Chapter 5

Concluding discussion and future perspectives

About a decade after nanopore DNA sequencing was demonstrated, nanopores have emerged as sensors for the single-molecule detection of proteins. The transition from DNA to protein sequencing is far from trivial and intensive research is required to adapt nanopores to peptide or protein detection. In this thesis we explored the use of biological nanopores for the detection of proteins and their modifications by nanopore peptide profiling. The nanopore signal contained information about the size, but also about the chemical properties of the peptides, indicating that nanopores can be used for the rapid identification of proteins and their modifications. However, before biological nanopore can be adapted to more complex samples and be used to complement MS measurements, some improvements to the pores, the analysis of the nanopore signal as well as the nanopore device itself should be explored.

Interactions between the peptide and the nanopore are key

A strong peptide-nanopore interaction is pivotal to accurately detect peptides, especially when detecting more complex mixtures of peptides. For accurate measurements, a dwell time of a millisecond is probably desired. However, free translocation of a peptide in the nanopore would occur on much shorter time scales. An estimation of the translocation velocity based on measurements of the diffusion coefficient of peptides[1,2] is given in box 1. The estimated dwell time of 0.1 µs for a freely translocating peptide is too short to be detected in a typical nanopore experiment. This fast translocation problem was circumvented by using peptides designed to reside in the nanopore, for instance by oppositely charged tails. Such studies can demonstrate the detection of peptides in the nanopore[3,4], however they fail to address the fast translocation of more natural peptides. In the studies presented in this thesis we mainly focused on the detection of proteogenic peptides with a variety of charge and chemical composition. Therefore, nanopore-peptide interactions were essential for accurate measurements. We showed that wild-type aerolysin and CytK pores badly detected peptides, but can be engineered to contain acidic-aromatic constrictions to increases the dwell time and resolution of peptides in the nanopore. A similar nanopore engineering strategy also improved the detection of peptides in FraC nanopores.[5] In addition, we showed that glycosylated peptides could only be detected in nanopores with an aromatic constriction in high electrolyte conditions.

Box 1: Estimation of the electrophoretic drift velocity of a freely translocating peptide

The electrophoretic drift velocity of a particle can be expressed as: $v = \mu * E$, where μ is the mobility of the peptide and E the applied electric field.[2] The peptide mobility can be calculated from its diffusion coefficient (D): $\mu = \frac{Z*|e^-|*D}{k*T}$, where Z is the charge of the peptide, e^- the elementary charge (1.6*10^{-19} C), k the Boltzman constant (1.38*10^{-23} J K^{-1}) and T the temperature (298 K).[2] The electric field can be expressed as: $E = \frac{\Delta V}{l_{pore}}$, where ΔV is the applied potential and l_{pore} is the pore length. Thus, the electrophoretic drift can be expressed as: $v = \frac{Z*|e^-|*D*\Delta V}{k*T*l_{pore}}$. Assuming a peptide with a charge of +1 in an electric field of 50 mV, a pore length of 10 nm and a diffusion coefficient of approximately 4*10^{-10} m^2 s^{-1} (as determined for Angiotensin II)[1], the electrophoretic drift velocity is estimated to be 0.08 m/s. This means a peptide will translocate a 10 nm long pore in approximately 0.1 µs.

In this work we found that a single-amino acid mutation in the lumen of an alpha helical nanopore can make a difference for peptide detection,[6,7] suggesting that peptide-nanopore interactions play an important role for peptide recognition. Furthermore, β-barrel nanopores, which have a more stable and long sensing region, allow more complicated engineering

strategies, including the introduction of new constriction sites.[8] It is thus likely that nanopores will be further engineered to improve their resolution.

Importantly, the use of enzyme complexes introduced right above a nanopore, such as the proteasome that was recently described,[9] may enable control over translocation velocity of the polypeptide and can circumvent the fast translocation problem. However, it is likely that further nanopore mutations will still be required to accurately decode the amino acid sequence of a translocating polypeptide chain, or to identify proteins based on peptide fragments if the protease within the nanopore-proteasome is activated. In addition, the labelling of specific amino acids may help to retrieve sequence information from peptides and proteins[10], however this process is time-consuming and looses a major advantage of nanopore technology with regards to other single-molecule protein detection techniques.

Multi-dimensional nanopore spectrometry

Nanopore events are mainly characterised by two parameters: their relative blockade and duration (dwell time). From these parameters, a two-dimensional nanopore spectrum can be constructed where each peptide is expected to converge to a cluster of events. Two-dimensional nanopore spectra were successful in detecting peptide mixtures from trypsinated proteins[11,12], however this two-dimensional spectrum is easily saturated with event clusters when mixtures of proteins are measured. The characterisation based on relative blockade and dwell time alone assumes the events to be rectangular waveforms with some random noise. In reality, however, the nanopore events are more complicated and may contain different ionic current levels, reflecting different positions of the peptide in the nanopore. Therefore, additional features such as the ionic current noise[13] or a frequency spectrum[14] may be retrieved from nanopore events. In addition, measurements at different voltages or pH may add a dimension to nanopore spectra. Machine learning algorithms are likely to be required to correlate the multi-dimensional nanopore spectrum to properties of the peptide and to allow the *a priori* prediction of nanopore spectra.

Nanopores interfaced with microfluidics

Nanopore experiments in this thesis were recorded by measuring single nanopores in artificial lipid bilayers. However, massive parallelisation is one of the strengths of next-generation sequencing techniques. Nanopores might therefore be integrated into microfluidic devices to measure multiple channels in parallel[15]. Such systems have been commercialised by companies such as Oxford Nanopore technologies and Nanion Technologies. Moreover, the integration with microfluidics might also allow the addition of a front-end HPLC system to separate the peptide mixture before it arrives to the nanopore.

The parallel reading of multiple channels will greatly increase the throughput of nanopore experiments, a consideration that becomes increasingly important with more complex samples. In addition, it is unlikely that one nanopore variant will be capable to measure and discriminate the variety of peptides that are typically present in complex samples. Nanopores-based microfluidics devices may provide the ability to measure samples with different nanopores at the same time. Each nanopore variant can then detect a specific property of the peptide, which adds more dimensions to the nanopore spectrum. Importantly, such device would generate a spectrum of chemical properties of peptides rather than a mass spectrum alone, and would eventually be able to relate the nanopore signal to the peptide sequence.

Outlook on nanopore protein identification and sequencing

The detection of peptides and their modifications in nanopores is moving away from model peptides towards more naturally occurring substrates. In this thesis a nanopore approach towards the detection and quantification of glycosylated proteins as well as the detection of isobaric peptide modifications was presented. These are examples of nanopore peptide profiling for the detection of natural peptides and proteins with important practical applications and show that nanopores can be used to complement mass-spectrometry experiments. Nanopores lack the sub-Dalton accuracy shown by MS measurements, however, they have other strengths. Notably, nanopores can be integrated into portable or bench-top devices for real-time measurements. Therefore, nanopore spectrometers have great potential to be used in the clinic for the rapid detection of proteins, peptides or other biomarkers. Future research should focus on the detection of protein modifications from more complex samples. Eventually, nanopores integrated with enzymatic complexes can be used to sequence polypeptide chains as they are translocated across the nanopore.

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