Surfactant-free purification of membrane proteins with intact native membrane environment

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Abstract

In order to study the structure and function of a protein, it is generally required that the protein in question is purified away from all others. For soluble proteins, this process is greatly aided by the lack of any restriction on the free and independent diffusion of individual protein particles in three dimensions. This is not the case for membrane proteins, as the membrane itself forms a continuum that joins the proteins within the membrane with one another. It is therefore essential that the membrane is disrupted in order to allow separation and hence purification of membrane proteins. In the present review, we examine recent advances in the methods employed to separate membrane proteins before purification. These approaches move away from solubilization methods based on the use of small surfactants, which have been shown to suffer from significant practical problems. Instead, the present review focuses on methods that stem from the field of nanotechnology and use a range of reagents that fragment the membrane into nanometre-scale particles containing the protein complete with the local membrane environment. In particular, we examine a method employing the amphipathic polymer poly(styrene-*co*-maleic acid), which is able to reversibly encapsulate the membrane protein in a 10 nm disc-like structure ideally suited to purification and further biochemical study.

Introduction

Anyone will tell you that the juiciest apples on an apple tree are always to be found at the very top, where only the most intrepid youth will venture, in full knowledge that he risks injury in order to attain the succulent flesh. Many would say that this analogy also describes the task that faces biochemists studying the form and function of a membrane protein. Not for them the low-hanging fruit of soluble proteins, but instead they risk long periods of frustration and slow progress in a quest to understand the inner workings of these intriguing biomolecules. Now, in a world where the supply of succulent low-hanging fruit has dwindled, more scientists are becoming interested in the challenges provided by the study of membrane proteins. But are membrane proteins worth the effort? The answer is an emphatic 'yes'! Membrane proteins are predicted to make up approximately 30% of gene transcripts in the natural world and control many fundamental processes within the life of the cell. The position of membrane proteins at the very periphery of the cell, at the interface of what is, and what is not cell, places them in an environment of pivotal importance to a wide range of cellular functions. Restricted to movement in the two-dimensional plane of the membrane, some membrane proteins function as guardians, actively controlling the movement of molecules into and out of the cell. Other membrane proteins act as signal transducers, detecting changes in the external milieu and reacting by initiating signalling cascades within the cell that trigger appropriate cellular responses [e.g. GPCRs (G-protein-coupled receptors) and ion channels]. However, it should not be thought these are the only functions of membrane proteins, as the membrane, within which these proteins themselves reside, is itself a dynamic and malleable entity, which needs to move and change shape to facilitate a number of cellular functions. Membrane proteins participate in the process acting both as simple membrane anchors for the force generating machinery (e.g. integrins) within the cell and as local modifiers of membrane curvature (e.g. dynamin and BAR-domain-containing proteins).

As can be appreciated, these are just some of the many functions of membrane proteins and their importance has not gone unnoticed within the pharmaceutics sector. The position of membrane proteins on the outer surface of the cell means that many are the target of drugs which act by modulation of cellular response via the membrane protein without the complexities of developing drugs that can transit the membrane barrier itself. For those drugs that do require entry into the cytoplasm, membrane proteins are also of great interest, as some membrane channels can aid the passage of drugs, whereas others actively pump drugs from the cell, greatly effecting efficacies. With all of these attributes, it is not at all surprising that membrane proteins have, for a long time,

Key words: amphipol, bicelle, membrane protein, nanoparticle, solubilization, surfactant.

Abbreviations used: AUC, analytical ultracentrifugation; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; GPCR, G-protein-coupled receptor; MSP, membrane scaffold protein; SMA, poly(styrene-*co*-maleic acid); SMALP, SMA lipid particle.

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intrigued bioscientists. However, should scientists outside the field examine the number of publications produced that include studies of the mechanism of membrane proteins, they would be surprised to find that progress in this research area lags far behind that made with soluble proteins. As an example, if one were to focus on arguably the apex of such work, high-resolution X-ray structures of proteins, one would find that, although tens of thousands of protein structures have been solved, currently fewer than 500 of these are of membrane proteins. From this, scientists may conclude that one or more technical issues reduce the ease with which X-ray studies can be performed on such biomolecules; and they would be correct.

Membrane proteins differ from soluble proteins in one significant way, they have evolved to be solvated in a complex pseudo-two-dimensional solvent, made up of the membrane itself and the immediate aqueous environment. A soluble protein, in contrast, has evolved to exist in the three-dimensional aqueous continuum. This means that the surface of the membrane protein is, in fact, split into spatially separate regions with different physicochemical properties that support interactions with the aqueous phase, the charged lipid headgroups and the hydrophobic membrane interior; each environment contributing stability to the functional protein fold. In contrast, soluble proteins only need to have a surface that complements the aqueous solvent. In many ways, it is this complexity that both intrigues and frustrates scientists studying membrane protein form and function as, in order to examine the native function of the protein, the complex lipid environment should ideally be maintained at all times. This is not a problem if the protein is studied *in situ*, i.e. in the cell, but many methods used to study the structural changes that underlie the function of membrane proteins rely on a reductionist approach in which samples contain only the protein of interest without other contaminating proteins or heterogeneities. This immediately poses a problem, namely how to separate proteins joined by a common membrane, while maintaining that membrane environment at all times? This problem has been the subject of an enormous amount of work in the biochemical community over the last 60 years and only now do we seem to be coming close to a solution.

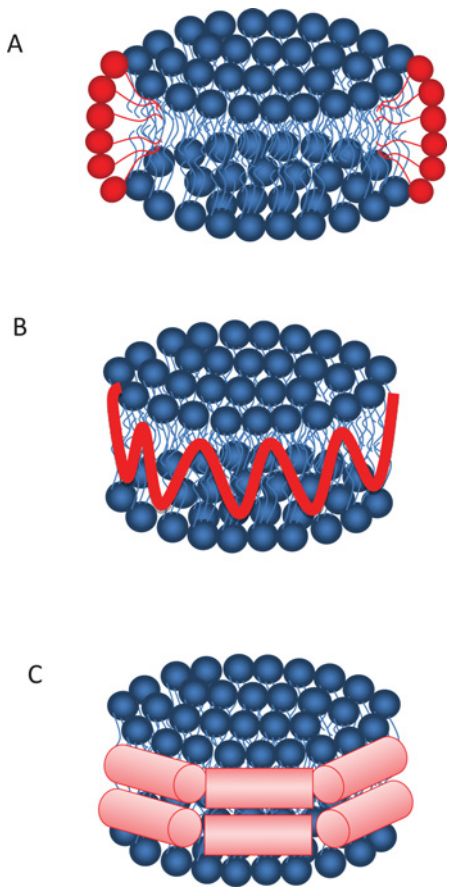
In the early days of membrane protein study, the importance of the membrane was perhaps not as well appreciated as it is now. Instead, the drive to produce pure membrane protein overrode many other issues. To achieve homogeneity, scientists employed the use of surface active agents (abbreviated to surfactants) that act to disrupt the membrane, breaking it down into smaller and smaller structures. The natural endpoint of this process being a level of disruption within which each membrane protein was separated from each other (see [1] for a review of membrane protein purification). In practice, these surfactants (erroneously referred to widely and less precisely as 'detergents', a detergent being defined as a formulation of surfactants and other components used in cleaning) not only broke down the lipids into small micelles, but also replaced the annular lipids that are closest to the membrane, forming a composite protein/surfactant micelle. This had the desired effect of producing a solution comprising self-contained discrete particles containing individual membrane proteins that could then be separated. However, in producing these particles, the complex membrane environment had been replaced by that of surfactant micelles. These micelles could be said to exhibit gross physicochemical properties similar to those of the membrane, as the hydrophilic surfaces of the membrane protein are exposed to the aqueous phase, while the hydrophobic surfaces are protected by the hydrophobic chains of the surfactant. This approximation appears to be valid for a number of proteins and still constitutes by far the most popular method for isolating and studying membrane proteins. However, the method suffers from a number of issues. First, the surfactant micelle is only able to provide an environment that is, at best, a rough approximate of that found in membranes. Studies using neutron-scattering methods show that the membrane is made up of a number of layers running perpendicular to the membrane normal [2,3]. Each of these has a specific property that, as mentioned above, is matched by the corresponding region of the membrane protein surface. Any simple surfactant will struggle to form a structure that exactly replicates this, hence leaving the membrane protein in a particle that only approximates to the native environment. Secondly, in addition to the complex layered nature that is common to all membranes, the constituents that make up the membranes themselves are highly variable. Even simple changes in phospholipid headgroup and acyl chain have profound effects on the physical properties of the membrane, which in turn alters the interaction with the embedded membrane protein [4–6]. Thirdly, it is also becoming clear that membrane proteins associate with regions within the cell membrane that contain certain lipids; and it is these lipids that help to maintain the function of the membrane protein [7–9]. An extreme example are the membrane proteins that inhabit lipid rafts. These are regions of the cell membrane that contain high levels of sphingolipids (particularly sphingomyelin) [10]. It can therefore be appreciated that, although membrane proteins are viewed by some as residing in a simple phospholipid bilayer, they are often segregated into explicit regions within the bilayer [11,12]. Again, it is clear that a simple surfactant micelle will not be able to completely replicate this complex environment. Finally, an issue that has to be addressed for surfactant solubilization is the presence of a lateral pressure within the membrane. It has been known for some time that there is a pressure within a bilayer with a directionality perpendicular to the membrane normal. This pressure has been shown to be important in maintaining the membrane protein fold [5,13]. Taken collectively, it is clear that it is an experimentally challenging task to replace the membrane environment with that of surfactant. This difficulty is amply illustrated by the number of detergent solubilizations that end with inactive proteins. Added to this, despite more than 50 years of research, the membrane protein solubilization literature is still littered with a large number of different surfactants and surfactant mixtures, none of which

has provided the 'universal' reagent for membrane protein purification solution. Perhaps it is time that another approach was used.

In the last 10 years, a new strategy to membrane protein purification has taken hold. This approach takes into account all of the new knowledge of the complexities of the phospholipid bilayer and the importance in maintaining activity of membrane proteins. The approach takes inspiration from the relatively new discipline of nanoscience and nano-self-assembly to produce a group of reagent systems that are joined by a common aim: to purify membrane proteins complete with the local membrane environment. This marks a radical departure from previous methods where the importance of the membrane was underestimated. Instead, the new method embraces the realization that the immediate membrane environment (also called the annular lipid environment) is part of the functional membrane protein itself. Each of these approaches aims to develop a reagent that, like a surfactant, breaks the membrane (complete with membrane proteins) up into smaller fragments which can then be purified. However, these new approaches aim to limit the level of disruption in order to maintain particles that are large enough to contain at least some membrane with the membrane protein. The size of these particles therefore has to be very carefully controlled: too big and you are left with particles containing many membrane proteins making purification impossible; too small and there will be no membrane. The consensus of the methods that have so far been described is that particle diameters between 10 and 20 nm are favourable. There is, however, considerable experimental divergence on how this particle should be produced, and in the remainder of the present review, we examine four distinct approaches currently being developed.

Figure 1 | Diagrammatic representation of three nano-encapsulation methods

(**A**) A lipid bicelle showing a lipid bilayer (blue) stabilized in discoidal form by cone-shaped lipids (red). For clarity, only the cone-shaped lipids stabilizing the sides of bicelle are shown. (**B**) Amphipols (red) stabilize the bilayer (blue) by wrapping around the circumference of the disc. (**C**) MSPs (red) also stabilize the bilayer by wrapping around the circumference of the disc.



Bicelles, amphipols and nanodiscs (Figure 1) and the SMA co-polymer (Figure 2)

Bicelles

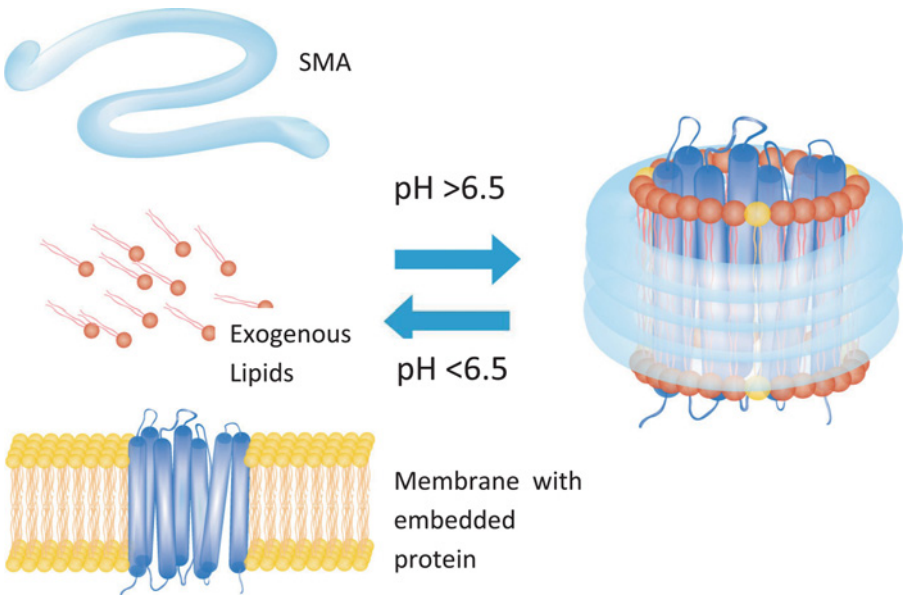
Bicelles are self-assembled disc-like structures that can be formed entirely from phospholipids and surfactants. Development of these particles is based on the observation that phospholipids with certain headgroup/acyl chain combinations favour the form of highly curved bilayers. When these lipids are mixed in the correct ratio with conventional lamella-forming lipids, a disc-like structure is formed which contains the lamella lipids surrounded by the non-bilayer forming lipids. There are a number of mixtures that exhibit this behaviour, perhaps most notably mixtures of DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) and DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), although more recently there has been significant success with systems that substitute the surfactant CHAPS {3-[(cholamidopropyl)dimethylammonio]propane-1-sulfonic acid} for DHPC. These systems have been used fairly extensively for biophysical studies of membrane proteins where the presence of some surfactants is tolerated. These have included NMR (e.g. [14]) and protein folding studies [15]. However, in practice, these systems are impossible to form directly from biological membranes containing the target protein. Instead, an intermediate surfactant solubilization step is required, meaning that this method is still hindered by the issues associated with surfactant use.

Amphipols

The utilization of amphipols is another method available to solubilize proteins. They are a milder substitute to conventional surfactants, allowing the membrane protein to remain surrounded by annular lipids, all within small hydrophilic complexes [16]. Amphipols are amphipathic polymers containing hydrophilic backbones with hydrophobic side chains. The polymer has the ability to hypercoil

Figure 2 | Diagrammatic representation of lipid and membrane protein encapsulation by SMA co-polymer

SMALPs are formed by the simple addition of the SMA co-polymer to the membrane containing the chosen membrane protein. At neutral/basic pH, a disc-like structure self-assembles, encapsulating the protein in a form amenable to purification methods. During this stage, it is also possible to add exogenous lipids and lipid-like molecules to the disc. At acidic pHs, the polymer disassociates from the particle to leave membrane containing the membrane protein.



around transmembrane regions of proteins, allowing them to stay folded. Amphipols (specifically A8-35) have been used to successfully solubilize a number of proteins, including GPCRs, while retaining function [17]. However, tests on the SERCA1a (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1a) protein with the polymer A8-35 show the amphipol can interfere with the protein's intrinsic ATPase activity [16]. Further problems also exist, with monodispersion of the polymer proving difficult to obtain. Acidic pH, bivalent cations and inadequate starting polymer concentrations can result in amphipol aggregations and hence inefficient membrane protein solubilization [17]. The use of amphipols in membrane protein purifications seems to be a useful approach, and further development to address the issues discovered for early studies is likely to lead to the development of a truly useful reagent.

Nanodiscs

Denisov et al. [18] have developed a 'nanodisc' technology that stabilizes proteins after they have been removed from the membrane with surfactants. Nanodiscs consist of an amphipathic helical lipoprotein acting as the MSP (membrane scaffold protein) that surrounds membrane bilayer fragments consisting of 130–160 lipids. Nanodiscs of approximately 150 kDa in size have the ability to associate with one single membrane protein, with the MSP acting as a hydrophobic platform for the protein to bind [19]. The nanodiscs are thought to have the same lipid make-up, as the bilayer, allowing easy integration to the native cell's membrane. They have been shown to maintain the folded protein during extraction from the surfactant. The requirement for a protein-based scaffold introduces a number of issues, including that, as proteins, they are inherently reactive, labile and expensive to make. The scaffold protein is essentially a contaminant to all downstream processes, adding a complicating component to remove later on. The protein will also interfere with scattering experiments and the nanodisc itself is hard to remove from the protein once bound [19]. Many variations of nanodisc technology exist (scaffold proteins of different sizes), with various protocols that change according to the initial surfactant used to remove the protein from the membrane. Despite some issues it is, however, likely that as these protocols develop the method will have widespread utility.

SMALPs [poly(styrene-*co*-maleic acid) lipid particles]

SMA [poly(styrene-*co*-maleic acid)] is a chemical entity that is well known in the plastics industry where it is used in a broad range of applications. However, our recent research has shown that the lower-molecular-mass variants of this polymer may have significant application in the purification of membrane proteins. The polymer itself is made of alternating hydrophilic (maleic acid) and hydrophobic (styrene) moieties, making it amphipathic. The degree of alternation varies within the polymer, with regions that are richer in either constituent of the polymer. At neutral pH, the low-molecular-mass polymers are soluble in water, but become insoluble at pH values less than 6.0 as the maleic acid group becomes protonated. The amphipathic properties of the polymer have been of interest to bioengineers for some

time, and applications have been found for the polymer in drug delivery [20] to enhance hydrophobic drug efficacy. However, our recent work [21] has shown that, in the presence of lamellar membranes, the SMA auto-assembles within the membrane itself to form disc-like structures that contain a lipid interior surrounded by an SMA annulus. This process produces a remarkably homogenous particle with a diameter between 9 and 11 nm. This behaviour closely matches that of the other nano-encapsulation methods detailed in the previous sections and offers the opportunity to use the material for membrane protein purification. Our initial trials have focused on using SMA to purify the naturally abundant proteins bacteriorhodopsin and PagP. In both experiments, the SMA was added directly to bilayers or micelles containing the proteins without the need for any prior treatment with surfactant. In each case, the SMA clarified the turbid membrane solution and was amenable to conventional protein purification methods. Initial examination of the final purified proteins showed native-like activity, demonstrating that the solubilization method preserved the structural integrity of the protein. A more in-depth study showed that the particles could be studied using a range of biophysical studies including CD, AUC (analytical ultracentrifugation), DSC (differential scanning calorimetry) and fluorescence. In each of these experiments the SMALPs containing the membrane proteins showed significant advantages over more conventional membrane protein preparations. For example CD studies of SMALP-solubilized material could be carried out at lower wavelengths than is possible with other systems that contain the membrane protein in native membrane (e.g. proteoliposomes). The resulting increase in signal to noise is attributed to the lack of light scattering from the SMALPs which have dimensions on a scale that is significantly lower than the wavelength range used in the experiment. Experiments using AUC showed that there was a correlation between the migration of the protein encapsulated within the particle and the mass of the protein. An approximation of the mass of the protein could therefore be determined by comparison of the mass of the protein in the SMALP with the mass of the SMALP alone. The promising nature of this initial work has led us to carry out a much more extensive study and we have currently succeeded in using the SMALP method to successfully purify a large variety of membrane proteins. We have also explored the use of the protein-containing SMALPs in a wider range of downstream applications. From these studies, we have learned that, once encapsulated, the particles are remarkably resilient, surviving both high temperatures ($>90^{\circ}C$) as well as desiccation by freeze drying to form powders. In addition, the pH-sensitivity of the encapsulation reaction has allowed us to disassociate the SMALPs in order to regenerate protein-containing lamella membranes, which can then be used for a number of other studies.

Given the recent progress in surfactant-free methodologies, the future of membrane protein study looks brighter than it has ever been. These new methods mean that preparations of membrane proteins will become easier to obtain, enabling more complex and imaginative studies of an ever-growing number of proteins. Furthermore, it might stimulate renewed interest from the biopharma/bioprocessing sectors in difficult protein targets (membrane-associated and other hydrophobic proteins) previously considered too difficult to manufacture efficiently. We believe that our SMALP method represents the latest step in these developments, providing, as it does, a method for directly solubilizing proteins from their native environment into a form that is highly amenable to many downstream studies.

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References

1 Lin, S. H. and Guidotti, G. (2009) Purification of membrane proteins. Methods Enzymol. **463**, 619–629
2 Wiener, M. C. and White, S. H. (1992) Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. Biophys. J. **61**, 434–447
3 Wiener, M. C. and White, S. H. (1992) Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. II. Distribution and packing of terminal methyl groups. Biophys. J. **61**, 428–433
4 Damianoglou, A., Rodger, A., Pridmore, C., Dafforn, T. R., Mosely, J. A., Sanderson, J. M. and Hicks, M. R. (2010) The synergistic action of melittin and phospholipase A_2 with lipid membranes: development of linear dichroism for membrane-insertion kinetics. Protein Pept. Lett. **17**, 1351–1362
5 Charalambous, K., Miller, D., Curnow, P. and Booth, P. J. (2008) Lipid bilayer composition influences small multidrug transporters. BMC Biochem. **9**, 31
6 Debnath, D. K., Basaiawmoit, R. V., Nielsen, K. L. and Otzen, D. E. (2011) The role of membrane properties in Mistic folding and dimerisation. Protein Eng. Des. Sel. **24**, 89–97
7 Lee, A. G. (2005) How lipids and proteins interact in a membrane: a molecular approach. Mol. Biosyst. **1**, 203–212
8 Bogdanov, M., Xie, J. and Dowhan, W. (2009) Lipid–protein interactions drive membrane protein topogenesis in accordance with the positive inside rule. J. Biol. Chem. **284**, 9637–9641
9 Paila, Y. D., Tiwari, S. and Chattopadhyay, A. (2009) Are specific nonannular cholesterol binding sites present in G-protein coupled receptors? Biochim. Biophys. Acta **1788**, 295–302
10 Pike, L. J. (2009) The challenge of lipid rafts. J. Lipid Res. **50** Suppl., S323–S328
11 Fullekrug, J. and Simons, K. (2004) Lipid rafts and apical membrane traffic. Ann. N. Y. Acad. Sci. **1014**, 164–169
12 Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. **1**, 31–39
13 Miller, D., Charalambous, K., Rotem, D., Schuldiner, S., Curnow, P. and Booth, P. J. (2009) *In vitro* unfolding and refolding of the small multidrug transporter EmrE. J. Mol. Biol. **393**, 815–832
14 Whiles, J. A., Deems, R., Vold, R. R. and Dennis, A. D. (2002) Bicelles in structure–function studies of membrane-associated proteins. Bioorg. Chem. **30**, 431–442
15 McKibbin, C., Farmer, N. A., Jeans, C., Reeves, P. J., Khorana, H. G., Wallace, B. A., Edwards, P. C., Villa, C. and Booth, P. J. (2007) Opsin stability and folding: modulation by phospholipid bicelles. J. Mol. Biol. **374**, 1319–1332
16 Picard, M., Dahmane, T., Garrigos, M., Gauron, C., Giusti, F., le Maire, M., Popot, J. L. and Champeil, P. (2006) Protective and inhibitory effects of various types of amphipols on the Ca^{2+}-ATPase from sarcoplasmic reticulum: a comparative study. Biochemistry **45**, 1861–1869

17 Popot, J. L. (2010) Amphipols, nanodiscs, and fluorinated surfactants: three nonconventional approaches to studying membrane proteins in aqueous solutions. Annu. Rev. Biochem. **79**, 737–775
18 Denisov, I. G., Grinkova, Y. V., Lazarides, A. A. and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. J. Am. Chem. Soc. **126**, 3477–3487
19 Leitz, A. J., Bayburt, T. H., Barnakov, A. N., Springer, B. A. and Sligar, S. G. (2006) Functional reconstitution of β_2-adrenergic receptors utilizing self-assembling Nanodisc technology. BioTechniques **40**, 601–606
20 Maeda, H., Bharate, G. Y. and Daruwalla, J. (2009) Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur. J. Pharm. Biopharm. **71**, 409–419
21 Knowles, T. J., Finka, R., Smith, C., Lin, Y. P., Dafforn, T. and Overduin, M. (2009) Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. J. Am. Chem. Soc. **131**, 7484–7485

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