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Hoechst likes to play hide and seek . . . use it with caution!

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ABSTRACT

We describe an unexpected feature of the dye Hoechst 33342 that may lead to misinterpretation of fluorescence assay results. When dissolved in aqueous solvent, Hoechst 33342 massively adsorbs on the polytetrafluoroethylene (PTFE)-coated stirrer and on the quartz cuvette. The interaction between the dye and the PTFE is stronger than that between the dye and the quartz. We show that Hoechst 33342 adsorption on quartz is due to its amphiphilic properties, as it depends on the buffer ionic strength and on pH. We suggest a procedure to detect and remove any residual Hoechst 33342.

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Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1*H*-benzimidazole trihydrochloride trihydrate) is a bisbenzimidazole dye that exhibits a poor blue fluorescence in aqueous medium, while it is highly fluorescent upon intercalation into DNA. In addition, this molecule exhibits an increased fluorescence when it partitions into hydrophobic environments such as detergent micelles or lipidic membrane bilayers. It was proposed in the late 1990s to be a model substrate for membrane protein transporters from the ATP binding cassette (ABC) family. Indeed, there is a correlation between the development of colchicine resistance and the decreased uptake of the dye [1]. This was later explained by the fact that Hoechst 33342 is a substrate for P-glycoprotein (P-gp), a membrane protein transporter overexpressed at the plasma membrane of highly multidrug-resistant cells and responsible for the active efflux of noxious compounds out of the cell [2]. Most notably, Shapiro and Ling [3] have shown that rhodamine 123 apparently "stimulates" the efflux of Hoechst 33342, and vice versa, and this has led to a model in which P-glycoprotein would contain at least two positively cooperating sites (dubbed "H site" and "R site" for their respective substrate specificities) for drug binding and transport.

As stated above, Hoechst 33342 is very sensitive to its environment. Hence, active transport can be deduced from time-course fluorescence measurements with proteoliposomes or from membrane vesicles derived from cells. This allowed settling the framework for a better understanding of P-gp as a paradigm of all ABC transporters. On a broader scale, this molecule is widely used as a screening tool for human absorption, distribution, metabolism, and elimination in the pharmaceutical industry [4]. A large number

of other ABC transporters have been studied using this compound, as well as many other membrane proteins outside of the ABC transport field [5]. Even if fluorescence artifacts have been described [6], this compound is a very useful tool in fields ranging from immunohistochemistry to membrane protein biochemistry.

We recently came across an unexpected feature that might lead to misinterpretations of fluorescence data if preliminary checks are not performed.

To perform the herein described assays, Triton X-100 and Hoechst 33342 were purchased from Sigma-Aldrich. Hoechst 33342 was resuspended into 25 mM sodium phosphate buffer, pH 7, and 100 mM CH₃COOK (Fig. 1) or into 25 mM sodium phosphate buffer at various pH (from pH 6.3 to pH 8.3) and NaCl ranging from 0 to 1 M (Fig. 2). Quartz cuvettes (Suprasil 101-QS) and polytetrafluoroethylene (PTFE)-coated stirrers were obtained from Hellma. Fluorescence measurements were conducted at 25.0 °C using a Jasco FP-6200 spectrofluorimeter. The measurements were performed using the time-course mode, setting measurements with excitation/emission wavelengths of 352 nm/500 nm.

While we were running fluorescence measurements ($\lambda_{\text{ex Hoechst 33342}} = 352 \text{ nm}$ and $\lambda_{\text{em Hoechst 33342}} = 500 \text{ nm}$), we noticed that the dye adsorbs on PTFE (a polymer coating of most magnetic stirrers) and on quartz. As shown in Fig. 1A, addition of detergent into a quartz cuvette containing a buffer stirred by a magnetic stirrer has, of course, no effect on the fluorescence signal. By contrast, when Hoechst 33342 is added to a rinsed cuvette containing the buffer and the stirrer, as expected, the fluorescence increases (Fig. 1B). To our great surprise, addition of detergent to the same cuvette, which was thoroughly rinsed, now led to a significant increase in fluorescence (Fig. 1C). We interpret this fluorescence increase as the desorption of Hoechst 33342 from PTFE and from quartz driven by the detergent. Indeed, Hoechst 33342 is highly

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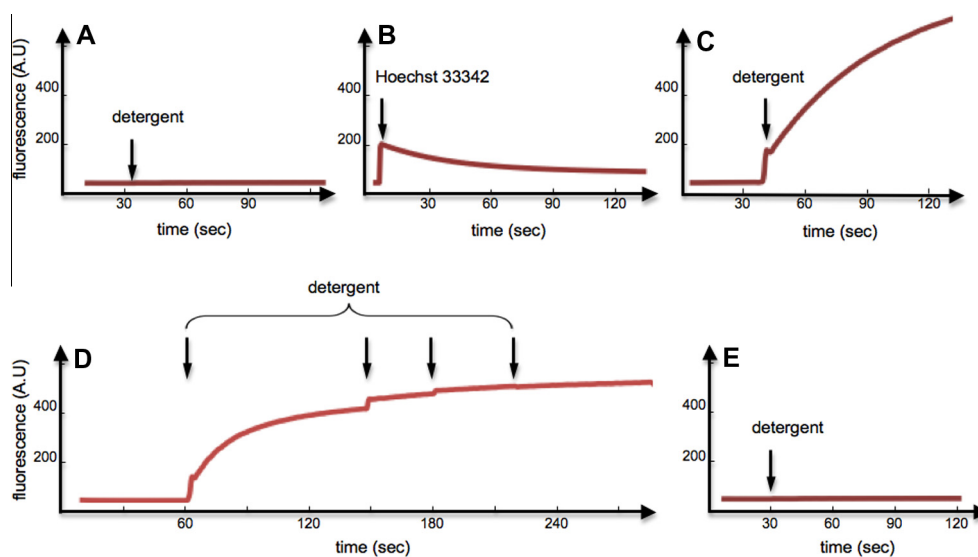


Fig.1. Hoechst 33342 adsorption and desorption; Hoechst 33342 fluorescence (λ_{ex} Hoechst 33342 = 352 nm and λ_{em} Hoechst 33342 = 500 nm) as a function of time. Each graph corresponds to a fluorescence measurement performed in the same quartz cuvette containing 2 ml of 25 mM sodium phosphate buffer, pH 7, and 100 mM CH_3COOK stirred by a PTFE-coated magnetic stirrer. The cuvette was extensively rinsed with distilled water between each measurement. (A) Addition of Triton X-100 (1% final). (B) Addition of Hoechst 33342 (1.25 μM final). (C) Addition of Triton X-100 (1% final). (D) Four additions of Triton X-100 (4% final). (E) Addition of Triton X-100 (1% final).

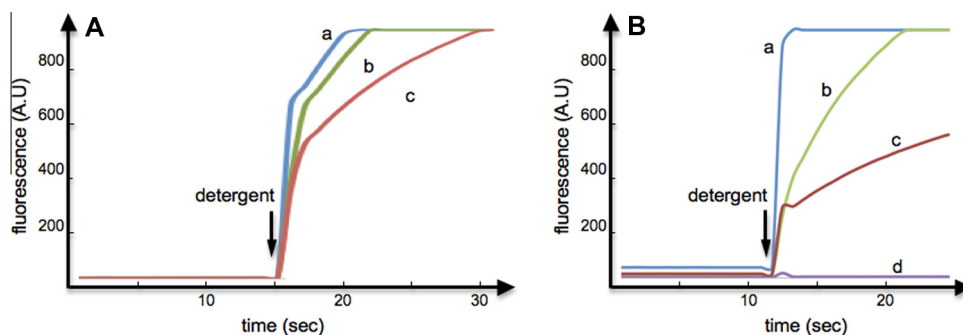


Fig.2. Hoechst 33342 desorption upon detergent addition. Hoechst 33342 fluorescence (λ_{ex} = 352 nm and λ_{em} = 500 nm) as a function of time. (A) Effect of pH during the incubation with PTFE. Hoechst 33342 (1.25 μM final) was previously added to a stirred quartz cuvette containing 25 mM sodium phosphate buffer, pH 6 (blue trace a); 25 mM sodium phosphate buffer, pH 7 (green trace b); and 25 mM sodium phosphate buffer, pH 8 (red trace c), then the cuvette and stirrer were thoroughly washed with water. Sodium phosphate buffer at pH 7 was added to the cuvette and the stirrer and Triton X-100 was added (0.1% final). (B) Effect of the ionic strength during the desorption by detergent. Hoechst 33342 (1.25 μM final) was previously added to distilled water (blue trace a); to 25 mM sodium phosphate buffer, pH 7, with an ionic strength of 200 mol/L (green trace b); to 25 mM sodium phosphate buffer, pH 7, with an ionic strength of 700 mol/L (red trace c); or to 25 mM sodium phosphate buffer, pH 7, with an ionic strength of 1200 mol/L (purple trace d). Triton X-100 was added (0.1% final) as indicated by the arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorescent in a hydrophobic environment, like a detergent micelle. Fortunately enough, after addition of adequate amounts of detergent (Fig. 1D), the desorption was complete as illustrated by the fact that the same cuvette rinsed with water and then subjected to detergent addition did not lead to any fluorescence increase anymore (Fig. 1E). The same trend was observed when emission spectra were recorded rather than time-resolved spectra (see Supplementary Fig. 1). Note that when we added liposomes instead of detergent, we observed the same tendency shown in Fig. 1C (data not shown). We repeated the experiments presented in Fig. 1 at various Hoechst 33342 concentrations (from 2.5 nM to 25 μM). It appears that at concentrations as low as 25 nM the detergent-mediated desorption of the dye gives rise to significant fluorescence levels (data not shown).

To determine if Hoechst 33342 adsorbs on PTFE or on quartz, we monitored the effect of detergent either on a Hoechst 33342-contaminated cuvette agitated by a clean stirrer (Supplementary Fig. 2C) or on a Hoechst 33342-contaminated stirrer in a clean

quartz cuvette (Supplementary Fig. 2D). First we checked that both cuvette and stirrer were Hoechst 33342-free (Supplementary Fig. 2A). Then we added Hoechst 33342 (Supplementary Fig. 2B) to allow the dye to distribute. To observe Hoechst 33342 desorption from quartz we used the same cuvette as for Supplementary Figs. 2A and B but a different, Hoechst 33342-free, stirrer: desorption of Hoechst 33342 from quartz (driven by a detergent addition) was instantaneous (Supplementary Fig. 2C). To observe Hoechst 33342 desorption from PTFE, we used the same stirrer as for Supplementary Figs. 2A and B but a different cuvette, Hoechst 33342-free. Desorption of Hoechst 33342 from PTFE (driven by detergent addition) was slower (Supplementary Fig. 2D).

We conclude that the biphasic Hoechst 33342 fluorescence increase shown on Fig. 1C is due to its slow desorption from PTFE and fast desorption from quartz. Hence, Hoechst 33342 seems to bind with stronger affinity on PTFE than on quartz.

To interpret the biphasic trend of the fluorescence signal observed upon addition of Hoechst 33342 (Fig. 1B), we discriminated

the contribution of the dye itself and that ascribable to the presence of the magnetic stirrer. It appears that the mere presence of Hoechst 33342 leads to a steady fluorescence signal and that the slow decrease is observed only when the PTFE-coated stirrer is present (for details, see [Supplementary Fig. 3](#)). We suggest that the slow decrease in fluorescence observed after Hoechst 33342 addition is due to the adsorption of the dye on the PTFE.

The Hoechst 33342 amphiphilic properties arise from the presence of two hydrophobic aromatic rings and from hydrophilic charges carried by protonated nitrogen atoms. It has been shown that water solubility is highly dependent on pH [7], see also [Supplementary Fig. 4](#). To confirm that Hoechst 33342 is indeed adsorbing onto PTFE via the hydrophobic effect, we evaluated the ability of Triton X-100 to desorb the dye after it had been incubated for 5 min in a cuvette at pH 6, at pH 7, or at pH 8 ([Fig. 2A](#)). This experiment confirmed that the higher the pH during the incubation (i.e., the more abundant the hydrophobic forms), the more difficult the desorption of the dye from PTFE. To confirm further that the interaction of the dye with PTFE is hydrophobic, we studied the effect of ionic strength on the detergent-driven desorption of Hoechst 33342 at a given pH. To work on this hypothesis, we incubated the stirrer, the cuvette, and Hoechst 33342 with a sodium phosphate buffer (25 mM, pH 7) with ionic strength ranging from 0 to 1 mol/L. We show in [Fig. 2B](#) that the buffer ionic strength had a dramatic effect on the desorption of Hoechst 33342 from PTFE and quartz. Those differences of desorption rate were due to the ionization state of the dye. It is known that the water solubility of zwitterionic or neutral molecules is increased by high salt concentration (to a certain point). If Hoechst 33342 is solubilized in a buffer that has a low ionic strength, the dye water solubility is low and then its interaction with PTFE and quartz is very strong: Hoechst 33342 adsorbs enormously. Therefore addition of detergent led to a massive increase in fluorescence ([Fig. 2B](#), blue trace a). In contrast, if Hoechst 33342 is now solubilized in a buffer with a high ionic strength, the water solubility of the dye is increased. As a consequence, the interactions between Hoechst 33342 and quartz are abolished and the dye does not adsorb. In such a case detergent addition did not lead to any fluorescence increase ([Fig. 2B](#), purple trace d).

Hoechst 33342 is a dye very sensitive to its environment. Hence, active transport can be deduced from Hoechst 33342 time-course fluorescence measurements with reconstituted proteoliposomes or from membrane vesicles derived from cells. We raise the problem that such fluorescence measurements can easily be contaminated by residual Hoechst 33342 adsorbed on PTFE and quartz. This is all the more true because liposomes, as well as detergent, do trigger the desorption of the dye.

Note that, in addition, the use of Hoechst 33342 is even more tricky because it can be present in its cationic, neutral, or zwitterionic form, depending on the pH and because its partition coefficient varies with its charge. Hence, investigations on Hoechst

33342 transport involving proteoliposomes energized by the proton-motive force must discriminate between actual protein-mediated transport and Δ pH-dependent movement of Hoechst 33342. From this point of view, negative controls including samples that contain nonfunctional proteins (obtained by site-directed mutagenesis for example) should be standard practice.

We conclude that Hoechst 33342 is a hazardous dye to use because of its amphiphilic properties and its ability to form hydrogen bonds. Because of what is described above, we highly recommend double-checking for the presence of Hoechst 33342 on PTFE and quartz by measuring Hoechst 33342 fluorescence after addition of detergent on the cuvette and stirrer before any fluorescence measurement. Incubation in an acidic solution with a high salt concentration will accelerate the washing process. This observation might be crucial in the context of *in vitro* membrane protein transport studies in which Hoechst 33342 is very commonly used as substrate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ab.2013.05.019>.

References

- [1] M.E. Lalande, V. Ling, R.G. Miller, Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells, *Proc. Natl. Acad. Sci. U.S.A.* 78 (1981) 363–367.
- [2] A.B. Shapiro, V. Ling, Extraction of Hoechst 33342 from the cytoplasmic leaflet of the plasma membrane by P-glycoprotein, *Eur. J. Biochem.* 250 (1997) 122–129.
- [3] A.B. Shapiro, V. Ling, Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities, *Eur. J. Biochem.* 250 (1997) 130–137.
- [4] F. Tang, H. Ouyang, J.Z. Yang, R.T. Borchardt, Bidirectional transport of rhodamine 123 and Hoechst 33342, fluorescence probes of the binding sites on P-glycoprotein, across MDCK-MDR1 cell monolayers, *J. Pharm. Sci.* 93 (2004) 1185–1194.
- [5] C.G. Blackmore, P.A. McNaughton, H.W. van Veen, Multidrug transporters in prokaryotic and eukaryotic cells: physiological functions and transport mechanisms, *Mol. Membr. Biol.* 18 (2001) 97–103.
- [6] B. Poolman, M.K. Doeven, E.R. Geertsma, E. Biemans-Oldehinkel, W.N. Konings, D.C. Rees, Functional analysis of detergent-solubilized and membrane-reconstituted ATP-binding cassette transporters, *Methods Enzymol.* 400 (2005) 429–459.
- [7] C. Alemán, A.M. Namba, J. Casanovas, Acid-base and electronic structure-dependent properties of Hoechst 33342, *J. Biomol. Struct. Dyn.* 23 (2005) 29–36.