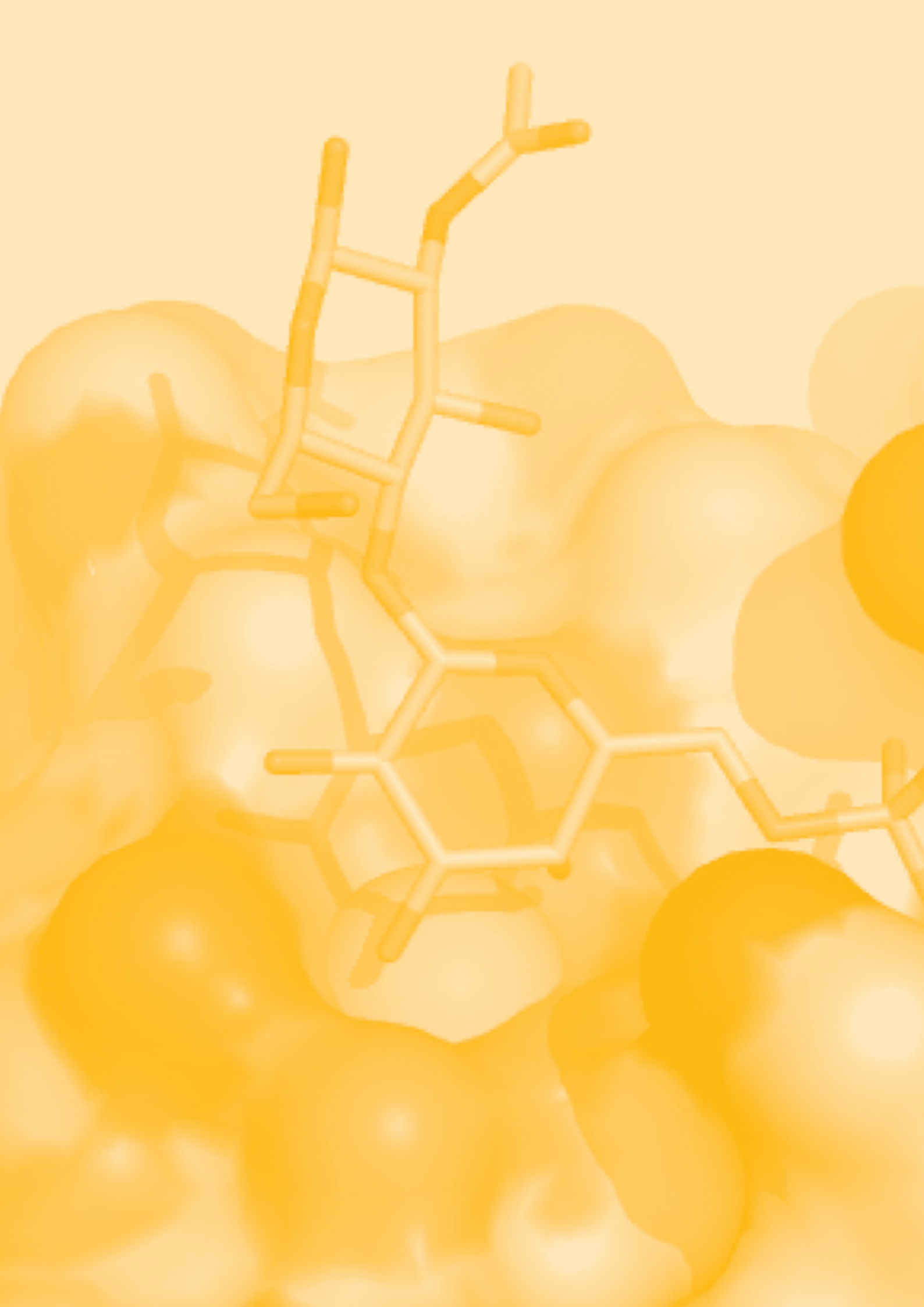

→ Continuous Chemical Systems
→ Glycoproteomics
→ Glycoimmunology
→ Syntethic Plant Carbohydrates
→ Structural Glycobiology
→ GPIS and Glycoproteins
→ Immunomics
→ Polymeric Biomimetics

BIOMOLECULAR SYSTEMS

Research in the Department of Biomolecular Systems

Peter H. Seeberger 14.11.1966
1989: Vordiplom
(Univ. Erlangen-Nürnberg)
1995: PhD, Biochemistry
(University of Colorado, Boulder)
1995-1997: Research Fellow
(Memorial Sloan-Kettering
Cancer Center)
1998-2003: Assistant and Associate
Professor (Massachusetts Institute of
Technology, MIT, Cambridge, USA)
2003-2009: Professor
(Swiss Federal Institute of Technology
(ETH) Zurich, Switzerland)
2003-2014: Affiliate Professor
(The Burnham Institute for Medical
Research, La Jolla, USA)
Since 2009: Director, Department of
Biomolecular Systems (Max Planck
Institute of Colloids and Interfaces)

Selected References:

[1] Zhang, K.; Kopetzki, D.; Seeberger, P.H.; Antonietti, M.; Vilela, F.; Surface Area Control and Photocatalytic Activity of Conjugated Microporous Poly(benzothia-diazole) Networks; Angew. Chem. Int. Ed. 2013, 52, 1432-1436.
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The Department for Biomolecular Systems conducts research at the **interface of chemistry, engineering, biology, immunology and medicine.** The approach is trans-disciplinary and interactive between the groups in the department that cover different areas of expertise. The core focus is the development of **synthetic methods** for the chemical synthesis of defined oligosaccharides. The compounds are the basis for **chemical tools** that aid **biological investigations** into the fundamental roles complex carbohydrates play in biological processes that underlie disease. Carbohydrate arrays greatly helped us to advance our understanding of **immunological aspects** of various infectious diseases. **Vaccine development** has resulted in innovation at all levels including the glycan portion, novel carriers, and novel modes of presentation to the immune system. Several glycoconjugate vaccine candidates have passed challenge studies in experimental animals and are being readied for human clinical trials in a spin-off company. Following an initial growth phase after the move from ETH Zurich in 2009, the department has reached a steady state. In 2014, four group leaders left the department. Dr. Laura Hartmann was promoted to W3 professor at the University of Düsseldorf after less than five years with us. Prof. Tyler McQuade left in 2013 after just one year with us after receiving an immensely attractive offer as program chief at the US Defense Agency Research Program Ageny (DARPA) while holding a professorship at Florida State University. Dr. Kerry Gilmore assumed the position as leader of the continuous flow group after proving himself for one year as project leader of the same group in 2014. Dr. C. Anish left for a leadership position at a top vaccine company in Holland following a very productive phase of the vaccine biology group. Dr. Lepenies who worked with his glycoimmunology group on the role of glycans *in vivo* will leave for a W2 professorship in Hannover in 2015. Currently, we are actively seeking a glycoimunologist to fill the void in this area.

Glycan sequencing and glycomics (Dr. Kolarich) helps to identify glycans of biological importance particularly on interfaces of the human body – skin and intestine. The synthesis and study of plant glycans is the focus of a new Emmy-Noether research group headed by Dr. Fabian Pfrengle. Our increased interest in establishing structure-function correlations of glycans is expanding. In addition to the Emmy-Noether group of Dr. C. Rademacher that is concerned with questions relating to structural immunology, in 2015 Dr. Ursula Neu will join our department to add strength in X-ray crystallography. Together, we are actively pursuing different questions in the glycosciences including the structure, function and biological role of sugars found on the surface of mammalian and bacterial cells particularly in the areas of immunology, biochemistry and human disease.

Materials aspects related to carbohydrates are continuing to be pursued in the department particularly since we are now able to produce ever larger, structurally defined polysaccharides.

Continuous-flow chemistry has seen immense successes in part due to a close collaboration to the chemical engineers of the group of Prof. Seidel-Morgenstern at the MPI in Magdeburg. The development of novel concepts for the modular production of pharmaceutically active ingredients is currently a key focus for the group. The department is engaged in collaborations with the Colloid Department concerning the use of supported catalysts.

Automated Synthesis of Carbohydrates

Automated glycan assembly, our core technology has reached a new level of sophistication. After many years of systematic improvements, the synthesizer as well as most reagents have been commercialized via the spin-off company **GlycoUniverse.** At the same time, the variety and complexity of oligosaccharides has been increased and the type of linkages that are now accessible has been drastically expanded.

Automated glycan assembly is becoming more and more a standard tool to prepare ever longer polysaccharides that enable investigations into new areas of biology as well as material sciences.

The first commercial automated synthesizer Glyoneer 2.1 that was based on work in the Biomolecular Systems Department

Synthetic Tools for Glycobiology
Access to synthetic oligosaccharides has given rise to tools such as glycan microarrays, glycan nanoparticles, and radioactively labeled glycans. These tools are now commonly used by the glycobiologists in the department to elucidate fundamental processes such as the entry mechanism of parasites into host cells.

Synthetic Carbohydrate Vaccines
We have established a comprehensive program targeting the development of fully synthetic carbohydrate vaccines. The team of Dr. C. Pereira produced a host of antigens found on the surface of pathogenic bacteria. Conjugations of these antigens with carrier proteins and with self-adjuvanting glycolipids performed extremely well in immunological and functional studies in several disease models in experimental animals. Several constructs have now reached a mature stage that resulted in preparation of a spin off company that is expected to be launched in 2015 to advance the different synthetic vaccines into human clinical trials.

Carbohydrate-based Nanotechnology
The attachment of carbohydrates to the surface to nanoparticles has been expanded further in efforts to use glycosylated materials for disease monitoring as well as treatment. Stroke models in rats have been a focus of recent activities, while the attachment to silicon nanoparticles is gaining momentum.

Glycoimmunology
Carbohydrate recognition by C-type lectin receptors influences key functions of dendritic cells such as antigen presentation, cytokine release, and the expression of co-stimulatory molecules. Since all of these processes impact T cell priming and differentiation, CLR targeting is a means to orchestrate an initiated immune response. To identify immune stimulatory and immune modulatory CLR ligands, a screening platform has been developed, followed by *in vitro* and *in vivo* assays. The extracellular domains of different CLRs were expressed as fusion proteins and used in conjunction with the glycan array technology for high-throughput screening of lectin/carbohydrate interactions. Novel binding partners of CLRs were identified and interactions with known ligands confirmed. Carbohydrate-protein interactions were further characterized by surface plasmon resonance (SPR) measurements. This platform brings together CLR ligand identification and their immunologic evaluation. Hence, it is a highly useful tool for the functional analysis of CLR ligands

Carbohydrate-carbohydrate interactions
As part of the Collaborative Research Centre (SFB) 765 ("Multivalency as chemical organization and action principle"), we focus on the characterization of carbohydrate-carbohydrate interactions. Interactions between carbohydrates are even weaker than carbohydrate/lectin interactions, thus are often hardly measureable. In this context we focus on biophysical and biological analyses of interactions between the tumor-specific carbohydrate antigens GM3 and Gg3 as well as GB4 and GalGB4. Efforts to employ these multivalent carbohydrate interactions for cell-specific targeting and imaging are underway.

Continuous Flow Chemistry
After our pioneering efforts since the turn of the century, continuous flow chemistry has now reached a stage where not single reactions but rather entire systems are under investigation. Over the past three years, the production of artemisinin-based anti-malaria medications has been a key example to demonstrate the power of the approach. A general approach to create chemical assembly systems has resulted in modular methods to create multiple medications. Among other successes, access to a key anti-HIV medication in fewer steps and higher yield has been a major breakthrough. The group leader, Dr. Kerry Gilmore has added chemical engineers to the team in order to further automate reaction optimization and to more intricately integrate in-line analytical methods.

Peter H. Seeberger
Director of the Department of Biomelecular Systems

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Development of Chemical Assembly Systems

Kerry Gilmore 10.06.1984
2002-2006: Bachelor of Science majoring in Chemistry
Bachelor of Science majoring in Biology (Roger Williams University, Bristol, RI, USA)
2006- 2012: Doctoral Thesis: Cyclizations of Alkynes. (Department of Chemistry and Biochemistry, Florida State University. Tallahassee, FI, USA)
2010: Fulbright Scholar
2012- 2014: Postdoctoral Scientist Biomolecular Systems Department, Max Planck Institute of Colloids and Interfaces.
Since 3/2014: Research Group Leader, Department of Biomaterials, Max Planck Institute of Colloids and Interfaces

Chemical synthesis traditionally takes a linear approach, developing both chemistries and technologies to achieve novel and more efficient routes towards specific targets. In recent years, flow chemistry has emerged as a useful tool to aid in a chemist's pursuits, accessing advanced structures and active pharmaceutical ingredients (APIs) in both step-wise and multi-step processes. Conceptually, however, the field has not advanced, as multi-step synthetic processes remain target oriented. This group has introduced a novel paradigm in organic synthesis aimed at advancing this field: chemical assembly systems **[1].** These systems consist of modular synthesis platforms **[2-4],** capable of being connected in an interchangable fashion. This non-iterative approach to automated assembly manifests itself into three fields of study in our group: (1) the development of novel reaction modules allows us to perform detailed methodological studies, accessing transformations and selectivities not previously realized; (2) when individual units are linked together, continuous, multistep synthesis can be achieved targeting specific compounds with the goal of low-cost, high-efficiency syntheses; (3) individual modules can also be arranged interchangeably, with the goal of developing divergent synthesis systems which allow access to a wide breathe of chemical space.

Methodology

The development of novel reaction modules is the core of our philosophy. Reaction modules are developed in one of two mindsets: either as a method to achieve selectivity/reactivity unachievable in a batch setting or to provide a required transformation within a given synthetic setting. One area where we have made a considerable impact is the application of photochemistry in a continuous synthesis setting **[5].** Specifically, in 2011 we developed a facile and reliable means of producing singlet oxygen photochemically in a flow reactor **[2].** This allowed for the rapid examination of a variety of transformations including the ene reaction. This important process was utilized to provide the first continuous synthesis of an anti-malarial medicine Artemisinin **[6, 7, Fig. 1].** The advantage of our developed reactor module as compared to previous photochemical set-ups is our ability to control the temperature down to -80° C, allowing us to control reactivity and achieve excellent selectivity. One example of this selectivity is that while secondary amines can be easily oxidized with singlet oxygen and trapped with a nucleophile, the corresponding primary amines instead give the product of oxidative coupling at room temperature. However, we have shown that at -50° C the desired aldimine can, for the first time, be efficiently trapped with a variety of nucleophiles **[3,8].** We were also the first to introduce a visible light-mediated single electron transfer flow system **[9],** accomplishing a variety of transformations using a home built reactor **(Fig. 1).** Recently, we have developed a means of accomplishing carbonyl and imine reductions using inexpensive sodium borohydride in flow **[4].** While this common reagent has been used for decades in batch systems, only expensive, soluble reductants could be used in flow. This module was developed in the context of a divergent synthesis system, described below.

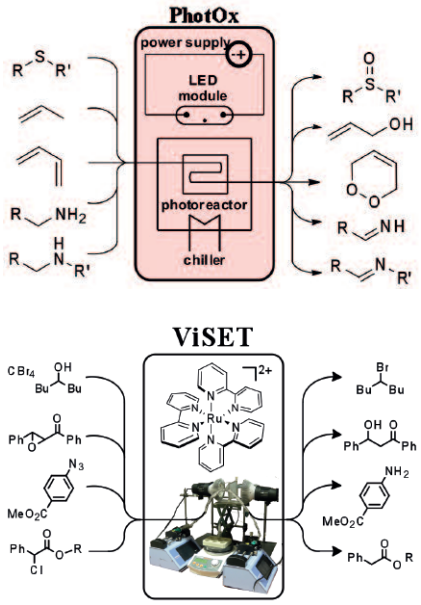

Fig. 1: Two examples of reaction modules which have been developed. These versatile, chemoselective units can be linked with others or used individually.

Target Oriented Synthesis

Continuous chemistry offers the inherent advantage of more efficient and less expensive production. With this in mind, this group, as well as others, have sought to develop continuous, multi-step syntheses for the production of active pharmaceutical medicines (APIs). By coupling our PhotOx module with a second reactor capable of an acid-catalyzed rearrangement, we were able to develop a synthetic process to produce artemisinin **[6, 7].** This WHO-critical medicine is currently obtained via extraction from the plant *A. annua* and the price fluctuates so erratically that demand is often not met. While artemisinin is now also being produced in limited quantities in a biotech/chemical manner, our developed process will help to expand production by converting a current plant waste product, dihydroartemisinic acid, into artemisinin – increasing production and helping to stabilize prices **(Fig. 2).** We have also developed several other processes for the synthesis of other APIs, including the anti-obesity drug Rimonabant as well as an upcoming publication featuring the shortest-ever synthesis of the HIV medicine Efaverinz **[10].**

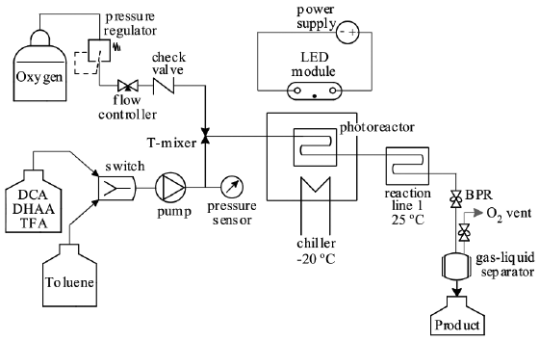

Fig. 2: Chemical engineering diagram for the developed continuous-flow process for the synthesis of artemisinin from the natural extract dihydroartemisinic acid (DHAA).

Systems Oriented Synthesis

The most efficient way to manufacture a given product, however, is not target oriented. Ideally, a process would be adaptable, divergent, and modular such that a variety of products can be made using a single system. While almost every other product worldwide is produced in this assembly line manner, pharmaceuticals are still produced in a stepwise batch manner, resulting in long production times and, critically, high costs. In 2014, we introduced a novel paradigm to organic synthesis, that of the chemical assembly systems (CAS). These processes rely on flow reaction modules, connected in series, to produce specific core structures. By modifying the reactants as well as the order of the reaction modules, a wide breadth of chemical space can be accessed in both a convergent and divergent manner. Two such systems have thus far been introduced **[11, 12].** The first expands our synthetic efforts past artemisinin to the derivatives utilized in anti-malaria treatments **(Fig 3).** The major breakthrough was the development of a means of not only reducing artemisinin to dihydroartemisinin inexpensively **[4],** but the coupling of this step to the synthesis of the final substrates, something which was previously unrealized. Inline IR monitoring of the process allowed for real-time information regarding the quality and robustness of the process to be monitored. In collaboration with Prof. Seidel-Morgenstern at the MPI in Magdeburg, we coupled this system to a continuous purification module, allowing for the continuous production of medicines which exceed WHO/FDA quality standards **[11].**

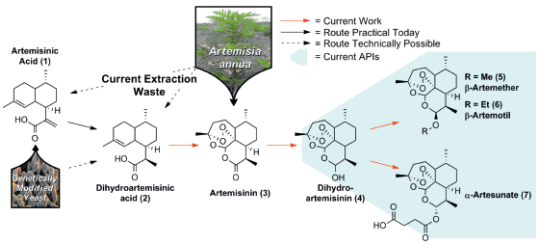

Fig. 3: A divergent chemical assembly system was developed to access all of the currently WHO-recommended treatments for malaria.

While a variety of medicines could be obtained using the above-described system, the structural diversity of the products obtained is low. We thus set out to develop a truly divergent system, where interchangeable modules allowed access to a wide breadth of structural classes. As such, a five-module system was developed, which could be connected in a several different combinations to access three different structural classes: β-amino acids, γ-lactams, and γ-amino acids **(Fig 4).** With judicious choices of starting materials, we were able to synthesize five different active pharmaceutical ingredients present in generic or patented medicines (Rolipram, Lyrica, Phenibut, Baclofen, Gabapentin) in good overall yields (49–75%). Importantly, neither purification nor modification of the reaction stream occurred between modules **[12].**

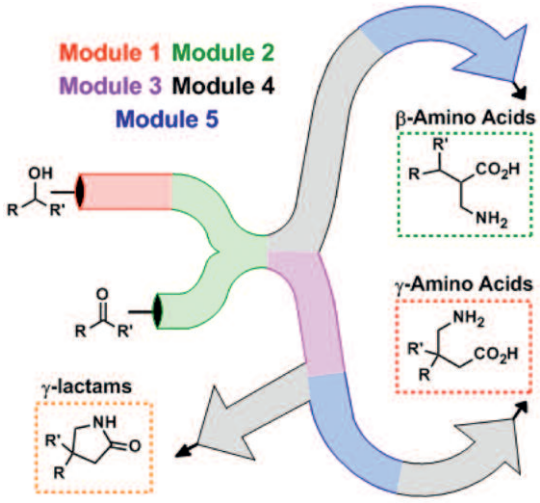

Fig. 4: A five module CAS was developed which accessed three structural classes of compounds. This system was used to produce five active pharmaceutical ingredients continuously in good overall yields.

This group attempts to advance the manner of which organic compounds are being produced through the development of chemical assembly systems. These flexible systems are comprised of flow reaction modules, which are developed either to fit a need within a given synthesis or as part of mechanistic investigations. We also utilize the inherent advantages of flow chemistry to devise alternate, more efficient syntheses of specific compounds, specifically medicines, with the aim of reducing both production time and cost.

K. Gilmore, C. Correia, D. Ushakov, M. Plutschack, S. Vukelić, G. Xiao, S. Chatterjee
kerry.gilmore@mpikg.mpg.de.

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Opening the Doors Towards Automated In-Depth Glycan Sequencing

Daniel Kolarich 25.03.1975
2000: Diploma, Food Science and Biotechnology (University of Natural Resources and Applied Life Sciences, Vienna)
2004: PhD, (University of Natural Resources and Applied Life Sciences, Vienna), Topic: Mass spectrometry based glycoproteomic analysis of GMO food crops and allergens from plants and insects
2005-2007: Postdoc, (University of Natural Resources and Applied Life Sciences, Vienna)
2007-2010: Post Doc, (Macquarie University, Sydney, Australia)
Since 09/2010: Group Leader Glycoproteomics Group, (MPI of Colloids and Interfaces, Berlin)

Glycoproteins are complex molecules where the DNA encoded protein sequence is further modified with specific sugar chains **[1].** A sophisticated cellular network of various enzymes performs these complex modifications on the protein sequences in a non-template manner, making any predictions which set of glycans is present on a specific protein at a specific time point impossible. However, the specific glycan structures present on particular cell surfaces and on specific proteins at a given time point are known to influence the functionalities of the respective cells & proteins, which becomes particularly important in the context of many major diseases. Despite the fact that a global change of glycosylation has been frequently reported in many different types of cancer and chronic inflammatory diseases such as inflammatory bowel diseases, the global impact of these dynamic glycosylation alterations are still largely not well understood – also due to the lack of reliable, sensitive and sufficiently selective methods to analyse specific glycosylation signatures within a single experiment from minute amount of clinical specimens. Within the Glycoproteomics group we are working on developing and automating methods for glycan and glycoprotein sequencing and applying these on clinically relevant challenges **(Fig. 1).**

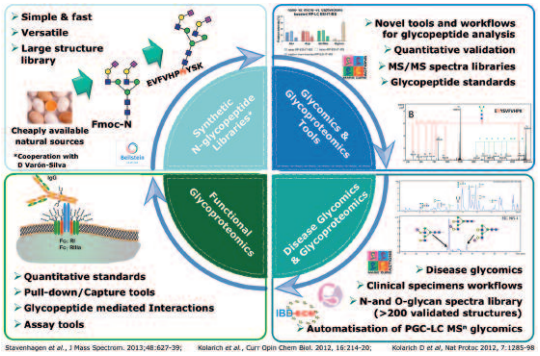

Fig. 1: Four major, interconnected research areas are followed within the glycoproteomics group in the efforts to sequence glycoconjugates.

The Technology Behind Mass Spectrometry Based Glycan Sequencing

Glycan structures are often built up by similar building blocks, however, in contrast to e.g. peptide or DNA oligomers, these different glycan building blocks can be linked either via different linkages (alpha or beta) or via different positions within a monosaccharide to form larger oligosaccharides. However, the type of linkage and its position influence the biological properties of these glycoconjugates, thus understanding the functional relevance of protein glycosylation requires accurate and selective methods for their sequencing.

Many of these glycoconjugates exhibit an exact similar chemical composition, making it difficult to separate and distinguish them by simple tandem Mass Spectrometry (MS/MS) approaches. Within the glycoproteomics group we are combining the selectivity benefits provided by porous graphitized carbon (PGC) liquid chromatography (LC) separation with the sensitivity and speed of MS/MS detection to separate, detect, characterise and relatively quantify such isobaric structure compounds **(Fig. 2) [2,3].**

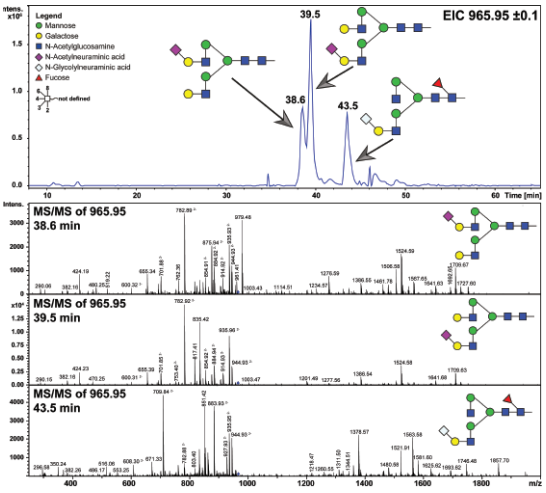

Fig. 2: Example for the separation and identification of an isobaric N-glycan present in porcine serum. Top: Extracted ion Chromatogram (EIC) showing the separation of the three N-glycans exhibiting exactly the same m/z value. Bottom spectra: Fingerprint tandem MS spectra allowing automated in depth structure assignment.

The Human Bowel N- and O-glycomes Show Section Specific Signatures

Chronic inflammatory bowel diseases (IBD), including Crohn s disease and ulcerative colitis, are affecting a large part of the society, but IBD onset and progression are comparably poorly understood. The colon mucosa is heavily glycosylated and lectins as well as glycan receptors were suggested to be involved in the aetiology of IBD. However, there is comparably little known about IBD related glycosylation. A step towards a better understanding of these diseases is the identification of disease specific glycosylation signatures by profiling large sample cohorts of colon tissue. Mucin glycosylation changes have been reported to occur over the entire healthy colon, but hardly any information is available on the bowel cell surface protein glycosylation.

As part of a larger EU-funded consortium (www.ibd-biom.eu) aiming to gain a better understanding of IBD onset and progression we are investigating bowel tissue specific glycosylation signatures. In order to identify disease specific global glycosylation features we first require a detailed map on the cell surface glycosylation of the colon, one of the largest organs of the human body. Using our PGC-LC ESI MS/MS based glycan sequencing platform we systematically analysed and deciphered N-glycan and O-glycan profiles of colon biopsies from IBD and control patients. From the biop-

sies which were taken from seven distinct positions between ileum and rectum the to date most comprehensive protein glycome map of the human bowel was established **[4].** In each biopsy more than 150 individual N-glycan structures and a similar number of O-glycans could be identified and characterised. We could show that distinct differences in the occurrence of specific glycosylation features occurred in a region specific manner (an example for a single glycan feature is shown in **Fig. 4**). This data provides crucial step forward in the on-going glyco-marker screening as this information allows a better matching between different disease vs. control samples and disease specific features can be more accurately distinguished from region specific ones. A key achievement in this undertaking was the establishment of a distinct N- and O-glycan MS/MS spectra library database for semi-automated glycan structure annotation. These developments are currently being applied in the on-going analysis of a larger dataset derived from 500 patient and the comparable number of control samples.

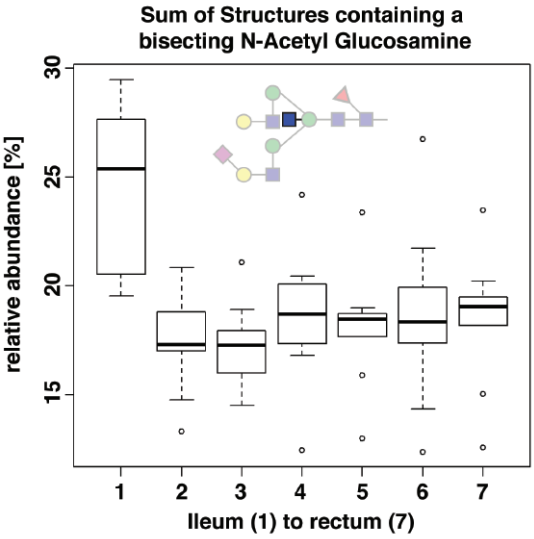

Fig. 4: Occurrence of N-glycans carrying a bisected N-acetyl glucosamine residue. The presence of this glycan feature is significantly increased in the ileum part of the colon.

Accessing Disease Glyco-Signatures from FFPE Histopathological Specimens

In order to study the diagnostic and/or prognostic potential of disease induced glycosylation alterations access to well-characterised samples is a crucial factor. Nevertheless, the availability of such well-defined clinical specimens in sufficient numbers often represents a serious obstacle in glyco-biomarker analyses. Therefore we explored the possibility to use formalin-fixed paraffin-embedded (FFPE) clinical specimens as an attractive alternative for glyco-biomarker research, given that the glyco-epitopes remain unaltered and sufficient glycan amounts can be obtained from conventional FFPE tissue sections frequently used by pathologists.

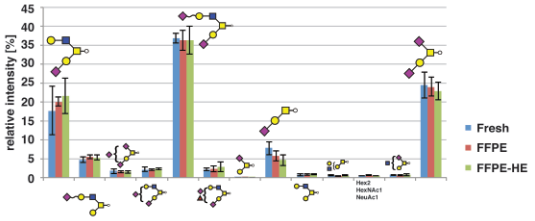

Fig. 3: O-glycan profiles obtained from different preparations of human heptacellular carcinoma specimens from a single donor. No statistically relevant differences in the qualitative and relative quantitative presence of the identified O-glycans were detected between fresh tissue, FFPE and FFPE slides stained with hematoxylin (FFPE-HE).

Our recent efforts have allowed us to establish a workflow for an in-depth glycomic PGC-LC ESI MS/MS based profiling of both, N-and O-glycans from single FFPE tissue sections as thin as 3 µm. We could show that unstained as well as hematoxylin and eosin (HE) stained FFPE tissue samples provide similar results and that these results are largely correlating to the data obtained from non-FFPE treated fresh material [5]. This enables us now to I) gain easier access to clinical specimens from FFPE storage repositories, II) easily obtain statistically significant numbers of clinical samples required for serious glyco-biomarker screening and III) work from specimens which have been evaluated by pathologists prior analysis.

Our recent developments represent an important step forward in the glycan sequencing of clinical samples, providing large datasets on the dynamics of disease induced glycosylation alterations. With this basic knowledge a better understanding on the functional aspects of protein glycosylation in health and disease will be achieved.

D. Kolarich, K. Alagesan, A. Almeida, H. Hinneburg, U. Möginger, F. Schirmeister,
daniel.kolarich@mpikg.mpg.de.

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C-type Lectin Receptors: Ligand Identification, Cell-Specific Targeting and Their Role in Infection and Inflammation

Bernd Lepenies 18.09.1978
1999-2004: Diploma, Biochemistry & Molecular Biology (University of Hamburg)
2005-2007: PhD, Biology ("summa cum laude") (Bernhard Nocht Institute for Tropical Medicine, Hamburg, and University of Hamburg) Thesis topic: Role of co-inhibitors CTLA-4 and BTLA in T cell regulation during malaria
2008: Postdoctoral Fellow (Swiss Federal Institute of Technology, ETH Zurich, Switzerland)
2009-now: Group Leader, Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces
2012-now: Project Leader, Collaborative Research Centre 765 (Multivalency, Freie Universität Berlin)
2014: Habilitation in Biochemistry (Freie Universität Berlin)
Thesis topic: C-type lectin receptors – ligand identification, cell-specific targeting and their role in infection and inflammation

Research of the Glycoimmunology group focuses on lectins. Lectins are carbohydrate-binding proteins that display high specificities for certain sugar moieties. We are interested in a specific lectin superfamily, termed C-type lectin receptors (CLRs). In innate immunity, CLRs serve as pattern recognition receptors that recognize invading pathogens, thus they provide a first line of defense in the body. CLRs are mainly expressed by antigen-presenting cells (APCs) such as dendritic cells and macrophages and often recognize carbohydrates in a Ca^{2+}-dependent manner. Engagement of CLRs by carbohydrate ligands may lead to APC activation, but may also dampen APC functions. Thus, CLRs are often crucial to initiate protective immune responses against pathogens, but they can also contribute to immune homeostasis **(Fig. 1).**

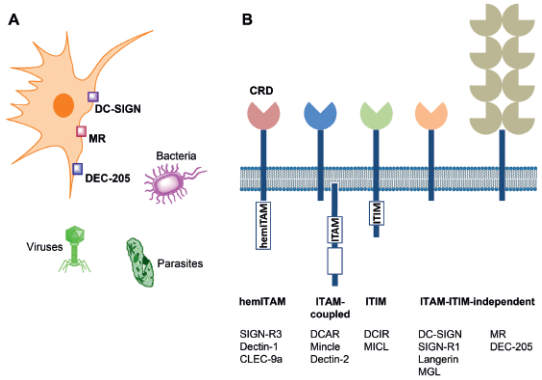

Fig.1: Myeloid C-type lectin receptors (CLRs) in innate immunity. A, CLRs such as DC-SIGN, Mannose receptor (MR), or DEC-205 serve as pattern recognition receptors in innate immunity and bind to carbohydrates on pathogens such as bacteria, viruses, or parasites. B, Myeloid CLRs share one or more homologous carbohydrate-recognition domains (CRD) that bind to carbohydrates often in a Ca^{2+}-dependent manner. The signaling pathway elicited upon CLR engagement depends on the cytoplasmic signaling motif. Figure reprinted from ***[1]*** *with permission from Elsevier.*

One major goal of our group is to identify yet unknown CLR ligands on pathogens and to further characterize these novel CLR-pathogen interactions on the molecular level. In addition, we use distinct carbohydrate ligands to target CLRs on APCs for a cell-specific delivery of drugs and vaccine antigens. Finally, we are interested in the role of CLRs *in vivo*. To this end, we employ murine infection and autoimmunity models to unravel the contribution of specific CLRs to immunity against infections and to clarify their role in immune homeostasis.

CLR Targeting for Cell-Specific Drug Delivery and Immune Modulation

Numerous APC functions may be influenced by CLR targeting such as antigen uptake and presentation, cytokine production, and/or the expression of co-stimulatory molecules **(Fig. 2).** As a consequence, the ligand recognition by CLRs on APCs impacts the subsequent T cell activation and also affects the $CD4^+$ T cell differentiation into T cell subsets. Thus, CLR targeting is a means to shape an initiated immune response and may be exploited for immune modulatory therapies **(Fig. 2).**

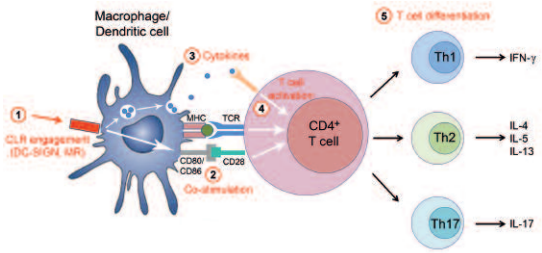

Fig. 2: CLR targeting as a means to shape an initiated immune response. Engagement of myeloid CLRs in innate immunity such as DC-SIGN or MR leads to receptor-mediated endocytosis (1). When an antigen is targeted to an antigen-presenting cell (APC), the antigen will be internalized, processed and presented by major histocompatibility complex (MHC) molecules to T cells. Moreover, signaling pathways may be provoked in APCs that lead to the expression of co-stimulatory molecules CD80/CD86 (2), and the production of cytokines (3). T cells recognize MHC-presented peptides through their T cell receptor (TCR) and are activated in the presence of a secondary, co-stimulatory signal mediated via the interaction of CD80/CD86 with CD28 (4). Cytokines released by APCs influence T cell differentiation and T cell effector functions (5). Figure reprinted from ***[1]*** *with permission from Elsevier.*

During the past years, the Glycoimmunology group has generated a comprehensive CLR library using eukaryotic expression systems. The library consists of so-called CLR-Fc fusion proteins in which the extracellular domain of each CLR is fused to the Fc part of human IgG1 molecules. By now, the library covers a high number of immunologically relevant CLRs. With the help of this library, we have identified several novel carbohydrate ligands of CLRs and have evaluated their potential for cell-specific antigen delivery and immune modulation. As a first step, the glycan array platform was used to screen for CLR-carbohydrate interactions **(Fig. 3).** To test whether the identified CLR ligands could be used for CLR targeting on APCs, selected carbohydrates were then coupled to the model antigen ovalbumin (OVA). The OVA neoglycoconjugates were used to stimulate APCs *in vitro* and were also employed for immunization studies *in vivo*. Indeed, the carbohydrate modification of OVA led to increased antigen targeting to APCs and impacted their cytokine profile as well as their antigen presentation capability **[2, 3].** Hence, we have demonstrated that the platform developed in our group can be used to screen for CLR ligands and their potential for immune modulation.

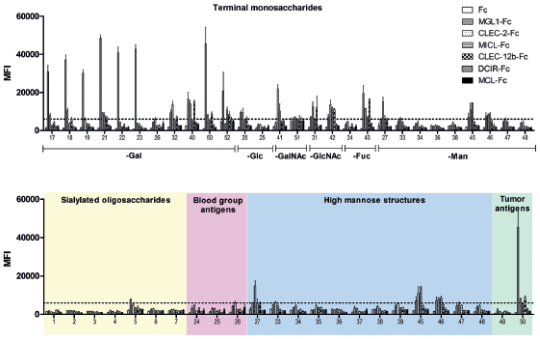

Fig. 3: Exemplary glycan array results for the CLR-Fc fusion proteins MGL-1-Fc, CLEC-2-Fc, MICL-Fc, CLEC-12b-Fc, DCIR-Fc, and MCL-Fc. The results are shown as mean fluorescence intensities (MFI)±SEM and are presented based on terminal monosaccharides recognized by the respective CLR-Fc fusion protein or based on the type of glycan structure. Figure reprinted from ***[2]*** *with permission from Elsevier.*

In a collaborative project with the MPI-DKTS in Magdeburg, we investigated how a differential glycosylation of influenza vaccine antigens impacted APC targeting and subsequent T cell activation **[4]**. We focused on influenza virus hemagglutinin (HA) since HA is the most abundant protein in the virus particle membrane and an essential component of most influenza vaccines. Indeed, HA glycosylation markedly impacted T cell activation *in vitro*. To analyze the impact of HA glycosylation *in vivo,* mice were immunized with the differentially glycosylated influenza virus variants. We observed a dramatic reduction in T cell activation and anti-HA antibody production when mice were immunized with the deglycosylated influenza virus variants. In conclusion, this study highlights the potential of "glyco-optimization" of antigens to increase their immunogenicity and to enhance vaccine potency.

Role of CLRs in Infection and Inflammation

A major focus of our work is the characterization of CLR functions *in vivo* in relevant murine models. Currently, we have established a number of CLR-deficient mouse lines and elucidate the role of specific CLRs in malaria as well as colitis models. In a recently published study, the contribution of the CLR Dendritic cell immunoreceptor (DCIR) to the pathogenesis of cerebral malaria was analyzed **[5].** Using *Plasmodium berghei* ANKA infection of mice, we found a crucial role for DCIR in cerebral malaria induction. DCIR$^{-/-}$ mice were protected from cerebral malaria and displayed markedly reduced leukocyte sequestration in the brain. Accordingly, DCIR$^{-/-}$ mice exhibited decreased TNF-α serum levels as well as a modulated activation of CD4^{+} and CD8^{+} T cells in spleen. Thus, DCIR is essential for cerebral malaria induction highlighting the importance of CLRs for innate immunity during malaria.

We have also analyzed the function of CLRs in the regulation of intestinal immunity. For instance, we have investigated the role of the murine CLR SIGNR3 in colitis pathogenesis **[6].** We found that SIGNR3 recognizes fungal species present in commensal microbiota. To analyze whether the SIGNR3/fungi interactions influence intestinal immunity, the model of chemically induced colitis was employed. In this model, SIGNR3$^{-/-}$ mice exhibited an increased weight loss accompanied by more severe clinical colitis symptoms compared to wild-type mice. The increased inflammation in SIGNR3$^{-/-}$ mice was caused by higher cytokine levels such as TNF-α in colon. This finding indicates that CLRs are involved in intestinal immune homeostasis and that dysfunction in commensal recognition by specific CLRs may contribute to colitis. We also analyzed binding of two other poorly characterized members of the CLR family, the Macrophage-restricted C-type lectin (MCL) and the Dendritic cell immunoreceptor (DCIR) to microbiota **[7].** Both CLRs bound to intestinal microbiota to a different extent and modulated the production of pro-inflammatory cytokines by APCs upon stimulation with heat-killed microbiota. In addition, these CLRs also impacted T cell responses in APC/T cell co-cultivation assays *in vitro*. However, MCL$^{-/-}$ as well as DCIR$^{-/-}$ mice exhibited only a slightly increased severity of disease in the murine model of chemically induced colitis compared to wild-type mice. The limited role of both CLRs *in vivo* may be due to cross-talk between different CLRs and partially redundant CLR functions in intestinal immunity. As a next step, we plan to investigate how multiple CLRs impact disease pathogenesis. Currently, we are also identifying distinct CLR ligands on commensal microbes.

The role of CLRs in antimicrobial immune responses seems to be evolutionarily conserved across species. Recently, we discovered that specific C-type lectins in the roundworm *Caenorhabditis elegans* are crucial for immunity during bacterial infection **[8].** Thus, CLRs may contribute to pathogen recognition in species of many phylogenetic clades.

Multivalent Targeting Approaches

Carbohydrate-lectin interactions and – even more – carbohydrate-carbohydrate binding events generally display low affinities. Consequently, multivalent approaches are often needed to exploit these weak interactions for cell-specific targeting and imaging. Further targeting approaches of our group include liver-specific drug delivery by targeting the asialoglycoprotein receptor expressed by hepatocytes as well as antibiotics delivery to bacteria for antimicrobial treatment **[9].**

B. Lepenies, M. Eriksson, J. Hütter, T. Johannssen, M. Maglinao, S.M. Miltsch, U. Vogel, A. Wagner, T. Wagner, S. Zimmermann
Bernd.Lepenies@mpikg.mpg.de.

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Synthesis of Plant Carbohydrates and their Biological Application

Fabian Pfrengle 26.11.1981
2001-2006: Diploma in Chemistry (with distinction) at the Freie Universität Berlin
2006-2010: Dissertation in Organic Chemistry (summa cum laude) at the Freie Universität Berlin, Advisor: Professor Hans-Ulrich Reißig
2007-2009: Predoctoral fellowship of the Fonds der Chemischen Industrie (FCI)
2010-2013: Research Associate in the Department for Chemical Physiology at the Scripps Research Institute in La Jolla, USA, Advisor: Professor James C. Paulson
2010-2012: Postdoctoral fellowship of the Deutscher Akademischer Austauschdienst (DAAD)
Since 09/2013: Research Group Leader in the Department of Biomolecular Systems at the Max Planck Institute of Colloids and Interfaces
2013-2015: Liebig fellowship of the Fonds der Chemischen Industrie (FCI)
Since 02/2015: Emmy-Noether fellowship of the DFG

Carbohydrates play crucial roles in the life cycle of plants, both as structural components and as important players in signaling events and energy provision **[1].** As a food source, plant carbohydrates can provide beneficial effects on the human immune system, but constitute also abundant immune determinants on allergens. Despite the strong impact of plant carbohydrates on human health, their chemical synthesis remains largely unexplored compared to the synthesis of mammalian and bacterial glycans. Our aim is to explore automated oligosaccharide synthesis **[2]** and chemo-enzymatic methods **[3]** for the generation of plant carbohydrate libraries as a powerful means for investigating their application in plant biology and biomedical research. In particular, two types of plant carbohydrates are synthesized: polysaccharide fragments of the plant's cell wall and *N*-linked glycans of plant glycoproteins. The synthesized plant carbohydrates are applied in the characterization of monoclonal antibodies derived from cell wall polysaccharides and in the development of improved methods for allergy diagnosis. In addition, the polysaccharide fragments are evaluated for their immunostimulatory potential. Together, the synthetic plant carbohydrates will provide a new toolbox for studying the role of carbohydrates in plant biology and their interaction with human health.

Characterization of Cell Wall Glycan-Directed Antibodies with Synthetic Plant Carbohydrates

A large amount of plant carbohydrates are located in the cell wall, which consists of a complex mixture of polysaccharides and other biopolymers assembled into a highly organized network that surrounds all cells. Many genes responsible for the biosynthesis of cell wall polysaccharides have been identified and detailed insight into the structure and function of plant cell wall polymers has been gained by high resolution imaging of cell wall microstructures **[4].** Monoclonal antibodies directed toward plant polysaccharide antigens are used by plant biologists as powerful molecular probes to detect the structural elements of glycans in the cell wall. However, the precise molecular structures recognized by the antibodies are unknown. The goal of the project is to exploit automated solid-phase synthesis for the rapid assembly of plant carbohydrate libraries and their application in the epitope mapping of monoclonal antibodies.

One of the main components of plant cell wall polysaccharides is the hemicellulose xylan, the second most abundant polysaccharide in nature. Xylans are dietary carbohydrates in everyday food that can provide medicinal benefits including immunomodulatory, anti-tumor, and anti-microbial effects. In addition, xylans are potential resources for the production of food additives, cosmetics, and biofuels. Although the structure of xylans varies between plant species, they all possess a common backbone consisting of β-1,4-linked D-xylopyranoses. This backbone structure may be partially acetylated and substituted with L-arabinofuranosyl or D-(4-*O*-methyl) glucuronyl residues.

We produced a library of eleven oligoarabinoxylans of different complexity by automated solid-phase synthesis and printed the compounds as microarrays for probing a set of 31 anti-xylan monoclonal antibodies for binding. We observed specific binding of the antibodies to the synthetic oligoarabinoxylans and the binding epitopes of several antibodies were characterized **(Fig. 1).** This work will serve as a starting point for future studies where libraries of synthetic plant oligosaccharides are screened for the binding of cell wall glycan-directed antibodies, generating the essential information required for interpretation of immunolabeling studies of plant cell walls.

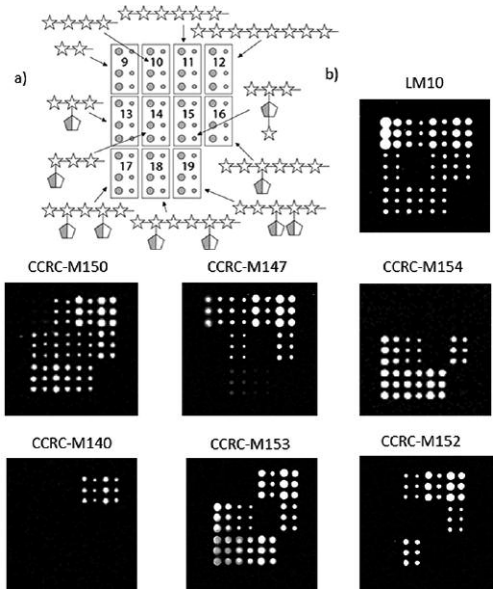

Fig. 1: Detection of oligoarabinoxylans by anti-xylan monoclonal antibodies (mAb): a) Printing pattern; b) Microarray scans. Representative scans of at least two independent experiments are shown. The intensity of the spots corresponds to the binding affinity of the respective mAb. The structures are drawn according to the CFG-nomenclature.

Evaluation of the Synthesized Polysaccharide Fragments for their Potential as Immunomodulators

Plant cell wall polysaccharides are important dietary carbohydrates in everyday food such as fruits and cereals. They are believed to exhibit beneficial therapeutic properties through modulation of innate immunity **[5],** but the molecular basis of their interaction with immune receptors remains largely unknown. We will evaluate synthetic polysaccharide fragments for their potential to stimulate immune cells. A long-term objective of the study is the identification of specific binding epitopes on immunomodulatory polysaccharides and of the receptors responsible for their recognition.

Chemo-Enzymatic Synthesis of Plant N-Glycans

Plants are not only an important part of the food chain, but can also cause pollen and food allergies **[6].** Many or most of the plant-based allergens we inhale or ingest are glycosylated with oligosaccharides that are potentially immunogenic. *N*-Linked glycans in plant glycoproteins include similar glycans as found in animals but are of limited diversity and feature several unique modifications such as an additional xylose or fucose residue. We plan on synthesizing a collection of plant *N*-glycans by enzymatic carbohydrate synthesis for various biological applications.

Construction of a Plant Carbohydrate Microarray

Current carbohydrate microarrays are strongly biased towards mammalian glycans and do not contain large numbers of bacterial or plant-specific oligosaccharides **[7].** To resolve this shortcoming, we will generate a comprehensive microarray containing synthetic plant carbohydrates. The microarray will be used for lectin binding studies, screening of the sera of allergy patients, and epitope mapping of monoclonal antibodies developed against plant cell wall polysaccharides **(Fig. 2).**

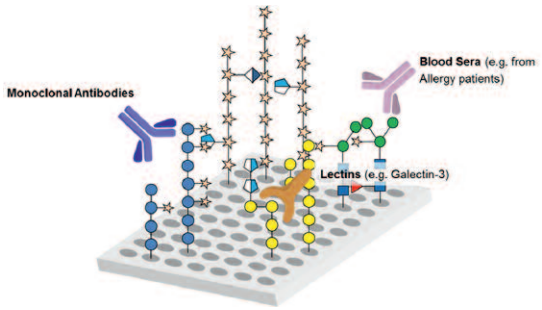

Fig. 2: Plant carbohydrate microarray for probing the binding specificities of monoclonal antibodies, antibodies from sera, and lectins.

F. Pfrengle, P. Dallabernardina, D. Schmidt, M. Bartetzko
Fabian.Pfrengle@mpikg.mpg.de

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Specific C-type Lectin Receptor Ligands

Christoph Rademacher 05.05.1979
2001-2004: Bachelor Studies in "Molecular Biotechnology" at the University of Lübeck, Germany
2004-2006: Master Studies in "Molecular Life Science" at the University of Lübeck, Germany
2006-2009: Doctoral Thesis: "Investigations into Viral Entry Mechanisms and Carbohydrate-Protein Interactions using NMR" (summa cum laude, Prof. Dr. Thomas Peters, Institute of Chemistry, University of Lübeck, Germany)
2007-2009: PhD stipend from the Fonds der chemischen Industrie.
2009-2011: Postdoctoral training at Department of Chemical Physiology, The Scripps Research Institute with Prof. Dr. James C. Paulson (La Jolla, CA, USA).
2009-2011: EMBO Long-Term Fellowship
Since 11/2011: Research Group Leader, Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces
2011-2012: Liebig Stipend from the Fonds der Chemischen Industrie
Since 06/2012: Emmy-Noether Research Group Leader

Carbohydrates cover every living cell and are a central biomacromolecular building block of life. Cell surfaces are decorated with a large diversity of glycans and by that determine many fundamental processes such as embryonic development, cell-cell communication and regulation of the immune system. Hence, it is not surprising to find glycan binding proteins in all organisms. Three major mammalian glycan binding protein families are important in immunobiology, namely Galectins, Siglecs and C-type lectin receptors (CTLRs). These lectins determine the response to incoming signals during pathogen recognition and killing, antigen processing, and tumor progression.

In particular, mammalian C-type lectin receptors have emerged as targets for immunomodulatory therapies. In contrast to their potential, the number of specific molecular probes modulating carbohydrate recognition by C-type lectin receptors is limited. The dendritic cell-specific intercellular adhesion molecules-3-grabbing non-integrin (DC-SIGN) is a well-studied CTLR and is expressed on dendritic cells and macrophages. A large array of pathogens such as Mycobacterium tuberculosis, Leishmania, hepatitis C virus, Ebola and HIV is recognized. For HIV, it was demonstrated that DC-SIGN promotes trans-infection of T cells and has since then drawn attention as a therapeutic target in anti-viral therapy **[1].** Another CLTR of high interest is Langerin. This lectin is expressed on Langerhans cells and dendritic cells and promotes pathogen uptake and antigen presentation. Due to its restricted expression pattern it also resembles an attractive receptor for targeted immune stimulation and delivery.

The overall goal during the present and upcoming period is the development of specific and high-affinity CTLR ligands for chemical biology purposes. For this, orthogonal routes are followed: (i) fragment-based lead discovery, (ii) computer-aided structure-based design, and (iii) receptor-based NMR techniques.

Druggability Assessment of CTLRs and Fragment-Based Design of Novel Lectin Ligands

The development of small molecule modulators of biological processes is an expensive endeavor and target receptor selection should be done carefully. The term 'druggability' refers to the ability of a target protein to bind a small molecule drug with high affinity and specificity. Several techniques have evolved over the recent years for the prediction of druggability of proteins and resemble a valid starting point in many drug discovery campaigns. Conversely, low druggability scores have been found to be a good indicator for a high failure rate during later stages of development. Both, computational and experimentally derived predictors of druggability have been pursued to evaluate the potential of CTLRs.

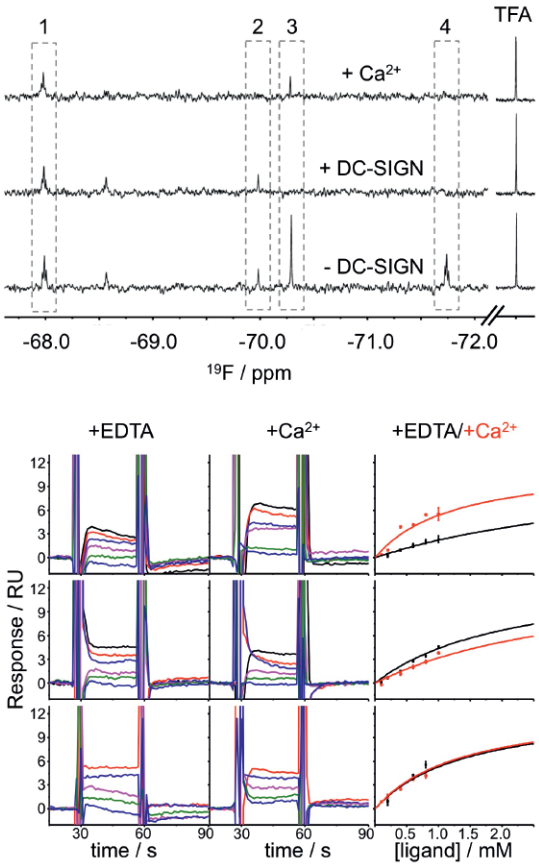

Fig. 1: Screening a fragment library against DC-SIGN. (upper) ^{19}F NMR-based screening of a selected panel of fragments is shown in absence and presence of DC-SIGN and in presence of calcium as a competing ligand for CTLRs (from bottom to top). Disappearance of signals in presence of protein highlights binding of fragments. Re-appearance after addition of calcium suggests that these fragments bind close to the carbohydrate recognition site. (lower) Hit validation was performed using SPR spectroscopy. Here, data of three fragments are shown, exemplifying binders being affected by presence of calcium ions ***[2].***

Computer-aided assessment of target druggability is an attractive method, as its resource requirements are limited. Scores can be deduced from available X-ray crystallographic data and many software tools are available to the community. These algorithms have a two-step process in common that first identifies binding sites and then scores their potential to bind a drug-like ligand. To predict the druggability of human CTLRs we compiled a set of 22 crystal structures and analyzed it using DogSiteScorer **[3].** Many mammalian glycan binding proteins have shallow and feature-less binding sites and in accordance with previous computational evaluations of the druggability of lectins, CTLRs have been found to show only limited potential to recognized drug-like molecules **[2].**

Next, we pursued experimental validation of our findings. One experimental approach to assess the druggability

of a target is screening of fragments of drug-like molecules. A diverse library of fragments ranging between 150 and 300 Da of molecular weight has been constructed and screened **(Fig. 1).** The advantage of using fragments instead of drug-like molecules is the large coverage of chemical space. It has been estimated that 1000 fragments can cover a similar chemical space as 10 trillion drug-sized molecules **[4].** Fragments have an intrinsically low affinity for their targets, requesting sensitive biophysical screening techniques for detection. We chose NMR spectroscopy for the primary screen as it has a remarkably low false-positive rate. In particular, ^{19}F NMR turned out to be highly sensitive and enabled us to detect 10 to 16 % hits in the first round of screening against three human CLTR targets, namely DC-SIGN, Langerin and MCL. These hit rates are a good indicator for a high druggability of the lectin receptors. As ligand observed NMR techniques allow identifying hits from mixtures without deconvolution, these hit structures were directly advanced to an orthogonal validation screen using SPR spectroscopy **(Fig. 1).** Overall, our results highlight limitations of current *in silico* approaches to druggability assessment, in particular with regard to carbohydrate-binding proteins. At the same time, our data indicate that small molecule ligands for a larger panel of C-type lectin receptors can be developed and the biophysical screening resulted in several staring points for future design of specific CTLR ligands **[2].**

Computer-Aided Carbohydrate-Based Design of C-Type Lectin Ligands

The availability of a few X-ray structures of CTLRs provides opportunity for rational ligand design **(Fig 3).** We employed the co-crystal structure of a CTLR with its natural carbohydrate ligand to elucidate the potential of carbohydrate derivatives as lectin ligands. In general, compared to the number of members of the C-type lectin fold family, the structural information is rather sparse. Still, a few members of the subfamily of myeloid CTRLs have been characterized by X-ray crystallography and NMR spectroscopy. The resolution of these structures is of sufficiently high resolution allowing *in silico* methods to be used to aid the search for small molecular probes for these lectins. CTLRs that obey a calcium-mediated recognition of glycans share a shallow binding site **(Fig. 2).** This not only explains the intrinsically low affinity of these cell surface receptors for their glycan ligands, but also imposes a challenge to any rational design of high affinity ligands for these proteins.

To overcome this challenge, structure-based *in silico* design is combined with carbohydrate chemistry to generate focused libraries of carbohydrate derivatives. Molecular Operating Environment (MOE) was employed to evaluate a database of 40,000 commercially available building blocks as substituents on a carbohydrate scaffold. Moreover, a synthetic strategy for the preparation of the selected analogs was established. ^{19}F R_2-filtered and ^{1}H saturation transfer difference nuclear magnetic resonance experiments were employed to determine the affinity and the bound conformation of the synthesized analogs.

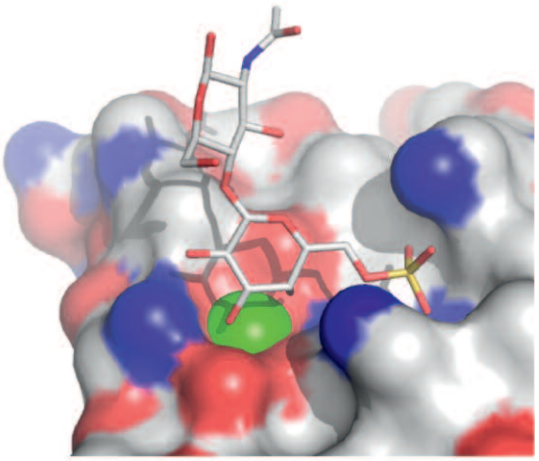

*Fig. 2: Langerin in complex with a natural carbohydrate ligand. The 6'-sulfo-galactose moiety is coordinated by the central calcium ion (green) in the binding site of Langerin **[5].***

Studying C-Type Lectin Interactions with Complex Glycans by Biomolecular NMR Spectroscopy

To expand our understanding of CTLR structural biology, we began studying these receptors employing techniques from solution NMR spectroscopy in presence of their natural glycan ligands. Complex glycan structures have evolved as versatile regulators of many aspects of health and disease such as in immune cell recognition, development, hormone activity, tissue organization, and metastasis. In general, many functions of carbohydrates in a biological context are tightly coupled to their recognition by glycan binding proteins (GBPs). In the context of immune cell regulation, CLTR recognise self- as well as non-self glycan structures. Only the side-by-side analysis of the recognition process at atomic resolution and functional studies in a relevant biological environment provides means to fully elucidate a carbohydrate structure-function relationship. Therefore, we apply biophysical techniques, such as nuclear magnetic resonance (NMR) and combine the results with our insights from computational analysis. These data give rise to hypotheses that are under current investigations in the laboratory.

C. Rademacher, J. Aretz, J. Hanske, J. Schulze, E-C. Wamhoff.
Christoph.Rademacher@mpikg.mpg.de.

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Chemical Biology of Glycosylphosphatidylinositols

Daniel Varón Silva 09.04.1974
1992-1998: Diploma in Chemistry, Department of Chemistry (National University of Colombia, Bogotá, Colombia)
1998-2002: Research associate, Department of Chemistry (Colombian Immunology Institute Foundation, Bogotá, Colombia)
2003-2007: PhD in Chemistry, Bioorganic Chemistry, (University of Bayreuth, Germany)
2007-2008: Postdoctoral Fellow, Laboratory of Organic Chemistry (Swiss Federal Institute of Technology, Zurich, Switzerland)
2009-2010: Postdoctoral Fellow, Department of Biomolecular Systems (Max Planck Institute of Colloids and Interfaces, Potsdam, Germany)
Since 06/2010: Group Leader, GPI and Glycoproteins group, Department of Biomolecular Systems (Max Planck Institute of Colloids and Interfaces, Potsdam, Germany)

Many eukaryotic proteins are attached to the cell membrane using glycosylphosphatidylinositols anchors (GPIs). GPIs are characterized by a conserved core structure containing a glycan pseudo-pentasaccharide, a phosphoethanolamine unit and a phospholipid. However they are usually modified with phosphates, glycans and lipid chains in a cell type dependent form **[1]**. The lipid moiety is variable and may include diacylglycerol, alkylacylglycerol or a ceramide, with chains of different length and degree of unsaturation **(Fig. 1).**

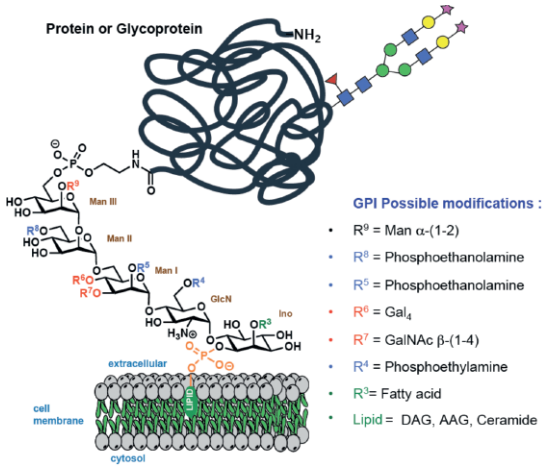

Fig. 1: Structure and possible modifications of GPI anchors. (DAG Diacylglycerol, AAG: Alkylacylglycerol)

The primary biological role of GPIs is to localize the attached molecules to the outer leaflet of the cell membrane **[2].** However, different studies show that GPIs play a role in the association of anchored proteins with lipid rafts and are, thereby, involved in diverse processes such as regulation of innate immunity and protein trafficking among others **[3].**

Development of Strategies to obtain GPIs

Studies to evaluate the role of GPIs and the structure-function relationship rely on the availability of good amounts of homogeneous glycolipids. To address this need we developed a synthetic strategy to obtain well-defined GPIs **[4].** Our strategy is based on modular assembly of common building blocks and relies on a fully orthogonal set of protecting groups. They enable the regioselective introduction of phos-phodiesters and efficient assembly of the glycans **(Fig 2)**. This strategy was used to obtain different and structurally distinct GPIs and GPI derivatives for biological and biophysical evaluations.

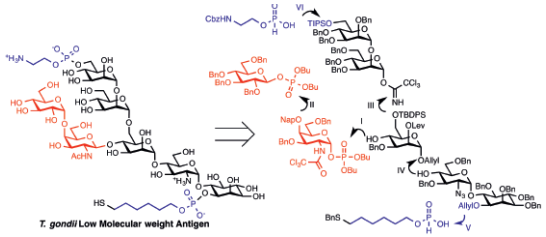

Fig. 2: Assembly sequence of the low molecular weight antigen from T. gondii using the general strategy.

The assembly sequence of the GPIs is dictated by the position of the protecting groups, which is kept constant across the set of common building blocks. The glycosylations are performed using similar coupling partners, making the reactions conditions transferable between different GPI syntheses **[5].**

GPI-Anchors as Diagnostic Tools

Protozoan parasites express highly amounts of non-protein-linked, free GPIs, and GPI-anchored proteins (GPI-APs) that may participate in the regulation of the host immune response during infections **[6].** However, in most cases, the heterogeneity and difficult isolation of pure GPIs have limited the evaluation of their function.

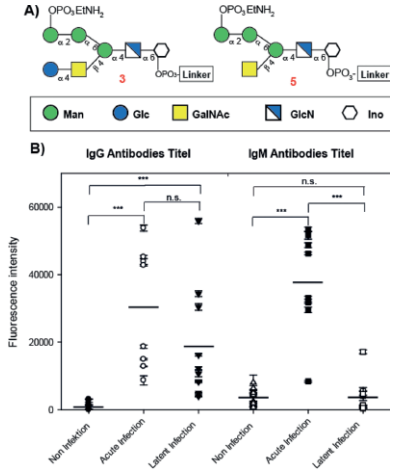

Fig. 3: Antibody levels determined by microarray analysis against LMA of T. gondii in sera samples from different toxoplasmosis stages. Black bars represent mean antibody levels

Two GPIs **3** and **5** of *T. gondii* and a series of GPI fragments either containing a single amine or thiol linker were synthesized and printed on epoxide modified glass slides **(Figure 3A)**. The resulting GPI-microarray was incubated with reference sera of patients with acute or latent toxoplasmosis and with seronegative individuals.

The screening results showed that all sera from non-infected patients contained undetectable or low levels of IgG and IgM antibodies directed against the printed GPIs or their substructures. In contrast, all sera from patients diagnosed with an acute toxoplasmosis showed high levels of IgG and IgM antibodies recognizing the full GPI structure. The sera samples of latently infected patients showed an IgG antibody binding pattern and signal intensities that are comparable

with analyses of sera from acutely infected humans **(Figure 3B)**; however, the IgM levels were considerably low.

These results are in accordance with reports describing that the immune response against GPIs in *T. gondii* infected humans is mainly directed against the free GPI **3**. Base on these results, the GPI **3** of *T. gondii* emerged as a suitable biomarker for the diagnosis of different stages of toxoplasmosis. The IgG level against the GPI **3** can be used to distinguish non-infected from *T. gondii* infected humans whereas the concentration of IgM antibodies binding the same carbohydrate may serve to differentiate latent and acute toxoplasmosis **[7].**

Biophysical Studies with GPI-fragments

Insights into the behaviour of GPIs and GPI-APs in cell membranes could contribute to the understanding of the roles GPIs play in biological processes. In this context, to evaluate the participation of GPIs in the formation of microdomains in the cell membrane, it was performed a comparative analysis of the structural arrangement in a series of 2D model membranes of three GPI-fragments (monolayers formed at the air/water interface). This study demonstrates that increase in the size of the head groups of the fragments from **6** to **7** and then **8** results in an increase in the in-plane area per molecule, which causes increase in the tilt of the alkyl chains and increase in surface pressure required for the transition to a non-tilted phase. While the trends observed are in line with what is expected for such a series of GPI fragments, the addition of a GlcN moiety in compound **8** causes dramatic changes in the structure of the monolayers **(Fig. 4) [8].**

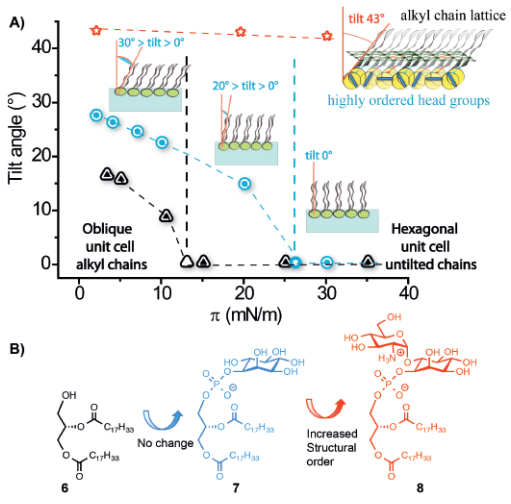

Fig. 4: (A) Comparative representation of the monolayer structural changes of three GPI-fragments (variation of the tilt angle of the alkyl chains (t) with the lateral surface pressure (p)) on PBS at 20 °C. (B) Chemical structures of the investigated GPI-fragments

Compounds **6** and **7** form ordered monolayers defined only by an alkyl chain lattice. In contrast, GPI-fragment **8** forms higher ordered monolayers characterized by two commensurate lattices: a lattice of the alkyl chains and a molecular lattice formed as a consequence of ordering of the head groups through interactions between glycans **[9].** These interactions are likely responsible for the partial segregation of GPI-fragment **8** when mixed with a liquid-disordered model membrane of POPC. When compound **8** is mixed with lipids that form ordered monolayer phases, such as **6** or **7**, hydrophobic interactions of the chains induce complete mixing of the two components. The mixed monolayers of **6** or **7** with **8** are homogenous with structures defined only by ordered alkyl chains and characterized by packing parameters of compounds without strong head group interactions. Further experiments to determine if this behaviour of **8** is applicable in real membranes and transferable to full GPIs are under process.

Synthesis of GPI-Anchored Proteins

To evaluate the effect of GPIs in the function and activity of GPI-anchored proteins, two protein splicing approaches have been used to attach synthetic GPIs to proteins: native chemical ligation (NCL) and protein trans-splicing (PTS).

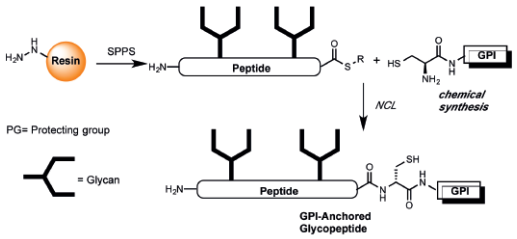

Fig. 5: Schematic representation for the synthesis of GPI-Anchored Glycoproteins

In the first strategy, GPIs containing a cysteine residue at Man III were obtained using the described strategy. To obtain the required protein thioesters, three proteins (GFP, IL-2 and PrP) fused at their C-termini to an intein domain were expressed in *E. coli* in optimized form. After establishing the best purification and folding conditions, the desired protein thioesters were formed. A NCL between the GPI and the protein delivered the GPI-APs. In the second strategy, a naturally split intein from *N. punctiforme* has been used **[10].** In this strategy, the proteins are expressed as fusion proteins with the N-terminal fragment of the split intein and submitted to PTS with GPI molecules linked to the C-terminal fragment of the split intein. With the established methods we have synthesized GPI-anchored GFP and PrP.

Besides the GPI-APs, these strategies allow also the synthesis of GPI-anchored glycopeptides **(Fig. 5).** To introduce the carbohydrate to the peptides we accomplish the Lansbury aspartylation between a solid phase bound, an activated peptide and an amino sugar. After the cleavage of the peptide from the resin, a conversion to the glycopeptide thioester, a NCL is performed between the two synthetic molecules to generate a GPI-anchored glycopeptides. Initial results were obtained with small sugars, however we are developing new methods that allow the introduction of large oligosaccharides into the peptides and proteins.

D. Varón Silva, R. Roller, S. Götze, M. Grube, M.A. Carrillo, B.-Y. Lee, D. Michel, M. Garg and A. Malik
daniel.varon@mpikg.mpg.de

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In Search of the Difference

Zoltán Konthur 18.06.1971
1993-1996: Bachelor of Science (Dual Honours degree) in Genetics and Microbiology (Sheffield University, UK)
1996-2000: Graduate student, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany
2000: Magister rerum naturalium majoring in Genetics (Paris Lodron University, Salzburg, Austria)
2000-2001: Scientific staff, Institute of Pathology, Freie Universität Berlin, Berlin, Germany
2001-2002: Scientific staff, Department of Vertebrate Genomics, Max Planck Institute of Molecular Genetics, Berlin, Germany
2002: Dotor rerum naturalium with "summa cum laude" (Paris Lodron University, Salzburg, Austria)
2002-2012: Research Group Leader, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany
Since 03/2013: Research Group Leader (part-time), Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces

The immune system protects us from foreign substances or pathogens by eliciting an immune response generating specific antibodies. However, in a variety of diseases – especially autoimmune disorders – the immune system dysfunctions leading to self-reactive (auto)antibodies. In some cases, these antibodies can cause severe damage to the body, while in other cases their presence is seemingly without consequence. Our knowledge about their role in disease progression, whether being of significance or simply a bystander effect is rather vague.

The major interest of the Immunomics group centers on the investigation of antibody-antigen complexes in autoimmune disorders. The scientific focus of the group centres on the analysis of V(D)J recombination patterns in immunoglobulin repertoires in healthy individuals and autoimmune patients and the elucidation of autoantigenicity patters in health and disease. The methodological portfolio includes the use of Next Generation Sequencing (NGS), Phage Display as well as Protein Array Technologies.

Immunoglobulin Repertoires of Health and Disease

Our goal is to explore whether there is a difference in the nature of how healthy individuals and autoimmune patients shape their antibody repertoires. The variety of immunoglobulin (Ig) paratopes for antigen recognition is a result of a V(D)J recombination mechanism in the heavy and the light chain of the antibody molecule, while a fast and efficient immune response is mediated by specific Ig-isotypes obtained through class switch recombination (CSR). Hence, we believe that it is not enough to analyse V(D)J recombination, but we need to address the effector function of an antibody encoded in the isotype as well, as it is of equal importance.

Since no adequate analytical tools were available to tackle this question, we have established a new method of yet unpaired sensitivity to amplify and sequence the expressed antibody repertoire of an individual. The method is based on V-gene independent amplification of rearranged immunoglobulin repertoires in combination with emulsion PCR to minimize primer- and PCR-induced bias. We first analysed the obtained sequences using the IMGT/High V-Qest online tool and developed a novel avenue of bioinformatic analysis based not only on information on V(D)J recombination but also on class-switch recombination of individual donors by incorporating isotype-specific analysis of the antibody sequences.

We sequenced the antibody repertoire of peripheral blood mononuclear cells from 14 healthy Caucasian donors of different age and gender. We found, that hierarchical clustering of the donors only according to the V(D)J recombination information revealed neither correlation by age nor gender. However, when CSR information was introduced into the analysis, for the first time, donors clustered hierarchically according to age. We could observe changes in Ig-isotype repertoires to be age-dependent indicating reduction of class-switch capability. This is in good agreement with recent findings suggesting that the dramatically reduced vaccination efficacy in elderly populations is not because of a lack of specific antibodies due to reduction of V(D)J recombination, but rather a problem in antibody titre and lacking specificity in the right immunoglobulin class to elicit response. Unexpected however is the fact that the decline of class-switch ability starts already relatively early. The age of fifty and beyond defines the onset of immune senescence **[1].** We are now extending our analyses to immunoglobulin repertoires of autoimmune patients with rheumatoid arthritis (RA) in collaboration with the Department of Rheumatology and Clinical Immunology of the Charité. We are comparing this data set with that obtained of healthy individuals to see whether there is a difference in the nature of V(D)J recombination patterns between the antibody repertoires of healthy individuals and autoimmune patients.

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Autoantigenicity Patters in Health and Disease

Everybody has circulating self-reactive antibodies in their blood. Although individual repertoires of autoantibodies can significantly overlap, they differ between healthy and diseased individuals. Differential analysis can lead to the identification of biomarker sets that can clearly separate different autoimmune diseases or even allow subdiagnosis of patients within a certain disease. A current project centres on RA and is conducted in close collaboration with the Department of Rheumatology and Clinical Immunology of the Charité. Applying protein array technology, we were able to find specific autoantibody profiles, which allow discrimination between early stages of RA and systemic lupus erythematosus (SLE). With the ongoing downstream characterisation and evaluation of the biomarkers in a BMBF-funded project we now are exploring their diagnostic value. The biomarkers can possibly not only discriminate between early stage RA and SLE, but may possibly also serve as prognostic marker, i.e. give clues about the progression of RA in those patients possessing such autoantibodies.

We apply two complementary screening technologies for the discovery of autoantigenicity patters, namely Protein Arrays and Phage Display. They comprise of different subsets of the human proteome and offer different means of selection. While most antigens on the array are denatured, the proteins on the bacteriophage surface are presented as folded structures. Our protein arrays consist of ~25.000 expression constructs of a human foetal brain cDNA library representing 2055 human genes in multiple copies. For phage display, we have now generated various versions of full-ORF libraries of 4452 genes applying the Gateway-technology. While the identity of each spot on the protein array is known, the phage display libraries require downstream processing. Selection is carried out in an iterative process based on affinity enrichment using patient-derived immunoglobulin fractions as selection targets. The identity of the enriched clones is revealed by NGS of the cDNA inserts. We have recently applied these screening technologies for the elucidation of autoantigenicity profiles of healthy donors, Multiple Sclerosis and Alzheimer's patients **[2, 3]**.

Expression of Recombinant Biomolecules in *L. Tarentolae*

Since most of the biological drugs marketed today are secretory proteins, novel routes of expression are of great interest to the biotech community **[4].** Within the frameworks of a BMBF project, we have explored the use of the protozoa *Leishmania tarentolae*, as an expression host for secretory molecules. *L. tarentolae* is a eukaryotic protozoa which is easily cultured and has been reported to glycosylate proteins in a more human-like manner than for instance yeast. To investigate this, we have generated a vector series for optimized secretion/expression using recombinant antibody fragments as an example **[5].** Additionally, we used these vectors for expression of bioactive human soluble amyloid precursor protein alpha (sAPPalpha) and analyzed its glycosylation pattern in cooperation with the glycoproteomics group (Dr. Daniel Kolarich). We could show for the first time that recombinantly expressed human protein in *L. tarentolae* can not only be N-glycosylated, but also O-glycosylated **[6, 7]**. Additionally, we have recently established a vector/strain combination, which allows the production of *in vivo* biotinylated recombinant molecules **[8].**

Zoltán Konthur, Yuliya Georgieva, Matthias Kaup, Stephan Klatt
zoltan.konthur@mpikg.mpg.de

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Precision Glycopolymers

Laura Hartmann 05.10.1979
1998-2001: Vordiplom majoring in Chemistry (Universität Köln, Germany)
2001-2004: Diplom majoring in Polymer Chemistry (Albert-Ludwigs-Universität Freiburg, Germany)
2004-2007: Doctoral Thesis: Synthesis of monodisperse, multifunctional poly(amidoamines) and their application as non-viral vectors for gene therapy (Max Planck Institute of Colloids and Interfaces, Department of Colloidal Chemistry, Golm, Germany)
2007-2009: Postdoctoral Scientist (Stanford University, Department of Chemical Engineering, Palo Alto, USA)
2009-2014: Emmy Noether research group leader (Max Planck Institute of Colloids and Interfaces, Department of Biomolecular Systems, Berlin/Golm, Germany)
2014: Habilitation in Polymer Chemistry: Precision Glycomacromolecules and Glycohydrogels: Synthesis and Applications in Biotechnology and Biomedicine. (Freie Universität Berlin, Germany)
Since 07/2014: W3-Professor for Macromolecular Chemistry (Heinrich-Heine-Universität Düsseldorf, Germany)

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Sugar Mimetic Ligands

Sugars play an important role in various areas of science and our everyday life. They are an important energy source and thus part of our nutrition. In plants and insects, polysaccharides are used as materials e.g. to build up a chitin shell but they also play an important role in many industrial applications from detergents to paints to plastics. Another area where sugars are gaining more and more attention is medicine. Sugars play a key role in any kind of cell-cell-interaction e.g. during infection, tissue growth or cell signalling. Such interactions are most often mediated by the sugar acting as a ligand binding to a specific protein receptor. However, the specific binding of a sugar ligand to a protein receptor is not easily obtained, as the binding affinity of a sugar to a protein is usually very weak. Nature overcomes this problem by using the so-called multivalency effect where several sugar ligands bind to several receptors at the same time. While it is highly important to understand the principle of multivalency in order to learn more about the role of sugar ligands in biology and medicine, this also opens an opportunity for a new class of molecules – the sugar mimetic ligands. Through the covalent attachment of several sugar ligands to an artificial scaffold and thus their multivalent presentation, we can obtain a molecule having a much higher affinity than the single sugar ligand itself. One important class of such sugar mimetic ligands are the glycopolymers carrying multiple sugar ligands along a polymeric backbone.

Glycopolymers have a great potential for various applications in biomedicine and biotechnology e.g. as antibacterial or antiviral therapeutics due to their increased affinity and other positive attributes such as their ease of synthesis and prolonged stability. However, most glycopolymers so far are optimized empirically and little is known about what makes a glycopolymer a good sugar ligand mimetic and thus a potential candidate in biomedical applications. The major limitation is the ill-defined nature of classical synthetic polymers as they are always obtained as polydisperse samples and do not allow for a sequence control of monomers within the polymeric scaffold.

Solid Phase Polymer Synthesis

Therefore, we introduced a new approach towards monodisperse, sequence-defined glycooligo- and polymers **[1].** The highly defined chemical structure of these so-called precision glycopolymers allows for detailed structure-property correlation studies. On the one hand, this will help to give new insights into the multivalent interactions of natural and sugar mimetic ligands and their role in cell-cell interactions. On the other hand, this will allow for the rational design and straightforward synthesis of novel glycopolymers for various applications especially in biomedicine and biotechnology. Our approach is based on the use of solid phase peptide synthesis **[1,2].** However, instead of amino acids, we use especially designed dimer building blocks. Stepwise assembly of the building blocks on a solid support allows for the straightforward control over the monomer sequence and yields monodisperse molecules **(Fig. 1).** The sugar ligands can be introduced in the same way, by covalent attachment to a functional side chain of one of the building blocks during solid phase synthesis **(Fig. 1).** Besides the exact control over the position of the sugar ligand on the precision glycooligomer scaffold, this also makes it possible to have the precise positioning of different sugars at different positions leading to so-called heteromultivalent glycooligomers **[3].**

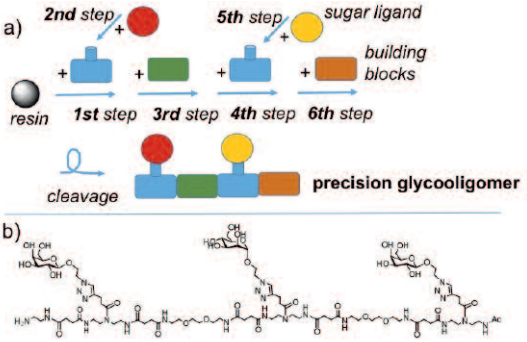

*Fig. 1: a) Schematic presentation of the solid phase polymer synthesis of glycooligo-/polymers. The stepwise assembly of tailor-made building blocks on a solid support and their functionalization with sugar ligands allows for the synthesis of monodisperse sequence-controlled or so-called precision glycooligomers. b) example for a heteromultivalent glycooligomer **[3].***

Currently we are expanding our building block library introducing a variety of functional groups in the scaffold such as degradable moieties, switchable units **[4]** or charges **[5].** Additionally, we can vary the architecture going from linear to branched **[6]** scaffolds as well as to peptidomimetic scaffolds with secondary structure motifs e.g. β-strands **[7].**

Fundamental Studies on Precision Glycopolymers Targeting Lectin Receptors

In order to obtain novel insights into the rational design of glycopolymers and the underlying concept of multivalency, we synthesized a series of glycooligomers varying the number, position and kind of sugar ligands as well as the chemical properties of the scaffold itself **[1-3].** We have found that depending on the structure of the glycooligomer, different binding modes can be observed for sugar ligand/protein receptor complex formation **(Fig. 2).** Specifically, we have observed a strong influence of statistical rebinding, where a higher local density of sugar ligands leads to an increase in binding probability and thus overall binding affinity. Another important effect, so far often neglected for sugar mimetic ligands, are the sterical contributions of all non-binding parts of the molecule. Non-binding parts e.g. of the scaffold or through non-binding sugar ligands attached to the scaffold can sterically shield binding pockets of the receptor and thus inhibit other ligands from binding on this site. This leads to an observed increase in binding affinity. Most of our structure-property correlation studies were performed with Concavalin A **(Fig. 2),** a well-characterized lectin receptor, but we could show that these results are also transferrable to other

lectin receptors such as PA-IL **[4].** Ongoing projects further extend these systematic studies e.g. towards glycooligomers with varying architecture.

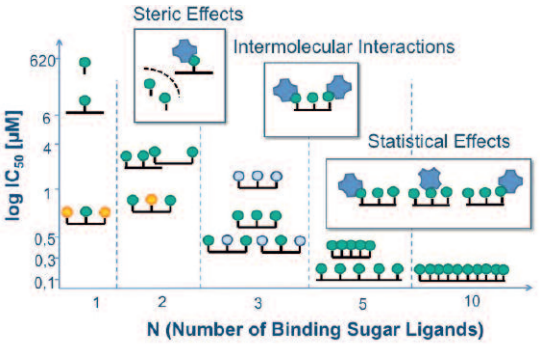

Fig. 2: Examples for different glycopolymers, their IC_{50} values and possible binding modes with ConA.

Glycopolymers in Biotechnology and Biomedicine

Especially for the transition from the fundamental studies towards the application of our precision glycooligomers in biotechnology and biomedicine, a key information is still missing. If we look at the ligand-receptor mediated contact of a bacteria and a cell, the glycooligomer has to compete with several ligand-receptor complexes at the cell-cell interface in order to inhibit the bacterial infection. However, this situation is not represented by any of the standard affinity assays mostly displaying one or both, ligand or receptor, in solution. Therefore, we developed a novel affinity assay mimicking more closely ligand-receptor complex formation between two surfaces and the inhibition of these complexes by sugar mimetic ligands **[8,9].**

The measuring principle is based on the adhesion and deformation of a hydrogel particle, a so-called soft colloidal probe (SCP), on a glass surface. Via reflection interference contrast microscopy (RICM), the contact area of the SCP on the surface can be detected and through a mathematical model (JKR-model) directly correlated to the adhesion energy. Here, the SCP is modified with a sugar ligand while the glass surface presents the protein receptor **(Fig. 3).** Through addition of increasing concentrations of an analyte in solution (e.g. a glycooligomer) and detection of the decreasing contact area, the half maximum inhibitory concentration (IC_{50} value) can be obtained. On the one hand, this set-up represents a simplistic model of cell-cell-contacts and allows for novel insights and systematic studies of ligand-receptor mediated adhesion at soft interfaces. On the other hand, this assay offers high sensitivity and specificity while using only simple and cost-efficient materials and equipment, e.g. in comparison to affinity assays based on surface plasmon resonance or isothermal calorimetry. In current projects, we therefore apply the SCP-RICM assay also to study other adhesion phenomena e.g. the self-healing of mussel-derived peptides **[10].**

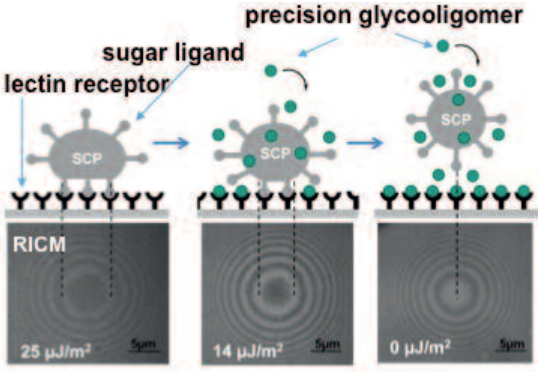

*Fig. 3: Schematic presenation of the SCP-RICM assay **[9].***

The results obtained from the SCP-RICM assay of different precision glycooligomers, are currently compared with the inhibitory concentration of the glycooligomer ligands in bacterial growth assays. Besides their use in antibacterial therapy, glycooligomers have been applied for cell specific targeting, as multivalent scaffolds in vaccine development and are currently under investigation for their use in antiviral treatments (unpublished results).

Following the same synthetic strategies and using the results obtained from the bacterial inhibition studies, we also developed novel bacteria binding magnetic particles **[11].** In contrast to the commercial system, the magnetic porous sugar-functionalized poly(ethylene glycol) particles (MAPOS) can bind more bacteria per particle and avoid non-specific interactions e.g. with serum proteins or other cells. MAPOS can therefore be used to isolate specific bacterial strains from solution e.g. in patients sample analysis.

L. Hartmann, F. Wojcik, D. Ponader, D. Pussak, S. Mosca, M. Behra, S. Igde, N. Ninnemann-Mujkic, H. Wang
laura.hartmann@hhu.de

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