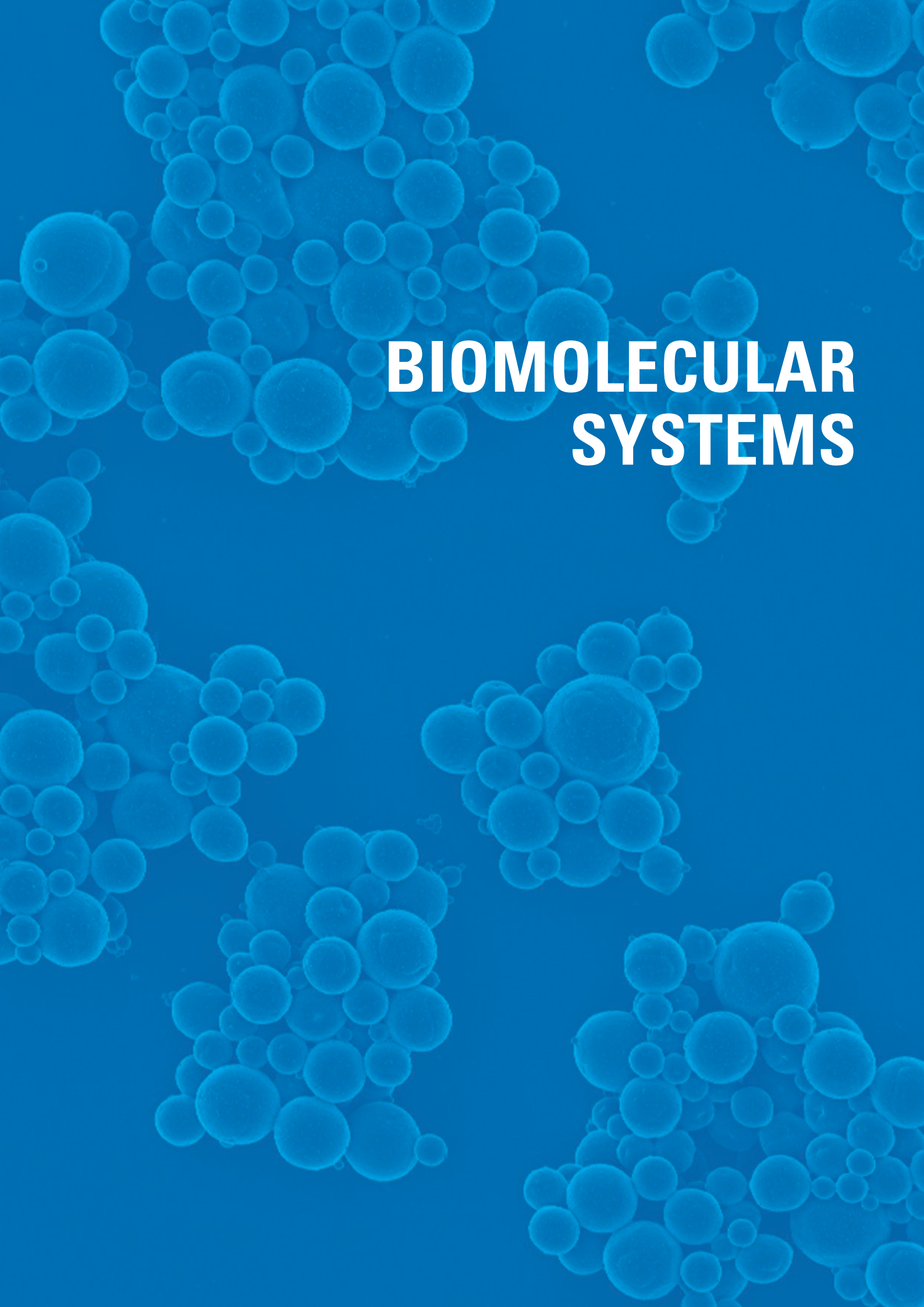
BIOMOLECULAR
SYSTEMS

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 Sanford-Burnham Institute for Medical Research, La Jolla, USA)
Since 2009: Director, Department for Biomolecular Systems (Max Planck Institute of Colloids and Interfaces, Potsdam)

The Department of 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 are the glycosciences where the development of *synthetic methods* for the automated assembly of defined polysaccharides remains a key interest. The glycans serve as *chemical tools* that aid *biological investigations* into the fundamental roles complex carbohydrates play in biological processes of disease. Carbohydrate arrays are now a routine tool to advance our understanding of *immunological aspects* of various infectious diseases. Insights into how the mammalian immune system recognizes oligosaccharides laid the foundation for *vaccine development* efforts concerned with the glycan portion, novel carriers, and novel modes of presentation to the immune system.

In summer 2015, the department moved finally to the new building in Potsdam to be in close proximity to the other departments. In anticipation of the move the size of the department was reduced by 20% but has now reached steady state again.

In the past two years, three group leaders left the department. Dr. Bernd Lepenies who led the *Glycoimmunology* group for six years accepted a W2 professorship in Hannover in 2015. In the summer of 2015, Dr. Claney Pereira who led the *Vaccine Chemistry* group joined the spin-off company *Vaxxilon* that received €30 Mio in funding as head of research and was joined by six senior postdocs. Dr. Kolarich who had been in charge of the *Glycoproteomics Group* assumed an Associate Professorship at Griffith University (Australia) at the end of 2016.

To build strength on the technology front, Dr. Felix Löffler joined the department in early 2016 and already raised a large BMBF grant to build his *Synthetic Array Technologies* Group. During the past two years three Emmy-Noether Groups were associated with the department: Dr. Christoph Rademacher s group is concerned with questions relating to structural immunology and he received an ERC Starting Grant in 2016. Dr. Fabian Pfrengle has built a strong group concerned with the synthesis and study of plant glycans and Dr. Ursula Neu adds strength in X-ray crystallography studies. On the biophysical front, Prof. Gerald Brezesinski joint our department following his official retirement from the institute to maintain the strength in X-ray studies of glycolipids in membranes.

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. Over the past two years our efforts in creating and understanding novel carbohydrate materials have grown significantly. Fueled by our ability to prepare polysaccharides as large as 50-mers, collaborations with the *Biomaterials Department* as well as the MPI in Stuttgart for structural investigations were set up. Continuous-flow chemistry has benefitted from a close collaboration with the *Colloids Department*. Materials from our colleagues are key catalysts for efficient transformations in the context of the synthesis of active pharmaceutical ingredients.

Automated Synthesis of Carbohydrates

Automated glycan assembly (AGA), our core technology, has reached a new level of sophistication. After the synthesizer as well as most reagents were commercialized via the spin-off company *GlycoUniverse*, we are in the process of developing even better instruments and methods. Most types of linkages are now accessible selectively and using ever shorter coupling cycles that allow for access to polysaccharides as long as 50-mers have been drastically expanded. AGA is now a standard tool to prepare diverse sets of ever longer polysaccharides that enable investigations into new areas of biology as well as material sciences.

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

Our long-standing program to develop synthetic carbohydrate vaccines yielded more than ten vaccine conjugates that passed challenge studies in experimental animals. The team produced a host of antigens found on the surface of pathogenic bacteria. A major focus in the past two years was placed on emerging hospital acquired infections. Conjugations of these antigens with carrier proteins and with self-adjuvanting glycolipids performed extremely well in immunological and functional studies in several disease models, particularly in *Streptococcus pneumoniae*. Several glycoconjugate vaccine candidates are being readied for human clinical trials in the spin-off company *Vaxxilon*.

Carbohydrate-based Nanotechnology

The attachment of carbohydrates to the surface of nanoparticles continues also in close collaboration with material scientists and medical researchers from many collaborating laboratories. Silicon nanoparticles equipped with glycans have proven highly attractive and yielded interesting in vivo results.

Glycoimmunology

Several lines of immunological investigations are being pursued. 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. Even after the departure of the Lepenies group this area of investigation is seeing intense study mainly by the Rademacher group. Novel binding partners of CLRs were identified and used as delivery agents into dendritic cells.

The glycan array facility we have established over the past two years is now engaged in investigations into glycan antigens responsible for different types of allergies (*e.g.* meat allergies), autoimmune diseases and adverse responses to carbohydrate-based drugs.

Synthetic Plant Glycans

The Emmy-Noether research group, employs automated glycan assembly and chemo-enzymatic methods for the generation of plant carbohydrate libraries as a powerful means for investigating plant biology. The synthesized plant carbohydrates are applied in the characterization of monoclonal antibodies derived from cell wall polysaccharides and cell wall glycan-deconstructing enzymes. In addition, the polysaccharide fragments are evaluated for their immunostimulatory potential. The synthetic plant carbohydrates will provide a new toolbox for studying the role of carbohydrates in plant biology and their interaction with human health.

Structural Glycobiology Group

The group around Dr. Rademacher has made great progress in fragment-based drug design to develop novel glycan binding protein ligands. Small heterocyclic fragments of drug-like molecules are screened using NMR and SPR-based protocols as well as chemical fragment arrays on solid supports. Actives are identified and evolved to higher affinity ligands. These studies go alongside with virtual screening and molecular modeling techniques to complement our insights and yield a more comprehensive picture of the interaction. Highly interesting ligands have been identified that hold significant promise for pharmaceutical and drug delivery applications.

GPIs and Glycoproteins

Five years ago, the group developed a general synthetic strategy to obtain glycosyl phosphatidyl inositol anchors (GPIs). By using this strategy, GPI molecules from parasites (*T. gondii, T. congolense, T. brucei* and *P. falciparum*) and from mammalian cells have been prepared. The resulting molecules have given rise to diagnostics fort he parasitic disease toxoplasmosis. To create even more complexmolecules, the group is focusing on the synthesis of homogeneous GPI-anchored proteins and glycoproteins. New ligation strategies to conect synthetic GPI-anchors with expressed proteins and methods for the incorporation of carbohydrates into the side chains of peptides and proteins.

Multivalent 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 and on the use of multivalent carbohydrate display on graphene surfaces. A method for the selective killing of pathogenic E. coli bacteria was developed. Efforts to employ multivalent carbohydrate-protein interactions for cell-specific targeting and imaging are underway.

Continuous Flow Chemistry

The past two years have seen two major breakthroughs in long-running projects. For one, the automated reaction optimizer was completed and allowed for the reliable and reproducible screening of up to 48 reactions per day. The data obtained using this instrument has provided fundamental insights into the nature of the glycosylation reaction. The modular assembly of complex molecules has yielded several active pharmaceutical substances and has benefitted greatly from the inclusion of novel catalysts obtained from the Colloids Department. Dr. Kerry Gilmore obtained significant funding to expand the group and further deepen the collaboration with the chemical engineering colleagues at the MPI in Magdeburg with a major focus to implement continuous purification methods.

Peter H. Seeberger
Director of the Department of Biomolecular Systems

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Automated Glycan Assembly

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)
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2003-2009: Professor (Swiss Federal Institute of Technology (ETH) Zurich, Switzerland)
2003-2014: Affiliate Professor (The Sanford-Burnham Institute for Medical Research, La Jolla, USA)
Since 2009: Director, Department for Biomolecular Systems (Max Planck Institute of Colloids and Interfaces, Potsdam)

The main focus of the Seeberger group since its inception in 1998 has been the development of automated oligosaccharide synthesis. In order to more rapidly procure synthetic glycans as tools for the glycosciences, all aspects of the assembly process have been improved systematically.

Instrument Development

Since 2014 commercial glycan synthesizers (Glyconeer 2.1®) are marketed by the spin-off company *GlycoUniverse*. By now several instruments have been placed in Europe, Asia and North America.

At the same time, the group is continuously improving all aspects of the automation process **[1].** Three homebuilt synthesizers are used to incorporate novel means to more quickly adjust the reaction temperature and to shorten the coupling cycles. The novel designs and improvements are tested in the context of syntheses of complex glycans. The coupling cycles have been shortened from over three hours to about 45 minutes recently to greatly accelerate the assembly of longer sequences.

Rapid Quality Control of Synthetic Oligosaccharides by Ion Mobility-Mass Spectrometry

With a greatly improved AGA, the bottle-neck shifted to glycan analysis and quality control. Methods such as nuclear magnetic resonance spectroscopy, although capable of assigning linkages, requires milligrams of material while mass spectrometry on the other hand can provide information on glycan composition and connectivity even for small amounts of sample, but cannot distinguish stereoisomers. We demonstrated that ion mobility-mass spectrometry (IM-MS), a method that separates molecules according to their mass, charge, size, and shape, can unambiguously identify glycan regio- and stereoisomers.

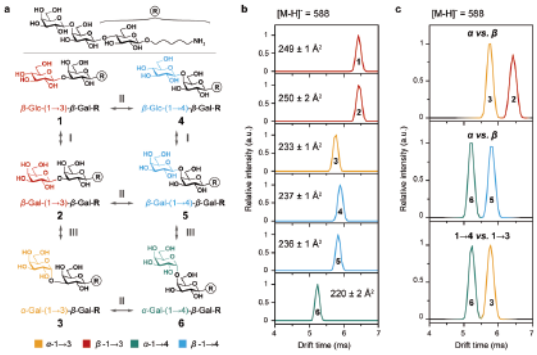

Figure. 1. Structure and IM-MS data of trisaccharides 1-6. (a) The synthetic trisaccharides 1 6 share the same disaccharide core and merely differ in the composition(I), connectivity(II), or configuration(III) of the last monosaccharide building block. (b) IM-MS arrival time distributions of 1-6 as [M-H]- ions. The number corresponds to the estimated CCS in the drift gas nitrogen. Although compositional isomers cannot be distinguished, connectivity and configurational isomers are clearly identified on basis of their CCS. (c) IM-MS arrival time distributions of isomeric mixtures show baseline separation between linkage- and stereoisomers.

Coexisting glycan isomers can be identified and relative concentrations as low as 0.1% of the minor isomer can be detected. In addition, the analysis is fast, requires no derivatisation and only small amounts of sample. IM-MS is an exceptionally effective tool for the structural analysis of complex carbohydrates that should become the standard for glycan characterization **[2].**

Incorporation of Sialic Acid Building Blocks

Over the past three years we have focused on extending the glycospace that can be prepared using automated glycan assembly.

The incorporation of sialic acid units via a sialic acid glycosyl phosphate building block was possible with high α-selectivity **[3].** The combination of automated glycan assembly (AGA) and enzymatic synthesis proved promising as five α(2,3)-sialylated glycans were prepared by rapid and high yielding assembly of the glycan backbones, while a sialyltransferase was used for high yielding and highly regio- and stereoselective sialylations **[4].**

Selective Incorporation of cis-Glycosidic Linkages

Previously, AGA was mainly employed to incorporate trans-glycosidic linkages, where C2 participating protecting groups ensure stereoselective couplings. Stereocontrol during the installation of cis-glycosidic linkages cannot rely on C2-participation and, anomeric mixtures are typically formed. We demonstrated that oligosaccharides containing multiple cis-glycosidic linkages can be prepared efficiently by AGA using monosaccharide building blocks equipped with remote participating protecting groups. The concept was illustrated by the automated syntheses of biologically relevant oligosaccharides bearing various cis-galactosidic and cis-glucosidic linkages.

Improvement of Overall AGA Protocols

After we had improved the automated assembly process in the past few years, a major focus was placed on creating an overall process involving the selection of building blocks all the way to purification and characterization. Using the commercial *Glyconeer 2.1* synthesizer we have been able to work out this.

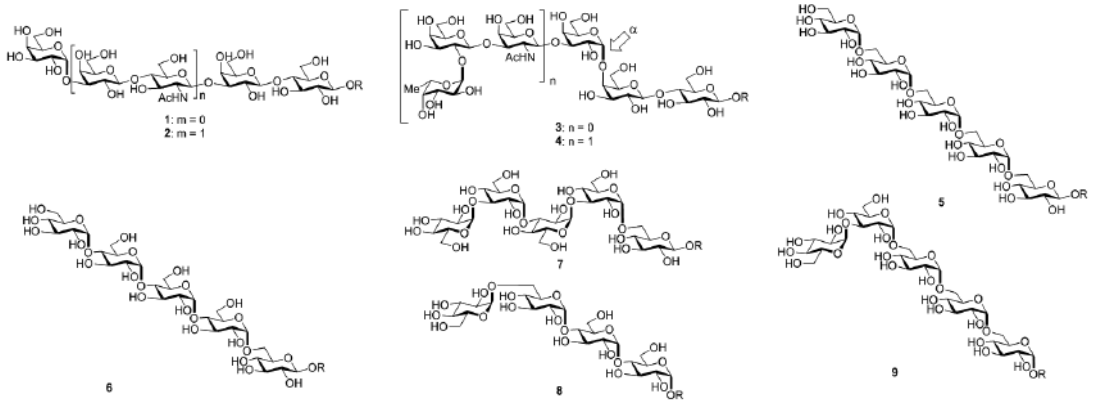

Figure 2. Oligosaccharides (1-9) containing different cis-glycosidic linkages were assembled by automated synthesis. OR = $O(CH_2)_5NH_2$

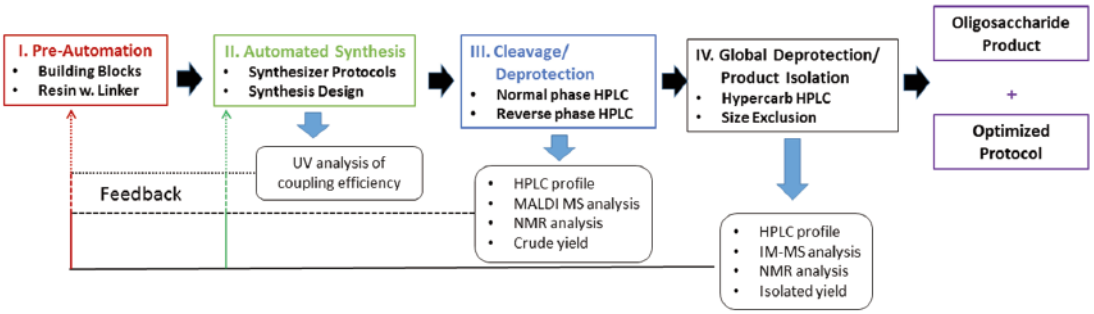

Figure 3. Work-flow for the automated synthesis, purification and analysis of complex glycans ***[13].***

AGA of Different Classes of Glycans

The AGA paradigm was challenged and expanded by assembly of different classes of glycans that served as synthetic tools or vaccine candidates for other groups in the department. Sulfated glycosaminoglycans remained of interest to us and the synthesis of chondroitin sulfate was further advanced **[6].** Streptococcus pneumoniae serotype 3 capsular polysaccharide antigens were prepared to assist the vaccine groups **[7].**The assembly of complex oligosaccharides related to blood group determinants was achieved to provide glycans for glycan array studies **[8].**

Oligo-N-acetyllactosamine glycan probes were prepared by AGA to help characterize adenovirus-glycan interactions as a basis for drug-delivery applications **[10].**

Seeberger, P. H.; Delbianco, M.; Kononov, A., Schuhmacher, F., You, Y.; Pardo, A.; Ghosh, Ch.; Fair, R.; Kottari, N.; Hahm, H.S.; Weishaupt, M.; Edupuganti, V.V.R.; Menova, P.; Lykke, L.
peter.seeberger@mpikg.mpg.de

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Controlled Conditions, Controlled Chemistry

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

Inarguably, the success of the vast majority of chemical transformations is reliant on the degree of control exhibited over a wide range of variables such as stoichiometry, temperature, reaction time, mixing, and exposure to light. Utilizing flow chemical techniques – where reagents are passed through a set of conditions via thin tubing as opposed to applying conditions to a round bottom flask – has allowed for achieving chemistries and efficiencies previously inaccessible. The modular nature of this technique has also facilitated the development of a novel means of chemical synthesis, which targets core functionalities as opposed to specific molecules, allowing for multiple derivatives to be produced with a single flow system.

At its core, the essential focus of an organic chemist's pursuits is control over molecules – whether this is expressed as regio- or chemoselectivity of functional group transformations, the formation and utilization of reactive intermediates, or precision in reaction conditions. For a number of applications, a significant increase in molecular and environmental (reaction conditions) control can be achieved using flow chemistry. This technique, where reagents are passed through tubing held at a precise set of conditions, is particularly advantageous in high temperature/pressure chemistries, multi-phasic systems (gas/liquid, liquid/liquid), very fast reactions, and photochemistry **[1].**

Flow chemistry is modular in nature, allowing for its components (pumps, mixers, reactors, etc.) to be arranged in any number of combinations. This built-in flexibility has allowed for a wide variety of applications. Recently, our group has utilized this technique to probe two major branches of organic synthesis: methodology (developing new reactions and studying their mechanisms) and multi-step synthesis (continuous and semi-continuous processes to produce active pharmaceutical ingredients (APIs)).

Methodology

Two areas of chemistry which are well suited for flow are the utilization of dangerous reagents and photochemistry. The reasons reactions using dangerous reagents are often converted to continuous flow processes are twofold: the amounts utilized at any one time are smaller due to the smaller volume of the reactor as compared to a batch process and the vastly-greater surface area that allows for excellent heat dissipation for exothermic reactions. One example is our recent work on the alpha-nitration of esters **[2],** where highly caustic fuming nitric acid and sulphuric acid are mixed to create a nitronium ion. By controlling the layout and the speeds which the reagents move through the modular system, this reactive intermediate can be trapped by a range of α-keto esters to give the desired product following treatment with methanol. The same reaction in batch was reported to "eject of the reaction material from the reaction vessel".

The second arena – photochemistry – is better suited for flow chemistry due to the limitations set by the Beer-Lambert Law, which describes the rapid decrease in the intensity of light when penetrating an absorbent medium. As such, by performing photochemistry in reactors thinner than this drop-off point (~1 mm), more rapid and efficient processes can be developed. Photochemistry is also reliant on a molecule being able to absorb light, generally through an extended π-system or a carbon-heteroatom double/triple bond. This limitation can be circumvented through the use of a photocatalyst, which is capable of utilizing photons to drive chemical transformations either through single-electron transfer (SET) processes or through energy transfer.

The first example of SET in flow was shown using a $Ru(bpy)_3$ catalyst **[3].** This simple flow set-up was shown to work for a range of both oxidative and reductive chemistries **(Fig. 1)**. Energy transfer processes nicely showcase the power of flow photochemistry, particularly for the generation of singlet oxygen. This allowed for the rapid examination of a variety of transformations and was utilized to provide the first continuous synthesis of the anti-malarial artemisinin.

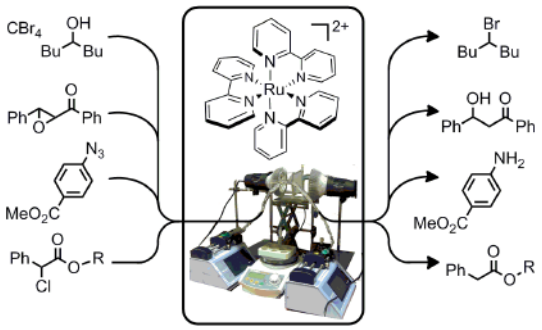

Fig. 1: The versatility of the photocatalytic SET reaction module, accessing both oxidative and reductive transformations.

The advantage of the flexible reactor module is the ability to control the temperature down to –80 °C, allowing for control of reactivity. This is critical in the singlet-oxygen-mediated oxidation of primary amines, where the resultant aldimine is immediately trapped by starting material to give the undesired secondary imine. However, at –50 °C the desired oxidation occurs cleanly, and can be trapped with a cyanide source to give the valuable α-aminonitrile functionality **[4].**

Coupled Modules

Molecular complexity can be quickly added by coupling two reactors together in either a continuous or semi-continuous process. This is advantageous when the product of the first reactor is unstable – as in the case of α-aminonitriles. One value-adding transformation is the hydrolysis of the nitrile portion of the molecule to give α-amino acids, achieved rapidly in flow (40 mins vs 48 h in batch) by reacting a concentrated HCl solution above its boiling point under pressure. A gas-liquid reactor can be exchanged for the HCl unit, allowing for carbon dioxide to react with the α-aminonitrile to give heterocycles called hydantoins **(Fig. 2) [5].**

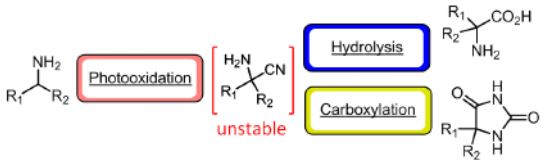

Figure 2: By coupling reaction modules together, molecular complexity can quickly be increased.

Target-Oriented Synthesis

Coupled reactors can also be used to design processes towards the synthesis of valuable small molecules such as APIs. For example, using precise control over reaction times, an ortho-lithiation of 1,4-dichlorobenzene using n-BuLi was achieved. This reactive intermediate was subsequently trapped by an acylating agent, and this serves as the first step in the shortest-ever (3 steps) semi-continuous synthesis of the HIV medicine Efaverinz **[6].**

Core-Functionality Targeted Synthesis

While processes can be developed to synthesize specific molecules, there exist a number of high-value molecules and APIs which share the same core functionalities. By coupling flexible, chemoselective reaction modules together it is possible that a multistep process can be created which targets structural cores – independent of the pendent functionalities. As these reaction modules are not dependent on the preceding or succeeding reactions, they can be interchanged to access different structural cores. This allows for an assembly-line approach to organic synthesis.

These chemical assembly lines can be arranged in in either a divergent or convergent manner. The first example is the extension of the artemisinin synthesis to yield all artemisinin-derivatives which serve as the WHO-recommended first-line treatments for Malaria. This three module process was further extended to include continuous purification, producing >99.5% pure material **(Fig. 3) [7]**.

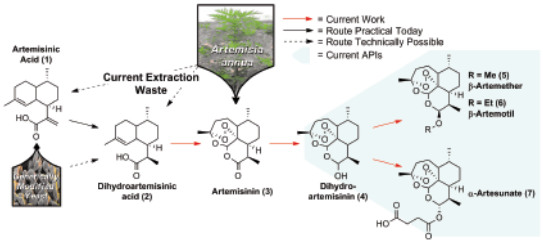

Figure 3: A divergent process accessing all four anti-malarial artemisinin derivatives.

The power of this approach was showcased in a series of publications utilizing a pool of eight reaction modules **(Fig 4) [8,9]**. These modules were arranged in divergent and convergent processes to produce five different structural cores and ten different active pharmaceutical ingredients. No intermediate purifications were used for any of the developed processes.

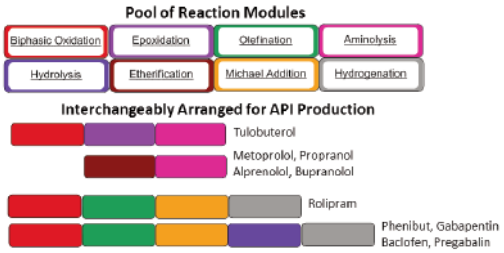

Fig. 4: A pool of reaction modules were utilized interchangeably to access five different structural cores and ten different APIs.

K. Gilmore, S. Chatterjee, A. Chenstova, S. Moon, M. Plutschack, B. Pieber, S. Vukeli
kerry.gilmore@mpikg.mpg.de

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The Glycome Represents a Largely Unmined Source for Disease Markers

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)
09/2010-2016: Group Leader Glycoproteomics Group, (MPI of Colloids and Interfaces, Berlin)
since 2017: Assoc Prof. and Research Leader at the Institute for Glycomics, Griffith University, QLD, Australia

Cell surface and body fluid proteins are extensively modified with specific sugar moieties, so called glycans. These glycans build the basis for a universal language cells use to communicate, but it is also abused by cancer cells through specific glycosignature modifications. Though all cells in the body share one alphabet, different organs use different dialects, manifested by the individual glycosignatures that cells impose on the proteins they express. Changes in protein glycosylation are also a universal hallmark of cancer and in several examples, such as hepatocellular carcinoma, specific glycosignatures are already being applied in the clinic to improve diagnostic sensitivity and selectivity (**Fig. 1**) **[1].** However, to date the full potential embedded in glycosignatures to detect, subtype, classify and grade different types of cancer has not been exploited to its full potential. This has also been due to technical limitations hampering accurate translation of these glyco-languages from limited amounts of available clinical tissue specimens. We have recently overcome these issues and developed highly sensitive and selective glycomics tools that enable us now to translate these dialects from formalin-fixed, paraffin embedded (FFPE) histopathological slides providing hitherto unprecedented insights into disease specific glycosignatures from minimal amounts of clinical material **[2].**

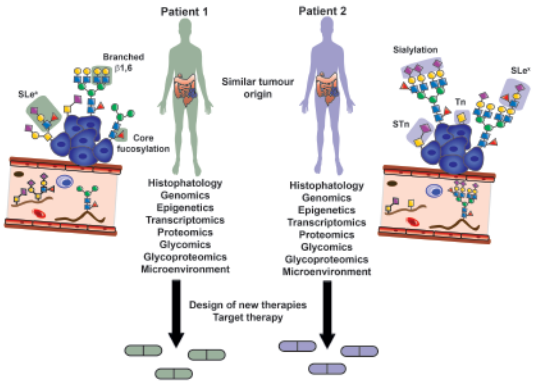

Fig. 1. Glycosylation signatures provide a global reflection on an individual's health/disease status and can function as predictive indicators for treatment success. A combination of different -omics strategies including glycomics & glycoproteomics will be essential for improving diagnosis and treatment personalisation. Figure taken from Almeida & Kolarich, BBA 2016 ***[1].***

The Technology behind PGC-nanoLC ESI-MS/MS Glycomics

Porous Graphitized Carbon nano Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry (PGC-nanoLC ESI-MS/MS) based glycomics is a highly selective and sensitive technology that enables an exact glycan sequencing. Glycan structures are built up by similar building blocks, but in contrast to e.g. peptide or DNA oligomers there are numerous possibilities how these different glycan building blocks are linked to each other. The type of linkage and its position, however, influence the biological properties of glycoconjugates. Our technologies thus provide a key asset to sequence these molecules to better understand their functional relevance.

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 have been combining the selectivity benefits provided by PGC-LC ESI MS/MS detection to separate, detect, characterise and relatively quantify such isobaric structure compounds (**Fig. 2**) **[2].**

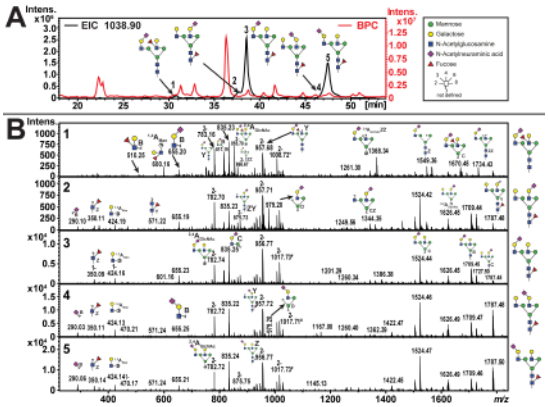

Fig. 2: Example for structure identification using PGC nano LC-ESI MS/MS. A: Base peak chromatogram (BPC, red trace) representing the N*-glycome obtained from FFPE preserved hepatic tissue. Extracted ion chromatogram (EIC, black trace) of an example* N*-glycan (Hex5HexNAc4NeuAcFuc, [M-2H]$^{2-}$ = 1038.9 Da) that is present in five different structure isomers. B: Individual product-ion spectra of the five Hex5HexNAc4NeuAcFuc isomers enabling differentiation and relative quantitation of the various N-glycan isomers. Figure taken from Hinneburg et al., MCP 2017* ***[2].***

FFPE Histopathological Tissue Slides can now be Used as a Source for Clinical Glycomics [2]

N- and *O*-glycans are attractive clinical biomarkers as glycosylation changes in response to diseases. The limited availability of defined clinical specimens impedes glyco-biomarker identification and validation in large patient cohorts. FFPE clinical specimens are the common form of sample preservation in clinical pathology, but qualitative and quantitative *N*- and *O*-glycomics of such samples has not been feasible to date. We have developed a novel approach to isolate and analyse the *N*- and *O*-glycome of FFPE clinical specimens that now allows a highly sensitive and glycan isomer selective characterisation of *N*- and *O*-glycans from histopathological slides. As few as 2000 cells isolated from FFPE tissue sections by laser capture microdissection were sufficient for in-depth histopathology-glycomics using porous graphitized carbon nanoLC ESI-MS/MS (**Fig. 3**). *N*- and *O*-glycan profiles

were similar between unstanined, hematoxylin and eosin stained FFPE samples but differed slightly compared with fresh tissue. With this method in hand we are now systematically investigating cancer glyco-biomarkers from FFPE histopathological tissues slides archived in pathology laboratories worldwide. The ability to investigate the differential glycome of disease and non-disease tissue of the very same patient from isolated tissues provides now unprecedented insights into disease associated glycan signatures.

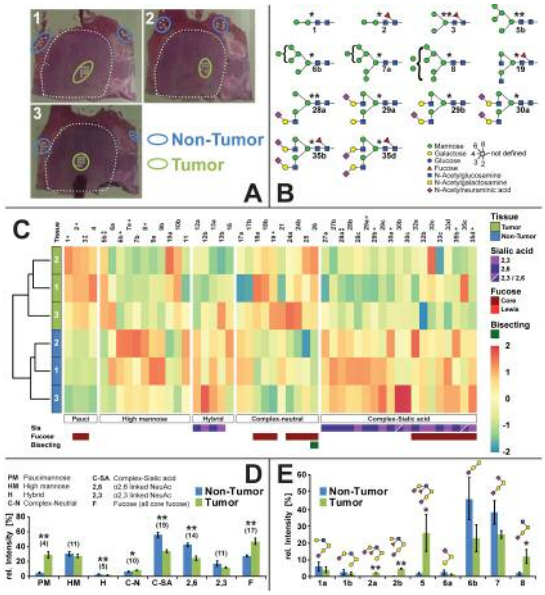

Fig. 3: N- *and* O*-glycome of hepatocellular carcinoma (HCC) and non-cancer hepatic tissue (NC). A: Representative image of FFPE preserved tissue sections of HCC used for isolation of HCC cells and surrounding non-cancerous tissue by laser capture microdissection. B:* N*-glycan structures exhibiting the largest expression level changes between HCC and NC tissue. C: Heat map obtained after unsupervised hierarchical clustering of the* N*-glycans detected from 2000 cells of HCC and NC hepatic tissue and sorted according to major structure categories, clearly showing the global changes present in the cancer cell* N*-glycome. D: Category comparison of the HCC and NC* N*-glycomes. The number of structures in each category is indicated above bars. E: Comparison of HCC and NC* O*-glycomes obtained from the very same material the* N*-glycomes were obtained. Sialyl Lewis X epitopes present on core 2 type* O*-glycans show a significant increase in HCC tissue, whereas core 1 type* O*-glycan levels are reduced. Figure taken from Hinneburg et al., MCP 2017* ***[2].***

Middle-down Glycoproteomics Uncovers the Specific Sites Preferred when Chemically Glycosylating Vaccine Proteins [3]

Production of glycoconjugate vaccines involves the chemical conjugation of glycans to an immunogenic carrier protein such as Cross-Reactive-Material-197 (CRM197). Instead of using glycans from natural sources recent vaccine development has been focusing on the use of synthetically defined minimal epitopes. While the glycan is structurally defined, the attachment sites on the protein are not. Fully characterized conjugates and batch-to-batch comparisons are the key to eventually create completely defined conjugates. We used a variety of mass spectrometric techniques to identify and characterise the specific sites modified during the conjugation process, showing that specific sites are preferred during the conjugation process. In particular regions close to the N- and C-termini were most efficiently conjugated (**Fig. 4**). These results help to ensure production consistency and provide better and safer products for the next generation of defined glycoconjugates vaccines **[3].**

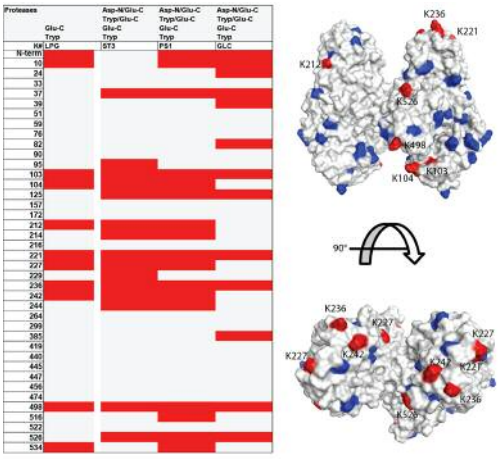

Fig. 4: Left: Heatmap showing the preferred lysine residues sites of CRM197 that are modified by chemical glycosylation (red). Right: 3D crystal structure of CRM197 dimer (PDB entry: 4AE0). Lysine residues are labelled blue. Lysine residues that were frequently found conjugated with a glycan in all the samples are labelled in red. Figure taken from Moginger et al., SciRep 2016 ***[3].***

In January 2017 the glycoproteomics group relocated to the Institute for Glycomics at Griffith University, Queensland, Australia

D. Kolarich, K. Alagesan, A. Almeida, H. Hinneburg, U. Möginger, F. Schirmeister
d.kolarich@griffith.edu.au

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C-type Lectin Receptors – from Glycan Arrays to Murine Models

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)
2009-2015: Group Leader, Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces
2012-2015: 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
07/2015-now: Appointment as Associate Professor (W2) for Infection Immunology at the University of Veterinary Medicine Hannover

A main research focus of the Glycoimmunology group has been on pattern recognition receptors (PRRs) in innate immunity. PRRs recognize evolutionarily conserved pathogen- or danger-associated molecular patterns. They are predominantly expressed by cells of the innate immune system and provide a first line of defense in the body. Since PRRs trigger endocytosis and may also provoke signaling pathways that impact antigen presentation, the expression of co-stimulatory molecules, and cytokine production, PRRs are often essential to activate adaptive immune responses **(Fig. 1)**.

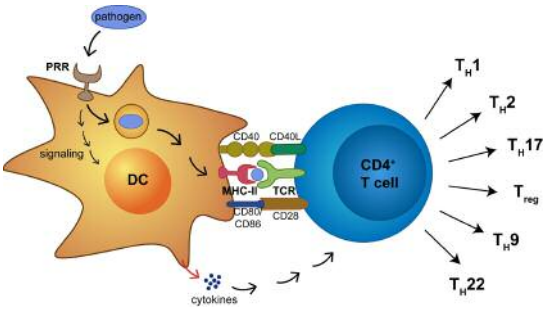

Fig. 1: Pattern recognition receptors (PRR) expressed by cells of the innate immune system such as dendritic cells (DC) bind to conserved pathogen-associated molecular patterns. This may lead to receptor-mediated endocytosis, antigen processing and presentation by major histocompatibility complex (MHC) molecules to T cells. In addition, signaling pathways are induced in DCs that induce the expression of the co-stimulatory molecules CD80/CD86 as well as cytokine production. Thus, subsequent T cell activation and differentiation may be influenced by signaling via PRRs. Figure designed by Dr. Julia Hütter.

Lectins represent a class of PRRs that are specialized to recognize glycan structures on pathogens and self-antigens. The Glycoimmunology group has been particularly interested in a large lectin superfamily called C-type lectin receptors (CLRs). CLRs often recognize carbohydrates in a Ca^{2+}-dependent manner and contribute to immunity by triggering a variety of cellular functions including antimicrobial responses, cytokine secretion, dendritic cell (DC) maturation, phagocytosis, antigen presentation, and T cell activation. Since some CLRs activate cellular functions whereas others inhibit intracellular signaling pathways, CLRs may be involved in both immune stimulation and immune suppression, thus they are promising targets for immune modulatory therapies **[1,2] (Fig. 2).**

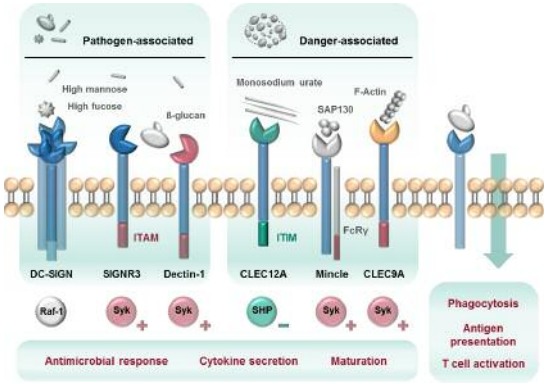

Fig. 2: Myeloid C-type lectin receptors (CLRs) in innate immunity. CLRs such as DC-SIGN, SIGNR3, Dectin-1, CLEC12A, Mincle, CLEC9A, among others, recognize pathogen- and also danger-associated molecular patterns. CLR ligation may trigger a variety of cellular functions including antimicrobial responses, cytokine secretion, phagocytosis, antigen presentation, and T cell activation. While some CLRs act as activatory receptors and stimulate cellular responses, others serve as inhibitory receptors, thus dampen immune responses. Figure designed by Dr. Timo Johannssen.

During the past years, we have generated and continuously extended a comprehensive library of CLR-Fc fusion proteins to identify yet unknown CLR ligands on pathogens and in libraries of synthetic glycans using ELISA-based methods, lectin arrays, and the glycan array technology. To this end, the extracellular part of the respective mouse CLR containing the carbohydrate recognition domain (CRD) was fused to the Fc fragment of human IgG1 molecules. The dimeric CLR-Fc fusion proteins were then transiently expressed in Chinese hamster ovary (CHO) cells, purified from the culture supernatant and used for comparative screenings.

A main focus has been on the role of innate immunity and in particular CLRs during infections **[3,4]**. With the help of the established CLR-Fc library, several previously unknown CLR ligands on bacterial pathogens or microbiota could be identified **[4,5]**. In one study, the CLR Macrophage-inducible C-type lectin (Mincle) was shown to bind to *Streptococcus pneumonia* in a Ca^{2+}-dependent, serotype-specific manner. Mechanistic studies using different Mincle-expressing cells as well as Mincle-deficient mice revealed a limited role of Mincle in bacterial phagocytosis, neutrophil-mediated killing, cytokine production, and antibacterial immune response during pneumonia **[4]**. However, this study demonstrates the utility of the generated CLR-Fc library to screen for novel CLR ligands on pathogens **(Fig. 3).**

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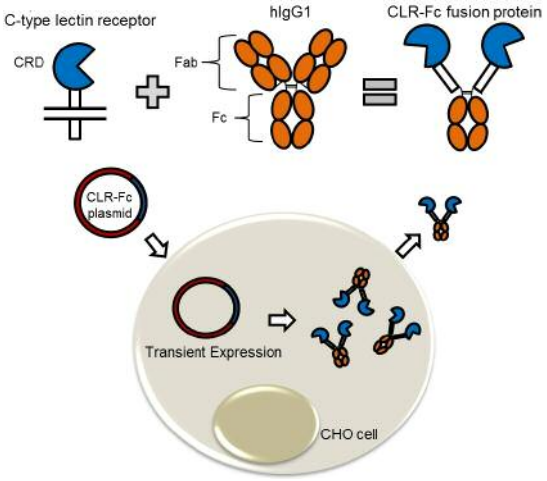

Fig. 3: Generation of the CLR-Fc fusion protein library. The extracellular part of the respective mouse CLR containing the carbohydrate recognition domain (CRD) is fused to the Fc fragment of human IgG1 molecules rendering dimeric CLR-Fc fusion proteins. They are transiently expressed in Chinese hamster ovary (CHO) cells and then purified from the culture supernatant. Figure designed by Joao Monteiro.

CLR Targeting for Drug and Vaccine Delivery

Since antigen targeting to DCs is a promising strategy to enhance the efficacy of vaccines, DC-expressed CLRs are attractive targets for cell-specific vaccine delivery. In addition, CLR targeting may be a means to enhance antigen presentation to T cells, thus promotes subsequent T cell activation. Numerous studies have focused on antibody-mediated CLR targeting, whereas glycan-based targeting approaches have only gained increasing attention during the last years. In fact, multivalent display of glycan CLR ligands on suitable carrier systems such as nanoparticles, dendrimers, polymers, or liposomes may have some advantages compared to antibody-mediated CLR targeting due to the easy tunability of ligand density and their spatial orientation on the carrier. Thus, glycan-based CLR targeting may indeed be a promising approach to manipulate cellular functions effectively **[6-8]**. Furthermore, various glycan ligands can be presented on one carrier to allow for targeting of different CLRs simultaneously. Interestingly, small structural glycan modifications have a marked impact on CLR binding, CLR-mediated endocytosis, and subsequent T cell activation. In a recent proof-of-principle study, glycoproteins displaying different biantennary *N*-glycans were analyzed for their binding to the previously established CLR-Fc fusion protein library **[6]**. Although both *N*-glycans only differed in the presence of an O-2 core xylosylation, they exhibited differential binding to selected CLRs which impacted targeting and uptake of the glycoproteins by DCs and also affected T cell activation in a DC/T cell co-cultivation assay. On the one hand, this study shows the utility of glycan-based DC targeting, but on the other hand it also highlights the marked impact of small differences in glycan structures on the targeting efficacy. Thus, glycan ligands of CLRs have to be tested in appropriate cell culture assays and *in vivo* models in order to evaluate their utility for targeted therapy and/or immune modulation **(Fig. 4)**.

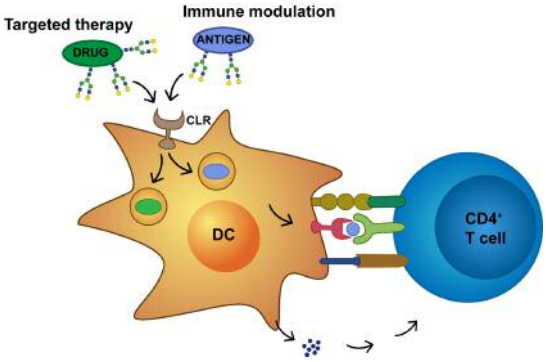

Fig. 4: Targeting of C-type lectin receptors (CLR) is a means to shape initiated immune responses. CLRs can be exploited for cell-specific drug and vaccine delivery into CLR-expressing cells such as dendritic cells (DC). Furthermore, signaling pathways may be provoked by CLR targeting that affect the expression of co-stimulatory molecules and cytokine production. As a consequence, T cell activation and differentiation may be influenced by CLR-mediated signaling. Thus, CLR targeting is a promising strategy to modulate immune responses. Figure designed by Dr. Julia Hütter.

Carbohydrate-carbohydrate Interactions

Glycan–lectin interactions are generally weak, thus multivalent binding is often crucial to exert biological effects. However, carbohydrate–carbohydrate interactions are even of ultralow affinity and are often difficult to detect. They are considered to be relevant during early embryogenesis as well as during the initial adhesion of melanoma cells to the endothelium thus contributing to metastasis formation. To analyze the function of carbohydrate–carbohydrate interactions more in depth, carbohydrate-capped silicon nanoparticles were used to measure the interaction between the cancer-associated glycosphingolipids GM3 and Gg3 **[10]**. Surface plasmon resonance experiments were performed to determine the binding affinity and cell binding studies revealed the relevance of this model carbohydrate–carbohydrate interaction *in vitro*. Thus, carbohydrate-capped silicon nanoparticles are useful tools for cell imaging and targeting and can be employed to analyze carbohydrate interactions of ultralow affinity in biophysical as well as cell biology studies.

B. Lepenies, J. Hütter, T. Johannssen, U. Vogel, S. Zimmermann
Bernd.Lepenies@tiho-hannover.de

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BIOMOLECULAR SYSTEMS

Synthetic Plant Carbohydrates

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. The synthesized plant carbohydrates are applied in the characterization of monoclonal antibodies derived from cell wall polysaccharides and cell wall glycan-deconstructing enzymes. 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.

Automated Glycan Assembly of Plant Cell Wall Oligosaccharides

Automated glycan assembly is a technology that was introduced by Prof. Peter H. Seeberger in 2001 **[4]**. Using this technology, rapid access to collections of defined oligosaccharides is provided. We recently assembled structurally related fragments of different plant cell wall polysaccharide classes from a few monosaccharide building blocks **(Fig. 1) [5-8]**. These synthetic glycans were applied in the characterization of cell wall glycan-directed antibodies and cell wall-degrading enzymes.

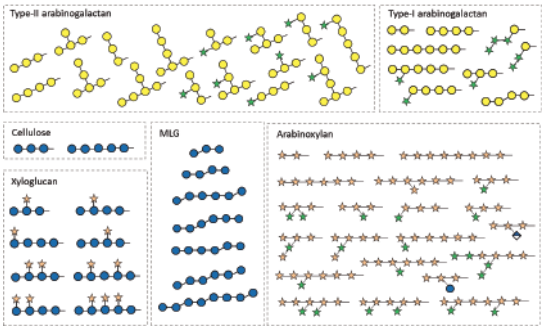

Fig. 1: Synthetic plant carbohydrates produced by automated glycan assembly.

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 **[9]**. 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 synthesize plant carbohydrate libraries for a comprehensive 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 b-1,4-linked D-xylopyranoses. This backbone structure may be partially acetylated and substituted with L-arabinofuranosyl or D-(4-O-methyl) glucuronyl residues.

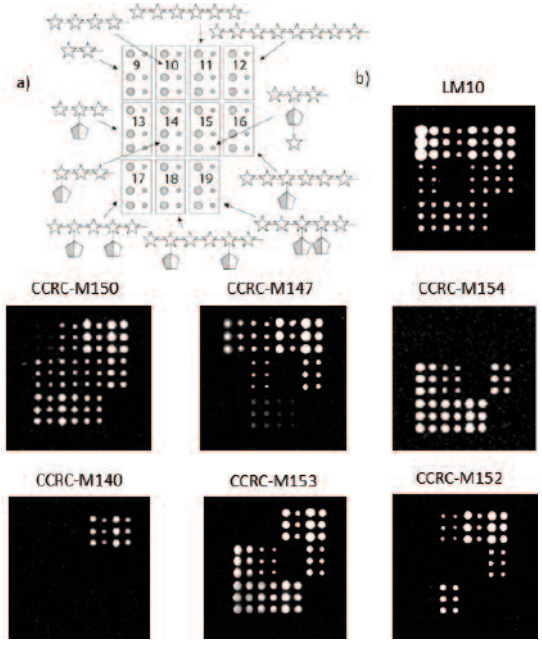

Fig. 2: Detection of oligoarabinoxylans by anti-xylan monoclonal antibodies (mAb): a) Printing pattern; b) Microarray scans. Representative scans of at least two independent experiments are shown. The intensity of the spots corresponds to the binding affinity of the respective mAb.

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. We observed specific binding of the antibodies to the synthetic oligoarabinoxylans and the binding epitopes of several antibodies were characterized **(Fig. 2)**. 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.

Active Site-mapping of Cell Wall-Degrading Enzymes Using Synthetic Plant Carbohydrates

Cell wall-degrading enzymes are essential in a variety of processes such as biomass conversion into fuels and chemicals and the digestion of dietary fiber in the human intestinal tract. We used synthetic plant carbohydrates to gain further insight into these enzymes by incubating the glycans with various glycosyl hydrolases followed by analyzing the digestion products by HPLC-MS. Synthetic arabinogalactan oligosaccharides enabled us for instance to determine the binding specificities of endo-galactanases **(Fig. 3)**. We discovered that the endo-galactanases recognize and hydrolyze arabinogalactan oligosaccharides of different lengths and arabinose substitution patterns.

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 **[10]**, 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.

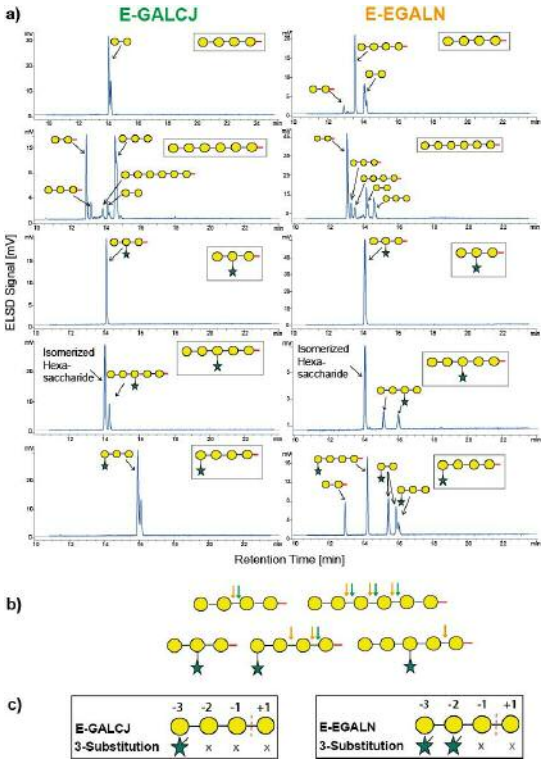

Fig. 3: Digestion of synthetic arabinogalactan oligosaccharides with the endo-galactanases E-GALCJ and E-EGALN and analysis of the resulting hydrolysis products by HPLC-MS. (a) HPLC analysis of the products after incubation of the respective oligosaccharides with the galactanases. (b) The cutting sites derived from (a) are summarized and indicated by arrows. (c) General requirements for arabinose substitutions relative to the cutting site of the galactanases. "X" denotes galactose residues that must not be substituted with arabinofuranose.

F. Pfrengle, P. Dallabernardina, D. Senf, M. P. Bartetzko, C. Ruprecht
Fabian.Pfrengle@mpikg.mpg.de

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Ligand-based Targeted Delivery to C-type Lectin Receptors

Christoph Rademacher 05.05.1979
2001-2004: Bachelor Studies in "Molecular Biotechnology" at the University of Lübeck, Germany (with honours).
2004-2006: Master Studies in "Molecular Life Science" at the University of Lübeck, Germany (with honours)
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
2012-2016: Emmy-Noether Young Research Group Leader

Many pharmaceuticals are administered systemically and act on the diseased as well as on the healthy tissue. To reduce dosing and consequently the risk for side-effects, it would be highly desirable to enable tissue- or even cell-type specific delivery of pharmaceuticals. In particular, the specific modulation of immune cells in cancer immunotherapy has become an attractive route. To enable a cell-specific delivery of immunomodulatory agents, immune cell receptors have been identified as excellent entry points into these cells. More precisely, carbohydrate binding receptors, such as C-type lectins expressed on myeloid cells of the innate immune system, emerged as a prime target receptors. These receptors have a narrow expression pattern and promote uptake and processing of a variety of antigens. Classically, these receptors have been addressed using antibody-based delivery approaches. Stimulants are conjugated to an anti-lectin antibody and following injection, these antibodies deliver their payload to a defined subset of immune cells ultimately activating the immune system. Alternatively, carbohydrate ligands have been applied to enable uptake of nanoparticles making use of the pattern recognition function of these C-type lectin receptors. Glycosylated, particulate antigens are recognized by these lectins and initiate uptake and processing. Unfortunately, mammalian carbohydrate binding proteins typically bind their ligands with low affinity and the specificity for simple carbohydrates is often limited. Consequently, synthetic molecules mimicking the carbohydrate scaffolds may provide higher affinity and selectivity.

The development these so-called glycomimetics as targeting ligands to enable targeted delivery to cells of the innate immune system has been the prime goal of our research efforts and will be described in the following.

Druggability Assessment of C-Type Lectins

Before embarking on a small molecule discovery campaign, it is important to estimate the potential of the targeted receptor to interact with small drug-like molecules. This so-called druggability assessment can be conducted using several methods. First, computational techniques have been quite popular using pocket detection algorithms to find and score suitable binding sites available on protein crystal structures. We performed such analysis using DoGSiteScorer, analysing a larger panel of C-type lectins **[1]**. We concluded that the vast majority of these targets should be considered either challenging or undruggable owing to their shallow and hydrophilic binding sites.

In contrast, experimental druggability assessment of a set of C-type lectins using NMR-based fragment screening suggested the opposite **[1]**. Here, we applied ^{19}F NMR, as it is a sensitive method with limited sample consumption. A high portion of fragments bound to the lectins and strongly suggested these C-type lectins are druggable.

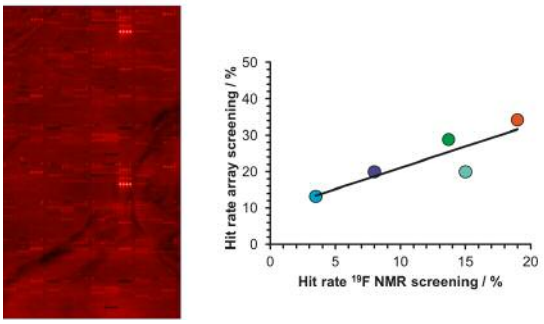

Fig. 1: Druggability assessment of C-type lectin receptors using chemical fragment arrays. Fragments of drug-like molecules were immobilised on a glass slide and probed with fluorescently labelled proteins (left). These data correlated well with previous findings of moderate to high hit rates for several protein targets including three C-type lectins (right). ***[1, 2].***

To further our insight into the druggability of C-type lectins, we developed so-called chemical fragment arrays. Small droplets containing fragments of are spotted on the surface of a glass slide and UV-activated, which led to covalent immobilisation of the fragment to the array **[2]**. Fluorescently labelled, multimeric and monomeric C-type lectin receptors bound to the immobilized fragments. As a result, the hit rates correlate with those found previously by ^{19}F NMR [1], which suggests that this fragment array method is well suited for druggability assessment with 1000-fold reduced protein consumption compare to NMR. Overall, these data suggested again a medium to high druggability of C-type lectins and initiated further research into the identification of targeting ligands.

Identification of Potential Targeting Ligands for Human Langerin

Experimental data from ^{19}F NMR and chemical fragment array screening during the druggability assessment provided enough evidence to continue our efforts to find targeting ligands for the human C-type lectin Langerin **[1, 2]**. For this, a suitable assay system had to be developed to guide the design process as the requirements for the identification of glycomimetic ligands for mammalian lectins are as follows: (i) a homogeneous assay is necessary, devoid of any washing steps as these might be too harsh and reduce reproducibility, (ii) such an assay has to provide binding site information, otherwise ligands binding to irrelevant sites might be identified, and (iii) the assay would preferentially yield thermodynamic information with high sensitivity. To this end, we have developed a ^{19}F NMR reporter displacement assay **[3]**. Here, a trifluoroacetamido mannose served as a sensitive reporter to probe for binding and competition with potential glycomimetics.

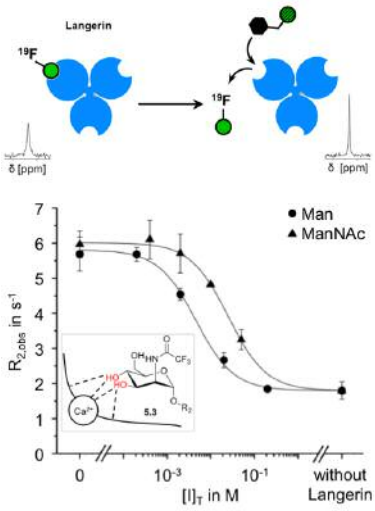

Fig. 2: ^{19}F NMR-based reporter displacement assay. Increased R_2 relaxation of a trifluoro group is measured upon binding to the C-type lectin Langerin. If the trifluoroacetamido mannose is displaced from the binding site by a competitor the R_2 relaxation decreases again. Titration studies using competitors yield thermodynamic inhibition constants. ***[3].***

Using this assay, two orthogonal screenings were conducted. First, an *in silico* screening was performed that evaluated a library of commercially available building blocks to increase the affinity of an existing carbohydrate ligand in the binding site of Langerin. Based on these insights, a selected panel of mannose derivatives was synthesized and tested. Ligands with 36-fold higher affinity than the natural ligand were identified. Second, 290 fragments were screened in cocktails of five and a fragment was identified to block the Langerin recognition site with millimolar affinity **[3]**.

An Allosteric Network Modulates the Calcium Affinity of Langerin

While the experimental druggability assessment suggested the availability of serval pockets that can harbour small molecule modulators of Langerin, our computational analysis did not **[1-3]**. Hence, we investigated the dynamics of the human Langerin **[4]**. Here, we focused on the molecular determinants of the affinity of Langerin for its cofactor Ca^{2+}. We expressed the carbohydrate recognition domain (CRD) in *E. coli* enabling ^{15}N and ^{13}C isotope enrichment, which allowed NMR resonance assignment. Following changes of these NMR resonances upon Ca^{2+} titration in a ^{1}H-^{15}N HSQC experiment, generated insight into a large network of amino acids involved in Ca^{2+} binding. Surprisingly, the network spans a large fraction in the protein, with many residues significantly distal from the Ca^{2+} site. Further analysis combining experimental mutagenesis and molecular dynamics simulations prompted us to propose an allosteric network modulating the function of Langerin. In particular, two single point mutations allowed to perturb the network, giving further insight into its function, which we concluded is to downregulate Ca^{2+} affinity. This modulation of the cofactor affinity by an allosteric network has important implication for the receptor function as the ligand and release under endosomal conditions needs to be tightly regulated. Overall, these data provided the first insight into such an allosteric network in a C-type lectin and opened new avenues to further development of potential drugs that modulate C-type lectins **[4]**.

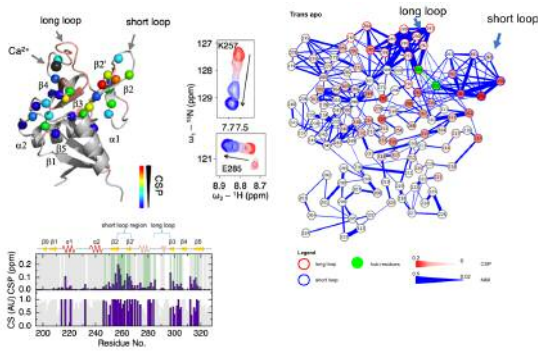

Fig. 3: The Ca^{2+} affinity of Langerin is modulated by an intradomain allosteric network. Binding of the cofactor Ca^{2+} to Langerin leads to chemical shift perturbation (CSP) of the backbone resonances of the carbohydrate recognition domain at several distal sites of the protein. Residues involved in this communication network are evolutionary conserved (CS). Analysing extensive molecular dynamics simulations using information theory yielded insight into the atomic details of this network (right). ***[4]***

Bacterial Polysaccharide Specificity of Langerin is Highly Species-dependent

Our insights into secondary sites being able to modulate the function of Langerin prompted us to look closer into the recognition of larger polysaccharide ligands. C-type lectin receptors such as Langerin are pattern recognition receptors that detect invading pathogens by carbohydrate patterns present on their surfaces. Therefore, these innate immune cell receptors are the product of a species-dependent evolutionary pressure. In this respect Langerin is of particular importance since it is conserved in many mammals with only moderate sequence variation. For example, humans and mice share 66% sequence identity. Based on our insights on the dynamics of the human Langerin and the observation that even single amino acid variations can potentially introduce significant changes in its biological function, we compared the human and the murine homolog side-by-side. We found that, while monosaccharide specificities remain almost unchanged between the two species, recognition of larger polysaccharides was significantly altered **[5]**. We solved the crystal structure of the murine Langerin to compare the molecular determinants and could deduce that minor changes at the trimeric interfaces might result in repulsion in the murine Langerin, which is not the case in the human homolog. At the same time a secondary binding site was proposed form our work explaining how the specificity might be altered for larger polysaccharides.

C. Rademacher, J. Aretz, H. Baukmann, J. Hanske, J. Schulze, E.-C. Wamhoff, R. Wawrzinek
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, 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 anchored to the outer leaflet of the cell membrane using glycosylphosphatidylinositol glycolipids (GPIs). GPIs are characterized by a conserved core structure containing a glycan pseudo-pentasaccharide, a phosphoethanolamine unit, and a phospholipid tail. The GPI core is usually modified with additional phosphates, glycans and lipid chains in a cell type dependent form. The phospholipid moiety is highly variable and may contain a diacylglycerol (DAG), an alkylacylglycerol (AAG)or a ceramide (CER), with alkyl chains of different length and degree of saturation **(Fig. 1)**.

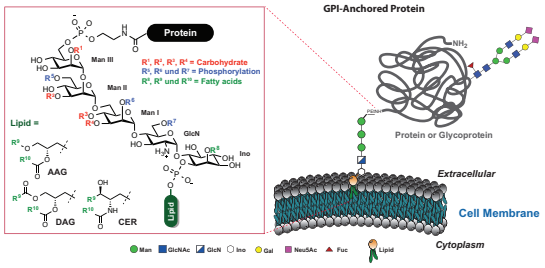

Fig. 1: Structure and possible modifications of GPIs

The primary and best known biological role of GPIs is to anchor the attached molecules to the cell membrane **[1]**. 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 the immune system of the host during parasitic infections and protein localization, among others **[2]**.

Development of Strategies to obtain GPIs

To evaluate the role of GPIs and their structure-function relationship is necessary the availability of good amounts of homogeneous glycolipids. To address this need we developed a synthetic strategy to obtain well-defined GPIs **[3]**. This strategy was used to obtain different and structurally distinct GPIs and GPI derivatives that allowed biological and biophysical evaluations of these molecules. Various GPIs are characterized by the presence of unsaturated lipid chains and require special conditions for their synthesis. More recently, we developed a new strategy to obtain GPI molecules bearing unsaturations. The strategy is based on the use of the 2-(naphthyl)methyl (NAP), an acid labile group, for the permanent protection during the synthesis, the use of the allyl and PMB ethers as orthogonal protecting groups to mask the positions requiring late-stage phosphorylation, and the NAP easy removal using high concentrated TFA **(Fig 2) [4]**. To demonstrate the applicability of this strategy, we synthesized the pseudo-disaccharide GlcN-Ino 1 from the GPI of the Trypanosoma cruzi parasite **[5]**, which unsaturated lipid moiety has been associated with the strong proinflammatory activity of the GPI from this parasite.

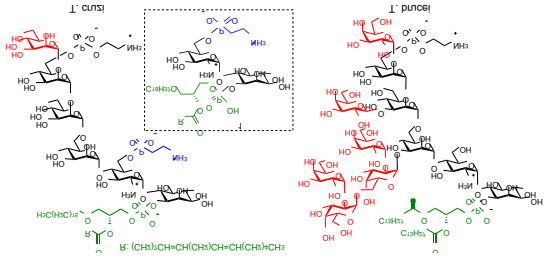

Fig. 2: Structures of the glycosylphosphatidylinositola from T. cruzi and T. brucei

GPIs as Vaccine Candidates for Toxoplasmosis

Protozoan parasites express high 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.

We investigated the immune response elicited in a BALB/c mouse model by two GPI glycans as vaccine candidates toward *T. gondii* using synthetic GPIs coupled to the carrier protein CRM197. We examined the generation of anti-GPI antibodies and the protective properties of the GPI-glycoconjugates in a lethal challenge study using the virulent *T. gondii* RH strain. Upon immunization, the mice raised anti-GPI antibodies that bind to the respective glycan structures on carbohydrate microarrays. **[7]** Unfortunately, these antibodies were mainly directed against an unspecific epitope of the glycocojugates including the cross-linker used for the attachment of the GPI to the carrier protein. Furthermore, antibodies *in vitro* binding studies suggest the lack of specificity toward cell surface exposed *T. gondii* GPI epitopes to be the reason for the failure of protection during the challenge studies. Consequently, future GPI-based vaccine candidates against toxoplasmosis, or any other disease, should rely on a conjugation without the use of immunogenic cross-linkers to generate a specific immune response against the desired carbohydrate antigens.

Structure of Glycosylphosphatidylinositols

NMR spectroscopy is a useful technique for the conformational analysis of carbohydrates in solution. However, it is not feasible to determine the dynamical conformation of carbohydrates solely on the basis of local conformational restraints derived from nuclear overhauser effects (NOEs) and scalar couplings. Recently, paramagnetic NMR techniques have been applied to provide long distance information for the characterization of carbohydrates conformations at the atomic level **[8]**. These techniques have been successfully applied to the conformational analysis of the disaccharides such as chitobiose, lactose, and also in breaking the pseudosymmetry of complex N-Glycans **[9]** .

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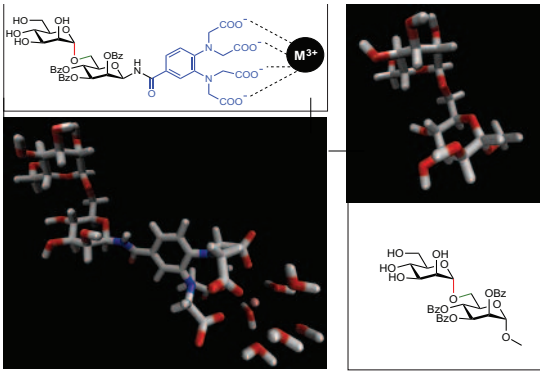

Fig. 3: Conformation of a dimannose with and without a TAG for the paramagnetic NMR.

To evaluate the effect of the core modifications in the structure and function of GPIs, we have applying this approach to perform conformational studies of GPI anchors fragments using lanthanides-assisted paramagnetic NMR spectroscopy in combination with molecular dynamics (MD) simulations. For this purpose, a metal chelating tag was synthesized and attached at the reducing end of different GPI glycan moieties of different length and carbohydrate constitution. We evaluated the effect of diverse paramagnetic metals (Dy^{3+}, Tb^{3+}, Yb^{3+}, Eu^{3+}) in paramagnetic tagged NMR spectroscopy, which in combination with molecular dynamics calculations deliver information about the conformation and flexibility of dimannose 1,2- and 1,6-linkages present in the glycan core structure of GPIs. These results confirmed the flexibility of the GPI structure around the 1,6-bond and delivered different conformations of GPIs for the elucidation of the structure-function relationship of GPIs, which may explain the formation of specific antibodies against the modifications on GPIs over other components of the GPI-core and help us to design GPI-based molecules for therapeutic applications.

Semi-Synthesis of GPI Anchored Proteins

To evaluate the effect of GPIs in the function and activity of GPI-anchored proteins, two ligation strategies have been used to attach synthetic GPIs to proteins: expressed protein ligation (EPL) and intein-mediated protein trans-splicing (PTS) **(Fig. 4)**.

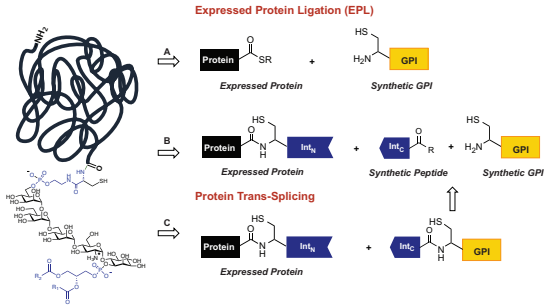

Fig. 4 Schematic representation for the semi-synthesis of GPI-anchored proteins

In the first strategy, GPIs containing a cysteine residue at the phosphoethanolamine residue were obtained using chemical synthesis. The active protein thioesters were isolated after folding an expressed fused protein of the protein of interest and an intein domain in the presence of thiols **(Fig 4,A)**, or they were generated in situ by the formation of an active intein domain using a mutated split-intein and the corresponding splicing reactions **(Fig 4,B)**. After establishing the best folding conditions, the desired protein thioesters were used in a EPL process with the Cys-containing GPIs to deliver the GPI-APs. In the second strategy, the proteins of interest are expressed as fusion proteins with the *N*-terminal fragment (Int^N) of the split intein from *N. punctiforme* **[10]**. The GPI molecules are ligated to a synthetic C-terminal intein fragment peptide by native chemical ligation. The GPI-APs are obtained by a trans-splicing process induced after folding the two split-intein fragments. With the established methods, we have synthesized diverse GPI-APs including the PrP, INF-α2 and GFP as well as some parasitic proteins containing mono- and bilipidated GPI molecules.

Besides the GPI-APs, we have also investigated the use of the NCL strategy for the synthesis of N-glycoproteins and for the synthesis of GPI-anchored glycopeptides. We evaluated different methods and established the combination of allyl / N,N-dimethylamino-phenacyl groups as suitable protecting groups for the side chain protection of aspartic acid and to obtain N-glycopeptides with variable glycosylation patterns. **[11]** The peptide thioesters were formed after cleavage of the glycopeptides from the resin. NCL has been applied to attach the glycopeptides to GPIs or to elongate the peptide chain to larger glycopeptides. Glycopeptides were obtained with small sugars and with complex N-glycans.

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

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Autoantigenicity Patters in Health and Disease

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
03/2013-09/2015: Research Group Leader (part-time), Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces
Since 10/2015: Research Group Leader, Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces

Everybody has circulating self-reactive antibodies in the blood. Although these individual repertoires of autoantibodies can significantly overlap, they differ between healthy and diseased individuals. Therefore, we believe that differential analysis can lead to the identification of biomarker sets that can clearly separate different diseases or even allow subdiagnosis of patients within a certain disease.

Autoimmune disorders are characterised by the presence of antibodies against a number of self-antigens. In some cases, these autoantibodies have a known pathophysiological role and are explicit drivers of the disease leading to tissue destruction. However, in many autoimmune disorders, their role is yet not understood and 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 centres on the investigation of antibody-antigen complexes in autoimmune disorders. The scientific focus is currently on the elucidation of autoantigenicity patterns in rheumatoid arthritis. The group is part of a consortium between a local small enterprise and the Charité and is financed exclusively by third party funding of the German Federal Ministry of Education and research (BMBF) and the Federal Ministry of Economic Affairs and Energy (BMWi). The aim of the work within this partnership is the characterisation of autoantibody profiles and the definition of patterns that can be used for differential diagnosis. The methodological portfolio includes primarily protein array and phage display technology, recombinant protein expression, as well as immunological methods.

Characterization of Autoantibody Repertoires in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a heterogeneous disease of presumably multifactorial but unknown aetiology. To date, the course of the disease as well as the response to specific treatment strategies is insufficiently predictable. RA cannot be cured, requires lifelong medication, frequently (>50%) causes work disability within the first ten years of disease and reduces life expectancy compared to the general population. Nevertheless, over the last years, the treatment of patients with RA has changed considerably. The new targeted therapies (biologics) can induce previously unachievable responses in subgroups of patients. Most recent studies suggest that especially "fast response" after initiation of treatment with biologics is an indicator of successful anti-inflammatory targeting. However, in long-term treatment relapses may occur and molecular mechanisms related to these flares are insufficiently understood. Furthermore, the goal of therapy is not only symptom relief, but in particular the prevention of long-term structural damage and functional decline. So far no personalized biomarkers exist, which can be used by the clinicians to decide which type of therapy shall be given, or which type of biologic drug in use is effective in an individual RA patient and can therefore be used to induce a fast remission.

A recent project in close collaboration with the Department of Rheumatology and Clinical Immunology of the Charité centred on the clinical validation of certain IgA autoantibody profiles, which can be used to identify TNFalpha inhibitor non-responders. We could show that measuring autoantibodies against a set of 5 autoantigens can identify 80% of therapy non-responders **[1, 2, 3]**. In another project, we used protein array technology to characterize autoantibody profiles in mouse models in RA **[4, 5, 6]**. We demonstrated, that the development of certain autoantibodies are toll-receptor dependent **[4]**. In yet another study, we looked into specific autoantibody profiles, which allow discrimination between early stages of RA and systemic lupus erythematosus (SLE). According to our current findings, the biomarkers may possibly also serve as prognostic marker, i.e. give clues about the progression of RA in those patients possessing such autoantibodies. We could show that detection of autoantibodies against certain heterogeneous ribonulceoproteins (hnRNPs) can be used to reduce the sensitivity gap of current standard biomarkers used in the initial serological diagnosis of RA **[7]**. We are currently finalizing a manuscript with our clinical partner where we have tested >1000 early RA patients with these markers. Another manuscript we are currently preparing shows that selected hnRNPs can distinguish between erosive and moderate forms of RA and therefore measuring autoantibodies against these markers can assist the clinician in his therapy decision.

The current research program is focussing on analysing the diagnostic potential of further hnRNPs for diagnostic purposes in the field of RA and their potential application as prognostic and predictive markers for therapy outcome. One major aspect is the investigation of (aberrant) post-translational modifications in this context. Within the project, we rely on our expertise in the production of recombinant proteins and antibodies in prokaryotic and eukaryotic hosts as well as the protozoan host *Leishmania tarentolae*. *L. tarentolae* is a promising host for the expression of recombinant proteins, as it has the ability to produce soluble proteins in the cytoplasm as well as glycosylated proteins utilising secretory pathways. We have explored this potential and could show for the first time, that O-glcosylation of a recombinant protein expressed in *L. tarentolae* can occur **[8]**.

The most recent project is devoted to the identification of autoantigenicity patterns that accompany therapy and therefore, might allow drawing predictive conclusions about therapy outcome **[9]**. Here, 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. The used protein arrays consist of ~25.000 expression constructs of a human foetal brain cDNA library. For phage display screening, we will use various full-ORF libraries and peptide libraries available in our laboratory. While the identity of each spot on the protein array is known, the phage display libraries require downstream processing. Phage display selection is an iterative process based on affinity enrichment using patient-derived immunoglobulin fractions as selection targets over several rounds. The identity of the enriched clones is revealed by sequencing of the DNA inserts **[10]**.

Zoltán Konthur, Ute Nonhoff
zoltan.konthur@mpikg.mpg.de

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Rational Approach towards Glycoconjugate Vaccines

Claney Lebev Pereira 17.05.1978
1996-1999: B.S, St. Aloysius College, Mangalore, India
1999-2001: M.S, Mangalore University, Mangalore, India
2001-2004: Scientist, Syngene International Pvt. Ltd, (BIOCON), Bangalore, India
2004-2005: Associate Chemist, Aurigene (Dr. Reddy's Laboratory), Bangalore, India
2005-2010: PhD, Emory University, Atlanta, GA, USA
2010-2012: Postdoc, Max-Planck-Institute of Colloids and Interfaces, Potsdam, Germany
2012-2015: Group Leader, Max-Planck- Institute of Colloids and Interfaces, Potsdam, Germany

Design of carbohydrate based glycoconjugate vaccine has not seen a huge change from its inception. Commercial vaccines are still rely on isolated natural capsular polysaccharide (CPS) present on bacterial surface. The vaccine subgroup is using a rational approach utilizing organic syntheses to identify key epitopes which are protective from these natural CPS. The approach relies on a library of oligosaccharides varying in length, type of monosaccharides at the reducing and non-reducing end, branching vs linear, frame shifts, neutral vs charged and many others. Using these unique set of glycans and employing glycan array and protective monoclonal antibodies for a given pathogen, we elucidate the most potent, immunogenic, antigenic and functional oligosaccharide that is then evaluated further using additional invitro and invivo experiments to identify a potential semi-synthetic vaccine candidate. The challenges associated in syntheses leads to further development of existing protocols or discovery of novel methods and reagents.

In an ongoing effort the ''vaccines'' research group is working on the chemical synthesis and biological evaluation of carbohydrates present on *Streptococcus pneumoniae, Yersinia pestis, Chlamydia trachomatis, Haemophilus influenzae type b, Leishmania donovani, Neisseria meningitidis, Salmonella typhi, Kleibsella pneumoniae, and Clostridium difficile.* Since carbohydrates are complex molecules the vaccine group is also refining the activators and glycosylation methods needed to put together these molecules using thier individual monosaccharides.

Streptococcus pneumoniae: The group is currently pursuing several serotypes and has finished the synthesis of a number of repeating units of the CPS of ST-1, ST-3 **[1],** ST-4 **[2],** ST-5, ST-8 and ST-12F (**Fig 1**). In all cases immunological evaluations have been followed up by functional evaluation either using the challenge studies or the standard surrogate opsonophagocytic killing assay (OPKA). This massive project opens up the possibility to better understand the roles played by various substituents like acetates and pyruvates (ST-4), rare sugars like pneumosamine (ST-5), conjugation methods, sugar loading, etc. on immunogenicity and antigenicity. We would like to address some key questions on glycoconjugate vaccine design so as to move away from empirical to rational way of designing glycoconjugate vaccines. The oligosaccharides also serve to evaluate potential stability issues, formulation development and to standardize the analytics needed to manufacture a glycoconjugate vaccine.

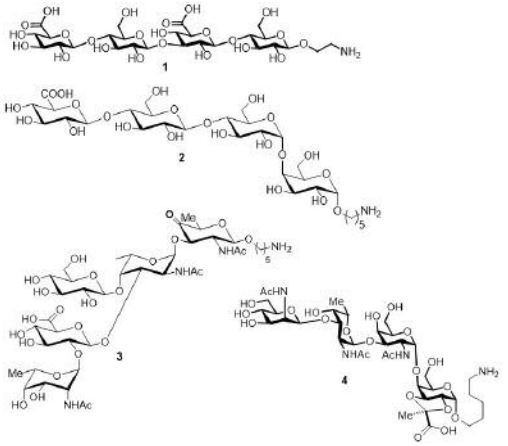

Fig. 1: Carbohydrate structures of S. pneumonaie that the ''vaccines'' research group has synthesized

Haemophilus influenzae: A library of synthetic oligosaccharides, based on the CPS repeating unit of Hibwere synthesized and subjected to immunological evaluation. The glycans were found to be immunogenic and showed cross reactivity to the natural CPS.

Clostridium difficile: Synthesis and immunological evaluation of the newly reported PS-III antigen wascarried out (**Fig 2**) **[3].** PS-III along with PS-I and PS-II antigens seem to be a promising vaccine candidate and would need to be further evaluated through an active as well as a passive challenge model using mAb specific for one of the three oligosaccharides.

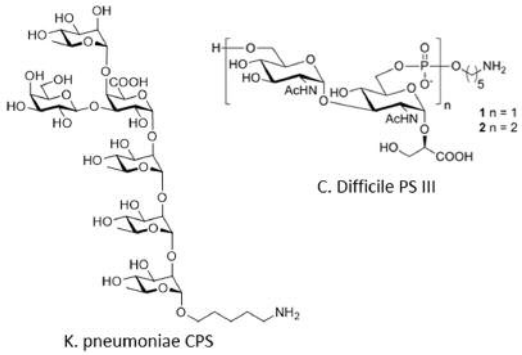

Fig. 2: Carbohydrate structures of pathogens that the ''vaccines'' research group has synthesized

Kleibsella pneumoniae: Infections caused by *K. pneumoniae* are becoming an important challenge due to the emergence of strains resistant to carbapenem antibiotics. Recently the CPS structure of ST258 clone was identified. Using this information the total synthesis of hexasaccharide repeating unit of the CPS of a carbapenem resistant *K. pneumoniae* was achieved (**Fig 2**). Immunization experiments and mAb generated cross reacted with the native CPS. In order to guage the potential as a vaccine candidate animal challenge models needs to be established and is currently underway for this very important medically unmet pathogen.

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Methodology: Glycosylation are still challenging reactions in oligosaccharide syntheses especially 1,2- cis. Given our interest to install an amine containing alkyl linker at the reducing end of an oligosaccharide, the selectivity and yield for such 1,2-cis glycosylations are governed by various factors that dictate the final steroselective outcomes. We developed a new methodology project using a unique fluorine containing linker that allowed us to get exclusive or better 1, 2 cis selectivity during glycosylations (**Fig 3**) **[4].** This approach is currently being tested on the syntheses of antigens using the automated glycan assembly were the 1,2-cis glycosylation are even more difficult to achieve.

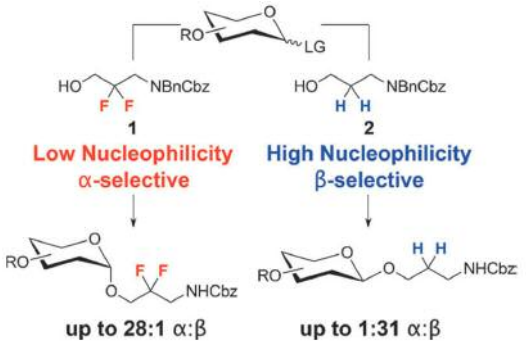

Fig. 3: Nucleophile directed stereocontrol in glycosylation

Other Projects: Along with the above mentioned work, we also are involved in methodology development for activation of glycosyl donors for glycosylation, mass based analysis of glycoconjugates **[5],** synthesis of glycans from lipopolysaccharides **[6],** modification of glycans to increase stability, syntheses of GSL based fully synthetic compounds and the syntheses of antigens from various other pathogens both in solution and using AGA.

Vaccine Chemistry:
C. L. Pereira, J. Y. Baek, M. Lisboa, S. G. Parameswarappa, B. Schumann, A. Calow, X. Fei-Fei, M. Emmadi, X. Guozhi, P. Menova, S. Awan. L. Lykke
claneylebev.pereira@mpikg.mpg.de

Vaccine Biology:
Peter H. Seeberger, F. Bröcker, A. Geissner, A. Reinhardt, P. Kaplonek, N. Khan, A. Wahlbrink.
peter.seeberger@mpikg.mpg.de

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