Reconstitution of the activity of RND efflux pumps into proteoliposomes: a quantitative approach

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Introduction

Over the years, bacteria have developed many mechanisms of resistance to antibiotics. This is due to various factors such as: strong dependence of people on antibiotics, bad prescription, as well as overuse in agro industries. Resistance relies on mechanisms like modification of the antibiotic target, degradation of the antibiotic by enzymes, alteration of the membrane permeability, short circuit of the targeted metabolic pathway, and finally the activation of efflux pumps (figure 1). The latter point is the major mechanism of resistance to antibiotics. Efflux pumps are membranous proteins which export substrates from the cytoplasm/periplasm to the extracellular medium of the bacteria.

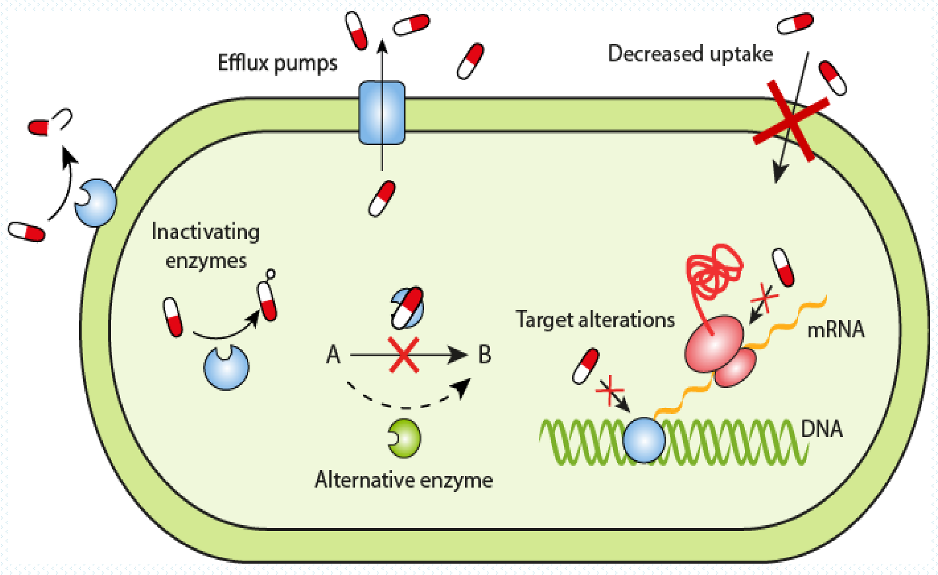


Fig. 1 : The different mechanisms of antibiotic resistance in bacteria.

There are different types of efflux pumps, classified by the way they transport their substrates, and by the energy needed to be active (ATP hydrolysis, proton motive force, ion counter-transport) (figure 2).
Efflux pumps from the RND (Resistance, Nodulation and Division), which use a proton gradient to be active, are composed of three proteins : the Outer Membrane Factor (OMF), a channel localized in the outer membrane, the Resistance-Nodulation-Division (RND) transporter in the inner membrane, and the Membrane Fusion Protein (MFP) which connects the previous proteins.

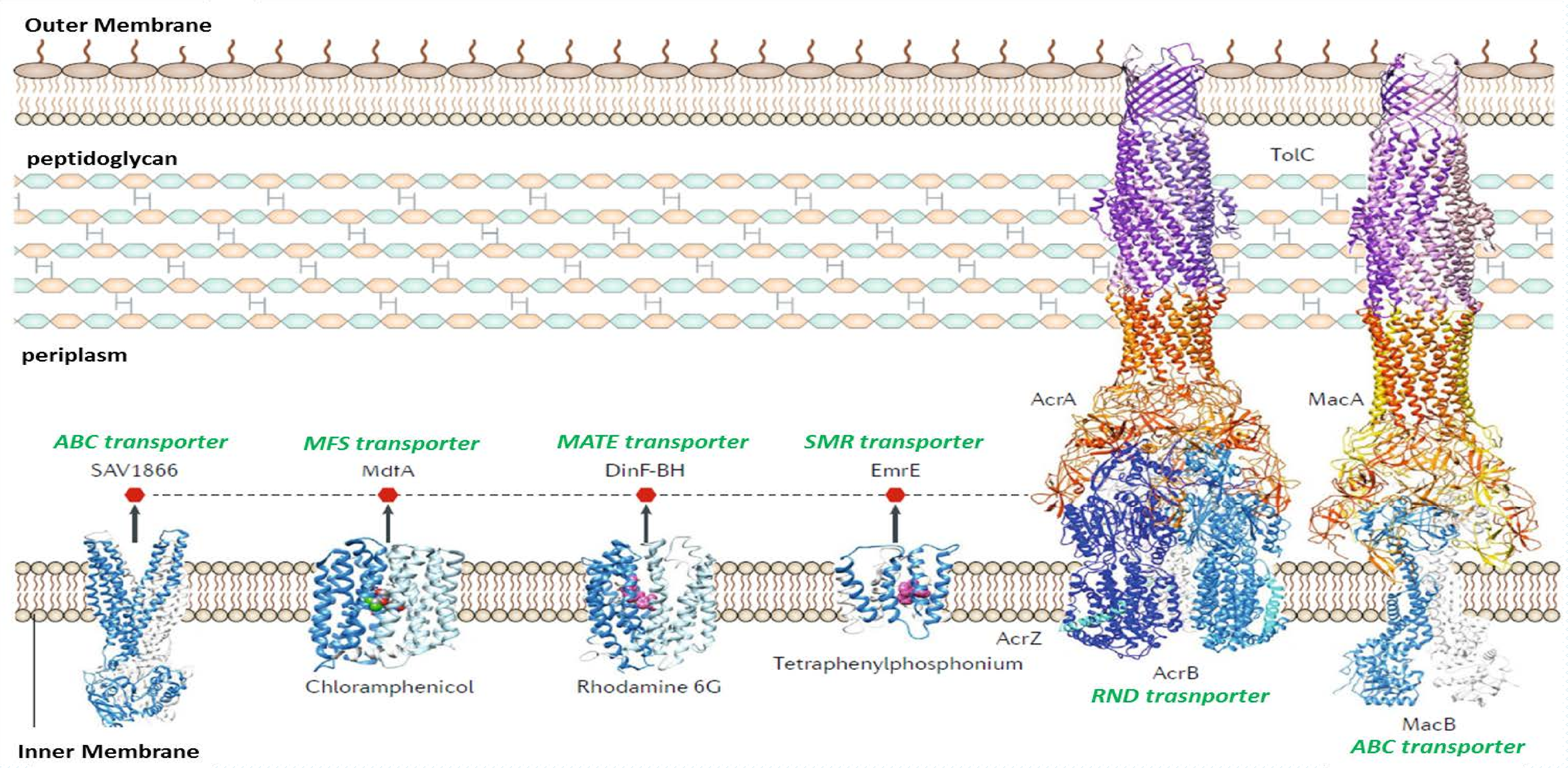


Fig. 2: Schematic representation of the main MDR-transporters in *Escherichia coli; Illustration from Du, Nature Review 2018.*

Here, we focus the MexA-MexB-OprM RND type efflux pump from *Pseudomonas aeruginosa*, a Gram-negative bacterium responsible for clinical diseases such as nosocomial pneumonia (OMS, 2017).

By reconstituting proteins into liposomes (producing so called proteoliposomes), we can measure activity of the pump: first we reconstitute MexA and MexB into one liposome, and then we reconstitute OprM into another liposome. This proof of concept has been established in 2014 by Alice Verchère and colleagues (figure 3):

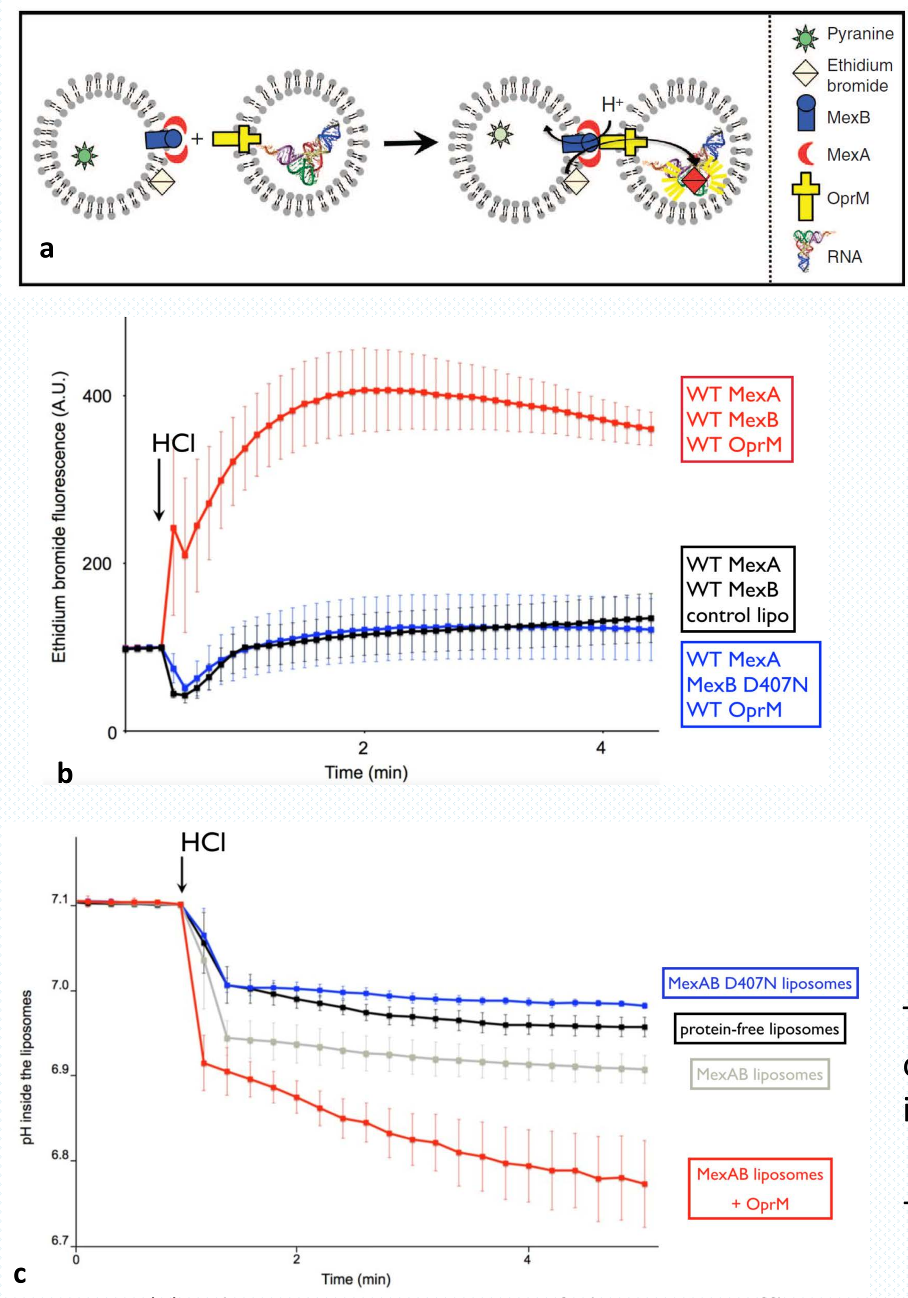
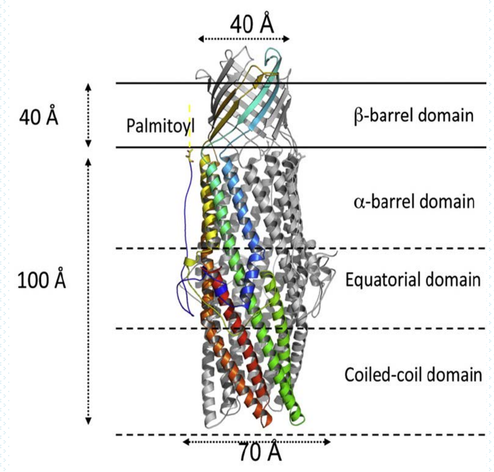
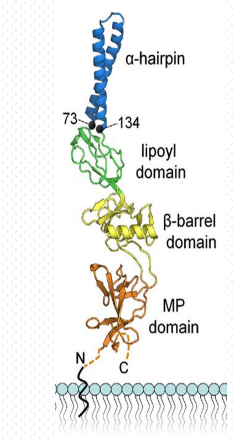


Fig. 3: (a) Schematic representation of the *in vitro* efflux pump assembly; (b) Ethidium bromide fluorescence and (c) liposome pH in function of time.

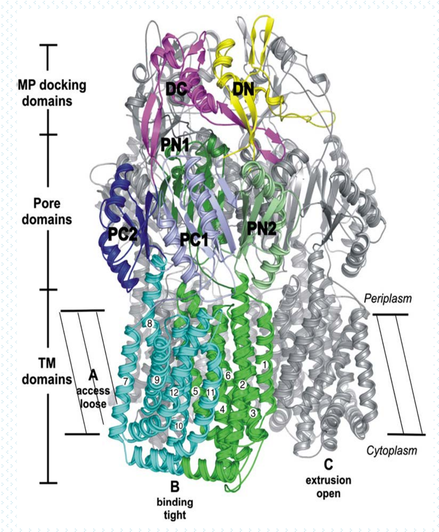
What do we know about the structures ?



OprM structure (150 kDa). Adapted from Phan et al Structure 2010.



MexA structure (40 kDa). Adapted from Greene FEBS 2013.



MexB structure (330kDa). Adapted from Sennhauser JBM 2009.

What we do not know

- Catalytics parameters (Km, Vmax, kcat,…).
- The number of H^+ needed for the transport of one molecule of antibiotic.
- How the recognition of substrate occurs.
- Direct inhibitors of this efflux pump.

Project aims

The aim of this project is to measure the velocity of transport of the efflux pump MexA-MexB-OprM when reconstituted into proteoliposomes. For this purpose, we have to:

- Prepare homogenous population of proteoliposomes and estimate the average of proteins per liposomes.
- Use a Stopped-flow apparatus to measure fast velocity of transport.

Methods

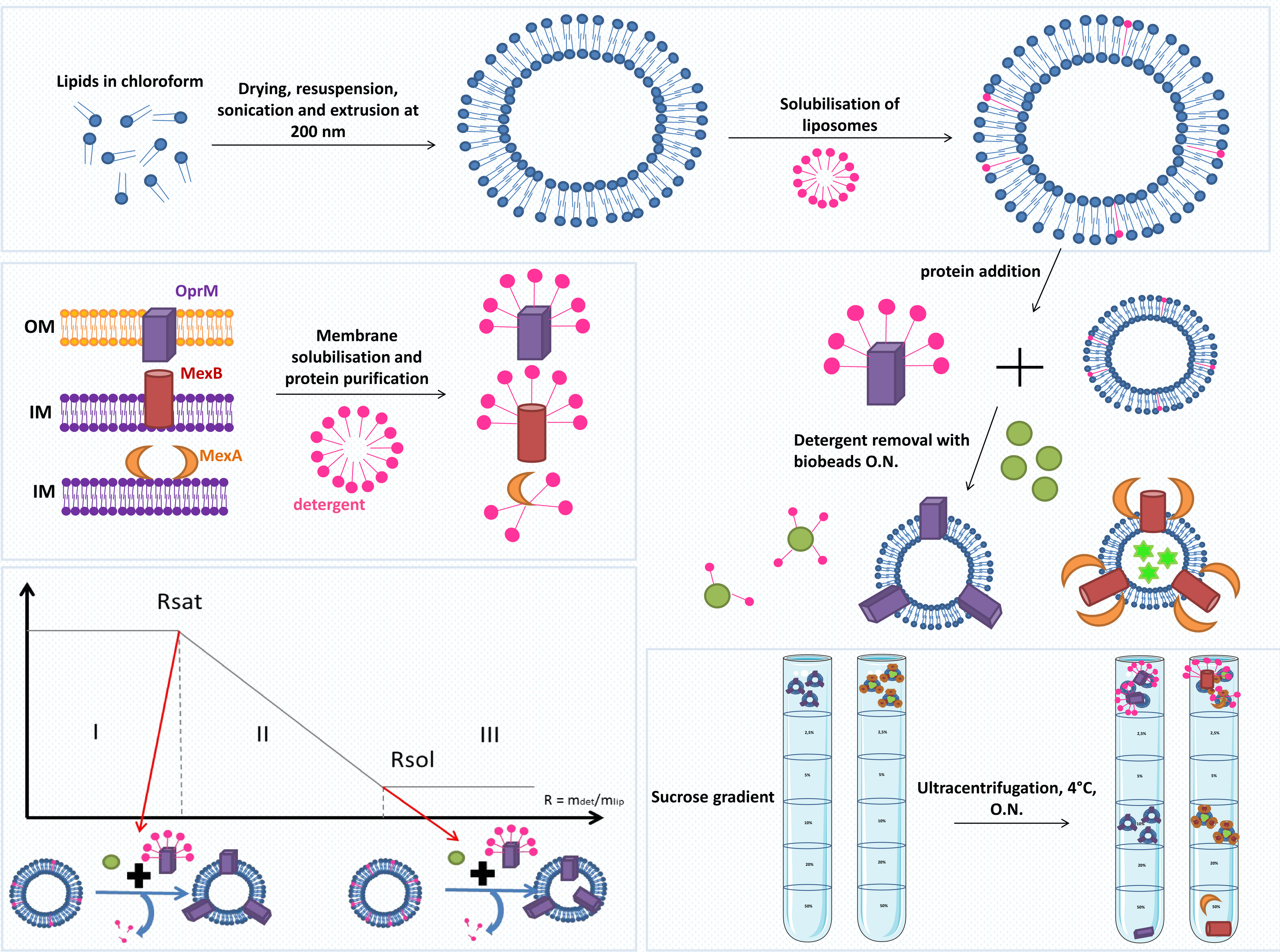


Fig. 4: Proteoliposome reconstitution and purification on sucrose gradient. : parameters that can be tuned

Results

DLS measurement on MexAB proteoliposomes

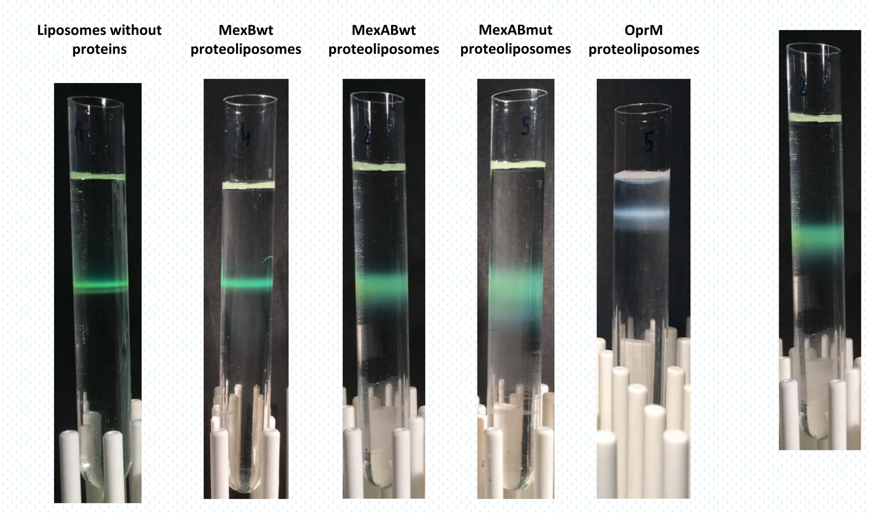
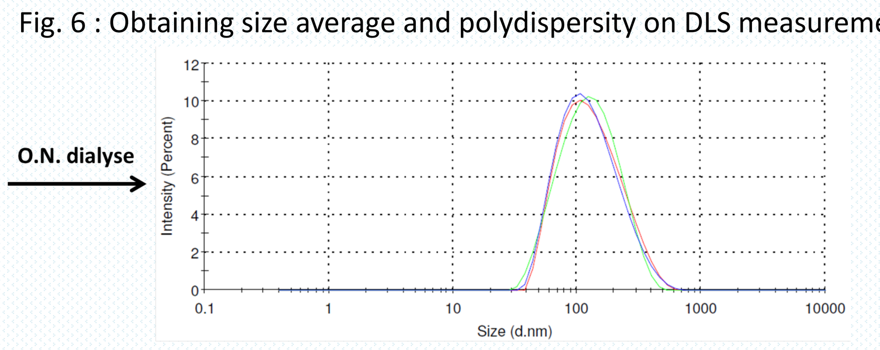


Fig. 5 : Pictures of proteoliposomes after an overnight sucrose gradient at 4°C ; from left to right: empty DOPC + cholesterol liposomes (containing pyranine); MexB proteoliposomes; MexAB proteoliposomes; empty DOPC liposomes; OprM proteoliposomes.

Fig. 6 : Obtaining size average and polydispersity on DLS measurement.



Size : 106,7
Polydispersity : 0,195

Fig. 7: Quantification of MexAB proteins incorporated into proteoliposomes (20 µL of proteoliposomes) on a SDS-PAGE.

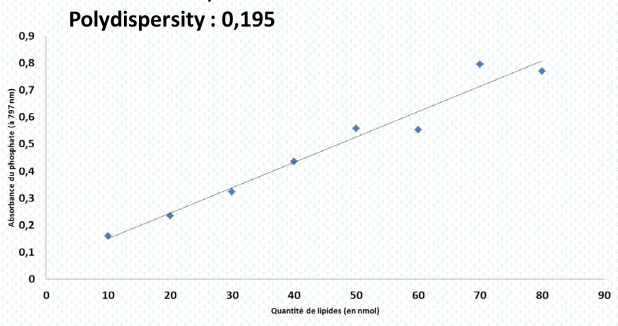


Fig. 8: Quantification of lipids constituting MexAB proteoliposomes in 20µL of proteoliposomes (phosphomolybdate colorimetric assay).

Characterisation of MexAB proteoliposomes by Negative-staining and cryo-EM

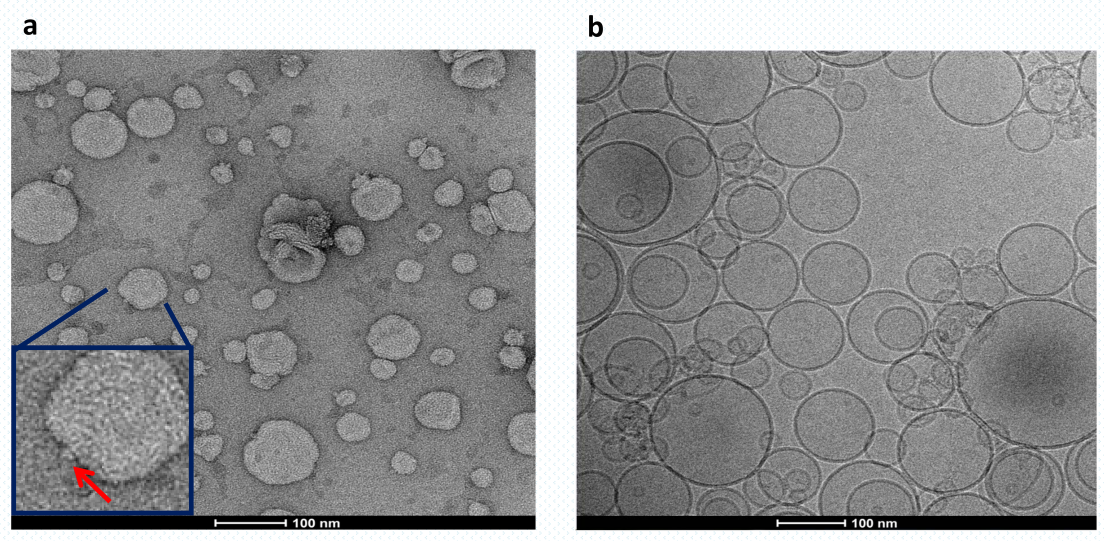
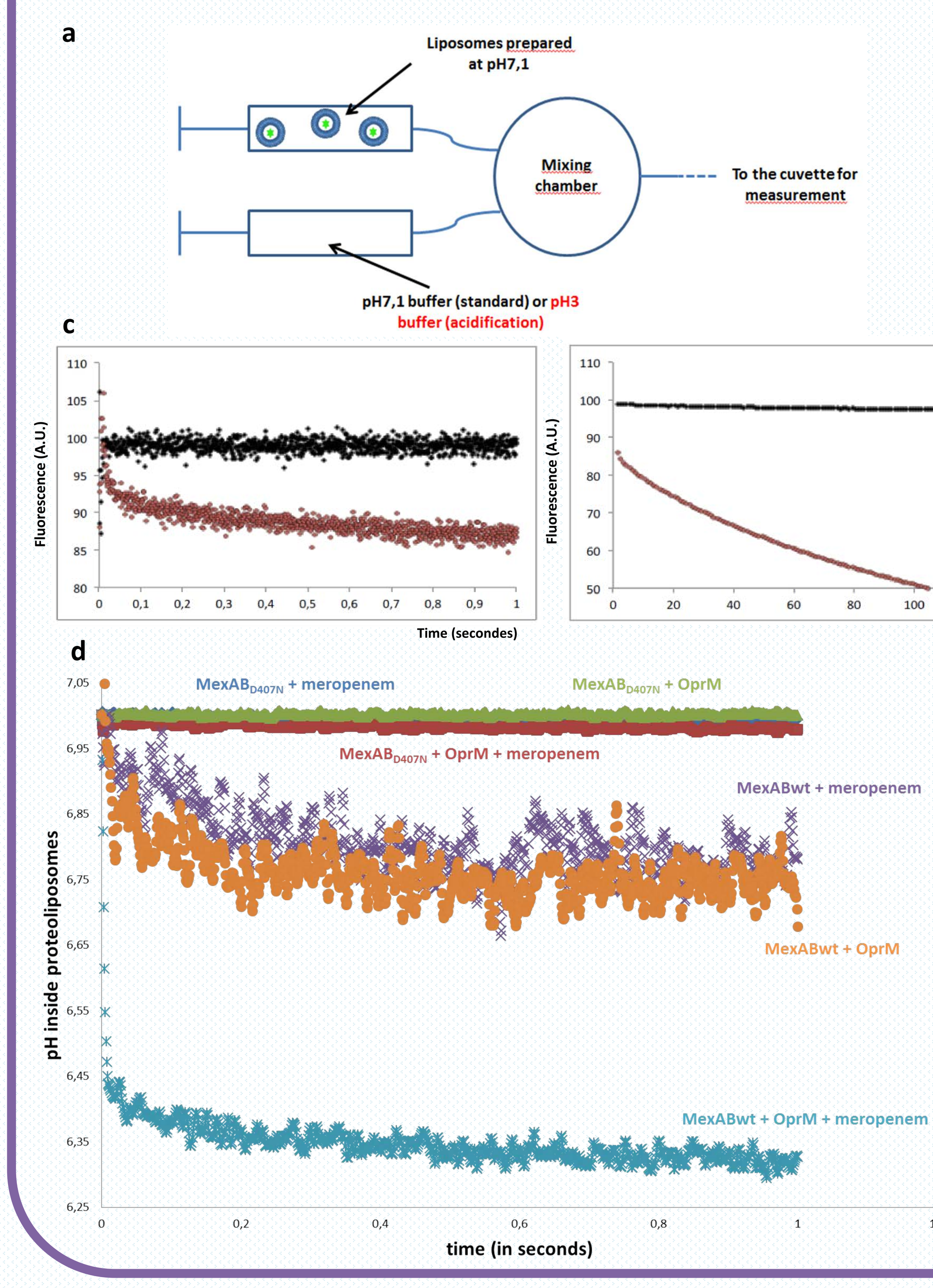


Fig. 9: Electron microscopy visualization of the proteoliposomes (coll. O. Lambert, CBMN, Bordeaux)
(a) MexAB proteoliposomes, Negative-Staining EM (uranyl acetate). (b) MexAB proteoliposomes, cryo-EM (Tecnaï F20 (FEI)).

Measure of the velocity of transport using Stopped-flow

b

DOPC 100%	DOPC 100% + cholestérol
DOPC 80% DOPG 20%	DOPC 80% DOPG 20% + cholestérol
DOPC 80% DOPE 20%	DOPC 80% DOPE 20% + cholestérol
DOPC 80% DOPE 10% DOPG 10%	DOPC 80% DOPE 10% DOPG 10% + cholestérol
DOPC 70% DOPE 30%	DOPC 70% DOPE 30% + cholestérol
DOPE 60% DOPG 40%	



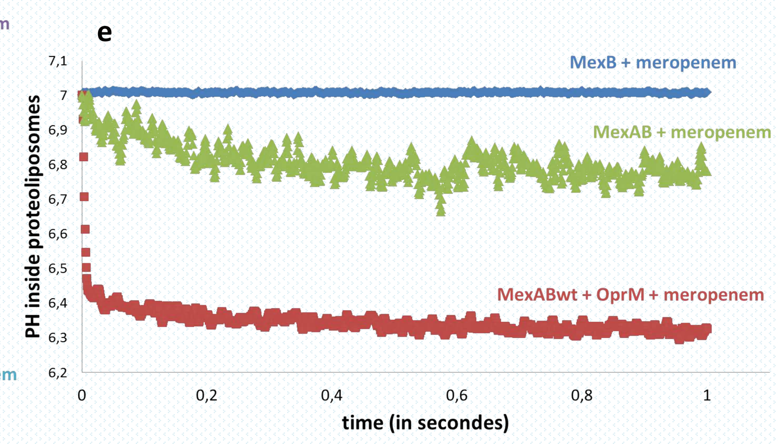


Fig. 10: Rationale of the assay.
(a) Principle of Stopped-flow; (b) Various lipids composition tested principle of protons diffusion through liposomes; (c) Measurement of pyranine fluorescence as a function of time, at pH7 (black) vs pH3 (red); (d) Acidification of different proteoliposomes in presence of meropenem and OprM proteoliposomes (e) Acidification of MexBwt, MexABwt and MexABwt + OprM in presence of meropenem.

Future directions

- Measure the rate of transport for different concentration of substrates.
- Measure the rate of transport for different substrates.
- Measure the velocity in presence of potential inhibitors in order to screen inhibitors.
- Measure the influence of membrane potential on the velocity of transport.

References

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Acknowlegments

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