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BBA Biomembranes Review

Encapsulated Membrane Proteins: A Simplified System For Molecular Simulation

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Abstract

Over the past 50 years there has been considerable progress in our understanding of biomolecular interactions at an atomic level. This in turn has allowed molecular simulation methods employing full atomistic modeling at ever larger scales to develop. However, some challenging areas still remain where there is either a lack of atomic resolution structures or where the simulation system is inherently complex.

An area where both challenges are present is that of membranes containing membrane proteins. In this review we analyse a new practical approach to membrane protein study that offers a potential new route to high resolution structures and the possibility to simplify simulations.

These new approaches collectively recognise that preservation of the interaction between the membrane protein and the lipid bilayer is often essential to maintain structure and function. The new methods preserve these interactions by producing nano-scale disc shaped particles that include bilayer and the chosen protein. Currently two approaches lead in this area: the MSP system that relies on peptides to stabilise the discs, and SMALPs where an amphipathic styrene maleic acid copolymer is used. Both methods greatly enable protein production and hence have the potential to accelerate atomic resolution structure determination as well as providing a simplified format for simulations of membrane protein dynamics.

1. Introduction - The membrane protein dichotomy.

Atomic resolution structures of proteins have revolutionised our understanding of many of the processes that underpin biology. The same structures have also enabled the growth of molecular simulation studies that have done much to uncover the complex structural dynamics that underpin protein function. However, progress across all protein classes has not been equal, with atomic resolution studies of membrane proteins, in particular, lagging significantly behind soluble proteins. For example extraction and purification of the first membrane protein (glycophorin A) did not occur

until more than 30 years after a similar feat was achieved for soluble proteins. The first high resolution structure of a membrane protein, that of the photosynthetic reaction centre, was not solved until 1984[1], nearly 20 years after this landmark was reached with soluble proteins[2]. This slow progress in the study of membrane proteins continues to the present day with only 1700 high resolution structures of membrane proteins compared to more than 110,000 of soluble proteins being found in the PDB in 2015[3]. This is despite membrane proteins having a clear and significant importance in biology The obvious question posed by this comparison is *Why has success in studying membrane proteins been so limited cf. that of soluble proteins?*

1.1 The challenge of membrane protein extraction and purification.

The real challenge in the production and study of membrane proteins occurs at the first step of the purification process: the need to separate the target protein from all other proteins present in the membrane. Over-expression of the chosen protein helps by increasing its proportion in the membrane, but to continue the purification process the membrane must be solubilised. Solubilisation of biological membranes is trivial using a detergent (or strictly speaking surface active agent, "surfactant") will lead to a rapid fragmentation of the membrane. The resulting solution contains a heterogeneous mix of 'detergent/lipid', 'detergent/protein' and 'detergent only' micelles. However, the general issue that has challenged membrane protein scientists for more than 40 years is that the target protein contained in the 'membrane protein/detergent' micelles often possesses low activity and/or stability. This makes the study of these proteins after purification very challenging.

1.2 Complexity of the bilayer: Why are detergents an imperfect solution?

At first sight the 'detergent/protein' micelle seems like the perfect solution. The protein is contained in a small particle made up of a reagent that has both hydrophobic and hydrophilic character. The hydrophobic moieties replace the acyl chains of the lipid in membrane stabilising the surfaces of the protein that naturally interact with the hydrophobic part of the membrane. The hydrophilic moieties ensure that the resulting mixed micelle is soluble in water. The real situation however, is somewhat different.

The first issue is that the membrane itself is a very complex structure made up of a number of physico-chemically distinct layers. It is in this complex layered environment that membrane proteins have evolved to function. This means that the outer surface of the membrane protein that is in contact with the membrane is not necessarily simply hydrophobic, but instead may contain polar amino acids which engage in complex interactions with the membrane in order to stabilise the conformation of the protein; perhaps most well-known of these being the positively charged arginine "snorkel"[4]. It is therefore clear that to maintain the activity of the protein any detergent system has to replicate this complex membrane structure as closely as possible. Such replication is seldom possible using the available detergents. This effect is further exacerbated when it is considered that most natural membranes are not made up of a single lipid type, but are mixtures of many different types of lipids. Such mixtures include phospholipids with different head-groups and acyl chains (varying in length and degree of unsaturation) as well as non-phospholipid constituents such as cholesterol. To replicate this is an almost impossible task for any single detergent or indeed combination of various detergents.

A second major issue with detergent solubilisation is that detergents themselves have the potential to disrupt the intramolecular interactions within a membrane protein that are essential for structural integrity. The choice of a detergent therefore always involves maintaining a delicate balance between successful solubilisation and retention of the target membrane protein's native structure. In practice this is very rarely achieved using detergents.

1.3 A new dawn in membrane protein solubilisation

In this review we discuss a range of new approaches to the production of membrane proteins that recognise the importance of the lipid bilayer in determining the correct structure and function of said species. These methods aim to preserve the lipid bilayer in the locality of the protein with the aim of preserving 'native-like' activity and stability. We describe how these techniques have developed from early proofs-of-concept that still require some use of detergent through to the most recent methods that dispense with detergent entirely. In the latter part of the review we describe how a wide range of structural and biochemical analytical techniques can be applied to the study of SMALP-encapsulated membrane proteins, and finally argue that the time is now ripe for these new particles to be the basis for molecular simulation studies.

2. The role of nanoparticles in membrane protein purification

In recent years, the basic format of an amphipathic molecule capable of solubilising a lipid bilayer and/or maintaining membrane proteins (MPs) in solution has been radically reinterpreted. The first development was amphipols (APs), amphipathic polymers invented by Popot[5] and coworkers that have been shown to bind tightly to MPs and preserve their solubility in water. Like detergents, APs largely replace the lipid bilayer leaving a protein/AP particle that is analogous to the protein/detergent micelle. More recently methods have been developed that attempt to preserve the membrane context around the protein. By achieving this scientists aim to more effectively preserve protein stability, structure and function.. At the forefront of this new movement are groups that have developed systems that extract disc shaped particles from membranes. These new particles share a common format: the MP sits in a disc-shaped piece of membrane (generally close to 10 nm in diameter) that is stabilised by a reagent that interacts with the edge of the disc. Two systems currently lead in this area and utilise different methods for stabilising the particles. First to show utility were groups that used membrane scaffold proteins (MSPs) as stabilising agents while later on the scene have been groups that use an organic polymer based on styrene maleic acid (SMA). In the next section both systems will be analysed.

2.1 Membrane scaffold protein (MSP) nanodiscs

The development of nanodiscs as a method for MP encapsulation was first reported in 2002 by Stephen Sligar and co-workers. Their nanodisc system contains three components,: the MP, a lipid bilayer and an amphipathic helical protein termed a membrane scaffold protein (MSP)[6].

MSPs are peptides derived from the human serum apolipoprotein A1. They consist of 22 residue peptides that form helical repeats punctuated by proline and glycine. When incorporated into nanodiscs it is thought that two molecules of MSP encircle a lipid bilayer (with embedded MP) forming a stabilizing belt around the nanodisc. Research has shown that the length of MSP used to produce the nanodiscs can be varied. When combined with different lipid contents the size of the resulting nanodisc can be controlled to give discs with diameters between 9.8 nm to 12.8 nm[7].

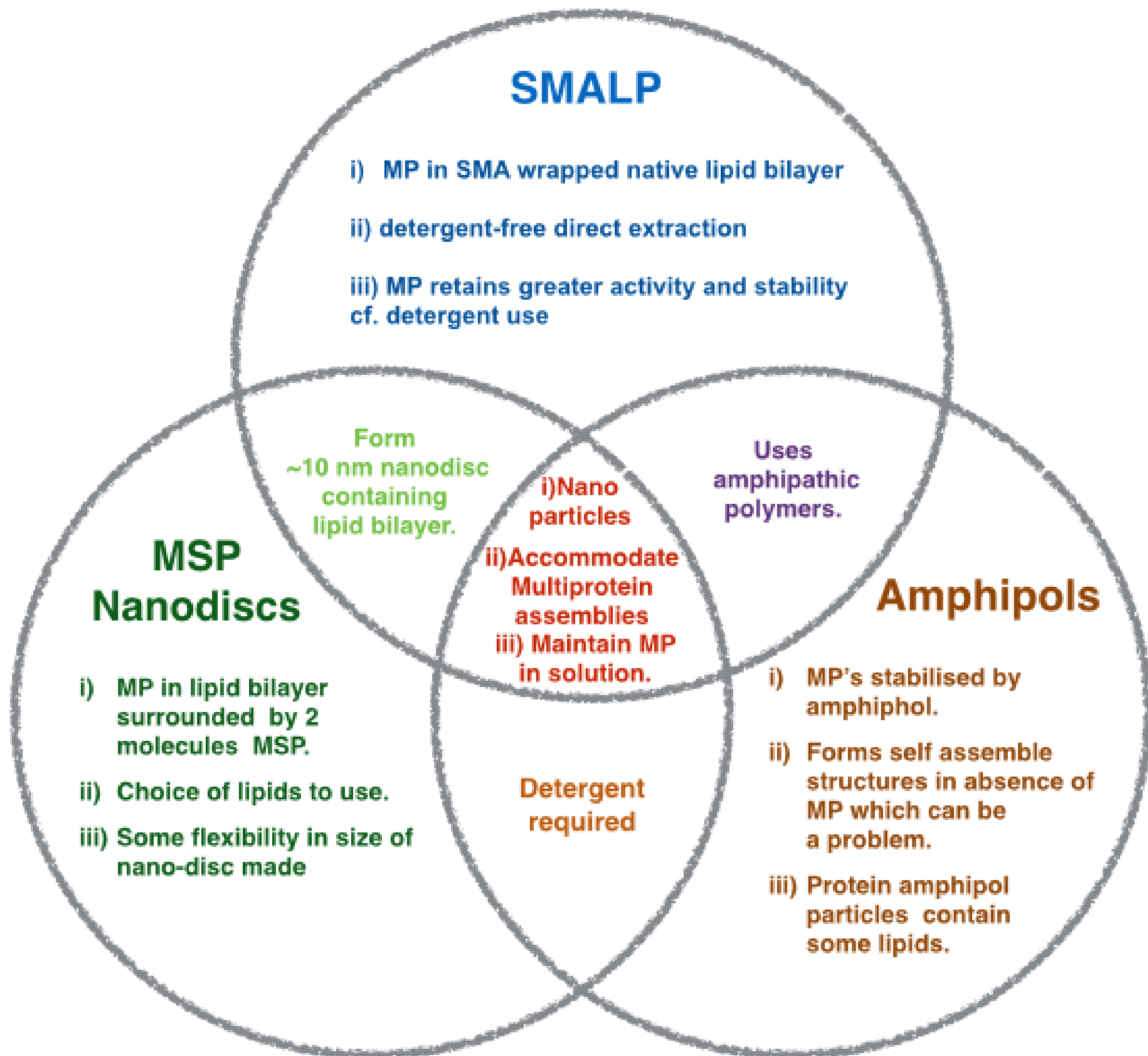
Since the first description of the MSP system[6], Sligar and colleagues subsequently demonstrated its successful application with a diverse range of MPs, including bacteriorhodopsin[8], GPCRs[9], cholera toxin[10], coagulation factors[11], cytochrome P450[12,13] and TAR receptor[14]. By showing this wide utility

Sligar and co-workers established the importance of nanodisc approaches in studies of MPs. However, close examination of the method used to make MSP nanodiscs highlights a significant limitation: the process relies on the solubilisation of the protein in a traditional detergent. Following purification, MPs are reconstituted along with lipid into the MSP discs. Therefore despite containing lipids, MSP nanodiscs are unlikely to represent the native lipid bilayer. Once lost it is very difficult, if not impossible, to recreate this unique environment.

***2.2** Styrene maleic acid lipid particles (SMALPs)*

MSP nanodiscs undoubtedly offer a solution, imperfect though it may be, to studying MPs in a lipid supported environment. More recently, a new method has emerged that employs 'styrene maleic acid copolymer (SMA)' to extract proteins from membranes[15]. Similar to the MSP system the resulting particles are disc-shaped, containing lipid and protein encircled by a belt of polymer, in this case SMA. Knowles and co-workers[15] first reported the use of SMA in the purification of PagP and Bacteriorhodopsin, and the phrase 'styrene maleic acid lipid particles (SMALPs)' was coined to describe the nano sized disc shaped particles formed in the process. Subsequently Orwick-Rydmark *et al*[16] used a related SMA polymer (containing a ratio of 3:1 Styrene to Maleic acid *cf.* 2:1 used in the Knowles study) to solubilise a MP in a particle they called a Lipodisq™. The major difference between the SMA- and MSP-based methods is that SMA polymers can be used to excise MPs directly from membranes, without at any stage in the protocol employing traditional detergents(**Fig.1)**. This means that the MP is effectively co-purified with its native lipid environment, providing a near-perfect solution to the production of MPs. The following sections discuss aspects of the SMA-based isolation system.

Figure 1. A comparison of SMALPs, MSP- nanodiscs, and amphipols for membrane protein purification.



3. Biophysical characteristics of SMALPs

A key question about lipid nanoparticles is *'do these small disc-shaped pieces of lipid truly replicate the properties of the larger lipid continuum?'* In order to answer this question, one must consider what physical characteristics define a lipid bilayer. For simplicity this can be considered in two ways. Firstly, the structural details of the bilayer,pertinent questions to address here are the following:

- Is the overall organisation of the bilayer in the disc the same as that in the membrane continuum?
- Are the hydrophilic head groups at the surface and the acyl chains buried in the core?
- Is the bilayer a planar structure? Or is it curved in some way?

The second aspect requiring consideration is bilayer dynamics. Here two main questions come to mind:

- Are the lipids free to diffuse?
- Are the various phase transitions exhibited by lipid bilayers preserved?

In the following section we discuss each of these in turn, using where available relevant examples from the literature.

3.1 The structure of SMALPs

Studies of SMALPs using small angle X-ray (SAXS) and neutron (SANS) scattering measurements show that the particles are indeed disc shaped. Measurements of the height of the disc (a dimension that should replicate that of an intact bilayer) provide a dimension that is similar to that of a bilayer formed of the equivalent lipid[17] (**Figure 2a**). Distinct headgroup and tail regions, characterised by different scattering length densities, can be discerned from these data, as well as a region filled with the hydrated polymer belt encircling the hydrophobic region at the edge of the disc. The SMALP discs appear to have a circular cross-section, in contrast to the elliptical cross-section reported for MSP-stabilized nanodiscs[17,18] [17,18]**Figure 2b**). This may be due to the larger degree of flexibility inherent in the polymer chains wrapping the lipid bilayer tails *cf.* that of the MSP, which has a defined secondary structure. SAXS and SANS experiments indicate that when prepared using the commercially available SMA polymer, the discs have a maximum diameter of 9.8 nm, while TEM images of dried nanodisc solutions stained with uranyl acetate show a bimodal population consisting of 11.1 ± 3.3 nm and 16.0 ± 3.0 nm objects[17]. This difference may be due to the orientation of the discs on the EM grid, capture of fluctuating objects via the drying procedure, and/or effects of the staining process *cf.* the time- and rotationally-averaged solution structures measured in scattering experiments[17].

Both SAXS/SANS and ATR-FTIR suggest that in the case of SMA-stabilized discs there is some penetration of the hydrophobic moieties of the polymer into the tail region of the lipid bilayer. In particular, data from polarised ATR-FTIR[17] shows that the styrene side chains of the SMA polymer lie

with their long axes parallel to the long axis of the lipid acyl chains. This implies some form of intercalation analogous to that observed when cholesterol interacts with the acyl chains of phospholipids[17]. Taken collectively these studies confirm that the dimensions of the bilayer encapsulated within the SMALP replicate those observed in intact cell membranes.

One unanswered question is whether the lipids simply fill the available space within the bounds of the encircling stabilizer, with dimensions set by the stabilizer (which appears to be the case for MSP stabilized discs)[19,20], or whether the stabilizer adjusts to encompass available lipids. The latter case, which appears to apply to SMA stabilized discs[21], is an equilibrium structure driven by line-tensions, molecular and bilayer curvature, and hydrophobic and hydrogen bonding This is a question that may be addressed by molecular dynamic simulations and is revisited in Section 5.

3.2 Phase transition behavior of lipid bilayers in discs

The phase behaviour of the lipids is the final aspect to consider when assessing whether a membrane encapsulated within a disc system (SMALP or MSP) mimics the lipid continuum. It has long been known that lipid bilayers undergo a range of phase transitions, the most physiologically important being the gel to liquid transition. The mid-point temperature of this transition has significant importance for MP function, in particular for those proteins where a conformational change is an essential part of function. For example, in the gel phase motions within and between individual lipids are much reduced. This has the effect of inhibiting any motions within a MP that might require a rearrangement amongst lipids close to the protein. In contrast, above the lipid transition temperature (in the liquid phase) there is an enhancement in the motions of the phospholipids, which has the potential to enable protein conformational changes to take place. It is therefore clear that should a membrane encapsulation system, such as those described here, change the transition temperature to any significant degree it could greatly alter the activity of the encapsulated MP. This is particularly pertinent for any membrane systems that exhibit a transition near room temperature (for example the transition temperature of DMPC is 24 ^{o}C), where even small change in transition temperature could significantly change the outcome of an experiment.

To assess the effect of encapsulation on lipid phase behaviour, differential scanning calorimetry (DSC) provides the gold standard (although Denisov *et al.* have complemented such measurements with

Laurdan generalised polarisation[20]). Using DSC significant studies have been made of the two major encapsulation methods (SMALP and MSP), as well as some analysis of the effect of subtle changes to each individual encapsulation reagent itself, e.g. looking at the effect of changes in styrene:maleic acid ratio used in SMALP formation. In the majority of studies one common observation is made. When unencapsulated lipid bilayers are studied using DSC the transition is very sharp, indicating that the phase transition has a very high degree of cooperativity. This is unsurprising given that the transition reflects a state change much like the melting of ice. However, similar transitions observed in encapsulated systems show a much broader change indicating a lower level of cooperativity. This is also not surprising given that the encapsulated system consists of isolated pieces of bilayer, which in general contain less than 150 lipid molecules. This is small *cf.* the number of cooperative units generally observed in lipid bilayers which extend into many thousands of lipid molecules. It is also possible that some element of this change may indicate that, of the 150 lipid molecules, those around the edge interact more strongly with the encapsulation system, which in the case of SMA involves interactions with styrene groups. These interactions may dampen the phase transitions of these lipids (effectively freezing them out) meaning that the effective number of lipid molecules that undergo the transition in the disc is significantly less than 150.

The second observation made from DSC data concerns the transition temperature itself. Compared to the observations made on the shape of the transition, it is here that different characteristics of the encapsulation system become apparent. A very comprehensive study of two MSP encapsulation methods by Denisov[20] showed that for DMPC and DPPC discs the phase transition temperature stays relatively close to that observed for the free bilayer. The discs made with DMPC displayed a phase transition close to 29 ºC *cf.* 24 ºC for the free bilayer, while the figures for DPPC discs are approximately 43.7 ºC *cf.* 41 ºC for the free bilayer. Interestingly, in both cases the transition temperatures in the disc are higher than that observed in the lipid continuum, suggesting that in some way the encapsulation inhibits melting of the bilayer. This could be either related to the interactions between the side chains of the encapsulation polymer and the lipids (as mentioned earlier), or alternatively result from some form of lateral compression of the membrane in the disc. Of the two explanations for the elevated transition temperature increased lateral compression is the more likely; given that the same effect is observed for larger discs formed using a different MSP. If side chain interactions were important then these larger discs should show a smaller effect as the ratio of outer lipids to inner lipids is reduced.

Similar studies have also been carried out for the SMALP system by ourselves[17] and others[22]. The earlier study carried out by Orwick *et al.* employed SMA with a 3:1 styrene: maleic acid ratio, and here again a broad temperature transition was observed. In the same report Orwick and co-workers described a reduction in the transition temperature for a DMPC disc of some 10 ^{o}C. One explanation for this reduction in transition temperature is that some disruption of the bilayer structure occurred which is likely related to higher styrene content of the SMA reagent employed. In comparison the later study, which used 2:1 ratio SMA polymer showed the smallest perturbation of the phase transition temperature of all the systems analysed amounting to decrease of 1 ^{o}C.

Some variation in the physical characteristics of the bilayers encapsulated in different systems aside, drawing all available data together clearly indicates that SMAs with a styrene: maleic acid ratio of a 2:1 perturb the bilayer to the smallest degree. In view of this observation the next section highlights the use of the SMA 2:1 SMALP system in the study of a range of MPs.

4. Use of SMALPs in the production of membrane proteins

Since its discovery the SMALP system has been used to produce a wide range of MPs. The technique has been shown to be versatile not only for MPs that are recombinantly expressed in a variety of prokaryotic and eukaryotic systems, but also for MPs from natural sources. In all of these examples, protein has been produced without the use of detergents at any stage during the protein purification process (Table 1). The MPs that have been successfully extracted using SMA include a range of structures including β-barrel and α-helical transmembrane (TM) regions, sizes from a single TM α-helix up to 36 TM helices, and functions including transporters, channels, GPCRs and enzymes. **(Figure 2c-d)**

Once proteins have been removed from the membrane in a SMALP, the advantage of this method is that they can then be treated like a soluble protein during purification. Using affinity tags, such as 6 - 8 × HIS tag, purification of the protein of interest can be achieved via immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC).

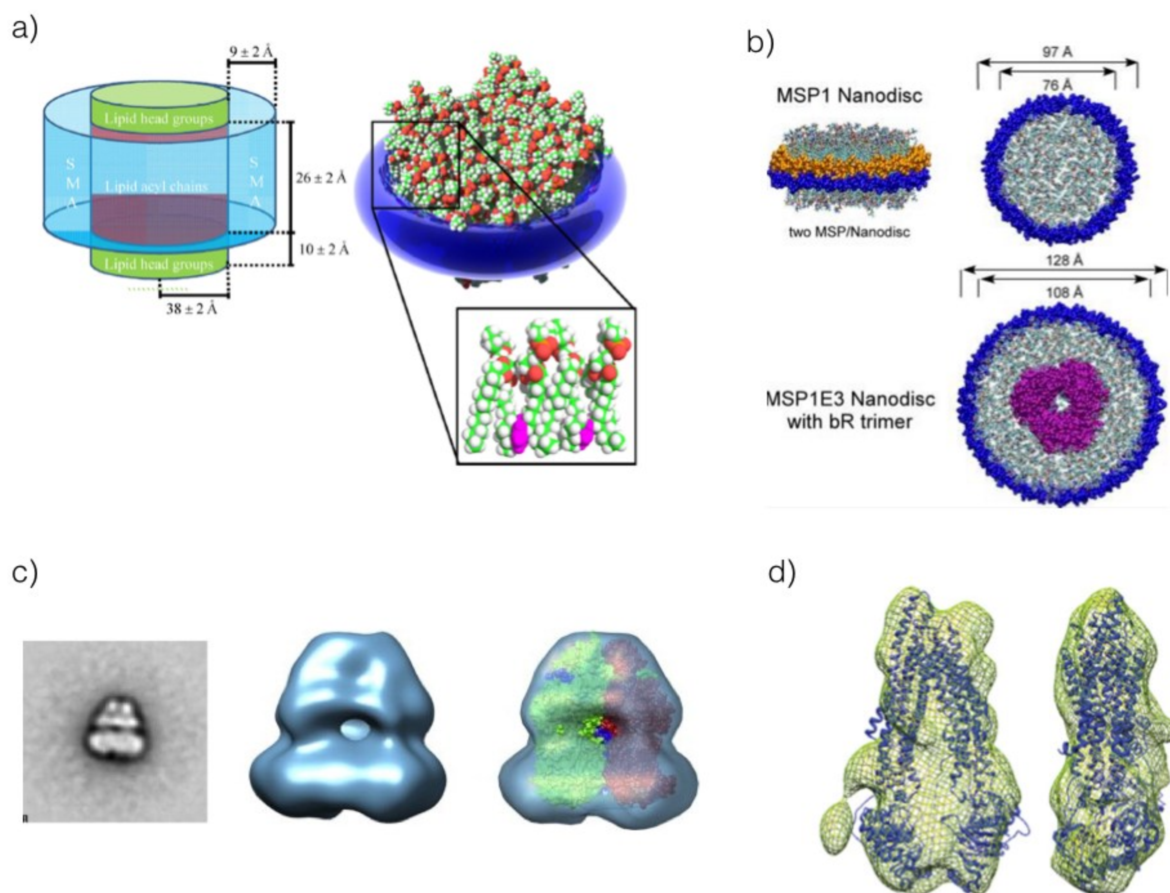
One of the unique features of the SMA approach is that the protein is extracted directly from a membrane with its annular lipids. As mentioned previously, the function of many MPs is highly

dependent on their membrane environment and may require interactions with specific lipid species. Several studies have shown that the lipids within purified SMALPs can be extracted and analysed[23–26], meaning it is possible to determine if a protein associates with a specific subset of the bulk lipids. Such insights are likely to be important for a more complete understanding of MP function or localisation. For example, it is possible that such studies could compare the annular lipids of a protein from 'healthy' versus 'diseased' tissues. A second feature of the SMA approach is that it is possible to isolate native complexes. If the proteins interact in the native membrane (and assuming the complex is not too large for a SMALP) then they can be co-extracted from the membrane[23,27].

There does appear to be a maximal size of protein/complex that can be extracted using SMA, simply because the size of the disc formed is limited, and the protein needs to fit within it. However, interestingly SMALPs have been used indirectly to purify a large complex of photosystem I-Light harvesting chlorophyll II from spinach thylakoid membranes. The PS I-LHC II complex is too large for the SMALP size, but instead researchers used SMALP solubilisation to remove key contaminants from the membrane, leaving a membrane preparation highly enriched for the complex[28].

Figure 2.

Comparison of membrane proteins in nanodiscs; a) Lipid-only SMALP, illustrating the dimension of the SMA belt and how the styrene groups of the polymer intercollate with lipid[17]; b) MSP showing lipid-only nanodisc, formed using two molecules of MSP1 two discs of different sizes are shown, MSP1E3 is used to create a larger size nanodisc which can accommodate the membrane protein Bacteriorhodopsin[7];c) Negative stain 3D reconstruction of SMALP AcrB, accommodates 36 transmembrane helices[29] d) Cryo-Em 3D reconstruction of SMALP P-glycoprotein ABCB1[30].



4.1 Structural and functional studies of membrane proteins encapsulated in SMALPs

The size and stability of the SMALP structure makes them amenable for many techniques used to study protein structure and function. When a protein is encapsulated in a SMALP both sides of the membrane are freely available in solution, which is advantageous for studies of ligand binding. This is quite unlike crude membranes or reconstituted proteoliposomes, where one side is not exposed to bulk solvent. Proteins encapsulated into SMALPs have been successfully used to study ligand binding to a range of different proteins, including GPCRs and transporters, and it has been shown that binding parameters are comparable to those observed in the natural membrane[29–31].

The small size of the SMALP particles means that unlike membranes or proteoliposomes they do not scatter light, and further, they contain a higher 'protein: lipid' ratio than proteoliposomes, attributes which are helpful for spectroscopic techniques. SMALP-encapsulated proteins have been studied using a wide range of spectroscopic techniques including absorbance spectroscopy[15,26] and fluorescence spectroscopy, utilising approaches such as tryptophan quenching, quenching of exogenous fluorescent labels and FRET[23,29,30]. Unlike MSPs, which use a protein 'belt', the SMA polymer does not interfere with spectroscopic measurements such as circular dichroism (CD), enabling study of the secondary structure and stability of MPs[15,24,30,31]. In recent years DEER-EPR (double electron-electron resonance - electron paramagnetic resonance) has been the applied to the study of changes in long range distances between residues that result from protein conformational changes. In these studies the lipid nanodisc structure (both MSP and SMALP), has been shown to improve signal quality *cf.* proteoliposomes, without sacrificing the lipid bilayer environment[16,32,33]. This data provides useful distance constraints for molecular modelling[34].

Structural studies of MPs in SMALPs have been successfully carried out using single particle electron microscopy (EM), using both negative stain[29] and cryo methods[30] (**Figure 2c-d**). One of the advantages of electron microscopy is that it does not require crystallisation of the protein and uses relatively small amounts of sample. Notably the SMA polymer does not dominate EM images *cf.* observations with particles using the MSP protein scaffold[29]. Given the recent technical advances in both generating and processing EM images it is quite plausible to think that atomic resolution structures will be achievable using the SMALP system in the future[35,36]. The application of other structural techniques to SMALP encapsulated proteins has yet to be demonstrated, although SAXS and SANS approaches should work well given the stable, monodisperse nature of the samples, and their track record for studies of lipid-only SMALPs[17].

Whether SMALP purified protein can be used to generate crystals is yet to be determined The signs are promising: proteins in SMALPs can be more easily concentrated than in detergents, generally display greater purity, and are monodisperse. One concern is whether the polymer can be easily removed without disrupting the protein; this remains to be seen.

Table 1. Comparison of the proteins purified using SMALPs.

Name	TM structure	Family/ function	Source	Recombinant /native	Polymer ratio S:M	Purified	Functional assay	Ref.
PagP	8-stranded β-barrel	Outer membrane enzyme	Purified, reconstituted proteoliposomes	Native (*E. coli*)	2:1	n/a	Enzyme activity (phospholipase)	[15]
Bacteriorhodopsin	7 TM α-helices	Light-driven proton pump	Purple membrane from *Halobacterium salinarum*	Native	2:1 and 3:1	Size exclusion	Spectroscopic characteristics	[15], [16]
Respiratory complex IV	19 TM α-helices	Cytochrome c oxidase	Mitochondria from *S. cerevisiae*	Native	3:1	-	Enzyme activity (ferrocytochrome c oxidation)	[26]
KCNE1	1 TM α-helix	Modulates function of certain K_v channels	Purified, reconstituted proteoliposomes	Recombinant (*E. coli*)	3:1	n/a	-	[33]
P-glycoprotein (ABCB1)	12 TM α-helices	Multidrug efflux, ABC superfamily	High Five insect cell membranes	Recombinant	2:1	Nickel affinity	Ligand binding (fluorescence)	[30]
MRP1 (ABCC1)	17 TM α-helices	Multidrug efflux, ABC superfamily	H69AR human cancer cell line	Native	2:1	Immuno-affinity	Ligand binding (radioligand)	[30]
MRP4 (ABCC4)	12 TM α-helices	Multidrug efflux, ABC superfamily	*Sf9* insect cell membranes	Recombinant	2:1	Nickel affinity	-	[30]
ABCG2 (BCRP)	2 x 6 TM α-helices	Multidrug efflux, ABC superfamily	High Five insect cell membranes	Recombinant	2:1	Nickel affinity	Ligand binding (fluorescence)	[30]
CFTR	12 TM α-helices	Chloride channel, ABC superfamily	*S. cerevisiae* membranes	Recombinant	2:1	Nickel affinity	-	[30]
PBP2/PBP2a	1 TM α-helix each	Penicillin binding proteins	*S. aureus* whole cells	Native	2:1	Immuno-precipitation	-	[27]
Photoreaction centre	11 (5 + 5+ 1) TM α-helices	Charge separation during	Purple bacterium *Rhodobacter*	Recombinant	2:1	Nickel affinity & size	Charge recombination (spectroscopic)	[25]

		photosynthesis	*sphaeroides*			exclusion		
KcsA	4 x 2 TM α-helices	Potassium channel	*E. coli* whole cells	Recombinant	2:1	Nickel affinity & size exclusion	Planar bilayer electrophysiology	[24]
AcrB	3 x 12 TM α-helices	Multidrug efflux, RND family	*E. coli* membranes	Recombinant	2:1	Cobalt affinity	Ligand binding (fluorescence)	[29]
A_{2A}R (adenosine 2A receptor)	7 TM α-helices	G-protein coupled receptor	*P. pastoris* membranes	Recombinant	2:1	Nickel affinity & size exclusion	Ligand binding (radioliagnd)	[31]
SecYEG	15 (10 + 3 + 2) TM α-helices	Protein translocation	*E. coli* membranes	Recombinant	3:1	Nickel affinity	Substrate binding (FRET)	[23]
ETK	2 TM α-helices	Tyrosine kinase	*E. coli* membranes	Recombinant	3:1	Nickel affinity	Auto-phosphorylation	[37]
TSPAN7	4 TM α-helices	Tetraspanin	*S. cerevisiae* membranes	Recombinant	3:1	-	-	[38]

5. Opportunity for use in Molecular Simulation

Molecular dynamics (MD) simulations can yield a picture of the *dynamics* of proteins in a range of environments at physiologically relevant temperatures. MD simulations of MPs are a relatively recent addition to an established history of MD simulations of proteins in general; nevertheless they have already proven to be a successful addition to the toolkit for studying biological membranes and the proteins that are associated with them[39,40]. Currently MD simulations are routinely used to study the evolution of a molecular system on microsecond timescales (we note that timescales that exceed these have been reported, but these usually require custom-built hardware)[41–43].

MD simulations are based on classical mechanics. The components of the simulation system (protein, lipids, water, etc.) and their interactions are described by a suitable molecular mechanics force-field. The force-field provides a link between the positions of particles in a molecular system and the potential energy of that system. Numerical integration of Newton's equations of motions for every atom or particle in the system enables the positions of the atoms to be recorded at suitable intervals yielding a trajectory, which describes the time evolution of the protein, lipid, and water molecules. Analysis of the trajectory in terms of e.g. protein conformational rearrangements, protein/ligand and

protein/lipid interactions, are then typically performed offline. Thus MD provides an ideal complement to structural and biophysical methods of characterising MPs.

Traditionally MD simulations of MPs have been performed at the atomistic (AT) level of resolution, where each atom is explicitly considered (with the exception of 'united atom' models in which non-polar hydrogen atoms are considered implicitly)[44]. These simulations have been successful for elucidating details of e.g. protein-ligand interactions, protein conformational changes that occur on nanosecond to microsecond timescales and for aiding the design of mutations for a range of applications. Coarse-grain simulations (CG), in which a number (usually ~ 4 as in e.g. the MARTINI force field) of heavy atoms are grouped together into pseudo-atoms, enable simulation of larger systems than atomistic simulations and also allow access to longer timescales at the cost of explicit all-atom detail[45,46]. Such models enable simulation of self-assembly processes and exploration of protein-membrane interactions that occur over many microseconds. Traditionally, one of the gaps between *in vitro* and *in silico* experiments of MPs has often been in the size of system studied, with *in vitro* usually being much larger than *in silico*. This is another issue that enhances the complexity of studying MPs as compared to their soluble cousins. In the latter case the system to be simulated needs only be a protein in a homogenous solvent. While for a MP there is the added need for a stable membrane, which in itself is chemically heterogenous alongside the aqueous solvent and the protein. To add more complexity a discrete portion of the membrane cannot be simulated as the effects of having exposed acyl chains at the edge of such a construct leads to instability. This means that either very large simulations that includes a vesicle (therefore lacking any edges) or implementation of periodic boundary conditions to simulate a membrane continuum. A key advantage of any of the disc-based systems is that they naturally bridge this gap; the nanodiscs are small enough to be simulated using MD, given they are ~ 10 nm in diameter and composed of ~ 120 lipids. This is well within the capabilities of state-of-the-art MD simulations at both the atomistic and CG levels.

In the context of the discussion above, it is useful to review the nanodisc simulations studies reported in the literature; these have included both atomistic and CG MD methodologies. Shih *et al* reported some of the earliest molecular dynamics simulations of MSP nanodiscs[47]. They performed atomistic simulations of nanodiscs composed of DPPC phospholipids surrounded by various lengths of the truncated human scaffold protein MSP1 (which contains the 200-residue lipid-binding C-terminal domain of apolipoprotein A-1). The nanodisc models were based on the earlier modelling and

simulation work of Klon *et al*.[48]. The nanodiscs contained 160 lipids, 80 in each leaflet of the bilayer and two belts of scaffold proteins. The initial alignment of the scaffold proteins proved to be rather crucial in these simulations as the restricted timescales accessible to atomistic simulations did not allow for realignment of the proteins in a more favorable configuration and thus out of plane deformations were observed with some lengths of scaffold protein. Interestingly, embedding bacteriorhodopsin, an integral MP into the nanodiscs did not induce out of plane deformations, however these discs did adopt a more rectangular overall morphology.

The authors concluded that while atomistic simulations timescale of nanoseconds, allow for the relaxation of the scaffold protein, they are not long enough to observe any potential changes in the scaffold protein alignment. Encouragingly, these simulations did show the potential utility of MD simulations in the future design of improved scaffold proteins.

To overcome the timescale limitations of atomistic simulations, in 2006 Shih *et al* reported a new coarse-grain (CG) protein-lipid model developed specifically with the aim of studying large, nanoscale systems[49]. One aspect of SMALP nanodisc structure where MD simulation can provide unprecedented levels of information is the self-assembly of the discs. To this end, in a subsequent paper the CG model was employed to study the self-assembly of high-density lipoprotein (HDL) nanoparticles from microsecond timescale simulations[50]. HDL assembly was found to proceed via two broad stages; firstly the aggregation of proteins and lipids driven by the hydrophobic effect was observed to occur over ~ 1 microsecond, this was followed by the optimization of the protein localisation and orientation, which is driven by increasingly specific protein–protein interactions which lead to the ordered 'double belt' arrangement that is characteristic of scaffold proteins in nanodiscs.

Recently,Debnath and Schäfer reported study of nanodiscs composed of DMPC phospholipids, surrounded by a 'belt' composed a major scaffold protein from human apolipoprotein A-1[51]. Self-assembly of the lipids and protein into nanodiscs was studied by CG-MD. Simulation of the nanodiscs at both atomistic and CG resolutions revealed that while on average lipids in the nanodisc have lower configurational entropy and higher acyl tail order than in a lamellar bilayer phase, the actual dynamic behaviour depends very much on the location of the lipids within the nanodisc. Lipids located at the centre of the nanodisc are highly ordered, whereas lipids that are in contact with the MSP proteins at the outer rim of the nanodiscs are highly disordered, due to disrupted packing. This study illustrates

the unique advantages of probing lipid behaviour within nanodiscs by molecular dynamics simulation. Vestegaard *et al.* reported a coarse-grain MD study, in which the properties of nanodiscs, bicelles and flat bilayers were compared. Their results showed that the extent of peptide solvation within bicelles and nanodiscs is strongly dependent upon location of the peptide within the membrane mimetic environment and that dynamics of membrane mimetics can be rather different when proteins are embedded compared to the protein-free environment. This observation is in agreement with other molecular dynamics simulations studies, which have also revealed the complex interplay between proteins and lipids[52,53].

While, to our knowledge there are only a few reported molecular simulation studies of SMALPS nanodiscs to date (two papers not discussed here are[54,55]), the field of nanoparticle simulation in general is growing at a rapid rate. Numerous studies of the interaction of fullerenes, carbon nanotubes, and dendrimers with lipid bilayers have been published in the last few years [56–60]. In addition, the state-of-the-art in terms of size and complexity of atomistic and coarse-grain membrane models is also rapidly increasing. At the atomistic level, studies of bacterial membranes in particular are now regularly incorporating the asymmetry and mixed lipid composition of *in vivo* membranes[61,62]. Similarly, at the coarse-grain level, state-of-the-art models of eukaryotic membranes now incorporate a range of lipids and multiple copy numbers of integral MPs[52,63].

5.1 *The opportunities available using SMALP based simulations.*

It is clear that the discrete nature and small unit size of disc-shaped encapsulation systems like SMALPs and MSPs provides a clear advantage for MD simulation approaches. However there are a number of practical aspects of these systems that have the potential to provide information that can be utilised by *in silico* modellers. In the following sections we highlight some of these opportunities.

Over the past few years biophysical data on both SMALP and MSP systems has begun to appear. In the case of the MSP system some MD simulation work has begun to unravel some of the fundamental aspects of the MSP structure and that of proteins contained within the particle. Similar work on the SMALP system is, in contrast, still very much in its infancy. Studies of SMALP structure so far carried out (and reviewed in previous sections) provides a number of observations that are testable in MD systems. For example the arrangement of the SMA chain with respect to the bilayer, including the orientation of the styrene moieties with respect to the acyl chains, the size and stability of the particle

itself and the influence of maleic acid charge on the structure. Some of these questions are clearly answerable using atomistic simulations but some, notably self-assembly of the particle itself, could require CG approaches. It is clear that in an ideal world these investigations should be carried out before those on protein loaded SMALPs to provide confidence that the simulations protocols adequately represent the basal particle.

Once these simulations have been established then other aspects of the SMALP system will be ripe for exploitation. The discrete nature of these disc system also means that there is some control over the lipid make-up of the bilayer in the particle. Recent published work[24] has shown that the lipid content with the disc can be determined to some degree. Our own recent unpublished work shows that application of the relevant mass spectrometry methods to SMALPs can provide details on the abundance of lipids with different headgroups and acyl chain types in the particle. Availability of this information means that simulations can be set up with more certainty that the membrane being simulated better matches reality. Over the last few years, MD simulations have also been particularly successful in identifying important lipid-protein interactions, especially in mixed lipid systems. Often these *in silico* experiments are difficult to replicate as generating membrane systems with new lipid makeups can be challenging. The reversibility of the SMALP system which allows easy transition between vesicle and disc forms means that lipid exchange experiments could be accomplished with more ease and certainty. For example a SMALP protein preparation could be returned to a vesicle state (by reducing pH to 6, which is below the pKa of SMA). Addition of a different lipid combined with some form of agitation will allow the formation of mixed vesicles that can be returned to the SMALP state by raising the pH. If the protein containing SMALPs are then separated (by simple affinity chromatography) then the mass spectrometry methods detailed above can be used to confirm the lipid exchange.

In addition to the reduced system size, a number of other properties make SMALPS and MD simulations ideal complementary techniques. For example it is practically possible to produce materials that contain differing numbers of proteins in a single SMALP. The activities of these materials can be tested and then compared to simulation. This would be particularly advantageous for studying the effect of monomerization/oligomerization on MP function, as the copy numbers of the proteins can be tightly controlled in both techniques. Membrane proteins have been demonstrated to have an increased stability and activity when immobilised in SMALPS nanodiscs when compared to

detergent-based methods. Given the short timescales accessible to MD simulations, this increased activity provides advantages when comparing data from experimental and computational methods.

Perhaps the most exciting opportunity for the future comes from the combination of new atomic resolution EM techniques with SMALPs and MD simulation. As has already been demonstrated SMALP-MP complexes seem to be well suited to study by EM. The advent of direct detection EM and hence the potential for near atomic resolution data provides a potentially rapid route to structural data sets that contain both protein and lipid. These provide almost ideal starting points for MD studies. Cryo-EM can also provide "snapshots" of the conformational subforms that are present in solution, unlike X-ray crystal structures that provide a single conformational form. This could provide MD simulation experts with snapshots in real trajectories, and statistical data as to the prevalence of those subforms in the bulk. As can be imagined such directly observed datasets provide ideal data for assessing MD simulation performance.

Thus, it can easily be envisaged that in the future MD simulation and experimental techniques will be used in conjunction with each other to obtain complementary insights into the dynamic behaviour of proteins embedded within SMALPs nanodiscs and to design novel scaffold proteins.

6. Conclusion.

For the latter part of the twentieth century membrane protein purification relied almost entirely on the use of detergents in order to produce pure preparations of these important biomolecules. These times were often intensely frustrating for the membrane biologist, with only a small subset of the membrane proteome being amenable to purification using detergent methods. Since the millennium a number of new approaches have appeared that aim to resolve the detergent issue. In this review we have charted the development of these new methods which have valued the retention of the lipid bilayer in the final membrane protein preparation. The latest chapter in this story has been the establishment of the SMALP method that removes the need for detergent entirely, ensuring that the local environment around the membrane protein is preserved. With the establishment of this method researchers can look forward to an era of new opportunities where large previously intractable parts of the membrane proteome become available for study. Inevitably this will lead to an expansion in our knowledge of the structure of MPs at the atomic level, which will make a plethora of simulation studies possible.

However, it is also clear that use of discrete proteolipid particles to solve this historical bottleneck offers even more to the molecular simulator. These new particles have the potential to provide structural data on not just the protein, but also the local membrane, an element almost entirely missing from existing structures of MPs. In addition, the relative simplicity of these systems (containing less than 150 lipids molecules and 9 kDa of polymer in addition to the protein) greatly simplifies the simulation system allowing longer simulations to be carried out.

In conclusion, these new encapsulation systems have the potential to revolutionise our knowledge of membrane protein structure and function and enhancing our understanding of biology at the atomic level.

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