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Review Article

Biophysical characterisation of SMALPs

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Membrane proteins such as receptors, ion channels and transport proteins are important drug targets. The structure-based study of membrane proteins is challenging, especially when the target protein contains both soluble and insoluble domains. Most membrane proteins are insoluble in aqueous solvent and embedded in the plasma membrane lipid bilayer, which significantly complicates biophysical studies. Poly(styrene-co-maleic acid) (SMA) and other polymer derivatives are increasingly common solubilisation agents, used to isolate membrane proteins stabilised in their native lipid environment in the total absence of detergent. Since the initial report of SMA-mediated solubilisation, and the formation of SMA lipid particles (SMALPs), this technique can directly isolate therapeutic targets from biological membranes, including G-protein coupled receptors (GPCRs). SMA now allows biophysical and structural analyses of membrane proteins in solution that was not previously possible. Here, we critically review several existing biophysical techniques compatible with SMALPs, with a focus on hydrodynamic analysis, microcalorimetric analysis and optical spectroscopic techniques.

Introduction

Membrane proteins are a major focus of biochemical and biophysical research and are one of the largest classes of drug targets. The study of membrane proteins whilst mimicking their native lipid environment has been notoriously difficult using biophysical techniques. This is often due to the use of chemical detergents during the purification process that remove the essential lipids surrounding the protein to form a micelle. This micelle formation results in proteins in non-native conditions [1].

In recent years alternative approaches to using detergents for membrane protein extraction have emerged. These methods incorporate the proteins in a lipid bilayer to increase protein stability and retain the protein's native activity. The first approach, and perhaps most used, is based on membrane scaffold proteins (MSP) which stabilise a disc of bilayer that contains the membrane protein [2,3]. The use of MSPs has demonstrated the utility of this extraction method but has two potential drawbacks. First, it still requires detergent extraction for protein extraction, which can destabilise the protein and reducing yield. Secondly, it is hard to ensure that the reconstituted lipid environment in the disc matches that in the native source membrane. A second approach using synthetic organic polymers has recently been developed that produces lipid containing particles. This technique resolves the issues associated with MSPs by directly extracting the protein from the native lipid into the polymer supported particle.

Poly(styrene-co-maleic acid) (SMA) is a synthetic co-polymer of alternating hydrophilic maleic acid and hydrophobic styrene moieties. SMA molecules associate with and stabilise lipid bilayers to form discoidal SMA lipid assemblies and produce membrane protein-containing particles (SMALPs) or nanodiscs [4,5]. Figure 1 is a representative image of a SMALP. Over the past decade SMALPs and other co-polymer derivatives have been utilised for the isolation, solubilisation, and purification of membrane proteins with an intact local lipid environment, often maintaining functional protein conformation and activity. This is attributable to the preservation of native lipid bilayer by the encapsulating polymer which is optimal for the analysis of proteins in their active physiological state [6]. The use of SMALPs is a beneficial tool to control the size of the resulting lipid–polymer disc, apply charges and introduce functional modifications to aid in biophysical studies [4].

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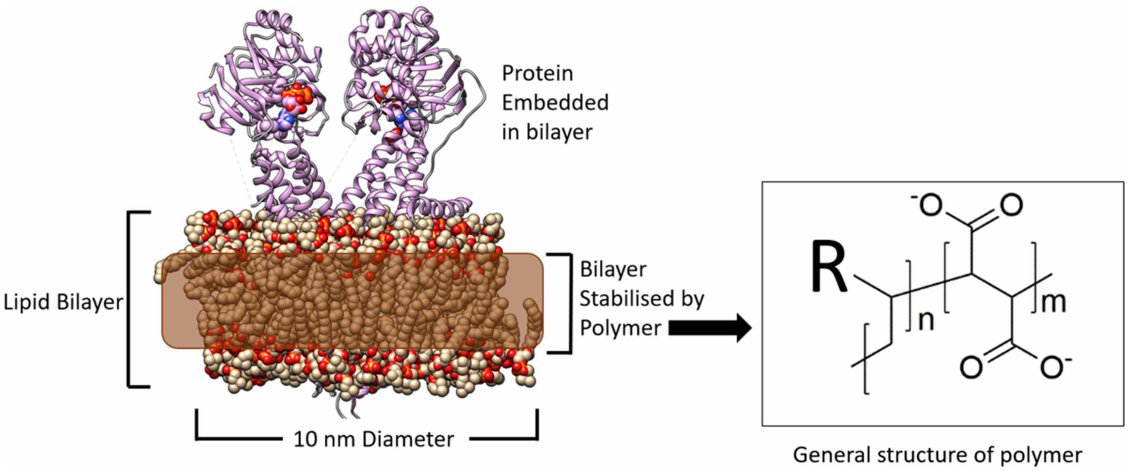

Figure 1. Diagrammatic representation of CFTR from chicken (PDB: 6D3R) [61] solvated by a 10 nm diameter DPMC membrane bilayer disc highlighting the morphology of polymer extracted lipid disc systems.

Lipids are shown as CPK colours in red and beige, and protein is shown as a ribbon representation in pink with ligand shown as CPK. The figure was created using Chimera [62].

SMALP and SMALP-related technologies enable biophysicists the ability to probe the mechanism of membrane protein function, often for the first time. This provides the exciting opportunity to unleash a plethora of biophysical techniques that have been honed for use on soluble proteins to interrogate the newly available samples. This will lead to advancing life sciences research, and the discovery and development of new therapies which target membrane proteins. In this minireview we discuss selected biophysical techniques used to characterise SMALPs and related co-polymers. Table 1 summarises the techniques discussed in this review, including typical sample consumption, advantages and limitations, and citations that describe its use for membrane protein and SMALP characterisation

Hydrodynamics analysis techniques for SMALPs

For the study of SMALPs it is important to ascertain hydrodynamic information regarding their size, shape, and homogeneity [7]. In this section we discuss the several biophysical techniques that assess the hydrodynamic properties of SMALPs.

Dynamic light scattering

Dynamic light scattering (DLS) measures the velocity at which particles are diffusing via Brownian motion in a solution [8]. As light hits a moving particle the intensity of the scattered light fluctuates due to these motions. Small particles diffuse quickly thus their signals fluctuate rapidly, while large particles produce signals that fluctuate slowly. The intensity of light scattering as a function of time can be plotted to produce an autocorrelation function and the diffusion coefficient is extrapolated. Particle shape and size distribution can be determined using the Stokes–Einstein equation (Figure 2). DLS rapidly determines the optimal conditions for SMALP nanodisc formation, stability, and quality at a low resolution.

DLS is used for benchmarking and validating encapsulation techniques, to characterise the effectiveness and homogeneity of lipid particles generated from SMA, poly(styrene-co-maleimide) (SMI) and diisobutylene/maleic acid (DIBMA) [9,10]. This has become more important with the increase in interest in the new modified polymers. Recent research [11] used DLS to investigate how the chain length of reversible addition-fragmentation chain transfer polymerisation (RAFT)-polymerised DIBMA with varying molar masses influenced the formations of discs and disc size of DIBMA lipid particles (DIBMALP) with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). DLS measurements indicated that all but the shortest polymer chains successfully produced lipid-only and protein-encapsulating nanodiscs. This study also demonstrated 3 to 7 kDa polymers showed greater solubilisation efficiency and cleaner separation. DLS has also been used to assess the effect of conditions on solubilisation. For example, a recent publication [12] demonstrated

Table 1 The biophysical techniques included in this minireview, with typical sample amounts, advantages and limitations of each method for the characterisation of SMALPs and other copolymers, and citation(s) on the use of that technique for the study of membranes, membrane proteins and SMALPs Part 1 of 2

Biophysical technique	Typical sample amount/ measurement	Advantages	Limitations	Relevant citations
Hydrodynamics:				
Dynamic light scattering (DLS)	40 microL — 1 ml, ≤1 mg/ml	• Rapid • Uses low concentrations and small volumes • Samples are recoverable	• A pure sample is required • Each sample species present in solution will contribute a proportional amount of scattering, • Highly sensitive to larger particles (i.e. aggregates or insolubilised material)	[8–15]
Size exclusion chromatography (SEC)	0.5–4% of total column volume	• High resolution separation of compounds in solution • Rapid	• No information regarding the stoichiometry of the lipid ratios. • Small impurities may remain even following fractionation.	[7,16–18]
Small angle X-ray scattering (SAXS)	10–100 microL or 1–2 mg/ml total protein	• Doesn't require a staining procedure • Can provide a more accurate size distribution than DLS or SEC.	• Lacks atomic resolution • Complex data analysis requiring specialist software following collection	[19–24]
Analytical ultracentrifugation (AUC)	80–400 microL	• Can be used to determine oligomerisation state • Doesn't require a staining procedure • Samples are recoverable	• Slow • Low throughput • Complex data analysis requiring specialist software following collection	[6,7,24–26]
Transmission electron microscopy (TEM)	4–10 microL, 0.1–3 mg/ml protein	• Uses low concentrations and small volumes • Can be used to determine bilayer thickness alongside diameter (nm)	• Issues with negative staining by phosphotungstic acid (PTA) staining. • Causes disc stacking when imaging DIBMA lipid-only discs however this orientation can be utilised to determine the disc width. • SMALP preparation or interactions occurring on the carbon surface of the grids during sample preparation for microscopy can cause problems in image processing due to the dominating effect of the nanodisc in alignment and classification.	[11,24,27–29]
Microcalorimetry:				
Differential scanning calorimetry (DSC)	0.1 mg to 2 mg	• Measure heat change related to unfolding/melting • Label free -no tags or chemical modifications • Non-optical • Non-fluorescent, data not affected by fluorescence artefacts • DSC can be done with pure biopolymers and complex mixtures • Direct measurement of T_M • Quantitative data • Can be performed in the presence of detergents and other additives • Minimal assay development	• A pure sample is required • Uses larger sample concentration and volumes compared with other biophysical assays • Low throughput	[22,30–40]

Continued

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Table 1 The biophysical techniques included in this minireview, with typical sample amounts, advantages and limitations of each method for the characterisation of SMALPs and other copolymers, and citation(s) on the use of that technique for the study of membranes, membrane proteins and SMALPs Part 2 of 2

Biophysical technique	Typical sample amount/ measurement	Advantages	Limitations	Relevant citations
Isothermal titration calorimetry (ITC)	0.05 mg to 5 mg	• Measure heat change related to binding and other biomolecular interactions • Label free -no tags or chemical modifications • Non optical • Non-fluorescent, data not affected by fluorescence artefacts • In-solution, no immobilisation • No size or molecular weight limits – can be used for ions, small molecules, large protein complexes and nanodiscs • Can be performed in the presence of detergents and other additives • Minimal assay development • Samples are recoverable	• A pure sample is required • Uses larger sample concentration and volumes compared with other biophysical assays • Low throughput	[41–50]
Optical spectroscopy:				
Absorbance spectroscopy	0.05 mg to 5 mg	• Simple to use and generically available • DIBMA polymers have less interference in the near UV region. • 245 nm peak of styrene allows SMA to be tracked during chromatography	• Styrene based polymers have some absorbance in the near UV (peak at 245 nm)	[39]
Circular dichroism (CD)	0.05 mg to 5 mg	• Provides rapid measurements of protein secondary structure (far UV) and hydrophobic core perturbations (near UV) • SMALP and DIBMALP methods provide samples with low CD background signals that allow high quality data to be collected	• Pure sample is optimal • Requires microgram quantities of protein • Low throughput (although 96 well plate systems are entering service) • Users need to be wary of including Cl^- ions in samples as these absorb far UV light. However, F^- can be substituted without altering lipid particle stability and structure. • Lacks atomic resolution information	[4,24,46,53–56]
Fluorescence spectroscopy	0.005 mg to 5 mg	• Provides a wide range of experimental options • Can provide information on: o Protein conformation change o Ligand binding o Protein folding o Solvent accessibility • Polymer reagents do not significantly interfere with either intrinsic or extrinsic fluorescence	• Lacks atomic resolution information • Requires a pure sample	[14,29,54,58–60]

that disc size and homogeneity was not dependant on DIBMA/lipid ratios. Multiple studies have used DLS to determine that the disc size (~20 nm) of DIBMALP when compared with SMALPs (~7 nm) increased lipid flexibility within the disc. Although this can contribute to lower protein yield, it is beneficial with proteins such as GPCRs, which require conformational change for correct protein functionality [13,14]. DLS can also be used

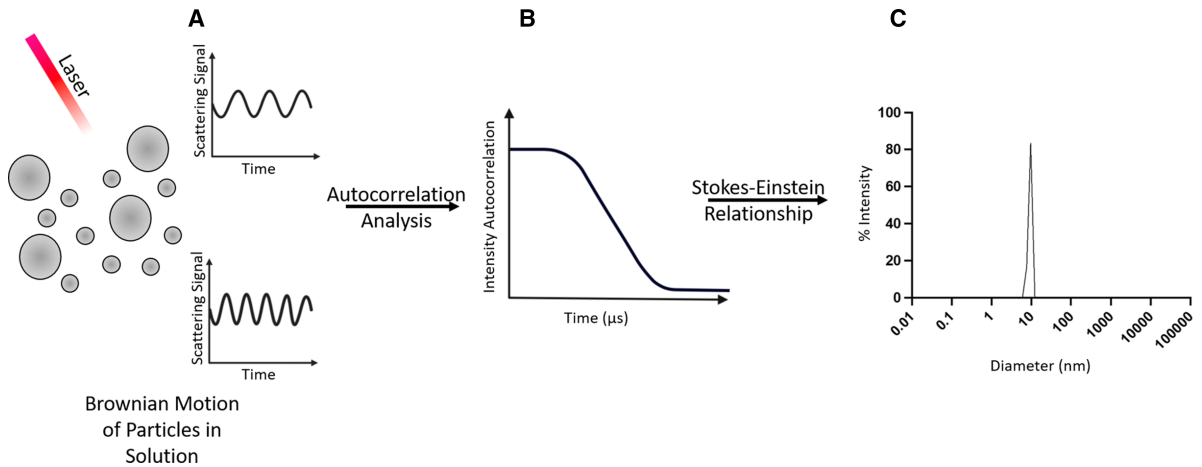

Figure 2. Overview of DLS. DLS measures the size of particles rotating in solution by recording how laser light is scattered over time.

(**A**) Particles move in solution based on the theory of Brownian motion and when light hits these particles the signal fluctuates, small particles generating faster flucations over time compared with the larger particles and this can be interpreted as an autocorrection function when plotted against time (**B**). The hydrodynamic radius of particles can be determined from the speed of rotations using the Stokes–Einstein equation. (**C**) DMPC solubilised using SMA to produce lipid-only nanodiscs using 2.5% SMA and 5 mg/ml DMPC, with an average diameter of 7.4 nm.

to monitor thermostability of polymer-encapsulated lipid and proteins [15]. This study focused on acid-compatible SMI lipid particles, which were shown to be highly stable with no biophysical changes up to 80°C but below 16°C and after multiple freeze-thaw cycles changes in particle diameter were observed [15].

Size-exclusion chromatography and size-exclusion chromatography-multi-angle light scattering

Size-exclusion chromatography (SEC) is a biophysical technique that separates species present in a solution based on the components' hydrodynamic radii as they pass through a gel matrix [16]. SEC is used to assess the mass and size tuneability of SMALPs by providing an estimate of their homogeneity and hydrodynamic radius (Figure 3A). Like DLS, SEC allows quick characterisation for the assessment of optimal disc-forming conditions. SEC fractionation also removes excess polymer from solubilised solutions and improves sample homogeneity [7]. SMA has poor stability in solutions of divalent cations, so the removal of excess polymer can reduce the amount of precipitation induced in these solutions.

SEC-MALS offers the combination of SEC with multi-angle light scattering (MALS) and is often utilised for the determination of polymer molecular weight parameters [17]. Unlike SEC, in SEC-MALS hydrodynamic volume is used to determine molecular weight rather than retention time. SEC identified which species were present in the population of SMALP-DMPC at different polymer concentrations during nanodisc assembly to decipher information on the phase behaviour of solubilisation [17]. A recent publication [18] utilised SEC-MALS for the molecular weight determination of AaFtsH and its lipid content within the nanodisc, determining a total of 246 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) molecules per nanodisc [18].

Small-angle X-ray scattering

To aid the low-resolution data obtained by DLS and SEC, small-angle X-ray scattering (SAXS) can be used. During SAXS monochromatic X-rays illuminate a solution, and the X-ray scattering is recorded by a detector. Once the scattering pattern of solvent has been subtracted, the remaining signal is attributable to the protein of interest and contains information about its shape and size [19–21]. SAXS can quantify polymer/lipid ratios within both lipid-only discs and membrane protein-containing discs [22].

SAXS can determine the size of SMALP supramolecular aggregates, assessing the impact of pH, ionic strength, polymer/lipid concentration and temperatures [23]. This study concluded that varying the

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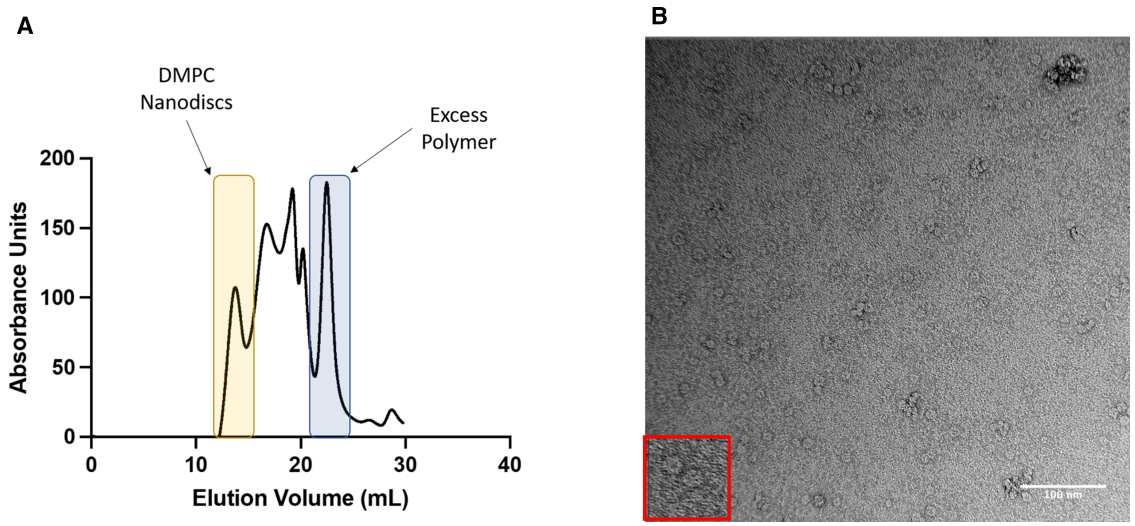

Figure 3. Representative data from characterisation of DMPC solubilised using SMA to produce lipid-only nanodiscs using 2.5% SMA and 5 mg/ml DMPC with SEC (A) and TEM (B).

(**A**) Representative chromatogram of DMPC liposomes solubilised with SMA 2 : 1 in a 1 : 1 (w/w) ratio following SEC using a Superdex 200 Increase 10/300 GL equilibrated in 50 mM Tris, 150 mM NaCl, and pH 7.4. Absorbance was measured at 280 and 254 nm. Fractions corresponding to purified SMALPs used for further analysis as confirmed by DLS. Purified SMALPs were contained within first peak corresponding to elution volume of 12–14 ml (orange) with a diameter of ∼10 nm and excess polymer eluted at 22–24 ml (blue). (**B**) Representative TEM of DMPC liposomes solubilised with SMA 2 : 1 following staining with 2% (w/v) uranyl acetate, showing single particles. Scale bar (100 nm) and diameter of single particles (7.85 nm). Magnified image in lower left shows representative single particles of SMA DMPC nanodiscs from the selected micrograph.

physiochemical properties of SMA across a range of conditions resulted in aggregates with different sizes based on the number of hydrophobic residues present, whilst the general shape remains the same. It was also shown that following an increase in temperature the particle diameter of styrene enriched SMA supramolecular aggregates increased along with radius of gyration [23]. This study can be expanded by using other biophysical techniques alongside SAXS to investigate the impact of bilayer thickness.

SAXS can also be used to elucidate preliminary protein structural data as seen in the structural study of SMALP-ZipA [24]. ZipA is a 40 kDa membrane protein involved in *E. coli* cell division. SAXS demonstrated SMALP-ZipA monodispersity and indicated a degree of flexibility in solution. Scattering intensities were analysed using the ATSAS data analysis package. The data were used to produce a dummy atom model displayed as spheres, and the dummy model was compared with a Cryo-electron microscopy map to verify protein structure.

Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) helps determine protein homogeneity, mass, shape, and hydrodynamic radius, and aids in the characterisation of stoichiometry and oligomerisation of proteins [25]. AUC is routinely used to study the degree of monodispersity in nanodisc preparations [6,24]. Unlike SEC, AUC provides data regarding the protein/lipid ratio. In AUC the solution of interest is sedimented at high centrifugal force and the process of sedimentation is monitored in real-time.

For the purposes of AUC, SMALP protein complexes can be treated like globular proteins to allow for the determination of size and oligomerisation state. This was first demonstrated by using SMALP-ZipA [7]. AUC analysis of purified SMALP-ZipA concluded that a single monomeric species was present at ∼70 kDa of which 30–35 kDa was attributable to lipid and SMA present within the nanodisc. In a different study, AUC was used by to determine the difference in sedimentation profiles of SMA in contrast with detergent isolated photosystem I (PSI) [26]. The generated AUC data demonstrated a lower sedimentation coefficient when using SMA for solubilisation, implying that within SMALP-PSI a greater lipid–protein ratio was present. The resulting sedimentation profile showed that SMALP-PSI consisted of pure trimeric particles.

Transmission electron microscopy

Transmission electron microscopy (TEM) is widely used to observe the general morphology and size of lipid-only and protein-containing particles [27], confirm the disc shape of SMALPs, and investigate conditions that can alter disc size and morphology. During TEM a beam of electrons is passed through a thin layer of sample mounted on a grid. For negative stain TEM the sample is coated with heavy metals to a detector to enhance the scattering of the electron beam (Figure 3B). As the electrons interact with the sample the resultant signals provide high resolution images that can indicate nanoscale features [28].

TEM was utilised for the study of homogeneity and size of DMPC liposomes solubilised using RAFT DIBMAs to corroborate data obtained by DLS [11]. Size distribution of the DIBMALP-DMPC particles was plotted as a function of molecular weight to demonstrate that molar mass had a non-significant effect on particle diameter. In recent years negative stain TEM has been utilised as a screening tool prior to Cryo-TEM of SMALP-protein complexes, ranging from 40 kDa to 1.2 mDa. One of the first studies to demonstrate the suitability of TEM for the study nano-encapsulated proteins embedded in a lipid environment used SMALP-AcrB, *an E. coli* inner membrane transporter, as a model system [29]. This study highlighted the caveats in sample preparation required when using SMALP-protein complexes (Table 1). Similarly, despite being on the minimum molecular weight limit typically used for Cryo-TEM studies, researchers [24] investigated the size and shape of SMALP-ZipA to determine that in solution monodisperse particles were present with a narrow size distribution.

Microcalorimetric analysis techniques for SMALPs

Microcalorimetry measures heat, is label-free and non-optical, and can often be performed with samples and conditions that are not amenable to other biophysical assays. In this section we explore how microcalorimetry has been or could be used to study SMALPs and membrane proteins.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) characterises the thermal and conformational stability of proteins, lipids, and other biopolymers [30,31]. DSC is used to study biological membranes [32,33], detergent-solubilised membrane proteins [34], protein–lipid interactions [35], and MSP nanodiscs [36].

DSC instruments used for biopolymer characterisation has the biopolymer solution (in buffer) placed in a fixed-in-place cell, and the corresponding buffer placed in a matched reference cell [30,31]. As the biopolymer is exposed to increasing temperature, it begins to unfold or undergo a phase transition, and the excess heat capacity (Cp) of the biopolymer increases. The thermal transition midpoint (T_M) is where 50% of the biopolymer is in its native (folded) conformation, and 50% is in its denatured (melted) conformation. The T_M is a 'peak' of a DSC thermogram (Figure 4A) and is considered a good indicator of stability — the higher the T_M, the biopolymer is more thermally stable.

DSC characterised the thermotropic gel-to-fluid phase transitions of DMPC in the absence and presence of SMA [22,37,38,39] and DIBMA [39,40]. A recent publication [37] showed DSC thermograms of DMPC with a highly cooperative gel-to-fluid phase transition at 24°C, typical of DMPC large unilaminar vesicles (LUVs) (Figure 4A). In the presences of SMA, the DSC thermograms were broadened, and the peak height decreased by a factor of ∼10. Changing the SMA concentration impacted the observed T_M; T_M increased when the polymer/lipid ratio was below the saturation boundary (where the first nanodiscs start to form), likely due to the stabilisation of gel over the fluid phase. At the SMA concentration where the DMPC were completely solubilised, the T_M decreased to ∼23°C. At SMA concentrations above the solubilisation boundary (where it is assumed there is complete nanodisc formation) T_M continued to decrease (Figure 4B). The authors speculated that the T_M decrease was due to 'looser' lipid particle packing in polymer-induced nanodiscs compared with LUVs [37]. DSC also showed the effect styrene/maleic acid ratio had on the T_M of the DMPC LUV (Figure 4B inset), likely due to different polymer packing [37].

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) directly measures the heat change associated with binding events, and other biomolecular interactions. ITC determines the binding affinity, typically represented as the dissociation constant (K_D), enthalpy of binding, and binding stoichiometry for protein–protein, protein–drug, protein–lipid,

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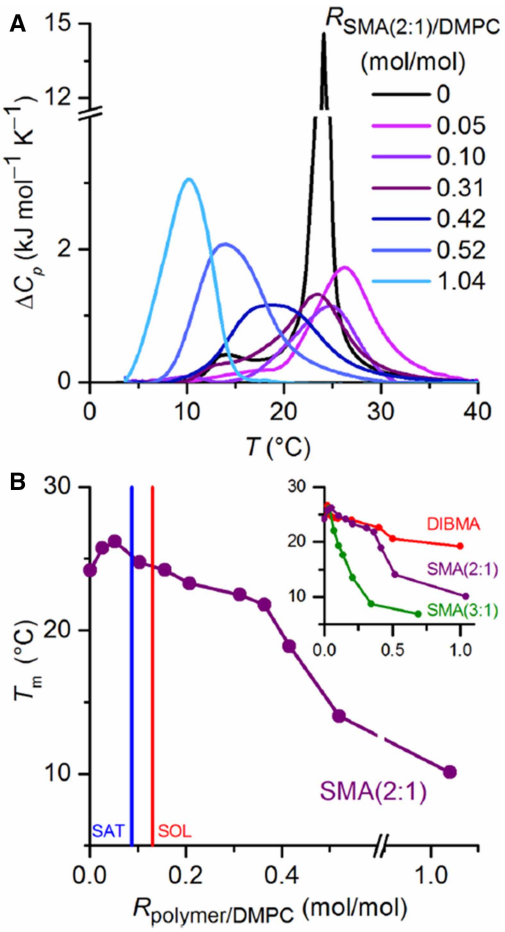

Figure 4. DSC thermograms showing Thermotropic phase behaviour of DMPC solubilised with SMA.
(**A**) DSC thermograms showing of 5 mM DMPC in a range of SMA concentrations. (**B**) and inset: Gel-to-fluid phase T_Ms of 5 mM DMPC in increasing concentrations of SMA at 2 : 1 or 3 : 1 ratio, or DIMBA. Vertical lines indicate the saturation (SAT) and solubilisation (SOL) boundaries of SMA. Reproduced from Ref. [37], published under Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/.

polymer–lipid, and other interactions [41,42]. ITC is used to study membrane proteins [43,44] and the solubilisation and reconstitution of lipid membranes [45–48].

ITC was used to measure the binding affinity of TonB to detergent-free MSP nanodiscs made with FluA, a TonB-dependent membrane receptor [49]. In the presence of ferricrocin, the observed K_D was ∼200 nanomolar [49]. ITC was also utilised to study the lipid binding properties of membrane-active peptide AA139 to anionic nanodiscs formed by circularised MSPs [50]. ITC isotherms showed weak binding of AA139 to nanodiscs in the micromolar range. This ITC data correlated to nuclear magnetic resonance binding data [50]. ITC showed that binding was driven by a significant enthalpic contribution (−6 kcal/mol) and a stoichiometry of four peptides per disc [50].

Similar ITC binding experiments can be done with polymer based nanodiscs made with SMA or other amphipathic polymers, which will advance the study of the thermodynamics of lipid–polymer and lipid–protein interactions. ITC can also be used to study the binding of ligands to the proteins embedded in the SMALPs and other nanodiscs.

Optical spectroscopic techniques for SMALPs

The biophysical techniques 'arsenal' for SMALP characterisation includes a range of spectroscopic techniques that have been successfully used in the past to investigate conformational changes within proteins. In this

section we will explore how spectroscopic methods might be, or have been, deployed on polymer encapsulated samples of membrane proteins. The brevity of the review precludes a detailed discussion of all forms of optical spectroscopy so for the scope of this review discussions will be limited to optical spectroscopy in the visible and ultraviolet part of the spectrum. This region encompasses electronic transitions of chromophores found in the side chains and back bone of the protein as well as those of prosthetic groups and co-factors.

Absorbance spectroscopy

Absorbance spectroscopy is probably the most used spectroscopic technique in biophysics. The Beer-Lambert law allows the method to provide a trivial method for quantifying chromophores (like proteins) in solution. However, when applied to membrane proteins in polymer–lipid particles like SMALPs several issues need to be acknowledged. Key amongst these are the absorbance signals that come from the polymer itself. Polymers that include aromatic groups, like the styrene in SMA, are likely to absorbance in the UV/visible region. The styrene group shows a significant peak at 245 nm which tails of rapidly towards 300 nm [51].

This means that simple recording of absorbance at 280 nm, often used to quantify protein needs to consider the styrene absorbance from the polymer It should be noted however that this effect can be somewhat minimised by using a higher wavelength (e.g. 295 nm, if tryptophans are present in your sample) to assess protein concentration. In comparison, DIBMA, which does not have styrenes within its structure does allow proteins to be quantified using a 280 nm absorbance reading [39].

Circular dichroism

Circular dichroism (CD) spectroscopy provides a data on the conformation of backbone and aromatic side chains of proteins by measuring the differential absorbance of left- and right-handed circular polarised light by chiral elements in proteins. Far UV CD (<230 nm) provides quantitative information on the presence of secondary structures (α-helix and β-sheet) in a protein while far UV CD (230–300 nm) provides non-quantitative information on the relative conformations of aromatic side chains [52].

One might expect that the presence of an absorbance signal from the polymer could disrupt CD, however from the very first SMALP paper it has been clear that CD is compatible for use with SMALP solubilised proteins. This is because although polymers like SMA and DIBMA absorb in the UV, their lack of chirality means that they have no CD signal (Figure 5). This means that CD can be used to provide invaluable data on the secondary structures within membrane proteins encapsulated in SMA [6,53,54]. We have also used the temperature dependence of near UV signals to analyse the stability of membrane proteins [6,53,54]. Previous work on CD of membrane proteins in detergents highlighted a significant challenge caused interfering signals from detergent absorbance and light scattering from micelles (detergent and protein:detergent) [55]. This led to significant issues recording far UV CD of such samples driving the use of intense light sources offered by Synchrotron sources.

In contrast, the smaller size and high homogeneity of polymer-lipid particles mean they have much lower scattering signals. In addition, solutions containing SMALP solubilised proteins do not contain any micellar material, unlike detergents, minimising the potential for interference. This means that membrane proteins solubilised using polymer-based methods are likely to yield higher quality CD data than equivalent samples solubilised in detergent. With the advantage that polymer encapsulation methods will allow CD data to be collected on a benchtop instrument to a low wavelength. This adds significantly to the information gained from the method, increasing the accuracy of deconvolution algorithms (like SELCON3 and CDSSTR) in determining the secondary structure of the protein. This is enhanced even further by using available membrane protein basis sets for the calculations [56].

Fluorescence spectroscopy

Fluorescence spectroscopy provides a third, and very powerful, option for researchers who are particularly interested in studying folding or ligand binding of membrane proteins. Protein fluorescence studies are split into two types, those exploiting the natural, or intrinsic, fluorescence and those exploiting a non-natural, or extrinsic fluorophore. Since the 1970s studies have exploited signals from both sources to explore conformational dynamics of proteins. The number and range of techniques is beyond this mini review but details of most of these are found in comprehensive reviews [57]. The fundamental process of fluorescence involves the absorbance of a photon of light by a fluorophore which then re-emits a photon of lower energy. Measurements

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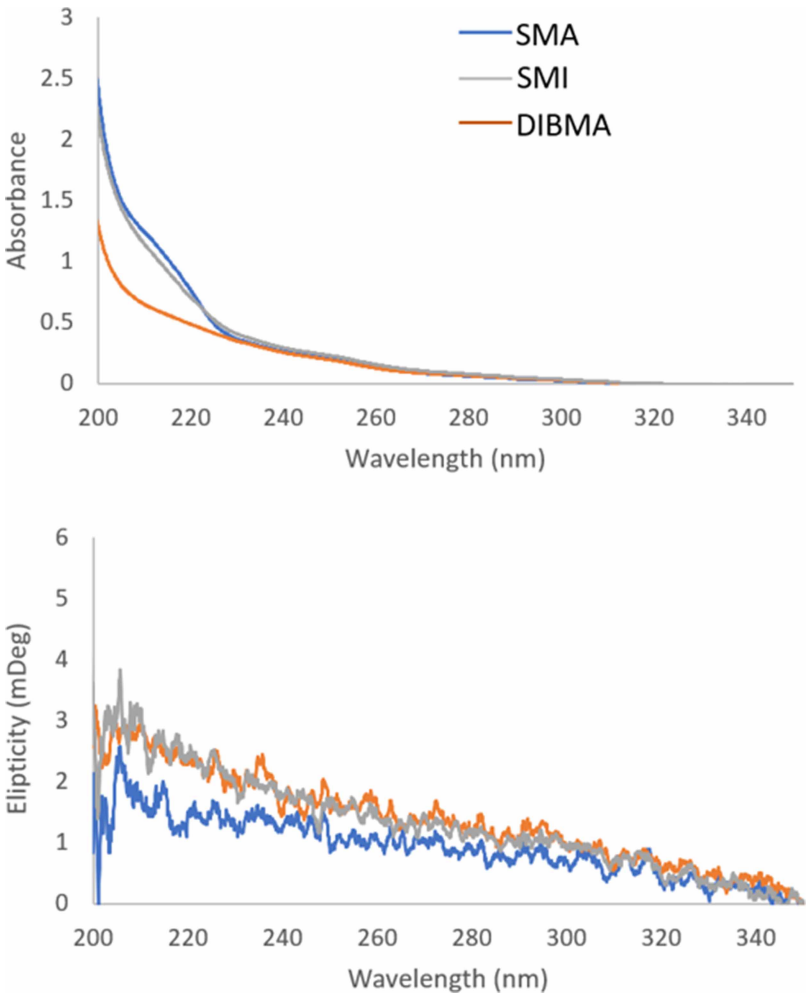

Figure 5. Polymer-only CD spectra. SMA (blue), DIBMA (orange) and SMI (grey) were prepared in 50 mM Tris, 150 mM NaCl, at a final concentration of 0.025% w/v.

Top, shows the CD signals from each sample which have no significant features. The slight upward slope of these data is an indication of scattering from polymer aggregates in the solution which do not occur when assembled into a disc. *Bottom*, shows the absorbance for the same samples. The presence of absorbing groups for styrene containing polymers can be seen between 200 and 220 nm with only minimal extra absorbance at 240 nm. Data collected on a JASCO J1500 spectropolarimeter.

of the intensity, time dependency and anisotropy of the emitted photon can then be used to provide information on the orientation, structural fluctuations, and solvent accessibility of the fluorophore. Such measurements offer relatively simple methods to explore the folding of proteins as well as ligand binding and the formation of multiprotein complexes [57].

The optically benign nature of the polymers used in membrane protein encapsulation (see Absorbance spectroscopy section) make them highly complementary to these techniques. This is reflected in the increasing numbers of papers that have used fluorescence and polymer encapsulation. One of the earliest SMALP studies demonstrated that intrinsic tryptophan fluorescence signals from SMALP solubilised KcsA could be measured and used to provide information on the structure and stability of the protein [54]. Other research showed that extrinsic fluorescence anisotropy could be used to measure ligand binding to SMALP solubilised AcrB [29], and a similar method measured ligand binding to an ABC transporter [13]. More recently fluorescence has been used to detect the conformational changes that occur upon binding, providing a method to probe the ligand-induced activation of GPCRs [58].

Each of these methods rely on the measurement of a population of solubilised proteins in solution, and recent publications used fluorescence of individual solubilised proteins to understand ligand binding. Fluorescence correlation spectroscopy methods measured ligand binding to a GPCR [14] and ABC-transporter [59] in SMALPs. These results show that polymer encapsulated membrane proteins offer ideal reagents for fluorescence-based studies. In the future methods based on resonance transfer (e.g. bioluminescence resonance energy transfer and fluorescence resonance energy transfer) will also come to the fore. It is also clear that exploitation of the ability to modify of the polymer [60] to include fluorophores could lead to an even wider range of experiments.

Summary

Membrane proteins represent 30–40% of the proteome of life and yet our knowledge of their structures and functions significantly lag soluble globular proteins. This inequality due to the difficulty in obtaining of pure, active membrane protein. This issue was significantly resolved by polymer mediated membrane protein extraction. SMA copolymer and other reagents have simplified membrane protein extraction and improved stability. This review looked at the progress made to use biophysical techniques in the study of polymer encapsulated membrane proteins: hydrodynamic (such as AUC and DLS), thermodynamic (DSC and ITC) and spectroscopic methods (CD, fluorescence, and absorbance). We highlight exemplars of best practice showing any methodological implications of using these new reagent systems. This review shows what can be done with polymer encapsulated membrane proteins so researchers to carry out more in-depth studies of their own membrane protein systems.

Perspectives

- **Importance of the field:** Membrane protein purification with poly(styrene-co-maleic acid) lipid particle (SMALP) assemblies are used to purify and solubilise membrane proteins, maintaining native protein structure and function.
- **Summary of current thinking:** Many biophysical techniques, normally used to study soluble proteins, can be now be used to characterise membrane proteins in SMALPs.
- **Comment on future directions:** Understanding the hydrodynamics, thermodynamics, conformation, and other biophysical properties of SMALPs will advance membrane protein research, and their biomedical applications such as drug discovery, development, and delivery.

Competing Interests

V.F. is employed full-time by Malvern Panalytical. T.R.D. provides paid consultancy to Orbiscope BV.

Author Contributions

S.A.N. and T.R.D. designed and performed research and analysed DLS, SEC, TEM and CD data. S.A.N., T.R.D., and V.F. wrote, revised and proofread the manuscript.

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Abbreviations

AUC, Analytical ultracentrifugation; CD, Circular dichroism; Cryo-EM, Cryo-electron microscopy; Cryo-TEM, Cryo-transmission electron microscopy; DIBMA, Diisobutylene/maleic acid; DIBMALP, Diisobutylene/maleic acid

lipid particle; DLS, Dynamic light scattering; DMPC, 1,2-Dimyristoyl-sn-glycero-3-phosphocholine; DSC, Differential scanning calorimetry; GPCR, G-protein coupled receptor; ITC, Isothermal titration calorimetry; K_D, Dissociation constant; kDa, Kilodalton; LUV, Large unilaminar vesicle; mDa, Megadalton; mol, Mole; MSP, Membrane scaffold protein; nm, Nanometre; PSI, Photosystem I; RAFT, Reversible addition-fragmentation chain transfer polymerisation; SAXS, Small-angle X-ray scattering; SEC, Size exclusion chromatography; SEC-MALS, Size exclusion chromatography-multi angle light scattering; SMA, Poly(styrene-co-maleic acid); SMALP, Poly (styrene-co-maleic acid) lipid particle; SMI, Poly(styrene-co-maleimide); TEM, Transmission electron microscopy; T_M, Thermal transition temperature; UV, Ultraviolet.

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