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# 1 Combining ionic diode, resistive pulse and membrane for 2 detection and separation of anti-CD44 antibody.

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12

## 13 **Abstract**

14 The need for antibody protein is growing for both diagnosis and therapy. In this work, we combine  
15 two strategies based on single nanopore sensing and multipore membrane to separate, detect  
16 and identify antibodies. This proof of concept was done for the anti-CD44. In a first step, a single  
17 nanopore with a bullet shape were designed using track-etching of polymer film and  
18 functionalized, with a PEG spacer and CD44 antigen. The detection of anti-CD44 was evidenced  
19 by an inversion of the current rectification. After demonstrating the ability to specifically detect  
20 the antiCD44 in a single nanopore, a multipore membrane with cylindrical nanopore was designed  
21 following the same protocol and used to elute a solution of anti-CD44. In a second step, the elution  
22 product was analysed using a single SiN nanopore showing that the anti-CD44 and CD44 antigen  
23 are extracted from the membrane. The analysis of the amplitude of the current blockade shows  
24 that the complex is dissociated.  
25

## 26 **1. Introduction**

27 The increasing need for the identification of proteins as biomarkers as well as their synthesis as  
28 therapeutic agents requires the development of innovative solutions for their analysis, production  
29 and identification [1,2]. The protein extraction from complex biological matrices is usually done  
30 using numerous methods including dialysis, centrifugation, electrophoresis, chromatography, 2D

31 gel or immunoprecipitation [3–5]. More recently, microfluidic techniques met a great interest  
32 because of the possibility of multiplexing channels and low volume required ( $10^{-9}$  to  $10^{-18}$  L) [6,7].  
33 This interest is emphasized by proteomic advances. They require that the separation is connected  
34 to a detector with a high sensitivity, providing information about the presence of the target  
35 protein [8]. Membrane chromatography for protein separation has emerged several decades ago  
36 for analysis [9,10] and more recently for production [11–14]. However, they stayed as a concept  
37 for protein analysis due to numerous bottlenecks such as low surface volume ratio, limiting the  
38 quantity of protein bonding [15,16]. This makes impossible to detect the eluted analyte directly  
39 from the membrane to common detection devices.

40 On the other hand, the sensing technology based on single polymers and solid-state nanopore  
41 has demonstrated numerous achievements in the area of protein detection, discrimination, and  
42 identification of assembly [17]. There are basically two ways to detect protein using such  
43 nanopore. The first one is based on ionic diode properties [18,19]. Such properties are usually  
44 obtained in asymmetrical (conical or bullet-like shape) nanopore made in polymer film using  
45 track-etching technique [20–23]. Because of the asymmetrical shape and the surface charge, the  
46 current-voltage (I-V) response is not linear [24–26]. Thus, when the target binds to the probe  
47 grafted inside the nanopore, it induces a modification of inner surface wall properties that is  
48 immediately followed by a modification of the ionic transport generating a change in the ionic  
49 diode signal [27,28]. This type of nanopore was previously used to detect several proteins such as  
50 lysozyme [29–34], antibodies[35], and biopolymers [36]. Despite the possibility to specifically  
51 detect a target, the ionic diode is not suitable for a fine characterization of proteins or provide  
52 information on their possible structural modification [17]. To do so, a second way can be used  
53 involving resistive pulse sensing (RPS) [37,38]. The RPS consists of applying a constant voltage  
54 through a single nanopore and record the ionic current. The passage to an analyte induces a

55 current perturbation characterized by an amplitude and duration. The resistive pulse has already  
56 been used for protein or antibody analysis [39–43]. The current perturbations provide a  
57 fingerprint of the protein or protein assembly [44–52]. The RPS sensing can be done at protein  
58 concentrations in the nM range and does not require labelling. This technique is also gaining  
59 interest since the development of dielectric breakdown manufacturing that allows single  
60 nanopore fabrication, making it more accessible than electrons or ions beam that require  
61 expensive facilities [53,54].

62 Combining these different single nanopore and classical membrane approaches could be an  
63 interesting way to detect, separate and characterize a protein with minimum steps and a go/no-  
64 go information during the separation step. It is interesting to note that the investigations  
65 combining single and multipore membranes mainly deal with osmotic energy production. This  
66 combined approach provides a fundamental understanding of ionic transport at the single  
67 nanopore scale and evaluation of the efficiency on multipore membranes [55,56]. Conceptually  
68 for proteins, the separation and detection steps will involve the use of a track-etched membrane  
69 that is scalable from a single nanopore membrane and high density of nanopore membrane ( $10^5$   
70 to  $10^{11}$  pores/cm<sup>2</sup>). Placed in paralleled, the single nanopore membrane "sensor" validate  
71 whether the analyte is present in the solution while multipore the membrane separates the  
72 analyte from the matrix. These two singles and multipore membranes can be produced and  
73 functionalized with a probe following exactly the same conditions [23,57], and thus the sensor  
74 have the same physico-chemical properties as separation membrane. In the second step, the  
75 target protein separated by the multipore membrane will be analysed using a single SiN nanopore  
76 by resistive pulse sensing.

77 Here, we demonstrate a new approach combining different single and multi-pore membrane  
78 technologies for separation, detection and analysis of antibodies. We selected the CD44 antigen

79 (AgCD44) and its associated antibodies (anti-CD44) due to their involvement in cancer [58] or as  
80 a monoclonal antibody-based therapeutic agent [59]. For this purpose, single pores are produced  
81 by track etching method and functionalized with AgCD44 as probe. Then, we demonstrate that  
82 the AgCD44 grafting does not alter its specificity to detect anti-CD44 before producing multipore  
83 membranes under the same conditions to selectively capture anti-CD44. In a second step, the  
84 complex anti-CD44/AgCD44 is eluted from the multipore membrane and analysed by a single SiN  
85 nanopore using the resistive pulse method. This second step will aim to demonstrate if the elution  
86 process maintains the complex AgCD44/anti-CD44.

## 87 **2. Materials and Method**

### 88 **2.1. Materials**

89 The 13  $\mu\text{m}$  thick PET films with biaxial orientations were purchased from Goodfellow (ES301130).  
90 EDC ([≥99.0%](#), 03449), HCl (30721) NaOH ([≥98%](#), 30620), MES ([≥99.5%](#), M8250), NaCl ([≥99.5%](#) ,  
91 S7653), Ethylenediamine ([≥99%](#), E26266), NiCl<sub>2</sub> ([98%](#), 339350), ethylenediaminetetraacetic acid  
92 (EDTA) ([98.5-101.5%](#), E5513), CD44 Antigen (APREST83079), BSA (A9418), lysozyme (05281),  
93 Avidin (A9275), Anti-avidin (B9655), Anti-BSA (SAB4301142), L-DOPA ([≥98%](#), D9629) and PBS tab  
94 buffer (P4417) were purchased from Sigma-Aldrich. NHS-PEG-NTA (228PG2-NSNT-2K) were  
95 purchased from Nanocs. KCl ([≥ 99.5%](#), POCL-00A) was purchased from Labkem. Dowfax 2A1 (lot  
96 #TO20884587-02) was purchased from EZkem. CD44 antibody (ANT-242) was purchased from  
97 PROSPEC.

### 98 **2.2. Membrane design**

#### 99 **2.2.1. Single and multipore track-etched membrane**

100 The 13  $\mu\text{m}$  thick PET films were irradiated by heavy swift ion Kr (9 MeV) at GANIL (Caen, France).  
101 The single pores were obtained from a single track while the multipore membranes were obtained  
102 with a fluency of  $10^8$  ion/ $\text{cm}^2$ [55]. To obtain a bullet-shaped nanopore (single and multipore  
103 membranes), only one side of the film was exposed to UV at 312 nm for 26 hours avoiding  
104 surfactant adsorption[60,61]. The chemical etching was done using a solution containing 6 M  
105 NaOH and 0.05% Dowfax, heated at 60°C for 6 min. Then, the pore was rinsed with ultrapure  
106 water into successive baths of 10 min, 15 min, 30 min, one hour and then overnight.. To obtain  
107 cylindrical nanopore (multipore membrane only), the irradiated polymer films were activated by  
108 12 h per side exposition to UV at 312 nm[62]. Then the chemical etching was done under NaOH  
109 4M solution at 40°C for 4 min to obtain nanopore with a diameter around 100 nm.

### 110 **2.2.2. Track-etched nanopore and membrane functionalization**

111 Single and multipore PET membrane were functionalized using the same procedure. The  
112 membrane (single or multipore) was immersed overnight in a solution of ethylenediamine (1:100)  
113 in MES buffer 0.1 M containing 0.05 M of EDC (pH was adjusted to  $4.5 \pm 0.5$  with HCl) [63]. Then,  
114 a small amount of NHS-PEG-NTA was added to 1 mL of PBS for 12 hours. The membranes (single  
115 and multipore) were then rinsed by Milli-Q water and incubated in a BSA solution at 1 mg/mL for  
116 3 hours. Next,  $\text{NiCl}_2$  solution 100 mM was added for 1 hour. Finally, CD44 antigen His-tag was  
117 added to the tip side of the nanopore and incubated for 3 hours. This was followed by incubation  
118 of the membrane with three different anti-CD44 (3  $\mu\text{g}/\text{mL}$ ) for 1 hour each. The washing solution  
119 was prepared with 500 mM imidazole and 100 mM EDTA, the pH was adjusted to 8.

### 120 **2.3. Ionic diode measurement**

121 The current-voltage (I-V) measurements were performed with an eONE XV amplifier. The current  
122 was measured by Ag/AgCl electrodes. One electrode is connected to the working electrode the

123 amplifier and the second one is connected to the ground. The working electrode and the ground  
124 were placed on the tip and the base side of nanopore respectively (Figure 1e). The I-V curves were  
125 recorded using PBS solution 1X after each step of the functionalization and antibody detection.

## 126 **2.4. Filtration**

127 For the filtration, 1.3 cm diameter multipore membranes (pore density  $10^8$  pore/cm<sup>2</sup>) were  
128 functionalized and then placed into a filtration system made of Teflon ( $r= 0.6$  cm) into which the  
129 sample is injected via a syringe. The filtration process was done using Harvard apparatus PHD  
130 2000 perfusion, and consists of three steps: first, the passage of 250  $\mu$ L of the anti-CD44 (3.37  
131  $\mu$ g/mL) at a flow rate of 167  $\mu$ L/h. Next, the membrane was washed with PBS before to remove  
132 any molecules not bound to the immobilized receptors. Finally, 250  $\mu$ L of the washing solution  
133 was passed through at a flow rate of 167  $\mu$ L/hr to remove the complex AgCD44/Anti-CD44.

134

## 135 **2.5. Resistive pulse sensing**

### 136 **2.5.1. SiN drilling and coating**

137 The single nanopore was drilled inside stressless SiN, 12 nm thick (Norcada) by dielectric  
138 breakdown using Northern Nanopore instruments. Briefly, the SiN film was washed in piranha  
139 ( $H_2SO_4:H_2O_2$ , 3:1) solution at 90°C for 1 hour. Then, it was rinsed with Milli-Q water and dried  
140 thoroughly using an air gun, before being placed in the microfluidic cell (Northern Nanopore). The  
141 system was then flushed with 600  $\mu$ L of propan-2-ol before being filled with deionized and  
142 degassed water. After that, the cell was filled with a solution of KCl (1 M)/ HEPES (8.3 mM), the  
143 pH is then adjusted to 8 by KOH (1 M). Then a potential ramp from 0 to 5 V followed by a slower  
144 ramp from 5 to 14 V was applied across the microchip until a pore opening is detected. Once the

145 nanopore is formed, the solution was changed to NaCl 1.5 M, HEPES 8.3 mM at pH 8 (adjusted  
146 using NaOH 1 M) for the conditioning step. The latter consists to apply a voltage box from -3 V to  
147 3 V for several cycles until the nanopore reached the desired diameter, here about 9.5 nm. After  
148 nanopore opening and characterization by measurement of the conductance, the nanopore was  
149 incubated with saturated L-DOPA ( $c = 10\mu\text{g}/\text{ml}$ ) solution in degassed deionized water for 2 hours.  
150 Then the current was measured for 1 min using -300 mV and +300 mV in order to assess the noise  
151 level of the nanopore.

### 152 **2.5.2. Protein detection and analysis**

153 Protein detection through SiN nanopore (diameter about 9.5 nm) was performed by resistive  
154 pulse using electrolyte solution NaCl 2 M, PBS 1X, pH 7.4. The solution of AgCD44, anti-CD44 and  
155 complex AgCD44/anti-CD44 use for calibration was prepared at 20 nM. The protein solution was  
156 placed on the half-cell connected to the working electrode, the ground was placed on the  
157 opposite side (figure 1) then applying a voltage of -300 mV for 5 min for each protein solution.  
158 The ionic current was recorded at sampling rate 200 kHz with a Bessel filter at 10 kHz. The data  
159 acquisition was performed with a HEKA EPC800 amplifier coupled with a LIH 8+8 acquisition card  
160 using patchmaster software (HEKA electronics, Germany). The current drop induced by the  
161 proteins were detected using a custom-made LabVIEW software “Peak Nano Tools”. The current  
162 traces were filtered at 5 kHz with a Butterworth filter. The threshold for event detection was  
163 determined as 6 times the standard deviation after correction of baseline fluctuations by a  
164 Savitzky–Golay filter. The current drop call events were characterized by a dwell time, and an  
165 amplitude of the blockade of the current as  $\Delta I/I_0$ .

### 166 **Fluorescence measurement**



167 The fluorescence correlation spectroscopy was performed using lab-made confocal  
168 spectrometer previously described [42]. Anti-CD44 was labelled with the alexa-fluor 640 under  
169 buffer NaCl 500 mM, Tris 5 mM, pH 7.5 at a concentration around 1 nM.

### 170 **3. Results and discussion**

#### 171 **3.1. Track etched design and functionalization**

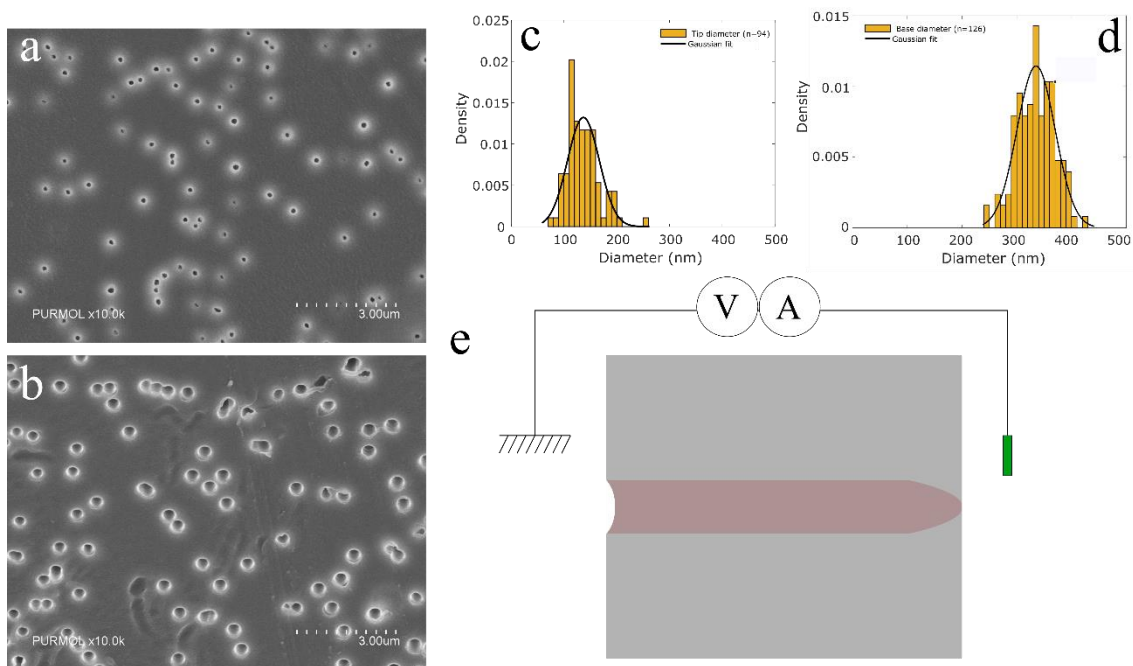
172 Single and multipore nanopore membranes were obtained by the track-etched technique on PET  
173 films (13  $\mu\text{m}$ ) To obtain the sensors, we designed a single asymmetric nanopore for their ionic  
174 diode properties. To this end, bullet-like shape nanopore was achieved by the addition of Dowfax  
175 2:1 surfactant in the etching solution thanks to the different etching rate due to the exposure or  
176 not of the PET surface to UV light [64] (Figure 1e). The diameters  $d_{tip}$  and  $D_{base}$  were determined  
177 by scanning electron microscopy (SEM) on multipore membranes with high pore density obtained  
178 under the same conditions (Figure 1a-b). For an etching time of 6 minutes at a temperature of 60  
179  $^{\circ}\text{C}$ , the histogram of measured  $d_{tip}$  and  $D_{base}$  are centred at 130 +/- 20 nm and 320 +/- 50 nm  
180 respectively (Figure 1c-d). Because it exists variability in nanopore size, each single nanopore was  
181 characterized by ionic conductance measurements. After the chemical etching, the I-V curves  
182 recorded in PBS 1X are non-linear due to the negative charge of the carboxylate moieties. The  
183 origin of this current rectification is the non-homogeneous distribution of ions along the nanopore  
184 as previously reported[65,66] . It depends on the electrolyte concentration and the nanopore  
185 shape and surface charge. Thereby, a weak modification of the nanopore surface state due to a  
186 functionalization step or the binding of an analyte induce a modification of I-V curve shape that  
187 can be characterized by a change of the rectification factor (eq 1)

$$188 R_f = \log \left| \frac{I_{(+1.5V)}}{I_{(-1.5V)}} \right| \quad (1)$$

189 Under our conditions, a positive value of  $R_f$  reflects a global negative charge of the nanopore  
190 surface. On the contrary, an inversion of  $R_f$  toward negative values is induced by a global positive  
191 surface charge of the nanopore. The functionalization of the single nanopore requires 4 steps  
192 (Figure 2a) before binding the AgCD44. Due to the variability of the nanopore, the rectification  
193 factors are given by the average value and the error bars correspond to the standard errors  
194 obtained on 8 independent single nanopores. For each I-V curve, at least 10 measurements were  
195 performed and averaged. The first functionalization step consists of adding ethylenediamine to  
196 the carboxylate moieties. The success of this step is evidenced by a decrease or an inversion of  
197 the  $R_f$  value due to a partial conversion of the  $\text{COO}^-$  of the PET by positive charges of the  $\text{NH}_3^+$   
198 moieties. In the second step, the NHS-PEG-NTA is grafted in the presence of EDC. We observe that  
199 the current rectification increases due to the replacing of  $\text{NH}_3^+$  moieties by negative charge of  
200 carboxylate moieties of PET-NTA. This confirms that the functionalization was successful. The next  
201 steps consist of the addition of the AgCD44 with a histidine tag after addition of  $\text{Ni}^{2+}$ . It is obvious  
202 that the addition of PEG as a spacer will not avoid the non-specific adsorption of AgCD44. Indeed,  
203 the reported density for carboxylic groups inside a PET nanopore is about  $0.2 \text{ COOH}/\text{nm}^2$  [67]. In  
204 addition, the successive functionalization steps do not have a 100 % yield. Considering that, there  
205 are spaces between the PEG-NTA chains where the AgCD44 can be adsorbed. This could lead to a  
206 modification of the nanopore surface and thus play a role in the  $R_f$  value.

207 There are several strategies to reduce non-specific adsorption [68]. The most effective is high  
208 density PEG grafting. This option is not suitable as it is limited to the number of grafting points in  
209 the nanopore, in our case, that would be the carboxylate moieties' density. Another solution is to  
210 adsorb BSA in the free spaces. We used this second strategy. After adsorption of BSA on the  
211 nanopore using a solution at  $1 \text{ mg}/\text{mL}$  in PBS, we notice a decrease in the  $R_f$ . This confirms the  
212 presence of free space between the PEG-NTA. We determined an optimal incubation time of 3

213 hours as after the measured rectification factor stays constant. Once BSA was adsorbed,  $\text{Ni}^{2+}$  was  
 214 added for 3h. The chelation of  $\text{Ni}^{2+}$  on the carboxylate moieties and tertiary amine of NTA  
 215 functions induces an inversion of the rectification factor due to an addition of positive charge in  
 216 the nanopore. We notice that the values of the current and the thus the  $R_f$  are not strictly identical  
 217 for all the single nanopores. This is due to the variability in the size of the nanopore and the  
 218 functionalization rates. However, the trends are identical regardless the nanopore.

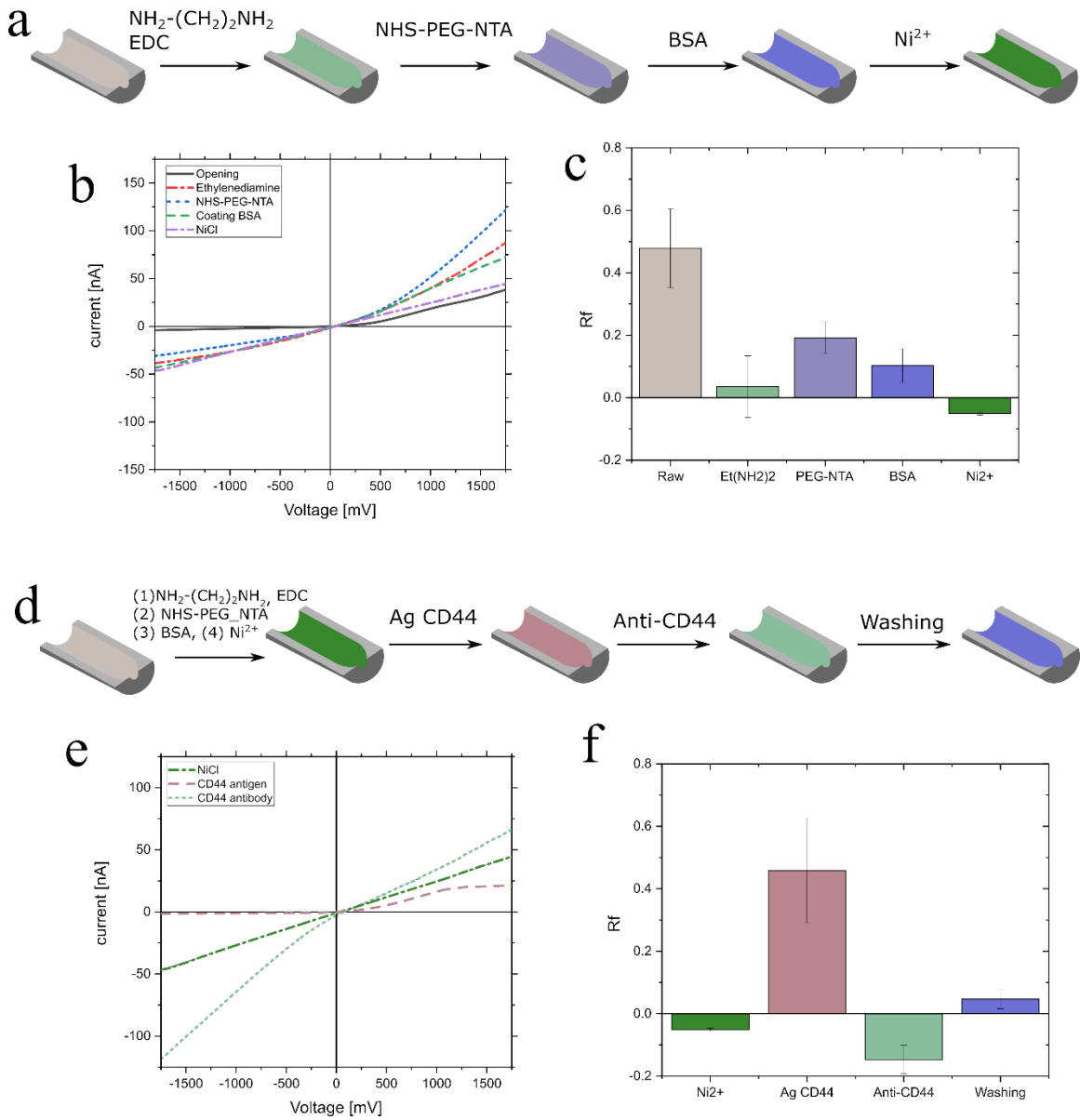


219  
 220 *Figure 1 : SEM of bullet shape multipore membrane open under the same condition that single*  
 221 *nanopore. (a) is the tip side and (b) the base and the associate histogram of diameter (c) and (d)*  
 222 *respectively. (e) Scheme of single bullet shape nanopore for the measurement of the current of*  
 223 *the ionic diode under an applied potential.*

### 224 **3.2. Anti-CD44 detection**

225 Our aim is to detect the anti-CD44. For this, the probe selected is the AgCD44 with a histidine-tag  
 226 because the two amines of imidazole moieties specifically chelate the  $\text{Ni}^{2+}$  loaded on the PEG-NTA  
 227 grafted inside the nanopore. The nanopore incubates for 3 hours in the AgCD44-his-tag solution

228 and then the I-V curves are recorded. We observe an inversion of the  $R_f$  which is in good  
229 agreement with a negative global charge of the AgCD44 at pH 7.2 (IEP 4.9 calculated from IPC).  
230 We therefore attempted to detect the anti-CD44 in PBS solution at a concentration of 3  $\mu\text{g/ml}$ .  
231 After incubation for 90 min, the  $R_f$  reverses toward negative values. We note at this point that the  
232 successive inversion of the  $R_f$  recorded after the  $\text{Ni}^{2+}$  addition, AgCD44 and antiCD44 occurred on  
233 all single nanopores ( $n=8$ ). This allows, despite the variability of the  $R_f$  values inherent to the  
234 distribution of single nanopore size (as shown for multipore membrane on figure 1), to control of  
235 the last steps of the functionalization and the detection of the antibody. We rinsed the nanopore  
236 with an imidazole solution that contains EDTA In order to release the AgCD44/anti-CD44 complex.  
237 After washing, the rectification reverses to reach a range close to the step before the addition of  
238  $\text{Ni}^{2+}$  suggesting that the complex is filtrated. However, this will be confirmed later by experiments  
239 using multipore membranes and further analysis of the filtrate solution.



240

241 *Figure 2 : (a) Illustration of the functionalization steps of the single and multipore nanopore*

242 *membranes (b) I-V curve obtained after each functionalization steps using PBS 1X (c) histogram*

243 *of mean  $R_f$  value obtained from 8 individual singles nanopores ( $d_{tip} = 130 \pm 20$  nm and  $D_{base} =$*

244  *$320 \pm 50$  nm). (d) Illustration of the detection and elution steps of the single and multipore*

245 *nanopore membranes (e) I-V curve obtained after each detection and washing steps (f)*

246 *histogram of mean  $R_f$  value obtained from 8 individual singles nanopore. The error bars are the*

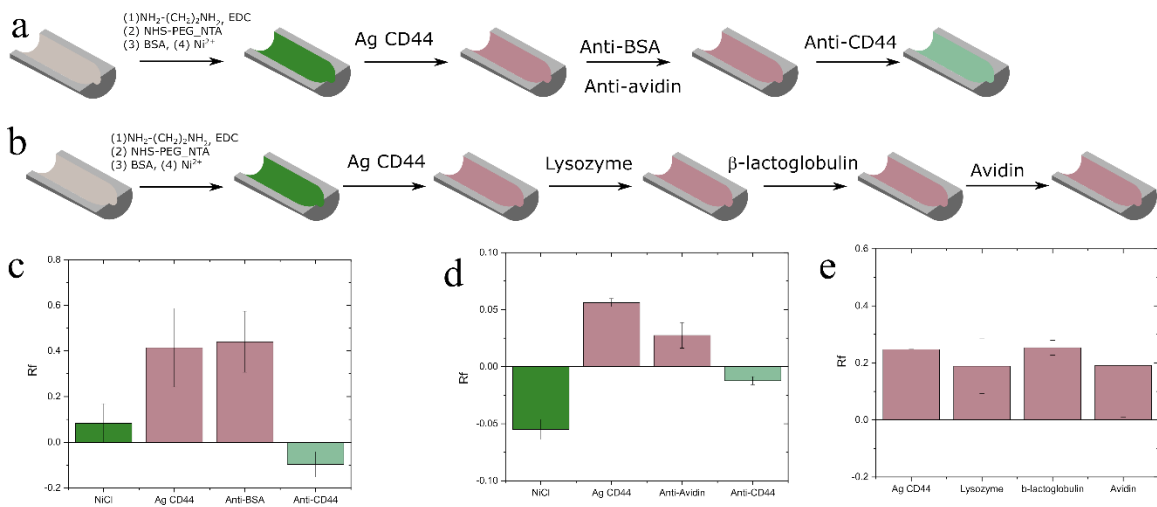
247 *means error obtain from the 8 individual singles nanopore.*

248

249 At this stage, we have to evaluate the selectivity of the single nanopore against antibodies and  
250 other proteins. After 3h incubation with anti-BSA (n=6 independent nanopore), we did not  
251 observe any inversion of the rectification factor (figure 3). This means that there is no BSA binding  
252 site accessible in the nanopore from the coating. Similar results were obtained using anti-avidin  
253 (n=3 independent nanopore). Regardless the antibody, a small variation of  $R_f$  may occur due to  
254 non-specific interactions or adsorption. Nevertheless, this does not compromise the efficiency of  
255 the nanopore sensor. Indeed, after incubation with anti-BSA or anti-avidin, the nanopore remains  
256 functional to detect anti-CD44 as shown by the inversion of the current rectification (figure 3).

257 We have also evaluated whether the addition of different proteins induces a disruption of the  $R_f$   
258 due to non-specific adsorption. For this purpose, a series of proteins was incubated in the AgCD44-  
259 functionalized nanopore. After washing with PBS, the I-V response remained the same, as the  
260 rectification factor (Figure 3e). This means that the selected proteins do not modify the surface  
261 properties of the nanopore and therefore their eventual adsorption can be neglected. After  
262 incubating these proteins, the nanopore sensor remains functional. However, it should be noted  
263 that without an optimal BSA coating, the  $R_f$  is modified after protein incubation.

264



265

266

Figure 3 : (a) Illustration of selectivity tests performed for single nanopore membranes against

267

(a) antibodies and (b) proteins (c) histogram of mean  $R_f$  value obtained from  $n$  individual single

268

nanopores after incubation with (c) anti-BSA ( $n=6$ ), (d) anti-avidin ( $n=3$ ) and (e) various proteins

269

( $n=3$ ). The error bars are the means error obtain from the  $n$  individual single nanopore. The

270

nanopore size are  $d_{tip} = 130 \pm 20$  nm and  $D_{base} = 320 \pm 50$  nm.

271

### 3.3. Separation by multipore membrane and sensing of eluted anti-CD44 at the single

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#### molecule scale

273

We have previously demonstrated on a single nanopore membrane that functionalization with

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AgCD44 was efficient in detecting anti-CD44. The washing with imidazole solution suggested that

275

the complex AgCD44/anti-CD44 should be removed from the pore. To verify this and thereby

276

validate our approach to antibody separation, we designed multipore membranes containing

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cylindrical pores of 100 nm of  $\pm 20$  nm diameter following strictly the same steps as the single

278

nanopore. After grafting the AgCD44, anti-CD44 solutions were filtered before washing with

279

imidazole\EDTA solution. The eluted solution obtained with unlabelled anti-CD44 was analysed

280

by single SiN nanopore of 9.7 nm diameter and 12 nm thickness obtained by dielectric breakdown.

281

In the first step, solutions of AgCD44, anti-CD44 and the complex anti-CD44/AgCD44 were

282 analysed by the nanopore. The current traces show for each sample current blockades that can  
 283 be assigned to the analyte. The amplitude of the current blockades for AgCD44 and anti-CD44 are  
 284 centred at 0.03 and 0.04 respectively. This may seem counter-intuitive given the difference in  
 285 volume of the two proteins (i.e.: 61 nm<sup>3</sup> and 356 nm<sup>3</sup> respectively). We notice, however, that the  
 286 size of anti-CD44 is larger than the nanopore and therefore only bumping events can be recorded.  
 287 This is confirmed by a shorter dwell time recorded for the anti-CD44 than the AgCD44 (Figure 4).  
 288 Conversely, the AgCD44 antigen can translocate into the nanopore. Based on the calculation  
 289 proposed by Yusko *et al.* [69], it is possible to obtain a theoretical value for the amplitude of the  
 290 current blockage by assuming a spheroidal oblate geometry of the AgCD44.

$$\frac{\Delta I}{I_0} = \frac{\Lambda \gamma}{\pi r_p^2 (l_p + 1.6r_p)} S\left(\frac{r_p}{2R_h}\right) \quad (2)$$

291 where  $\Lambda$  is the protein volume,  $\gamma$  is a scaling factor,  $S\left(\frac{r_p}{2R_h}\right)$  correction factor that calculates the  
 292 effective hydrodynamic radius  $R_h$  of the protein inside the nanopore (equation 4).

$$S\left(\frac{r_p}{2R_h}\right) = \frac{1}{1 - 0.8\left(\frac{R_h}{r_p}\right)^3} \quad (3)$$

293 In the equation 2,  $\gamma$  depends on the shape of the protein, for a spherical one it is equal to 1.5.  
 294 For a spheroid, the value of  $\gamma$  depends on the orientation (parallel  $\parallel$  or perpendicular  $\perp$ ) of the  
 295 protein relative to the electrical field. Form factors relative to the orientation  $\gamma_{\parallel}$  and  $\gamma_{\perp}$  are given  
 296 by equation 5 [69].

$$\gamma_{\parallel} = \frac{1}{1 - n_{\parallel}} \text{ and } \gamma_{\perp} = \frac{1}{1 - n_{\perp}} \quad (4)$$

297 Where  $n_{\parallel}$  and  $n_{\perp}$  are dependent on the ratio  $m$  of two hydrodynamic radii. The antibodies have  
 298 an oblate shape  $m=a/b$ . The form is given by the equation 5 and 7



$$n_{\parallel} = \frac{1}{1 - m^2} \left[ 1 - \frac{m}{\sqrt{1 - m^2}} \cos^{-1}(m) \right] \quad (5)$$

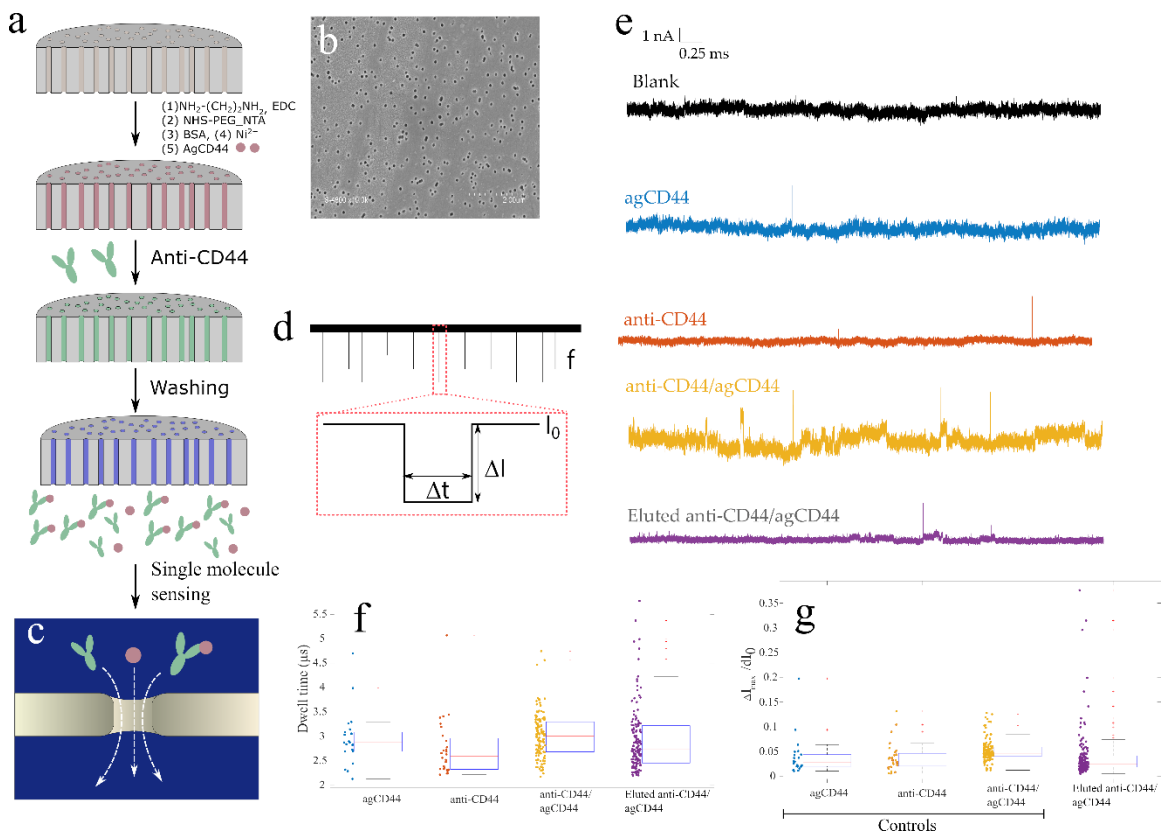
$$n_{\perp} = \frac{(1 - n_{\parallel})}{2} \quad (6)$$

299

300 Taking as radius  $a = 2$  nm and  $b = 2.7$  nm (from the PBD structure), the expected  $\Delta I/I_0$  (according to  
 301 a parallel and perpendicular orientations 0.05 and 0.09 respectively) are larger than measured  
 302 one (centred to 0.03). The experimental values lower than expected could be due to a change in  
 303 the structure of the protein under the effect of the electric field in the pore [70]. We also observe  
 304 that the dwell time is ms scale is larger than the theoretical one as previously reported for the  
 305 experiment conducted at sampling rate of 200 kHz [42,71]. This was assigned to the ability to  
 306 detect only the protein that interacts with the nanopore [42]. The  $\Delta I/I_0$  obtained for the  
 307 AgCD44/anti-CD44 complex are centred on 0.06 (Figure 4). This shift toward larger values can be  
 308 attributed to a larger volume of the complex than that of the anti-CD44, thus inducing a larger  
 309 blocking amplitude during the bump. Interestingly, the dwell time increase regarding the  
 310 antiCD44 that can be assigned to the larger volume of the complex and thus an increase of the  
 311 diffusion coefficient.

312 The eluted solution from the multipore membrane was then analysed by the same single SiN  
 313 nanopore. To do so, we measured current blockade during 25 min with a frequency rate of 0.11  
 314 events/s. Even if this capture rate is low, it confirms that proteins are eluted from the membrane.  
 315 The analysis of the  $\Delta I/I_0$  reveals that the distributions are centred on 0.03. This corresponds well  
 316 with the expected values for non-complexed anti-CD44 and AgCD44. In addition, the distribution  
 317 of dwell time is spread suggesting that translocation and bumping are recorded. These results  
 318 could be explained by the dissociation of AgCD44/anti-CD44 complex during the washing process.

319 In order to confirm this, filtration experiments with anti-CD44 labelled with alexa fluor 647 were  
 320 performed. In order to observe a fluorescence signal in the elution product, we used a confocal  
 321 system to perform fluorescence correlation spectroscopy measurements. First, a fluorescence  
 322 signal was observed for the filtration product confirming the presence of anti-CD44 in the elution  
 323 product. Self-correlation function analysis did not reveal a significant increase in the diffusion  
 324 coefficient of antiCD44 due to its complexation with AgCD44. This is consistent with the nanopore  
 325 analysis which suggests that the washing with imidazole\EDTA allow the elution of the anti-CD44  
 326 and AgCD44 but separates the two entities.



327  
 328 *Figure 4 : a. Scheme of the filtration process through a multipore membrane. b. scanning*  
 329 *electron microscope image of the multipore membrane, c. Scheme of the detection process by a*  
 330 *solid state nanopore, d. Scheme of the resistive pulse showing an event having a current*  
 331 *blockade  $\Delta I/I_0$  and dwell time  $\Delta t$ , e. Traces of each of the measured proteins (AgCD44 in blue,*

332 *anti-CD44 in orange, the complex in yellow) and the eluted solution in violet shown here by the*  
333 *membrane trace, f. Boxplot for the logarithm of the dwell time of all the events obtained from*  
334 *each protein and the filtered membrane solutions, e. boxplots for the current blockade of all the*  
335 *protein and filtered solutions.*

## 336 **4. Conclusion**

337 In summary, we designed single nanopores ionic diode sensors to detect anti-CD44 by a simple I-  
338 V curve measurement. The use of a bullet-like shape allows a good reproducibility, despite  
339 variability inherent to the individual measurement. The multipore membrane obtained under  
340 strictly the same conditions allowed the capture of the antibodies and their analysis by two  
341 methods (nanopore and FCS). The elution product contains the uncomplexed antibody. Thus, we  
342 show that it is possible to design a membrane based on single and multipore track-etched  
343 technique for the separation of antibodies with an associated sensor allowing the parallel  
344 detection of the antibody capture from the bulk solution. Next, using SiN nanopore sensors,  
345 further analysis on the elution product allows to obtain information on the assembly of the two  
346 entities antibody and antigens. We expect that the combination of a multipore membrane with  
347 the ionic diode sensor in a microfluidic system could provide a novel versatile nanopore-based  
348 device for an unprecedented fast preparation of analytical samples or the production of  
349 antibodies. In addition, the parallel detection of traces of a target antibody could allow a pre-  
350 control of the sample before a deeper and costly analysis.

351

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357

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