Nanopore spectrometry for the detection of proteins and their modifications

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DOI:
10.33612/diss.568483425

Chapter 6

Summary

The first part of **chapter 1** provides a general background to the field of proteomics, discusses techniques to detect proteins as well as their limitations and focusses on single-molecule techniques that have recently been developed. Proteomics heavily relies on tandem mass spectrometry (MS) for the identification of proteins, but a significant number of proteins and their proteoforms have never been detected. This 'Dark Proteome' may be analysed with more precise, single-molecule techniques. In the last decade, several single-molecule techniques to identify proteins have been developed, which all use labelling strategies to detect the position of specific amino acids.

In the second part of **chapter 1**, nanopores are presented as a label-free single-molecule technique for the identification of proteins. Nanopores are tiny holes in thin membranes that identify molecules at the single-molecule level as they are lodged inside the pore. Nanopores have been successfully used for DNA sequencing, and may find a broader application as protein sequencers. However, protein sequencing poses significantly more challenges than DNA sequencing from the biophysics point-of-view. Control over capture and translocation is difficult due to the chemical heterogeneity of peptides and proteins. Nevertheless, studies show that nanopores can be used to capture folded proteins, allowing the determination of proteins size and dynamics. In addition, nanopores can be functionalised with enzymatic complexes to unfold and transport polypeptide chains. Finally, nanopores can be used as spectrometers for the detection of proteins by peptide profiling.

In **chapter 2** the detection of proteogenic peptides in β-barrel nanopores is studied. We found that wild-type aerolysin and Cytotoxin K (CytK) pores captured peptides from trypsinated lysozyme badly. However, the introduction of an acidic residue together with an aromatic residue greatly increased the dwell time of peptides in the nanopore and hence improved the resolution for peptides. Such acidic-aromatic sensing region likely interacts with the peptide via π-π stacking and cation-π interactions. This effect was most significant when the aromatic residue was placed in close proximity to the acidic residue. Using aerolysin and CytK nanopores with acidic-aromatic sensing regions, peptides from trypsinated lysozyme could be identified in the nanopore spectrum. Measurement of the ion selectivity in the pores revealed that peptide capture in aerolysin was governed by the electro-osmotic flow, whereas the capture in CytK was governed by electrophoresis. Despite the difference in the main driving force behind the capture of peptides, a very similar set of mutations improved the detection of peptides, indicating that the introduction of acidic-aromatic sensing regions might also be used to improve peptide detection in other β-barrel nanopores.

In **chapter 3** we study the use of nanopores for the detection of protein glycosylation. Despite the ability of Fragaceatoxin C (FraC) nanopores to capture a wide range of peptides, we found

that hydrophilic glycopeptides translocated too quickly to be accurately detected by the nanopore. A combination of high salt, low pH and an acidic-aromatic constriction increased the dwell time of glycopeptides and allowed their discrimination based on the nanopore signal. Using these conditions, we detected arginine rhamnosylation reactions on cyclic peptides and showed that the extent of rhamnosylation can be quantified using biological nanopores. Finally, we showed that the presence of rhamnosylation on elongation factor P (EF-P) can be detected and quantified from the peptide profile after digestion by the protease Lys-C. With this study we showed the direct quantification of protein glycosylation using nanopores, indicating that nanopore spectrometry can be used to detect and quantify post-translational modifications (PTMs) in proteins.

The use of nanopore spectrometry to detect PTMs is further studied in **chapter 4**, where we study the detection of isobaric peptide modifications in biological nanopores. Isobaric peptide modifications are extremely difficult to detect using approaches based on mass spectrometry. We found, however, they may be detected by their nanopore signal. First, we show that peptides with the same mass, but different chirality, yield very different event clusters in the nanopore spectrum. This allowed for the identification of a single *D*-amino acid difference in Enkephalin peptides. Interestingly, $CytK^{Wt}$ could not to detect the difference between *L* and *D* peptides, but several Lys128 mutants could, indicating that peptide-pore interactions are important for the discrimination. The difference in nanopore signal likely reflects different positions of the peptides inside the nanopore. Finally, we demonstrate the use of nanopores for the direct detection of lanthipeptide ring formation in peptides, circumventing otherwise tedious experiments. Nanopore experiments might eventually be used in the development of therapeutic and antimicrobial peptides as a quick method to detect isobaric peptide modifications.

In **chapter 5** a concluding discussion and some future perspectives on the use of nanopores for protein identification and sequencing is presented. In the work presented in this thesis, interaction between the peptide and the nanopore proved to be important for the accurate detection of peptides in biological nanopores. Nanopore engineering can further enhance or tune these interactions to achieve even better peptide separation in the pore. In addition, significantly more information could be retrieved from the nanopore events by looking at multi-dimensional nanopore spectra, rather than the relative blockade versus dwell time spectra that are most often presented in literature. The integration of nanopores into microfluidic devices may increase the throughput and may allow the detection of the substrates by different pores at the same time. Such integrated nanopore spectrometer can eventually be used in the clinic for the rapid detection of proteins, peptides or other biomarkers.