Targeted diazotransfer to proteins
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APPENDIX

Summary
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6.1 SUMMARY

The research presented in this thesis was conducted at the Chemical Biology workgroup of the Stratingh Institute for Chemistry at the University of Groningen. The name of this workgroup was given throughout the four years of conductance of the PhD thesis work. It was changed from the former name Bio-organic Chemistry workgroup. This name change is exemplary for a general trend observed in the natural sciences, aiming at a larger focus on interdisciplinarity in research but it also takes account of an increased appreciation chemists have shown towards understanding the living world on a molecular level by using a chemist’s mind-set and building up on the established methodologies. On the bright side of this trend stands the creation of an innovative research environment and the invention of several novel strategies to answer nagging questions underlying the molecular mechanisms of what may constitute life itself.

A main pillar in the early years of this new interdisciplinary research field has been the transfer and development of methodologies, or in other words, the appropriation of the molecular tools necessary to answer the underlying mechanistic questions. One of the great innovations of this crucible of disciplines is bioorthogonal chemistry. It is based on the idea that certain chemical entities, even though well-known to the studied chemist, simply do not occur in any of the organisms known to man. In some cases, these xenobiotic chemical groups come in pairs of reaction partners that react selectively with each other but are inert to the plethora of other chemical entities present in the context of a chemical environment found inside a cell. These pairs are what ultimately enables bioorthogonal chemistry.

The azide functionality as a reaction partner constitutes one of such bioorthogonal chemical groups. It can be seen as a chemoselective handle in complex media that reacts smoothly with terminal or strained alkynes (Sharpless-Meldal, Bertozzi), or certain phosphines and phosphites (Staudinger type). It is essentially inherent to the nature of this strategy that the xenobiotic groups do not occur in the biological context, in which they are thought to be applied for further studies. Therefore, one of the main challenges in the method development for using bioorthogonal chemistry is the instalment of such groups into biomolecules. The idea behind the here presented research is to introduce the azide chemically into proteins by means of small molecule probes. Small molecule chemical probes are excellent tools to target proteins within complex experimental settings. Due to the nature of many protein classes, especially enzymes, to bind chemical ligands selectively, a reactive probe can make use of a selectivity- and efficiency-enhancing proximity effect to modify the biomolecule it is targeting. If one end of the probe binds with a ligand to the protein of interest, the other end of the probe can modify its target chemically. In the case of the probes applied
in this thesis’ research, the diazotransfer reagent imidazole-1-sulfonyl azide is tethered to the directing group moiety (i.e. the ligand). The thus obtained probes will enable a targeted diazotransfer reaction to primary amines within proteins. These primary amines occur on lysine side chains and the protein’s N-terminus. The amines are converted into azides via diazotransfer upon probe binding.

The aim of the introduction (Chapter 1) is to give the reader the ability to integrate the presented research (Chapters 2 - 5) into a broader historical context. It describes the emergence of the research field chemical biology as an independent entity among the classical natural science disciplines of physics, chemistry and biology. The main focus is stressed on two important concepts that have shaped chemical biology and at the same time still contribute to its rapid transformation. These two concepts are bioorthogonal chemistry and the methodology for the introduction of xenobiotic groups into all classes of common biomolecules, often to enable the implementation of the former to complex biological systems and research questions.

In Chapter 2 the proof-of-concept of the new strategy for the selective and site-specific introduction of a bioorthogonal handle into proteins is demonstrated. Based on the diazotransfer reagent imidazole-1-sulfonyl azide a probe was developed. The design of the probe is based on the tethering of this reagent to the ligand moiety biotin. With the so obtained small molecule probe DtBio, different biotin binding proteins could be equipped with the azide functionality. This example introduces a novel class of protein labelling probes, the targeted diazotransfer reagents. In this sense the probe is a combination of the concept of proximity enhanced protein labelling and bioorthogonal chemistry for protein manipulation in complex biological settings. Besides the well-established (strept) avidin-biotin system, the membrane located biotin binding protein BioY, an S-component of the ECF small molecule membrane transporter complex, was modified with the azide and visualised via fluorescence.

In Chapter 3 the strategy of the targeted diazotransfer method for protein labelling is extended to an analytical method suitable for target deconvolution. This approach is based on chemical enrichment of azide bearing molecules via copper catalysed alkyne-azide cycloaddition (CuAAC), employing an immobilized cleavable linker. The linker was synthesized around a triazene motif. The approach is combined with modern tandem mass spectrometry for the subsequent protein analysis. The capture-and-release strategy with the bifunctional linker was coined clinker pull down (clinker: a clickable and cleavable linker). In this way diazotransfer sites on proteins can be identified unambiguously on the peptide level. The combination of the target selective diazotransfer reaction via the probe DtBio with chemical enrichment of the azide bearing peptides originating from an enzymatic digest of
the modified protein allows for precise and rapid identification of the modification site as demonstrated for streptavidin and a new target of the probe DtBio, the biotin ligase BirA. With this tool in hand, not only target identification of novel ligands/inhibitors will become feasible in the future, but also the idea of multiplexing experiments, making use of stable isotope labelling of the clinker fragment is at hand.

In Chapter 4 the targeted diazotransfer concept is transferred to a second probe-protein pair. The zinc containing model protein carbonic anhydrase II was targeted by a probe bearing the benzenesulphonamide moiety as ligand. A set of probes, termed DtBSu-n, was used to study the probe efficiency and selectivity to its respective protein target in reaction mixtures like cell lysates. A focus is put on structural aspects of the probe-protein interaction, which is supported by data obtained in experiments with fluorescence labelling, tandem mass spectrometry, also involving the previously introduced clinker tool (Chapter 3), and further corroborated with structure elucidation via X-ray crystallography of the probe-protein complex. The combined data of the mass spectrometry experiments and the protein X-ray structures provide evidence for a second probe binding site on the N-terminal end of the protein, as has been discussed previously in the literature. Additionally, the metal catalyst that is involved in the diazotransfer reaction was evaluated more thoroughly. The obtained results suggest the use of zinc as a viable and more benign alternative to copper for the application of the diazotransfer reaction in more complex systems, especially when transferred to cell culture experiments in the future.

In Chapter 5 a concept is introduced that goes towards the development of protein labelling probes with a new strategy. This strategy is based on the modular construction of the probe molecule that allows for a more rapid production of targeted protein probes. By combining hydrazide- or alkoxamine-bearing ligand molecules with aldehyde-bearing reactive groups in solution, probes can be prepared in a combinatorial manner just prior to protein labelling applications. This approach potentially allows for a rapid screening of the best protein-of-interest – protein-labelling-strategy combination. The modular nature of this approach allows for the construction of probes based on the diazotransfer protein labelling strategy with different ligand combinations but it can be expanded to other covalent protein labelling strategies, as well. This technology will lead to a quick initial screening for novel protein targets.

The presented work demonstrates how the focus on one protein labelling strategy can lead to the construction and thorough testing of several useful probes; a task that easily fills four years of research in the lab. The gained insights further aim at the development of technologies based on targeted diazotransfer probes. The methodological character of the research presented, opens up doors for applications to answer more challenging biological
questions, for instance, in the explanation of observed phenotypes in cell culture scans. A first step towards this goal would be thus the transfer of the targeted diazotransfer method to cell culture experiments. A step which has already been initiated by the testing of alternative to and less cytotoxic catalysts than copper. The combined evidence collected in Chapter 4, hinting at a second ligand binding site in carbonic anhydrase II, which was already postulated and divisively discussed in literature, was a truly serendipitous finding. It underpins the benefits of the collection of additional, complementary data sets and the careful analysis of the obtained results despite a seemingly straight-forward initial interpretation. Such a drive should always be given room during a focused and stringent PhD research program.
6.2 SAMENVATTING

Het onderzoek in dit proefschrift werd uitgevoerd in de Chemische Biologie groep van het Stratingh Instituut voor Chemie aan de Rijksuniversiteit Groningen. Voorheen had de afdeling de naam Bio-organische chemie, maar deze is tijdens het vierjarige onderzoekstraject veranderd. Deze naamsverandering is een van de vele voorbeelden van de trend in de natuurwetenschappen om interdisciplinair onderzoek te stimuleren. Daarnaast toont het ook de vergrote waardering van chemici voor het begrijpen van de natuur op moleculair niveau. Als gevolg van deze trend ontstond een innovatieve onderzoeksomgeving die als doel heeft het ontwikkelen van nieuwe technieken die gebruikt kunnen worden om prangende vragen betreffende moleculaire mechanismen die de basis vormen van het leven te beantwoorden.

Een hoeksteen van deze nieuwe interdisciplinaire benadering was het overbrengen van kennis over en het ontwikkelen van chemische methoden die gebruikt kunnen worden door levenswetenschappers. Met andere woorden, het verschaffen van de moleculaire gereedschappen die nodig zijn om de onderliggende mechanistische vragen te beantwoorden. Een van deze nieuwe methodologieën is bioorthogonale chemie. Het is gebaseerd op het idee dat bepaalde chemische functionele groepen, ondanks welbekend bij chemici, simpelweg niet voorkomen in de natuur. In sommige gevallen komen deze xenobiotische chemische groepen voor in koppels van reactiepartners die selectief met elkaar reageren, maar chemisch inert zijn voor de vele andere processen in de cel. Deze koppels maken uiteindelijk het toepassen van bioorthogonale chemie mogelijk.

De azide is een voorbeeld van een dergelijke bioorthogonale chemische groep. Ze kan gezien worden als een chemoselectief handvat in complexe media die probleemloos reageert met eindstandige alkynen, alkynen met hoge ringspanning en met fosfine reagentia. Inherent aan de strategie is dat xenobiotische groepen niet in de specifieke biologische context voorkomen. Daarom is een van de grote uitdagingen in bio-orthogonale chemie het inbouwen van dergelijke groepen in biomoleculen. Het idee achter het in dit proefschrift beschreven onderzoek is het chemisch introduceren van een azide groep aan eiwitten door het gebruik van een kleine moleculaire probes. Dergelijke probes zijn uitermate geschikt om specifieke eiwitten te bestuderen in een complexe experimentele context. Vele eiwitten, maar vooral enzymen, binden selectief chemische liganden selectief en dit kan gebruikt worden voor de om een reactieve groep in de nabijheid van een eiwit te brengen. Door een probe te ontwikkelen met aan een uiteinde van een ligand en het andere uiteinde een reactieve groep kunnen doeleiwitten heel specifiek worden veranderd. In het geval van het onderzoek in dit proefschrift is gebruik gemaakt van het diazo-transfer reagens imidazole-1-
sulfonyl azide, welke gekoppeld is aan een sturende groep (zoals een ligand). De verkregen probes zullen het mogelijk maken een specifieke diazo-overdracht plaats te laten vinden van de probe naar primaire aminen in de eiwitten, waarbij het amine wordt omgezet in een azide. Deze primaire aminen komen voor op lysine zijketens en de N-terminus van eiwitten.

Het doel van de introductie (hoofdstuk 1) is om de lezer het vermogen te geven om het gepresenteerde onderzoek (hoofdstukken 2 - 5) in een bredere historische verband te plaatsen. Het beschrijft het ontstaan van het chemische biologie onderzoeksveld als een onafhankelijke stroming tussen de klassieke natuurwetenschappen zoals natuurkunde, scheikunde en biologie. De focus is gelegd op twee belangrijke concepten die de chemische biologie vorm hebben gegeven en op hetzelfde moment nog steeds bijdragen aan de snelle ontwikkeling. Deze twee concepten zijn bioorthogonale chemie en de methodologie voor het introduceren van xenobiotische groepen in alle classes van algemene biomoleculen, vaak om het eerste concept te implementeren in complexe biologische systemen.

In hoofdstuk 2 wordt een nieuwe strategie voor het selectief en specifiek introduceren van een bioorthogonaal handvat in eiwitten beschreven. Gebaseerd op het diazo-transfer reagens imidazole-1-sulfonyl azide werd een nieuwe probe ontwikkeld. Het ontwerp van de probe is gebaseerd op het verbinden van dit reagens met biotine als ligand. Met de daaruit verkregen probe DtBio werden verschillende biotine bindende eiwitten gefunctionaliseerd met de azide groep. Dit voorbeeld introduceert een nieuwe klasse van eiwit markerende probes; de ligand-gestuurde diazo-transfer reagentia. In deze context is de probe een combinatie van het ligand gestuurde eiwit labeling en bioorthogonale chemie voor eiwit manipulatie/aanpassing in complexe biologische context. Naast het alom bekende (strept)avidine-biotine systeem, kon het in het membraan voorkomende biotine bindende eiwit BioY (een S-component van het extracellulaire vocht transport complex) met de azide worden gemodificeerd en gevisualiseerd door middel van fluorescentie.

In hoofdstuk 3 wordt de strategie van de ligand gestuurde diazo-transfer methode voor eiwit labelling uitgebreid tot een analytische methode die geschikt is voor identificatie van eiwitten. Deze benadering is gebaseerd op chemische verrijking van azide dragende moleculen via koper gekatalyseerde alkyn-azide cycloadditie (CuAAC) met afsluitsbare alkyn gefunctionaliseerde vaste dragers. Om de verrijkte peptiden af te splitsen werd er een triazen motief ingebouwd tussen de vaste drager en het alkyn. De methode is gecombineerd met moderne tandem massa spectrometrie voor de eiwit analyse. De ontwikkelde strategie werd clinker pull down genoemd. Met deze methode kunnen diazo-transfer plekken op het eiwit op peptide niveau geïdentificeerd worden. De combinatie van een eiwit selectieve diazo-transfer reactie met de DtBio probe en een chemische verrijking van de azide bevattende peptides maken het mogelijk om precies en snel de modificatieplek
te identificeren. De strategie werd met succes gebruikt op het in hoofdstuk 2 beschreven doelwit streptavidin en een nieuw doelwit van de DtBio probe, namelijk the biotine ligase BirA. Niet alleen maakt de beschreven techniek identificatie van de eiwitten die aan andere liganden/remmers binden mogelijk, maar door gebruik te maken van stabiele isotopen in het clinker fragment kunnen zouden meerdere samples tegelijkertijd moeten kunnen worden geanalyseerd.

In hoofdstuk 4 werd het diazotransfer concept toegepast op een tweede probe-eiwit koppel. Door gebruik te maken van een benzeensulfonamide groep als ligand konden probes ontwikkeld worden voor het zink bevattende model eiwit carbonaatdehydratase II. Een set van verschillende probes, genaamd DtBSu-n, is gebruikt om de efficiëntie en selectiviteit van de probes voor het specifieke eiwit te bestuderen. Er werd met name geconcentreerd op de structurele eigenschappen van de probe-eiwit interacties door middel van experimenten met fluorescente labelling, tandem massaspectrometrie en structuuropheldering met Röntgendiffractie. De gecombineerde data van de massaspectrometrie experimenten en de eiwit Röntgendiffractie geven bewijs voor een tweede probe bindingsplek aan de N-terminus van het eiwit, zoals eerder in de literatuur is voorgesteld. Daarnaast is het effect van de katalysator in de diazotransfer reactie verder bestudeerd. De verkregen resultaten tonen aan dat zink een goed een niet-toxisch alternatief voor koper is in complexere systemen en deze condities zullen in de toekomst gebruikt kunnen worden met celcultures.

In hoofdstuk 5 wordt een nieuw concept geïntroduceerd voor de ontwikkeling van eiwit labellende probes. Deze nieuwe strategie is gebaseerd op het bouwen van probes welke bestaan uit verschillende fragmenten. Dit zou het mogelijk maken om binnen korte tijd nieuwe eiwit probes te maken. Door hydrazide- of alkoxyamine-dragende liganden te combineren met aldehydes zouden probes voor het labelen van eiwitten in een combinatoriële manier gemaakt kunnen worden. Deze aanpak zou tot een snelle identificatie kunnen leiden van de beste ligand-reactieve groep combinatie voor een interessant eiwit. De modulaire basis van deze methode maakt het mogelijk om probes te maken gebaseerd op de diazo-transfer eiwit labelling strategie met verschillende ligand combinaties, maar het zou ook tot andere strategieën uitgebreid kunnen worden.

Het hierin gepresenteerde werk laat zien hoe de focus op één eiwit-labellings strategie kan leiden tot de bouw en het veelvuldig testen van verschillende nuttige probes, een taak die makkelijk vier onderzoeksjaren in het laboratorium kost. De verkregen inzichten richten zich verder op de ontwikkeling van technologieën gebaseerd op diazotransfer probe. De nieuwe onderzoeksmethodologieën openen de deur voor toepassingen om ingewikkelde biologische vragen te beantwoorden, bijvoorbeeld de verklaring van verschillende fenotypen. Het in hoofdstuk 4 verkregen bewijs dat zinspeelt op een tweede ligand bindingsplek, wat al was
gepostuleerd en veelvuldig besproken in de literatuur, was een toevallige vinding en laat zien hoe belangrijk nieuwschierigheidgedreven onderzoek is als deel van een gericht PhD onderzoeksprogramma.
Appendix

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Apologies for being incomplete

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