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REVIEW

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Detergent removal by non-polar polystyrene beads

Applications to membrane protein reconstitution and two-dimensional crystallization

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Abstract Detergent removal from lipid-protein-detergent micellar solutions is the most successful strategy for reconstitution of integral membrane proteins into proteoliposomes or into two-dimensional crystals. This review establishes the potential of polystyrene beads as a simple alternative to other conventional detergent removing strategies such as dialysis, gel chromatography and dilution. Kinetics and equilibrium aspects of removal of different detergents by hydrophobic adsorption onto polystyrene beads have been systematically investigated. A mechanism of adsorption onto polystyrene beads is proposed and provide useful information about the use of these beads in reconstitution experiments. The usefulness of this detergent removal strategy to produce quasi-ideal proteoliposomes is evaluated by considering the morphology and the size of the reconstituted vesicles, the homogeneity in size and protein distribution, the final protein orientation and the permeability of resulting proteoliposomes. Finally, the advantages of detergent removal by polystyrene beads as an alternative to conventional dialysis in two-dimensional crystallization trials are evaluated through review of recent structural reconstitution studies.

Key words Bio-Beads · Detergents · Liposomes · Proteoliposomes · Reconstitution · 2D crystallization · Membrane proteins

Abbreviations $C_{12}E_8$ n-dodecyl-octaethylene-glycol monoether · *DOM* 1-o-n-dodecyl-β-D-maltoside · *OG* 1-o-n-octyl-β-D-glucopyranoside · *HG* Hecameg, 6-o-(n-heptyl-carbamoyl)-methyl-α-D-glucopyranoside · *Chaps* 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulfonate · *Chapso* 3-(3-cholamidopropyl-dimethylammonio)-2-hydroxy-1-propanesulfonate · *TX 100* TritonX100 · *cmc* critical micelle concentration

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Introduction

Reconstitution of integral membrane proteins into phospholipid vesicles to form functioning proteoliposomes or to produce two-dimensional crystals amenable to structure analysis of membrane proteins provides a powerful tool in membrane research.

In the first place, membrane protein reconstitution into liposomes has been, and should remain, a potentially powerful tool that can be used to identify and characterize the mechanism of action of membrane proteins. In particular, it has played a central role in studies of membrane proteins with a vectorial transport function. More generally, through biophysical approaches including nuclear magnetic resonance, electron spin resonance, infra-red and fluorescence spectroscopies, it has led to important information about lipid-protein and protein-protein interactions as well as topological features of proteins in the membrane (Bloom and Evans 1991, Marsh 1993, Blume 1996, Konig and Sackmann 1996). The principles and practice of membrane protein reconstitution have been covered in detail and a number of reviews are available concerning different classes of membrane proteins such as receptors, energy-transducing membrane proteins, substrate carriers, and ion motive ATPases (Eytan 1981, Racker 1982, Casey 1984, Levitzki 1985, Villalobo 1990, Cornelius 1991, Rigaud et al. 1995).

In the second place, reconstitution of membrane proteins in two-dimensional crystals has opened the way to solve their structures (Kühlbrandt 1992, Jap et al. 1992, Heymann et al. 1997). Indeed, electron crystallography has developed for the past few years to that point that it represents an excellent alternative to X-ray diffraction studies of 3D crystals as a method for high resolution structural analysis of membrane proteins. Atomic models of bacteriorhodopsin (Henderson et al. 1990) and light-harvesting complex (Kühlbrandt et al. 1994) have been proposed based on 3 dimensional maps at 3.5 Å resolution. Besides these two examples, many other 2D crystals have led to medium or low resolution

maps and work towards determining the structure of different proteins at high resolution is making good progress (Toyoshima et al. 1993, Cyrklaff et al. 1995, Walz et al. 1997).

However, in many instances, the ability to investigate functional aspects or to solve structures of membrane proteins through the use of reconstitued systems, are limited by the fact that method for producing high quality proteoliposomes or 2D crystals have not advanced in step with the technical capability of biochemical, biophysical and molecular biology techniques on the one hand and electron microscopy and image analysis methods on the other hand. Thus it appears that one limiting factor in obtaining molecular information is related to the lack of reproducible methods of reconstitution. Therefore an enormous effort is required for new approaches to be evaluated, refined and applied to available proteins in order to make reconstitution even more important as a tool for further structure-function relationship studies at a molecular level.

In this context, the most frequently used strategy for proteoliposome and 2D crystal preparation consists of starting with the purified protein and a suitable combination of lipids, both solubilized in detergent. Next the detergent is removed from these lipid-detergent and lipid-protein-detergent micellar solutions, resulting in the progressive formation of lipid bilayers in which the proteins are incorporated. In addition to the large number of parameters that critically affect bilayer formation, such as the nature of the protein, of the lipid and of the detergent, the buffer composition and temperature, an important key factor is related to the detergent removal step. For example, the three phases of proteoliposome formation, namely bilayer closure, protein incorporation and post-vesiculation growth, as well as the three phases of crystal production, namely nucleation, growth and cessation of growth are dependent upon the rate of detergent removal and the level of residual detergent (Rigaud et al. 1995, Engel et al. 1992, Dolder et al. 1996). Furthermore, the residual detergent level is an important parameter since it determines the residual permeability of reconstituted vesicles and may also interfere in protein-protein and lipid-protein interactions.

This review deals with the different strategies commonly used to remove detergent from lipid-protein-detergent mixtures and establishes the potential of hydrophobic adsorption onto polystyrene beads as a simple powerful alternative to other conventional methods for removing detergent and for obtaining reconstituted membranes. It will focus on the mechanisms by which polystyrene beads adsorb detergents and general guides or rules to remove detergents will be proposed on the basis of the broad and systematic assessment performed in our laboratory on the reconstitution of different classes of membrane proteins (Rigaud et al. 1988, Richard et al. 1990, Levy et al. 1992, Pitard et al. 1996a, b, Hao et al. 1994, Cladera et al. 1996, Rigaud and Levy 1996, Rigaud et al. 1997). Finally the efficiency and relevant advantages of this strategy to produce liposomes, proteoliposomes and 2D crystals will be considered and discussed in relation to recent functional and structural studies.

Strategies for detergent removal

From the analysis of the abundant literature concerning the insertion of membrane proteins into liposomes four basic strategies can be outlined which are derived mainly from those used to prepare pure phospholipid vesicles (for reviews see Szoka and Papahadjopoulos 1980, Rigaud et al. 1995): 1) organic solvent-mediated reconstitutions including ethanol injection, ether infusion and reverse phase evaporation techniques; 2) mechanical means including sonication, french-press, or freeze-thaw techniques; 3) direct incorporation of proteins into pre-formed liposomes by using destabilizing agents acting as "impurities" (Jain and Zakim 1987); 4) detergent-mediated reconstitutions.

The most commonly used strategy for proteoliposome preparation is that involving the use of detergents, since most membrane proteins are extracted and purified through the use of detergents (Helenius and Simons 1975). The standard procedure involves co-micellization of membrane proteins and phospholipid in detergent with the subsequent removal of the detergent. As the detergent concentration decreases to a critical level, the proteins spontaneously associate with phospholipids to form proteoliposomes. Thus, in order to be suitable for reconstitution procedures, detergents, in addition to fulfilling the requirements of protein solubilization, must also be readily removable.

There are various methods for detergent removal based on the following physicochemical properties of detergents: critical micelle concentration (cmc), which is defined as the concentration at which the detergent monomers form micellar aggregates; micellar size, which is directly related to the number of detergent molecules in a micelle; the hydrophilic-liphophilic balance, which is an empirical value related to the amphiphilicity of a detergent (Helenius and Simons 1975).

Dialysis

Detergents with high cmc's are easily removed by dialysis. It is generally assumed in dialysis experiments that only the monomeric form of a detergent diffuses through the dialysis bag and that the rate of removal will depend upon the monomeric detergent concentration gradient across the bag. In this context, it has been shown that the rate of detergent removal by dialysis relies on the relative cmc of the detergent, with more rapid removal for a higher cmc. It is also the reason for the 100-fold greater retention of detergents such as Triton X100, with a very low cmc, as compared to cholate or deoxycholate (Allen et al. 1980). Addition of polystyrene beads outside the dialysis bag, to maintain the external concentration of dialyzed detergent as low as possible, can decrease the dialysis time and re-

duce the number of changes of buffer generally required during dialysis (Philipot et al. 1983). Using a flow-through dialysis cell can be advantageous as the rate of detergent removal can be carefully controlled and the dialysis time significantly decreased (Milsmann et al. 1978, Engel et al. 1992).

The dialysis procedure leads to relatively uniformly sized vesicles, but is mainly limited to the use of dialysable detergents with high cmc's and involves long time exposure of the protein to the detergent, which can be deleterious.

Gel chromatography

Detergents with high cmc's generally form small micelles and can be removed by gel filtration techniques. In this case, the initial lipid-detergent-protein micellar mixtures are eluted through a gel-exclusion column: as the mixture passes through the resin, proteoliposomes are formed which separate because they do not enter the pore gels and they elute from the column in the front peak, well before the detergent. Depending upon the size of the micelles, gel columns ranging from Sephadex G25 to Sephadex G200 have been used. Detergent removal by gel filtration has been shown to be more efficient than removal by dialysis: after a single gel filtration passage on Sephadex G200, the amounts of deoxycholate or cholate retained are similar to those retained after almost 2 weeks or 50 h dialysis, respectively (Allen et al. 1980).

The most significant advantage of this strategy is its rapidity, avoiding long periods of contact between detergent and protein, which however turns into a disadvantage in terms of inhomogeneous protein incorporation and heterogeneous size distribution of the reconstituted preparations (Racker 1982). Another drawback of gel exclusion chromatography may be related to a dilution of the samples which makes the method unsuitable for 2D crystallization trials.

Dilution

Another procedure for obtaining proteoliposomes from micellar solutions consists of diluting the reconstitution mixture. Dilution lowers the detergent concentration below its cmc and proteoliposomes form spontaneously. Detergents with high cmc's have been used and generally the dilution was followed by centrifugation of the diluted proteoliposomes. Although employed with some success for the reconstitution of different classes of membrane proteins, this strategy has failed in many cases (Racker et al. 1979). The rate of dilution has been shown to drastically affect the homogeneity of protein distribution and the morphology of the reconstitution product. In some cases, it has been shown that, with fast rates of dilution, this method does not form sealed vesicles at all, but membrane fragments (Champeil et al. 1982, Almog et al. 1986). Although this can be useful to analyze activity (e.g. ATPase activity by an ATPase) to be measured in the absence of any vectorial transport process, this turns into a disadvantage when analyzing transported species (Champeil et al. 1982). In this context, the rate of dilution can be controlled using a peristaltic pump or progressive addition of the dilution buffer (Jackson and Litman 1985, Dolder et al. 1996). Finally, a drawback of this strategy may be related to the residual detergent which should be, in many cases, be removed by other procedures.

Hydrophobic adsorption onto polystyrene beads

Detergents with low cms's, which consequently form large micelles, are not readily removed by dialysis, gel chromatography or dilution but can be efficiently removed through adsorption on hydrophobic resins. The method was introduced by Holloway (1973), using SM2 Bio-Beads to remove Triton X100 from protein solutions in order to avoid interference of the detergent during protein assay measurements. Later, other resins such as amberlites XAD 1, 2, 4, 7 or IRC50 were used to remove detergents with low cmc's (Cheetman 1978, Scheule and Gafney 1981, Horigome and Sugano 1982, Moriyama et al. 1983, Ueno et al. 1984, Lemaire et al. 1987). The batch procedure, where the amount of added beads can be easily controlled, is the most convenient way to remove a detergent by adsorption onto polystyrene beads. Some examples appear in the literature using packed columns: in this case, rate of elution, size of the column, and number of column passages have to be optimized (Ueno et al. 1984, Dierks and Kramer 1988). However the main disadvantage of the columns as compared to the batch procedure is related to the dilution of the sample.

Characteristics and mechanisms of hydrophobic adsorption onto Bio-Beads SM2

Bio-Beads SM2 (registered name for polystyrene beads from Bio-Rad; Richmond, California) are macroporous divinyl benzene cross-linked polystyrene beads with a high surface area for adsorbing organic materials from aqueous solutions. They are available with different polarities and different surface characteristics. Bio-Beads SM2 and SM4 are non-polar polystyrene beads with average pore diameters of about 90 and 40 Å, respectively, while Bio-Beads SM7 are of intermediate polarity with an average pore diameter of 90 Å. Bio-Beads SM2 are the most commonly used beads and adsorption of detergents onto these neutral porous copolymers is thought to be mediated by hydrophobic bonds. Polar forces appear not to be involved since changes in pH or ionic strength have no effect on adsorption (Cheetman 1978).

Although the simple method originally described by Holloway (1973) to remove Triton X100 appeared very simple and efficient, several authors have thought, or found, that the method had inherent drawbacks in liposome

and proteoliposome reconstitution. These include: i) incomplete detergent removal; ii) large loss of lipids and/or proteins which also adsorb onto the beads; iii) formation of multilamellar structures attributed to fast detergent removal.

Owing these limitations, often dogmatic in the absence of systematic physico-chemical studies, only few examples of reconstitutions by this strategy have appeared in the literature in the last decade (Chiesi et al. 1978, Dierks and Kramer 1988, Kramer and Heberger 1986, Wolosin 1980, Baldwin et al. 1980, Cornelius and Skou 1984). However, recent studies from our laboratory have demonstrated that all the difficulties reported above could be minimized or overcome (Levy et al. 1990 a, b, Pitard et al. 1996 a, b, Cladera et al. 1997, Rigaud et al. 1997, Lambert et al. 1998). On the basis of systematic studies, we have analyzed the mechanisms underlying detergent adsorption onto Bio-Beads SM2 and general rules for the use of polystyrene beads in proteoliposome and 2D crystal production can be proposed.

Detergent adsorption

Incomplete detergent removal by copolymer beads might be due to different factors: (1) saturation of the bead capacities; (2) too slow detergent adsorption; (3) adsorption of only micellar and not monomer species. In order to answer these questions, we have analyzed in detail both kinetic and equilibrium aspects of detergent binding to Bio-Beads SM2 (although we have used radioactive labelled detergents, detergent removal can also be quantitatively analyzed through the use of chemical assays or spectroscopic measurements).

Binding capacity

The first parameter to know in using Bio-Beads is related to the adsorptive capacity of beads for detergents, i.e. the determination of the maximal amount of detergent that can be adsorbed per gram of beads. This adsorptive capacity can be easily determined by analyzing the time course of detergent removal from a solution containing a fixed amount of detergent and various amounts of beads

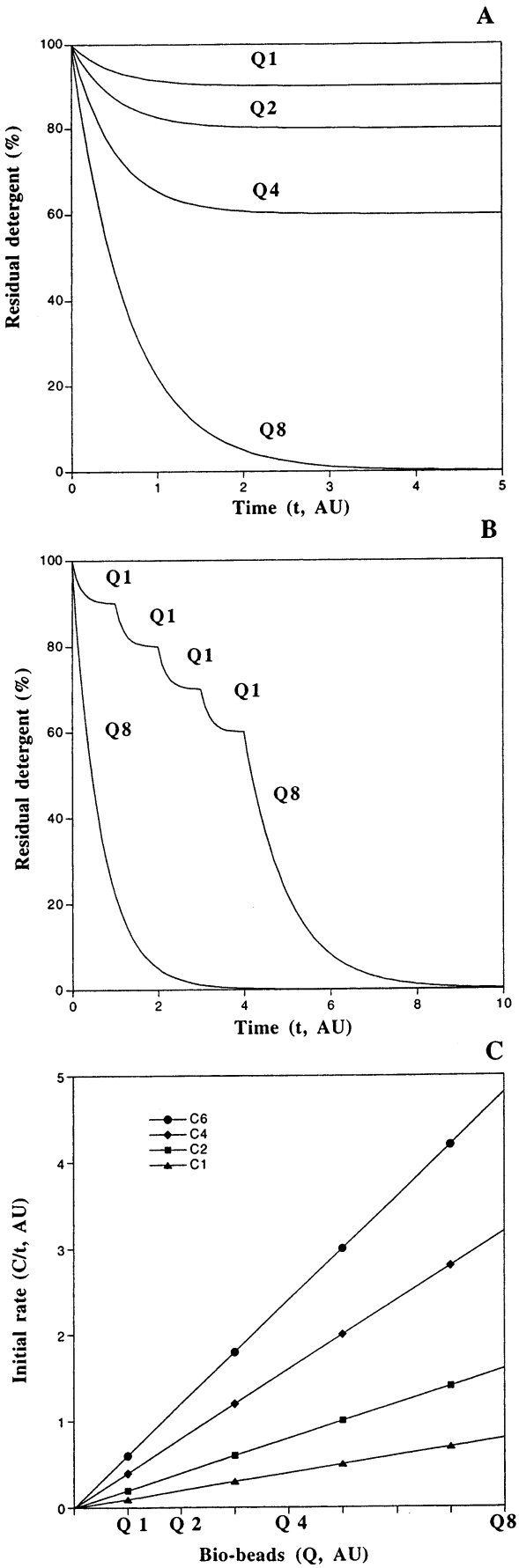

Fig. 1A–C Detergent adsorptive capacities of Bio-Beads. Panel **A** Schematic representation of the time course of detergent removal by different amounts (Q_8=2.Q_4=4.Q_2=8.Q_1 mg) of Bio-Beads. Binding capacities of Bio-Beads were determined from the plateau values observed at low bead concentrations. Panel **B** Use of Bio-Beads to control the rate of detergent removal. Detergent can be removed either by adding all the beads in one batch above their adsorptive capacity or by adding small successive amounts of beads. Panel **C** Initial rates of detergent removal as a function of bead concentration. **C** corresponds to different initial detergent concentrations. All panels are schematic simulated representations derived from previous published data on different classes of detergents (Levy et al. 1990 a, b, Rigaud et al. 1997, Lambert et al. 1998)

Table 1 Adsorption capacities of Bio-Beads SM2 for detergents, phospholipids and proteins

Compound	Adsorptive capacity (mg/g beads)
Detergent:	
Triton X100	185
$C_{12}E_8$	190
Dodecyl maltoside	105
Cholate	80
Chaps, chapso	85
Hecameg	110
Octyl glucoside	117
Phospholipid:	
Liposomes	1
Lipid-detergent micelles (Rsol)*	2
Lipid-detergent micelle (3×Rsol)	4
Lipid-detergent-protein micelles	0.5–1
Protein:	
BR, Ca^{++}ATPase, F0F1 ATPase, melibiose permease, cytochrome b6f	0–0.2

* Rsol represents the effective detergent to lipid ratio needed for total solubilization of preformed liposomes
Values of adsorption capacities were from references (Levy et al. 1990 a, b, Rigaud et al. 1997)

(Fig. 1A). For each detergent there is clearly an optimal detergent to bead ratio beyond which detergent removal cannot be improved. At low bead to detergent ratios, plateau values are reached which allowed us to determine the adsorptive capacities of the beads. Adsorptive capacities, from our studies on different detergents, are summarized in Table 1. The data reported demonstrate that Bio-Beads SM2 can absorb any type of detergent examined whatever their cmc's, the adsorptive capacity depending upon the chemical structures of the hydrophilic and hydrophobic moieties of the detergent molecules, with a much lower capacity for ionic detergents.

One important and general observation from these studies of adsorptive capacities is that, as expected for an adsorption behaviour, the rate of detergent removal has been shown to be proportional to the amount of beads (Fig. 1C). A limiting factor in the rate of detergent removal by Bio-Beads is the availability of the free bead surface: in other words, the accessibility of detergent to the pores of the beads is hampered by the pre-fixation of other detergent molecules and raising the bead concentration is expected to accelerate detergent removal. A striking advantage of this adsorptive property is to allow a simple control of the rate of detergent removal (Fig. 1B): rapid detergent removal can be obtained by adding beads at or above the adsorptive capacity while detergent removal can be made slower by adding small successive portions of beads instead of adding the amount needed for complete detergent removal in one batch. Control of this parameter can be essential for reconstitution experiments in which the effect of detergent removal rate needs to be varied and controlled (see section IV).

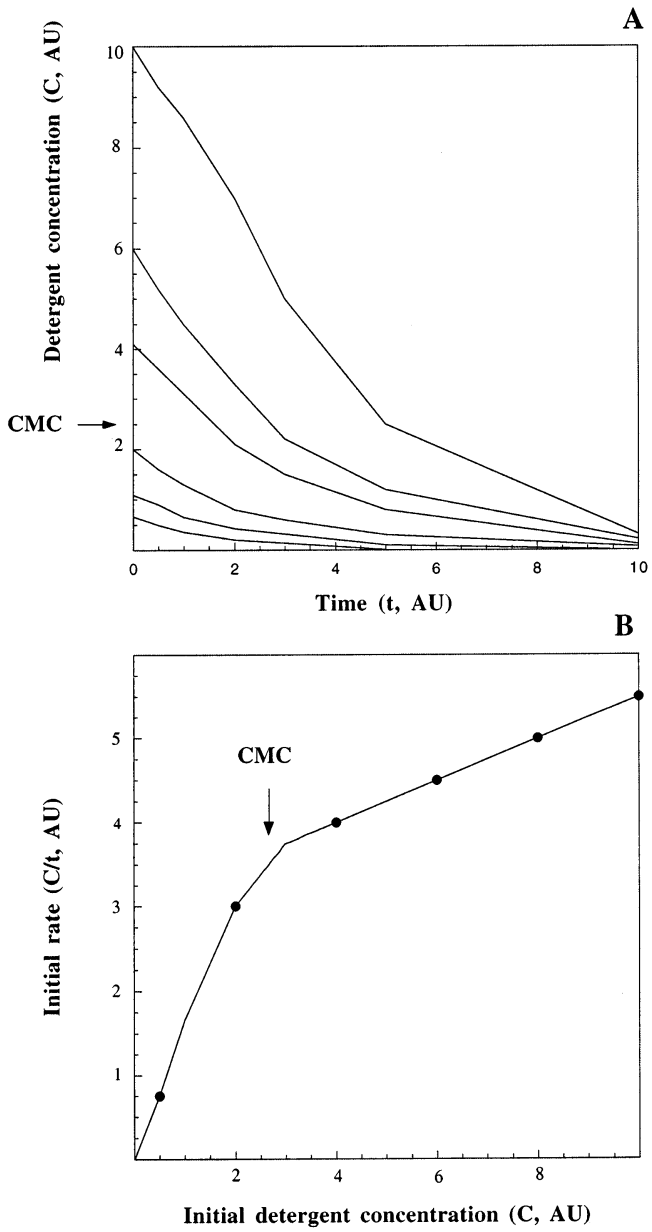

Fig. 2A, B Monomeric and micellar detergent adsorption onto Bio-Beads. Panel **A** Kinetics of detergent adsorption as a function of detergent concentration. The same amount of beads is added to solutions containing detergent concentrations above and below the cmc. Panel **B** Initial rates of detergent removal as a function of detergent concentration. The *arrow* indicates the detergent cmc deduced from such plots. All panels are schematic simulated representations derived from previous published data on different classes of detergents (Levy et al. 1990a, b, Rigaud et al. 1997, Lambert et al. 1998)

Monomer versus micelle adsorption

Another important point to be stressed from our data on the adsorptive capacity was that, provided that the amount of Bio-Beads exceeded their adsorptive capacities, virtually all detergent initially present could be removed. Whatever the detergent analyzed, less than 0.02 mM of residual detergent could be detected after adsorption on beads, i.e.

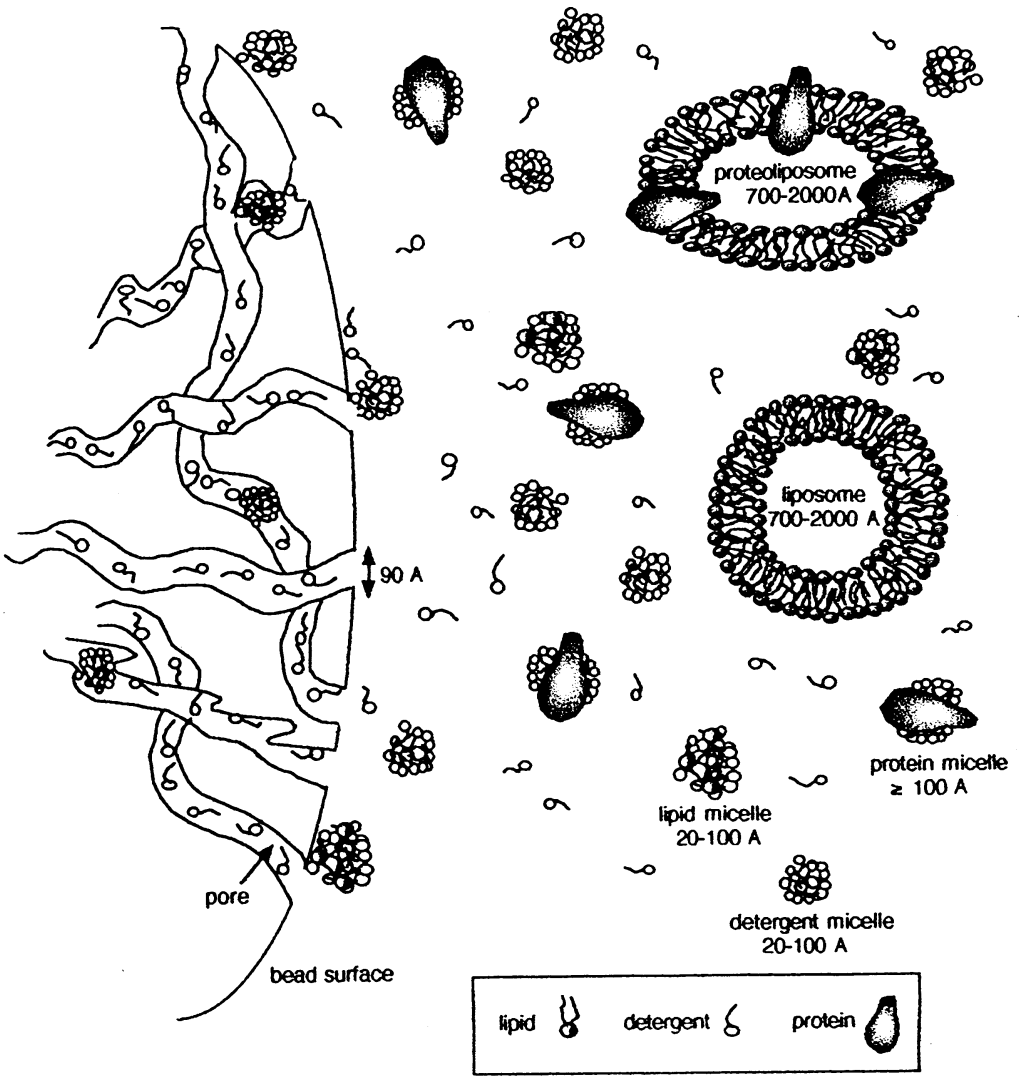

Fig. 3 Proposed mechanisms of adsorption onto Bio-Beads. Adsorption of detergents, lipids and proteins depend upon the accessibility within the pores of the beads of detergent monomers, binary or ternary micelles, liposomes or proteoliposomes

well below the critical micellar concentrations of the detergents. This result contradicted previous earlier incomplete studies suggesting that copolymer beads adsorbed only the micellar species and not the monomer, leading to anomalous retention of detergent outside the bead pores (Holloway 1973, Cheetman 1978).

To compare the rates of detergent removal in monomeric and micellar forms, experiments can be performed as a systematic function of detergent concentration at a constant bead concentration (Fig. 2A). From our different studies with various detergents, the relationship between the initial rate of detergent removal and the initial detergent concentration can be schematically represented according to Fig. 2B. It is clear that the rate is linearly related to detergent concentration with a typical breakpoint, characteristic of two kinetic regimes. The breakpoint in the curve has been related to the respective cmc's of the detergents, the first slope reflecting the rate of monomeric detergent adsorption and the second slower one reflecting the rate of micellar adsorption. In a process involving dissociation of micelles into monomers and adsorption of the monomeric form onto Bio-Beads, the rate of detergent removal should have been constant above the break point. Thus, the different slopes, generally observed, indicate a 3-fold faster absorption of monomers as compared to micelles: a simple interpretation would be to consider that the access to the pores of the beads is limited when the detergent is in a micellar form while monomeric detergent can easily penetrate the pores of the beads (see Fig. 3).

Effect of temperature on detergent adsorption

For a process with a positive entropy such as hydrophobic adsorption, an increase in the rate of detergent removal with temperature is expected. Since reconstitution experiments often need to be performed at low temperature to avoid possible protein denaturation, it was of interest to examine the binding efficiency of Bio-Beads SM2 as a function of temperature.

Although the total absorptive capacity was found to be independent of temperature, the rates of absorption

were found to be strongly dependent on temperature. Whatever the detergent analyzed, rates of adsorption doubled every 15 °C and Arrhenius plots of the initial rates of detergent removal indicated an activation energy of about 7 kcal · mol^{-1} (Levy et al. 1990, Rigaud et al. 1997).

From a technical point of view, these results also show the possibility of optimizing the rate of detergent removal during reconstitution experiments by simple control of the temperature of the incubation medium since, for example, the rates of detergent adsorption are about 3 times faster at 25°C as compared to 4 °C.

Phospholipid adsorption

The Bio-Bead method has for a long time been considered to be unsatisfactory for reconstitution trials because it was thought to result in large losses of lipids by hydrophobic adsorption onto the beads. Thus to obtain efficient reconstitutions conditions, it was crucial to analyze and minimize the losses of lipids during detergent removal.

Such analysis led to a number of interesting observations in line with a typical hydrophobic adsorption of lipids onto Bio-Beads (Levy et al. 1990 b, Rigaud et al. 1997). In particular, the rates of phospholipid adsorption were found to be dependent upon the initial lipid concentration and/or the initial amount of beads. However, the most striking feature was the low adsorptive capacity of beads towards lipids, which was found to be about 2 orders of magnitudes lower than for detergents (Table 1). Significantly, the adsorptive capacities for phospholipids were found to depend upon the initial detergent to phospholipid ratio: values less than 1 mg of lipid per gram of beads were obtained when starting from pre-formed liposomes, increasing to about 2 mg of lipid per gram of beads when starting from solubilized liposomes, and up to 4 mg when starting from lipid-detergent ratios well above that needed for solubilization. In the same framework, the adsorptive capacity of lipids from micellar solutions was found to decrease significantly in the presence of high protein concentrations, i.e. in 2D crystallization experimental conditions.

It is of value for reconstitution experiments to properly use the beads having such characteristic properties in terms of detergent and lipid adsorption. Since the adsorptive capacity of Bio-Beads towards phospholipid is 100–200 times lower than for detergents, lipid loss can be minimized by using the lowest Bio-Bead to detergent ratio needed for complete detergent removal, i.e. at the adsorptive capacity. The problem of phospholipid adsorption can be even more reduced on the basis of the differences in lipid adsorptions from micelles or from liposomes: this consists in exposing solubilized samples to repeat small portions of beads in order to go through the micelle to vesicle transition in the presence of few beads and, once the vesicles are formed, to increase the number of beads, the adsorption of lipids being lower in the form of liposomes.

However, since 2D crystallization trials are generally performed at low lipid to protein ratios, weak lipid adsorption may have profound effects. Thus, we have tried to overcome lipid adsorption by incubating the beads with an excess of sonicated liposomes prior to using them for detergent removal. Loss of lipid is almost entirely avoided on lipid pre-saturated beads with no significant change in detergent removal (Rigaud et al. 1997).

Finally, one another interesting observation was that the rates of detergent removal were always found to be slower in the presence of lipid, i.e. in reconstitution experimental conditions. This delayed uptake rate of detergent in the presence of phospholipids can be accounted for in many ways; a) prior binding of lipids to the pores gives rise to a decay in detergent removal; b) decrease in the detergent cmc's in the presence of lipids; c) only unbound detergent interacts with beads and removal of detergent associated with lipids is limited by the rate of exchange between the free detergent and detergent bound to lipids. Whatever, it should be stressed that all these parameters have only a slight influence on the rate of detergent adsorption which was generally found to decrease by less than 20% in the presence of lipids.

Protein adsorption

Adsorption of solubilized membrane proteins during detergent removal by Bio-Beads is also a parameter important to control. From our studies of proteoliposome reconstitution and 2D crystallization with proteins having very different hydrophilic/lipophilic balances, it appears that the adsorptive capacity of Bio-Beads SM2 for membrane proteins is very low and almost negligible, whatever the detergent used to solubilize the protein (Rigaud et al. 1995, 1997). Among the proteins analyzed one can cite: very hydrophobic proteins such as bacteriorhodopsin and melibiose permease with 7 and 12 transmembrane α helices respectively; proteins with a hydrophilic part equivalent to the hydrophobic one such as Ca^{++} ATPase and cytochrome $b_6 f$; proteins with a very large hydrophilic part such as F_0F_1 ATPases. Such low adsorptive capacities could again be explained by assuming a restricted accessibility of protein detergent micelles to the pores of the beads. In this context, previous studies on adsorption capacities of Bio-Beads SM2 for soluble proteins also indicated very low adsorptive values, the highest value of 10 mg protein/gram beads being reported for cytochrome c as compared to 0.2 mg for bovine serum albumin (Moriyama et al. 1984).

Mechanisms of adsorption onto Bio-Beads

A reasonable explanation for the different adsorptive capacities for detergents, lipids and proteins would be to consider the accessibility of detergent monomers, binary or ternary micelles, liposomes or 2D crystals to the volume and/or surfaces within the pores of the absorbent beads (see Fig. 3).

In this context, it should be stressed that the surface area available for adsorption onto beads is mainly represented

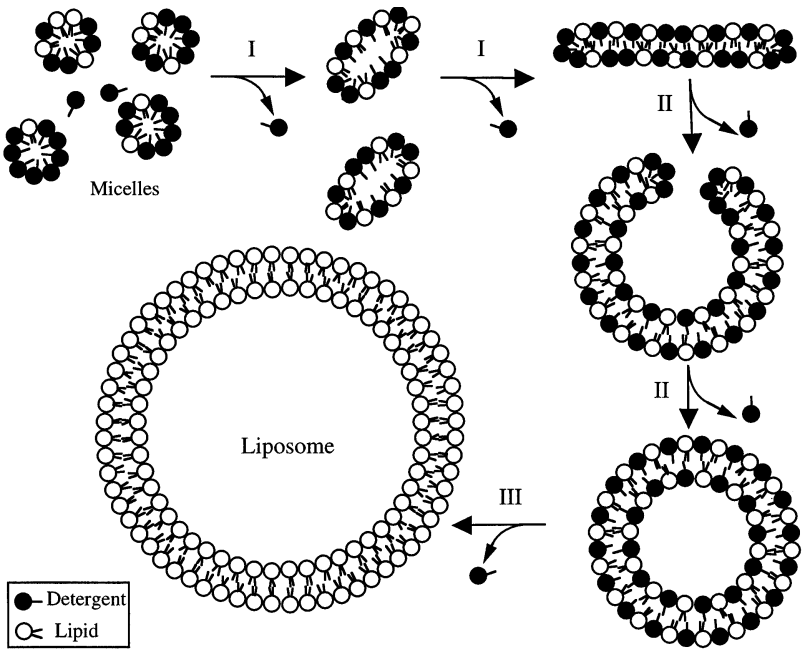

Fig. 4I–III Schematic representation of the different aggregational states and micellar-lamellar phase transformations in detergent-mediated liposome formation. Detergent removal from lipid-detergent micelles causes the transformation of small micelles to larger ones (**I**), which bend upon further detergent removal to form curved mixed micelles. Subsequently, detergent-saturated vesicles are formed (**II**) which undergo size growth through fusion and lipid-exchange mechanisms (**III**), leading ultimately to larger unilamellar liposomes

by the surface area within the pores and that the external surface of the beads is only a small fraction of the adsorptive surface. For Bio-Beads SM2, taking a mean bead size of about 750 µm and a total surface area of about 300 m^2/g of beads (Bio-Rad catalog), it can be calculated that the surface area within the pore is about 10^4 higher than the external surface of the beads. Thus it can be easily foreseen that the accessibility within the pore will be a crucial parameter in determining the adsorptive capacity. In the case of Bio-Beads SM2 (20–50 mesh), the average pore diameter of the beads is reported to be 90 Å and this has to be compared with the reported sizes of detergent micelles: about 20–40 Å diameter for ionic detergents and 90–100 Å for detergents with low cmc's such as TX100, $C_{12}E_8$ or DOM (Helenius and Simons 1975, Moller and Lemaire 1993). From such a comparison, it can be concluded that the access and/or the diffusion of micelles within the pores of the beads will be hampered and/or limited, explaining the large differences in the rates of monomer and micelle adsorption. This also explains the low phospholipid adsorption onto beads: due to their very low cmc of about 10^{-8} M, phospholipids exist either in the form of lipid-detergent micelles, up to 2 times larger than pure detergent micelles, or in the form of liposomes with diameters of 800–2000 Å. Few lipid-detergent micelles can be adsorbed within the pores and furthermore, upon detergent removal from these micelles, the lipids rapidly re-associate into liposomes which have no access to the pores of the beads and could only be adsorbed on the external surface. A similar interpretation can be advanced to explain the negligible protein adsorption since proteins exist either as large lipid-detergent-protein micelles, or as proteoliposomes or 2D crystals which cannot enter the low average diameter pore of the beads and can only be adsorbed to the surface of the beads.

Use of Bio-Beads in proteoliposome reconstitution

Any method for reconstitution of membrane proteins into closed vesicles should fulfill a number of important criteria. Besides the need for conditions that preserve the activity of the protein under study, the following criteria should be considered: the morphology and the size of the reconstituted proteoliposomes, the homogeneity in size and protein distribution, the final protein orientation and the permeability of the resulting proteoliposomes. Although some of these criteria depend mainly on the nature of the detergent, the protein and the lipid, as well as on the ionic conditions, the temperature and the precise conditions of initial detergent solubilization, we believe that the particular procedure used to remove the detergent is essential since it controls: 1) the sequences of events during the transformation of initial mixed micelles into closed bilayers containing the protein. This may play a role in determining the morphology and the size of the reconstituted vesicles as well as protein incorporation and orientation; 2) the residual detergent level which affects the residual permeability and the stability of the protein after reconstitution.

Size homogeneity and unilamellarity of reconstituted vesicles

In the light of a model proposed by Lasic (1988) for vesicle formation by detergent depletion techniques, three steps occur in the overall process of micellar to lamellar transformation: micellar equilibration including micellar growth by fusion or lipid exchange, vesiculation (bilayer closure) and post-vesiculation size growth, due to the residual detergent in the vesicles. The basic concepts, sche-

matically illustrated in Fig. 4, are as detergent is removed from micellar solutions, a series of micelle-micelle interactions is initiated to minimize the unfavourable energy resulting from consequent exposure of lipids and/or protein hydrophobic regions to the aqueous medium. This result in large mixed disk-like structures whose edges are coated with detergent. When they have grown past a critical radius, a subsequent bending of large micelles to form curved micelles occurs. At a critical micelle size, the amplitude of the bending is sufficient to cause bilayer closure and thus vesicle formation. These initially formed vesicles still undergo size transformation processes as long as the level of residual detergent remains high. Many results are consistent with the above simple model scheme of liposome formation, although different intermediates between micelles and closed bilayers were proposed involving the formation of large bilayered aggregates or long extended rod-like structures (Vinston et al. 1989, Walter et al. 1991, Silvius 1992, Lambert et al. 1998).

In line with this general model, slower detergent removal is expected to produce larger vesicles because micelle fusion (Lasic 1988), lipid exchange (Almog et al. 1986) and post vesiculation (Ueno et al. 1984) are not instantaneous processes. For example, Almog et al. (1986) reported that the size and homogeneity of lipid vesicles obtained by dilution of micellar cholate-lipid solutions depended upon the rate and extent of dilution: rapid dilution to very low cholate concentrations produces a stable population of small vesicles while less extensive dilution leads to the formation of vesicles that subsequently grow in size.

The size and unilamellarity of liposomes reconstituted by detergent removal with Bio-Beads have been analyzed in detail by electron microscopy either by negative staining, freeze fracture and/or more recently cryo-transmission electron microscopy. For most detergents analyzed, reconstituted liposomes or proteoliposomes consisted of unilamellar vesicle populations whatever the rate of detergent removal, i.e. whatever the initial bead to detergent ratio (only one exception to this rule has been recently reported by Lambert et al. (1998) who have shown, in dodecylmaltoside-mediated reconstitution, a large propensity for multilamellar liposome formation upon slow detergent removal). In these conditions, mean sizes of the reconstituted vesicles were dependent upon the nature of the detergent initially present: mean diameters of about 150–200 nm for Triton X100, 50–70 nm for cholate or deoxycholate, 100–150 nm for OG, 80 nm for $C_{12}E_8$, 120 nm for DOM and 120–150 nm for Chaps or Chapso-mediated reconstitutions. However, in line with the model proposed by Lasic, the final size distribution of the reconstituted vesicles is critically dependent upon the rate of detergent elimination: at high rates of detergent removal, a population of very small unilamellar vesicles (about 25 nm) formed whose proportion increased with speed of removal (Levy et al. 1990 a, b, Lambert et al. 1998).

From all these considerations, it appears that the rate of detergent removal is a key parameter in reconstitution studies since it interferes with the morphology and the size homogeneity of resulting vesicles. In this context, the use of Bio-Beads has been shown to be a convenient and reproducible way for preparing unilamellar and homogeneous preparations of liposomes or proteoliposomes from mixed micellar mixtures. The batch procedure with successive additions of beads allows control of the rate of detergent removal, which is essential, and prevents the formation of heterogeneous preparations in terms of size and/or multilamellarity (the general principle for liposome formation is to use a low bead to detergent ratio in the first step of reconstitution to promote the micellar to lamellar transition in not less than about 3 hours. After such a transition, bead concentration can be increased up to the adsorptive capacity to remove residual detergent from detergent-containing liposomes).

Protein distribution and orientation

The parameters governing the homogeneous distribution and orientation of proteins in proteoliposomes have been analyzed in detail in the course of our thorough systematic studies on the reconstitution of different membrane proteins using Bio-Beads as a way to control and vary the rate of detergent removal. All our data can be integrated into a general model including the nature of the detergent, the nature of the protein and also the effects of the rate of detergent removal.

With regard to proteoliposome formation, i.e. protein-lipid association during detergent removal from mixed micelles, two main mechanisms were proposed (Fig. 5): i) Mechanism A: vesicle formation and protein incorporation are separate events. Upon detergent removal, the structures formed depend upon the relative stability of mixed lipid-detergent and lipid-protein-detergent micelles. If ternary micelles are preferentially detergent-depleted (mechanism 2 a), lipid-protein-rich structures are first formed in the early stages of detergent removal. Then, upon further detergent removal, liposomes are formed by depletion of binary micelles and finally the protein can be incorporated in the detergent-doped liposomes. On the other hand, if binary lipid-detergent micelles are preferentially detergent-depleted (mechanism 1 a), liposomes are first formed by partial detergent removal and, only upon further detergent removal, is the protein transferred from ternary micelles to the detergent-doped liposomes; ii) Mechanism B: the protein simply participates in the membrane formation process, which corresponds to the micellar-lamellar phase transformation and the simultaneous coalescence of mixed micelles.

The rate of detergent removal has been demonstrated to be a key factor in determining the importance of these two mechanisms, i.e. in determining the sequence of events during detergent-mediated membrane protein reconstitution and consequently in determining the mode of protein incorporation (Levy et al. 1992, Jackson et al. 1988). Upon rapid detergent removal, protein incorporation occurs during vesicle formation: both types of micelles are simultaneously detergent-depleted, they coalesce and fuse, resulting in a mixing of the different components and a final

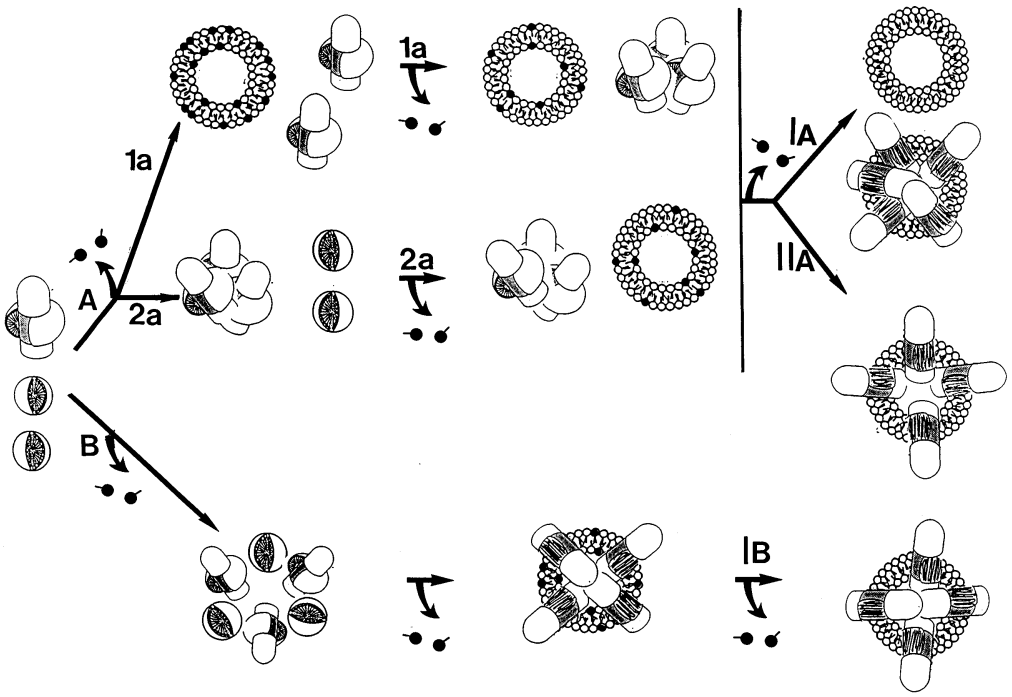

Fig. 5A, B Schematic representation of the different mechanisms by which proteins can associate with phospholipid during detergent-mediated reconstitutions. The scheme depicts possible sequences of events leading to proteoliposome formation upon detergent removal from a mixture of lipid-detergent and lipid-protein-detergent micelles. **A** of this scheme corresponds to preferential detergent removal from binary micelles (*1a*) or ternary micelles (*2a*). This leads in both cases to an intermediate state in which coexist detergent-saturated liposomes and lipid-protein-detergent aggregates. If the detergent is able to incorporate the protein into preformed liposomes, an homogeneous proteoliposome preparation, with asymmetric protein orientation, will be produced (*IIA*). If the detergent is unable to incorporate the protein, pure liposomes and protein-rich liposomes will be produced (*IA*). **B** of this scheme corresponds to homogeneous and simultaneous detergent removal from all micelles, leading to an homogeneous proteoliposome preparation with a symmetric protein orientation (*IB*)

homogeneous proteoliposome preparation (Mechanism B, IB). Upon slow detergent removal, vesicle formation and protein incorporation are two separate processes (Mechanism A). The homogeneous distribution of the protein among all the liposomes will then depend upon the ability of the detergent to incorporate the protein from lipid-detergent-protein complexes into preformed detergent-doped liposomes. If the detergent is unable to incorporate the protein, an heterogeneous proteoliposome preparation consisting of a mixture of pure liposomes and protein-rich proteoliposomes will be produced (Mechanism A, IA). If the detergent is able to incorporate the aggregated protein, an homogeneous proteoliposome preparation will be produced (Mechanism A, IIA). Protein incorporation into the pre-formed detergent-doped liposomes has been shown to depend upon the nature of the detergent and of the protein: indeed, such an incorporation depends upon the ability of the detergent to destabilize the bilayer for protein incorporation but also upon the state of protein aggregation in the complexes from which the protein has to be transferred.

In addition, besides the homogeneity of protein distribution an important consequence of the mechanisms is related to the final orientation of the protein: proteoliposomes formed by coalescence of the different types of micelles have been shown to have a more random protein orientation than proteoliposomes formed by direct incorporation of the protein into preformed liposomes which have been shown to have an asymmetrical protein orientation (Rigaud et al. 1995, Eytan 1981, Helenius et al. 1981).

Thus, in conclusion, whatever the protein to be reconstituted and the detergent to be used, it is of value to have a strategy for detergent removal in which the rate can be controlled and varied in order to optimize the reconstitution process in terms of orientation and homogeneous protein distribution.

Passive permeability of reconstituted liposomes

In reconstitution procedures involving detergents, the ability to totally remove the detergent is important. If recovery of biological properties such as transport is the aim, the presence of residual detergent is critical since detergents have been shown to drastically increase bilayer permeability at concentrations well below their cmc. From another point of view, if the purpose of reconstitution is to perform biophysical studies such as NMR, ESR or fluorescence spectroscopy, the presence of a few molecules of detergent may also be critical, leading to a significant disordering of hydrocarbon chains and polar head groups of phospholipids.

The most striking feature of our studies on liposomes or proteoliposomes obtained by the Bio-Beads strategy, is that the liposomes are impermeant to divalent ions (SO_4^{--}, PO_4^{--}, Ca^{++}) and slightly permeant to monovalent

ions (H^+, K^+, Na^+). The measured H^+ permeability of the liposomes was approximately 10^{-5} cm · s^{-1} (Levy et al. 1990a, b), larger than those of liposomes prepared using organic solvents but much smaller than those reported for other liposomal preparations (Deamer 1993).

Such low permeabilities are clearly an improvement in the detergent-mediated reconstitution procedures, especially when studying the functional characteristics of energy transducing membrane proteins. Among the important advantages of the proteoliposomes reconstituted during the course of our studies one can cite: 1) large light-induced ΔpH by bacteriorhodopsin-containing liposomes (2.5 pH units), among the highest values reported to date for such proteoliposomes (Rigaud et al. 1988); 2) high steady state Ca^{++} accumulation corresponding to lumenal concentrations of about 20 mM by Ca^{++} ATPase proteoliposomes without the need of precipitating agent such as oxalate or phosphate (Levy et al. 1992, Rigaud and Levy 1996); 3) demonstration that the Ca^{++} ATPases behave as Ca^{++}/H^+ countertransports (Levy et al. 1990c, Hao et al. 1994); 4) rates of ATP synthesis by artificially imposed μH^+ constant for 10–20 s as compared to 200 ms in previously reported reconstitutions of chloroplast F_0F_1 ATPase (Richard et al. 1990); 5) light-induced ATP synthesis by proteoliposomes co-reconstituted with bacteriorhodopsin or Photosystem I and F_0F_1 ATPases in the range of the highest values reported to date (Pitard et al. 1996, Cladera et al. 1996); 6) possibility to study the role of transmembrane electrical potentials in Ca^{++} ATPase functioning (Yu et al. 1993).

Biological activity

Another important parameter that can critically affect the result of a reconstitution experiment is the final activity of the protein, which can be drastically dependent upon the time during which the detergent has been in contact with the protein. In this context, the use of Bio-Beads for detergent removal is a powerful strategy since it deals with rates of detergent removal much faster than dialysis. For detergents with high cmc's which are readily removable by dialysis in about 24–48 hours, only 3–5 hours are maximally needed for a good reconstitution through the use of Bio-Beads. For example this has been shown to be important in OG-mediated reconstitution of Ca^{++} ATPase, a protein easily denaturated by this glycosylated detergent and which needs relatively fast detergent removal during reconstitution mediated by this detergent (Levy et al. 1990c, Levy et al. 1992). The time parameter is even more important for detergents with low cmc's for which dialysis is relatively inefficient since it requires many weeks for complete removal and can be deleterious for membrane proteins.

An important consequence of the use of Bio-Beads for total and relatively fast removal of detergents with very low cmc's has been to reevaluate their role in reconstitution studies. In particular, it should be stressed that Triton X100, which was considered for a long time to be a non-efficient detergent for reconstitution, has been shown to be very efficient for reconstitution of different energy-transducing membrane proteins (Rigaud et al. 1995). The same considerations can now apply to the use of $C_{12}E_8$ and dodecylmaltoside, other low cmc detergents difficult to remove by other conventional techniques.

Use of Bio-Beads in 2D crystallization of membrane proteins

Apart from a few membrane proteins that occur as regular arrays in native membranes, bacteriorhodopsin being the only example of very well ordered 2D arrays, and other 2D crystals produced within the membrane upon extraction of lipids or proteins, most of the 2D crystals have been produced from isolated purified proteins (for reviews see Kühlbrandt 1992, Jap et al. 1992, Heyman et al. 1997).

To date, the most successful strategy employed for 2D crystallization relies on the general method of reconstitution of membrane proteins into lipid vesicles but at low lipid-protein ratios. This method involves removing the detergent from a micellar solution containing the purified protein and a suitable combination of lipids.

The most common method for detergent removal in 2D crystallization is dialysis. Microdialysis has been used most frequently with a simple device consisting of a glass capillary tube bent by 90° near one end and sealed with a dialysis membrane (Kühlbrandt 1992). More sophisticated devices for growing 2D crystals by dialysis have been developed by Engel et al. (1992), based on flow through dialysis. The dialysis method has been applied to many membrane proteins, but presents a disadvantage for detergents with low cmc's which are difficult to remove and at best require a long time for dialysis.

More recently, dilution has been demonstrated to be efficient in producing 2D crystals of membrane proteins (Dolder et al. 1996, Tsiotis et al. 1996), but again this method is mainly restricted to detergents with high cmc's and furthermore does not allow complete detergent removal. Because of problems of sample volume and sample dilution, detergent removal by gel chromatography has never been used in 2D crystallization trials.

Surprisingly, hydrophobic adsorption of detergents onto polystyrene beads as an alternative for detergent removal has been scarcely used (Wingfield et al. 1979, Li and Hollingshead 1982, Bottcher et al. 1992, Akiba et al. 1996). This may be related to the often dogmatic view of the drawbacks of this strategy, as reported above.

In order to design a new successful strategy that would serve as a general guide for 2D crystallization, we have recently examined in detail the removal by Bio-Beads SM2 of different detergents classically used in membrane protein solubilization and reconstitution. The following requirements have been systematically explored for further production of 2D crystals: a) adsorptive capacity and level of residual detergent; b) effect of temperature; c) phospholipid and protein adsorption. The usefulness of detergent removal by Bio-Beads in crystallization trials was demon-

strated through the production of 2D crystals of different prototypic membrane proteins, with radically different hydrophilic/lipophilic balances.

Different new crystals could be produced for Ca^{++} ATPase from sarcoplasmic reticulum in $C_{12}E_8$, melibiose permease from *Escherichia coli* in DOM and cytochrome b_6f from *Chlamydomonas reinhardtii* in Hecameg (Rigaud et al. 1997).

Significantly, in the case of cytochrome b_6f, very large, ordered flat crystals were produced with optical diffraction spots up to 10 Å resolution, demonstrating for the first time the potential of Bio-Beads in producing 2D crystals useful for high resolution analysis. A projection map has been calculated at 8 Å resolution in negative staining (Mosser et al. 1997). As for proteoliposome reconstitution, the possibility to vary and control the rate of detergent removal was demonstrated to be very useful in 2D crystallization trials. For example, 2D crystallization experiments of Ca^{2+} ATPase were performed at different rates of detergent removal, leading to two different crystal morphologies (Lacapere et al. 1997). When the detergent was removed rapidly in 30 minutes by adding an excess of beads, tubular crystals between 0.2 and 0.5 µm length and about 0.2 µm in diameter were produced. When the detergent was removed in 5 hours by adding the same amount of beads in several steps, thin flat multilamellar crystals composed of a stack of two-dimensional crystalline lamellae ranging between 0.5 and 2 µm in diameter were produced. These 2D crystals of Ca^{2+} ATPase produced by complete detergent removal and without the need for chemical agents to induce the crystallization represent yet other types of crystals as shown by image analysis and may be a key step to structure determination of this P-type ATPase (Lacapère et al. 1997, Stokes and Green 1990).

In the same framework, different types of 2D crystals of melibiose permease were produced depending upon the rates of detergent removal by varying the temperature. 2D crystallization experiments performed at 4 °C allowed complete detergent removal in about 4–5 hours: in these conditions, vesicles of approximately 50 nm diameter can be produced, growing gradually to large aggregated vesicles (0.5–1 µm diameter) containing ordered regions, diffracting to about 20 Å. Interestingly, when crystallization was performed under similar conditions except that detergent removal was carried out at room temperature (equivalent to a three fold increase in the rate of detergent removal) many thin crystalline tubular structures (40 nm width, few µms length) were identified.

As another strategy for 2D crystallization, Bio-Beads have also been shown to be very efficient to analyze the process of 2D crystallization of Ca^{++} ATPase induced by vanadate (Young et al. 1997). In this case, Ca^{++} ATPase proteoliposomes were first formed by detergent removal, leading to densely protein-rich liposomes, and 2D crystallization was induced by vanadate. It was shown that the rate of detergent removal was important to produce protein-rich liposomes and unidirectional protein orientation. These two reconstitution criteria, obtained by slow detergent removal with Bio-Beads, were shown to be essential to produce further tubular crystallization of the Ca^{++} ATPase upon vanadate addition. The tubular crystals, similar to those obtained in native sarcoplasmic reticulum vesicles, were shown to be suitable for helical reconstruction and open the way to the crystallization of other P-type ATPases which will require reconstitution for further crystallization, given their low density in their native membranes.

Finally, through collaborative studies, the strategy of detergent removal by Bio-Beads was recently demonstrated to be efficient in producing 2D crystals of many other membrane proteins, solubilized in different detergents (OG, DOM, TX100, Nonyl-N-methylglucamide) (Lacapère et al. 1997, Saint et al. 1998).

Again, as for proteoliposome formation, an important advantage in the use of Bio-Beads for detergent removal is to allow rather rapid 2D crystallizations, avoiding long time of contact between proteins and detergents. For most proteins analyzed in our laboratory, 2D crystallization could be observed immediately after the short incubations with Bio-Beads as compared to weeks when using conventional dialysis.

Conclusions

The present review has been aimed at investigating the optimal conditions for detergent adsorption onto Bio-Beads and at evaluating the usefulness of this strategy for membrane protein reconstitution and 2D crystallization.

By analyzing the mechanisms of hydrophobic adsorption onto Bio-Beads, it has been possible to provide useful information about the use of these beads in reconstitution experiments involving detergent removal. The obvious consequence of these studies was that the disadvantages related to the use of this strategy for liposome or proteoliposome reconstitution as well as for 2D crystallization could be overcome or minimized provided that the adsorptive properties of the beads for detergents, lipids and proteins were carefully controlled.

Among the advantages of this strategy as an alternative to conventional methods for detergent removal such as dialysis, dilution or gel chromatography, one can stress:

1) a general use of this strategy for all kinds of detergents classically used in membrane protein solubilization, purification and reconstitution (ionic or non ionic detergents, low or high cmc detergents).
2) an easy control of the rate of detergent removal which is crucial for controlling protein insertion, protein orientation and size homogeneity of the reconstituted preparations.
3) an efficiency for total detergent removal which allows one to produce impermeant proteoliposomes and avoids artifactual interactions when studying lipid-protein or protein-protein interactions.
4) a relatively rapid strategy which avoids prolonged contact of proteins with detergents.

Table 2 Recent reconstitutions of membrane proteins using Bio-Beads SM2 as detergent-removing agent

Protein	Function	Reference
Bacteriorhodopsin	Light-induced H^+ pumping	Rigaud et al. 1988
Ca^{++}ATPase (SR)	ATP dependent Ca^{++}/H^+ co-transport	Levy et al. 1990c
Ca^{++}ATPase (plasmic)	ATP dependent Ca^{++}/H^+ co-transport	Hao et al. 1994
F0F1 ATPase (chloroplasts)	Artificial DMH^+-driven ATP synthesis	Richard et al. 1990
F0F1 ATPase (mitochondria)	ATP synthesis and hydrolysis	Groth and Walker 1996
Bacteriorhodopsin+F0F1 ATPase	Light-induced ATP synthesis	Pitard et al. 1996
Photosystem I	Light-induced H^+ transport	Cladera et al. 1996
Photosystem I+F0F1 ATPase	Light-induced ATP synthesis	Cladera et al. 1996
Band 3	Anion transport	Boulter et al. 1994
P-glycoprotein	ATP dependent drug transport	Dong et al. 1996
Heme-copper oxydase	H^+ pumping	Verkhovskaya et al. 1997
Ocytocin receptor	Hormone binding	Klein and Farenholtz 1994
Lactose permease	Lactose transport	Knol et al. 1996
Alkaline phosphatase	Phosphate hydrolysis	Angrand and Roux 1997
Mechano sensitive chanel	Ion chanel	Martinac et al. 1996
Stomatitis virus envelope	Fusion activity	Paternostre et al. 1997
Glutamate carrier	Aminoacid transport	Dierks and Kramer 1988
Fhua	Phage fixation and DNA transport	Plançon et al. 1997

An important consequence is that this procedure of detergent removal is demonstrated to be successful for the reconstitution of different classes of membrane proteins solubilized in various detergents. Reconstituted proteoliposomes with the highest biological activities reported up to now have been produced through the use of Bio-Beads. Table 2 reports the diverse successful reconstitutions reported in the literature over the past few years which include a very diverse range of membrane proteins reconstituted through this strategy.

Therefore, we propose in conclusion that the method analyzed in this review satisfies criteria that make the procedure a powerful alternative strategy to dialysis or dilution for reconstitution experiments. This approach will hopefully enhance the success rate of reconstitution experiments, proteoliposomes or 2D crystals, and facilitate future studies on other membrane proteins.

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