Discrimination of Supramolecular Chirality using a Protein Nanopore

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Supramolecular chirality may emerge from self-assembly processes to yield architectures that differ only in the topological arrangement of their constituent parts. Since the properties of the resulting enantiomeric assemblies are identical, purification and characterisation can be challenging. Here, we have examined the hypothesis that the intrinsic chirality of a protein nanopore can be exploited to detect supramolecular chirality. Transient blockages in the ion current flowing through a single membrane-spanning α-haemolysin nanopore were shown to discriminate between M₄L₆ tetrahedral coordination cages of opposing chiralities. The single-molecule nature of the approach facilitated direct access to the rates of association and dissociation with the nanopore, which allowed the concentrations of the enantiomeric supramolecular assemblies to be determined in situ. Thus, we have established that a protein nanopore can be used to discriminate the chiral topologies of supramolecular assemblies, even when they are too large to fully enter the nanopore.

Introduction

Chirality is ubiquitous in chemistry and biology. As such, the discrimination and separation of stereoisomers is vital. Diasterotopic relationships have long been exploited to discriminate between stereoisomers. For example, covalent derivatisation with chiral reagents can be used to distinguish between chiral centres that are identical in every other regard. Diastereotopic relationships can also be manifested in a non-covalent supramolecular context, as illustrated by stereoselective synthesis, chiral HPLC and the use of chiral shift agents in NMR spectroscopy. However, the control and characterisation of supramolecular chirality in self-assembled systems becomes more challenging as chemists seek to construct increasingly complicated assemblies. Indeed, the expression, recognition and control of supramolecular chirality is essential for life. Bringing together these biological and synthetic supramolecular aspects, we reasoned that intrinsically chiral transmembrane protein nanopores might be utilised as detectors of supramolecular chirality.

Here we have examined the utility of an α-haemolysin (α-HL) protein nanopore to discriminate the supramolecular chirality of tetrahedral coordination cages (Fig. 1). Enantiopure cages, and mixtures thereof, were interrogated at the single-molecule level by monitoring changes in the transmembrane ion current passing through a single protein pore under an applied potential (Fig. 2). Differences between the magnitudes of the ion current blockages (Figs. 2 and 5), and the kinetics and thermodynamics of binding (Fig. 3) were evaluated for both chiral forms of the supramolecular cage and discussed in the context of the relative dimensions of the cage complex and the nanopore (Fig. 4).

Chirality is a key aspect in biological signal transduction that has inspired synthetic transmembrane messengers. Similarly, the chiral discrimination of molecules small enough to enter membrane-spanning nanopores has been previously demonstrated. To date, it is not yet known whether nanopore-based chiral sensors are amenable to the study of
larger supramolecular assemblies, particularly those that are too large to enter the nanopore. Furthermore, the advantage of using atomically precise protein nanopores in the enantio-
detection of small molecules has been counterbalanced by the
need to employ genetically modified proteins.\textsuperscript{18-20}

Results and discussion

We selected the pairing of the transmembrane protein
nanopore, wild-type \(\alpha\)-haemolysin (\(\alpha\)-HL), and a previously
reported chiral Ga(III) cage for our investigation of
supramolecular enantiodiscrimination (Fig. 1).\textsuperscript{28} These
coordination cages possess supramolecular chirality due to the
two possible propeller-like arrangements of the ligands around
each metal centre (\(\Lambda\Lambda\Lambda\Lambda\) and \(\Delta\Delta\Delta\Delta\), Fig. 1B). Like many other
cages constructed from rigid bis(bidentate) ligands and
octahedral metal ions, these complexes assemble exclusively as
the homochiral racemate at the expense of the other possible
diastereoisomers.\textsuperscript{29-36} It has previously been established that
the selected Ga(III) tetrahedral cages are water soluble, and
that conversion between the homochiral forms is negligible
under basic conditions.\textsuperscript{37} The cages bear twelve negative
charges meaning that they can be driven towards the nanopore
under an applied electric field.\textsuperscript{38} Furthermore, the dimensions
of the tetrahedral cage and the \textit{cis}-opening of \(\alpha\)-HL nanopore
are similar (~2.3 nm vs. ~2.6 nm).

At the start of our investigations we synthesised a racemic
mixture of the tetrahedral cages\textsuperscript{28} for nanopore analysis. In the
initial nanopore experiments, a planar lipid bilayer was painted
across a 100 µm aperture separating two wells of buffered
solution (1 M KCl, 30 mM Tris-DCl, pD 7.6 in D\textsubscript{2}O). A single \(\alpha\)-HL
nanopore was introduced into the bilayer,\textsuperscript{39} as indicated by the
characteristic ionic current flowing through the nanopore at an
applied transmembrane voltage of +100 mV (\(I_0\), Fig. 2, left).

Upon the addition of ~100 nM of a racemic mixture of the
tetrahedral cage to the \textit{cis}-side of the bilayer (Fig. 1A), temporal
blockages of the ion current were observed at two discrete
levels (\(I_b\), grey bars in Fig. 2A). Data collated from multiple
experiments that consisted of several thousand blockage events
revealed two Gaussian distributions in the residual ion current
(\(I_b/I_0\)), consistent with two distinct classes of blockage event.
The possibility that these two classes arose from multiple cages
interacting with the pore simultaneously was ruled out, since
the ratio of the two events was concentration independent (Fig.
S15, ESI\textsuperscript{†}). Thus, the two classes of blockage in the presence of
a racemic mixture of the \(\Lambda\Lambda\Lambda\Lambda\) and \(\Delta\Delta\Delta\Delta\) tetrahedral cages was
consistent with our initial hypothesis that a protein nanopore
may be able to discriminate supramolecular chirality at the
single-molecule level.

Encouraged by these preliminary findings, we set out to
confirm the ability of the approach to discriminate the chirality
of tetrahedral cages. Enantiopure samples of both the \(\Lambda\Lambda\Lambda\Lambda\)
and \(\Delta\Delta\Delta\Delta\) cages were obtained using established procedures.\textsuperscript{37, 40}

Pleasingly, only one discrete blockage event class was
observed for each enantiopure \(\Lambda\Lambda\Lambda\Lambda\) and \(\Delta\Delta\Delta\Delta\) cage sample

(Fig. 2B–C). Moreover, the residual currents of these individual
peaks were coincident with the two classes of event observed
for the racemic mixture (Fig. 2B–C cf. Fig. 2A). Hence, we
confirmed that \(\alpha\)-HL is capable of discriminating the opposing
supramolecular chirality of two otherwise chemically identical
Ga(III) tetrahedral cages.

Having established that the discrimination of
supramolecular chirality was possible based on the current
blockage, we sought to examine the underlying kinetics and
thermodynamics of the recognition process, which might be
expected to significantly differ between enantiomers. Indeed,
single-molecule methods allow the direct observation of
association/dissociation kinetics.\textsuperscript{41} A series of nanopore
analyses were performed in which the concentration of each
chiral form of the tetrahedral cage was varied between 25 and
are difficult to predict.43

functions (Figs. S20–S21, ESI†). For both enantiomers, count histograms and fitted to single exponential decay times at each concentration. Event durations and inter-event durations (<200 ms) of the blockage events, combined with the marginal differences in the rates of association and dissocation (Table 1), indicate that the coordination cages interact transiently with the cis-opening of the nanopore without completely entering or translocating.45–47 The scaled diagram shown in Fig. 4A shows that the longest diameter of the cage (2.3 nm) is slightly narrower than the cis-opening of the pore (2.6 nm), but wider than the trans-opening (2.1 nm). However, the space filling model does not take into account the solvation shell surrounding both the protein and the highly-charged cage. Indeed, dynamic light scattering experiments gave a hydrodynamic diameter of 2.5 ± 0.7 nm for the cage in D2O (Fig. 4B). Thus, the size analysis and the characteristics of the blockage events indicate that entry of the cage into the wider vestibule of the pore is largely occluded. Nonetheless, deeper current blockages were occasionally observed that often lasted for tens of seconds under a continued applied potential. Such deeper events were distinct from non-specific gating events and showed a qualitative concentration dependence, suggesting that they may have arisen from inclusion of the cage within the vestibule of the pore (Figs. S10–13, ESI†). In contrast, no significant current blockages were observed when cages were added to the opposite side of the membrane that contained the even narrower trans-opening of the α-HL pore (Figs. 4 and S14, ESI†). Interestingly, the ability of ion current to discriminate the supramolecular chirality of a proportionally large cage species during transient interactions with the cis-entrance of the pore, rather than inclusion within the pore, raises the intriguing possibility that protein nanopores may provide a platform for the analysis of even larger constructs at the single-molecule level.

Figure 3. (A–D) Association and dissociation kinetics of the ΔΔΔΔ and ΔΔΔΔ cages with an α-HL nanopore. The rate constant \( k_{\text{on}} \) was obtained from the slope of the linear fit of \( 1/\tau_{\text{on}} \) versus [cage] for ΔΔΔΔ (A) and ΔΔΔΔ (B). The rate constant \( k_{\text{off}} \) was obtained from the intercept of the graph \( 1/\tau_{\text{off}} \) versus [cage] for ΔΔΔΔ (C) and ΔΔΔΔ (D). Experiments were performed in 1 M KCl, 30 mM Tris-DCl, pD 7.6 in D2O at 293 ± 2 K with an applied potential of +100 mV.

Table 1. Residual ion currents, kinetic and thermodynamic data for α-HL•cage complexation determined from nanopore experiments performed in 1 M KCl, 30 mM Tris-DCl, pD 7.6 in D2O at 293 ± 2 K with an applied potential of +100 mV.

<table>
<thead>
<tr>
<th></th>
<th>ΔΔΔΔ cage</th>
<th>ΔΔΔΔ cage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual current, ( I_{0}/I_{0} )</td>
<td>0.76 ± 0.013</td>
<td>0.66 ± 0.011</td>
</tr>
<tr>
<td>Rate of association, ( k_{\text{on}} / \text{M}^{-1} \text{s}^{-1} )</td>
<td>7.5 ± 0.3 × 10^7</td>
<td>6.5 ± 0.1 × 10^7</td>
</tr>
<tr>
<td>Rate of dissociation, ( k_{\text{off}} / \text{s}^{-1} )</td>
<td>31 ± 3</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>Association constant, ( K_{\text{a}} / \text{M}^{-1} )</td>
<td>2.4 ± 0.3 × 10^7</td>
<td>1.8 ± 0.3 × 10^6</td>
</tr>
</tbody>
</table>

100 nM. Each nanopore analysis was performed at least three times at each concentration. Event durations and inter-event durations (\( \tau_{\text{off}} \) and \( \tau_{\text{on}} \) respectively) were plotted as frequency-count histograms and fitted to single exponential decay functions (Figs. S20–S21, ESI†). For both enantiomers, \( \tau_{\text{off}} \) was found to be independent of cage concentration, whereas \( \tau_{\text{on}} \) was linearly dependent on concentration (Fig. 3). These concentration dependencies confirmed the bimolecular nature of the interaction between each tetrahedral cage and the nanopore.42 Thus, the rate constants of dissociation, \( k_{\text{off}} = 1/\tau_{\text{off}} \), and association, \( k_{\text{on}} = 1/\tau_{\text{on}}(\text{cage}) \), could be determined for each enantiomer (Table 1). Intuitively, the intrinsic diastereotopic nature of the α-HL•cage complex might be expected to result in markedly different binding characteristics. However, only marginally different \( k_{\text{on}} \), \( k_{\text{off}} \) and \( K_{\text{a}} \) values were observed (Table 1). Thus, unambiguous assignment of cage chirality was only possible using ion current blockages that result from electrostatic and steric factors, which are difficult to predict.43

Figure 4. (A) Scaled model of a Ga(III) cage complex overlaid with the crystal structure of α-HL.44 (B) Dynamic light scattering measurement of a racemic sample of the Ga(III) cage complex in D2O 293 ± 2 K.
Figure 5. (A–C) Event distributions generated for enantio-enriched samples. Experiments were performed in 1 M KCl, 30 mM Tris-DCl, pH 7.6 in D2O at 293 ± 2 K with an applied potential of +100 mV.

Table 2. Determination of cage enantiomer concentrations in enantio-enriched samples.

<table>
<thead>
<tr>
<th>[cage]total/nM</th>
<th>30:70</th>
<th>30:70</th>
<th>70:30</th>
<th>70:30</th>
<th>Unknown</th>
</tr>
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<tbody>
<tr>
<td>Λenantiomers</td>
<td>1715</td>
<td>501</td>
<td>4763</td>
<td>3148</td>
<td>1723</td>
</tr>
<tr>
<td>ΔΛtotal</td>
<td>3753</td>
<td>1060</td>
<td>2411</td>
<td>1565</td>
<td>425</td>
</tr>
<tr>
<td>[Λ]on/nM</td>
<td>22 ± 2</td>
<td>30 ± 3</td>
<td>47 ± 5</td>
<td>65 ± 7</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>[Δ]on/nM</td>
<td>55 ± 6</td>
<td>72 ± 7</td>
<td>28 ± 3</td>
<td>37 ± 4</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>ΔΛtotal</td>
<td>28.72</td>
<td>29.71</td>
<td>63.37</td>
<td>64.36</td>
<td>78.22</td>
</tr>
</tbody>
</table>

*Determined by 1H NMR spectroscopy.

Significantly, the single-molecule nature of the approach enables facile *in situ* determination of enantiopurities. Since the *k*on of each enantiomer can be determined from a racemic mixture of enantiomers that populate discrete current levels, then the concentration of each enantiomer can be determined by simply counting events. Equation 1 describes the general relationship between the event counts and the concentration of an individual enantiomer (see ESI† section 4 for derivation).

\[
\Delta\Lambda = \frac{\Lambda_{\text{obs} \cdot k_{\text{on}}}}{\Lambda_{\text{obs} \cdot k_{\text{on}}^\Lambda \cdot \Lambda_{\text{obs} \cdot k_{\text{on}}^\Delta}}} \cdot [\text{cage}]_{\text{total}} \quad (1)
\]

We demonstrated the validity of this approach by determining the absolute concentrations of known mixtures of enantio-enriched samples (Fig. 5, Table 2 and ESI† section 4). Furthermore, the technique proved useful during our own investigation by revealing that a sample that was intended to be enantiopure was, in fact, contaminated with 22% of the other enantiomer (Table 2, “Unknown” column). It is important to emphasise that the nanopore-based approach can determine the enantiopurity of a sample without a 100% pure reference sample, as required by ensemble analytical methods. Thus, we have established nanopore analysis as a complementary approach to existing methods such as circular dichroism\(^a\),\(^b\) for the detection supramolecular chirality.

Conclusions

In conclusion, we have demonstrated the general principle that an intrinsically chiral protein nanopore can serve as a detection element to discriminate the chirality of otherwise identical supramolecular entities. More specifically, we showed the magnitude of the ion current blockages arising from the transient association of tetrahedral Ga(III) cages with an α-haemolysin nanopore provided unambiguous assignment of the individual enantiomers. The single-molecule nature of the approach presents a distinct advantage over traditional ensemble-averaged techniques that cannot easily determine whether a sample is enantiopure or enantio-enriched, thus side-stepping the challenges associated with enantiopurification.\(^c\),\(^d\) Direct access to kinetic parameters relating to the association/dissociation of individual cages with the nanopore allowed the concentrations of cage enantiomers to be determined from a single experiment on a timescale of minutes. Thus, such an approach may be amenable to the *in situ* analysis of dynamic supramolecular systems,\(^e\),\(^f\) such as those associated with chiral amplification phenomena.\(^g\)\(^h\)\(^i\) Significantly, the small magnitude of the observed current blockages was consistent with transient interactions with the pore opening rather than inclusion within the pore. As such, this preliminary study should encourage the future interrogation of even larger supramolecular architectures using nanopores.

Acknowledgements

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Notes and references

\(\ddagger\)D2O was used to enable NMR characterisation at all stages of the experiments.


