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► **To cite this version:**

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HAL Id: hal-04154813

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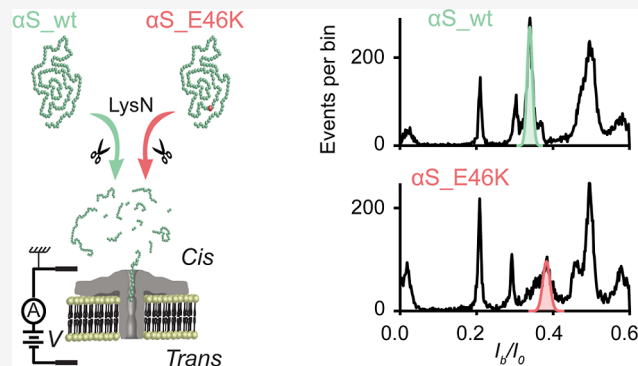
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1 Discrimination between Alpha-Synuclein Protein Variants with a 2 Single Nanometer-Scale Pore

3 Mazdak Afshar Bakshloo, Safia Yahiaoui, Matthieu Bourderioux, Regis Daniel,
4 Manuela Pastoriza-Gallego, John J. Kasianowicz,* and Abdelghani Oukhaled*

5 **ABSTRACT:** Alpha-synuclein is one of several key factors in the
6 regulation of nerve activity. It is striking that single- or multiple-
7 point mutations in the 140-amino-acid-long protein can change its
8 structure, which leads to the protein's aggregation and fibril
9 formation (which is associated with several neurodegenerative
10 diseases, *e.g.*, Parkinson's disease). We recently demonstrated that a
11 single nanometer-scale pore can identify proteins based on its ability
12 to discriminate between protease-generated polypeptide fragments.
13 We show here that a variation of this method can readily
14 discriminate between the wild-type alpha synuclein, a known
15 deleterious point mutation of the glutamic acid at position 46
16 replaced with a lysine (E46K), and a post-translational modification
17 (*i.e.*, tyrosine Y39 nitration).

18 **KEYWORDS:** *alpha-synuclein, nanopores, Parkinson's disease, protein identification, post-translational modifications*



19 ■ INTRODUCTION

20 Alpha-synuclein (α S) is a protein located primarily in
21 neurons,^{1–3} where it aids in the regulation of synaptic vesicle
22 trafficking and neurotransmitter release.^{4,5} In dysfunctional
23 states, it is associated with several neurodegenerative diseases
24 (synucleinopathies), including Parkinson's disease (PD),^{6,7}
25 multiple system atrophy,^{8–11} dementia with Lewy bodies
26 (DLB),^{12–16} and others.^{17,18} In these cases, α S, which is
27 intrinsically disordered,¹⁹ typically accumulates and aggregates
28 into fibril structures^{20–24} in Lewy bodies.³ It is the latter form
29 that induces cytotoxic effects, which are assumed to be
30 responsible, in part, for the symptoms noted above. Some
31 evidence suggests that α S can interact with relatively short
32 amyloidogenic proteins (*e.g.*, A β 40 and A β 42).^{25,26}

33 Alpha synuclein's sequence is highly conserved in many
34 species,^{27–29} and in humans it is composed of 140 amino
35 acids.^{30,31} A rather striking finding is that a point mutation of
36 the guanosine to adenosine at position 209 (G209A) in the
37 SNCA gene encoding for α S, which causes an A53T amino
38 acid change in the N-terminal repeat segment of the protein, is
39 associated with PD.^{32–34} After the initial study, it was shown
40 that additional missense mutations of α S, including A53E,³⁵
41 A30P,³⁶ and E46K,³⁷ were linked to PD as well.

42 We previously demonstrated that an ion channel formed by
43 aerolysin (AeL) can discriminate between different proteins
44 based on how their protease-cleaved fragments partition into
45 the channel's nanometer-scale pore and thereby reduce its
46 ionic conductance.^{38,39} For example, trypsin-induced frag-

ments from three different full-length proteins (myoglobin, 47
lysozyme, and cytochrome C, with molecular masses between 48
12 and 16.9 kg mol⁻¹) produce ionic current signal patterns 49
that act as characteristic fingerprints of each protein. Here, we 50
show that the method can also distinguish between proteolytic 51
fragments from wild-type α S, one of the deleterious point 52
mutants mentioned above (E46K), and a post-translational 53
modification (PTM) of α S (Y39 nitration). This method 54
should be versatile because the use of multiple proteases allows 55
the targeted cleavage of the α S protein into detectable peptide 56
fragments of desired length carrying the mutation(s) or 57
PTM(s) of interest, thereby permitting the discrimination of 58
various forms of α S protein. 59

60 ■ RESULTS

The primary sequence of full-length wild-type (*i.e.*, native) α S 61
(α S_wt) is shown in Scheme 1. To produce the desired 62
enzyme-induced cleavage fragments for α S_wt and the single 63
point mutant version (α S_E46K), we used the recombinant 64
zinc metalloprotease LysN from *Grifola frondose*, which 65
cleaves proteins at the amino terminus side of lysine 66

Received: March 11, 2023

Accepted: May 18, 2023

Scheme 1. Full Length Human α -Synuclein Sequence^a

10 20 30 40 50
 MDVFMKGLSK AKEGVVAAAE KTKQGVAAEA GKTKEGVLYV GSKT**KEGVVH**
 60 70 80 90 100
GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKQDL
 110 120 130 140
 GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEP EA

^aThe sequence of 140 amino acids in α S, with the first proteolytically cleaved fragment of interest (highlighted in yellow) and the single-point amino acid mutation studied here (α S_E46K, emphasized in red).

67 residues.^{40–42} Thus, LysN cleavage of wild-type α S would
 68 theoretically create 16 total polypeptides with varying numbers
 69 of amino acid residues and composition, of which 13 are
 70 unique (Table 1). Note that one of these fragments, which

Table 1. LysN Cleavage Products of Wild-Type α S (α S_wt)^a

Length	Sequence	Mass (g mol ⁻¹)	Charge @ pH 7.5
1	K	146.2	1
2	KA	217.27	1
2	KT	247.29	1
2	KT	247.29	1
2	KF	247.29	1
2	KT	247.29	1
4	KGLS	403.48	1
5	MDVFM	641.8	-1
5	KDQLG	559.62	0
9	KEGVVAAAE	872.97	0
9	KQGVAAEAG	829.91	0
9	KEGVLYVGS	951.09	0
13	KEGVVHGVATVAE	1295.46	-1
16	KTVEGAGSIAAATGFV	1478.67	0
20	KEQVTNVGGAVVTGVTAVAQ	1928.17	0
39	KNEEGAPQEGILEDMPVDPD NEAYEMPSEEGYQDYEP EA	4416.59	-13

^aThe protease cleavage fragment in which the point mutation E46K occurs is highlighted in yellow, and the two amino acids are emphasized in red colored text. The three 9-amino-acid-long fragments highlighted in light orange are discussed later in this study.

71 contains 13 amino acids highlighted in yellow (residues 45 to
 72 57, inclusive), is where the E46K point mutation considered
 73 here would be located. Thus, as we demonstrate below, the
 74 ability to discriminate between the two versions of this
 75 particular fragment, α S_wt and α S_E46K, is a key to
 76 differentiate between these versions of the full-length protein.
 77 The physical properties of this fragment's two variants are
 78 listed in Table 2. Note that the (α S_wt)₁₃ fragment has 13
 79 amino acids, whereas (α S_E46K)₁₂ has 12, because the latter's
 80 full-length protein includes an additional LysN cleavage site at
 81 residue 46. The net charges on each of the polypeptide

Table 2. Properties of the Two α -Synuclein Fragments Studied Here^a

Fragment	Length (amino acids)	Sequence	Mass (g mol ⁻¹)	Charge @ pH 7.5
(α S_wt) ₁₃	13	KEGVVHG V ATVAE	1295.46	-1
(α S_E46K) ₁₂	12	K GVVHG V ATVAE	1166.32	0

^aThe altered amino acid in the point mutant is highlighted in red colored text.

fragments are -1 and 0 for the α S variants (α S_wt)₁₃ and
 (α S_E46K)₁₂, respectively.

Because protein-based nanopores can differentiate between
 slightly different molecular species,^{43–60} including pro-
 teins,^{38,39,61} we used the nanometer-scale pore formed by the
 protein *Aeromonas hydrophila* AeL^{62,63} in an attempt to
 discriminate between the two α S polypeptides in Table 2.

In the protein nanopore-based analytical method,⁴⁷ an ion
 channel is formed in a planar lipid bilayer membrane, and the
 target species are typically added to one of the two aqueous
 electrolyte solutions bathing the membrane. Individual
 molecules that enter the pore transiently reduce the ionic
 current due to volume exclusion and, in certain instances to the
 reversible binding of mobile ions to the molecules.⁴⁹ The
 degree by which the single channel current is reduced, and the
 distribution of residence (or dwell) times for the molecules in
 the pore provide information about the target molecules'
 physical and chemical properties.⁴⁹ The relatively long dwell
 times of single polypeptides in the pore are a consequence of
 the interaction between the two.

We first show how enzymatically cleaved fragments of wild-
 type α S (α S_wt) and the E46K single-point mutant version
 (α S_E46K) interact with a single AeL nanopore. Figure 1A
 illustrates schematically that the LysN-induced cleavage of

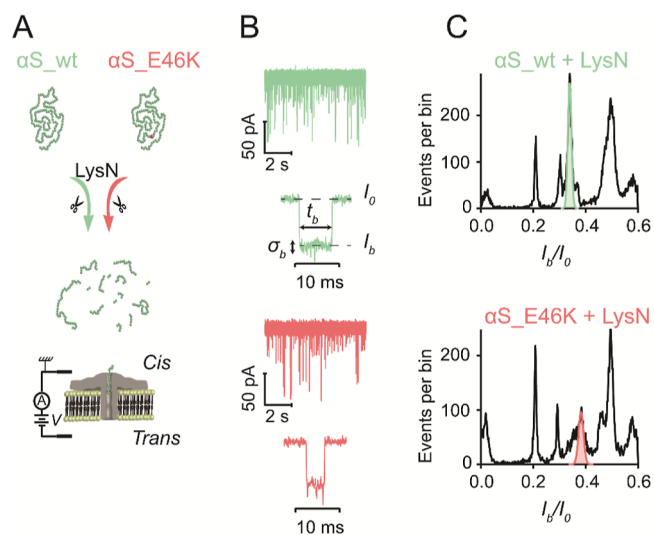


Figure 1. Nanopore-based discrimination of protease fragments of wild-type α -synuclein (α S_wt) and a point mutant version of the protein (α S_E46K). (A) Cleavage of either full length α S_wt or α S_E46K by protease LysN produces well-defined polypeptide fragments. Polypeptide fragment samples of either α S_wt or α S_E46K are added to the electrolyte solution surrounding one side (*cis*) of an AeL nanopore. A transmembrane voltage V drives fragments into the pore and thereby reduces the pore's ionic conductance. (B) Protease-induced fragments of α S_wt or α S_E46K cause short-lived reductions in the ionic current (typical single blockade events for each of the samples are shown); I_o , I_b , σ_b , and t_b are the mean open current, the mean blocked current, the standard deviation of the blocked current, and dwell time for each event, respectively. (C) Histogram of the I_b/I_o values for the cleaved samples of α S_wt (top) or α S_E46K (bottom). The colored peaks correspond to a Gaussian fit to those segments of the data. All data were recorded at $T = 22$ °C, $[KCl] = 2.5$ M, $[CaCl_2] = 1$ M, $[HEPES] = 25$ mM, pH 7.5, and the transmembrane potential was $V = +80$ mV. The data shown here are for a single experiment, but the results were highly reproducible ($n = 3$).

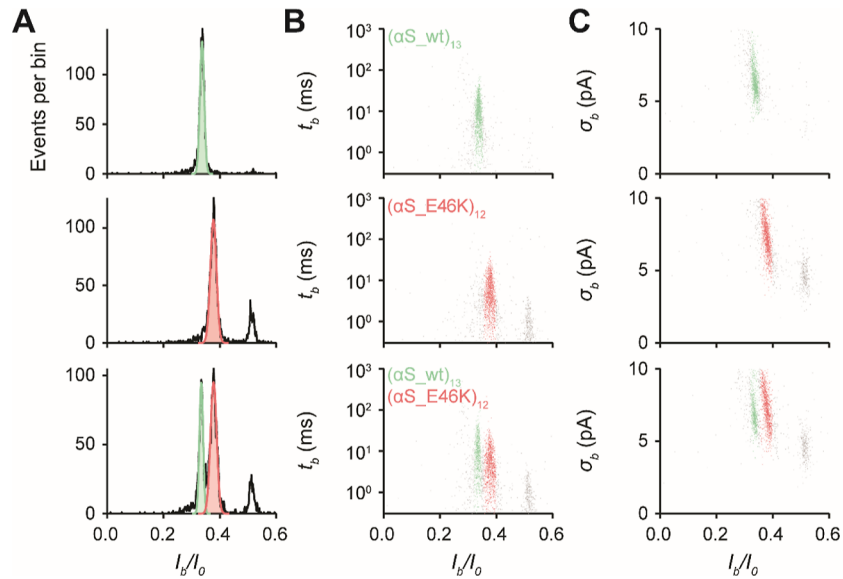


Figure 2. Nanopore-based discrimination of the synthetic short polypeptides $(\alpha S_{wt})_{13}$ and $(\alpha S_{E46K})_{12}$ (Table 2). (A) Single channel ionic current blockade value histograms, (B) dwell time (t_b) vs current blockade value (I_b/I_0) event plots, and (C) blocked ionic current standard deviation event plots for $(\alpha S_{wt})_{13}$ only (top); $(\alpha S_{E46K})_{12}$ only (middle); or an equimolar mixture of both $(\alpha S_{wt})_{13}$ and $(\alpha S_{E46K})_{12}$ polypeptides in the same sample (bottom). Gaussian fits to the predominant single peaks for the $(\alpha S_{wt})_{13}$ (green) or $(\alpha S_{E46K})_{12}$ (red) samples are shown. All data were recorded at $T = 22^\circ\text{C}$, $[\text{KCl}] = 2.5\text{ M}$, $[\text{CaCl}_2] = 1\text{ M}$, $[\text{HEPES}] = 25\text{ mM}$, $\text{pH } 7.5$, and the transmembrane potential was $V = +80\text{ mV}$. These data were obtained in single experiments but were highly reproducible in $n > 3$ replicates.

106 either of the full-length proteins produces well-defined
 107 polypeptide fragments (listed in Table 1). An aliquot of one
 108 protein's fragments is added to the aqueous phase bathing the
 109 *cis* side of a membrane that contains a single AeL nanopore,
 110 and a voltage V is applied across the membrane, which drives
 111 polypeptides into the pore nanopore one at a time, thereby
 112 causing brief but characteristic reductions in the pore's ionic
 113 current (Figure 1B). Three parameters for each event are
 114 recorded: the relative current blockade value (I_b/I_0 , where I_0
 115 and I_b are the ionic currents through a fully open and
 116 polypeptide-occupied pore, respectively), the dwell time of the
 117 molecule in the pore (t_b), and the ionic current standard
 118 deviation (σ_b) (Figure 1B).

119 The histograms of the relative current blockade values I_b/I_0
 120 for each of the two cleaved protein fragments (Figure 1C)
 121 show that a single AeL nanopore can discriminate between the
 122 LysN-induced polypeptide fragments from full-length wild-
 123 type (αS_{wt}) and the point mutant (αS_{E46K}). In addition to
 124 the discriminatory capability of the I_b/I_0 histogram (Figure
 125 1C), the proteins can also be qualitatively differentiated based
 126 on the dwell time- and standard deviation-event plot data
 127 (Figure S1, Supporting Information section).

128 In Figure 1C, there are fewer distinct peaks than one might
 129 expect based on the predicted action of the protease on the
 130 target protein. There are several hypothetical reasons that
 131 might account for that observation. First, proteolytic digestions
 132 are generally not 100% efficient in either the complete
 133 processing of the target protein or in the production of every
 134 polypeptide fragment. Second, it is possible that aggregation of
 135 either the target protein or some of the fragments could occur
 136 even though the experimental conditions (*i.e.*, a relatively short
 137 digestion time of 3 h, the pH was basic at pH 10, and no
 138 stirring was applied) are not conducive to that process
 139 (aggregated fragments would likely not enter the pore).
 140 Third, some of the predicted polypeptide fragments may not
 141 enter the pore due to an excessively high entropic cost (*i.e.*,

their secondary structures may limit the partitioning of some
 142 polypeptides into the highly confined geometry of the nanopore).
 143 We are currently investigating this issue experimentally.
 144

The green and red Gaussian fits to the single peaks in the I_b/I_0
 146 histogram data (Figures 1C and S1) are located at $I_b/I_0 =$
 147 0.34 ± 0.01 and 0.38 ± 0.01 for the samples derived from full-
 148 length (αS_{wt}) and (αS_{E46K}), respectively. Because the
 149 only polypeptide fragments that would contain the amino acids
 150 that are different in the two full-length proteins are (αS_{wt})
 151 and (αS_{E46K})₁₂ (Tables 1 and 2), it follows that those
 152 particular fragments are the likely cause of those two different
 153 peaks.
 154

To test that hypothesis, we studied the effects of synthetic
 155 polypeptides (αS_{wt})₁₃ and (αS_{E46K})₁₂ individually on the
 156 AeL nanopore. Figure 2A (top) shows that there is one peak in
 157 the I_b/I_0 histogram for (αS_{wt})₁₃ located at 0.34 ± 0.01 . The
 158 results obtained with (αS_{E46K})₁₂ (Figure 2A, center) show
 159 that there are two peaks in its I_b/I_0 histogram: a predominant
 160 one located at 0.38 ± 0.01 and a secondary peak at $0.51 \pm$
 161 0.01 . Note that the locations of the single peak for (αS_{wt})₁₃
 162 and the more probable peak for (αS_{E46K})₁₂ are situated at
 163 the same I_b/I_0 values as the Gaussian fit peaks from LysN-
 164 cleaved full-length (αS_{wt}) and (αS_{E46K}), respectively
 165 (Figure 1C). Note that the ionic current blockade dwell time
 166 (Figure 2B, center) and standard deviation (Figure 2C, center)
 167 vs the blockade depth (I_b/I_0) event plots provide two
 168 additional means to discriminate between these two synthetic
 169 polypeptides. Lastly, the bottom panels in Figure 2A–C show
 170 that the effects of (αS_{wt})₁₃ and (αS_{E46K})₁₂ on the AeL
 171 nanopore current are independent of each other, which
 172 suggests that the two different polypeptides do not interact
 173 significantly with each other in the bulk aqueous phase. The
 174 histograms shown in Figure 2, which were produced with
 175 equimolar concentrations of (αS_{wt})₁₃ and (αS_{E46K})₁₂
 176 polypeptides, have qualitatively similar blockade event rates.
 177

Table 3. Physical Properties of the Three 9-Amino-Acid-Long Polypeptides Predicted from LysN Cleavage of the Full-Length α S_wt Protein (from Table 1) and of the PTM Y39 Nitration Version^a

Polypeptide Abbreviation	Sequence	Length (amino acids)	Mass (g mol ⁻¹)	Charge @ pH 7.5
(KE wt)	KEGVVAAAE	9	872.97	-1
(KG wt)	KQGVAAEAG	9	829.91	0
(Y39 wt)	KEGVLYVGS	9	951.09	0
(Y39 NO ₂)	KEGVLY (NO ₂) VGS	9	996.08	0

^aThe polypeptide abbreviations, which are specific to this study, are color-coded to aid interpretation of the electrophysiology data illustrated in Figure 3.

178 These results suggest that even though these two fragments are
179 only slightly different in sequence, mass, and length (Table 2),
180 the AeL nanopore can easily discriminate between them and
181 thus between the LysN-cleaved versions of the full-length
182 native and (α S_E46K) point mutant proteins (Figure 1).

183 Although the E46K point mutation correlates with some
184 cases of PD and DLB, not all the patients affected by
185 synucleinopathies present with it. Other reported neuro-
186 degenerative cases are associated with certain PTMs of α S.
187 Thus, these PTMs, which include phosphorylation of
188 S129,^{64,65} nitration,⁶⁶ O-GlcNAcylation,⁶⁷ sumoylation, or
189 ubiquitination,⁶⁶ are also used as biomarkers for PD and DLB.

190 LysN proteolysis of full-length α S_wt would theoretically
191 produce three unique 9-amino-acid-long polypeptides (Table
192 1), which we label here as (KE wt), (KG wt), and (Y39 wt).
193 The physical properties of those fragments and those of a Y39
194 nitration PTM of the latter one (Y39 NO₂) are listed in Table
195 3. Given that nanopores have proven useful for discriminating
196 between different size RNA oligonucleotides,⁴⁵ synthetic
197 polymers,^{46,49} polypeptides,^{68,69} and characterizing PTMs in
198 proteins,^{70–73} we tested whether the AeL nanopore can
199 discriminate between these three identical-length wild-type
200 protein fragments and a PTM nitration version.

201 Figure 3A–C shows the AeL nanopore electrophysiology
202 data for the three 9-amino-acid-long polypeptide fragments
203 predicted from LysN proteolysis of wild-type α S protein, each
204 produce characteristic and differentiable distributions. The
205 polypeptide fragment (KE wt) produces events characterized
206 with one peak at $I_b/I_0 = 0.48 \pm 0.01$ in the relative current
207 blockade value histogram (Figure 3A left). Adding the (KG
208 wt) polypeptide to the sample causes a new peak to appear at
209 $I_b/I_0 = 0.57 \pm 0.02$ (Figure 3B left). The subsequent addition
210 of the third fragment type (Y39 wt) caused a broadening of the
211 peak caused by (KE wt) due to an additional peak at $I_b/I_0 =$
212 0.49 ± 0.02 (Figure 3C left). Finally, adding the Y39 nitration
213 PTM polypeptide (Y39 NO₂) caused another distinct peak at
214 $I_b/I_0 = 0.42 \pm 0.02$ (Figure 3D left).

215 While the I_b/I_0 histogram data allow the discrimination
216 between all four of these polypeptides, their corresponding
217 dwell time (t_b) event plots (Figure 3A–D, center) provide
218 additional discriminatory capability. The latter plots show that
219 the first two polypeptide variants, (KE wt) and (KG wt), have
220 similar dwell time distributions with mean residence times $\langle t_b \rangle$
221 ~ 0.3 and 0.5 ms, respectively (Figure 3B center). Both the
222 third polypeptide variant (Y39 wt) and its Y39 nitration PTM
223 version (Y39 NO₂) have mean dwell times that, at $\langle t_b \rangle \approx 5.4$
224 and 7.9 ms, respectively, are about an order of magnitude
225 longer than those for the other two polypeptides. Moreover,
226 note that the clustered t_b event plots for each of the four
227 variants are further distinguishable because their distributions
228 cluster at different I_b/I_0 values. Moreover, the standard
229 deviation (σ_b) event plots (Figure 3A to 3D right) for each

variant are also distinct. Note further that the first three 230
polypeptides (Figure 3C) are also discriminated when the 231
whole α S protein variants discussed above are digested 232
(Figures S2–S4 for α S_wt and S5 to S7 for α S_E46K). 233

Another relevant biomarker is a phosphorylation on serine 234
residue S129. However, the length of the peptide fragment 235
generated by LysN that includes S129 (Table 1) is longer than 236
the pore's length and therefore not within the detectable and 237
discernible length range for polypeptide discrimination with 238
the AeL nanopore.^{68,69} We overcame that limitation by 239
changing the protease to cleave the relevant fragment into 240
one that is sufficiently short. Specifically, we used another 241
enzyme, AspN peptidyl-Asp metalloendopeptidase from the 242
Gram-negative bacteria *Pseudomonas fragi*,^{74–77} which cleaves 243
at the amino terminus side of aspartic acid residues. This 244
process should produce the polypeptide fragments listed in 245
Table 4. 246

Our goal here was to determine whether the AeL nanopore 247
can discriminate between wild-type α S and a PTM 248
phosphorylation of serine residue at amino acid 129 (S129). 249
AspN should produce a 14-amino-acid-long polypeptide that 250
contains S129. This fragment and its phosphorylated version 251
are listed in Table 5. 252

Figure 4 illustrates the AeL nanopore electrophysiology data 253
for the (S129wt)AspN and phosphorylated PTM variant 254
(S129_pho)AspN proteolysis fragments. The individual 255
polypeptide fragment (S129_wt)AspN produces two peaks 256
in the I_b/I_0 histogram at 0.18 ± 0.01 and 0.14 ± 0.01 (Figure 257
4A, left). The phosphorylated fragment (S129_pho)AspN 258
produces also two peaks at $I_b/I_0 = 0.20 \pm 0.02$ and 0.09 ± 0.01 259
(Figure 4B, left). When both polypeptides were detected with 260
the AeL nanopore from a co-mixed sample, two well-defined 261
populations were observed, which clearly show the pore's 262
ability to discriminate between the minor components of 263
(S129_wt)AspN and (S129_pho)AspN (Figure 4C, left). In 264
addition, the dwell time (Figure 4A–C, center) and ionic 265
current standard deviation (Figure 4A–C, right) event plots 266
provided further discriminatory capability for this particular 267
PTM. Note that the dominant peaks in the phosphorylation 268
data overlap strongly. Nevertheless, the lesser peaks still 269
provide a means to differentiate between the different species. 270

DISCUSSION 271

Previous studies on nanopore-based detection of individual 272
polymers enabled sequencing DNA,^{45,46,51,52,54,56} simultaneous 273
detection of several different proteins,⁷⁸ discriminating 274
between polymers based on their size,^{46,49,53,57,79} and 275
identifying several different proteins from their protease- 276
induced fragments.^{38,39,61} The work described herein repre- 277
sents a significant extension of the latter method. Specifically, 278
we used two different proteases (LysN and AspN) and the AeL 279

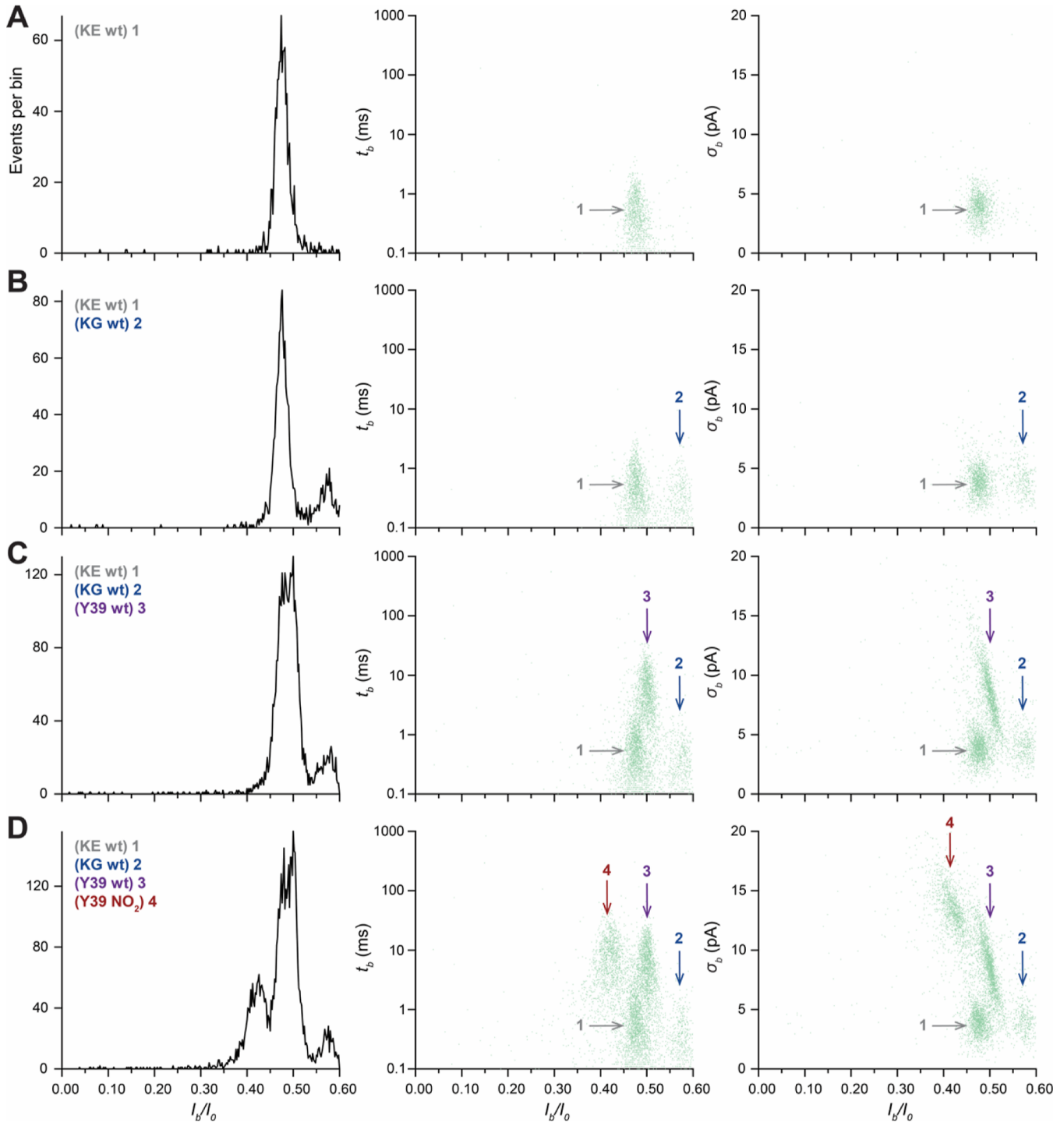


Figure 3. AeL nanopore-based discrimination of the three 9-amino-acid-long polypeptides predicted from LysN proteolysis of full-length α S_{wt} (Tables 1 and 3) and the PTM (Y39) nitration of one of the polymers (Table 3). The plots are histograms of the relative ionic current blockade value, I_b/I_0 (left), dwell time–current blockade value event plots (center), and ionic current standard deviation–current blockade value event plots (right) for a successive addition of (A) (KE wt), (B) (KE wt) + (KG wt), (C) (KE wt) + (KG wt) + (Y39 wt), and (D) (KE wt) + (KG wt) + (Y39 wt) + (Y39 NO₂). For clarity, the results that correspond to each polypeptide variant are indicated with arrows matching their respective color-code. The data shown is from a single experiment but was highly reproducible for $n > 3$ replicates.

280 nanopore to discriminate between wild-type α -Synuclein and
 281 three different versions of it (*i.e.*, a single-point mutation E46K
 282 and two different PTMs, Y39 nitration and S129 phosphor-
 283 ylation) that are implicated in several neurodegenerative
 284 diseases, including Parkinson’s and DLB. The method shown
 285 here offers the possibility to detect various forms of proteins
 286 with a single nanopore through its versatility. This is made

possible because of multiple selective proteases allow the
 precise cleavage of proteins into detectable and discriminable
 polypeptide fragments of the desired length bearing the
 mutation(s) or MTP(s) of interest.

We note that not all of the blockade events shown in Figure
 1 are necessarily due to polypeptide translocation. Specifically,
 some of the events might instead be caused by polypeptides

Table 4. Seven Polypeptide Fragments Predicted from AspN Proteolysis of Full-Length Wild-Type α S Protein^a

Length (amino acids)	Sequence	Mass (g mol ⁻¹)	Net Charge @ pH 7.5
1	M	149.21	0
2	DP	230.22	-1
4	DMPV	460.55	-1
6	DYEPEA	722.71	-3
14	DNEAYEMPSEEYQ	1661.67	-5
17	DQLGKNEEGAPQEGILE	1826.94	-4
96	DVMMKGLSKAKEGVVAAAEEKTKQGVAAEAGKTKEGV LYVGSKTKEGVVHGVATVAEKTKEQVTNVGGAVVTG VTAVAQKTVEGAGSIAAATGFVKK	9516.8	-5

^aThe 14-amino-acid-long variant, highlighted in yellow, and a phosphorylated variant of it (Table 5) were analyzed with a single AeL nanopore (Figure 4).

Table 5. Two Variants (Wild-Type and S129 Phosphorylation) of the Predicted 14-Amino-Acid-Long AspN-Cleaved Fragment from the Full-Length α S_{wt} Protein

Polypeptide	Length (amino acids)	Sequence	Mass (g mol ⁻¹)	Charge @ pH 7.5
(S129_wt)AspN	14	DNEAYEMPSEEYQ	1661.67	-5
(S129_phospho)AspN	14	DNEAYEMPS(phospho)EEYQ	1741.63	-7

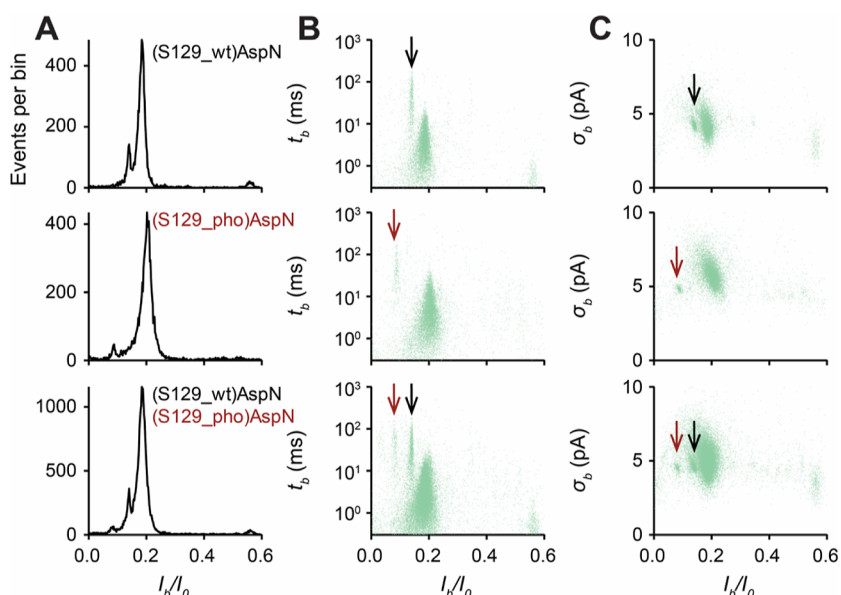


Figure 4. AeL nanopore-based discrimination of two 14-amino-acid-long polypeptides (α S_{wt})AspN₁₄ and its phosphorylated version (α S_{S129})AspN₁₄_phospho. The single channel ionic current blockade value histograms (left), dwell time (t_b) vs current blockade value (I_b/I_0) event plots (center), and blocked ionic current standard deviation event plots (right) for (A) (S129_wt)AspN only, (B) (S129_phospho)AspN only, or (C) a mixture of both (S129_wt)AspN and (S129_phospho)AspN polypeptides in the same sample. For clarity, the results that correspond to each polypeptide variant are indicated with arrows matching their respective color-codes. All data were recorded at $T = 22$ °C, $[KCl] = 2.5$ M, $[CaCl_2] = 1$ M, $[HEPES] = 25$ mM, pH 7.5, and the transmembrane potential was $V = +50$ mV. The data shown are for single experiments but were highly reproducible in replicate ($n > 3$) experiments.

294 that enter and leave the pore from the sample side. Also, there
295 are two known processes that can drive polypeptides into and
296 possibly through the aerolysin channel. These include
297 electrophoresis (e.g., as was the case for single-stranded DNA
298 transport through the α HL nanopore)⁴⁵ and electroosmosis,
299 which can drive polypeptides with any net charge (positive,
300 negative, or neutral) into and possibly through the pore.³⁹

301 This method for the label-free identification of mutations
302 and PTMs, which should work for many other proteins, could
303 be a useful tool for rapid diagnoses at the point of care in
304 medical facilities if it is integrated into a low-cost, miniaturized,
305 high-throughput analytical platform. However, there are several
306 technological hurdles and levels of systems integration (e.g., of

microfluidics-based protein separation,^{80,81} etc.) that will need
to be resolved or employed. 307
308

EXPERIMENTAL SECTION 309

Protein Synthesis. His SpinTrap mini columns were purchased 310
from GE Healthcare Life Science (Chicago, IL, USA), IPTG 311
(R0392), HEPES buffer (BP310) from Thermo Fisher Scientific 312
(Waltham, MA, USA), and Vivaspin 10 kDa cut-off filters (VS0101) 313
from Sartorius (Gottingen, Germany). LysN protease (L101) was 314
purchased from ImmunoPrecise Antibodies (Victoria, Canada), 315
trypsin-agarose (T1763), imidazole (104716), potassium chloride 316
(P5405), calcium chloride (223506), trifluoroacetic acid (TFA, 317
T6508), and decane (457116) were purchased from Merck 318
(Darmstadt, Germany). Diphtanoyl-phosphocholine (DPhPC, 319

320 850356P) was purchased from Avanti Polar Lipids (Alabaster, AL,
321 USA).
322 KEGVVHGVATVAE, KGVVHGVATVAE, KEGVVAEE,
323 KQGVAAEAG, KEGVYLVGS, KEGVY(NO₂)LVGS, DNEAYEMP-
324 SEEQYQ, and DNEAYEMPS(pho)EEGYQ polypeptides (purity ≥
325 98%) were purchased from ProteoGenix (Schiltigheim, France), and
326 recombinant αS_{wt} and αS_{E46K} (purity ≥ 95%) were purchased
327 from rPeptide (Watkinsville, GA, USA).
328 **Protein Digestion.** Recombinant αS_{wt} and αS_{E46K} were
329 dissolved in a 0.1 M glycine, 16 mM NaOH buffer at pH 10. The
330 proteins were digested by LysN in a 1:50 ratio (protein/LysN, mass/
331 mass) for 3 h at 37 °C. LysN cleavage was stopped by quenching the
332 sample using 0.1% TFA. The subsequent fragmented proteins were
333 filtered using a 10 kDa cut-off membrane at 15,000 x g 4 °C for 1 h to
334 withdraw undigested, aggregated proteins, and native LysN protease.
335 The fragmented samples were stored at -20 °C until their use.
336 **Aerolysin Production.** AeL is a pore-forming toxin from *A.*
337 *hydrophila* produced as a precursor protein, proaerolysin (ProAeL),
338 which forms soluble dimers. Recombinant wild-type ProAeL was
339 produced in *Escherichia coli* BL21 using the pET22b-PA vector.
340 ProAeL is produced after IPTG induction and localizes into the
341 bacteria's periplasm. The periplasmic fraction containing soluble
342 ProAeL dimers was extracted using an osmotic shock. Because the
343 recombinant protein is C-terminal His-tagged, ProAeL was further
344 purified by affinity chromatography using Ni-Sepharose MiniSpin
345 columns, and elution was made by adding imidazole. The
346 recombinant ProAeL purity (about 99%) was determined by SDS-
347 polyacrylamide gel electrophoresis and Coomassie blue staining. The
348 ProAeL concentration was estimated by optical absorbance at 280
349 nm. ProAeL (3 μg) was activated by 0.25 units of trypsin-agarose in
350 25 mM HEPES pH 7.5 for 30 min at 25 °C, as previously reported.⁸²
351 **In Silico Modeling of Protein Digestion.** While it is possible to
352 predict which polypeptide fragments will be produced by LysN by
353 inspection of the protein's sequence, we used an *in silico*
354 fragmentation model of the αS protein that was calculated using
355 the expasy PeptideCutter software (https://web.expasy.org/peptide_cutter/). The codes of αS_{wt} (P37840) and αS_{E46K} (P37840,
356 VAR_022703) proteins are available in a free Uniprot database
357 (<https://www.uniprot.org>).
358 **Nanopore Recording.** A vertical lipid bilayer electrophysiology
359 setup (Warner Instruments, Hamden, CT, USA) was used for all the
360 experiments with AeL. The lipid bilayer was formed by painting and
361 spontaneous thinning a film of diphytanoyl-phosphocholine, DPhPC
362 (Avanti Polar Lipids, Alabaster, AL, USA), in decane (Merck,
363 Darmstadt, Germany) at 10 mg/mL over a 150 μm diameter aperture
364 on a Teflon support separating the *cis*- and *trans*-compartments. The
365 aqueous solutions on both sides of the membrane contained 1 mL of
366 2.5 M KCl 1 M CaCl₂, 25 mM HEPES at pH 7.5 (Merck, Darmstadt,
367 Germany). Two matched Ag/AgCl electrodes (Alfa Aesar, Ward Hill,
368 MA, USA) were used to apply +80 mV or +50 mV across the
369 membrane and measure the ionic current. The *cis*-compartment is
370 defined as the virtual ground. After forming the lipid bilayer, activated
371 aerolysin was added to the *cis*-compartment at ≈ 50 ng/mL final
372 concentration. Once a single AeL nanopore formed, a fragmented αS
373 protein (αS_{wt} or αS_{E46K} digested by LysN) or an αS peptide
374 sample was added to the *cis*-compartment. The final concentration of
375 fragmented protein is 3.5 μM each of αS_{wt} and αS_{E46K}. The final
376 concentration of (KE wt), (KG wt), (Y39 wt), and (Y39 NO₂)
377 polypeptides is 5 μM and for (S129_{wt})AspN, (S129_{pho})AspN is
378 30 μM.
379 Single-channel current recordings were performed using an
380 Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale,
381 CA, USA) in the whole-cell mode with a CV-203BU headstage. The
382 signal was filtered using the internal 4-pole Bessel filter at a cut-off
383 frequency of 5 kHz. The data were digitized at 100 kHz and current
384 digital resolution of 30.5 fA/bit state, using a DigiData 1550B A/D-
385 converter (Molecular Devices) controlled by Clampex 10.2 software
386 (Molecular Devices). The solution temperature was held at 22.0 ± 0.5
387 °C for all the experiments with a Peltier device controlled by a bipolar

temperature controller (CL-100, Warner Instruments) coupled to a
liquid cooling system (LCS-1, Warner Instruments).

Data Analysis. Data analysis was performed using Igor Pro 6.12A
software (WaveMetrics, OR, USA) with in-house routines. The
approach relies on a statistical analysis of the properties of the analyte-
induced current blockades, involving at least several hundred (more
typically thousands) events. The detection of each individual current
blockade in a nanopore current vs time recording is based on a single
current-threshold (*Th*) method. A blockade event is detected when
the current magnitude becomes smaller than *Th*, until it returns to a
value greater than *Th*. The beginning of the blockade is defined as the
first point of current increase after having monotonically decreased
below *Th*, and its end as the last point of current decrease before
monotonically increasing above *Th*. This defines the range of points
used to compute the characteristic quantities of the blockade, such as
dwell time *t_b*, mean residual current value *I_b*, and the standard
deviation *σ_b* of the residual current. Here, *Th* = *I₀* - 5*σ₀*, where *I₀* and
σ₀ are the mean value and standard deviation of a Gaussian fit of the
open-pore current distribution, respectively.

Histograms of the relative mean residual current *I_b/I₀* are
constructed with a bin width of 0.002. *I_b/I₀* values constitute the
main criterion to discriminate populations of blockades induced by
enzymatically produced polypeptide fragments from the αS protein
variants. *I_b/I₀* population *j* is defined as the subset of blockades whose
I_b/I₀ values fall in the range $\langle I_b/I_0 \rangle_j \pm 1.96\sigma_j$, where $\langle I_b/I_0 \rangle_j$ and σ_j are
respectively the mean value and standard deviation of a Gaussian fit of
the distribution of *I_b/I₀* values in the corresponding histogram peak.
This range includes 95% of the Gaussian distribution.

The mean blockade duration $\langle t_b \rangle_j$ is determined by fitting the
distribution of $\ln[\langle t_b \rangle_j]$ values with a single-exponential probability
density model, where the maximum of the distribution occurs at
 $\ln[\langle t_b \rangle_j]$. The mean standard deviation of the residual current $\langle \sigma_b \rangle_j$ is
the mean value of a Gaussian fit of the distribution of $\langle \sigma_b \rangle_j$ values. In
the case where the distribution of $\langle \sigma_b \rangle_j$ presents different sub-
populations, each sub-population is Gaussian-fitted and associated
with its own mean value.

MALDI Instrumentation. MALDI-TOF mass spectra were
acquired using a Bruker UltraFLEXtreme mass spectrometer (Bruker,
Billerica, MA, USA) equipped with a Smartbeam 2 Nd/YAG laser
(Bruker) operating at 355 nm. Spectra were acquired using
FlexControl software (version 3.4 Bruker). The reflectron mode
was used with a mass range of *m/z* 100 to 600. The fixed instrument
voltages were ion source 1, 20.00 kV; ion source 2, 17.85 kV; lens,
7.90 kV; reflector 1, 20.80 kV; and reflector 2, 10.80 kV. All mass
spectra were processed using FlexAnalysis software (version 3.4,
Bruker). Pulsed ion extraction was set to 170 ns. Matrix suppression
cut off mass was set to 500 *m/z*. Mass calibration was performed using
peptide calibration standard from the Bruker Starter Kit (P/N
8208241).

MALDI Sample Preparation. The 2,5-dihydroxybenzoic acid
(DHB) matrix was prepared at 20 mg/mL in 30% acetonitrile (v/v)
in water with 0.1% trifluoroacetic acid (TFA). Polypeptides were
diluted to a working concentration of 1 μM in 30% acetonitrile (v/v)
in water with 0.1% TFA as well. Samples were mixed 1:1 with DHB
matrix prior to spotting 1 μL on an MTP anchorchip 384 target (P/N
8280790) and allowed to air dry at room temperature.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at
<https://pubs.acs.org/doi/10.1021/acscchemneuro.3c00164>.

Discrimination between αS_{wt} and αS_{E46K} proteins;
reproducibility of αS_{wt} protein fragmentation and
(αS_{wt})₁₃ identification; reproducibility of αS_{E46K}
protein fragmentation and (αS_{E46K})₁₂ identification;

453 MS analysis of α S_{wt} protein fragmentation; and MS
454 analysis of α S_{E46K} protein fragmentation (PDF)

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483 Author Contributions

484 M.A.B., J.J.K., and A.O. conceived the project, designed the
485 experiments, organized and wrote the manuscript. S.Y. and
486 M.A.B. performed the nanopore experiments. M.A.B. analyzed
487 the data from the nanopore experiments. M.B., R.D., and
488 M.A.B. conceived mass spectrometry experiments and analyzed
489 data from them. M.P.G. and M.A.B. elaborated the protocol of
490 α S enzymatic degradation. M.P.G. produced the aerolysin
491 nanopore material.

492 Notes

493 The authors declare no competing financial interest.

494 ■ ACKNOWLEDGMENTS

495 This work was supported in part by the Agence Nationale de la
496 Recherche ANR (ANR-17-CE09-0032-01 to A.O.), the NIST
497 Office of Law Enforcement Standards, and a Marie
498 Skłodowska-Curie/Freiburg Institute for Advanced Studies
499 Senior Fellowship (both to J.J.K.). We thank F. Gisou van der
500 Goot (Ecole Polytechnique Federale de Lausanne, Switzer-
501 land) for providing the pET22b-proAL plasmid containing the
502 pro-aerolysin sequence.

503 ■ ABBREVIATIONS

504 α S, alpha synuclein; DLB, dementia with Lewy bodies; PD,
505 Parkinson's disease; AeL, Aerolysin; PTM, post-translational
506 modification; KCl, potassium chloride; CaCl₂, calcium chloride

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