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### **Toward electrophoretic separation of membrane proteins in supported n-bilayers**

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## Toward electrophoretic separation of membrane proteins in supported n-bilayers

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Electrophoresis, Membrane, Mobility, Proteins, Separation

**ABSTRACT:** Membrane proteins are key constituents of the proteome of cells but are poorly characterized, mainly because they are difficult to solubilize. Proteome analysis involves separating proteins as a preliminary step towards their characterization. Currently, the most common method is “solubilizing” them with sophisticated detergent and lipid mixtures for later separation *via*, for instance, sodium dodecyl sulfate polyacrylamide gel electrophoresis. However, this later step induces loss of 3D structure (denaturation). Migration in a medium that mimics the cell membrane should therefore be more appropriate. Here, we present a successful electrophoretic separation of a mixture first of two and then of three different membrane objects in supported *n*-bilayers. These “objects” are composed of membrane proteins Sulfide Quinone Reductase and  $\alpha$ -Hemolysin. Sulfide Quinone Reductase forms an object from three monomers together and self-inserts into the upper leaflet.  $\alpha$ -Hemolysin inserts as a spanning heptamer into a bilayer or can build stable dimers of  $\alpha$ -Hemolysin heptamers under certain conditions. By appropriately adjusting pH it proved possible to move them in different ways. This work holds promise for separating membrane proteins without losing their 3D structure, thus their bioactivity, within a lipidic environment that is closer to physiological conditions and for building drug/diagnostic platforms.

## 1 Introduction

Membrane proteins play an important role in biological processes. Located at the boundary surface between the inside and the outside of the cell, they play many roles, acting as a gate in active/passive transport of ions/molecules through membranes and thereby ensuring reception/transmission of signals, modeling and adhesion, intercellular recognition, extracellular matrix binding, enzymatic activity, etc. Although they constitute roughly 30% of the proteome of a mammalian cell, i.e. close to 30 000 proteins, only 1040 ( $\approx 3.5\%$ ) 3D structures are known<sup>1</sup>. Yet membrane proteins represent 50% of pharmaceutical targets, knowledge of 3D structure being crucial to researchers seeking to design better-targeted drugs.

This lack of characterization stems from issues with purifying, crystallizing and functionally reconstituting membrane proteins. The main reason is that they are extremely difficult to handle in a water environment without denaturing them. In practice, a mixture of detergents and lipids must be used to stabilize the 3D structure. In addition, the environment is very difficult to reproduce in a fully biomimetic manner, and can wind up being completely different, for example with a separation method like SDS-PAGE. In that case, because of denaturing conditions, the 3D structure is completely lost. As a successful alternative, methods like Blue Native and Clear Native PAGE<sup>2-6</sup> involve one-step isolation of membrane proteins using nonionic detergents such as dodecyl maltoside, Triton X-100 or digitonin under conditions enabling to solubilize biological membranes. By carefully selecting and adjusting the detergent and its concentration, it is possible to create conditions that are not harmful to the membrane proteins or the complexes, owing to the presence of some of the lipids from the solubilized membranes. Then, separated bands can be excised and run in another dimension for final separation with various denaturing techniques (SDS-PAGE, doubled SDS-PAGE and IEF/SDS 3D PAGE).

Pioneering work has been done to apply electrophoresis to achieve biomolecule separation in model membrane. The authors showed that both the electrophoretic and electroosmotic forces could be altered independently of one another and then can be used to precisely control the movement of membrane bound species<sup>7</sup>. Later they developed electrophoretic- electroosmotic focusing of membrane-bound proteins in SLB containing charged lipids<sup>8</sup>.

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3 Some years ago, another strategy was used<sup>9</sup>. In this work, the authors were able to report progress  
4 in the knowledge of membrane proteins, by identifying complexes playing vital biological roles in  
5 *Saccharomyces cerevisiae*. They identified complexes with 1590 putative integral, extrinsic and  
6 lipid-anchored membrane proteins using tandem affinity purification and mass spectrometry.  
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9  
10 More recently, Okamoto et al. separated for the first time two membrane pore-forming proteins in  
11 multilayer lipid membranes at one pH<sup>10</sup>. Embedding the membrane proteins in a medium which  
12 partially mimics their natural environment meant that they could be separated without  
13 denaturation. Bao et al have reported a 25-fold increased concentration of trans-membrane  
14 protein–proteorhodopsin incorporated into supported lipid bilayers using electric fields<sup>11</sup>.  
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19 In the field of separating membrane proteins, another interesting technique was developed by the  
20 Fredrik Höök's group. They demonstrated affinity capturing and local enrichment of membrane  
21 proteins by using a fluid lipid bilayer as the mobile phase. Specific membrane-protein capturing  
22 and enrichment was accomplished by moving with a hydrodynamic flow the membrane protein  
23 inserted in a supported bilayer over a capture region<sup>12</sup>. An alternative to this method consists in  
24 targeting the desired membrane protein contained in proteoliposome with nanoparticles. Then  
25 proteoliposomes are merged into a continuous supported bilayer. Through the extended  
26 nanoparticle tag, a weak hydrodynamic force could be applied, inducing directed in-membrane  
27 movement of targeted membrane protein exclusively. This enabled selective thousand-fold  
28 enrichment of the targeted membrane protein while preserving a natural lipid environment<sup>13</sup>  
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37 We propose here to carry out electrophoretic separation of a mixture of two objects at differing  
38 pHs in differing lipidic systems (different chemical nature and number of bilayers) and then to  
39 separate a mixture of three objects: the two previous objects plus a dimer of one of them. These  
40 objects are composed of membrane proteins Sulfide Quinone Reductase (SQR) or  $\alpha$ -Hemolysin  
41 ( $\alpha$ -HL), model proteins easily handled in aqueous solution before insertion. Sulfide Quinone  
42 Reductase forms an object from three monomers together with some detergent and self-inserts into  
43 the upper leaflet of the bilayer.  $\alpha$ -Hemolysin inserts as a spanning heptamer into the bilayer or can  
44 build stable dimers of  $\alpha$ -Hemolysin heptamers under certain conditions. In previous papers<sup>14-17</sup> we  
45 measured some physico-chemical properties (diffusion coefficient, electrophoretic mobility, and  
46 charge) of these two different objects alone, taken as models in such an environment. Sulfide  
47 Quinone Reductase (SQR) and  $\alpha$ -Hemolysin ( $\alpha$ -HL) were chosen because they are very different:  
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3 one is monotopic (inserts in one leaflet); the other is transmembrane (spans the two leaflets). This  
4 increases the probability that they will have different electrophoretic mobilities. Another reason is  
5 that they are easy to handle because soluble as monomers in aqueous solution which auto-assemble  
6 and then self-insert into the bilayer. About  $\alpha$ -HL and its ability to form multimers, this method  
7 shows that it could be a way for separation of multimeric ion channels.  
8  
9

10 We use supported phospholipidic  $n$ -bilayers of two different lipids with  $n$  which can be set to 1, 2  
11 or  $\cong 35$ . The method was “learning when walking »: i) building one single bilayer ( $n=1$ ) with a  
12 Langmuir-Blodgett trough leads to a well-defined basic system; ii) a double bilayer ( $n=2$ ) provides  
13 another test system; iii) a multibilayer ( $n \cong 35$ ), very easy to build by simply rehydrating a  
14 desiccated film, will be useful for quick applications. It is a third system to test reproducibility of  
15 results and might improve membrane protein loading capacities for what we hope will become  
16 routine separation.  
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19 Starting with a mixture of known objects, conditions can be chosen so as to immobilize either one  
20 or the other object, move them in opposite directions, etc. Later, starting with an unknown mixture,  
21 after a screening of the pHs, separating conditions can be chosen in accordance with each situation.  
22 Furthermore, these systems can be used as a drug or diagnostic platform, once proteins have been  
23 separated.  
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## 34 35 36 **2. Results and Discussion**

### 37 38 **2.1 Characterization of the different systems**

39 Because lipids of our SLBs have phosphatidylcholine head groups, saturated aliphatic backbones,  
40 and because diffusion coefficients, mobilities, quantitative analysis of protein incorporation have  
41 been measured in the fluid phase, there is no difference between them going from a system to  
42 another. This was expected and there is no reason to observe different behaviour under electric  
43 field as reported<sup>18-19</sup>, perhaps due to the confined geometry.  
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49 The different systems of our study are summarized in Table 1.  
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52 **Table 1:** Systems, pH and incubation temperature in different supported lipidic bilayer systems  
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Mixture	Conditions	DMPC single bilayer	DMPC double bilayer	DMPC Multibilayer	EggPC Multibilayer
of 2 Objects	pH	6.8; 8.3; 9.2	-----	6.8; 8.3; 9.2	6.8; 7.2; 8.3; 9.2
	Incubation T(°C)	33	-----	33	33
of 3 Objects	pH	-----	9.2	-----	-----
	Incubation T(°C)	-----	33 °C ( SQR & $\alpha$ - HL) then 22 °C to obtain $\alpha$ -HL dimers	-----	-----

### 2.1.1 Mixture of two objects

For the mixture of the two objects, we used different membrane models and as shown in the table 1, we incubated both SQR and  $\alpha$ -HL in a single bilayer of DMPC, a multibilayer of DMPC and a multibilayer of EggPC. After incubation of SQR over a single bilayer of DMPC, the support was placed under a fluorescence microscope and a density of 426 spots/mm<sup>2</sup> was observed. After incubation of  $\alpha$ -HL and washing, the spot density observed for  $\alpha$ -HL was 177 spots/mm<sup>2</sup>. The order of magnitude of the spots densities was reproducible whatever the system.

### 2.1.2 Mixture of three objects

We did this experiment on a double bilayer of DMPC as indicated in the table 1. As an illustration of what was described in the previous paragraph, we observed 392/mm<sup>2</sup> spots *versus* 426 spots/mm<sup>2</sup> (mixture of 2) for SQR, a reproducibility of insertion to that of the protein alone, which saturated at around 400 spots/mm<sup>2</sup> in [16]. There was the same trend for  $\alpha$ -HL with 182 spots/mm<sup>2</sup> *versus* 177 spots/mm<sup>2</sup> (mixture of 2) and  $\approx$ 180 spots/mm<sup>2</sup> for the protein alone<sup>14</sup>. For the dimer of heptameric  $\alpha$ -HL, we found 130 spots/mm<sup>2</sup>, which is in good agreement with the 139 spots/mm<sup>2</sup> obtained after 24 h incubation<sup>16</sup>. Number of spots varied from one experiment to another by around 5 %.

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3 In addition, these reproducible densities shed light upon the mechanism of dimer formation: the  
4 dimers are built independently, their formation does not consist in dimerization of already-inserted  
5 heptameric  $\alpha$ -HL.  
6  
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### 8 9 **3.1.3 Identification of objects**

#### 10 11 **Fluorescence intensity in arbitrary unit (a.u)**

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13 In previous work<sup>14-17</sup>, we observed an average intensity close to 38 arbitrary unit (a.u.) for trimeric  
14 SQR and heptameric  $\alpha$ -HL. The same intensity was observed here, corresponding to the label  
15 content of each object. For the dimer of heptameric  $\alpha$ -HL alone, the average intensity was  $\approx 70$   
16 a.u, roughly twice the previous intensity, as expected.  
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19  
20 In the mixture of two objects, we observed spots having an average intensity of 38 a.u. In the  
21 mixture of three objects, we observed spots having the same intensity and spots having an average  
22 intensity of 67-70 a.u, indicating the presence of the three objects.  
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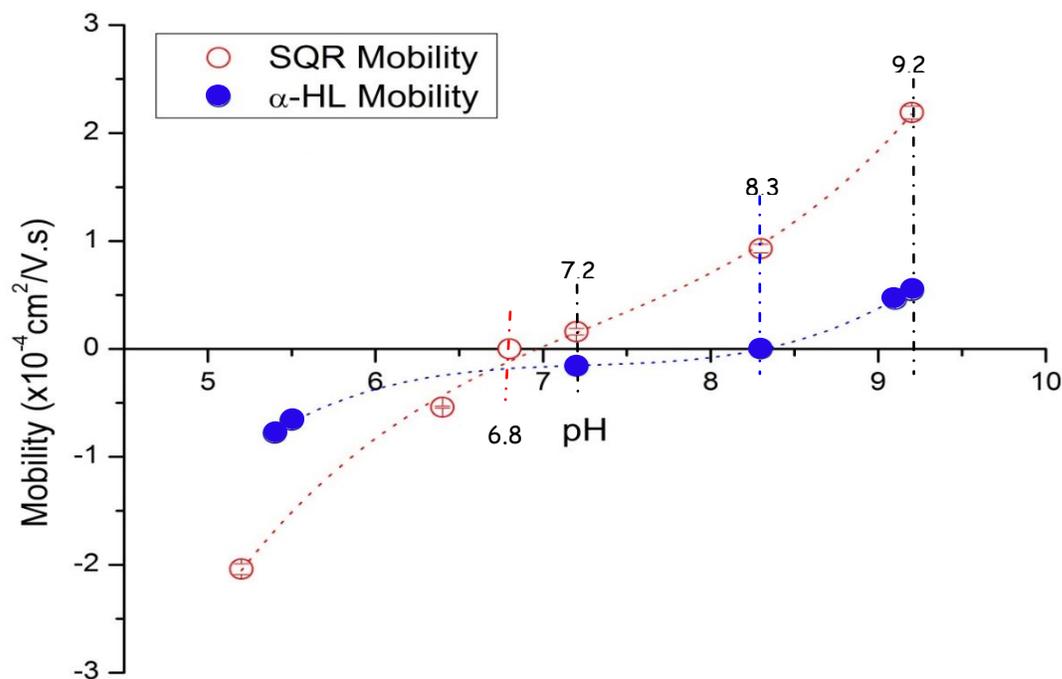
#### 25 26 **Differentiation between SQR and $\alpha$ -HL**

27  
28 For both mixtures (2 or 3 objects):  
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- 30 • SQR was incubated first, then videoed and analyzed. Recording free particle trajectories  
31 with our video setup, and treating them with Image J developed by the MOSAIC GROUP  
32 at ETH Zurich [25], we determined diffusion coefficients  $D$  of SQR at zero electric field  
33 (with the plugin), finding values of  $D$  in good agreement with previous reports<sup>16</sup>.  
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- 36 • Next,  $\alpha$ -HL was incubated. Then, we put the electric field on and observed two populations:  
37 each had its own mobility, similar to when it was measured on protein alone<sup>14-15</sup>. This  
38 enabled us to distinguish SQR from  $\alpha$ -HL.  
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- 41 • No video measurements of diffusion coefficients were performed for  $\alpha$ -HL but total spot  
42 density was observed to have increased, indicating that SQR and  $\alpha$ -HL were now inserted  
43 in the same bilayer.  
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- 46 • Each determination (fluorescence intensity and electrophoretic mobility) on each video  
47 was carried out on 10 spots at least. Using these determinations, we obtained a position  
48 pattern for a given object that was then used, in a sort of feedback loop, to selectively color  
49 the spots on the figures of the article (SQR in red,  $\alpha$ -HL in blue) for easier discrimination.  
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### 3.2 Relevant pHs for protein separation

We wished to visualize, and thus to predict, which pH(s) could be used to separate the objects. Our previous studies<sup>15, 17</sup> provided data on how electrophoretic mobility of each protein depends on pH, enabling us to draw Figure 1 to compare them.



**Figure 1:** Electrophoretic mobility of trimeric SQR alone (open red circles) and heptameric  $\alpha$ -HL alone (full blue circles) as a function of pH (the error bar is  $\pm 10\%$ ).

We determined four relevant pH values for testing:

- 7.2: objects should move in opposite directions;
- 9.2: the two objects (later, the three) should race each other in the same direction, SQR being the fastest;

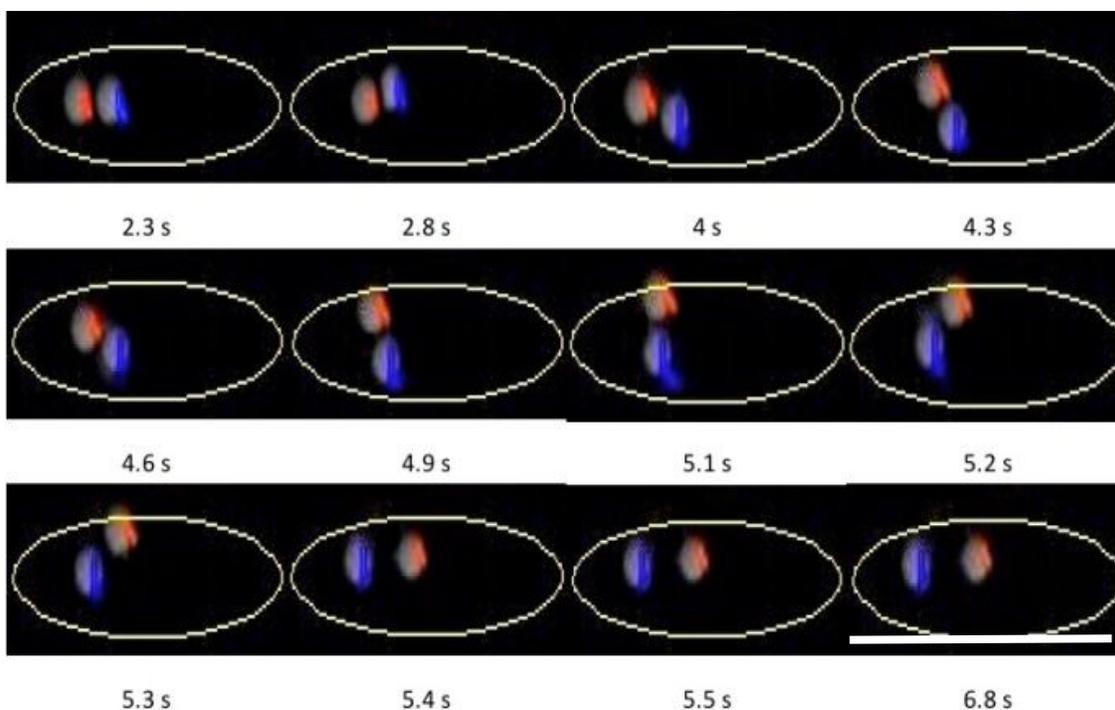
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3 - 6.8 and 8.3 (SQR and  $\alpha$ -HL, respectively): one object should be immobile because the pH  
4 corresponds to its pI;  
5  
6

7 That is what was observed. To help visualize the differing behaviors, in addition to the videos  
8 (Supplementary Information S1-S5) a series of snapshots is presented in figures 2-3. SQR objects  
9 are colored red,  $\alpha$ -HL objects blue, and dimer  $\alpha$ -HL objects yellow, as are the arrows illustrating  
10 their motion.  
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### 14 3.3 Separation of two objects (trimeric SQR + heptameric $\alpha$ -HL)

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16 At pH 7.2, both proteins have mobility, but in opposite directions since they have opposite charges  
17 (see fig.1). The complete sequence is shown in video 1 (Supplementary information S1). They  
18 frequently appear to simply go their own way, ignoring the existence of other species. However,  
19 there are some situations where two different objects approach each other closely enough to  
20 collide, with potentially varying results. The question is: will they join together and stop  
21 migrating? Or will they migrate as one object with a mobility that is derived from their opposite  
22 mobilities?  
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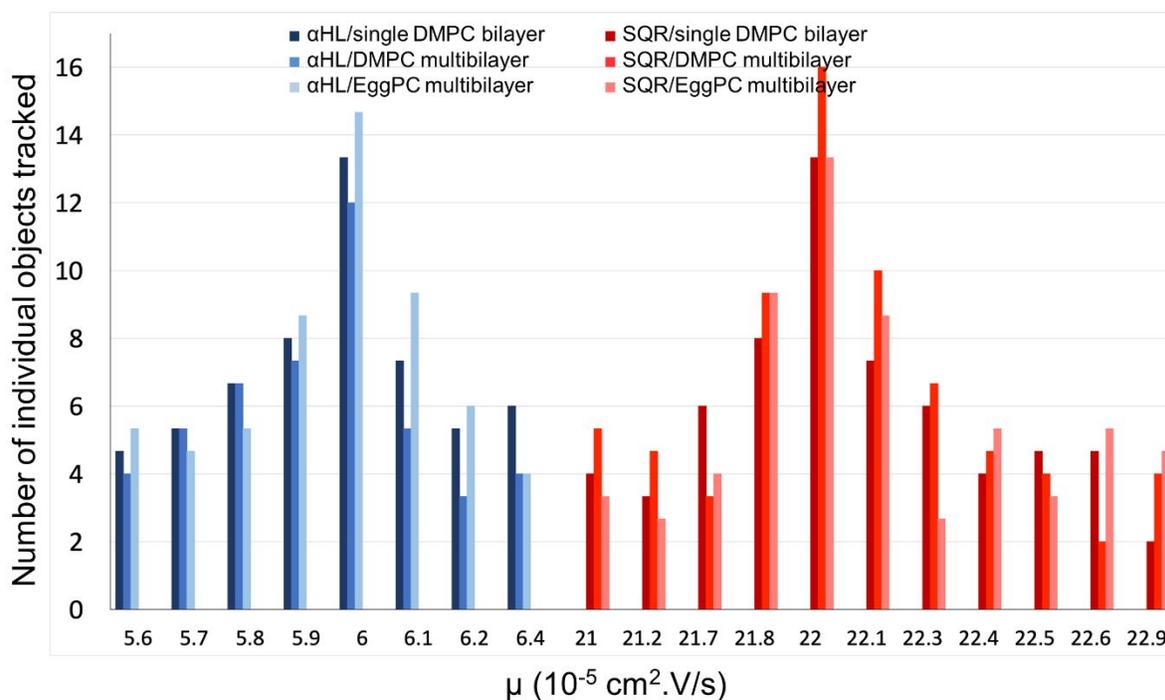
29 We were able to capture these particular situations (bottom/left and top/right, yellow ellipses in  
30 video 1). We focus on one of these events from the movie, detailed in figure 2: we extracted some  
31 snapshots, time interval 2.3 s to 6.8 s, from video 1 (Supplementary information S1).  
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3 **Figure 2:** Zoom on SQR (red) bumping into  $\alpha$ -HL (blue) inserted in EggPC supported multibilayer  
4 at pH = 7.2 under a 3 V/cm electric field with the time interval 2.3 s to 6.8 s extracted from video  
5 1 (S1). Scale bar: 50  $\mu$ m.  
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11 In the movie, the objects start to bump into each other at first, bounce, and finally manage to find  
12 a way to glide past each other before following separate paths. The striking feature is that, even  
13 though they have opposite charges, they do not stick to each other. This is a very interesting result  
14 since it proves that, even with opposite charges, separation is still possible. This is one of the key  
15 points that such a platform could perform the separation for multiple biomolecules. We suggest  
16 that they do not stick to each other because each object is surrounded by a belt of bound lipids.  
17 This is supported by previous studies which have established that the folding, structure and  
18 function of membrane proteins are influenced by their lipid environments and that lipids can bind  
19 to specific sites<sup>20,21</sup>  
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27 The complete sequence at pH 9.2 is shown in video 2 (Supplementary information S2). As figure  
28 1 shows, the difference in mobility is maximal, meaning that this is the pH at which separation is  
29 fastest and most efficient for this system. At this pH, all objects have a negative charge and race  
30 toward the positive electrode as shown by the red (for SQR) and blue (for  $\alpha$ -HL) arrows. SQR  
31 migrates faster ( $\mu_{\text{SQR}} = 2.2 \times 10^{-4} \text{ cm}^2/\text{V} \cdot \text{s}$ ) than  $\alpha$ -HL ( $\mu_{\alpha\text{-HL}} = 6 \times 10^{-5} \text{ cm}^2/\text{V} \cdot \text{s}$ ), the mobilities being  
32 the same as when the proteins were alone. SQR either overtakes  $\alpha$ -HL easily while migrating when  
33 they are in distinct lanes or may overtake by changing lanes when SQR catches up with  $\alpha$ -HL on  
34 the same trajectory (see yellow ellipse on video 2 around  $t = 13$  s). Again, we observe no sticking  
35 to each other or overcrowding that would prevent separation. A histogram of distribution of  
36 measured electrophoretic mobility including the number of individual proteins tracked (SQR -  
37 different red- and  $\alpha$ HL -different blue-) at pH 9.2 is provided in figure 3.  
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**Figure 3:** Distribution of measured electrophoretic mobility as a function of the number of individual objects tracked (distributions in nuances of blue for  $\alpha$ HL and in nuances of red for SQR)

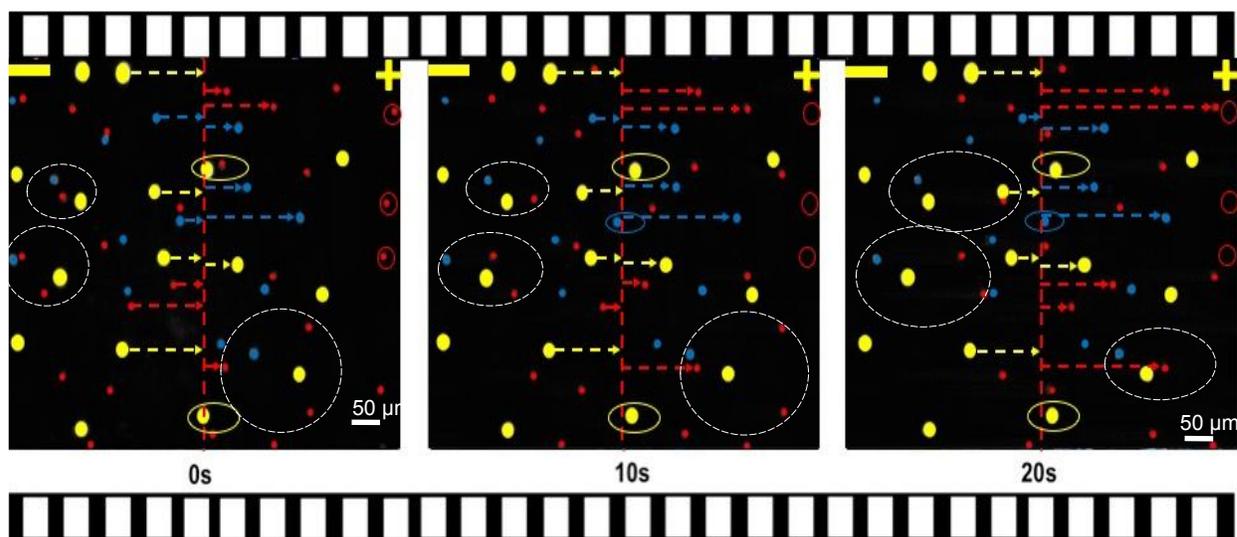
This distribution results from the analysis of 3 experiments carried out at 3 different places on the same sample (around 60 spots followed). Beyond natural dispersion of a measure, this distribution can come from variation of the label content of the objects (two dyes on a monomer or two labeled monomers in an object). The overall variation is for each mobility is close to 10 %.

At pH 6.8, the SQR is motionless, while the  $\alpha$ -HL moves toward the negative electrode (see video 3- Supplementary information S3). At this pH,  $\alpha$ -HL (blue spots) has a positive charge whereas SQR (red spots) has reached its pI (isoelectric point)<sup>15, 17</sup>. We measured the mobility of  $\alpha$ -HL, and found  $\mu = -1.9 \times 10^{-5} \text{ cm}^2/\text{V} \cdot \text{s}$  alike when it was alone<sup>15</sup>. The next experiment is intended to reverse roles for SQR and  $\alpha$ -HL. At pH = 8.3,  $\alpha$ -HL reached its pI and SQR had a net negative charge. The SQR moves toward the positive electrode (see video 4- Supplementary information S4) but the  $\alpha$ -HL is immobile. We measured the mobility of SQR, finding a value of  $1.0 \times 10^{-4} \text{ cm}^2/\text{V} \cdot \text{s}$ , alike when it was alone<sup>17</sup>.

All these scenarios at differing pHs will lead to the separation of the proteins.

### 3.4 Separation of three objects (trimeric SQR + heptameric $\alpha$ -HL+ di-heptameric $\alpha$ -HL)

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3 As a final experiment, we prepared a mixture of three objects: trimeric SQR, heptameric  $\alpha$ -HL and  
4 dimer of heptameric  $\alpha$ -HL. We constructed a system composed of a double bilayer into which we  
5 inserted these three objects, as described in the Materials and Methods section. The experiment  
6 was run at pH 9.2, where all objects are negatively charged. They race each other toward the  
7 positive electrode (video 5- Supplementary information S5). In this video, the different objects  
8 have different shades of gray (trimeric SQR and heptameric  $\alpha$ -HL: light gray; the dimer of  
9 heptameric  $\alpha$ -HL: brilliant gray). In figure 4, as illustration of video 5, trimeric SQR and  
10 heptameric  $\alpha$ -HL are shown in their usual color, red and blue respectively, and the third object, the  
11 dimer, is in yellow (same color code for the arrows). Circles/ellipses illustrate situations where the  
12 three objects (red, blue and yellow) are close at the beginning and where distances between them  
13 increase with time.  
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41 **Figure 4:** Images of fluorescent SQR (red),  $\alpha$ -HL (blue) and dimeric  $\alpha$ -HL (yellow)  
42 electrophoretic migration recorded at 0 s, 10 s and 20 s extracted from video 5 (S5). The vertical  
43 red dotted line is an arbitrary reference line to highlight the position change of objects. Scale bar:  
44 50  $\mu$ m.  
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48 The fastest is SQR and the slowest is the dimer. Each object has its mobility value, determined  
49 during previous work on protein alone. This experiment shows again that it is possible to  
50 electrophoretically separate a mixture of membrane proteins in supported lipid bilayers in a short  
51 time and with a fairly low electric field.  
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55 All the mobilities recorded during these experiments are presented in table 2.  
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**Table 2:** Mobilities  $\mu$  of the different objects as a function of pH and  $x$ , the number of species present in the mixture. Values of  $\mu$ :  $\pm 10\%$ .

Mobility ( $10^{-5}\text{cm}^2/\text{V.s}$ ) of	SQR			$\alpha\text{HL}$			dimer- $\alpha\text{HL}$	
	$x=1$ (alone)	$x=2$ (+ $\alpha\text{HL}$ )	$x=3$ (+ $\alpha\text{HL}$ , dimer)	$x=1$ (alone)	$x=2$ (+SQR)	$x=3$ (+ SQR, dimer)	$x=1$ (alone)	$x=3$ (+ SQR, $\alpha\text{HL}$ )
<b>5.2-5.5</b>	-20.6	-	-	-8.2	-	-	-	-
<b>6.4</b>	- 6.0	-	-	-	-	-	-	-
<b>6.8</b> (pI of SQR)	0	0	-		-1.9	-	-	-
<b>7.2</b>	1.6	1.6	-	-1.9	-2.1	-	- 1,0	-
<b>8.3</b> (pI of $\alpha\text{HL}$ )	9.9	10.0	-	0	0	-	-	-
<b>9.2</b>	22.0	22.2	21.8	5.8	6.0	6.1	-	1.9

### 3. Conclusion

This work provides new insights into the feasibility of an electrophoretic separation of membrane proteins in single, double and multi supported bilayers that can be routinely used starting from a cell or a cell lysate. This separation occurs at low electric field. Beyond separation to establish 3D structure of individual membrane proteins, thus keeping their bioactivity, these systems can be used as test platform for drug or diagnostic. It reveals that SQR remains immobile (when at its pI) while  $\alpha\text{-HL}$  can migrate alone or *vice versa*, which is achieved by suitably adjusting the pH of the buffer. We also demonstrated that when proteins are charged (mixture of two or three objects), separation is feasible due to the differing mobility that results from their differing structures and charges. If the proteins interact while migrating, they do not stick together, probably due to the existence of bound lipids which act as a bumper, avoid each other and continue migrating. This is a very interesting result since even in these situations, separation occurs. Compared to standard

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3 electrophoretic methods, we observed clear separation of the three objects in substantially shorter  
4 distances (microns) and times (seconds), and with a weak field (3V/cm). We hope to generalize to  
5 other membrane proteins after solving the next challenge which is to insert an unknown membrane  
6 protein mixture from living systems into the supported multibilayer. We suggest two methods as  
7 potentially feasible: direct fusion of cells on an already-present supported bilayer or healing an  
8 intentionally-created scratch in a multibilayer with a low-detergent solubilized biological  
9 membrane. In addition, this multibilayer system, easy to build using rehydration of a dried lipid  
10 film, could increase membrane protein loading capacities.  
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## 20 **4 Materials and Methods**

### 21 **4.1 Sample**

#### 22 **4.1.1. Supports**

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26 The slides used as supports were 2.5x 2.5 cm<sup>2</sup>, either mica cleaved on both sides just before use  
27 (JBG-Metafix, France) to support the double bilayer or glass (Marienfield, cut edges, France) to  
28 support the single bilayer or the multibilayer. They were cleaned by immersion for 10 min in  
29 freshly prepared alcoholic NaOH, then thoroughly rinsed with ultra-pure water and sonicated 3  
30 times for 5 min in ultra-pure water.  
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#### 35 **4.1.2. Lipids**

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37 All lipids were purchased from Avanti Polar Lipids (Coger, France) and used without further  
38 purification. Supported single and double bilayers were prepared using 1,2-dimyristoyl-sn-  
39 glycerol-3-phosphocholine (DMPC, Mw 734 g·mol<sup>-1</sup>). Supported multibilayers were prepared  
40 using either DMPC or L- $\alpha$ -phosphatidylcholine (EggPC, Mw 770 g·mol<sup>-1</sup>).  
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45 The single bilayer and double bilayer of DMPC were built using the Langmuir-Blodgett transfer  
46 technique (for details, see [22]). Lipid molecules solubilized in chloroform (~1 mg/ml) were  
47 deposited on the water subphase (18 M $\Omega$ cm, MilliQ, 15 °C) of a Langmuir trough (KSV  
48 Minitrough 361 mm  $\times$  74 mm, Finland), equilibrated for 10 minutes to allow complete solvent  
49 evaporation and then compressed to 30 mN/m. Transfer ratios were close to 1.  
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3 For the double bilayer on mica, the fourth monolayer, closing the second bilayer, was transferred  
4 using the Langmuir-Schaefer (LS) technique. Transfer ratios were close to 1 for the first three  
5 monolayers and 0.8 for the fourth monolayer.  
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8  
9 The multibilayer was formed *via* the lipid film hydration method. 20  $\mu\text{L}$  of lipids (either DMPC  
10 or EggPC) in chloroform (1mg/mL) were first desiccated under vacuum for 1 h on a glass slide.  
11 Then the slide was positioned in our incubation cell (a Teflon<sup>TM</sup> cylinder embedded in steel, 1 cm  
12 diameter) and the film was rehydrated at room temperature with ultrapure water (18 M $\Omega$ -cm,  
13 MilliQ) for 30 min. Obtaining a multibilayer can be difficult. Several articles detail how  
14 Multilamellar Vesicles (MLV) can be produced *via* rehydration of dry lipidic film. We noticed  
15 that it usually involves supplying energy to the system, for instance through strong agitation or  
16 electric fields. Failing this, the outermost bilayer of a rehydrated DOPC film will spontaneously  
17 peel off (possibly bilayer by bilayer). However, it is also reported that peeling does not occur when  
18 starting from DPPC in gel phase<sup>23</sup>. More recently, Relat-Gobena et al<sup>24</sup> carried out AFM  
19 experiments on supported rehydrated multibilayer stacks in buffer solution. Keeping the same  
20 buffer without rinsing, they avoided turbulences and thus detachment of the lipid stacks.  
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30 In our system, we obtain a supported multibilayer and this is ensured by the following:

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- Removal, washing and addition to the Teflon cell were performed very gently and along the wall of the cell, to minimize turbulence.
  - Working in pure water on glass increases electrostatic interactions because of the low ionic strength. This helps stabilize the rehydrated multibilayer at the glass surface.
  - Qualitatively, under the microscope, the sample appeared flat: there was no need to adjust the  $z$  direction to remain in focus, and no sign of the existence of slowly “swimming” MLVs.

45 Additionally, the electrophoretic mobilities of our objects were analogous when measured in single  
46 or double bilayers. Experiments were performed in replicate reproducibly in all systems (at least  
47 twice, sometimes more).  
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50 Assuming a lipid head molecular area<sup>25</sup> of 0.63 nm<sup>2</sup>, given the amount of dried lipids (20  $\mu\text{L}$  from  
51 a solution 1mg/mL) and the area exposed to rehydration (a circle 1 cm in diameter), we estimated  
52 a number of bilayers close to  $\approx 35$  in the multibilayer.  
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3 Supported Lipidic Bilayers (SLB) were kept under water throughout the experiment and used  
4 directly after preparation.  
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### 6 7 **4.1.3. Proteins**

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9  $\alpha$ -Hemolysin ( $\alpha$ -HL), extracted from *Staphylococcus aureus*, was purchased from Sigma Aldrich  
10 Inc and used without further purification.  
11

12  
13 Sulfide Quinone Reductase (SQR) was extracted from *A. aeolicus* membrane with dodecyl- $\beta$ -D-  
14 Maltoside (DDM) and purified as previously described<sup>26</sup>. Following purification, SQR was  
15 dialyzed against 50 mM HEPES buffer pH 7.2, 0.1% DDM using Vivaspin ultrafiltration spin  
16 columns (Sartorius stedim), concentrated at about 2 mg/ml and stored at -20 °C.  
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#### 20 21 **4.1.3.1 Protein Labeling**

22  
23 We labelled the proteins using a kit from Molecular Probes (Invitrogen) to covalently link  
24 Alexafluor™ 488 fluorescent dye to the protein<sup>27</sup>. Alexa Fluor® dyes are reactive molecules used  
25 to add a fluorescent label to the primary amines (R-NH<sub>2</sub>) of proteins. It provides the combination  
26 of water solubility and pH insensitivity between pH 4 and 10 for compatibility in diverse biological  
27 environments. We used Alexa Fluor® 488 TFP (tetrafluorophenyl) ester because TFP esters are  
28 less susceptible to spontaneous hydrolysis during conjugation reactions. The initial concentration  
29 of the protein was 0.4 mg/ml in PBS 1x (with DDM in the case of SQR), the reaction time was 15  
30 min for  $\alpha$ -HL and 40 min for SQR. This yields an average labelling of 0.2 mole Alexa 488/mole  
31 protein.  
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34  
35 After purification, the solution containing the labelled protein was mixed with the proper amount  
36 of a solution of the same protein unlabelled, to reach a final solution of  $\alpha$ -HL with 1/7 labelled  
37 monomer and a solution of SQR with 1/3 labelled monomer. This rather low-labelled monomer  
38 content rules out any disturbance of the insertion mechanism of the protein and of the dynamics  
39 of the final object. Final aliquots of protein (0.2  $\mu$ M) in Phosphate Buffer (PB) 20mM and NaCl  
40 100 mM pH 7 were stored at -18 °C before use (with DDM in the case of SQR). We used the same  
41 label Alexa Fluor® 488 for both proteins because our camera is black and white, we have acquired  
42 the skill of the labelling process and our optical setups are designed for blue excitation and green  
43 emission.  
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#### 54 55 **4.1.3.2 Protein Insertion**

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3 SQR inserts as a trimeric integral monotopic (lipid-anchored in only one leaflet) membrane protein  
4 at the end of the process<sup>28-29</sup> and we define it as the first object.

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7 Incubated over a fluid bilayer,  $\alpha$ -HL monomers in solution assemble as heptameric pre-pores at  
8 the surface of the bilayer, turning into transmembrane (spanning) heptameric pores<sup>30-31</sup>. We define  
9 it as the second object.

11  
12 Incubating  $\alpha$ -HL over a lipid bilayer consisting of a mixture of fluid/gel regions (half transition)  
13 promotes the formation of dimers of  $\alpha$ -HL heptameric pores<sup>14</sup>, we define it as the third object of  
14 our protein mixture.

16  
17 The composition of the aliquots leads on average to one fluorescent label per object (the  
18 heptameric pore for  $\alpha$ -HL and the SQR trimer for SQR) and to two fluorescent labels per dimer of  
19 heptameric  $\alpha$ -HL.

#### 21 22 23 24 **4.1.3.3 Preparing a system containing a mixture of 2 objects: trimeric SQR and single** 25 **heptameric $\alpha$ -HL**

26  
27 The volume over a slide with the bilayer (or multibilayer) positioned in our incubation cell was 1  
28 ml. First, 700  $\mu$ l of water were removed, leaving the bilayer under the remaining 300  $\mu$ l, and 300  
29  $\mu$ l of an SQR aliquot were added. This final volume of 600  $\mu$ l of protein (SQR 0.1  $\mu$ M in PB  
30 10mM NaCl 100 mM, pH 7.2) was incubated for 12 hours at 33  $^{\circ}$ C (bilayer in the fluid phase),  
31 after which 300  $\mu$ l of the supernatant were discarded and the excess of SQR was gently removed  
32 by adding and pipetting 500  $\mu$ l of distilled water 10 times.

33  
34 The sample was returned to the incubation cell and again immersed under 1 ml of distilled water,  
35 700  $\mu$ l of which were removed again. Then 300  $\mu$ l of an aliquot of  $\alpha$ -HL were added to the  
36 incubation cell following the same protocol (12 hours at 33  $^{\circ}$ C).

#### 37 38 39 40 41 **4.1.3.4 Preparing a system containing a mixture of 3 objects: SQR, single heptameric $\alpha$ -HL,** 42 **and dimer of heptameric $\alpha$ -HL**

43  
44 The incubation was carried out over a DMPC double bilayer. The first two steps were as described  
45 above for the mixture of two objects. Formation of dimers of heptameric  $\alpha$ -HL depends on  
46 temperature and time. To obtain inserted dimers of heptameric  $\alpha$ -HL, the solution was brought  
47 back to 22  $^{\circ}$ C, a temperature where gel and fluid lipid regions coexist and which is thus conducive  
48 to the formation of dimers of heptameric  $\alpha$ -HL. An extra 12 hours of incubation was carried out,  
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3 which added to the initial 12 h incubation of  $\alpha$ -HL gave a total of 24 hours, found to be optimal to  
4 obtain a large number of dimers<sup>14</sup>.

#### 7 **4.1.4 Systems**

9 The temperature used to measure electrophoretic mobilities was 35 °C for all systems, a  
10 temperature where bilayers are fluid. For a given species, for a given pH, all mobilities were  
11 identical whatever the system (single bilayer, double bilayer, and multibilayer, composed of  
12 EggPC or DMPC) and identical to the value measured on the object when alone and not in a  
13 mixture.  
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### 18 **4.2 Videomicroscopy**

#### 20 **4.2.1 Cell**

21 The sample (supported bilayer + inserted proteins) was transferred underwater in a thermostated  
22 electrophoretic cell (see [15] and Supplementary information S1).  
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#### 27 **4.2.2 Video**

28 An inverted fluorescence microscope (Leica) and a camera (Hamamatsu C2400-77: 768 x 494  
29 pixels, 30 MHz) equipped with a fluorescence intensifier suitable for fluorescence imaging  
30 (magnification x 400) were used to carry out protein velocity measurements. Videos of migration  
31 of proteins under electric field (3V/cm) at different pHs are available in the Supplementary  
32 information files S1-S5 (video 1 to 5). As illustrations, series of snapshots extracted from videos  
33 at different times are shown in the Results and Discussion section (figures 2-3). Briefly, every time  
34 after applying the electric field during 20 s to record one experiment, we flush the cell with fresh  
35 buffer to remove electrolysis products, moved to another place over the sample. The setup is ready  
36 to record a new experiment.  
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### 45 **4.3 Electrophoretic mobility**

46 The resulting effective mobility of an object ( $\mu_{\text{eff}}$ ) is the sum of several contributions [11]:  
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$$50 \mu_{\text{eff}} = \mu_E + \mu_{\text{lipid}} + \mu_{\text{EOF}} \quad (\text{eq. \#1})$$

51 We showed that changing the sign of the protein by changing the pH reversed the direction of its  
52 migration. We also showed that the pI of the proteins was unchanged by the presence of Alexa  
53 Fluor® 488. Both arguments mean that the contribution of the charge of the dye to the total charge  
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3 of the object is negligible. Therefore,  $\mu_E$  is the mobility of the charged object, depending on its  
4 charge and its size. Because phosphatidylcholine groups are globally neutral,  $\mu_{lipid}$  is also  
5 negligible. Lastly, the contribution of electroosmosis (EOF) is also negligible due to the ionic  
6 strength and the “coating of the surface by the bilayers as reported by Monson et al<sup>7</sup>.  
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10 We thus get:

$$\mu_{eff} = \mu_E \quad (\text{eq. \#2})$$

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13 We obtain the mobility of the object directly by simply dividing its velocity by the field value.  
14  
15 Drift velocity measurements were performed as described in Harb et al.<sup>15</sup>. Briefly, the velocity of  
16 at least 10 spots measured on three different locations in the bilayer from each sample was obtained  
17 by dividing distance traveled by the objects by time. Reproducibility was within 10 %. The primary  
18 source of error was the measurement of the displacement, due to the resolution of the microscope  
19 ( $\pm 1 \mu\text{m}$ ).  
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38 The authors have declared no conflict of interest.

## 39 **Supporting Information.**

40 S1 to S5: Video 1 to 5 (.avi file)

41  
42 S6: Schematic diagram of the video/electrophoretic cell and its description. Protocol to make  
43 videos (text and diagram, pdf)  
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### 55 **Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## ABBREVIATIONS

DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; EggPC, L- $\alpha$ -phosphatidylcholine (Egg, Chicken); SLB, Supported Lipidic Bilayer; SQR, Sulfide Quinone Reductase,  $\alpha$ -HL, Alpha Hemolysin.

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