Harnessing nanopores for single-molecule enzymology and protein sequencing

Giovanni Maglia

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Outline

• Nanopore technology
• Single-molecule nanopore enzymology and metabolite sensing
• Sequence identification of proteins and peptides
• Control of transport across nanopores
Protein transport across nanopores

Secretion

Degradation

Un-folded  Folded

Ubiquitin chain

SEC Pathway Post-translational
SRP Pathway Co-translational
TAT Pathway Post-translational
SecYEG Translocon
TAT Translocon
Biological nanopore

- Water soluble monomer that assembles on membrane
- Oligomers
- Toxins (cell army)
- Porins (control of membrane traffic)
- Highly stable
- dsDNA and protein analysis
Biological nanopore technology

Ionic solution

Lipid bilayer
Nanopore technology

- ~ $10^9$ ions per seconds
- Single molecule ($20^{21}$ molecules in a tablet of ibuprofen)
Trapping Forces: Electrophoresis

- $F_{EP} = EQ$
- $F_{EP}$ is strong and extends outside the nanopore
- For DNA $\sim 10$ pN under $+150$ mV
- Proteins are usually not strongly charged
Electroosmotic flow

• Brownian force, a few pN
• Protein are weakly charged: Electro-osmosis dominates
• The field extends outside the nanopore
Single-molecule DNA sequencing

- A positive applied potential stretches the DNA
- A molecular machine steps base-by-base
- A constriction in the nanopore recognize nucleobase(s)
Protein analysis with nanopore: challenges

• Non-uniform charge. How amino acids with opposite charge can translocate at a fixed potential?
• How proteins enter the nanopore?
• Will proteins or amino acids can be recognized by nanopore currents?
• Will folded proteins interact / unfold inside a nanopore?
Applications: protein sequencing

Motor protein

- Unfold a protein
- Feed it through a nanopore amino acid-by-amino acid
- Single-molecule sequencing
  - low abundance proteins
  - Protein modifications
Protein Mapping

Labeling

- 25,000 protein sequences of proteins in the human proteome
- Proteins can simply be identified for biomarker detection
Identification of Folded Proteins

Folded proteins

• Proteins might not need unfolding
• Ionic current can be used to recognize sub-populations of proteins (e.g. different nanopores identify different size of proteins)
• Purification / Separation, can be integrated in a microfluidic device
Single-Molecule enzymology

Folded proteins

- Single-molecule enzymology
  - Native proteins
- Metabolite detection
  - Multiplexing

Biological sample
This talk

• Single-molecule enzymology and detection of metabolites

• Recognize of peptides during translocation across the nanopore in single-molecule protein sequencing
Nanopores

αHL  MspA  FhuA  OmpG  AeL  ClyA

β-barrel transmembrane region

FraC  Φ29p  ClyA

α-helical transmembrane region
Nanopores

Frac

6 nm

1.2 nm

ClyA

5.5 nm

3.3 nm
Applications

Unfolded-folded protein analysis

Folded protein analysis

Frac

ClyA
A nanopore test-tube

- Do protein enter the nanopore (they are not highly charged)?
- Can the confinement of proteins inside the nanopore be controlled?
- Can we observe the binding of analytes inside the nanopore?
- Is confinement changing the properties of the proteins?
  - Protein surface effects
  - Is confined water the same as bulk water?
Protein entry

- At $-V$, proteins enter the nanopore (current block)
- Electro-osmosis is important
- Protein charge is not (very) important: Electroosmosis dominates
- Proteins enter and remain inside the nanopore for ms-hours
Proteins inside the nanopore

- Thrombin (35 kDa): hours
- GFP (27 kDa): ms
- SBD (25 kDa): minutes
- AlkB (23 kDa): minutes
- DHFR (19 kDa): ms
- Lysozyme (14 kDa): us

- Size charge and shape are important
- All give a distinguished current signal
- Limits: ~35<protein<~14 kDa proteins
SBD proteins

- Venus flytrap protein domains
- Large conformational changes
- Many, e.g. glucose and glutamine

25 kDa

open

closed

Asparaginine
Capture

- Proteins spontaneously enter the nanopore but only at negative applied potentials
- Nanoscale trap
- Proteins are confinement depends on the protein

[Diagram showing proteins entering through a nanopore with a highlighted constriction]
\( K_D = 470 \text{ nM (350 nM with FRET)} \)

- **Off rate** = \( 9.1 \text{ s}^{-1}, (4.2 \text{ s}^{-1} \text{ from FRET}) \)
- **On rate** = \( 1.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1} (2.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1} \text{ from FRET}) \)

- **Intrinsic dynamics and conformational changes**
• **Intrinsic dynamics and conformational changes**

\[ 1.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1} \]

\[ 9.1 \text{ s}^{-1} \]

\[ \text{K}_d = 0.47 \pm 0.03 \mu \text{M} \]
FRET Studies

- $K_D = 350 \text{ nM from FRET, } 470 \text{ nM nanopore (0.2 } \mu\text{M bulk experiment)}$
- Off rate $4.2 \text{ s}^{-1}$ from FRET, $9.1 \text{ s}^{-1}$ from nanopore
- On rate $= 2.4 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ from FRET, $2.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ nanopore
- Neutral analyte almost bulk/like concentration
Confinement

• Intrinsic dynamics of proteins appear to be almost identical than in bulk

• The charge of molecules influence the diffusion through the nanopore (more to be done)

• The surface charge of the nanopore and confinement does not appear to be a big issue (more to be done)
Why

• Nanopores can be interfaced with electronic devices
• If enzymatic reaction can be observed, any biological active molecule can be detected
• Analogue to digital converter
• Small volumes, high sensitivity, wearable sensors.
Other SBD protein tested:

• Glucose binding protein behave the same as SBD1

• The case of SBD2 (binds glutamine)
SBD2

- The signal switches between two levels
- Conformational dynamics or different conformations inside the nanopore
- One conformation from FRET experiments
SBD2

- Closed conformation is a different level
- The off rate is identical from both level
- Most likely two conformations inside the nanopore
FRET Studies

- Intrinsic dynamics
- SBD2 $K_D^Q = 1.1 \mu M$ (0.9 $\mu M$ with ITC) : 0.8 $\mu M$ with nanopore

- On rate $= 3.8 \times 10^7$ s$^{-1}$ M$^{-1}$ : $3.7 \times 10^7$ s$^{-1}$ M$^{-1}$ (level A) $3.8 \times 10^7$ s$^{-1}$ M$^{-1}$ (level C) with nanopore

- Off rate 17.2 s$^{-1}$ : 39.8 s$^{-1}$ with nanopore
Different orientations?
Orientation

Orientation A
- T256E
- S358K
- T256E + S358K

Orientation B
- T256K
- S358E
- T256K + S358E
SBD2 tumbles inside the pore

- Proteins inside the pore are oriented
- The EF and EOF most likely keep the protein in the middle of the nanopore
- The orientation of the protein can be selected
Detection from bodily fluids

- The nanopore serve as a natural filter for large molecules
- Wearable devices

Blood, sweat, urine...
Detection from bodily fluids

- Glucose can be detected directly from sweat, urine and blood
- Good comparison with other methods
- Multiple analytes can be detected simultaneously
- Wearable devices?
Peptide analysis and sequencing

- FraC nanopores
- Ideal shape to study peptide

6 nm

Constriction of 1.2 nm
Peptide analysis and sequencing

- FraC nanopores
- Ideal shape to study peptide
- Small constriction, ideal to sequence proteins
- **Issue**: can peptide be translocated at a fixed applied potential? Can a EOF >> EF be engineered?
Biomarker analysis

- 5 protein-peptide biomarkers
- Different size, shape and folds
Protein Capture

pH 7.5, 1 M KCl

Capture only at -V

No translocation
- Electro-osmosis is important
- Protein can be trapped for a few seconds
- Protein do not translocate across the nanopore
- Proteins are ‘pressed’ against the constriction
Peptide Capture

- An helix kept folded by disulfide bridges
- Formal charge -2
Peptide Capture

pH 7.5, 1 M KCl

No Capture
The constriction prevents the translocation of negatively charged peptides.

Translocation is observed when the constriction is positive.

However, a charged constriction is necessary to induce an electro-osmotic flow.

SOLUTIONS?
Magic pH 4.5

• All peptides are captured

• Important for protein sequencing applications
β2-Microglobulin

11.6 kD, pI=5.6

- The protein translocate across the nanopore
- It cannot be captured at pH 7.5
- The alpha helices probably move to let the protein pass
EGF human
Endothelin 1

6.2 kD, pI=4.5

• Translocation is above a threshold potential

2.5 kD, pI=4.1
Angiotensin I

- The blockades are very short
- Probably the limit of detection with FraC nanopores

1.3 kD, pI=7.9, 12 AA
Polypeptide recognition

• Small differences can be observed
Conclusion

- Ionic current through nanopores can be used to study proteins
- Single-molecule enzymology
  - Native proteins
  - High-bandwidth
  - Conformational dynamics
  - Binding Constants
  - Protein confinement is not an issue
- Biomarker detection
  - Protein: will require a purification step
  - Metabolites with protein adaptor: no purification
  - Sensing device for metabolite and glucose
- Multiplexing
  - Multiple nanopores to detect many proteins
  - Panel of hundreds metabolites
- Wearable devices
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PhD and Post-doc positions available

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