Interaction of detergents with biological membranes: Comparison of fluorescence assays with filtration protocols and implications for the rates of detergent association, dissociation and flip-flop

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Abstract

The present study mainly consists of a re-evaluation of the rate at which C12E8, a typical non-ionic detergent used for membrane studies, is able to dissociate from biological membranes, with sarcoplasmic reticulum membrane vesicles being used as an example. Utilizing a brominated derivative of C12E8 and now stopped-flow fluorescence instead of rapid filtration, we found that the rate of dissociation of this detergent from these membranes, merely perturbed with non-solubilizing concentrations of detergent, was significantly faster ($t_{1/2} < 10$ ms) than what had previously been determined ($t_{1/2} \sim 300–400$ ms) from experiments based on a rapid filtration protocol using 14C-labeled C12E8 and glass fiber filters (Binding of a non-ionic detergent to membranes: flip-flop rate and location on the bilayer, by Marc le Maire, Jesper Møller and Philippe Champeil, Biochemistry (1987) Vol 26, pages 4803–4810). We here pinpoint a methodological problem of the earlier rapid filtration experiments, and we suggest that the true overall dissociation rate of C12E8 is indeed much faster than previously thought. We also exemplify the case of brominated dodecyl-maltoside, whose kinetics for overall binding to and dissociation from membranes comprise both a rapid and a slower phase, the latter being presumably due to flip-flop between the two leaflets of the membrane. Consequently, equilibrium is reached only after a few seconds for DDM. This work thereby emphasizes the interest of using the fluorescence quenching associated with
Introduction

Since the early seventies, natural membranes were used to study interactions of membrane proteins and lipids with detergents [1–3]. Sarco-endoplasmic reticulum vesicles (SR) prepared from fast-twitch muscle contain the Sarco-Endoplasmic Reticulum Ca\(^{2+}\)-ATPase isoform 1a (SERCA1a) at very high density and purity, making this natural sample of particular interest for studying the effect of detergent on a membrane protein [4–6]. In 1984, Ueno, Tanford and Reynolds reported that when reconstituting membranes from detergent-solubilized SR, and using polystyrene hydrophobic beads for trapping of the non-ionic detergent octaethylene-glycol-dodecylether (C\(_{12}\)E\(_{8}\)), it was virtually impossible to get rid of almost half of the initially bound C\(_{12}\)E\(_{8}\). The authors then suggested that this particular detergent experienced an extremely slow flip-flop (over a period of days) from one side of the membrane to the other [7]. At variance, in subsequent experiments in our laboratory, performed using a rapid filtration unit for measuring the kinetics of detergent removal from biological membrane vesicles or pure lipid vesicles, previously deposited on glass fiber filters, C\(_{12}\)E\(_{8}\) removal from such vesicles was found to reach a state of complete removal with an apparent half-time of 300–400 ms, and similar results were also obtained with multi-layered lipid vesicles (Fig 5 of ref. [8]). It was therefore concluded that the results of Ueno et al. had to be attributed to an unrecognized artefact(s), possibly trapping of very small fragments of the hydrophobic beads with their bound detergent inside the just-reconstituted membrane vesicles. We still think this could have been the case. Nevertheless, we now also think that our previous conclusion that overall dissociation of C\(_{12}\)E\(_{8}\) from the membranes was proceeding with a half time of 300–400 ms [8] has to be revised, too.

We indeed more recently performed new experiments on this issue, but now using a stopped-flow fluorescence assay with brominated detergent analogues. This method is based on the fact that the intrinsic fluorescence of protein tryptophan (Trp) residues located inside the membrane may get quenched by the bromine atoms residing on the hydrophobic chain of such detergent molecules in the vicinity of these Trp residues: the rate of fluorescence quenching can therefore reveal the rate of detergent binding to the membranes, while fluorescence will of course recover if the brominated detergent dissociates from the membrane [9]. In fact, exploiting fluorescence quenching by brominated analogues has previously made it possible to study the interaction of brominated lipids with transmembrane proteins. For instance, brominated lipids was recently used to measure binding constants of lipids to channels [10,11], and even earlier for evaluating their interactions with SERCA1a [12], including in the presence of non-brominated detergent [13]. The SERCA1a from SR native membranes is a particularly appropriate target for such Trp fluorescence quenching studies, since this single polypeptide chain of 994 residues contains 13 tryptophan residues among which 12 are located rather symmetrically in the transmembrane domain [14], mostly at the lipid-protein interface [12,15]. Brominated analogues of two different detergents were used: one derived from octaethylene-glycol-dodecylether (C\(_{12}\)E\(_{8}\)) and the other one from the widely used non-ionic detergent dodecyl-maltoside (DDM).

Surprisingly, stopped-flow results with 5,6-Br\(_2\)C\(_{12}\)E\(_{8}\) were not consistent with the results obtained in 1987 using rapid filtration and \(^{14}\)C-labelled C\(_{12}\)E\(_{8}\); we found that upon strong
dilution into detergent-free buffer of membranes previously incubated with non-solubilizing concentrations of the brominated 5,6-Br2C12E8, the recovery of the protein intrinsic fluorescence was very fast (with half times shorter than 10 ms), suggesting that dissociation of this detergent from the membranes is probably much faster than what we deduced originally from the filtration experiments with non-brominated C12E8 [8].

Yet, when the brominated (and again 14C-labelled) analogue of C12E8 was used in rapid filtration experiments, we obtained results consistent with those previously obtained for the non-brominated compound, with a slow apparent dissociation rate for the different molecules. We then became aware of a previously unsuspected methodological bias in our past and present filtration experiments, a bias arising from the combination of the local crowding of the membranes adsorbed onto the filters with the favourable partition of detergent into these filter-loaded membranes and the limited range of perfusion rates achievable with the rapid filtration equipment. As a consequence, and based on our new data with the brominated analogue, we now suggest that the exit of C12E8 from the membranes into the water phase is much more rapid than previously envisioned, and indeed occurs with a half time not larger than a few milliseconds.

We also here report the fluorescence quenching and de-quenching experiments performed with another detergent, 5,6-Br2DDM. In those experiments, fluorescence changes during detergent binding to or dissociation from SR membranes revealed both fast components, as for 5,6-Br2C12E8, and also much slower components specific for DDM. We then briefly discuss association, dissociation and flip-flop rates for these two detergents.

Materials and methods

The brominated detergents used in the present study, octaethylene-glycol-5,6-dibromododecylether (5,6-Br2C12E8, MW = 696 g.mol⁻¹) and dibromo-dodecyl-β-D-maltoside (5,6-Br2DDM, MW = 668 g.mol⁻¹) were synthesized as previously described [9,16], including in 14C-labelled form. Non-brominated C12E8 (MW = 538 g.mol⁻¹) was obtained from Nikko and non-brominated DDM (MW = 510 g.mol⁻¹) was from Anatrace Inc.. In contrast to non-brominated compound, dry 5,6-Br2C12E8 had an oily and yellowish appearance (presumably due to the bromine atoms themselves, since ¹H NMR spectra confirmed the absence of detectable impurities), but this did not prevent complete solubilization at concentrations as high as 100 mg/ml (like the non-brominated C12E8). Detergent stock solutions were subsequently prepared in deionised water at concentrations of 100 mM (for instance, 55 mg/ml and 70 mg/mL for the unbrominated and brominated 5,6-Br2C12E8, respectively), together with more dilute solutions (e.g. 5.5 mM for C12E8, 10 mM for DDM), and more concentrated ones if desired (e.g. 100 mg/mL C12E8, i.e. 186 mM, or 200 mg/ml DDM i.e. 390 mM). These nominal values (±10%), based on weight measurements, were in agreement with independent estimates based on measurement of the polyethyleneglycol contents of the detergent via its reaction with ammonium cobalto-thiocyanate and extraction into ethylene dichloride [17–19].

The biological membranes used here were sarcoplasmic reticulum (SR) vesicles extracted from rabbit fast twitch muscle (and containing about 0.5 g lipids/g membrane proteins), as in [8] and [20]. SR vesicles were prepared during the period from 2006 to 2009 and stored at -80°C until being used, under conditions that do not alter their biological properties, i.e. in the presence of 0.3 M sucrose. Three different SR preps were used for the present experiments, which were performed over several years, with no loss of ATPase activity observed on this period. Membrane preparation from the rabbit was carried out in strict accordance with the recommendations and after agreement from the Ethic committee of the "Commissariat à l’Énergie Atomique et aux Énergies Alternatives" (CEA agreement #E 91 272 106; see S1
NC3Rs ARRIVE Guidelines Checklist). All surgery was performed after killing rabbit by bleeding after a blow to the neck with a metal bar, as quickly as possible to minimize suffering (for a detailed procedure of subsequent steps, see [21]). The concentration of SR membranes in each experiment is expressed in terms of their protein contents, i.e. in μg of protein/mL. In all experiments the buffer contained 100 mM KCl, 1 mM MgCl₂ and 50 mM Tes-Tris at pH 7.5 and 20˚C (designated as “pH 7.5 buffer”), together with 0.05 mM free Ca²⁺ (0.1 mM total Ca²⁺ and 0.05 mM EGTA) to optimize preservation of SERCA1a, the main protein of the SR membranes.

For each detergent, its critical micellar concentration (cmc) at pH 7.5 was estimated using 40 μM methyl orange, as previously described [22,23]. Light scattering (at 290 nm) and Trp fluorescence (λ_ex and λ_em at 290 nm and 340 nm, respectively) of the membranes, in the absence or presence of detergent, were measured as previously described (see e.g. [15]), and sometimes simultaneously, using a Spex Fluorolog equipped with two independent monochromators in the “T” configuration. In some cases also, light scattering and Trp fluorescence changes were measured during slow continuous dilution inside the cuvette of the contents of a detergent-containing mechanically-driven syringe. In those cases, detergent delivery from the syringe (containing a 5.5 mM C₁₂E₈ or a 10 mM DDM solution) into the 2 ml spectrophotometer cuvette was performed at 400 μl/hour, resulting within half an hour in a final addition of 0.5 mM or 0.9 mM detergent together with an up to 10% dilution of the membranes. Note that this detergent delivery procedure not only makes it possible to collect a large number of data points, but also minimizes the artefactual transient solubilization of membranes which might occur when a droplet of concentrated detergent is added from an ordinary pipet [24].

Stopped-flow experiments were performed using a Biologic SFM 3 equipment (see e.g. [25]), but here with mixing in different volume-to-volume ratios of the contents of the two syringes. The nominal dead-time of the machine is about 3 ms. The excitation wavelength was 290 nm, and fluorescence emitted at 340 nm was detected using a combination of filters (MTO J324 + A340).

Rapid filtration measurements were performed using a Biologic equipment, as in [8], and Whatman GF/F glass fiber filters. Such filters have pore diameters larger than the typical diameter (0.06–0.3 μm) of SR vesicles, and therefore retain the SR membranes thanks to adsorption of these membranes onto the pore walls. The total volume of “wetting fluid” in such filters is ~100 μL, but membranes are loaded onto the filter using a funnel of diameter smaller than the one of the filter, and they probably adsorb mainly onto the walls of the central pores of the filter, say, within ~50 μL fluid. The diameter of the syringe delivering the perfusion fluid is intermediate. Perfusion rates were 2–4.5 ml/s (faster rates for shorter periods).

Remember that in the presence of a low, non-solubilizing (i.e. only “perturbing” [4,26]) concentration of detergent, the total concentration of this detergent is equal to the sum of its free concentration (in the water phase) and its bound concentration (inserted in the membranes, but here expressed per ml of water phase), the latter, at a given free detergent concentration, being dependent on the amount of membranes [27]). The detergent binding isotherms used here, for planning and roughly estimating free and bound concentrations of the brominated detergents under the various situations explored in Figs 2 and 5, were deduced (see e.g. [27,28]) from the membrane concentration-dependent shifts in the detergent-dependent light scattering curves of Figs 1 and 4. They were similar for both versions of each detergent, and consistent with the binding isotherms already published for the non-brominated versions of either C₁₂E₈, [4,8] or DDM [29].
Fig 1. Comparison of C_{12}E_8 and 5,6-Br_2C_{12}E_8 properties: Critical micellar concentration, steady-state interaction with SR vesicles as deduced from light scattering, and effects on protein intrinsic fluorescence. (A) Detergent cmc (arrow) for C_{12}E_8 and 5,6-Br_2C_{12}E_8, as determined from the spectral changes of 40 μM methyl orange (expressed as ΔA_{415} nm - ΔA_{500} nm, in absorbance units) in the presence of increasing concentrations of these detergents. (B) Perturbation by C_{12}E_8 and 5,6-Br_2C_{12}E_8 of the 90° light scattering (at 290 nm) by SR vesicles (at 4 μg protein/ml). In Panels A and B, closed symbols correspond to 5,6-Br_2C_{12}E_8, open symbols correspond to non-brominated C_{12}E_8. (C and D) Perturbation by C_{12}E_8 (C) and 5,6-Br_2C_{12}E_8 (D) of light scattering by SR vesicles, as in Panel B, but here recorded upon continuous delivery of concentrated detergent from a small syringe into the spectrophotometer cuvette, and in the presence of different concentrations of membranes (20, 50 or 100 μg/ml of protein, short dash, long dash, and continuous lines, respectively). Recorded signals were not corrected for the resulting small dilution of membranes (10% at 0.5 mM detergent). The few data points for “negative” detergent concentrations correspond to data recorded before actuation of the syringe. (E and F) Perturbation by C_{12}E_8 (E) and 5,6-Br_2C_{12}E_8 (F) of the intrinsic fluorescence signal for SR vesicles under the above conditions (continuous delivery of detergent and in the presence of different concentrations of membranes, again at 20, 50 or 100 μg/ml of protein). In Panels D and F, after addition of Br_2C_{12}E_8 up to 0.44 mM to the 100 μg/ml SR suspension, increasing amounts of non-brominated C_{12}E_8 were finally added to the ~2 ml suspension, up to about 6 mM (2, 2, 4, 8, 16, and finally 32 μl of a very concentrated solution of C_{12}E_8~100 mg/ml, i.e. 186 mM). Blank values (buffer only, in the absence of membranes) were subtracted from the “fluorescence” signal, but detergent-induced dilution and photolysis were not corrected for.

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Results

Brominated detergents have previously been described as useful tools to study detergent-membrane interaction, thanks to their fluorescence quenching properties [9]. In the present experiments (Fig 1), we first ascertained that brominated 5,6-Br₂C₁₂E₈ detergent had physicochemical properties very similar to those of the parent detergent, C₁₂E₈. Indeed we found a fairly similar cmc for the brominated or unbrominated detergent, ~75 μM (arrows in Fig 1A), as tested with methyl orange [22,23], and fairly similar abilities to perturb light scattering by

![Diagram](https://d oi.org/10.1371/j ournal.pone.0222932.g002)
sarcoplasmic reticulum (SR) membrane vesicles (Fig 1B). At low, non-solubilizing concentra-
tions, such light scattering changes may reflect changes in membrane shape as well as changes
in the refraction index of the membrane vesicles, while at higher concentrations, they mainly
reflect solubilization of the membranes down to poorly scattering detergent/protein/lipid s
mixed micelles, as previously described for other non-brominated detergents [4,13,26]. Fur-
thermore, we observed similar abilities of the brominated and the non-brominated detergents
to partition into the membranes, as determined from the fact that at increasingly higher con-
centrations of membranes, light scattering curves were shifted to the right (see e.g. [9,28]) in a
quantitatively fairly similar manner (see Fig 1C for \( \text{C}_{12}E_8 \) and Fig 1D for \( 5,6\text{-Br}_2\text{C}_{12}E_8 \)). At
low, non-solubilizing as well as high concentrations, brominated detergent was also exhibited
the typical influence of \( \text{C}_{12}E_8 \) on the enzymatic activity of the \( \text{Ca}^{2+}\)-dependent ATPase SER-
CA1a, the main protein present in these membranes [4,13].

Of major importance, the brominated \( 5,6\text{-Br}_2\text{C}_{12}E_8 \) was able to quench the intrinsic fluores-
cence of the Trp residues of the proteins in these membranes in a detergent- and membrane
concentration-dependent manner (Fig 1F). This quenching can be assigned to contact or very
short-distance fluorescence quenching by the bromine heavy atoms [12,20] located on the
hydrophobic chain of this detergent, as previously observed for brominated phospholipids
[9,12,13] as well as for other brominated detergents like dibromo-dodecylmaltoside [9,20]. At
the intermediate plateau level, corresponding to saturation of membranes by detergent (just
before their solubilisation), the extent of fluorescence quenching by the membrane-bound bro-
minated detergent was already very significant (close to 40–50%), presumably because in SER-
CA1a, most Trp residues are embedded in the transmembrane region, and located very close
to its hydrophobic surface [14]. Fluorescence quenching was further increased at solubilizing
detergent concentrations, and such quenching could be reversed by addition of excess non-
brominated \( \text{C}_{12}E_8 \) at the end of the experiment, as expected (Fig 1F, right side). In contrast,
non-brominated \( \text{C}_{12}E_8 \) only marginally interfered with the Trp intrinsic fluorescence (Fig 1E):
the minor decrease in signal illustrated in Fig 1E mainly corresponds to detergent-induced dilution and time-dependent photolysis, which have not been corrected for in Fig 1E & 1F. It should be noted that when detergent was added to the membranes at non-solubilizing concentrations, it nevertheless interacted with both sides of the membrane on the time-scale of the present experiments, since transmembrane flip-flop of C\textsubscript{12}E\textsubscript{8} has been shown to be faster than 300–400 ms [8].

The contact and dose-dependent quenching exerted by 5,6-Br\textsubscript{2}C\textsubscript{12}E\textsubscript{8} on the fluorescence of membrane-embedded Trp residues made it possible to take advantage of stopped-flow fluorescence for measuring the kinetics of 5,6-Br\textsubscript{2}C\textsubscript{12}E\textsubscript{8} binding to, or dissociation from, these membranes. Binding experiments were the first ones to be performed, and results are illustrated in Fig 2A. As a preliminary control, SR membranes were first mixed with buffer alone, to reveal the Trp fluorescence reference level in the absence of brominated detergent (about 7 volts, top trace in panel A). SR membranes were then mixed with two non-solubilizing concentrations of 5,6-Br\textsubscript{2}C\textsubscript{12}E\textsubscript{8}, resulting in free detergent concentrations of 38 μM (intermediate trace in panel A) and 57 μM (bottom trace in panel A), respectively (see figure legends for details). In both cases, the Trp fluorescence level dropped rapidly, with observed half times shorter than 10 ms. Note that the recorded traces begin (at t = 0) significantly below the control level of 7 V,
presumably because the initial fluorescence drop occurred within the dead time of the stopped-flow equipment (a few ms), and this makes it impossible to more precisely estimate the true rate constants of the fluorescence changes in Panel A. However, it is clear that 5,6-Br₂C₁₂E₈ insertion into the membranes is very fast, even at the non-solubilizing concentrations used here. Recording the traces over longer periods did not reveal anything except the expected photolysis.

In a second step, dissociation experiments were performed, in which SR membranes pre-incubated with 5,6-Br₂C₁₂E₈ were mixed with detergent-free buffer, with significant dilution. For the lower trace illustrated in panel B of Fig 2, concentrated SR membranes, pre-incubated with a relatively high but nevertheless non-solubilizing concentration of 5,6-Br₂C₁₂E₈ (200 μM total, i.e. 46 μM free, a concentration lower than both the cmc and the critical solubilisation concentration (csc) of C₁₂E₈ [30]), were mixed with buffer alone, at a ratio of 1:4.5 v:v as for the binding experiments in Panel A. Fluorescence recovery was completed after a few tens of milliseconds only. At the end of the recording, the fluorescence level still remained below that of the control experiment in Panel A, in agreement with the fact that upon reaching the final

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At this stage, we also investigated another brominated detergent, 5,6-Br₂DDM. Just like 5,6-Br₂C₁₂E₈, 5,6-Br₂DDM has properties similar to those of its non-brominated parent, DDM. The very similar cmc (0.14–0.16 mM) and solubilisation efficiency of 5,6-Br₂DDM and DDM have recently been published, together with their similar perturbing effects on ATPase activity (see supplemental information in [20]). Observed effects of the two versions of DDM on light scattering by SR, shown in Fig 4A and 4B are rather similar, although 5,6-Br₂DDM has a more marked tendency to induce a transient increase in light scattering at intermediate concentrations of detergent. Note that such an increase has already been observed for a number of detergents in the region where their binding to membranes, and more especially to pure liposomes, becomes cooperative and solubilisation starts. It is probably due to macroscopic reorganization and transient aggregation of the detergent-saturated membranes, e.g. opening and fusion of the vesicles [24,30]. As anticipated from the present results obtained with 5,6-Br₂C₁₂E₈ (Fig 1D) or those obtained previously with other brominated analogues of DDM [9], 5,6-Br₂DDM was also able to quench the fluorescence of SR Trp residues (Fig 4D) even before membrane solubilisation. This quenching is even larger than in the case of 5,6-Br₂C₁₂E₈ (compare Fig 4D vs Fig 1F).

Stopped flow experiments were then performed to reveal the kinetics of 5,6-Br₂DDM binding or dissociation. The kinetics observed for 5,6-Br₂DDM binding or dissociation again comprised a very rapid phase, within milliseconds, poorly resolved by our stopped-flow equipment. While signals were stable after 100 ms for 5,6-Br₂C₁₂E₈ (Fig 2B–2D), the kinetics for 5,6-Br₂DDM binding or dissociation also comprised a slower phase, observable both over 0.5 s (Fig 5A and 5B) and still observed if signal recording is extended over a few seconds (see Fig 5C and 5D). Over such a longer period of time, the slow component in the dissociation trace (Fig 5D) was still present but less obvious than in the association trace (Fig 5C). A possible reason for this particular behaviour will be discussed later.
Discussion

One of the main conclusions drawn from this work is that caution needs to be taken when designing a method for measuring the rate of dissociation of detergent from membranes. Our first attempt in 1987 using a rapid filtration method had suggested for C$_{12}$E$_4$ a half time for dissociation in the 300–400 ms range, both from egg phosphatidylcholine unilamellar or multilamellar vesicles and from protein-containing sarcoplasmic reticulum (SR) membrane vesicles (Fig 5 in [8]). But stopped-flow experiments using 5,6-Br$_2$C$_{12}$E$_8$ now reveal a much shorter half time (a few milliseconds), hardly distinguishable from the dead time of the stopped-flow equipment itself (Fig 2B–2D of the present paper), despite the fact that [$^{14}$C]5,6-Br$_2$C$_{12}$E$_8$ behaves like [14C]C$_{12}$E$_8$ in rapid filtration experiments (Fig 3 of the present paper). We propose a tentative explanation for this discrepancy below.

But before doing so, it is perhaps worth to address a conceivable objection to our fluorescence recovery experiments performed with 5,6-Br$_2$C$_{12}$E$_8$: as it is only the fluorescence of Trp residues which is monitored in these experiments, it might be argued that such stopped-flow experiments only reveal the possibly fast rate of dissociation of those detergent molecules which are in contact with the proteins, and not the dissociation rate of bulk detergent molecules present elsewhere in the lipid phase and possibly located too far from the protein Trp residues to be efficient quenchers. We in fact consider this possibility as highly unlikely, for three reasons: (1), detergent molecules in contact with membrane proteins probably exchange position very rapidly with the “bulk” detergent molecules, so that on the millisecond time scale it is a homogenous population; in fact, even phospholipid molecules rapidly exchange positions: lipids which were originally described as “immobilized” when they were first studied by electron spin resonance techniques with sub-microsecond timescale sensitivity, were subsequently found to be indistinguishable from “bulk” lipids when they were examined by nuclear magnetic resonance techniques (especially deuterium NMR) with millisecond time scale sensitivity (e.g. [32,33]); (2), for exerting significant fluorescence quenching on the SERCA1a Trp residues which is monitored in these experiments, it might be argued that such stopped-flow experiments only reveal the possibly fast rate of dissociation of those detergent molecules could be more easily exchangeable than bulk detergent molecules simply embedded within the membrane lipids; (3), stopped-flow fluorescence binding and dissociation experiments similar to the ones reported for 5,6-Br$_2$C$_{12}$E$_8$ in Fig 2, but performed with another non-ionic brominated detergent, 5,6-Br$_2$DDM, reveal for both binding and dissociation the existence of much slower components of fluorescence changes (Fig 5, see further discussion below) than for 5,6-Br$_2$C$_{12}$E$_8$. Similar slow components were also mentioned with another brominated version of DDM, 7,8-Br$_2$DDM [9] and they had been considered to reflect the previously suggested slow flip-flop of DDM compared with C$_{12}$E$_4$ [30]. The conclusion of such an observation with Br$_2$DDM necessarily is that using brominated detergents and a fluorescence quenching or de-quenching assay cannot per se be responsible for the absence of slow events during dissociation of 5,6-Br$_2$C$_{12}$E$_8$. So the rapid kinetics illustrated in Fig 2B–2D probably do reflect rapid overall dissociation of 5,6-Br$_2$C$_{12}$E$_8$ from the membrane while a different detergent, e.g. 5,6-Br$_2$DDM, may have different properties regarding its kinetics of overall interaction with the membrane and in particular its kinetics of transmembrane flip-flop (see further discussion below).

Back to the methodological issue raised by comparing the very fast events observed in 5,6-Br$_2$C$_{12}$E$_8$ fluorescence de-quenching dissociation experiments and the seemingly much slower dissociation of $^{14}$C-labelled 5,6-Br$_2$C$_{12}$E$_8$ (or C$_{12}$E$_8$) in rapid filtration experiments, we now think that the rapid filtration experiments with $^{14}$C-labelled versions of C$_{12}$E$_8$ give
artefactual results. In such filtration experiments, the local concentration of the membrane vesicles adsorbed onto the glass fiber filter is very high. Consequently, the resulting partition of detergent, here C_{12}E_{8}, between water and the membranes is very much in favour of the bound detergent. For instance, with either 80 or 100 μg protein loaded onto each filter (as in the present Fig 3 experiments, or those in the Fig 5 experiments of [8]), this ratio is ~1/12 or ~1/15 (mol free in water/mol bound to membrane), as calculated using the same detergent binding isotherm for C_{12}E_{8} as the one in Fig 2 of [8] and estimating the fluid volume around the filter-loaded membranes to about 50 μL as mentioned under Materials and Methods. Therefore, even if the volume of buffer perfused within a certain period seems to be enough to efficiently rinse the pore contents (e.g. if the filter is perfused with buffer during a 300 ms period at a perfusion rate of 3.3 ml/s, leading to a perfused volume of 1 mL), the perfused buffer will efficiently wash out only the fraction of detergent which is free, while the bound detergent, even if assumed in relatively rapid equilibrium with the free one, will only drop moderately. Therefore, the total amount of detergent on the filter will decrease unduly slowly, with a rate of no significance for the possibly very fast "true" rate of detergent dissociation from membrane. The same artefact will also show up in detergent binding experiments. In 1987, binding of non-brominated C_{12}E_{8} to SR membranes measured by rapid filtration, was found to be seemingly slow (Fig 6 in our previous work [8]), but the perfusion rates provided by the filtration equipment were in fact too slow to reveal the fast binding now detected (Fig 2A) by stop-flow fluorescence quenching upon binding of brominated C_{12}E_{8}. Ironically, the change in intrinsic fluorescence occurring upon binding of non-brominated C_{12}E_{8} to SR membranes had already been found to be very fast in 1987 (Fig 6C in our previous work [8]), but this observation was unfortunately misinterpreted. Avoiding these perfusion rate artefacts would require to reduce considerably the amount of membranes loaded onto the filter, but such reduction would be incompatible with keeping a fair signal over noise ratio. Binding of detergent to the filter, in the absence of membranes, is indeed quite significant, even though glass fiber filters are more favourable than other types of filter from the point of view of this non-specific binding. Note that non-specific adsorption is a common problem for brominated versions and radioactively-labelled versions of detergents (either DDM or C12E8): such detergents easily adsorb onto many surfaces (not only to our glass fiber filters, but also various materials, nitrocellulose, Falcon tubes, etc . . .), even at low concentrations, and with specific kinetics: such adsorption processes of course complicate precise comparisons between different experiments (and particularly, in our stopped-flow fluorescence experiments, comparison of amplitudes). In contrast, in previously published rapid filtration experiments studying dissociation of ^{45}\text{Ca} initially bound to SERCA1a, potential artefacts due to limited perfusion rates and local crowding did not show up, because ^{45}\text{Ca} dissociation was measured in the presence, in the perfusion buffer, of either an excess of a strong chelator (EGTA), or an excess of non-radioactive calcium (^{40}\text{Ca}) [34]. In such experiments, the nitrocellulose filters used do not adsorb calcium, so that calcium binding is restricted to the SR membrane whereas detergents can bind to the entire surface of the filter.

At this point, it is perhaps time to enlarge our discussion and tentatively discuss in more detail the various steps which may influence the observed overall signals in our fluorescence quenching and de-quenching experiments, namely: (i) the detergent movements between the water phase and the external monolayer of the membrane whose kinetics are governed by rate constants $k_{\text{ass}}$ and $k_{\text{diss}}$, respectively. For simplicity we will neglect the binding and dissociation events occurring on the internal side of the vesicles, since they probably affect an only small fraction of the signal; (ii) the detergent movements from one monolayer of the membrane to the other are governed by rate constants $k_{\text{flip}}$ and $k_{\text{flop}}$. For simplicity we will assume that these two rate constants are equal. The exact numbers and quantum yields of Trp residues within
each monolayer, as well as and their exact degree of accessibility to detergent, will of course also influence the amplitude of the fluorescence changes during these various steps. Although our experiments do not allow us to determine all these parameters, especially because part of the fluorescence signal is lost within the dead-time of the stopped-flow equipment, they are maybe enough to make the following hypothesis.

Firstly, in the case of 5,6-Br$_2$DDM, the overall fluorescence quenching curves observed, with both a fast initial component and a subsequent slower component, can probably be accounted for by assuming initial rapid binding of 5,6-Br$_2$DDM to the external monolayer (within a few milliseconds, see Fig 5A), followed by a relatively slow flip-flop (over a few seconds at 85 μM free 5,6-Br$_2$DDM, see Fig 5C), as previously suggested for DDM [30]. The slower phase was less obvious in dissociation experiments than in binding experiments probably because of the relatively slow rate of flip-flop for 5,6-Br$_2$DDM in perturbed membranes which gets even slower when both membrane monolayers progressively become almost completely free of detergent. The flip-flop rate of DDM may indeed depends significantly on other membrane components around, including for example transmembrane proteins. It might also explain why biological membranes can be easily solubilized by DDM, while pure liposomes get solubilized much more slowly [13].

Secondly, in the case of 5,6-Br$_2$C$_{12}$E$_8$, because of the absence of such a slow phase in the fluorescence quenching or de-quenching traces, half times corresponding to all rate constants must be equal to or shorter than a few milliseconds, but our equipment and data themselves do not allow us to decide whether these few milliseconds correspond to the half time for flip-flop, or to the one for true dissociation or association. Our 1987 filtration experiments with multilayered liposomes excluded that C$_{12}$E$_8$ flip-flop could be considered as the rate-limiting step, but only when compared with the 300–400 ms time-scale considered in our initial work [8]. However, on the timescale of a few milliseconds only determined in the present experiments, flip-flop of C$_{12}$E$_8$ might well be the rate-limiting step: a half time of a few milliseconds for 5,6-Br$_2$C$_{12}$E$_8$ flip-flop, with even faster rates for “true” dissociation or binding, would provide a fair explanation for the apparent concentration-independence of observed rate constants in Fig 2. Theoretical computations of C$_{12}$E$_8$ binding and dissociation in model systems using molecular dynamics simulations on a millisecond time-scale might help to address this possibility, but at this stage the idea that flip-flop might be the rate-limiting step for the movements of both C$_{12}$E$_8$ and DDM (although at a much faster step in the case of C$_{12}$E$_8$), seems to be reasonable.

To summarize, use of brominated C$_{12}$E$_8$ combined with stopped-flow measurements, instead of rapid filtration experiments using radiolabelled C$_{12}$E$_8$, allowed us to reach a safer conclusion concerning the dissociation rate of C$_{12}$E$_8$ previously bound to SR membranes. In the case of C$_{12}$E$_8$, this dissociation is much faster (half time of a few milliseconds, or even shorter) than the one previously estimated from the earlier rapid filtration experiments [8]. In the case of DDM, the rate of binding to or dissociation from membrane is probably fast but a slower flip-flop rate significantly limits overall binding or dissociation. The present work therefore fully confirms the interest of using brominated detergents and the accompanying quenching of membrane protein intrinsic fluorescence for studying detergent/membrane interactions [9].

Supporting information
S1 NC3Rs ARRIVE Guidelines Checklist.
(PDF)
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