Proteopolymersomes: *In vitro* production of a membrane protein in polymersome membranes

Madhavan Nallani,[a),b)] Mirjam Andreasson-Ochsner,[a)] Cherng-Wen Darren Tan, and Eva-Kathrin Sinner[c)]
*Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research), 3 Research Link, Singapore 117602*

Yudi Wisantoso and Susana Geifman-Shochat
Nanyang Technological University, School of Biological Sciences, 60 Nanyang Avenue, Singapore 637551

Walter Hunziker[d)]
*Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), 61 Biopolis Dr, Singapore 138673*

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Polymersomes are stable self-assembled architectures which mimic cell membranes. For characterization, membrane proteins can be incorporated into such bio-mimetic membranes by reconstitution methods, leading to so-called proteopolymersomes. In this work, we demonstrate the direct incorporation of a membrane protein into polymersome membranes by a cell-free expression system. Firstly, we demonstrate pore formation in the preformed polymersome membrane using α-hemolysin. Secondly, we use claudin-2, a protein involved in cell-cell interactions, to demonstrate the *in vitro* expression of a membrane protein into these polymersomes. Surface plasmon resonance (Biacore) binding studies with the claudin-2 proteopolymersomes and claudin-2 specific antibodies are performed to show the presence of the *in vitro* expressed protein in polymersome membranes. © *2011 American Vacuum Society*. [DOI: 10.1116/1.3644384]

I. INTRODUCTION

Cells are able to isolate and use multiple complex reaction pathways by compartmentalizing them in membranes. Such biological membranes are complex assemblies of lipids, proteins and carbohydrates that not only allow for cellular compartmentalization, but also through the control of the permeability allow cells to communicate with each other and the external milieu. Through lipid-protein, protein-protein and protein-ligand interactions, membranes are involved in many vital cellular processes, such as signal transduction, ion transport, cell-cell recognition and stimulus detection.[1] In addition, integral membrane proteins constitute 30–40% of all human genome encoded proteins. All of these observations underscore the key importance of membrane proteins as diagnostic and pharmaceutical drug targets.[2] However, little is known about the structure/function relationships for most integral membrane proteins because it remains a challenge to characterize them by current analytical methods. This is mainly a consequence of the difficulty to express membrane proteins and isolate them in their functional conformation, which typically requires embedding them into an appropriate model membrane.

Recently, we have reported the *in vitro* synthesis and spontaneous incorporation of functional membrane proteins into artificial tethered lipid bilayer planar membranes to overcome the difficulties of purification, isolation and reconstitution.[3] Tethered lipid membranes or similar liposomal systems have been established as model platforms for membrane protein characterization.[4] However, for realistic applications in, for example biosensing, lipid based systems are limited due to the labile nature of lipid assemblies. To overcome the issues of stability, block copolymers have been explored as more stable alternatives, both in vesicular (polymersomes)[5] and in planar membrane form.[6] The possibility of inserting membrane proteins into block copolymer membranes has been shown by reconstituting isolated membrane proteins, in particular bacterial porins, in block copolymer vesicles.[7–9]

Here, we report the first successful insertion of an integral membrane protein into block copolymer membrane vesicles (proteopolymersomes) by a cell-free *in vitro* synthesis method (Fig. 1). The ability to incorporate membrane proteins into a stable matrix, directly, via *in vitro* synthesis opens up new possibilities for their characterization. It may also overcome issues such as cytotoxicity, misfolding and aggregation which occur in conventional over-expression and reconstitution of membrane proteins[10] into lipid membrane models. We used polymersomes made of polybutadiene-polyethyleneoxide (PBD-PEO)[11] as an expression platform and claudin-2 (Cldn2), a membrane protein involved in cell-cell interaction,[12] as a model membrane protein. Staphylococcal α-hemolysin was used as a control to demonstrate the spontaneous insertion of a membrane protein into the PBD-PEO membrane.

II. EXPERIMENT

A. Polymersome and liposome preparation

The synthesis and polymersome preparation of the diblock copolymer polybutadiene-polyethylene oxide ($[PBD]_{21}$-

[a)]Authors contributed equally.
[b)]Electronic address: nallanim@imre.a-star.edu.sg.
[c)]Electronic address: sinnere@imre.a-star.edu.sg.
[d)]Electronic address: hunziker@imcb.a-star.edu.sg.

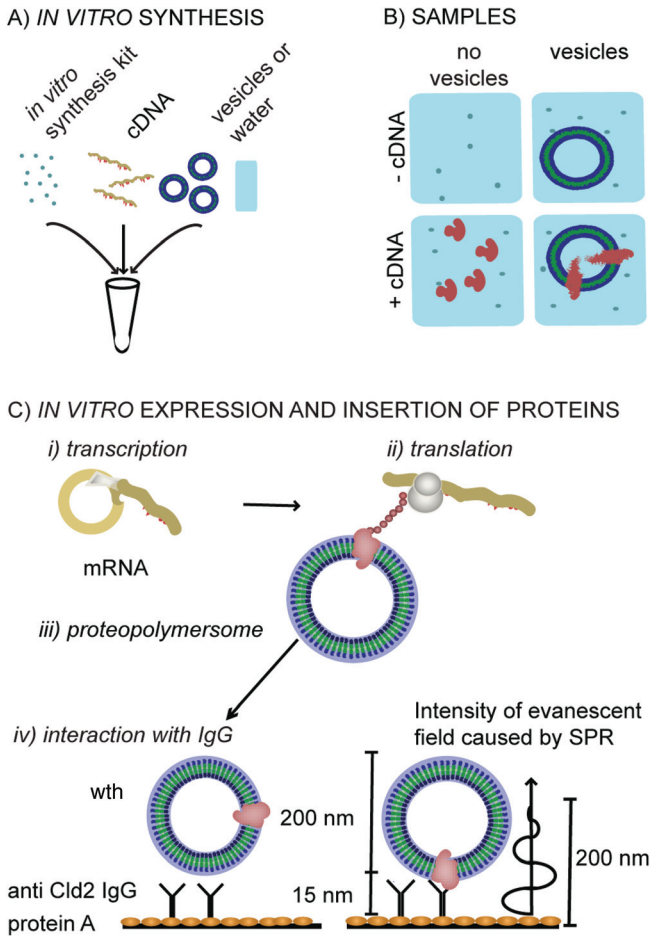

FIG. 1. (Color) Schematic representation of proteopolymersomes (*in vitro* production and insertion of membrane protein in polymersome membrane). (a) The in vitro synthesis kit, the cDNA, and vesicle solution are mixed together in order to start the expression of the membrane protein. The term vesicle refers here to both polymersomes as well as liposomes. (b) Summary of the samples we used during this study. (c) Expression of membrane protein into the polymersome and interaction of proteopolymersomes with anti-Cldn2 IgG immobilized via Protein A onto a Biacore CM5 chip. The binding of Cldn2-containing polymersomes to the surface was monitored by SPR, which uses the evanescent field to detect changes in the refractive index in the linear regime of the evanescent field, which is approximately 200 nm in distance to the surface.[20]

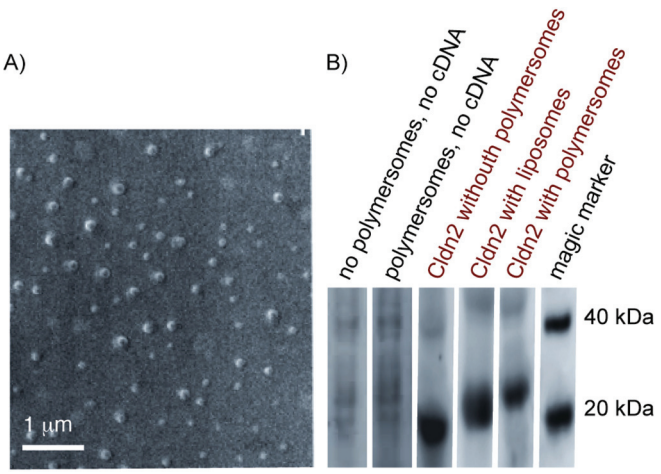

FIG. 2. (Color) (a) SEM image shows PBD-PEO polymersomes with a diameter of about 150−200 nm. These images were taken after purifying them from the in vitro synthesis reaction mixture. (b) Representative Western blot of Cldn2 expressed in the presence or absence of PBD-PEO polymersome or liposomes.

$[PEO]_{12}$, named here PBD-PEO) has been described in detail elsewhere.[11,13] Briefly, PBD-PEO polymersomes (10 mg/ml; 1.0 ml) were prepared by dissolving 10 mg of PB-PEO in 300 μL of tetrahydrofuran (THF) and the solution was added drop wise to 700 μl of ultrapure water. The mixture was subsequently left at room temperature for at least 12 h. The resulting suspension was purified by dialysis against ultrapure water (Spectrum Laboratories, Inc; MWCO 50 000) to remove THF. For characterization of the PBD-PEO polymersomes, scanning electron microscopy (SEM, Joel FESEM JSM6700F) and dynamic light scattering (DLS, Brookhaven BI-APD) were performed. The polymersomes were in the range of 150–200 nm as shown in Fig. 2(a). This agreed well with polymersome sizes reported in the literature.[11]

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, USA. DOPC was extruded following a modified protocol from MacDonald *et al.*[14] Briefly, 5 mg of DOPC was dissolved in chloroform, dried under nitrogen flow and rehydrated in 1 ml of PBS. Extrusion (LiposoFast extruder, Avestin) through two layers of polycarbonate membranes, each with 100 nm pore size, produced nominally 100 nm unilamellar vesicles.

B. Insertion of α-hemolysin into preformed polymersomes

For calcein leakage assays, calcein (Sigma Aldrich) was encapsulated inside the polymersomes at self-quenching concentration (30 mM). Polymersomes encapsulated with calcein were dialyzed to remove the nonencapsulated probe. 20 μl of α-hemolysin monomer solution (0.5 mg/ml in NaCl/MOPS buffer (0.1 M NaCl/0.01 M MOPS, *p*H 7), was added to the polymersomes in a 96 well plate and the increase in the fluorescence intensity due to calcein leakage was monitored (excitation 495 nm, emission 515 nm) by TECAN plate reader.

C. *In vitro* production of claudin-2 (Cldn2) in polymersomes (proteopolymersomes)

The use of cell-free extracts for the cell-free expression of protein is described in literature and commonly used in basic research, molecular diagnostics and high through put screening.[15,16] We employed this method, based on a coupled transcription/translation system, in which complementary DNA (cDNA) encoding the protein of interest is used as a template (see Fig. 1). Briefly, cDNA encoding the human membrane protein, claudin-2 (Cldn2), was inserted into a pTNT T7-promoter expression vector using standard molecular biology techniques. The resultant plasmid was then transformed and amplified in DH10α *Escherichia coli* culture, extracted and finally purified, using standard molecular biology techniques. *In vitro* synthesis of the Cldn2 was then carried out using a wheat germ extract cell-free expression

system (Promega, USA) by adding the amplified plasmid as well as the polymer or lipid vesicles as carrier membranes to the kit. As a control, the same reaction mix without added cDNA was used. The reaction mixtures were prepared according to the supplier's instructions and incubated at 37°C for 90 mins [Fig. 1(a)]. Depending on the type of vesicles (lipid or polymer vesicles) and the presence or absence of cDNA, different samples were produced as summarized in Fig. 1(b).

The polymersomes were filtered to remove impurities from the *in vitro* expression system and also to remove the non-incorporated Cldn2. For this, the polymersome suspension was filtered twice using Amicon centrifugal filtration cartridges (Durapore PVDF 0.1 μm, Millipore, 100 nm cut off) (Eppendorf Mini Plus, 3000 rpm, 3 min). The polymersomes in the supernatant were then resuspended in 100 μl HBS-EP buffer (10 mM Hepes *p*H 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P-20; GE Healthcare) and collected for Western blot or Biacore analysis. Electrophoresis (Nu PAGE 10% Bis-Tris Gel, Invitrogen) and Western blot (Western Breeze, Invitrogen) were performed according to the supplier's manual and the signal intensities of the bands were analyzed using *FUJIFILM Multi Gauge v3.2* image analysis software.

D. Characterization of proteopolymersomes by SPR

The surface plasmon resonance (SPR) experiments were performed on a Biacore 3000 instrument (GE Healthcare). HBS-EP buffer was used.

To capture the polymersomes on the Biacore CM5 chip (Dextran Matrix) (GE Healthcare), Protein A (GE Healthcare) was first immobilized onto the surface using an amine coupling procedure. For this purpose, the carboxylic groups on the surface matrix were activated with a mixture of 0.2 M 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide (GE Healthcare) at 10 μl/min for 10 min. The reactive succinimide esters formed reacted spontaneously with the amine groups of Protein A (20 μg/ml, level of immobilization 2500 RU). Remaining reactive ester groups were inactivated with ethanol-amine (1 molar, *p*H 8.5; GE Healthcare) (10 μl/min, 10 min).

To prevent any unspecific protein interaction, remaining exposed membrane surfaces were then blocked by incubating with bovine serum albumin (BSA, 10 mg/ml in HBS-EP buffer, Sigma, 160 μl @ 5 μl/min). Subsequently, the antibody of interest (monoclonal mouse anti-Cldn2, 1:50 in HBS-EP buffer, Abnova or nonspecific mouse IgG, 1:50 in HBS-EP buffer, Santa Cruz) was injected into the flow cell (65 μl @ 2 μl/min for the anti-Cldn2 IgG, for the nonspecific IgG until saturation of the surface was reached). After stabilization of the baseline, the proteopolymersome solution or pure protein was injected (10 μl @ 2 μl/min). For regeneration of the Protein A surface, 10 mM HCl and 10 mM NaOH was used. As a control experiment, a pure Protein A surface without antibodies was used. SPR data (Δ RU) were normalized against the amount of IgG adsorbed onto the protein A surface. Shown plots are the result of double-referencing against the control surface without antibody immobilization as well as against blank polymersomes ($\Delta\ RU = \Delta\ RU_{\text{surface with antibodies}} - \Delta\ RU_{\text{control surface without antibodies}} - \Delta\ RU_{\text{protein expressed without cDNA}}$). Δ RU was measured at the end of each injection and average values with standard error of the mean were calculated from three independent experiments except the control with liposomes (from two independent experiments).

III. RESULTS AND DISCUSSION

A. Expression of membrane protein in presence of polymersomes

Polymersomes were prepared as described in the Experimental section. After *in vitro* expression, we purified the polymersomes from components of the *in vitro* expression system by filtering with a size cut-off of 100 and characterized them by SEM (see Experimental section). As shown in Fig. 2(a), the proteo-polymersomes are present in the supernatant and the filtering process did not destroy them.

Western blot analysis was performed to confirm the expression and incorporation of the membrane protein into the polymersomes [Fig. 2(b)]. *In vitro* expressed Cldn2 was detected, using Cldn2-specific antibodies, either in the presence or absence of polymersomes as a band of apparent molecular mass of ~22 kDa.[17] Interestingly, the apparent mass of Cldn2 incorporated into the polymersomes was slightly higher compared to that in the absence of polymersomes. This possibly reflects a change in the mobility of the protein due to interaction with the polymer during polyacrylamide gel electrophoresis. Similar changes in electrophoretic mobility have been observed for porins inserted into polymersomes.[18]

In addition, we compared the signal intensities of the bands from the Western blots to determine if the presence of polymersomes or liposomes influenced the expression level of Cldn2 [Fig. 2(b)]. No difference in expression level in the presence of polymersomes or liposomes was however detected. This suggested that the measurements by SPR could be compared since similar amounts of membrane protein were expressed in the samples.

B. Incorporation of membrane proteins into polymersomes

In order to confirm the insertion of membrane proteins into preformed PBD-PEO polymersomes, calcein was encapsulated at self-quenching concentrations. A monomeric solution of the bacterial pore-forming toxin, staphylococcal α-hemolysin (αHL) was then added to the polymersomes. αHL forms an heptameric transmembrane pore in the presence of membranes and should lead to calcein release.[19] Indeed, a clear increase in fluorescence was observed upon addition of αHL which must result from the leakage of calcein by pore-formation in the polymersome membrane. Addition of NaCl/MOPS buffer only did not show any

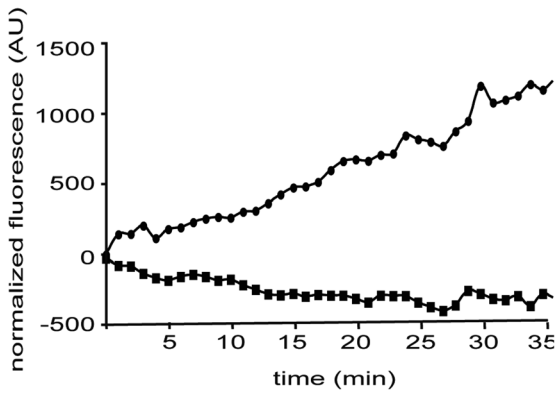

FIG. 3. Increase in fluorescence intensity of calcein due to the dilution from a self-quenching environment (●). Dilution results from the diffusion to the exterior of vesicles through α-hemolysin pore. As a control experiment, MOPS/NaCl buffer was added and no increase in the fluorescence was observed (■).

increase in fluorescence (Fig. 3). Based on this result, we are confident that the membrane protein could be directly incorporated into PBD-PEO polymersomes.

C. *In vitro* synthesis of membrane protein and incorporation into polymersomes

The presence of the *in vitro* synthesized Cldn2 into the polymersomes was then characterized by monitoring the binding of specific antibodies against Cldn2 using a surface plasmon resonance (SPR)[20] biosensor (Biacore[21]). Specifically, the carboxy methylated dextran surface of the sensor chip was first functionalized with protein A, which in turn was functionalized with different immunoglobulins (IgG) [Fig. 1(c)]. Subsequently, either Cldn2-containing polymersomes or polymersomes without Cldn2 (as a control) were injected over the antibody-functionalized surface and polymersome adsorption on the surface was monitored by SPR as a function of time.

As shown in Fig. 4(a), the SPR sensorgram of the Cldn2-containing polymersomes shows a significant increase of RU ($\Delta RU_{monoclonal} \approx 127 +/- 14$), indicating an interaction of the proteopolymersome with the mouse monoclonal anti-Cldn2 IgG-modified surface. Since the concentration of the polymersome and Cldn2 in the suspension cannot be measured accurately on the chip surface, it is not possible to calculate the kinetic constants or affinity of the interactions and we are currently investigating this in more detail. However, it is clear from these results that Cldn2 is present in the polymersome membrane and is specifically recognized by the anti-Cldn2 IgG (see also below).

To further demonstrate the specificity of the Cldn2-containing polymersomes interaction with the anti-Cldn2 IgG surface, several additional control experiments were conducted. To determine the effects of nonspecific binding, polymersomes containing *in vitro* synthesized G protein-coupled odorant receptor 5 (OR5) were applied to the anti-Cldn2 surface. These showed dramatically reduced adsorption (approximately one order of magnitude decrease,

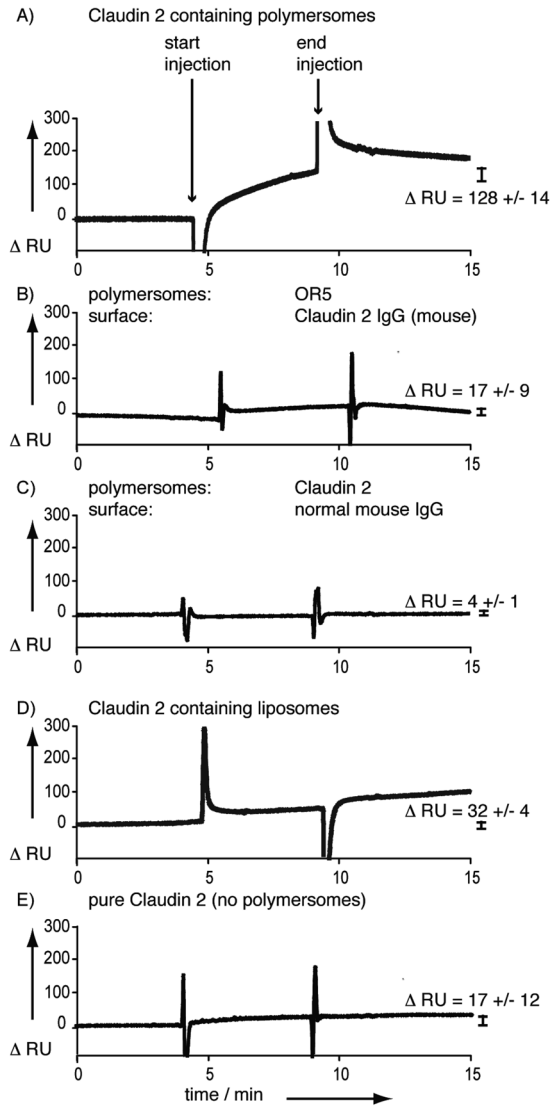

FIG. 4. Representative SPR sensorgrams obtained from the injection of Cldn2-containing polymersomes binding to antibody functionalized chips. (a) Cldn2-containing proteopolymersomes resulted in a significant change of RU indicative of binding to the anti- Cldn2 IgG functionalized surface. (b) Sensorgrams obtained by injection of OR5 containing polymersomes onto anti-Cldn2 functionalized sensor chip surfaces as a control for the unspecific interaction of a protein with the anti-Cldn2 IgG. (c) Cldn2-containing polymersomes interacting with unspecific monoclonal IgG as a control for the unspecific interaction of Cldn2 with an IgG. (d) Cldn2-containing liposomes binding to the anti-Cldn2 IgG functionalized surface. E) Cldn 2 synthesized in the absence of polymersomes. The SPR measurements of the unspecific IgG and OR5 did not show in a significant interaction of the polymersome with the surface.

($\Delta RU_{monoclonal} \approx 17 +/- 9$)) onto the anti-Cldn2-functionalized surface when compared to the polymersomes carrying Cldn2 [Fig. 4(b)]. In addition, to probe the nonspecific interaction of Cldn2 with a normal IgG, the Protein A surface was modified with a nonspecific (or normal) mouse IgG and exposed to the Cldn2-containing polymersomes. Almost no change in RU (Δ RU $\approx 4 +/- 1$) and therefore no binding of the Cldn2-containing polymersomes to the nonspecific mouse IgG was detected [Fig. 4(c)]. These control experiments verify the specificity of the observed interaction

between the Cldn2-containing polymersomes and the anti-Cldn2 IgG-functionalized biosensor chip.

Cldn2 synthesized in the presence of liposomes also showed a change in RU (Δ $RU_{monoclonal} \approx 32 +/- 4$) [Fig. 4(d)]. This specific interaction with the anti-Cldn2 IgG-functionalized surface was much lower than the binding response monitored for Cldn2-containing polymersomes but exceeded that of Cldn2 synthesized in the absence of a membrane system (Δ $RU_{monoclonal} \approx 17 +/- 12$) [Fig. 4(e)]. Because the expression level of Cldn2 in all three samples above were comparable from the Western blots [Fig. 2(b)], we tentatively conclude that only the polymersomes are amenable to analysis by SPR because of their enhance stability. It should be noted however that it is difficult to quantitatively compare the responses obtained by the binding of free Cldn2, Cldn2-containing polymersome and Cldn2-containing liposomes and only qualitative conclusions can be drawn. First of all, the SPR signal decays exponentially with the distance to the sensor surface (complete decay at around 200 nm)[20,22] while the size of the polymersome is ~150–200 nm as characterized by dynamic light scattering (data not shown) and SEM [Fig. 2(a)]. Therefore only part of the polymersome may be present in the SPR detection range. Second, the presence of the polymersome itself contributes to the SPR signal.[20] Finally, steric hindrance due to the size of the polymersome, differences in the capacity of the polymersome, the inability of free Cldn2 to penetrate the dextran matrix and differences in stability between polymersomes and liposomes also affect signal intensity.

IV. SUMMARY AND CONCLUSIONS

From the data presented here we conclude that the expression of an *in vitro* synthesized integral membrane protein, such as Cldn2, in polymer membranes is a successful strategy to generate membrane like assemblies. Presence of the protein was demonstrated by its interaction with specific antibodies. Thus, as an alternative to cellular expression commonly used to characterize integral membrane proteins, we show the feasibility of the *in vitro* synthesis and presence in relatively stable membrane mimics. Compared to incorporation into intrinsically labile lipid bilayer membranes (liposomes), polymersomes offer a new and more stable alternative for studying integral membrane proteins[23] and to develop screening protocols using a relatively robust system.[6] We are currently investigating proteopolymersomes for screening applications.

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