane. Also show is Rv1698 (MctB), hypothesised to be a membrane pore-forming protein. (*Based on diagram from Rowland & Niederweis, 2012.)*[12]

Conclusion

With what has been discovered so far in copper homeostasis in pathogenic bacteria, copper detoxification is clearly a key feature of bacterial physiology. While no two strains will have the exact same physiology, broadly this detoxification can be grouped into three mechanisms: Copper export, sequestration and modification. Currently our knowledge of copper homeostasis in pathogens remains largely incomplete. Incomplete knowledge of bacterial physiology and a lag between discoveries and their application in clinical practice, have contributed to the current issue of antibiotic resistance. As our innate immune systems use copper against invading pathogens, the risks of using copper in healthcare should be assessed, particularly if selecting for copper tolerance in hospital settings, might render our own immune responses less effective at killing pathogens. This assessment would rely on an understanding of copper homeostasis in pathogens, which can only be achieved through scientific research. As copper utilising organisms, we can learn a lot from our pathogens and potentially use this knowledge in future, in medical and pharmaceutical applications of copper.

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Genetic Evidence for the Involvement of Immune Processes in Alzheimer's Disease

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Alzheimer's disease (AD) is a common neurodegenerative disease. Despite the prevalence of this disease, current treatments are ineffective in preventing disease progression. Previously, research has focused on amyloid processing as a clinical target, but "omics" approaches such as genome-wide association studies (GWAS) have identified alternative processes involved in pathogenesis which may represent clinical targets. Immune processes are common to many neurodegenerative diseases and have also been associated with AD. In the past, it was unclear if inflammation was causative or a side effect of disease. However, GWAS data supports the hypothesis that immune involvement is a causative factor in disease. This review summarises genetic evidence for immune involvement in disease pathogenesis, and describes several clinical interventions targeting these processes currently under investigation.

Introduction

Alzheimer's disease (AD) is a primarily age-related neurodegenerative disease and the principle form of dementia. In 2015, approximately 44 million people worldwide were affected, and this is predicted to almost double by the year 2030.[1] However, current treatments are symptom-based, and do very little to inhibit disease progression.[2] A more detailed understanding of the causes of pathology would allow the design of targeted therapies, which could prove to be more successful.

AD is classified into two forms: early-onset AD (EOAD) and late-onset AD (LOAD). Although both forms of disease are characterised by stereotyped pathology, primarily amyloid-β plaques and neurofibrillary tangles, there are significant differences between them.[1] EOAD onset occurs before 65, and a small number of causative mutations in key genes have been identified. In contrast, LOAD is much more heterogeneous, with onset after 65 and heritability estimates between 60-

80%.[3] Until recently, efforts to develop therapies had been focused on the amyloid processing pathway, which had been identified as central to pathology in EOAD by linkage analysis and pedigree studies of affected families.[4] However, EOAD only explains 2-10% of all cases, while LOAD is more common and more complex in its heritability and causes.[1]

Genome-wide association studies (GWAS) have been used to identify mutations associated with AD. The most penetrant risk variant is the *APOE* ε4 allele, but this only accounts for 30% heritability. A systems-based approach has been used to try to identify categories of genes and molecules that may be involved in AD pathology.[5] Although abnormal immune processes had previously been characterised in AD brains, it was unclear if this was a by-product of other forms of pathology, or if it directly contributed to disease.[6] Immunity and inflammation has been identified by both GWAS and transcriptomic analysis as being central to LOAD pathology, which suggests they do contribute to disease onset. The utility of related pathways and molecules as clinical targets is currently under investigation.

The Amyloid Hypothesis and Disease Biology

Autosomal dominant AD accounts for less than 1% of disease occurrence, but insights from the genetics of this form of disease have had a major impact on understanding of disease biology.[1] The most commonly mutated genes in familial AD are amyloid precursor protein (*APP*), and presenilin 1 and 2 (*PSEN1* and *PSEN2*).[7] These converge on the amyloid processing pathway, which is strongly suggestive of a causative role for amyloid pathology in disease progression.

APP can be processed by two possible pathways: the constitutive, nonamyloidogenic pathway, involving α- and γ-secretases, and the amyloidogenic pathway, involving β- and γ-secretases (figure 1).[8] Presenilins are components of the γ-secretase. The amyloidogenic pathway produces amyloid-β isoforms of different lengths, which vary in their tendency to form aggregates, and hence amyloid plaques. APP processing doesn't produce a single isoform, but the familial AD mutants may influence the processing pathway so the ratio of longer, more aggregation prone isoforms to shorter ones is increased, i.e. the ratio of $A\beta_{1\text{-}42}$ to $A\beta 1_{\text{-}40}$ is increased.[1,9] These analyses have led to the hypothesis that amyloid processing is central to AD pathology.

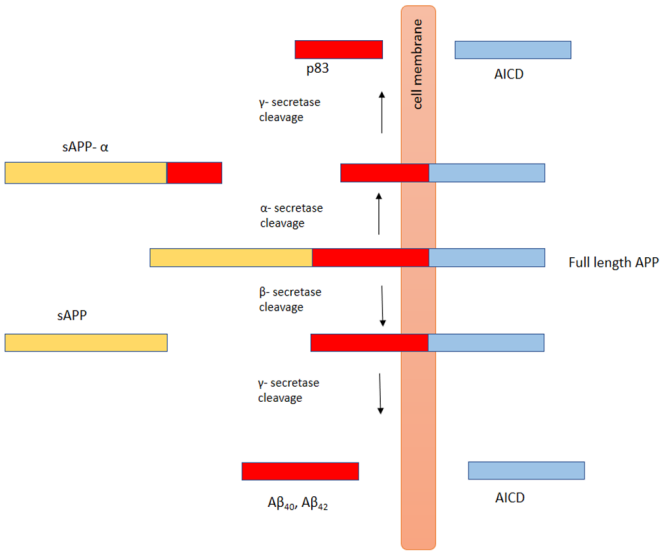

Figure 1: Schematic of APP processing pathway. Non-amyloidogenic pathway involves α- and γ-secretases; involves β- and γ-secretases. Amyloidogenic pathway APP: amyloid precursor protein; sAPP: soluble APP; AICD: amyloid precursor intracellular domain; p83: shorter cleavage product of non-amyloidogenic pathway. *Adapted from Zhang et al (2011).*

Hyper-phosphorylated forms of the microtubule-associated protein tau is also a hallmark of both AD and other neurodegenerative diseases. This protein forms extracellular neurofibrillary tangles, which is associated with neurodegeneration.[10] Although the precise role of amyloid-β and tau neurofilaments is yet to be described, it is thought they may interact in some way to mediate neurotoxicity.[11]

The genes discussed above converge on the amyloid processing pathway as a cause of AD. However, therapies targeting this pathway have not led to improved care.1 The genetic complexity of LOAD suggests other factors influence disease risk and progression, and a greater understanding of this is necessary to comprehend pathology. The use of high-throughput sequencing and systems biology approaches have allowed a more detailed understanding of the pathways involved in AD.

Late-Onset AD and Genome-Wide Association Studies

LOAD is a genetically more complex and more heterogeneous disease than familial EOAD.[1] Unlike EOAD, nearly all cases do not have a single identifiable genetic cause, but involve lots of factors modulating disease risk. Therefore, different approaches are necessary to identify factors accounting for the estimated 60-80% heritability

of this form of disease. The advent of high-throughput technology has allowed for the use of "-omics" to identify possible underlying genetic causes and pathways involved in pathogenesis.[12] Genome-wide association studies (GWAS) are used to identify SNPs associated with disease. As well as the most significantly associated variant (the APOE ε4 allele) at least 19 other risk loci have been identified.[13]

However, these only explain a proportion of heritability observed.[14] A complementary, systems-based approach has been developed, which identifies particular gene ontology categories enriched in GWAS datasets, and hence possible underlying pathways in disease.[15,16] In addition, possible gene interactions and gene networks can be identified, and a transcriptomic approach identifies categories of genes that are up or down-regulated in disease.[5,14,17] Although a proportion of disease heritability is identified by GWAS, these "system-based" approaches can be a sensitive way to identify underlying pathways in disease progression, which can be used to evaluate variants and identify possible targets for clinical intervention.[16] However, this approach is limited by the functional annotations available - unless a signal is well annotated in the databases being used, it will not be detected.[15] This needs to be considered when data is assessed.

Pathways or categories of genes identified from these studies can be used to identify possible biological processes involved in disease progression. These categories can then be investigated more closely to identify possible disease mechanisms and therapeutic targets. Categories such as immunity and inflammation have been significantly associated with disease, and represent promising therapeutic targets.

Microglial Activation

A common feature of neurodegenerative disorders is neuroinflammation, and immune-related genes are important contributors to LOAD.[16–19] Microglia-mediated immunity is important both in the phagocytosis of amyloid-β and the secretion of inflammatory mediators. Therefore, microglia are directly relevant to amyloid pathology, but also to the inflammation characteristic of many neurodegenerative disorders.[20]

Rare coding variants in *TREM2* and *ABI3* have been associated with LOAD pathology.[14] *ABI3* is primarily associated with actin cytoskeletal remodelling, a process which has also been implicated in disease pathogenesis, but it has also been reported to be involved in interferon signalling.[14,21] TREM2 is a lipid-sensing receptor that can detect phosphatidylserine, which is exposed on damaged cells. Two independent risk variants were identified in *TREM2* (R62H and R47H), located in exon 2, the predicted ligand-binding domain of the receptor. The *TREM2* R47H mutation has also been associated with disease by GWAS, with an odds ratio of approximately 2.9, almost rivalling that of *APOE* ε4.[13,22] An odds ratio is the ratio of probability of disease in carriers of the mutation to probability of disease in people without the mutation. Mutations in this domain would result in an impairment

of receptor function and hence a loss of signalling. This loss of signalling reduces microglial M2 activation, which is involved in the recognition of debris from lipid membranes and amyloid deposits.[23] *Krasemann et al.* (2017) also identified a TREM2 signal important in mediating changes in microglial phenotype as neurodegeneration progressed.[24] Hence, lesions in TREM2 can result in deficits in amyloid clearance.

Other components of this signalling cascade include *SPI1*, a transcription factor involved in microglial activation and *CSF1R*, mutations in which are associated with white matter disease. Pharmacological targeting of *CSF1R* switches the profile of microglia from pro- to anti-inflammatory, and prevents progression of AD-like pathology in mice.[25] This evidence suggests dysfunctional TREM2 signalling contributes to some cases of LOAD, and targeting elements of this pathway such as CSF1R may be a valuable therapeutic strategy.

Although polymorphism in TREM2 has been found to be associated with AD, its role is controversial. *Krasemann et al.* (2017) report *Trem2*$^{-/-}$ mice suppressed inflammatory signals upregulated in disease, and *TREM2* upregulation is associated with an AD-associated *CD33* variant[24,26]. However, *Sims et al.* (2017) and *Wang et al.* (2015) report that variants in TREM2 are associated with disease progression.[14,23] The role of microglia in both phagocytosis of amyloid-β and in inducing inflammation suggest TREM2 signalling might be difficult to classify as "good" or "bad", and further investigation using specific *TREM2* risk variants is needed to clarify involvement in disease.

TYROBP is a binding partner of TREM2 also implicated in AD progression.[16,17] According to *Zhang et al.* (2013), *TYROBP* is significantly upregulated in LOAD brains and its differential expression between cases and controls is more extreme than other microglial markers.[16] This provides further evidence for the importance of microglia in AD, particularly TREM2-TYROBP signalling.

Zhang et al. also investigated the effect of TYROBP dominant-negative truncation on gene expression profiles in mice. It was found TYROBP was upstream of several pathways, but the most significant effect of the truncated gene product was that autophagy-associated genes were downregulated in mice with wild-type TYROBP, whereas they were upregulated in mice with the dominant-negative mutation.[16] This suggests autophagy and hence inflammation are relevant to AD pathology. In addition, mutations in *TYROBP* or *TREM2* can cause Nasu-Hakola disease (a Mendelian neuroinflammatory disease), which further suggests a role for inflammation in AD pathology.[27]

Phaogcytosis

Microglia are phagocytic cells, and phagocytosis contributes to amyloid-β plaque clearance. *ABCA7* has been associated with both EOAD and LOAD.[28,29] ABCA7

is a lipid transporter, and is highly expressed in microglia but not neurons or astrocytes, which suggests it may play a role in mediating phagocytosis.[30] *De Roeck et al.* (2017) identified several premature termination mutations in EOAD that increased familial AD load. None of the identified variants passed study-wide multiple testing correction, but three SNPs were identified as being "nominally significant".[28] In addition, variants in this gene have been associated with LOAD by GWAS, with an odds ratio of approximately 1.17.[29] ABCA7 knockout in mouse models of disease results in increased plaque load, which has been suggested to be due to defective microglial phagocytosis.[28,31]

Inflammation

The relationship between impaired phagocytosis and disease risk is easy to understand, as there is a direct connection to amyloid-β pathology and clearance. However, inflammation is a key feature of many neurodegenerative diseases, and microglial-induced inflammation may contribute to AD.[32] *Zhang et al.* (2013) categorised genes from gene-regulatory networks in post-mortem brain tissue of AD patients and controls into functional modules, and then dissected the immune/microglia module further to identify processes in AD pathogenesis. The most significant modules were complement and Fc. These gene ontology terms relate to immune signalling processes that can result in an inflammatory response. *TYROBP* was in the complement module, along with *MS4A6A* and *CD33*, which had previously been identified by GWAS as containing variants associated with AD.[13,29] *MS4A6A* variants are associated with hippocampal atrophy and inflammation.[33,34] *TREM2* and *CSF1R* were in the Fc module. This provides convergent evidence for the importance of microglial and immune processes in AD, which provide candidates for therapeutic targets.

Variants in other immune-related genes have been associated with AD by GWAS.[13,15] The most significant new association identified by *Lambert et al.* (2013) was within the *HLA-DRB5-DRB1* region, which is involved in immunocompetence. In addition, variants within this region have also been associated with other neurodegenerative diseases such as Multiple Sclerosis and Parkinson's disease.[18,19]

Complement component (3b/4b) receptor 1 (*CR1*) variants have also been found to be associated with LOAD.[35] C3b binding to its receptor mediates phagocytosis. Another complement component found to be associated with amyloid deposition, and hence AD pathology is *CD88*.[36] This is the receptor of C5a, a pro-inflammatory mediator.[37] The involvement of both phagocytosis and inflammatory processes provide further evidence that immunity plays an important role in AD pathogenesis. In fact, complement components have been investigated as a possible novel therapeutic target, which is discussed in the next section.[38]

Clinical Relevance

The genetic evidence for microglial involvement in AD has led to the identification of potential therapeutic targets relevant to phagocytic pathways and microgliosis. Current treatments under investigation mainly focus on familial AD and the characteristic amyloid pathology of disease.[2] For example, the inhibition of β- and γ-secretases involved in the processing of APP to amyloid-β has been investigated,[39,40] and other groups have worked on upregulating α-secretases involved in the non-amyloidogenic pathway.[41] However, it has been reported that γ-secretase inhibitors can cause serious side effects, due to its involvement in Notch processing.[42] Notch is a signalling receptor involved in neuronal differentiation, but is also expressed on adult cells and is thought to be involved in memory.[43] Further work has been done to investigate the utility of "notch-sparing" inhibitors, but most demonstrated poor efficacy.[42] Therefore, alternative therapeutic options need to be investigated.

Genes related to immunity and inflammation are enriched in LOAD.[16] Therefore, pathways enhancing inflammation and microgliosis may be appropriate therapeutic targets in AD. *Landlinger et al.* (2015) investigated the utility of immunisation against the C terminal epitope of the complement component C5a.[38] C5a can induce tissue injury when inappropriately activated.[44] It was found that immunisation reduced C5a from circulation, reduced microgliosis and improved memory retention in mouse models. However, later stage vaccination had no influence on amyloid burden in the mouse brain, highlighting the need for early diagnosis and early treatment of this disease. It has been reported that administration of C5a receptor antagonists may work as an alternative approach, but immunisation allows prolonged effects using booster vaccinations, whereas more regular administration of receptor antagonists would be required.[38,44]

The TREM2 signalling pathway has been proposed as an attractive therapeutic target for AD, as it includes several genes with variants implicated in AD. Targeting of *CSF1R*, which has been associated with TREM2 signalling, has been shown to prevent progression of AD pathology in mice.[14,16,25] However, conflicting reports of TREM2 involvement in AD means further investigation is necessary to evaluate its suitability.

The genetic variants associated with LOAD as risk factors are useful in defining relevant pathways that may be targeted in AD therapy. Although the approaches to therapy discussed above would not necessarily be useful to all patients, personalised AD therapy and patient genotype may become increasingly useful in defining clinical approaches. In addition, risk variants can be useful in diagnostics. Although association of individual variants with disease is quite weak, an analysis of individual genotypes could be useful in generating a "hazard score", or likelihood that individuals have the disease.[45,46] This score can be used to identify individuals at high risk of developing AD, and can also be predictive of rate of cognitive decline.

Conclusion

Although EOAD and LOAD have common features, they differ in causes, age of onset and heritability. Therefore, treatments based on familial AD genetics may not be particularly useful in treating LOAD, which is the more common form of disease. Inflammation has been shown to be a common feature of AD and other neurodegenerative diseases, but it was uncertain if it was causative or a by-product of other pathological processes. GWAS have been a useful tool to identify risk variants in disease, some of which are related to immune processes such as microgliosis. Validation of these variants in animal models and in vitro experiments suggest they are relevant to pathogenesis.

This review has discussed the evidence for the involvement of immune-related genes both directly in amyloid processing and in pro-inflammatory processes. These processes represent promising targets for clinical intervention, and further investigation into how variants in genes such as *TREM2* contribute to pathology will allow for further development of therapy. Other gene ontology categories such as synaptic transmission, lipid and sterol metabolism and the cytoskeleton have also been significantly associated with AD, which may also provide clinical targets.

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TS
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Gut Brain Interactions in the Regulation of Behaviour

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The gut-brain axis is a bidirectional communication system as the gut and brain affect each other in various ways. Colonisation of the gut by bacteria is critical for the development of healthy gut microbiota, which is important for the brain to be able to function efficiently. The gut and brain interact in different ways such as through immune, neural and endocrine pathways. Psychobiotics may hold the key for treating a dysfunctional gut or restoring healthy microbial diversity within the gut.

Introduction

From the moment we are born, we are colonized by a diverse range of microorganisms. There are approximately 100 trillion bacteria in the body of a human, with an estimated 80% of these bacteria residing in the gut.[1] It therefore comes as no surprise that the gut microbiota has an impact on the functioning of the body. The gut microbiota can be defined as the variety of microorganisms which occupy the span of the gastrointestinal tract.[2] While some lay people still believe that microorganisms such as bacteria have a negative effect on one's health, research has shown otherwise indicating that a substantial amount of microorganisms have symbiotic relationships with their host. For example, many microorganisms are responsible for both activation and suppression of the inflammatory response.[3] Microorganisms also benefit its host in ways such as augmenting the response of its host to a disease or improving the ability of the host to effectively extract nutrients from the food it consumes.[4]

The gut-brain axis can be defined as the bidirectional signalling that takes place between the central nervous system (CNS) and the brain.[5] Accumulating data is suggesting that the gut microbiota can communicate with the CNS through the nervous, endocrine and immune systems.[6] Many of the specific mechanisms of the gut microbiota-brain axis are not yet fully understood. However, there is a vast array of evidence from animal along with some human studies which have shown that the gut microbiota plays an important role in brain and cognitive

development.[1] Disruption to the microbial balance in the gut, which is commonly known as dysbiosis, has been linked to various problems such as obesity, malnutrition, neurological disorders and cancer.[7] In the past 10 years there has been a noticeable increase in the number of papers published on the gut microbiota and the gut-brain axis. Newly discovered links between abnormal gut microbiotas and disease as well as the various impacts of the gut on the brain have heightened the interest in this field. This review will address what is known about how the gut microbiota and brain communicates. The importance of the interactions that occur between the gut microbiota and the brain and how they impact the body will also be addressed.

Complexity and Development of the Gut Microbiota

The total amount of bacteria in the gut microbiota represents about 0.3% of the overall body weight, it also contains 100 times the amount of genetic material that can be found within all of the cells of the human body.[8,9] There are six major bacterial phyla that reside in the gut and these are Firmicutes, Bacteroidetes, Proteobacteria, Actinomycetes, Verrucomicrobia, and Fusobacteria. Firmicutes and Bacteroidetes being the most prominent of the six phyla.[10]

Initial colonization of the gut by microbes occurs as soon as an infant is born and exposed to a rather complex microflora; the initial microbiome of an infant will usually have a maternal signature.[11] Abnormal or lack of microbial colonization in new-borns has been linked with long-term effects on host metabolism and reduced development of the immune system.[12] The main determining factor for the shaping of the microbiome composition of early life is the method of birth. Babies born naturally obtain microbes such as Lactobacillus and Prevotella which resemble the mother's vaginal microbiota. Whereas babies that are born by way of Cesarian section possess mainly 'skin' type bacteria such as Staphylococcus, Corynebacterium, and Propionibacterium.[13] It seems that the nutrition infants receive early on also has an effect on the early development of the gut microbiota. Various studies have shown that breastfeeding is directly associated with increased levels of IgA and the amount of Bifidobacterium present in the gut. On the other hand, infants that are fed formula during their first four weeks exhibit a decrease in overall amount of bacterial species.[14] During an infant's first year of life the gut microbiota is constantly changing, it is only when they are approximately 12 months old that they reach an adult-like gut microbiota composition, although this process can differ among individuals.[15]

Mechanisms of Interaction

Neural Pathways

There are two main neural pathways which allow the gut to communicate with the brain. The first pathway involves direct exchange of information between the gut and brain by the interactions that occur between the autonomic nervous system and the vagus nerve (which extends from the head to the abdomen). The second pathway involves bidirectional interactions between the gut and brain through the enteric nervous system in the gut and both the autonomic nervous system and vagus nerve within the spine.[1] The neural complex for regulating gut function involves both the intrinsic and extrinsic nervous system and forms a hierarchic four-level integrative organization system. The intrinsic nervous system is responsible for managing gut functions while the extrinsic nervous system is controlled by the brain and is responsible for muscle contractions which moves food along the digestive tract.

The first level of this complex system is the enteric nervous system which is characterized by myenteric ganglia, submucosal plexus, and enteric glial cells all of which can be found within the gut. The intrinsic primary afferent neurons in the enteric nervous system control reflexes that occur locally such as the migrating motor complex and the peristaltic reflex. The main enteric motor neurons and interneurons which are excitatory are cholinergic, although some dopaminergic neurons which have been linked to intestinal motility can also be found within the gut. The second level is the prevertebral ganglia which modulates many of the peripheral visceral reflex responses that occur within the gut such as secretion, absorption and motility.[16,17] The third level is the autonomic nervous system which can be found within the spinal cord and the brainstem with the nucleus tractus solitarius and dorsal motor nucleus of the vagus nerve, which serves to receive and send various neural signals to both the afferent and efferent fibers of the vagus nerve. The fourth and final level is what is known as the higher brain centres (complex parts of the brain which include the cerebral cortex). Information from the cortical and subcortical centres (which includes the basal ganglia) funnels down to specific brainstem nuclei, from where various gut functions are controlled.[16] This neural network, which is responsible for connecting the gut with the brain at various levels, is one the structural bases for the function of the microbiota gut-brain axis.[10] Damage and irregularities to the various levels which have been mentioned can have an impact on the regulation of gut function, including local gut reflexes, and external neural control.[16]

Direct neural communication between the gut microbiota and the brain occurs mainly through the vagus nerve (bacteria stimulates afferent neurons of the enteric nervous system), this provides a route through which microorganisms within the gut can exert an impact on brain functions.[18] Various studies have shown that primary afferent pathways through the vagus nerve mediate interactions between microbes within the gut and the CNS. The studies conducted recognised

the introduction of c-fos, a proto-oncogene, in vagal sensory neurons along with post vagotomies as a potential neural mechanism of these interactions.[19] The vagal signal from the gut is capable of stimulating the anti-inflammatory response, which can prevent against pyosepticemia (infection of the blood with various types of bacteria) which is caused by microorganisms.[20]

Neuroendocrine Pathways

The main neuroendocrine pathway known is the hypothalamic–pituitary–adrenal (HPA) axis, activation of this pathway occurs in response to physical and psychological stress.[11] The HPA axis involves interactions between the three endocrine glands mentioned above. Pro-inflammatory cytokines are known to be important stimulators of the HPA axis.[5] It was a study conducted by *Sudo et al*.[21] which suggested that the gut microbiota has a role in the development of the HPA axis. In this experiment germ-free (GF) mice were exposed to a mild restrain stress. It was shown that the mild stress induced an excessive release of corticosterone and adrenocorticotrophin hormone (ACTH) in comparison to the specific pathogen free (SPF) controls. Interestingly, the stress response that was induced in the GF mice was somewhat reversed by recolonization with faecal matter from the SPF animals. Furthermore, the stress response was also fully reversed by monoassociation with *B. infantis* over an extended time course. This study presented a clear indication that the microbes which are contained within the gut are crucial for the development of a fitting stress response to occur later in life. It also showed that there is a crucial time period in the early days of life when colonization of the gut microbiota needs to take place in order to establish normal development of the HPA axis. In the same study, decreased levels of brain derived neurotrophic factor (BDNF) were seen in both the cortex and hippocampus of GF animals in comparison to the SPF controls. BDNF is a key neurotrophin that is important for neuronal growth and survival.[21]

Hyperactivity of the HPA axis is known as one of the most definite biological causes for severe cases of depression.[22] In a study conducted it was shown that rats which had activated stress circuits exhibited anxiety and depressive-like behaviours. However, when the stressful stimulus was removed HPA hyper-reactivity was normalised which caused reversal the abnormal behaviours. This was shown by measuring their internal corticosterone levels.[23]

Gut-Immune Signalling

One of the key functions of the gut microbiota is its involvement in the development of the neuroimmune system.[24] The neuroimmune system involves interactions between the immune system and the CNS. Interestingly, changes in early life to the gut microbiota has been associated with predisposition to immune disorders.[24]

The gut microbiota has a large amount of potential messenger molecules such as primary metabolites which include microbe-associated molecular patterns (MAMPs) as well as Pathogen-associated molecular patterns (PAMPs). Secondary

metabolites such as bile acids can also be found within the gut microbiota. These are produced by the microbial fermentation of various food components or alteration of host molecules.[25] Excluding the MAMPs and PAMPs, there are many more microbial metabolites.[26] These metabolites are known to have an impact on the immune system and because of this have the potential to travel via the blood stream to the brain. An imbalance in the relationship between the gut microbiota and the immune system signifies a stressful state which, if communicated to the brain, will produce a systemic stress response within the organism.

The effect of MAMPs and PAMPs (lipopolysaccharide (LPS) in particular), on the brain through various immune pathways have been widely studied. As well as having an effect on brain function and behaviour, PAMPS acting through the pattern recognition receptor (PRR) toll-like receptor-4 (TLR4) have been associated with contributing to the pathogenesis of cerebrovascular disease.[25,27] There have been some microbial metabolites - for instance LPS, tested on humans. For example, intravenous injection of LPS into healthy human subjects was shown to increase the circulating levels of IL-10, IL-6, TNF-a, soluble TNF receptor, IL-1 receptor antagonist and cortisol. This was linked with increased body temperature, anxiety, adverse mood, reduced memory performance and hyperalgesia.[25]

Microorganisms and Brain Function

It was previously thought that microbes and the brain seldom interacted apart from occurrences when the rare pathogen would breach the blood-brain barrier (BBB). A good example of a well-known pathogen which was known breach the BBB is rabies. But until rather recently a lot of the microbes in the gut were uncharacterised and it was not known how much of an impact microorganisms could have on the brain.[28]

Since the gut-brain axis concept has arisen, the impact that microorganisms exert on the brain has become progressively more recognised. Signals generated from microbes have the ability to regulate essential functions in healthy humans. As we have seen from the mechanisms of interactions discussed, many diseases and health problems that occur within the body are due to disturbances to the microorganisms within the gut.[29] Changes to gut microbiota composition have been linked with alterations in behaviours related to mood, pain and cognition.[30] In another study conducted it was discovered that faecal microorganism transplantation changed the behaviour of the receiver mice to that of the donor.[31] *Desbonnet et al.* also showed that the microorganisms within the microbiota are essential for the development of normal social behaviours to occur in GF mice.[32]

Treatment of the Gut: Psychobiotics

Psychobiotics are a compelling new phenomenon which provide mental health benefits when ingested. Psychobiotics include both probiotics which are live bacteria and prebiotics which aid in the development of beneficial bacteria found within the gut.[33] As has been discussed earlier there are a lot different pathways relating the health of the gut to the brain. This has created a possible therapeutic potential for using probiotics against neurodegenerative diseases,[34] such as multiple sclerosis and schizophrenia. It may also provide a therapeutic target for other health problems such as anxiety, depression and autism that have been associated with a dysfunctional gut-brain axis.[4,10,29]

Conclusion and Future Directions of the Field

It is clear that the time has come for the gut and brain to be recognised as a bidirectional and highly co-operative system rather than two isolated entities. The impact this system has upon the health and behaviour of organisms cannot be overlooked. Various studies have identified the possible role of the gut microbiota in a variety of health problems. Many of these health problems occur as a result of alteration to the populations of microorganisms which can be found in the gut. The importance of microbial diversity within the gut is becoming increasingly recognised as key component for the general health and well-being of an organism. Increasing evidence is also suggesting that pathogenic microorganisms contribute to neurodegeneration.[35] However, more research needs to be done so that a healthy gut microbiota can be distinguished from the type of gut microbiota which can be seen in disease or in organisms with mental health problems. A lot of studies have provided excellent insights into how the gut and brain communicate but the specific details of a lot of these interactions are still unknown, further research on the interactions which occur are imperative to the progression of this field. As the gut-brain axis is a rather new concept it is important to note that correlation does mean causation. The knowledge of how the different gut-brain interactions take place as well as the underlying mechanisms may introduce an exciting new therapeutic target for health problems associated with the gut-brain axis.

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The Role of Invariant Natural Killer T Cells in Cancer Immunotherapy

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Innate T cells are a unique family of leukocytes that exhibit both innate and adaptive immunological functions. These 'hybrid' cells play a crucial role in bridging these two immunological families through their ability to activate both innate immune cells such as Macrophages, Dendritic Cells (DCs), and Natural Killer (NK) cells, as well as adaptive immune cells such as T and B cells. This review will focus primarily on the role of invariant Natural Killer T (iNKT) cells, one of the best characterised of the innate T cell family, in an immunological setting. Due to their multifaceted effector functions, and their ability to activate multiple immune cells, their role in disease has become a point of interest for many researchers. In particular, their role in cancer and their antitumour effects has attracted much attention, and is a keen area of focus for potential immunotherapies. This review collates the research in this field to date, and outlines the future directions and prospects of iNKT immunotherapies.

Introduction

Conventional T cells are very well characterised and are primarily known for their ability to recognise peptide antigens presented by Major Histocompatibility Complex (MHC) Class I and II. MHC is a molecule expressed on cell surfaces that interacts with immune cells to stimulate immune responses. MHC I is expressed on all cells in the body (except sometimes cancer cells) and MHCII is expressed by Antigen Presenting Cells (APCs). Natural Killer T cells, unlike conventional T cells, are characterised by their ability to recognize lipid antigens presented by the class Ib MHC molecule, CD1 (see Fig. 1). In the early days of discovery for this cell type, it was originally postulated that murine NKT cells were a sub-population of T-cells that constitutively expressed the NK cell marker NK1.1. Though this has since proven not to be the case, the name NKT cells has remained in use for both

humans and mice.

There are two distinct types of NKT cells: Type I or invariant NKT cells (iNKT cells) and Type II or variant NKT cells (vNKT cells). iNKT cells are restricted to monomorphic CD1d and are stimulated constitutively by lipid antigen α-galactosylceramide (α-GalCer).[1] α-Gal-Cer is a synthetically modified compound that was discovered in the marine sponge *Agelas mauritianus*. It is a very potent iNKT activator, and while it is thought that lipids as potent don't occur in physiological settings, it is very useful in research settings as an iNKT cell stimulant. As with all T cells, they possess a T cell receptor (TCR) with an α and a β chain. The invariant α-chain in iNKTs is encoded by the recombinant gene sequence Vα14Jα18 in mice and Vα24Jα18 humans. This receptor combination is not found in any other T-cell, thus making it a hallmark of iNKT cells. It is accompanied by a restricted selection of β chains - usually Vβ8.2 in mice and Vβ11 in humans.[2] Type II NKT cells bind CD1a, b, and c, and - akin to iNKT cells - recognise lipid and glycolipid antigens presented by CD1d. However, they differ from iNKT cells in that they neither recognise nor are stimulated by α-GalCer.[3] For the purpose of this review, discussion of NKT cells will refer to Type I NKT or iNKT cells only.

The innate-style characteristics of iNKT cells stems from their 'poised effector state'[4] referring to the large amounts of cytokines, or chemical messengers, they can release in response to other pro-inflammatory cytokines and other danger signals. The presence of preformed mRNA in the cytosol allows for the production of a vast array of cytokines as IFNγ, TNF, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-21 and GM-CSF within hours of stimulation.[5,6,7] All of the above listed cytokines help propagate the immune response in the body by helping activate other immune cells.

Two signals are typically required for iNKT activation, the strength of each influencing the effector role of the cell. First is the CD1d-lipid antigen-TCR stimulation, the second being cytokine release from the APC. In the presence of a strong TCR signal, whereby there is a high affinity by the TCR for the lipid-CD1d complex, a weak cytokine signal is observed.[7] I would speculate that due to the high specificity for that antigen, the iNKT streamlines its effector functions to favour adaptive-like behaviour, such as direct cytotoxicity of target cells with that antigen.

Contrastingly, when a weak TCR signal is received there is low specificity of that iNKT for that antigen. Thus, to compensate, a strong innate-like cytokine signal is received which promotes innate-like immune responses such as cytokine release and transactivation of other innate cells including macrophages and neutrophils. However, it has been shown that iNKT cells can be activated by IL-12 and IL-18 alone, without TCR stimulation.[6,8]

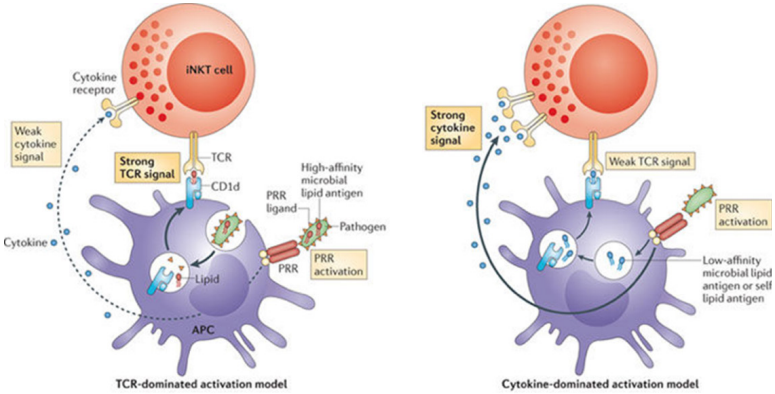

Figure 1: Activation of iNKT cells. iNKT cells can be activated via two mechanisms: 1) T cell receptor (TCR) mediated recognition of CD1d presenting lipid or glycolipid antigens by antigen-presenting cells (APC) via (left) and/or 2) via cytokine release by the APC in response pathogen recognition receptor (PRR) recognition (right). These two pathways can occur on their own or concurrently, depending on the affinity of the CD1d/lipid antigen complex for the TCR. *Figure obtained from Brennan et. al (2013) Nat. Rev. Immunol* [7]

Many APCs can express CD1d, such as dendritic cells (DCs), neutrophils, macrophages and B cells, meaning they have the capacity to stimulate iNKT cells. As well as iNKT activation, APCs receive reciprocal activation when TCR-CD1d signalling occurs, stimulating the maturation and migration of DCs for example, thus making them more effective APCs to other immune cells such as T cells.[9] This activated pair can transactivate other leukocytes such as NK cells via IFNγ (iNKT derived) and IL-12 (APC derived) signalling. Conventional T cell differentiation and activation is also aided by this iNKT-DC interaction. The release of cytokines such as IL-4, IL-17a, IL-10 and IFNγ by the iNKT cell helps drive these two processes indirectly, while the activated DC makes direct contact with the T cell, stimulating it via its own TCR.[7]

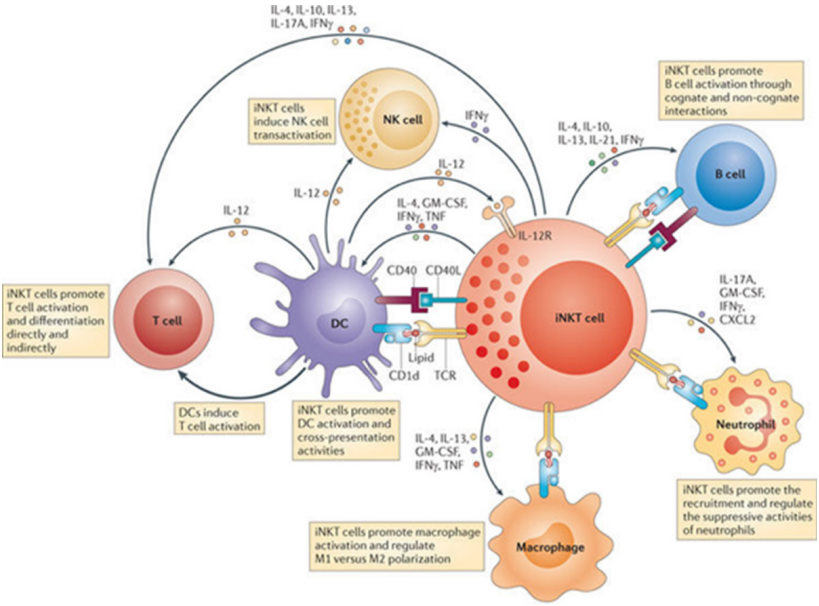

Figure 2: iNKT interactions with other leukocytes: iNKT cells are activated by APCs expressing CD1d, including DCs, Macrophages, Neutrophils, and B cells. Activated iNKT cells provide reciprocal activation to the APC they are in contact with, often promoting and amplifying their immunologic effects. iNKT cell stimulation via DCs cause the release of cytokines that promote T cell activation, differentiation, and proliferation, while the co-activated DC activates the T cell directly via MHC-TCR interaction. This pair can also transactivate NK cells via the presence of IL-12 and IFNγ. iNKT-Macrophage interaction promotes Macrophage activation and regulates M1/M2 polarisation via iNKT release of IL-4, IL-13, GM-CSF, IFNγ and TNF. iNKT-Neutrophil interaction promotes neutrophil recruitment via CXCL12 release and regulates their suppressive activity. Finally, cognate and non-cognate interactions between B cells and iNKT's promote B cell activation. This is also aided by the release of IL-4, IL-10, and IFNγ. *Figure obtained from Brennan et.al (2013) Nat. Rev. Immunol.*[7]

There are different subsets of iNKT cells determined by the profile of cytokines they produce upon stimulation by an APC. For example, NKT1, NKT2 and NKT17 cytokine profiles mimic those of CD4+ T-helper 1, Th2, and Th17 cell subsets, respectively.10 A proposed classification system for the subsets of iNKT cells showing the markers they typically exhibit, activated transcription factors, their location of highest density in the body, and cytokine profile can be seen in Fig.3. Unlike conventional T cells, iNKT subtypes aren't plastic and just display characteristics that are regulated by the tissue environment.

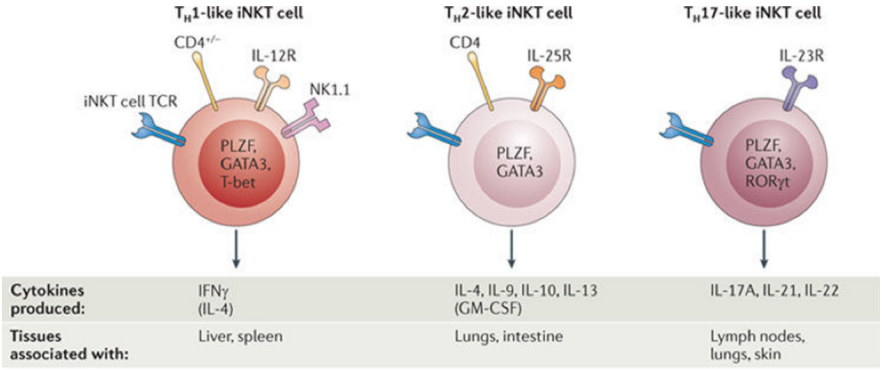

Figure 3: Subsets of iNKT cells. A proposed classification system for iNKT subsets is that by their receptor expression, transcription factor activation, cytokine profile, and associated tissues. Th-1 like iNKT cells express IL-12R, the NK marker NK1.1, and can be CD4+/-. Signalling via these receptors upregulates GATA3 and T-bet, resulting in a Th1- like cytokine profile comprising mostly of IFNγ. Th-2 like iNKT cells are CD4+ and express IL-25R in addition to the standard iNKT cell TCR. Signalling via IL-25R upregulates PLZF and GATA3 results in a Th-2 like cytokine profile including IL-4, IL-10 and IL-13. Finally, Th-17 like iNKT cells express IL-23R and uniquely upregulate RORγt, resulting in the production of IL-17A, IL-21, and IL-22, all typically associated with a Th17 type cytokine profile. *Figure obtained from Brennan et.al (2013) Nat. Rev. Immunol*[7].

The Role of iNKT Cells in Anti-Tumor Immunity

iNKT cells have been found to have both protective and harmful roles in the body, depending on disease pathology. Examples of such pathologies include: autoimmune disease,[11] microbial infection,[12] allergy,[13] and cancer.[14] The role of iNKT cells in cancer and particularly their scope for potential immunotherapies is of particular interest for this review.

iNKT cells exhibit indirect anti-tumour responses via their adjuvant activity; the large amounts of IFNγ they produce upon DC stimulation activates both NK cells and CD8+ cytotoxic T cells (see Fig. 3(a)). This allows for elimination of both MHC negative tumours via NK cells, and MHC positive tumours by CD8+ T cells.[15] In iNKT cell-sufficient mice, it was observed that administration of α-GalCer resulted in iNKT activation preceding the polyclonal expansion and activation of T, B and NK cells within four hours.[9,16]

Another method by which iNKT cells exhibit anti-tumour effects is through their ability to infiltrate the tumour microenvironment (TME), preventing angiogenesis and killing tumour promoting cells such as Tumour Associated Macrophages

(TAMs) and Myeloid-Derived Suppressor Cells (MDSCs)(see Fig. 3(b)).

iNKT cells can also directly kill tumour cells upon TCR stimulation by expressing CD178, perforin and releasing granzymes into the immunological synapse between the iNKT and the tumour cell[17] (see Fig. 3(c)). The potency of this reaction appears to correlate with the presence of CD1d and stimulatory lipid antigens.[18] Given that the interstitial fluid surrounding tumours is ripe with free fatty acids as a result of their increased lipid synthesis,[19] there is potential that this lipid-rich TME could affect iNKT activation. Stress ligands on the tumour cell ligate the iNKT receptor NKG2D, providing co-stimulation for TCR signalling and enhancing its cytotoxic outcome.[20] Blocking either one of CD1d antigen presentation, TCR signalling, or perforin expression was seen to result in a very significant reduction in cell cytotoxicity by iNKT cells in vitro.[21]

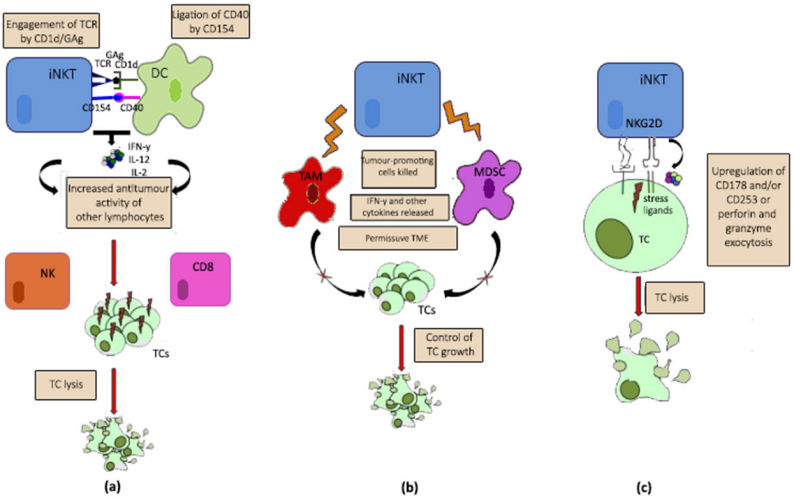

Figure 4: Direct and indirect anti-tumour effects of iNKT cells: (a) iNKT stimulation and subsequent IFN-γ production activates NK and CD8+ T cells. These cells in turn exhibit their cytotoxic capacities, lysing tumor cells (TCs). (b) iNKTs kill tumour promoting cells such as Tumour Associated Macrophages (TAMs) and Myeloid Derived Suppressor Cells (MDSCs). By infiltrating the tumour microenvironment (TME), angiogenesis can be prevented, limiting oxygen and nutrient supply to the tumour. (c) Direct killing of TC via perforin/granzymes release and/or NKG2D signalling upon stimulation by stress ligands expressed by the transformed cell. *Figure adapted from Nieda et.al (2004) Blood*[22].

It is known that chronic inflammation is a hallmark of cancer,[23] and by producing large amounts of the anti-inflammatory cytokine IL-10, particularly in adipose tissue,[24] iNKT cells exhibit a preventative role in tumour immunity. In obese patients, where iNKT cell levels are severely depleted, increased chronic inflammation and

thus higher rates of cancer are observed.[24]

The Role of iNKT Cells in Cancer Immunotherapy

Given the multi-faceted nature of their anti-tumour effects, it is unsurprising that iNKT cells have been targeted for potential cancer immunotherapies.

The first attempt involved the administration of α-GalCer directly to the patients, to stimulate iNKT cells in vivo in a Phase I clinical trial.[25] Though the range of doses used were well tolerated with low toxicity, unfortunately no clinical effect on solid tumours was observed. This could potentially be due to the very low probability of: a) an APC coming in contact with an α-GalCer molecule in the blood, b) the APC then coming in contact with and activating an iNKT cell and c) the subsequently activated iNKT cell coming in contact with tumour cells.

Building on this study, immature monocyte-derived DC's from metastatic cancer patients were pulsed *ex vivo* with α-GalCer. These DC's were then reintroduced into the patients' bloodstream where they presented this lipid antigen to host iNKT cells, activating them. This method is known as adoptive transfer. The results of this study observed significant increase in serum IFNγ and IL-12, as well as T and NK cell activation. In addition to this, a decrease in tumour necrosis was observed in one patient and tumour biomarkers was seen in a two patients lasting up to 12 months.[26] While this was a promising initial study, further development was required to optimise iNKT activation and function.

Next, the same study was repeated, but this time the DC's that had been pulsed with α-GalCer were allowed to mature before adoptive transfer took place. A very promising >100-fold increase in iNKT numbers was demonstrated in all participating patients. This increase lasted for over 6 months.[27] This method was employed in a number of small Phase I studies for patients with recurrent and advanced non-small cell lung cancer[28] and head and neck cancers.[29] Overall, the results showed increased IFNγ and iNKT levels in a portion of patients, and stabilisation of disease in even fewer. That being said, there was minimal toxicity associated with these treatments.

Phase IIa clinical trials then progressed for these studies, which observed prolonged median survival times when used in conjunction with standard treatments such as chemotherapy, radiation therapy, and surgery.[2,28]

One drawback of the above outlined methods is that, for not fully understood reasons, iNKT cell numbers tend to be depleted or suppressed in cancer patients.[30] Thus, the administration of α-GalCer pulsed DC's will only have limited efficacy if there are no cells to stimulate. This allowed for the emergence of a new approach, whereby autologous Peripheral Blood Mononuclear Cells (PBMC's) from lymphoma patients were stimulated *ex vivo* in anti-CD3, IL-2, and IFNγ in

an attempt to boost iNKT numbers before adoptive transfer back to the patients. An average of 20% enrichment was observed, but while these cells had very high cytotoxic capacity in vivo, only two of nine patients showed disease stabilisation, and another two showed a partial response.[31]

One of the more recent immunotherapy strategies for iNKT cells involves a combination of the above two methods; administration of in vitro expanded iNKT cells and α-GalCer-pulsed DC's simultaneously. This method saw partial clinical responses or disease stabilisation in 7 out of 8 patients.[32]

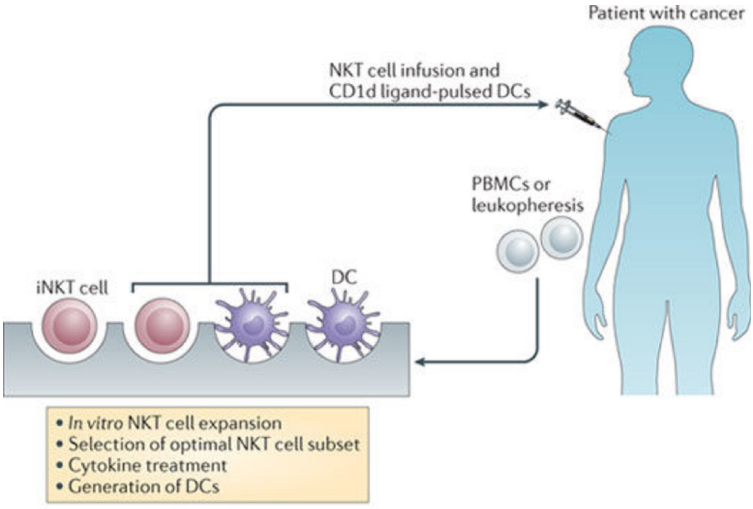

Figure 5: Current immunotherapy strategy for iNKT cells. PBMC's are taken from the patient, and iNKT cells and DCs are isolated and expanded in vitro. Optimal subsets of iNKT cells are selected and treated with IL-2, IFNγ and anti-CD3. Simultaneously, DC's are pulsed with α-GalCer. A combination of the two cell infusions is reintroduced to the patient via adoptive transfer, where the enriched iNKT cells can exert their therapeutic effect. *Figure obtained from Vivier et. al (2012) Nat. Rev. Immunol.*[14]

Future Directions for this Research

Because of the depleted numbers of iNKT cells in cancer patients and their commonly immunosuppressed states, finding new methods of expanding these defective cells is an important task. The future of iNKT cell based immunotherapies is set to take advantage of pioneering research in induced pluripotent stem (iPS) cells. Pluripotency can be induced in mature donor iNKT cells, creating iNKT-iPS cells. These iNKT-iPS cells could potentially generate unlimited amounts of

iNKT cells suitable for transfer to patients. Current studies using mouse models have successfully transferred iNKT-iPS derived iNKT cells without any signs or symptoms of graft versus host disease (GvHD), unlike what is observed when CD4+ T cells are transferred.[15,33] The absence of GvHD is a huge factor in the promising nature of iNKT-iPS immunotherapy.

Finally, exploiting new research in chimeric antigen receptors (CAR's), iNKT cells could potentially be engineered to express CAR's specific to their target cancers e.g. GD2 CAR on iNKT cells specifically combat neuroblastoma in mice.[34] Combining the above two therapies could mark a new era in the effectiveness of iNKT's in treating disease.

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The Development of Optogenetics and its Use in *Drosophila melanogaster*

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The ability to selectively manipulate specific subsets of neurons to explore their function and connectivity has been a long sought-after goal in neuroscience. With the advent of modern optogenetics, this goal has been realised. Researchers around the globe are using these sophisticated techniques to probe neural circuits in a large variety of organisms. The fruit fly Drosophila melanogaster has been a popular model organism in neuroscience research for decades. This is in part due to its genetic similarity with humans, short life span, and ease of maintenance; but especially because of its genetic tractability. This trait also makes it an ideal testing ground for optogenetics, with powerful molecular tools allowing for the expression of light-activated ion channels in designated neuronal circuits with relative ease. This review will discuss the beginnings and development of modern optogenetics and its applications in the fruit fly.

Introduction

Since Galvani's early experiments with "animal electricity" in frog legs,[2] the direct stimulation of nervous tissue in order to elicit desired behaviours has been used as an experimental technique throughout the history of neuroscience. Probing neurons in such a way can grant many useful insights into the functions and anatomy of neural circuits; however, traditional techniques often involved the use of electrodes. While temporally precise, stimulation via electrode in vertebrates often results in the activation of a number of different cell types indiscriminately because of their shared anatomical location, posing challenges for resolving roles of specific neuronal cell types in processing. In invertebrates, separation and selective stimulation of individual circuits is easier, but has its limitations due to the technical difficulty of the recording. It was Francis Crick who first suggested that, in order to assemble a general theory of mind, we must identify a method

by which we might selectively turn off or on the firing of all neurons of a specific type in the brain; in doing so, he said, "our view of ourselves… will be totally transformed".[3] Crick suggested that an ideal signal for this tool would be light, but admitted that this speculative tool was "far-fetched". But in fact, just a few years later, a powerful system was developed that achieved exactly this.[4]

The field of optogenetics is a relatively recent development, and involves the exogenous expression of light-driven molecules on excitable tissue, allowing its activity to be driven or silenced by light.[4] The kinds of tissues that have been targeted with these techniques range from muscles to glia; but the most widespread use of these tools is in the selective activation of neurons. Such tools have conferred unprecedented control over neuronal activity, and allow probing of specific neuronal circuits to improve understanding of how different classes and subsets of neurons work to perform such complex functions - or, conversely, how their dysfunction may cause pathology. In particular, the use of directly light-gated cation channels such as channelrhodopsin-2 (ChR2)[5] allows for optical control of neural activity that is temporally precise to the same level and scale as actual neural computation.[4]

The potential for the use of optogenetics in *Drosophila* research was immediately apparent upon its inception. Such a system, in concept requiring only the exogenous expression of optogenetic molecules on targeted tissue (though practically, its use in *Drosophila* necessitates the addition of all-*trans*-retinal as a food supplement), lends itself well to use in an organism with such an expansive genetic toolbox, well-equipped to deliver such molecules to the desired neural circuits. The tools provided by rapidly advancing optogenetic technology, together with *Drosophila*'s genetic tractability, will allow researchers to make huge strides in elucidating previously unexplored functions and mechanisms of *Drosophila* neural circuitry.

Early Efforts in Optogenetics

Before the discovery of the directly light-gated ChR2, pioneering efforts in optogenetics involved more complex light-driven systems. As mentioned previously, the problem with methods of neuronal stimulation in vertebrates at the time lay within their lack of cell-type specificity. Stimuli were largely anatomically targeted, e.g. using electrodes or focused light beams,[6] meaning either large groups of functionally diverse neurons were targeted indiscriminately, or stimuli positions had to be carefully controlled, leading to complications with behavioural experiments. It was Gero Miesenböck who first recognised that perhaps an indigenous biological tool would be better for study of biology, and suggested that these problems with specificity might be better resolved if specificity was encoded biologically.[7] Equipping only target cells with a "receiver" for a generally-broadcast stimulus would allow for simultaneous activation of multiple neurons, disregarding their spatial distribution, and allowing for selective manipulation of

behaviour.

Miesenböck's group developed an elegant system for this, involving a phototrigger consisting of ligand-gated ion channels, genetically targeted to specific tissue, and activated by photochemically 'caged' ligands that could be liberated by a flash of light.[8] However, this system required multiple components, limiting its applicability. These light-driven currents also operated at quite large timescales, taking several seconds to switch on and off. Miesenböck himself acknowledged the need for millisecond temporal control over a stimulus in order to operate at physiological clock speeds – indeed, this realisation formed the basis of his decision to use light as a stimulus due to its speed and temporal precision – and admitted that the ideal tool would be "a structurally simple ion channel that can be gated directly by an electromagnetic signal, such as light." However, he conceded that, at the time, such an ideal "had not been found in nature or engineered in the laboratory".[7]

The Advent of Modern Optogenetics

In 2003, a paper was published identifying a directly light-gated cation channel from the green alga *Chlamydomonas reinhardtii*, which functioned in almost exactly the manner that Miesenböck was to lament the lack of a year later.[5] Microbial opsins like bacteriorhodopsin and halorhodopsin had been known about and studied extensively since the 1970s for their fascinating light-driven mode of action.[9,10] These proteins require retinal, an organic co-factor related to vitamin A, which upon absorption of a photon isomerises from the all-trans configuration to the 13-cis configuration to initiate phototransduction.[11] However, photoisomerisation of these opsins generally triggers a cyclic reaction sequence involving a series of intermediates, eventually triggering the opening of an ion channel via a soluble messenger.[12] Cycling through this cascade makes the response of the channel slower than would be desired if one was aiming to harness these for optical control of neurons at physiological rates.

However, this new type of microbial opsin – the previously mentioned ChR2, used to drive phototaxis in *C. reinhardtii* – combined the light-sensing properties of these proteins with the ability to directly mediate electrical conductance: it was an ion channel with an intrinsic light sensor. Upon absorption of a photon, a light-induced isomerisation of all-*trans*-retinal to 13-*cis*-retinal causes a conformational change in the channel, opening the pore to generate a permeability for cations (Figure 1.).[5] The authors were quick to recognise this dual-function protein's potential for heterologous expression in animal cells, and demonstrated its use as a powerful tool to rapidly depolarise both frog oocytes and mammalian cells simply via illumination. A crucial feature of this channelrhodopsin was its temporal precision – it could be stimulated incredibly rapidly (<50μs) after a flash of light, and the transiently-opened pore closed within milliseconds.[5] This, together with its

successful, functional expression in animal cells, immediately attracted the interest of researchers as a possible tool for fast, targeted neuronal stimulation.

The first group to describe the use of this channelrhodopsin for such temporally precise optical control of neurons was *Boyden et al.* in 2005.[4] They used lentiviral gene delivery to express ChR2 in mammalian neurons, and were able to photostimulate these with high-speed optical switching. ChR2 could drive neuronal depolarisation in a safe, non-invasive manner, and demonstrated significantly faster voltage control than previous experiments in optical control of neuronal activity.[13] In fact, it allowed precise spatiotemporal control of individual spikes on a millisecond-scale – the same timescale that is occupied by actual neural computation. This technology could also be genetically targeted to allow probing of specific neuron subclasses within a heterogeneous neural circuit using cell-specific promoters, making it a hugely powerful and applicable tool for the alteration of neural processing.[4] Thus, the first single-component optogenetic tool had been developed, fulfilling the widely sought-after goal for a method by which scientists might selectively manipulate genetically discrete neuronal types, and doing so with a system that lent this method unprecedented temporal control.

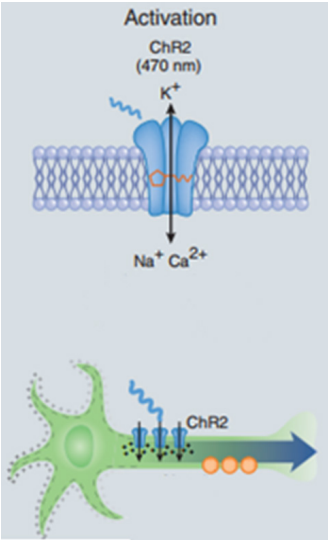

Figure 1: Optogenetic activation of a neuron expressing ChR2. The upper diagram illustrates the photo-activated opening of the ChR2 pore and its subsequent cation permeability. The lower diagram shows this activation of ChR2 molecules expressed in a neuron; the ensuing influx of cations into the neuron, triggering an action potential; and the propagation of this action potential along the axon. The blue wave indicates the light stimulus. *Adapted from Pastrana*[1].

Developments in Optogenetics

Since the discovery and implementation of ChR2, optogenetics groups across the globe have conducted large-scale genomic screens and used systematic mutagenesis to identify dozens of new variations and types of these microbial opsin proteins, vastly expanding and optimising the optogenetic toolbox to allow for a

huge diversity of experimental conditions and configurations.[14] For instance, some light-activated ion pumps like NpHR (a yellow light-activated halorhodopsin from Natronomonas pharaonic) pump chloride rather than cations and may be used for inhibition rather than excitation, granting the ability to mediate both neuronal activation and neuronal silencing in a single system.[15]

Other opsin variants that have been explored are opsins that are activated by different wavelengths of light. If the different wavelengths of light used for activation are sufficiently spectrally separated, this can allow for simultaneous control of separate circuits within the same organism, activated by different colours of light; and thereby granting huge insights into the interactions of different pathways involved in encoding information in the brain. This was successfully achieved in 2014, by *Klapoetke et al.*, who demonstrated two-colour independent optical excitation of separate neural populations in mouse brain slice using two newly identified opsins: Chrimson and Chronos.[28] Other opsin variants have altered ion channel properties; some remain open for longer time periods, or close more quickly after cessation of a light pulse. Some are more light-sensitive or have higher amplitudes when expressed in neurons, or take longer to inactivate.[14]

Applications of Optogenetics

Optogenetics has provided a method of controlling neurons in systems as complex as live mammals to stimulate excitation and inhibition in a temporally precise manner, with fast deactivation of the optogenetic tools upon cessation of light. Such a powerful tool has proved extremely useful in studies of physiology and behaviour, elucidating how specific neuronal types mediate brain functions spanning from the most basic homeostasis to advanced cognitive functions; such as learning, wakening, somatosensation, movement, and breathing.[16] Optogenetics has also been used to illuminate aspects of disease pathology, especially in psychiatric and neurodegenerative disease,[17] and has even shown potential as a therapeutic modality; for example, rescuing motor deficits in mouse models of Parkinson's via optogenetic activation of the basal ganglia direct pathway.[18]

Optogenetic tools have also proved extremely useful in the mapping and probing of neural circuits in the brain, illuminating the pathways of information flow in the brain and their connectivity. Such optogenetic probing has also helped to identify circuit activity and electrical patterns, the opposing roles of interacting cell types in complex behaviours, and the underlying principles of brain functional organisation.[16]

Optogenetics in *Drosophila* - an Ideal Model Organism

Ever since Thomas Hunt Morgan's pioneering experiments on inheritance in the

early twentieth century, the fruit fly *Drosophila melanogaster* has proved to be an invaluable model organism for biomedical research. It is hugely versatile, inexpensive to maintain in the laboratory, and not subject to the animal licensing laws that can pose obstacles for research into vertebrates.[19] More importantly, most fundamental biological pathways crucial for development and survival have been evolutionarily conserved between *Drosophila* and humans, meaning that research into *Drosophila* often readily translates into applications in humans.[20]

Drosophila also has a relatively short life cycle, with its life span lasting only about 40-50 days. This allows complex genetic experiments which would take much longer in vertebrates to be examined for phenotypes across all stages of the life cycle in just a few weeks.[19] This short life span also makes the fruit fly very well-suited for analyses in experiments related to ageing and neurodegenerative disease.

Most importantly of all, more than a century of study of *Drosophila* has bestowed on it a vast genetic toolbox whose diversity far outstrips that of any other multicellular organism. There are many powerful genetic tools for studying disease which may be used in *Drosophila* with comparative ease, greatly assisting research and making the generation of transgenic flies expressing genes of interest relatively simple.[19] Their small size and quick propagation rate also allow researchers to analyse large numbers of genetically identical animals easily. This genetic tractability has made *Drosophila* very attractive for experiments using optogenetics, in which ChR2 or some other microbial opsin variant must be exogenously expressed in *Drosophila* neurons. In particular, the GAL4/UAS system for targeted gene expression, first developed by Brand and Perrimon in 1993,[21] has proved itself to be especially useful in *Drosophila* optogenetics.

Optogenetic Research in *Drosophila*

Many researchers quickly recognised the value of the channelrhodopsin-mediated, single-component optogenetic approach upon its inception in 2005.[4] It provided a gleaming solution to a major goal in neuroscience: understanding the link between specific neuronal circuits and complex behavioural outcomes. The utility of optogenetics in examining this link was thoroughly explored by *Zhang et al*., who demonstrated that optogenetic stimulation of nociceptive neurons in transgenic *Drosophila* expressing ChR2 could elicit a 'pain-like' response, as well as showing that similar activation of various other sensory and motor neurons could trigger many other established responses.[22]

Aside from simply proving the existence of this neuronal-behavioural link, many other studies used optogenetics to gain insights into these links' underlying structure and function. One of the first studies in which optogenetic stimulation was used to probe defined neuronal populations investigated the basis of appetitive versus aversive odorant learning in *Drosophila*. Here the authors used optogenetic stimulation of the dopaminergic and octopaminergic/tyraminergic neurons,

paired with an odour stimulus, to demonstrate that these two neuron classes antagonistically modulate aversive and appetitive learning, respectively.[23] Another paper then investigated this *Drosophila* appetitive/aversive odorant learning at the receptor, using optogenetic stimulation of single olfactory receptor neurons (ORNs) to produce the illusion of an odour stimulus, and showing that olfactory avoidance behaviour was controlled by activation of a specific class of ORNs.[24] In another example of the utility of optogenetics in the resolution of roles of specific neurons in behaviour, a later paper investigated the innate startle response in *Drosophila* by optogenetic activation of the acj6 neurons, and confirmed the importance of cholinergic neurons in this response.[23, 25]

In the years following the establishment of ChR2 as a reliable and versatile optogenetic tool, many papers were published illustrating the diverse applications of its implementation in *Drosophila*. However, one initial hurdle facing the use of optogenetics in the fruit fly was ChR2's blue-shifted activation spectrum, which requires blue light as an optogenetic stimulus. Light of this wavelength has poor cuticle penetration in the adult fly[26] and induces visual system-mediated behavioural artefacts that can complicate results[27]. In order to circumvent this, researchers often use channelrhodopsins activated by red light, to which the *Drosophila* visual system is not sensitive; the current most prominent of which in *Drosophila* optogenetics is CsChrimson.[28]

One group which has done significant work with CsChrimson in *Drosophila* is the Card lab at Janelia Farm, whose research uses optogenetics to probe the *Drosophila* giant fibre system.[29, 30] This is a neural circuit coding for the escape response which has been made both compact and robust by millennia of evolution alongside predators. When CsChrimson is expressed in the giant fibre interneurons, a simple pulse of red light causes the fly to execute a modular escape jump sequence, providing an elegant illustration of the ability of optogenetics to probe neuronal circuits and precisely connect them with complex, observable behaviours. The Card lab has used this tool to gain new insights into the precise mechanisms of the giant fibre system, with findings such as the discovery of a bimodal aspect to the escape behaviour, and a spike-timing mechanism for action selection between the two modes[29]; as well as a system of linear, giant fibre-mediated feature integration that influences this action selection.[30]

Conclusion

The tools offered by modern optogenetic advances have provided neuroscientists with their biggest opportunity yet to explore and describe the brain in its entirety. While this is perhaps still an ambitious goal for the human brain, the number of neurons in the *Drosophila* nervous system is smaller by three orders of magnitude - and so we may not be quite so far off from achieving this goal in the fruit fly. The long-term goal of using model organisms is often to gain knowledge that may have

translational uses in humans; and the unassuming *Drosophila*, with its tractable nervous system, may prove to be the most advantageous focus for optogenetic research.

Acknowledgements

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Progress in Developing Therapies for β-Haemoglobinopathies Using Gene Editing Technologies

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Recently developed gene editing technologies have the ability to make precise changes in the genomes of human cells. Efforts have been made to apply gene editing technologies to develop targeted therapies for diseases with known genetic causes, resulting in a number of clinical trials. These efforts typically aim to use gene editing technologies to disrupt a particular gene or to mediate the site-specific integration of a gene, for therapeutic benefit. β-haemoglobinopathies are a group of disorders caused by mutations affecting the β-globin gene and include sickle cell disease and β-thalassaemia. It has been estimated that over 300,000 children are born with a severe haemoglobin disorder annually, creating a pressing need for effective therapeutic approaches. In this review, a number of studies that highlight the progress made in applying gene editing technologies to develop therapies for β-haemoglobinopathies will be discussed.

Introduction

Gene editing technologies enable targeted changes to be made to the genomes of unicellular and multicellular organisms. These technologies include those based on zinc-finger nucleases (ZFNs) and the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system. Each of these technologies uses programmable nucleases i.e. proteins that can be guided to specific loci and have the potential to generate DNA double-strand breaks (DSBs) at the loci that they are guided to (see Figure 1).[1] DSBs induce cellular DNA repair mechanisms, such as non-homologous end-joining (NHEJ) and homology-directed repair (HDR).[2]

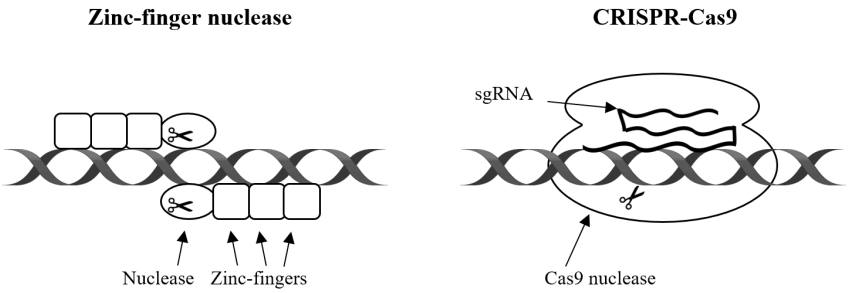

Figure 1: Zinc-finger nuclease and CRISPR-Cas9 gene editing technologies. A zinc-finger nuclease (ZFN) consists of DNA-binding zinc-finger domains linked to a nuclease domain. The zinc-finger domains allow a ZFN to recognise a specific DNA sequence. The Cas9 nuclease can be guided to specific genomic sequences by a single guide RNA (sgRNA). Both technologies can generate a targeted DNA double-strand break. *Figure adapted from Yin et al..*[3]

NHEJ is the main mechanism that repairs DSBs in mammalian cells.[4] As a result of a DSB, two DNA ends are generated. In NHEJ each of these DNA ends is identified and bound by a loading protein which facilitates the docking of the nuclease, polymerases and ligase of NHEJ.[5] Once docked, the nuclease makes the two DNA ends compatible by degrading short regions of DNA; the polymerases add deoxynucleoside triphosphates in a template-dependent or template-independent manner and the ligase joins the DNA ends together. These enzymes function in any order meaning that multiple rounds of DNA degradation and addition are possible.[6] This process is error-prone and can cause small insertion or deletion mutations (indels) at the DSB site which has been repaired.[4] The introduction of indels into the coding sequence of a gene can cause a frameshift resulting in a premature stop codon coming into frame. This could lead to the mRNA transcript produced from the gene being degraded by nonsense-mediated decay or could lead to the synthesis of a truncated protein product.[7] Thus, the introduction of indels can potentially inactivate genes. Gene editing technologies that can make DSBs at precise genomic loci, have the potential to activate the NHEJ DSB repair mechanism, which can cause indels to be introduced at the DSB site, potentially resulting in the desired inactivation of a specific, pre-determined gene.

In contrast to NHEJ, HDR repairs a DSB using an endogenous or exogenous DNA donor template which has homology (sequences that are the same or similar) to sequences adjacent to the DSB site.[8] By recombination, HDR can replace the sequence at the DSB site with the sequence of the donor template that is found between the regions of homology.[9] Donor templates can be engineered to contain insertions (potentially of a whole transgene), deletions and alterations in comparison with the sequence at the genomic site intended to be changed. Supplying engineered donor templates while using gene editing technologies to make precise DSBs can activate the error-free HDR mechanism, which has the potential to lead to the insertion,

deletion or alteration of a sequence at a particular site.[10] ZFNs and CRISPR-Cas based technologies are capable of introducing precise DSBs which can lead to the introduction of indels at specific genomic loci via the NHEJ mechanism or can lead to exact changes being made in a sequence via a donor template and HDR.

ZFNs can recognise a specific DNA sequence and generate a DSB at that sequence. ZFNs consist of DNA-binding zinc-finger domains linked to a nuclease domain derived from the restriction enzyme, FokI.[1] The zinc-finger domains allow a ZFN to recognise a specific sequence. Each zinc-finger domain can bind to approximately 3 specific base pairs (bp) of DNA and linking an array of these domains together can allow an engineered protein to recognise a DNA sequence of 9-18 bp.[11] The nuclease domain of FokI has to form a dimer in order to generate a DSB and has been further engineered to only generate a DSB when a heterodimer is formed.[12] To generate a DSB at a specific position a ZFN pair is required. A FokI nuclease domain is joined to a set of zinc-finger domains designed to bind one flank of the desired DSB site and a compatible FokI nuclease domain is joined to a different set of zinc-finger domains designed to bind to the opposite flank of the desired DSB site.[1] As a ZFN monomer can recognise a sequence of 9-18 bp, in total a ZFN pair can recognise 18-36 bp, potentially meaning that a DSB could be targeted to occur at a unique site in a genome.[12]

In contrast to ZFNs, the CRISPR-Cas gene editing system is guided to specific genomic sequences by RNA. The bacterial CRISPR-Cas9 system functions by incorporating foreign DNA at CRISPR loci. These loci can then be transcribed into RNA which targets the nuclease Cas9 to cleave foreign DNA, providing resistance to the foreign DNA (e.g. bacteriophage infection). Transcripts produced from the CRISPR locus, known as CRISPR RNAs (crRNAs), associate with a scaffold-like RNA known as trans-activating CRISPR RNA (tracrRNA).[13] After processing, the mature crRNA-tracrRNA complex can associate with Cas9. Cas9 can then be directed to generate a DSB at a foreign sequence complementary to the crRNA (known as a protospacer), positioned next to a short sequence referred to as a protospacer adjacent motif.[10]

Understanding the basic biology of CRISPR-Cas9 has paved the way for its adaptation as a gene editing technology. After the demonstration that a single artificial RNA (known as a single guide RNA or sgRNA) could direct Cas9 to generate site-specific DSBs[14], a number of groups went on to show that CRISPR-Cas9 could be adapted to enable targeted gene editing in human cells.[15–17] For example, *Jinek et al.* expressed Cas9 (containing a nuclear localisation signal) and a sgRNA targeting the clathrin light chain gene in human embryonic kidney cells and found evidence of the generation of targeted DSBs at the gene, leading to NHEJ.[16]

There are many potential applications of gene editing technologies arising from their ability to precisely target nucleic acids. These include applications in agriculture[18,19] industrial biotechnology[9], imaging[20], pathogen detection[21] and in controlling disease vectors.[22,23] Gene editing technologies have also been applied in

the generation of cellular and animal models, which can help develop insights into disease progression and the functions of genes.[8] Many diseases have a genetic basis – over 3,000 human genes have been associated with Mendelian disorders and ~500 genes are associated with susceptibility to infections or complex diseases.[3] Given this, prospective clinical applications of gene editing technologies have recently been explored by many groups. One approach, therapeutic *ex vivo* gene editing, involves removing cells from a person's body, making a change or multiple changes to the DNA of those cells using gene editing technologies, and then transplanting the edited cells into a patient for therapeutic benefit. This review will discuss studies that illustrate current progress in applying gene editing technologies to develop *ex vivo* therapies, specifically for β-haemoglobinopathies.

β-Haemoglobinopathies

β-haemoglobinopathies are a group of disorders caused by mutations affecting the β-globin (HBB) gene and include sickle cell disease (SCD) and β-thalassaemia.[24] It has been estimated that over 300,000 children are born with an inherited haemoglobin disorder every year.[25] A sickle cell allele missense mutation results in a hydrophobic valine being present at position six of the encoded protein, rather than the hydrophilic glutamic acid usually found at this position. Individuals homozygous for this mutation would be expected to suffer from the blood disorder, SCD. The amino acid change results in the formation of defective haemoglobin tetramers which can polymerise leading to the disruption of the shape of erythrocytes. These deformed cells can block blood vessels which can lead to intense pain.[26] Approximately 10% of individuals with SCD are eligible for a bone-marrow transplant which can cure the disease.[27] A number of preclinical studies have explored the application of gene editing technologies to develop *ex vivo* therapies, with the hope that in the future more people with SCD can be treated.

Preclinical Application of Gene Editing Technologies to Treat β-Haemoglobinopathies

One proposed gene editing strategy involves correcting the HBB missense mutation in patient haematopoietic stem/progenitor cells (HSPCs) via HDR followed by transplantation of these edited cells back into the patients. *Hoban et al.* (2015) electroporated HSPCs with mRNA encoding ZFNs targeted to the HBB gene and an oligonucleotide donor template, containing the correct HBB sequence. They reported that 18.8% of cells had undergone oligonucleotide-templated gene modification, but that 33.3% of cells had NHEJ-mediated indels.[28]

Dever et al. used CRISPR-Cas9 to target the HBB gene. This group began by electroporating a ribonucleoprotein complex (consisting of Cas9 and a sgRNA targeting

HBB) into HSPCs and then supplying a single-stranded adeno-associated virus (AAV) vector containing a GFP gene flanked by regions homologous to HBB.[24] AAV vectors are small viruses that have been widely used to shuttle genes into organisms due to their diverse tissue tropism and low risk of pathogenicity.[3] After delivering the AAV donor and Cas9 ribonucleoprotein, using FACS, the researchers observed a population of cells that had high levels of GFP expression.[24] They reported that 92% of clones derived from this population had the AAV donor sequence copied into them. As GFP wouldn't be suitable as a reporter in the clinic, it was replaced with a truncated nerve growth factor receptor (which is expressed on the cell surface), and a population of cells that had high expression of this receptor could be enriched for using targeted magnetic microbeads. The ability to purify cells with high levels of targeted integration via the presence of the receptor may, in the words of the researchers, "prove valuable in a clinical setting for removing untargeted HSPCs that will be in competition with homologous-recombination-edited HSPCs for engraftment and re-population after transplantation".[24]

SCD-patient derived HSPCs were electroporated with Cas9 RNP and transduced with an AAV donor template including a correct copy of the HBB gene and the truncated nerve growth factor receptor (see Figure 2).[24] On average, 11% of HSPCs obtained from a given SCD patient were successfully edited to include a correct copy of the HBB gene. A population of SCD-patient derived HSPCs was noted to have high expression of the truncated nerve growth factor receptor and extrapolating (as this was a preclinical study), the suggestion would be that in a clinical setting these cells could be purified using the magnetic microbeads specific to the receptor and then it would be these purified cells that could be transplanted back into a patient. It has been reported that the group behind this work are planning to move into clinical trials in the near future.[27]

In contrast to SCD, more than 200 different mutations have been implicated in β-thalassaemia pathology.[29] As HDR and NHEJ compete with each other to repair a DSB, aiming to use HDR to repair the HBB gene may be problematic. In parallel to SCD allele correction via HDR, NHEJ will create indels at a certain frequency. These indels could result in the unintended generation of a β-thalassaemia allele.[4] Another gene editing strategy that bypasses this issue by not targeting HBB, has been proposed for the treatment of both SCD and β-thalassaemia.

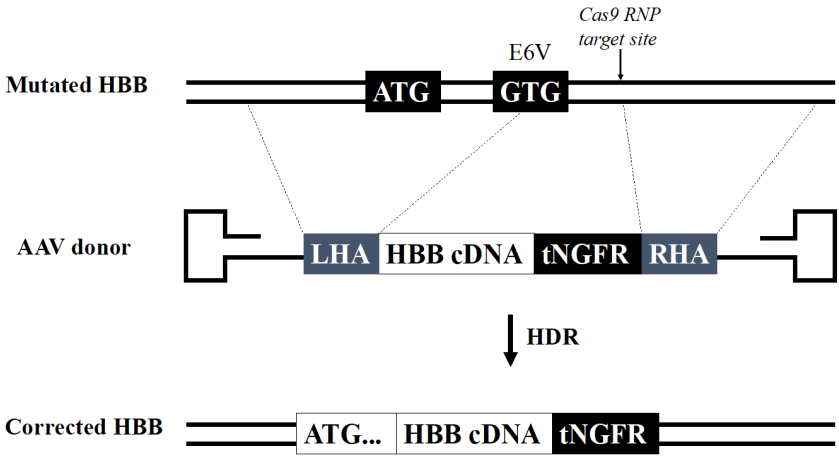

Figure 2. Targeting HBB for homology directed repair using an AAV cDNA donor. Schematic representation of an AAV donor template encoding a functional copy of HBB and truncated nerve growth factor receptor (tNGFR). HBB and tNGFR are flanked by left and right homology arms (LHA and RHA). The sickle cell allele contains a missense mutation (GAG to GTG) encoding valine (V) instead of glutamic acid (E) at position six of β-globin. The Cas9 ribonucleoprotein (RNP) generates a targeted DNA double-strand break inducing homology directed repair (HDR). PolyA signals and promoter of tNGFR omitted for simplicity. *Figure adapted from Dever et al. (2016).*[24]

Preclinical Efforts to Increase γ-globin

Around the time of birth, humans normally switch from making foetal haemoglobin using γ-globin, to producing adult haemoglobin using β-globin. Data has suggested that increased levels of foetal haemoglobin (after birth) can reduce the severity of SCD and β-thalassaemia.[30] γ-globin expression is normally repressed in adults by the transcription factor BCL11A.[31] An enhancer which specifically controls BCL11A expression in erythroid cells has been identified.[32] As individuals that have deletions in the BCL11A gene itself can have neurological defects, targeting the erythroid-specific enhancer of BCL11A for disruption using gene editing technologies may spare the loss of BCL11A expression in other cell types.

Canver *et al.* (2015) used CRISPR-Cas9 to specifically disrupt the erythroid-specific enhancer, leading to increased expression of γ-globin in a erythroid precursor cell line.[33] Furthermore, they introduced Cas9 and a pair of sgRNAs to delete the enhancer in mice, and reported that unlike conventional Bcl11a knockout mice that die soon after birth due to neurological and immunological problems, enhancer-deleted mice were born healthy and displayed less repression of γ-globin.[33] Sangamo Therapeutics are currently carrying out preclinical studies using ZFNs to disrupt this enhancer and a number of other companies, including CRIS-

PR Therapeutics and Editas Medicine, are carrying out preclinical studies using CRISPR-Cas9, aiming to increase γ-globin expression in edited cells.[8,27]

Preclinical Application of 'Base Editor' to Treat β-Haemoglobinopathies

A new adaptation of the CRISPR-Cas9 system, referred to as 'base editor', has recently been used in human embryos to change a β-thalassaemia-causing point mutation in HBB.[34] The first base editor arose when a fusion of a catalytically dead Cas9 (dCas9) and a cytidine deaminase enzyme was engineered.[35] dCas9 doesn't generate a DSB, but can be guided to a specific sequence by a sgRNA. Naturally occurring cytidine deaminases can catalyse the conversion of cytosine to uracil, which lacks only a single methyl group in comparison with thymine. A dCas9-cytidine deaminase fusion can be targeted to a particular site by a sgRNA, where it can mediate the conversion of cytosine to uracil. After DNA replication or repair this could result in a cytosine to thymine (or guanine to adenine) substitution.[35]

Following on from being the first group to report using CRISPR-Cas9 to edit human embryos,[36] *Liang et al.* are the first to report using base editor on human embryos, targeting the HBB gene in both instances.[34] Using base editor, they found that in 8 of 20 embryos, one or two of the HBB alleles had been corrected – but these eight embryos appeared to be mosaics, meaning that not every cell in a given embryo was corrected. New base editors were recently reported that can convert thymine to cytosine (or adenine to guanine) in genomic DNA,[37] and adenine to inosine (which is read as guanine) in RNA;[38] expanding the gene editing technology toolkit.

Conclusion

Gene editing technologies can make targeted changes in genomes. Many researchers are attempting to harness this ability to correct mutations underlying genetic diseases. β-haemoglobinopathies can be caused by a single mutation, making them an attractive target for gene editing. Mutations in the HBB gene have been specifically corrected in preclinical models using ZFN and CRISPR-Cas gene editing technologies. Base editor is a new CRISPR-Cas adaptation which has been demonstrated to be capable of fixing a β-thalassaemia-causing point mutation in human cells. An alternative treatment strategy being explored, aims to reactivate γ-globin expression, to serve as a substitute for defective β-globin. Hopefully in the near future, preclinical studies using gene editing to treat β-haemoglobinopathies will translate into benefit for individuals with these diseases.

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T Cell Immunometabolism: New Frontiers in the Fight against Cancer?

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During an immune response, immune cells can undergo rapid shifts in metabolism to support a required functional output. The role of cellular metabolism in controlling the activation, differentiation, proliferation and effector functions of immune cells has only recently been appreciated; and the field of immunometabolism represents a considerable pillar in efforts to develop new therapies against disease. This is especially true for cancer, particularly in contexts where the tumour microenvironment (TME) causes immunosuppression by disrupting immune cell metabolism. Development of therapies to promote efficient metabolic reprogramming in exhausted immune cells to support a reciprocal immune response is the crux of metabolic immunotherapy against cancer. The critical role of T cells in anticancer immunity has cemented them as fundamental targets in cancer immunotherapy. Recent efforts to define T cell metabolism in terms of their activation states, differentiation lineages, proliferative capacity and effector functions has unveiled new potential targets in enhancing their anticancer activity. Manipulation of T cell metabolism to enhance desired effector functions may soon emblematise cancer immunotherapy, particularly in the context of tumour-induced immunosuppression.

Introduction

In a New York Times interview in 2016, Nobel Laureate James Watson commented that identifying genes involved in tumorigenesis has been "remarkably unhelpful" in developing therapies against cancer. He continued, saying "I never thought, until about two months ago, I'd ever have to learn the Krebs cycle. Now I realize I have to."[1]

This sentiment has been echoed by many cancer researchers worldwide, reflected in the recent growing interest in the role of metabolism in regulating immunity.[2] The contemporary shift towards enhancing our immune system's natural anticancer capabilities has become an attractive option, as immunotherapy against cancer has shown unprecedented success in various clinical settings.[3]

T cells have become a fundamental target in cancer immunotherapy due to their pivotal role in orchestrating an anticancer immune response. The use of inhibitory monoclonal antibodies against immune checkpoint receptors such as programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte associated protein-4 (CTLA-4), as well as adoptive transfer of chimeric antigen receptor (CAR) T cells, have recently been approved for clinical use to overcome the immunosuppressive effects of the TME and reinvigorate metabolically-exhausted T cells.[4-6]

More recently, immunometabolism has emerged as an exciting field in cancer immunotherapy, and other diseases which involve immune dysfunction and metabolic deregulation, including obesity and type 2 diabetes. Immunometabolism is concerned with the way in which cellular metabolic pathways regulate the growth, proliferation, activation, differentiation and effector functions of immune cells.[2] Deciphering the role of cellular metabolism in the regulation of T cell biology allows us to exploit new therapeutic targets in the treatment of disease, as well as manipulate the way in which they become activated, differentiate into specific subsets, and carry out their effector functions to our advantage in anticancer immunity. As cellular metabolism is becoming increasingly more apparent to be integrally linked to immune cell function, it represents a paramount strategy for treating a disease which is responsible for almost 1 in 6 deaths worldwide each year.[7]

Immunometabolism and the Warburg Effect

Although the primary goal of cellular metabolism is the generation of ATP for the maintenance of homeostasis, it has a secondary function in the regulation of cell growth and proliferation.[8] Catabolic processes can consist of many intermediary steps, and these provide biosynthetic precursors for the production of amino acids, nucleotides, lipids and other biomolecules necessary for growth and proliferation.[9]

Glucose is the primary fuel utilised by mammalian cells, and is metabolised through two integrated pathways – glycolysis and oxidative phosphorylation (OxPhos). Glycolysis involves a series of enzymatically-regulated steps, whereby glucose is broken down into two molecules of pyruvate, generating two molecules of ATP.[10] Pyruvate is then transported to the mitochondria, where it is converted to acetyl-CoA and oxidised in the Krebs cycle to generate NADH and FADH2. Exergonic transfer of electrons from these reducing agents to the electron transport chain (ETC) – a series of electron-accepting protein complexes embedded in the inner mitochondrial membrane – facilitates translocation of protons to the

intermembrane space. The resulting electrochemical potential generated across the inner membrane acts as the driving force of ATP synthase, giving rise to up to 34 ATPs per molecule of glucose in the process of OxPhos.[11,12] The efficiency of this process in generating ATP coheres OxPhos as the primary process for homeostatic maintenance in mammalian cells.

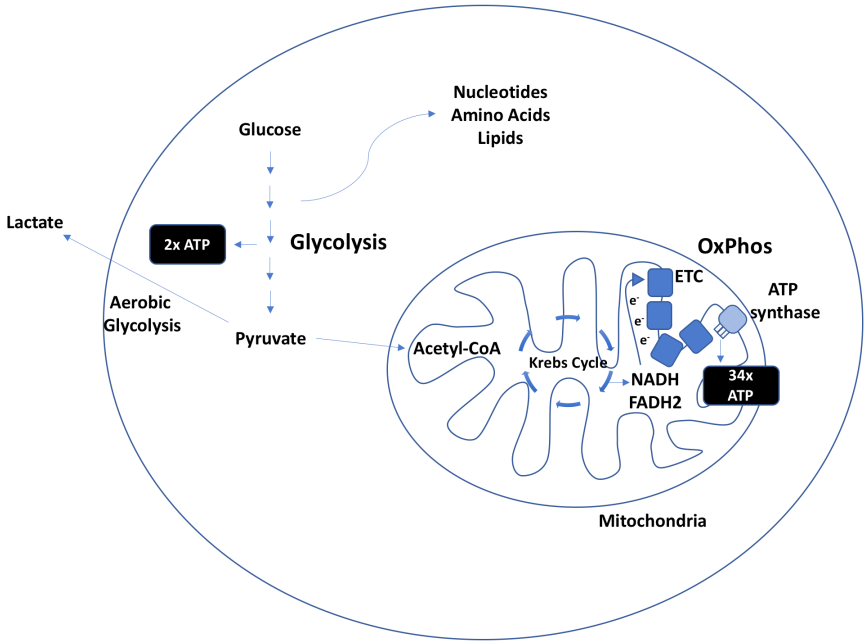

Figure 1: At the basal level, glucose is integrally metabolised by glycolysis and OxPhos, generating high levels of ATP for cellular homeostasis. Upon activation, immune cells preferentially upregulate aerobic glycolysis for the provision of biosynthetic precursors in the production nucleotides, amino acids, lipids and other molecules necessary for activation, proliferation and effector function acquisition. *Adapted from Loftus and Finlay (2015).*[13]

The first observation of a perturbation in cellular metabolism being associated with malignancy came from German biochemist Otto Warburg in 1923.[8] Widely disregarded at the time, Warburg proposed a shift in the metabolic equilibrium of cancer cells from primarily relying on oxidative metabolism to primarily engaging in aerobic glycolysis; the process whereby glucose is metabolised to pyruvate and then lactate – even in the presence of oxygen – before being secreted from the cell. This metabolic reprogramming, that is, upregulation of glycolytic enzymes and glucose transporters to attain a state of elevated glycolytic flux, is thought to be induced by mutated oncogenes and tumour suppressor genes[14,15], and has become

known as the Warburg Effect.[8] Although this shift towards a less efficient means of producing ATP seems counterintuitive, upregulation of glycolytic machinery and export of lactate facilitates sustained and elevated glycolysis; increasing ATP production and the provision of biosynthetic intermediates for cell growth and proliferation. Tumour-derived lactate also polarises local immune cells towards anti-inflammatory phenotypes, such as the conversion of proinflammatory M1 macrophages to their pro-tumorigenic M2 counterparts. Thus, the role of metabolism is repurposed in cancer cells towards tumour augmentation, as well as adapting to adverse environmental conditions like hypoxia, rather than solely on ATP production.[8]

However, this metabolic reprogramming is not unique to cancer cells. There has been growing research interest in the distinct metabolic profiles adopted by immune cells for their activation, proliferation, effector molecule production and longevity; and strategies that can be used to manipulate these.[2,10] T cells have been of particular interest, due to their importance in orchestrating a wide range of immune responses. Lymphocytes must have significant plasticity in their metabolism due to the diverse activation states and effector functions which they employ, and different T cell subsets exhibit distinct metabolic profiles to match their specific needs.[16] Like cancer cells, activated T cells, such as cytotoxic CD8+ T cells, engage in aerobic glycolysis to improve their proliferative capacity for clonal expansion and generate biosynthetic precursors for effector molecule production.[17] In comparison, memory T cells primarily engage in fatty acid oxidation (FAO), indicative of how cellular metabolism and phenotypic function are linked. A comprehensive understanding of how T cell metabolism affects an immune response – particularly in cytotoxic CD8+ T cells – may provide new targets for exploitation in cancer immunotherapy.

Metabolic Regulation of T Cell Activation

Because T cells progress through such diverse activation states throughout the course of an immune response, they need to be able to dynamically reprogram their metabolism to match their required functional output. Metabolic regulation of effector function acquisition in T cells allows rapid interchange between states of quiescence and activation; thereby implying differential metabolic signatures between naïve, effector and memory T cells.[18]

Naïve T cells (T_N cells) are those which have not yet encountered their cognate antigen, and continuously recirculate throughout the periphery and secondary lymphoid tissue such as the lymph nodes.[19] This seemingly interminable process of recirculation requires minimal output in terms of proliferation, growth and effector functions, and thus the immediate purpose of T_N cell metabolism is subsistence and longevity.[20] Generation of ATP through OxPhos constitutes ~96% of cellular ATP in T_N cells and is the most efficient way of survival until activation.[21]

Three signals are required to induce the transition from a naïve state to an effector state in T cells: ligation of antigen-loaded major histocompatibility complex (MHC) of antigen presenting cells (APC) to the T cell receptor (TCR), CD28-CD80/86 interactions, and cytokines from the local microenvironment.[22] Transduction of the signal resulting from appropriate T cell stimulation leads to dramatic metabolic change characteristic of an effector T cell (T_{EFF} cell).[2] One T_N cell can clonally expand into millions of T_{EFF} cells, a feat which requires an abundance of biosynthetic material.[23] Metabolic reprogramming from oxidative metabolism to aerobic glycolysis facilitates this rapid growth and proliferation through the provision of glycolytic intermediates, as seen in transformed cells.[16] For example, pyruvate kinase, an enzyme which catalyses the final step of glycolysis, exists as one of two isoforms – M1 or M2 – generated by differential splicing of its mRNA transcript. Although T_N cells express both at rest, the M2 isoform rapidly accumulates upon T cell activation. Interestingly, the M2 isoform is much less efficient than its M1 counterpart, and thus causes a build-up of its substrate, phosphoenolpyruvate, intracellularly. This amassment of phosphoenolpyruvate induces the accumulation of other glycolytic intermediates within the cell, which, as proposed by *Pearce et al*., can then be shunted into biosynthetic pathways, including the pentose phosphate pathway in nucleotide synthesis, in support of T_{EFF} growth and proliferation.[24]

Mammalian Target of Rapamycin Complex I (mTORC1) is a serine/threonine kinase and master regulator of cellular metabolism and immunity, and has been shown to be instrumental in T cell metabolic reprogramming.[25,26] Activation of mTORC1 by mitogens such as interleukin-2 (IL-2) triggers protein synthesis and upregulation of many proinflammatory T cell effector molecules, including perforin, granzyme B and interferon-γ (IFN-γ).[27,28] This increase in effector molecule production is facilitated by mTORC1-mediated glycolytic reprogramming, through the upregulation of transcription factors hypoxia inducible factor-α (HIF-1α) and c-Myc; key regulators of glycolytic machinery.[2] mTORC1 also induces the upregulation of L-selectin and CCR7, necessary for T_{EFF} homing to the tumour site from secondary lymphoid tissue.[16,25] Repurposing of metabolism towards immunoregulation allows acquisition of a T_{EFF} phenotype necessary for anticancer immunity, which may eventually become exploitable for cancer immunotherapy.

Once an immune response is resolved, most T_{EFF} clones apoptose to prevent autoimmunity. Some cells remain, however, and adopt a memory phenotype (T_{MEM} cells) so that a rapid recall response can be initiated in the case of reinfection or cancer recurrence. Like T_N cells, the primary focus of T_{MEM} cells is longevity. Although both use OxPhos to generate ATP, T_{MEM} cells predominantly employ FAO to generate acetyl-CoA in driving the Krebs cycle and subsequently OxPhos, producing 106 ATP molecules per fatty acid burned.[29] *Van der Windt et al.* have shown that this process is mediated by IL-15-induced mitochondrial biogenesis, facilitating the elevated spare respiratory capacity seen in T_{MEM} cells, as well as providing more sites for FAO to take place.[30] Interestingly, T_{MEM} cells do not use endogenous fatty acids for this process, and instead engage in glycolysis-driven *de novo* fatty acid synthesis. This process allows T_{MEM} cells to concurrently employ both glycoly-

sis and OxPhos, perhaps increasing the efficacy of a rapid recall response.[13]

The unique metabolic signatures adopted by T cells in different activation states can directly mould their phenotype. By understanding the switches that denote a change in T cell activation state, we can identify targets to manipulate a T cell response in an anticancer context.

Tumor Immunology & Challenges of the TME

The immune response to cancer involves a repertoire of both innate and adaptive immune cells working synergistically to eliminate cancer cells. Immunosurrveillant cells, namely natural killer (NK) cells, NKT cells and γδ T cells, initially recognise neoplastic tissue and produce IFN-γ; a proinflammatory cytokine which initiates a cascade of responses.[31-33] Recruitment of APCs like dendritic cells (DCs) and macrophages facilitates the uptake and presentation of tumour antigens to lymphocytes. These then carry out effector functions at tumour site, including direct cytotoxicity and secretion of cytokines like IFN-γ and TNF-α.

Solid tumours consist of cancer cells, fibroblasts, endothelial cells and infiltrating immune cells.[34] Local alterations in nutrient and oxygen availability because of Warburg metabolism and excessive proliferation by cancer cells makes the tumour microenvironment a hostile site for T_{EFF} cells. Lack of glucose to fuel aerobic glycolysis results in diminished effector functions, including IFN-γ production and cytotoxicity.[35] Hypoxia reduces local IL-2, impairing T cell activation, as well as activating HIF-1α. *Shi et al.* have also described the role of cellular metabolism in defining T cell lineage, identifying HIF-1α as a metabolic regulator of Th17 cells differentiation through activation of RORγt; resulting in increased IL-17 and subsequent low-grade inflammation in the TME, which may exacerbate immunosuppression.[36] Lactate secretion by the tumour generates an acidic microenvironment, negatively regulating T cell proliferation and cytokine production due to decreased glycolysis.[37] Depletion of nutrients that feed into metabolic pathways such as glutamine and tryptophan by tumour products also contributes to T_{EFF} suppression.[38]

Chronic stimulation of T_{EFF} cells in the TME eventually results in metabolic exhaustion and expression of inhibitory checkpoint receptors like CTLA-4 and PD-1. These challenges represent a significant barrier to normal T_{EFF} function by directly restricting their metabolic capacity and subsequently their ability to become activated.[39] Development of metabolic immunotherapies to exploit these compromised pathways may reinvigorate exhausted T cells to reengage in glycolytic metabolism in the enhancement of anticancer responses.

Strategies to Exploit T Cell Metabolism in Cancer Immunotherapy

A number of therapies targeting T cells are already in use in clinical settings, including anti-CTLA4 (ipilimumab) and anti-PD-1 (nivolumab) treatment for metastatic melanoma.[45] CTLA-4 and PD-1 are immune checkpoint receptors which downregulate T_{EFF} cell responses by disrupting the PI3K-Akt-mTOR axis and subsequent reprogramming to glycolytic metabolism.[45] Ipilimumab and nivolumab received FDA approval in 2011 and 2014 respectively, and mediate their effects through blockade of CTLA-4/PD-1 signalling to promote Warburg metabolism and reinvigorate exhausted T_{EFF} cells in the TME.

Secretion of IL-10 and TGF-β by regulatory T (Treg) cells causes local immunosuppression in the TME by inducing an anti-inflammatory phenotype in tumour-infiltrating immune cells to promote cancer persistence and metastasis.[40] Strategies to manipulate T cell metabolism may be effective in polarising T_N cells towards a proinflammatory phenotype and minimise the pro-tumoural effects of Treg cells. Etomoxir, a drug which inhibits mitochondrial enzyme carnitine palmitoyl transferase-1 (CPT1), has been shown to block Treg cell differentiation due to disrupted lipid oxidation.[41] Similarly, metabolic manipulation may allow us to influence differentiation of other CD4+ T_{EFF} subsets. Proinflammatory Th1 and Th17 cells, for example, require glycolytic metabolism to mediate their effects[16], and manipulation of metabolism may enable us to control the relative numbers of immune cells present in the TME.

Induction of T_{MEM} cells is the end goal of any vaccine. Recent efforts to develop vaccines against cancer exemplifies the importance of devising mechanisms to enhance T_{MEM} formation. In a seminal paper, Pearce and colleagues described the importance of mitochondrial architecture ultrastructure for driving T_{MEM} cell formation; with T_{EFF} cells displaying discrete, dispersed mitochondria with punctate cristae, in contrast to fused networks of mitochondria with closely spaced cristae in T_{MEM} cells.[41] Thus, drug-induced mitochondrial remodelling may represent a mechanism whereby T_{MEM} cells can be enhanced therapeutically.

Combination of these therapies with other regimens may promote synergistic anticancer activity, exemplified by the numerous clinical trials evaluating the efficacy of immune checkpoint blockade in combination with chemotherapy and radiotherapy.[42,43] As we learn more about T cell immunometabolism, we create new opportunities to develop treatments targeting specific pathways controlling effector functions, differentiation lineages and activation state.

Discussion & Conclusion

It is clear that immunometabolism is a promising field in the development of cancer immunotherapy. The integral relationship between metabolism and

immune cell activity provides us with insights into the requirements for different T cell subsets to become activated, proliferate and carry out effector functions, as well as the mechanisms whereby tumours can cause immunosuppression. Understanding how these pathways are regulated will be instrumental in the advancement of metabolic immunotherapy. For example, enhancing the activity of T_{EFF} and T_{MEM} cells, whilst blunting that of Treg cells, is a desirable scenario in the TME. Anticancer T_{EFF} cells include Th1 cells and CD8+ cytotoxic T cells, which are known to preferentially upregulate aerobic glycolysis in a proinflammatory context. Although both T_{MEM} and Treg cells primarily engage in fatty acid oxidation for survival, perhaps T_{MEM} cells can be preferentially induced through engineering mitochondrial architecture towards a fused network phenotype. Furthermore, targeting the mechanisms whereby the TME mediates its adverse effects on T cells, including reduced nutrient bioavailability and an acidic and hypoxic microenvironment, may prove beneficial in alleviating immune cell exhaustion and promote acquisition of normal proinflammatory effector functions.

Immunotherapy against cancer is prevalent now more than ever in clinical settings. We have already seen the introduction of immune checkpoint blockade and adoptive cell transfer of CAR T cells in certain cancers. These therapies arose from enhancing our immune system's natural anticancer capabilities, reflective of the primary objective in immunometabolism.

Overall, manipulation of T cells to achieve an appropriate metabolic profile to promote specific effector functions – even in the adverse TME – is the key to metabolic immunotherapy against cancer.

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Natural Sciences

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Letter from the Editor

The articles I received this year were truly phenomenal, and I cannot thank all the submitting authors enough for making the selection process as difficult as it was. The four reviews published here are the product of immense effort from the authors and the advisors. From botany to geology, these reviews showcase the broad range of topics that the natural sciences cover and their intimate connection with one another. Every element of the natural sciences is ultimately governed by climate, and it is my hope that the reader notices how climate change will affect the topics of all four of these reviews, and that this highlights the potential impact that increased interdisciplinary research could have on our understanding of and predictions for the future.

Terra Nova: Subglacial Volcanism and the West Antarctic Ice Sheet is a review of all the geological analyses of the impact of climate change on our marine-based ice sheets, and the potential for hidden volcanoes exacerbating these impacts. It provides just one element of proof of climate change within a myriad offered by scientific literature, and is an essential technical approach to predicting our future. I believe the author has mastered this approach, blending technical facts with insightful commentary.

Plenty More Fish in the Farm discusses the multiple impacts environmental degradation can have on Atlantic salmon, and from this one can infer the additional strains that climate change may impose. Aside from direct effects, the melting of ice sheets will indirectly modify marine systems and thereby modify the success of the Atlantic salmon farms and farming techniques discussed in this thorough review. The breadth of disciplines involved in this review is a testament to the author's superb understanding of the scope of the issue.

The Role of Green Walls and Green Roofs in Urban Farming addresses the ever-increasing rate of urbanisation, loss of food security, and the importance of interdisciplinary solutions. As climate change is predicted to cause sea level rise and intensify storm systems, more and more rural people will become environmental refugees and congregate in urban environments. Arable land will be lost and, with-

out urban farming, food production will likely decrease and famine may occur. Urban farming offers a unique solution to loss of food security and technological innovations in terms of efficient closed-loop systems, a point that this author communicates effectively.

Unravelling the Psychoactive Nature of Morning Glories describes how alkaloids found in morning glories can be studied to elucidate possible production of safer and more effective medicines. The topic is intrinsically fascinating, and the author has infused their knowledge of plant sciences into every aspect of the paper. Climate change is predicted to decrease biodiversity, and one of the symptoms of species loss will be the loss of medicines derived from extinct species. It is therefore essential to mitigate climate change if we want to provide better medical treatment through botanical methods.

Although these types of connections can be hard to make and may even seem tenuous at times, they are essential to understanding our world at present and in the future. The breadth, quality and accessibility of the articles published here showcase the importance of interdisciplinary research in the natural sciences, and I believe that the reader will find them as intriguing as I did.

Isabel Jorgensen
Natural Sciences Editor
Trinty Student Scientific Review 2018

Plenty More Fish in the Farm: The Environmental Imact of Domesticating Atlantic Salmon

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With world populations set to increase by 80 million each year, food security and economic stability has become increasingly uncertain. To ensure a stable food supply for this growing population, the fish farming industry is becoming more widespread than ever. Atlantic salmon, one of the most popular products that fish farming produces, is a major growth area for national economies. In Ireland alone, there is forecasted 78% increase in the production of Atlantic salmon by 2020 (3). This rapid expansion in fish farming could potentially pose significant threats to marine environments and human health if left unchecked. In order to safeguard against these threats, this report aims to promote awareness surrounding the impacts of fish farming, highlighting the core problems within the sector while also suggesting solutions for them. To do this, three major impacts will be explored that Atlantic salmon farming has been reported to have in countries such as Ireland, Canada, Greenland and Norway - benthic pollution, chemical accumulation and genetic regression in wild fish stocks. Best management practices can be employed through an understanding of the ecology of marine environments and new technologies can help mitigate the impacts that Atlantic fish farming can have. Additionally, this report will demonstrate the need for improved assessment methods of agro-chemical bioaccumulation and its impact on human health and the marine environment, and further research on the effects of fish farm's escapees on the wild type genetic pool.

Introduction

Through the myth of "The Salmon of Knowledge", the image of the salmon has forever been embedded culturally and historically in Ireland's national identity[1]. However, in recent years the decline in Atlantic salmon stocks (*Salmo salar*) is threatening to confine the existence of salmon to myths and legends - or to that of farms[2]. Countries which share the Atlantic Ocean at its coast have seen a declining trend in *Salmo salar* in recent years[2]. Rivers in Atlantic Canada and Quebec have witnessed a 27% decrease in the number of Atlantic salmon returning to rivers in 2015[2]. In 2016, Greenland for the first time was unable to reach their small fishing quota for Atlantic salmon[2]. Similarly, Ireland has seen a dwindling number of Atlantic salmon returning to rivers to breed, falling from 20% in 1980 to just 5% in 2017[3] . According to Inland Fisheries Chief Executive, Dr. Ciaran Byrne, the decline is being attributed to increasing ocean temperatures, pollution, numbers of by-catch, and the spread of disease from fish farms that have been trying to fill the vacuum these dwindling fish stocks have left[3].

With the world's population set to increase by 80 million each year, the Food and Agriculture Organization has estimated that we will need to produce 70% more food globally by 2050[4]. Meeting these demands has become increasingly difficult due to climate change, the availability of land, and the limited amounts of natural resources such as phosphate fertilizers and freshwater available to help intensify agricultural production[4]. Therefore, fish farming in recent years has been presented as a more attractive option for increasing food production. In the 1960s, sea cage culture was first used in Norway for Atlantic Salmon in order to raise stocks to a marketable value[5]. Due to favourable conditions such as deep waters and strong currents surrounding Norway, their investment in sea cage culture was an economic success and soon the farming of Atlantic salmon spread laterally to Scotland, Ireland, Canada, America and even Australia[5]. Today, the growth of fish farms is still paramount to countries' economies and food production. In Ireland alone, aquaculture and fish farming is flagged as a growth area with a 78 % increase forecasted for farmed Atlantic salmon by 2020[3]. However, as the popularity of fish farming grows, it is essential that we evaluate the impact it is having on marine environments. Without the conservation of our oceans, fish stocks could potentially collapse.

Environmental Impacts

Benthic Environment

As fish farms are localised structures, the accumulation of organic matter in the surrounding benthic environment is an important factor to consider when assessing fish farms' environmental impact. In fish farms, a considerable amount of waste faeces and food pellets are generated during production and accumulate

on the bottom of the seafloor[6]. An influx of organic sediment to the benthic environment can alter the chemical and physical characteristics of the sediment present[6]. Consequently, this can disrupt faunal communities in the area due to the rise of anoxic conditions as well as the outgassing of methane and hydrogen sulphide – disturbing the ecological community on a whole[7]. In a study of 168 Norwegian salmon fish farms, total organic carbon content in sediment samples found directly under production sites was significantly higher than control benthic sites with no farming above them[6]. Of these samples taken from under cages, 32% showed significant degradation mainly attributed to a rise in acidification and anoxic conditions[6]. A loss in faunal biodiversity is also illustrated by a drop in the Shannon-Wiener diversity index when compared to reference sites[6]. However, the severity of these effects lessens 50-100 m away from the direct impact site and is dependent on the rate of nutrient loading in addition to the depth and currents present in the marine environment[6]. Therefore, a site's oceanography and rates of feeding are crucial factors to take into account when trying to mitigate the damaging effects on the benthic environment.

Chemical Inputs

A large variety of chemicals are introduced to fish farming systems in order maintain healthy captive fish. Sea lice are one of the major pests plaguing fish farms as they thrive in their confined space[8]. They have infested nearly half of Scottish salmon fish farms and have cost the industry 300 million pounds[9]. In 2016, Norway reported to have lost 60,000 tonnes of salmon directly affected by infestations of sea lice[9]. The control of sea lice is paramount to keeping salmon fish farms healthy and so they are treated with pesticides. A mixture of hydrogen peroxide and deltamethrin via bath treatments is generally used to do this, along with in-feed treatment of emamectin benzoate[10]. Although hydrogen peroxide has a rapid decomposition and consequently has little environmental impact, both deltamethrin and emamectin benzoate are classed as a "Dangerous Substance" under Directive 2006/11/EC[10]. As a result of their toxic nature, Environmental Quality Standards (EQS) are implemented to control how much of these toxins enter the environment[10]. However, these EQS figures are unique to the EU and differ from country to country - with some having none in place at all. Researchers recently tested salmon fish farms for antiparasitic medicines in Norway and found that the level of sea lice pesticides exceeded that of UK standards[8]. Norway fish farms supply over half of the world's population with farmed salmon, yet Norway has no EQS set for pesticides such as deltamethrin, emamectin, and three other sea lice treatments[8,11]. Furthermore, pesticides for sea lice treatment were also found in blue mussels, cod, shrimp and brown crabs surrounding the salmon farms, which is of particular concern for marine organisms with shells as it interferes with shell formation[8]. Pesticide usage is evidently growing in fish farms, with a reported 10 fold increase according in the last 18 months in Scottish salmon farms[9]. This could potentially give rise to a higher resistance in sea lice, worsening their infestations further in the coming years.

Antibiotics are also frequently used in fish farms to curb the spread of disease, such as amoebic gill disease (AGD), which is widespread in Atlantic salmon farms in Ireland, Scotland, Norway and even Tasmania[12]. AGD is characterized by white lesions on the gills of fish and is typically treated with 2-3 hour baths in an antibiotic solution[12]. Reports in Ireland state that the antibiotic baths in the past have lasted up to 6 hours[10]. Although the report states the use of antibiotics is tightly regulated, it is hard to know the lasting effects that will occur as a result of its usage[10]. The bioaccumulation of antibiotics in fish and the health risk posed to humans varies with species and with the specific substance being used as treatment[13]. For instance, when grass carp are treated with the antibiotic ciprofloxacin, it poses no human health risk; however when red-tail prawns are treated with the antibiotic erythromycin–H_2O there was a reported potential human health risk[13,14]. At present, more study needs to be done on Atlantic salmon and the common antibiotics used for their treatment in order to conclusively determine that consumption of farmed salmon poses a human health risk. However, studies have shown that the continuous application of antibacterial agents can build up in marine sediments and lead to the development of antibiotic resistant bacteria[7]. This holds greater long term effects for marine life as well as ourselves.

Wild Salmon Stocks

The escape of farmed Atlantic salmon also poses a threat towards the wild salmon populations. The introgression between domesticated salmon genes and wild types can cause a decrease in fitness, productivity, genetic diversity, and resilience to disease[15]. The phenotypic differences can be observed in Figure 1.

Figure 1: An escaped Atlantic farmed salmon in Norway with the wild population[15]. *Credit: Rune Muladal.*

With repeated cases of farmed salmon escapes and inbreeding, the fitness of the wild Atlantic population may decrease so much that it can threaten extinction in extreme case[15]. In Ireland, between the years of 1996-2004 approximately 415,000 farmed Atlantic salmon were reported to have escaped into coastal waters[15]. Large storm events have been associated with increased escape events[15]. For instance in 2014, escapee figures reached that of 230,000 for farmed Atlantic salmon in Ireland[15]. No numbers at present have been released for 2017, during which Irish shores were hit by Hurricane Ophelia last October. In 2013, the Marine Institute issued a statement saying that fish farm escapes were at a low level and contribute little to spawning stocks in Ireland[16]. Contrary to this statement, Inland Fisheries Ireland stated that there were concerns over the amount of escaped Atlantic salmon and their effect on wild stocks[17]. This was following a number of fish escapes being unreported in 2017, even though fish farmers are required to report any escapes under their operating licence[17]. Inland Fisheries Ireland have been collecting data on the farmed salmon captured in 2017 to help consolidate and quantify the risk to wild native stock[17].

Discussion and Conclusion

There is a clear impact of Atlantic salmon fish farms on the environment. The massive surge in fish farming over the past few decades will likely continue to develop, calling for increased understanding of its ecological implications as well as "best management practices" to be put in place to safeguard wild salmon stocks. However, one can argue that fish farming practices, especially chemical inputs, are comparable to terrestrial agriculture and therefore should have similar guidelines. The benefits of fish farming are clear: a reliable food supply and commodity for countries and a boost in regional economies in many rural areas[16]. With this being said, in order for this industry to be viable, safe and inexpensive, the ecology of marine systems must be fully appreciated.

The most damaging effects towards benthic environments can be negated by best management practices and understanding the oceanography of the fish farm's location[6]. Choosing a sustainable rate at which feed is issued along with a site that has significant depth and currents present is vital practice to reduce the impact on benthic environments from waste matter as it spreads it over a larger scale[6]. This, along with sustainable stocking densities can help reduce the impact that total organic carbon has on the seafloor[6]. Additionally, new technology is continuously being created to help manage fish farms better. For instance, researchers from Universidad Politécnica de Madrid and University of Florence have developed a robotic fish called "Ituna", which is equipped with biosensors capable of measuring water quality levels in real time but without disturbing the fish present[18]. This technology can also allow for tighter control on the environmental impacts fish farms have.

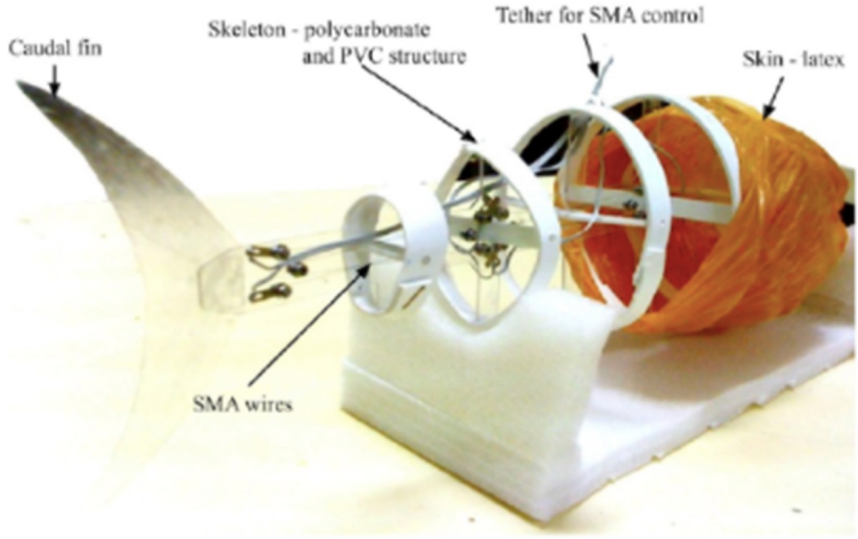

Figure 2: New robotic fish technology. Image adapted from *Rossi,* 2011[18]

In relation to chemical inputs in fish farms, it appears there needs to be more research done on specifically Atlantic salmon and sea lice and its effects on human health. However, it is clear that international standards of Environmental Quality Standards are needed. Additionally, the genetic diversity of wild Atlantic stocks needs to be fully protected by implementing better farming structures so escapes are less likely to happen - especially during periods of stormy weather. Furthermore, all escape incidents should be fully reported so that there can be an accurate consensus on the effects that fish farms have on wild stocks. This will maximise the chance that wild stocks of Atlantic salmon have of recovering, but also help evaluate the structural issues that may need to be redeveloped in fish farming practices. With these practices in place, Atlantic salmon both wild and farmed can be persevered and managed safely for generations to come.

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The Role of Green Walls and Green Roofs in Urban Farming

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Green roofs and green walls are roofs and walls that are partially or completely covered in soil and vegetation, typically implemented in urban environments. Green roofs and greens walls provide a suitable platform for urban farming to occur and can play a key role in agricultural activities. They also have numerous other benefits such as storm-water management, reduction in local temperatures, improvement of air and water quality, and economic advantages, that can operate in a positive feedback loop to boost urban agriculture. The problems that may arise include water use in drought-prone regions, integration with the urban landscape, and food contamination. This will be addressed in the review.

Introduction

Urban farming is the process of cultivating, processing and distributing crops from a small plot of land within a larger urban area. It is proposed as an environmentally and economically sustainable answer to global challenges including urbanization, public health, food security, nutrition and climate change. It is particularly relevant in the developing world where these issues are most prevalent. Due to the rapid urbanisation of cities, vast areas of natural landscapes have been be replaced with impervious structures from development. This has disturbed or completely eliminated the natural ecological systems of these habitats, which are vital for the maintenance of balanced ecosystems[1]. Not only does this degrade the natural environment, but it also leads to pollution and health problems. Urban poverty has also increased with urbanisation. Urban dwellers are more likely to suffer from food insecurity due to a lack of access[1]. While most rural dwellers produce their own food or have access to locally produced food, urban dwellers are more dependent on non-local food purchases. This leaves them more vulnerable to rising food prices, with many poor households surviving on cheap, highly-processed high calorie foods that lack nutritional value. Urban agriculture has a multitude of benefits that tackle these issues. It provides direct access to staple crops, fresh fruit and vegetables

and even livestock products which improves dietary quality and therefore health of community. It reduces local food prices by removing post-farm costs such as transportation, storage, marketing etc. while providing job opportunities for urban dwellers by generating income, especially for urban poverty groups[2,3]. It also has a major role in urban greening which is vital for environment conservation. Increased urban green spaces can contribute to ecosystem remediation and reduction of greenhouse gases, which would also benefit the well-being of the community.

Green Roofs and Walls

The term "green roofs", also known as "living roofs", refers to a 'roof' of a building which is partially or completely covered in soil and vegetation[5]. Germany is the world leader in green roof technology, having first developed the modern green roof in the 1960's[5]. There are three distinct classifications of green roofs: intensive, semi-intensive and extensive, as outlined by the German Landscape Construction and Development Research Society[6]. Extensive green roofs are the most common. They have a maximum depth of 15 cm and incorporate lightweight media that is made from recycled materials such as broken roof tiles, rather than traditional soil[7]. Their light weight means they can be implemented on flat or sloped gradients and are suitable to most sites[1]. All three types of green roofing have similar constructive components and can all support urban farming activities. The conventional green roofs consist of different layers. A root barrier layer prevents damage to the underlying structure, drainage layer facilitates the removal of excess water and a filter fabric prevents the drainage layer from clogging up the growing media and vegetation layers[4].

The term "green walls", also known as "living walls" or vertical gardens, refers to vegetation grown directly on a building façade or grown on a freestanding structure that is attached or adjacent to the wall[8]. They can be indoors or outdoors and can be used for food production in urban farming. Building facades lend themselves to the production of climbing crops such as beans and passion fruits. Other innovative designs, such as container/felt systems, allow other food crops with a more lateral spread to be grown vertically.

Environmental Benefits

The use of green technology in urban agriculture also has a number of environmental benefits for adapting urban areas to current climatic conditions and future climate change. They provide many ecological and economic benefits such as storm water management, energy savings and mitigation of the urban heat island effect. Natural processes that are fundamental to our environment, e.g.

water and nutrient cycles and solar radiation are managed effectively by a balanced ecosystem. However, with urbanisation these natural ecosystems are disrupted or eliminated in replace of impervious structure for urban development, which has resulted in many ecological and environmental problems.

Stormwater Management

First, the ground can no longer absorb precipitation, reducing infiltration into groundwater[15]. When rain falls on forest, it goes through its natural hydrological cycle, where water percolates shallow and deep aquifers or is released into back into the atmosphere through evapotranspiration. This means nearly 100% of the water is absorbed and runoff is rare[15]. This is not the case in urban areas where the majority of land is covered in impervious surfaces. This inhibits water absorption and the majority of rainfall accumulates as runoff. High rainfall or storm events can have detrimental effects on municipal sewer systems. If the storm water exceeds a sewage system's capacity, they overflow into rivers, allowing raw waste to enter natural water systems[1]. Therefore, a direct link exists between runoff from impervious structures and pavings and the degradation of stream water quality, which has major implications for humans who rely on rivers for drinking water supply, irrigation systems and stream biodiversity. It can also increase the probability of flooding downstream if channel capacities are exceeded, which can cause damage to properties, infrastructure and humans. Green roofs and walls can help mitigate these problems. Green roofs are known to reduce and delay roof runoff which lowers the peak rate, reducing the pressure on stormwater infrastructure[15]. They absorb rainwater which can then be taken up by the vegetation and released back into the atmosphere through evapotranspiration or retained in the soil. In studies,the soil and vegetation on green roofs retained approximately 75% of rainwater, meaning only 25% became runoff. In a study done in 2004, Liu found that run off volumes decreases from between 73% and 85% depending on rain intensity[16]. A study in Berlin showed that run-off from conventional impermeable roofs was almost the same as precipitation amount (288mm), whereas run off from green roofs was only 50- 100 mm, depending on the soil medium and age of roof[17]. In addition to the overall volume reduction, runoff from green roofs does not manifest immediately. The soil medium retains the rainwater until it becomes saturated and then the water slowly percolates through the vegetation layer into the drainage outlet[17].T he length of delay depends on many factors such as the composition of soil medium, rain intensity and duration and wetting history. Green roofs have been found to delay runoff by 95 minutes in light rain and 24 minutes in heavy rain. Moran et al. found that for 60% of rainfall events, a minimum delay of 30 minutes was achieved by green roofs[18]. The result of this is a decrease of peak flow that, when combined with the reduction of storm water volume, diminishes the erosional power of the runoff. This consequently reduces the pressure on stormwater infrastructure, preventing overflow of sewage into rivers and mitigating flooding events. Additionally, some studies found that green roof runoff contained less stormwater contaminant than conventional roofs and also found that green roofs had the ability to neutralise acid rain[19]. This

improvement in stormwater quantity and quality could have other indirect benefits to urban agriculture as it would improve urban stream water quality which urban farmers use for irrigation, essentially creating a positive feedback loop.

Urban Heat Island

Another major environmental benefit that could be attained with the use of green roofs and walls in urban agriculture is the mitigation of the urban heat island (UHI). Urban areas tend to have higher temperatures than surrounding rural areas, mainly due to the high density of low albedo structures and building and the lack of vegetation which absorbs solar radiation[20]. This leaves urban areas more vulnerable to the effects of global warming, which has implications for urban farmers as increased temperatures can have adverse effects on crop productivity[21]. The introduction of urban green infrastructure such as green roofs or green walls can help reduce this effect by returning green vegetation to urban areas. This vegetation can intercept incoming solar radiation and also retain a considerable amount of solar energy for utilization in photosynthesis. This provides a cooling effect, helping to reduce temperatures and therefore the UHI[22].One study found that maximum daytime temperatures in humid cities could be significantly reduced by implementation of vertical greenery (green walls), by 8.4ºC in Hong Kong, by 10ºC in Athens and 3.4ºC in Riyadh[23]. This would thus reduce heat stress for both the residents and the vegetation of urban areas.

Potential Problems

Although urban agriculture has the potential to overcome many urban and environmental problems, there are many barriers restricting this sector's growth. Three key issues will be addressed: water use, integration with the urban environment, and food contamination. Water use, particularly in drought-prone regions, intense competition for space with other forms of economic developments, and the potential of food contamination, particularly in heavily polluted cities, must be addressed in order to implement urban agriculture[4]. Coincidentally, implementing green roofs and green walls in urban agriculture could provide a viable solution to these problems. They are innovative solutions that increase green spaces in built-up areas, without interfering with other economic practices. They reduce competition for space at ground level because they are built on the roofs or walls of already established structures. This also reduces the potential of food contamination by avoiding polluted urban soils.

Water Use

Water scarcity is a global problem and there is immense pressure on large cities to provide sufficient water for drinking and domestic use to their entire population. Urban farms put additional pressure on this water supply as they rely on it for irrigation. Hot arid climates are particularly vulnerable to droughts, potentially hindering the success of urban farming in Africa and the Middle East. Green technology can alleviate this problem by incorporating innovative ways to

reuse water. Green walls are often irrigated in a recirculating system, relieving the pressure on the city's water supply and reducing, if not elimination, water wastage. Interior green walls can also be irrigated using in-house grey water. Grey water makes up a large proportion of water pollution and can be harmful if it enters waterways. Despite this, it is known to contain nutrients that are beneficial to the growth of most crops[13]. Therefore, one way to improve urban agriculture in drought stricken poor communities is to reuse treated grey water for the irrigation of food crops on green walls. Although there is a potential health risk with using untreated grey water in normal urban agriculture, studies have shown that green walls can be engineered to treat it, removing potentially harmful substances. For example, a study found that coco coir and perlite soil media was successful in the removal of pollutants from greywater[14]. Therefore, the ability of green walls to filter and reuse grey water could have many benefits for urban agriculture, particularly in water-stressed regions.

Integration with the Urban Environment

Another issue with urban agriculture is the lack of space. Incorporation of green roofs and green walls into new and existing urban developments increases the potential agricultural area while preventing competition for land with other urban economic developments that may be more profitable and therefore more appealing to land owners. Providing food to an urban centre from nearby agricultural areas is often insufficient and food must be transported long distances to reach consumers in urban areas. For example, New York City's food supply is sourced from an average 74 km away and production in New York state alone can only feed 55% of the population[9]. This is due to land scarcity and a lack of formal land rights that prevent urban farming in New York state. If farming could occur within the urban centre, the ecological footprint of food production could be greatly decreased and cheaper and fresher food would be made accessible to urban dwellers. A large proportion of roofs in large cities are flat and the vast majority of vertical space is unutilised. This could accumulate to a great deal of land for potential agricultural use. Already existing roofs and walls could be retrofitted with green technology, which would provide the much needed space for food production[4]. It is important to note that some roofs and walls may not have the structural integrity or load capacity to support green roof without additional structural support that may be expensive. However, those that are suitable for retrofitting and or new construction could greatly contribute to food production and the surrounding areas would reap the benefits.

Remediation of Food Contamination

Another major obstacle for urban agriculture is food contamination. The main sources of contamination are the soils where food is grown, water that is used for irrigation, and air pollution. Firstly, land available for urban agriculture may already be contaminated with heavy metals and other chemicals from previous industrial or excavation practices. Urban farmers, particularly in the developing world, may not have access to clean water for irrigation. Thus, wastewater

irrigation is a common practise for food crops in urban farming. Due to the rapid urbanization and industrialisation in the 20th century, there has been an increased amount of toxic chemicals making their way into wastewaters[10]. This practise can therefore contaminate the soil and the crop adversely affecting the safety of food crops. Toxic metals, originating from various natural processes, anthropogenic activities and atmospheric emission can make their way into soils.When these soils are used for agriculture, they can cause heavy metal accumulation that may be taken up by food crops and stored in the plant tissue. Consumption of high levels of heavy metals are known to have negative effects on the human body and are often associated with cancer and other diseases in the kidney and liver[11]. Air pollution of toxic chemicals and trace elements is also greater in urban and industrial areas, potentially leading to greater uptake of these contaminants into crops[12]. The use of green technology can reduce these health concerns. They do not require the use of local, potentially contaminated soils. The soil media used in green roof and walls is usually engineered rather than transplanted from ground level. This media has a higher permeability and lower cation exchange rate, making it less vulnerable to the accumulation of heavy metals and other toxic chemicals[4]. This reduces the uptake into crops and therefore reduces the risk of food contamination and subsequent health problems. Additionally, the distance from the major atmospheric pollution sources i.e. roadways is also known to affect the extent of pollution. Green roofs are mainly situated several stories above ground level, providing a large distance between crop production and the main sources of urban air pollution.

Conclusion

In conclusion, this report found that green technologies such as green walls and roofs have immensely beneficial potentials in urban agriculture. They provide the much needed spaces for these activities to occur, improving food security and accessibility, especially for poor urban dwellers. Additionally, they help mitigate the major problems associated with traditional urban farming such as food contamination and water scarcity. The reduction of food contamination reduces the risk of health problems by providing safe and nutrition food to urban dwellers. Green technology also reduces the pressure on urban water supplies through the engineering of recirculative irrigation systems and the filtering and reuse of greywater. Finally, along with these direct benefits to urban agriculture, green technology can have substantial environmental benefits that help tackle the effects of climate change. They can play a significant role in stormwater management by reducing and delaying stormwater run-off and can also reduce the urban heat island effect. This is of particular importance as future climate predictions suggest unprecedented temperature and precipitation changes. Green walls and green roofs are major components of the green revolution that is evolving all around the world. With more and more research and awareness going into green technologies

such as these, there is optimism that a more sustainable and environmental friendly society can be achieved.

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Unravelling the Psychoactive Nature of Morning Glories

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Morning glory is the common name for many species of common garden plant, some of which contain ergot alkaloids. Ergot alkaloids are chemically similar to LSD, resulting in a long history of cultural use, particularly in Central and South America, where they were used in religious rituals. Today, ergot alkaloids have a diverse range of applications in medicine, such as in the treatment of migraines, induction of uterine contractions, and in the treatment of Parkinsons. Currently, the production of these compounds relies heavily on infecting grains with ergot fungus (Claviceps purpurea), which in the past has been responsible for mass-poisonings and is likely the cause of medieval outbreaks of St. Anthony's fire. In this review the history of the study of these plants in context of their alkaloids will be discussed, and possible areas of future research will be highlighted. Understanding how these plants obtain their alkaloids may shed light on the evolutionary and ecological processes that may exist, but might also allow us to use that mechanism to our advantage in the production of more effective and safer medicines.

The History of Morning Glories and Their Use

Plants from the family *Convolvulaceae* have had a long history of ritualistic and ceremonial use in Central and South American cultures[1]. The seeds of *Turbina corymbosa* (syn. *Rivea corymbosa*) which were called '*Ololiuqui*' in the native Aztec language Nahuatl, were often consumed in a spiritual context, leading to bizarre visions used for prophetic purposes and divination as Richard Evan Schultes observed. This led to an effort to try to characterise the active components of this plant and closely related plants also used in such ceremonies (in particular *Ipomoea violaceae*). It was later found by Albert Hoffman, who had previously worked for the Sandoz chemical company and had discovered LSD, that these seeds contained a chemical in the same class of secondary metabolites, called ergot alkaloids[2].

This was problematic as it challenged the idea of chemotaxonomy, that similar chemical compounds are produced by related organisms. These newly discovered ergot alkaloids were extraordinarily similar to those derived from the ergot fungus (*Claviceps purpurea*) which often infects cereal grains, but their presence in dicotyledonous plants was unknown[3]. There are three possibilities that may explain this disjunct occurrence of ergot alkaloids: first, horizontal gene transfer of the genes responsible for producing these alkaloids (likely from a *Clavicipitaceous* fungus to the plant), second, the metabolic pathway for the production of these alkaloids evolved twice separately, and third, there may be a fungus-plant symbiosis that produces the alkaloids[4]. To date, 79 *Ipomoea* species have been analysed for their alkaloid contents, with 23 testing unambiguously positive for ergoline-alkaloids, 15 testing with ambiguous and contradictory results, and 41 testing negative for ergoline-alkaloids[3]. This is significant, as it indicates some genus-level relationship between the occurrences of the alkaloids at the very minimum, rather than the previously tested species being anomalous. These alkaloids have to date mainly been detected in the *Ipomoeae* tribe of the Morning glory family, but there have been reports of ergot-positive species of *Convolvulus*, a species of *Stictocardia*, and even from a parasitic *Cuscuta* species[5].

Ergot Alkaloid Use and Significance

Ergot alkaloids show a huge diversity in nature, with the three main classes being defined as simple clavines, lysergic acids and their amides, and ergopeptines, all of which contain physiologically active members[6]. This is an incredible diversity of compounds that can not only help us understand the various evolutionary processes that led to them, but can also be adapted for medicinal purposes themselves. While some, such as ergometrine, have been used as uterotonics to aid contractions during childbirth and to help with postpartum bleeding, others have been used in the treatment of migraines and Parkinsons disease[7, 8]. Possibly the most famous ergot alkaloid is the semi-synthetic LSD, which was important culturally and for therapeutic uses during the 1950's and 60's in particular[9]. With such a high natural diversity of alkaloids and an enormous potential for physiologically active natural alkaloids and semi-synthetics, it is quite possible that further research in this area may provide us with an array of useful drugs.

It is important to be wary however, as ergot poisoning (also known as ergotism) has been seen en masse in both humans and livestock[10]. This has typically been due to infection of rye or other grain by *Claviceps purpurea*, but certain types of ergot poisoning known as fescue toxicosis and ryegrass stagger are seen in livestock without being connected to ergot infected grain[11]. It was found that many monocotyledonous plants contained ergot alkaloids, all of which are in the order *Poales*. This is puzzling at first, but upon closer inspection it was found that the grasses that the livestock were grazing on (*Festuca arundinacea* and *Lolium perenne*) contained endophytic fungi related to ergot that resulted in fescue toxicosis and

ryegrass stagger respectively[12]. Endophytes are fungi and bacteria that live within plant tissues without causing pathogenicity, and while these can change from mutualistic or ommensal relationships to pathogenic or parasitic relationships, this is heavily dependent on environmental factors. These fungi have been studied quite extensively as the ergot alkaloids often occur in grass species that livestock might graze on, thereby reducing weight gain and fertility, and causing hyperthermia, convulsions, gangrene of the extremities and possibly death[14]. These fungi are in the genus *Epichloë* (with asexual forms being called *Neotyphodium spp.*), and range from antagonistic fungi that infect new hosts to mutualistic endophytic fungi that are vertically transmitted from parent plant to the seed. The benefit for the plant of passing on these mutualistic endophytic fungi seems to be increased resistance to a wide range of herbivores (due to the wide range of ergot alkaloids), increased drought tolerance and, potentially, improved growth and nutrient acquisition[15]. However, the presence of these endophytic fungi in the same family as ergot gives indication that there may be similar endophytic species living in the Morning Glory family, resulting in these alkaloids found in some of the species.

Ergot Alkaloids in Plants; A Fungal Symbiosis

In 2011, a new fungal genus was discovered on the *Convolvulaceae*, growing as an epibiont on the leaf surface of young leaves[16]. This genus had two species isolated from *Ipomoea asarifolia* and *Turbina corymbosa*, and were called *Periglandula Ipomoeae* and *Periglandula turbinae* after their respective hosts. These fungi were seen to associate with the secretory oil glands on the upper leaf surface of the young leaves. Through various fungicide treatments and growth trials, it was observed that these epibiotic fungi were needed for the production of ergot alkaloids, but that they contained very little of the alkaloids themselves[17]. Despite the absence of ergot alkaloids, it seems the fungus contained the gene responsible for the production of the alkaloids, indicating that there must be some sort of metabolic communication between the fungus and its host in terms of producing these alkaloids[18]. The fact that the seeds of these species, *T. corymbosa* in particular, have been used ritualistically for their alkaloid content raises the question of whether alkaloids may have been translocated to the seeds to increase their concentration, and how the fungus itself might be passed on via the seed if it has not been observed to penetrate the plant tissue.

It has been shown that the synthesis of the ergot alkaloids is in the leaves through grafting experiments between plants that are known to be producers of the alkaloids and plants that are known to be devoid of them (*I. tricolor* and *I. nil* respectively)[19]. This means that, once the alkaloids have been produced, they are translocated to the seeds, where they are far more concentrated than in any other tissue of the alkaloid containing species. This allocation of alkaloids and other bioprotective compounds to the seed of a plant has been hypothesised to be because it confers a significant fitness advantage to the survival of the seed and seedling, as it deters

herbivores from damaging and consuming the plant when it is most vulnerable[20]. The question of transmission of the fungal symbiont is important too, as it has been shown to be vertically transmitted to the seed from the parent, which is thought to be indicative of its adaptive value to the plant as it would otherwise not persist[21]. To date, there has been little research done on the exact transmission of Periglandula from parent plant to seed, and how an apparently epibiotic fungus never observed to penetrate the leaf surface could be transmitted to seed.

More recently, in 2015, it was found that eight additional *Convolvulaceae* species that produced ergot alkaloids contained members of *Periglandula*, and that these were a monophyletic group with similar chemotaxonomy[22]. What is interesting is that this study was the first to find *Periglandula* in species from Asia, Australia, Africa and North America, with some hosts being vines, some shrubs and some even from outside of the tropics. It was observed that, whenever ergot alkaloids were present, there was a species of *Periglandula* that was associated with the host plant and vice-versa. This indicates that the 23 unambiguously ergot alkaloid positive species noted by Eich are, with an overwhelming likelihood, also associated with a species of *Periglandula*, or at least a related fungus. It has however been noted that the species *Ipomoea tricolor* does not exhibit the epibiotic mycelium on the upper leaf surface as is characteristic of the genus *Periglandula*, despite being amongst the most widely used species for its alkaloids[23]. This further complicates the relationship this plant family has with these fungi, as it doesn't seem that all the symbioses behave in the same manner, but that there can be similarity between species from vastly different geographic regions and of different life forms. This may also indicate that rather than just having epibiotic *Periglandula* species, the Morning Glories containing ergot alkaloids may form symbioses with endophytic *Clavicipitaceous* fungi similar to the *Epichloë* and *Neotyphodium spp.* found in many grass species. This may be problematic, as the fungi in Morning Glories have been near impossible to culture in the lab, making identification of endophytes difficult. Considering the global distribution of the *Convolvulaceae* and their disjunct distribution, phylogenetic analyses of both the host and fungus may provide new insight into the evolution of the plants[24]. One species (*Ipomoea pes-caprae*) having been noted to contain ergot alkaloids shows a pantropical distribution, and the identification and mapping of the potentially associated fungus may provide some interesting new insights to diversification of these fungi in relation to their hosts.

Future Research

Considering that the *Ipomoeas* alone are estimated to contain between 600-700 species[25], one can imagine that many more *Clavicipitaceous* fungi are in symbiosis with these plants, and that using alkaloid production is a good proxy of these symbioses. There may even be complicated relationships seen between the associated fungi and their hosts, leading to fungal hybridisation and diversification of alkaloids as seen with the in grasses[26]. To date, *Periglandula* species have not been cultured in

vitro with great success, but culturing in vitro in association with plant cell culture has been shown to be somewhat successful[16]. This process is quite labour intensive however, and while hyphae were observed in the cell culture, ergot alkaloids were only observed once cell differentiation had been induced into the plant cells[17]. This indicates high specialisation and further metabolic communication between the fungus and its host that needs to be explored.

It seems that in the future it would be useful to assess more of the species within the *Convolvulaceae*, in particular in the *Ipomoea* tribe, for their alkaloid content. This may allow for the mapping of the alkaloid profiles to the host taxonomically, potentially giving us some indication of the evolutionary past of this symbiotic relationship. Following from this, one could screen the species with these alkaloids for ergot alkaloid producing fungi, as it has been seen that alkaloids have always been associated with a *Clavicipitaceous* fungus. These fungi could be epibiotic or endophytic, and may associate with various plant structures, but it seems that vertical transmission through the seed is typical, indicating that further inspection of the seeds of Morning Glories may be of use. This would give high priority to determining the exact mode of fungal transmission from the host to the seed too, as it is still unknown how these fungi exactly interact with their host plants to allow for vertical transmission from host to seed. Furthermore, it was through chemotaxonomic investigation that *Argyreia nervosa* was shown to contain enormous concentrations of ergot alkaloids when compared to species of *Ipomoea*, and through further investigation of these host plant species we may discover species with an even higher concentration or even novel ergot/indole alkaloids. In 2010 there was 20,000 kg of ergot alkaloids produced, half of which was produced via growing of grain and infecting it in the field, and half of which was produced via fermentation in bioreactors[27]. Elucidation of novel alkaloids in some Morning Glory species, and potentially even highly productive fungi may pave the way for new medicines and more efficient production of these alkaloids, and with over 80 known ergot alkaloids already being known it is likely that further bioactive alkaloids exist that could be of tremendous use to modern medicine[28].

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Terra Nova: Subglacial Volcanism and the West Antarctic Ice Sheet

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The integrity of the West Antarctic Ice Sheet (WAIS) is one of the most significant factors contributing to the rise in sea levels predicted by most climate change models. It is the last marine-based ice sheet in existence and is fragile. It sits in a geologic basin fringed by fracturing ice shelves, atop a divergent continental rift system. The West Antarctic Rift System (WARS) affects the stability of the WAIS through geothermal flux and subglacial volcanism.

To this point, most studies of the WAIS have been unaware of the presence, activity and effects of subglacial volcanism on West Antarctic ice flow due to the complications of studying the underlying terrain through kilometers of ice. As a result, most climate change models do not account for the effects of subglacial volcanism on ice volume or basal melting.

This paper seeks to consider the evidence regarding West Antarctic subglacial volcanism and the effect of geothermal flux on basal melting, glacier flow, isostatic depression and the overall integrity of the WAIS, with a particular emphasis on the consequences for climate change.

Introduction

West Antarctica is a focus of global-warming anxiety, heroic romanticism, and diverse geophysical, biological and chemical research. An area of 1.97 x 106 km2 west of the Transantarctic Mountains, West Antarctica and what lies below its ice is an evolving mystery with consequences for climate change, biodiversity and oceanography[1].

This paper considers the dynamics of the West Antarctic Ice Sheet (WAIS), which

covers approximately 97% of West Antarctica and drains into the Weddell, Bellinshausen, Amundsen and Ross Seas[1]. Beneath the WAIS is the West Antarctic Rift System (WARS), a volcanic rift system active for 35 Ma, running from the Transantarctic Mountains to Marie Byrd Land, and from North Victoria Land to the base of the Antarctic Peninsula[2,3].

West Antarctica

West Antarctica differs notably from East Antarctica by its elevation; while much of the bed of the East Antarctic Ice Sheet (EAIS) is above sea level, the WAIS bed is up to 2km below sea level in the Ross and Weddell embayments[3,4]. Without the unique features of the geologic basin it sits in, which traps ice between uplifted mountains and plateaus but allows drainage to the ocean through the shallow Ross and Weddell embayments, the WAIS would likely never have formed[3]. Geologically, West Antarctica is significantly less stable than East Antarctica, a Precambrian craton at least 500 Ma (million years) old[1,5]. The WAIS lies upon a puzzle of at least 4 major crustal blocks, relics of the breakup of Gondwanaland: an area of thin volcanically active continental crust with ongoing fracturing and magmatic pluming, a Southern border to the circum-Pacific 'Ring of Fire'[1-3].

Dissimiliarities exist between the WAIS and the EAIS as well[1]. The last ice sheet of its type, a marine-based ice sheet in a geologic basin, the WAIS is over 2 km thick in many places but averages 1.0 – 1.3 km[1,3,4,6,7]. The weight of the ice has caused isostatic depression of 0.5 km in West Antarctica[4]. In contrast, though the EAIS displays greater isostatic depression (1.0 km) and greater maximal thickness (4.7 km), the average elevation of the EAIS is significantly higher than that of the WAIS[1,4,7,8]. The shapes of the ice sheets reflect their basal geologic dissimilarity and differences in ice flow: the EAIS has a vaguely domed shape, flatter in the interior and sloping steeply down towards the coast; whereas the WAIS is more fragile with a shallower, convex-up centre, which changes to concave-up and flattens towards the margins[1,9].

Figure 1: A map of Antarctica. *Figure adapted from L.Ivanov (51)*

Volcanic Features

Studies have identified 138 volcanoes throughout West Antarctica, principally aligned along the main axis of the WARS[3,10]. Many of these volcano identifications are on the basis of bed-elevation data from the BEDMAP2 digital elevation model (DEM), as two-thirds of the volcanoes of the WARS do not protrude above the surface of the ice[10,11]. The volcanic cones extracted from BEDMAP2 data in van Wyk de Vries *et al.* are identified on the basis of height (at least 100 m prominence from the surrounding terrain) and ratio of width to length (< 1.5), and are consistent with shield volcano morphology[10]. More may exist, as the ice cover has prevented a comprehensive survey and the BEDMAP2 DEM is significantly limited in its ability to capture granular data[4,10]. Additonally, volcanic features that are less topographically prominent, such as tindar (glaciovolcanic tephra ridges formed by fissure eruptions), or are older and thus more eroded by glacial flow, may have been missed by studies to this point due to the difficulty of mapping and conclusively identifying such features under the ice[10,12,13].

Only two volcanoes are definitively known to be currently active in Antarctica, because their eruptions were visible above the ice: Mount Erebus and Deception Island[2,10,14]. Mount Erebus lies atop the mantle plume that formed Ross Island; the

island is volcanic, comprised of Erebus, the dormant Mounts Terror and Bird and a number of smaller peaks and craters[2]. Erebus is one of only a few volcanoes with a lava lake open to the sky and has erupted continuously since 1972, ejecting lava bombs and exploding massive gas slugs[2,10,15]. While Erebus has a magma chamber of undetermined volume 500 m below the surface, it is releasing CO_2 from the mantle, more than 16 km below the surface[15]. Deception Island, a collapsed caldera, produced by crustal rifting in the South Shetland Islands, has erupted regularly for centuries, most recently in 1967-70[14,16,52]. The 1969 subglacial fissure eruption destroyed the British and Chilean observatories with pyroclastic material and meltwater flooding[14,16].

According to geologists, both previous studies and recent seismic activity have suggested that Marie Byrd Land, where many of the newly-discovered cones are concentrated, may also be another currently active subglacial volcanic hotspot[3,10,17]. Twenty-nine of the cones identified by van Wyk de Vries *et al*. in Marie Byrd Land exceed 1 km in prominence[10]. Marie Byrd Land itself may be uplifted mostly or entirely due to magmatic plumes, the result of volcanism beginning approximately 35 Ma ago[3,10,18].

Analogous Rift Systems

Analogous volcanic rift systems and areas of stretched continental crust can provide clues to the behavior of crustal rifts and volcanoes underlying the WAIS. Comparably-sized regions with similar traits include the East African Rift System and the Basin and Range Province of the western USA and Mexico[1]. The East African Rift System is a collection of seismically-active rift valleys along more than 6300 kilometers of the splitting African Plate, running south from the Afar Triangle (Eritrea, Djibouti, Ethiopia) to the North-Tanzanian divergence (Kenya, Tanzania) and from Lake Albert (Uganda, Democratic Republic of the Congo) to Lake Malawi (Malawi, Tanzania, Mozambique). It includes most of the major lakes of East Africa, as well as a majority of the elevation over 1200m in Africa along the NNE-oriented continental ridge[19]. The Basin and Range Province is an area of relatively thin crust, up to 1000 kilometers wide, over a system of extensional faults stretching from Idaho to Mexico and covering most of Utah and Nevada[20,21]. Both the East African Rift System and the Basin and Range Province are associated with thin extended crust, high heat flux, and a high degree of seismic activity and volcanism[19,21]. Like the East African Rift System, the observed volcanism of the WARS is also aligned along the rift axes[10].

In other major rift systems, stretched crust is associated with increased volcanism, but due to the difficulties of studying the lithosphere beneath the thick Antarctic ice, the volcanic activity of the WAIS is comparatively little-known[10]. The glaciovolcanic nature of the WARS can be understood through the better-studied glaciovolcanism of Iceland. While the WAIS is much thicker than Iceland's ice caps,

the volcanism of the WARS may have a similar impact on the dynamics of the WAIS as the volcanoes of Iceland do on Icelandic glaciers[1]. Common factors include the impact of heat flux on basal melting; the effect of basal melting on glacial flow; subglacial lakes and drainage; and *jökulhlaups* (glacial outburst flooding as the result of geothermal heating or subglacial volcanic eruptions)[12,18,22-27].

Impact of Subglacial Volcanism

Movement of ice sheets in Antarctica is dominated by basal-slip motion, counter to the standard model of ice sheet flow, which assumes motion principally driven by internal deformation and disregards the impact of coastal regions[23]. Basal slip may be a product of subglacial topography or the weight of the ice itself, both of which generate friction; the sediments and qualities of the subglacial bed; and geothermal flux and subglacial eruptions, which directly contribute to basal melting[9,10,12,23,28,29].

Subglacial eruptions and increased geothermal flux not only affect ice flow through basal melting, but also have a first-order impact on ice volume[11,22,29,30]. While basal melt and glacial lubrication contribute to the destabilisation of the WAIS and to the risk of major breakups and loss of ice, subglacial volcanism directly melts ice, which flows through subglacial drainage systems directly into the Southern Ocean.

Rapid unloading of ice over a spreading rift can cause long-term volcanic consequences as well[31]. Increased melt causes decompression, which speeds magma to the surface, increasing the frequency of eruptions; for example, Iceland shows increased eruptions that coincided with the deglaciation at the end of the Last Glacial Maximum[31,32]. Besides the direct impact of eruptions on ice and volcanic aerosols on the atmosphere, ash and other eruptive material that makes it to the surface can affect the ice-albedo feedback, altering insolation and accelerating ice melt further[9,29,32].

The melting of the WAIS as a result of climate warming may trigger increased volcanic activity, causing in turn a devastating feedback loop. Isostatic rebound – land masses 'bouncing back' from glacial compression – reduces pressure on magma, causing more eruptions and increased subglacial heat[29,33]. This melts ice at the bottom of glaciers, contributing directly to ice loss and increasing its speed as it flows into the ocean, contributing to isostatic rebound[34].

However, a volcanic system could actually be beneficial in the fight against ice loss and thus climate change. Volcanic deposits form subglacial 'protuberances,' slowing down ice flow and retreat by form drag and rough basal texture[10,28]. The melting of the WAIS itself could also contribute obliquely to combating climate change, through a complex ecological scheme which studies suggest contributed to the Last Glacial Maximum: iron-rich Antarctic meltwater feeds phytoplankton (photosynthetic algae) near the ocean's surface, which consume large amounts of CO_2[35]. Eventually the phytoplankton die, sinking to the bottom of the ocean

and sequestering carbon there[35,36]. It is not currently known to what degree phytoplankton might offset atmospheric CO_2. Other confounding factors in studies include air-sea CO_2 flux, particularly in the under-saturated Southern Ocean, and mid-oceanic rifts, which release CO_2 from deep within the mantle through volcanic eruptions and also cause it to be sequestered in carbonates in the newly-formed ocean floor[37-40]. This subduction of ocean crust is currently a net carbon sink, sequestering 1.8 – 2.9 x 1010 kg C per year, but its value in slowing climate change due to atmospheric CO_2 is limited, as this value is greatly below the human-driven emission rate of 8.7 – 9.8 x 1012 kg C per year and complete precipitation of carbonates in new crust can take a few tens of millions to 100 million years[37-43].

Discussion and Conclusions

The stability of the WAIS has serious consequences for global sea levels[44]. Marine-based ice sheets like the WAIS are regarded as the most prone to rapid ice loss[45]. West Antarctica is currently contributing about 10 percent of the annual global sea level rise but the WAIS has enough water to raise global sea levels by 3-6 m[1,6,44,46]. The effects of rising oceans with storm surge currently threaten 270 million people globally[44]. A 1 m rise in sea levels, consistent with projections for climate change by 2100, would threaten 450 – 670 million people with storm surge, exacerbated by increasingly-strong tropical storms, displace 145-300 million people from coastal areas and inundate 2.2 x 106 km2 of coastline[34,44,47].

Despite the identification of conical edifices, the volcanoes underlying the WAIS may or may not be active regardless of extensional rift activity[10,17,48]. High regional geothermal heat flux and evidence of subglacial volcanism suggests current activity; however, the unusually low elevation of the WARS and the low quantity of basalt tephra recovered in the area suggest an inactive rift[22,30,10]. Although eruptions may not be currently occuring, heat flux through the thin crust can still have an effect on basal melting[30]. The difficulty in studying the edifices and beds underlying the WAIS make it difficult to evaluate to what degree subglacial volcanism is affecting the WAIS now.

Similarly, without finer-grained and more conclusive data on the activity of the WARS and its effect on the stability of the WAIS, it is difficult to correct models for ice dynamics and ice loss[34]. Chris Rapley, head of the British Antarctic Survey notes, "Current computer models do not include the effect of liquid water on ice sheet sliding and flow," which means the impact of basal melting is likely to exceed current predictions of ice loss[49]. The geothermal heat flux from the WARS contributes to the basal melt and high speed of ice streams flowing into the Ross and Weddell Seas[9]. Also confounding attempts at modelling is that, over time, the various ice streams of the WAIS have altered course and speed: one stream feeding the Ross Ice Shelf suddenly stopped flowing 150 years ago and others have been slowing[9]. On the human scale of time, sediment and water saturation are the major

forces affecting the flow of ice; in the long-term, global climate is[9]. BEDMAP2 has improved our ability to identify probable volcanic edifices, but additional seismic, radar and satellite data is absolutely critical to understanding the terra nova beneath the West Antarctic ice[50].

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Chemistry

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Letter from the Editor

Chemistry is known for being a very broad area of study and often is referred to as the central science. This volume of the Trinity Student Scientific Review echoes this thought. The reviews published tru-ly show how current chemical research is based on improving the world around us and the lives of people in it, from finding new can-cer therapies and technologies to reduce our dependence on fossil fuels, from personal to global issues. This volume's reviews include a discussion of the advances made to chemical protein synthesis via native chemical ligation. This area of study is attracting a huge amount of interest to further improve our methods of synthesising proteins and to deepen our understanding of their function and structure. In addition, the advantages and limitations of Perovskite Solar Cells are discussed. These have the potential to make ener-gy production more sustainable and efficient. Aggregation Induced Emission Nanoparticles and Antibody-Drug Conjugates are also re-viewed in this volume. These are new cancer detectors and therapies which have the potential to show reduced toxicity and improved tumour targeting specificity when compared to many current cancer treatments.

The TSSR could not happen without the support of many people within the college community. I would like to extend my thanks to Dr. Mike Southern, the chemistry academic advisor who is always extremely supportive of the journal and generous with his time and support. I would also like to thank all the peer reviewers, Dr. Eoin Scanlan, Dr. Max Garcia-Melchor, Dr. Richard Hobbs and Mr. John J. Magan for the time and effort they gave to ensure the high standard of scientific rigour that is seen in the TSSR each year. Their support of this publication is appreciated by both the authors and the editorial team.

Finally, I would like to thank all the students who submitted to the Chemistry Section of the journal this year. Every year, students take time on top of their coursework to write an article on a topic that they find interesting. This year the standard of articles was very impressive. The TSSR hopes to encourage all undergraduates to think beyond their course and to think independently about ongoing re-

search. I believe the following reviews will show that we have succeeded our goal and display the diligence and talent of undergraduate chemistry students in the Trinity College. These students, from a range of courses, Nanoscience, Chemistry and Medicinal Chemistry, show exceptional drive and enthusiasm when it comes to scientific research. This volume of the TSSR is a great representation of the capabilities of students in Trinity College.

Eleanor Windle
Chemistry Editor
Trinty Student Scientific Review 2018

Anitibody-drug Conjugates: The Future of Cancer Therapies?

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Medicinal Chemistry

Cancer, with millions of cases reported and millions of deaths attributed to it each year, is one of the worst diseases humanity has ever had to face. Despite the massive amounts of funding and man hours invested in the pursuit of treatments for the various types of cancer, in the UK alone someone dies as a result every four minutes.[1] Although somewhat limited, progress in this field has been made, with many cancers having some form of treatment available, whether that be curative or other. Antibody-Drug conjugates (ADCs) are options for cancer treatment which have been explored for decades, but only recently reached a point where approval for clinical implementation is being achieved. ADCs encompass a powerful anti-cancer drug linked to a large protein known as an antibody. These antibodies bind specifically to other proteins on the surface of cancerous cells and, in combination with the linker, provide the means for the attached drug to be released into the cell and exert its effect. The specific nature of the antibody component is what gives ADCs an advantage over classical examples of chemotherapy drugs, where nearly as many non-cancerous cells are killed as are cancerous ones. The reduction in "innocent" cell death while still effectively treating the disease is what makes this such a conceptually attractive idea. Although, as will be discussed, there are still some large problems to overcome with regard to the practical application and also the production of these compounds.

Introduction

The magic bullet concept, as proposed by Paul Ehrlich,[2] is based on the idea of targeted treatment of disease. One of the largest problems faced in the treatment of cancer is the systemic toxicity caused by many of the most common forms of treatment, for example chemotherapy.[3] This has made the development of safer, more focussed cancer therapeutics one of the major challenges of modern medicine.[4]

It is currently the opinion of many, that antibody-drug conjugates (ADCs) may be the therapeutic agents to bring Ehrlich's vision to fruition. Three such compounds have recently been approved by the Federal Drug Administration (FDA), Kadcyla®, Adcetris®[4] and Besponsa®. The most recent of which, Besponsa, approved in 2017, came after almost a 5 year drought where, despite masses of research and funding, no new ADCs made it to the market.

ADCs are comprised of a monoclonal antibody bound, via a cleavable or non-cleavable linker, to a cytotoxic drug.[4] Antibodies bind specifically to proteins expressed by cells. The intention is, through the specificity of the antibody, a payload of anticancer drug can be delivered solely, or mostly, to malignant cells. This would ideally reduce systemic toxicity to the patient and increase the therapeutic index of the drug,[2] all while still effectively combating the disease. Although conceptually simplistic, the implementation of such a concept had eluded researchers for quite some time. As mentioned, only three ADCs are currently on the market, the first (BV) only since 2011,[5] despite decades of research.[4]

There are many parameters which must be considered when designing an ADC. These include selection of linker, drug and antibody; the selection of the site of conjugation; the expression profile of the target receptor and many more.[5] The early failures of ADCs can be attributed to poor parameter selection and, to a certain extent, lack of technology. The development of Monoclonal antibodies, and, hence, more feasible tumour targeting, by Köhler and Milstein[6] brought with it the founding of ADC programmes by many pharmaceutical companies.[7] The prospects for ADCs today are extremely positive with many currently in phase I, II and III clinical trials.[8]

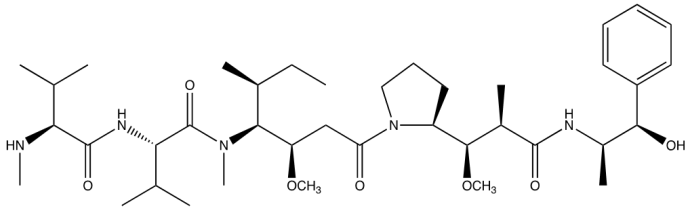
This literature review will discuss four areas surrounding antibody-drug conjugates, using Seattle genetics' ADC Adcetris®. Adcetris®, also known as Brentuximab Vedotin (BV), is used to treat relapsed Hodgkin's lymphoma and anaplastic large cell lymphoma. The areas to be discussed are: the selection of the antibody, drug and linker; the mechanism of action; the large scale production of ADCs and the advantages and disadvantages of ADCs. Furthermore, the extent to which the emergence of ADCs as anticancer therapeutics has revolutionised the field of cancer therapy will be evaluated.

Selection of Drug, Antibody and Linker

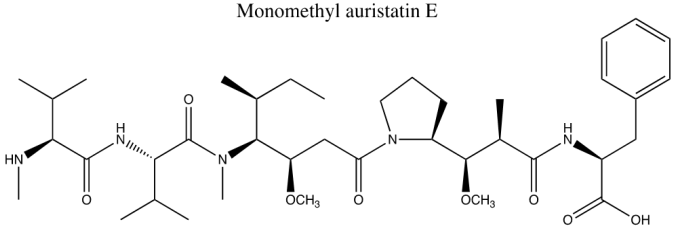
These are, arguably, the most essential parameters of the ones mentioned above and also for ADCs in general. Each part of an ADC must meet certain requirements to ensure the safety of the patient and also the efficacy of the drug itself. Slight changes in any one could result in entirely different pharmacodynamic and pharmacokinetic properties, as well as changes in the toxicity, efficacy and other properties of the drug. All three parts are of equal importance.

ADCs use highly cytotoxic drugs, which are capable of disrupting cellular function to the point of cell death. Drugs viable for incorporation to an ADC must satisfy three main parameters: the drug must have a strong cell toxicity; it must possess the appropriate modified site for conjugation to the antibody and finally, it must have a defined mechanism of action.[8] Broadly speaking, there are two main categories of anti-cancer drug: DNA and RNA targeting drugs and microtubule-active agents.[8] Of the three ADCs approved by the FDA, Adcetris® and Kadcyla® use tubulin-disrupting agents and Besponsa® uses a DNA-targeting drug. ADCs are designed to mitigate the damage caused to non-cancerous tissue, this permits the use of drugs several orders of magnitude more potent than other less specific chemotherapeutic treatments.[9] The systemic toxicity these drugs would cause without this selectivity would make them far too dangerous to use on patients alone.

Monomethyl auristatin E (MMAE) is the tubulin-disrupting drug used in Adcetris®. As indicated by its name, MMAE falls into the auristatin family of synthetic analogues of Dolastatin 10 - an antineoplastic, naturally occurring compound.[10,11] MMAE is a pentapeptide composed of unnatural amino acids, which, along with MMAF, was developed in an attempt to widen the therapeutic window of Dolastatin 10, in order to achieve the optimum activity for use in an ADC.[12] As a tubulin-disrupting drug, its function is to bind and inhibit the polymerisation of microtubules in cells.[13] Many cellular functions rely on the viability of these structures and apoptosis can be induced in the afflicted cell as a result of such a disruption.



Monomethyl auristatin E



Monomethyl auristatin F

Figure 1- MMAE and MMAF; synthetic analogues of Dalastatin 10.[14]

Humanized monoclonal antibodies, are compounds which bind specifically to proteins, known as receptors, expressed on the surface of cells.[15] They are produced synthetically by combining monoclonal antibodies harvested from mammalian cells (Chinese hamster ovaries in the case of Adcetris®)[16] with a small part of a human antibody.[17] Antibodies are what give ADCs their specificity and the criteria they must meet is rigorous. The selected antibody must preferentially target cancer cells through an overexpressed antigen, and have a high binding affinity for that antigen.[8] Once bound, the antibody must then be capable of inducing endocytosis.[8] Furthermore, the antibody must have low immunogenicity.[18] Finally, the conjugation of the antibody to the drug must cause no change to the activity of the drug or the affinity of the antibody for the antigen.[18] BV uses the cAC10 anti-CD30 antibody, which binds selectively to the CD30 antigen.[16]

The linker, which conjugates the antibody to the drug, has the largest effect on the pharmacokinetic and pharmacodynamic properties of the ADC.[19] The linker must be stable in blood plasma conditions for long enough to allow it to arrive, intact, to the desired cell, but release the drug once inside.[8] These requirements must be met in order to maintain the efficacy of the ADC.[20] Adcetris® uses a valine-citrulline dipeptide linker which is joined to MMAE through an aminobenzyloxycarbonyl spacer at the N-terminus.[7] Acid labile and disulphide linkers were also considered, but the propensity ADCs have for journeying through lysosomes (which contain high concentrations of protease enzymes) led to selection of a peptide linker instead.[7] Another reason for this selection is the stability of this type of linker in the absence of protease enzymes. After ten days of incubation in human plasma, it was found that only 2% of the drug is released.[21]

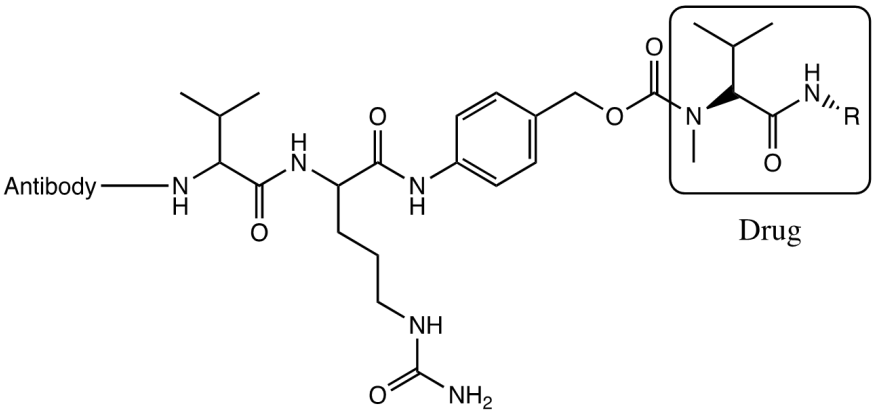


Figure 2: Valine-Citruline dipeptide linker and the aminobenzoyloxycarbonyl spacer.

In summary, BV combines the highly potent tubulin-disrupter monomethyl auristatin E with the cAC10 CD30-binding antibody via a protease-sensitive cleavable Val-Cit dipeptide linker. This can be represented schematically as follows:

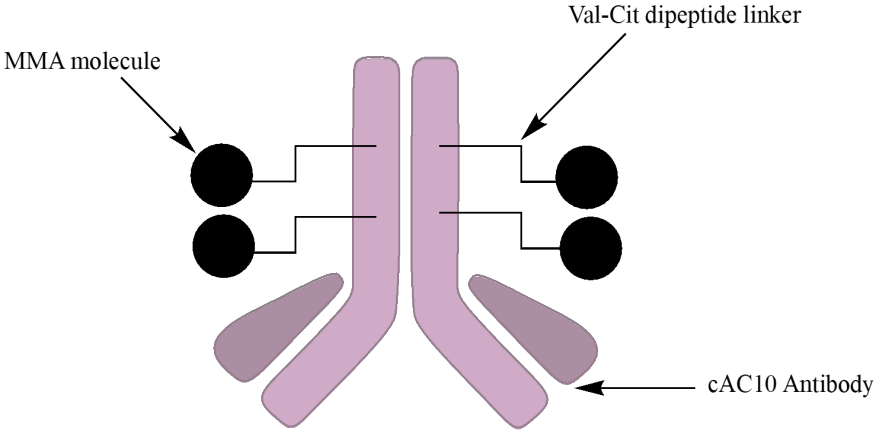


Figure 3: Schematic representation of Adcetris®[19].

Mechanism

The multistep process[21] which constitutes the pharmacological activity of BV can be summarised into three main stages: 1. Attachment and uptake, 2. Drug release and 3. Drug action. As previously stated, the key objective of an ADC is to eradicate cancer cells while leaving normal tissue largely unaffected.[11] This makes knowledge of the mechanism of action of paramount importance. The general mechanism for any ADC follows the steps outlined above. Firstly, the antibody binds its receptor on the cell surface. Following this, endocytosis is induced and the whole ADC is taken into an endosome. The drug is released into the cytosol by one of two methods[8] and allowed to exert it's action. The cell dies as a result of this action.

Attachment and uptake is mediated by the antibody component of the compound.[22] In the case of Adcetris®, the antibody binds the CD30 antigen, which is part of the Tumour Necrosis Factor (TNF) receptor family and is overexpressed in anaplastic large cell lymphoma and Hodgkin's disease.[22] More importantly, expression of CD30 does not appear on any other healthy human tissue outside of the immune system.[22] In line with this proposed mechanism, control studies showed no binding to CD30 negative cells.[22]

Binding to the receptor causes a biological cascade reaction, resulting in receptor-mediated endocytosis of the ADC.[23] Once inside the endosome or lysosome, either the linker or the antibody itself is digested.[8] In the case of non-cleavable linkers, the antibody is enzymatically broken down and the drug is released into the cytosol.[8] In the case of cleavable linkers, as used in Adcetris®, the linker is broken down as a result of the intracellular environment it has been taken up into.[24] Non-

covalent interactions between protease enzymes, such as cathespin B, promote nucleophilic attack of water at the carbonyl carbon, resulting hydrolysis of the peptide bond.[12] Self-immolation (self-degradation by resonance) of the subsequent para-aminobenzyl species (the spacer) releases the active MMAE molecule [Figure 4].[12] Adcetris® takes advantage of the ability of protease enzymes to recognise the dipeptide bond and, more importantly, their abundance in the cytosol of cells relative to the blood.8

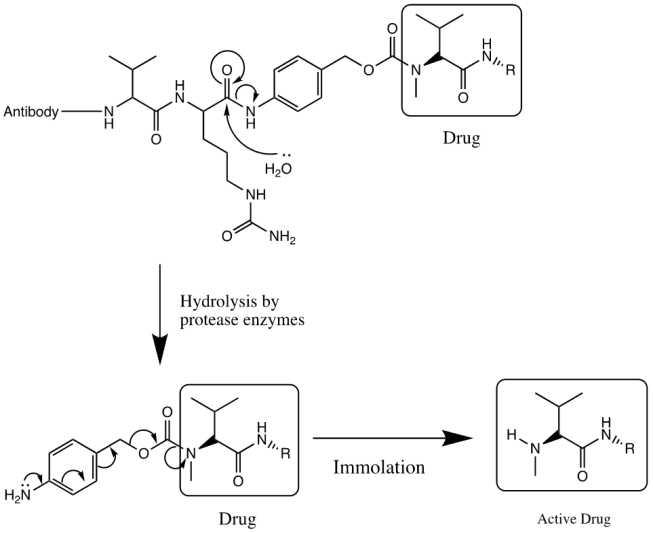


Figure 4: Mechanism of linker cleavage and release of active drug.

As stated, the two main categories of anti-cancer drug are DNA-disrupting drugs and Tubulin-disrupting drugs - MMAE is tubulin-disrupting. Eukaryotic cells all contain microtubules, which form part of the cytoskeleton and play an extremely important role in many cellular processes- one of those being mitotic spindle formation, a crucial aspect of cell division.[25] A disruption in the function of microtubules could interrupt this process and threaten the viability of the cell. Microtubules are comprised of polymerised heterodimeric α,β-tubulin subunits.[26] Their abundance is in equilibrium with depolymerised, or 'free' tubulin. MMAE reversibly binds 'free' tubulin at the β-subunit in a region known as the vinca-binding domain, through a number of non-covalent interactions.[26] This causes aggregation as opposed to polymerisation, the result of which is the formation of misshapen microtubules which are not viable.[25] The maintenance of the equilibrium is key for cell survival, and this sort of disruption often leads to cell death.[25]

Large-scale Production

While antibody-drug conjugates may offer a new and improved form of cancer treatment, the manufacturing of such compounds presents a new set of production challenges. The chemical synthesis, logistics of large-scale production and safety of the operating personnel are tasks which require the implementation of highly specialised, unique, and therefor costly, infrastructure. Furthermore, the installation of a diverse, multidisciplinary workforce is paramount to the production process.[27] Many operations involving ADC production are classified by SafeBridge® as containment category 4[28] - meaning exposure to the compounds being handled could result in death. Because of this, only a handful of contract manufacturing organisations (CMO's) have the facilities required to carry out the production of ADCs.[29] Aside from the actual dangers encountered on the production line there are other difficulties such as the raw material supply chain and analytical support for the process.

ADCs have an extremely elaborate raw material demand, significantly more than other biopharmaceuticals.[27] The sourcing of the antibody, linker and cytotoxic drug are often from multiple vendors and therefor a lot of coordination is needed. This results in an increase in total project hours and therefor overall cost of the operation.[27] As the antibodies are harvested from mammalian cells, extensive virus testing is required to ensure there is no carry through of any infective agents. This will also have implications on the purification process later down the line, as any microbes that are present will have to be removed.[27] The nature of the cytotoxic drug, whether it is fully or semi-synthetic, is also of relevance. There are a limited number of CMO's with the facilities to produce semi-synthetic drugs, meaning that availability of the drug itself can be a limiting factor on the rate of production.[27]

MMAE can be prepared by total synthesis,[30] which means functionality can be easily included or excluded from the molecule. The utility of this is exemplified by the inclusion of the terminal amine functionality which allows facile joining of the spacer and linker to the drug via a simple peptide bond forming, condensation reaction. Following modifications to the disulphide bonds that link the 'heavy' and 'light' chains of the antibody, it can be covalently bound to the linker-spacer-drug complex.[7] These alterations have an added bonus, as inter-chain disulphide bridges are prone to metabolic reduction,[31] additionally the activity of the antibody is not affected.[32] Each antibody is conjugated to either 2, 4 or 6 MMAE molecules[4] (with an average of 4[7]). Irrespective of its heterogeneity, Adcetris® performed excellently in phase II clinical trials for both the cancers it is used to treat, and as a result experienced accelerated approval by the FDA.[7]

One of the most demanding component of the large scale production of ADCs is the analytical support required. This aspect is particularly taxing as it requires multi-disciplinary instruments and employees, due to the fact that both small and large molecules need to be analysed.[27] Analytical equipment is necessary not

only to validate the product, but also to ensure the safety of the employees on the production line. As a consequence of the potency of the drugs employed, devices capable of detecting between 10^{-9} and 10^{-12} grams of toxic substances are necessary to ensure the workers are not exposed to dangerous amounts of the drug.[27]

All of the above must be considered when designing a process which culminates in the output of the desired product with the required purity, all while preventing contamination of the workers or the general environment all at a cost-effective price.[29]

Advantages and Disadvantages

This targeted approach of cancer treatment brings with it some enormous advantages, but also a few disadvantages. The advantages have been mentioned several times through this review in all of the various sections. The disadvantages come mainly from a logistics perspective, as there are very few with regards to the theory of antibody-drug conjugates as anticancer agents.

One of the main issues arising with this line of treatment is the unwanted immunogenicity associated with biotherapeutics. Prevention of this is attempted by "humanizing" the harvested monoclonal antibodies.[33] The complexity of ADCs mean that, despite this, and several risk assessment strategies, many patients do develop an immune response to the drug.[34] One method of qualitatively analysing an immune response in the host for a particular drug is done through measuring the incidence of antidrug antibodies (ADA's). Although limited clinical data is available, these show that ADA prevalence for Adcetris® is approximately 37% compared to approximately 5.3% in Kadcyla®.[34]

Other disadvantages of ADCs include the price of production of such compounds, which is substantial,[27] and, because of their complex nature, the actual desired product can be difficult to obtain from a synthetic standpoint.[4] The limited stoichiometric control we currently have over the outcome of the product brings with it many other issues from a medicinal chemistry standpoint[35]: Optimisation of a drug relies on consistent synthesis of a product with a defined chemical structure, thereby allowing analysis of the activity of the compound after the alteration of functional groups, etc. relative to the original molecule. This is crucial when attempting to improve the properties of a drug.[35] As mentioned in the last section, the safety of those on the production line is of great concern and must be considered a disadvantage if ADCs are going to be widely available to the public.

The advantages to antibody-drug conjugates are copious, and the improvement in patient care they potentially bring with them, make the disadvantages mentioned above seem almost trivial. This kind of treatment could bring an end to the horrific side effects associated with conventional cancer regimens and cause a notable decrease in the cancer mortality rate. Adcetris® is the first ever approved treatment

for anaplastic large cell lymphoma and the first for Hodgkin's disease in over three decades.[21]

Conclusion

Although much progress has been made in this field there is a lot still to be done. While approved drugs like Adcetris®, Besponsa® and Kadcyla® show the potential ADCs have, examples such as Pfizer's drug Mylotarg® being removed from the market in 2010[36] serve as a reminder of the progress yet to be made. It is still fair to say, based on the core concept alone, that antibody-drug conjugates have revolutionised the field of cancer therapy, and with the number of ADCs currently in the pipeline, the prospects for this field of treatment appear to be very promising.

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Aggregation Induced Emission Nanoparticles for Cancer Detection and Therapy

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Chemistry

Fluorescent nanoparticles (FNPs) are emerging as efficient bioprobes for cancer resulting from their ability to target tumours both passively and actively using the enhanced permeability and retention effect and surface functionalised tumour targeted ligands. However, FNPs suffer from a aggregation-caused quenching of their emission at high concentrations, limiting their in vivo applications. Aggregation-induced emission (AIE) is a phenomenon through which fluorophores are weakly emitting in dilute solutions but become strongly emitting in concentrated solutions and in solid state. This is the inverse of the aggregation-caused quenching that occurs with most conventional fluorophores. Molecules that display AIE have their non-radiative pathways removed upon aggregation, resulting in their enhanced fluorescence. By developing FNPs that incorporate AIE, powerful systems for cancer detection and treatment can be developed. This review explores the application of AIE NPs in cancer diagnosis, treatment via drug delivery, and photodynamic therapy.

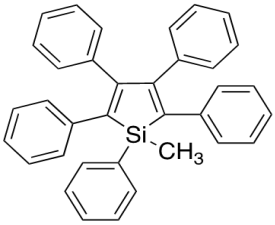
Introduction

In 2014, cancer accounted for 14.6% of total human deaths.[1] Early detection is crucial for effective treatment, but is extremely difficult as tumours must be large before they can be detected.[2] Ultrasound imaging does not provide high enough sensitivity, X-Ray and computed tomography (CT) scans expose patients to high risk radiation, and magnetic resonance imaging (MRI) scans are very expensive to run. Fluorophores activated using aggregation-induced emission can provide a solution to the issues preventing early cancer detection, with the advantages of low cost, high signal-to-noise ratio, and low toxicity.[2]

For many years, studies of fluorescent molecules were carried out in dilute

conditions allowing for the precise measurement of photophysical parameters without interaction of chromophores.[3] These molecules had the ability to luminesce in dilute solutions, but once the concentrations were increased, the luminescence could not be observed due to the formation of aggregates in solution. This effect is termed "Aggregation-Caused Quenching" (ACQ). Most fluorophores consist of planar aromatic rings, and it is these molecules that are most susceptible to ACQ. While the increased π-conjugation does lead to increased luminescence on a dilute scale, aromatic rings can undergo strong π-π stacking interactions when aggregates are formed at high concentrations. This leads to the formation of excimers that provide a non-luminescent pathway to relaxation, and thus, ACQ. This effect is extremely unfavourable to certain real-world applications. As a result, many attempts have been made to prevent the formation of aggregates, although this leads to many new problems and has achieved little success.[4]

In 2001, a silole derivative (Figure 1) was found to be weakly emitting in dilute solution and became strongly luminescent in concentrated solution and solid state.[5]



1-methyl-1,2,3,4,5-pentahenylsilole (**1**)

Figure 1: The silole derivative found to undergo AIE in 2001.

In contrast to traditional fluorophores, **1** is non-planar. It is believed that this deviation is what causes the unusual effect termed 'Aggregation-Induced Emission' (AIE), which has the inverse effect of ACQ. **1** contains 'propeller' like benzene rings which rotate in solution and undergo dynamic intermolecular rotations. These provide a non-radiative decay pathway for excited states in dilute solutions. Non-planar rings also prevent the molecules from undergoing π-π stacking interactions when it is forming aggregates in concentrated states. This, coupled with the propellers being unable to rotate due to the physical constraints, leaves no non-radiative pathway open, and leads to the emission of light from the molecule. It has been confirmed that this "Restriction of Intramolecular Rotation" (RIR) is the key to the AIE phenomenon.[6] Since the discovery of the silole derivative, many more fluorophores have been designed using RIR in molecules which undergo AIE, opening up a world of technological applications for the phenomenon.

Fluorescent nanoparticles are prime candidates for the future of cancer detection. Their nano size results in an enhanced permeability and retention (EPR) effect which

allows passive targeting of tumours.[7] Furthermore, fluorescent nanoparticles can be modified with tumour specific ligands to encourage binding on tumour cells, resulting in increased cellular uptake. However, these molecules commonly suffer from ACQ, preventing them from replacing traditional techniques. By developing fluorescent nanoparticles using AIEgen molecules (fluorophores with AIE effects), superior systems can be developed that do not suffer from ACQ.

Research has shown that AIE imaging systems show enhanced photo-bleaching resistance, high luminosity, large absorptivity, enhanced bio-compatibility, and are free from random blinking when compared to conventional organic dyes and quantum dots.[8] These attributes mean that AIE based nanoparticles can be used in a non-invasive, high contrast manner for cancer cell detection, long term cell tracing, and tumour imaging.[9]

AIE Nanoparticles for in vivo Cancer Diagnosis

Emission of fluorescence probes in the near-infrared (NIR) region (λ > 650 nm) is highly desired as light of this wavelength can penetrate biological tissue more effectively compared to visible light.[10] Therefore, for optimum in vivo fluorescence imaging, potential probes must fluoresce in the NIR as well as meet the following requirements: (i) optimum morphology, size, and surface chemistry to achieve the EPR effect, (ii) surface functionalisation for specific tumour targeting, and (iii) low cytotoxicity, and in vivo toxicity.

AIE functionalised nanoparticles (NPs) have distinct advantages over other fluorescence imaging probes as due to their unique optical properties they meet every requirement outlined above. In contrast, quantum dots - the most widely used nanomaterials in biological research - are extremely cytotoxic and do not fluoresce in the NIR.[11]

One case of AIE NPs designed to emit in the NIR were synthesised by the Zhu group which used quinolone-malononitrile (QM) as an AIE building block to design and synthesise a series of AIEgens (Figure 2).[12] All QM compounds showed fluorescence in the NIR in tetrahydrofuran (THF) and H_2O mixtures with a large water fraction. In addition, the fluorescence of the QM compounds were found to be dependent on their aggregation.

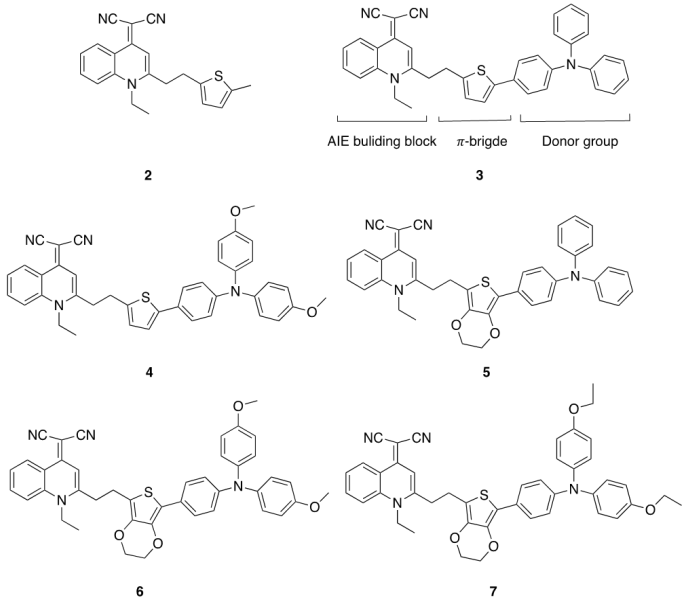


Figure 2: The series of QM derivatives.

Extending the π-conjugation of the QM compounds leads to emission spectrums in the NIR. This was done by attaching strong π-donors to the thiophene moiety. Interestingly, upon changing the electron donating groups and thiophene π – bridge, the morphology of the organic nanostructures could be changed. Transmission electron microscopy (TEM) revealed that aggregates of **2**, **3**, and **4** formed mircorods whereas aggregates of **5**, **6** and **7** were spherical with mean dimensions of 80 – 200 nm. Of the compounds synthesised, **3** and **6** were most typical rod-like and spherical shapes, and therefore chosen for in vivo tumour imaging. Both **3** and **6** displayed low cytotoxicity and high photo bleaching resistance. **3** and **6** nanoaggregates were injected intravenously into tumour-bearing mice and monitored using non-invasive in vivo fluorescence imaging. **3** aggregates were rapidly distributed via blood circulation and displayed a clear fluorescence after 30 minutes. In contrast, 6 aggregates showed superior EPR and a distinct NIR fluorescence was detected in the tumour after 30 minutes.

The Zhu group further explored the effect of morphology and size of AIE nanomaterials by developing **3** hybrid NPs.[13] These NPs were developed by encapsulating **3** via block copolymer polystyrene-b-poly (acrylic acid) (PS- b -PAA), shell-crossing linking, hydrolysis with organic silane and further PEGylation. The resulting **3** hybrid NPs (**3@PNPs**) displayed uniform spherical morphology coupled with enhanced AIE-emission and high photostability. Interestingly, **3@PNPs** showed enhanced tumour targeting specificity, compared to **3**, demonstrating the effect of nano-sized enhanced EPR and surface functionalisation of PEG chains on

accumulation in tumour tissues.

This work performed by the Zhu group signifies the importance of morphology and size of AIE nanomaterials in designing effective probes for passive tumour-targeted imaging via the EPR effect.

Fluorescence dual-modality imaging is the practise of combining a high resolution imaging techniques, for example, MRI or X-Ray computed tomography, with fluorescence.[14] This is highly advantageous as it is possible to overcome the limitations that would face each imaging technique if used alone. A dual-modal MRI nanoprobe, TPE-2Gd (**8**) was reported by *Tang et al.* for both magnetic and fluorescence imaging.15 A tetraphenylethene (TPE) fluorophore is responsible for the aggregation-induced emission properties of the probes, which is connected to two gadolinium (Gd) diethylenetriaminepentaacetic acid moieties (Figure 3).

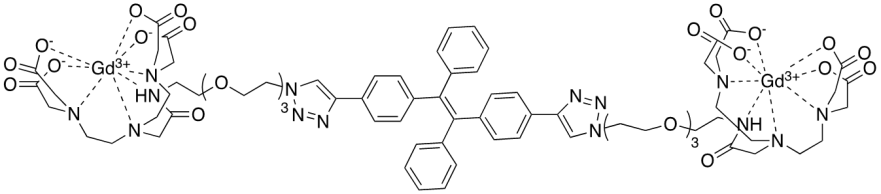


Figure 3: Dual model MRI-contrast agent, TPE-Gd

Due to the hydrophobic and hydrophilic properties of TPE and Gd respectively, the probes aggregate into NPs in aqueous mediums at high concentrations, with high fluorescent intensity. **8** exhibited low cytotoxity, and high photoresitivity. Moreover, as an MRI contrast agent **8** showed similar longitudinal relaxivity in water as the commercial agent, Magnevist®. **8** showed high specificity, with MR imaging remaining hyperintense up to 150 min post injection in the liver. **8** can be easily excreted from the body as the NPs undergo disassembly in solution. AIE nanoparticles are therefore prime candidates for the development of fluorescent dual-modality imaging, owing to their ability to incorporate two functionalities in a single molecule.

AIE Nanoparticles for Drug Delivery

AIE nanoparticles have the potential to not only act as diagnostic probes, but also theranostic agents. Theranostics is a term first coined to describe a material that combines diagnosis, treatment, and follow up of a disease.[16] Theranostics is thought to be the future of cancer therapy and AIE nanoparticles are prime candidates for the development of such systems. A promising avenue of in vivo theranostics

involves using drug loaded AIE NPs for delivery of therapeutic agents.

One such system was developed using coordination-driven self-assembly to synthesise an AIE tetraphenylethene-based metallacage (Figure 4).[17] The coordination of the metallacage results in RIR of the TPE moieties, resulting in a highly fluorescent system.

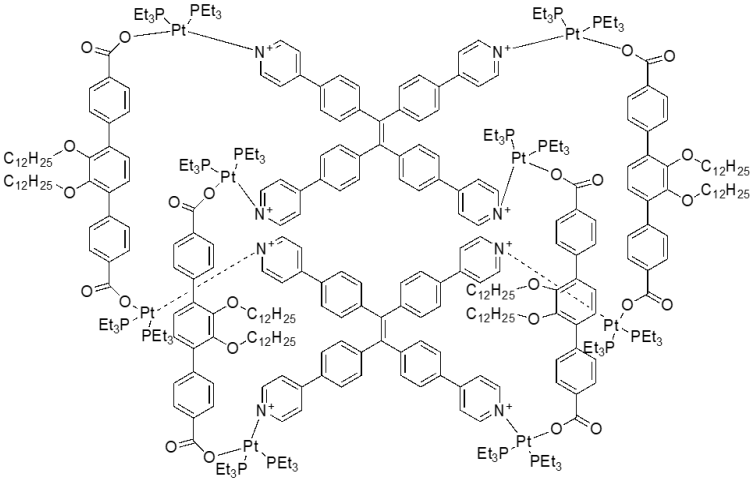


Figure 4: AIE tetraphenylethene-based metallacage

By treating the assembly with mPEG-DSPE and biotin-PEG-DSPE, which act as encapsulation matrices, metallacage loaded-nanoparticles (MNPs) are obtained. The resulting MNPs show excellent colloidal stability, spherical nanostructure, and targeting ability. Cancer cells overexpressing biotin receptors via receptor-mediated endocytosis were selectively targeted by the MNPs, which lead to superior anti-tumour efficiency of the MNPs when compared to clinically available Pt(II) based drugs.[17]

Another method developed by *Huang et al.* is an AIE-active amphiphilic supramolecular brush copolymer with the ability to self-assemble into supramolecular nanoparticles (SNPs) in aqueous solution.[18] The SNPs emitted an intense fluorescence caused by the TPE moieties of the supramolecular brush copolymer. The SNPs can act as a nanocarrier of commercial anti-cancer drug Doxrubicon (DOX), which is released via intracellular reductase in a low pH environment. Upon encapsulation of DOX, the emission of both the SNPs and the drug are quenched, and upon release of DOX, emission resumed. This allows for real-time drug tracking and visualisation of drug release. Drug loaded SNPs also have shown encouraging tumour suppression in mice, which indicates the potential of such compounds in cancer therapy.

Photodynamic Therapy Using AIE NPs

Photodynamic therapy (PDT) is a non-invasive, highly specific treatment that has been applied to certain cancers such as carcinoma.[19] The principle of PDT involves irradiating a photosensitizer (PS) which generates the reactive oxygen species (ROS), singlet oxygen.[20] The mechanism of this system involves promotion of an electron from a singlet ground state, (S_0), to a singlet excited state, (S_1).[21] This is followed by intersystem crossing to form an excited triplet state (T_1). The excited triplet state can then react one of two ways - via a type I or II path way. Type I involves the reaction of an electron and a substrate to generate radicals, that further react with oxygen and produce hydrogen peroxide. Type II requires the energy of the excited triplet state to be transmitted to triplet oxygen ($^3O_2$) which generates highly reactive and toxic singlet oxygen ($^1O_2$) and results in the death of cancerous cells. The type II pathway is generally adopted by PS. Effective delivery of light to the PS determines the efficiency of the therapy.

Porphyrin and phenylthiazinium derivatives are the most widely used PS.[22,23] Unfortunately, these compounds absorb light in the visible or ultraviolet region, which overlaps with tissue absorption, and leads to a short penetration depth of illumination light and an overall ineffective treatment.[24] Furthermore, the rigid planar structures of these PS undergo π-π stacking which quenches their ability to form ROS.[25]

However, AIEgens have been shown to be more effective at producing ROS when in an aggregate state, coupled with fluorescence enhancement, making them more efficient PS compared to porphyrin and phenylthiazinium.[26] Using TPE as a building block, *Ramaiah et al*. designed a series of NIR fluorescent AIEgens (**9-11**) by utilising the TPE's ability to produce singlet oxygen while also being AIE active.[27] (Figure 5)

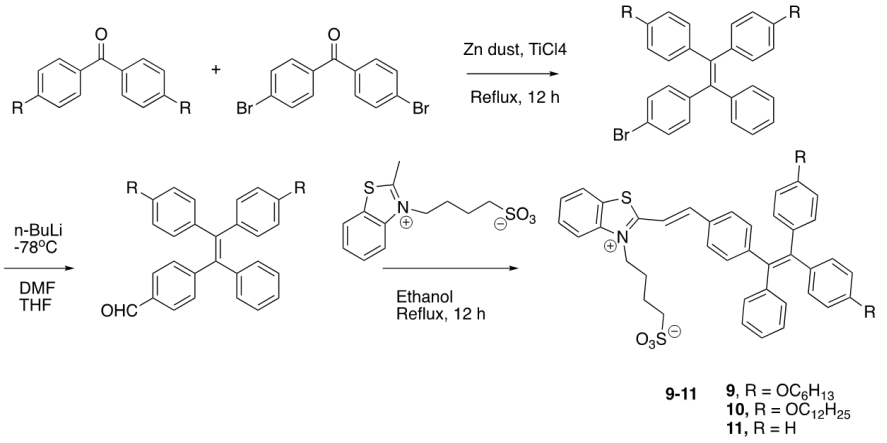


Figure 5: The synthesis of TPE derivatives **9-11**.

Interestingly, molecules **9-11** displayed the unique ability to self-assemble into spherical shaped nanoparticles of dimensions 10±5 nm. TPE nanoparticles showed NIR fluorescence emission, the ability to produce significant yields of singlet oxygen, efficient cellular uptake, and localisation in the cytoplasm in vitro. TPE-NPs showed negligible dark toxicity, and upon irradiation, had a photodynamic effect attributed to the singlet oxygen generation.

Encouraged by these results, mice that had been implanted with luciferase expressing human prostate cancer cells (PC3), were regularly injected with TPE-NPs of TPE conjugate **9**, and subject to regular irradiation of light. Tumours treated using PDT showed reduced growth compared to controls, demonstrating the power of PDT as a tool for cancer therapy.

Conclusions

Much progress has been made in the area of cancer diagnostics and therapy using AIE NPs. They offer clear advantages over traditional fluorescent nanoparticles as not only do they not suffer from ACQ, but also display intense fluorescence, low cytotoxity and in vivo toxicity, resistance to photobleaching, and can produce large amounts of ROS. Moreover, AIE NPs can be modified with tumour targeting ligands to enhance their specificity.

Future work of AIE NPs should focus on developing new AIEgens with a high degree of NIR emission and large multiphoton absorption sections. This would allow for in vivo tumour imaging, something which has yet to be achieved. AIE NPs also offer a superb platform for the development of PDT, a highly promising future cancer treatment. By designing AIE NPs that can overcome the lack of penetrating light and presence of oxygen in tumours, while also generating sufficient ROS, a revolutionary treatment can be applied.

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Chemical Protein Synthesis via Native Chemical Ligation

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Chemistry

Chemical protein synthesis (CPS) allows the preparation of proteins with atomic precision. The expedient and reliable synthesis of proteins remains one of the grand challenges of chemistry. The discovery of native chemical ligation (NCL) greatly expanded our synthetic capabilities, pushing the size limitations of CPS to new limits. Major advancements in NCL have been made since its inception, and have forced the technology towards its full potential. This short review aims to give the reader a brief overview of NCL, the key advancements made to the technology, the current frontiers of NCL, and a possible alternative.

Introduction

Proteins can be defined as macromolecules consisting of many amino acid units linked via amide bonds. Proteins are ubiquitous in life, with a multitude of functions, particularly as catalysts, in which case they are referred to as enzymes. For the chemist Emil Fischer, one of the grand challenges of the 20th century was the total chemical synthesis of enzymes. While this ultimate goal was not achieved by Fischer, his work did lead to the establishment of methodology for amide bond formation between α-amino acids and the importance of enantiopurity in synthetic peptides (essentially small proteins that show little rigidly defined 3-dimensional structure).[1] Unfortunately, progress in peptide synthesis was relatively sluggish for many decades. This was due to major problems associated with solution-phase peptide synthesis. Racemisation during the activation of the amino acid C-terminus was commonly observed, and purification and characterization of the products was found to be incredibly challenging. The most serious issue was that of solubility. Protected peptides were found to have very poor solubility in virtually all organic solvents. This led to reactions characterised by poor kinetics and yields. It was thus the opinion of many chemists at the time that there was an inherent barrier to the chemical synthesis of high molecular weight peptides.[2]

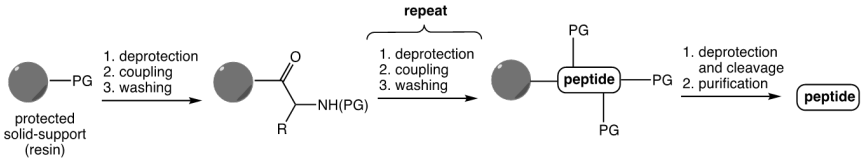


Figure 1: SPPS workflow. PG = protecting group.

Fortunately, this all changed with the invention of solid-phase peptide synthesis (SPPS) by Robert Bruce Merrifield.[3] This was a revolutionary concept in synthetic chemistry, and was acknowledged with the 1984 Nobel Prize in Chemistry to Merrifield.[4] The concept of SPPS is to attach a peptide to a solid-support (e.g. polystyrene beads), carry out the coupling chemistry on the peptide linked to the support, wash and repeat, cleave the peptide from the solid support, and then purify it. The workflow of SPPS is illustrated in Figure 1. While SPPS was met with some resistance (mainly for conceptual reasons), its reliability and expedience led to its universal adoption. It circumvented the conventional problems of arduous solution - phase peptide synthesis, and streamlined the synthesis of high molecular weight peptides. In fact, it was so reliable and operationally simple that it quickly became automated.[5] Today SPPS is used routinely and has been optimised to a high degree, with Fmoc-based SPPS being the most popular form.[6] However, as the reader may have already realised, when we speak of SPPS we speak only of peptides. Limitations in SPPS are inevitable, even with the most highly optimised SPPS conditions. Impurities bound to the support tend to accumulate due to incomplete reactions and impurities in the reagents and solvents. SPPS has been stretched to the limit with the preparation of peptides of ca. 50 amino acids;[2] though the limits are in truth dependent on the nature of the target peptide and the skill and knowledge of the practitioner. Therefore, the question is how we are to access proteins (and large peptides).

In this short review, advancements in synthetic methodology that have facilitated the reliable chemical synthesis of proteins will be discussed. There will be a particular emphasis on NCL and its modifications (ligation-desulfurisation and ligation-deselenisation,). Auxiliary mediated methods (AML) have received much attention in recent years, but are not mentioned in this review, though it has been recently the subject of a review by *Scanlan et al.* in 2017.[7] An alternative to NCL, α-ketoacid hydroxylamine (KAHA) ligation, will also be briefly discussed. It is the objective of this work to give the reader a brief overview of synthetic transformations for CPS, and to point towards particularly important methodological gaps to be filled.

Native Chemical Ligation (NCL)

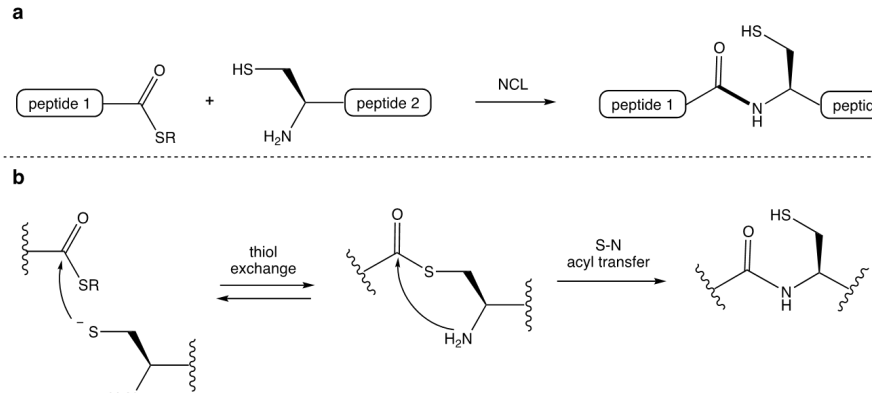
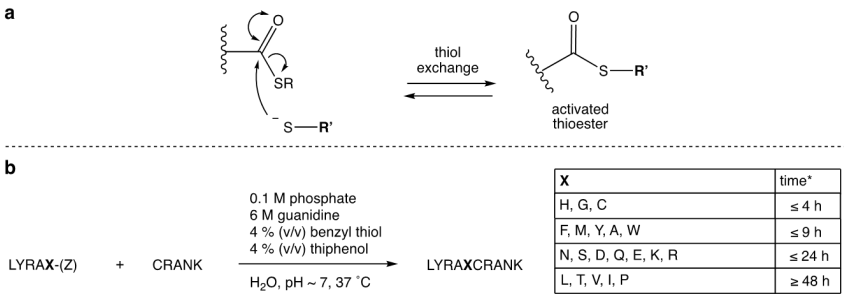


Figure 2: a) NCL transformation. b) Simplified NCL mechanism

In order to overcome the inherent size limitation of SPPS new technology was needed. To this end, *Kent et al.* at the Scripps Research Institute in 1994 reported on the synthesis of proteins via NCL (Figure 2a).[8] It involved the ligation (linking) of two peptides, one containing a thioester *C*-terminus and the other containing a cysteine (C, Cys) *N*-terminus. This was a major advancement in the field of CPS. NCL could be used to ligate two unprotected peptides chemoselectively under aqueous conditions! This was first demonstrated with the synthesis of human interleukin 8 (IL-8), a 72-amino acid polypeptide.[8] The mechanism of NCL (Figure 2b) involves an initial reversible nucleophilic attack on the thioester, expulsion of a thiolate, and a subsequent irreversible S-N acyl shift via a geometrically favoured 5-membered ring transition state; though larger rings are also possible (*vide infra*). The mechanism essentially uses an entropic "trap" to force amide bond formation.



X	time*
H, G, C	≤ 4 h
F, M, Y, A, W	≤ 9 h
N, S, D, Q, E, K, R	≤ 24 h
L, T, V, I, P	≥ 48 h

Figure 3: a) In situ thioester activation. b) Model NCL peptide ligations and kinetic measurements various junctions. time* = time taken for full conversion to product. (Z) = 2-(3-mercaptopropanamido)-4-methylpentanamide (MPAL).

It was reported that the addition of an excess of exogenous thiol could accelerate the rate of NCL dramatically.[9,10] This is due to in situ thiol-exchange to generate an active (reactive) thioester. (Figure 3a). *Dawson et al.* explored the substrate scope of NCL, and found that ligations were possible for all *C*-terminal thioesters of the 20 common natural amino acids (Figure 3b). However, it was found that ligations at valine (V), isoleucine (I) and proline (P) were particularly sluggish, requiring > 48 h and resulting in poor conversions.

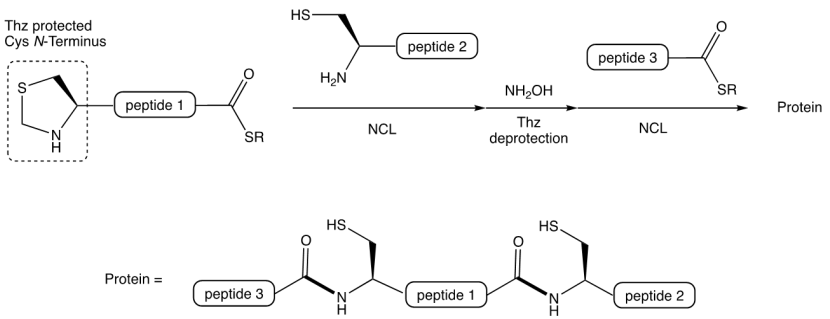


Figure 4: One-pot three-segment ligation with thiazolidine (Thz) protecting group.

In order to access larger proteins via NCL, sequential ligation procedures were developed.[11] The first sequential ligation procedures involved ligation, purification, deprotection, purification, ligation, and then purification. This was of course inefficient, as the purification steps were deleterious to the yields. There was therefore a need to develop a one-pot three-segment ligation procedure, involving ligation, deprotection, ligation, and then purification. *Kent et al.* first

reported on the use of a one-pot three segment ligation procedure (Figure 4) in the synthesis of crambin.[12] Other strategies, including photodeprotection and kinetic control, have been developed, though the thiazolidine (Thz) approach remains the most popular due to its reliability. Impressively, *Kent et al.* have extended the thiazolidine approach to a one-pot four-segment ligation procedure, which they demonstrated in the synthesis of ribonuclease A.[13] Notable problems with the thiazolidine approach include the need to adjust the pH (twice) and the use of hydroxylamine, which can sometimes react with the thioesters (inhibits NCL).[14] An ideal procedure would involve sequential ligations all in the same buffer with no protecting groups; and be reliable.

Muir et al. greatly expanded the scope of NCL with the invention of expressed protein ligation (EPL).[15] This method utilises molecularly engineered cells to produce thioester proteins, which can be subsequently used in NCL. This method greatly expanded the size limitations of NCL, in addition to the scope of substrates, as one can install a variety of unnatural attachments onto the expressed protein thioesters.[16]

Despite the power of NCL as a synthetic tool, an obvious limitation thus far has been the requirement of an *N*-terminal Cys. This severely limits the substrate scope of NCL, as Cys is a very low abundance amino acid in life (ca. 1.7 %).[17] Much of the motivation behind the work described in the next two sections originates from this limitation of NCL.

Ligation - Desulfurisation

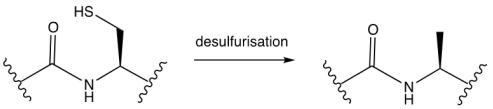


Figure 5: Desulfurisation of Cys (Cys → Ala).

To improve the substrate scope of NCL, procedures for desulfurisation of Cys were developed (Figure 5). The first procedure was reported by *Dawson et al.* in 2001 and involved the use of a heterogeneous catalyst (Raney Ni or Pd on a solid-support) and hydrogen gas.[18] This was a major conceptual advancement, giving access to Cys-free alanine (A, Ala) containing junctions via NCL. However, low yields and unselective reductions rendered this procedure unreliable. The need for hydrogen gas was also unfavourable (hazardous). *Danishefsky et al.* later uncovered an eco-friendly and operationally simple desulfurisation procedure, utilising a radical initiator, tert-butyl mercaptan (t-BuSH) as a hydrogen source, and tris(2-carboxyethyl)phosphine (TCEP).[19] This remains the most popular procedure for desulfurisation and deselenisation (*vide infra*) due to its operational simplicity and functional group tolerance.[7]

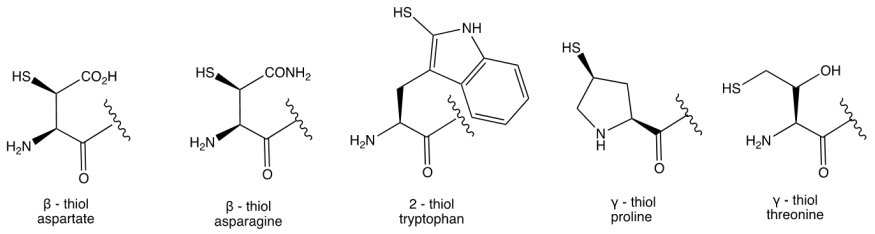


Figure 6: Selected examples of thiol-containing amino acid derivatives for ligation-desulfurisation.

Despite Ala being more common than Cys, it would be desirable further expand the substrate scope of NCL. Thiol-containing amino acid derivatives were thus explored (e.g. Figure 6). Thiol-containing amino acids for NCL have now been prepared for 15 of the 20 common natural amino acids.[7,20] By conducting NCL with an *N*-terminus containing one of the derivatives in Figure 5 and following up with desulfurisation (Figure 5), one can gain access to a large variety of amino acid junctions, thus expanding the substrate scope of NCL dramatically. However, due in part to lack of commercial availability, the application of these derivatives has not yet been widely adopted; their syntheses are also quite tedious.[20]

A problem associated with NCL procedures is often the need to carry out ligation followed by purification before carrying out desulfurisation. The reason for this is the use of excess exogenous thiols, which are incompatible with the desulfurisation conditions. *Payne et al.* disclosed a method to circumvent this problem, which utilised an alternative volatile thiol additive.[21] This allowed one to carry out one-pot ligation-desulfurisations, an important advancement. Interestingly, a recent report described the use of β-thiolactones for NCL, which work on the basis of release of ring strain to facilitate intermolecular thiol-exchange, thus requiring no exogenous thiol additives.[22]

The use of desulfurization was integral in the impressive total chemical synthesis of a single glycoform of erythropoietin reported by *Dankishefsky et al.* in 2013.[23] This was an important advancement, as it demonstrated the power of NCL in the preparation a homogeneous post-translationally modified biomacromolecule; something that even nature struggles with.

Ligation-desulfurisation represented an important advancement in CPS. However, it is by no means perfect. One important issue is that of regioselective desulfurisation. A protein subjected to desulfurisation conditions will undergo global desulfurisation of thiols (with the exception of Aspartate (D, Asp)).[24] This was an issue, and while Cys is a rare amino acid, it is often an integral amino acid for protein structure and function. The use of an acetamidomethyl (Acm) protecting group has partially solved the problem,[25] though not entirely, as the

Acm protected Cys residues cannot be used in NCL, and solubility issues can also arise.[26] Another issue with ligation-desulfurisation is sluggish kinetics, at both the ligation and desulfurisation stages. Furthermore, some amino acids are not accessible via desulfurisation (e.g. serine). To alleviate these problems ligation-deselenisation was developed, which is the topic of the following section.

Ligation - Deselenisation

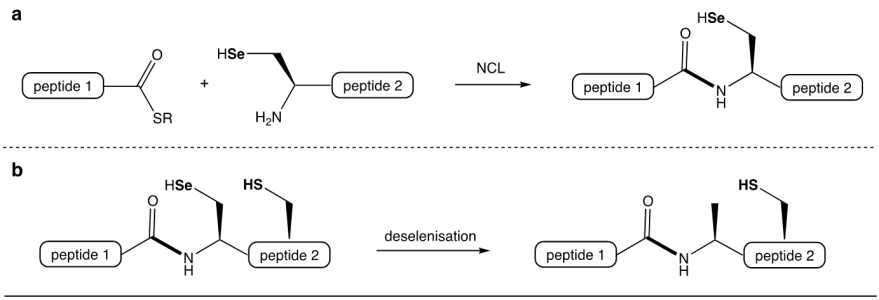


Figure 7: a) NCL with Sec. b) Chemoselective deselenisation

Selenocysteine (U, Sec), the Se homologue of cysteine, is the 21st amino acid.[27] It was reported in 2001 that selenocysteine could engage in NCL (Figure 7a).[28] However, it was not until *Dawson et al.* reported on the chemoselective deselenisation of a protein containing Sec and Cys that interest in Sec ligation chemistry was renewed (Figure 7b).[29]

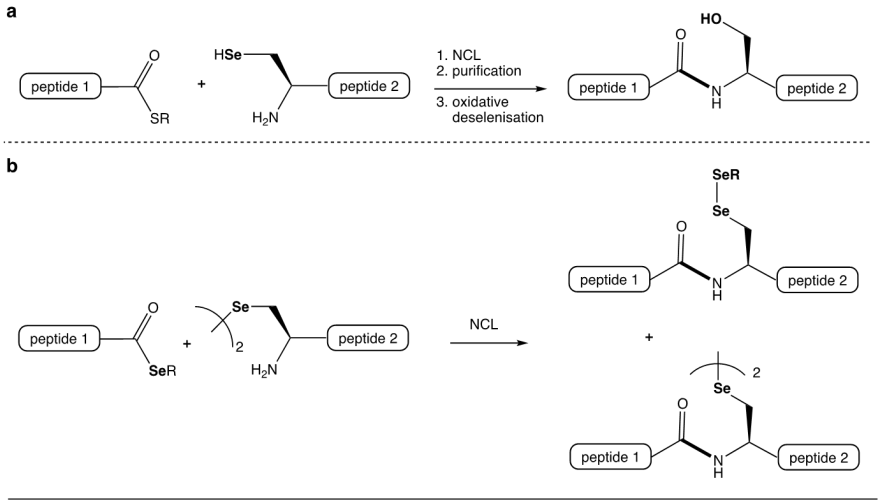


Figure 8: a) Ligation - oxidative deselenisation. b) Sec - selenoester ligation

Sec ligation chemistry has recently seen major advancements. A procedure for ligation-oxidative deselenisation, which allows rapid access to Sec/Cys-free serine junctions via NCL, has been reported (Figure 8a).[30] Threonine junctions were also investigated, but this led to a mixture of diastereoisomers. A one-pot modification to this procedure was later developed to allow concomitant ligation-oxidative delselenisation.[31] An impressive report by *Payne et al.* in 2015 disclosed rapid additive-free ligation with Sec and selenoesters (Figure 8b). The mechanism of this ligation is unclear. It does not appear to be radical based or be dependent on selenoester electrophilicity, but rather seems to depend on selenide solubility. Regardless, the authors concluded that no mechanism could be confirmed in the absence of further experiments. A one-pot ligation-deselenisation variant of this procedure was later reported.[32] The power of this procedure is the excellent kinetics, with most ligations going to completion in ca. 60 seconds. This compares quite favourably with thiol based NCL (see Figure 3b), which often takes longer than 5 hours. The high reactivity of selenoesters was very recently taken advantage of to prepare superoxide dismutase via a one-pot four-segment ligation procedure.[33]

It is clear from the above discussion that there is much promise in ligation-deselenisation. There is still much more to investigate. Possible avenues include kinetically controlled NCL wherein one could use sequential Sec-ligation and Cys ligation coupled with protecting group strategies (e.g. Thz) for the expedient high yielding synthesis of large proteins. Moreover, there is a need to prepare and investigate the use of more selenol-containing amino acids to gain rapid access to more amino acid junctions. It is also important that these selenol containing amino acids be made commercially available, so that this chemistry can be used outside

of just a handful of specialised labs. Furthermore, with the recent discovery that proteins containing an *N*-terminal selenocysteine can be prepared via EPL,[34] it is important that the preparation of selenoesters via EPL be investigated. This would greatly expand the generality of ligation-deselenisation with selenoesters by greatly expanding the chemical space that could be explored with this chemistry.

α-Ketoacid-Hydroxylamine (KAHA) Ligation: NCL Replacement?

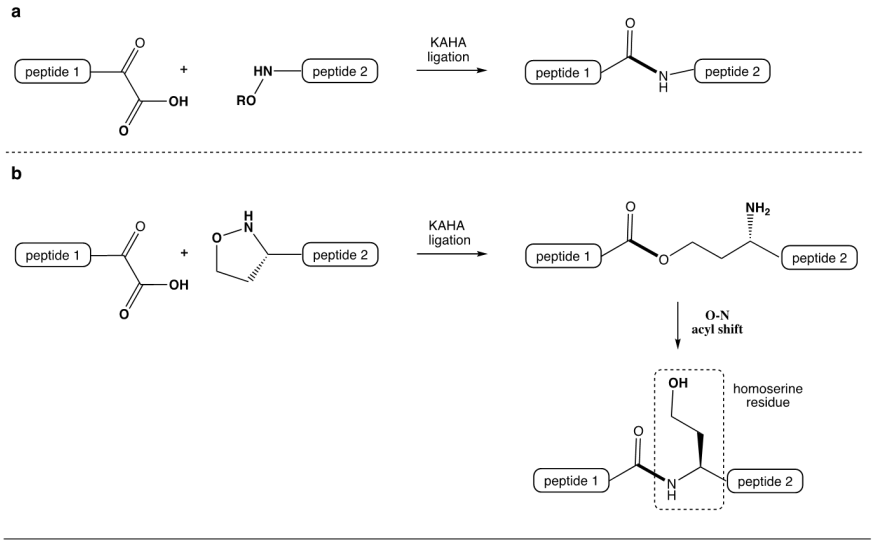


Figure 9: a) KAHA ligation. b) Optimised KAHA ligation.

Bode et al. have developed a ligation technology where peptides containing a C-terminal α-ketoacid can be coupled to peptides containing an *N*-terminal hydroxylamine (Figure 9a).[35] This method has been developed and optimised (Figure 9b).[36] The reaction initially forms an excess of a depsipeptide (ester instead of amide).[35] This has been found to be more of an advantage than a curse, as it often increases water solubility, making purification quite straight forward. However, the procedure utilises a cyclic hydroxylamine, which leads to an unnatural homoserine amino acid. This has not yet been found to be detrimental to the structure or function of the target proteins prepared via KAHA ligation. However, as a practitioner, the incorporation of unnatural amino acid into a target may not be desirable. While previous cases may not have revealed any detrimental impacts on structure or function, each target is different. This is particularly true if one is preparing an

enzyme where an unnatural amino acid could have a major impact on function.

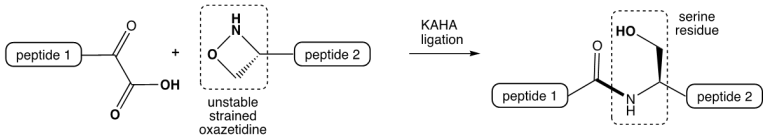


Figure 10. Serine forming KAHA ligation.

To circumvent the issue of homoserine, *Bode et al.* investigated the installation of serine-containing junctions using KAHA ligation (Figure 10). Unfortunately, the requisite unprotected oxazetidine was found to be too unstable, and thus not practical for use in CPS.[37] Furthermore, the requirement for special resins and amino acids has been a major hurdle in the adoption of KAHA ligation by others. The requisite materials have yet to be made commercially available. It therefore appears that KAHA ligation will not be replacing NCL as the dominant ligation technology in CPS anytime soon.

Summary and Outlook

We have seen here that NCL represented a major advancement in the field of CPS. It has allowed chemists to ligate large unprotected peptide fragments chemoselectively in water, thus greatly expanding the size limitation of CPS, opening the floodgates for chemists to investigate protein structure and function. We have seen major advancements in NCL since its inception. Mild and efficient desulfurisation has provided access to a wide variety of amino acid junctions. Sequential ligations using a variety of strategies have provided access to substrates far beyond the scope of single peptide ligations, as for example was demonstrated by the total chemical synthesis of homogeneous EPO. EPL has greatly expanded the size and substrate scope of NCL. Ligation-deselenisation has provided a solution to the issues of global desulfurisation. Sec-selenoester NCL has been demonstrated as a rapid transformation for more expedient CPS. Many avenues remain unexplored in regards to Sec-selenoester NCL, such as further selenol-containing amino-acid derivatives, and particularly in the area of EPL. Furthermore, given the success of Se ligation chemistry, it may be worthwhile to explore the homologous Te chemistry in NCL.

It is integral that reagents for NCL be made commercially available to allow chemists outside the ivory towers of specialised labs to utilise the methods developed in those labs. It may also be instructive if user manuals were to be published, as NCL often involves quite operationally complex procedures, difficult for the non-expert to conduct. It would thus be a positive development if the CPS community were to embrace recent calls to adopt new technologies for data sharing such as pictures

and video tutorials.[38]

As long as proteins remain the products of genes, they will remain of interest to mankind. It is thus important that we continue to investigate their synthesis so that we can understand their structures and functions, and how these are both related. Such advancements in understanding will inevitably benefit our society, be it through the discovery of novel and potent therapeutics or new and improved materials. It is also important that we realise the inherent limitations of our current synthetic methods. Consider this: one of the largest ever chemically synthesised proteins in recent times comprises 203 amino acids.[39] Now consider this: the largest known protein in nature, titan, comprises ca. 30,000 amino acids. Clearly, major advancements are needed if we are to ever dream of mastering the art of CPS.

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Perovskite Solar Cells: The Future is Bright for Solar Technology

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The global energy and environmental crisis is an issue which affects every single person on this planet. The worsening, adverse effects of this critical situation are only highlighting the urgent need for an alternative, clean energy supply. Perovskite solar cell research has been attracting incredible amounts of attention in the scientific community in recent times, appearing to present the most promising route to a clean, sustainable, renewable energy source. This review focuses on the necessity of an alternative to fossil fuel energy, the fundamental reasons why solar energy is the blatant solution, and the mechanism, properties and characteristics of perovskite solar cells, which are the origin of their popularity in renewable energy research. In addition, the barriers to the widespread deployment of this technology, namely stability and hysteresis, are examined. Strategies to overcome these obstacles, which are currently being researched, are identified and discussed, as well as the need for increased rigour and standardisation of testing conditions, for convincing demonstrations of PSC performance and stability.

Introduction

Global annual energy consumption has been increasing at an astonishing rate, particularly over the course of the last century,[1,2] with an astronomical 170 trillion kilowatt-hours (kWh) of energy consumed globally in 2017 alone (Fig.1). This is the energetic equivalent of 100 billion barrels of crude oil, or 25 billion tons of coal, corresponding to a power consumption of:

$$(1.7\times1014\ \text{kWh/yr})(3.6\times106\ \text{J/kWh})(3.169\times10{-}8\ \text{yr/s}) = 1.94\times1013\ \text{W} \quad (1)$$

Moreover, this global power consumption, currently at 19.4 terawatts (TW), is predicted to increase by a futher 28% by the year 2040.[2]

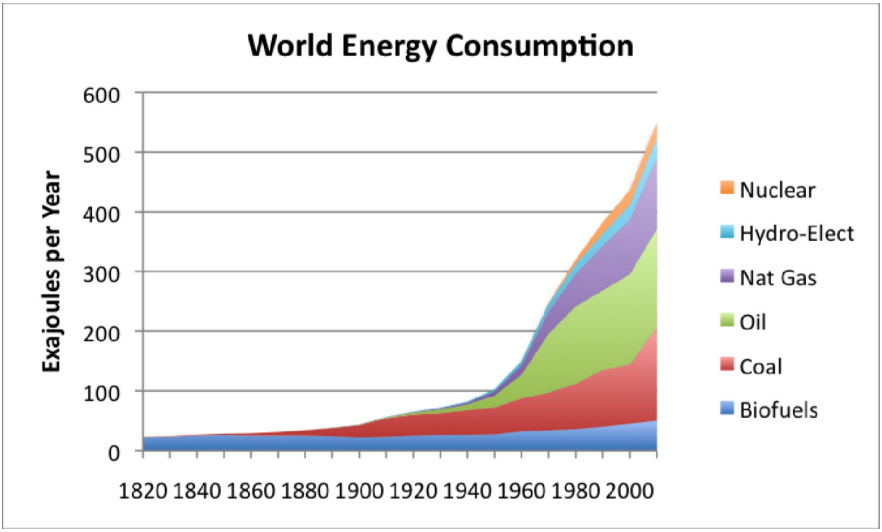

Figure 1: Annual Global Energy Consumption (1820-2010). (*Based on BP Statistical Data from 1965 Onwards*[3,4])

Sadly, a massive 86% of this energy supply originates from fossil fuels (as illustrated in figure 1).[1] Carbon dioxide (CO_2) emissions from the combustion of these fossil fuels account for the largest share of atmospheric greenhouse gases, one of the root causes of climate change and the environmental crisis[5]. Evidently, an alternative, sustainable, clean energy supply is needed to cope with the ever-growing global energy demand and to halt the human perpetuation of climate change and the environmental crisis.

Alternative Energy Source

Almost all of Earth's available sources of energy originate, directly or indirectly, from the Sun. The main process of collecting this energy, which has been in place for billions of years, is in fact photosynthesis, from which fossil fuels derive. This rudimentary viewpoint begs the question; why not skip the middleman (i.e. fossil fuels) and streamline the supply chain, by acquiring our energy directly from the Sun, as plants do? Again, adopting this fundamental yet reasonable approach to the energy problem, we ask: how much energy does the Earth actually receive from the Sun? Solar irradiance, that is, the power per unit area received in the form of electromagnetic radiation, has been measured to be 1360.8±0.5 W/m^2, at the surface of the Earth's atmosphere[6]. Accounting for the attenuating effects of atmospheric absorption, terrestrial rotation (i.e. day and night cycles), weather conditions and the latitude dependency of the solar angle, this culminates in an

average solar irradiance on the surface of the Earth of 130 W/m^2 (as recorded over the years 1990-2004)[7,8]. Given that the average radius of the Earth is 6,371 km[9], and that the surface area of a sphere is $4\pi r^2$, the total solar irradiation (irradiance times area) may be approximated as:

$$(130\ \text{W/m2})(4\pi\times(6.371\times106\ \text{m})2) = 5.63\times1016\ \text{W} \approx 60{,}000\ \text{TW} \qquad (2)$$

This value for the solar power supply is several orders of magnitude larger than the current global power consumption of 19.4 TW. An exciting result, which highlights the potential that solar energy possesses and the need for advanced materials capable of harvesting this energy.

Solar Technology

Photovoltaic (PV) technology is by far the most widely used form of solar energy technology today, constituting 98% of the global solar generation capacity as of 2013,[10] and it is believed by many to be the future of solar technology. PV technology is based on the 'photovoltaic effect': the creation of voltage and electric current in a material upon exposure to light.[11] An essential component of PV devices is the 'p-n junction'; an interface between two types of semiconductor materials, p-type (containing an excess of positive hole charge carriers) and n-type (containing an excess of negative electron charge carriers). As illustrated in figure 2, electrons in the valence band of a semiconducting material are excited to the higher-energy conduction band via the absorption of a photon (quantum of light energy). The presence of a p-n junction in the semiconductor creates a slope in the resulting energy bands, and so the promoted electron can subsequently "roll down" through the depletion zone of the junction into a lower energy band, rather than simply recombining with a positive hole as would occur otherwise.[12]

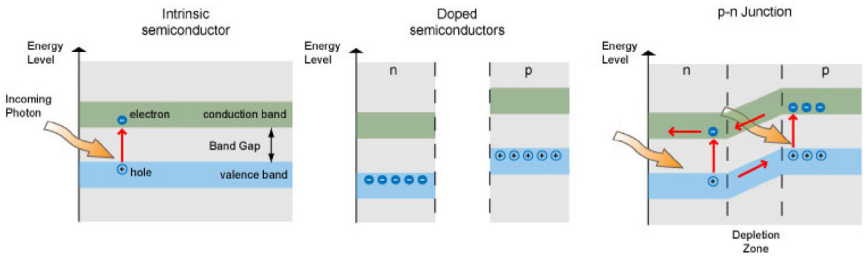

Figure 2: Photovoltic Effect in Semiconduction Solar Panels.[12]

This flow of charge generates a DC 'photo-current' which is then converted to the commonplace AC electricity (through the use of electrical inverters) and distributed on the power network. PV solar cells are powered directly by light, can operate near ambient temperature with no moving parts, and they are completely

scalable without loss of efficiency. For example, a 5 m^2 PV array is theoretically no less efficient per unit area than a 5 km^2 array. This contrasts with other solar technologies, which lose efficiency with reduced scale.[13]

Barriers to Solar Energy

The world record for PV solar cell efficiency, which was last set in December 2014 at the Fraunhofer Institute for Solar Energy Systems in Germany, was achieved using multi-junction concentrator solar cells, with an efficiency of 46%.[14] Clearly, neither the efficiency of modern solar technology nor the magnitude of the solar energy supply is the defining blockade to the use of solar energy at this time. The greatest barrier to widespread use of solar technology in the present day is the fact that modern solar technologies remain far too expensive for widespread deployment. Operational costs of PV solar panels are in fact quite low, coming in at about 8 cent/kWh, which is conservatively forecasted to drop to 4-6 cent/kWh in 2025 and on to 2-4 cent/kWh in 2050 (depending on annual sunshine levels).[15] Given that the current price of electricity here in Ireland is 24 cent/kWh[16], these are attractively low operating costs. Unfortunately, however, the actual cost of production of efficient solar panels remains quite expensive.

The root of this production expense is attributed to the scarcity of certain critical elements which are essential components in modern PV technologies. Examples of these critical elements include Tellurium for Cadmium Telluride (CdTe), and Gallium, Indium and Selenium for CIGS (Copper Indium Gallium Selenide) cells.[13] Another is crystalline silicon (c-Si), which despite the abundance of Silicon in the Earth's crust, has a large cost associated with it due to the difficulty of the necessary purification process.

Two main solutions to this supply barrier imposed by critical elements are currently being explored. Firstly, these limitations could be circumvented by reducing the proportion of critical materials in PV devices, while attempting to maintain cell efficiency. However, for many of the commercial PV technologies currently available, the necessary reductions in critical material concentration are unrealistically large, with large-scale deployment inhibited by physical and practical limits on the thickness of layers in the devices. For instance, thinner films may be unable to absorb sunlight fully, or may facilitate the development of short circuits in large-area devices.[13]

Alternative Thin-Film PV Technology

Another approach, currently being explored by research groups worldwide, is the development of thinfilm PV technologies with substantially lower critical material requirements. These emerging technologies utilise more abundant, alternative

active materials, such as high-production-volume primary metals, for example lead (Pb).

There currently exists a stark contrast between the material demands for commercial and emerging thinfilm PV technologies. For example, colloidal quantum dot (QD) PV cells require the equivalent of only 3 hours of global sulfur production (amount of sulfur produced globally per unit time), and 10 days of global lead production, to produce enough lead sulphide QD solar cells to yield a 10 terawatt power output (10 TWp).[17]

On the other hand, to yield an equivalent power capacity using current commercial PV solar cell technologies would require 12 years of global silver (Ag) production for c-Si PV,[18-20] 1800 years of global tellurium (Te) production for CdTe[19-21] and 710 years of global indium (In) production, 1200 years of global gallium (Ga) production and 650 years of global selenium (Se) production for CIGS cells.[19,20,22]

Perovskite, copper zinc tin sulfide (CZTS), organic and dye-sensitised solar cells (DSSC) all require elements which are produced in abundant quantities, and are therefore likely to be suitable for large scale-up and widespread deployment, compared to current commercial thin-film PV technologies (such as CdTe and CIGS). However, low efficiency ratings, poor stability, and the possibility of manufacturing limitations currently limit the economic practicality of these emerging thin-film PV technologies, rendering them important targets for research.

Perovskite Solar Cells

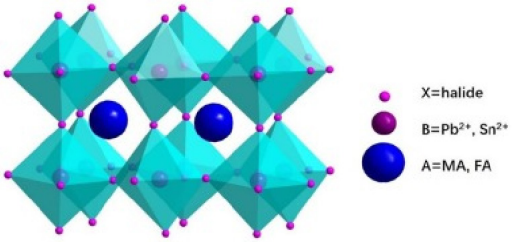

Figure 3: Perovskite Crystal Structure.[24]

The strongest contender for the next generation of alternative PV technology is the perovskite solar cell (PSC). Perovskite refers to the crystal structure ABX_3 (where A and B are cations (with A much larger than B) and X is a halide anion). The crystal structure of perovskite is ideally cubic, made up of corner-sharing BX_6 octahedra with the "A" cations situated at the interstices, themselves surrounded by 8 octahedra, as shown in figure 3. By varying the ions A^+, B^{2+}, and X^-, the optical and electronic properties of the crystal can be adjusted and fine-tuned.[24] The

most-researched perovskite structures for solar cells are hybrid organic-inorganic halides, due to their desirable properties, such as tunable bandgaps,[25] high optical absorption coefficients,[26] low exciton binding energies (<10 meV),[27] long charge-carrier diffusion lengths (and thus high mobilities),[28] as well as low-temperature solution processability.[29]

In hybrid organic-inorganic halide perovskite, A is an organic cation such as methyl ammonium ($CH_3NH_3^+$ = 'MA') or formamidinium ($HC(NH_2)_2^+$ = 'FA'); B is a metallic cation, such as lead (Pb^{2+}) or tin (Sn^{2+}); and the halide (X^-) may be chlorine (Cl^-), bromine (Br^-) or iodine (I^-).

The strong optical absorption of perovskite nanocrystals is due to the presence of anti-bonding interactions between s and p orbitals,[30] and this allows the perovskite layer in hybrid organic-inorganic solar cells to be quite thin - in the range of 400 nm. These s-p anti-bonding interactions occur as a result of the perovskite structure, shown above in figure 3.

The p-orbitals of the halide atoms are directed towards the large s-orbitals of the lead (or tin) atoms, resulting in a large degree of overlap and therefore strong bonding and anti-bonding interactions. As depicted in the energy band diagram in figure 4, this yields a valence band maximum (VBM) which is higher in energy than the halide (in this case, iodine) p-orbitals.

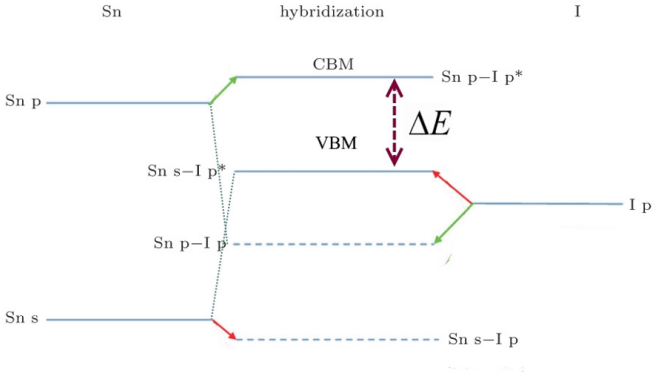

Figure 4: Energy Band Diagram of Perovskite.[31]

Consequently, there is only a small difference in the energies of the conduction band minimum (CBM) and the VBM, rendering the effective mass of positive holes (in this case; iodine vacancies) close to that of electrons. This enables substantial diffusion lengths and high mobility for electron-hole pairs in the material,[32] which is imperative for achieving high-efficiency solar panels The reduced band gap of perovskite contributes to its large optical absorption coefficient (in the range of $10^4 - 10^5 cm^{-1}$)[33], as it allows for absorption over the entire visible solar emission spectrum.[32] This is due to the fact that the direct energy gap of ΔE = 1.55 eV[33]

permits the absorption of UV and visible light wavelengths of up to 800 nm, as witnessed in the IPCE (incident photon conversion efficiency) spectra of perovskite cells, shown in figure 5. Again, this is an important property required for high-efficiency PV technologies. This capability of perovskite to produce high-efficiency solar devices, using relatively abundant materials, is the primary driving factor behind its fast-growing popularity in the emerging thin-film PV industry.

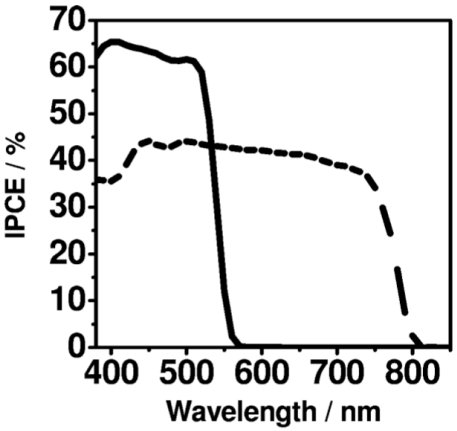

Figure 5: IPCE spectra for $CH_3NH_3PbBr_3/TiO_2$ (solid) and $CH_3NH_3PbI_3/TiO_2$ (dashed) Perovskite Cells.[30]

Perovskite: Future Concerns

The most significant concern for the practical application and commercial viability of PSCs is the stability of these devices.[34] Unfortunately, the stability of PSCs is relatively low, compared to other solar cell technologies. This is due to their strong sensitivity to environmental factors such as oxygen, water, UV radiation and temperature.[35] Additionally, PSC systems are known to exhibit hysteresis in their current-voltage response, primarily due to the trapping of charges and voltage-induced ionic migration. Moisture (H2O) is detrimental to PSCs as the crystallinity of the perovskite structure is damaged by the presence of excess H2O, for which the hydrolysation mechanism is depicted in equation 3 below.

$$\mathrm{CH_3NH_3PbI_3}(s) \xrightarrow{\mathrm{H_2O}} \mathrm{PbI_2}(s) + \mathrm{CH_3NH_3I}(aq) \qquad (3)$$

In addition, it has been observed that exposure to Oxygen or UV radiation can accelerate this decomposition process.[35] Hysteresis in the current-voltage (I-V) behaviour of PSCs, as first reported in 2014[36] and depicted in figure 6, is another concern for the future of PSC technology. When hysteresis occurs, the currentvoltage relation depends not only on the present applied voltage, but also

on the history of applied voltages, and this can have a substantial negative impact on the power conversion efficiency (PCE) of the solar cell, as the current response may become attenuated with larger degrees of hysteresis.

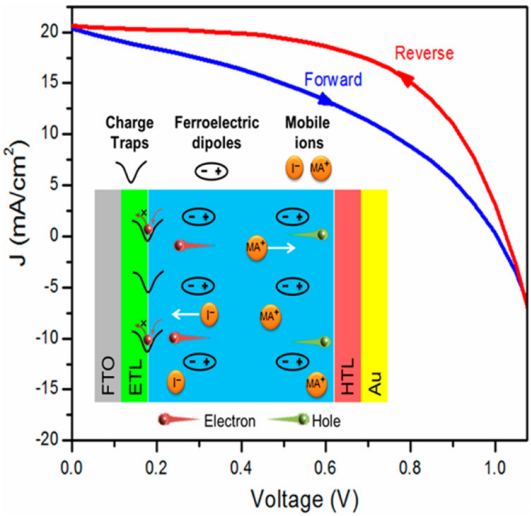

Figure 6: Current-Voltage Hysteresis Curve of Perovskite.[37]

A number of investigations have been carried out to determine the physical origin of this phenomenon, which is believed to be caused by the trapping of charge carriers at the layer interfaces,[38] ionic displacement,[39] ferroelectric behaviour[40] or a combination of these effects.[41]

The requirement of lead in many PSC devices is also a significant issue, due to its toxicity, and the search for lead alternatives is a considerable challenge that many researchers are currently facing.[42]

In relation to solutions for these issues, there exists several strategies that may be pursued to enhance the stability of PSCs.

Regarding the problem of I-V hysteresis, employment of a mesoporous (containing pores with diameters of 2-50 nm) titanium dioxide (TiO_2) scaffold to host the perovskite layer (depicted in figure 7) has been shown to massively attenuate the effects of hysteresis.[32] A TiO_2 scaffold can also strongly increase the carrier-collection efficiency (CCE) at the layer interfaces of the PSC, which is imperative for high performance devices. The formula for CCE is given as:

$$CCE = \eta_{coll} = (1 + (d^2/L_d)^2)^{-1} \quad (4)$$

where d^2 is the mean square displacement required for a charge carrier to reach the perovskite/TiO_2 contact, from the position at which it is photo-generated, and L_d is the diffusion length. A mesoporous architecture in the TiO_2 scaffold usually

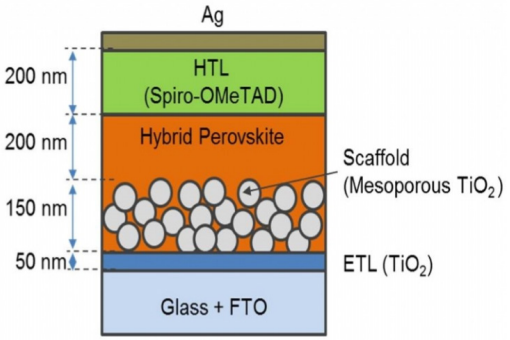

Figure 7: PSC with Mesoporous TiO_2 Scaffold.[43]

reduces d to less than 10 nm, and so a diffusion length of 100 nm (typical in perovskite sheets[44]) yields a CCE of:

$$\eta_{coll} = (1 + (10/100)^{2})^{-1} = (1.01)^{-1} \approx 99\% \quad (5)$$

In contrast to this, a typical non-mesoporous, planar film architecture with a thickness of 250 nm, possesses a CCE of less than 50%.[45]

In relation to the issue of instability upon exposure to moisture, efforts are generally focused on encapsulating the perovskite, by making the top charge transporting layer of the PSC moisture resistant.[46] Several approaches of this type have been explored, including the use of a thin blocking layer between the perovskite and hole-transporting material (HTM), such as Al_2O_3, which does not decrease the efficiency of the device.[47] Additionally, moisture-blocking hole transporters and hydrophobic carbon electrodes have also been investigated as possible solutions, with varying degrees of success.[48,49] However, as of 2018, these solutions are not entirely sufficient, and the combination of moisture, light and heat still results in the eventual degradation of the perovskite layer, despite the inclusion of these modified components,[46] and so more research on possible solutions to the moisture-instability issue is required.

It should be noted, however, that in several investigations of these proposed solutions to instability and hysteresis, the devices were not exposed to multiple stressors (humidity, UV light, elevated temperature etc.) simultaneously. Thus, it is difficult to accurately extrapolate the effectiveness of these modifications under more rigorous conditions, and as such, further research is necessary to fully evaluate the viability of such strategies.[23]

Conclusion

Solar energy appears to be the most auspicious solution to the energy crisis

currently facing humanity, despite the considerable barriers currently standing in the way of widespread production and deployment. Perovskite solar cells display many promising, desirable characteristics that should, in theory, result in their employment as the next generation of solar technology. Tunable bandgap, strong absorption, simple manufacturing process and, importantly, high efficiency are all properties which make PSCs the most likely path to success in the pursuit of a clean, renewable energy source. Significant impediments to this evolutionary breakthrough in human technology, however, are the issues of stability, hysteresis and lead toxicity. Nonetheless, it is the confident opinion of much of the scientific community, this author included, that with adequate research, time and money, this obstacle can and will be overcome, in order to achieve a brighter, sustainable future for all.

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Physics

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Letter from the Editor

Physics is, in some ways, the broadest discipline in science. All scales of scientific concern, from the smallest measurements possible, to considerations on a universal scale, can be explored within the framework of physics. In a more practical sense, physics can be employed across many fields to the benefit of the world as a whole. Much of the groundbreaking work done in modern science - be it the most culturally significant, headline-catching, and/or the most scientifically impactful - is done in the field of physics. As new advances are continuously made which define and redefine our view of the world around us, it is an incredibly exciting time to enter the world of physics research.

Physics rarely functions entirely within an intellectual space. While much research is done with the view of solving specific problems or achieving certain goals, it is rare that any research, no matter how how trivial, novel, or abstract, stays solely within the confines of academia.

The reviews published in the physics section of this year's edition of the Trinity Student Scientific Review are a reflection of this ethos. Covering diverse topics in material sciences, electronics, and astrophysics, these reviews take differing approaches to experimentation, and have different motivations for research. Each review shares a common theme of the tangible real-world benefits of research in these topics. This was something seen in all submissions for publication, of which we received a record-breaking number this year, with those selected being particularly noteworthy for both the utility of the research reviewed within, and the broad scope of topics they presented as a whole.

Discussions on the push to miniaturise electrical capacitors, and to identify high-temperature superconductors, are essential in understanding the effects such breakthroughs would have on our everyday lives. The work done into superlubricity could open up entirely new fields of research in nano mechanics and nano machinery. As the desire to explore space is rekindled in popular culture, the need

for an adequate understanding of how fire behaves in space, and safety features to deal with such, continues to grow.

I would like to take the opportunity to thank the authors and referees who put in countless hours of hard work and effort, in pursuit of the goal of making this edition of the TSSR physics section as high a quality as previous volumes have been. Most importantly, I would like to thank Prof. Mauro Ferreira of the School of Physics here in Trinity for serving as academic advisor. His guidance and input proved invaluable across all steps of bringing this volume of the TSSR to fruition. We are very proud to present this work as a testament to the ability and talent of the students of the TCD School of Physics.

Andrew Connolly
Physics Editor
Trinity Student Scientific Review 2018

Extra-terrestrial Fire Safety: Ongoing Research into Behaviour of Fire in Microgravity Environments

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Fire exhibits different behaviour in microgravity environments compared to its standard behaviour in Earth's gravity. This presents a challenge when designing and implementing procedures to combat any outbreak of fire on spacecraft such as the International Space Station. The shape and propagation of fire differs as a result of this reduced gravity. The lack of convection leads to a uniform rate of flame-propagation and a significant change in the size and concentration of products formed as a result of any combustion reaction. The current decade has seen several studies into behaviour under these conditions, such as NASA's FLEX and SAFFIRE experiments. These investigations aimed to study the nature and propagation of fires in microgravity environments as well as fire containment and suppressant measures. This knowledge is especially pertinent looking forward to the future of space exploration as missions venture further from Earth.

Introduction

Space travel is a complicated process with great potential for disaster. In the confined conditions of a spacecraft fire is a deadly hazard, even more so than on Earth. Escape from such a disaster is more difficult and the consequences, both scientifically and financially, are vast and dire. This review aims to examine the difference between terrestrial and extra-terrestrial fires as well as the underlying reasons for these differences. It will also detail past and current investigations into the nature of combustion in microgravity environments, and the results of such research.

Terrestrial vs. Extra-terrestrial Fires

Fire is a result of combustion. On Earth, many of the properties associated with fire exist as a consequence of Earth's gravity. Therefore, under different gravitational conditions one should expect the properties of fire to vary, and indeed this is the case. The traditional teardrop shape associated with a flame is a result of a buoyant upward flow. There is a large density gradient present in such a flame due to the high temperatures involved and as such, the components of lower density rise, with those of higher density sinking to give the characteristic shape. In microgravity environments such as those of extra-terrestrial spacecraft, upward buoyant flow is almost completely eliminated, giving rise to a spherical flame shape[1].

Aside from their shape, the behaviour of flames also differs under conditions of microgravity. The ejection of vapour or liquid globules from fires was observed during the Wire Insulation Flammability Experiment (WIF), the report for which was published in 1994[2]. These globules were propelled from the initial flame, set in a sample of burning wire insulation. Results differed from those in terrestrial gravity, in which these globules would sink and cool instead of being ejected. This behaviour presents an obvious safety hazard as these could ignite other nearby materials or cause injury to nearby astronauts.

Artificial environments such as those of spacecraft exhibit another crucial difference to Earth, namely the absence or near-absence of convection in the atmosphere. This is particularly relevant when discussing the manner in which fire forms and propagates, as well as its products. Considering the formation of fires, the lack of convection limits the heat transfer between objects and their surroundings. If an object is therefore subject to thermal stress, its temperature would increase more rapidly than otherwise expected on Earth. This presents a hazard in a spacecraft environment as the probability of a component overheating and subsequently igniting is increased, as was shown in the aforementioned WIF study[2].

The manner in which fire propagates in a microgravity environment is different to that on Earth. Under the convective conditions of Earth, buoyancy-induced flows are generated in the area around the flame, hereafter referred to as the flame zone. The convective conditions induce the spreading of the flame and supply continuous oxygen to the flame zone, thus allowing it to propagate. Under conditions of microgravity, it was found by Friedman that the static environment also allows for the propagation of flames. The flame spread was observed to be roughly uniform, however, this rate was seen to be far slower than that on Earth with rates of 15% the Earth rate recorded[3]. The lack of convection was also found to affect the limiting concentration of oxygen necessary for the flame spread for the burning of thin paper with a value higher in microgravity than on Earth. It was concluded that microgravity led to a reduced flammability range for the materials under consideration compared to their flammability ranges on Earth. One caveat regarding this conclusion, as mentioned by Friedman, is that in atmospheres containing higher concentrations of oxygen these differences in the rate of flame

spreading decreased. This is due to the supply of oxygen by convection decreasing in importance as a factor impacting the propagation of the fire[3].

Finally, when studying the effects of microgravity on fires, a difference in the products produced can be observed. When studying smoke produced by fluorinated wire in microgravity, Paul *et al.* analysed the particles present and determined that the size of the particles depended on the gravity of the environment in which they were produced. The morphology and elemental composition, however, were concluded to be independent of gravity. The average size of the particles observed in microgravity was found to be approximately double that of those particles produced in Earth's normal gravity. Additionally, it was observed that the particles produced in microgravity exhibited a larger range of particle sizes than those produced in normal gravity[4]. Several reasons were postulated for this difference in particle properties, for example, the difference in structure of the burning wire. In normal gravity, the wire's insulation was found to fall away from the combustion region due to gravity, whereas in microgravity the insulation stayed in the burning region, leading to a higher concentration of hot gas. This was determined to be the cause of the increased and more varied particle size. The comparatively long length of the residence time – the length of time for which the front of the fire occupies one point – compared to that on Earth, and the lower mass transport rates were also cited as reasons for the differences observed between the microgravity and normal gravity particle production[3].

The gaseous products of combustion were also investigated. A 1995 study[5] determined that light gases, such as carbon monoxide, carbon dioxide and methane were produced in much larger proportions through combustion in microgravity compared to Earth's gravity. The experiment examined smouldering, defined as "a non-flaming surface combustion reaction that takes place in the interior of porous combustible materials"[5]. In this test smouldering of polyurethane foam under microgravity conditions showed that the proportion of these light gases was significantly higher than on Earth. For example, in all cases studied in microgravity the amount of carbon monoxide produced fell in the range of 89 to 3,900 ppm. This was in stark contrast to the amount produced under normal gravity which was measured as less than 6 ppm. A similar, though less dramatic, trend was observed with carbon dioxide, where the amount was consistently found to be double that observed in normal gravity. Several other gasses, such as propene and methane were only weakly detected in normal gravity but were clearly observed in microgravity[5].

To summarise, it is clearly detailed that microgravity conditions greatly affect the formation, propagation and products of fire and combustion. There are distinct differences between combustion in Earth's normal gravity and that in microgravity.

Flame Extinguishment Experiment (FLEX)

NASA's Flame Extinguishment Experiment aimed to investigate "the effectiveness of fire suppressants in microgravity and quantify the effect of different possible

crew exploration atmospheres on fire suppression"[6]. The results were extensive, detailing conclusions on suppressant type, fuel type, droplet details and oxygen mole fraction.

Oxygen mole fraction and droplet diameter were examined together by Dietrich *et al.*[7]. Heptane and methanol were used as fuels which were ignited in microgravity in a controlled atmosphere in a combustion chamber designed for the experiment. The burn time was 20 seconds, after which the droplet was engulfed by a spherical flame of dimensions 2.5-4.0mm and the fuel droplet reduced in size until it was either consumed or the flame extinguished. Radiative and diffusive extinction were observed and these different extinction types exhibited different results as the oxygen mole fraction was changed. Radiative extinction refers to extinction due to heat loss from the flame zone, whereas diffusive extinction refers to extinction caused by insufficient residence time[8]. As the oxygen mole fraction was reduced, the diameter of those droplets that underwent diffusive extinction increased, while that of the droplets which underwent radiative extinction decreased. It was also shown that the heptane droplets exhibited "unique burning behaviour"[7]. When a hot flame underwent radiative extinction around a droplet of heptane, the droplet was not consumed but instead continued to burn, however, as a cool flame. This refers to a weak flame associated with fuel-air mixtures in multi-stage ignition. Cool flame gives off very little heat as combustion does not occur to completion, instead the molecules present break down and recombine to form stable compounds[9]. These results greatly improved the understanding of how such droplets undergo combustion – information that will inform future fire-safety measures on spacecraft. This is a further example of the benefits of conducting such research, as this effect is not observed in normal gravity. The implications of this will be discussed later in this review.

This cool flame behaviour of heptane droplets was further investigated by Farouk *et al.*[10]. The investigation induced this behaviour under a variety of conditions to understand the underlying mechanism that causes the transition between the hot combustion extinction and the second stage of low temperature combustion. Combustion was induced at high pressures and a cycle of hot-cool combustion was observed; the droplet initially underwent hot flame combustion before transitioning to cool flame combustion and back several times. Their simulations indicated that the multi-cycle phenomenon depended on the relative rates of diffusive extinction and heat generation that occurred in the cool flame stage. It was also determined that the environmental factors, such as the size of the droplets and the fuel properties would affect changes in these relative rates, thus affecting the absence or presence of the cycles.

The implications of these results are outlined by Nayagam *et al.*[11]. The immediate implication for fire safety in spacecraft was the need to consider the cool flame behaviour of the fuel droplets, and to reconsider procedures that consider hot flames only, as to omit cool flames presents a safety risk. These cool flames may, as previously outlined, transition back to hot flames, dependant on environmental

factors, and therefore cannot be ignored when designing safety protocols.

It is clear that any fire-control protocols put in place for spacecraft based purely on terrestrial research into combustion would be extremely short-sighted. There are effects present in a microgravity environment that do not appear in normal gravity and to ignore them would be a significant oversight. As such, it is important to continue such research under appropriate conditions.

A look to the future: SAFFIRE

In recent years, further study has been undertaken into the nature of combustion, and the management of fire in microgravity environments, as organisations like NASA prepare for longer term missions that take payloads and astronauts further away from Earth than any previous missions. Long term missions would require that, if a fire was to break out, the crew or the craft be able to control it without evacuation back to Earth. The current fire prevention procedure in place for the International Space Station (ISS) is that, if the fire were to become uncontrollable, any astronauts would evacuate to Earth[12]. Recent research has aimed to collect information to improve simulations of these events to allow for better planning.

NASA's Spacecraft Fire Experiment (SAFFIRE) is a series of experiments designed to investigate the propagation of fire in space, as well as the flammability of certain materials commonly used in spacecraft and related scenarios. To minimise the risk associated with combustion-based experiments taking place on the ISS, these experiments were set up in the Cygnus spacecraft which delivers supplies to the ISS. The experiments were initiated after the delivery of the supplies when the craft was no longer connected to the ISS. As of the date of this review, three of the planned six experiments have been completed. SAFFIRE-I and SAFFIRE-III, the first and the third experiments examined the propagation of fires in microgravity under the influence of different airflows. SAFFIRE-II tested the flammability limits of common materials used in the construction of spacecraft. Research is ongoing as three further stages of the experiment have been approved, and the results will greatly inform the next generation of fire-safety protocols[13].

Conclusions

In the near future, as human space travel progresses beyond Earth's immediate vicinity and deeper into the solar system, it is imperative that robust and thorough fire-safety protocols are implemented in all spacecraft. Decades of research have established the existence of significant differences between the behaviour of fire in microgravity environments and in Earth's gravity. The difference in gravity and the resulting change in convection compared to Earth greatly impacts the formation and propagation of fire as well as the results of any combustion. Any protocols set in place should therefore consider all available information and discard any Earth-bias in favour of results proven to be valid for microgravity environments.

The safety of the payload in any long-term space exploration must be ensured to avoid any loss of life or research. These findings will allow for the introduction of appropriate protocol to support humanity's future forays away from our home planet.

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High-T_c Superconductors: The Story so Far

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Physics

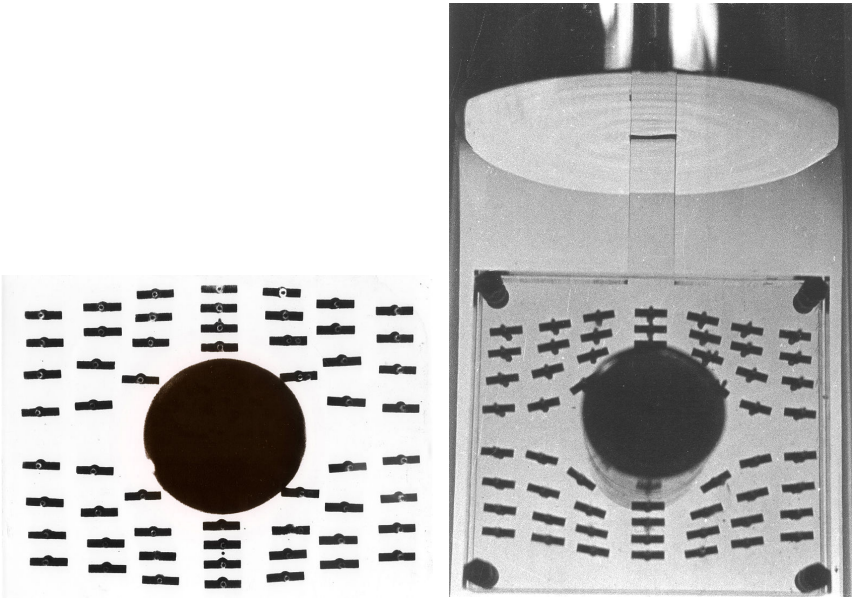
Superconductors are a class of materials which exhibit zero electrical resistance when cooled below a certain critical temperature, T_c. There exists a category of superconductor, known as a high-temperature superconductor (HTS), which can have a T_c greater than the boiling point of liquid nitrogen. Development of such superconductors is of increasing commercial interest due to the rising cost and scarcity of liquid helium[1], which is required to operate conventional, low-temperature superconductors (LTS). The first microscopic theory for conventional superconductors was the Bardeen-Cooper-Schrieffer (BCS) model which proposed that the superconducting electrons form what are known as Cooper pairs due to electron-phonon interactions. However, the mechanism for high-temperature superconductivity is a topic of much debate[2]. In this review we will explore some of the more prominent theories in the field, which are primarily modifications of BCS theory with the Cooper pairing being mediated by some boson other than the phonon. We will focus mainly on a class of high-T_c superconductors known as the cuprates. Finally, we will explore some of the potential uses of high-temperature superconductors from large-scale energy storage devices to superconducting magnets for use in NMR and MRI systems.

Introduction

On April 8th 1911, Dutch physicist Heike Kamerlingh Onnes was investigating the electrical properties of mercury wire immersed in liquid helium. Below 4.2 K, he observed the flow of DC current without resistance[3]. He dubbed this phenomenon superconductivity. Superconductivity differs from normal conductivity in that for a normal conductor the electrical resistance decreases gradually with temperature with a residual resistivity at 0 K due to defects.

In a superconductor the resistance behaves similarly to an ordinary conductor

down to a certain point where it drops abruptly to zero. Superconductivity is caused by the complete expulsion of magnetic field from the material below a critical temperature, T_c. This is known as the Meissner effect[4].



(a) $T > T_C$ (b) $T < T_C$

Figure 1: The expulsion of an applied magnetic field B from a superconductor as it is cooled from 4.2 K to 1.6 K ($T_c \approx 3$ K for Sn), this is called the Meissner Effect[5].

This can be observed indirectly by measuring the magnetic field distribution around a superconductor as it enters its superconducting state. The increase in the flux outside the material indicates a near total cancellation of internal magnetic fields (as flux is conserved)[6]. This result led to the conclusion that superconductivity could not be understood as a simple idealization of classical, perfect conductivity. A new model was needed to explain the phenomenon. This was first done with the phenomenological equations developed by brothers Fritz and Heinz London in 1935[7]. By modelling electrons as free particles under the influence of a uniform external magnetic field, they arrived at (eq.1) and (eq.2), known as the London equations:

$$\frac{\partial \boldsymbol{j_s}}{\partial t} = \frac{n_s e^2}{m}\boldsymbol{E} \tag{1}$$

$$\nabla \times \boldsymbol{j_s} = -\frac{n_s e^2}{m}\boldsymbol{B} \tag{2}$$

Where $\mathbf{j}_s$ is the supercurrent density, n_s is the number density of supercurrent carrier (superconducting electrons), and e and m are the electronic charge and mass respectively. From these equations, the Londons showed that the magnetic field strength in the bulk of a superconductor decays exponentially with depth, z:

$$\boldsymbol{B_z} = \boldsymbol{B_0} e^{-z} \tag{3}$$

Below the London penetration depth, the conductivity can be considered to be infinite.

A mathematical, physical model was later developed in 1950 by Vitaly Ginzburg and Lev Landau[9]. They argued that the Gibbs free energy of a superconductor near T_c could be expressed in terms of a complex-order field parameter, ψ, which is non-zero below T_c and is related to the density of superconducting electrons, as well a magnetic vector potential, $\boldsymbol{A}$. Minimising the Gibbs free energy with respect to these parameters yields the Ginzburg-Landau equations[8]:

$$\alpha\psi + \beta|\psi|^2\psi + \frac{1}{2m}(-i\hbar\nabla - 2e\boldsymbol{A})^2\psi = 0 \tag{4}$$

$$\nabla \times \boldsymbol{B} = \mu_0 \boldsymbol{j_s}$$
$$\boldsymbol{j_s} = \frac{2e}{m}[\psi^*(-i\hbar\nabla - 2e\boldsymbol{A})\psi + \psi(-i\hbar\nabla - 2e\boldsymbol{A})\psi] \tag{5}$$

These equations can be used to calculate several characteristic values of a superconductor, including the penetration depth, λ (eq.6):

$$\frac{1}{\lambda^2} \equiv 4e^2\frac{|\psi_0|^2}{m}\mu_0 \tag{6}$$

The coherence length, ξ (eq.7):

$$\xi^2 \equiv \frac{\hbar^2}{2m|\alpha|} \tag{7}$$

The ratio of the penetration depth to the coherence length is called the Ginzburg-Landau parameter, κ, and is independent of temperature.

Bardeen-Cooper Shrieffer Theory

The first microscopic theory of superconductivity was Bardeen-Cooper-Schrieffer (BCS) theory (1957), which describes superconductivity as an effect caused by Cooper-pairs condensing into a Bose-Einstein condensate, forming a highly collective condensate in reciprocal, momentum space[10]. In the case of con- ventional superconductors, Cooper pairs are coupled electrons resulting from the interaction between the electrons and the phonons of the ion lattice[11]. Cooper initially showed this by imagining two electrons above the Fermi surface of a metal, assuming an attraction between them, and solving the Schrödinger equation. He determined the energy of the electrons to be:

$$E = 2E_F + E \tag{8}$$

Where

$$E = -2\hbar\omega_D \exp[2/N(E_F)V] \tag{9}$$

and ω_D is the Debye frequency of the lattice, and V is the attractive potential. As $\varepsilon < 0$, this shows that the pair of electrons always bind when there is any attractive potential between them. This paired electronic state has an energy lower than the Fermi level, meaning they are bound.

A simplified, classical description of Cooper pairing is as follows. Consider electrons in a metal acting as free particles. The attractive force between the electrons and ion lattice partially distorts the lattice, increasing the positive charge density in the region around the electron and attracting other electrons. At long distances, this attraction can overcome Coulombic repulsion. The pairing energy is very low (10^{-3} *eV*), so significant numbers of Cooper pairs are only observed in superconductors at low temperatures. This is a long-range interaction with the coupling interaction occurring over several hundred nanometres, orders of magnitude greater than lattice spacing. Electrons are fermions with $S = 1/2$, but the total spin of a Cooper pair is $S = 0$, making it a composite boson, meaning more than one Cooper pair can occupy the same quantum state (Pauli exclusion principle).

In BCS theory, one considers the formation of many Cooper pairs. This results in a

band-gap opening in the continuous spectrum of electronic energy states, (i.e. all excitations to the system must possess some minimum energy). Small excitations such as those due to electron scattering are forbidden, hence, they can flow through the material as a superfluid experiencing zero resistance. It should be noted that BCS theory reduces to Ginzburg-Landau theory near T_c[12].

This model can be validated experimentally via the isotope effect[13]. BCS theory predicts that heavier ions will lead to lower T_c as they won't be displaced as much by the electrons as their lighter counterparts ($\omega_D \propto (m_{ion})^{-1/2}$), resulting in a smaller binding energy for the Cooper pair. These results were confirmed experimentally by measuring the T_c of ^{202}Hg and ^{198}Hg[14]. More evidence for BCS theory includes the exponential rise in specific heat near T_c suggesting an energy gap. At low temperatures, C_v is strongly suppressed as there are no thermal excitations remaining, however, prior to reaching T_c, C_v becomes even greater than that of a normal conductor (as it varies with $e^{-\alpha/T}$ rather than linearly)[15].

McMillan determined the upper limit of T_c to be ≈ 42 K[16], however, in 1986, high-temperature superconductors were discovered which exceeded this limit. Such superconductors, which cannot be explained by BCS theory, became known as "unconventional" superconductors or UcS (note that the cuprates were the second class of UcS discovered after the Heavy fermions in 1978[17]).

Superconductor Classifications

There are four main methods of classification for superconductors:

1. Response to applied magnetic field: In a Type-I superconductor, superconductivity is lost when a field greater than a critical value H_c is applied. This is because the Gibbs free energy of the superconducting state varies quadratically with the magnetic field, whereas, the normal state free energy is field independent (i.e. above H_c it is thermodynamically favourable to return to the normal state). It is possible to obtain an intermediate state consisting of a baroque pattern of normal regions carrying magnetic field and superconducting regions carrying no magnetic field (figure 2).

In Type-II superconductors, increasing the applied field past H_{c1} leads to a mixed (or vortex) state. In such a state, increasing amounts of magnetic flux penetrate the material, but there remains no resistance to DC current flow provided the current isn't too great. This mixed state is caused by vortices in the electronic superfluid called fluxons (as their flux is quantised in units $\Phi_0 = h/2e$). Above a greater applied field strength, H_{c2}, superconductivity is lost.

2. Theory of operation: The term conventional superconductor refers to one whose operation can be explained by BCS theory, whereas, a UcS operation cannot be explained by BCS theory.

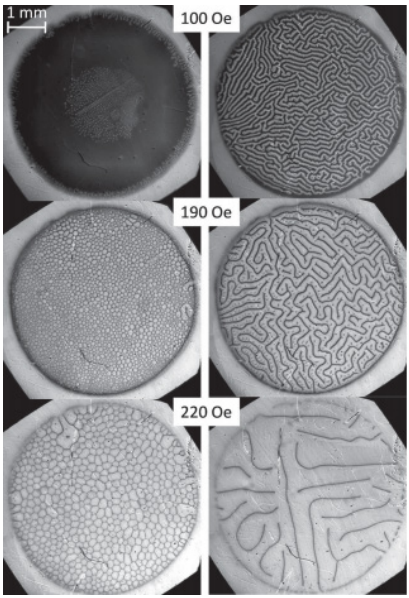


Figure 2: Intermediate states of a lead crystal at 5 K in increasing magnetic fields.[18]

3. Critical temperature: Superconductors can be either low or high- temperature. A HTS has a T_c above the limit predicted by BCS theory (42 K). The conventional superconductor with the highest critical temperature is MgB_2 with T_c = 39 K[19].

4. Material: There are a several materials which exhibit superconductivity including chemical elements, alloys, ceramics, iron pnictides, and organic superconductors.

High-Temperature Superconductors

In 1986, J. Georg Bednorz and K. Alex Muller were investigating the superconductivity of a type of ceramics known as cuprates. They discovered that the critical temperature of $La_{5-x}Ba_xCu_5O_{5(3-y)}$ was around 35 K, greater than predicted by BCS theory[20]. This new class of superconductors became known as high-temperature superconductors (HTS). The significance of the discovery was immediately recognized, with Bednorz and Muller winning the Nobel Prize in Physics the following year[21]. As of 2015, the highest known critical temperature is that of metallic hydrogen sulfide (H_2S) under incredibly high pressure (150 GPa)[22], with $T_c \approx 203$ K. Theoretical work suggests that a T_c of 280 K could be achievable by replacing some of the sulfur atoms with phosphorus[23].

Cuprate Superconductors

Cuprate superconductors have a multi-layered perovskite crystal structure which is both oxygen-deficient and distorted. A key feature of this structure are alternating layers of cuprate (CuO_2) planes and planes consisting of CuO chains (figure 3), with the copper atoms existing in both +2 and +1 oxidation states respectively. The superconductivity is believed to occur within such planes, and adding more layers leads to an increase in the critical temperature. Unsurprisingly, this structure is highly anisotropic, with the electrical resistivity in the plane perpendicular to the cuprate layers being much greater than the resistivity in the plane parallel to the layers.

Undoped cuprate compounds are Mott insulators, materials which should be conductors according to conventional electronic band theory methods, but in reality are insulators due to electronic interactions which the theories do not take into account.

At sufficiently low temperatures the compounds transition into an antiferromagnetic state resulting from repulsion between the electrons. To reach a superconductive state, cuprate compounds must be doped with electrons or holes. The similarities between the $d_{x^2-y^2}$ orbital of the antiferromagnetic (Mott insulator) state of the Cu^{2+} ions and their superconducting state suggests that electron-electron interactions play a more significant role in HTS than electron-phonon interactions. Cuprate superconductors do not demonstrate the strong isotope effect predicted by BCS theory and observed in LTS. The temperature vs. doping phase diagram of a cuprate superconductor exhibits a 'pseudogap' as shown in figure 4, an energy gap near the Fermi level.

Following Bednorz and Muller's discovery in 1986, cuprate superconductors developed rapidly. It was found that the critical temperature of barium- doped La_2CuO_4 increased to above 40 K under pressure[26]. By replacing the lanthanum atoms with smaller yttrium atoms, the first superconductor with $T_c > 77$ K (b.p. of liquid nitrogen) was discovered, YBCO[27]. This was of great significance as it did not require liquid helium to reach its superconductive state.

Similar cuprate superconductors exist in which the yttrium of YBCO is replaced with bismuth, thallium, or mercury. The highest critical temperature recorded under ambient pressure conditions was 135 K, which was measured for $HgBa_2Ca_2Cu_3O_{8+x}$[28].

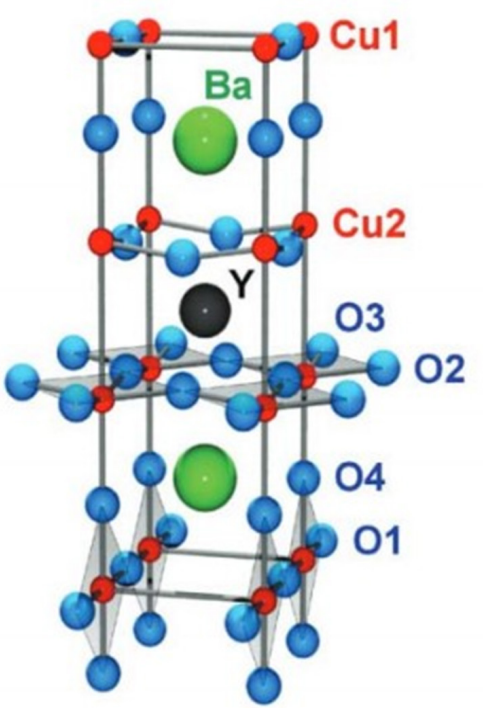


Figure 3: The crystal structure of YBCO.[24]

The advantages of cuprate superconductors their ability to withstand high applied currents and magnetic fields, and their high T_c (no liquid helium required). However, commercial applications have been limited as cuprates are brittle ceramics which cannot be easily formed into wires or other useful shapes. An important technique used for the characterisation of complex electronic structures such as cuprate superconductors is scanning tunneling microscopy (STS). STS was developed in part by Irish physicist J.C. Seamus Davis[29,30], who later went on to use the technique to characterise the physical[31] and electronic properties of BSCCO[32-34].

There are several projects either underway or in the planning stages which will utilise HTS for energy applications. These include the Holbrook superconductor project in Long Island (power transmission cables[35]), the Hydra project in New York (HTS power transmission cables with a built-in fault current limiter), and the Tres Amigas superstation in Clovis, New Mexico

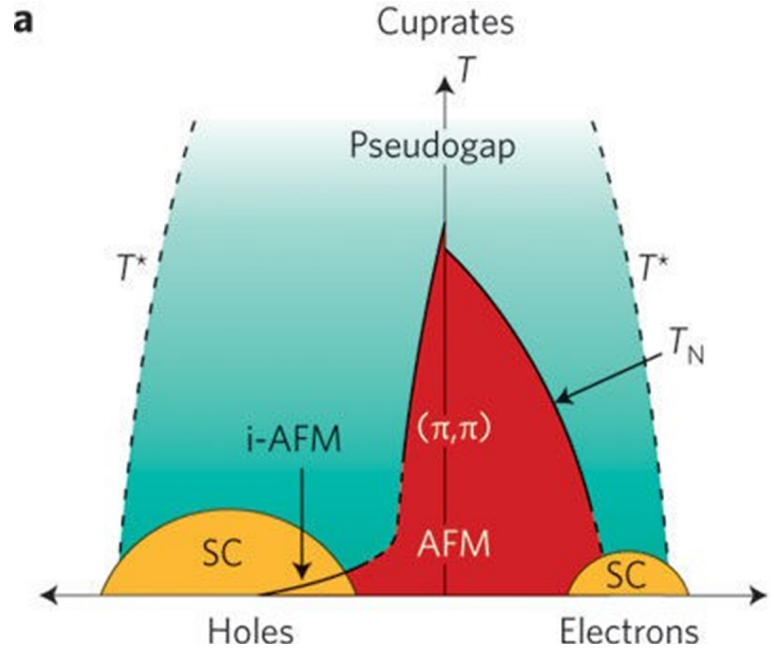


Figure 4: Phase diagram of a cuprate superconductor. The red region labelled "AFM" is antiferromagnetic Mott insulating, the yellow region marked "SC" is the superconducting, the blue region is the "Pseudogap" explained below, and the white region is the metallic regime.[25]

(HTS power market hub and energy storage system[36]). There is also hopes to one day replace the superconducting magnets in MRI and NMR systems with HTS alternatives[35].

Unconventional Superconductivity Theories

The theories of UcS differ from BCS theory mainly in their explanations of how the attractive potential between electrons which form Cooper pairs arises. One of the interesting characteristics on HTS are their strongly correlated d-electrons, as opposed to the s-electrons in conventional superconductors. In cuprates, the two-dimensional behaviour of the individual layers may also contribute to the superconductivity. The two most popular models for such behaviour are the weak-coupling model, in which antiferromagnetic spin fluctuations in the cuprate layers give rise to a pairing wave function with d-wave symmetry[38], and the interlayer-coupling model, in which the individual layers exhibit conventional superconductivity with s-wave symmetry and an additional tunnelling interaction[39].

In 1986, an experiment was performed attempting to determine the symmetry of the pairing wave function by measuring the flux quantization of a three-grain ring of YBCO at a junction interface as Cooper pairs tunnel through a Josephson junction[40]. A half-integer flux would be indicative of d-wave symmetry. The results of the experiment were ambiguous, and defects within the superconductor were believed to be responsible. A similar experiment was performed in which both a sample containing no defects and one with maximal defects were considered. This experiment demonstrated a half-integer flux, indicating d-wave symmetry[41].

However, YBCO has an orthorhombic crystal structure, meaning there may be an inherent s-wave component. The same group later determined that this s-wave component contributed about 3% to the wave-function mixture[42]. They also found pure d-wave symmetry in a thallium-based cuprate superconductor[43]. The fact that superconductivity occurs near a second-order antiferromagnetic phase transition suggests that spin fluctuations may mediate the Cooper pairing. Inelastic neutron scattering experiments on cuprates have shown a magnetic resonance below T_c which has an energy approximately proportional to the superconductive pairing energy gap, Δ [44], suggesting that magnetic resonance is somehow connected to the formation of Cooper pairs. Underdoped cuprate superconductors exhibit a 'pseudogap' which has yet to be adequately explained[45]. In this pseudogap, the density of states around the Fermi level decreases, the strength of this pseudogap is α $1/T_c$, another clue as to the origin of UcS. Cooper pair formation in BCS superconductors isn't overly sensitive to non-magnetic impurities[46], however, this is not the case in UcS. In such superconductors, the pairing is a function of the momentum vector, $\boldsymbol{k}$, of the electrons above the Fermi surface, but any impurities will result in a mixing of $\boldsymbol{k}$ values, inhibiting pair formation.

Figure 5 shows a linear relationship between the superconducting transition temperature and a characteristic temperature associated with spin fluctuations. This is highly indicative that the Cooper pairing in UcS involves an exchange of spin fluctuations[48,49]. It has been shown theoretically that this pairing interaction is antiferromagnetic spin-fluctuation mediated in the case of Hubbard-like models[50].

In the undoped cuprates, the electrons of the planar Cu atoms are in the d^9 state, these orbitals are split by the Jahn-Teller distortion from the surrounding oxygen atoms. This results in a partially filled $d_{x^2-y^2}$ which can form a covalent bond with the surrounding oxygen p_x and p_y orbitals. The cuprate therefore has the following orbitals: bonding, non-bonding, and

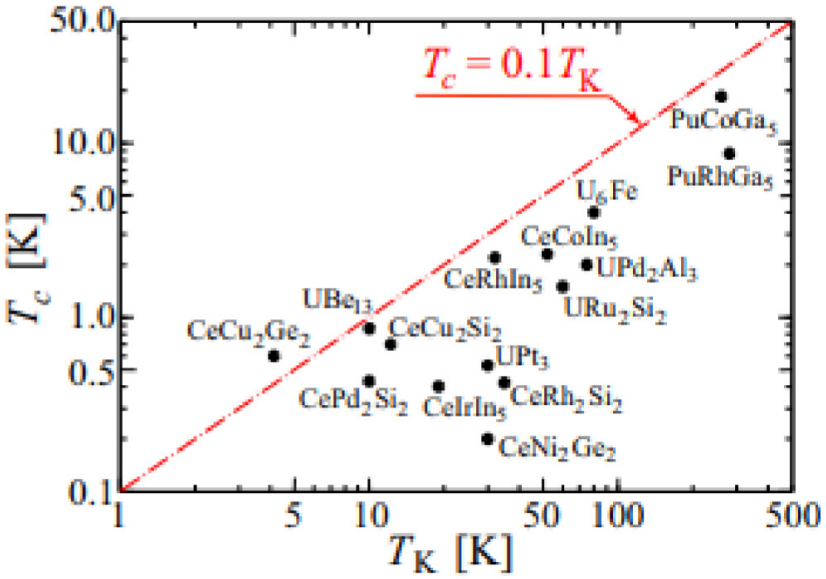


Figure 5: Relationship between the superconducting critical temperature, T_c, and characteristic temperature of the spin fluctuations, T_0[47].

half-filled anti-bonding. In the doped cuprate superconductors, there is an on-site Coulombic repulsion that is greater than the bandwidth of the anti-bonding electronic band, making energetically favourable for the electrons to be localized to an atom (this is why conventional band theory methods fail to characterise Mott insulators).

This Coulombic repulsion results in the further splitting of the anti-bonding band into two Hubbard bands separated by a few electron volts, the lower of which is completely full, and the higher completely empty. Such a system could be described by a three-band Hubbard model accounting for both the copper and oxygen orbitals[51,52], however, work by Fuchun Zhang and Thomas Rice (Irish-American physicist) suggest that a single-band model would suffice to describe doped cuprates. This is due to the formation of a singlet between the hole on the square of oxygen atoms and the hole on central Cu^{+2} atom (Zhang-Rice Singlet)[53].

The Hubbard model and similar t-J model for strongly correlated systems can be built upon, resulting in the resonating valence bond (RVB) theory. RVB theory was originally developed to describe organic compounds[54], and later re-purposed by P.W. Anderson to describe the cuprate superconductors[55,56]. In this model, the binding electrons can act as a mobile Cooper pair and form a condensate, leading to superconductivity as described by BCS theory.

Conclusions

Since the discovery of superconductivity over 100 years ago and the Londons' initial attempt to explain it 24 years later, our understanding of the phenomena at play has increased tremendously. The models for conventional superconductivity, both phenomenological (Ginzburg-Landau theory) and microscopic (BCS theory), are well established and adequately explain the phenomena observed in conventional superconductors (Meissner effect, isotope effect, etc.).

The microscopic origins of HTS remains a highly contentious field. Most UcS theories share a common theme, a modified BCS model with different causes of Cooper pair condensation. Despite our lack of insight into the exact mechanism, the future of HTS is bright, with several large-scale projects on the horizon.

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MIM Capacitors, Shockingly Important

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Metal insulator metal capacitors, since their discovery, have not only been critical for energy and data storage but also in computation, analogue-digital conversion and radio frequency communications. As was seen for transistor devices since the 1960s, the drive for smaller, more efficient devices has facilitated the funding and development of improved MIM capacitors. The increasing demand for portable devices has lead to the International technology roadmap for semiconductors setting out requirements for MIM capacitors until 2024 including capacitance densities > 12f F/μm^2, leakage current densities < 10^{-8} A/cm^2 and quadratic coefficients of capacitance < 100ppm/V^2. This review investigates approaches carried out to achieve these goals simultaneously focusing on the selection of materials of the dielectric, the combination of different dielectrics, and the optimisation of geometric structure utilised.

Introduction

Capacitors are used in energy storage[1], RF communication[2], memory devices[3], anti-counterfeiting[4], digital-analogue conversion[5], and decoupling[6]. Almost anything electronic will require a capacitor, and often devices that you would not expect to contain electronics contain them as security features. However due to the required capacitance values for most applications the devices are large and occupy a significant proportion of the available chip area. This is disappointing for such ubiquitous passive elements. With the push for minimisation, capacitors must also scale down. This is a critical step towards system on a chip technology.

Initial *Leyden jar* styled capacitors consisted of a glass jar covered internally and externally with layers of metal foil with the glass and air acting as the dielectric with a gap at the lip of the jar preventing the shorting of device. These were only capable of the production of capacitances of 1.1*nF*. The invention of radio, which demanded higher capacitance and high quality factor, low inductance, led to a

shift towards planar construction methods leading to the wide spread adoption of parallel plate styled capacitors. These planar devices could then be rolled to reduce their footprint area. So in the same way that the calls of technology required that new dielectric materials and geometries to be adopted to produce capacitors of suitable capacitance and quality factor, it now demands the production of high capacitance density devices that behave ideally with variation of voltage. Capacitors operate under the same mathematical basis regardless of their physical mode of operation, defined by equation 1. This gives rise to the definition of the Farad, 1F is the capacitance produced when 1C of charge is separated by a potential of 1V. For parallel plate capacitors this can be calculated using equation 2.

$$C = \frac{Q}{V} \tag{1}$$

Where Q is Charge and V is voltage.

$$C = \frac{c_0 K A^2}{d} \tag{2}$$

Where c_0 is the permittivity of free space, K is the dielectric constant, A is the effective area of the device and d is the distance between the two plates of the device.

The energy stored across a capacitor is calculated as 3:

$$W = \frac{1}{2} C V^2 \tag{3}$$

It is desirable to reduce the footprint area of devices so that more can be packed into the same space allowing for more energy to be stored in the same amount of space. To achieve the same capacitance there must also be an increase in effective area through an increase in areal density. This allows for the overall area of the capacitor, as treated by equation 2, to be maintained while the footprint area of the device normal to its surface is reduced. The International Technology Roadmap of Semiconductors[7] (ITRS) have set out a series of requirements for MIM capacitors. Capacitance densities > $12 fF/\mu m^2$ have been targeted for 2024, in addition to leakage current densities of $10^{-8} A/cm^2$ and quadratic voltage coefficient of capacitance < $100 ppm/V^2$.

To produce a high capacitance, a device would need to have a dielectric of high-K, such as HfO_2 with a K value of 19.6[8], a large effective surface area and a small distance between the plates. If a high capacitance were to be achieved then a lower voltage would be required to store the same amount of energy. This however, assumes that the capacitor behaves ideally. Empirically, it has been seen that high-K dielectrics display a non-linear response to the variation of applied potential. The

magnitude of these variations are further dependent on temperature, dielectric thickness, and device stress[9]. This leads to an empirical definition of capacitance density:

$$C_d = \frac{C}{A^2} = \frac{c_0 K}{d} \tag{4}$$

Where:

$$C_d(V) = C_{d0}(\alpha V^2 + \beta V + 1) \tag{5}$$

Where α is the quadratic voltage coefficient of capacitance (VCC), β is the linear VCC and C_{d0} is the capacitance density at zero applied potential. α and β can have both positive and negative values, indicating a deviation from the classical capacitance value as defined in Equation 1.

This results in capacitors which utilise high-k dielectrics behaving poorly. As such it is desirable to construct devices so that α is minimised and the behaviour of the device is more predictable. This presents challenges to increasing capacitance by the selection of dielectrics. To increase capacitance, a higher K dielectric can be selected but care must be taken to avoid non-ideal behaviour of the dielectric. Similarly the reduction in the thickness of the dielectric results in both the increase in the value of α as well as facilitating breakdown and leakage mechanisms reducing the quality of the device as well as its longevity. The effect of thickness on α has been shown empirically[10,11] as:

$$\alpha \propto \frac{1}{d^2} \tag{6}$$

Effectively preventing the downward scaling of dielectric thickness to increase capacitance. Additionally lower-k dielectrics such as SiO_2 under such scaling do not retain the expected properties, as leakage current increase as thickness decreases[12], suggesting the necessity of new dielectric materials to be produced if dimensional changes are to be made to future devices; a thicker layer of a higher-K dielectric would be able to achieve the same capacitance at a lower leakage current. If capacitance is considered in terms of the footprint area required to produce it, it follows that if a third dimension were employed that the areal density of capacitance could be increased for a given footprint area. This would allow for the production of devices with higher capacitance densities without the corresponding increase in leakage or α due to the quadratic dependence on area.

To fulfil the ITRS roadmap goals, the geometry of the devices must be such that a densely structured device is produced, minimising thickness and maximising

effective device area; the dielectric must be chosen so that the VCC values are minimised while retaining a sufficiently large K to maximise the capacitance. It is also required that the devices are stable with a low leakage current. Various leakage mechanisms are possible, many of which are quantum mechanical in nature, while others can arise from manufacturing processes. Due to the dependence of tunneling on the width of a barrier, it is necessary to minimise the leakage and VCC while still maximising the capacitance value creating difficulty in the selection of dielectric thickness due to the conflicting dependences.

Dialectric Material's Structure's Effect

It is possible to combine different dielectric materials in order to control the properties of the resulting capacitor. It is known that high-K dielectrics tend to have highly positive α values[13,14] while other materials have negative α values such as SiO_2[15]. If placed in series, these α values can negate each other. The devices however need not be separate. The different materials can instead be layered on top of each other to achieve the same behaviour in a smaller footprint. The thickness of the layers can be tuned in order to minimise α to almost zero. It was shown by *Kim et al.*[16] that it is possible to produce capacitors with high capacitance densities $\approx 6fF/\mu m^2$ while displaying the desired low α value of $14ppm/V^2$ through the use of layered HfO_2/SiO_2 dielectric materials, allowing for the positive quadratic term of one material to counteract the negative quadratic term of another to produce linear behaviour. Similar effects of the behaviour of dielectrics were observed in a comparative study of $Al_2O_3/HfO_2/Al2O_3$ (AHA) and $SiO_2/HfO_2/SiO_2$ (SHS) multi-layer systems by *Park et al.*[17]. It was observed that the AHA system displayed a capacitance density of $8fF/\mu m^2$ and the SHS system a capacitance density of $5.1fF/\mu m^2$. The AHA system was also able to maintain a low current density, $50nA/cm^2$, leakage at 1V, only one order of magnitude larger than the ITRS target for 2024. The quadratic VCC terms for SHS and AHA respectively were $31.9ppm/V^2$ and $694.1ppm/V^2$. This corresponds to literature indicating that SiO_2 dielectrics have negative α values[15,18] and Al_2O_3 dielectrics have positive α values[19].

In further studies, *Park et al.*[20] compared $SiO_2/ZrO_2/SiO_2$ (SZS) and $ZrO_2/SiO_2/ZrO_2$ (ZSZ) dialectrics, where for each dielectric laminated structure the total thickness of each material is approximately constant. It is observed that for SZS that $\alpha = 42ppm/V^2$ while for ZSZ layered structures $\alpha = -1094ppm/V^2$. This suggests that α is not a bulk property of the dielectric and instead depends on the ordering of the layers of the dielectric and their interactions with the electrode. Considering that ZrO_2 as a higher-k dielectric material than SiO_2, it has a higher defect density and a lower barrier height[21]. Further suggesting that a surface interaction with the metal is responsible for VCC. A capacitance density of $7.2fF/\mu m^2$ was recorded for both dielectric stacks, indicating that it is a bulk property corresponding with the expected behaviour of capacitors in series.

The use of multilayer structures is not isolated to tuning the linearity of capacitors. *Wu et al.*[22] have taken advantage of dielectric material selection to reduce the leakage current associated with high- K dielectrics instead of reducing the effects on VCC. This is due to high-K dielectrics possessing a low band offset between the dielectric and the metal, the corresponding unstable interactions arising as a result. Therefore, by surrounding the high-K dielectric with films of other high-K dielectric materials, it will increase the quality of the interface and reduce the leakage current due to the increase the barrier height. Additionally, it has been observed that high-K dielectric materials have a greater number of bulk traps and interface states than lower-K dielectrics after the application of an electrical stress, the addition of an interface layer of such a dielectric may reduce these negative effects.

Three Dimensional Thinking for a Two Dimensional Problem

Three dimensional structures allow for higher capacitance density for the same areal footprint. It is possible to produce three dimensional structures for capacitors in numerous ways, including imprinting, etching of pores/troughs, and bottom up synthesis. In works carried out by *Hourdakis et al.*[23], thin films of Al on Si were imprinted using a Si stamp to produce trenches of $1.8\mu m$ increasing the effective area by 58%. Atomic layer deposition (ALD), photolithography, and anodisation were used to produce capacitors both on the flat, unimprinted, surface and inside the trenches. This produced capacitors of areal capacitance density greater than the unimprinted equivalents. More significantly it was observed that for devices of equivalent capacitance density $10fF/\mu m^2$ the imprinted device produced a α value 45% smaller. This allows for the reduction of α in capacitors without the use of multilayer or laminated structures which would result in the reduction capacitance density, due to the use of a lower-K dielectric in the multilayer.

The selective anodisation and electrochemical etching of pores produces pores of well defined geometries and depth was studied by *Banerjee et al.*[24]. Allowing for the production of devices from a chemical process instead of a physical process. Producing devices with less precision but in greater yields. These pores can then be filled with MIM layers using ALD and anodisation techniques. In studies carried out on epitaxial Al, pores containing MIM layers of $TiN/Al_2O_3/TiN$ were formed. The resulting capacitor displaying a capacitance density of $36fF/\mu m^2$ and a leakage current of $< 3nA/cm^2$ [4].

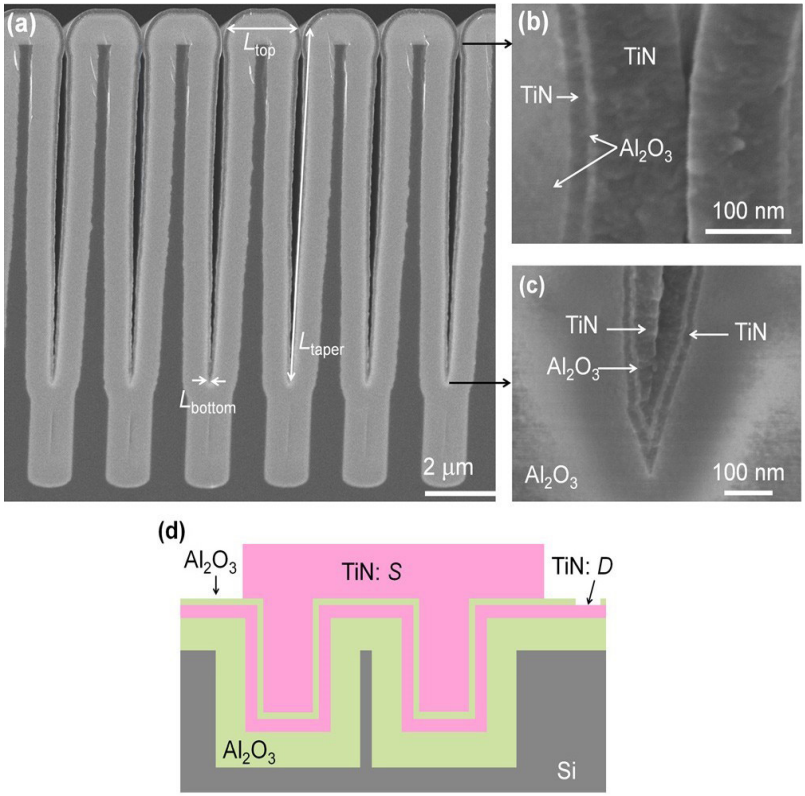

Figure 1: (A) Cross section of X-SEM image of pore TiN/Al_2O_3/TiN device. (B,C) High magnification image of TiN/Al_2O_3/TiN layered material structure at top (B), and bottom (C) of the pores. (D) Idealised schematic of pore array capacitor. *Adapted from Burke et al.*[4]

These top down self-assembly approaches allow for the production of finely packed capacitors without the use of stamps or lithographic tools, simplifying their production, reducing the cost, and increasing the scalability.

Bottom-up growth of capacitors is also possible through the production of vertically aligned carbon nanofibers, on the surface of a silicon substrate, acting as bottom electrodes where they can then be coated with an Al_2O_3 dielectric through ALD. Sputtering of Ti and Au on top of the alumina completes the MIM capacitor. It is observed to produce a capacitance density for the areal foot print of the device of 11-15nF/mm^2 corresponding to the use of 71% of the surface area of the nanofibers[25]. The benefit of this approach is similar to that of the pore synthesis. It sacrifices the well-defined geometries available through lithography or imprinting in favour of scalability.

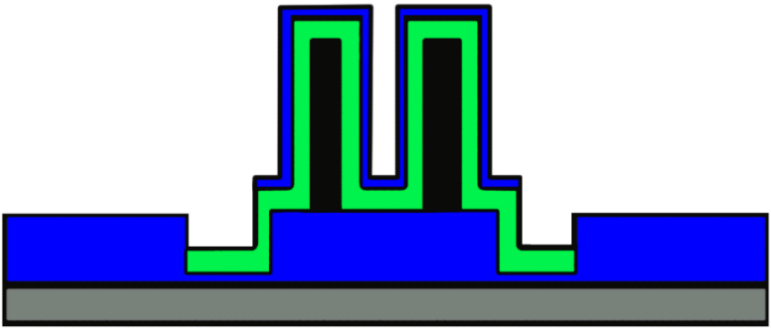

Figure 2: Cross section of geometry of vertically aligned carbon nanofibre grown capacitors, with Black: Carbon nanofibres, Green: Dielectric, Blue: Metal contact. *Adapted from Saleem et al.*[25]

Further attempts to increase the capacitance density have been carried out by the production of multilayered stacked capacitors inside pores, achieving capacitance densities of $440nF/mm^2$ and leakage currents of $10nA/mm^2$ at 3V. This allowed for several capacitors to be produced in a single pore and for greater control of their connections. This is due to the well-controlled thickness of the layers produced by ALD.[26]

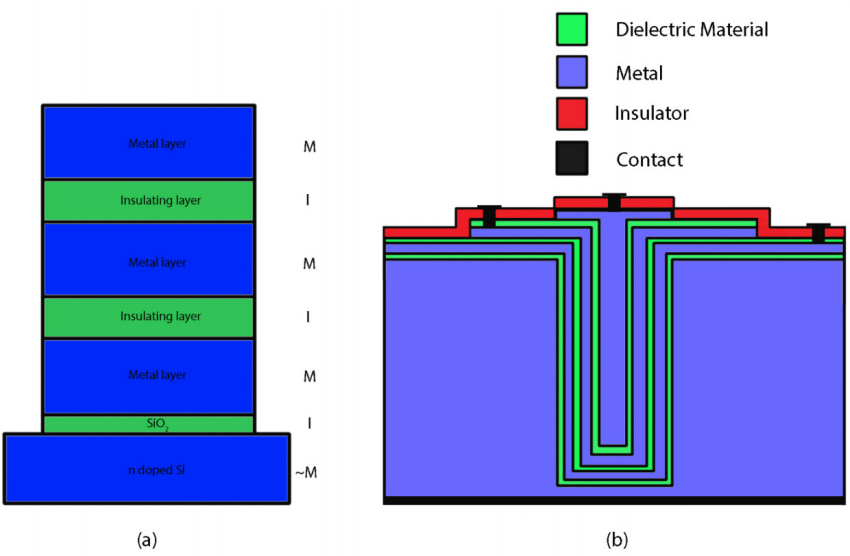

Figure 3: (A) Atomic layer deposition material order to produce capacitors. (B) Schematic of multi layer pore capacitor device. *Adapted from Klootwijk et al.*[26]

Conclusions

As the polarization of a dielectric is intrinsic in the operation of a capacitor it stands to reason that the dielectric material is critical in its future developments. It has been shown that high-k dielectrics are needed if thin film capacitors are to be produced. The high-k dielectrics present novel challenges of non-ideal capacitance-voltage dependence. Through material design and layering it is possible to utilise non-ideal high-k dielectrics to produce a capacitor that exhibits almost ideal behaviour. It has also been shown that through the targeted selection of dielectric pairings, that leakage current densities can be reduced through thin coatings of a dielectric of a different material than that of the main body of dielectric on the metal plates used in the capacitor construction.

To produce high-areal-capacitance-densities devices suitable for on chip applications, it will be necessary for devices to occupy a three dimensional structure. This has been achieved through the imprinting of thin films to produce trenches, through the chemical etching of pores and through the growth of geometrically controlled nanostructures used as both electrodes and to increase surface area. Beyond these approaches more complex geometries have been produced including the layering of discrete capacitor devices inside trenches capable of cumulatively producing large areal capacity densities. Due to the large capacitance density produced, the required dielectric thickness to produce a given capacitance in a given area is relaxed, allowing for the reduction in α.

Currently multilayered dielectric, three dimensional capacitors have not been achieved in literature. They present the potential to produce low leakage currents, ideally behaving capacitors and high capacitance density. If design limitations and can be surpassed, this region of further study has the potential of fulfilling the ITRS roadmap requirements for 2024 for MIM capacitors simultaneously.

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A Slippery Slope: Superlubricity and its Application in Nanotechnology

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Friction is the force that resists the movement of two objects that are in solid contact with one another. The phenomenon of mechanical friction is essential in industry and everyday life alike. But friction also has the drawback of limiting energy efficiency and damaging components of machinery. Approximately 30% of energy generated in transport and up to 20% of the energy in industry is used overcoming frictional forces, costing up to 1.4% of the GDP in some countries[1]. Lubrication or replacement of damaged components is trivial at the macroscale, but energy dissipation due to friction is a limiting factor in the development of nanotechnology. Surfaces at the nanoscale are much rougher in practice than we can perceive at everyday length scales. Manufacturing nanodevices such as nanomotors requires us to minimise the energy dissipation in order to them suitable for practical applications. The phenomenon of superlubricity, where the frictional forces between two surfaces vanish, has been proposed since the 1990s. Research in this area has grown considerably since the discovery of graphene with many fascinating phenomena due to ultra-low friction being reported. Research into superlubricity has led to the development of nanodevices such as motors and sensors. This progress has implications for fields as diverse as medicine and astrophysics, and is likely to continue well into the future.

Friction

Friction is the force that opposes the motion of a body. In classical mechanics, there are two distinct types of friction, static friction and kinetic friction[2]. Static friction prevents the sliding motion between two surfaces and is expressed as $|F| \leq \mu_s N$. Kinetic friction slows the sliding motion between two surfaces and is

given by $F = \mu_k N$ and is independent of the sliding velocity. N is a perpendicular normal force holding the surfaces together and μ is a coefficient that depends on the material. The coefficient of kinetic friction μ_k is less than that of static friction μ_s. This relationship works well for objects on the macro scale, but the interaction between surfaces of nanoscale materials is much more complex.

The Prandtl-Tomlinson model presents a highly simplified interaction where a particle is dragged across a surface[3].

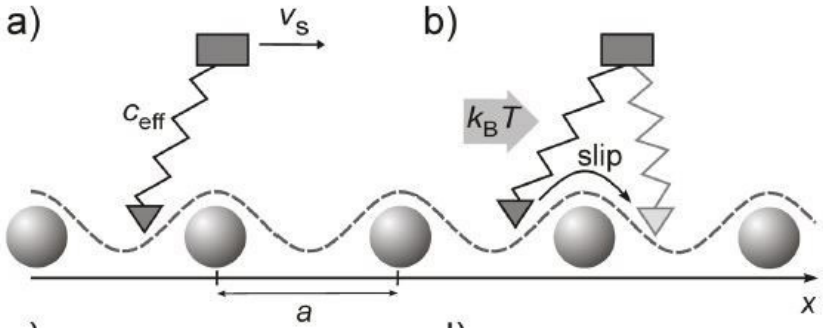

Figure 1: A representation of the Prandtl-Tomlinson model of the interaction between surfaces. *Schwarz U.D, Holscher H. Exploring and Explaining Friction with the Prandtl–Tomlinson Mode[3] Figure 1a).*

A particle connected via a spring to a mass travelling in one dimension is dragged over the surface, which is represented by a periodic potential. In physical terms, the particle represents an atom, the spring approximates an atomic bond, and the mass is the bulk mass which the atom belongs to. The periodic potential corresponds to the positioning of atoms on the surface, a maximum potential occurs at the position of another atom.

This configuration leads to a "stick-slip" motion as the particle is dragged across the potential. The particle moves very slowly up the sides of the potential barrier before moving quickly forward once it reaches the top. This model describes how friction arises from interactions on an atomic scale. These interactions therefore play an important role when investigating materials on the scale of nanometres.

History of Superlubricity

The term "superlubricity" was coined in a 1993 paper by Shinjo and Hirano[4,5] to refer to the vanishing of frictional forces under certain circumstances. They investigated the Prandtl-Tomlinson model with a chain of particles coupled to a substrate potential, by metallic bonding or van Der Waals forces for example. Figure 2 represents a simplified example of this system. If the chain of particles

were to be 'commensurate' with the surface below (ie. the particles of the chain resting in the valleys of the surface below), then the attempt to drag the chain across the surface would result in the same "stick-slip" motion predicted by the Prandtl-Tomlinson. However, if the chain was oriented in such a way that the particles are 'incommensurate' with the surface below, the particles instead line up with the atoms of the surface and the potential barrier each atom must overcome is greatly reduced. Therefore, when the chain slides over the substrate, the "stick-slip" motion is less pronounced than in the commensurate orientation.

Figure 2: Representation chain of particles upon a substrate potential, incommensurate to the potential. *van den Ende J, de Wijn J.A. The effect of Temperature and Velocity on Superlubricity*[5] *Figure 1.*

Using this theoretical model with kinetic energy terms, the friction was analysed as a function of the degree of coupling and the velocity of the chain. They determined that, under certain conditions, the chain could slide almost indefinitely without experiencing kinetic friction. This was called superlubricity.

In 1997, an experiment performed by Shinjo and Hirano[6] experimentally observed superlubricity between a clean Si(001) surface and a clean W(011) surface. The force of friction measured when the surfaces were in the predicted superlubric condition was found to be below the resolving power of $3x10^{-9}$ N which their equipment could measure. In comparison, the force measured in the friction region was found to be approximately $8x10^{-8}$ N, so the friction was reduced by at least one order of magnitude.

However, a 2000 paper by Consoli[7] disputed some of the findings of Shinjo and Hirano. The paper presented a model showing that the superlubricity of the system will break down over time due to dissipative resonance involving phonons. The small size of the system and low sliding velocities in the Shinjo and Hirano experiment meant that this effect was not observed.

One of the most significant experiments was performed by Dienwiebel *et al.*[8] in 2003. A tungsten-tipped friction force microscope capable of detecting forces as low as 15 pN was scanned across a graphite surface. The tip recorded a stick-slip motion consistent with the lattice period of the graphite and very low frictional forces on the order of 20 pN. They rotated the substrate relative to the tungsten tip and noticed there was much higher friction at certain angles of rotation (Figure 3).

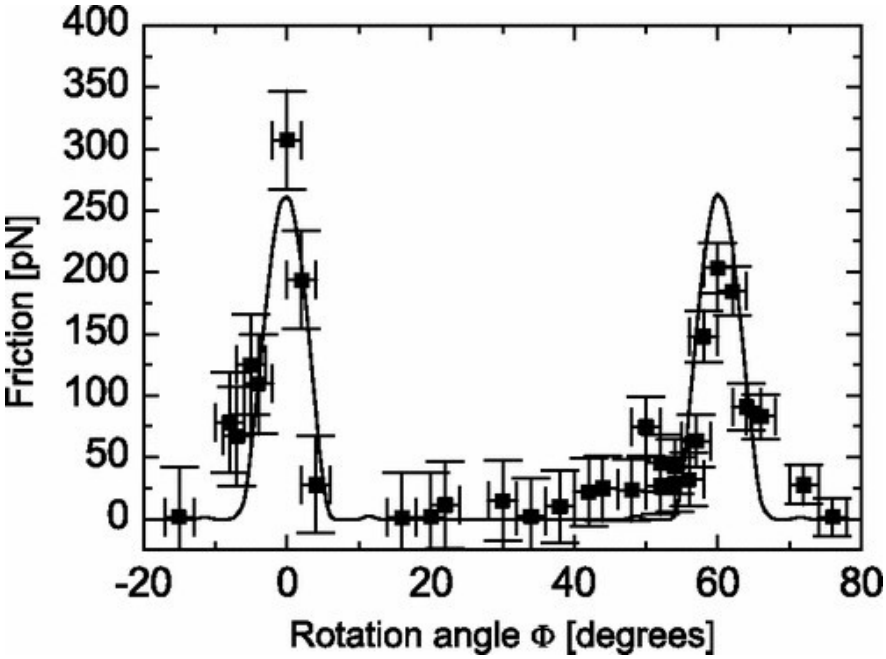

Figure 3: Friction measured by a tungsten tip sliding across a graphite substrate as a function of the angle of rotation of the substrate. *Dienwiebel M, Verhoeven G. Superlubricity of Graphite* [8] *Figure 3.*

There are two orientations where friction reaches a maximum, likely corresponding to loose graphite flakes at the surface becoming commensurate with the graphite substrate. Figure 3 shows that this high-friction orientation repeats after a rotation of 60°, corresponding with the symmetry of layers in a graphite lattice.

Interesting Mechanisms due to Superlubricity Self Retraction of Graphite

One of the consequences of very low friction at an interface is that motion can be caused by even very small forces. Spontaneous motion due to superlubricity has been observed after shearing a graphite mesa at the microscale[9]. Figure 4 shows an experiment in which a graphite mesa (a raised structure with steep sides obtained through lithography) was sheared and underwent self-retraction.

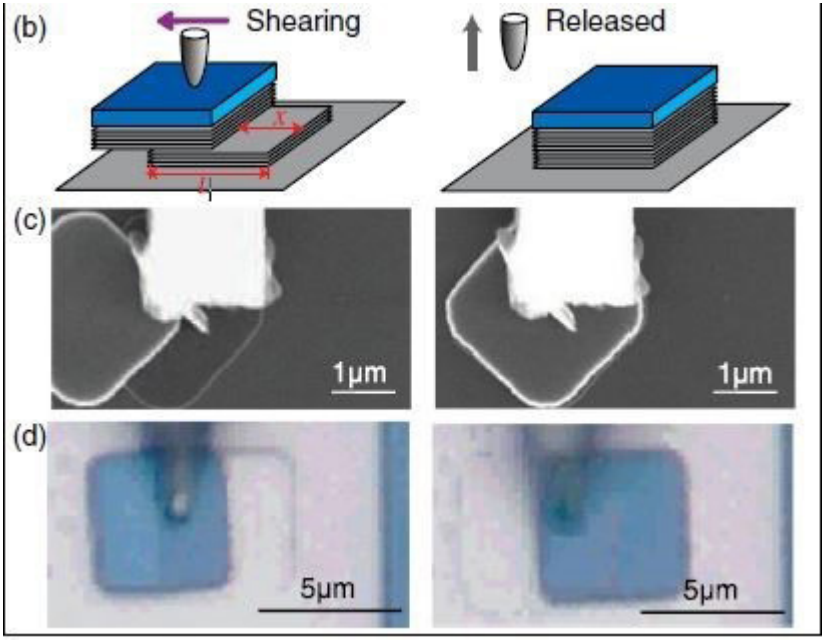

Figure 4: (b) Illustration of a mesa being partially sheared with a micromanipulator, to form a self-retracting flake on a graphite platform. When the microtip is raised to release the flake, it automatically returns to its original position on the mesa.(c) Observation of this process in a vacuum in a SEM. (d) Observation of the same process under ambient conditions with an optical microscope. *Liu Z, Yang J. Observation of Microscale Superlubricity in Graphite9 Figure 1 b), c), d)*

A tungsten microtip shears the mesa, increasing the surface area and therefore the surface free energy. When the tip releases, the flakes can move due to thermal motion or van der Waals forces. Once a graphite flake moves to a position of lesser surface energy, it is unlikely to move back to a position of higher energy. The net result of this is that the mesa returns to its lowest energy position, a single stack. In other words, the mesa spontaneously retracts once the tip is removed.

Sliding and Telescoping of a Double-Walled Carbon Nanotubes (DWCNT)

A similar effect is seen in DWCNTs, where the lowest energy position is when the inner CNT is contained entirely within the outer shell. In this position, there is no energetically preferred orientation of the nanotubes[10].

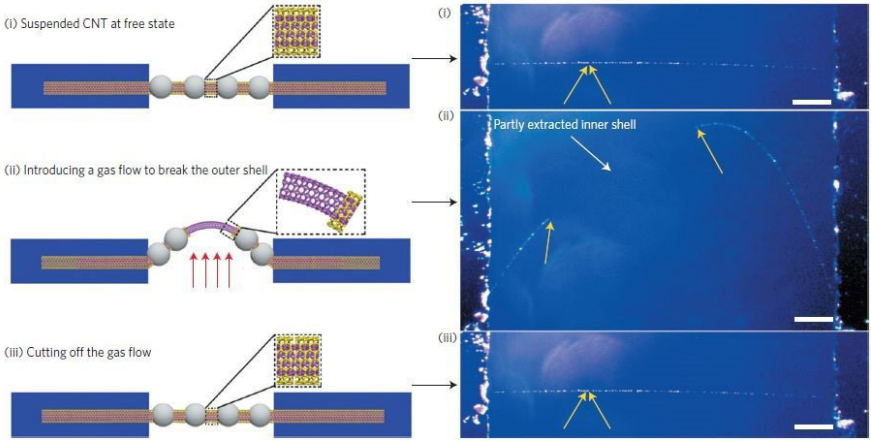

Figure 5: Illustrations (left) and optical images (right) showing inner-shell sliding processes for ultralong DWCNTs (scale bars in (i)–(iii), 100 mm). Yellow arrows in (i)–(iii) indicate the TiO2 nanoparticles at the breaking point of the outer shell[10]. *Figure 1 f)*

Scrolling of Graphene onto Fullerene String

Graphene is a two-dimensional sheet of sp^2-bonded carbon atoms in a hexagonal lattice, graphite consists of multiple layers of graphene. A fullerene string, a group of fullerene molecules connected by a binding molecule, placed on a graphene nanosheet (GNS) becomes enfolded within the sheet[11]. Van der Waals interactions between the fullerene molecules and graphene allow the GNS to scroll onto the fullerene. Figure 6b demonstrates how this mechanism evolves with time.

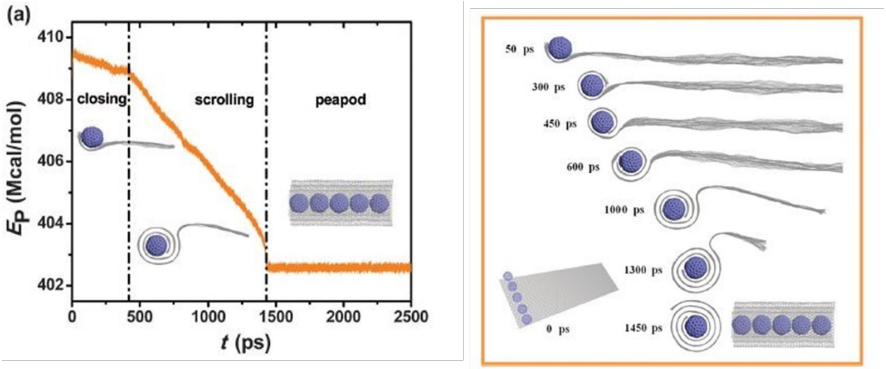

Figure 6: Scrolling of GNS onto fullerene strings. (a) The potential energy (E_p) of the whole system as a function of simulation time in the process of helical wrapping.[11] *Xu S, Fu H. Novel scroll pea pod produced by spontaneous scrolling of graphene onto fullerene string*[11] *Figure 3 a)*

(b) Spontaneous scrolling of a GNS onto five C180 balls to form a novel scroll pea pod.[11] *Figure 3 a)*

The fullerenes end up enfolded within the GNS like peas in a pod. This occurs because the lowest energy position of the GNS is folded onto itself in a AB stacking configuration. Figure 6a shows the reduction in potential energy at each stage of the mechanism.

Recent Developments

The isolation of graphene by Professors Andre Geim and Kostya Novoselov in 2004, as well as improvements in equipment has led to an explosion of research into the phenomenon of superlubricity. Graphene has displayed remarkable electronic properties as well superlubric tendencies due to its structure. The study of graphene as well as CNTs has been crucial to the development of nanoscale electromechanical systems (NEMS) such as nanomotors and nanosensors.

Nanomotors

The superlubric properties of multi-walled CNTs (MWCNTs) allow for them to be used as nanomotors. As there is no energetically preferred position for the inner tubes within the system, they are free to rotate and slide along the axis of the outer tubes. Harnessing these motions has led to the development of both rotary and translational nanomotors. The negligible amount of friction opposing the motion of the tube means that its motion can be influenced by controlling the conditions of the system. A rotary motor operates by the rotation of the tubes with respect to each other.

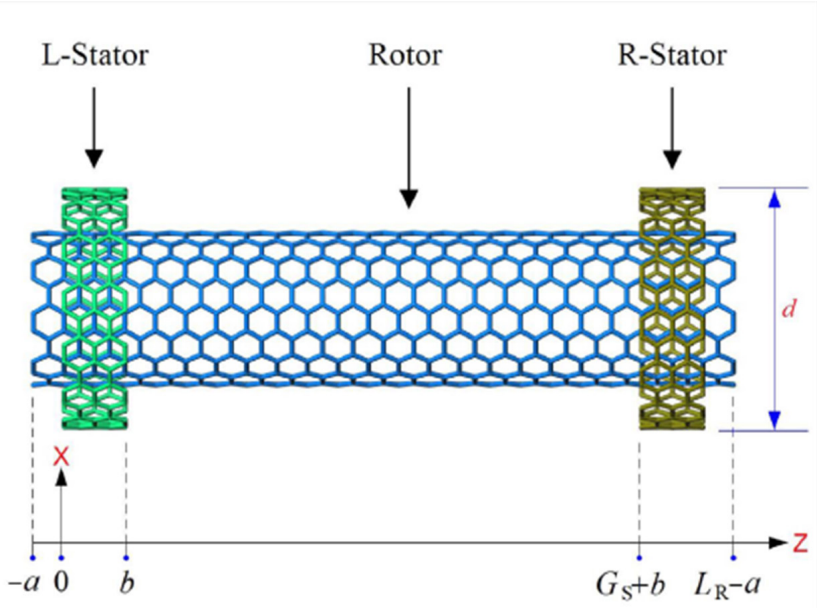

Figure 7: A CNT rotary motor. *Cai K, Wan J. Quantitative control of a rotary carbon nanotube motor under temperature stimulus*[12] *Figure 1 a)*

Figure 7 shows a rotary motor based on a DWCNT where much of the outer tube has removed in order to form stators (stationary portion of a motor). *Cai et al.*[12] found that a greater environmental temperature resulted in a higher rotational speed of the rotor. With no friction to oppose this rotation, the speed is therefore very sensitive to the temperature. This could allow us to fine-tune the speed at which the motor operates, giving a level of flexibility when integrating these motors into nanotechnology.

Temperature can also be used to influence linear nanomotors. Linear motors based on MWCNTs can act as nano-oscillators capable of GHz frequencies[15]. This is due to the telescoping of inner tubes followed by self-retracting, which is only possible in the presence of ultra-low friction. Experiments have shown that the range of frequencies produced is dependent on the diameters of the CNTs[16].

The potential applications for components such as nanomotors and nano-oscillators in future technology are endless. Their ability to power complex nanomachines could revolutionise medicine as well as our ability to manipulatematter at the micro and nano level.

Nanosensors

Development of nanosensors has a clear advantage over larger sensors, their sensitivity allows for greater accuracy and their size means they can be used safely in the human body. Researchers have been able to apply some of the mechanisms resulting from ultra-low friction to sensor technology. Nanosensors are also desirable for use in spacecraft as component weight is a major consideration when launching satellites or probes.

Take, for instance, the nanoscale inertial measurement unit (IMU) that has been developed which utilises the self-retraction of graphite[13].

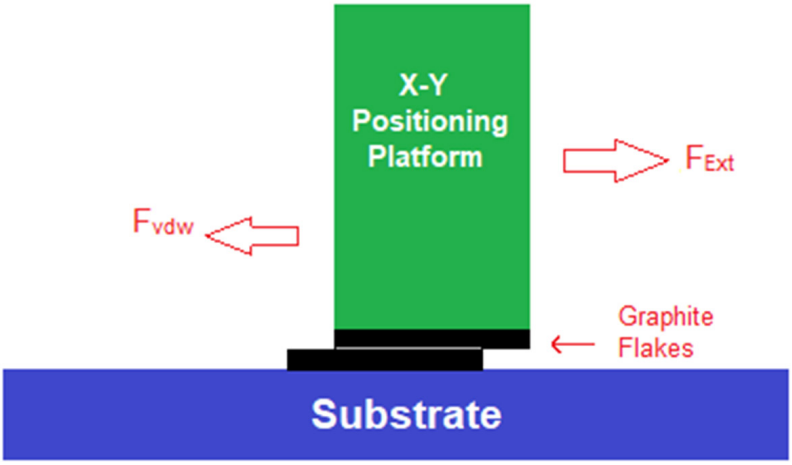

Figure 8: IMU with graphite flake. The x-y positioning platform slips due to external force (F_{ext}) and is restored due to the van der Walls forces (F_{vdw}).

An external force applied to the graphite flake causes it to telescope before retracting back to its original position once the force is removed. The positioning platform is used to detect displacement in the X-Y direction.

Once again, the lack of resistance to motion means that the system is very sensitive to external forces. This technology could be used in motion sensors, in experiments that require the measurement of extremely weak forces or in spacecraft as a low-weight component.

Macro-Scale Superlubricity

While microscale and nanoscale superlubricity has implications for nanotechnology, there have been results that suggests that the advantages of ultra-low friction may

be realised on a larger scale.

A high power pulsed plasma enhanced chemical vapour deposition technique was used to deposit graphene layers in a unique structure similar to a human fingerprint[14], as seen in (Figure 9).

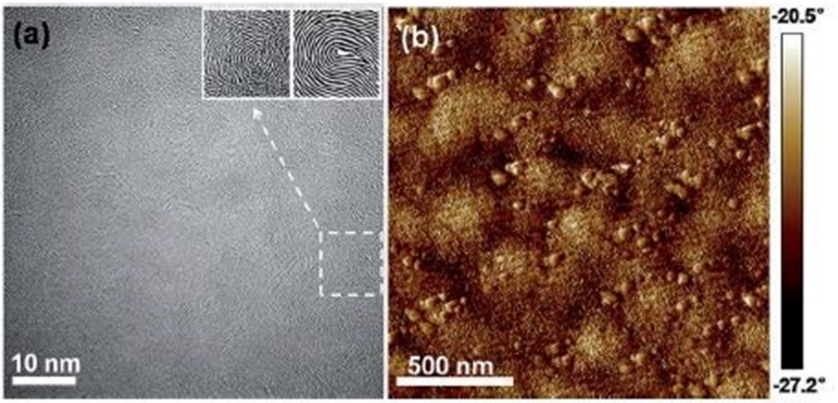

Figure 9: (a) High-resolution transmission electron images of the fingerprint-like carbon films. Insets: carbon fingerprint and human fingerprint pattern. (b) An AFM phase image of the fingerprint-like carbon film deposited on a silicon wafer. *Gong Z, Shi J. Engineering-scale superlubricity of the fingerprint-like carbon films based on high power pulsed plasma enhanced chemical vapour deposition [14] Figure 1*

The structure was found to result in hardness, elastic recovery and a low friction coefficient in both dry and humid conditions. Although not yet tested on the macroscale, the researchers who developed the films believe they could be used on the engineering scale. If these thin films could be used as a lubricant, the savings to industry due to increased energy efficiency could be dramatic.

Conclusion

Our understanding of how surfaces interact on the nanoscale has grown rapidly over the last two decades. The phenomenon of superlubricity, first hypothesised in the early 1990s, is now at the cutting edge of modern technology. Researchers have observed fascinating phenomena due to ultra-low friction, from the self-retraction in graphite and graphene to the sliding and rotation of MWCNTs. Not only have we achieved an understanding as to how van der Waals forces, thermal motion and other effects cause these mechanisms, but we are now beginning to manipulate them into useful components. We have harnessed the motion of MWCNTs to create nanoscale motors and oscillators, the self-retraction of graphite is being used as the

basis of inertial sensors and graphene films may soon become an engineering scale lubricant. We are already investigating how to optimise the function and minimise the energy dissipation of these components by influencing their environments, temperature and CNT diameter have already been used to fine-tune the operation of nanomotors. We will likely see research into the effect of applying electromagnetic fields or more complex substrates on the behaviour of these superlubric regimes. Understanding and utilising superlubricity is bringing us closer to the creation of complex nanoelectronics and systems. These advancements would revolutionise various fields of science. The cost of sending advanced probes into space would be less prohibitive as the mass of the instruments is reduced. Material scientists could utilise nanomachines to manipulate matter with never before seen precision. Minimising the obstructions of friction is allowing science to push the limits of technology further than ever before.

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