University of Groningen

In Singulo Biophysics

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DOI:
10.33612/diss.213651070

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Buzón Rodriguez, P. (2022). *In Singulo Biophysics: accessing the Dynamics of Intracellular Processes at the Molecular and Cellular Levels*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. https://doi.org/10.33612/diss.213651070

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Summary and Perspectives

6.1. SUMMARY

The understanding of the mechanisms behind the dynamics of biological processes is crucial to build a more comprehensive picture of the principles governing living systems. At the microscopic level, local fluctuations and perturbations shape the stochastic behaviour of these natural processes, demanding experimental access to the action of their individual players, e.g., biomolecules, supramolecular complexes, organelles. The experimental and conceptual challenges involved in the study of life with such desired sensitivity and resolution are at the edge of the latest technical and theoretical developments. This thesis presents a variety of biophysical strategies to study biomolecular processes that are dynamic in essence. Great consideration has been devoted to the design of novel experimental procedures that would allow the analytical scrutiny of the targeted processes at the molecular and cellular levels. Thus, Chapter 1 includes some personal comments on the complexity associated with studying living systems and an introduction to the techniques and methodologies employed in this thesis.

Self-assembly processes are of the essence of some of the most fundamental features exhibited by biological systems. Some viruses, for instance, have the capability to form new viral particles through the mere interaction of their individual components, synthesized by the host cell. The formation of a closed protein shell, the viral capsid, serves as an excellent example of an irreversible process that proceeds through reversible non-covalent interactions (Chapter 2), which is a ubiquitous mechanism in biological systems.

Chapter 3 unveils key mechanisms on the dynamics of Hepatitis B virus (HBV) assembly by combining single-molecule manipulation with high spatiotemporal resolution techniques. We show that the capsid protein of HBV is able to condense single-stranded DNA molecules by converting stabilization energies gained from favourable protein-protein and protein-DNA interactions. These detailed mechanisms were achieved by applying a combination of force spectroscopy strategies on individual DNA molecules. The assembly mechanism inferred from our single-molecule observations relies on the formation of key assembly intermediates that exhibit compact arrangements, that is, maximizing protein-protein contacts. Importantly, high-speed atomic force microscopy also revealed that these early assembly intermediates, although small, preserved the preferred curvature of the viral capsid. These compelling features seem to be of major importance mediating genome condensation during HBV assembly.

Another highly dynamic process with notable biological significance is that of genome accessibility in eukaryotes. In these living organisms, genome functionalities are regulated

by a multitude of cellular factors acting on the nucleosome, the minimal unit of organization in eukaryotic genomes. Nucleosomes are built from two copies of each core histone (H2A, H2B, H3, H4), which associate to form a histone octamer that wraps ~146 base pairs of double-stranded DNA.

Chapter 4 focuses on the action of two distinct histone chaperones, SET/template-activating factor-Iβ (SET/TAF-Iβ) and Nucleophosmin 1 (NPM), which represent the two most conserved histone chaperone folds. Particularly, this chapter presents the study of these two chaperones mediating histone-DNA interactions in nucleosomes as well as for isolated histones. Histone chaperones are known to play a major role during nucleosome assembly and disassembly; however, we show that both SET/TAF-Iβ and NPM preferentially interact with partially dismantled nucleosomes, suggesting that their primary role lies in nucleosome assembly. This structural specificity was disclosed thanks to the unique property of optical tweezers and fluorescence microscopy that allowed us to control the wrapping state of nucleosomes while detecting individual chaperone binding events. Furthermore, by combining kinetic and equilibrium experiments we were able to capture SET/TAF-Iβ and NPM acting on DNA-bound histone through the specific recognition of non-native histone-histone and histone-DNA interactions. These observations revealed molecular insights into histone eviction and shielding activities of the chaperones, proposing a general and conserved mechanism of histone chaperones to maintain genome integrity.

In Chapter 5 the focus moves towards cell dynamics, where a kinetic study on macrophage activation at the single-cell level is presented. Macrophages are highly dynamic cells with key roles in immune system responses and tissue homeostasis. These cells polarize into different phenotypes under diverse stimuli, which essentially direct their function. The initial stage of polarization is activation, a complex process that typically involves coordinated signaling patterns. In this chapter, we uncover in real time the production of reactive oxygen species upon chemical stimulation, as a proof of principle to study macrophage activation kinetics of individual cells. Our method, which relies on the optical trapping of cells, highlights the contribution of cell adhesion and cell-cell communication modulating macrophage responses. Moreover, single-cell measurements by fluorescence microscopy disclose some degree of heterogeneity among different cells, as well as substantially faster kinetics when compared with other methods that rely on averaged readouts. Ultimately, the experimental work presented in this chapter demonstrates the importance of targeting the behavior of individual cells for their accurate kinetic characterization.

6.2. SAMENVATTING

Het begrijpen van de mechanismes achter dynamische biologische processen is cruciaal om de grondbeginselen van levende systemen in kaart te kunnen brengen. Op microscopisch niveau vormen lokale fluctuaties en verstoringen het stochastische gedrag van deze natuurlijke processen. Experimentele technieken zijn hierdoor essentieel om de werking van de individuele componenten, zoals bijv. biomoleculen, supramoleculaire complexen en organellen te doorgronden. De experimentele en conceptuele uitdagingen die het bestuderen van leven met een dergelijke gevoeligheid en scherpte met zich meebrengen, kunnen slechts door middel van de nieuwste technieken en theoretische ontwikkelingen overwonnen worden. Dit proefschrift beschrijft verschillende biofysische strategieën om dynamische, bio-moleculaire processen te bestuderen. Een substantieel deel is gewijd aan het ontwerpen van nieuwe experimentele methoden die het mogelijk maken om op een analytische en kritische wijze specifieke processen te onderzoeken op moleculair en cellulair niveau. Hoofdstuk 1 bevat een aantal persoonlijke opmerkingen wat betreft de complexiteit die geassocieerd is met het bestuderen van levende systemen en een introductie tot de technieken en methodes die zijn gebruikt in dit proefschrift.

Zelfassemblage is één van de meest fundamentele kenmerken van biologische systemen. Bijvoorbeeld, in het geval van veel virussen kunnen nieuwe virusdeeltjes enkel en alleen worden gevormd door de interacties tussen hun individuele bouwstenen, die gesynthetiseerd zijn door de gastheercel. Het ontstaan van een gesloten eiwitmantel, het virus capside, is een uitgesproken voorbeeld van een onomkeerbaar proces dat verloopt via omkeerbare, niet-covalente interacties (Hoofdstuk 2). Dit lijkt een breder vertegenwoordigd mechanisme in biologische systemen.

Hoofdstuk 3 legt de belangrijkste mechanismen bloot van de dynamica van Hepatitis B virus (HBV) assemblage, door het combineren van enkel-molecuul manipulatie met hoge spatiotemporele resolutie technieken. We laten zien dat de capside eiwitten van HBV in staat zijn om enkelstrengs DNA moleculen te condenseren, gedreven door de vrije energie toename resulterend uit voordelige eiwit-eiwit en eiwit-DNA interacties. Het assemblage mechanisme afgeleid uit onze enkel-molecuul observaties berust op het ontstaan van belangrijke geassembleerde tussenproducten die een compacte vorm laten zien, waarin de eiwit-eiwit contacten maximaal zijn. Belangrijk is dat, ofschoon de tussenproducten klein zijn, deze de krommingsradius van het uiteindelijke virale capside behouden. Het lijkt erop dat deze fascinerende kenmerken van groot belang zijn voor genoomcondensatie tijdens HBV assemblage.

Een ander zeer dynamisch, en biologisch significant, proces, is de toegankelijkheid van het genoom in eukaryoten. In deze levende organismen wordt genoomfunctionaliteit

gereguleerd door meerdere cellulaire factoren die van invloed zijn op het nucleosoom, de kleinste structurele vorm van het eukaryotische genoom. Nucleosomen zijn gevormd door twee kopieën van elk kern histon (H2A, H2B, H3, H4) met elkaar te verbinden tot een histon octameer, welke daarna ~146 baseparen van dubbelstrengs DNA omwikkelt.

Hoofdstuk 4 focust op de werking van twee verschillende histon chaperonnes, SET/template-activating factor-Iβ (SET/TAF-Iβ) en Nucleophosmin 1 (NPM), die twee van de meest voorkomende histon chaperonne structuren representeren. Dit hoofdstuk bespreekt met name hoe deze twee chaperonnes de histon-DNA interacties beïnvloeden in zowel nucleosomen als geisoleerde histonen. Histon chapperonnes staan er bekend om een cruciale rol te spelen tijdens (de-)assemblage van nucleosomen. Wij tonen echter aan dat SET/TAF-Iβ en NPM beide overwegend met gedeeltelijk ontmantelde nucleosomen interactie vertonen, wat suggereert dat hun voornaamste rol in het assemblage proces ligt. Verder, door kinetische en evenwichtsexperimenten te combineren, zijn wij in staat de interactie tussen SET/TAF-Iβ en NPM, en DNA-gebonden histonen vast te leggen door middel van de specifieke herkenning van niet-primaire histon-histon en histon-DNA interacties. Deze observaties resulteren in moleculaire inzichten van de chaperonnes met betrekking tot destabilisatie en afschermen van de histonen, wat wijst op een algemeen mechanisme om genoomintegriteit te behouden.

De focus in Hoofdstuk 5 wordt verlegd naar celdynamica, waarin een kinetische studie naar macrofaagactivatie op enkel-cel niveau wordt gepresenteerd. Macrofagen zijn zeer dynamische cellen met een sleutelrol in immuunreacties en weefselhomeostase. Deze cellen polariseren in verschillende fenotypes onder invloed van verschillende stimuli, wat bepalend is voor hun uiteindelijke functie. De eerste stap van polarisatie is activatie, een complex proces gekenmerkt door gecoördineerde signaalpatronen. In dit hoofdstuk ontrafelen wij in real-time de productie van reactieve zuurstofcomponenten die met behulp van chemische stimulatie gevormd worden. Dit werk is een proof-of-principle studie voor het bestuderen van de kinetica van macrofaagactivatie op het niveau van individuele cellen. Onze methode belicht de bijdrage van celadhesie en cel-cel communicatie, om zo macrofaagreacties na te bootsen. De enkel-cel metingen laten een zekere heterogeniteit zien tussen de cellen, evenals substantieel snellere kinetica in vergelijking met methoden gebaseerd op het middelen van meetresultaten. Samengevat demonstreert het experimentele werk in dit hoofdstuk het belang van een focus op het gedrag van individuele cellen voor een correcte karakterisering van hun kinetica.

Translation by Martijn Middelkamp

6.3. PERSPECTIVES

Biological systems are characterized by their dynamic change in stability. Viruses, for instance, show drastic changes during different stages of their life cycle. Viral particle assembly and genome encapsidation might occur irreversibly during the final stages of the viral life cycle, while genome uncoating and capsid disassembly are typically found in early stages, after the first virus-cell interactions have taken place. *In vitro*, capsid assembly can be modeled by a cascade of weak—several K_BT per protein contact—reversible reactions[1]. However, the formation of other finite-size homoprotein complexes has been shown to proceed through irreversible intermediate reactions, with negligible disassembly contributions[2]. Recently, single-particle kinetic experiments of viral particles have revealed the high cooperativity exhibited during the viral assembly process, where most individual particles are formed within one to two minutes[3]. In this short time, a supramolecular structure with icosahedral symmetry that comprises hundreds of identical proteins have been spontaneously formed. How is the system *steered* to avoid exploring all unproductive but available configurations? Indeed, this is still an open question, where the viral assembly problem has been compared with Levinthal's paradox enunciated in the field of protein folding.

Chapter 3 revealed key thermodynamic and structural features of the assembly process, but what about its kinetics? In this context, the presence of dispersed high affinity RNA aptamers in the viral genome, named packaging signals, has proven very successful to model the assembly of certain viruses[4]. However, not all viral protein seems to exhibit such specificity for their genome, and RNA topology has shown to play a critical role[5, 6]. In the simplified case of empty capsid assembly, assembly without the genome, the number of assembly intermediates is drastically decreased. Recently, the kinetics of empty capsids have been successfully described by applying the principle of maximum entropy to select a minimal number of assembly intermediates[7, 8]. *In vivo*, experiments have shown that assembly takes place under a continuous increase in capsid protein concentration[9]. Indeed, a protein concentration ramp was used to describe virus assembly kinetics by applying the packaging signal model through Gillespie simulations[10]. May these observations be pointing towards the need of a protein flux, a flux of matter into the system, to achieve efficient assembly? Therefore, could the emerging theories of non-equilibrium self-assembly[11] be applicable in this context? I believe that these theories in combination with experimental techniques that provide both high temporal resolution and single-particle sensitivity, e.g. the ones presented in this thesis and by Garmann *et al*.[3], will certainly change the way we understand the self-assembly of viruses.

The same dynamic stability seen for viral particles applies to chromatin fibers. Nucleosomes must remain in place to avoid DNA accessibility, contributing to block downstream processes such as transcription and replication[12]. However, under different conditions, a particular set of nucleosomes might need to be dismantled and removed to facilitate accessibility to certain genes[13]. Therefore, nucleosome stability needs to be balanced and finely orchestrated over time, which is typically associated with ATP consumption[14]. In addition to the action of ATP-dependent chromatin remodelers and histone chaperones, nucleosome homeostasis is coupled to highly regulated signaling cascades that include the chemical modification of histones, which has severe implications in nucleosome dynamics[15]. Altogether, these observations feature the presence of pivotal non-equilibrium processes[16], which are difficult to realize under simplified *in vitro* conditions. In Chapter 4 we have shown that the lack of these key regulatory processes can unbalance the activity of histone chaperones. Specifically, chaperones showed specificity interacting with partially disrupted nucleosomes, suggesting that their role on nucleosome disassembly might be linked to the action of other cellular factors. However, it remains unclear whether the chaperones need other binding partner/s, the chemical modification of histones, or the combination of both, in order to recognize intact nucleosomes. The results presented in this thesis may primarily propose new questions regarding histone chaperone activities; while providing a powerful strategy, based on optical tweezers and single-molecule fluorescence, to build upon by increasing the complexity of the biological system.

The two example presented above serve to highlight the presence of dynamic structures at the microscopic level. In addition, these structures give a justification for the appearance of irreversible processes in order to modulate their stability in time and place. However, these phenomena are not exclusive of molecular systems, on the contrary, they are seen in higher order systems such as cells and organisms[17]. Ultimately, life is maintained through the continuous consumption of free energy.

In the cellular context, the characterization of such molecular processes becomes even more challenging. Any perturbation induced to the system will likely produce a complex response involving a plethora of cellular mechanisms. A good example of that is the production of reactive oxygen species (ROS), which has been identified as a major signaling pattern in a multitude of cellular pathways[18]. In Chapter 5, the production of ROS by chemically stimulated macrophages was measured under diverse conditions. Our observations revealed the contribution of cell adhesion and cell-cell communication hindering ROS production, although the molecular basis of such behavior could not be identified. Interestingly, our measurements showed that the levels of ROS produced in the cytoplasm reach saturation

after ~5 min and are maintained for another 15 min, a time-span at which the external concentration of the chemical inducer is substantially depleted. Unfortunately, the reversibility of the process, ROS level decrease, could not be addressed due to limitations associated with our cell trapping procedure. Nevertheless, these observations highlight the presence of hysteresis in the system, as the ROS signal remained constant under decreasing levels of stimulation, which could be a critical feature of macrophage activation mechanisms. Overall, we show the great potential that cell trapping has in the study of cellular dynamics, which is likely to bring substantial advances to the field in the near future.

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LIST OF PUBLICATIONS

1. Buzón P, Velazquez-Cruz A, Gonzalez-Arzola K, Diaz-Quintana A, Diaz-Moreno I, Roos WH. (**2022**) Histone chaperones exhibit conserved functionality in nucleosome remodeling. bioRxiv 2022.01.13.476140 (*preprint*)

2. Maity S, Trinco G, Buzón P, Kodera N, Ngo KX, Ando T, Slotboom DJ, Roos WH. (**2022**) High speed-atomic force microscopy reveals a three state elevator-mechanism in the citrate transporter CitS. Proc. Natl. Acad. Sci. U.S.A. 119: (6) e2113927119

3. Buzón P, Maity S, Christodoulis P, Wiertsema MJ, Dunkelbarger S, Kim C, Wuite GJL, Zlotnick A, Roos WH. (**2021**) Virus self-assembly proceeds through contact-rich energy minima. Sci. Adv. 7: eabg0811

4. Vasse GF*, Buzón P*, Melgert BN, Roos WH, van Rijn P. (**2021**) Single cell reactomics: real-time single-cell activation kinetics of optically trapped macrophages. Small Methods. 5: 2000849 (**contributed equally*)

5. Buzón P, Maity S, Roos WH. (**2020**) Physical virology: From virus self-assembly to particle mechanics. WIREs Nanomed. Nanobiotechnol. e1613

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ACKNOWLEDGEMENTS

First of all, I would like to thank my promotors Wouter Roos and Patrick van Rijn for their support in completing this thesis. Special thanks goes to you Wouter, for your guidance and the many opportunities you gave me during my stay in your group. I particularly appreciated the freedom and your endless support to new ideas, good new ideas, which made me evolve as a researcher. Secondly, I would like to thank the assessment committee, Patrick Onck, Patrick van der Wel and Nynke Dekker, for their critical reading of my thesis and the feedback they provided.

The work presented here would not have been possible without the fruitful collaboration and contribution of many researchers I had the pleasure to work with. I would like to express my gratitude to Gwenda Vasse and Barbo Melgert (UMCG), Alejandro Velázquez Cruz, Katiuska González Arzola, Antonio Díaz Quintana and Irene Díaz Moreno (University of Sevilla-CSIC), Gijs Wuite (VU Amsterdam), and Steven Dunkelbarger, Christine Kim and Adam Zlotnick (Indiana University). Special thanks to you, Adam, for the way you embraced me as part of your group during my stay in your lab, and the support you are still providing to my scientific career.

Next, I would like to thank the people I have closely worked with in the Roos Group. Sourav, thank you for the great experiments, discussions, and times in general we enjoyed together. Martijn and Yanping, thanks for your fresh and new viewpoints. Viktor, thanks for your always useful suggestions and support to my fluorescence experiments. I would also like to thank the students I had the pleasure to work with, who taught me a lot and helped me to shape my research: Monique, Panos, Felix, Valerie and Oscar. In addition, I would like to thank the whole Molecular Biophysics Group for creating a beautiful atmosphere to do research: Melissa, Yuzhen, Guus, Ceri, Ignacio, Chris, Laura, Rafael, Adéla, Sajitha, Anja and Margriet. I would like to add here the van der Wel Group: Mustapha, Irina, Pushpa, Greeshama, Alessia and Raffaella. And extend my gratitude to Rifka Vlijm and her Group: Thomas and Eline. Also, thanks to the former members of the department I had the great pleasure to meet: Thorben, Giorgos, Kostas, Marijn, Jelle, Jochem, Jasper, Yichen, Florence, Yusran, Atieh, Nikos and Martin. I would like to include here Mariska and Margherita, from Gijs Wuite Group, thanks for your essential input on optical tweezers experiments.

I would like now to express my biggest gratitude to the people who first gave me the opportunity to do research in the University of Granada: Irene Luque, José Martínez and María del Mar García. And to my colleagues at that time who showed me what research really is: Ángel Pey, Bertrad Morel and Francisco Castillo. Thanks for the great times we had together as well.

Gracias Papá, Mamá, Juan José, por vuestro cariño y apoyo incondicional, y por darme una educación basada en el esfuerzo y la tolerancia. Gracias Anouk, Joaquín, que sois mi vida.