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A novel approach for the crystallization of soluble proteins using non-ionic surfactants

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Abstract

Crystallization trials using three polyoxyethylene surfactants as precipitating agents are described. Of the eight soluble proteins screened, five were successfully crystallized at the first attempt. These included lysozyme, catalase, ferritin, ribonuclease A and ubiquitin. Further work suggested that these surfactants could also be suitable for cryo-crystallographic analysis of crystals. At the concentrations used in the crystallization trials [10–40%(*v*/*v*)], they are capable of promoting the formation of non-crystalline glasses at cryogenic temperatures (77 K). This would facilitate crystal mounting and allow the minimization of crystal irradiation damage. Results from this study also suggest that proteins remain stable at high concentrations of these surfactants [40%(*w*/*v*)] and over long time periods (>1 month). A number of membrane proteins were also screened for crystallization. These included photosystems I and II and light harvesting complexes I and II from spinach and bacteriorhodopsin from *Halobacterium halobium*. The trials were unsuccessful both in the absence and presence of heptane-1,2,3-triol and over a wide range of surfactant concentrations.

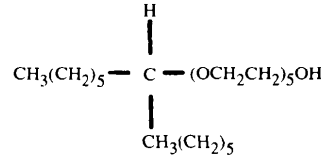
1. Introduction

The routine crystallization of proteins in general and membrane proteins in particular, is an important objective for current structural research. Whilst a variety of protein precipitating agents such as polyethylene glycol (PEG), ammonium sulfate and methyl pentane diol are added to protein solutions to induce crystallization, there is no general understanding of the molecular processes involved. It is commonplace for mixtures of two water-soluble polymers to give separate solution phases above specific polymer concentrations because of unfavourable polymer *A*/polymer *B* interactions. This is probably why PEG is effective in promoting protein crystal formation, but here the crystal forms rather than an isotropic solution. For membrane proteins there is the particular problem of keeping the protein in solution after solubilization from the membrane because of the hydrophobic domain(s). Detergents (surfactants) are employed to solubilize the protein, but this acts against the desired 'insolubility' required for crystallization. Non-ionic (polyoxyethylene) surfactants are employed for this purpose. They are known as mild detergents, having a minimal influence on the protein conformation. It is known that water-soluble polymers and concentrated non-ionic surfactants form separate aqueous phases (Zhang *et al.*, 1994; Cabane *et al.*, 1996), because the surfactant micelles act like a second polymer species in promoting phase separation. Thus, it is possible that non-ionic surfactants could be employed instead of PEG in promoting soluble protein crystallization. In addition, the surfactants could be used to solubilize membrane proteins and could also produce crystals. In this study we have selected three non-ionic surfactants to screen for macromolecular crystallization. All were *n*-alkyl polyoxyethylenes and will be referred to as surfactant *A*, $C_6C_5EO_5$ (pentaoxyethylene mono-6-dodecyl ether); surfactant *B*, C_8EO_4 (tetraoxyethylene mono-*n*-octyl ether); and surfactant *C*, DiC_7EO_9 (glycerol-1-nonaoxyethylene-2,3-diheptyl ether). Fig. 1 shows the chemical formulae of the three surfactants.

The surfactants were chosen either because they exhibit extensive lamellar phase formation or because their molecular structure closely resembles other surfactants which show this. Thus, the micellar environment should be fairly similar to that of a bilayer membrane. In addition, the materials have low cloud temperatures, which should also assist in the formation of separate surfactant and protein phases. The phase behaviour of all three surfactants has been characterized (*A* and *C*, Thompson *et al.*, 1996; *B*, Mitchell *et al.*, 1983). Phase transitions occur as a function of concentration of the surfactant and temperature.

Non-ionic surfactants at low concentrations, *e.g.* β-octyl glucoside at 0–1.5%, have been reported to influence the growth of soluble protein crystals in a positive manner and in some instances alter the crystal habit or the crystallographic unit cell (McPherson, Koszelak, Day, Robinson *et al.*, 1986, McPherson, Koszelak, Axelrod, Day, Williams *et al.*, 1986). However, the use of surfactants has mostly been limited to membrane protein crystallization (Michel, 1982, 1991). It is well documented that detergent–protein interactions are a very important factor in the crystallization of membrane proteins, as is the choice of detergent (Michel, 1991). The structure of the photosynthetic reaction centre from *Rhodopseudomonas viridis* revealed electron density for only one detergent molecule (*N*,*N*-dimethyldodecylamine-*N*-oxide or LDAO) (Michel, 1982).

Surfactant *A*



Surfactant *B*

$CH_3(CH_2)_7(OCH_2CH_2)_4OH$

Surfactant *C*

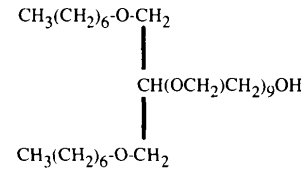


Fig. 1. The chemical formulae of the three *n*-alkyl polyoxyethylene surfactants.

Table 1. *Summary of the HEWL crystallization trials*

All trials consisted of a simple three-component mixture of buffer, protein and surfactant in water. For trials at pH 4.5, 0.1 *M* sodium acetate was employed; at pH 5.5 and 6.5, 10 m*M* MES (2-[*N*-morpholino]ethanesulfonic acid) was used; and trials at pH 7.0, 7.5 and 8.0, 10 m*M* HEPES (*N*-[2-hydroxyethyl]piperazine-*N*-[ethenesulfonic acid]) was present. For each set of crystallization conditions summarized in each row, the optimal combination of temperature and surfactant concentration (for batch method) and the optimal pH (for sitting-drop method) is listed in the final column. The estimated global optimum for all HEWL trials was assigned as an initial surfactant *B* concentration of 30%(*w*/*v*), an initial HEWL concentration of 80 mg ml^{-1} incubated at 283 K (see Table 2).

Initial concentration of HEWL (mg ml^{-1})	pH	Temperature (K)	Incubation time (d)	Surfactant	Initial concentration of surfactant [%(*v*/*v*)]	Percentage of cryst. success (total No. of trials)	Optimal conditions determined
80†	7.0	277, 283, 293	13	*A*	10, 20, 30, 40	28 (60)	20% *A*, 283 K
80†	7.0	277, 283, 293	13	*B*	10, 20, 30, 40	18 (60)	30% *B*, 283 K
80†	7.5	277, 283, 293	13	*A*	10, 20, 30, 40	40 (60)	20% *A*, 283 K
80†	7.5	277, 283, 293	13	*B*	10, 20, 30, 40	50 (60)	30% *B*, 283 K
80†	8.0	277, 283, 293	13	*A*	10, 20, 30, 40	7 (60)	20% *A*, 283 K
80†	8.0	277, 283, 293	13	*B*	10, 20, 30, 40	25 (60)	30% *B*, 283 K
80†	4.5	277, 283, 293	4	*B*	10, 20, 30, 40	17 (60)	40% *B*, 283 K
80†	4.5	277, 283, 293	7	*B*	10, 20, 30, 40	33 (60)	20% *B*, 283 K
80†	4.5	277, 283, 293	11	*A*	10, 20, 30, 40	25 (60)	20% *A*, 293 K
80†	4.5	277, 283, 293	11	B	10, 20, 30, 40	58 (60)	20% *B*, 283 K
80†	4.5	277, 283, 293	11	*C*	10, 20, 30, 40	33 (60)	10% *C*, 293 K
20‡	4.5, 5.5, 6.5, 7.5	293	16	*A*	20	25 (20)	4.5
20‡	4.5, 5.5, 6.5, 7.5	293	16	*B*	20	75 (20)	4.5
20‡	4.5, 5.5, 6.5, 7.5	293	16	*C*	20	25 (20)	4.5
40‡	4.5, 5.5, 6.5, 7.5	293	16	*A*	20	75 (20)	4.5
40‡	4.5, 5.5, 6.5, 7.5	293	16	*B*	20	75 (20)	4.5
40‡	4.5, 5.5, 6.5, 7.5	293	16	C	20	50 (20)	4.5

† Batch method was employed ‡ Vapour diffusion method was employed.

Roth *et al.* (1989) studied the size of the detergent ring surrounding this reaction centre in its crystallized form and the LDAO/reaction centre (RC) ratio value was found to be 110, a value more than two times lower than the value of 260 needed for the RC's solubilization (Gast *et al.*, 1994). In work carried out on the structure of the detergent phase in the *Rhodobacter sphaeroides* (strain Y) reaction centre crystals (Roth *et al.*, 1991) it was reported that β-octylglucoside formed a belt-shaped micelle around the hydrophobic 'waist' of the complex with interconnections between belts *via* micellar bridges. The studies illustrate common behaviour with respect to detergent packing and implications for membrane protein crystal growth (Ford, 1992). In recent work, Landau & Rosenbusch (1996) reported a novel rational approach for obtaining well ordered three-dimensional crystals of bacteriorhodopsin, a membrane protein, using quasisolid lipidic cubic phases. Hexagonal bacteriorhodopsin crystals diffracted to 3.7 Å resolution.

Because protein crystals are highly susceptible to radiation damage when studied at or near room temperature, it is desirable to study them at as low a temperature as possible (Haas, 1968; Haas & Rossmann, 1970). Rapid freezing in the presence of cryo-protectants prevents the formation of ice crystals and the observation of ice rings in the X-ray diffraction pattern.

2. Materials and methods

2.1. *Materials*

Hen egg-white lysozyme (HEWL), horse heart cytochrome *c* (cyt *c*), bovine red blood cell ubiquitin, bovine pancreas ribonuclease A, horse spleen ferritin and bovine liver catalase were all obtained from Sigma. Carnitine acetyl transferase was obtained from Boehringer Mannheim and heptane-1,2,3-triol (HT) from BDH Chemicals Ltd. All proteins were salt free, with the exception of ferritin which contained 0.15 *M* NaCl. Surfactants *A*, *B* and *C* were specially synthesized at Unilever Research Laboratories, Port Sunlight, England, as monodisperse surfactants. The purity of surfactants *A* and *C* were >96% as determined by NMR (Thompson *et al.*, 1996). Surfactant *B* was determined to be >98% pure (Mitchell *et al.*, 1983), and both surfactants *A* and *B* exhibited no pre-critical micelle concentration (c.m.c.) dip in the surface tension plot, indicating the absence of any surface active impurities. It has been demonstrated that such impurities can associate strongly with membrane proteins and must be removed prior to crystallization trials (Lorber *et al.*, 1990).

2.2. *The batch method*

The method employed has been described previously (Ford & Cochrane, 1993). Small glass tubes of diameter 1 mm were formed by pinching off and sealing the ends of long Pasteur pipettes. 5 µl of protein sample was loaded into the tubes using a Hamilton microsyringe. Then 5 µl of surfactant solution was layered on top of the protein in order to form a sharp interface. The tube was left unsealed at one end in order to allow a slow concentration of the sample by water evaporation. Evaporation rates were approximately 0.1, 0.3 and 0.5 µl d^{-1} at 277, 283 and 293 K, respectively. Protein solutions were buffered to pH values recommended to be optimal for their crystallization (McPherson, 1980) as detailed in the §3.

2.3. *Vapour diffusion by sitting drop*

Vapour diffusion by sitting drop was carried out as described by McPherson (1989). Crystal Clear Strips (Hampton Research)

Table 2. *Summary of successful crystallization conditions of some soluble proteins using the batch method with the estimated global optimum conditions of crystal formation and growth*

Protein	Lysozyme	Ubiquitin	Ribonuclease A	Ferritin	Catalase
Source	Hen egg white	Bovine red blood cell	Bovine pancreas	Horse spleen	Bovine liver
Initial concentration (mg ml^{-1})	20.0, 40.0 and 80.0	25.0	10.0, 20.0 and 40.0	40.0	70.0
pH	4.5, 5.5, 6.5, 7.5	5.6	5.6	7.4	7.4
Buffer	HEPES, MES and sodium acetate	MES	MES	HEPES	HEPES
Temperature (K)	277, 283, 293	277, 283, 293	277, 283, 293	277, 283, 293	277, 283, 293
Initial surfactant concentration [%(*v*/*v*)]	10, 20, 30, 40 *A*, *B* & *C*	10 & 20 *A*	20, 30, 40 *A* & *B*	10, 20, 30 *A* & *B*	10, 20, 30 *A* & *B*
Global optimum conditions	30% *B*, 283 K, 80 mg ml^{-1} protein	10% *A*, 293 K	30% *A*, 293 K, 40 mg ml^{-1} protein	10% *B*, 293K	20% *B*, 293 K

were employed, requiring 7 µl of sample and 100 µl for the reservoir. The droplet consisted of 3.5 µl of protein in buffer and 3.5 µl of surfactant solution and were mixed immediately (see Table 1). The plates were found to be less suitable as they were prone to rapid drying and were also very birefringent under cross-polarized light which made crystal detection with crossed polarizers difficult.

2.4. *Crystallization conditions*

Crystallization trials for HEWL were carried out at 277, 283 and 293 K at varying pH and protein concentration ranges, as detailed in Table 1. The membrane protein trials were carried out in the presence and absence of HT [final concentrations of 1, 2, 3, 4 and 5%(*w*/*v*) HT].

2.5. *Glass formation of surfactant solutions*

Each surfactant was screened for glass formation. Cryo-loops were dipped into the surfactant solution of interest and then frozen rapidly at 77 K by plunging into liquid nitrogen. The final surfactant concentrations screened were 5, 10, 15 and 20%(*v*/*v*).

2.6. *Specific activity of HEWL*

Specific activity of HEWL was measured by the method of Thomas *et al.* (1996). 10 µl aliquots of various HEWL samples were added to a 1 ml reaction mixture containing 3 mg *Micrococcus luteus* per 10 ml of 40 m*M* sodium phosphate buffer, pH 7.0. Activity was measured by a decrease in absorbance from 1.0 AU at 450 nm.

3. Results

Table 1 shows a summary of the crystallization trials of HEWL. It shows no strong pH dependence over the neutral range. In general, crystals grew more rapidly at lower pH as in previous reports of HEWL crystallization (McPherson, 1980; Durbin & Feher, 1986) (Table 1). Crystals were usually observed first for surfactant *B* followed by *A* and finally *C*. The efficiency of *B* for the crystallization of HEWL was also reflected when lower initial HEWL concentrations were tested, with a significantly greater number of successful trials for *B* at 20 mg ml^{-1} lysozyme. HEWL crystals grown in all three surfactants were mounted in buffer containing 60% surfactant, and X-ray diffraction was recorded on beamline 9.5 at SRS Daresbury

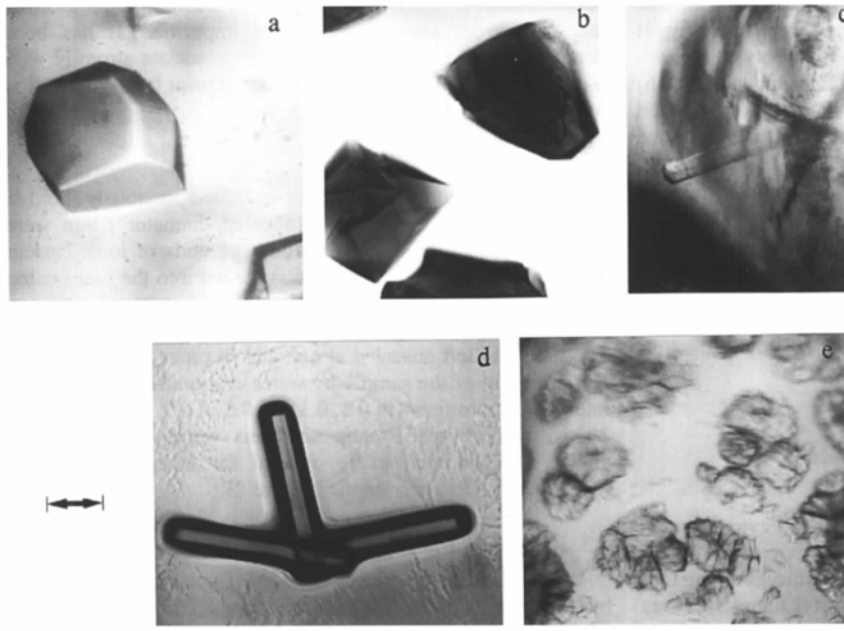


Fig. 2. Crystals of soluble proteins. (*a*) Lysozyme crystals grown from a 30% *B* (initial concentration) solution. (*b*) Ferritin crystals grown from a 10% *B* solution. (*c*) Ubiquitin crystal grown from a 10% *A* solution. (*d*) Catalase crystals grown from a 20% *B* solution. (*e*) Ribonuclease A crystals grown from 30% *A* solution. Scale bar represents 100 µm.

(UK). All the crystals diffracted to a higher Bragg resolution than 3 Å. Unit-cell parameters and space group were refined for all surfactants using *DENZO* (Otwinowski, 1986) and the space group and dimensions of, $a = 38.37$, $b = 79.71$, $c = 79.98$ Å and $\alpha = 90.03$, $\beta = 90.58$, $\gamma = 89.99°$ (surfactant *A*-grown crystal), $a = 37.91$, $b = 78.50$, $c = 78.56$ Å and $\alpha = 89.81$, $\beta = 89.63$, $\gamma = 89.80°$ (surfactant *B*-grown crystal) and $a = 37.92$, $b = 79.76$, $c = 79.74$ Å and $\alpha = 89.50$, $\beta = 89.68$, $\gamma = 89.69°$ (surfactant *C*-grown crystal) were consistent with the tetragonal space group $P4_32_12$ (Phillips, 1967).

Specific HEWL activity was measured for control and three month old surfactant-exposed crystal samples. No effect on activity was observed (activities of 250×10^3 units mg^{-1}) up to surfactant concentrations of 30%. Above this concentration, a decrease in the activity was recorded, suggesting some denaturation due to surfactant over these prolonged time scales.

Crystallization trials were extended to include a range of soluble proteins as well as a few membrane proteins that were available in the laboratory. None of the membrane protein trials produced useful crystals suitable for X-ray diffraction, and in some cases (bacteriorhodopsin and photosystem II photosynthetic complexes) some evidence for rapid denaturation was implied by colour changes. In contrast, crystals were obtained for several of the soluble proteins tested, and Table 2 summarizes the crystallization conditions and surfactants used and Fig. 2 shows examples of the crystals grown. The data suggests that this approach would be a suitable alternative in crystallization screens of soluble proteins.

Controls were carried out in order to check that the surfactants were giving rise to crystals by themselves rather than acting as co-precipitants in very concentrated protein solutions. Crystals only appeared in almost completely dehydrated control trials, and this only occurred with HEWL. In all other cases, no crystals appeared in control samples lacking surfactant. In addition, transfer of crystals from the protein-rich mother liquor to a protein-free solution during mounting of crystals for X-ray diffraction did not lead to dissolution of the crystals. However, crystal dissolution was immediate when the mounting buffer contained no surfactant or surfactant at a lower concentration than in the crystal mother liquor. These results strongly suggest that the surfactants had a direct role in the crystallization.

With the exception of ferritin, all protein solutions used in trials were salt free, eliminating the possibility that salt could be acting as a precipitant for these proteins. For ferritin trials, the salt concentration was low (0.05 *M* NaCl), and would not be expected to strongly influence the crystallization trials.

The surfactants showed the propensity to influence the growth of ice crystals in surfactant-containing solutions. Vitrified, transparent frozen solutions were noticed after plunging into liquid N_2. All surfactants gave a clear glass at concentrations between 5 and 20%(*v*/*v*) when used with a 0.2–0.3 mm diameter cryo-loop, but only surfactant *C* formed clear glasses when used with a 0.5–0.7 mm diameter cryo-loop.

4. Discussion

Surfactants are a necessary requirement for the crystallization of membrane proteins in order to solubilize them and prevent random aggregation of their extended hydrophobic waists (which would normally be membrane embedded) (Michel, 1991). However, surfactants have also been investigated as additives to crystallization trials of soluble proteins where it is thought they may prevent heterogeneous aggregation (Ducruix & Giegé, 1992; McPherson, Koszelak, Axelrod, Day, Robinson *et al.*, 1986). Here we show that it is possible to crystallize soluble proteins using surfactants as precipitants at much higher concentrations than used in previous studies where they were employed as additives (Garavito & Picot, 1990). Furthermore we show that the surfactants can promote glass formation, and hence may be suitable for direct transfer of crystals to temperatures of 100 K used for cryo-X-ray diffraction studies. Clearly, it is advantageous to be able to record diffraction data in the same medium in which the crystals are grown.

Although surfactants are useful for the crystallization of soluble proteins, they have not been successful for the membrane proteins in this study. This work suggests that membrane proteins are actually more sensitive to denaturation than are soluble proteins. This observation is worthy of further study, and it might be expected that an understanding of this surfactant sensitivity may help to optimize membrane protein crystallization trials in the future.

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