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Solid-State NMR Analysis of Polysaccharide Hydrogels, Biomimetic Extracellular Matrices, and Tissues

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CHAPTER 6

Summary and Perspectives

Solid-state NMR (ssNMR) is a powerful technique for analyzing intricate systems like multicomponent hydrogels and intact tissues at the atomic level. Throughout this thesis, I aimed to leverage ssNMR to examine HMW-HA hydrogels, particularly focusing on their interactions with extracellular matrix (ECM) components and HA cell surface receptors. Additionally, the ssNMR techniques were applied to investigate the composition and structural properties of intact tissues from various animal organs, aiming to elucidate the tissue clearance induced tissue shrinkage.

Method development to produce isotopically enriched HMW-HA

Chapter 2 of this thesis focuses on developing of a new method for producing isotopically enriched HMW-HA and its subsequent characterization using different spectroscopy and chromatography techniques. HA is a polysaccharide found abundantly in the ECM of vertebrate cells, and its properties make it highly attractive for biomedical applications, particularly in hydrogels. However, despite its importance, understanding the molecular underpinnings of HA structure-function relationships, especially for HMW-HA, has been limited by technical challenges in analysis of such long polysaccharides. Magic angle spinning (MAS) ssNMR, emerged as a valuable tool for analyzing intact HMW-HA samples. Yet, studying naturally abundant (unlabeled) HMW-HA polysaccharides presents limitations for advanced ssNMR experiments. To address this gap, the chapter described efforts to produce isotopically enriched HMW-HA using bacterial host systems. By incorporating ^{13}C and ^{15}N isotopes into HA during bacterial fermentation, labeled HMW-HA samples were obtained. Optimization of fermentation conditions, including culture volume and aeration, was conducted to maximize HA yield and minimize the presence of contaminants, such as lactic acid. The produced isotopically enriched HMW-HA samples were characterized using various analytical techniques, including liquid chromatography mass spectrometry (LC-MS), gel permeation chromatography (GPC) and solution-state NMR spectroscopy. LC-MS analysis confirmed the metabolic incorporation of isotopes into the HA structure, with partial and full labeling observed. For the first time, we've developed a method to produce isotopically enriched HMW-HA and characterize it using ssNMR to get valuable insights into the structural and functional properties of HA. This approach opens new avenues for studying HA-based materials in biomedical and industrial applications, facilitating advanced analyses of HA interactions with receptors and other ECM components as studied in chapters 3 and 4.

Characterization of HMW-HA hydrogels with biomimetic environmental conditions

Chapter 3 provided insights into the conformational dynamics of HMW-HA. The study delved into probing the conformational dynamics of HMW-HA under various hydration conditions and within an ECM-like environment using advanced MAS ssNMR. ^{13}C-labeled HMW-HA allowed for atom-specific analysis, revealing multiple conformations, particularly in the N-acetylglucosamine (GlcNAc) moiety, under different hydration levels. Notably, preferential hydration of specific parts of the HA polymer was observed. Additionally, the study extends its analysis to HMW-HA within an ECM-like environment, elucidating changes in flexibility and conformation induced by ECM interactions and hydration. The HMW-HA-ECM complex exhibited distinct mechanical properties compared to pure HMW-HA. Further analysis highlighted the sensitivity of GlcNAc carbons to changes in hydration and ECM interactions, with increased dynamics observed in the GlcNAc moiety. Interestingly, GlcNAc carbons C4 and C6 displayed structural heterogeneity, potentially reflecting regions crucial for HA recognition by binding proteins. Overall, the combination of ^{13}C-enrichment and MAS NMR offered atom-resolution insights into HA flexibility and its capacity to undergo structural changes in response to hydration and ECM interactions, with implications for biomedical and pharmaceutical applications. The findings suggest the significance of GlcNAc moiety in responding to environmental cues and its implications for HA interactions with binding proteins.

Chapter 4 of the thesis provided a comprehensive examination of the interaction dynamics between HMW-HA and HA binding proteins such as CD44, with a particular focus on their response to enzymatic degradation by hyaluronidase enzyme. Through multidimensional ssNMR employing ^{13}C-labeled HMW-HA and unlabeled CD44-HABD, the study elucidates the structural and dynamic changes in HMW-HA, induced by their binding. Notably, the interaction makes HMW-HA slightly more rigid, with specific carbons displaying decreased mobility, as revealed by relaxation properties analysis. Subsequent in-situ degradation experiments unveil that there might be a competition between hyaluronidase and CD44 for binding sites on the HA chain, resulting in slower and less efficient degradation in the presence of CD44-HABD. The ssNMR experiments corroborate these findings, highlighting the protective role of CD44 in enzymatic cleavage sites. Moreover, the hydrogel to liquid transition of HA,

monitored via ssNMR, demonstrated distinct digestion rates between HMW-HA alone and in complex with CD44-HABD, further emphasizing the role of CD44 in modulating enzymatic cleavage efficiency. This detailed analysis contributes to a deeper understanding of biomolecular interactions within the ECM and holds potential implications for the development of therapeutic interventions targeting HA-proteins interactions and degradation related pathologies.

ssNMR applications on mechanism of tissue shrinkage during tissue clearance

Chapter 5 presents an application of the techniques developed and applied in Chapters 2 through 4. In this chapter, I applied many of the same experimental approaches used in HA studies, performing in situ analyses on tissue biopsies. This chapter is crucial in linking the thesis together, demonstrating how the methodologies used for HMW-HA hydrogels can be applied to more complex systems, such as tissues. We investigated hydration, dehydration, and lyophilization behaviors, focusing on dynamics that may play a crucial role in these processes. The application of these techniques to tissues lays a strong foundation for future research, where combining HA labelling with in-situ tissue and cellular analysis could provide insights into the HA-ECM of tumor tissues.

In summary chapter 5 presents a detailed study on understanding the mechanisms underlying tissue shrinkage during tissue clearance procedures. The study employs ssNMR techniques to analyze various tissues and explore how their compositions change during the clearance process, shedding light on the molecular mechanisms of tissue clearing and associated shrinkage issues. The study begins with a comparative analysis of biomolecules in intact tissues biopsies derived from various sources, including chicken breast, cow loin meat, sheep liver, sheep kidney, and sheep tail fat. Through 1D ssNMR experiments, the relative content of proteins, carbohydrates, and lipids in these tissues were assessed, revealing distinct profiles among the samples. Subsequently, the focus shifts to examining the impact of tissue clearance, particularly using the BABB (3D imaging-compatible solvent-based tissue clearing) method, on chicken breast tissue. Through a series of ssNMR experiments, changes in lipid and protein content and dynamics were elucidated. The results demonstrated a substantial reduction in lipid content post-clearance, while protein dynamics are notably affected, with proteins exhibiting increased mobility in the presence of BABB. Further analysis using water-edited ssNMR revealed alterations in water-protein interactions induced by BABB, with implications for tissue hydration and protein stability. Interestingly, differential responses among protein types to the clearance

process were observed, suggesting a complex interplay between BABB, proteins, and water molecules. Overall, the research provides insights into intact tissue characteristics and highlights the multifaceted effects of tissue clearance on mechanism of tissue shrinkage and the consequences of tissue clearance methods for biomedical research.

Perspectives

The exploration of HA hydrogels opens a myriad of opportunities across various domains, with particular emphasis on 3D tissue culture, drug delivery systems, and insights into cellular interactions at the atomic scale. These applications not only hold promise for advancing our understanding of disease mechanisms but also offer avenues for innovative therapeutic interventions. In the area of 3D tissue culture, the utilization of isotopically enriched HMW-HA hydrogels presents a sophisticated approach to monitor pathological and physiological activities. By leveraging advanced ssNMR techniques, insight into the interactions between cells and their microenvironment within these 3D hydrogels can be obtained with atomic resolution. This capability holds significant potential for elucidating complex processes such as cancer cell migration, progression, and intercellular interactions, thereby deepening our understanding of tumor behavior in both health and disease contexts [1,2]. Moreover, the role of HA in drug delivery and cancer therapy is well-established [3,4], yet the mechanisms governing drug release and diffusion remain incompletely understood [5–7]. Combining ssNMR with ^{13}C-enriched HA hydrogels offers a promising approach to deciphering these intricate processes, could provide valuable insights into how drugs interact with targeted cells or systems within the body. The interplay between HA and cell surface receptors, as well as its degradation mechanisms, holds critical implications for diseases such as cancer and arthritis [1,2]. Leveraging the methodologies developed within this thesis, can be used delve deeper into understanding the dynamic interactions between ECM components, HA binding proteins and HA degradation. Such insights may pave the way for the development of targeted interventions aimed at modulating these interactions for therapeutic benefit.

The developed solid-state nuclear magnetic resonance (ssNMR) toolkit for studying intact tissue presents various potential applications in pathological and disease contexts. However, despite the potential offered by ssNMR techniques, challenges persist, including the intrinsic low sensitivity of NMR for unlabeled tissues and the limited resolution for cellular studies [8]. Recent advancements such as magic angle spinning dynamic nuclear polarization (MAS-DNP) hold promise

for overcoming these limitations [8,9]. By hyperpolarizing nuclear spins, MAS-DNP enhances sensitivity, enabling the characterization of unlabeled intact tissue materials or native cells with unprecedented detail and accuracy. Combining these technological advancements with existing toolkits may advance research in unlabeled intact tissue biopsies, offering new opportunities in fundamental science and clinical applications.

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Nederlandse Samenvatting

Solid-state NMR (ssNMR) is een krachtige techniek om ingewikkelde systemen zoals multicomponent hydrogels en intacte weefsels op atomair niveau te analyseren. In dit proefschrift heb ik ssNMR gebruikt om hyaluronzuur hydrogels met een hoog moleculair gewicht (HMW-HA) te onderzoeken, waarbij ik me vooral heb gericht op hun interacties met componenten van de extracellulaire matrix (ECM) en receptoren van het HA-celoppervlak. Daarnaast werden de ssNMR-technieken toegepast om de samenstelling en structurele eigenschappen van intacte weefsels van verschillende dierlijke organen te onderzoeken, met als doel het ophelderen van de door weefselopruiming geïnduceerde weefselkrimp.

Methodeontwikkeling voor toepassingen op HA-hydrogels

Hoofdstuk 2 van dit proefschrift richt zich op de ontwikkeling van een nieuwe methode voor de productie van isotopisch verrijkt HMW-HA en de daaropvolgende karakterisering met behulp van verschillende spectroscopie- en chromatografietechnieken. HA is een polysaccharide dat overvloedig voorkomt in het ECM van gewervelde cellen, en zijn eigenschappen maken het zeer aantrekkelijk voor biomedische toepassingen, met name in hydrogels. Echter, ondanks het belang van HA, is het begrijpen van de moleculaire onderbouwing van HA structuur-functie relaties, vooral voor HMW-HA, beperkt door technische uitdagingen in de analyse. Magic angle spinning (MAS) ssNMR kwam naar voren als een waardevol hulpmiddel voor het analyseren van intacte HMW-HA monsters. Het bestuderen van natuurlijk overvloedige (ongelabelde) HMW-HA polysacchariden heeft echter beperkingen voor geavanceerde ssNMR experimenten. Om deze lacune aan te pakken, beschrijft het hoofdstuk inspanningen om isotopisch verrijkt HMW-HA produceren met behulp van bacteriële gastheer systemen. Door het opnemen van 13C en 15N isotopen in HA tijdens bacteriële fermentatie, werden gelabelde HMW-HA monsters verkregen. De fermentatiecondities, waaronder kweekvolume en beluchting, werden geoptimaliseerd om de opbrengst van HA te maximaliseren en de aanwezigheid van verontreinigingen, zoals melkzuur, te minimaliseren. De geproduceerde isotopisch verrijkte HMW-HA monsters werden gekarakteriseerd met behulp van verschillende analytische technieken, waaronder vloeistofchromatografie massaspectrometrie (LC-MS), gelpermeatiechromatografie (GPC) en oplossingsstatus NMR spectroscopie. LC-MS analyse bevestigde de metabolische opname van isotopen in de HA-structuur, waarbij gedeeltelijke en volledige labeling werd waargenomen. Voor het eerst hebben we een methode ontwikkeld om isotopisch verrijkt HMW-HA te produceren en het te karakteriseren met

ssNMR om waardevolle inzichten te krijgen in de structurele en functionele eigenschappen van HA. Deze aanpak opent nieuwe wegen voor het bestuderen van HA-gebaseerde materialen in biomedische en industriële toepassingen en vergemakkelijkt geavanceerde analyses van HA-interacties met receptoren en andere ECM-componenten zoals bestudeerd in hoofdstuk 3 en 4.

Karakterisering van HMW HA-hydrogels met biomimetische omgevingscondities

Hoofdstuk 3 gaf inzicht in de conformationele dynamiek van HMW-HA. De studie onderzocht de conformationele dynamiek van HMW-HA onder verschillende hydratatiecondities en in een ECM-achtige omgeving met behulp van geavanceerde MAS ssNMR. 13C-gelabeld HMW-HA maakte atoomspecifieke analyse mogelijk en onthulde meerdere conformaties, met name in het N-acetylglucosamine (GlcNAc) gedeelte, onder verschillende hydratatieniveaus. Met name werd preferentiële hydratatie van specifieke delen van het HA-polymeer waargenomen. Daarnaast breidt het onderzoek de analyse uit naar HMW-HA in een ECM-achtige omgeving, waarbij veranderingen in flexibiliteit en conformatie veroorzaakt door ECM-interacties en hydratatie worden opgehelderd. Het HMW-HA-ECM complex vertoonde andere mechanische eigenschappen dan puur HMW-HA. Verdere analyse benadrukte de gevoeligheid van GlcNAc koolstof aan veranderingen in hydratatie en ECM interacties, met verhoogde dynamiek waargenomen in de GlcNAc molecuul. Interessant is dat GlcNAc-koolstoffen C4 en C6 structurele heterogeniteit vertoonden, wat mogelijk regio's weerspiegelt die cruciaal zijn voor HA-herkenning door bindende eiwitten. Over het geheel genomen bood de combinatie van 13C-verrijking en MAS NMR atoomresolutie inzichten in de flexibiliteit van HA en zijn vermogen om structurele veranderingen te ondergaan als reactie op hydratatie en ECM-interacties, met implicaties voor biomedische en farmaceutische toepassingen. De bevindingen suggereren het belang van het GlcNAc-deel in het reageren op omgevingsfactoren en de implicaties voor HA-interacties met bindende eiwitten.

Hoofdstuk 4 van het proefschrift bevatte een uitgebreid onderzoek naar de interactiedynamiek tussen HMW-HA en HA-bindende eiwitten zoals CD44, met speciale aandacht voor hun respons op enzymatische degradatie door het enzym hyaluronidase. Door middel van multidimensionale ssNMR met 13C-gelabelde HMW-HA en ongelabelde CD44-HABD, geeft de studie inzicht in de structurele en dynamische veranderingen in HMW-HA, geïnduceerd door hun binding. Met name de interactie maakt HMW-HA iets stijver, met specifieke

koolstofverbindingen die een verminderde mobiliteit vertonen, zoals blijkt uit de analyse van de relaxatie-eigenschappen. Daaropvolgende in-situ afbraakexperimenten onthullen dat er een competitie zou kunnen zijn tussen hyaluronidase en CD44 voor bindingsplaatsen op de HA-keten, wat resulteert in een langzamere en minder efficiënte afbraak in aanwezigheid van CD44-HABD. De ssNMR-experimenten bevestigen deze bevindingen en benadrukken de beschermende rol van CD44 in enzymatische splitsingsplaatsen. Bovendien liet de overgang van hydrogel naar vloeistof van HA, gecontroleerd via ssNMR, verschillende verteringssnelheden zien tussen HMWHA alleen en in complex met CD44-HABD, wat de rol van CD44 bij het moduleren van de enzymatische splitsingsefficiëntie verder benadrukt. Deze gedetailleerde analyse draagt bij aan een beter begrip van biomoleculaire interacties binnen het ECM en heeft mogelijke implicaties voor de ontwikkeling van therapeutische interventies gericht op HA-eiwit interacties en afbraak gerelateerde pathologieën.

ssNMR-toepassingen op het mechanisme van weefselkrimp bij weefselverwijdering

Hoofdstuk 5 presenteert een toepassing van de technieken die in de hoofdstukken 2 tot en met 4 zijn ontwikkeld en toegepast. In dit hoofdstuk heb ik veel van dezelfde experimentele benaderingen toegepast die gebruikt zijn in HA studies, door in situ analyses uit te voeren op weefselbiopten. Dit hoofdstuk is cruciaal om het proefschrift met elkaar te verbinden en laat zien hoe de methodologieën die zijn gebruikt voor HMW-HA hydrogels kunnen worden toegepast op complexere systemen, zoals weefsels. We onderzochten hydratatie, uitdroging en vriesdrogen, waarbij we ons concentreerden op de dynamica die een cruciale rol kan spelen in deze processen. De toepassing van deze technieken op weefsels legt een sterke basis voor toekomstig onderzoek, waarbij het combineren van HA-labeling met in-situ weefsel- en cellulaire analyse inzicht zou kunnen geven in het ECM van kankerweefsels.

Samenvattend presenteert dit hoofdstuk 5 een gedetailleerd onderzoek naar de mechanismen die ten grondslag liggen aan weefselkrimp tijdens procedures voor weefselverwijdering. Het onderzoek maakt gebruik van ssNMR-technieken om verschillende weefsels te analyseren en te onderzoeken hoe hun samenstelling verandert tijdens het klaringsproces, wat licht werpt op de moleculaire mechanismen van weefselopruiming en bijbehorende krimpproblemen. Het onderzoek begint met een vergelijkende analyse van biomoleculen in intacte weefselbiopten afkomstig van verschillende bronnen,

waaronder kippenborst, runderlendevlees, schapenlever, schapennieren en schapenstaartvet. Door middel van 1D ssNMR-experimenten werd de relatieve inhoud van eiwitten, koolhydraten en lipiden in deze weefsels beoordeeld, waarbij verschillende profielen tussen de monsters naar voren kwamen. Vervolgens verschuift de aandacht naar het onderzoeken van de invloed van weefselopruiming, met name met behulp van de BABB-methode (3D imaging-compatible solvent-based tissue clearing), op kippenborstweefsel. Door middel van een serie ssNMR-experimenten werden veranderingen in de vet- en eiwitinhoud en -dynamica opgehelderd. De resultaten toonden een substantiële vermindering aan van het lipidegehalte na het opruimen, terwijl de eiwitdynamica aanzienlijk werd beïnvloed, waarbij eiwitten een verhoogde mobiliteit vertoonden in de aanwezigheid van BABB. Verdere analyse met behulp van water-edited ssNMR onthulde veranderingen in water-eiwit interacties geïnduceerd door BABB, met implicaties voor weefselhydratatie en eiwitstabiliteit. Interessant genoeg werden verschillende reacties tussen eiwittypes op het opruimingsproces waargenomen, wat wijst op een complexe wisselwerking tussen BABB, eiwitten en watermoleculen. Over het geheel genomen biedt het onderzoek inzicht in intacte weefseleigenschappen en benadrukt het de veelzijdige effecten van weefselopruiming op het mechanisme van weefselkrimp en de gevolgen van weefselopruimingsmethoden voor biomedisch onderzoek.

Popular English summary

Hyaluronic acid (HA) is a naturally occurring substance in our body, particularly in the spaces between cells, helping to keep tissues hydrated, support communication between cells, and maintain their structure. HA, especially in its larger form (high molecular weight), is excellent at holding water, creating a gel-like structure that keeps tissues stable and functioning properly. Beyond normal functions, HA plays an important role in healing wounds and managing inflammation. It's also linked to diseases like cancer and osteoarthritis. In cancer, for example, HA builds up in tumors, making treatment less effective, while in osteoarthritis, it breaks down, causing joints to deteriorate faster and leading to pain. My research focused on understanding how HA works in its natural state and how it breaks down in response to different environments. To do this, we developed a special method using advanced solid state NMR techniques to study HA at the molecular level, revealing different structures and behaviors of HA hydrogels in various conditions. We also explored how HA interacts with other components in the body, such as proteins and enzymes that degrade it. This knowledge could help in creating better therapies for diseases where HA plays a crucial role. Additionally, we used the developed solid-state NMR methods to study tissue biopsy samples, focusing on how molecules change during tissue clearance procedures. This research could lead to improvements in medical imaging techniques and other medical applications.

Populaire Nederlandse samenvatting

Hyaluronzuur (HA) is een natuurlijk voorkomende stof in ons lichaam, met name in de ruimtes tussen cellen, die helpt om weefsels gehydrateerd te houden, de communicatie tussen cellen te ondersteunen en hun structuur te behouden. HA, vooral in zijn grotere vorm (hoog moleculair gewicht), is heel goed in het vasthouden van water, waardoor een gelachtige structuur ontstaat die weefsels stabiel houdt en goed laat functioneren. Naast de normale functies speelt HA een belangrijke rol bij het helen van wonden en het beheersen van ontstekingen. Het wordt ook in verband gebracht met ziekten zoals kanker en artrose. Bij kanker stapelt HA zich bijvoorbeeld op in tumoren, waardoor de behandeling minder effectief is, terwijl het bij artrose wordt afgebroken, waardoor gewrichten sneller verslechteren en pijn ontstaat. Mijn onderzoek richtte zich op het begrijpen hoe

HA werkt in zijn natuurlijke staat en hoe het afbreekt in reactie op verschillende omgevingen. Hiervoor ontwikkelden we een speciale methode met geavanceerde vaste-stof-NMR techniek om HA op moleculair niveau te bestuderen, waardoor verschillende structuren en gedragingen van HA-hydrogels in veranderende omstandigheden zichtbaar werden. We onderzochten ook de interacties van HA met andere componenten in het lichaam, zoals eiwitten en enzymen die het afbreken. Deze kennis kan helpen bij het ontwikkelen van betere therapieën voor ziekten waarbij HA een cruciale rol speelt. Daarnaast gebruikten we de vaste-stof NMR-methode om weefselbiopsiemonsters te bestuderen, waarbij we ons concentreerden op hoe moleculen veranderen tijdens het klaren van weefsel. Dit onderzoek zou kunnen leiden tot verbeteringen in medische beeldvorming en andere gezondheidsgerelateerde toepassingen.

Publications

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