Proof of Concept Awards– Outcomes of Projects funded by the Metals in Biology Network

ID number	**POCMiB002**
Title	Enhancing *E. coli* for optimal cofactor insertion into heme and iron-sulfur cluster proteins
Academic (lead) Partner	Nick Le Brun, University of East Anglia
Industrial Partner	Mark Blight, Biocatalysts Ltd
Public summary	Metals as cofactors of proteins and enzymes are essential for all of life. Many metalloproteins have properties that are useful outside of the cell, for example in the synthesis of useful materials or medicines. However, to exploit this, the metalloprotein must be purified with the metallo-cofactor fully incorporated; incomplete incorporation results in major inefficiencies in the production process. A strategy to improve cofactor insertion is to more carefully match protein synthesis with cofactor synthesis/insertion. While in some cases this can be achieved by manipulation of the growth conditions, for example by slowing down protein synthesis, for commercial processes where protein yields are key, important economic and production benefits should be achievable through the engineering of cell factories to increase their capacity to incorporate the cofactor. In this project, the academic partners, who are experts in iron metabolism and iron-protein chemistry, propose to generate strains of E. coli that have significantly increased capacity to incorporate iron sulfur clusters and heme into a range of protein targets. These strains, together with growth protocols developed to use them to best effect, will be tested for the production of examples of commercially relevant iron-sulfur and heme proteins by the commercial partner.
Start date	1 August 2015
Public summary of project outcomes	Enzymes and proteins are widely used in commercial processes, for example in the food industry, for the production of fine chemicals, in the detergent industry, as medicines, in diagnostic kits and in emerging fields such as bioenergy. Metals as cofactors of proteins and enzymes are essential for all of life, and metallo-proteins/enzymes represent a significant subgroup of commercially important enzymes/proteins. A potential problem for the use of metallo-enzymes is that their activity is wholly dependent on the cofactor (which is usually the active site at which the catalytic process occurs) and so the efficiency of production is dependent on the efficiency of cofactor insertion. Expression systems are capable of producing a target peptide up to 50% of total cell protein; in many cases, the metallo-cofactor and insertion apparatus of the cell cannot keep up with protein synthesis. This results in major inefficiencies in the production process. An obvious answer is to carefully match protein and cofactor synthesis/insertion. This can be achieved, in some cases at least, by slowing down the rate/reducing the extent of, protein synthesis such that the cofactor pathways can better keep up. However, this is unlikely to be suitable for a commercial context where achieving maximum yield is key. In 2015 we applied for and were awarded a PoC grant from the

	BBSRC Metals in Biology Network in Biotechnology and Bioenergy (Metals in Biology NIBB) to work alongside Biocatalysts Ltd, an SEM based in Cardiff that produces enzymes for various sectors, to tackle the problem of low cofactor insertion into proteins. Approximately half of the enzymes products of Biocatalysts are metallo-enzymes and efficient cofactor insertion is a problem that is impacting on their business. One of these, a heme- containing P450 enzyme, alone has significant (>£500,000 per annum) potential for Biocatalysts, but this opportunity is currently hindered by poor production titres. In order to realise the commercial potential, it is vital that Biocatalysts increase the heme incorporation levels in the enzyme, thereby reducing the required production level and, in doing so, bringing the process economics to a commercially viable level. Although initially aimed at both heme and iron-sulfur cofactors, following advice, we focussed entirely on the enhancement of heme incorporation.The project ran from August 2015 until May 2016. We explored several different strategies, including over-expressing parts of the heme biosynthetic pathway and expressing membrane bound importers capable of taking up intact heme from the growth medium. Through the excellent efforts of the PDRA employed on the project, Dr Robert Green, we have made significant progress towards our goal. Using Escherichia coli (a workhorse for protein production), we have generated a system that gives ~3-fold enhancement of heme incorporation into two very different 'test' heme proteins (one bacterial, one human). Preliminary results with a third 'test' protein indicated that the effect is reproducible for other heme containing proteins. Furthermore, up to now we have not seen any negative effects on yield of protein. These are all important properties for a process that could be commercially useful.

ID number	**POCMiB016**
Title	Proline Hydroxylases for Biocatalysis
Academic (lead) Partner	Chris Schofield, University of Oxford
Industrial Partner	Daniel Brookings, UCB Celltech
Public summary	Biocatalysis is a successful method for the industrial production of small- or macro-molecules and can enable production of molecules that are difficult, expensive or often impossible to synthesise. Metallo-enzymes (biological molecules that use metals to catalyse chemical reactions) are abundant in nature and are used by microbes for biosynthesis of small-molecules such as antibiotics or for the modification of biological macromolecules. The long term objective of this project is to develop and provide access to metallo-enzymes useful for biocatalysis in cells on an industrial scale. In collaboration with UCB Celltech we will focus on enabling discoveries that will support useful bio- catalysis and bio-transformations for the generation of end products or intermediates involved in the manufacture of biofuels, pharmaceuticals, fragrances, or flavours and also for the late stage diversification of tools for drug discovery (i.e. compound libraries) in a sustainable manner (i.e. Green Chemistry). There is a current lack of

	accessible libraries of oxygenase enzymes suitable for use in biocatalysis and little information on how their activity is limited by metal binding in cells. Our wide range of resources will allow us to efficiently explore and engineer novel methods for accessing industrial production of new chemical entities.
Start date	12 November 2015
Public summary of project outcomes	Enzymes are incredibly powerful biological catalysts that already have widespread applications in industry and the home. In microorganisms they make an enormous range of natural products, some of which are incredibly useful medicines, e.g. the penicillins. One of the strategies nature uses in the biosynthesis of many natural products is to modify a common intermediate to give multiple other products, which may have very different biological functions. Harnessing the power of such late stage modification has potential to generate many molecules from a single drug candidate. Such a process could expedite drug discovery by enabling the efficient discovery of optimised molecules. However, the natural enzymes are often not suitable for such use, because they have evolved to catalyse the production of specific products. What is needed are less selective enzymes, that can be subsequently optimised to be made selective if need be. Our BBSRC NIBB work concerned studies on a family of metal dependent enzymes that add oxygen, or sometimes chlorine or bromine, atoms to drug-like small-molecules and proteins. To accomplish these reactions the oxygenases employ oxygen from the atmosphere and iron in its ferrous form, as well as a common small-molecule metabolite, 2-oxoglutarate (2OG). Our BBSRC NIBB project principally focused on a specific subfamily of these enzymes that catalyse the addition of oxygen, or hydroxylation, to proline, an amino acid with a 5-membered ring. In one line of investigation we explored the proline hydroxylases for its selectivity towards different ring sizes and substitutions. We found that they can catalyses the hydroxylation of an unexpectedly wide range of rings, including tricyclic ring structures; some of these products are precursors for conversion into potential antibiotics. We also explored how these hydroxylases bind iron, using both assays for product formation and by X-ray crystallographic analyses of ‘mutant’ enzyme structures. Interestingly, we found that the proline hydroxylases can work with only two, rather than the normal three, points of attachment (ligand) of iron to the protein. These results inspired us to study metal binding by enzymes in cells (employing mass spectrometry) and to study variations on iron binding by other types of hydroxylases. In one case we found the hydroxylase can work with only one protein ligand.

ID number	**POCMiB022**
Title	New routes for expression of heme protein targets
Academic (lead) Partner	Emma Raven, University of Leicester
Industrial Partner	Andreas Kuglstatter, Roche Innovation Center
Public summary	Heme-containing enzymes are a mainstay of industrial biotechnology, and the industry depends on fundamental improvements in methodology emerging from academic groups

	around the world to harness the potential of their investments in biopharmaceuticals, bioenergy, biocatalysis and drug design. For a number of complex reasons, the interactions between industry/biotechnology and academic laboratories are often less facile and less extensive than they could be, so that new (often specialist and/or unpublished) information is not transferred fluently to industrial partners. Our overall objective is to use this project to develop new refolding methodologies for expression of difficult (insoluble) heme protein targets, and to set up an on-going dialogue between industrial and academic partners with mutual cognate interests in specific heme enzyme targets. The methodologies that we develop will open up new avenues for industry partners in cases where they have intractable (insoluble) protein targets.
Start date	27 November 2015
Public summary of project outcomes	In this project, new expression methods were developed for a range of different heme enzymes that are not readily expressed using conventional methodologies in E. coli. Targets from the Raven laboratory were used as a “test bed” for other heme systems. Human CLOCK protein was included amongst the targets, which is important in circadian control and thus an important drug target.

ID number	**POCMiB024**
Title	Tailoring the in planta synthesis of metal nanoparticles for production of high-value catalysts
Academic (lead) Partner	Neil Bruce, University of York
Industrial Partner	Richard Bate, Yorwaste Ltd
Public summary	Platinum group metals (PMGs) are used in an ever-expanding arrange of technologies and demand is spiralling upwards. PGMs are rare, exist in low concentrations and expensive to mine. It is essential that these metal reserves are utilised and recycled responsibly, not dispersed and lost into the environment. Plants can take up metals from their environment, and, in the case of PGMs, can deposit them as nanoparticles within their tissues. Nanoparticles have remarkable properties, when compared to the bulk of the same metal, which have been exploited. For example palladium nanoparticles are important catalysts for many pharmaceutical applications. Although currently synthesised chemically, we have shown that plants containing palladium nanoparticles can also be used to make efficient biocatalysts. These biocatalysts utilise carbon-neutral plant biomass, reduce processing steps by using the nanoparticles together with the plant material and concentrate valuable metals from waste sources. The addition of specific peptides (very small proteins) to solutions of metals increases nanoparticle formation, and alter size and shape; factors that can be used to optimise catalysts for different processes. This proposal is to investigate if expression of peptides in plants can be used to increase the formation, and control the size of, plant-derived biocatalysts.
Start date	1 November 2015
Public summary of project outcomes	We have achieved our objectives to investigate NP formation using synthetic peptides and analyse catalytic activity in the

	subsequently pyrolysed NP-containing plant biomass. Our promising ICP-OES, TEM and catalysis results demonstrate that the expression of synthetic peptides in plants can be used to alter gold NP size and subsequent catalytic activity in planta. As part of our third objective, to determine if plants could be used to selectively take up PGMs from sweeper wastes, we have shown that sweeper wastes contain detectable levels of valuable metals. However, our studies show that further work is needed to understand the phytotoxicity behind these wastes so that they can be optimised to allow plant growth. Our wider studies indicate that synthetic biology could be used to develop plants that can selectively take up PGMs from sweeper wastes.

ID number	**POCMiB011**
Title	Mag-Tag: magnetite nanoparticle affinity tags for industrial biotechnology protein purification
Academic (lead) Partner	Sarah Staniland, University of Sheffield
Industrial Partner	Mark Blight, Biocatalysts Ltd
Public summary	Enzymes are protein catalysts which can perform highly specific biotransformations to convert starting materials into desired (often) complex products using mild, aqueous reaction conditions. These key capabilities are difficult to achieve with conventional catalysts, making enzymes ideally suited to the industrial manufacture of foodstuffs, biofuels, pharmaceuticals and a range of other industrial biotechnology. The current challenge to the widening the use of enzymes is the expense of producing them. Large scale industrial purification of enzymes is often prohibitively costly due to the need for expensive, highly functionalised purification resins. This cost barrier limits the use of enzymes to industrial applications where unpurified, crude enzymes are suitable, or where the end product is sufficiently prized to enable the cost of purification to be recouped. We propose a revolutionary, cheap, universally applicable, enzyme purification method to widen the use of purified enzymes in industry. We will use protein fusion-tag technology to purify enzymes directly from crude preparations using cheap, unfunctionalised magnetic iron-oxide nanoparticles, which can then be bulk purified through magnetic separation. By substantially reducing the costs of purification we seek to make enzymes an affordable, green and sustainable method of producing a wide range of products.
Start date	1 October 2015

Public summary of project outcomes	Proteins perform a wide variety of different functional roles in biological systems and represent a rich source of biological reagents for industrial processes. Enzymes for instance, carry out highly specific biotransformations, converting substrate molecules into products using typically benign, aqueous conditions. Replicating the activity of these enzymes synthetically is extremely challenging, thereby making enzymes exceptionally valuable reagents for a variety of industries. Enzymes are usually over expressed in a suitable host system, such as the bacterium E. coli. However, once the desired enzyme has been produced in sufficient quantities it has to be enriched and formulated to the end user's requirements. A major challenge in the production of industrial enzymes is the difficulty in enriching the target enzyme from all the unwanted cellular components. Conventionally, a fusion tag, such as a poly-histidine sequence, would be incorporated onto one of the termini of the proteins and a compatible affinity resin would capture it from the lysate. This type of approach works extremely well, with high specificity and reliability. However, for industrial enzyme production these bead based resins are prohibitively expensive due to the complex and expensive surface chemistry required on each bead. We were successfully awarded proof of concept funding from the Metals in Biology Network in Biotechnology and Bioenergy (BBSRC NIBB) in 2015 to investigate this area. The funding allowed us to work alongside Cardiff based industrial enzyme producer Biocatalysts Ltd to test a potential alternative to commercial, expensive protein purification resins. We identified a protein, which we had analysed as part of a previously funded BBSRC project, which has a high binding affinity and selectivity for certain magnetic materials. Being able to bind to magnetic materials directly rather than to a heavily functionalised coating significantly reduces the cost of the purification matrix, potentially making bulk enzyme purification economically viable. Being able to magnetise the target protein in this way means external magnetic fields can be used to actuate and control the purification process easily and remotely. We used our affinity protein as a fusion tag to a test protein, GFP (green fluorescent protein), as this allowed us to track the binding and release of the target via simple fluorescence measurements. The project ran for just over 12 months. During this time we made the GFP fusion construct and showed that the presence of the magnetic material binding tag had no detrimental impact on production of the GFP. We optimised a simple synthetic route to the fabrication of cheap magnetic nanoparticles and demonstrated that the target fusion protein was able to bind to this under industrially relevant conditions, namely crude cell lysate with a high optical density. Fluorescence measurements showed that we could successfully capture the GFP fusion protein from the lysate, out-competing other proteins within the sample. Importantly, we were able to show that it was possible to recover the GFP from the nanoparticles after binding and clean-up.

ID number	POCMiB019
Title	Light-activated caged-iron chelators for skin photoprotection based on the natural product pulcherriminic acid
Academic (lead) Partner	Charareh Pourzand, University of Bath
Industrial Partner	Timothy Miller, Croda Europe
Public summary	At present, there is a significant need to counteract the cellular mechanisms that cause skin damage upon prolonged exposure to the UV component of sunlight. Exposure of skin cells to UVA, the oxidizing component of sunlight promotes the generation of harmful reactive oxygen species and leads to an immediate release of labile iron and susceptibility to both oxidative membrane damage and necrotic cell death. Research at Bath since 2006 has resulted in the synthesis and biological validation of light-activated protective compounds (i.e. light-activated caged-iron chelators, CICs) that respond to the UVA-component of sunlight. Upon activation by sunlight these 'intelligent' compounds release an active iron trapping agent (iron chelator) to remove the potentially harmful free labile iron released in skin cell and thereby to protect against iron-catalysed oxidative damage and cell death. A critical requirement for CIC technology is readily available, chemically tractable iron chelators, in which the iron-binding motif can be reversibly modified (caged). In this context, we plan to isolate and modify (cage) the pulcherrimic acid, a natural product from the yeast M. pulcherrima with iron chelating activity and subsequently evaluate its photoprotective activity against UVA-induced iron damage in cultured skin cells.
Start date	28 September 2015
Public summary of project outcomes	This project aimed to demonstrate that the natural iron chelator pulcherrimic acid (PA) derived from the yeast Metschnikowia pulcherrima (Mp) may be used as the basis for the development of novel light-activated skin photoprotectants. For this purpose, three overlapping work packages were designed to first evaluate the photoprotective potential of either isolated PA from yeast culture or the chemically synthesized PA and closely related analogues (PAA) against UVA damage in skin monolayer culture. The next step was to synthetically modify either the isolated PA from the yeast or the synthetic PA and its analogues in order to obtain UVA-activated caged derivatives in which the iron-binding motif of the chelator was temporally blocked by a photolabile caging group. The final step was to biologically evaluate the photoprotective potential of the caged PA/PAA compounds against UVA damage in skin cells. The project started by producing pulcherrimin and purifying PA from the culture grown in non-sterile conditions. Studies on lab scale showed that carbon sources like glucose and saccharose are able to trigger the production of pulcherrimin by the Mp yeast. The production of around 150mg/L of pulcherrimin was achieved on a 10L scale culture of an over-producing strain of Mp (DH5) in non-sterile conditions using an air-lift reactor. An optimization of the purification protocol for pulcherrimin was necessary for an effective recovery. Also, the purification of PA proved to be challenging due to the unstable nature of the compound that requires stringent conditions of cold

	temperature and protection from sunlight. In addition to isolated PA from the yeast, synthetic authentic samples of PA were successfully prepared in the chemistry laboratory, thus permitting the initial evaluation of this natural product as a photoprotective agent. The synthetic approach devised was robust and scalable, and should be suitable for the preparation of a range of amino-acid derived PA analogues and caged compounds. The project also demonstrated promising chemical routes for the synthesis of various light-activated caged PA derivatives. Nevertheless further chemical development work is required to build on the promising results obtained so far in order to obtain light-activatable caged compounds either from a versatile compound generated from the Mp-derived PA, or directly from the latter, both as renewable feedstocks. Although, the natural PA extracted from the Mp yeast was found indistinguishable from the authentic synthetic material, due to lack of optimum purity of PA isolated from the yeast, the biology work concentrated only on the highly pure synthetic PA. The results showed that PA is not cytotoxic per se when exposed to cultured primary skin fibroblasts overnight up to the concentration of 50uM. PA at concentrations in the range of 20-30uM provided a significant photoprotection against UVA-induced damage and cell death. Photoprotection by PA was much superior to that offered by the clinically used bidentate iron chelator Deferiprone at equimolar concentration of 20uM. These promising results provide the proof of concept for the potential development of photolabile caged PA as topical sunscreen ingredients against the damaging effects of solar UVA radiation.

ID number	**POCMiB029**
Title	Engineering metal dependent biotin synthase for the biotechnological production of biotin
Academic (lead) Partner	Christof Jaeger, University of Nottingham
Industrial Partner	Adriana Botes, VideraBio Ltd
Public summary	Biotin (vitamin B7) is an essential cofactor in bacteria, fungi and plants. It is used in vitamin food supplements and primarily for the enhancement of animal feed. Its production is challenging and particularly expensive with sales prices of ~$1600/kg. In this project we aim to demonstrate a potential way towards a sustainable and cost effective biotechnological production of biotin. We will initiate engineering the key bottleneck enzyme biotin synthase which is responsible for an iron sulfur cluster-induced sulfur insertion reaction. We are going to investigate the first steps into rational computational enzyme design in alignment with the development of automated high throughput, multidimensional, in vivo assays for this enzyme for use in our on-site robotics suite. Our approach will act as starting point for not only rational informed directed evolution strategies, but also will integrate regulatory elements for the repair mechanisms of the host cells.
Start date	15 July 2017

Public summary of project outcomes	The essential cofactor Biotin (Vitamin B7) has industrial use in the enhancement of animal feed, and in the pharmaceutical and biotechnology industries. Its production is particularly challenging and costly, favouring development of a sustainable and cost-effective biotechnological approach towards producing biotin. The enzyme biotin synthase, containing a delicate iron-sulfur cluster, represents one key bottleneck in the biosynthetic pathway producing biotin. However, engineering this radical SAM enzyme, which catalyses the complex and chemically difficult transformation of dethiobiotin via sulfur insertion into a non-activated carbon chain, remains highly challenging. During this BBSRC NIBB PoC project, fundamental development of high throughput, multidimensional biotin detection assays, making use of our on-site robotics suite at the Synthetic Biology Research Centre (SBRC), was achieved, in alignment with proof of concept computational investigations to support a rational enzyme design approach. We tested and successfully developed two biotin assays; one based on a previously described Corynebacterium glutamicum indicator strain, alongside a second assay making use of a biotin analogue, 4-Hydroxyazobenzene-2-carboxylic acid (HABA), bound to Avidin (HABA/Avidin). We have found that both the C. glutamicum-based biosensor assay (detection range between 0-1 µg.L-1) and the HABA/AVIDIN (detection range between 1-50 µg.mL-1) have the potential to be adapted for automation of large library screens in multi-titre plate format. The C. glutamicum-based bioassay measured cell growth (Abs600), both with biotin prepared from stock (0 to 5 µg.L-1) and biotin produced in E. coli cells, by lysing the E. coli cells and serially diluting the cytoplasmic fractions. A standard curve was obtained using biotin concentrations between 0 and 1µg.L-1, in two-fold dilution series. Manual loading of the microtiter plates, as well as preparing the dilutions and plates using the liquid handling unit of the robotic suite, demonstrated the biotin concentration was directly proportional to the ability to support C. glutamicum growth in the range from 0 and 1 µg.L-1. In the second affinity-based assay, the amount of free HABA reflects the amount of biotin that is present, once it is displaced from the Avidin. This quantity is measured by the change in absorbance at 500 nm. Sigma/Aldrich-supplied HABA/Avidin reagent (Product No. H2153) was utilised with the provided standard protocol. The assay was optimised for 96-well microtiter plate format with biotin standards added to HABA/Avidin reagent in eight replicates for each concentration, alongside HABA/Avidin-only and HABA/Avidin with media as blanks and background, respectively.The computational approaches focused on targeting potential mutation sites of BioB that influence the redox reactivity of one or both iron sulfur clusters and thus potentially influence sulfur insertion and cluster repair kinetics. For that reason scripts have been developed to analyse the electrostatic effect of the protein environment (due to directional polarity) on the cluster. This type of analysis now makes it possible to spot individual amino acids that influence the field significantly, without being directly

	in contact with the active site. These amino acids will be taken forward to mutation studies.

ID number	**POCMiB028**
Title	Arginine-terminated LPMOs: a new face in biomass breakdown?
Academic (lead) Partner	Paul Walton, University of York
Industrial Partner	Novozymes A/S
Public summary	The efficient conversion of abundant biomass into liquid biofuel is of vital importance in meeting the world's energy demands. Until recently, however, and despite its unrivalled calorific potential it has not been possible to take abundant biomass, which is composed mostly of lignocellulose, and industrially convert it through to bioethanol. The reason for this is the chemical recalcitrance of the cellulosic biomass. Of the available methods, the use of enzymes to perform the breakdown looks promising, especially enzymes called, lytic polysaccharide monooxygenases (LPMOs). LPMOs have overturned our understanding of biomass conversion as they boost significantly the conversion of biomass to ethanol. This particular project aims to study a whole new exciting class of metal- containing LPMOs which do not contain the usual active site amino acids (histidine), thereby offering new insight into how biology performs the conversion of biomass, and consequently humankind's ability to use biomass as a sustainable fuel source.
Start date	1 July 2017
Public summary of project outcomes	This project examined the structure and reactivity of a new class of lytic polysaccharide monooxyegenase (LPMO) enzyme. These new enzymes were expressed in eukaryotic systems to high yield by the company. The objective of the work was to establish whether the enzyme required metal ions for maximal activity and—if so—how those metal ions interacted with the enzyme. A further objective of the work was to establish whether the enzyme was active on lignocellulosic substrates with an oxidoreductase type action. A series of metal-binding studies was performed using isothermal calorimetry experiments, from which it was determined that metal binding was weak and non-specific, unlike the canonical class of other LPMOs. EPR studies showed that the enzyme bound copper in an unusual fashion. The dependence of activity on metal ions did not show a clear trend of activity. Notwithstanding the ostensible low activity, structures showed that the new class of LPMOs forms unusual interactions with lignocellulosic type substrates near the active site. This is now an area of investigation.

ID number	**ISCFPOCMiB032**
Title	Site-specific bioconjugate chemistry for antibody-nanoparticle conjugates
Academic (lead) Partner	Lu Shin Wong
Industrial Partner	Stephen Kilfeather, Aeirtec Ltd

Public summary	Fluorescence-based immunosorbent assays have become a key technology for the detection and quantification of biomolecules, and have found application in a range of fields from the testing of microbial contamination (in water, chemical, food and drug production), biomarkers (in medical diagnostics and drug discovery) and in biomedical imaging. This project will develop production methods for metal nanoparticle-antibody conjugates that are robust and scalable, which would be needed for commercial implementation. These hybrid metal-biomolecule materials offer advantageous spectroscopic properties that could greatly increase detection sensitivity. It is envisaged that such nanoparticle-antibody conjugates will themselves be highly sought-after industrial biotechnology products for the applications noted above. In addition, they will be utilised in the development of new biologics and peptides. Indeed, the development of such products will be greatly enhanced and accelerated by incorporation of very sensitive and faster monitoring assays that these conjugates will enable.
Start date	1 November 2017
Public summary of project outcomes	This Proof-of-Concept grant was used to develop a collaborative relationship on the production of protein-metallic nanoparticle conjugate materials, for use in Aeirtec's diagnostics platform. From an academic perspective, the grant was used to part-fund the research undertaken by students in Dr. Wong's laboratory. In particular, it part-funded a postdoctoral researcher working on the analysis of gold nanoparticle aggregation and a PhD student involved in the chemical synthesis of linker molecules that will enable the attachment of protein molecules to the nanoparticles. Aeirtec has benefited from this interaction through access to chemistry in relation to our capacity to generate a metallic-protein microparticle surface. The interaction has now set a direction for incorporation of metals alongside proteins, and forms the basis of the continued research by the PhD student.

ID number	**ISCFPOCMiB035**
Title	Adding value to biocatalytic hydroxylation products for synthesis and drug discovery
Academic (lead) Partner	Jeremy Robertson, University of Oxford
Industrial Partner	Jason King, Oxford Biotrans Ltd
Public summary	This project exploits the special chemical properties of a set of complex biological catalysts (enzymes) that contain iron. The enzymes will be produced in growing engineered E. coli cells, and the iron centre, when bound to oxygen from the air, is uniquely able to introduce a polar ‘handle’ onto organic molecules. We intend to use these iron-containing enzymes to produce compounds of value to the pharmaceutical industry whose drug discovery campaigns usually begin by screening compound collections for ‘hits’ or, in fragment-based approaches, the features of weakly-active ‘fragment’ compounds are combined to identify promising ‘leads’. This depends crucially on access to small organic molecules, whose nature dictates the trajectory of the whole drug discovery campaign. The highest chance of identifying leads that develop

	successfully into successful drugs arises when the initial compound/fragment collection is structurally diverse; therefore, we aim to diversify compound collections in a two-stage process that mimics the biosynthesis of known medicines such as the anti-cancer drug taxol. Stage one is the above-mentioned enzymatic introduction of the 'handle'; in stage two, the chemical properties of this handle will be exploited to introduce features that promote favourable interactions with drug targets.
Start date	1 November 2017
Public summary of project outcomes	Preparative-scale hydroxylations of selected nitrogen heterocycles were achieved. Around 300 mg of ethyl (3S,4S)-N-Boc-4-hydroxypiperidine-3-carboxylate were produced with an engineered cytochrome P450 from Bacillus megaterium (P450BM3). Following preliminary screening and optimisation studies with isolated enzyme preparations, the 1- and 2-hydroxy derivatives of N-Boc-8-azaspiro[4,5]decane were produced selectively with P450BM3 mutants A and B, respectively. These reactions were then repeated successfully as whole-cell processes using E. coli cells expressing these enzymes. Initially, 85 mg samples of the bare spirocycle were dissolved in 50 mL of the half-concentrated fermentation solution containing cells expressing mutant A or B. For A, 91% conversion of the starting material was achieved after 43 h; for B, 85% conversion was achieved after just 3 h and the reaction was complete within 20 h; the cells were still active and were re-used in an identical reaction (74% conversion after 72 h). Both reactions scaled well to 340 mg substrate; 90% conversion was reached within 24 h and 48 h for the reactions with mutants A and B, respectively. The reaction with A (that was slow on the smaller scale) was a direct scale-up from the 85 mg reaction; the reaction with B employed the same cell titre but at the original fermentation concentration. These proof-of-concept reactions provided synthetically useful outcomes, with reactions processing 1.70–3.4 g of substrate per litre of culture. With the enzymatic hydroxylation phase of the project completed successfully, attention turned to scoping a proposed method for elaborating the metabolites into a variety of derivatives based on tethered C–H insertion reactions, projecting to eventual production of a 'library' of elaborated fragment molecules for synthesis and drug discovery. Such reactions have not been reported for piperidine substrates of the type produced in the project; therefore, model studies were conducted with N-protected 4-hydroxypiperidine. Derivatives were prepared bearing N-Boc or N-toluenesulfonyl protecting groups in combination with either 4-diazoacetate (-OCOCH=N2), -carbamate (-OCONH2), -toluenesulfonyloxycarbamate (-OCONHOTs), or -sulfamate (-OSO2NH2) substitution. Variants of Du Bois' Rh(II)-catalysed nitrenoid and carbenoid insertion conditions were surveyed. With the N-Boc diazoester, C–H insertion gave the spirocyclic β-lactone and the fused γ-lactone in a 65:35 ratio, respectively (68% combined yield). The N-Boc and N-toluenesulfonyl carbamate variants afforded primarily the corresponding piperidinones which indicates insertion solely into the α-C–H

	bond with the resulting product being unstable with respect to elimination. The N-Boc sulfamate derivative formed, apparently, three C–H amination products which are currently assigned as two stereoisomers of the five-membered cyclic sulfamate (β-insertion) and the product of γ-insertion, a six-membered 'bridged' cyclic sulfamate. This work has provided insight into the likely outcomes in conformationally-biased molecules. Carbamate and diazoacetate derivatives of the hydroxylated metabolites described in the first paragraph have been prepared in readiness for their C–H insertion chemistry to be studied following ongoing work to find alternatives to the Du Bois conditions.

ID number	**ISCFPOCMiB041**
Title	Biosynthesis of bimetallic nanoparticles for fine and speciality chemical production
Academic (lead) Partner	Jon Lloyd, University of Manchester
Industrial Partner	Nigel Powell, Johnson Matthey plc
Public summary	This project combines biotechnologists from the University of Manchester and industrial catalysis experts at Johnson Matthey, a multinational speciality chemicals and sustainable technologies company. This "Proof of Concept" award will exploit the ability of microorganisms to produce bimetallic nanocatalysts for fine and speciality chemical production. Metal-reducing bacteria are able to recover a wide range of metals from process environments as catalytically active nanoparticles. By taking advantage of the unique biochemistry of metal-reducing bacteria, we will produce highly reactive and tunable metallic nanoparticles for speciality chemical production. These will include bimetallic nanoparticles that offer advantages over monometallic catalysts due to the combination of properties from the presence of two metals (rather than one) and from additional properties due to the synergy of the two metals, offering increased efficiency and specificity for speciality chemical production. This novel biotechnological process also offers a simple, cost-effective, environmentally friendly synthesis route for bimetallic catalyst production.
Start date	1 November 2017
Public summary of project outcomes	Building on from a successful NIBB Business Interaction Voucher project with Johnson Matthey, we have continued to investigate the biosynthesis of novel bimetallic nanoparticles for fine and speciality chemical production. Electron microscopy revealed that different metals have varying affinities for forming bimetallic nanoparticles and that the bimetallic nature is also affected by the order the metals are supplied to the bacteria. In addition, we found that the pH buffer used during synthesis can exert some control over the formation of the bimetallic nanoparticles. This knowledge will help us tailor these products going forward. Several of the biosynthesised nanoparticles showed promising catalytic activity. Although they did not perform to the same level as a commercial catalyst, this project has provided us with valuable insights into the optimisation of these bionanocatalysts which we are continuing to explore.

ID number	ISCFPOCMiB038
Title	Arginine terminated LPMOs—a new face in biomass breakdown? Follow-on funding
Academic (lead) Partner	Paul Walton, University of York
Industrial Partner	Jens Nielsen, Novozymes
Public summary	The generation of fuels/commodity chemicals from sustainable biomass hinges on a single key issue. This issue is that biomass (e.g. wood, plant matter) is very hard indeed to break down in a controlled manner, severely hindering its sustainable conversion into useful materials such as bioethanol and other chemicals. It is an issue which has bedevilled the bio-based industry. However, in a major breakthrough in 2010/11 we discovered copper-containing *lytic polysaccharide monooxygenases* (LPMOs) which are natural enzymes that can break down cellulose (a plant based polysaccharide) in a highly efficient manner. LPMOs are now used in biorefineries to generate bioethanol and have transformed the industry. In this project, we seek to maximise the ability of a new type of LPMO to break down woody biomass. If the ability of this enzyme to convert lignin could be harnessed then it would be a significant addition to the biomass industry.
Start date	1 December 2017
Public summary of project outcomes	This project examined the reactivity and chemistry of a new type of biomass-degrading enzyme drawn from the wider family of LPMO (lytic polysaccharide monoxygenase) enzymes. The new form of the enzyme is intriguing as it does not contain the usual amino acids at its active site and therefore suggest that it is active on a new range of biomass components. In this project we examined the activity of this new LPMO using UV/vis spectroscopy along with EPR. We also determined the structure of the enzyme, gaining particular insight into how it interacts with its substrates. This finding opens up the possibility of tailoring the enzyme to carry out new types of reactions which are of importance to biomass degradation. While we are not able to report the details of the findings due to IP reasons, the project was successful in that we were able to develop a new type of assay for this class of enzymes and we were also able to demonstrate that they possessed a new type of enzymatic activity on a range of substrates.