Cell Chemical Biology
Meeting Report

Protein Chemistry Looking Ahead: 8th Chemical Protein Synthesis Meeting 16-19 June 2019, Berlin, Germany

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The 8th Chemical Protein Synthesis meeting took place in Berlin in June 2019, covering broad topics in protein chemistry, ranging from synthetic methodology to applications in medicine and biomaterials. The meeting was also the culmination of the Priority Program SPP1623 on "Chemoselective Reactions for the Synthesis and Application of Functional Proteins" funded by the German Science Foundation (DFG) from 2012 to 2018. We present highlights from presentations at the forefront of the field, grouped into broad themes that illustrate how the field of protein chemistry is looking ahead to new discoveries and applications.

Protein Chemistry Looking Ahead

"Standing on the shoulders of giants" is a fitting, if somewhat cliché, description of the recent 8th Chemical Protein Synthesis (CPS) meeting held at the Max Planck Society Harnack House in Berlin-Dahlem. This venerable location that boasts having hosted Nobel Prize winners including Fritz Haber, Otto Hahn, and Albert Einstein became the venue for the biennial symposium, which is a highlight in the conference cycle of chemical biologists from around the world. The CPS meeting covers broad topics in protein chemistry, ranging from synthetic methodology to applications in medicine and biomaterials. This year it was hosted by Christian Hackenberger (FMP and Humboldt University of Berlin) and fellow committee members Jeffrey Bode (ETH Zürich), Ashraf Brik (Technion Haifa), Lei Liu (Tsinghua University), and Tom Muir (Princeton University) (Figure 1A). In addition to the high quality scientific talks and ample opportunities for interaction, there was an opera performance at the speakers' dinner. Conference participants were also treated to tours "On the Trail of Nobel Prize Winners" or "Female Pioneers of Science" to learn some of the history of the beautiful grounds of Harnack House.

Building on founding work in peptide synthesis, native chemical ligation and protein conjugation (Kent, 2017), the field of chemical protein synthesis has matured to produce faster, more versatile ligation methodologies. Synthetic methods and genetic code expansion techniques have yielded a smorgasbord of synthetic, semi-synthetic and *in vitro* proteins with natural and unnatural modifications (Bondalapati et al., 2016; Conibear et al., 2018; Lang and Chin, 2014; Schumacher and Hackenberger, 2014). Both the technologies and proteins themselves are finding a continually expanding range of applications in cell biology and medicine. Grouped into broad themes, we give a sample of highlights from talks at the forefront of the field presented at the 8th CPS (Figure 1B). The meeting was also the culmination of the Priority Program SPP1623 on "Chemoselective Reactions for the Synthesis and Application of Functional Proteins" funded by the German Science Foundation (DFG) from 2012 to 2018. The SPP1623 program was led by Christian Hackenberger and Dirk Schwarzer (University of Tübingen) and brought together a cooperative interdisciplinary network of scientists from all over Germany. The SPP1623 program has also provided sponsorship for previous CPS meetings and many of the talks featured new discoveries made and collaborations forged through this program.

Exploring New Tools for Peptide Synthesis, Ligation and Protein Modification

Manufacturing customized proteins with natural and artificial functionalities remains an important goal, and there are still significant challenges associated with synthesis efficiency, as well as functional group compatibility and site selectivity of protein modifications. Therefore, it is not surprising that new methods for peptide synthesis, ligation and selective protein modification were a major topic at this CPS meeting. Starting with new technologies for amide bond formation, the rapid preparation of long polypeptides was illustrated by Bradley Pentelute (Massachusetts Institute of Technology). A fully automated flow-based synthesizer ("Amidator") developed in his group forms amide bonds in seconds, assembling long peptides of up to 200 amino acids in a much shorter time than other available instruments. As an extension of flow chemistry to biocatalysis, Christof Niemeyer (Karlsruhe Institute of Technology) introduced self-immobilizing biocatalysts such as multi-enzyme cascades. Flow chemistry not only accelerates peptide synthesis, but also enables fast native chemical ligation reactions and in-line photodesulfurization, as demonstrated by Richard Payne (University of Sydney). Payne also reported a new method to install a challenging proline-proline junction. Using diselenide-selenoester ligation, his group was able to achieve the total synthesis of the proline-rich proteins submaxillary gland androgen-regulated protein

Figure 1. Protein Chemists Meet at Harnack House
(A) Organizing committee and keynote speakers in front of Harnack House. Left to right: Christian Hackenberger, Annette Beck-Sickinger, Tom Muir, Ashraf Brik, and Philip Dawson (also on the organizing committee but not in the picture: Jeffrey Bode and Lei Liu).
(B) Opening plenary session of the 8th CPS meeting, Hahn-Hörsaal, Harnack House.

3B and lumbricin-1 (Sayers et al., 2018). Selenium also played a central role in Norman Metanis' (The Hebrew University of Jerusalem) presentation on the synthesis of a diselenide-containing human insulin. In comparison with disulfide-containing natural insulin, the fully synthetic derivative displays enhanced foldability and stability. Following up on the topic of new ligation methods, Xuechen Li (University of Hong Kong) reported on a serine-threonine ligation and a chemoselective P-B desulfurization. This mild desulfurization method relies on a combination of TCEP (tris(2-carboxyethyl)phosphine) and borohydrate ($NaBH_4$ or $LiBEt_3H$) and is proposed to proceed via an *in-situ*-generated phosphine-borane complex (Jin et al., 2017). Notably, this method can also be used for site-selective deuteration of proteins.

A chemo-enzymatic approach for ligation was developed by Dirk Schwarzer (University of Tübingen), using engineered sortase and sortase motifs for multi-peptide assemblies. Jeffrey Bode (ETH Zürich) presented on an enzymatic, highly sequence-specific isopeptide-labeling method for native proteins with thioesters as probes, which was applied to dual labeling of a Trastuzumab antibody-binding fragment (F_{ab}). Bode also reported on new modifications of the KAHA-ligation, as well as traceless templated amide bond ligation using acylboronate-hydroxylamine reactivities (Osuna Galvez and Bode, 2019). Further on enzymatic labeling methods, Aymelt Itzen (University of Hamburg) presented on covalent protein modification via phosphocholination enabled by the bacterial enzyme AnkX from *Legionella pneumophila*. Frank Bordusa (Martin-Luther-University Halle-Wittenberg) discussed his research on a highly specific trypsin variant, a so-called "Trypsiligase," which enables selective conjugation and modification of both N- and C-terminal residues.

Not only enzymatic, but also numerous chemical methods are under investigation for site-specific modification of proteins. Philip Dawson (Scripps Institute), in his keynote lecture, presented on a selenomethionine-mediated incorporation of Freidinger lactams, which enable posttranslational backbone modifications. As solvent restrictions are a major challenge for biopolymer modification, Dawson also reported on new synthetic methods for DNA encoded libraries (DEL) using reversible adsorption to solid support (Flood et al., 2019). This method takes advantage of the reversible binding of DNA to an inert quaternary ammonium support and thereby enables the use of organic solvents for DEL synthesis. The concept of selective protein modification by ligand-directed synthesis was presented by Itaru Hamachi (Kyoto University), who employs *N*-acyl-*N*-alkyl sulfonamide as a reactive group for the rapid and selective modification of a lysine residue proximal to the respective ligand binding site. Huan Wang (Nanjing University) presented on dinitroimidazoles as trifunctional bioconjugation reagents for protein functionalization and peptide macrocyclization. Furthermore, he introduced his work on Pd-catalyzed peptide modification for cyclization reactions. Another application of palladium in protein chemistry was developed by Ashraf Brik (Technion, Haifa), which entails tuning the chemoselectivity of palladium complexes in aqueous medium to selectively deprotect several cysteine protecting groups. He and his group ultimately demonstrate the applicability of their method by the successful synthesis of an activity-based probe of

ubiquitinated histone H2A with either a nonhydrolysable or native isopeptide linkage. Joseph Fox (University of Delaware) presented on tetrazine ligations for the fast and inducible labeling of proteins (Zhang et al., 2016). Furthermore, he reported on a straightforward synthesis of tetrazine probes as well as an *in vivo* pro-drug strategy. Two researchers in the "Rising Stars" session, Monika Raj (Auburn University) and Hannes Mikula (TU Wien) also focused on site-specific labeling of proteins and peptides. Hannes Mikula presented on advancements for the tetrazine-based bioorthogonal bond cleavage, aiming for an ultrafast cleavage and release reaction for application in biological systems. Monika Raj reported on novel secondary amine selective peptide bioconjugation, which should ultimately enable the investigation of these modifications in natural systems.

Unveiling the Role of Posttranslational Modifications

Posttranslational modifications (PTMs) are used by nature to exponentially enlarge the information contained in the genome and to finely tune the properties and functions of proteins. Chemical synthesis and semi-synthesis methods give access to proteins that are specifically modified with PTMs, thus providing us with tools for structural and activity studies and with probes for investigating the biological relevance of PTMs.

Preparation and use of probes to study ubiquitination and ubiquitin (Ub) interactions were discussed during the meeting. Lei Liu (Tsinghua University) described the development of K27-linked di-ubiquitin probes to study, via fluorescence resonance energy transfer and X-ray crystallography, how Ub-interacting proteins can specifically recognize K27 Ubs, which have an isopeptide bond (or mimic) buried inside the di-Ub module (Pan et al., 2019). Satpal Virdee (University of Dundee) reported the semi-synthetic preparation of engineered conjugates and their use as sensors of Ub E3 ligase activity, providing information on the mechanism of action of these important enzymes (Pao et al., 2018). An investigation of the interactome of poly-Ub chains was presented by Andreas Marx (University of Konstanz). He illustrated how several homogeneous triazole-linked poly-Ub chains were prepared using copper(I)-catalyzed azide-alkyne cycloaddition. These poly-Ub analogs were used in affinity-based proteomics to identify previously unknown interacting partners of Ub chains. Another PTM that involves the attachment of a small protein is SUMOylation. Champak Chatterjee (University of Washington) illustrated the use of chemical and semi-synthetic approaches to study the effect of SUMOylation in repressing transcription by recruiting erasers that remove acetylation on histones.

Synthesis of homogeneous site-specifically glycosylated proteins enables us to elucidate the effect of one of the most abundant and complex PTMs on protein structure, properties and function. Yasuhiro Kajihara (Osaka University) reported on a method based on thioacid oxidation for generating polypeptides without the use of protecting groups (Okamoto et al., 2019). The reaction proceeds via oxidative formation of a diaminoacyl-disulfide from an α-amino thioacid. This intermediate undergoes an intramolecular S- to N-acyl transfer, thus generating a peptide bond. Co- and homo-oligomers of amino acids could be obtained from oxidation of the corresponding α-amino thioacids. Yasuhiro Kajihara also described the chemical synthesis of erythropoietin (EPO) variants carrying di- and triantennary homogeneous N-glycans and how the size of the glycan influences protein folding. The larger hydration sphere of triantennary glycan structures appears to shield the protein from solvent exposure, as shown in deuteration experiments, possibly providing a shielded environment in which the protein can fold. Synthesis of EPO glycoforms was also the subject of Carlo Unverzagt's (University of Bayreuth) presentation. He elucidated the total chemical synthesis of complex core-fucosylated N-glycans, and the strategies adopted toward the effective synthesis of highly pure EPO carrying three complex N-glycans (Luber et al., 2018). Hironobu Hojo (Osaka University) illustrated the chemical synthesis of glycoproteins and hydrophobic proteins, such as caveolin, exploiting the solubilizing effect of isoacyl functional groups. Christian Becker (University of Vienna) presented the synthesis of prion protein variants modified with lipids and polyethylene glycol (PEG) chains as glycan mimics, and an evaluation of their ability to form fibrils and interact with membranes.

Preparation of proteins carrying other PTMs was also described during the CPS meeting. Philip Cole (Harvard Medical School) reported on the semi-synthesis of site-specifically phosphorylated Akt kinase and the role of modifications on its activation. Functional studies of selectively phosphorylated, but also methylated and lipidated small G proteins obtained via semi-synthesis approaches were presented by Yongxiang Chen (Tsinghua University) in the Rising Stars session.

The role of PTMs on α-synuclein in the development of Parkinson's disease is still not fully elucidated. Hilal Lashuel (EPFL Lausanne) described the use of synthetic α-synucleins carrying PTMs in studies to assess the therapeutic potential of targeting PTMs. He and his group are elucidating the role of these PTMs in regulating the different steps involved in α-synuclein fibril formation and transition to Lewy body inclusion in neurons. James Petersson (University of Pennsylvania) presented the preparation of fluorescently labeled α-synuclein, which provided new insights into its fibrillation tendency and fibril structure.

Chemical Protein Synthesis for Antibody Therapeutics

The wealth of developments in bioconjugation techniques has been especially transformative in the production of next-generation biologics and vaccines, in particular for bi- and multi-specific antibodies. Mark Howarth (University of Oxford) described a recently reported application of his SpyTag/SpyCatcher pair, and newer generations such as the SnoopLigase platform, to the synthesis of modular, multivalent vaccines. Using a different approach, Hiroaki Suga (University of Tokyo), described the Mirabody® and Addbody®, currently being developed by his new company MiraBiologics. Grafting of an antibody or Fc fragment into high-affinity cyclic peptide sequences selected against a target using the RaPID system developed by the Suga lab allows construction of multivalent biologics. In the case of the Mirabody, up to 12 loop positions can be varied on a single fragment, allowing the possibility of multi-protein targeting. An alternative strategy for generating bispecific molecules is that of Norbert Sewald and coworkers (University of Bielefeld) who have developed an enzymatic method to produce dual-tagged DARPins. Two mechanistically distinct formylglycine-generating enzymes, one oxygen-dependent and the other S-adenosyl-L-methionine-dependent, are used to append two different molecules to a single DARPin scaffold via generation of the aldehyde-containing

amino acid C^{α}-formylglycine (Krüger et al., 2018). Synthesis and conjugation strategies for immune monitoring were discussed by Adel ElSohly (Genentech). He described the use of major histocompatibility complex class I (MHC I) tetramers for tracking CD8 T cell responses, the development of a SpyTag/SpyCatcher platform for better T cell staining, and the sortase-mediated N-terminal labeling of MHC II mimics for CD4 T cell monitoring.

In addition to altering the specificity of biologics with peptidic fragments, new bioconjugation methods allow the selective attachment of payloads to antibodies to form antibody drug conjugates. Harald Kolmar (TU Darmstadt) described elegant work from his laboratory exploiting hydrophilic dextrans as a scaffold for the attachment of multiple toxic payloads to a single antibody, a so-called "dextramab." The dextrans are decorated with a defined average number of azides to allow the addition of an alkyne-conjugated drug of choice by biocompatible click chemistry. This approach has recently been used to develop a conjugate that multimerizes death receptor 5, resulting in apoptosis (Schneider et al., 2019). In general, drug conjugation requires stable and selective linkage of drugs to antibodies. To this end, Heinrich Leonhardt (LMU Munich) described the recent development of ethynylphosphonamidates for cysteine-selective conjugation. Notably, this leads to a linkage that is substantially more serum stable than the commonly used maleimide linkage chemistry (Kasper et al., 2019). Linker chemistry can also be an important factor in extending the serum half-life of biologic drugs. Thomas Høeg-Jensen highlighted how Novo Nordisk have been working to develop alternatives to PEG that are homogeneous and biodegradable.

Peptides as Modulators of Protein-Protein Interactions

The ability of synthetic peptides to inhibit challenging protein-protein interactions was illustrated by a number of speakers at this year's conference. Annette Beck-Sickinger (University of Leipzig) opened the conference with an overview of her laboratory's efforts to target G protein-coupled receptors (GPCRs), here with a focus on neuropeptide Y receptors. A combination of cell-free protein synthesis, photo-crosslinking, X-ray crystallography and other biophysical techniques allowed characterization of the interactions of agonists and antagonists with these receptors, with the potential to enable structure-guided drug design. Christina Schroeder (University of Queensland) described targeting of another membrane protein, SCN9A, a sodium channel for which mutations result in a loss of ability to feel pain. She nicely illustrated how the specific mode of channel inhibition is also important for the exact mode of action, highlighting the complexity in developing drugs to modulate receptor activity. Selective gating modifier toxins do not always fully block activation. By contrast, pore blockers, which are effective at preventing activation, tend to be unselective in their action. Kristian Strømgaard (University of Copenhagen) described the transformation of a micromolar peptidic inhibitor of the PSD-95/NMDA receptor interaction currently in phase 3 trials into a low nanomolar, highly selective inhibitor through dimerization. Excitingly, in collaboration with Avilex Pharma, this new compound is about to enter phase 1 trials for the treatment of stroke (Bach et al., 2019). These talks demonstrated that for extracellular receptors, peptides are already beginning to live up to their promise of being potent and selective drug molecules.

Toward Selective Chemistry in Cells and beyond

Advances in chemical biology have yielded a variety of methods to modify proteins and make protein-based therapeutics such as those described above, but it can be challenging to deliver these molecules into cells for intracellular activity. As an example of getting large proteins into the cytosol of mammalian cells, Ronald Raines (Massachusetts Institute of Technology) described how masking anionic patches on proteins by esterification of carboxyl side chains could enable them to penetrate cells, as demonstrated for human ribonuclease 1. On entering the cells, the esters are hydrolyzed by endogenous esterases, restoring enzyme activity. Helma Wennemers (ETH Zürich) introduced how peptide-coated platinum nanoparticles functionalized with glucose are selectively taken up by hepatic cancer cells and become cytotoxic on oxidation.

Visualization of biological processes in cells is greatly facilitated by the protein chemistry toolkit, which enables us to label proteins with precise spatial and temporal control. This application was illustrated in work presented by Oliver Seitz (Humboldt University of Berlin), who described how templated coiled-coil protein interactions followed by native chemical ligation could be used to covalently label GPCRs on cell membranes (Lotze et al., 2018). Two-color pulse-chase labeling allowed for real-time imaging of different populations of human neuropeptide Y2 in the same cell. The inverse electron-demand Diels-Alder reaction has also been used as a biorthogonal reaction for labeling proteins in cells. Richard Wombacher (Heidelberg University) described the development of new tetrazine motifs with increased stability and tailored diene substrates as fluorogenic probes for intracellular live cell protein imaging at high resolution. Instead of labeling proteins, Valentin Wittmann (University of Konstanz) explained how the inverse electon-demand Diels-Alder reaction could be used in metabolic glycoengineering for visualizing carbohydrates on cell surfaces. Using three orthogonal reactions—inverse Diels-Alder, photoclick and copper-free click—they were able to label three different glycans and visualize them simultaneously on cell surfaces. Also harnessing the power of light, but in this case to control protein function and location with high precision in live cells, Yaowen Wu (Umeå University) described progress in chemo-optogenetic methods using photoactivatable and photoswitchable chemically induced dimerizsation (pCID and psCID) systems.

A complementary approach to selective chemistry in live cells is to make use of the cell's own translational machinery to introduce selective functional groups, thus avoiding the need to get synthetic components into cells. Henning Mootz (University of Münster) described recent work in his group on engineering a genetically encoded photoswitchable intein. An *ortho*-nitrobenzyl-tyrosine residue is incorporated by genetic code expansion and allows for triggering of the intein splicing reaction using light, with potential applications in protein regulation (Bocker et al., 2019). Also making use of split inteins, Tom Muir (Princeton University) and Yael David (Sloan Kettering Cancer Center) spoke about their work in chemical modification of chromatin. In recent work, the "N-TAIL" strategy using transpeptidase enzymes and split inteins was used to attach a synthetic biotinylated peptide to histone H3 in isolated protein, histone H3 in a nucleosome complex and histone H3 in nuclei isolated from cells (Thompson

Figure 2. Inspiring Venues for Scientific Discussion and Networking
(A) Vibrant discussions at the poster sessions (despite the heat!), Goethe Saal, Harnack House. (B) Gala dinner and awarding of poster prizes, watched over by dinosaur fossils at the Naturkundemuseum, Berlin.

et al., 2019). Genetic code expansion, used to incorporate unnatural amino acids into inteins as above, was also employed in work described by Kathrin Lang (TU Munich) to mimic site-specific inducible protein ubiquitination and SUMOylation in cells. An azide-masked glycine-glycine sortase tag attached to a lysine side chain by an isopeptide bond was incorporated into proteins using genetic code expansion. On induction via Staudinger reduction, a ubiquitin derivative bearing a sortase motif was ligated site-specifically to the protein, allowing for the study of the biological functions of ubiquitination. In work presented by Edward Lemke (University of Mainz), genetic code expansion was restricted to sub-cellular locations, demonstrating how membrane-less artificial organelles can be used for protein engineering (Reinkemeier et al., 2019). By spatially enriching components of the translational machinery in phase-separated droplets, the genetic code could be expanded for only selected mRNAs.

Conclusion and Outlook

The 8th CPS meeting highlighted the definitive transition of chemical protein synthesis from a niche discipline to a mature and rapidly growing field. The interdisciplinary approaches and diverse applications presented during the meeting demonstrated once more the high profile of the research and the contribution that peptide and protein chemistry is giving in deepening our understanding of complex biological processes. Nevertheless, challenges remain both in the chemical methods and biological applications, as well as in bringing these two disciplines together. Despite advances made in the last decades in methods for the synthesis of long, complex macromolecules, synthesis of homogeneous proteins carrying PTMs and the preparation of bioconjugates still often involves lengthy synthesis efforts and significant losses during purification. It is encouraging to see a growing number of collaborations between specialist synthesis groups and biologists using synthetic proteins to answer biological questions. Addressing intracellular targets with synthetic protein and peptide therapeutics is still hampered by difficulties in getting chemically modified proteins across biological barriers and is an area that will benefit from interdisciplinary approaches.

Applying novel ligation chemistries and ever more complex synthetic and semi-synthetic proteins to live cells in order to understand and manipulate biological processes is a fundamental goal of chemical biology. Many challenges arise when translating chemistry into biological systems, however several talks at the CPS meeting provided glimpses of how this can be achieved. Some of the genetic expansion techniques presented are showing promising advances in this area. Standing on the shoulders of those in the field who develop new reactions and tools, we expect that future CPS meetings will see increasing examples, such as those above, of extending protein chemical synthesis into live cells and even organisms.

The icing on the cake of this highly successful event was the gala dinner, which was held in the imposing presence of the dinosaur fossils at the Naturkundemuseum in Berlin (Figure 2), with new connections consolidated over dinner and a celebration of the poster prize winners. Poster prizes were generously

sponsored by several journals and societies and were awarded to: Alice Baumann, Sergej Schwagerus, and Marc Kasper (Humboldt Univerisity Berlin/FMP, ACS Bioconjugate); Raphael Hofmann (ETH Zürich, ACS Chemical Biology); Susanne Mayer (TU Munich, Nature Reviews Chemistry); Nina Hentzen (ETH Zürich) and Benjami Oller-Salvia (Ramon Llull Univeristy, Royal Society of Chemistry); Anselm Schneider (Frei University Berlin/FMP, German Chemical Society and Gemeinsame Fachgruppe "Chemische Biologie"); and Hendrik Schneider (TU Darmstadt, Angewandte Chemie). Well-deserved recognition was also given to Katrin Wittig, who has been key in the organization of both the SPP1623 program and the CPS meeting and to Christian Hackenberger for organizing an outstanding program and bringing together both emerging and well-known protein chemists.

The 9th CPS meeting will take place in Nagoya, Japan, in June 2021 with Jeffrey Bode as chair of the organizing committee. We are already looking forward to hearing what the exciting field of chemical protein synthesis will bring next.

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AUTHOR CONTRIBUTIONS

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