GPCR–styrene maleic acid lipid particles (GPCR–SMALPs): their nature and potential

Mark Wheatley[2]*, Jack Charlton*, Mohammed Jamshad*, Sarah J. Routledge†[1], Sian Bailey*, Penelope J. La-Borde*, Maria T. Azam*, Richard T. Logan*, Roslyn M. Bill†, Tim R. Dafforn* and David R. Poyner†

*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

†School of Life and Health Sciences, Aston University, Birmingham B4 7ET, U.K.

Abstract

G-protein-coupled receptors (GPCRs) form the largest class of membrane proteins and are an important target for therapeutic drugs. These receptors are highly dynamic proteins sampling a range of conformational states in order to fulfil their complex signalling roles. In order to fully understand GPCR signalling mechanisms it is necessary to extract the receptor protein out of the plasma membrane. Historically this has universally required detergents which inadvertently strip away the annulus of lipid in close association with the receptor and disrupt lateral pressure exerted by the bilayer. Detergent-solubilized GPCRs are very unstable which presents a serious hurdle to characterization by biophysical methods. A range of strategies have been developed to ameliorate the detrimental effect of removing the receptor from the membrane including amphipols and reconstitution into nanodics stabilized by membrane scaffolding proteins (MSPs) but they all require exposure to detergent. Poly(styrene-co-maleic acid) (SMA) incorporates into membranes and spontaneously forms nanoscale poly(styrene-co-maleic acid) lipid particles (SMALPs), effectively acting like a 'molecular pastry cutter' to 'solubilize' GPCRs in the complete absence of detergent at any stage and with preservation of the native annular lipid throughout the process. GPCR–SMALPs have similar pharmacological properties to membrane-bound receptor, exhibit enhanced stability compared with detergent-solubilized receptors and being non-proteinaceous in nature, are fully compatible with downstream biophysical analysis of the encapsulated GPCR.

Introduction

G-protein-coupled receptors (GPCRs) form the largest class of membrane proteins in the human genome with >800 unique GPCRs. These receptors regulate a plethora of physiological responses which has made them the primary focus for therapeutic intervention. Such is the importance of GPCRs to cell signalling and health that ~50 % of all prescribed drugs and ~25 % of the top-selling drugs generate their effects by modulating the function of members of this receptor family [1]. The natural activators of GPCRs are very diverse in their physico-chemical characteristics ranging from photons and small biogenic amines to peptides and large glycoproteins. Despite this pronounced diversity in the nature of the agonists, these receptors share a conserved protein architecture comprising a bundle of seven transmembrane (TM) helices linked by extracellular loops (ECLs) and intracellular loops (ICLs) [2]. GPCRs have been subdivided into families on the basis of sequence conservation [3], with three of these families being of particular importance: the rhodopsin/β-adrenergic receptor family (Family A), the secretin receptor family (Family B) and the metabotropic glutamate receptor family (Family C). The largest of these families by far is Family A.

GPCRs are highly dynamic in their conformation

It was long-thought that GPCRs were simply on/off switches which existed in two conformations, with the 'on' conformation being induced by the binding of an agonist. The activated receptor then activated a specific G-protein to initiate an intracellular signal. In contrast, it was thought that antagonists did not induce this conformational change but merely occupied the binding site thereby preventing activation by agonist. Subsequently, it was proposed that rather than acting as simple on/off switches, GPCRs existed in an equilibrium between the inactive (R) conformation and the active (R*) conformation and that agonists and inverse agonists stabilized the conformations R* and R respectively. It is now recognized that GPCR signalling is extremely complex and that the receptors can exist in a spectrum of conformational states. Individual GPCRs can signal

Key words: adenosine receptor, detergent-free, G-protein-coupled receptor (GPCR), membrane protein solubilization, protein thermostability, poly(styrene-co-maleic acid) lipid particle (SMALP).

Abbreviations: $A_{2a}R$, adenosine A_{2a} receptor; $A_{2a}R$–DDM, DDM-solubilized $A_{2a}R$; $A_{2a}R$–SMALP, SMALP-solubilized $A_{2a}R$; DDM, *n*-dodecyl-β-D-maltopyranoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; MSP, membrane scaffolding protein; SMA, poly(styrene-co-maleic acid); SMALP, SMA lipid particle; $V_{1a}R$, V_{1a} vasopressin receptor.

[1] **Present address: Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, U.K.**

[2] **To whom correspondence should be addressed (email m.wheatley@bham.ac.uk).**

through multiple intracellular cascades by activating more than one class of G-protein and can also initiate G-protein-independent signalling such as β-arrestin-dependent GPCR activation of mitogen-activated protein kinase (MAPK) [4]. Some agonists activate multiple signalling cascades equally whereas other agonists are 'biased' in that they selectively activate one signalling pathway over another. Furthermore, the pharmacological classification of an individual ligand binding to a GPCR can be dictated by the signalling system being assayed. For example, the peptide SPG binds to the V_{1a} vasopressin receptor (V_{1a}R) which stimulates MAPK but blocks V_{1a}R-mediated inositol phosphate signalling [5]. Full agonists and partial agonists possessing different sub-sets of functional groups stabilize different receptor conformations [6,7] and establish different networks of hydrogen bonds in the binding site [8]. Consequently, instead of possessing just one R* conformation, activated GPCRs can sample a wide spectrum of distinct active receptor conformations (R^*, $R^{*\prime}$, $R^{*\prime\prime}$ etc.), with different efficacies for different signalling systems. The precise receptor conformation stabilized by a particular ligand will dictate that ligand's pharmacological profile. A completely new level of conformational complexity is presented by allosteric ligands, which bind to sites which are discrete to the classical (orthosteric) binding site. Allosteric ligands alter the receptor conformation upon binding thereby tuning the GPCR signalling up or down [9]. From what has been said above, it can be appreciated that the normal functioning of a GPCR requires the receptor protein to populate a wide range of conformational states. Defining these multifarious conformational states and characterizing the transition between states is a pre-requisite to fully understanding GPCR signalling. Such experiments usually require purified GPCR. However, the flexible and highly dynamic nature inherent in the receptor protein presents a serious obstacle to such studies.

Studying isolated GPCRs – some problems

In common with integral membrane proteins in general, GPCRs have evolved to exist embedded in a lipid bilayer. This requires the protein to stably interact with two discrete physico-chemical environments simultaneously; (i) the aqueous phase plus charged lipid headgroups of the membrane and (ii) the hydrophobic membrane interior. Both of these environments contribute to the overall functional protein fold of the receptor. When GPCRs are studied *in situ* this is not a problem, but it becomes a problem when experiments require the receptor to be extracted from the complex environment of the plasma membrane. This has universally required the use of a class of surfactants commonly referred to as detergents. Although detergents are very good at molecular dispersal and in gross terms the detergent micelles approximate to the physico-chemical properties of a membrane, micelles are actually poor mimics of the plasma membrane bilayer. The solubilization process strips away the annular lipid in close association with the protein and it is thought that this removal of lipid from membrane proteins may be the most common cause of solubilization-induced loss of function [10]. The lipid components of native cell membranes are very heterogeneous with respect to structure, head group and acyl chains and membrane proteins can have a specific requirement for a particular lipid. It is well-established that GPCRs can be affected by the nature of juxtaposed lipid. Cholesterol can modulate receptor conformation [11] and function [12,13]. Indeed a specific cholesterol binding site incorporating a 'cholesterol consensus motif' has been proposed for some GPCRs following the identification of cholesterol in GPCR crystal structures [14]. Very recently, it has been shown that phospholipids can act as allosteric regulators of GPCRs with phosphatidylglycerol and phosphatidylethanolamine favouring active and inactive conformational states of the β_2-adrenergic receptor (β_2-AR) respectively [15]. Disruption of the lipid bilayer by detergent not only removes these structural nuances, it also removes the lateral pressure exerted by the bilayer structure on the embedded membrane proteins. This lateral pressure has been shown to be important for maintaining the native fold and activity of membrane proteins [16,17]. It would be expected that removal of lateral pressure would be particularly disruptive to integral membrane proteins that are conformationally dynamic, such as GPCRs. Consequently, detergent solubilization of membrane proteins typically results in protein instability and progressive loss of function. Continued presence of detergent is required to prevent aggregation of detergent-solubilized protein despite the detrimental stability implications cited above. These problems are reflected in the relatively very small number of high-resolution crystal structures for membrane proteins compared with soluble proteins.

Strategies for studying isolated GPCRs

A significant advance was made by the development of a new detergent which was less perturbing to GPCR conformation [18] but receptor stability remained an issue. One approach to increasing the stability of GPCRs in detergent is to alter the receptor sequence to generate a modified protein with significantly greater tolerance to detergent exposure. This has been achieved by two different methodologies: (i) a programme of extensive mutagenesis was used to develop a series of specific point mutations that generated a stabilized receptor (referred to as a StaR) in a specific conformation [19] and (ii) a directed evolution method was developed using *Escherichia coli* that allowed the direct selection of GPCRs stable in a pre-selected detergent from libraries containing >100 million variants [20]. As an alternative strategy, the solubilizing detergent used initially to extract the receptor has been replaced with amphipathic polymers (amphipols) [21] or by a stabilizing engineered β-sheet peptide [22]. Sligar and co-workers have developed protocols for encapsulating detergent-solubilized GPCRs in a lipid disc stabilized by an annulus of membrane scaffolding proteins (MSPs) derived

Figure 1 | **Poly(styrene-co-maleic acid).**

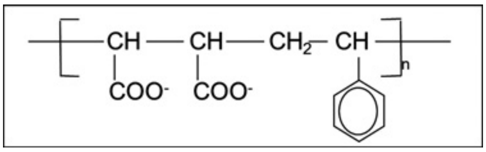
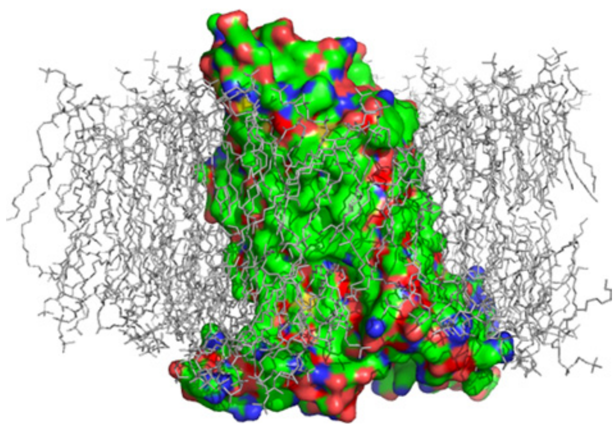


Figure 2 | **An $A_{2a}R$–SMALP**

A schematic representation of an $A_{2a}R$–SMALP viewed from within the plane of the membrane. Only the receptor (coloured) and lipid molecules (grey) are shown.



from apolipoproteins, thereby replacing the detergent micelle with a phospholipid bilayer [23].

Detergent-free extraction of GPCRs within a nanoscale native lipid bilayer

Historically there has been an absolute requirement for detergent to solubilize GPCRs despite the problems this causes. In an ideal situation, the 'solubilization' would remove the GPCR from the membrane still embedded in a nanoscale section of the native bilayer, thereby preserving the closely-associated annular lipid and lateral pressure. This is now possible using poly(styrene-co-maleic acid) (SMA) (Figure 1) which incorporates into membranes and spontaneously forms nanoscale poly(styrene-co-maleic acid) lipid particles (SMALPs), effectively acting like a 'molecular pastry cutter'. In 2009, SMA polymer with a 2:1 ratio of styrene to maleic acid was shown to solubilize membrane proteins [24]. Biophysical analysis of SMALPs revealed that the SMA polymer forms an annulus surrounding and stabilizing a disc of lipid bilayer (~10 nm diameter), possessing the expected thickness of a cell membrane, with the styrene rings of the polymer intercalated between the lipid acyl chains and the maleic acid likely to interact with the lipid headgroups [25]. The behaviour of lipids in a 2:1 SMA polymer SMALP was bilayer-like, as the transition temperature for gel to liquid phase transition of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was only 1 °C lower in the SMALP. A 3:1 SMA polymer has also been used to solubilize proteins including the *Halobacteria* proton pump bacteriorhodopsin [26]. However, the DMPC transition temperature in a 3:1 SMA polymer SMALP was considerably reduced (~10 °C) indicating a perturbation of the lipid bilayer. This could be important as membrane fluidity could impact on the conformational flexibility of dynamic proteins like GPCRs. We used 2:1 SMA polymer to solubilize the adenosine A_{2a} receptor ($A_{2a}R$) and purify it to homogeneity [27]. $A_{2a}R$-expressing membranes of the yeast *Pichia pastoris*, at a final concentration of 40 mg/ml (wet weight) were incubated with SMA (2.5 % w/v final concentration) for 2 h at 25 °C with gentle stirring. Non-solubilized material was sedimented at 100000 $\times$ ***g*** for 1 h at 4 °C, to yield a supernatant containing SMALP-solubilized $A_{2a}R$ ($A_{2a}R$–SMALP) [27]. This was the first time that a GPCR had been solubilized and purified in the total absence of detergent at any stage. The efficiency of extraction of active $A_{2a}R$ expressed in HEK293T cells using our SMALP approach was directly compared with that of *n*-dodecyl-β-D-maltopyranoside (DDM), a detergent commonly employed to solubilize GPCRs. The total binding of the $A_{2a}R$–SMALP was 2.0 ± 0.24 pmol/mg of protein ($n = 3$) equivalent to a yield of 23.3 ± 2.7 % compared with the original HEK293T cell membrane preparation. This was similar to the recovery seen from detergent (DDM) solubilization of $A_{2a}R$ (27.6 ± 11.4 %, $n = 3$). However, it was noted that the recovery with $A_{2a}R$–SMALP was less variable than the DDM-solubilized $A_{2a}R$ ($A_{2a}R$–DDM) [27]. It has been our experience that the extraction of GPCR–SMALPs is equally effective from a range of commonly employed GPCR expression systems, including yeast (*P. pastoris*), mammalian cells (HEK293T and COS-7 cells) and insect cells (*Sf*9 cells). Given that the $A_{2a}R$ encapsulated in the SMALP had been embedded in native membrane (Figure 2) throughout the solubilization process, we hypothesized that it would display increased stability compared with detergent-solubilized $A_{2a}R$. We made a direct comparison between the thermostability of $A_{2a}R$ solubilized by DDM and $A_{2a}R$–SMALP, using radioligand binding assays with [^{3}H]ZM241385 to monitor preservation of receptor conformational integrity. We established that the $A_{2a}R$–SMALP displayed increased thermostability over $A_{2a}R$–DDM to a range of challenges including storage at 37 °C and storage at 4 °C. Repeated freeze–thaw cycles are particularly damaging to detergent-solubilized GPCRs. $A_{2a}R$–DDM lost all binding capability after just one freeze–thaw whereas the $A_{2a}R$–SMALP binding capability was undiminished after five freeze–thaw cycles [27]. Such is the stability of the $A_{2a}R$–SMALP that it could be freeze–dried on to a tube and when re-hydrated with buffer retained ~70 % of its binding capability. The improved stability of the GPCR was independent of the source of receptor as this phenomenon was observed with $A_{2a}R$–SMALP generated from either the yeast *P. pastoris* or mammalian HEK293T cells. The lipid composition of yeast and mammalian cells differ, particularly with respect to cholesterol which is replaced by ergosterol in yeast [28] but the thermostability endowed by the SMALP on $A_{2a}R$ was comparable between the two expression systems.

GPCR–SMALP facilitates biophysical analysis

A great strength of the GPCR–SMALP is that the copolymer stabilizing the nanoparticle is non-proteinaceous in nature. Consequently it does not interfere with analysis of the encapsulated GPCR protein using biophysical methods such as CD [27]. The GPCR–SMALP would also facilitate the use of spectroscopic analysis of GPCR conformational changes using endogenous tryptophan fluorescence or introduced fluorescent moieties such as IAEDANS or bimane. The SMALP particles may however be too large for NMR currently. In contrast, a range of biophysical methods cannot be easily applied to GPCRs embedded in MSP-nanodiscs, as the discs effectively have an inherent contaminant in the form of the stabilizing scaffolding protein which can interfere with studies on the reconstituted GPCR protein.

Conclusion and future perspectives

A range of self-assembly systems has been developed which can be applied to studying and purifying GPCRs, including bicelles, amphipols, MSP-stabilized nanodiscs and SMALPs [29] but only the approach exploiting SMALPs is totally detergent-free, retains lateral pressure and preserves the native annular lipid environment of the receptor throughout. It is anticipated that GPCR–SMALPs will facilitate our understanding of fundamental GPCR molecular mechanisms using biophysical techniques. GPCR–SMALPs may also have general utility in a range of receptor-based assays linked to drug discovery.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council [grant numbers BB/I020349/1 (to M.W. and T.R.D.) and BB/I019960/1 (to D.R.P. and R.M.B.)].

References

1 Lagerström, M.C. and Schiöth, H.B. (2008) Structural diversity of G-protein-coupled receptors and significance for drug discovery. Nat. Rev. Drug Discov. **7**, 339–357 CrossRef PubMed

2 Wheatley, M., Wootten, D., Conner, M.T., Simms, J., Kendrick, R., Logan, R.T., Poyner, D.R. and Barwell, J. (2012) Lifting the lid on G-protein-coupled receptors: the role of extracellular loops. Br. J. Pharmacol. **165**, 1688–1703 CrossRef PubMed

3 Schiöth, H.B. and Fredriksson, R. (2005) The GRAFS classification system of G-protein-coupled receptors in comparative perspective. Gen. Comp. Endocrinol. **142**, 94–101 CrossRef PubMed

4 Azzi, M., Charest, P.G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M. and Piñeyro, G. (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. Proc. Natl. Acad. Sci. U.S.A. **100**, 11406–11411 CrossRef PubMed

5 MacKinnon, A.C., Tufail-Hanif, U., Wheatley, M., Rossi, A.G., Haslett, C., Seckl, M. and Sethi, T. (2009) Targeting V1a vasopressin receptors with [Arg6, D-Trp7,9, NmePhe8]Substance P (6–11) identifies a strategy to develop novel anti-cancer therapies. Br. J. Pharmacol. **156**, 36–47 CrossRef PubMed

6 Swaminath, G., Xiang, Y., Lee, T.W., Steenhuis, J., Parnot, C. and Kobilka, B.K. (2004) Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. J. Biol. Chem. **279**, 686–691 CrossRef PubMed

7 Swaminath, G., Deupi, X., Lee, T.W., Zhu, W., Thian, F.S., Kobilka, T.S. and Kobilka, B. (2005) Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. J. Biol. Chem. **280**, 22165–22171 CrossRef PubMed

8 Warne, T., Moukhametzianov, R., Baker, J.G., Nehme, R., Edwards, P.C., Leslie, A.G.W., Schertler, G.F. and Tate, C.G. (2011) The structural basis for agonist and partial agonist action on a b1-adrenergic receptor. Nature **469**, 241–245 CrossRef PubMed

9 Wootten, D., Christopoulos, A. and Sexton, P. (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. Nat. Rev. Drug Discov. **12**, 630–644 CrossRef PubMed

10 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 CrossRef PubMed

11 Muth, S., Fries, A. and Gimpl, G. (2011) Cholesterol-induced conformational changes in the oxytocin receptor. Biochem. J. **437**, 541–553 CrossRef PubMed

12 Gimpl, G., Burger, K. and Fahrenholz, F. (1997) Cholesterol as modulator of receptor function. Biochemistry **36**, 10959–10974 CrossRef PubMed

13 Pang, L., Graziano, M. and Wang, S. (1999) Membrane cholesterol modulates galanin-GalR2 interaction. Biochemistry **38**, 12003–12011 CrossRef PubMed

14 Hanson, M.A., Cherezov, V., Griffith, M.T., Roth, C.B., Jaakola, V.P., Chien, E.Y., Velasquez, J., Kuhn, P. and Stevens, R.C. (2008) A specific cholesterol binding site is established by the 2.8 Å structure of the human β2-adrenergic receptor. Structure **16**, 897–905 CrossRef PubMed

15 Dawaliby, R., Trubbia, C., Delporte, C., Masureel, M., Van Antwerpen, P., Kobilka, B.K. and Govaerts, C. (2016) Allosteric regulation of G protein-coupled receptor activity by phospholipids. Nat. Chem. Biol. **12**, 35–39 CrossRef PubMed

16 Charalambous, K., Miller, D., Curnow, P. and Booth, P.J. (2008) Lipid bilayer composition influences small multidrug transporters. BMC Biochem. **9**, 31 CrossRef PubMed

17 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 CrossRef PubMed

18 Chae, P.S., Rasmussen, S.G., Rana, R.R., Gotfryd, K., Chandra, R., Goren, M.A., Kruse, A.C., Nurva, S., Loland, C.J., Pierre, Y. et al. (2010) Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. Nat. Methods **7**, 1003–1008 CrossRef PubMed

19 Serrano-Vega, M.J., Magnani, F., Shibata, Y. and Tate, C.G. (2008) Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. Proc. Natl Acad. Sci. U.S.A. **105**, 877–882 CrossRef PubMed

20 Scott, D.J. and Plückthun, A. (2013) Direct molecular evolution of detergent-stable G-protein-coupled receptors using polymer encapsulated cells. J. Mol. Biol. **425**, 662–677 CrossRef PubMed

21 Banères, J.L., Popot, J.L. and Mouillac, B. (2011) New advances in production and functional folding of G-protein-coupled receptors. Trends Biotechnol. **29**, 314–322 CrossRef PubMed

22 Tao, H., Lee, S.C., Moeller, A., Roy, R.S., Siu, F.Y., Zimmermann, J., Stevens, R.C., Potter, C.S., Carragher, B. and Zhang, Q. (2013) Engineered nanostructured β-sheet peptides protect membrane proteins. Nat. Methods **10**, 759–761 CrossRef PubMed

23 Bayburt, T.H. and Sligar, S.G. (2010) Membrane protein assembly into Nanodiscs. FEBS Lett. **584**, 1721–1727 CrossRef PubMed
24 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 CrossRef PubMed
25 Jamshad, M., Grimard, V., Idini, I., Knowles, T.J., Dowle, M.R., Schofield, M., Sridhar, P., Lin, Y.-P., Finka, R., Wheatley, M. et al. (2014) Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins. Nano Res. **8**, 774–789 CrossRef
26 Orwick-Rydmark, M., Lovett, J.E., Graziadei, A., Linholm, L., Hicks, M.R. and Watts, A. (2012) Detergent-free incorporation of a seven-transmembrane receptor protein into nanosized bilayer lipodisq particles for functional and biophysical studies. Nano Lett. **12**, 4687–4692 CrossRef PubMed
27 Jamshad, M., Charlton, J., Lin, Y.-P., Routledge, S.J., Bawa, Z., Knowles, T.J., Overduin, M., Dekker, N., Dafforn, T.R., Bill, R.M. et al. (2015) G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. Biosci. Rep. **35**, e00188 CrossRef PubMed
28 Finean, J.B., Coleman, R. and Michell, R.H. (1978) Membranes and their Cellular Functions, 2nd ed., Blackwell Scientific Publications, Oxford, UK.
29 Jamshad, M., Lin, Y-P., Knowles, T.J., Parslow, R.M., Harris, C., Wheatley, M., Poyner, D.R., Bill, R.M., Thomas, O.R.T., Overduin, M. and Dafforn, T.R. (2011) Surfactant-free purification of membrane proteins with intact native membrane environment. Biochem. Soc. Trans. **39**, 813–818 CrossRef PubMed

Received 12 February 2016
doi:10.1042/BST20150284