Mechanosensation at the molecular level
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Chapter 7

Summary and Future Perspectives

Nederlandse Samenvatting

Acknowledgement
Summary and Future Perspectives

Mechanotransduction, the conversion of physical force into biochemical information, is fundamental to development and physiology. For example, in the vascular system, pressure and shear stress from pumping blood influence the morphology and pathology of the heart and vasculature. Bone is shaped by forces from gravity and muscle contraction. Hearing and touch are based on neural responses to pressure. Physical forces regulate a broad array of physiological processes, and dysregulation of mechanical responses contributes to major human diseases.

Extensive research has been done to uncover how mechanical forces are transduced into biochemical signals and how mechanotransduction affects cellular function. At the molecular level, mechanosensation is accomplished by mechanosensitive channels, which are sensors of the cells sensing the mechanical stimulus in the cell membrane. Despite its prevalence in many biological processes, molecular mechanism underlying mechanosensation is still unknown.

Our research aims at a better understanding of mechanosensation at the molecular level by using one of the “simplest” mechanosensors: Mechanosensitive channel of Large Conductance (MscL) from bacterium Escherichia coli. We engineered MscL and investigated its gating mechanism by employing different methodologies (Chapters 2, 3 and 4). While learning more about its molecular mechanism, we applied our findings to develop two-component drug release system from liposomes (Chapter 5). Engineering MscL aside, we also engineered amphiphiles that are permeable to ions, mimicking ion channels (Chapter 6).

**Ion Mobility Mass Spectrometry Reveals Global Conformational Changes of MscL during its gating**

Based on patch clamp electrophysiology and short-range interaction of MscL amino acids, it was modeled that MscL undergoes large conformational changes during its transition from the closed to the open state. However, until now, these global changes could not be observed directly. In chapter 2, we studied MscL gating by Ion Mobility Mass Spectrometry (IM-MS). By activating MscL to different magnitudes and following the resulting changes in the rotationally averaged collision cross-sections, we could monitor the resulting global conformational changes and relate them to the mechanosensing mechanism. This work also showed that MscL can gate in the absence of a lipid bilayer. We supported this observation by employing electron paramagnetic resonance spectroscopy (EPR) and electron microscopy (EM) and concluded that the gating associated structural changes are intrinsic to the protein, and are not dictated by the membrane.
**MscL is a jigsaw puzzle**

Till now, the homopentameric nature of MscL restricted the use of two different cysteine-specific probes (i.e., one to activate the channel, the other to observe the resulting conformational changes) within the same pentamer at desired ratios. We solved this challenge in chapter 3, by dissociating and re-associating MscL subunits in a controlled manner. We could obtain MscL labeled with two different cysteine-specific probes (a light switch to trigger MscL gating and a paramagnetic spin label to follow the resulting conformational changes) at desired ratios. By this method, we gate MscL into defined intermediate states and monitor the resulting structural changes by EPR.

**Watching the helical movements of MscL during its gating**

After seeing the global structural changes starting from an early stage of channel gating, we also investigated these changes locally. In chapter 4, we designed an environment-sensitive fluorescent probe that is sensitive enough to report the polarity changes in the microenvironment of the protein during MscL gating. By conjugating this probe to the individual cysteine residues in the pore forming TM1 helix, we could determine the polarity profile of the channel in the closed and different sub-open states. By this way, we could identify conformational changes at different parts of the pore-forming TM1 helix at various sub-open transitions of MscL during its gating. Our data forms the basis of a gating model of MscL from the onset of mechanosensation and will allow us to back-model the membrane forces that might generate such movements of MscL.

**A click reaction activates MscL embedded liposomes**

Having control over the gating of MscL, in chapter 5, we develop a two-component release system with MscL embedded liposomes. Spatio-temporal control over the release of liposomal content allows interfering with many processes within the body, including cancer imaging and therapy. In our bioorthogonal strategy, interaction of two specific components allowed the activation of the liposomes and resulted in the release of liposomal content. One component was chemically labeled MscL that is embedded in the liposomes, while the other component was a tetrazine that is added to the solution externally. Inverse electron-demand Diels-Alder reaction between these two components gate MscL so that release from liposomes could occur when and where desired. We believe that such a specific reaction could be promising in targeted delivery and/or controlled release of therapeutic agents.

**Ion Permeable Lipid Bilayers: A paradox?**

Functioning of ion channels in cells fascinated not only biologists but also chemists and material scientists. Many efforts have been focused on mimicking ion channels with synthetic ones towards generating artificial cells with controllable membrane
permeability. Hence communication. Recently, the permeability of the lipid bilayer itself has also been modified. In chapter 6, we developed amphiphiles, which self-assemble into ion-permeable bilayers. Our amphiphiles form not only stable planar lipid bilayers, but also cell-sized containers. Conductance and fluorescence dequenching ensemble measurements together with single molecule electrophysiology show that the bilayers allow the passage of small ions but retain large anions, and they can accommodate a biological ion channel in its functional form. Our results hold promise for the generation of an electro-chemical gradient across the lipid bilayer and the development of artificial cellular systems.

**Perspectives**

This thesis focused on understanding the gating mechanism of MscL, as a model to understand mechanosensation at the molecular level. Till now, the major experimental challenge was to stabilize MscL at an early sub-open state from the onset of mechanosensation and study the conformational changes during the transition from the closed to that state. We solved this problem by developing and applying new methods. With these methods, we elucidated both global and local conformational changes starting at a very early stage of channel gating. Now it is time to look back and correlate what kind of forces can generate these conformational changes. How is the force sensed and transduced? Do all mechanosensitive channels behave the same? Or does the protein–lipid–water junction hold even more secrets? Much work lies ahead if the answers to these questions are to be found; as Ching Kung says, ‘perhaps, just like after a long spell of rain, the floodgates of knowledge are about to be opened’...
Nederlandse Samenvatting

Mechanotransductie, de omzetting van fysieke kracht in biochemische informatie, staat aan de basis van de ontwikkeling en fysiologie van organismen. In het vasculaire systeem bijvoorbeeld, beinvloeden druk en schuifspanning, veroorzaakt door het pompen van bloed, de morfologie en pathologie van het hart en het stelsel van bloedvaten. Bot krijgt zijn vorm door de zwaartekracht en de kracht van het samenrekken van spieren. De reacties van neuronen op fysieke druk staan aan de basis van het gehoor en de tastzin. Fysieke krachten regelen een breed scala aan fysiologische processen en de ontregeling van mechanische reacties draagt bij aan belangrijke menselijke ziekten.

Uitgebreid onderzoek is uitgevoerd om te ontrafelen hoe mechanische krachten worden omgezet in biochemische signalen en hoe mechanotransductie de functie van de cel beinvloedt. Op moleculair niveau veroorzaken mechanosensitieve kanalen, celsensoren die de mechanische stimulans in de cellombraan voelen, de mechanosensatie. Ondanks het voorkomen in vele biologische processen is het moleculaire mechanisme dat ten grondslag ligt aan mechanosensatie nog onbekend.

Ons onderzoek richt zich op een beter begrip van mechanosensatie op moleculair niveau, met behulp van een van de "eenvoudigste" mechanosensors: het "Mechanosensitive channel of Large conductance" (MscL) van de bacterie Escherichia coli. We veranderden MscL en onderzochten het openingsmechanisme door gebruik te maken van verschillende methoden (hoofdstuk 2, 3 en 4). Terwijl we meer leerden over het moleculaire mechanisme, pasten we onze bevindingen toe in de ontwikkeling van een tweecomponenten geneesmiddelgiftesysteem met liposomen (hoofdstuk 5). Daarnaast hebben we ook iondoorlaatbare amfifielen die ionkanalen nabootsen, ontworpen (hoofdstuk 6).

Ion Mobility Mass Spectrometry onthult algemene vormveranderingen van MscL gedurende de openingstijd.

Gebaseerd op de patch clamp elektrofysiologie en de korte afstand interactie van aminozuren in MscL, kon worden gemodelleerd dat MscL grote conformationele veranderingen ondergaat tijdens de overgang van de gesloten naar de open toestand. Tot nu toe konden deze globale veranderingen niet direct worden waargenomen. In hoofdstuk 2 onderzochten we het openen van MscL door middel van Ion Mobility Mass Spectrometry (IM-MS). Door MscL op verschillende niveaus te activeren en daarna de daaruit voortvloeiende veranderingen in de zogenaamde rotationally averaged collision cross-sections te volgen, konden we de resulterende globale conformatieveranderingen volgen en relateren aan het mechanisme van mechanosensatie. Dit werk toonde ook aan dat MscL als poort kan fungeren in de afwezigheid van een lipide dubbellaag. We ondersteunden deze waarneming met electron paramagnetische resonantie spectroscopie (EPR) en electronen
microscopie (EM) en concludeerden dat de opening behorende bij de structurele veranderingen inherent aan het eiwit zijn, en niet worden gedicteerd door het membraan.

**MscL is een legpuzzel**

Tot nu toe beperkte het feit dat MscL een homopentameer is het gebruik van twee verschillende cysteine-specifieke probes (een om het kanaal te kunnen activeren, de andere om de resulterende conformatieveranderingen waar te kunnen nemen) in dezelfde pentameer in vantevoren bepaalde verhoudingen. Deze uitdaging hebben we opgelost in hoofdstuk 3, door MscL gecontroleerd te dissociëren en te re-associëren. Hiermee konden we MscL labelen met twee verschillende cystine-specificieke probes in de gewenste verhouding (een lichtschakelaar om MscL gating aan te kunnen schakelen en een paramagnetische spinlabel om de resulterende conformatieveranderingen te kunnen volgen). Met deze methode konden we MscL openen in gedefinieerde tussenliggende toestanden en konden we vervolgens de daaruit voortvloeiende structurele veranderingen met EPR volgen.

**Het observeren van de helix-actige bewegingen van MscL tijdens het openen**

Nadat we de algemene structurele wijzigingen vanaf een vroeg stadium van het openen van het kanaal hadden geobserveerd, hebben we ook de meer lokale veranderingen onderzocht. In hoofdstuk 4 ontwierpen we een milieu-gevoelige fluorescente probe die gevoelig genoeg is om de polariteit van de veranderingen in de micro-omgeving van het eiwit tijdens het openen van MscL vast te stellen. Door het conjugeren van deze probe met de afzonderlijke cysteïneresiduen in de porievormende TM1 helix, konden we het polariteitprofiel van het kanaal in zowel de gesloten als verschillende sub-openingstoestanden bepalen. Op deze manier kunnen we conformatieveranderingen in de verschillende delen van de porievormende TM1 helix op verschillende sub-openingsovergangen van MscL tijdens het openen van het kanaal identifieren. Onze gegevens vormen de basis van een openingsmodel van MscL vanaf het begin van mechanosensatie en zal ons toestaan de membraankrachten die zulke bewegingen van MscL kunnen genereren terug te modelleren.

**Een klikreactie activeert liposoomingesloten MscL**

Nu we controle hebben over de gating van MscL, ontwikkelden we in hoofdstuk 5 een twee-componenten afgifte systeem met in liposomen ingesloten MscL. Spatio-temporale controle over de afgifte van de inhoud van liposomen maakt het mogelijk te interfereren met vele processen in het lichaam, waaronder het in beeld brengen en de behandeling van kanker. In onze bio-orthogonale strategie maakte de interactie van twee specifieke componenten de activatie van de liposomen en het vrijkomen van liposomale inhoud mogelijk. Een van de componenten is chemisch gelabeld MscL dat is ingebed in liposomen, terwijl de andere component
een tetrazine is, die extern wordt toegevoegd aan de oplossing. Een Inverse elektron-demand Diels-Alder-reactie tussen deze twee componenten opent MscL zodat vrijlating uit de liposomen kan optreden waar en wanneer gewenst. Wij geloven dat een dergelijke specifieke reactie veelbelovend kan zijn voor gerichte aflevering en / of gecontroleerde afgifte van therapeutische middelen.

**Ion permeabele lipidedubbellagen: een paradox?**

Het functioneren van ionenkanalen in cellen fascineert niet alleen biologen, maar ook chemici en materiaalwetenschappers. Veel pogingen zijn gericht op het nabootsen van ionenkanalen met synthetisch gegenereerde kanalen met als doel het maken van kunstmatige cellen met regelbare membraanpermeabiliteit, en daarmee communicatie. Onlangs is het ook gelukt de doorlatbaarheid van de lipidebilaag zelf te wijzigen. In hoofdstuk 6 hebben we amfifielen ontwikkeld, die zichzelf assembleren tot ionendoorlatende dubbellagen. Onze amfifielen vormen niet alleen stabiele vlakke lipidedubbellagen, maar ook containers ter grote van een cel. Geleiding en fluorescentie dequenching metingen samen met single molecule electrofysiologie laten zien dat de bilagen de passage van kleine ionen toelaten maar grote anionen tegenhouden, en ze kunnen een biologische ionkanaal in de functionele vorm accommoderen. Onze resultaten beloven het genereren van een elektrochemische gradiënt over de lipide bilaag en de ontwikkeling van kunstmatige cellulaire systemen.

**Vooruitzichten**

Dit proefschrift richt zich op het begrijpen van het openingsmechanisme van MscL, als een model om mechanosensatie op moleculair niveau te begrijpen. Tot nu toe was de grote experimentele uitdaging om MscL te stabiliseren op een vroege sub-geopende toestand vanaf het begin van mechanosensatie en om de conformationele veranderingen tijdens de overgang van de gesloten naar die toestand te bestuderen. We hebben dit probleem opgelost door nieuwe methoden te ontwikkelen en toe te passen. Met deze methoden hebben we zowel lokale als globale conformationele veranderingen die beginnen in een zeer vroeg stadium van de opening van het kanaal, opgehelderd. Nu is het tijd om terug te kijken en te correleren wat voor krachten deze vormveranderingen kunnen genereren. Hoe wordt de kracht gevoeld en getransduceerd? Gedragen alle mechanosensitieve kanalen zich hetzelfde? Of houdt het eiwit-lipide-water junction nog meer geheimen verborgen? Er ligt veel werk in het verschiet als de antwoorden op deze vragen gevonden willen worden; zoals Ching Kung zegt 'misschien, net als na een lange periode van regen, staan de sluizen van de kennis op het punt om te worden geopend "...
Acknowledgement

Now it is time to complete this story... But I know that it would never be complete without mentioning the people who has been a part of it.

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Anna... My dear Anna... It took so much time before I started to understand your EPR language. But now I do, you should be proud of me! My feelings to EPR did not change much. How can they when I grow 10 L fermentor, do thousands of purifications (ok a bit exaggerated) and hundreds of chromatofocussing (again a bit exaggerated) and give you one eppendorf and after 1 min EPR experiment you tell me 'Duygu there is not enough protein here'?! Still at the end we managed to have chapter 3! Jokes aside, I am so happy that I met and collaborated with you. Thank you for being a great support for me all the time.

Nobina, my hectic Nobina... Your ambition towards your work is great! I wish you all the best in the future. I have no doubt you will be very successful.

One thing I learnt during my PhD is interdisciplinary approach can solve many problems at once. Collaborations make the research rich and satisfying. So I would like to thank here all the people who contributed so much to this thesis.

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And Marwah, my last but the most enthusiastic student who wants to know everything! On my last days in the lab, I am very happy that I pass the torch to someone like you. You will achieve so many things in your career.

Membrane Enzymology was a dynamic group. It has changed a lot during my PhD. So many people were there when I started.

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I wish you all the best for the rest of your time in the group.

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Now it is complete.

Time to take off.

Time to leave this story behind.

With a smile on my face, time to look ahead...

THE END