



Original article

# A liposomal method for evaluation of inhibitors of H<sup>+</sup> – coupled multidrug transporters



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ABSTRACT

**Introduction:** This paper describes a novel technique, Fluorosomes, applied to investigating the interaction of antimicrobials with proton driven microbial efflux transporters. These transporters remove toxic compounds from the cytoplasm, including antibiotics and are involved in antibiotic resistance.

**Methods:** To assess transporter activity we developed a methodology to generate a proton gradient across Fluorosome membranes into which selected purified fully active efflux transporters were reconstituted. The interior of the Fluorosome particle (a unilamellar liposome) contains a fluorescent drug sensing probe whose fluorescence is quantitatively quenched by transporter substrates. Using an injecting fluorescence plate reader to initiate a proton gradient and to monitor subsequent fluorescence change, real time transport kinetics can be followed and transport inhibition characterized.

**Results:** Fluorosomes containing the *Escherichia coli* EmrE efflux pump demonstrated transport of two known EmrE substrates, ethidium and methyl viologen upon creation of a proton gradient. For Fluorosomes containing the inactive EmrE mutant, E14Q, no transport was observed. When the gradient was fully collapsed by the addition of nigericin, full inhibition of substrate transport was observed. The IC<sub>50</sub> for nigericin inhibition of ethidium was shown to be 0.71 μM.

**Discussion:** We have for the first time prepared and validated a single bacterial efflux pump assay, Fluorosome-trans-EmrE, that faithfully mimics properties of the transporter in vivo. It is faster than whole cell screens, simple to use, amenable to robotics, and reports on very specific targets. We have demonstrated proof of principle with EmrE and have created the first of an intended series of proton driven Fluorosomes.

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## 1. Introduction

Many antibiotics are ineffective because they are actively removed (effluxed) from the target organism by multidrug efflux transporters (MDTs) (Li, Plesiat, & Nikaido, 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005). This is the case for bacteria such as *Escherichia coli* (Ec), a major Gram-negative pathogen responsible for enteric and urinary infections. Candidate inhibitors of the MDTs have been reported, but none of them have yet reached the market, probably because they are limited by their lack of selectivity and also that they inhibit specific mammalian pumps (Lomovskaya & Bostian, 2006; Lomovskaya et al., 2001; Nakayama et al., 2003; Opperman et al., 2014; Van Bambeke, Pages, & Lee, 2006). Here we provide a novel tool to tackle some of the existing problems by looking at the direct action of inhibitors on critical bacterial transport proteins.

We have adopted the Fluorosome platform to study proton driven bacterial transport systems. The Fluorosome method was originally developed to measure the passive diffusion of compounds across

membrane bilayers (Fix & Melchior, 2002). It was then extended to determine the interaction of drugs with mammalian ATP-driven multidrug resistant pumps such as the p-glycoprotein (Melchior et al., 2012) and recently the ABCG2 (BCRP) and ABCB11 (BSEP) transporters.

To adapt the Fluorosome platform to proton driven bacterial pumps, we chose the EmrE transporter as a model transporter. Numerous efflux transporters in Gram-negative bacteria, especially the AcrAB-TolC system in EC and the MexAB-OprM system in *Pseudomonas aeruginosa* (Pa), have been studied as targets for the reversal of specific antibiotics (Lomovskaya & Bostian, 2006; Lomovskaya et al., 2001; Nakayama et al., 2003; Opperman et al., 2014; Van Bambeke et al., 2006). While in Gram-negative bacteria, the AcrAB-TolC (and MexAB-OprM) efflux systems are considered to be the major multidrug resistance transporters (MDTs) (Li et al., 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005; Pos, 2009), it is estimated that in a bacterium such as Ec there are additionally 20 different proteins or protein complexes that function as MDTs, each of them conferring resistance to a slightly divergent, but partially overlapping set of drugs (Nishino & Yamaguchi, 2001). These proteins, based on amino acid sequence similarity, belong to five classes of transporters and have been designated as the: 1) ATP-binding cassette (ABC); 2) resistance nodulation–division (RND);

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3) multidrug and toxic compound extrusion (MATE); 4) small multidrug resistance (SMR); and 5) major facilitator (MFS) superfamilies (Li et al., 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005). We have shown that such a large variety of these multidrug transporters (MDTs) work in a concerted mode rather than independently (Tal & Schuldiner, 2009). Transporters such as the EmrE, MdfA and MdtM supply the substrates to the AcrB–TolC complex – they move drugs from the cytoplasm to the periplasm or the outer leaflet of the membrane from where the AcrB–TolC complex expels them directly into the external medium (Tal & Schuldiner, 2009). The central role of the AcrB–TolC complex stems from the fact that it catalyzes the last step in a multistep process where a large variety of MDTs take part. Indeed, we have shown that in EmrE and mdfA double chromosomal knockouts, the AcrB–TolC complex cannot provide resistance to a spectrum of toxic compounds (Tal & Schuldiner, 2009). The specificities of the MDTs are very broad and overlap each other like the slats of a fan, so that they form a very strong and efficient barrier that prevents accumulation of a wide variety of xenobiotics. The rationale of our approach is to develop a medium to high throughput liposomal assay to identify inhibitors of MDTs that can be used as adjuvants to existing antibiotics. For the above reasons, we have chosen one of the above MDTs, the EmrE, as a model transporter to develop our new Fluorosome assay.

Criteria for a useful antimicrobial screening assay for inhibitors of antibiotic efflux pumps would include the following; specificity, speed, simplicity of instrumentation and application, amenability to robotics, high throughput and low cost. Using the EmrE as a prototype transporter, we have developed a novel screening platform that fulfills the abovementioned criteria. This assay also obviates analytical problems arising from the presence of a periplasmic space.

## 2. Materials and methods

### 2.1. Fluorosome manufacture

EmrE and its inactive mutant E14Q were isolated and, together with total *E. coli* lipids (Avanti Polar Lipids, Alabaster, AL), were reconstituted as proteoliposomes essentially as previously described (Yerushalmi, Mordoch, & Schuldiner, 2001) at a protein to lipid ratio of approximately 1.1 µg protein/mg lipid (wt/wt). These proteoliposomes were converted into Fluorosomes by procedures described in the literature (Melchior et al., 2012). For this study, Fluorosomes were manufactured in “Loading buffer” (0.15 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.015 M Tris, pH 7.5). Fluorosome size was determined by photon correlation spectroscopy (PDDLS/Cool Batch, Precision Detectors, Franklin, MA) to be ~200 nm. Their bilayer integrity was confirmed by measurement of the passive permeability coefficients (PC) of test drugs amiloride (PC ~ 0.5 × 10<sup>-7</sup> cm/s) and propranolol (PC ~ 12 × 10<sup>-7</sup> cm/s).

### 2.2. Measurements

Measurements were made on Fluorosome-*trans*-EmrE, containing reconstituted wild type EmrE in their bilayers, and Fluorosome-*trans*-E14Q, containing reconstituted inactive mutant, E14Q, in their bilayers, with the drug sensor BSA–fluorescein (Sigma-Aldrich) in the aqueous interior of the Fluorosome. Transport was monitored in 384 well plates with a NOVOSTAR injecting fluorescence plate reader (BMG Labtech, Cary, NC). Measurements were made at 23 °C in well mode with excitation at 485 nm and emission monitored at 520 nm. To initiate proton driven substrate transport, 2.5 µl of Fluorosomes in Loading buffer were rapidly injected into 97.5 µl of “Reaction buffer” (140 mM K<sub>2</sub>SO<sub>4</sub>, 5 mM MgCl<sub>2</sub>, 10 mM Tricine, 10 mM Tris (pH 8.5)) containing either 25 µM ethidium plus 1 µM valinomycin or 500 µM methyl viologen as substrates. To quantitate the results, initial curve slopes (rate of substrate transport) were calculated after Fluorosome injection using BMG's Mars analysis package.

### 2.3. Compounds

Methyl viologen (paraquat), ethidium, nigericin, and valinomycin were purchased from Sigma-Aldrich. Methyl viologen and ethidium were added to buffer from water and nigericin and valinomycin from DMSO. The drug plate used for the nigericin IC<sub>50</sub> study was formed using a SOLO Robotic Pipettor (Hudson Robotics, Springfield NJ). The IC<sub>50</sub> value was calculated using Prism 5 software (GraphPad, San Diego, CA).

## 3. Results

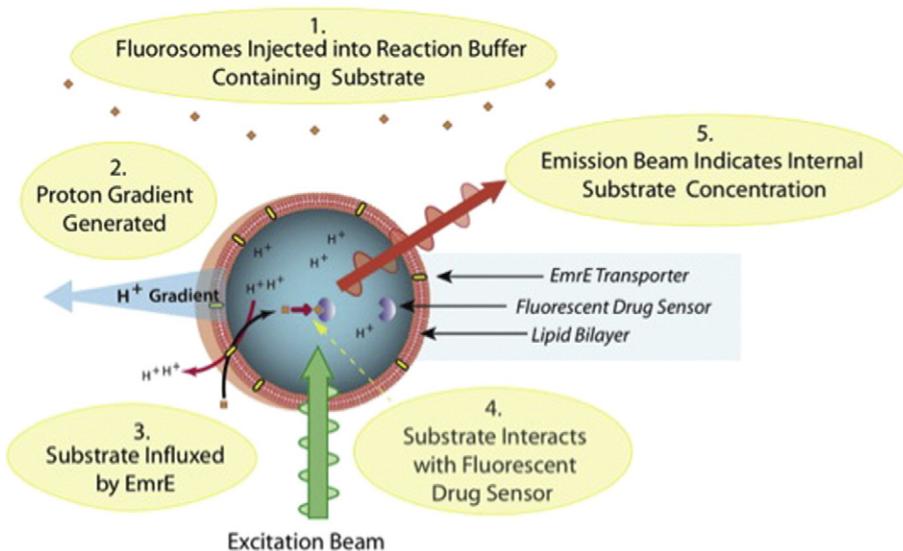
### 3.1. Background

Fluorosomes are unilamellar vesicles containing selected transporters in their bilayers and drug sensors (i.e. BSA-fluorescein) in their aqueous interiors (Melchior et al., 2012). Fig. 1 illustrates the principles involved in measurements made for proton driven Fluorosome transport. In order to produce a Fluorosome system employing a proton gradient as the driving force for protein mediated transport, Fluorosomes were manufactured in “Loading buffer” (see Material and methods section). Use of this buffer results in the interior of the Fluorosomes containing ammonium in equilibrium with ammonia and protons. When the Fluorosomes are injected (diluted) into an ammonium-free medium “Reaction buffer” (Material and methods section), the free ammonia diffuses rapidly out of the Fluorosome interior, leaving behind a proton. This diffusion of ammonia out of the Fluorosome shifts the equilibrium promoting further generation of ammonia and protons, which in turn will diffuse out leaving behind further protons. Thus a proton gradient is created. EmrE utilizes the pH gradient thus generated to drive the uptake of a substrate in exchange for two protons (Schuldiner, 2009).

Two model substrates were used in this study, methyl viologen and ethidium (Schuldiner, 2009). Both of these compounds interact with the drug sensor BSA–fluorescein resulting in an immediate large decrease in fluorescence. In the case of the model substrate methyl viologen, a bilayer impermeant divalent cation, EmrE utilizes the pH gradient generated as described above to drive the uptake a molecule of methyl viologen in exchange for two protons. Fig. 2 shows in real time the fluorescence change as the substrate methyl viologen is transported into Fluorosome-*trans*-EmrE following Fluorosome injection into wells containing Reaction buffer. The consequent methyl viologen transport results in a large downward slope in fluorescence. This reflects the entrance of substrate into the aqueous interior of the Fluorosome, where it rapidly quenches the fluorescence of the sensor BSA–fluorescein contained in the particle (green and red lines). The change in fluorescence vs. time indicates the extent and speed at which transport occurs during a given run. In the absence of substrate transport, i.e. when the Fluorosome injection is made into a buffer with the same composition as the Loading buffer so that no pH gradient is generated (blue line), or when the gradient is collapsed by the addition of nigericin, an H<sup>+</sup> and K<sup>+</sup> ionophore, full inhibition of substrate transport is observed (brown line) as indicated by the lack of quenching. DMSO was added as a control (red line) because nigericin is added in DMSO.

To demonstrate the specificity of Fluorosomes containing EmrE, Fluorosomes were created under the same conditions as those employed in Fig. 2, but lacking EmrE. When injected into Reaction buffer, no fluorescence quenching was observed (not shown). Similarly, when Fluorosomes were prepared with the inactive EmrE mutant, E14Q, upon injection into Reaction buffer practically no substrate-induced quenching of the fluorophore was observed (Fig. 3).

We also tested transport of ethidium, a different model substrate, which is monovalent and more hydrophobic than methyl viologen. The transport of ethidium at 25 µM is shown in Fig. 4. The omission of valinomycin is seen to result in a reduced rate of transport. As the transport of monovalent substrates is electrogenic, this results in the

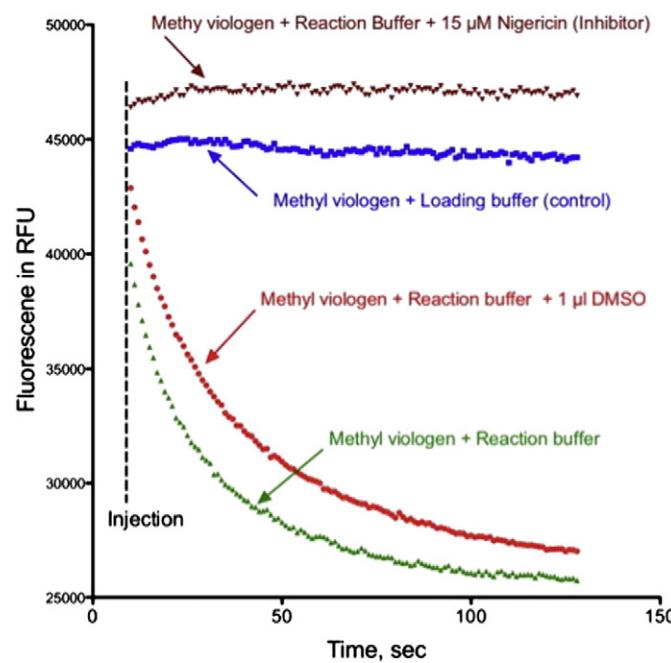


**Fig. 1.** Steps involved in the Fluorosome-*trans*-EmrE assay of pH gradient-driven drug efflux. Fluorosomes manufactured in “Loading buffer” have in their interiors ammonium in equilibrium with ammonia and protons. 1. Fluorosomes are injected (diluted) into fluorescence plate reader wells containing an ammonium-free medium “Reaction buffer”. 2. Free ammonia diffuses rapidly out of the Fluorosome interior, leaving behind a proton. This shifts the equilibrium promoting further generation of ammonia and protons, which in turn diffuses out leaving behind further protons. 3. EmrE utilizes this proton gradient to drive the uptake of substrate. 4. Fluorescence is monitored as substrate enters the Fluorosome, and interacts with the encapsulated drug sensor. 5. Decreasing fluorescent signal indicates increasing internal substrate concentration.

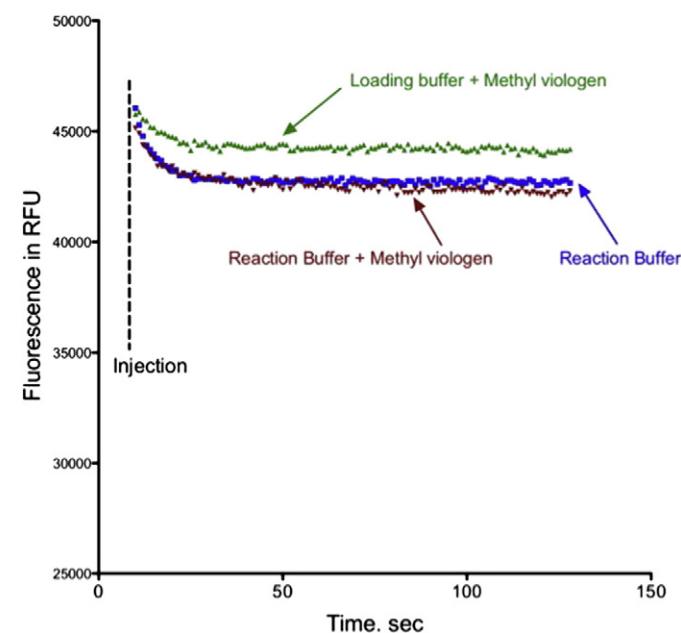
generation of a membrane potential that reduces substrate transport. The presence of valinomycin allows the influx of potassium into the Fluorosome as the monovalent cation ethidium is transported into the Fluorosome in exchange for 2 protons, thus avoiding the buildup of a membrane potential that would otherwise slow down ethidium

transport. As expected, the  $H^+ / K^+$  ionophore nigericin at 15  $\mu M$  greatly reduced Fluorosome-*trans*-EmrE transport activity for both the substrates ethidium and methyl viologen (Fig. 4). For Fluorosomes containing the inactive EmrE mutant, E14Q, the same low level of methyl viologen transport occurs in Reaction buffer, with and without nigericin, as it does when the Fluorosomes are injected into Loading buffer where there is no proton gradient.

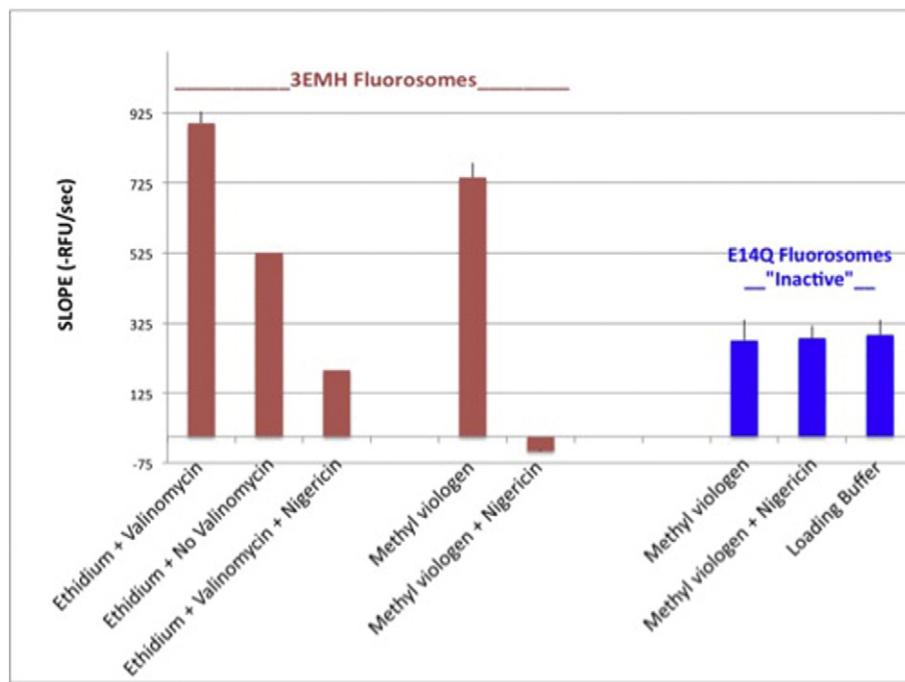
In order to demonstrate use of the assay for rapid  $IC_{50}$  determinations, EmrE activity in Fluorosome-*trans*-EmrE was determined with



**Fig. 2.** Proton driven transport of methyl viologen into Fluorosome-*trans*-EmrE monitored by fluorescence quenching. Transport commences upon injection of Fluorosomes containing “Loading buffer” into “Reaction buffer” which establishes a proton gradient between the interior and exterior of the Fluorosomes. Methyl viologen, and when indicated DMSO and nigericin, are present in the reaction buffer. Entrance of methyl viologen into the Fluorosome particle results in a quenching of the encapsulated fluorescent drug sensing probe, seen as a decrease in relative fluorescence units (RFU). Nigericin by collapsing the proton gradient prevents methyl viologen uptake and thus a level trace is seen.



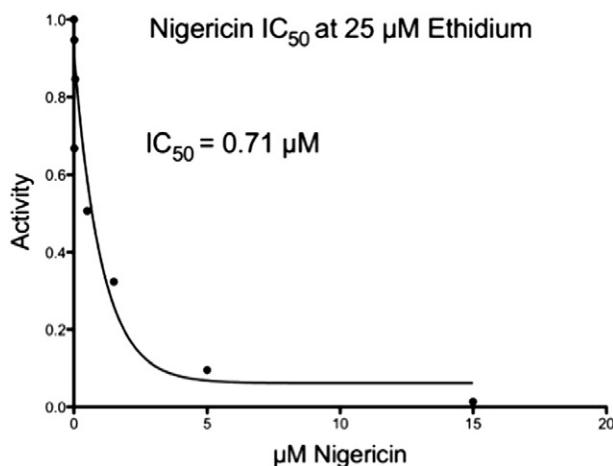
**Fig. 3.** Control studies on proton driven transport of methyl viologen into Fluorosome-*trans*-EmrE containing the inactive EmrE mutant E14Q. Upon injection of these Fluorosomes manufactured in “Loading buffer” into “Reaction buffer”, thereby establishing a proton gradient, little or no transport (decrease in fluorescence) is seen relative to Fluorosomes containing the wild type transporter (Fig. 2). (The small fluorescence change observed in all cases is artifactual.)



**Fig. 4.** Histogram of transport by Fluorosome-*trans*-EmrE containing either wild type EmrE or the inactive mutant transporter, E14Q, for the substances ethidium and methyl viologen with and without the inhibitor nigericin. Transport is given as the initial slope of fluorescence change upon establishment of a proton gradient between the inside and outside of the Fluorosome particle.

ethidium as substrate ( $25 \mu\text{M}$ ) in the presence and absence of various concentrations of nigericin. All data were generated in less than 3 min and are shown in Fig. 5. Nigericin at increasing concentrations, up to  $15 \mu\text{M}$ , is seen to progressively reduce Fluorosome-*trans*-EmrE transport activity with an  $\text{IC}_{50}$  of  $0.71 \mu\text{M}$ .

The stability of Fluorosome-*trans*-EmrE was tested by performing assays with methyl viologen on a single batch of Fluorosomes over a period of three weeks (Fig. 6). During this time, the Fluorosomes were stored at  $4^\circ\text{C}$ . After three weeks, 40% activity remained, sufficient for viable assays. The Fluorosome vesicle itself is stable at  $4^\circ\text{C}$  for well over 2 months as determined by photon correlation spectroscopy and measurement of its passive permeability to standard compounds (results not shown; see Materials and methods section).

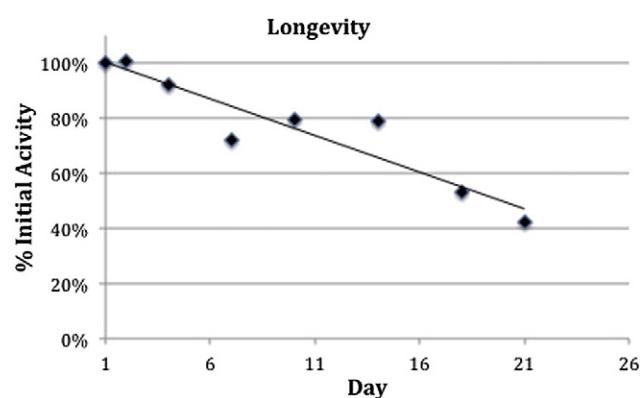


**Fig. 5.** Activity-concentration plot of the inhibition by nigericin of ethidium transport by EmrE using the Fluorosome-*trans*-EmrE assay. Activity is given by the initial slope of fluorescence change at each concentration of nigericin. Values were normalized relative to full activity at  $0 \mu\text{M}$  nigericin.

#### 4. Discussion

We have prepared and validated a single bacterial efflux pump assay, Fluorosome-*trans*-EmrE, that faithfully mimics properties of the transporter in vivo. This is an excellent assay for a medium to high throughput screen. It is faster than whole cell screens and is directed to very specific targets that have not been yet studied for the purpose of development of novel inhibitors. The well-studied EmrE transporter was used as a proof of principle and we will now prepare Fluorosomes with other microbial multidrug transporters.

In this assay, the interior of these Fluorosomes contains a fluorescent drug sensor, BSA-fluorescein, which allows the substrate transport to be followed in real time in a basic injecting fluorescence plate reader. The assay was developed such that proton generation within the Fluorosome produces a gradient that drives the EmrE transporter. The fluorescent sensor used is a general sensor but specific ones for given



**Fig. 6.** The stability of Fluorosome-*trans*-EmrE activity over a period of three weeks. During this time, the Fluorosomes were stored at  $4^\circ\text{C}$  and assays were carried out using methyl viologen as substrate.

antibiotics can be used as well. As substrate is pumped into the interior of the Fluosome, it interacts with the encapsulated drug sensor, quantitatively quenching fluorescence. In practice, single well measurements made sequentially in well mode can be completed in less than 20 s each, permitting moderate to high throughput analyses. With more sophisticated instrumentation, simultaneous injections could be made and throughput would be substantially increased.

In this particular Fluosome assay, two validated EmrE substrates are employed, ethidium and methyl viologen. Both interact strongly with the particular sensor chosen for this study, BSA-fluorescein. There is no need for the antibiotics to interact with this sensor, since what we desire to observe is the inhibition of transport of chosen model substrates by these small molecules and not the interaction of the antibiotics themselves with the sensor. Any interaction of an antibiotic passively entering the Fluosome and interacting with the sensor would be noted in control runs. This unlikely situation would then be treated as a background value and the transport rates corrected accordingly.

Fluosome-trans-EmrE was validated with two EmrE substrates, methyl viologen (paraquat) and ethidium, with and without valinomycin. Transport was not observed under any conditions with the inactive mutant of EmrE, E14Q or in the absence of a proton gradient, the driving force for accumulation. We showed that the assay can be used with a variety of substrates transported either in an electroneutral or electrogenic mode.

Fluosomes lost activity slowly and very gradually, retaining 40% of original activity after 3 weeks of storage at 4 °C. This activity, however, was still adequate enough for viable assays. The Fluosome particle itself is stable for several months. Loss of activity most probably is a consequence of the slow loss of activity of the EmrE protein.

One of the problems of previously developed microbial transporter inhibitors was their high toxicity due to their interaction with mammalian multidrug transporters. We have previously developed Fluosomes containing the mammalian multidrug transporter P-glycoprotein (Melchior et al., 2012) and are currently developing Fluosomes containing the mammalian transporters BCRP (ABCG2) and BSEP (ABCB11). We are, therefore, capable of testing candidates from bacterial inhibitor screens with the latter mammalian-derived assays to screen for more specific and minimally toxic inhibitors. The development of further varieties of Fluosomes containing both microbial and mammalian transporters is underway.

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