Development and Application of Ligation and Labelling Techniques in Glycobiology

Doctoral thesis for obtaining the academic degree Doctor of Natural Sciences (Dr. rer. nat.)

submitted by
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1. Referent: Prof. Dr. Valentin Wittmann
2. Referentin: Prof. Dr. Tanja Gaich
Es gibt Augenblicke, da gelingt uns alles.
Kein Grund zu erschrecken: Das geht vorüber.

Jules Renard
Danksagung

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<tr>
<td>AAC</td>
<td>Azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>2-AA</td>
<td>2-aminobenzoic acid</td>
</tr>
<tr>
<td>2-AB</td>
<td>2-aminobenzamide</td>
</tr>
<tr>
<td>2-AP</td>
<td>2-aminopyridine</td>
</tr>
<tr>
<td>4MPA</td>
<td>4-methyl-2-phosphonoaniline</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin-converting enzyme 2</td>
</tr>
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<td>ADMI</td>
<td>2-azido-1,3-dimethylimidazolium hexafluorophosphate</td>
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<tr>
<td>AE</td>
<td>Anion-exchange</td>
</tr>
<tr>
<td>AF555</td>
<td>Alexa Fluor™ 555</td>
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<tr>
<td>ANTS</td>
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<tr>
<td>ASC</td>
<td>Human adipose stromal cells</td>
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<tr>
<td>ASGP-R</td>
<td>Asialoglycoprotein receptors</td>
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<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCN</td>
<td>Bicyclononyne</td>
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<td>BCN</td>
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</tr>
<tr>
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<td>Butenyloxycarbonyl</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblat growth factor</td>
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<td>Btl</td>
<td>But-3-enoyl</td>
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<td>BTTES</td>
<td>3-(4-((Bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)propane-1-sulfonic acid</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CEY</td>
<td>Combined equilibrium yield</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine monophosphate</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>cRGDfK</td>
<td>Cyclo(Arg-Gly-Asp-D-Phe-Lys)</td>
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<td>CuAAC</td>
<td>Cu(I)-catalysed azide-alkyne cycloaddition</td>
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<tr>
<td>CY</td>
<td>Combined yield</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DAinv</td>
<td>Inverse-electron-demand Diels-Alder</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger associated molecular patterns</td>
</tr>
<tr>
<td>DAP</td>
<td>2,6-diaminopyridine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidin-2-phenylindol</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
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<td>DIBO</td>
<td>4-dibenzocyclooctynols</td>
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<td>DIFO</td>
<td>Difluorinated cyclooctyne</td>
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<td>DMB</td>
<td>1,2-diamino-4,5-methylenedioxybenzene</td>
</tr>
<tr>
<td>DMC</td>
<td>2-chloro-1,3-dimethylimidazolinium chloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>EWG</td>
<td>Electron-withdrawing group</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC-DBCO</td>
<td>Fluorescein-conjugated dibenzocyclooctyne</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetyl galactosamine</td>
</tr>
<tr>
<td>GBPs</td>
<td>Glycan-binding proteins</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>Heoc</td>
<td>Hexenyoxyxycarbonyl</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<td>-----------</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency viruses</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Hxl</td>
<td>Hex-5-enoyl</td>
</tr>
<tr>
<td>IE</td>
<td>Incorporation efficiency</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>k</td>
<td>reaction constant</td>
</tr>
<tr>
<td>$k_h$</td>
<td>Hydrolysis rate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetyl mannosamine</td>
</tr>
<tr>
<td>MBLs</td>
<td>Mannose-binding lectins</td>
</tr>
<tr>
<td>MGE</td>
<td>Metabolic glycoengineering</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>N-acetyl neuraminic acid</td>
</tr>
<tr>
<td>Neu5Ac9Ac</td>
<td>N-acetyl-9-O-acetyleneuraminic acid</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>N-glycolyl neuraminic acid</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OEG</td>
<td>Oligo ethylene glycol</td>
</tr>
<tr>
<td>OST</td>
<td>Oligosaccharyltransferase</td>
</tr>
<tr>
<td>$p$</td>
<td>para</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline Tween</td>
</tr>
<tr>
<td>PDA</td>
<td>Para-phenylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>Peoc</td>
<td>Pentenyloxycarbonyl</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PNGase F</td>
<td>Peptide-N-glycosidase F</td>
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<tr>
<td>Ptl</td>
<td>Pent-4-enoyl</td>
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<tr>
<td>pyBOP</td>
<td>Benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphat</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycin-aspartic</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed-phase</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>Severe acute respiratory syndrome coronavirus type 2</td>
</tr>
<tr>
<td>sCy3</td>
<td>Sulfo-Cyanine 3</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>Sia</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>SPAAC</td>
<td>Strain-promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>STD</td>
<td>Saturation transfer difference</td>
</tr>
<tr>
<td>Strep</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>TAMRA</td>
<td>Carboxytetramethylrhodamine</td>
</tr>
<tr>
<td>TBTA</td>
<td>Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine</td>
</tr>
<tr>
<td>TCO</td>
<td>Trans-cyclooctenes</td>
</tr>
<tr>
<td>TEG</td>
<td>Tetra(ethylene glycol)</td>
</tr>
<tr>
<td>THPTA</td>
<td>Tris(3-hydroxypropyltriazolylmethyl)amine</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TMTH</td>
<td>3,3,6,6-tetramethyl-thiacycloheptyne</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>TSP</td>
<td>Trimethylsilylpropanoic acid</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine-5'-diphosphate</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
</tbody>
</table>
1. Introduction

Can you remember a time more life-changing and extreme than the current coronavirus pandemic? To date it has taken more than 3.8 million lives worldwide (status June 2021\textsuperscript{[1]}), devastated economies across the globe and changed lives forever. From toilet paper hoarding to limits on gatherings, the pandemic’s immediate effects on our surrounding environment is omnipresent. Researchers all across the globe are racing to develop ways to treat and prevent new infections. Many of these treatments focus on blocking the virus’ ability to latch onto and infect cells in the body. SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2) attaches to cells using its spike (S) proteins. The surface of the spikes is densely glycosylated, each spike trimer displaying 66 N-linked glycosylation sites (Figure 1.).\textsuperscript{[2]} The S protein binds carbohydrate-mediated to the angiotensin-converting enzyme 2 (ACE2) receptor, a molecule that sits on the surface of human cells. After binding to ACE2, the virus undergoes a structural change that allows it to fuse with the cell.\textsuperscript{[3-4]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Overlay of snapshots from simulation of the S glycoprotein with site-specific glycosylation. The glycans are shown in ball-and-stick representation in green, dark yellow, orange and pink. Image taken from Grant \textit{et al.}.\textsuperscript{[5]}}
\end{figure}

This is only one of many examples underlining the importance of protein glycosylation. Thus investigation of protein glycosylation, and of course not merely viral protein glycosylation, is of great importance for deeper understanding of the important role glycoproteins have in many physiological and pathophysiological processes.\textsuperscript{[6-8]} In order to investigate and understand the processes glycans and their corresponding binding-partners are involved in, they firstly have to be identified. This investigation and identification, however, has proven to be difficult, for one because of the vast amount of natural carbohydrates occurring which can be linked at various sites and in different stereochemistry leading to a vast diversity of possible linear and branched glycoconjugates. For another, because glycans are secondary gene products and are not controlled by a direct genetic code. The bottleneck in investigating glycans is that natural glycans can only be isolated in small amounts and cannot be amplified like e.g. DNA,
and that the synthesis of glycans is very challenging.\textsuperscript{[9]} An elegant method, which in high-throughput manner and only with minute amounts of carbohydrate sample analyses carbohydrate interaction is the microarray technology.\textsuperscript{[10]} In this method carbohydrate libraries are immobilised on a solid surface and are incubated with enzymes, antibodies, lectins, viruses, or even whole cells to characterise their binding specificities and affinities for up to serval hundred glycans at once. For efficient immobilisation, the carbohydrates are functionalised with a linker, mostly at their reducing end in a chemoselective reaction with nitrogen nucleophiles, like oxyamines. Oxyamines are especially well suited as glycoconjugates are gained in high yields, in one step and under mild aqueous conditions. Profound understanding and consequently investigation of ligation reactions is therefore important preparatory work for glycan analysis.

A highly valuable method to non-destructively investigate glycosylation pattern in and on cells is metabolic glycoengineering (MGE).\textsuperscript{[11-15]} For this, chemically modified carbohydrates are metabolically incorporated into glycoproteins. Due to the unnatural modification, a so-called reporter group, bioorthogonal ligation reactions for analysis and visualisation of the targeted glycoproteins can be performed. This method has been used with different carbohydrate derivatives\textsuperscript{[11,13,16-17]} different bioorthogonal ligation reactions\textsuperscript{[18]} and it can be used to visualise carbohydrate on the cell surface\textsuperscript{[18-20]} as well as in the extra cellular matrix\textsuperscript{[21]}.

In this thesis the focus is on three important aspects for better and more profound understanding of glycans, glycoconjugates and glycoconjugation. Firstly, on the investigation of the bioorthogonal light-induced 1,3-dipolar cycloaddition of a tetrazole and an alkene for application in MGE. Secondly on the generation and investigation of extracellular matrices equipped with additional functions by using MGE and the microarray technology for later application in wound care. And thirdly on the systematic investigation of the oxyamine ligation reaction for more efficient glycan ligation under aqueous conditions and without the need for external catalysts.
2. State of Knowledge

2.1. Glycosylation

One important reaction, making life on earth possible, is the unique reaction of assimilation of green plants. Sugar produced in this reaction is not only source of all carbohydrates, but also directly or indirectly, of all other components of living organisms. Plants and animals rely heavily on carbohydrates (e.g. starch, glycogen and sucrose) as a source of energy. Apart from this important nutritional role, carbohydrates also serve as a macromolecular structural compound (e.g. cellulose, chitin), they are a component of the energy transport compound adenosine triphosphate (ATP), they are one of three essential components of DNA and RNA and they are essential for recognition sites on cell surfaces. Glycobiology investigates these functions of sugars, of which many are not yet well understood. The challenge lies in defining the biological functions of sugars attached to biomolecules inside the cell and on the cell membrane, in investigating in which way structural features determine and regulate these biological functions and in determining how these functions are carried out. Carbohydrates which are located on the outside of the cell surface form the so called glycocalyx. When they are linked to biomolecules like proteins or lipids they are referred to as glycoproteins and glycolipids or in general as glycoconjugates; the carbohydrate part is called glycan. These conjugates are obtained via glycosylation i.e., the process linking carbohydrates covalently to the target molecules. This attachment of carbohydrates is an important posttranslational modification, crucial for the survival of most organisms, as e.g. without glycosylation some proteins fold incorrectly or are unstable (see chapter 2.1.1. Significance of Protein Glycosylation). There is no principal function of protein- and lipid-linked glycans. They serve several functions on enzymes, hormones, transporters and structural elements. Glycosylation is an enzyme-mediated reaction carried out site-specifically during the transport of the glycoconjugate through the rough endoplasmic reticulum (ER) and the Golgi apparatus, with no template or direct genetic code. There are eight different monosaccharides (D-glucose, D-mannose, D-galactose, L-fucose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-neuraminic acid, and D-xylose) known to be contained in eukaryotic glycans, which can be linked at various sites and in different stereochemistry leading to a vast diversity of possible linear and branched glycoconjugates. Enzymes can identify the structure of glycans and add and/or remove single monosaccharides, creating structurally different glycans. These small differences between basically identical molecules are called microheterogeneity (see chapter 2.2. Biosynthesis of Glycans/ 2.2.2. N-Glycoprotein Biosynthesis). The structural differences of carbohydrate mono- and oligomers have a major impact on their chemistry and biology, making firstly the amount of information that carbohydrates can carry significantly more than that of proteins and secondly enlarge the
repertoire of protein’s characteristics, to a greater diversity than encoded on the genome. This makes research on glycans a challenging task.\textsuperscript{[25]}

Besides glycosphatidylinositol(GPI)-anchored proteins, the two most frequent chemical linkage types between carbohydrates and proteins are \textit{N}-glycosidic and \textit{O}-glycosidic bonds. In \textit{N}-glycans, there always is a \textit{\beta}-\textit{N}-glycosidic linkage between the \textit{N}-acetylglucosamine of the core pentasaccharide (\(\text{Man}_3\text{GlcNAc}_2\)) to an asparagine (Asn) residue of the polypeptide chain (Asn-Xaa-Ser/Thr, with Xaa = any amino acid but prolin). The structure of \textit{N}-linked glycans usually falls within three major types: the high-mannose, the complex and the hybrid type. They share the core pentasaccharide and differ mainly in their branching patterns (Figure 2.1.\textsuperscript{[23,26]}) In \textit{O}-glycoproteins the sugar portion is \textit{O}-glycosidically linked to an OH-group in the side chain of a serine (Ser) or threonine (Thr) residue in the peptide moiety.\textsuperscript{[26]} As \textit{O}-glycans do not share a common core region, different types of \textit{O}-glycosylation can be defined, depending on the type of monosaccharide that is attached directly to the peptide. For example, in mucin-type glycans the glycan chain starts with \textit{N}-acetyl-galactosamine (GalNAc) \textit{\alpha}-\textit{O}-glycosidically linked to the protein.\textsuperscript{[24]}

\textbf{Figure 2.1.}: The three general types of eukaryotic \textit{N}-glycans: A high mannose, B complex, and C hybrid. Each \textit{N}-glycan contains the common core \(\text{Man}_3\text{GlcNAc}_2\text{Asn}\). D \textit{O}-glycans, mucin-type linked via GalNAc to either Ser or Thr.

\textbf{2.1.1. Significance of Protein Glycosylation}

As mentioned above, glycosylation is important for protein processing, as it affects the three-dimensional structure of proteins and thereby influences the proteins function, stability, immunogenicity, circulatory half-life, and consequently drug efficiency.\textsuperscript{[8]} It is estimated that up to 50\% of all proteins in nature, in particular secreted and transmembrane proteins of eukaryotes, are glycosylated. 90\% of these glycoproteins are \textit{N}-glycosylated.\textsuperscript{[24,27-28]} Their main tasks include the regulation and/or enhancement of physicochemical properties of proteins, signalling pathways and adhesion processes.\textsuperscript{[7]} Apart from their valuable physiological role, glycans are also involved in pathophysiological processes. To give an impression of the significance to understand protein glycosylation, some examples are given here:
Information on “Age” is stored within glycans. An example is the disposal of old erythrocytes from the blood. As erythrocytes age their glycocalyx is gradually desialylated, which increases the number of exposed galactose moieties on its surface. This allows asialoglycoprotein receptors (ASGP-R) in the liver to identify old erythrocytes and induce their destruction.[29-30] Crucial physicochemical properties like correct protein folding is mediated by proteins that bind to the glycan part of glycoproteins, so called glycan-binding proteins (GBPs) or lectins. Membrane-bound calnexin and its soluble homologue calreticulin are examples hereof. In the ER these lectins bind to incomplete or incorrect folded N-glycoproteins and assist as chaperons the correct folding. Furthermore, they prevent incorrectly folded proteins from leaving the ER.[31-32]

Also, viruses, bacteria and fungi express an enormous array of GBPs. An example is the toxin from *Vibrio cholerae* (cholera toxin) or the shiga toxin, produced by *Shigella dysenteriae*. Their glycan-binding subunits allow the toxin to combine with membrane glycoconjugates and deliver the active toxic subunit across the membrane.[24]

Furthermore, glycosylation can enhance protein stabilisation as well as protein dynamic and activity. The glycosylated Ribonuclease (RNase) B shows superior stability compared to its non-glycosylated form RNase A. In contrast to the lower stability of RNase A, its enzymatic activity is three times higher than that of RNase B.[33]

An example of N-glycans modelling an adhesion process is found in the glycoproteins of the HIV viral coat, GP120. This heavily glycosylated protein is essential for the initial contact between the HI virus and a host cell by mediating the adhesion. GP120 enables, together with the host cell’s CD4 protein, a membrane fusion between host cell and virus particle. Also, the heavy glycosylation on GP120 acts as a natural barrier, defending itself from immune cells and antibodies, which makes recognition and elimination of the HIV virus difficult for the immune system.[34-35]

For the immune system to respond to an invading pathogen, it needs to first identify the threat as “non-self”. It was found that many of the “pathogen-associated molecular patterns” (PAMPs) and “danger associated molecular patterns” (DAMPs) are glycoconjugates, and their immune receptors are lectins. One example are the mannose-binding lectins (MBLs), which recognise foreign glycan patterns present on microbes and injured host cells.[36-37] To conceal themselves from the immune system some pathogens even synthesise glycans mimicking the host cells glycosylation pattern.[38-39]

Changes in the natural glycosylation pattern of glycoproteins can play an important role in diseases such as rheumatoid arthritis (RA). In RA patient’s serum, among others, a reduction in galactose in immunoglobulin G (IgG) N-glycans and an increase in fucose residues in α1-acid glycoprotein have been observed. This indicates that RA may be a glycosylation-related
disease, reflecting changes in the intracellular processing, or degradation of \( N \)-linked oligosaccharides.\(^\text{[40-41]}\) Also, glycans are involved in cell-cell communication and cell trafficking; for example in the navigation and recruitment of leukocytes into different tissues.\(^\text{[42]}\) The involvement of glycans in cancer is still under investigation. Because of dysregulated enzymes tumour cells show significant alterations in their cell surface glycosylation pattern. An increase of certain sialylated glycans is a well-known characteristic of cancer cells and altered sialylation has long been associated with metastatic cell behaviour. Understanding these changes is therefore extremely important so that glycoproteins could be used as biomarkers thereby improving diagnostics and therapeutic strategies.\(^\text{[43-45]}\) It becomes clear from these examples that understanding and investigating protein- glycan interaction is of huge interested and an important topic to be investigated.

2.2. Biosynthesis of Glycans

The synthesis of glycans starts with the monosaccharides, the basic building blocks of carbohydrates. There are different ways to obtain them. They can be synthesised by the cell itself or imported from the outside into the cell, where they are firstly phosphorylated and then reacted with a nucleotide triphosphate resulting in a nucleotide-activated carbohydrate. Glycosyltransferases transfer the activated carbohydrates onto proteins or growing glycan chains. Monosaccharides can also be obtained by the salvage pathway, i.e. through breakdown of oligosaccharides in lysosomes and subsequent transfer of monosaccharides to the cytosol. A third way is the interconversion of nucleotide-activated carbohydrates achieved by multiple enzymatic activities. This so-called interconversion pathway plays a major role in maintaining stable intracellular levels of nucleotide-activated sugars under conditions where the supply of specific monosaccharides may be limited. As enzyme activity varies by cell type and intracellular compartment, different cells can synthesise glycoproteins with different glycan structure. The glycosylation process seems chaotic, but in fact is a series of very ordered, consecutive reactions, each step and enzyme activity dependents on the completion of the preceding enzymatic reaction.\(^\text{[24]}\) Scheme 2.1. gives an overview over the biosynthesis and interconversion of common monosaccharides. It shows the complexity of glycoconjugate biosynthesis, the high number of enzymes involved and the many interconversion possibilities. Every branching points give scientists the opportunity to interfere with the system at different stages and with different sugar derivatives.
Scheme 2.1.: A) Biosynthesis and interconversion of monosaccharides. The relative contributions of each pathway under physiological conditions are unknown. ( ) donors; ( ) monosaccharides; ( ) control points. Taken from "Essentials of Glycobiology, 3rd edition[24]

B) carbohydrate symbol representations modified by the CFG[46] and the editors of "Essentials of Glycobiology"[24].

For this thesis, mainly the biosynthesis of sialic acids (Neu5Ac) and the synthesis of N-glycoproteins are of interest and are discussed in more detail.

### 2.2.1. Sialic Acid Biosynthesis

Sialic acid is a carbohydrate where the C1 position is a carboxylate group that is ionized at physiological pH giving it a negative charge. They are mostly attached to the terminal ends of glycans on the glycocalyx, where they are involved in defence and communication mechanisms of the cells. They shield recognition sites and serve as anti-recognition agents, for example against pathogens. They also are involved in multiple interaction and recognition events, for example with hormones, lectins and antibodies.[47-48] Sialic acids are also referred to as neuraminic acids and about 50 different types are known. The most common one is N-acetylneuraminic acid (Neu5Ac), followed by N-glycolylneuraminic acid (Neu5Gc) and N-acetyl-9-O-acetylneuraminic acid (Neu5Ac9Ac).[47] The biosynthesis of CMP-activated sialic acid (CMP-Sia) is described hereinafter, focusing only on Neu5Ac (Figure 2.1.).[24,48] Its
precursor N-acetylmannosamine (ManNAc) can be obtained from the extracellular environment or by enzymatic conversion from GlcNAc by GlcNAc-2-epimerase or from UDP-GlcNAc by UDP-GlcNAc-2-epimerase. Latter also catalyses, the conversion of ManNAc to ManNAc-6-phosphate as ManNAc-6-kinase. This enzyme is inhibited by CMP-Neu5Ac, exemplifying the high order and tight regulation of biosynthetic pathways.\textsuperscript{[48-49]} ManNAc-6-phosphate is then converted by the Neu5Ac-9-phosphate synthase using phosphoenolpyruvate (PEP) to Neu5Ac-9-phosphate, which is dephosphorylated by Neu5Ac-9-phosphate phosphatase to become the sialic acid Neu5Ac. Neu5Ac is synthesised in the cytosol and is activated to CMP-Neu5Ac by CMP-Neu5Ac synthase in the nucleus. CMP-Neu5Ac is transported to the cytosol and subsequently to the Golgi lumen by the CMP-Sia transporter where it serves as a donor substrate to sialyltransferases that incorporate it at the nonreducing end of mature glycans. There are 20 different sialyltransferases that transfer CMP-Sia onto a glycoprotein or glycolipid.\textsuperscript{[48]} The completed glycoconjugate can be transferred, for example, to the cell membrane. Removal of sialic acids is catalysed by four different sialidases.\textsuperscript{[24]} The interconversion of ManNAc to Neu5Ac enables scientists to incorporate unnatural sialic acids without actually synthesising them, by simply offering unnatural ManNAc derivatives to the cells. As Scheme 2.1. shows GlcNAc derivatives can be use, too. These derivatives however have different functions and UDP-GlcNAc is not only a precursor for ManNAc but can also be converted to UDP-GalNAc and is most importantly an intermediate for the O-GlcNAcylation of proteins.\textsuperscript{[48]} An application of modified carbohydrate derivatives is the modification of glycoproteins using metabolic glycoengineering (see chapter 2.3. Metabolic Glycoengineering).

### 2.2.2. **N-Glycoprotein Biosynthesis**

All glycans, thus also N-glycans are not encoded by a DNA template but are secondary gene products assembled by various enzymes. This, in combination with possible microheterogeneity can result in specific proteins with very different glycosylation patterns (see chapter 2.1 Glycosylation). Scheme 2.2. shows a summary of N-linked glycosylation, the exact process is much more complex. It can roughly be divided into three stages:

1. Synthesis of a dolichol phosphate-linked heptasaccharide precursor on the endoplasmic reticulum (ER) surface. Translocation by a flipase to the luminal face of the ER and further elongation of the core structure. Transfer by an oligosaccharyltransferase (OST) from the dolichol phosphate anchor to nascent peptides emerging from membrane-bound ribosomes where they are attached to asparagine side chain.
(2) Synthesis and remodelling of branched oligosaccharide chains by trimming and adding carried out in the Golgi complex by Golgi glycosyltransferases.

(3) Secretion of glycoprotein into the cytosol or transport to the plasma membrane.\cite{24,50}

**Scheme 2.2.** Schematic representation of N-glycoprotein biosynthesis in eukaryotes. 

- **A)** Glycosylation of dolichol phosphate.
- **B)** Translocation of the glycan precursor across the ER membrane.
- **C)** Elongation of the glycan structure.
- **D)** Transfer of the glycan to the nascent peptide chain at membrane-bound ribosomes.
- **E)** Transport of the glycoprotein to the Golgi complex and remodelling of the N-linked glycan.
- **F)** Secretion into the cytosol or transport to the plasma membrane. Adapted from Schwarz et al.\cite{50}

### 2.3. Metabolic Glycoengineering (MGE)

#### 2.3.1. Bioorthogonal Ligation Reactions for MGE

Metabolic glycoengineering (MGE) is a technique that investigates glycosylation by utilising the cells ability to metabolise and incorporate synthetic monosaccharides bearing unnatural chemical groups into the cellular architecture. The introduced derivative is taken up into the cell, transformed by several enzymatic steps (see chapter 2.2. Biosynthesis of Glycans) and incorporated instead of the natural one, resulting in e.g. cell-surface glycans bearing unnatural functional groups (Scheme 2.3.). Peracetylated monosaccharides much easier penetrate the cell membrane and are thus commonly used in cell experiments.\cite{51-52} Inside the cell, acetylesterases cleave off the acetyl protecting groups, yielding the unprotected, unnatural monosaccharide. However, there are limits to the incorporation, as N-acetyl mannosamine (ManNAc) 6-kinase does only accept carbohydrate derivatives carrying slightly modified and small N-acyl groups.\cite{13} Aside from ManNAc derivatives, other carbohydrates such as GalNAc (N-acetyl galactosamine)\cite{16,53}, L-fucose\cite{54-55} or GlcNAc (N-acetyl glucosamine)\cite{11} derivatives have also proven to be accepted.
Metabolic glycoengineering of unnatural ManNAcX derivative and ligation reaction of corresponding unnatural sialic acid derivative.

REUTER and co-workers reported 1992 on ManNAc derivatives with unnatural N-acyl side chains (N-propanoyl, N-butanoyl, and N-pentanoyl).[11] 1997 the group around BERTOZZI investigated an unnatural derivative of ManNAc containing a ketone group. This carbohydrate was converted to the corresponding sialic acid and incorporated into cell surface glycan chains, resulting in a cell surface bearing ketone groups.[12,56-57]

After successful incorporation, a molecule bearing a complementary reactive functional group is introduced and will react with the modified carbohydrate moiety. For a ketone group that would be a hydrazide. The ketone group allows to covalently link the molecule bearing the hydrazide moiety to the cell surface,[12,56] this is called a bioorthogonal ligation reaction. Ligation reactions enable researchers to selectively link molecules, immobilise substrates on surfaces, or attach moieties like probes or fluorescent dyes. The labelled cells can then be visualised and analysed using confocal fluorescence microscopy, flow cytometry or various other analytical methods. Bioorthogonal ligation reactions have become very popular as scientists are very interested in developing methods to study the dynamics and functions of biomolecules in their native surroundings. To be classified as a bioorthogonal reaction several conditions have to be fulfilled: the reactants must be highly selective between functional groups and mutually reactive, but are not allowed to cross-react or interact in any way with biological functionalities or reactions in the cell. Reactants as well as forming products have to be stable and nontoxic in physiological settings. Reaction conditions must be adaptable to the cellular environment and preferably the reaction should be fast.

Hereinafter bioorthogonal ligation reactions successfully applied in MGE are described. The group of BERTOZZI with the already mentioned ketone-hydrazide reaction were the first to establish a ligation reaction in MGE (Scheme 2.4.A). The ketone (or aldehyde) situated on the side chain of a sugar selectively reacts with a hydrazide yielding a stable hydrazone. The
usage of this reaction in living organisms is not possible as a pH of 5–6 is required for an adequate reaction rate.\cite{13,58}

Alternatively, to ketones/ aldehydes, the group around BERTOZZI used azides. These react with triarylpophosphines bearing an ester group situated ortho to the phosphorus, yielding in an aza-ylide intermediate. After an intramolecular cyclisation the ester reacts with the nucleophilic azaylde, forming a covalent amide bond.\cite{59-60} This Staudinger ligation (Scheme 2.4.B) is the first truly bioorthogonal reaction. It is quite slow and it needs high concentrations of the cytotoxic phosphine reagent as this is easily oxidized by air,\cite{60} but the reaction works in water, at room temperature and neutral pH, enabling application in living cells.\cite{61-62}

\begin{center}
\includegraphics[width=\textwidth]{Scheme24.png}
\end{center}

\textbf{Scheme 2.4.:} (A) Ketone-hydrazide ligation and (B) Staudinger ligation.

Another ligation reaction, utilising the small, bioorthogonal azide group is the azide-alkyne cycloaddition (AAC). HUISGEN developed this 1,3 dipolar cycloaddition in 1963. He used azides as 1,3-dipoles to react in a [3+2] dipolar cycloaddition with alkynes.\cite{63} In 2002 SHARPLESS and MELDAL both found copper(I) to be a suitable catalyst.\cite{64-65} The Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC, Scheme 2.5.A), also known as “click-chemistry”, gives at room temperature, with high reactivity and in high yields chemically robust triazoles with exclusive 1,4-regioselectivity. The azide as well as the alkyne are small and can easily be used as chemical reporters for tagging cells. But as copper(I) is cytotoxic, the application of CuAAC in MGE is difficult. More suitable for biological systems are copper-chelating ligands. Polytetrazole TBTA was the first copper(I) stabilizing ligand\cite{66}, over the course of years more were developed: e.g. THPTA\cite{67} being more water soluble or BTSES\cite{68} enhancing the reaction rate.

Another kind of AAC, a copper free variant, is the strain-promoted azide-alkyne cycloaddition (SPAAC, Scheme 2.5.B). In this method, an alkyne is activated by ring strain, for example cyclooctyne. SPAAC has a lower reaction rate than CuAAC, but as copper is not needed as a catalyst no cytotoxicity is observed,\cite{69} enabling its use in living systems. Several strained alkyne derivatives have been developed and applied as ligation partners for azides; e.g. DIFO (difluorinated cyclooctyne)\cite{70}, DIBO (4-dibenzocyclooctynol)\cite{71}, BCN (bicyclo[6.1.0]nonyne)\cite{72}
or TMTH (3,3,6,6-tetramethyl-thiacycloheptyne)\textsuperscript{[73]}. Strained cyclooctynes, for example DIBO, can lead to unspecific background staining due to a reaction with thiols.\textsuperscript{[74]} To reduce this background staining fluorogenic probes like fluorescein-conjugated dbenzocyclooctyne (FC-DBCO)\textsuperscript{[75]} and also click-activatable fluorescent probes, meaning probes that are only fluorescent after the reaction, have been developed.\textsuperscript{[76-77]}

\[ \text{Scheme 2.5.: Azide-alkyne cycloaddition in the (A) copper-catalyzed and (B) strain promoted variant.} \]

A different catalyst-free ligation reaction is the inverse-electron-demand Diels-Alder (DAinv) reaction, which was reported independently by FOX\textsuperscript{[78]}, WIEßLER\textsuperscript{[79]}, and HILDEBRAND\textsuperscript{[80]} as a bioorthogonal ligation reaction. Here the chemical reporter group is an alkene reacting with a tetrazine (Figure 2.2.A).\textsuperscript{[81]} The formed highly strained bicyclic intermediate reacts in a retro Diels-Alder reaction yielding 4,5-dihydropyridazine. Due to nitrogen release the reaction is irreversible and following air oxidation leads to the corresponding pyridazines. Insight into mechanistic and kinetic aspects of the DAinv reaction was given by the group around SAUER,\textsuperscript{[82-85]} Kinetics are determined by the HOMO\textsubscript{dienophile} (alkene) – LUMO\textsubscript{diene} (tetrazine) gap. A smaller energy difference between the frontier molecular orbitals accelerates the reactions rate, thus for the DAinv reaction electron-rich dienophiles and electron-poor dienes are most beneficial. The reaction rate of cycloalkenes, e.g. norbornenes, trans-cyclooctenes (TCO) or cyclopropenes is considerably higher than of acyclic alkenes. This is due to their ring strain being released upon reaction (Figure 2.2.B).\textsuperscript{[83,85-88]} Lately bioorthogonal turn-on probes linked to tetrazine\textsuperscript{[89-91]} and photo-caged Q-rhodamines and fluoresceins linked to proteins\textsuperscript{[92]} were developed. By significantly increasing the signal-to-background ratio, they facilitate imaging targets inside living cells. In addition to alkenes also some strained alkynes like BCN or cyclooctyne react with tetrazines in a DAinv reaction (Figure 2.2.B).\textsuperscript{[93-96]} other strained alkynes like DIBO do not react with tetrazines.\textsuperscript{[96]} This gives the opportunity to use certain alkenes—
alkynes pairs in one experiment, conducting the DAinv reaction and the SPAAC at the same time and orthogonal to each other.\cite{97} Because in MGE only small reporter groups are readily accepted by the biosynthetic machinery, the main disadvantage of the DAinv reaction is the size of tetrazines and strained alkenes (or alkynes), in comparison to azides or alkynes. Attractive bioorthogonal reporters used for the DAinv reaction are terminal alkenes\cite{98} (also incorporated in unnatural amino acids\cite{99}) and especially the small cyclopropenes independently explored by the groups of DEVARAJ\cite{100} and PRESCHER\cite{97} and applied for MGE\cite{101-105}.

A newer addition to the toolbox of bioorthogonal ligation reactions is the light-induced 1,3-dipolar cycloaddition of a tetrazole and an alkene (short photoclick reaction). The concept was first reported by HUISGEN and SUSTMANN between a 2,5-diphenyltetrazole and methyl crotonate in benzene in 1967.\cite{106} In 2008 it was reinvestigated by the group of LIN and firstly reported as a bioorthogonal ligation reaction.\cite{107-108} The photoclick reaction starts with a light induced decomposition of tetrazoles. By releasing molecular nitrogen, a nitrile imine intermediate is generated, which reacts with various unactivated alkenes and alkynes through a 1,3-dipolar cycloaddition yielding in stable, fluorescent pyrazoline compounds (Scheme 2.6).\cite{109} The presence of this short-lived and highly reactive nitrile imine intermediate was discovered by HOLM in 1980.\cite{110}

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**Figure 2.2.** A) Inverse-electron-demand Diels-Alder (DAinv) reaction. Only one dihydropyridazine tautomer is depicted. B) Strained alkenes and alkynes used in the DAinv reaction.
Scheme 2.6.: Photoactivated 1,3-dipolar cycloaddition of a tetrazole with an alkene yielding in a pyrazoline.

In this reaction, the two $\pi$-electrons of the alkene (LUMO) and the four electrons of the dipolar compound (HOMO) are involved, forming the new bonds of the pyrazoline compound. Electron-withdrawing groups (EWG) on the dipolarophile (= alkene) favor the interaction of the dipolarophiles LUMO with the dipoles HOMO, whereas electron donating groups on the dipolarophile have the opposing effect.\[111\] The rate of the reaction can also be accelerated by the use of strained alkenes like norbornenes\[112\] or cyclopropenes\[113-114\].

The photoclick reaction can theoretically yield in two isomers, the 4- and the 5-substituted pyrazolines. Due to electronic effects the majority of reactions favor the 5-substituted pyrazoline.\[115\] By using mild conditions the 5-substituted pyrazoline is obtained exclusively, simplifying analysis significantly.\[116-117\] Once the tetrazole photoclick reaction to the pyrazoline compound is completed, consecutive reactions such as its oxidation to the corresponding pyrazole are also reported.\[116\]

Since nitrile imines are unstable, they have to be generated in situ.\[116-117\] Multiple ways are reported: in 1962 the basic activation of hydrazonoyl chlorides at room temperature or the thermolysis of tetrazoles at 150 – 160°C, followed by nitrogen release were reported.\[118\] Latter has the advantage that loss of nitrogen makes the reaction irreversible. In the later 1960s the basic activation of $\alpha$-nitro phenylhydrazones\[119-120\] and the photolysis of tetrazoles\[120\] were also reported. For the basic activation as well as for the photolysis applies that the first step, which yields the nitrile imine, is rate determining.\[121\]

A great advantage of the photo activation is that the reaction can be started at a chosen time and location, thus temporal as well as spatial control over the reaction is gained. Furthermore, stable and fluorescent pyrazolines are formed, which can be readout and imaged directly without the requirement of further purification. Also, the photoclick reaction has several advantages over other click reactions: for example, it does not require a metal catalyst; tetrazole-based molecules are gained from short synthetic routes and can be activated simply by use of a handheld UV lamp; also the reaction is monitored easily as fluorescent pyrazoline cycloadducts form and the only byproduct is nontoxic nitrogen.\[122\] But, several side reactions occur due to the high reactivity of the nitrile imine. Hydrolysis\[123-124\], dimerisation of nitrile imine yielding tetrazines\[116\] and reactions with amino acid residues\[125\] (e.g. tryptophan\[126\]) were observed and reported. To keep in mind is that all of these studies were performed in absence
of the most compatible reaction partners of nitrile imines, which are electron deficient alkenes. Thus, in presence of a reactive dipolarophile, by utilising the selectivity among the differently reactive alkenes and by choosing suitable reaction conditions the side reactions should be negligible. Also the usage of UV light for tetrazole activation is toxic for cells, even though only short irradiation times (seconds to minutes) are necessary for the photoclick reaction some damage is caused. Using tetrazoles with lower excitation energy is beneficial. Such naphthalene- and thiophene-based tetrazoles were developed by the group of LIN with excitation wavelengths up to 700 nm (Chart 2.1.).[113,127-129]

2.3.2. Applied Metabolic Glycoengineering

As said at the beginning of the chapter, different monosaccharide derivate are used in MGE. Because mannosamine derivatives are well accepted by the cellular machinery and are mainly incorporated on the cell surface as terminal sialic acids of N-glycans and because sialic acids are present in large number, they can easily be analysed by microscopy and are thus used in most experiments, especially when a new method has to be established.[16,62,103,134-136] Based on the aforesaid findings of REUTTER[11] mostly the N-acyl group of the sugar is derivatised. For example ketones (Ac₄ManNLev[56]), azides (Ac₄ManNAz[59]), alkynes (Ac₄ManNAI[137], Ac₄ManPoc[135]), various terminal (e.g. Ac₄ManNPT[98], Ac₄ManNBeoc[20]) and cyclic alkenes (Ac₄ManNCyc[101], Ac₄ManNCyc[102], Ac₄ManNCp[104]) as well as isonitriles (Ac₄ManN-o-Iso[138]) and the diazo group (Ac₄ManDiaz[139]) have been introduced as chemical reporter groups (Chart 2.2.). The azido sugar Ac₄ManNAz is the most commonly used derivative to label cells[67,75,140-141], mice[142-143], zebrafish embryos[144] as well as enveloped viruses[145] with different ligation reactions. Specific proteins can be analysed by using Förster resonance energy transfer (FRET) microscopy.[146-147]

To circumvent the enzymatic interconversion steps of MGE and the associated difficulties, sialic acid derivatives were employed directly.[148-149] For example BCN conjugated sialic acid for the DAINV reaction within live zebrafish embryos[93] or CMP activated azido sialic acid in the SPAAC for selective exo-enzymatic labelling with a sialyltransferase.[150] MGE was also used for photocrosslinking, where instead of the chemical reporter a diazirine (Ac₄ManNDAz,
Chart 2.2.) was incorporated which is photoactivated and covalently traps glycoprotein interactions.[151]

Regardless of the incorporated reporter group and the applied ligation reaction, MGE faces yet another challenge as the monosaccharides are metabolically transformed into one another (see chapter 2.2. Biosynthesis of Glycans); e.g. UDP-GlcNAc into UDP-GalNAc[53,152] and vice versa as well as GlcNAc into ManNAc and reverse. The group of Pratt was able to avoid this interconversion by introducing a 6-azido GlcNAc derivative (Ac₃6AzGlcNAc), which is selectively incorporated into O-GlcNAcylated proteins.[153]

An attractive applicability of different bioorthogonal ligation reactions is their possible orthogonality.[154-155] Different monosaccharide derivatives bearing different reporter groups are used in orthogonal ligation reactions in a way that two unnatural carbohydrate derivatives can be detected in one experiment, even on one cell. When two fluorescent dyes are used this is called dual labelling strategy. Among the first to try this method was the group of Bertozzi who incorporated Ac₄ManNLev together with Ac₄ManNAz[141] and also Ac₄ManNLev together with Ac₄GalNAz[58] and labelled them using the ketone-hydrazide and the Staudinger ligation. In 2013, the group around Rescher showed by using differently substituted methyl cyclopropene model proteins, that the tetrazole photoclick reaction and the tetrazine DAinv reaction are compatible in one experiment.[114] The Wittmann group exploited the orthogonality of Ac₄ManNPl and Ac₄GalNAz[88] as well as Ac₄ManNCyc and Ac₄GlcNAz[102] and the DAinv reaction and the SPAAC were used in one experiment. Also, in 2019 the Wittmann group was the first to use the photoclick reaction to label carbohydrates for MGE, in the course of this they also performed triple orthogonal labeling of glycans by applying the photoclick reaction, the DAinv reaction and the SPAAC in one experiment.[18]
Mostly mass spectrometry, Western blot analysis and microscopy are used to study the incorporation level into cells. The WITTMANN group also used another method, the DMB (1,2-diamino-4,5-methyleneoxybenzene) labelling reaction by which the incorporation efficiency (IE) of sialic acids can be determined. The labelling efficiency is not only dependent on the reaction rate of the bioorthogonal ligation reaction but also on the extent by which the natural sialic acids are replaced by the modified ones, thus the acceptance of the unnatural derivative by the biosynthetic machinery of the cell, thus the IE. DOLD et al. used DMB labelling for the detection and quantification of sialic acid derivatives after MGE with various azide- and alkene-modified ManNAc, GlcNAc, and GalNAc derivatives. They found for decreasing side chain length of the unnatural monosaccharides an increase for the IE, and a decrease of the rate of the DAinv reaction. Thus a certain chain length is an optimal balance between IE and reactivity, visible by the highest labelling intensity on the surface of the cells.\[19\] The WITTMANN group showed that the maximal IE is achieved between 24 – 48 hours after addition of the monosaccharides.\[156\] Furthermore, certain GlcNAc derivatives are converted to sialic acid, leading to the cell-surface staining, whereas the corresponding GalNAc derivatives are not.\[105\] This might be due to additional enzymatic steps required to convert GalNAc derivatives to the corresponding GlcNAc derivatives (see chapter 2.2.1. Sialic Acid Biosynthesis).\[156\] In HEK293T cells they found that the investigated sugars induce an increased sialic acid biosynthesis. Cell fractionation experiments showed that the cell interior fraction has the largest contribution to the observed increase in sialic acid levels whereas sialic acid levels in the membrane fraction are only moderately increased.\[156\]

### 2.4. Cellular Adhesion

All cells in solid tissue are surrounded by extracellular matrix (ECM). The ECM is a gel-like three-dimensional structure composed of proteins and polysaccharides released by cells into space between them. ECM organisation and ECM components differ from tissue type to tissue type, which enables each specialised ECM to carry out tissue-specific roles. The ECM provides cells with a medium for extracellular communication assisted by cell adhesion molecules (CAMs), with the possibility of cell migration during cell development and wound repair and with a stable position in tissues through cell matrix adhesion. In the dynamic process of cell adhesion cells and ECM, adjacent cells or cells and substrates form contacts with each other. Adhesion plays an important role in cell communication and regulation and is of great importance in the development and maintenance of tissues. The adhesion process is mediated by specific interactions between CAMs, which are specialised transmembrane glycoprotein complexes such as selectins or integrins and their appropriate ligands.\[157\] Selectins are carbohydrate-binding proteins (lectins). When for example tissue is infected, endothelial cells in the venules express selectins. The carbohydrate coating of leukocytes adhere to the lectins
Integrins are cell-surface receptors, important for the regulation of communication between cells and their environment and they play various roles in differentiation. Many integrins recognise the tripeptide motif arginine-glycin-aspartic (RGD). This motif can be found in many components of the ECM like laminin, collagen, and fibronectin. RGD mediates cell adhesion by mechanically anchoring cells.

An example of carbohydrate involvement in cell adhesion are galectins, which bear one carbohydrate binding domain. E.g., Galectin-3 interact with the Thomsen-Friedenreich disaccharide on cancer-associated mucin1, promoting the adhesion of cancer cell to endothelial cells by revealing adhesion molecules that are otherwise concealed.

As cell adhesion is of fundamental importance, the study of this field has been widely explored in cellular biology, biomedicine, and engineering. Every application has its own requirements regarding cell adhesion; these requirements depend on the specific applications of the cells. For example: Artificial heart valves and blood vessels must not adhere cells or plasma proteins, because of risk of thrombosis and embolism. But materials used in scaffolds for tissue generation or implants should promote cells’ adhesion and proliferation. Because carbohydrates take part in essential functions in biological systems and interact with various biomolecules, they are sought-after biomaterials. Polysaccharides, such as chitosan or hyaluronic acid, have been used as biomaterials for many years, because of their ability to support and control tissue growth, to mimic the ECM and their biocompatibility.

Polysaccharide biomaterials have been used in a variety of tissue engineering fields including skin, cartilage, cardiovascular, neural and hepatics. They have also found application in orthopaedic implants where they have shown to have a positive effect on cell viability on titanium surfaces.

2.5. Glycan Release and Analysis

For investigation of afore-said important protein-glycan interactions (see chapter 2.1. Glycosylation) and to monitor changes in glycosylation, generally two approaches can be used. One approach is to analyse intact glycoproteins and smaller glycopeptides. In this approach the analytes are separated for example by reversed-phase high-performance liquid chromatography (RP-HPLC) or size exclusion chromatography (SEC) and subsequently analysed by mass spectrometry (MS) with for example electrospray ionisation - time of flight (ESI-TOF) or matrix-assisted laser desorption ionization (MALDI). In the second approach the whole glycan is released from the protein. When studying glycan structures and structure-activity relationships of glycoproteins this approach is often beneficial because of the different physicochemical properties of carbohydrate and proteins. For this procedure certain criteria should be met: The release strategy should be nonselective regarding the type of
glycan (e.g. N- or O-glycan) for a representative glycan profile. The released glycans should not be modified and peptide and glycans should be separable from each other. A huge advantage of this approach to investigate glycosylation is, that glycans with a free reducing end are obtained. This facilitates subsequent derivatisation and ionisation for analysis of the glycans and is therefore very desirable. Also the deglycosylated protein is more easily digested by proteolytic enzymes for peptide analysis. However, complete glycan release is difficult to realise and unreleased glycans are left undetermined.

To assist and improve HPLC- and MS-based analysis of the released glycans, they are mostly functionalised by chemical methods like fluorescent labelling, or permethylation. This is done to improve stability for more fragile sialylated glycans or generally to enable downstream protein binding experiments on e.g. microarrays. The glycans can be separated by methods like hydrophilic interaction chromatography (HILIC), anion-exchange (AE)- or RP-HPLC or capillary electrophoresis (CE) and then be analysed by MALDI-TOF-MS or ESI-MS/(MS). There are two main possibilities to cleave glycans off proteins, either chemically or enzymatically. In the chemical approach unreduced O- and N-linked oligosaccharides are released using hydrazinolysis. At 60 °C O-linked oligosaccharides are selectively released with fresh anhydrous hydrazine followed by N-linked oligosaccharides at 100 °C. Disadvantage of this method is the deacetylation of N-acetyl sugars components and the use of toxic and highly explosive anhydrous hydrazine. A standard method for O-glycan release is β-elimination, carried out under alkaline/reducing conditions. For the enzymatic release strategy there is a growing number of enzymes, like endoglycosidases or glycosamidases used to release glycoproteins oligosaccharides. As there is no universal O-glycosidase available that can cleave all O-glycans, the enzymatic release strategy is mostly used for N-glycans, where for example peptide-N-glycosidase F (PNGase F) cleaves the amide bond between the innermost GlcNAc residue and the asparagine (Asn) side chain of the peptide/protein. The glycan is released as a hemiaminal which hydrolyses under aqueous conditions to the hemiacetal, whilst Asn is converted to aspartic acid (Asp, Scheme 2.7.). Freshly released glycosyl amine can also be directly labelled with for example a succinimidyl ester-activated fluorophore for analysis or using NHS-ester amine chemistry with mass tags. The released glycan can be analysed as the intact glycan or further cleaved with exoglycosidases into individual monosaccharide units or small oligosaccharides for structural analysis.
2.6. Functionalisation of Reducing Carbohydrates

In principle there are two ways to obtain glycans for investigating their important properties: Either by chemical synthesis or by using natural occurring ones. Even though chemical synthesis of mono- and oligosaccharides has gotten faster\cite{205-206}, it remains a time-consuming and laborious task, also because of the extensive use of protecting group strategies and because synthetic scale-up is difficult.\cite{9} Consequently, commercially available or naturally occurring carbohydrates with the resulting convenient loss of protecting group strategies are often used and functionalised to assist and improve further analysis.\cite{207} Here, the focus will be on methods to functionalise the reducing end of unprotected carbohydrates, as in this manner functionalised glycoconjugates are versatile usable e.g. for the generation of carbohydrate microarrays.

2.6.1. DMC-promoted Glycosylation

Glycosyl azides are key synthetic intermediates in carbohydrate chemistry as well as important precursors of glycoarrays\cite{208-209} and glycoconjugates\cite{210-212}. In 2009 the group of SHODA introduced a method to directly and selectively generate glycosyl azides from unprotected sugars mediated by 2-chloro-1,3-dimethylimidazolinium chloride (DMC) in aqueous solution.\cite{213} This method can also be used to generate dithiocarbamates\cite{214}, and aryl thiols\cite{215-216} in presence of the corresponding nucleophiles (Scheme 2.8.A). In 2014 the FAIRBANKS group presented an improved method to obtain anomeric glycosyl azides. They used an azido derivative of DMC, 2-azido-1,3-dimethylimidazolium hexafluorophosphate (ADMI) which conveniently provides the anomeric activating agent as well as the nucleophilic azide (Scheme 2.8.B). Subsequently they added alkyne, CuSO₄ and L-ascorbic acid and were able to obtain in a two-step one-pot method glycosyl click products.\cite{217}
Scheme 2.8.: A) Activation of an unprotected reducing carbohydrate with DMC for the generation of glycosyl azides (top) and thioglycosides (middle) and aryl thiols (bottom) under aqueous conditions.\cite{213, 214, 216} B) Direct two-step one-pot access to glycoconjugates through in situ formation of the glycosyl azide followed by click reaction.\cite{217}

The DMC-promoted glycosylation method has been used for the synthesis of glycopeptides\cite{218} and sugar nucleoside diphosphates\cite{219}. Saccharides have been functionalised in this manner with oligo ethylene glycol (OEG) linkers for the generation of carbohydrate microarrays,\cite{220} glycosyl azides were generated for site-specific glycoconjugation of protein via bioorthogonal tetrazine cycloaddition.\cite{221} A disadvantage of this method of glycosylation is that DMC is very hygroscopic and unstable under aqueous conditions,\cite{222} as well as that sugar concentrations of around 100 mM are needed to achieve high yields.\cite{223}

2.6.2. Glycosyl Amine Formation

The anomic center of reducing carbohydrates can be converted into amines resulting in N-glycosylamines. Interest in glycosylamines originates mostly from the need of scientists for access to N-glycoprotein material and the knowledge of the strong N-glycosyl amide bond between GlcNAc-Asp by which N-Glycans are anchored to polypeptides in glycoproteins (see chapter 1. Glycosylation),\cite{224} from the need of rapid reaction conditions of released N-glycans with labelling reagents for analysis,\cite{203} and from the knowledge of glycosylamines nucleophilicity, enabling them to create together with functionalised linkers oligosaccharide microarray.\cite{225} The main disadvantage of glycosylamines is that they are unstable and thus prone to dimerisation, hydrolysis, and isomerisation.\cite{226-228} Different glycosyl amine synthesis routes were developed over time: In 1913 GARRETT published the synthesis of glucosyl amines with ammonia.\cite{229} 1986 KOCHETKOV made glycosyl amines accessible by treating unprotected and fully protected reducing sugars with 50 times excess of ammonium bicarbonate for 6 days (Scheme 2.9.A).\cite{227} Drawbacks of the KOCHETKOV method are long reaction times and difficult product purification due to huge excess of reagent.\cite{230} FLITSCH have shown that microwave irradiation can accelerate this reaction.\cite{231} LIKHOSHERSTOV et al. improved the reaction by
substituting ammonium bicarbonate with ammonium carbamate (Scheme 2.9.B). This method has the advantage of easy product isolation, as it precipitates as a carbamic acid salt.[230] The salt state also prevents hydrolysis and diglycosylamine formation, if the amount of water is minimised.[230,232] HACKENBERGER et al. used this method of selective amination of unprotected sugar derivatives to transform crude chitobiose to 1,β-aminochitobiose.[233] A different approach to obtain glycosyl amines is the reduction of glycosyl azides with either hydrogen and palladium on charcoal (Scheme 2.9.C),[234] propanedithiol,[235] or Staudinger reduction[236].

![Scheme 2.9.: Synthetic routes for the preparation of glycosyl amines. A) Kochetkov conditions[227] B) Likhosherstov improved conditions,[230] and C) reduction of glycosyl azides[234-235].](image)

### 2.6.3. Reductive Amination

In this functionalisation method an primary amine condenses with the aldehyde tautomer of a reducing carbohydrate to form an imine in equilibrium with an glycosyl amine, the unstable imine can be reduced using e.g. sodium cyanoborohydride or 2-picoline-borane[237] to a stable secondary and acyclic amine (Figure 2.10.).

![Scheme 2.10.: Functionalisation by reductive amination of reducing carbohydrate via imine to stable amine.](image)

The primary amine that attacks the aldehyde tautomer mostly has a label attached that allows direct and quantitative detection of the carbohydrates by e.g. measurement of fluorescence or UV-absorbance intensity. Various labels have been used for the reductive amination of glycans. Already in the 1980s 2-aminopyridine (2-AP) was used as a label for fluorescence labelling of glycosaminoglycans.[238-239] Commonly applied labels are 2-aminobenzamide (2-AB),[240] 2-aminobenzoic acid (2-AA)[241-242] or 2-aminonaphthalene trisulfonic acid (ANTS).[242-243] 2-AB lacks negative charges and is commonly applied in chromatographic analysis, the 2-
AA label carries one negative charge, making it more suitable for applications like MALDI analysis.\[244-245] Direct coupling of compounds via reductive amination is attractive for glycoconjugate formation due to the irreversible formation of a covalent bond. The sometimes slow coupling rate and/or inefficient yield can significantly be increased by changing the temperature and pH of the reaction and by the addition of salts.\[246-247] But high amounts of salt entail the need for purification.\[245] The versatility of this method can be enhanced by using bifunctional reagents, such as 2,6-diaminopyridine\[248] (DAP) and derivatives thereof,\[249] in this way glycans can, for example, be coupled and then immobilised on succinimidyl ester-modified glass slides\[248]. Literature reports that some proteins bind exclusively to the ring-closed form of carbohydrates,\[250] thus the permanent acyclic structure of the functionalized carbohydrates obtained in a reductive amination reaction is disadvantageous for some applications.

2.6.4. Hydrazide Ligation

This type of ligation reaction also utilises the equilibrium between the hemiacetal and aldehyde form of reducing carbohydrates. The aldehyde is chemoselectively attacked by the acylhydrazide under acidic aqueous conditions, yielding acyclic (E)-/(Z)-acylhydrazones in equilibrium with the thermodynamically favoured cyclic \( \beta \)-N-glycosyl hydrazide (Scheme 2.11.).\[251-252] The \( \alpha \)-effect of the additional heteroatom in hydrazines and hydrazides (N-N), cause a higher reactivity\[253-254] and product stability\[255], which makes these reagents superior to primary amines in carbohydrate functionalisation.

\[ \text{Scheme 2.11.: Reaction of reducing carbohydrate with acylhydrazide to (E)-/(Z)-acylhydrazone in equilibrium with the favoured (E)-/} \]

\[ \beta \]-N-glycosyl hydrazide.\[251-252] \]

In contrast to the reductive amination where exclusively acyclic product forms, the great advantage of the hydrazide ligation is the formation of cyclic hydrazide. It was shown in microarray experiments that these cyclic glycoconjugates are recognised by lectins.\[256-257] The Hydrazide Ligation is applied for sensitive analysis of glycans by MALDI-TOF-MS.\[258] SHINOHARA et al. used hydrazide tagging of oligosaccharides, to introduce a biotin tag to immobilise glycans on streptavidin-coated carriers for biomolecular interaction studies.\[259] Hydrazides are also used as enzyme inhibitors,\[260] and to generate glycosyl hydrazides as O-glycosylation precursors.\[261] Drawbacks in comparison with oximes, are the slow reaction of hydrazides and lower hydrolytic stability of hydrazones.\[255]
2.6.5. Oxyamine Ligation

Already in 1882 oximes and their formation have been examined.\textsuperscript{[262-265]} In the early 90s with the generation of artificial peptides\textsuperscript{[266]}, glycopeptides and lipopeptides\textsuperscript{[267]} the oxyamine ligation has become more and more popular. This chemical ligation is very versatile and has since been used for many applications: Aminooxylated carbohydrates have been used as building blocks for glycoconjugates\textsuperscript{[268]} and as building blocks for the synthesis of natural products. Neo-glycopeptides and -proteins\textsuperscript{[269-270]} have been generated and reducing sugars have been ligated for e.g. the glycorandomization of digitoxin\textsuperscript{[271-272]}. The ligation has been successfully used for surface functionalisation, like labelling cell surface sialic acid-containing glycans on living animal cells,\textsuperscript{[273]} to immobilise carbohydrates on surfaces for isolation,\textsuperscript{[274-275]} for the generation of microarrays,\textsuperscript{[250,256-257,276-278]} to modify gold nanoparticles,\textsuperscript{[279-280]} and biomaterial scaffolds,\textsuperscript{[281]} for mass spectrometrical analysis,\textsuperscript{[282]} or to tag native carbohydrates for analysis with, for example fluorescence detection,\textsuperscript{[283-284]} fluorescence quenching\textsuperscript{[285]}, or PET labeling\textsuperscript{[286-287]}. The oxyamine ligation and its equilibrium state has been studied by several research groups for example by HPLC\textsuperscript{[288]}, or by NMR spectroscopy\textsuperscript{[250,280,288-293]} by taking samples at certain time points to get information about the reaction's current state.

The oxyamine ligation commences with a proton-catalysed nucleophilic attack of a primary oxyamine on the aldehyde tautomer of the reducing carbohydrate yielding a hemiaminal. (Scheme 2.12.A). After the elimination of water, acyclic oximes ((E)- and (Z)-configuration) in equilibrium with cyclic N-glycosides (α- and/or β-configuration) form. The acyclic oximes form the major share of the ligation products whereas the cyclic N-glycosides form the minor.\textsuperscript{[289,294-295]} Minor amounts of N-furanosides (not shown) can also be observed. All steps of the oxyamine ligation are reversible. The isomerisation of the open chain oximes runs via the closed forms. The final product composition of the reaction is not predictable through the reaction mechanism; it depends primarily on the monosaccharide unit, the thermodynamically most stable products are observed in higher ratios.\textsuperscript{[295-296]}

\[A) \text{H}_2\text{N} \text{OR}^1 \longrightarrow \text{HO} \text{N} \text{OR}^1 \text{H} \text{OH} \text{O} \text{H} \text{O} \text{hemi} \text{aminial} \text{H}_2\text{O} \text{H}_2\text{N} \text{OR}^1 \text{H} \text{OH} \text{O} \text{H} \text{O} \text{hemi} \text{aminial} \text{H}_2\text{O} \text{(E)-(Z)-oximes} \text{N-glycoside} \]

\[B) \text{H}_2\text{N} \text{OR}^1 \longrightarrow \text{HO} \text{N} \text{OR}^1 \text{H} \text{OH} \text{O} \text{H} \text{O} \text{hemi} \text{aminial} \text{H}_2\text{O} \text{H}_2\text{N} \text{OR}^1 \text{H} \text{OH} \text{O} \text{H} \text{O} \text{hemi} \text{aminial} \text{H}_2\text{O} \text{N-glycoside} \]

\textbf{Scheme 2.12:} Formation of oximes by ligation of reducing carbohydrates with A) primary or B) secondary oxyamines. Via the hemiaminal and after water elimination, the respective oximes and N-glycosides are formed; all compounds are in equilibrium with each other.\textsuperscript{[289]} Figure adapted from Baudendistel et al., 2016\textsuperscript{[289]}. 
Hydrolysis of oximes is obtained by reversing the order of the reaction steps; the back-reaction is initiated by protonation of the imine nitrogen. It was reported in 1997 that the reduced lone-pair repulsion between the two adjacent heteroatoms of the oxime and the increased electron density at C-1, due to the delocalised α-heteroatom lone-pair across C–N–X, causes an stabilising effect on the sp² state and thus enhance the hydrolytic stability of oxime (and hydrazone) neo-glycoconjugates. RAINES et al. showed in 2008 that the hydrolytic stability derives mainly from the negative inductive effect of the α-heteroatom attached to the imine-forming nitrogen. Thus, oximes show greater hydrolytic stability than corresponding hydrazones, as the electronegativity of the α-oxygen is higher than of the α-nitrogen. Furthermore is has been shown that electron-rich neo-glycoconjugates (e.g. of xylose) are hydrolysed more rapidly than electron-poor conjugates (e.g., GlcNAc), which corresponds to the readiness for the formation of the iminium species.

Whether acyclic glycoconjugates are recognised and bound by their interaction partners is still under discussion. Au fond literature states that only the unmodified core-monosaccharide unit, thus the cyclic form is recognised and bound by its interaction partners. FEIZI and co-workers showed by saturation transfer difference (STD) NMR, that only ring-closed oxime-linked fucose was recognised by a specific lectin, from a mixture that also contains the ring-opened forms. THYGENSEN et al. reported on a complete enzymatic consumption of maltooligosaccharide oximes by glucoamylase. As only the cyclic glycoconjugate can be hydrolysed, the complete turnover was explained by a shift in equilibrium towards consumed, cyclic tautomer upon protein binding. Thus, they suggest that the acyclic oximes can re-equilibrate to the cyclic form, if the cyclic form is removed from equilibrium.

To ensure the formation of cyclic products, secondary oxyamines (N,O-dialkyl-oxyamines) can be used as nucleophiles, they solely lead to cyclic N-glycosides (Scheme 2.12.B). During ligation with these oxyamines a hemiaminal forms. By water elimination it is transformed into a positively charged N-alkyl-N-oxyalkyl glycosyliminium ion which instantly tautomerises into the thermodynamically most stable cyclic glycoconjugate. Dependent on the carbohydrate an equilibrium of N-pyranosides (typically β configuration) and N-furanosides is obtained (Figure 2.16.B). Hence secondary oxyamines have found many application for example in the preparation of therapeutic neoglycoconjugates, or the formation of glycosyl acceptors in enzymatic synthesis. Ligation reactions with secondary oxyamines proceed with considerably lower equilibrium constants (Keq) than the reaction for oxime formation. To compensate for this, large excess of oxyamine, high reagent concentrations of sugar or nucleophile or both are applied. In a direct comparative study the group around JENSEN showed that the conformationally locked cyclic forms, derived from secondary oxyamines, translate into a higher observed affinity towards glycan-binding proteins. LIU et al. on the
other hand reports a hindrance of the core monosaccharide for recognition upon introducing a methyl group to the aminooxy linker attached to oligosaccharides.\[250\]

### 2.6.5.1. Catalysis for Oxyamine Ligation

That the formation of oximes from aldehydes can be catalysed by aniline has already been demonstrated in 1962 by Jencks,\[302\] Jensen applied this nucleophilic catalysis in 2010 firstly on carbohydrates. He found that the ligation reaction rate can be accelerated up to about 20-fold\[303\]. This rate enhancement is especially significant in the formation of neoglycoconjugates from slow reacting carbohydrates (e.g., GlcNAc). The aniline catalysis is more complex for reducing carbohydrates than for normal carbonyl compounds (Scheme 2.13.). That is because of additional equilibria like between the aldehyde form (B) and the cyclic hemiacetal (A) or between forming intermediates like cyclic N-phenyl glycosylamine (E), both influence the reaction rate with possible rate-limiting intermediates.\[302-304\]

![Scheme 2.13.](image)

Scheme 2.13.: General mechanism of aniline catalysed ligation of reducing carbohydrates with primary oxyamines. A) A reducing carbohydrate, B) its aldehyde tautomer, C) hemiaminal formed with aniline, D) glycosyl imine after dehydration, *DH* the protonated state, E) its glycosyl amine tautomer, F) aminal after oxyamine attack, G) (E)-/ (Z)-oximes, H) α- and β-N-glycosides, J) hemiaminal after oxyamine attack on B) via the uncatalysed reaction pathway and *BH* the protonated state.\[303\]

The reactive aldehyde (B) is mainly masked as unreactive hemiacetal (A, >99%) or in equilibrium with the hydrate (not shown). When B is attacked by aniline, it is converted into hemiaminal (C) and after dehydration into imine intermediate (D), the imine can be trapped as non-electrophilic glycosyl amine tautomer (E) which is a poor electrophile\[305\]. The two important benefits of the catalyst are firstly that it shifts the equilibrium towards reactive imine (D) without affecting the equilibrium for oxime formation, so increasing the amount of the imine intermediate relative to the amount of aldehyde present in the uncatalysed pathway.\[295\] And secondly the formed imine (D, pKₐ ~ 3)\[306\] with its higher basicity is much easier protonated than the carbonyl species (B, pKₐ ~ -4)\[307\]. Thus, the imine is a superior electrophile compared
to the aldehyde and provides high amounts of electrophilic iminium species \( (*DH) \) compared to the minor amounts of oxonium species \( (*BH) \), thereby facilitating the attack of an \( \alpha \)-nucleophile.\(^{\text{261}}\) The nucleophilic catalysis was used in the generation of neoglycopeptides,\(^{\text{303}}\) fluorescence labeled glycofoldamers\(^{\text{284}}\), oligosaccharide conjugates\(^{\text{293}}\) and studied with pyridinium and acetate as co-catalysts\(^{\text{308}}\).

The \( \alpha \)-effect nitrogen of an aminoxy and aniline have similar \( \text{pK}_a \) of about 4.6.\(^{\text{302,309}}\) The \( \text{pK}_a \) of hemiaminal \( (J) \) formed with the aminoxy is usually 5–6 units below the \( \text{pK}_a \) of the parent amine of the aminoxy, whereas the \( \text{pK}_a \) of the glycosyl imine \( (D) \) is only 2 units below that of aniline.\(^{\text{310}}\) Thus \( D \) is protonated under ligation conditions (pH 4–5), whereas \( J \) is almost completely deprotonated.\(^{\text{311}}\) Aniline catalysed ligation reactions are performed at pH 4–5, because this pH range also strikes a balance between the beneficial increase in protonated carbonyl and the protonation of the amine nucleophile counteracting this effect. This pH sensitivity narrows the field in which the aniline catalysis can be applied. A broader pH range for catalysis, especially pH range 7–8, would be desirable for applications like biocatalysis.

Over the years a new-generation of aniline-derived catalysts were developed and tested for the purpose of optimising the nucleophilic catalysis.\(^{\text{303,312-315}}\) Aniline derivatives with a high \( \text{pK}_a \) would provide higher concentrations of the protonated, reactive imine \( (D) \) at neutral pH and consequently be more effective catalysts. \( \text{ØSTERGAARD et al.} \) found para-phenylenediamine (PDA) to be a superior catalyst for aldose oxime formation at pH 7. They hypothesise that protonation of the distal amino group of the PDA-imine species leads to a destabilisation of the imine and enhances its leaving group properties, it would so perform two tasks as a strong nucleophile and as a good leaving group through proton exchange reaction.\(^{\text{312}}\) PDA has found application in protein labelling.\(^{\text{314,316}}\) PEGylation and immobilisation\(^{\text{317}}\) The downside of PDA is its oxidative instability causing its conversion to \( \rho \)-benzoquinones and its cytotoxicity.\(^{\text{318}}\) To date, different kinds of catalysts have been developed including for example bifunctional catalyst. Bifunctional in the sense of a catalyst containing two functional groups playing a direct role in catalysis (e.g. 4-methyl-2-phosphonoaniline, 4MPA)\(^{\text{313,319}}\); and in the sense of a catalyst acting as buffer and as catalyst simultaneously.\(^{\text{320}}\) Evidence was found that proton donating groups \( \text{ortho} \) to the nucleophilic amine group in aniline derivatives provide general acid/base catalysis which leads to the important reactive iminium intermediate.\(^{\text{319,321}}\)

All in all, the oxyamine ligation is a highly efficient, powerful and mild method for functionalising carbohydrates and glycans with tags or reactive groups for analysis and surface immobilization and can be deployed in order to answer biochemical and biomedical questions.
2.7. Carbohydrate Microarrays

The concept of “microarray technology” was developed in the early 1990s. Because of its high number of tests per time unit, its comparatively low required sample quantities and the good automation capacity it fast developed into a well-used tool in research. Since 2002 carbohydrate microarrays have become valuable tools to systematically investigate carbohydrate structures for particular function, like carbohydrate associated binding events.[322-325] Binding events with a vast array of biological targets including proteins, antibodies, enzymes, lectins, viruses and cells can be studied in a high-throughput manner. Additionally, interaction inhibition, binding preference, enzyme activity and structure-function relationships of carbohydrates for physiological as well as pathological processes are efficiently detected.

To generate carbohydrate microarrays, carbohydrates are mostly immobilised on a solid surface in a certain formation. (Scheme 2.14.)

Different materials have been used for carbohydrate microarrays: e.g. glass,[327-328], gold[329] or polystyrene.[330] Because glass 1) has low background fluorescence, 2) is chemical inert, 3) is resistant to high temperatures and 4) is cheap and easily available,[331] it is often the material of choice. The glass surface is mostly coated, e.g. with nitrocellulose[323], 3-D hydrogel-NHS esters[332] or hydrazides[333]. A fundamental aspect for successful glycan microarray application is accessibility to a large native glycan library or promising synthesis strategies to synthesis one; keeping in mind that the former method entails extraction and purification from biological sources and the latter laborious and time-consuming multi-step synthesis (chapter 2.5. Glycan Release and Analysis). Most carbohydrate structures need to be functionalised with a moiety in order to react with the solid support.[278] There are three ways to do so (Scheme 2.15.): A) by functionalising the core monosaccharide of the glycan during organic synthesis. B) by enzymatic synthesis and following reaction of a bifunctional linker to the glycan. C) by releasing glycans from a protein, followed by reaction with bifunctional linker. For details regarding
functionalisation of reducing carbohydrates, see chapter 2.6.: Functionalisation of Reducing Carbohydrates. REICHARDT further derivatised and altered carbohydrate libraries after linkage to the microarray by using glycosyltransferases.[334-336]

**Scheme 2.15.:** Functionalisation methods of carbohydrates for immobilisation on a surface: A) chemical synthesis of functionalised glycans, B) enzymatic synthesis of glycans followed by functionalisation or C) glycan release followed by functionalisation.[337] Figure adapted from PAULSON[338], Protein from JONES[339]. FG = functional group, X = NH\sub{2} and after hydrolysis OH.

Because of the vast amount of literature on carbohydrate microarrays, hereinafter only some commonly used immobilisation techniques, detection methods and applications for microarrays are described in detail.

### 2.7.1. Immobilisation

Immobilisation methods for carbohydrate microarray chips can be categorised by non-covalent (Figure 2.3.) and covalent interactions (Figure 2.4.) between the glycans and the array surface. These two techniques can be further split into site-specific attachment yielding well-ordered surface structures and site-nonspecific attachment resulting in random surface structures. Depending on the immobilisation method, sugar compounds can, but must not always be synthetically modified, making time-consuming and labour-intensive modification of sugars superfluous.

#### 2.7.1.1. Non-Covalent Immobilisation

Non-covalent, site-nonspecific immobilisation methods were amongst the first reported for glycan arrays.[323,325] Different immobilisation concepts have been utilised: WANG immobilised free polysaccharides on nitrocellulose membrane through adsorption.[323] HSIEH-WILSON exploited electrostatic interactions in the fabrication of polysaccharide arrays on poly-L-lysine coated slides.[340] MAYER used a DNA-directed immobilisation strategy, where carbohydrates...
are covalently attached to an oligonucleotide probe, while a complementary DNA sequence is immobilised on a solid surface.\textsuperscript{[340-341]} Regardless their site-nonspecific, random orientations on the array, the glycans can maintain their antigenicity when screened with potential interaction partners.\textsuperscript{[338]} Although it is also reported that binding of some potential interaction partners to non-specific attached carbohydrates is restricted due to steric hindrance for the interaction partner.\textsuperscript{[326]}

Non-covalent, site-specific immobilisation techniques were for example applied with neoglycolipids attached to polyvinylidene difluoride or nitrocellulose membranes via hydrophobic interactions,\textsuperscript{[324,342]} by using a fluorous-based method in which fluorous tag-glycan conjugates bind to fluoroalkylated surfaces via fluorous–fluorous interactions,\textsuperscript{[343-344]} or by utilising the strong biotin-streptavidin interaction, were biotin-conjugated glycans were immobilized on the streptavidin-coated surface.\textsuperscript{[252,345-347]}

The limitation of the non-covalent attachment strategy is that it only works best with a larger contact area between glycan/linker and the solid support. The reason is that this area must be large enough to provide sufficient adsorption to the surface. Smaller-sized glycans/linkers mostly attach too weakly and are easily lost during washing steps.\textsuperscript{[326]} The success of an individual method is of course mostly depended on the quantity of binding affinity provided by that method.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure23.png}
\caption{Strategies for non-covalent carbohydrate immobilisation. Attachment of A) polysaccharides through adsorption, B) polysaccharides by electrostatic interactions, C) tagged monosaccharides via fluorous–fluorous interactions, D) biotinylated glycans via biotin-streptavidin interaction, E) carbohydrates by DNA-complementary DNA interaction.\textsuperscript{[326,338]}}
\end{figure}
2.7.1.2. Covalent Immobilisation

Covalent, site-nonspecific carbohydrate immobilisation can be achieved by several means: Free carbohydrates are linked to a solid support derivatised by photolabile groups, e.g. aryltrifluoromethyldiazirine which reacts after light irradiation as a highly reactive carbene,[348] or phthalimide groups which under UV irradiation form triple state carbonyl oxygens that abstract hydrogen atoms from carbohydrates, followed by radical recombination.[349-350] Or immobilisation by complexation of boronic acids with 1,2- or 1,3-diols of sugars.[351] The potential adverse effect of site-nonspecific, random orientations of carbohydrates as described above is also problematic for covalent immobilisation.[326]

Covalent, site-specific immobilisation is the most extensively developed method for glycan microarrays preparation. It is suitable for creating microarrays containing carbohydrates from simple monosaccharides up to oligosaccharides. Glycans mostly need to be equipped with functional groups at the anomeric position which then react selectively with counter reactive groups on the derivatised surface, thereby irreversibly anchoring the glycan to the surface. Consequently, glycans as well as the solid support require chemical modifications. The length and type of bond between glycan and surface has shown to affect the binding affinity of interaction partners towards immobilised carbohydrates as well as the nonspecific adsorption of the interaction partners.[322,352] Varying lengths of linkers provide different accessibility for interaction partners to the attached carbohydrates. Glycans attached by hydrophilic linkers show better binding properties for proteins than those conjugated by hydrophobic linkers.[322,352]

A large share of covalent immobilisation methods for glycan arrays is thiol and amine chemistry. Already in 2002 Shin reported on the covalent immobilisation of maleimide-functionalised glycans to thiol-derivatised slides.[322,352] In 2003 the inverse arrangement of thiol functionalised saccharides on maleimide-modified slides was reported.[327,353] As thiol-functionalised substances readily undergo air oxidation this strategy requires careful handling. Amine chemistry for immobilisation is widely applied in the microarray technology. Some examples are amine/cyanuric chloride coupling,[354-355], amine/N-hydroxysuccinimide-ester coupling[10,356] or amine/epoxide coupling[357-359]. Interesting because of their known bioorthogonality, the inverse-electron-demand Diels-Alder (DAinv) reaction and the Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC) have also been applied for glycan immobilisation. The DAinv reaction was used to immobilise dienophile-functionalised saccharides, bearing terminal alkenes or norbornenes (not shown), onto tetrazine-modified glass slides,[360] while the CuAAC was utilised to either use azide functionalised carbohydrates on alkyne modified surfaces, or inverse with alkyne functionalised carbohydrates on azide modified surfaces.[209,361-363] An elegant option to covalently and site-specifically attach free and unmodified reducing carbohydrates on a surface is the use of the hydrazone and oxime formation applying hydrazide- and aminooxy-modified surfaces. A drawback for this method is
that the free carbohydrates require an incubation time of 12 h at 50 °C.\textsuperscript{[256-257]} The reverse combination has also been exploited.\textsuperscript{[364-365]} To keep in mind when using the oxyamine ligation is that this reaction mainly yields acyclic oxime structures and literature states that only the unmodified core-monosaccharide unit, thus the cyclic form, is recognised and bound by interaction partners (see chapter 2.6.5. Oxyamine Ligation).

Figure 2.4.: Strategies for covalent carbohydrate immobilization: Attachment of A) maleimide-functionalised carbohydrates to a thiol-modified surface, B) dienophile-functionalised carbohydrates to a tetrazine-modified surface, C) amine-functionalised carbohydrates to a NHS ester-modified surface, D) amine-functionalised carbohydrates and E) hydrazide-functionalised carbohydrates to an epoxide-modified surface, F) azide-functionalised carbohydrates to an alkyne-modified surface, G) unmodified glycans to an phthalimide-modified surface by UV irradiation.\textsuperscript{[326,338]}
2.7.2. Detection Methods

A variety of methods to detect binding events on glycan microarrays have been developed. The most common methods utilise fluorophores: by directly labelling glycans with a fluorophore or by using a fluorophore-labelled reagent that either directly binds to glycans or to a tag attached to the glycans (e.g. biotin-streptavidin interaction).[326] Opportune for utilising fluorophores is that microarray scanners with fluorescence detection are available in many laboratories. Limitations are that modifications to glycans such as biotinylation can reduce activity or alter binding selectivity,[366] that for newly discovered glycans fluorophore-labelled secondary reagents are not available and also that fluorophores are often light sensitive (bleaching over time) and prone to oxidative degradation.[326] Other methods to study binding events include: analysis by MS, where an enzymatic reactions on the slide surfaces that result in a change in mass are monitored,[367-368] surface plasmon resonance (SPR) imaging, which measures kinetics and thermodynamics of binding events,[369-370] or detection of radioactivity[371-372] or electrochemoluminescence[373].

2.7.3. Applications

The scope of application for glycan microarrays in biological and biomedical research is large. The range of possible interaction partners stretches from lectins, enzymes over pathogens to whole cells.[326] Glycan-binding proteins (GBPs) such as lectins are found in plants, most microbes and animals,[374] and have been extensively studied and characterised using the microarray technology.[10,352,375-376] Well-characterised plant lectins like wheat germ agglutinin (WGA) and concanavalin A (ConA) are also important tools to control and ensure integrity and quality of carbohydrate arrays.[377] The specific binding of mammalian lectins to glycans mediates a wide range of essential biological processes. Evaluating these lectins and identifying their binding partners using microarrays can thus be useful in the development of therapeutics. Examples are the human DC-SIGN and DC-SIGNR; C-type lectins present on the surface of macrophages and dendritic cells, able to increase viral infection by binding to glycans present on the surface of viruses and parasites.[326,346,378-379] A nice example to show the impact of carbohydrate microarray investigations on identifying new GBPs is work by Feizi on a protein called malectin.[380] It was only known, that this protein is expressed in many different tissues and present across many species,[381] which suggests an important biological role. Microarray analysis showed strong and selective binding to a di-glucosyl-N-glycan (Glc₂Man₇). This finding not only provides main evidence for glycan binding property of malectin, but also provides important information over its biological function. Because Glc₂Man₇ is an intermediate in the biosynthesis of N-glycans, this suggests a role for malectin in the production and quality control of glycoproteins in the ER. Along with lectins and antibodies glycan microarrays have been used to probe binding of viruses and whole cells.[382] Even though analysis can be more complex due to the presence of more than one GBP, they
are presented in a natural context with natural spacing and orientation of their binding sites, which is not always provided for individual lectins or antibodies. Glycan microarrays have been used to study binding properties of various influenza and parainfluenza strains. Microarrays have become a valuable tool to gain insights into the different interaction partners of glycans, the different types of interaction glycans mediate and the underlying biology thereof.
3. Aim of Project

3.1. Part I – Metabolic Glycoengineering using the Photoclick Reaction

Metabolic glycoengineering (MGE) is a powerful technique that allows scientists to modify and analyse the structure of glycans and gives insight into cellular processes. Non-natural analogues of N-acetylmannosamine (ManNAc) intercept the biosynthetic pathway for sialic acid (Sia) and subsequently become metabolically incorporated into sialoglycoconjugates instead of the natural ones. The hereby unnatural, functional groups present can then react with molecules bearing complementary reactive functional groups in bioorthogonal ligation reactions. One example is the light-induced 1,3-dipolar cycloaddition (aka photoclick reaction) of a tetrazole and an alkene, where a nitrile imine is generated in situ and reacts with an alkene forming a fluorescent pyrazoline.\[^{18,109,113}\] Wittmann and co-workers were the first to use the photoclick reaction in MGE. In a first step, they reacted a tetrazole-biotin derivative with metabolically incorporated unnatural sugars, subsequently they reacted the biotin moiety with a streptavidin-dye conjugate. They successfully applied the reaction, but observed background staining in the negative control of their biological assays.\[^{18}\] The first aim of this project was to synthesise a tetrazole fluorescent dye conjugate that should allow to label the incorporated unnatural carbohydrates in one step reducing undesired background staining (Figure 3.1.A). A linker for improved water solubility and a fluorescent, water soluble dye for detection should be included in the molecule. With the tetrazole-dye conjugate in hand, its fluorogenic properties and its reactivity with suitable alkenes should be investigated in NMR (nuclear magnetic resonance) studies and UV-VIS studies with respect to reaction speed and conjugate stability and reactivity. Further, the conjugate should be applied in MGE, ligation conditions should be optimised and the conjugate’s suitability for visualisation and analysis of carbohydrate labelling should be shown in cell assays using confocal fluorescence microscopy.

![Figure 3.1.: A) tetrazole-dye conjugate, comprising of a tetrazole, a water-soluble dye and linker. B) tetrazole for detailed investigation the spatial control of the photoclick reaction.](image)

The second aim of this project was the investigation of the valuable temporal and spatial control feature of the photoclick reaction, as the reaction can be started at a defined time and location by irradiation of the tetrazole to the corresponding highly reactive nitrile imine. How good or precise the spatial control over the photoclick reaction actually is, has not been investigated
yet. To investigate this, suitable unnatural monosaccharide derivates should be incorporated into the cells using MGE. Then a tetrazole[127], activatable by laser light (Figure 3.1.B) should be dispersed over these cells and turned into the nitrile imine by use of a laser installed on a confocal fluorescence microscope. This setting should allow real time observations of the formation and dispersion of fluorescent pyrazoline. The limits of the spatial control over the photoclick in biological assays can be explored in this manner.
3.2. Part II – Production and Application of a Glyco-Functionalised Extracellular Matrix

The treatment of particularly deep and large wounds for example after burns or chronic wounds remains a major challenge to this day. Desirable would be a biological material gained from endogenous cells that is well tolerated and promotes wound healing. The aim of this project is to develop such a novel material based on tissue-specific extracellular matrix (ECM) of patients’ own cells. In cooperation with Hochschule Reutlingen and Universität Stuttgart a so-called clickECM should be generated, optimised and analysed (Figure 3.2.). The clickECM should be equipped with additional functions and could so be used for numerous applications in various areas of medical technology, like immobilisation on artificial skin substitutes.

In this thesis, the focus ought to be on the treatment of deep wounds. Since the clickECM will be produced by patient's own subcutaneous cells in vitro, it should provide an optimal environment for cells, with a tissue-specific composition and excellent bioactivity. The projects first goal was the generation of a library of galactosamine and mannosamine derivatives

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**Figure 3.2.:**

A. Growth of ECM in suitable cells, followed by cell lysis.
B. Preparation of clickECM by incorporation of synthesised monosaccharides in harvested ECM.
C. Incorporation of synthesised linker-oligosaccharides adducts in clickECM.
D. Integration and subsequent gelation of polymerlinkers.
E. Evaluation of obtained material.
F. Intended application as a custom-fit wound implant for deep wounds.

Picture F taken from WITTMANN.\(^{387}\)
bearing an unnatural reporter group. The monosaccharides should be incorporated into the ECM using MGE, creating the clickECM. The ECM is harvested from human adipose-derived stromal/stem cells (ASCs). The reporter groups should than be reacted in bioorthogonal ligation reactions, for example the inverse-electron-demand Diels Alder (DAinv) reaction. Via such a reaction, cell adhesion-promoting agents like oligosaccharides or growth factors should be coupled to the clickECM. To check oligosaccharides for their potential activity in promoting cell proliferation and cell migration, microarrays should be prepared and the adhesion of ASCs shall be monitored on the carbohydrate microarrays using microscopy. For creation of the microarrays an oligosaccharides library shall be used. To link the detected bioactive oligosaccharides with the clickECM a trivalent linker should be synthesised. The linkers should comprise of two functional groups, one to react with the oligosaccharides and one for reaction with the reporter groups of the incorporated sugars in the clickECM and a fluorescent dye for detection. A divalent linker without the fluorescent dye shall be needed for the final application of the finished material. The final step of the project should be the introduction of photocross-linkable polymers into the ECM to create a material that can, after liquid application, be turned into a hydrogel. Hydrogels provide optimal filling of the wound bed and allow an almost free diffusion of oxygen, carbon dioxide, nutrients and proteins to preserve the viability and function of the encapsulated cells.
3.3. Part III – Investigation of the Oxyamine Ligation

Desirable attributes of ligation reactions used in assembly and modification of biomolecules is among others fast kinetics. Because biomolecules are often present at low concentrations, effective labelling depends highly on the reaction rate of the applied ligation reaction. To compensate for slow reaction kinetics high concentrations, large excesses of reactants, elevated temperatures or catalyst are used. Often applied in carbohydrate chemistry is the oxyamine ligation, a covalent coupling of reducing glycans with aminooxy nucleophiles to form carbohydrate oximes in equilibrium with N-glycosyloxamines. Under certain conditions reaction rates of the oxyamine reaction are slow. Rate enhancement of the oxyamine ligation is possible by aniline or general nucleophilic catalysis. The catalytic intermediate of the aniline catalysed oxyamine ligation reaction is a glycosyl imine (Scheme 3.1.B). Glycosyl imines are also obtained when N-glycans are enzymatically released from N-glycoproteins (Scheme 3.1.A). The gained imines are easily protonated to the iminium species which is very susceptible to an oxyamine attack and can be directly used as the catalytic intermediate without the need for a catalyst at the same pH.

The aim of this work was to systematically investigate the reaction process and the reaction kinetics of the oxyamine ligation reaction performed with glycosyl amines at different pH values, with different concentrations and different equivalents of reactants, using $^1$H-NMR spectroscopy. GlcNAcNH$_2$ should be used as a model compound. The acquired data set of this novel strategy to obtain oximes should then be compared to data acquired using the conventional oxyamine ligation reactions with reducing carbohydrates, in this case GlcNAc with and without different nucleophilic catalysts, conducted and monitored under the same settings and reaction conditions as for the glycosyl amines studies. In cooperation with PROF. DR. U. STEINER fitting functions should be approximated to the obtained data points and rate constants should be calculated and compared for every performed ligation reaction.

Scheme 3.1.: Formation of oximes, either by A) ligation of enzymatically released glycosyl amines with oxyamine or by B) aniline catalysed ligation of reducing carbohydrates with oxyamine.
Aim of Project
4. Results and Discussion

4.1. Part I – Metabolic Glycoengineering using the Photoclick Reaction

Bioorthogonal reagents and reactions transform our understanding of biomolecules and living systems and have revealed important new insights into biomolecule structure and function. Thus, continued development and discovery of bioorthogonal reactions promises to refine our view of biological systems and facilitate new insights. The light-induced 1,3-dipolar cycloaddition of a nitrile imine and an alkene (aka photoclick reaction) is a recent addition to the toolbox of bioorthogonal ligation reactions and has firstly been applied to MGE by the WITTMANN[18] group. They incorporated Ac4ManNAcryl into the glycocalyx of cells and reacted the acryl moiety with an in situ generated nitrile imine-PEG-biotin conjugate, obtained by irradiation at 302 nm from the corresponding tetrazole-PEG-biotin conjugate. In a second step they reacted the biotin moiety with a streptavidin-Alexa Fluor™ 555 (StrepAF555) conjugate. Unfortunately, the photoclick reaction resulted in some background staining in the negative controls of the cell assays. We hypothesised that a conjugate of tetrazole and dye, thus labelling in one step could result in less background staining and thus further improve this valuable method.

4.1.1. Synthesis of Tetrazole-Dye Derivatives

The tetrazole-dye conjugate consists of a tetrazole core, a linker enhancing water solubility and a dye for detection. For the tetrazole core the same two literature known tetrazoles 1[388] and 2[389] as used in the WITTMANN group before were chosen (Figure 4.1.).[18] Both derivatives are 2,5-diaryl-substituted with a methoxy group, which is in para-position on either of the aryl rings. The electron donating group increases the reaction rate and allows derivatisation of the tetrazoles.

![Figure 4.1.: Structures of tetrazoles 1 and 2.](image-url)

Besides their biological application with metabolically incorporated unnatural sugars on cells[18], these tetrazoles have been used to chemically functionalise proteins with incorporated homoallylglycine inside mammalian cells[390], they have been reacted with unnatural amino acids containing terminal alkenes in bacteria[122] and with cyclopropenes in mammalian cells[113]. They also found application in spatiotemporally controlled real-time fluorescence
imaging of mitochondria and lysosomes[539] and were used to generate crosslinked protein nanocarriers[532].

The synthesis of 1 was performed according to Ito et al. (Scheme 4.1.A).[538] Benzenesulfonyl chloride was reacted with 4-methoxybenzhydrazide 2 resulting in the hydrazine 3. The hydrazine was reacted with thionyl chloride to the corresponding chloride 4, which was reacted with phenylhydrazine to yield 1 in an overall yield of 23%. 2 was synthesised according to Ito et al. (Scheme 4.1.B).[539] Benzaldehyde 5 was reacted with benzenesulfonyl hydrazide resulting in a phenylsulfonylhydrazone 6. The hydrazone 6 was reacted with an in situ prepared arene diazonium salt followed by a cyclisation in pyridine yielding 2 in an overall yield of 59%. Both tetrazole derivatives (1 and 2) were then treated with BBr₃ to cleave the methyl group (7 and 8), after literature known procedure in quantitative yields.[539] Next, the alcohols were activated with para-nitrophenyl chloroformate yielding carbamates 9 and 10 according to Schart et al. in yields of 70% and 77% (Scheme 4.1.).[539] The activated tetrazole 9 was reacted with Boc-amino-PEG₃-amine 11 (Scheme 4.2.A). To enhance water solubility, I chose polyethylene glycol (PEG) with three repeats for a linker – the same length as already successfully applied in the photoclick reaction by the Wittmann group[18]. Purification via flash column chromatography yielded tetrazole-PEG-amino-Boc in yields of 74% for 12. The Boc protecting group was removed under acidic condition in quantitative yields and the tetrazole-PEG-amine 13 was reacted without further purification with sulfo-Cyanine 3 (sCy3) NHS-ester 14. Deciding for a suitable dye, I took into account the equipment at the microscopy facility and the absorption and emission spectra of the dye. A fundamental quality of the applied dye should be, that it is not affected in any way by the wavelength converting the tetrazole into the corresponding nitrile imine. E.g. the dye should neither lose its fluorogenic properties nor shift its absorption maximum upon irradiation. Thus, the dye’s absorption spectrum must not overlap with the tetrazoles absorption spectrum. I chose sCy3, as firstly it very likely meets these requirements (absorption maximum at 548 nm) and secondly is also well water soluble, due to the attached sulfo groups. After RP-HPLC purification the tetrazole-dye conjugate was obtained in 56% (15, Tet1-sCy3).

Tet2-sCy3 18 was synthesised following the same synthetic route (Scheme 4.2.B). The activated tetrazole 10 was reacted with Boc-amino-PEG₃-amine 11 yielding in tetrazole-PEG-amino-Boc 16 in 77%. The Boc protecting group was removed under acidic condition in quantitative yields and the tetrazole-PEG-amine 17 was reacted without further purification with sulfo-Cyanine 3 (sCy3) NHS-ester 14. After RP-HPLC purification the tetrazole-dye conjugate 18 was obtained in 62%.
**Scheme 4.1.:** A) Synthesis of activated Tetrazole 9. Benzenesulfonyl chloride was reacted with 2 to hydrazine 3. 3 was reacted with thionyl chloride to the corresponding chloride 4, which was reacted with phenylhydrazine yielding Tet1 (1). 1 was reacted with BBr₃ yielding 7, which was activated yielding 9. B) Synthesis of activated Tetrazole 10. Benzaldehyde 5 was reacted with benzenesulfonyl hydrazide to 6. 6 was reacted with an *in situ* prepared arene diazonium salt. After cyclisation Tet2 (2) was gained. 2 was as reacted with BBr₃ yielding 8, which was activated yielding 10.
Scheme 4.2.: A) Synthesis of tetrazole-PEG-sCy3 15. The activated tetrazole 9 was reacted with linker 11 yielding carbamate 12. 12 was Boc-deprotected yielding 13 and was reacted in an amidation with sCy3 NHS-ester 14 yielding 15. B) Synthesis of tetrazole-PEG-sCy3 18. The activated tetrazole 10 was reacted with linker 11 yielding carbamate 16. 16 was Boc-deprotected yielding 17 and was reacted in an amidation with sCy3 NHS-ester 14 yielding 18.
The sCy3 NHS-ester 14 was synthesised following literature known procedure\cite{393-394}, starting from the two indoline building blocks (Scheme 4.3.), kindly provided by O. BAUDENDISTEL. The carboxylic acid-derivatised indolenine 19 was added to diphenylformamidine and acetic anhydride, then the second indolenine 20 was added yielding sCy3 21 in 74 % yield. 22 was activated into a succinimidyl ester (14) with disuccinimidyl carbonate in 34% yield.

Scheme 4.3.: A) Synthesis of sCy3 NHS-ester 14. Indolenines 19 and 20 reacted with diphenylformamidine to sCy3 21. 21 was activated with disuccinimidyl carbonate yielding sCy3 NHS-ester 14.

With both tetrazole-dye conjugates Tet1-sCy3 and Tet2-sCy3 in hand, I started investigating the photoclick reaction using firstly Tet1-sCy3.

4.1.2. Analysis of the Optical Properties of Tet1-sCy3

In order to determine the optical parameters of Tet1-sCy3 and the corresponding pyrazoline derivative, a preparative scale photoclick reaction of Tet1-sCy3 and CHexNAcryl 22, a model compound with acryl moiety, was performed (Scheme 4.4.). The acryl moiety is known to be highly reactive in the photoclick reaction.\cite{18,395} The tetrazole-dye conjugate was dissolved in ethanol, mixed with 1.5 equivalents 22 and irradiated at 302 nm with a hand-held UV-lamp placed directly on top of the reaction vessel for 100 minutes. The product crystallised in ethanol and was filtered off. The pyrazoline-dye conjugate 23 was gained in 90% yield. The acryl model compound was synthesised according to literature from cyclohexylamine and acryloyl chloride.\cite{396}
Results and Discussion

Scheme 4.4.: A) Synthesis of pyrazoline-PEG-sulfoCy3 23.

The optical properties of the tetrazole-dye conjugate 15 were measured using a 10 μM solution in dimethyl sulfoxide (DMSO), for the pyrazoline-dye conjugate 23 a 15 μM solution in DMSO was used. The absorption spectra were measured from 200 to 800 nm (Figure 4.2.A.). Tet1-sCy3 shows an absorption maximum at 280 nm for the tetrazole part of the molecule and an absorption maximum at 550 nm for the sCy3 dye part. Tet1-sCy3 shows absorbance at 302 nm, thus 302 nm is a suitable wavelength for conversion of Tet1-sCy3 to the corresponding nitrile imine. The absorption spectrum of the pyrazoline-dye conjugate 23 shows an absorption maximum at 270 nm for the pyrazoline part and also the absorption maximum at 560 nm for the dye part of the molecule. The emission spectra were measured from 200 to 800 nm (Figure 4.2.B), the compounds were irradiated at 360 nm. For the Tet1-sCy3 no emission signal was found for the tetrazole part of the molecule, but an emission maximum at 585 nm for the dye part. The emission spectrum of the pyrazoline-dye conjugate 23 shows an emission maximum at 440 nm for the pyrazoline part and also the emission maximum at 585 nm for the dye.

Figure 4.2.: Measurements with a 10 μM solution of tetrazole-dye 15 and a 15 μM solution of pyrazoline-dye 23 in DMSO with solvent correction A) Absorption-spectra of tetrazole and pyrazoline derivatives. B) Fluorescence spectra of tetrazoles and pyrazolines; samples excited at 360 nm.
These measurements verify, that the dye is well suited to the application, as the zone of absorption of tetrazole and dye do not overlap. To further examine if the dye is affected by the irradiation at 302 nm and possibly shifts the absorption maxima or loses its fluorescence, the sCy3 NHS-ester was tested in a UV-VIS study (Figure 4.3.). The dye was dissolved in water to a 20 μM solution and a UV-VIS spectrum was recorded. The solution was then irradiated at 302 nm with a hand-held UV-lamp for 1 minute, the distance between UV-lamp and the UV-VIS cuvette being 1 cm. Subsequently the next UV-VIS spectrum was recorded. This was continued until a total irradiation time of 10 minutes was reached. The data shows that the dye is not affected by the irradiation, the absorbance stays at the same intensity and the absorbance maxima is not shifted.

At 280 nm the absorbance spectra of the sCy3 NHS-ester 14 (Figure 4.3.) and of Tet1-sCy3 (Figure 4.2.A) show a slight absorbance. The two measurements cannot be compared directly as they were performed in different solvents. Nonetheless, because of this observation it should be taken into account, that it is possible that sCy3 absorbs light at 302 nm, thus maybe the tetrazole part of the tetrazole-dye conjugate is not converted to the corresponding nitrile imine with the same speed as for example the tetrazole of the tetrazole-biotin conjugate (used in two step labelling) is. It is certain, because of the preformed preparative scale photoclick reaction of Tet1-sCy3 with 22, that definitely not all light is absorbed by the dye, and that the pyrazoline-dye conjugate 23 does form, however the question about the speed of transformation has to be kept in mind.

Figure 4.3.: Absorption-spectra of a 20 μM solutions of sCy3 NHS-ester 14 in water with solvent correction.

Knowing the fluorogenic properties of Tet1-sCy3 and 23 I started investigating the photoclick reaction with the new tetrazole-dye conjugate in biological assays.
4.1.3. Biological Investigations

HEK293T cells were seeded and allowed to attach overnight. The cells were incubated with either 100 µM Ac₄ManNAcryl 24, Ac₄ManNCp 25, Ac₄ManNCyoc 26 (Figure 4.4.) or DMSO in the negative control. These sugars were chosen, because it is known from literature that all three work well in MGE, and additionally that Ac₄ManNAcryl, Ac₄ManNCp work well in the photoclick reaction thus yield good cell membrane staining. In contrast, for Ac₄ManNCyoc no membrane staining is expected according to literature.¹⁸,¹⁰⁵ Thus Ac₄ManNCyoc works as a kind of additional negative control.

![Alkene modified sugar derivatives](image)

**Figure 4.4.**: Alkene modified sugar derivatives 24, 25 and 26 used in the biological assays to test Tet1-sCy3 with the photoclick reaction.

After 48 hours 100 µM Tet1-sCy3 was added and the cells were irradiated for 30 seconds with a hand-held UV-lamp (302 nm) placed directly on top of the *ibidi* imaging chamber. The cells were then washed and a fluorescence readout was performed using confocal fluorescence microscopy. Unfortunately, all cells including the ones incubated with Ac₄ManNCyoc as well as the negative control showed the same amount of fluorescent membrane staining (Figure 4.5.).

To further investigate these findings, the cell assays were repeated under varying reaction conditions with different combinations of tetrazole-dye conjugate Tet1-sCy3 concentration and irradiation times (Table 4.1.).

**Table 4.1.**: Investigation of reaction conditions for photoclick reaction with Tet1-sCy3 and metabolically incorporated alkene modified monoamine derivatives 24, 25, 26 in HEK293T cells.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Concentration Tet1-sCy3 [µM]</th>
<th>Irradiation time [sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>5</td>
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<tr>
<td>4</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
Results and Discussion

Figure 4.5.: MGE with Ac₄ManNAcryl 24, Ac₄ManNCp 25 and Ac₄ManNCyoc 26 labelled using the photoclick reaction. HEK293T cells were incubated with either 100 μM sugar 24, 25, 26 or DMSO as solvent control for 48 h. Living cells were reacted with Tet1-sCy3 (30 sec at 302 nm). Nuclei were stained with Hoechst33342. Scale bar 30 μm.

All experiments, except entry 6, showed identical results: the same amount of membrane staining for all 3 investigated sugar derivatives as well as for the negative control. For entry 6 the cells were not irradiated and no membrane staining was detected. This shows that the tetrazole-dye conjugate is not entangled in e.g. the glycocalyx of the cells but is completely washed away prior to confocal fluorescence microscopy.

In a next step, I performed the originally established method from the Wittmann group[18] (two step labelling) together with my new system, in order to be able to directly compare the two methods. I cultivated HEK293T cells and incubated them with 100 μM Ac₄ManNCp (24) or DMSO. After 48 hours 50 μM tetrazole-biotin or Tet1-sCy was added and the cells were irradiated for 30 seconds with a hand-held UV-lamp (302 nm) placed directly on top of the ibidi imaging chamber. Cells with tetrazole-biotin conjugate were subsequently treated with
StrepAF555. All cells were washed and a fluorescence readout was performed using confocal fluorescence microscopy. Analysis of the data for cells incubated with Ac₄ManNCp and treated with tetrazole-biotin/ StrepAF555 showed nice fluorescent membrane staining like published by the WITTMANN group[18] and low background staining (Figure 4.6.A). And like in my previous experiments all cells treated with Tet1-sCy3 showed the same amount of staining (Figure 4.6.B). Now, being able to directly compare the two methods it became evident that the supposedly high background staining of the reaction with Tet1-sCy3 is in fact less general staining of glycocalyxes in comparison to the positive staining of cells treated with Ac₄ManNCp and tetrazole-biotin/ StrepAF555 (Figure 4.6.).

![Figure 4.6: MGE with Ac₄ManNCp 24 labelled using the photoclick reaction. HEK293T cells were incubated with 100 μM sugar 24 or DMSO as solvent control for 48 h. Living cells were reacted with A) tetrazole-biotin followed by StrepAF555 and B) Tet1-sCy3. Irradiation for 30 sec at 302 nm, nuclei were stained with Hoechst33342. Scale bar 30 μm.](image-url)
Results and Discussion

Possible explanations for these findings could be:

- Nearly all 302 nm light is absorbed by the dye and only minimal light is left to convert the tetrazole to the corresponding nitrile imine.
- Nitrile imine is generated and reacts with the dye instead of the alkene in a sort of cyclisation reaction, thus no binding to glycocalyx and the conjugate is washed off.
- Sterical reasons.

For further insight, I analysed and investigated Tet1-sCy3 and the photoclick reaction in NMR studies.

4.1.4. NMR studies

The NMR studies were conducted as follows: for each experiment a tetrazole derivative and if needed a cyclopropene model compound (CHexNCp\textsuperscript{1} \cite{397}, 27) were dissolved in DMSO-d\textsubscript{6} and/or ethanol-d\textsubscript{6}. All compounds were mixed in a Suprasil\textsuperscript{®} quartz NMR tube and a \textsuperscript{1}H-NMR spectrum was recorded. The NMR tube was then irradiated at 302 nm with a hand-held UV-lamp for 5 minutes, the distance between UV-lamp and NMR tube being 1 cm. Subsequently the next \textsuperscript{1}H-NMR spectrum was recorded. This procedure was followed until an irradiation time of 30 minutes was reached, after that the irradiation time between each NMR measurement was extended to 10 minutes until a total irradiation time of 100 minutes was reached. For analysis a distinctive signal of the investigated compounds was integrated. for CHexNCp the resonance of the vinyl protons at 7.38 ppm, for tetrazole Tet1 the resonance of the aromatic protons at 8.66 ppm, for Tet1-sCy3 the resonance of the tetrazole aromatic protons at 8.17 ppm and the resonance of the sCy3 protons at 8.33 ppm (CH group), 7.79 ppm (aromatic protons) and 4.08 ppm (CH\textsubscript{2} group) (Figure 4.7.). The solvent peak was used as an internal standard for calibration of the distinctive signal intensities. For this calibration the integrals of all measured distinctive peaks in one experiment were divided by the integral of the solvent peak and the signal was analysed and compared to the other experiments. CHexNCp was chosen because it is known from previous studies that this cyclopropene derivative works well in the photoclick reactions and also facilitates the analysis of the recorded NMR spectra compared to its sugar analogue.\textsuperscript{18,397}

\textsuperscript{1} Compound synthesised in my master thesis, University of Konstanz, 2017.
Figure 4.7.: Investigated protons of the used compounds are shown in red. CHexNCp: vinyl protons at 7.38 ppm. Tet1 the aromatic protons at 8.66 ppm. Tet1-sCy3 the tetrazole aromatic protons at 8.17 ppm, the sCy3 protons at 8.33 ppm (CH group), 7.79 ppm (aromatic protons) and 4.08 ppm (CH2 group).

First, only Tet1-sCy3 was investigated in order to examine the sensitivity of the new tetrazole-dye conjugate to light irradiation at 302 nm (Figure 4.8, blue curves). This measurement was then compared to a kinetic study with Tet12 [997] (Figure 4.8, pink curve). It is evident from the integral decrease that Tet1-sCy3 vanishes and presumably converts into the nitrile imine in the same manner as Tet1, Tet1 being marginal faster than Tet1-sCy3. Thus the 302 nm light is not absorbed by the dye in any interfering way but reaches the tetrazole and initialises its conversion to the nitrile imine. Also, this measurement suggests, that the dye is not affected by the irradiation, evident by the lack of integral reduction.

Figure 4.8.: Integral quantity analysis of Tet1-sCy3 in comparison with Tet1. Integrals were calibrated to an internal reference. Total irradiation time of 100 min, irradiated at 302 nm with a hand held UV-lamp.

2 NMR study performed in my master thesis, University of Konstanz, 2017.
Next, 1 equiv. Tet1-sCy3 was reacted with 1.5 equiv. CHexNCp to investigate the reactivity of the new tetrazole-dye conjugate with a reaction partner known to be highly reactive and unaffected by the irradiation (Figure 4.9., pink curves). This reaction was compared to the reaction of 1 equiv. Tet1 with 1.5 equiv. CHexNCp (Figure 4.9., blue curves)\(^3\). Upon irradiation the integrals for both tetrazoles reduce in the already observed manner. The integral for CHexNCp in reaction with the nitrile imine of Tet1 is reduced to 0.21, not to zero as it is used in excess. Because a 1.5 equiv. excess is used, a reduction to 0.33 is to be expected, the inaccuracy in integral reduction is likely due to the precipitating product inside the NMR tube, changing the width of the peaks, making integration more difficult. The integral for CHexNCp in reaction with the nitrile imine originating from Tet1-sCy3 is only reduced to 0.61. These results affirm the ones from the biological investigation, the new tetrazole-dye conjugate does not react as effective with the provided alkene as Tet1 does.

![Figure 4.9.](image-url) Integral quantity analysis of 1.0 equiv. Tet1-sCy3 in reaction with 1.6 equiv. CHexNCp, in comparison with the reaction of 1.0 equiv. Tet1 with 1.6 equiv. CHexNCp. Integrals were calibrated to an internal reference. Total irradiation time of 100 min, irradiated at 302 nm with a hand-held UV-lamp.

To get another indication if the nitrile imine does react with the dye in a kind of cyclisation reaction instead of with the provided alkene, an NMR study with 2,3 equiv. Tet1 reacting with 1,6 equiv. CHexNCp in presence of 1,0 equiv. sCy3 NHS-ester was performed (Figure 4.10.). In this setting the dye is no longer attached to the tetrazole, thus a cyclisation reaction is far less likely. The data shows that the integral of the tetrazole and the cyclopropene derivatives decrease in the already observed manner, whereas the integral for the dye stays

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\(^3\) NMR study performed in my master thesis, University of Konstanz, 2017.
again unchanged. Thus, also this measurement suggests that the nitrile imine does not interact with the dye but reacts with the provided alkene.

![Graph](image)

**Figure 4.10.** Integral quantity analysis of 2.3 equiv. Tet1 in reaction with 1.6 equiv. CHexNCp 27, in presence of 1.0 equiv. sCy3 NHS-ester 14. Integrals were calibrated to an internal reference. Total irradiation time of 100 min, irradiated at 302 nm with a hand-held UV-lamp.

Summarising, the NMR suggest show that the new Tet1-sCy3 conjugate is converted to the corresponding nitrile imine upon irradiation, that the nitrile imine assumedly does not react with the dye and, together with the UV-VIS study, that the dye is not affected by irradiation at 302 nm. The lower reactivity of the Tet1-sCy3, apparent in both the NMR studies and the biological assays could be due to sterical reasons. Despite the fact that the PEG linker is flexible and the utilised length has been successfully used in MGE, it might be that the linker is too short to bridge the distance between the bulky dye and the reporter group on the glycans of the glyocalyx. Thus, the highly reactive nitrile imine might be too shielded and has, before it reaches the alkene, already found a different reaction partner.

The new Tet1-sCy3 conjugate was synthesised to reduce background staining in cell assays. But as it firstly does not show any improvement in that respect and secondly does react to a lesser extent with the provided alkene, the investigation was stopped at this point.
4.1.5. Investigation of the Temporal and Spatial Control of the Photoclick Reaction

An interesting and valuable feature of the photoclick reaction is the possibility of temporal and spatial control over the reaction. Even though the photoclick reaction is often advertised with the spatiotemporal control, this feature and its limits have never been investigated closely. The exact time point when the reaction starts, is generally when all reactants are present. For the photoclick reaction this is when the tetrazole is converted to the corresponding nitrile imine under UV irradiation, a time point which is very easily selectable and controllable simply by turning on the light source. Also, as soon as the light is turned off the reaction stops and only already formed nitrile imine can react further. Spatial control over reactions is rare. A reaction is mostly only confined by the container it is performed in, being it a cell culture dish for biological assays or a flask for synthetic chemistry. Because the reaction only takes place in the exact area of irradiation the spatial control over the photoclick reaction is reliant on the kind of light source used. In case of a hand-held UV lamp a big area is irradiated, however when a laser beam is used, only a very small area is irradiated. Meaning, applications where a single cell or even small compartment of an individual cell need to be highlighted become available. Because the nitrile imine is highly reactive, the zone in which the reaction will take place is likely to be similar to the irradiation area. This area maybe only slightly be expanded by the diffusion rate of the nitrile imine, until it finds a suitable reaction partner.

I wanted to investigate the possibility of spatiotemporal control of the photoclick reaction using MGE. Because Ac₄ManNAcryl 24 is a known excellent reaction partner for the photoclick reaction[18], I chose to use this carbohydrate for metabolic incorporation. For the tetrazole derivative I chose to a literature known laser-activatable bithiophene-substituted tetrazole 28 with extended π-systems, which turns at 405 nm irradiation into the corresponding nitrile imine and reacts in the 1,3 dipolar cycloaddition into a red-emitting pyrazoline 29[127] (Scheme 4.5.).

Scheme 4.5.: Workflow to investigate the spatiotemporal control of the photoclick reaction by using MGE. Ac₄ManNAcryl 24 is metabolically incorporated in HEK293T cells. The acryl moiety reacts in the photoclick reaction with the nitrile imine, generated upon irradiation from tetrazole Tet-bithio to the corresponding, fluorescent pyrazoline 29.
4.1.5.1. Synthesis of Ac₄ManNAcryl and Bithiophene Tetrazole

Ac₄ManNAcryl 24 and Tet-bithio 28 were synthesised under my supervision by L. ARNOLD and during his bachelor thesis by N. MAURER[398]. For the synthesis of the acrylamide derivative 24, the amine of mannosamine hydrochloride was Boc-protected[399] and the alcohols were peracetylated, the protected sugar was gained in 28% over two steps. The Boc protecting group was removed under acidic conditions and the sugar was gained as amine TFA salt. The amine was neutralised and reacted with acryloyl chloride yielding Ac₄ManNAcryl 25 in 15% over two steps.[18] Tetrazole Tet-bithio was synthesised according to literature known procedures (Scheme 4.6.). 2,2'-bithiophene 30 was reacted with n-butyllithium and trimethylborate to 2,2'-bithiophene-5-boronic acid 31 in 71% yield.[400] Iodobenzene diacetate 32 was tosylated to hydroxy(phenyl)iodo tosylate 33 in 74% yield.[401] The tosylate 33 and boronic acid 31 were reacted yielding phenyl(bithiophen-2-yl)iodonium salt 35 in 95% yield.[128] Cinnaminitrile 35 was reacted with sodium azide and trimethylamine to yield (E)-5-styryl-2H-tetrazole 36 in 96%.[127] Tetrazole 29 was obtained in a Cu¹¹-catalysed cross-coupling of the styryl-tetrazole 36 and the iodonium salt 34 in 14% yield.[127]

Scheme 4.6.: Synthesis route for Tet-bithio 29, starting from cinnaminitrile 35, iodobenzene diacetate 32 and 2,2'-bithiophene 30.

With all needed derivatives in hand, we could start to investigate the spatiotemporal control of the photoclick reaction in biological assays.
4.1.5.2. Biological Investigations

HEK293T cells were seeded and allowed to attach overnight. The cells were incubated with 100 μM Ac₄ManNACryl (24) or dimethyl sulfoxide (DMSO) in the negative control, after 48 hours 100 μM Tet-bithio was added. Under the confocal fluorescence microscopy small areas (8–30 μm) of the ibidis imaging chamber were irradiated with a laser beam for 4 sec at 405 nm, indicated by white circles (Figure 4.11. and 4.12.) and images were taken immediately after. In these early investigations no washing steps were performed after irradiation.

In a first approach, we irradiated cells all metabolically functionalised with the acryl moiety. One batch of cells was treated with Tet-bithio, the other one not. The obtained images for cells treated with Ac₄ManNACryl and Tet-bithio show fluorescent staining in the irradiated area, cells only treated with Ac₄ManNACryl show no fluorescent staining in the irradiated area (Figure 4.11.).

![Image](image_url)

**Figure 4.11.**: Investigation of the spatiotemporal control over the photoclick reaction, comparison before and after irradiation of Tet-bithio for 4 sec. at 405 nm. HEK293T cells were incubated with 100 μM Ac₄ManNACryl 24 for 48 h. Living cells were reacted with A) no tetrazole derivative and B) Tet-bithio. Scale bar 30 μm, irradiated area indicated by white circle.

In a second approach, we irradiated cells metabolically functionalised with the acryl moiety and cells without that functionality. All cells were treated with Tet-bithio. The obtained images for cells treated with Ac₄ManNACryl and Tet-bithio show again fluorescent staining in the irradiated area; cells only treated with Tet-bithio show marginal fluorescent staining in the irradiated area (Figure 4.12.).
Results and Discussion

Figure 4.12.: Investigation of the spatiotemporal control over the photoclick reaction, comparison before and after irradiation of Tet-bithio for 4 sec. at 405 nm. Living HEK293T cells were treated with A) no sugar derivative, B) 100 μM Ac₄ManNAcryl 24 for 48 h. Scale bar 30 μm, irradiated area indicated by white circle.

The data shows, that the short irradiation period of the cells (4 sec.) is sufficient to turn enough Tet-bithio into the highly reactive nitrile imine to observe fluorescent staining. The time span between irradiation and scanning of the cells for fluorescence imaging is also only a few seconds, these few seconds are also already enough for the photoclick reaction to take place, thus for pyrazolines to form. This shows the high reactivity of the nitrile imine and underpins the assumption that the reactions zone is only limited by the diffusion rate of the nitrile imine.

The obtained images also show that not only the cell membrane is stained, but the whole irradiated area of the cells. This indicates that tetrazole Tet-bithio or its resulting nitrile imine might be able to penetrate the cell. The WITTMANN group solely obtained cell membrane staining with Ac₄ManNAcryl and the photoclick reaction.[18] It could be that their used tetrazole-biotin conjugate and its corresponding nitrile imine cannot cross the cell membrane. Or it could be that their tetrazole-biotin and/or nitrile imine derivative does in fact penetrate the cell, but as the used streptavidin-dye conjugate cannot enter the cell, they could not detect any internal staining. Literature states, that because of the high reactivity of the nitrile imine several side reactions can occur.[116,402-405] Thus the nitrile imine reacts partly with other molecules in the culture medium or the aqueous buffer in the irradiation zone above the cells. Because imaging is done seconds after irradiation these fluorescent by-products are still present in the irradiation zone. A washing step after irradiation could be useful to remove these unbound by-products. This will likely also reduce the background staining obtained for the unmodified cells treated with Tet-bithio (Figure 4.12.B).
To this end, at only the beginning of the investigation of the temporal and spatial control over the photoclick reaction, we could successfully demonstrate in biological assays the high and exact control that can be gained over the reaction.
4.2. Part II – Generation and Application of a Glyco-Functionalised Extracellular Matrix

The natural environment of cells is the extracellular matrix (ECM), cell-specific matrices can be obtained from in vitro culture of the respective cells. ECMs are investigated and already used as biomaterials in many different applications, e.g. as cell-influencing coatings.[174,406-407]

Many of these applications require the modification of the ECM with individually addressable functional groups. Metabolic glycoengineering (MGE) is a valuable tool to incorporate chemically modified monosaccharides into natural intra- and extracellular oligosaccharide structures of the cell while the ECMs integrity is preserved. These functionalities can subsequently be addressed by bioorthogonal chemical ligation reactions, for visualising and tuning the chemical and physical properties of metabolically modified glycoconjugates. Building on earlier work[21] and in cooperation with Hochschule Reutlingen and Universität Stuttgart, we aimed to generate, optimise, and analyse a so called clickECM. A material consisting of ECM equipped with additional properties to enhance cell proliferation and migration, for application in wound care.

Some data that is presented in the following chapter 4.2. is also part of a publication “An Advanced ‘clickECM’ that Can be Modified by the Inverse-Electron Demand Diels-Alder Reaction” submitted to ChemBioChem, some of the figures and schemes are transferred or modified from this publication.

4.2.1. Synthesis of a Carbohydrate Library

To be able to apply the well-established bioorthogonal DAinv ligation reaction[19-20,136] in a dienophile-functionlised ECM, I synthesised a library of novel (Ac₄GalNPtl, Ac₄GalNBeoc, Ac₄GalNPeoc, Ac₄GalNHeoc) and literature known unnatural N-acetylgalactosamine (GalNAc) and N-acetylmannosamine (ManNAc) derivatives with carbamate- and amide-linked terminal alkenes and amide-linked cyclopropanes for incorporation into the ECM using MGE. The series of GalNAc derivatives shown in Chart 4.1.A was synthesised starting from galactosamine hydrochloride. The series of ManNAc derivatives shown in Chart 4.1.B was synthesised starting from mannosamine hydrochloride.
Results and Discussion

Chart 4.1.: Library of alkene-modified A) galactosamine and B) mannosamine derivatives for incorporation into the ECM using MGE. Btl = but-3-enoyl, Ptl = pent-4-enoyl, Hxl = hex-5-enoyl, Beoc = butenloyloxycarbonyl, Peoc = pentylenloyxycarbonyl, Heoc = hexenloyxycarbonyl.

All sugars, except Ac₄ManNAcryl 24 and Ac₄GalNAcryl 37, were obtained by reacting the respective hexosamine hydrochloride with the corresponding alkenyl succinimidyl carbonate, followed by acetylation in good yields (Scheme 4.7). 24 and 37 were obtained by literature known procedure[18] as described in chapter 4.1.5.1. Synthesis of Ac₄ManNAcryl and Bithiophene Tetrazole).

Scheme 4.7.: Synthesis route for alkene-modified monosaccharides, exemplary for galactosamine derivatives. The hexosamine hydrochloride was reacted with the corresponding alkenyl succinimidylic carbonate, followed by acetylation with acetic anhydride. R= Btl, Ptl, Hxl, Beoc, Peoc and Heoc moieties.
4.2.2. Evaluation of the Biocompatibility of the Dienophile-Functionalised Carbohydrates and Generation of Functionalised ECM (clickECM)

The dienophile functionalised carbohydrates were then tested at Hochschule Reutlingen by S. NELLINGER on their biocompatibility and suitability for MGE. For this, they were applied to stem cells derived from adipose tissue (adipose-derived stromal/stem cells, ASCs). The ASCs are isolated from human tissue samples obtained from patients undergoing plastic surgery. Because ASCs are permanently present and can be obtained by minimally invasive procedures, they are very suitable for the intended application. Firstly the cytotoxic properties of the carbohydrates were examined using established viability assays. Lactate dehydrogenase (LDH) release (cell death) and resazurin turnover (metabolic activity) were measured. Both assays showed no cytotoxic properties for all examined monosaccharides, which is a prerequisite for their use to modify the ECM. Interestingly, ASCs treated with the cyclopropene modified monosaccharides (Ac₄GalNCp) exhibited a significant lower amount of released LDH compared to the other derivatives, meaning this carbohydrate seems to be the most suitable candidate and is therefore used in further investigations. The underlying cause for this finding however is not known.

Next, the dienophile-functionalised carbohydrates should be metabolically incorporated into the ECM (Figure 4.13.A). For this, ASCs were seeded and kept in cell culture for 4 days. Sodium ascorbate was added periodically to enhance synthesis and secretion of matrix molecules. After 4 days the cells were treated with 100 μM of a synthesised monosaccharide (Ac₄GalNCp). After 72 h incubation the cells were lysed, the dienophile-functionalised ECM was isolated and washed. After isolation the ECM was concentrated and homogenised. For detection of the alkene groups homogenised ECM was incubated with 50 μM biotinylated tetrazine for 1 h at rt followed by StrepAF555 for 20 minutes at rt. Then, a fluorescence readout was performed using confocal fluorescence microscopy. Unfortunately, ECM with alkene modification and ECM without alkene modification showed the same amount of matrix staining, no visible difference between the two (Figure 4.13.B and 4.13.C).
Results and Discussion

Figure 4.13: A) Workflow for MGE with ASCs and dienophile modified monosaccharide, followed by cell lysis and detection using tetrazine-biotin and StrepAF555. Fluorescence readout of B) ECM functionalised with Ac₄GalNCp 44 and labelled using the DAinv reaction. C) negative control, ECM without Cp modification, DAinv reaction performed. The ASCs were incubated with 100 μM sugar 44 or DMSO as solvent control for 72 h. ClickECM was reacted with 50 μM biotinylated tetrazine followed by StrepAF555. Scale bar 30 μm.
To further investigate these findings, the cell assays were repeated and varying conditions for the ligation reaction were screened:

- We chose a different streptavidin-dye adduct for the labelling procedure. The biotinylated tetrazine was reacted with StrepCy3, but also with this dye we observed matrix staining in the negative control.

- We tried a tetrazine-TAMRA (carboxytetramethylrhodamine) adduct, to obtain labelling in one step, but again observed matrix staining in the negative control.

- In order to determine if the dyes might get entangled in the ECM and thereby cause the background staining, we solely reacted the ECM with StrepAF555. With this setting we did not observe matrix staining, thus the dyes do not get entangled.

- We also tried to saturate the ECM with tetrazine-PEG-OH, followed by treatment with tetrazine-PEG-biotin/StrepAF555, tetrazine-PEG-biotin/StrepCy3 and only StrepAF555. In both set-ups with StrepAF555 we observed matrix staining in the negative control, in the set-up with Cy3 we did not observe matrix staining in the negative control.

All in all, these findings do not fit together, no clear statement can be made about why and when labelling occurs and about why it occurs so inconsistently. Also, the alkene modified monosaccharides have never before been used to functionalise ECM, so it has never been proven that the ECM is in fact alkene-functionalised. Thus, matrix staining of ASCs treated with dienophile modified monosaccharide could consequently also be a wrong positiv signal, and in reality no alkene modifications are found in the ECM. The utilised labelling system is clearly unsuited for reliably detecting the differenciation between alkenes modified ECM and unmodified ECM. A more reliable detection system is absolutely necessary.

Such a system was found by using streptavidin linked to horse radish peroxidase (HRP) and subsequent treatment with TMB (3,3′,5,5′-tetramethylbenzidine) (Figure 4.14.A), a known and reliable detection system for ELISA (enzyme-linked immunosorbent assay). For this detection method the clickECM was treated with 50 µM biotinylated tetrazine for 1 h at rt. The samples were washed and incubated with 6.6 µg/mL StrepHRP. TMB was added to the samples and after colour change the reaction was stopped using acidic conditions, this again changes the solutions colour from blue to yellow. The absorbance of the supernatant was then measured using a plate reader at 450 nm. Values of the unmodified ECM (coECM) were set as 100 % and values of the modified ECMS were normalised to the coECM. As Figure 4.14.B shows, not only the cyclopropene modified monosaccharide but also several other terminal alkene modified derivatives were tested with the new assay. These results show, that firstly we now operate with a reliable and robust detection system for the clickECM and secondly that the dienophile modified monosaccharides have definitely been metabolically incorporated into the
Results and Discussion

ECM. Moreover, the obtained data shows that the TMB turnover for the cyclopropene modified ECM is significantly higher compared to the other dienophiles. In Figure 4.14.C a comparison between clickECMs modified with Ac₄GalNCp or Ac₄ManNCp, harvested from three different donors (see Figure 4.14.C, numbers 59/76/78) is shown. It is again evident that the ECM is significantly and consistently modified with cyclopropene moieties. The data also shows, it does not matter whether the ASCs are treated with mannosamine or galactosamine derivatives, a much stronger impact has the donor from which the ASCs are obtained from. That might be due to irregularities in the ECM. Firstly because the clickECM is not gained from cell lines but from primary ASCs and secondly because the ASCs derive from different donors and every person has an individual composition of ECM\(^{408}\), thus there ought to be differences visible. Nonetheless the clickECM from every donor shows significant modification with the cyclopropene moiety. To be in accordance with former work\(^{21}\), all following experiments with modified clickECM, including the preparation of ECM-gum hybrid hydrogels were conducted with galactosamine derivatives.

![Diagram A)
](image1)

**Figure 4.14.:** A) Workflow of a new method for detection of the dienophile functional groups incorporated into the clickECM using firstly tetrazine-biotin, followed by HRP and its reaction with TMB. B) Comparison of relative absorbance of alkene modified clickECM and unmodified ECM.
Figure 4.14.: C) Comparison of relative absorbance of Ac$_4$GalNCp 44 and Ac$_4$ManNCp 25 and unmodified ECM from three different doners (59/76/78).

4.2.3. Synthesis of a Trifunctional Linker for Functionalisation of the clickECM

Oligosaccharides are involved in many processes of cell adhesion and recognition. In order to promote these processes in the later application of the clickECM in wound care, the clickECM should be functionalised with suitable oligosaccharides. The workflow to covalently link oligosaccharides to the clickECM is depicted in Figure 4.15.A. For this a linker had to be synthesised, as the linker has to meet certain requirements it needs the following features (Figure 4.15.B): A primary oxime moiety, for functionalising reducing carbohydrates. The oxime ligation reaction is chosen because it produces stable glycoconjugates under aqueous conditions and in high yields,\cite{255} and is also well established within this research group and optimal reaction conditions for functionalisation are known.\cite{289,332} The oxime is attached to the linker backbone via a tetra(ethylene glycol) (TEG) spacer that increases its water solubility. Also connected to the backbone via a TEG spacer is a tetrazine derivative. It reacts in the DAinv reaction with the alkenes metabolically incorporated into the ECM and so covalently links matrix and oligosaccharides. Lastly, a fluorescent dye is attached for detection of already minimal amounts of glycoconjugates linked to the clickECM. I chose the sCy3 dye because it water soluble and stable.\cite{409} The backbone is lysine (lys), through three stable amide bonds all functionality are connected here. For later application in patients’ wounds, the dye is of course no longer needed and nor wanted and will therefore not included in the linker 51 anymore.
Figure 4.15.: A) Workflow for immobilising oligosaccharides on the dienophile modified clickECM using a synthetised linker 51. B) Structure of the trifunctional linker for functionalisation of reducing carbohydrates consisting of a primary oxyamine for glycoconjugation, two TEG spacers to improve water solubility, a tetrazine for reaction with the alkene modified clickECM, a fluorescent dye for detection, and a lysine backbone to connect all functional groups via stable amide bonds. $R = \text{H}$ or glycanchain, $R' = \text{H}$ or pyrimidinyl.
I synthesised two linker derivatives 67 and 68 incorporating a different tetrazine each (Scheme 4.8.). The synthesis of the monoaryl tetrazine derivative 55 was performed according to JIMÉNEZ-MORENO et al.[410] 4-Cyanobenzoic acid 52 was reacted with hydrazine hydrate and formamidine acetate 53 yielding 54, after oxidation with sodium nitrite 55 was gained in 18% yield. The biaryl tetrazine derivative 57 was in stock in the research group and was only purified by flash column chromatography. Both tetrazine derivatives were reacted in a first pyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphat) peptide coupling with an Boc-amino-PEG3-amine linker. Purification via flash column chromatography yielded tetrazine-PEG-amino-Boc 57/58 in yields of 40% (monoaryl, 57) and 83% (biaryl, 58). The Boc protecting groups were removed under acidic condition, and the tetrazine-PEG-amines 59 and 60 were reacted without further purification with an Boc protected lys-sCy3 conjugate 62 in a second pyBOP peptide coupling. The tetrazine-PEG-lys-sCy3 derivatives 63 and 64 were obtained after HPLC purification in yields of 60% (monoaryl, 63) and 69% (biaryl, 64) over two steps. The Boc protecting groups were removed under acidic condition and the tetrazine-PEG-lys-sCy3 derivatives 65 and 66 were reacted with an Boc-aminoxy-PEG3-COOH linker in a third pyBOP peptide coupling, yielding tetrazine-PEG-lys-sCy3-PEG-aminoxy-Boc 67 and 68 in yields of 60% (monoaryl, 67) and 60% (biaryl, 68) over two steps. The boc protected lys-sCy3 adduct 62 was obtained by reacting boc-protected lysine 67 with the sCy3 NHS-Ester 14 in 67% yield. The sCy3 NHS-ester was synthesised following literature known procedure, as described in chapter 4.1.1.: Synthesis of Tetrazole-Dye Derivatives.

The Boc protecting group was not yet removed from the final compounds 67 and 68. First suitable, cell adhesion promoting oligosaccharides had to be identified.
Scheme 4.8.: Synthesis of trivalent linker 67 and 68, with aminooxy, tetrazine and sCy3 functionality for ligating, immobilising and detecting oligosaccharides on an alkene modified clickECM. R = H or pyrimidinyl.
4.2.4. Investigation of Cell Adhesion on Carbohydrate Microarrays

The influence of various oligosaccharides on cell adhesion was evaluated by screening covalently and site-specifically immobilised oligosaccharides on activated glass slides using the microarray technology. In order to immobilise sugars and enable screening they need to be functionalised with a reactive group. Such an functionalised oligosaccharide library was kindely provided by O. BAUDENDISTEL.\textsuperscript{[332]} He synthesised a trivalent linker \textit{69} (Figure 4.16.B) consisting of a sCy3 dye for detection, an oxyamine functional group for reaction with the reducing oligosaccharides and an amine functionality for reaction with the coated glass slides (Figure 4.16.A). This linker was reacted in an oxime coupling with 25 reducing carbohydrates, ranging from mono- to decasaccharides. The obtained library includes carbohydrate structures with diverse sizes and stereochemistry, some derivatives containing hydrolysis prone fucose- and sialic acids (Figure 4.16.C-I). For easier comprehension the oligosaccharides are depicted in their carbohydrate symbol representations, modified by the CFG\textsuperscript{[46]} and the editors of “Essentials of Glycobiology”\textsuperscript{[24]}.

4.2.4.1. Assembly and Screening of Carbohydrate Microarrays

The glycoside library was designed for immobilisation on amine reactive surfaces. I used Nexterion\textsuperscript{®} H slides provided from Schott. These slides are coated with a thin film 3D hydrophilic polymer activated with \textit{N}-Hydroxysuccinimide (NHS) esters to provide covalent immobilisation of amine groups by forming amide bonds (Scheme 4.9.). The polymer-based layer is very resistant to non-specific binding and allows molecules to be immobilised in a quasi-liquid environment whilst maintaining their native structure.\textsuperscript{[411]} The general workflow for the generation of carbohydrate microarrays and their biological application comprises four steps (Scheme 4.9.).

1) Non-contact printing or spotting of the substrate, i.e., the glycoconjugate library, onto glass slides.
2) Incubation of the slides for efficient immobilisation/ formation of the amide bond.
3) Washing to remove unbound substrate and deactivation of remaining NHS-esters on the glass surface.
4) Incubation of the immobilised glycoconjugates, in this application with ASCs, to detect possible cell adhesion.
Figure 4.16: Preparation of oligosaccharide library by functionalisation of 25 reducing carbohydrates with trivalent oxyamine linker 69, produced and kindly provided by O. BAUDENDISTEL. A) Reaction scheme of oxyamine ligation of reducing carbohydrates. B) Molecular structure of trivalent linker 69. The established library is categorised in C) di-, D) tri-, E) tetra-, F) penta-, G) hexa-, H) octa-, I) decasaccharide glycoconjugates.
Results and Discussion

Scheme 4.9.: General workflow for carbohydrate microarray assembly: The functionalised glycoconjugates are spotted on the activated glass surface by a non-contact microarray printer. There, the terminal primary amine of the linker reacts with the NHS-ester modified surface, forming stable amide bonds. The immobilised structures are then incubated with ASCs to monitor possible cell adhesion by fluorescence and light microscopy.

For non-contact printing the lyophilised glycoconjugates were dissolved in plotting buffer to a starting concentration of 2 mM. These solutions were then diluted with 2 mM ethanolamine in plotting buffer, resulting in glycoconjugate concentrations of 2 mM (undiluted), 1 mM and 0.5 mM. Then, the samples were plotted with a non-contact printer applying 15 droplets (à 4 nL) of glycoconjugate solution per spot to the surface, creating spots with a diameter of approximately 1 mm. In total three replicates of the three molar dilutions were plotted to provide one block of 3x3 for each sample (Figure 4.17.A). As a positive control, commercially available peptide sequence cRGDfK (cycl(Arg-Gly-Asp-D-Phe-Lys and the glycoprotein fibronectin, which contains the RGD motif, were also immobilised. Both were spotted in 2 mM concentration, cRGD in a block of 3x2, fibronectin in two blocks of 3x3. The RGD binding sequence was chosen because it is a component of the ECM that specifically promotes cellular adhesion.412] All blocks were printed in rows numbered from 1–3 and columns termed with letters (A-J), so forming a grid on the glass surface to determine the exact position of every individual glycoconjugates (Figure 4.17.). The blocks were distributed randomly on the array to avoid that glycoconjugates of similar size or carbohydrate composition are next to each other on the array. Also the three different concentrations of linker-glyco-conjugate were spotted in different orders. For example Figure 4.17.A, panel 1A, concentration order from left to right: 2 mM, 1 mM, 0.5 mM. In contrast panel 1H, concentration order from left to right: 1 mM, 0.5 mM, 2 mM. The integrity of the microarrays, i.e. successful immobilisation of the glycoconjugates on the slides was checked by a fluorescence readout of the sCy3 dye of the trivalent linker (Figure 4.17.A). This allowed to detect defect slides before they are used in
subsequent time-consuming cell experiments. In this manner I could show proper immobilisation of all glycoconjugates and well defined and separted spots. In row 1 column F and row 2 column F some spots are smeared, but nonetheless are for these two glycoconjugates also correct spots present, thus cell adhesion can still be investigated for all 25 glycoconjugates.

The following cell experiments were performed at Hochschule Reutlingen by S. NELLINGER. The functionalised slides were incubated with ASCs for 3 and 5 h in cell media with and without fetal bovine serum (FBS) as a universal growth supplement of cell culture media. The slides were then washed and possibly adhered cells were fixed with paraformaldehyde. The microarrays were then mounted with mounting medium before scanning the slides using a light microscope. As a result, the ASCs showed only specific adhesion on spots with immobilised cRGDFK (Figure 4.17.B+C). All the other samples, including fibronectin did not lead to adhesion of ASCs.

The data shows that only cRGDFK enhances cell adhesion, the investigated oligosaccharides seem not to do so, or only in such a small extent that is not detectable on microarrays. As a future alternative to the oligosaccharides a growth factor, like the basic fibroblat growth factor (bFGF), could be incorporated into the clickECM and covalently linked to the modified matrix via the DAinv reaction. Growth factors are signalling molecules that are principle mediators in tissue regeneration.
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Figure 4.17.: Carbohydrate microarray spotted with 25 glycoconjugates, fibronectin (P1) and cRGDIK (P2). 

A) Fluorescence readout of immobilised sCy3-containing glycoconjugates (row 1 A-H, row 2 A-I, row 3 B-I), ethanolamine as negative control (2J), cRGDIK as positive control (3A) and fibronectin (1J) at 532 nm excitation and detection above 575 nm. 

B) Fluorescence readout after incubation with ASCs. Only on the positive control (cRGDIK) ASCs adhered. Slides were mounted with FluoMount, nucleus stained with DAPI (4',6-Diamidin-2-phenylindol). 

C) Magnification of A3, cRGDIK spots with adhered ASCs using fluorescence and light microscopy, scale bar 200 μm.
4.2.5. Properties of the clickECM

Lastly dienophile and azide modified clickECMs were investigated in view of the influence the incorporated functional groups have on the ECM as a biomaterial and whether the functional groups have an impact on cellular behaviour. Latter has to be examined because a complete reaction of all introduced functional groups in the ECM in the bioorthogonal ligation reaction cannot be ensured. These investigation were performed at Hochschule Reutlingen and Universität Stuttgart.

First a hydrogel material with poly(ethylene glycol) diacrylate (PEG-DA) framework was prepared. In this method a metabolically incorporated acryl functional groups (see chapter 4.2.1. Synthesis of a Carbohydrate Library), could be used for interlinkage. Investigations of this material showed firstly, that the UV irradiation for generation of the PEG-DA framework causes substantial damage to the DNA of the cells and secondly, that the achievable interlinkage of the hydrogel material is already efficient without the additional crosslinking acryl groups in the ECM. Thus an alternative material which does not need UV irradiation for generation of the framework was needed. Henceforth gellan gum-hybrid hydrogels were produced and the ECM was incorporated in these hydrogels. Gellan gum is a water-soluble polysaccharide produced by bacteria and used in many tissue engineering approaches. It is known that gellan gum does not influence ASCs behaviour,[413-414] meaning that possibly observed changes are directly linkable to the ECM. The hybrid hydrogels were prepared of 1% gellan gum and 0.25%wt homogenised ECM, either control ECM without modification (coECM), ECM with azide modification (AzECM) or ECM with cyclopropene modification (CpECM) were used. In accordance with former work[21] only the galactosamine derivatives (thus Ac₄GalNAz and Ac₄GalNCp 44) were used for the preparation of the alkene-modified ECM. As a negative control, 1% gellan gum hydrogels without ECM supplementation were prepared (w/o ECM).

It is well known that stiffness has a high impact on cellular behaviour.[415-416] To exclude inconsistent stiffness as a cause for different cellular behaviour, rheological measurements were performed with the ECM-gellan gum-hybrid hydrogels (coECM, AzECM, CpECM) and the negative control (w/o ECM). For this the liquid hydrogels were covered in phosphate-buffered saline (PBS) with magnesium and calcium (PBS+) to induce cross-linking and were left for swelling for 72 h at rt. Evaluation of stiffness (\(G'\), \(G''\), Figure 4.18) of all different hydrogels showed no differences. The amount of ECM used in this study is very likely too low to have an impact on hydrogel stiffness.
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Figure 4.18.: Biological application of the modified ECM in 3D hydrogels, w/o ECM (with no ECM), coECM (ECM without modification), AzECM (azide modified) and CpECM (cyclopropene modified). ECM-gellan gum-hybrid hydrogels with 1 % gellan gum, 0.25%wt homogenised ECM. Rheological measurements, evaluation of stiffness of the material.

For determination of the cellular response to the modified ECM within the hydrogels, ASCs were encapsulated in the hydrogels (Figure 4.19.). Therefore, hydrogels with 1 % gellan gum, 0.25 %wt ECM and 300.000 ASCs per 100 µL hydrogel were prepared. The liquid hydrogel solution was again covered with PBS+ to induce cross-linking. After 30 min incubation at 37 °C, PBS+ was changed to culture medium. Hematoxylin and eosin (HE) staining was performed and ensure a homogenous distribution of ECM within the hydrogels (Figure 4.19.A–first row).

A homogenous distribution of ECM is important because it directly impacts the number of possible interactions between cells and ECM and thus indirectly influences the magnitude of influence the ECM has on cellular survival, proliferation and differentiation. Because the amount of ECM within the hydrogels is relatively low the distribution of the ECM particels is of even greater importants for the cell experiments. Figure 4.19.A, HE staining shows a homogenous distribution for coECM, AzECM and CpECM. On day 3 of cell culture, live-dead staining was performed and analysed for cellular survival and proliferation of the encapsulated ASCs. Figure 4.19.A shows live/dead staining of the encapsulated ASCs, viable cells were stained with FDA (fluorescein diacetate (in green)) and dead cells were stained with PI ((propidium iodide) in red). The cellular survival and relative proliferation were quantitatived by image analysis software on basis of the images of live-dead staining. For cellular survival the number of viable and dead cells were determined and shown as percentage. It was observed that ASCs in hydrogels with ECM exhibited a higher survival rate compared to the control without ECM. Within the samples containing ECM no significant differences could be observed between the different modifications or unmodified ECM (Figure 4.19.B). Proliferation was investigated by comparing the percentage of total cell number on day three relative to day zero. Figure 4.19.C shows again that there are no significant differences between the hydrogels without modification (coECM) and with modifications (AzECM and CpECM), thus the functionalisations have no negative effect on cellular survival and proliferation and are therefore suitable for functionalising the ECM and the intended application. Also on day 3 after
hydrogel preparation the metabolic activity of the encapsulated ASCs was investigated using a resazurin assay. Figure 4.19.D shows the semi-quantitative determination of metabolic activity, no differences between the hydrogels with ECM and the control without ECM were detected. Results were calculated based on the number of viable cells per hydrogel and values were normalized to the control hydrogel without ECM.

All in all these results show, that the incorporated functional groups in the ECM have no negative impact on encapsulated ASCs and do not affect their cellular behaviour. Thus the obtained cell-derived ECM material can now further be equipped with additional functions with bioactive molecules like growth-factors or the material could be further cross-linked by using corresponding linker molecules. The great advantage over the previously reported functionalisation of a clickECM by CuAAC\(^{[21]}\) is the independence of any catalyst which might exhibit cytotoxic effects or has an negative impact on the ECM's cell production.

**Figure 4.19.:** Biological application of the modified ECM in 3D hydrogels, w/o ECM (with no ECM), coECM (ECM without modification), AzECM (azide modified) and CpECM (cyclopropene modified). ECM-gellan gum-hybrid hydrogels with 1 % gellan gum, 0.25%wt homogenised ECM, and encapsulated ASCs (300.000 cells/ 100µL hydrogel) A) Determination of the cellular response, hematoxylin and eosin (HE) staining to ensure homogenous distribution, live-dead (PI) staining and FDA (Fluorescein diacetate) staining, nuleus staining with Hoechst 33342. Scale bar: 200µm.
Results and Discussion

Figure 4.19.: Biological application of the modified ECM in 3D hydrogels, w/o ECM (with no ECM), coECM (ECM without modification), AzECM (azide modified) and CpECM (cyclopropene modified). ECM-gellan gum-hybrid hydrogels with 1% gellan gum, 0.25%wt homogenised ECM, and encapsulated ASCs (300,000 cells/ 100µL hydrogel) B) Investigation of cellular survival, C) proliferation and D) metabolic activity of the encapsulated ASCs.
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4.3. Part III – Investigation of the Oxyamine Ligation

Carbohydrates are the most abundant and divers biomolecules on earth and are heavily involved in post-translational protein modification. For the analysis of carbohydrate mixtures and carbohydrate interactions, a reaction which allows modification of glycans with high chemoselectivity and in high yields is needed. Often applied to this end is the oxyamine ligation, a covalent coupling of reducing glycans 70 with aminooxy nucleophiles to form acyclic carbohydrate oximes 71 (\((E)\)- and \((Z)\)-configuration) in equilibrium with cyclic N-glycosyloxyamines 72 (\(\alpha\)- and/or \(\beta\)-configuration) (Figure 4.30.). In some cases for instance with certain carbohydrates \([289]\) (GlcNAc) and higher pH (\(\geq 7\)) reaction rates are slow. Rate enhancement of the oxime formation is possible by nucleophilic catalysis or aniline (and derivatives thereof) catalysis, reported 2006 by Dawson.\([311]\] This finding was then applied to carbohydrates in 2010 by Jensen.\([303]\] The central catalytic intermediate of the aniline catalysed oxyamine ligation reaction is the glycosyl imine 74. The catalytic strength lies in the fact that this phenyl imine 74 is more easily protonated than the parent carbonyl compound, which facilitates the attack of the oxyamine. If N-glycans are enzymatically released from N-glycoproteins glycosyl amines are obtained (Scheme 4.10.). The imine tautomer 73 of the released glycosyl amine is even easier protonated to the iminium species than the phenyl imine 74, therefore is very susceptible to an oxyamine attack and a much more efficient intermediate for the oxime formation, maybe also at higher/neutral pH. The easier protonation of 73, caused by a \(pK_a\) decrease, can be estimated by comparing the \(pK_a\) decrease caused by the phenyl group between glucosyl amine (5.6) and glucosyl aniline (1.5). According to that, we expect the \(pK_a\) of 73 to be several pH units higher than that of 74. Thus glycosyl amines are very well suited and can be directly be used as the catalytic intermediate of the oxyamine ligation reaction without the need for a catalyst at the same pH.
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Scheme 4.10.: Interrelationship of reaction pathways of the uncatalysed, the aniline catalysed and the glycosyl amine oxyamine ligation reactions and the enzymatic cleavage of N-glycans from N-glycopeptides. Asp= asparagine.

Building on work from O. BAUDENDISTEL,[332] I investigated the oxyamine ligation reaction using glycosyl amine GlcNAcNH$_2$ 75. This compound was chosen as a model compound, because it matches the terminal carbohydrate of released N-glycans and is easily available in contrast to the scarcely available N-glycans. Because GlcNAcNH$_2$ is prone to hydrolysis,[417] GlcNACN$_3$ 76 serves as a shelf stable precursor (kindly provided by M. SCHÖWE), the amine is obtained by hydrogenation of the azide (Scheme 4.11.).[418]

Scheme 4.11.: Reduction of GlcNAcN$_3$ 76 with hydrogen and Pd/C in MeOH yielding GlcNAcNH$_2$ 75.
4.3.1. Hydrolytic Stability of Glycosyl Amine GlcNAcNH₂

When investigating N-glycans released from glycoproteins, they are commonly used after complete hydrolysis from GlcNAcNH₂ 75 to GlcNAc 70. When using GlcNAcNH₂ in the oxyamine ligation, hydrolysis of GlcNAcNH₂ is a not negligible side reaction. Thus I first investigated the hydrolytic stability of GlcNAcNH₂ in an ¹H NMR study under aqueous conditions in buffered systems at different pH values with deuterated ammonium acetate buffer (ND₄OC(O)CD₃) for pH 5–7 and deuterated phosphate buffer (KD₂PO₄) for pH 8 to a final concentration of 5–10 mM GlcNAcNH₂. First investigations on the hydrolysis of GlcNAcNH₂ were also performed by O. BAUENDISTEL. The ammonium buffer was chosen because ammonia is released during the reaction, thus by using this buffer system the equilibrium of the reaction should be shifted towards the starting materials and the hydrolysis rate should decrease. I tested this theory by comparing deuterated ammonium acetate buffer with sodium acetate buffer at pH 5 and 6. For pH 5 the hydrolysis rate was the same with both buffer systems, most likely because hydrolysis is so fast, the effect the ammonium has is neglectable. At pH 6 an effect is visible, hydrolysis proceeded faster in sodium acetate buffer than in ammonium acetate buffer. Based on these results all oxyamine ligation reactions were conducted in ammonium acetate buffered systems (see appendix Figure 9.1. and 9.2.).

GlcNAcNH₂ was freshly prepared for each experiment and its integrity checked by NMR. The reaction was monitored via ¹H NMR spectroscopy, immediately after dissolution of the carbohydrate in buffer. Due to the release of NH₃ the stability of the buffered system, thus steadiness of pH was ensured by checking the pH after the experiments. An external TSP (trimethylsilylpropanoic acid) standard allowed calibration of the obtained integrals, thus calculation of the concentration of glycosyl amine present in solution. The percentage of remaining GlcNAcNH₂ was plotted over time. A logarithmic x-axis was chosen for a better resolution of the high reaction speed at the beginning of the reaction (Figure 4.20.).

As expected the hydrolysis rate of GlcNAcNH₂ is fastest at pH 5 and becomes increasingly slower with increasing pH, since hydrolysis proceeds via the generation of an iminium ion and release of ammonia which are both acid catalysed. To precisely compare the hydrolysis at different pH, mathematical functions were fitted to the obtained data and calculations were performed. This was done by U. STEINER, he determined hydrolysis rates (kₕ) for every hydrolysis at pH 5–8, also t₀.₁ values were determined as the time when 10 % of GlcNAcNH₂ is still intact. (Table 4.2.).
Table 4.2.: Calculated hydrolysis rates and $t_{0.1}$ values of GlcNAcNH$_2$ in buffered system at different pH. $k_h$ = hydrolysis rate. *The exponential decay constants given refer to the final 60-70% of decay. For details see appendix Part III, Kinetics of Hydrolysis.

<table>
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<tr>
<td>4</td>
<td>8</td>
<td>0.050*</td>
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Figure 4.20.: Hydrolytic stability of GlcNAcNH$_2$ 75 in aqueous buffer at pH 5–8 over time. Glycosyl amine 75 was dissolved in either 500 mM ND$_4$OC(O)CD$_3$ (pH 5–7) or 500 mM KD$_2$PO$_4$ (pH 8) and the starting material was monitored via $^1$H NMR spectroscopy. The hydrolysis rate decreases with increasing pH.

Summarising, I showed the different hydrolysis progressions of GlcNAcNH$_2$ from pH 5 to 8, hydrolysis proceeding fastest at pH 5 due to the hydrolytic stability of GlcNAcNH$_2$ increasing with increasing pH.
4.3.2. Oxime Formation with Glycosyl Amine GlcNAcNH$_2$

Having gained knowledge over the hydrolytic stability of GlcNAcNH$_2$ I next examined the oxime formation of GlcNAcNH$_2$ 75 reacting with ethoxyamine using $^1$H NMR spectroscopy. I chose $^1$H NMR spectroscopy as analytical method, because it allows quantification of all three forming products during ligation in real time and regardless of their stability during workup. The yields of the individual ligation products ((E)- and (Z)-oximes 71, N-glycoside 72, Figure 4.12.) were added up to the combined yields (CY) in dependence on the reaction time (for an example see appendix, Figure 9.3. and 9.4.). As some resonances in the recorded NMR spectra are in close proximity to the huge water signal, I performed multipoint baseline correction for every single recorded NMR spectra in order to obtain nicely baseline separated peaks. U. STEINER again performed all calculations and fitted mathematical functions to the obtained data. The kinetic data and fits follow from a simplified kinetic model, with three processes described by pseudo first order kinetics: $k_1$, $k_1$ for the reversible reaction from amine 75 to product 71/72, $k_h$, $k_h$ for the reversible hydrolysis of amine 75, $k_2$, $k_2$ for the reversible product formation 75 from GlcNAc 70, formed by hydrolysis or used as a starting material, respectively (see appendix for more detailed information).

![Scheme 4.12.](image)

Scheme 4.12.: Reaction scheme for the oxyamine ligation reaction of GlcNAcNH$_2$ with ethoxyamine to E/Z oxime and β-N-pyranoside in buffered systems, at 39°C and pH 5–8.

I started the investigation of the oxyamine ligation reaction with 36 mM GlcNAcNH$_2$ and 180 mM (5 equiv) ethoxyamine in the pH range from 5–8, in buffered systems (for pH 5–7 ND$_4$OC(O)CD$_3$, for pH 8 KD$_2$PO$_4$) at 39°C, to match the data obtained for the hydrolytic stability of GlcNAcNH$_2$. After monitoring the reaction via $^1$H NMR spectroscopy, the combined yields (CY) were plotted over reaction time (Figure 4.21.). For comparison, I also performed the ligation reaction with 36 mM GlcNAc and 180 mM (5 equiv) ethoxyamine with and without aniline-$d_7$ catalysis (100 mM) with same set-up and under the condition as for the oxyamine ligation with GlcNAcNH$_2$.

As Figure 4.21.A shows, at pH 5 the ligation reaction is so fast, that even at the first data point (taken after 6 min) CY was close to the combined equilibrium yield (CEY) and thereafter increased only slightly further. In comparison, the reaction of GlcNAc resulted in a CY of only 24% after 1 h and the aniline-catalysed reaction with GlcNAc slightly faster under these conditions with a CY of 33% after 1 h. The rate constant $k_1$ of the reaction of GlcNAcNH$_2$ is
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25 h\(^{-1}\) which is 80 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k_2 = 0.30\ h^{-1}\)) and 69 times higher than of the aniline-catalysed GlcNAc ligation (\(k_2 = 0.35\ h^{-1}\)). At pH 6 (Figure 4.21.B), the reaction of GlcNAcNH\(_2\) reached a plateau of approx. 84% CY in less than 1 h, whereas CY of the reaction of GlcNAc was only 4.5% and that of the aniline-catalysed reaction of GlcNAc was 7% after 1 h. The rate constant \(k_1\) of the reaction of GlcNAcNH\(_2\) is 8 h\(^{-1}\) which is 160 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k_2 = 0.050\ h^{-1}\)) and 100 times higher than the rate constant of the aniline-catalysed GlcNAc ligation (\(k_2 = 0.080\ h^{-1}\)). At neutral pH (Figure 4.21.C), the reaction of GlcNAcNH\(_2\) reached a plateau after approx. 2 h of 72% CY (64% CY after 1 h). The combined yields of the reaction of GlcNAc after 1 h were 0.5% (uncatalysed) and 2% (aniline-catalysed). The rate constant of the reaction of GlcNAcNH\(_2\) (\(k_1 = 1.5\ h^{-1}\)) is 150 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k_2 = 0.010\ h^{-1}\)) and 54 times higher than of the aniline-catalysed GlcNAc ligation (\(k_2 = 0.030\ h^{-1}\)). Even at pH 8 (Figure 4.21.D), the ligation reaction is significant for GlcNAcNH\(_2\) (24% CY after 1 h), although the plateau is reached after 60 h with a CY of 70%. Both, the uncatalysed (0.4% CY after 1 h) and the aniline-catalysed reaction (0.5% CY after 1 h) are too slow to be useful. The rate constant \(k_1\) of the reaction of GlcNAcNH\(_2\) is 0.37 h\(^{-1}\) which is 70 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k_2 = 0.005\ h^{-1}\)) and 58 times higher than of the aniline-catalysed GlcNAc ligation (\(k_2 = 0.006\ h^{-1}\)).

These experiments clearly demonstrated the superiority of glycosyl amine ligation over aniline-catalysed GlcNAc ligation, and although hydrolysis and ligation are competing reactions, high ligation yields are obtained with GlcNAcNH\(_2\) after short reaction times.
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A – pH 5

- GlcNAcNH₂
- GlcNAc (aniline cat.)
- GlcNAc (uncat.)

B – pH 6

- GlcNAcNH₂
- GlcNAc (aniline cat.)
- GlcNAc (uncat.)
Figure 4.21.: Reaction of 36 mM 70 or 75 with 5 equiv. ethoxyamine, 100 mM catalyst at pH 5–8, 39°C. The CY for the reaction products (71 and 72) were obtained from $^1$H spectra and plotted over reaction time. Curve fitting was performed follow from a simplified kinetic model, with three processes described by pseudo first order kinetics (for more details see appendix).
When cleaving glycans of proteins only smallest amounts of glycans are obtained. Thus, ligation reactions in biological applications are commonly carried out in low concentrations. To match these conditions better, I next determined reaction kinetics at concentrations of 5 mM GlcNAcNH₂ and 50 mM (10 equiv.) ethoxyamine at pH 5 to 8 (Figure 4.35.), the data was obtained under the exact same conditions as described above for the higher concentration’s measurements. For further comparison, I also performed the ligation reaction with 5 mM GlcNAc and 50 mM (10 equiv) ethoxyamine with and without aniline-d₇ and para-phenylenediamine-d₄ (PDA) as catalysts (100 mM).

Figure 4.22.A shows, that at pH 5 the ligation reaction is still very fast and reached a plateau at about 70% CY after only a few minutes. In comparison, the reaction of GlcNAc resulted in a CY of only 5% after 1 h, the aniline-catalysed reaction in a CY of 21% and the PDA-catalysed reaction in a CY of 25% after 1 h. The rate constant $k_1$ of the reaction of GlcNAcNH₂ is 8 h⁻¹ which is 157 times higher than the rate constant of the uncatalysed reaction of GlcNAc ($k_2 = 0.051$ h⁻¹) and 32 times higher than the rate constant of the aniline-catalysed GlcNAc ligation ($k_2 = 0.25$ h⁻¹) and 33 times higher than the rate constant of the PDA-catalysed GlcNAc ligation ($k_2 = 0.30$ h⁻¹) In this kinetic measurement of GlcNAcNH₂ a kind of wave movement is visible (between 0.5–20 h). A first plateau is reached after about half an hour, the CY then slightly decreases and starts the ascent to the final plateau yield which is reached after about 5 h. Formally, the kinetic curve, when drawn over a log(t) scale, as is the case in Figures 4.21. and 4.22., exhibits three inflection points. Based on a system of linear differential equations for describing the kinetics, such a behaviour can only be expected if at least four species are involved. If that is the case, the fourth species probably originates from the product side – to explain the first “overshooting” of the curve (Figure 4.22.A, from 0.5–3 hours), before the product concentration rises again to what will eventually be the final combined equilibrium yield (CEY). We hypothesise that this species $X$ is a consecutive or side-product to the main oxime ligation products (71, 72), perhaps an aminal 77 formed upon addition of a further ethoxyamine molecule (Scheme 4.13.). The generated products react in part further to species $X$, an equilibrium is generated between the two. Later, the hydrolysed GlcNAc also turns into product, which generates the increase to the second stage, whilst the three products (71, 72) still remain in equilibrium with species $X$. The kinetics has been treated based on a reaction scheme entailing such a side reaction pathway (see appendix for more detailed information). Thereby, a smooth simulation of the observed kinetics is possible. Up to this point, species $X$ has not been conclusively identified and remains unrecognised by ¹H-NMR as a distinctive signal, likely because is the concentration of $X$ is too low to be dected within reasonable measuring time and/or the distinctive signal is hidden under der broad solvent peak.
Species **X** might be something like:

![Diagram of species X]

**Scheme 4.13.** Proposed species X. Arising from a second nucleophilic attack of the ethoxyamine on the oximes, in equilibrium with the other products 71, 72.

At pH 6 (Figure 4.22.B), the ligation reaction with GlcNAcNH₂ reached a plateau of 65% CY after 1 h. Whereas the reaction of GlcNAc has a CY of 0.3% at the same time point, the aniline-catalysed reaction of GlcNAc a CY of 4% and the PDA-catalysed reaction of 18% after 1 h. The rate constant \(k₁\) of the reaction of GlcNAcNH₂ is 5.0 h\(^{-1}\) which is 417 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k₂ = 0.012\) h\(^{-1}\)), 125 times higher than the rate constant of the aniline-catalysed GlcNAc ligation (\(k₂ = 0.040\) h\(^{-1}\)) and still 25 times higher than the rate constant of the PDA-catalysed GlcNAc ligation (\(k₂ = 0.200\) h\(^{-1}\)). The observation of this set of kinetic measurements was continued until all four reactions reached the equilibrium (in total ~500 h). With these long measurements I was able to check the general validity of my measurement and analysis methods. As all four different approaches have the same amount of carbohydrate (5 mM GlcNAcNH₂ or GlcNAc), all four have to lead to the same amount of product in equilibrium, thus to the same combined equilibrium yield (CEY). As visible in Figure 4.22.B this is the case, all four different approaches lead to a CEY of about 90%. The PDA-catalysed reaction reached the CEY after 10 h, the aniline-catalysed reaction and the uncatalysed reaction of GlcNAc, as well as the reaction with GlcNAcNH₂ reach the CEY after ~200 h. At neutral pH (Figure 4.22.C), the reaction of GlcNAcNH₂ reached a CY of 36% after 1 h and a plateau of 65% after 6 h. The combined yields of the reaction of GlcNAc after 1 h were 0% (uncatalysed) and 5% (aniline-catalysed), 9% (PDA-catalysed). The rate constant \(k₁\) of the reaction of GlcNAcNH₂ (\(k₁ = 0.55\) h\(^{-1}\)) is even 275 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\(k₂ = 0.002\) h\(^{-1}\)), 55 times higher than the rate constant of the aniline-catalysed GlcNAc ligation (\(k₂ = 0.010\) h\(^{-1}\)) and 6 times higher than the rate constant of the PDA-catalysed GlcNAc ligation (\(k₂ = 0.090\) h\(^{-1}\)). Even at pH 8 (Figure 4.22.D), the ligation...
reaction is significant for GlcNAcNH₂, after 50 h a CY of 50 % is reached, whereas both the uncatalysed and the aniline-catalysed reaction still have a CY of 0 %. The PDA-catalysed ligation reaction has also reached a CY of 50 % after 50 h. The rate constant $k_1$ of the reaction of GlcNAcNH₂ is $0.060 \, \text{h}^{-1}$ which is 86 times higher than the rate constant of the uncatalysed GlcNAc ligation ($k_2 = 0.0007 \, \text{h}^{-1}$) and 4 times higher than the rate constant of the PDA-catalysed ligation ($k_2 = 0.014 \, \text{h}^{-1}$). The rate constant for the aniline-catalysed ligation was too slow to be determined. Also the measurements at lower concentrations show the superiority of glycosyl amine ligation over aniline-catalysed GlcNAc ligation. The optimum of this advantage of the glycosyl amine ligation is found at a pH of 6 with not much loss until pH 7, which indicates that the distinct optimum should be between these two pH values. Under the chosen conditions and concentrations, PDA catalysis of the ligation with GlcNAc has shown to be superior. The products are yielded faster and in higher yield than the glycosyl amine ligation.
Results and Discussion

A – pH 5

B – pH 6
Figure 4.22.: Reaction of 5 mM 70 or 75 with 10 equiv. ethoxyamine, 100 mM catalyst at pH 5–8, 39°C. The CY for the reaction products (71 and 72) were obtained from $^1$H spectra and plotted over reaction time. Curve fitting was performed follow from a simplified kinetic model, with three processes described by pseudo first order kinetics (for more details see appendix).
Table 4.3.: Combined yield (CY) and rate constant ($k_{obs}$) for the oxyamine ligation reactions with sugar concentrations of 36 mM and 5 equiv. of ethoxyamine or 5 mM and 10 equiv. of ethoxyamine according to Figures 4.22. and 4.21.. $k_{obs} = k_1$ or $k_2$.

<table>
<thead>
<tr>
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<th>catalyst (100 mM)</th>
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<th>5 mM sugar</th>
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<td></td>
<td>CY after 1h [%]</td>
<td>$k_{obs}$ [h$^{-1}$]</td>
<td>CY after 1h [%]</td>
</tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>GlcNAc</td>
<td>--</td>
<td>0.4</td>
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Hydrolysis of GlcNAcNH$_2$ gains bigger impact at lower carbohydrate concentrations (36 mM compared to 5 mM), this causes two stages in the overall oxyamine ligation reaction. These two stages are nicely visible in Figure 4.22.B (red data points) due to the extended measurement time. The first stage lasts until about 1 h after start of the reaction, when ~60% GlcNAcNH$_2$ has reacted in the fast oxyamine ligation reaction to the ligation products 71 and 72, and the remainder percentage of GlcNAcNH$_2$ has hydrolysed. The second stage is the reaction of the hydrolysis product GlcNAc in a slower oxyamine ligation reaction to the ligation products, until equilibrium is reached. Figure 4.22.B gives the impression that from about 1 –
10 h the ligation reaction nearly comes to a halt; this is due to the logarithmic timescale. In fact, the reaction rate is only slower with GlcNAc than with GlcNAcNH₂ and the reaction rate is also slower because of the lower concentration of reactive carbohydrate (~60% GlcNAcNH₂ has already reacted in the ligation reaction). This “hydrolysis effect” is of course present in all kinetic measurements with GlcNAcNH₂. The effect is the reason why, even though a plateau is reached for a kinetic measurement with GlcNAcNH₂, the reaction is mostly not yet in equilibrium, but only the starting material GlcNAcNH₂ has changed to GlcNAc and the reaction continues at a slower rate, thus the combined yield (CY) and not the combined equilibrium yield (CEY) is reached, which is why and the x-axis is designated CY.

I increased the equivalents of alkoxyamine from 5 to 10 equivalents for two reason. For one to push the equilibrium towards the product side and for another to try to counteract the slower reaction rate caused by the simultaneously used lower concentrations of carbohydrate (from 36 mM to 5 mM). Rate law (I) states that the reaction rate v, in contrast to the rate constant k, is influenced by the concentrations of the reactants, thus the ligation reactions at lower carbohydrate concentrations (5 mM) proceed slower than the reactions at higher carbohydrate concentrations (36 mM).

\[ v = k \cdot [A]^a \cdot [B]^b \]  

By comparing the obtained data sets (Figure 4.21. and 4.22.), is becomes apparent that the higher equivalents of nucleophile (from 5 to 10 equiv.) cannot compensate the slowdown caused by the general lower concentration of reactants. The hydrolysis’ reaction rate of GlcNAcNH₂ can be considered constant for both concentration sets, because the aqueous buffer is present in a huge excess. But because at lower concentrations (5 mM) the reaction rate of the GlcNAcNH₂ oxyamine ligation reaction is lower (see also Table 4.3.), hydrolysis (even though with the same rate constant) gains a bigger influence because GlcNAcNH₂ remains longer unreacted in solutions and thus prone to hydrolysis, which then causes an lower CY for the first stage of the kinetic measurements with 5 mM GlcNAcNH₂ and 5 equiv. ethoxyamine in comparison to 36 mM GlcNAcNH₂ and 10 equiv. ethoxyamine. But still, the experiments at lower carbohydrate and alkoxyamine concentrations clearly demonstrated the superiority of glycosyl amine ligation over aniline-catalysed GlcNAc ligation.

For catalysis to be effective, aniline and PDA, have to be added in high concentrations. Both compounds are toxic and can cause the formation N-arylglycosylamine side products. Furthermore, PDA is chemically unstable, leading to deeply coloured solutions after reaction times of only a few hours and precipitate formation (see appendix Figure 9.5.). Thus, usage of the nucleophilic catalysis, especially with PDA comes with severe disadvantages. However, as Figure 4.22.A-D shows, PDA catalysis of the oxyamine ligation reaction with GlcNAc is under the observed conditions (5 mM carbohydrate, 10 equiv. ethoxyamine) and in the
considered pH rage superior to the ligation reaction using 5 mM GlcNAcNH₂ and 10 equiv. ethoxyamine. As explained above, the reactions speed can be accelerated by increasing the equivalents of nucleophile. Thus in order to further enhance the reactions speed and in case of the reaction with GlcNAcNH₂ push back the immediate impact the hydrolysis has on the CY, I conducted kinetics measurements with 5 mM carbohydrate (GlcNAcNH₂ or GlcNAc) with 100 mM (20 equiv.) and 250 mM (50 equiv.) ethoxyamine, uncatalysed, aniline- and PDA-catalysed (100 mM) at pH 6 (Figure 4.23). The data was also obtained under the exact same conditions as described above.

Pleasantly, these measurements with higher equivalents of nucleophile increased the yield in the first stage of the reaction significantly to a CY of 76% after 1 h (with 20 equiv. nucleophile, Figure 4.23.A) and even 89% after only 30 min (with 50 equiv. nucleophile, Figure 4.23.B). For comparison, the aniline-catalysed and the uncatalysed reaction of GlcNAc resulted in yields well below 5% at the same points of time for both 20 and 50 equiv. of oxyamine. The CY of the PDA- catalysed reaction of GlcNAc after 1 h is 76% (with 20 equiv. nucleophile) and 89% (with 50 equiv. nucleophile). For the reaction with 20 equiv. nucleophile, the rate constant \( k_1 \) of the reaction of GlcNAcNH₂ (\( k_1 = 8.0 \text{ h}^{-1} \)) is 533 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\( k_2 = 0.015 \text{ h}^{-1} \)), 133 times higher than the rate constant of the aniline-catalysed GlcNAc ligation (\( k_2 = 0.060 \text{ h}^{-1} \)) and 35 times higher than the rate constant of the PDA-catalysed GlcNAc ligation (\( k_2 = 0.230 \text{ h}^{-1} \)). For the reaction with 50 equiv. nucleophile, the rate constant \( k_1 \) of the reaction of GlcNAcNH₂ (\( k_1 = 12.0 \text{ h}^{-1} \)) is even 200 times higher than the rate constant of the uncatalysed reaction of GlcNAc (\( k_2 = 0.060 \text{ h}^{-1} \)), 109 times higher than the rate constant of the aniline-catalysed GlcNAc ligation (\( k_2 = 0.110 \text{ h}^{-1} \)) and 43 times higher than the rate constant of the PDA-catalysed GlcNAc ligation (\( k_2 = 0.280 \text{ h}^{-1} \)). As shown by O. BAUDENDISTEL, access nucleophile can easily be removed by use of an aldehyde-functionalised scavenger resin\[^{332}\], thus the utilised large accesses shut not be problematic in future application.
Results and Discussion

Figure 4.23: Reaction of 5 mM 70 or 75 with (A) 20 equiv. (B) 50 equiv. ethoxyamine, 100 mM catalyst at pH 6, 39°C. The CY for the reaction products (71 and 72) were obtained from 1H spectra and plotted over reaction time. Curve fitting was performed follow from a simplified kinetic model, with three processes described by pseudo first order kinetics (for more details see appendix).
5. Summary and Outlook

Ever since scientists discovered the important roles carbohydrates take in many physiology as well as pathological processes, glycoscience has become of great general interest in academia and industry. Three different aspects, under the general topic of glycoscience were investigated in this thesis:

- The investigation of the photoclick reaction for application in metabolic glycoengineering.
- The generation of an advanced clickECM for application in wound care (in cooperation with Hochschule Reutlingen and University of Stuttgart).
- The examination of the oxyamine ligation reaction using glycosylamine, for accelerated ligation rates.

5.1. Part I - Metabolic Glycoengineering using the Photoclick Reaction

In order to try and reduce the background staining occurring in the negative control of biological assays of the photoclick reaction of a tetrazole and an alkene-functionalised glycocalyx, two tetrazole-dye adducts (15, Tet1-sCy3 and 18, Tet2-sCy3) were synthesised. The optical properties of Tet1-sCy3 and of the corresponding pyrazoline-dye conjugate 23 were investigated. It was found, that the dye is well suited for the application because its intensity and absorbance maxima are not affected by the irradiation at 302 nm and because the zones of absorption of dye and tetrazole do not overlap. Tet1-sCy3 was then applied in biological assays. Via MGE three mannosamine derivatives (Ac₄ManNAcryl 24, Ac₄ManNCp 25, Ac₄ManNCyoc 26) were incorporated into the glycocalyx of HEK293T cells and subsequently labelled by the photoclick reaction using Tet1-sCy3 (Scheme 5.1.). Confocal fluorescence microscopy was used to detect membrane staining. Unfortunately, the cells in negative control showed the same amount of matrix staining as the cells fed with the three sugar derivatives. By directly comparing the new system (Tet1-sCy3) with the published system by SCHART et al.[18] (Tet-biotin/StrepAF555) it became apparent that Tet1-sCy3 does in fact not cause a higher background staining, but generally less staining compared to the published procedure.
To further investigate this finding, NMR studies were performed. These studies confirmed the hypothesis that Tet1-sCy3 reacts in a lesser extend with the provided alkene than does Tet1. The NMR studies further support the findings from the UV-measurements that the irradiation light of 302 nm is not absorbed by the dye, thus the nitrile imine is generated upon irradiation. Also the NMR studies indicated that the generated nitrile imine does not react in a sort of cyclisation reaction with the dye. The new Tet1-sCy3 conjugate was synthesised to reduce background staining in cell assays, but as it firstly does not show any improvement in that respect and secondly does react to a lesser extent with the provided alkene, the investigation was stopped at this point.

A unique and valuable feature of the photoclick reaction is the possibility of temporal and spatial control over the reaction. To investigate these features, Tet-bithio 28 (Figure 5.1.A) was synthesised following literature known procedures and applied in MGE using mannosamine derivative Ac₄ManNAcryl 24. The 405 nm laser of the confocal fluorescence microscope was used to irradiated Tet-bithio for 4 sec. directly on top the acryl-modified cells, inducing the photoclick reaction. The fluorescent pyrazoline 29 was detected and for the first time the extent of the spatial control the photoclick reaction provides was visible (Figure 5.1.B). To this end, at only the beginning of the investigation of the spatial control over the photoclick reaction, the high and exact control that can be gained over the reaction could successfully be demonstrated in biological assays.
Figure 5.1.: A) molecular structure of Tet-bithio 29. B) Investigation of the spatiotemporal control over the photoclick reaction, comparison before and after irradiation of Tet-bithio for 4 sec. at 405 nm. HEK293T cells were incubated with 100 μM AcManNAcryl 24 for 48 h. Living cells were reacted with Tet-bithio. Scale bar 30 μm, irradiated area indicated by white circle.

The protocols of the biological assays can now be varied and improved in many places, e.g. with the addition of washing steps removing unspecific staining, with different concentrations of tetrazole bithio-Tet 29, different lengths of irradiation time or laser intensity. To be able to control the time and especially the place when a chemical reaction will take place, can be important for the development of applications: e.g. self-repair in the human body by ligating growth factors or cytokines to cells for assistance in wound healing processes; applications like customised surface patterning of biomaterials in for example extracellular matrix (ECM) gels; or generally by utilisation of the spatiotemporal control feature to investigate molecular processes in cell biology.

5.2. Part II – Generation and Application of a Glyco-Functionalised Extracellular Matrix

This project started with the synthesis of a library of new and literature known unnatural N-acetylgalactosamine (GalNAc) and N-acetylmannosamine (ManNAc) derivatives with carbamate- and amide-linked terminal alkenes and amide-linked cyclopropenes for incorporation into the ECM of primary adipose-derived stromal/stem cells (ASCs) using MGE. The biocompatibility of the dienophile-functionalised carbohydrates was tested to be good, all investigated carbohydrates showed no cytotoxic properties, thus a functionalised ECM was generated, the so-called clickECM (Figure 5.2.A). The incorporation into the ECM was checked by reacting the alkene modified ECM with tetrazine-biotin in a DAinv reaction followed by labelling with a Streptavidin-dye adduct. This detection system turned out to be unreliable, as
some of the assays showed the same amount of staining in the negative control ECM as in the modified clickECMs. The literature known and reliable detection system of streptavidin linked to HRP with subsequent treatment with TMB was then tested and has proven to give stable results (Figure 5.2.B). With this system it could be proven that the alkenes were successfully incorporated into the ECM, with the cyclopropene modified ECM showing the most absorption compared to the other clickECMs. The data also showed, that whether the ASCs are treated with mannosamine or galactosamine derivatives does not have a significant impact on the amount of incorporated carbohydrate. A much stronger impact has the donor from which the ASCs are obtained from, this is likely due to the fact that the ECM is gained from primary cells and not from cell lines. The gained clickECM was then investigated in view of the influence the incorporated functional groups have on the ECM as a biomaterial and whether the functional groups have an impact on cellular behaviour. For this gellan, gum-hybrid hydrogels were produced and the ECM was incorporated in these hydrogels (Figure 5.2.C). Various tests and measurements like rheological measurements for evaluation of the material's stiffness, HE-staining to ensure homogenous distribution of the ECM inside the hydrogel, proliferation as well as cellular survival experiments of the encapsulated ASCs were performed. All measurements showed that the incorporated functional groups in the ECM have no negative impact on encapsulated ASCs and do not affect their cellular behaviour.

It is known that oligosaccharides are involved in cell adhesion and recognition processes. Thus, the next goal was to develop and synthesise a trivalent linker for further functionalisation of the clickECM with suitable oligosaccharides for the later application as wound care material. The linker consists of an oxyamine functional group for reacting in an oxyamine ligation with reducing oligosaccharides, of an tetrazine functionality for a DAinv reaction with the clickECM and a dye for detection, latter is of course neither needed nor wanted in the final application and is only included in the linkers structure for research purposes. The synthesis of this trivalent linker was completed for two tetrazine derivatives 67 and 68 (Figure 5.2.E) in 8 (67) and 7 steps (68). The screening for suitable oligosaccharides was performed using the microarrays technology. For this an already existent library consisting of 25 functionalised oligosaccharides ranching from mono- to decasaccharides was used. Via a similar trivalent linker (with an amine instead of a tetrazine functionality) the oligosaccharides were covalently and site-specifically immobilised on activated glass slides via non-contact printing. These functionalised slides were then incubated with ASCs, unfortunately only on the positive control, functionalised with the peptide sequence cRGDfK cells adhered, on all saccharides spots no cells were found (Figure 5.2.D).

The obtained cell-derived ECM hydrogel can now further be equipped with additional functions with bioactive molecules like growth-factors. For example, the already mentioned basic fibroblat growth factor (bFGF), which could be incorporated into the clickECM by covalent
linkage to the modified matrix via the DAinv reaction. The bFGF growth factor for one promotes the differentiation of ASCs into fat cells and for another is involved in the formation of vascular structures (vascularisation). Both features would be very beneficial in a material for the treatment of deep wounds where the development of fat cells and blood vessel systems is needed. The material could also be further cross-linked by using corresponding linker molecules.

**Figure 5.2.**: Workflow of performed tasks in Part II, the generation and application of a glyco-functionalised extracellular matrix A) Metabolic incorporation of alkene-functionalised monosaccharides into harvested ECM. B) Detection via Step-HRP and TMB of incorporated alkenes in clickECM. C) Preparation and characterisation of gellan gum-hybrid hydrogels. D) Investigation of cell adhesion on carbohydrate microarrays E) Molecular structure of trivalent linker 67 and 68 with R= H or pyrimidinyl.
5.3. **Part III – Investigation of the Oxyamine Ligation**

In part III of this thesis the oxyamine ligation i.e., the reaction of carbohydrates with oxyamines was investigated. When N-glycans are cleaved of proteins, glycosyl amines are obtained, which over time hydrolyse to the corresponding reducing sugar. The glycosyl amines are easily protonated to the iminium species and are thus very susceptible to an oxyamine attack. It was systematically investigated if and how much glycosyl amines enhance the speed of the ligation reaction via real-time $^1$H-NMR spectroscopy. These measurements were compared to experiments performed with the corresponding reducing sugar (GlcNAc), both in catalysed as well as uncatalysed oxyamine ligation reactions. All measurements were carried out in aqueous buffered systems, at pH values ranging from 5-8. As model compound GlcNAcNH$_2$ 75 was used, obtained from the shelf stable precursor GlcNAcN$_3$ 76 by hydrogenation.

First the hydrolytic stability of GlcNAcNH$_2$ was tested. The hydrolysis rate of GlcNAcNH$_2$ is fastest at pH 5 and becomes increasingly slower with increasing pH, because hydrolysis is an acid catalysed process. Next the oxime formation of 36 mM GlcNAcNH$_2$ 75 reacting with 5 equiv. ethoxyamine was monitored, and the yields of the individual ligation products ($^E$- and $^Z$-oximes 71, $N$-glycoside 72) were added up to the combined yields (CY) and plotted against the reaction time (Figure 5.4.A). Also 36 mM GlcNAc 70 reacting with 5 equiv. ethoxyamine in an uncatalysed and in an aniline-catalysed ligation reaction (100 mM catalyst) were measured and plotted over time. Comparing the calculated rate constant showed that by using GlcNAcNH$_2$ instead of GlcNAc as starting material, the reaction speed is accelerated up to 160 times. Comparing rate constants of GlcNAcNH$_2$ with the aniline-catalysed reaction of GlcNAc the reaction’s speed is accelerated up to 100 times.

To match conditions of a possible biological application better, all measurements were repeated at lower concentrations with 5 mM GlcNAcNH$_2$ or GlcNAc reacting with 10 equiv. ethoxyamine and catalyst concentrations of 100 mM (Figure 5.3.B). The equivalents of nucleophile were increased to try to counteract the slower reaction rates caused by the lower concentrations. Comparing the calculated rate constant showed that by using GlcNAcNH$_2$ instead of GlcNAc as starting material, the reaction speed is accelerated up to 417 times. Comparing rate constants of GlcNAcNH$_2$ with the aniline-catalysed reaction of GlcNAc the reaction’s speed is accelerated up to 125 times when using GlcNAcNH$_2$. Hydrolysis of GlcNAcNH$_2$ gains bigger impact at lower concentrations, causing two stages in the overall oxyamine ligation reaction (Figure 5.3.B). The first stage, the reacting of GlcNAcNH$_2$ in a fast oxyamine ligation reaction and simultaneously hydrolysis to GlcNAc. The second stage, hydrolysed GlcNAc reacting in a slower oxyamine ligation reaction until the equilibrium is yield. The amount of hydrolysis can be forced back or rather the reactions speed can be accelerated significantly by using high equivalents of nucleophile (50 equiv.) for the ligation reaction. Under
these conditions the reaction rate is so fast, that the oxyamine ligation reaction with \( \text{GlcNAcNH}_2 \) is instantly finished, before significant amounts of \( \text{GlcNAcNH}_2 \) are hydrolysed. Under these condition the ligation with \( \text{GlcNAcNH}_2 \) is also much faster to the ligation reaction of \( \text{GlcNAc} \) catalysed with PDA as a catalyst (Figure 5.3.C). Because of the particular case at pH 5, 5 mM \( \text{GlcNAcNH}_2 \) ("overshooting" of the equilibrium, Figure 4.22.A ) we expect a fourth species in the equilibrium and hypothesise that this might be the aminal formed upon addition of a further ethoxyamine molecule to the oximes. However, to this date this species has not been conclusively identified. All experiments clearly show the superiority of oxyamine ligation with glycosyl amine over the uncatalysed as well as the aniline-catalysed \( \text{GlcNAc} \) oxyamine ligation, and although hydrolysis and ligation are competing reactions, high ligation yields are obtained with \( \text{GlcNAcNH}_2 \) after only short reaction times. When large excesses of nucleophile are used the ligation with \( \text{GlcNAcNH}_2 \) is even significantly faster than the ligation reaction catalysed by first-rate catalyst PDA.

\[ \text{GlcNAcNH}_2 \]

\[ \text{GlcNAc} \]

\[ \text{GlcNAc} \text{ (aniline cat.)} \]

\[ \text{GlcNAc} \text{ (uncat.)} \]

\[ \text{GlcNAc} \text{ (PDA cat.)} \]

**Figure 5.3.:** Comparison of different oxyamine ligation reaction. A) 36 mM, carbohydrate, 5 equiv. Ethoxyamine, 100 mM catalyst, pH 6, 39°C. B) 5 mM, carbohydrate, 10 equiv. Ethoxyamine, 100 mM catalyst, pH 6, 39°C. C) 5 mM, carbohydrate, 50 equiv. Ethoxyamine, 100 mM catalyst, pH 6, 39°C.
6. Zusammenfassung und Ausblick

Seitdem Wissenschaftler die wichtige Rolle von Kohlenhydraten in vielen physiologischen und pathologischen Prozessen entdeckt haben, ist die Glykowissenschaft von großem allgemeinem Interesse in Wissenschaft und Industrie. Drei verschiedene Aspekte, unter dem übergeordneten Thema Glykowissenschaften, wurden in dieser Arbeit untersucht:

- Die Untersuchung der Photoclick-Reaktion zur Anwendung im metabolischen Glykoengineering.
- Die Generierung eines neuartigen clickECM-Materials für die Anwendung in der Wundversorgung (in Kooperation mit der Hochschule Reutlingen und Universität Stuttgart).
- Die Untersuchung der Oxyamin-Ligationsreaktion unter Verwendung von Glykosylaminen für beschleunigte Ligationsraten.

6.1. Part I - Untersuchung der Photoclick-Reaktion zur Anwendung im metabolischen Glykoengineering

Zusammenfassung und Ausblick


Zusammenfassung und Ausblick

Photoclick-Reaktion, die hohe und exakte Kontrolle, die über die Reaktion gewonnen werden kann, in biologischen Assays erfolgreich demonstriert werden.

![Molekülstruktur von Tet-Bithio 29.](image)


6.2. Part II – Erzeugung und Anwendung einer glyko-funktionalisierten extrazellulären Matrix


Es ist bekannt, dass Oligosaccharide an Zelladhäsions- und Erkennungsprozessen beteiligt sind. Das nächste Ziel war daher die Entwicklung und Synthese eines trivalenten Linkers zur weiteren Funktionalisierung der clickECM mit geeigneten Oligosacchariden für die spätere

6.3. **Teil III – Untersuchung der Oxyamin-Ligation**


Zusammenfassung und Ausblick

Abbildung 6.4.: Vergleich verschiedener Oxyamin-Ligationsreaktionen. A) 36 mM, Zucker, 5 Äquiv. Ethoxyamin, 100 mM Katalysator, pH 6, 39°C. B) 5 mM, Zucker, 10 Äquiv. Ethoxyamin, 100 mM Katalysator, pH 6, 39°C. C) 5 mM, Zucker, 50 Äquiv. Ethoxyamin, 100 mM Katalysator, pH 6, 39°C.
7. Experimental Section

7.1. Chemical Synthesis

7.1.1. General Methods

Chemicals and solvents were purchased from Merck (Sigma-Aldrich), Acros Organics, TCI, abcr, Carbosynth, Roth, Carbolution, peptides international, Deutero or Lumiprobe and were used without further purification. All technical solvents were distilled prior to usage. If necessary, reactions were carried out under inert atmosphere (nitrogen) using Schlenk techniques. Dry solvents were obtained by common methods (dichloromethane: CaH₂, chloroform: CaH₂, diethylether: Na/K, tetrahydrofuran: Na/K) or purchased from Acros Organics (methanol, dimethyl sulfoxide, dimethyl formamide) or Iris Biotech (peptide grade DMF).

Reactions were monitored by thin layer chromatography (TLC) using aluminium sheets pre-coated with silica gel 60 F254 (Merck) with detection by UV light (λ = 254 nm). Additionally, acidic ethanolic p-anisaldehyde solution, ethanolic ninhydrin solution or basic KMnO₄ solution, followed by gentle heating, were used for visualisation.

Preparative flash column chromatography was performed using silica gel 60 M from Macherey-Nagel or with an MPLC-Reveleris X2 system from Büchi. Solvent mixtures are expressed as volume ratios (v/v).

Semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a LC20A Prominence system (high-pressure pumps LC-20AT, auto sampler SIL-20A, column oven CTO-20AC, diode array detector SPD-20A, controller CBM-20A, software LC-solution) from Shimadzu under the following conditions. Solid phase: Nucleodur 100-5 C18ec from Macherey Nagel (21.1 x 250 mm); mobile phase: gradient of acetonitrile with 0.1 % formic acid (solvent B) in water with 0.1 % formic acid (solvent A), flow: 9 mL min⁻¹.

Analytical RP-HPLC-MS was performed on an LCMS2020 Prominence system (high-pressure pumps LC-20AD, auto sampler SIL-20AT HAS, column oven CTO-20AC, UV/Vis detector SPD-20A, fluorescence detector RF-20A, controller CBM-20A, ESI detector, software LC-solution) from Shimadzu under the following conditions. Solid phase: Macherey-Nagel Nucleodur C18 Gravity or Phenomenex Kinetex C18 100 A; mobile phase: gradient of acetonitrile with 0.1 % formic acid (solvent B) in water with 0.1 % formic acid (solvent A), flow: 0.4 mL min⁻¹.

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature (if not stated otherwise) with an Avance III 400, Avance III 600 or Avance Neo 800 instrument from
**Bruker.** Chemical shifts are reported in ppm relative to solvent signals (CDCl$_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.16$ ppm, DMSO-$d_6$: $\delta_H = 2.50$ ppm, $\delta_C = 39.5$ ppm, methanol-$d_4$: $\delta_H = 4.87$ ppm, 3.31 ppm, $\delta_C = 49.0$ ppm, D$_2$O $\delta_H = 4.79$ ppm, sodium 3-(trimethylsilyl)propanoate-$2,2,3,3$-$d_4$: $\delta_H = 0.00$ ppm, $\delta_C = 21.0$ ppm). The multiplicity of the resonances was abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublet), ddd (doublet of doublet of doublet), t (triplet), dt (doublet of triplet), q (quartet), dq (doublet of quartet), quin (quintet), m (multiplet), br. (broad signal). Signal assignments were carried out by two-dimensional $^1$H, $^1$H and $^1$H, $^{13}$C correlation spectroscopy (COSY, HSQC, and HMBC). Analysis of spectra was performed with the software MestReNova v.14.1.0 from Mestrelab Research S.L.

**High-resolution mass spectra** (HRMS) were recorded on a microTOF II instrument from Bruker in positive and negative mode. The ionization method was electrospray (ESI) and for detection the time of flight (TOF) method was used. Analysis of recorded mass spectra was performed using the software Xcalibur by Thermo Fischer Scientific.

**UV/VIS absorption** was measured with a Cary 50 instrument from Varian and Cary WinUV scanning kinetics software.

**Fluorescence** spectra were recorded on a Cary Eclipse Fluorescence Spectrophotometer from Agilent.

For **UV-irradiation** a hand-held UV-lamp (UVM-18EI Series UV Lamp, 8 Watt, 302 nm) from UVP was used.

**pH values** were measured and adjustment were performed using a 827 pH lab from Metrohm.
7.1.2. Part I - Metabolic Glycoengineering using the Photoclick Reaction

The following derivatives were synthesised according to published procedures:
Activated Tet1 9 [18,388], Activated Tet2 10 [18,389], NHS-sCy3 14 [393-394], CHexNAcryl 22 [396], CHexNCp 27 [397,422], Ac₄ManNAcryl 24 [18], Ac₄ManNCp 25 [104], Ac₄ManNCyoc 26 [102], Teth-bithio 29 [127-128,400-401].

**Tet1-PEG-Boc (12)**

Tert-butyl (1-oxo-1-(4-(2-phenyl-2H-tetrazol-5-yl)phenoxy)-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate

![Chemical structure of Tet1-PEG-Boc (12)](image)

Activated Tet 9 (180 mg, 0.44 mmol, 1.0 equiv) and linker 11 (210 mg, 0.65 mmol, 1.5 equiv) were dissolved in 5 mL dry dichloromethane and DIPEA (0.30 mL, 1.76 mmol, 4 equiv) was added. After 3 h the mixture was washed with NaHCO₃, 1M HCl and dried over MgSO₄. The solvent was removed under reduced pressure and the mixture was purified by flash column chromatography (petroleum ether/ethyl acetate 1/10). 12 was gained in 60%.

¹H NMR (400 MHz, CDCl₃): δ (ppm) = 8.03 (dd, J = 2.1, 7.5 Hz, 2H, C⁴/C⁵H); 7.97 (d, J = 8.9 Hz, 2H, C⁵⁷H); 7.35 (t, J = 7.2 Hz, 2H, C²³H); 7.28 (t, J = 6.9 Hz, 1H, C¹¹H); 7.08 (d, J = 8.9 Hz, 2H, C⁶⁹H); 5.85 (s, 1H, N¹⁰H); 4.76 (s, 1H, N²¹H); 3.44 (m, 10H, C¹³⁻¹⁸H₂); 3.30 (t, J = 5.9 Hz, 2H, C¹⁸H₂); 3.21 (q, J = 6.0, 12.0 Hz, 2H, C¹¹H₂); 3.00 (t, J = 6.3 Hz, 2H, C²⁰H₂); 1.66 (quin, J = 5.8, 12.1, 18.2 Hz, 2H, C¹²H₂); 1.53 (quin, J = 6.3, 12.5, 18.9 Hz 2H, C¹⁹H₂); 1.23 (s, 9H, C²²⁻²⁴H₃).

¹³C NMR (400 MHz, CDCl₃): δ (ppm) = 165.3, 156.2, 154.0, 152.2, 133.9 (C₄₅H₁); 130.6 (C¹); 129.2 (C²⁻³); 122.8 (C⁶⁹H); 121.0 (C⁵⁷H); 70.7, 70.3, 70.0, 69.6 (C¹³⁻¹⁸); 40.0 (C¹¹); 38.7 (C²⁰); 29.8 (C¹²); 29.1 (C¹⁹); 28.3 (C²²⁻²⁴).
Tet2- PEG-Boc (16)

Tert-butyl (1-oxo-1-(4-(5-phenyl-5H-2,4-tetrazol-2-yl)phenoxy)-6,9,12-trioxo-2-azapentadecan-15-yl)carbamate

Activated Tet 10 (46 mg, 0.114 mmol, 1.0 equiv) and linker 11 (73 mg, 0.228 mmol, 1.5 equiv) were dissolved in 5 mL dry dichloromethane and DIPEA (0.27 mL, 1.60 mmol, 14 equiv) was added. After 3 h the mixture was washed with NaHCO₃, 1M HCl and dried over MgSO₄. The solvent was removed under reduced pressure and the mixture was purified by flash column chromatography (petroleum ether/ethyl acetate 1/10). 16 was gained in 60%.

¹H NMR (400 MHz, CDCl₃): δ (ppm) = 7.99 (dd, J = 2.2, 7.9 Hz, 2H, C⁴⁵H); 7.93 (d, J = 8.9 Hz, 2H, C⁶⁷H); 7.35 (m, 3H, C¹⁵H); 7.10 (d, J = 9.0 Hz, 2H, C⁸⁹H); 5.82 (t, J = 8.9 Hz, 1H, N¹⁰H); 4.70 (s, 1H, N²¹H); 3.43-3.34 (m, 10H, C¹³⁻¹⁷H²); 3.28 (t, J = 6.0 Hz, 2H, C¹⁸H²); 3.18 (q, J = 6.0, 11.9 Hz, 2H, C¹¹H²); 2.97 (t, J = 6.5 Hz, 2H, C²⁰H²); 1.63 (quin, J = 6.0, 11.8, 18.0 Hz, 2H, C¹²H²); 1.49 (quin, J = 6.1, 12.3, 18.6 Hz 2H, C¹⁹H²); 1.19 (s, 9H, C²²⁻²⁴H₃).

¹³C NMR (400 MHz, CDCl₃): δ (ppm) = 165.1, 156.1, 153.9, 151.9, 133.9 (C⁴⁵H); 129.0 (C²⁻³); 127.9 (C⁴⁵H); 122.7 (C⁸⁹H) 120.8 (C⁶⁷H); 70.7, 70.2, 70.1, 69.5 (C¹³⁻¹⁸H); 40.0 (C¹¹H); 38.7 (C²⁰H); 29.7 (C¹²H); 29.0 (C¹⁹H); 28.5 (C²²⁻²⁴H₃).
**Tet1-sCy3 (15)**

1-(1,17-dioxo-1-(4-(2-phenyl-2H-tetrazol-5-yl)phenoxy)-6,9,12-trioxo-2,16-diazadocosan-22-yl)-3,3-dimethyl-5-sulfo-2-(((E)-3-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium

C₅₄H₇₇N₈O₁₂S₂⁺

1083.43 g/mol

Tet1-PEG-NHBoc 12 (1.2 mg, 0.00205 mmol, 1 equiv) was dissolved in 500 µL DCM and 500 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product Tet2-PEG-NH₃*TFA 13 was used without further purification.

13 (1.2 mg, 0.00200 mmol, 1.2 equiv) was dissolved in 100 µL dry DMF, DIPEA (3.7 µL, 0.0200 mmol, 12 equiv) was added, the yellowish mixture was stirred for 10 min at rt than NHS-sCy3 14 (2 mg, 0.00272 mmol, 1 equiv) was added. After stirring in the dark for 18 h the solvent was removed under reduced pressure and the mixture was purified by RP-HPLC (40-45%). 15 was gained in 56%.

**1H NMR (600 MHz, DMSO-δ₆):** δ (ppm) = 8.33 (t, J = 13.3 Hz, 1H, C₃₂H); 8.16 (t, J = 8.7 Hz, 4H, C₄⁵/₆/₇H); 7.89 (t, J = 5.6 Hz, 1H, N¹⁵H); 7.80 (s, 4H, C₂⁹/₃⁷H); 7.76 (t, J = 5.2 Hz, 1H, N²¹H); 7.69 (m, 4H, C₂³/₂₈/₃₈H); 7.63 (t, J = 7.6 Hz, 1H, C¹H); 7.37 (m, 4H, C⁸/₉/₂⁷/₃₉H); 6.48 (dd, J = 13.2, 2.4 Hz, 2H, C₃₂/₃₄H); 4.09 (t, J = 7.0 Hz, 2H, C₂⁶H₂); 3.65 (s, 3H, C₄₀H₃); 3.53 – 3.48 (m, 6H, C₁₄/₁₆/₁₇H); 3.46 – 3.44 (m, 4H, C¹₃/₁₅H₂); 3.37 (m, 2H, C₁₆H₂); 3.14 (q, J = 6.6 Hz, 2H, C¹¹H₂); 3.05 (q, J = 6.9 Hz, 2H, C²⁰H₂); 2.05 (t, J = 7.1 Hz, 2H, C²²H₂); 1.72 (m, 4H, C¹₂/₂₅H₂); 1.69 (s, 12H, C₃₀/₃₁/₃₅/₃₆H₂); 1.36 (p, J = 8.5 Hz, 2H, C²₄H₂).

**¹³C NMR (600 MHz, DMSO-δ₆):** δ (ppm) =174.7, 173.9, 171.5, 164.0, 153.8, 153.1, 145.8, 145.7, 142.5, 141.8, 140.0, 136.1, 123.0 (C quánt.); 149.6 (C³); 130.2 (C¹); 130.1 (C²³); 127.8 (C₆/⁷); 126.2, 126.1 (C₈/₉H); 122.6 (C⁰/⁹/₁₀H); 119.8 (C⁴/₅/₂⁷/₃₇); 110.5 (C⁷/₉/₁₀); 103.3, 102.7 (C₁₂/₃/₄); 69.7 (C¹⁴/₁₇); 69.5 (C₁₅/₁₆); 68.0 (C¹₈); 67.8 (C¹₃); 48.8 (Cューart); 43.7 (C₂₆); 37.8 (C¹¹); 35.6 (C²⁰); 35.0 (C²²); 31.5 (C₄₀); 29.3 (C₁₂/₁₉/₂₅); 27.4, 27.1 (C⁵₁₀/₃₁/₃₅/₃₆); 25.7 (C₂₄); 24.9 (C²₃).

**HRMS (ESI-MS):** m/z calcd. for C₅₄H₇₇N₈O₁₂S₂⁺: 1083.4314 [M⁺]; found: 1145.3842 [M+Na+K⁺].
**Tet2-sCy3 (18)**

1-(1,17-dioxo-1-(4-(5-phenyl-2H-tetrazol-2-yl)phenoxy)-6,9,12-trioxa-2,16-diazadocosan-22-yl)-3,3-dimethyl-5-sulfo-2-((E)-3-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium

![Chemical Structure of Tet2-sCy3 (18)](image)

C_{54}H_{77}N_{15}O_{12}S_{4}^{+}

1083.43 g/mol

Tet2-PEG-NHBoc 16 (1.6 mg, 0.00273 mmol, 1 equiv) was dissolved in 500 µL DCM and 500 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product Tet2-PEG-NH3*TFA 17 was used without further purification.

17 (1.6 mg, 0.00267 mmol, 1.2 equiv) was dissolved in 140 µL dry DMF, DIPEA (4.7 µL, 0.027 mmol, 12 equiv) was added. The yellowish mixture was stirred for 10 min at rt than NHS-sCy3 14 (2.76 mg, 0.00375 mmol, 1 equiv) was added. After stirring in the dark for 18 h the solvent was removed under reduced pressure and the mixture was purified by RP-HPLC (40-45%). 18 was gained in 62%.

**1H NMR (600 MHz, DMSO-d$_6$):** δ (ppm) = 8.33 (t, J = 13.2 Hz, 1H, C$_{33}$H); 8.16 (t, J = 8.4 Hz, 4H, C$_{45/67}$H); 7.93 (t, J = 5.0 Hz, 1H, N$_{10}$H); 7.80 (s, 4H, C$_{29/37}$H); 7.76 (t, J = 4.7 Hz, 1H, N$_{21}$H); 7.69 (t, J = 7.9 Hz, 2H, C$_{28/38}$H); 7.63 – 7.59 (m, 3H, C$_{1/2/3}$H); 7.43 (d, J = 8.7 Hz, 2H, C$_{69}$H); 7.38 (t, J = 8.6 Hz, 2H, C$_{37/39}$H); 6.48 (dd, J = 2.6, 13.3 Hz, 2H, C$_{32/34}$H); 4.09 (t, J = 6.5 Hz, 2H, C$_{39}$H$_2$); 3.64 (s, 3H, C$_{40}$H$_3$); 3.55-3.49 (m, 10H, C$_{14/17}$H$_2$); 3.45 (m, 2H, C$_{13}$H$_2$); 3.14 (q, J = 6.2 Hz, 2H, C$_{11}$H$_2$); 3.04 (q, J = 6.0 Hz, 2H, C$_{20}$H$_2$); 2.04 (t, J = 6.9 Hz, 2H, C$_{22}$H$_2$); 1.71 (m, 4H, C$_{12/25}$H$_2$); 1.69 (s, 12H, C$_{30/31/35/36}$H$_3$); 1.57-1.52 (m, 4H, C$_{19/23}$H$_2$); 1.35 (p, J =8.6 Hz, 2H, C$_{24}$H$_2$).

**13C NMR (600 MHz, DMSO-d$_6$):** δ (ppm) = 174.7, 173.9, 171.5, 164.4, 153.7, 152.1, 145.7, 145.6, 142.5, 141.8, 140.0, 132.8, 126.3 (C$_{quart.}$); 149.6 (C$_{33}$); 129.3 (C$_{2/3}$); 126.6 (C$_{6/7}$); 126.2, 126.1 (C$_{28/38}$); 123.2 (C$_{6/9}$); 119.9 (C$_{4/5}$); 119.7 (C$_{29/37}$); 110.5 (C$_{27/39}$); 103.3, 102.7 (C$_{32/34}$); 69.7 (C$_{14/17}$); 69.5 (C$_{15/16}$); 68.0 (C$_{18}$); 67.8 (C$_{13}$); 48.8 (C$_{quart.}$); 43.7 (C$_{26}$); 37.8 (C$_{11}$); 35.7 (C$_{20}$); 35.1 (C$_{22}$); 31.5 (C$_{45}$); 29.3 (C$_{12/19/25}$); 27.3, 27.1 (C$_{30/31/35/36}$); 25.1 (C$_{24}$); 24.9 (C$_{23}$).

**HRMS (ESI-MS):** m/z calcd. for C$_{54}H_{77}N_{15}O_{12}S_{4}^{+}$: 1083.4314 [M]$^{+}$; found: 1107.4266 [M+Na+H]$^{3+}$. 
Pyrazoline-sCy3 (23)
1-(1-{4-({5-(cyclohexylcarbamoyl)}-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenoxy}-1,17-dioxo-6,9,12-trioxo-2,16-diazadocosan-22-yl)-3,3-dimethyl-5-sulfo-2-{((E)-3-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium

C_{63}H_{82}N_{17}O_{35}S_{2}^+  1209.50 g/mol

Tet1-sCy3 15 (3 mg, 0.00248 mmol, 1 equiv) was dissolved in 1 mL ethanol and 22 (0.57 mg, 0.00372 mmol, 1.5 equiv) was added. The mixture was irradiated at 302 nm with a hand-held UV-lamp placed directly on top of the reaction vessel for 100 minutes. The product crystallised in ethanol. It was filtered off, dried and used without further purification. The pyrazoline-dye conjugate 23 was gained in 90% yield.

Nuclear Magnetic Resonance Kinetic Experiments with Tet-sCy3

The needed derivatives (tetrazole, cyclopropene model compound or sCy3 NHS-ester) were dissolved in a mixture of DMSO-d$_6$ and/or EtOH-d$_6$ and mixed in a Suprasil® quartz NMR tube. A $^1$H-NMR spectrum was recorded, the NMR tube was then irradiated at 302 nm with a hand-held UV-lamp for 5 minutes, the distance between UV-lamp and NMR tube being 1 cm. Subsequently the next $^1$H-NMR spectrum was recorded. This procedure was followed until an irradiation time of 30 minutes was reached, after that the irradiation time between each NMR measurement was extended to 10 minutes until a total irradiation time of 100 minutes was reached. For analysis, a distinctive signal of the investigated compounds was integrated. The solvent peak was used as an internal standard for calibration of the distinctive signal intensities. For this calibration, the integrals of all measured distinctive peaks in one experiment were divided by the integral of the solvent peak and the signal was analysed and compared to the other experiments. For CHexNCp 27 the resonance of the vinyl protons at 7.38 ppm, for tetrazole Tet1 the resonance of the aromatic protons at 8.66 ppm, for Tet1-sCy3 the resonance of the tetrazole aromatic protons at 8.17 ppm and the resonance of the sCy3 protons at 8.33 ppm (CH group), 7.79 ppm (aromatic protons) and 4.08 ppm (CH$_2$ group). The relative integrals were plotted against time using Origin 9.8.0.200.
Experimental Section

Exemplary kinetic measurement:

Figure 7.1.: Exemplary kinetic measurement performed with Tet1 and sCy3 NHS-ester 14. Recorded spectra stacked and plotted over time. Total irradiation time of 100 min, irradiated at 302 nm with a hand-held UV-lamp in DMSO-d$_6$/EtOH-d$_6$. 
7.1.3. Part II – Generation and Application of a Glyco-Functionalised Extracellular Matrix

The following derivatives were synthesised according to published procedures:

- \text{Ac}_4\text{ManNBTl}\[423]\,
- \text{Ac}_4\text{ManNPtl}\[424]\,
- \text{Ac}_4\text{ManNHxl}\[425]\,
- \text{Ac}_4\text{ManNBeoc}\[20]\,
- \text{Ac}_4\text{ManNPeoc}\[20]\,
- \text{Ac}_4\text{ManNHeoc}\[20]\,
- \text{Ac}_4\text{GalNBTl}\[423]\,
- \text{Ac}_4\text{GalNHxl}\[426]\,
- \text{Ac}_4\text{GalNAcryl}\[427]\,
- \text{monoaryl-tetrazine}\[410]\.

**General procedure for the synthesis of dienophile-modified GalNAc derivatives:**

Galactosamine hydrochloride (3.3 g, 15.3 mmol, 1 equiv.) was dissolved in dry MeOH (90 mL) and NaOMe (0.5 M in MeOH, 31 mL, 1 equiv.) was added. The reaction mixture was stirred for 1.5 h at rt and a solution the alkene derivative (39/41/42/43) (1.04 equiv) in dry MeOH (90 mL) was added. After having been stirred at rt for 18 h, the solvent was removed under vacuum and the residual brown syrup was dissolved in pyridine (40 mL). Acetic anhydride (14 mL, 150 mmol) was added, and the mixture was stirred for 18 h. The solvent was removed under vacuum, the residue was dissolved in DCM and washed with aqueous KHSO₄ (3 x), sodium bicarbonate (2 x) and brine (1 x). The organic layer was dried over MgSO₄ and concentrated resulting in a dark brown solid which was purified by silica gel chromatography (petroleum ether/ethyl acetate) yielding the corresponding GalNAc derivative.

\text{Ac}_4\text{GalNBeoc} (41)

\[(3R,4R,5R,6R)\cdot6\cdot(\text{acetoxymethyl})\cdot3\cdot((\text{but}-3\cdot\text{en}-1\cdot\text{yl oxy})\cdot\text{carbonyl})\cdot\text{amino})\cdot\text{tetrahydro-2H-pyrant-2,4,5-triyl triacetate}

The title compound was synthesised with but-3-en-1-yl succinimidyl carbonate according to the general procedure and obtained as a colourless solid (8.0 g, 63 %) as a mixture of anomers (α/β = 3.2/1).

**TLC:** \(R_f = 0.33\) (petroleum ether/ethyl acetate 1:1).

\(\alpha\)-anomer: \(^1\text{H NMR} (400 \text{ MHz, CDCl}_3): \delta = 6.23 \text{ (d, } J = 3.5 \text{ Hz, } 1\text{H, C}^1\text{H}), 5.70–5.80 \text{ (m, } 1\text{H, C}^9\text{H}), 5.42 \text{ (d, } J = 2.3 \text{ Hz, } 1\text{H, C}^4\text{H}), 5.19–5.03 \text{ (m, } 3\text{H, C}^3\text{H, C}^{10}\text{H}), 4.64 \text{ (d, } J = 9.7 \text{ Hz, } 1\text{H, NH}), 4.41 \text{ (dt, } J = 3.4, 11.4 \text{ Hz, } 1\text{H, C}^2\text{H}), 4.22 \text{ (m, } 1\text{H, C}^5\text{H}), 4.04-4.15 \text{ (m, } 4\text{H, C}^7\text{H, C}^8\text{H}), 2.35–5.33 \text{ (m, } 2\text{H, C}^9\text{H}), 2.16 \text{ (s, } 2\text{x3H, CH}_3), 2.02 \text{ (s, } 2\text{x3H, CH}_3) \text{ ppm}; \ ^{13}\text{C NMR} (100 \text{ MHz, CDCl}_3): \delta = 170.8, 170.3, 168.8, 155.9 \text{ (C=O), 133.9 (C}^3\text{), 117.2 (C}^1\text{), 91.5 (C}^1\text{), 68.5 (C}^5\text{), 68.0 (C}^3\text{), 66.7 (C}^4\text{), 61.2, 64.5 (C}^6\text{, C}^7\text{), 48.6 (C}^2\text{), 33.3 (C}^9\text{, 20.9, 20.6 (CH}_3) \text{ ppm.}}
β-anomer: $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 6.17$ (d, $J = 4.7$ Hz, 1H, C$^1$H), 5.69–5.80 (m, 1H, C$^9$H), 5.32–5.28 (m, 1H, C$^3$H), 5.18–5.03 (m, 4H, NH, C$^5$H, C$^{10}$H$_2$), 4.52–5.47 (m, 1H, C$^2$H), 4.18–4.12 (m, 5H, C$^4$H, C$^7$H$_2$, C$^6$H$_2$), 2.35 (m, 2H, C$^8$H$_2$), 2.12 (s, 3H, CH$_3$), 2.07 (s, 2x3H, CH$_3$), 2.02 (s, 3H, CH$_3$) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 170.5$, 169.9, 169.3, 168.9, 155.9 (C=O), 133.9 (C$^9$), 117.2 (C$^{10}$), 93.9 (C$^1$) 78.9 (C$^5$), 73.9 (C$^3$), 68.6 (C$^4$), 64.5 (C$^7$), 62.0 (C$^6$), 57.8 (C$^2$), 33.2 (C$^8$), 20.6, 20.7, 20.8, 21.1 (CH$_3$) ppm

HRMS (ESI-MS): m/z calcd. for C$_{19}$H$_{27}$NO$_{11}$: 446.1657 [M+H]$^+$; found: 468.1471 [M+Na]$^+$.

**Ac$_3$GalNPeoc (42)**

(3R,4R,5R,6R)-6-(acetoxyethyl)-3-(((pent-4-en-1-yl)oxy(carbonyl)amino)tetrahydro-2H-pyran-2,4,5-triyl triacetate

![Chemical Structure](image)

The title compound was synthesised with pent-4-en-1-yl succinimidy carbonate according to the general procedure and obtained as a colourless solid (4.5 g, 49 %) as a mixture of anomers (α/β = 2.2/1).

**TLC:** $R_f = 0.5$ (petroleum ether/ethyl acetate 1:1).

α-anomer: $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 6.21$ (d, $J = 3.5$ Hz, 1H, C$^1$H), 5.71–5.81 (m, 2H, C$^{10}$H$_2$), 5.40 (d, $J = 2.9$ Hz 1H, C$^4$H), 5.21–5.17 (m, 1H, C$^3$H), 5.05–4.96 (m, 2H, C$^{11}$H$_2$), 4.70 (d, $J = 9.5$ Hz, 1H, NH), 4.44–4.40 (m, 1H, C$^9$H), 4.24–4.19 (m, 1H, C$^8$H), 4.10–4.01 (m, 4H, C$^6$H, C$^7$H$_2$), 2.14 (s, 6H, CH$_3$), 2.06–2.07 (m, 2H, C$^9$H$_2$), 1.99 (s, 3H, CH$_3$) 2.00 (s, 3H, CH$_3$), 1.64–1.71 (m, 2H, C$^8$H$_2$) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 170.8$, 170.3, 170.1, 168.8, 155.9 (C=O), 137.3 (C$^{10}$), 114.9 (C$^{11}$), 91.3 (C$^1$), 68.4 (C$^5$), 68.1 (C$^3$), 66.8 (C$^4$), 64.9, 61.2 (C$^6$, C$^7$), 48.6 (C$^2$), 29.9 (C$^8$), 28.1 (C$^9$), 20.8, 20.7, 20.6 (CH$_3$) ppm

β-anomer: $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 6.19$ (d, $J = 4.7$ Hz, 1H, C$^1$H), 5.84–5.74 (m, 1H, C$^{10}$H), 5.34–5.30 (m, 1H, C$^3$H), 5.12–4.97 (m, 3H, NH, C$^5$H, C$^{11}$H$_2$), 4.54–4.49 (m, C$^9$H), 4.25–4.04 (m, 5H, C$^4$H, C$^6$H$_2$, C$^7$H$_2$), 2.17 (s, 3H, CH$_3$), 2.11–2.08 (m, 8H, CH$_3$, CH$_3$, C$^9$H$_2$), 2.04 (s, 3H, CH$_3$), 1.74–1.67 (m, 2H, C$^8$H$_2$) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 170.7$, 170.3, 170.1, 168.9, 156.2 (C=O), 137.8 (C$^{10}$), 115.2 (C$^{11}$), 93.3 (C$^1$), 78.8 (C$^5$), 73.9 (C$^3$), 68.6 (C$^4$), 65.3 (C$^7$), 61.5 (C$^6$), 57.6 (C$^2$), 29.6 (C$^8$), 27.8 (C$^9$), 21.1, 21.2, 21.0, 20.8, 20.7 (CH$_3$) ppm

HRMS (ESI-MS): m/z calcd. for C$_{20}$H$_{29}$NO$_{11}$: 460.1813 [M+H]$^+$; found: 482.1628 [M+Na]$^+$.
**Ac4GalNHeoc (43)**

(3R,4R,5R,6R)-6-(acetoxyethyl)-3-(((hex-5-en-1-yl)oxy)carbonylamino)tetrahydro-2H-pyran-2,4,5-triyl triacetate

The title compound was synthesised with hex-5-en-1-yl succinimidyl carbonate according to the general procedure and obtained as a colourless solid (5.6 g, 76%) as a mixture of anomers (α/β = 2.6/1).

TLC: \( R_f = 0.45 \) (petroleum ether/ethyl acetate 1:1).

**α-anomer:**

\[ ^1H \text{NMR (400 MHz, CDCl}_3\]): \( \delta = 6.21 \text{ (d, } J = 3.5 \text{ Hz, } 1H, C^1H) \), 5.81–5.70 (m, 1H, C\(^{11}H\)), 5.40 (d, \( J = 2.3 \text{ Hz, } 1H, C^4H\)), 5.21–5.11 (m, 1H, C\(^3H\)), 5.00–4.92 (m, 2H, C\(^{12}H_2\)), 4.68 (d, \( J = 9.6 \text{ Hz, } 1H, \text{ NH}\)), 4.42–4.36 (m, 1H, C\(^5H\)), 4.23–4.18 (m, 1H, C\(^5H\)), 4.12–4.01 (m, 4H, C\(^6H_2\), C\(^7H_2\)), 2.14 (s, 2x3H, CH\(_3\)), 2.06–2.03 (m, 2H, C\(^{10}H_2\)), 2.00 (s, 2x3H, CH\(_3\)), 1.59 (m, 2H, C\(^8H_2\)), 1.45–1.37 (m, 2H, C\(^9H_2\)) ppm. \(^{13}C \text{NMR (100 MHz, CDCl}_3\): \( \delta = 170.9, 170.4, 169.9, 168.9 \) (C=O), 156.1 (C\(^{15}H\)), 138.3 (C\(^{11}H\)), 114.9 (C\(^{12}H\)), 91.6 (C\(^{1}H\)), 68.6 (C\(^5\)), 68.1 (C\(^3\)), 66.9 (C\(^4\)), 65.5 (C\(^7\)), 61.4 (C\(^5\)), 48.7 (C\(^2\)), 33.3 (C\(^{10}H\)), 28.4 (C\(^8\)), 25.1 (C\(^9\)), 20.8, 20.7 (CH\(_3\)) ppm.

**β-anomer:**

\[ ^1H \text{NMR (400 MHz, CDCl}_3\): \( \delta = 6.16 \text{ (d, } J = 4.7 \text{ Hz, } 1H, C^1H) \), 5.81–5.70 (m, 1H, C\(^{11}H\)), 5.32–5.28 (m, 1H, C\(^2H\)), 5.21–5.11 (m, 2H, C\(^3H\)), 4.95 (m, 2H, C\(^{12}H_2\)), 4.52–4.47 (m, 1H, C\(^3H\)), 4.20–4.01 (m, 5H, C\(^4H\), C\(^6H_2\), C\(^7H_2\)), 2.06–2.03 (m, 2H, C\(^{10}H_2\)), 2.11 (s, 3H, CH\(_3\)), 2.07 (s, 2x3H, CH\(_3\)), 2.02 (s, 3H, CH\(_3\)), 1.59 (m, 2H, C\(^9H_2\)), 1.45–1.37 (m, 2H, C\(^8H_2\)) ppm. \(^{13}C \text{NMR (100 MHz, CDCl}_3\): \( \delta = 170.9, 170.4, 169.9, 168.9 \) (C=O), 156.1 (C\(^{15}H\)), 138.3 (C\(^{11}H\)), 114.9 (C\(^{12}H\)), 94.1 (C\(^{1}H\)), 79.0 (C\(^5\)), 74.1 (C\(^3\)), 70.4 (C\(^4\)), 68.0 (C\(^7\)), 62.2, 61.3 (C\(^5\)), 57.9 (C\(^2\)), 33.3 (C\(^{10}H\)), 28.4 (C\(^8\)), 25.1 (C\(^9\)), 21.2, 21.1, 21.0, 20.8, 20.7 (CH\(_3\)) ppm.

**HRMS (ESI-MS):** \( m/z \) calcd. For C\(_{21}\)H\(_{31}\)NO\(_{11}\): 474.1970 [M+H]\(^+\); found: 496.1786 [M+Na]\(^+\).
**Ac₄GalNPtI (39)**

(3R,4R,5R,6R)-6-(acetoxymethyl)-3-(pent-4-enamido)tetrahydro-2H-pyran-2,4,5-triyl triacetate

![Chemical Structure](image)

C₁₉H₂₇NO₁₀

429.16 g/mol

The title compound was synthesised with succinimidyl pent-4-enoate according to the general procedure and obtained as a colourless solid (6.5 g, 70 %) as a mixture of anomers (α/β = 2.4/1).

**TLC:** $R_f = 0.2$ (petroleum ether/ethyl acetate 1:1).

α-anomer: $^1$H NMR (400 MHz, CDCl₃): $\delta = 6.21$ (d, $J = 3.6$ Hz, 1H, C¹H), 5.79–5.69 (m, 2H, C¹⁰H₂), 5.55 (m, 1H, NH), 5.39 (m, 1H, C⁴H), 5.19–5.16 (m, 1H, C³H), 5.04–4.95 (m, 2H, C⁹H₂), 4.73–4.68 (m, 1H, C²H), 4.23–4.20 (m, 1H, C⁵H), 4.10–4.01 (m, 2H, C⁶H₂), 2.33–2.28 (m, 2H, C⁸H₂), 2.23–2.19 (m, 2H, C⁷H₂), 2.14 (s, 3H, CH₃), 1.99 (s, 3H, CH₃) 2.00 (s, 3H, CH₃) ppm.

$^{13}$C NMR (100 MHz, CDCl₃): $\delta = 172.3, 171.0, 170.3, 170.2, 158.8$ (C=O), 136.6 (C¹⁰), 115.7 (C⁹), 91.3 (C¹), 68.6 (C⁵), 68.5 (C⁶), 67.8 (C³), 66.7 (C⁴), 61.3 (C⁶), 46.8 (C²), 35.5 (C⁷), 29.2 (C⁸), 20.9 (CH₃), 20.6 (CH₃) ppm.

β-anomer: $^1$H NMR (400 MHz, CDCl₃): $\delta = 5.70$ (d, $J = 8.7$ Hz, 1H, C¹H), 5.81–5.73 (m, 1H, C¹⁰H), 5.38 (m, 2H, NH, C⁴H), 5.10–4.98 (m, 3H, C³H, C⁹H₂), 4.50–4.43 (m, 1H, C²H), 4.20–4.09 (m, 1H, C⁶H₂), 4.02–4.00 (m, 1H, C³H), 2.36–2.31 (m, 2H, C⁸H₂), 2.24–2.21 (m, 2H, C⁷H₂), 2.17 (s, 3H, CH₃), 2.12 (s, 3H, CH₃), 2.05 (s, 3H, CH₃), 2.01 (s, 3H, CH₃) ppm. $^{13}$C NMR (100 MHz, CDCl₃): $\delta = 172.4, 170.7, 170.1, 164.6$ (C=O), 136.5 (C¹⁰), 115.7 (C⁹), 93.0 (C¹), 71.9 (C⁵), 70.3 (C³), 70.3 (C⁶), 66.3 (C⁴), 61.3 (C⁶), 49.6 (C²), 35.8 (C⁷), 29.2 (C⁸), 21.0, 20.8, 20.6, 20.5 (CH₃) ppm.

**HRMS (ESI-MS):** $m/z$ calcd. For C₁₉H₂₇NO₁₀: 430.1708 [M+H]⁺; found: 452.1524 [M+Na]⁺.
Synthesis of trivalent linkers

Lys(NHBoc)-sCy3 (62)

1-(6-((5-((tert-butoxycarbonyl)amino)-5-carboxypentyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((E)-3-((E)-1,3,3-trimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate

BOC-Lys-OH 61 (1 mg, 0.0039 mmol, 1.2 equiv) was dissolved in 100 µL dry DMF, DIPEA (5.6 µL, 0.026 mmol, 12 equiv) was added, the mixture was stirred for 10 min at rt than NHS-sCy3 14 (2 mg, 0.0027 mmol, 1 equiv) was added. After stirring in the dark for 18 h the solvent was removed under reduced pressure and the mixture was purified by HPLC (30-45%). Pure product 62 was obtained in 67 % yield.

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) =8.34 (t, $J = 13.3$ Hz, 2H, C$_{24}$H); 7.80 (s, 2H, C$_{19/29}$H); 7.76 (t, $J = 5.5$ Hz, 1H, N$_{11}$H); 7.67 (t, $J = 7.7$ Hz, 2H, C$_{18/30}$H); 7.39 (t, $J = 7.4$ Hz, 2H, C$_{17/31}$H); 6.91 (broad s, 1H, N$_{5}$H); 6.49 (dd, $J = 6.2$, 13.3 Hz, 2H, C$_{23/25}$H); 4.10 (t, $J = 6.8$ Hz, 2H, C$_{16}$H); 3.78 (q, $J = 4.3$, 8.0 Hz, 1H, C$_{6}$H); 3.65 (s, 3H, C$_{32}$H$_2$); 2.99 (q, $J = 6.0$, 6.1 Hz 2H, C$_{10}$H$_2$); 2.04 (t, $J = 7.2$ Hz , 2H, C$_{12}$H$_2$); 1.70 (s, 12H, C$_{21/22/27/28}$H$_2$); 1.60 – 1.52 (m, 6H, C$_{7/13/15}$H$_2$); 1.36 (s, 9H, C$_{1/2/3}$H$_3$); 1.33 (m, 2H, C$_{9}$H$_2$); 1.28 – 1.23 (m, 4H, C$_{8/14}$H$_2$).

$^{13}$C NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) =174.88, 173.99, 171.72, 156.2 145.88, 145.71, 142.60, 141.88, 140.05, (C$_{quart.}$); 149.70 (C$_{24}$); 126.84 (C$_{18/30}$H$_2$); 119.84 (C$_{19/29}$H$_2$); 110.62 (C$_{17/31}$H$_2$); 103.39, 102.77 (C$_{23/25}$); 77.90 (C$_4$); 48.93 (C$_{20/26}$); 43.83 (C$_{16}$); 38.16 (C$_{15}$); 35.21 (C$_{12}$); 31.56 (C$_{32}$); 30.58 (C$_{15}$); 28.82 (C$_{14}$); 28.21 (C$_{12/13}$); 27.45, 27.24 (C$_{21/22/28}$H$_2$); 26.78 (C$_{13}$); 25.84 (C$_7$); 25.01 (C$_9$); 23.04 (C$_8$).
**Experimental Section**

**Tetrazine(mono)-PEG$_3$-NHBoc (57)**

Tert-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate

![Chemical Structure](https://via.placeholder.com/150)

Tz-COOH 55 (30 mg, 0.15 mmol, 1 equiv) was dissolved in 1.6 mL dry THF. DIPEA (70.0 µL, 0.4 mmol, 2.5 equiv) and HATU (79.0 mg, 0.02 mmol, 1.25 equiv) were added and the mixture was stirred for 1 h at 0°C and 1 h at rt. NHBoc-PEG$_3$-NH$_2$ (77 mg, 0.24 mmol, 1.5 equiv) was added and the mixture was stirred for 1 h at rt. After the reaction was complete the solvent was removed under reduced pressure and the residue dissolved in ethyl acetate (5 mL). The organic layer was washed with citric acid (5%aq, 3x 5 mL), water (1x 5 mL), aqueous NaCl (1x 5 mL) and dried over MgSO$_4$ and concentrated resulting in a red solid which was purified by flash column chromatography (DCM + 5%acetone), 57 was obtained in 40 % yield.

$^1$H NMR (400 MHz, CDCl$_3$): δ (ppm) = 8.69 (d, $J = 8.2$ Hz, 2H, C$_{Tz}$H); 10.3 (s, 1H, C$_{Tz}$H); 8.06 (d, $J = 8.3$ Hz, 2H, C$_{Tz}$H); 4.89 (broad s, 1H, N$_1$H); 7.45 (broad s, 1H, N$_1$H); 3.72 – 3.59 (m, 10H, C$_2/5$-$8$H$_2$); 3.51 (dd, $J = 3.6$, 5.8 Hz, 2H, C$_4$H$_2$); 3.46 (t, $J = 6.0$ Hz, 2H, C$_9$H$_2$); 3.20 (t, $J = 6.4$ Hz, 2H, C$_{11}$H$_2$); 1.97 (p, $J = 5.7$ Hz, 2H, C$_{10}$H$_2$); 1.75 (p, $J = 6.3$ Hz, 2H, C$_{15}$H$_2$); 1.42 (s, 9H, C$_{13-15}$H$_3$).

$^{13}$C NMR (400 MHz, CDCl$_3$): δ (ppm) = 166.2, 166.1, 138.9, 135.3, 78.8, (C$_{Quar}$); 158.0, 133.9, 128.4, 128.2 (C$_{2}$); 71.1, 70.6, 70.5, 70.3, 69.6, (C$_{4-9}$); 39.5 (C$_{2/11}$); 29.9, 28.8, 28.5 (C$_{3/10/13-15}$).

HRMS (ESI-MS): m/z calcd. for C$_{24}$H$_{39}$N$_8$O$_6$: 505.2769 [M+H]$^+$; found: 505.2623.
**Tetrazine(mono)-PEG₃-lys(NHBOc)-sCy₃ (63)**

1-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-18-((tert-butoxycarbonyl)amino)-1,17,24-trioxo-6,9,12-trioxo-2,16,23-triazanonacosen-29-yl)-3,3-dimethyl-2-((E)-3-((E)-1,3,3-trimethyl-5-sulphonatoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate

![Chemical Structure](image)

\[C_{60}H_{81}N_{10}O_{14}S_2\]

1230.48 g/mol

Tz-PEG-NHBOc 57 (5 mg, 0.01 mmol, 1 equiv) was dissolved in 400 µL DCM and 400 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product 59 was used without further purification.

Boc protected lys-sCy3 conjugate 62 (5.9 mg, 0.007 mmol, 1 equiv) was dissolved in 200 µL dry DMF and 200 µL dry THF. DIPEA (1.8 µL, 0.018 mmol, 2.5 equiv) and HATU (3.7 mg, 0.01 mmol, 1.34 equiv) were added and the mixture was stirred for 2 h at rt in the dark. Tz-PEG-NH₃⁺TFA 59 (5 mg, 0.01 mmol, 1.34 equiv) was added and the mixture was stirred for 1.5 h at rt in the dark. The solvent and DIPEA were removed under reduced pressure and the product was purified by RP-HPLC (40-80%). Pure product 63 was obtained in 60% yield.

\[^{1}H\text{ NMR}\ (800 \text{ MHz, DMSO-}d_6): \delta \text{ (ppm)} = 10.64 \ (s, 1\ H, C^1) ; 8.70 \ (t, J = 5.3 \text{ Hz}, 1\ H, N^6) ; 8.57 \ (d, J = 8.2 \text{ Hz}, 2\ H, C^{23} \text{ or } C^{45}) ; 8.35 \ (t, J = 13.3 \text{ Hz}, 1\ H, C^{39}) ; 8.11 \ (d, J = 8.2 \text{ Hz}, 2\ H, C^{23} \text{ or } C^{45}) ; 7.81 \ (dd, J = 1.4, 5.8 \text{ Hz}, 2\ H, C^{35/43}) ; 7.75 \ (t, J = 5.2 \text{ Hz}, 1\ H, N^{17}) ; 7.72 \ (t, J = 5.6 \text{ Hz}, 1\ H, N^{27}) ; 7.68 \ (td, J = 1.5, 8.4 \text{ Hz}, 1\ H, C^{34/44}) ; 7.39 \ (td, J = 1.8, 8.3 \text{ Hz}, 2\ H, C^{33/45}) ; 7.68 \ (td, J = 1.5, 8.4 \text{ Hz}, 1\ H, N^6) ; 6.72 \ (d, J = 8.1 \text{ Hz}, 1\ H, N^{19}) ; 6.52 \ (dd, J = 7.0, 13.4 \text{ Hz}, 2\ H, C^{38/40}) ; 4.10 \ (t, J = 7.6 \text{ Hz}, 2\ H, C^{32}) ; 3.65 \ (s, 3\ H, C^{46}) ; 3.79 \ (q, J = 8.5 \text{ Hz}, 2\ H, C^{18}) ; 3.53 – 3.43 \ (m, 12\ H, C^{8/14}) ; 3.35 – 3.29 \ (m, 2\ H, C^{7}) ; 3.12 – 3.02 \ (m, 2\ H, C^{6/8}) ; 2.98 – 2.94 \ (m, 2\ H, C^{26}) ; 2.04 \ (t, J = 7.7 \text{ Hz}, 2\ H, C^{28}) ; 1.78 \ (q, J = 6.4 \text{ Hz}, 2\ H, C^{9}) ; 1.75 – 1.71 \ (m, 2\ H, C^{31/23}) ; 1.70 \ (s, 12\ H, C^{36/37/41/42}) ; 1.62 – 1.58 \ (m, 2\ H, C^{15}) ; 1.57 – 1.53 \ (m, 2\ H, C^{29}) ; 1.35 \ (s, 9\ H, C^{20/22}) ; 1.31 \ (t, J = 7.3 \text{ Hz}, 2\ H, C^{25}) ; 1.30 – 1.20 \ (m, C^{30/24})\]
$^{13}$C NMR (800 MHz, DMSO-d$_6$): δ (ppm) = 174.1, 173.9, 172.0, 171.7, 165.4, 165.1, 158.3, 155.3, 146.0, 145.8, 141.9, 141.3, 140.3, 140.1, 138.2, 134.1 (C$_{\text{quat.}}$); 149.9 (C$_{39}$); 128.0 (C$_{2/3}$ or C$_{4/5}$); 126.3 (C$_{34/44}$); 120.4 (C$_{43/35}$); 111.0 (C$_{33/45}$); 103.8 (C$_{38/40}$); 69.8, 69.6 (C$_{10}$-$14$); 68.1 (C$_{9}$); 54.4 (C$_{18}$); 49.0 (C$_{47/48}$); 43.9 (C$_{32}$); 38.2 (C$_{46/26}$); 36.8 (C$_{7}$); 35.8 (C$_{16}$); 35.2 (C$_{28}$); 29.3 (C$_{8/15}$); 28.9 (C$_{24}$); 28.2 (C$_{20/22}$); 27.4 (C$_{36/37}$ or C$_{41/42}$); 27.3 (C$_{36/37}$ or C$_{41/42}$); 26.8 (C$_{23/31}$); 25.8 (C$_{30}$); 25.0 (C$_{29/25}$).

HRMS (ESI-MS): m/z calcd. for C$_{60}$H$_{83}$N$_{10}$O$_{14}$S$_{2}$+: 1231.5526 [M+H]$^+$; found: 1231.5522.

Tetrazine(mono)-PEG$_3$-lys-sCy$_3$-PEG$_3$-aminooxy-Boc (67)

1-(20-((1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-6,9,12-trioxapentadecan-15-yl)carbamoyl)-2,2-dimethyl-4,18,26-trioxo-3,6,9,12,15-pentaoxa-2-triazahentriacontan-31-yl)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate

Tz-PEG-sCy$_3$-NH$_2$Boc 63 (5 mg, 0.004 mmol, 1 equiv) was dissolved in 400 µL DCM and 400 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product 65 was used without further purification.

Boc-aminooxy-PEG$_3$-COOH (1 mg, 0.003 mmol, 1 equiv) was dissolved in 100 µL dry DMF. DIPEA (0.7 µL, 0.007 mmol, 2.5 equiv) and HATU (1.5 mg, 0.004 mmol, 1.34 equiv) were added and the mixture was stirred for 2h at rt in the dark. Tz-PEG-sCy$_3$-NH$_3^+$TFA 65 (5 mg, 0.004 mmol, 1.34 equiv) was added and the mixture was stirred for 1.5 h at rt in the dark. The solvent and DIPEA were removed under reduced pressure and the product was purified by RP-HPLC (30-60%). Pure product 67 was obtained in 60% yield.
**Experimental Section**

^{1}H NMR (800 MHz, DMSO-d$_6$): $\delta$ (ppm) = 8.70 (t, $J$ = 5.4 Hz, 1H, N$_6$H); 8.57 (d, $J$ = 8.2 Hz, 2H, C$_{23}$H or C$_{45}$H); 8.35 (t, $J$ = 13.4 Hz, 1H, C$_{49}$H); 8.11 (d, $J$ = 8.5 Hz, 2H, C$_{23}$H or C$_{45}$H); 7.98 – 7.92 (m, 2H, N$_{19/28}$H); 7.82 (t, $J$ = 5.8 Hz, 1H, N$_{17}$H); 7.81 (dd, $J$ = 1.5, 5.8 Hz, 2H, C$_{44/54}$H); 7.72 (t, $J$ = 5.7 Hz, 1H, N$_{36}$H); 7.68 (td, $J$ = 1.6, 8.0 Hz, 1H, C$_{43/55}$H); 7.39 (td, $J$ = 1.3, 8.2 Hz, 2H, C$_{42/56}$H); 6.52 (t, $J$ = 12.7 Hz, 2H, C$_{48/50}$H); 4.18 – 4.08 (m, 5H, C$_{41/57}$H$_2$, C$_{18}$H); 3.79 – 3.44 (m, 26H, C$_9$-$14/21$-$27$H$_2$); 3.38 – 3.35 (m, 2H, C$_7$H$_2$); 3.12 – 3.02 (m, 2H, C$_{19}$H$_2$); 2.97 – 2.94 (m, 2H, C$_{35}$H$_2$); 2.37 (m, 2H, C$_{20}$H$_2$); 2.04 (t, $J$ = 7.1 Hz, 2H, C$_{37}$H$_2$); 1.78 (p, $J$ = 6.4 Hz, 2H, C$_8$H$_2$); 1.75 – 1.72 (m, 2H, C$_{40}$H$_2$); 1.70 (s, 12H, C$_{46/57/52/53}$H$_3$); 1.58 – 1.53 (m, 2H, C$_{45}$H$_2$); 1.57 – 1.53 (m, 2H, C$_{39}$H$_2$); 1.46 – 1.41 (m, 2H, C$_{32/34}$H$_2$); 1.35 (s, 9H, C$_{29}$-$31$H$_3$); 1.35 (t, $J$ = 7.7 Hz, 2H, C$_{39}$H$_2$); 1.31 (t, $J$ = 7.2 Hz, 3H, C$_{58}$H$_3$); 1.25 – 1.20 (m, C$_{33}$H$_2$).

$^{13}$C NMR (800 MHz, DMSO-d$_6$): $\delta$ (ppm) = 174.1, 173.9, 171.6, 171.5, 170.0, 165.4, 165.2, 163.1, 158.3, 156.1, 146.0, 145.8, 141.9, 141.3, 140.1, 138.2, 134.1, 132.4 (C$_{quart}$); 149.9 (C$_{49}$); 128.2, 127.7 (C$_{1/2/3/5/5}$); 126.3 (C$_{43/55}$); 120.0, 119.9 (C$_{44/54}$); 110.7, 110.5 (C$_{42/56}$); 102.9, 102.8 (C$_{48/50}$); 79.6 (C$_{30}$); 74.8 (C$_{19}$); 69.9, 69.8, 69.5, 69.7, 69.6, 69.5, 68.3, 68.0, 67.0 (C$_{9/14/21/27}$); 66.8 (C$_{34}$); 52.4 (C$_{18}$); 48.9 (C$_{45/51}$); 43.9 (C$_{41}$); 38.3 (C$_{35/57}$); 36.8 (C$_{11}$); 35.8 (C$_{16/20}$); 35.2 (C$_{37}$), 31.8 (C$_{32/34}$); 29.3 (C$_{8/15}$); 28.8 (C$_{33/58}$); 28.0 (C$_{29/31}$); 27.4, 27.3 (C$_{46/47/52/53}$); 26.8 (C$_{40}$); 25.8 (C$_{39}$); 25.0 (C$_{38}$); 22.8 (C$_{33/58}$).

HRMS (ESI-MS): m/z calcd. For C$_{70}$H$_{101}$N$_{11}$O$_{19}$S$_2$: 1463.6717 [M+H]$^+$; found: 1463.6718.
Tetrazine(biaryl)-PEG$_3$-NHBoc (58)

Tert-butyl (1-oxo-1-(4-((pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)phenyl)-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate

Tz-COOH 56 (66 mg, 0.24 mmol, 1 equiv) was dissolved in 200 µL dry DMF (peptide grade), NMM (58 µL, 0.53 mmol, 2.2 equiv) and pyBOP (135 mg, 0.26 mmol, 1.1 equiv) were added, the mixture was stirred for 10 min at rt than H$_2$N-PEG$_3$-NHBoc (83 mg, 0.26 mmol, 1.1 equiv) was added and the mixture was stirred for 2 h. DCM (10 mL) was added and the mixture washed with aqueous NaHCO$_3$ (3x 20 mL) and water (3x 10 mL). The organic layer was dried over MgSO$_4$ and concentrated resulting in a red solid which was purified by silica gel chromatography (ethyl acetate + 5% acetone). Pure product 58 was obtained in 83 % yield.

$^{1}$$H$ NMR (400 MHz, CDCl$_3$): δ (ppm) = 9.17 (d, $J = 4.8$ Hz, 2H, C$_{Tz}$H); 8.81 (d, $J = 8.0$ Hz, 2H, C$_{Tz}$H); 8.10 (d, $J = 8.0$ Hz, 2H, C$_{Tz}$H); 7.62 (t, $J = 4.8$ Hz, 1H, C$_{Tz}$H); 7.49 (broads, 1H, N$_{1}$H); 5.01 (broads, 1H, N$_{12}$H); 3.75 – 3.64 (m, 10H, C$_{2/5-8}$H); 3.55 (t, $J = 4.7$ Hz, 2H, C$_{4}$H); 3.50 (t, $J = 6.1$ Hz, 2H, C$_{5}$H); 3.20 (broads, 2H, C$_{11}$H); 1.97 (p, $J = 5.7$ Hz, 2H, C$_{3}$H); 1.75 (p, $J = 6.3$ Hz, 2H, C$_{10}$H); 1.43 (s, 9H, C$_{13-15}$H).

$^{13}$C NMR (400 MHz, CDCl$_3$): δ (ppm) = 166.1, 164.1, 163.2, 159.4, 158.4, 156.1, 139.1, 139.1, 133.6, 128.9, 128.7, 128.2, 128.0, 122.6 (C$_{quat}$); 158.5 (C$_{Tz}$); 78.9, 70.9, 70.5, 70.3, 70.2, 69.5, (C$_{4-9}$H); 39.3, 38.4 (C$_{2/11}$); 29.7, 28.8, 28.6, 28.3 (C$_{3/10/13-15}$).

HRMS (ESI-MS): m/z calcd. for C$_{28}$H$_{38}$N$_{8}$O$_{6}^+$: 583.2987 [M+H]$^+$; found: 583.2994.
Tetrazine(biaryl)-PEG$_3$-lys(NHBoc)-sCy3 (64)

1-(18-((tert-butoxycarbonyl)amino)-1,17,24-trioxa-1-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)phenyl) -6,9,12-trioxa-2,16,23-triazanonacosan-29-yl)-3,3-dimethyl-2-((E)-3-((E)-1,3,3-trimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate

Tz-PEG-NHBoc 58 (3.4 mg, 0.057 mmol, 1 equiv) was dissolved in 200 µL DCM and 200 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product 60 was used without further purification.

Boc protected lys-sCy3 conjugate 62 (3.6 mg, 0.0043 mmol, 1 equiv) was dissolved in 100 µL dry DMF (peptide grade), NMM (1.2 µL, 0.011 mmol, 2.5 equiv) and pyBOP (3 mg, 0.0057 mmol, 1.34 equiv) were added, the mixture was stirred for 10 min at rt in the dark, than Tz-PEG-NH$_2$·TFA 60 (3.4 mg, 0.057 mmol, 1.34 equiv) was added. After stirring for 2 h in the dark the solvent was removed under reduced pressure and the product was purified by RP-HPLC (40-80%). Pure product 64 was obtained in 69 % yield.

$^1$H NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) = 9.19 (d, $J = 4.8$ Hz, 2H, C$_{Tz}$H); 8.74 (t, $J = 5.6$ Hz, 1H, N$_1$H); 8.66 (d, $J = 8.2$ Hz, 2H, C$_{Tz}$H); 8.33 (t, $J = 13.4$ Hz, 1H, C$_{36}$H); 8.13 (d, $J = 8.2$ Hz, 1H, C$_{Tz}$H); 7.83 (q, $J = 4.6$ Hz, 1H, C$_{Tz}$H); 7.79 (s, 2H, C$_{31/41}$H); 7.73 (m, 2H, N$_{23}$H, N$_{12}$H); 7.67 (td, $J = 7.6$ Hz, 2H, C$_{30/42}$H); 7.38 (t, $J = 8.6$ Hz, 2H, C$_{29/43}$H); 6.47 (dd, $J = 13.5, 5.7$ Hz, 2H, C$_{35/37}$H); 4.09 (t, $J = 7.4$ Hz, 1H, C$_{28}$H$_2$); 3.78 (m, 1H, C$_{13}$H); 3.52 – 3.35 (m, 14H, C$_2$/4-9H$_2$); 3.14 – 3.04 (m, 2H, C$_{11}$H$_2$); 2.95 (q, $J = 6.1$ Hz, 2H, C$_{22}$H$_2$); 2.03 (t, 2H, C$_{24}$H$_2$); 1.79 (p, $J = 6.1, 5.6$ Hz, 2H, C$_{3}$H$_2$); 1.72 (m, 2H, C$_{27}$H$_2$); 1.68 (s, 12H, C$_{33/34/39/40}$H$_3$); 1.59 (m, 2H, C$_{19}$H$_2$); 1.54 (m, 2H, C$_{25}$H$_2$); 1.43 (m, 2H, C$_{19}$H$_2$); 1.34 (s, 9H, C$_{16-18}$H$_3$); 1.32 – 1.27 (m, 4H, C$_{21/26}$H$_2$); 1.32 (m, 2H, C$_{20}$H$_2$).

$^{13}$C NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) =174.8, 174.3, 173.9, 171.7, 165.4, 163.2, 162.8, 158.9, 155.2, 142.6, 141.9, 139.6, 137.3, 137.3, 133.7 (C$_{quar}$); 158.9, 158.5 (C$_{Tz}$); 149.7 (C$_{36}$);
128.2, 128.1 (C\textsuperscript{Tz}); 126.2, 126.1 (C\textsuperscript{30/42}); 122.9 (C\textsuperscript{Tz}); 119.8, 119.7 (C\textsuperscript{31/41}); 110.6 (C\textsuperscript{29/43}); 103.3, 102.7 (C\textsuperscript{35/37}); 77.9 (C\textsuperscript{15}); 69.7, 69.6, 69.5, 69.4, 68.3, 68.2, 67.2 (C\textsuperscript{4-9}); 54.2 (C\textsuperscript{14}); 48.8 (C\textsuperscript{32/38}); 43.7 (C\textsuperscript{28}); 38.1 (C\textsuperscript{22}); 36.8 (C\textsuperscript{13}); 35.1 (C\textsuperscript{24}); 31.6 (C\textsuperscript{19}); 29.2 (C\textsuperscript{11}); 28.9 (C\textsuperscript{26}); 28.1 (C\textsuperscript{16-18}); 27.3, 27.1 (C\textsuperscript{33/34/39/40}); 26.6 (C\textsuperscript{27}); 25.7 (C\textsuperscript{21}); 24.9 (C\textsuperscript{25}); 22.7 (C\textsuperscript{20}).

HRMS (ESI-MS): m/z calcd. for C\textsubscript{64}H\textsubscript{84}N\textsubscript{12}O\textsubscript{14}S\textsubscript{2}+: 1308.5671 [M+H]+; found: 1308.5672.

**Tetrazine(mono)-PEG\textsubscript{3}-lys-sCy3-PEG\textsubscript{3}-aminoxy-Boc (68)**

(E)-2-((E)-3-1-(2,2-dimethyl-4,18,26-trioxo-20-((1-oxo-1-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)phenyl)-6,9,12-trioxo-2-azapentadecan-15-yl)carbamoyl)-3,6,9,12,15-penta-oxa-5,19,25-triazahentriacontan-31-yl)-3,3-dimethyl-5-sulfo-3H-indol-1-ium-2-yl)allylidene)-1,3,3-trimethylindoline-5-sulfonate

\[
\begin{align*}
\text{C}_{73}\text{H}_{101}\text{N}_{13}\text{O}_{19}\text{S}_{2} & = 1527.68 \text{ g/mol} \\
\end{align*}
\]

Tz-PEG\textsubscript{3}-lys(NHBoc)-sCy3 \textbf{64} (6.1 mg, 0.0072 mmol, 1 equiv) was dissolved in 500 µL DCM and 500 µL TFA was added. The mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure and the residue was co-evaporated with toluene and ethyl acetate. The crude product \textbf{66} was used without further purification.

The oxyamine linker (0.64 mg, 0.0019 mmol, 1 equiv) was dissolved in 200 µL dry DMF. NMM (0.52 µL, 0.0047 mmol, 2.5 equiv) and pyBOP (1.2 mg, 0.0023 mmol, 1.2 equiv) were added and the mixture was stirred for 10 min at rt in the dark. Tz-PEG\textsubscript{3}-lys-sCy3*TFA \textbf{66} (3 mg, 0.0023 mmol, 1.3 equiv) was added and the mixture was stirred for 1.5 h at rt in the dark. The solvent and NMM were removed under reduced pressure and the product was purified by RP-HPLC (40-60%). Pure product \textbf{68} was obtained in 60% yield.

\[^1\text{H} \text{NMR} (600 \text{ MHz, DMSO-d}_6): \delta (ppm) = 9.20 (dd, J = 1.5, 4.9 Hz, 2H, C\textsuperscript{Tz}); 8.74 (t, J = 5.4 Hz, 1H, N\textsuperscript{1}H); 8.66 (dd, J = 1.6, 8.4 Hz, 2H, C\textsuperscript{Tz}); 8.33 (t, J = 13.3 Hz, 1H, C\textsuperscript{43}H); 8.14 (dd, J}
Experimental Section

$^1$H NMR (600 MHz, DMSO-$d_6$): $\delta$ (ppm) = 174.8, 173.8, 171.6, 171.4, 169.9, 166.2, 166.1, 145.9, 142.9, 139.9, 138.2, 128.2, 128.0, (C$^{\text{quat}}$); 150.1 (C$^4$); 158.5, 133.7, 128.2, 128.1 (C$^T$); 126.0 (C$^{38/48}$); 120.0 (C$^{39/47}$); 110.5 (C$^{37/49}$); 102.6 (C$^{42/44}$); 74.7 (C$^5$); 69.7, 69.3 (C$^{4/9/16-22}$); 67.9 (C$^2$); 66.8 (C$^6$); 52.4 (C$^{30}$); 40.5 (C$^{36}$); 38.2 (C$^3$); 36.8 (C$^{28}$); 35.7 (C$^{32}$); 35.1 (C$^{11}$); 31.8 (C$^{50}$); 29.2 (C$^{3/10/27/29/33/34}$); 28.8 (C$^{35}$); 27.9 (C$^{24-26}$); 27.3 (C$^{40/41/45/46}$).

HRMS (ESI-MS): m/z calcd. for C$_{73}$H$_{102}$N$_{13}$O$_{19}$S$_2$: 1528.6851 [M+H]+; found: 1528.6858.
**Carbohydrate Arrays**

Optimisation of the buffer compositions as well as establishing conditions for immobilisation and detection of glycoconjugates on Nexterion® H slides was performed by O. Baudendistel.[332,428]

**Buffer Preparation**

**Phosphate buffer** (150 mM): A 150 mM phosphate buffer was prepared by dissolving 52.3 g Na₂HPO₄·12 H₂O and 480 mg NaH₂PO₄·2 H₂O in 1 L ultrapure water. The pH was adjusted to 8.5 with NaOH.

**PBST buffer** (150 mM, 137 mM NaCl, 2.7 mM KCl, 0.01 % Tween 20): 4.00 g NaCl and 100.6 mg KCl were dissolved in 500 mL phosphate buffer to give a final concentration of 137 mM NaCl and 2.7 mM KCl. After addition of 50 µL Tween 20, the pH still was at pH 8.5 without any adjustment.

**Dilution buffer** (PBST buffer with 2 mM ethanolamine): 30 µL Ethanolamine were added to 250 mL PBST buffer and the pH adjusted to 8.5 with conc. HCl.

**Deactivation buffer** (PBST buffer with 50 mM ethanolamine): 1.5 mL Ethanolamine were added to 500 mL PBST buffer and the pH adjusted to 8.5 with conc. HCl.

**HEPES buffer**: 4.77 g HEPES, 17.53 g NaCl, and 29.4 mg CaCl₂ were dissolved in 2 L ultrapure water to a final concentration of 10 mM HEPES, 150 mM NaCl, and 0.1 mM CaCl₂. The pH was adjusted with NaOH solution to pH 7.5.

**HEPES wash buffer**: 1.0 mL Tween 20 was added to 1 L HEPES buffer (0.1% v/v). If necessary, the pH was adjusted to 7.5 with HCl or NaOH.

**Generation of Carbohydrate Microarrays (Nexterion H)**

The obtained glycoconjugates were diluted in PBST buffer to final concentrations of 2 mM, 1 mM and 0.5 mM. The glycoconjugate solutions (35 µL each) were transferred into a 384-well plate on ice. Then, microarrays were spotted on succinimidyl ester-activated Nexterion® H glass slides using an automated non-contact array printer Nanoplotter 2 from GeSiM. During array generation the working plate with the glass slides was kept at 10 °C with a cryostat from Lauda and a humidity between 69–71 % with a Humidity Control II from Lucky Reptile connected to a humidifier from Hobby. The samples were transferred in 15 droplets with a final volume of 4 nL to the surface and three replicates per concentration were prepared. After finished spotting, the microarrays were incubated over a NaCl solution (0.5 M) at RT for 90 min in the dark. To block the unreacted succinimidyl ester surface, the arrays were rinsed with ultrapure water and incubated in 50 mL deactivation buffer for 90 min on an orbital shaker. The
glass slides were again rinsed with ultrapure water and washed with MeOH (2 x 50 mL) and ultrapure water (2 x 50 mL) for 1 min each, dried with nitrogen, and stored at RT in the dark. To check the quality of the microarrays, the fluorescence of the immobilised Cy3- labelled glycoconjugates (kindly provided by O. BAUENDISTEL, for molecular structure see Figure 4.16.) was analysed at 532 nm excitation wavelength with a Tecan scanner.
7.1.4. Part III – Investigation of the Oxyamine Ligation

The following derivative was synthesised according to published procedures: GlcNAcNH$_2$[418]

NMR Kinetic Experiments with Glycosyl Amines

Preparation of Deuterated Buffers and Reagents

Deuterated ammonium acetate buffer

To prepare 5 mL buffer, CD$_3$COOND$_4$ (from deuter) was dissolved in 4 mL D$_2$O. Subsequently, the pH was adjusted with CD$_3$COOD or NaOD in D$_2$O (40 % w/v) to the desired value and then D$_2$O was added to reach the final volume of 5 mL. To determine the pH, a pH-meter which had been calibrated in non-deuterated buffers, was used. The instrument displayed a pH* value, which had to be converted to the real pH according to formula (1)[429].

\[ pH = 0.9291 \times pH^* + 0.421 \]  

(1)

Deuterated ammonium acetate buffer containing Oxyamine

This buffer was prepared following the procedure described above with ethoxyamine*HCl added to a final concentration of 50 mM, 100 mM, 180 mM or 250 mM.

Deuterated ammonium acetate buffer containing TSP

This buffer was prepared following the procedure described above with [D$_4$]sodium 3-(trimethylsilyl)propanoate-2,2,3,3 (TSP) added to a final concentration of 2 mM.

Deuterated ammonium acetate buffer containing aniline

This buffer was prepared following the procedure described above with [D$_7$]aniline added to a final concentration of 100 mM.

Deuterated ammonium acetate buffer containing PDA

This buffer was prepared following the procedure described above with [D$_7$]p-phenylenediamine added to a final concentration of 100 mM.

Deuterated potassium phosphate buffer

To prepare 5 mL buffer, KD$_2$PO$_4$ was dissolved in 4 mL D$_2$O. Subsequently, the pH was adjusted with CD$_3$COOD or NaOD in D$_2$O (40 % w/v) to the desired value and then D$_2$O was added to reach the final volume of 5 mL.
**Deuterated potassium phosphate buffer containing Oxyamine**

This buffer was prepared following the procedure described above with ethoxyamine·HCl added to a final concentration of 50 mM, 100 mM, 180 mM or 250 mM.

**Deuterated potassium phosphate containing TSP**

This buffer was prepared following the procedure described above with [D₄]sodium 3-(trimethylsilyl)propanoate-2,2,3,3 (TSP) added to a final concentration of 2 mM.

**Deuterated potassium phosphate containing aniline**

This buffer was prepared following the procedure described above with [D₇]aniline added to a final concentration of 100 mM.

**Deuterated potassium phosphate containing PDA**

This buffer was prepared following the procedure described above with [D₇]p-phenylenediamine added to a final concentration of 100 mM.

**Preparation of glycosyl amines**

The 2-Acetamido-2-deoxy-β-D-glucopyranosyl azide 76 (kindly provided by M. SCHÖWE) was hydrogenated to the glycosyl amine as described in chapter 4.3. Part III – Investigation of the Oxyamine Ligation. Subsequently the catalyst was removed with a syringe filter and the filtrate was aliquoted. For instance, 21 mg azide and 10 mg palladium catalyst were suspended in 2.0 mL MeOH and hydrogenated. After filtration, the mixture was divided in three aliquots (about 400 µL each) into 4 mL glass vials. The solvent was removed under reduced pressure and the aliquots were lyophilized for at least 3 h. The amount of glycosyl amine was determined by weighing the glass vial Tara and additionally dissolving one aliquot in D₂O to determine the concentration against the external TSP standard in a ¹H-NMR spectrum. The crude product was used without further purification.

**Monitoring the Reaction of Ethoxyamine with Glycosyl Amine**

Freshly prepared 2-acetamido-2-deoxy-β-D-glucopyranosyl amine (GlcNAcNH₂) was dissolved in deuterated buffer containing ethoxyamine to the desired final concentration, e.g. 5 mM glycosyl amine and 50 mM ethoxyamine. The reaction mixture was transferred into a 5 mm NMR tube and the reaction was followed by ¹H NMR spectroscopy. Spectra were recorded with 1 scan (with Avance Neo 800 instrument) or 4 scans (with Avance III 600 instrument) every 150 sec and a pulse angle of 30°. A relaxation delay of 20 secs was used, corresponding to more than five times T1 (relaxation time). For integration of ¹H spectra, only well-separated resonances were used. For every spectrum in which the used resonances were not basis line separated, a multiple points basis line correction was performed. The integrals were converted
to concentrations using the known concentration of added standard (TSP). Combined yields of products were plotted against time (logarithmic scale) using Origin 9.8.0.200.

**Monitoring the Reaction of Ethoxyamine with N-Acetylglucosamine (GlcNAc)**

GlcNAc was dissolved in deuterated buffer containing ethoxyamine to the desired final concentration. Then, the experiment was carried out as described in above "Monitoring the Reaction of Ethoxyamine with Glycosyl Amine".

**Monitoring the Hydrolytic Stability of Glycosyl Amine**

Freshly prepared 2-acetamido-2-deoxy-β-D-glucopyranosyl amine (GlcNAcNH₂) was dissolved in deuterated buffer, the reaction mixture was transferred into a 5 mm NMR tube and the reaction was followed by ¹H NMR spectroscopy as described above above “Monitoring the Reaction of Ethoxyamine with Glycosyl Amine”. The amount of remaining glucosyl amine was plotted against time (logarithmic scale) using Origin 9.8.0.200.
7.2. Biological Section

General Methods

AlexaFluor™ 555-labelled streptavidin and Hoechst33342 were purchased from Invitrogen, tetrazine-Cy3 was purchased from Jena Bioscience. Confocal fluorescence microscopy was performed with a Zeiss LSM 880 instrument equipped with a 40x1.4 NA Plan-Apochromat oil immersion objective and a GaAsP detector array for spectral imaging. The obtained data were analysed by using Fiji/Image J software.

Cell culture

The human embryonic kidney cell line 293T (HEK293T cells) was grown in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), 100 units mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin (Gibco). Mycoplasma contamination was negatively tested using the Venor®GeM Classic Kit (Minerva Biolabs). Cells were incubated under carbon dioxide (5 %) in a water-saturated incubator at 37 °C. Cells were diluted every 3 to 4 days by washing with PBS buffer and detaching with trypsin and EDTA.

Human adipose stromal cells (ASCs) were cultivated at Hochschule Reutlingen.

Sugar stock solutions

The sugars were prepared as stock solutions (100 mM) in DMSO and stored at -20 °C. They were freshly diluted into media on the day of the experiment.

Confocal fluorescence microscopy

The wells were coated with 0.01% poly-L-lysine (Sigma) in phosphate-buffered saline (PBS) for 1 h at 37 °C (or overnight at 4°C) and rinsed with PBS. 50 000 cells/well were seeded in 4-well ibiTreat μ-Slides (ibiidi) and allowed to attach overnight. Cells were then incubated with 100 μM of the corresponding derivative (stock solutions of 100 mM in DMSO) for 48 h. DMSO was added as solvent control.

DAinv reaction

HEK293T cells were washed two times with PBS and then treated with Tetrazine–biotin (1-3 h, 100 μM for cyclopropane) at 37 °C. After two washes with PBS, cells were incubated with AlexaFluor™-labeled streptavidin (6.6 μg mL⁻¹) and Hoechst33342 (10 μg mL⁻¹) for 20 min at room temperature in the dark. Cells were washed three times with PBS, and DMEM was added for microscopy.
**Photo-click reaction**

Cells were washed two times with PBS and then treated with 50 μM Tet1-sCy3 or tetrazole-biotin (stock: 50 mM in DMSO). Cells were irradiated for 30 sec with a hand-held UV-lamp (302 nm) which was directly placed on top of the 4-well ibiTreat μ-Slides (ibidi). All cells were washed twice with PBS, cells treated with tetrazole-biotin were incubated with AlexaFluor555™-labeled streptavidin (6.6 μg mL⁻¹) for 20 min at room temperature in the dark and again all cells were incubated with Hoechst33342 (10 μg mL⁻¹) for 20 min at room temperature in the dark. Cells were washed three times with PBS, and DMEM was added for microscopy.
8. Bibliography


Bibliography


Bibliography


9. Appendix

Part I - Metabolic Glycoengineering using the Photoclick Reaction

\[ \text{H NMR (400 MHz, CDCl}_3\text{) of 12.} \]

\[ \text{\textsuperscript{13}C NMR (100 MHz, CDCl}_3\text{) of 12.} \]
$^{1}H$ NMR (400 MHz, CDCl$_{3}$) of 16.

$^{13}C$ NMR (100 MHz, CDCl$_{3}$) of 16.
$^1$H NMR (400 MHz, D$_2$O) of 15.

$^{13}$C NMR (100 MHz, D$_2$O) of 15.
$^1$H NMR (600 MHz, DMSO) of 18.

$^{13}$C NMR (150 MHz, DMSO) of 18.
Part II - Generation and Application of a Glyco-Functionalised Extracellular Matrix

$^1$H NMR (400 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNBeoc.

$^{13}$C NMR (100 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNBeoc.
$^1$H NMR (400 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNPeeo.

$^{13}$C NMR (100 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNPeeo.
$^1$H NMR (400 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNHeoc.

$^{13}$C NMR (100 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNHeoc.
$^1$H NMR (400 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNPtl.

$^{13}$C NMR (100 MHz, CDCl$_3$) of $\alpha/\beta$-Ac$_4$GalNPtl.
$^1$H NMR (800 MHz, D$_2$O) of 62.

$^{13}$C NMR (200 MHz, D$_2$O) of 62.
\[^{1}\text{H NMR (400 MHz, CDCl}\text{3)}\text{ of 57.}\]

\[^{13}\text{C NMR (100 MHz, CDCl}\text{3)}\text{ of 57.}\]
$^1$H NMR (400 MHz, CDCl$_3$) of 58.

$^{13}$C NMR (100 MHz, CDCl$_3$) of 58.
$^1$H NMR (800 MHz, DMSO) of 63

$^{13}$C NMR (200 MHz, DMSO) of 63.
\[^1\)H NMR (600 MHz, DMSO) of 64.\]

\[^{13}\)C NMR (150 MHz, DMSO) of 64.\]
\[^1\text{H NMR (800 MHz, DMSO)}\] of 67

\[^{13}\text{C NMR (200 MHz, DMSO)}\] of 67.
$^1$H NMR (600 MHz, DMSO) of 68.

$^{13}$C NMR (150 MHz, DMSO) of 68.
Part III – Investigation of the Oxyamine Ligation

Comparison hydrolysis rates in different buffered systems

Figure 9.1.: Hydrolysis of GlcNAcNH₂ at pH 5, in 200 mM ND₄OC(O)CD₃ and 200 mM NaOC(O)CD₃.

Figure 9.2.: Hydrolysis of GlcNAcNH₂ at pH 6, in 200 mM ND₄OC(O)CD₃ and 200 mM NaOC(O)CD₃.
Exemplary summating of all three ligation products:
((E)- and (Z) oximes and N-glycosyloxyamine)

Figure 9.3.: Oxyamine ligation reaction of 36 mM GlcNAc with 180 mM ethoxyamine in 500 mM ND₄OC(O)CD₃ at pH 5.

Figure 9.4.: Oxyamine ligation reaction of 36 mM GlcNAcNH₂ with 180 mM ethoxyamine in 500 mM KD₂PO₄ at pH 8.
Figure 9.5.: Photographs of uncatalysed and catalysed oxyamine ligation reactions taken at different time points. **A)** uncatalysed oxyamine ligation in equilibrium, **B)** aniline-catalysed oxyamine ligation in equilibrium, **C)** PDA-catalysed oxyamine ligation in equilibrium, **D)** PDA-catalysed oxyamine ligation directly after start of the reaction, **E)** PDA-catalysed oxyamine ligation after 15 min of reaction time.
Kinetic Treatment of obtained kinetic data of oxyamine ligation reaction
(all calculations were performed by U. Steiner)

For a kinetic treatment of the amine ligation kinetics, the general reaction scheme (see
Scheme 9.1.).

was reduced to the simplest form possible, comprising only the following three species (kinetic
notation in parentheses): aminosugar 75 (A), GlcNAc 70 (B) and oxim product 71 + 72 (P).
The processes connecting these species were assumed to be of pseudo first order kinetics
with pertinent first order rate constants as given in the following scheme:

\[ \begin{align*}
A & \xrightleftharpoons[k_{-1}]{k_1} P \\
A & \xrightleftharpoons[k_{-h}]{k_h} B \\
B & \xrightleftharpoons[k_{-2}]{k_2} P
\end{align*} \]

(S1a-c)

Direct Ligation of GlcNAc

Ligation of GlcNAc just represents a fraction of the general scheme in equation (S1):

\[ B \xrightleftharpoons[k_{-2}]{k_2} P \]  

(S1c)

This is a simple equilibration kinetics and is described by the following time-dependence:

\[ p(t) = \frac{k_2}{k_2 + k_{-2}} (1 - \exp[-(k_2 + k_{-2})t]) \]  

(S2)

The determination of \( k_2 \) and \( k_{-2} \) by exponential fitting is straightforward
Ligation of GlcAcNH₂

There are six rate constants in this case. It should be noted, however, that, for thermodynamic consistence, these cannot be chosen independently because the three chemical equations represent a cyclic reaction system:

\[ A \rightleftharpoons P \rightleftharpoons B \rightleftharpoons A \]

and the equilibrium constant for one half cycle: \( A \rightleftharpoons P \rightleftharpoons B \) must be equal to the equilibrium constant for the other half cycle: \( B \rightleftharpoons A \). This requirement leads to the following equation for \( k_\text{h} \)

\[
k_\text{h} = k_\text{h} \frac{k_2 k_{1\text{h}}}{k_1 k_2}
\]  

The kinetic processes of eq. (S1) are represented by the following system of first order linear differential equations, wherein \( a, b \) and \( p \) denote the concentrations of the species \( A, B \) and \( P \), respectively:

\[
\begin{align*}
\dot{a}(t) &= -k_1 a(t) - k_2 a(t) + k_{-3} b(t) + k_{-3} p(t) \\
\dot{b}(t) &= k_1 a(t) - k_2 b(t) - k_{-3} b(t) + k_{-2} p(t) \\
\dot{p}(t) &= k_1 a(t) + k_2 b(t) - k_{-3} p(t) - k_{-2} p(t)
\end{align*}
\]

The general solution of this system of linear differential equations can be represented as a sum of a constant and two exponential functions. If the rate constants of the two processes from \( A \) to \( P \) and from \( B \) to \( P \) are sufficiently different a diagram of the product concentration versus a log(r) shows a double step functions with a first plateau representing the product yield from \( A \) directly, while the hydrolysis product \( B \) is still unreacted. It is then followed by a step to a second plateau representing the total product yield. If \( k_1 \) is not significantly greater than \( k_2 \), an intermediate plateau will not be visible. In some cases, \( k_2 \) may be so small that the reaction seems to stop on the first plateau.

To solve the equations (S2) the software package Mathematica\(^{[430]}\) was used. Finding the best fits of the rate constants was facilitated by using the graphical interactive Manipulate function. A working example is given in Figure 9.6.

First guesses of the rate constants are taken in descending order of the size. In Fig. Figure 9.6.A, the value of \( k_1 \) is set in such a way as to achieve best agreement in the beginning of the ascending part of the product curve. This leads to an overshooting in the later part and the plateau of the product curve. Adjusting the value of \( k_0 \) (Figure 9.6.B) can settle the second part of the rise of the product curve including its first plateau. There is a further increase of the product curve from the maximum to the final equilibrium value. Now, the value of \( k_2 \) is adjusted
such that the time of the second inflection point of the product curve is met (Figure 9.6.C).

However, this leads to a further increase in the product yield. The final adjustment is done using $k_1$ and $k_2$. Their absolute values cannot be fixed unambiguously. To achieve the correct final level $Y$ of the product yield, the two rate constants must fulfill the following linear correlation:

$$
k_{-2} = k_2 \frac{1-Y}{Y} - \frac{k_2}{k_1} k_{-1}
$$

(S4)
Figure 9.6: Stages of graphical determination of rate constants (case glycosyl amine in Figure 4.22.B) For explanation cf. text. Rate constants are given in units of h⁻¹.
Thus, one of them can be chosen rather freely. For the example given, the relation between $k_2$ and $k_1$ is shown as a diagram in Figure 9.6. If $k_1$ is chosen close to zero, the rise kinetics to the second plateau remains determined by $k_2$. If, however, $k_1$ reaches the order of $k_2$, the second rise becomes faster (see Figure 4.23B). Thus, we fixed $k_1$ to about 1/10 of the value of $k_2$. The corresponding values of $k_1$ and $k_2$ are listed in Table 9.1/9.2. together with their boundaries defined by equation (S4).

**Ligation of GlcAcNH₂ – Extended Reaction System**

In one case (reaction of 5 mM GlcNAcNH₂, 10 equiv. ethoxyamine, pH 5) there is a third inflection point in the kinetic product curve. Such a behavior cannot be represented by a function with two exponentials only, but needs a third exponential. The minimum requirement of the kinetic scheme is a fourth species X. This species must be produced from the ligation product. Therefore, we extended the reaction scheme as follows

\[
\begin{align*}
A & \xrightarrow{k_1} P \\
A & \xleftarrow{k_{-1}} B \\
B & \xrightarrow{k_2} P \\
P & \xleftarrow{k_{XP}} X
\end{align*}
\]

with the pertinent system of differential equations:

\[
\begin{align*}
\dot{a}(t) &= -k_1 a(t) - k_{hA} a(t) + k_{-h} b(t) + k_{-1} p(t) \quad (S6a) \\
\dot{b}(t) &= k_1 a(t) - k_{-h} b(t) + k_2 b(t) + k_p p(t) \quad (S6b) \\
\dot{x}(t) &= k_{XP} p(t) - k_{XP} x(t) \quad (S6c) \\
\dot{p}(t) &= k_1 a(t) + k_2 b(t) - k_{-1} p(t) - k_{-2} p(t) - k_{pX} p(t) + k_{XP} x(t) \quad (S6d)
\end{align*}
\]

A curve fit based on this mechanism is shown in Figure 9.7, given together with the values of the rate constants and the triexponential fit-function. There are three rate constants (time constants):

(i) 10.5 h⁻¹ (5.7 min), (ii) 0.60 h⁻¹ (1.7 h), (iii) 0.031 h⁻¹ (32 h) corresponding (i) to the formation of the product P from A (GlcNAcNH₂) and, simultaneously, of some amount of the hydrolysis product B (GlcNAc), (ii) to the formation of an equilibrium amount of X from P and (iii) to the formation of P, with an equilibrium concentration of X, from B.

Regarding the rate parameters, we note that $k_1$ can be varied in a wide range without changing the product yield. However the final increase of the signal is shifted to shorter times (i.e. the
third time constant becomes shorter) as $k_1$ approaches the order of magnitude of $k_2$. A value of $k_1 = 0.001 \text{ h}^{-1}$ does not yet change the kinetics noticeably, but seems of plausible order of magnitude (as compared to $k_2$ and $k_h$).

Speculating about the chemical nature of the form X, that is obviously not visible by a distinct NMR signal, we suggest an addition of the reagent oxyamine to form the aminal (see Scheme 4.13.).

![Figure 9.7: Fit of the product formation kinetics shown in Figure 4.22.A for the ligation reaction of GlcNAcNH₂. The fit is based on the mechanism (S5) with the system (S6) of kinetic equations.](image_url)
Table 9.1.: Rate constants in units of h\(^{-1}\) for oxyamine ligation reactions with sugar concentrations of 36 mM and 5 equiv of ethoxyamine to Figure 4.21. in Results and Discussion Part III.

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Table 9.2.: Rate constants in units of $h^{-1}$ for oxyamine ligation reactions with sugar concentrations of 5 mM and 10 equiv of ethoxyamine to Figure 4.22. in Results and Discussion Part III.

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$^{(1)}$ In this case, kinetics shows an intermediate minimum (dip) that is incompatible with the simple reaction scheme. Data refer to fit with an extended kinetic model, involving a side-product X in equilibrium with the products.
Kinetics of Hydrolysis

Most of the kinetics at pH 5 – 7 are not monoexponential, indicating that the process is not a one-step reaction. During the first 10 – 30% of hydrolysis, the kinetics is much faster than for the rest. Thus, except for the case of pH 5, biexponential fits were employed. The obtained fits and parameters are given in Figure 9.8.

![Figure 9.8: Data and fit curves for GlcNAcNH₂ hydrolysis at various pH. Except for pH 5, the curve fits are biexponential. Beyond the given time limits, the curves decay monoexponentially with the smaller of the two exponential decay constants.](image)