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Nanoscopic Leg Irons: Harvesting of Polymer-stabilized Membrane Proteins with Antibody – Functionalized Silica - Nanoparticles

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Silica – based nanoparticles (SiNPs) are presented to harvest complex membrane proteins, which have been embedded into unilammelar polymersomes *via* membrane assisted protein synthesis (iMAP). Size – optimized SiNPs have been surface-modified with polymer – targeting antibodies, which are employed to harvest the protein – containing polymersomes. The polymersomes mimick the cellular membrane. They are chemically defined and preserve their structural – functional integrity as virtually any membrane protein species can be synthesized into such architecture *via* the ribosomal context of a cellular lysate. The SiNPs resemble 'heavy leg irons ' catching the polymersomes in order to enable gravity – based, generic purification and concentration of such proteopolmyersomes from the crude mixture of cellular lysates.

The cell-based production of membrane proteins comes always along with issues of, aggregation, misfolding, often low yield expression and potential cytotoxicity. Mastering those issues, purification often requires lysis of cells and keeping the membrane proteins in solution by the use of surfactants[1,2]. However, the membrane proteins most often loses structural – functional integrity and are often degraded by proteases. Characterization and long term storage plans are often rendered impossible when it comes to the class of membrane proteins. At present, careful selection of the reconstitution methods used for protein production.[3] As an alternative to surfactant stabilization, we present cell-free synthesis and co-translational insertion of membrane proteins into artificial membranes as an interesting alternative [4–6]. We introduced the method as *in vitro* membrane-assisted protein synthesis (iMAPS) replacing lipid membranes by polymer membranes[7–10]. Such polymeric membranes self-assemble into the form of robust 2D structures [8,11] that can either be tethered to a surface, or formed into spherical vesicles often referred to as polymersomes[8,9,12]. These have successfully even been applied as antigen presenting matrix for vaccination by Nallani et al. [13] in combination with conventionally reconstituted membrane protein species.

However, iMAPS – involves cell lysates, which inherently comprise a complex environment containing membrane remnants, soluble proteins and metabolics of various kinds. As such, it becomes necessary to apply drastic purification measures. So far, liposomes and proteoliposomes, which are lipid vesicles with membrane proteins embedded, had been available – those are often purified using density gradient ultracentrifugation[14–16] or high-speed ultracentrifugation[17]. These methods suffer from either resulting in dilution of the desired protein or in disintegration as liposomes are exposed to destructive shear forces over extended periods of time[14–17]. Alternatively, without shear forces being involved, one could purify His_6-tagged membrane proteins using Ni-NTA supports[10,18] or membrane protein-GFP fusion constructs for fluorescence detection size-exclusion chromatography[19]. Choosing polymeric membrane analogues, we presented the application of centrifugal microfiltration (involving the commercially available Amicon® filters) in order to purify proteopolymersomes from cellular lysates. The method of centrifugal microfiltration and as such has recently been reported by us to be useful in the purification of membrane proteins integrated into polymersomes[6]. However, recent problems with remaining contaminating materials corrupting the structural – functional integrity from the membrane protein of interest pushed us in the development of a novel strategy for proteopolymersome purification.

We propose using a modified immunoprecipitation strategy based on silica nanoparticles decorated with antibodies that have been raised against the respective polymer material forming the proteopolymersomes. In our case, anti-PEG antibodies, targeting the polymer itself, and anti-VSV

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antibodies, able to bind to the used proteins, were immobilized onto the surface of colloidal silica nanoparticles (SiNPs). The resulting antibody-functionalized SiNPs are effectively used for 'harvesting' the membrane protein species of interest. Following the one-step membrane protein synthesis procedure facilitated by iMAPS, such modified SiNPs are added to the iMAPS reaction mix and allowed to bind their targets (Fig. 1).

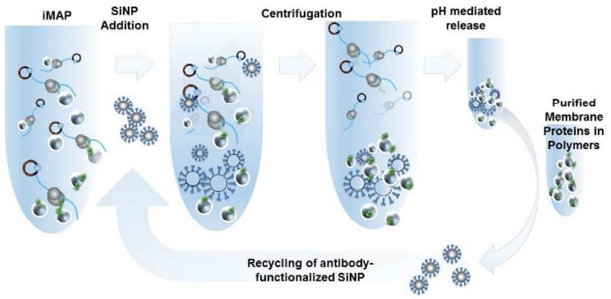

Fig. 1 Schematic overview of iMAP in presence of polymersomes, followed by addition of anti-polymer – functionalized silica nanoparticles (SiNP). Centrifugation of the SiNP with attached proteopolymersomes results in sedimentation of SiNPs with attached proteopolymersomes. Removal of supernatant containing cell lysate components followed by a pH - mediated dissolving of immunocomplex, harvesting of purified proteopolymersomes and regeneration of antibody - functionalized SiNPs for repeating cycles.

Subsequently, the SiNPs would facilitate sedimentation of the immunocomplexes, made from SiNPs and proteopolymersomes, using centrifugation at forces below 1, 700 x g over periods of only minutes. Acidic (10 mM Glycine/HCl, pH 2) as well as alkaline (10 – 100 mM NaOH, pH 12-13) treatment is subsequently carried out in order to dissolve the immunocomplex and 'free' the proteopolymersomes from the nanoparticle load (see supporting information). The resultant proteopolymersomes are subjected to further characterization procedures. Those harsh release conditions were chosen to ensure an efficient release as well as to reduce the non-specific protein adsorption to the polymer surface.

SiNP were synthesized using Stöber's method[20] resulting in spherical, monoparticles (see Fig. 2). SiNP with a diameter of about 550 nm were synthesized by an appropriate selection of NH_3, H_2O and tetraethyl orthosilicate in ethanol. This size was ideal for our use as the nanoparticles were small enough to maintain colloidal stability in PBS, yet provided sufficient mass to enable centrifugation at low forces achievable with standard table-top centrifuges. Furthermore, being three fold larger than the 200 nm polymersomes, the interstices of densely-packed SiNP would still provide sufficient space for polymersome integrity and interaction. In the preparation procedure of the polymersomic giant unilaminar vesicles (pGUVs) we added sucrose as this has been described to enhance stability[21]. Furthermore due to formation of huge immunocomplexes, observable by phase contrast microscopy, a lower centrifugal speed was applicable for pGUVs than for 200 nm sized polymersomes.

The SiNP produced were modified with 3-aminopropyl (trimethoxy) silane (APTES) to introduce primary amine groups, suitable for peptide coupling chemistry for antibody binding (Fig. 2A). In the next step, an incubation of the SiNP with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) was performed. These compounds usually activate carboxylic groups, such as are found in proteins, in order to form peptide bonds with amine groups. However in order to prevent crosslinking between antibodies molecules, we decided to treat only the silica nanoparticle surface instead. We immersed the SiNP in EDC/NHS 0,5M and 0,1M in H_2O for 10 min, and transferred the SiNP after sedimentation into antibody containing buffer solution. This procedure resulted in presence of EDC and NHS only in the unstirred layer being around the surface of the SiNP resulting in covalent binding only when antibodies were in close proximity to the SiNP surface and not among each other. Deactivation of residual active groups was achieved by incubation with 1 M ethanolamine for another 10 min. Surface modifications to the nanoparticles were monitored by zeta potential analysis and were represented as changes in the surface charge (see ESI). Dynamic light scattering yielded a hydrodynamic diameter for the unmodified SiNP of about 540 nm.

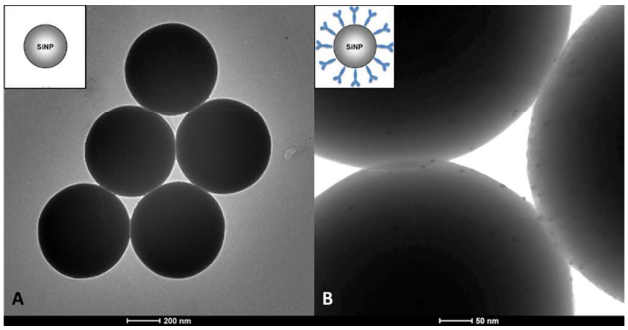

Fig. 2 A: TEM of SiNP surface-modified with APTES and mouse α-VSV antibodies (Sigma). B) TEM of SiNP after binding of immunogold-labelled goat anti-mouse IgG (Sigma, 10 nm gold particle size). The small black spots indicate the presence of gold nanoparticles, and hence, the mouse α-VSV antibody.

The amphiphilic block – copolymer tested by us for producing the polymersomes is PBD-1200-PEO-600 (PolymerSource). This molecule consists of a hydrophobic poly(butadiene) domain conjugated to a hydrophilic poly(ethylene oxide) domain. The monoclonal rabbit antibody raised against poly-ethylene-glycol (α-PEG) is able to bind specifically to the hydrophilic poly(ethylene oxide) domain and is therefore suitable for this harvesting endeavor. The ability of α-PEG-modified SiNP to immunoprecipitate polymersomes was evaluated in two ways: First of all, phase-contrast microscopy was used to determine if pGUVs are co-localized with the anti-PEG antibody - SiNP forming an immunocomplex (Fig. 3A). As a second strategy to show the immunocomplex formation, fluorescent dye labelled polymersomes were used (see ESI).

The polymersomic GUVs were sedimented at 600 x g for 1 min and treated with 100 mM NaOH for 10 min in order to release the polymersome – SiNP immunocomplex. Followed by

another centrifugation step to separate the SiNPs and release pGUVs. The microscopical images in phase contrast mode present the polymersomes as individual spheres as the SiNPs are disconnected (Fig. 3B).

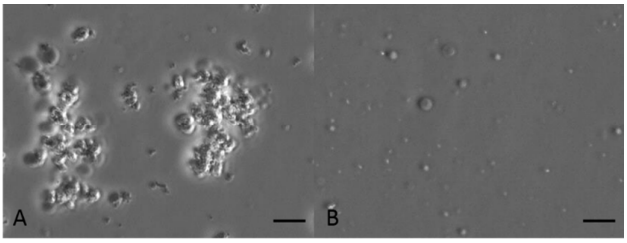

Fig. 3. A) Phase contrast micrograph showing pGUVs of several µm in size forming an immunocomplex with the smaller silica nanoparticles (SiNP) B) Micrograph showing released pGUVs present as individual, non-connected pGUVs after 100 mM NaOH treatment (scale bar; 10µm).

Additionally, we present production and stabilization of unlabeled membrane protein species from various origins. In iMAPS, membrane proteins are reproducibly synthesized in the presence of polymeric membranes as a robust and chemically defined materials bypassing regulating mechanisms of a cell, but still implying the quality control and insertion mechanism of proteins, being processed in a cellular context. We present examplic preparations in Fig. 4 from volume batches of 10µl of cellular lysate (wheat germ) – for LHCII, this resulted in relatively high protein yields as shown in Fig. 4, for the human claudin 2, the synthesis level in the wheat germ extract was substantially lower but still reproducible and clearly detectable on a standard western blot. We could observe the polymeric matrix to stabilize the incorporated membrane protein species from protease degradation over several days up to weeks as we visualized pGUVs with LHCII proteins incorporated after several days, observing specific interactions of antibodies raised against an affinity tag of the protein by standard immunogold labelling procedure.

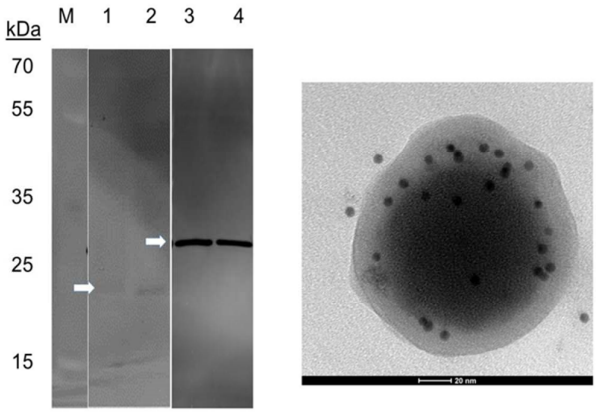

Fig. 4 On the left side: Examplic Western blot of immunoprecipitated LHCII proteopolymersomes containing the human claudin 2 (lane 1 and 2) and the plant protein LHCII in lane 3 and 4. Comparison between precipitation assay using a 'generic', polymer-specific antibody assay, namely α-PEG-functionalized SiNP *versus* a protein – specific ('non-generic') α-VSV-functionalized SiNP assay. The signals on the western blot indicate successful synthesis for both membrane protein species and sufficient interaction with the respective SiNP – functionalized antibodies. α-VSV-SiNP for sedimentation, Protein standard: PageRuler Plus Prestained Protein ladder. **On the right side:** Transmission electron microscope (TEM) image of an immunogold-labelled proteopolymersome with integrated light harvesting protein (LHCII). LHC functionalized immunoprecipitated proteopolymersomes were labelled by primary, monoclonal murine α-VSV antibodies secondary immunogold labelled anti-mouse IgG, followed by crosslinking within 2.5% glutaraldehyde and incubation in contrast agent, 1% OsO4.

To determine if recovered antibody-modified SiNPs are able to repeatedly immunoprecipitate polymersomes, α-PEG-SiNPs were used to immunoprecipitate LHCII-proteopolymersomes in a 'recycling experiment' (Fig. 5). 5 µl polymersome preparations, functionalized by IMAPS into proteopolymersomes, were incubated for 1 h with 100 µg of α-PEG-modified SiNPs and incubated with overhead shaking at 60 rpm. The polymersome – SiNP complexes where then centrifuged at 1 700 x g for 5 min. The pelleted complexes were resuspended and incubated for 15 min with either 10 µl of 10 mM glycine/HCl pH 2 and alternatively 10 µl of 100 mM NaOH in order to release the immunocomplex between proteopolymersomes and SiNPs. After another centrifugation step the supernatants from each recovery process were analyzed using the standard Western blot immuodetection method while the SiNPs were reused. The respective blots showed successful immunoprecipitation after recycling of α-PEG-SiNPs (Fig. 5). The different LHCII signal intensities between the different rounds of recovery can be explained by batch to batch variations of LHCII expression efficiency, representing the very similar efficiency of the harvesting/release procedure. Optimal purification efficiency was achieved using an α-PEG-modified SiNP to polymersome ratio of 20:1, e.g. 100 µg of α-PEG-SiNP used for harvesting 5 µg of polymersomes. Both pH changes result in the efficient release of the immunocomplex as shown in the Fig. 5, demonstrating the efficiency of pH shift for the release of the desired proteopolymersomes after the harvesting procedure and - at the same time – enabled recycling of the antibody – functionalized SiNPs as the pH increase was moderate, and the antibodies did not lose their binding capacity.

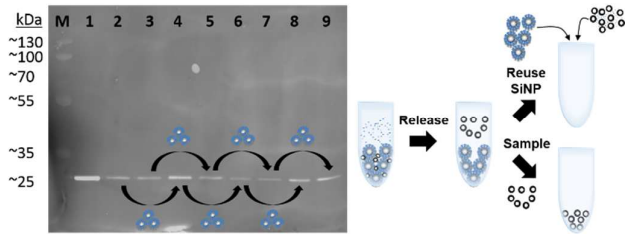

Fig. 5 Elution of polymersomes from α-PEG-SiNP and repeated use for immunoprecipitation based harvesting. α-PEG-SiNP were used to immunoprecipitate LHCII proteopolymersomes with integrated LHCII. The proteopolymersomes were then recovered using treatment with either 100 mM NaOH or 10 mM glycine/HCl. The recovered antibody-modified SiNP were then used two more times to immunoprecipitate LHCII proteopolymersomes. The supernatants from each recovery process were analyzed using Western blot. Lane M: PageRuler Plus Prestained Protein ladder. Lane 1: unpurified reaction mix containing LHCII produced in the absence of polymersomes. In lanes 2, 4, 6 and 8 elution with 10 mM glycine/HCl pH2 and successful reuse of the α-PEG-modified SiNP is shown. While in lanes 3, 5, 7 and 9, elution with 100 mM NaOH and reuse of the α-PEG-modified SiNP proved to be as successful as the acidic treatment.

The efficiency of our anti-PEG-SiNP-based immunoprecipitation method in order to purify LHCII proteopolymersomes was compared to the efficiency of centrifugal microfiltration. Both strategies are dependent on

the use of polymersomes as robust matrices, hosting the protein of interest.

Centrifugal microfiltration has been described for the purification of proteopolymersomes from transcription-translation reaction mixtures.[6] This method relies on the membrane sieving of proteopolymersomes from the rest of the reaction mixture. For this to be effective, the diameter of the proteopolymersomes needs to be larger than the cut-off size of the filters used. However, it should be noted that polymersomes in the same dimensions as the filter pore sizes might deform and penetrate the filter pores and thus be lost in the collection procedure. Furthermore, the efficiency and efficiency of purification by centrifugal microfiltration is dependent on the amount and size distribution of the polymersome sample. The length of time required for complete filtration also depends on the volume of the respective sample. Furthermore, localized concentration of polymersomes at the filter surface would result in blockage and severely reduce efficiency of contaminant removal.

Western blotting and staining of total protein content in a Coomassie stained – polyacrylamide gel electrophorese (PAGE), indicated increased purification efficiency by the SiNP-based immunoprecipitation strategy (Fig. 6) versus the microfiltration – based purification procedure. Employing centrifugal microfiltration, we observed comparable amounts of background protein levels but much lower LHCII levels. The centrifugal microfiltration strategy resulted in only approximately 7% of the immunoprecipitation method, which can be considered as a trade – off for the circumstance, that neither any antibody material nor any nanoparticles have to be involved in the microfiltration procedure but a standard AMICON® filter.

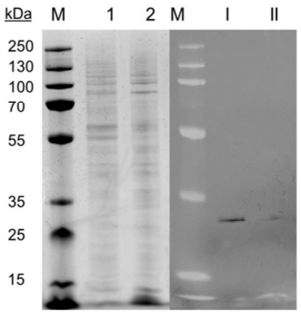

Fig. 6 Coomassie staining and immunoblotting experiment of Proteopolymersomes, purified *via* centrifugal microfiltration versus α-PEG-modified SiNP immunoprecipitation. Lane M appears twice and represents the protein standard in apparent molecular weights, indicated in kDA. All sample lanes contain the result of a 10 µl transcription-translation reaction mixture with cDNA coding for the LHCII protein. After purification *via* immunoprecipitation, employing α-PEG-modified SiNP, each sample was exposed to a standard gel electrophoresis (PAGE), followed by Coomassie staining. Lane 1 represent the sample experiencing immunoprecipitation, Lane 2 represent total protein staining after microfiltration – based purification. Lane I and II present the respective samples transferred onto a nylon membrane and exposed to an immunoblotting experiment.

The presented SiNPs-based immunoprecipitation method renders a straight forward approach for membrane protein synthesis and isolation of membrane proteins combined by a one-step purification procedure with the SiNPs involved reversibly forming an immunocomplex allowing for regeneration of the antibody – functionalized SiNPs for effective harvesting. The procedure is applicable for any membrane protein species with known cDNA sequence. The use of an antibody that targets the material of the polymeric membrane material, rather than the membrane proteins also allows membrane protein purification even if a specific antibody is not available. Furthermore the optimal purification and release conditions need to be adjusted just once and not for every monoclonal antibody raised against the protein of interest. The membrane protein of interest in the polymersomal matrix appears secured in chemically defined and robust proteopolymersomes. Subsequent elution from the SiNPs yields intact proteopolymersomes that may be used for vaccination or – as a perspective for the emerging field of membrane protein-related device fabrication – in robust, polymeric surfaces presenting membrane proteins for sensing and actuating.

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