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Compatibility of detergents with the microbatch-under-oil crystallization method

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Detergents are required to solubilize integral membrane proteins and are common components of the solutions used to crystallize these molecules. It has been unclear whether these detergents are completely compatible with the oils used in the microbatch-under-oil crystallization technique, because they might conceivably be lost from solution by partitioning into the oil phase. The partitioning of the detergents *n*-octyl-β-D-glucopyranoside and Fos-Choline-12 into two different oils used for microbatch crystallization experiments has been examined. It was found that vigorous mixing and prolonged incubation of the aqueous detergent solutions with the oils leads to small losses of detergent (approximately 5% of the total detergent mass); however, gentle mixing that is more typical of the mixing encountered in a crystallization experiments leads to negligible loss of detergent.

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1. Introduction

Integral membrane proteins represent a frontier area of structural biology: while accounting for perhaps one third of all proteins encoded in the genome, they represent fewer than 1% of the proteins of known structure. High-throughput crystallographic methods offer a means of increasing the number of membrane-protein crystal structures and redressing this imbalance (Stevens, 2000), but these methods must be tailored to the unique properties of membrane proteins if they are to succeed.

Membrane proteins are typically insoluble in any single solvent because their surfaces contain both polar and apolar regions. However, they can be rendered soluble in aqueous solutions through the use of detergents. The hydrophobic portions of the detergent molecules adsorb onto the protein's apolar surface, producing a protein–detergent complex (le Maire *et al.*, 2000; Garavito & Ferguson-Miller, 2001). To date, the majority of membrane-protein crystals have been obtained by direct crystallization of such protein–detergent complexes from detergent-containing solutions.

The microbatch-under-oil technique is a relatively new method of protein crystallization (Chayen *et al.*, 1990, 1992). It entails introducing a drop of protein solution and a drop of precipitant solution into a vessel containing a water-immiscible oil. Being denser than the oil, the protein and precipitant droplets move to the bottom of the reservoir and mix; the oil prevents water evaporation and reduces the amount of oxygen that can reach the droplet. In a variation on the original method, a water-permeant oil can be used, allowing slow concentration of the drop (D'Arcy *et al.*, 1996, 2003). The latter approach allows the experimenter to sample a range of precipitant concentrations in a single experiment, in a manner analogous to vapor diffusion. The microbatch-under-oil method has many advantages for high-throughput crystallization screens, being easily automated and allowing the use of small-footprint high-density microwell plates (Luft *et al.*, 2001).

We questioned whether crystallization under oil is compatible with the detergents used for membrane-protein crystallization. Specifically, we wondered if significant quantities of the detergent would partition into the oil phase, thereby reducing the amount of detergent available in the aqueous phase for maintaining protein solubility and risking protein aggregation and/or denaturation. Indeed, while at least one example of a successful membrane-protein crystallization under oil is known (Hankamer *et al.*, 1992), it has been suggested in this case that crystallization might be driven by removal of detergent into the oil phase (Chayen, 1997). To determine whether this might occur, we examined the partitioning of two different detergents into two of the oils commonly used in microbatch experiments, namely paraffin oil and silicon oil. Aqueous solutions of detergents were mixed with the oils and incubated for roughly 10 d; the concentration of the

detergent in the aqueous phase was then assayed to determine whether any significant changes had occurred.

2. Materials and methods

Two detergents, *n*-octyl-β-D-glucopyranoside (OG) and Fos-Choline-12 (FC-12), were chosen (Fig. 1). They represent two different families of detergents commonly employed in membrane-protein biochemistry: the neutral alkyl glycosides and the zwitterionic alkyl choline derivatives. Their critical micellar concentrations (CMCs) are 18 and 1.5 m*M*, respectively; CMC values for detergents commonly used in membrane-protein crystallization typically fall in the range 0.1–20 m*M*. Deuterated versions of both are commercially available; d_{17}-OG (deuterated on the alkyl chain only) and perdeuterated FC-12 (d_{38}) were used. All detergents were obtained from Anatrace Inc. (Maumee, OH, USA). The oils examined were paraffin oil and a 1:1 mixture of paraffin oil and silicon oil, marketed under the name 'Al's Oil'. Both oils were obtained from Hampton Research (Laguna Niguel, CA, USA; catalog Nos. HR3-411 and HR3-413, respectively).

Solutions of ^{1}H- and ^{2}H-containing detergents in water were prepared at concentrations corresponding to four times the critical micellar concentration [2.1 and 0.21%(*w*/*w*) for OG and FC-12, respectively]. 1 ml of the ^{1}H-detergent solution was placed in a vial with 2 ml of oil; the vial was tightly sealed and the contents were mixed. Mixing was either vigorous (vortexing for 10 s) or gentle (inversion of the vial three times). Control vials were also prepared containing detergent but no oil. All experiments were conducted in triplicate. The vials were incubated at room temperature for 9–11 d. The vials were weighed before and after incubation to measure any evaporation that might have occurred; the mean loss of mass for all vials during the incubation was 4 ± 3 mg. Corrected detergent concentrations were calculated assuming the loss of mass was entirely owing to water evaporation. (It cannot be proven that oil did not evaporate as well, but in any event the total loss of mass is small and this correction has a negligible effect on the outcome.) After the incubation, an aliquot of the aqueous phase was removed, weighed, and mixed with a weighed aliquot of deuterated detergent solution. This mixture was subjected directly to electrospray ionization mass spectrometry (Waters Micromass-ZQ system). 28 scans of total positive-ion counts were acquired per sample at a rate of one scan per second. Counts corresponding to the ^{1}H- and ^{2}H-detergent ions were normalized to the internal reference standard and integrated using the *MassLynx* 3.1 software package (Waters), yielding a raw ^{1}H/^{2}H ratio. The raw ratio was then normalized by dividing by the ^{1}H/^{2}H ratio observed for the control vials (*i.e.* those vials in which no oil was added to the detergent solution). Hence, a normalized ratio of 1.0 implies no loss of detergent into the oil phase, while ratios less than 1.0 signal partitioning of detergent into the oil. These normalized ratios are shown in Fig. 2.

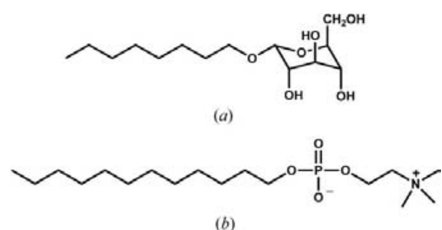

Figure 1
Detergents used in this study. (*a*) *n*-octyl-β-D-glucopyranoside (OG), (*b*) Fos-Choline-12 (FC-12).

3. Results

Two different protocols were used for mixing the detergent solutions with the oils. The vigorous mixing protocol involved rapid vortexing for 10 s; this invariably led to the formation of a foam in the oil phase that persisted for the course of the experiment. This protocol was chosen as a 'worst-case' scenario, as it represents a degree of mixing far exceeding anything likely to be encountered in a crystallization experiment. The stable foams produced by vortexing are expected to contain large interfacial surface areas that will attract surfactant, so some loss of detergent in this case would not be surprising. Even in the vigorous mixing case, however, loss of detergent into the oil phase was minimal: reductions of approximately 5% in the total detergent concentration were seen, but only the OG/Al's Oil combination displayed a difference between the oil-treated sample and control that was significant at the level of $p < 0.05$ (Fig. 2).

The gentle mixing protocol is more representative of the type of mixing that actually occurs during a microbatch-under-oil crystallization experiment. In this case, no statistically significant partitioning of detergent into the oil phase can be measured.

In our experiments, the ratio of the surface area of detergent solution in contact with oil to the volume of the detergent solution is lower than that encountered in a microbatch crystallization protocol. Hence, the insignificant loss seen in our gentle mixing experiments might be thought to be an underestimate of the actual loss that would occur in a crystallization experiment. However, because the foam formed by vigorous vortexing (with its very large interfacial area) causes a decrease of only a few percent in the detergent concentration, we do not expect this surface/volume effect to lead to significant detergent loss.

4. Conclusions

We demonstrate that for two commonly used and representative detergents, *n*-octyl-β-D-glucopyranoside and Fos-Choline-12, very low to negligible levels of the detergent

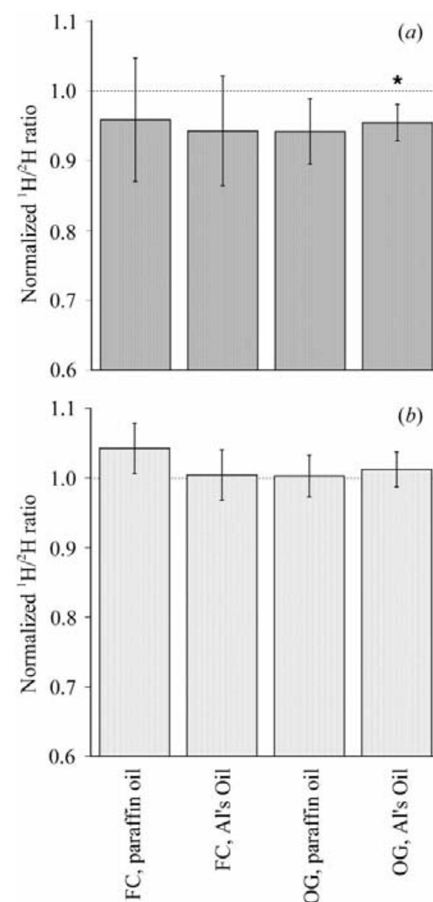

Figure 2
Normalized ^{1}H/^{2}H ratios for detergent-partitioning experiments. Detergent samples were incubated with the oils indicated, using either vigorous mixing (*a*) or gentle mixing (*b*). Aliquots were then withdrawn and spiked with deuterated detergents and the hydrogen:deuterium ratio was determined by mass spectrometry. FC denotes Fos-Choline-12; OG denotes *n*-octyl-β-D-glucopyranoside. Ratios are normalized using the ^{1}H/^{2}H ratio observed in control experiments containing no oils; ratios less than 1.0 imply a loss of detergent from the aqueous phase. Error bars represent the standard error calculated from three independent measurements; a star indicates a ratio that differs significantly from 1.0 (Student's *t*-test, $p < 0.05$).

partition into either of two oils commonly used in microbatch crystallization experiments. This implies that the microbatch-under-oil technique can be used safely with membrane proteins dissolved in these detergents, without fear of significant detergent depletion owing to diffusion into the oil phase.

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