Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information for:

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

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Experimental Section

Silica nanoparticle formation

Silica nanoparticle formation was based on the Stöber process.[1] For the production of 50 ml of 550 nm sized silica nanoparticles (SiNPs), 3.13 ml of tetraethyl orthosilicate (98%, Sigma-Aldrich), 10.61 ml ultrapure water, 2.9 ml ammonium hydroxide (30-33%, Sigma-Aldrich) and 33.36 ml ethanol (96%, Merck) were mixed in a one-step reaction. The mixture was rigorously stirred with a magnetic stirrer for 4 hours within a sealed bottle to prevent evaporation of the solvent and changes to the chemical composition. The reaction mix was then centrifuged at 1 700 x g for 10 min and the pellet was washed by resuspension and centrifugation three times with ethanol and three times with ultrapure water before drying under vacuum under the same centrifugation parameters as before. The size of the resultant nanoparticles was dependent on the amount of ammonia present in the solution.

All incubations were performed at room temperature unless stated otherwise. If not stated otherwise, centrifugation condition is 1 700 x g for 5 min.

Surface modification of SiNPs

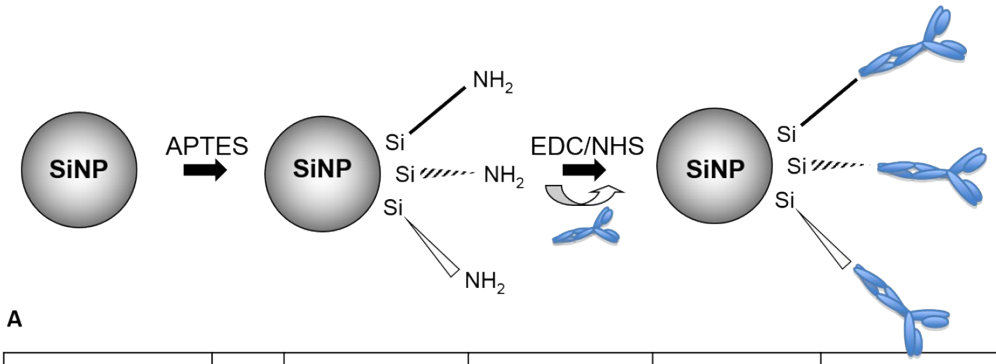
10 mg of dry SiNPs were taken up in 1 ml of 96% ethanol, sonicated until the SiNPs were dissolved (37 kHz and 80 W) and then centrifuged. All centrifugation steps in this following procedure was performed at 1 700 x g for 5 min. The pellet was resuspended in 950 µl of 96% ethanol and 50 µl of (3-aminopropyl) triethoxysilane (APTES) (99%, Sigma-Aldrich) followed by 14–16 h incubation on an overhead shaker at 60 rpm. The SiNPs were centrifuged again and washed with 1 ml ethanol to remove excess APTES. The nanoparticles were resuspended in 1 ml of ultrapure water and stored at 4°C until further usage at a particle concentration of 10 mg/ml.

For the coupling of antibodies to the nanoparticle surface, 100 µl of the amino-functionalized SiNPs solution were pelleted by centrifugation and subsequently resuspended in 500 µl of ultrapure water containing 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (≥99%, Roth) and 0.1 M N-hydroxysuccinimide (NHS) (98%, Sigma) and incubated on an overhead shaker for 10 min before being

centrifuged again at 1 700 x g for 5 min. Supernatant was discarded and the pellet was resuspended by sonication in 1 ml of 10 mM Glycine (≥99%, Promega)/HCl (Roth) pH 5 containing 1 µg of antibody, either monoclonal rabbit anti-poly-ethylene-glycol (α-PEG)(C/N: ab51257, ABCAM) or monoclonal mouse anti-vesicular stomatitis virus G (α-VSV) (C/N: V.5507 ,Sigma-Aldrich), respectively. The combination of SiNPs with the respective antibody was incubated for 1 h on an overhead shaker at 60 rpm before pelleting by centrifugation 1700 x g for 5 min. The SiNPs were then resuspended in 1 ml of 1 M ethanolamine (≥99%, Roth) and incubated for 10 min to deactivate any residual activated carboxyl groups. After centrifugation and pelleting, the SiNP were resuspended repetitively with ultrapure water to wash away residual ethanolamine. The pelleted SiNPs were finally resuspended in 100 µl of phosphate-buffered saline (PBS) resulting in a final concentration of 1 mg per 100 µl antibody-conjugated SiNPs and stored at 4°C until further usage.

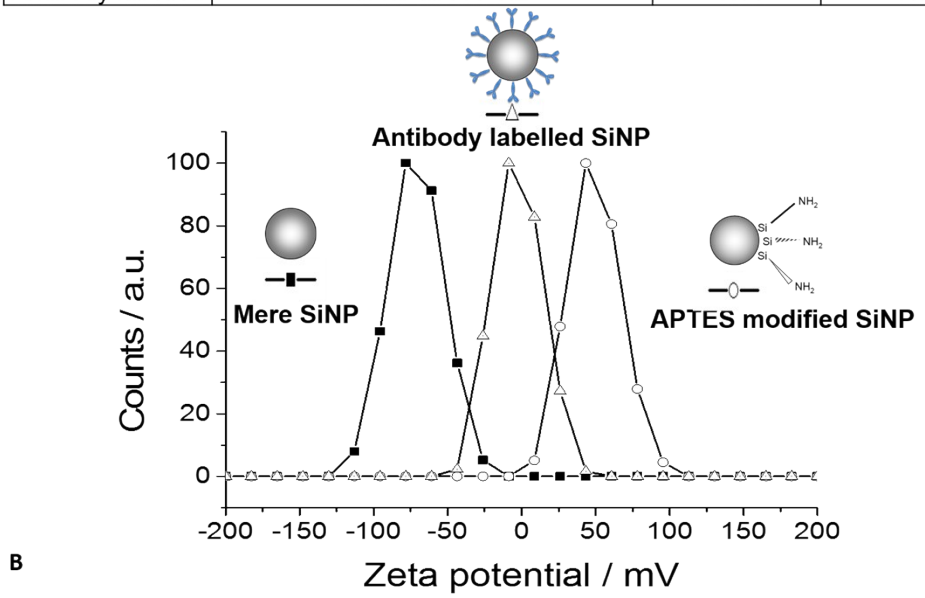
DLS and Zeta potential measurement before and after SiNP surface modification

Modification of the silica surface was monitored by measurements of dynamic light scattering (DLS) and zeta potential with the Zetasizer device (Malvern Instruments, Zetasizer Nano-ZS) (Fig. 1S). The measurements were performed in ultrapure water as well as 96% ethanol at a concentration of 1 mg/ml of SiNPs. Three analyses with multiple runs (18 for DLS, 12 for zeta potential) were performed. The DLS measurements were made within disposable microcuvettes (Roth, z = 8,5 mm) while for zeta potential measurements disposable zeta potential cells (DTS 1060/1070) were used.



A

	Size [nm]	Standard deviation [nm]	Poly- dispersity Index (PdI)	Zeta potential [mV]	Standard deviation [nm]
Pure	540.2	85.08	0.157	-49.1	10.6
APTES	570.3	82.57	0.157	31.8	16.6
Antibody labelled	PdI far above 0.2			-3.36	11.2



B

Fig. 1S A) Schematic overview of the SiNP modification. B) DLS and Zeta potential measurement profiles demonstrate that DLS and Zeta potential measurement can be successfully used to monitor the surface modification.

Visualization of SiNP surface modification by transmission electron microscopy (TEM)

Briefly, 10 µg (corresponding in ca. 10µl sample volume) of α-VSV-modified SiNPs were incubated in 1 ml PBS with 1 µg/ml immunogold-labelled goat anti-mouse IgG (C/N: G7777, Sigma, 10 nm gold particle size) for 1 hour prior to centrifugation for 5 min at 1 700 x g. One centrifugal washing step in 1 ml PBS (5 min at 1 700 x g) was performed to get rid of residual unbound goat anti-mouse IgG before resuspension in 10 µl of PBS was performed. The suspension was adsorbed for 15 min on a TEM copper grid. Residual

suspension was subsequently removed and the grid was dried in air over night at room temperature before analysis.

Visualization of LHCII in polymersomes by transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), LHCII proteopolymersomes, synthesized with 0.4 M trehalose, were prepared as described later and immunogold-labelled using rabbit anti-LHCII antibodies (1 µg/ml in Carl Roth Roti®-Block, Mainz, Harald Paulsen) as the primary antibody and immunogold-labelled goat anti-rabbit antibody (5 nm colloidal gold, Sigma, 1:100 diluted in Roti®-Block, Carl Roth) as the secondary antibody. The incubation and blocking of the TEM grids was done in a similar manner as Western blotting described later. Antibody incubation times were reduced to 30 min each, followed by 15 min of 2.5% glutaraldehyde fixation. For the staining of the polymersome membranes 1 h incubation with 1% OsO4 and three subsequent washing steps in ultrapure water were performed.

Formation of polymersomes and chloroplast-pigment extract-containing polymersomes

The polymer poly(butadiene) (PBD_{1200}-PEO_{600}, PolymerSource) was either dissolved as is or for identification of presence of polymersomes, incubated in crude total pigment extract derived from peas, at a pigment to polymer molar ratio of 1:200, in chloroform (≥99%, Roth). Crude total pigment was extracted from peas as described by Paulsen et al.[2] Aliquots of each chlorophyll preparation were dried into a thin pigment-polymer film in a glass round-bottom flask using a rotary evaporator and subsequently rehydrated in ultrapure water to a final concentration of 5 mg/ml. Each rehydration was subjected to five freeze-thaw cycles using a liquid nitrogen bath as well as an ultrasonic waterbath at 37°C in order to form unilamellar polymersomes[3]. For a uniform size distribution, the polymersomes were extruded 20 times through a 200 nm membrane filter (polycarbonate, 0.75´´, AVESTIN) and characterized using DLS and transmission electron microscopy (TEM).

Polymersomic Giant Unilaminar Vesicles (GUVs) were formed from poly(butadiene) in 0.4 M sucrose (≥99.5%, Roth) at 37°C with the Nan]i[on Vesicle Prep Pro® chamber at a

frequency of 5 Hz, amplitude of 3 V, a rise time of 1 min, main time of 120 min and a fall time of 5 min.

Fluorescence of pigmented polymersomes was analyzed with the Luminescence Spectrometer (LS 55, PerkinElmer Instr.) using disposable microcuvettes (Roth, z = 8,5 mm). Chlorophyll fluorescence was excited at wavelengths from 350 nm to 480 nm while fluorescence emission was recorded at 670 nm. This wavelength correlates to the maximal fluorescence of chlorophyll a that had been integrated into the membrane of the polymersomes, as the major component of the added pigment extract.

Synthesis of the examplic membrane protein species and formation of proteopolymersomes

To demonstrate the efficacy of our method of immunoprecipitation, we aimed to purify polymersomes with embedded membrane proteins. Respective cDNA with the coding sequence for an N-terminal Vesicular stomatitis virus glycoprotein (VSV) were used in the coupled transcription and translation system from wheat germ: The TNT® Quick Coupled Transcription/Translation system (L4140, Promega) was employed to express the pea derived (*Pisum sativum*) Light Harvesting Complex II (LHCII) under regulation of a T7 promoter. The reaction mix of a total volume of 10µl were composed according to the suppliers instructions, however, the suppliers recommendation advises for 25 – 50µl total volume, whereas in our hands, 10µl was sufficient for the immunoprecipitation methods, resulting in ca. 200ng of desired protein, visualized by westernblotting experiments. For the formation of proteopolymersomes, 2 µg of polymersomes (average size 200nm) was added to 10 µl of reaction mix for co-translational insertion of the LHCII into the polymer membrane. The preparations were then incubated for 90 min at 30 °C and shaken at 350 rpm. The final proteopolymersome/cell lysate sample was either stored at 4°C up to several weeks or directly processed by microfiltration/immunoprecipitation for purification.

Electrophoresis and Western blot analyses

All samples for electrophoresis were diluted 1:1 with 2 x NuPAGE® (Life Technologies) gel loading buffer (prepared from NuPAGE® LDS Sample Buffer (4x) and NuPAGE® Reducing Agent (10x)) and incubated at 70°C for 10 min. Each was then loaded into a

10% NuPAGE® Bis-Tris gel and reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the MOPS NuPAGE® SDS Running Buffer at a constant voltage of 200 V and 400 mA for 55 min.

Protein in the Bis-Tris gel was transferred to a nitrocellulose membrane using the iBlot™ System (Invitrogen) at 20 V for 7 min. The membrane was blocked for 1 h with gentle agitation using Odyssey™ Blocking Buffer, then incubated for 1 h with gentle agitation with monoclonal mouse anti-vesicular stomatitis virus G (α-VSV) (C/N: V.5507 ,Sigma-Aldrich) diluted 1:10 000 in Odyssey™ Blocking Buffer. This was followed by washing of the membrane with PBS supplemented with 0.01% Tween 20® (PBST) for 5 min. This was repeated 4 more times. Subsequently, infra-red dye-labeled goat anti-mouse IgG (C/N: 926-68021, IRDye 800CW, LI-COR Biosciences) was diluted 1:10 000 in a mixture of Odyssey™ Blocking Buffer diluted with PBS at a ratio of 1:1. The membrane was incubated for another 1 h with gentle agitation. Finally, the membrane was washed three times with PBST and twice with PBS. Once the membrane was completely dried, it was scanned using the Odyssey™ CLx infrared system (LI-COR Biosciences).

Immunoprecipitation of pigmented polymersomes and polymersomic GUVs

We validated the harvesting steps on microscopical scale using conventional phase contrast light microscopy of polymersomes interacting with α-PEG-SiNPs as ‘anchoring’ structures. Briefly, 100 µg of α-PEG-SiNPs were incubated with GUVs in 1 ml of PBS and incubated on an overhead shaker at 60 rpm for 1 h, followed by centrifugation at 600 x g for 1 min. The resulting pellet was gently resuspended in 20 µl of ultrapure water and 5 µl was used for microscopy. From the images, we learned that a strong connecting network from the SiNP had been formed, interconnecting the large polymersomes.

To evaluate the efficiency of immunoprecipitation using antibodies targeting polyethylene glycol the polymer membrane, 5 µg of 200 nm-sized pigment-containing polymersomes were diluted with 1 ml of PBS and incubated with 0, 10, 25, 50, 75, 100 and 125 µg of α-PEG-modified SiNPs for 1 h on an overhead shaker. After centrifugation for 5 min at 1, 700 x g the supernatant was collected and analyzed for chlorophyll fluorescence. The

data (Fig. 2S/A) indicates maximal supernatant fluorescence in absence α-PEG-SiNP. However, taking different samples with increased amounts of α-PEG SiNPs material, harvesting the polymersomes resulted in a decrease of fluorescence. Quantities of α-PEG-SiNP greater than 50 µg did not result in further reduction of fluorescence. This observation indicates a clearance of the pigmented polymersomes from the supernatant by the α-PEG-SiNP immunocomplex formation.

In case of polymersomic GUVs release the formed immunocomplex was sedimented at 600 x g for 1 min, supernatant discarded and the pellet treated with 20 µl 100 mM NaOH for 10 min in order to release the polymersome – SiNP immunocomplex. Subsequent centrifugation at 600 x g for 1 min was done to separate GUVs and SiNPs.

In case of fluorescent labelled polymersomes the sediment of each pigmented polymersome-SiNP clusters was resuspended in 100 µl of 10 mM NaOH (≥99%, Gerbu) for 10 min and centrifuged again. The supernatant was collected and analyzed for chlorophyll fluorescence. We observed substantial increase of fluorescence as a function of releasing the polymersomes from the SiNPs back into the bulk phase. Figure 2S/B depicts the fluorescence measurements of the very same samples, employed for the fluorescence decrease in the supernatant as function of SiNP – polymersome complex formation, shown in Fig. 2 S/B.

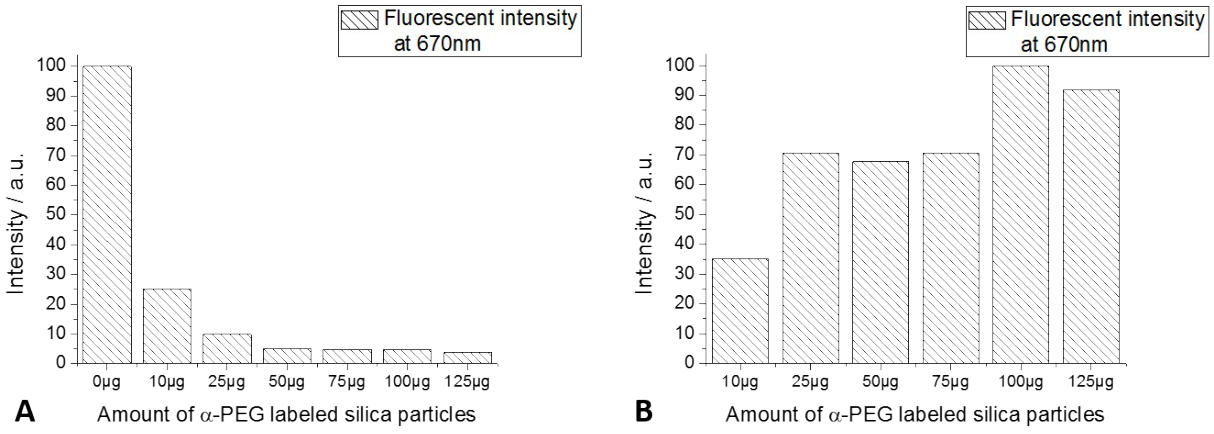


Fig. 2S A) Fluorescence analyses of supernatant after immunoprecipitation of pigmented polymersomes. Different amounts of α-PEG-SiNP were tested for their efficiency in immunoprecipitating 5 µg of 200 nm pigmented polymersomes. Fluorescence was normalized against the sample set maximum and indicates the presence of pigmented polymersomes. Analysis of the supernatants following immunoprecipitation shows a decrease in fluorescence at 670 nm with an increase in the amount of α-PEG-SiNP used. B) Fluorescence signals of chlorophylls, embedded in polymersome matrix after pH increase.

Immunoprecipitation of proteins and proteopolymersomes and reusability of antibody – functionalized SiNPs

Proteopolymersomes produced in transcription-translation reaction mixtures of 10 – 20 µl were first incubated for 1 h with 100 µg of α-PEG-modified SiNPs for each 5 µg of polymersomes and incubated with overhead shaking at 60 rpm. The mixtures where then centrifuged at 1 700 x g for 5 min and the pellets were used directly for electrophoresis. Alternatively, the pellet was resuspended and incubated for 15 min with either 10 µl of 10 mM glycine/HCl pH 2 or 10 µl of 100 mM NaOH to release the antibody-bound LHCII and Cldn2 proteopolymersomes. The supernatants were further treated for 1 h as described above with α-VSV-modified SiNPs. This allowed us to capture LHCII and Cldn2 that had not integrated into the polymersome membranes. In case of Cldn2, after SiNP based polymersome removal, Tween 20® (Sigma-Aldrich) had to be added to the supernatant up to a final concentration of 0.1% Tween 20® in order for the non-integrated protein to be immunoprecipitated. The samples were centrifuged again at 1 700 x g for 5 min and the pellets were used directly for electrophoresis. Furthermore, the reusability of the SiNP after 10 mM glycine/HCl pH 2 or 10 µl of 100 mM NaOH was tested.

Testing of unlabeled APTES-modified SiNP resulted into no detectable immunoprecipitation with of proteopolymersomes with the SiNPs.

To determine if recovered antibody-modified SiNPs are still able to immunoprecipitate polymersomes, α-PEG-SiNPs were used to immunoprecipitate LHCII-proteopolymersomes. The proteopolymersomes were recovered dissolving the immunocomplex with either 100 mM NaOH or 10 mM glycine/HCl. The recovered antibody-modified SiNPs were then used two more times to immunoprecipitate LHCII-proteopolymersomes. The supernatants from each recovery process were analyzed using the standard Western blot.

Comparison of centrifugal microfiltration and immunoprecipitation for proteopolymersome purification

For Amicon® centrifugal microfiltration 10 µl of transcription-translation reaction mixture containing 5 µg of LHCII proteopolymersomes were diluted with 500 µl of PBS, then loaded into an Amicon® centrifugal microfiltration cartridge (Ultrafree®-MC-VV, Durapore® PVDF 0.1µm). The samples were then centrifuged at 600 x g until all the solution had filtered through the cartridge. The retentates were resuspended in 20 µl PBS.

For antibody-modified SiNP immunoprecipitation 10 µl of transcription-translation reaction mixture containing 5 µg of LHCII proteopolymersomes were treated with 100 µg of α-PEG-SiNPs in 1 ml of PBS. The mixtures were incubated on an overhead shaker at 60 rpm for 1 h, then centrifuged at 1 700 x g for 5 min. The pellets were resuspended in 20 µl PBS.

Samples prepared using both methods of purification were denatured and electrophoresed as described above. Subsequently, the Bis-Tris gels were removed from the plastic casing and rinsed for 5 min in deionised water. The water was then replaced with 20 ml of SimplyBlue™ SaferStain for total protein staining and the gel was incubated for 1 h with gentle agitation. The SimplyBlue™ SaferStain was replaced with deionised water and the gel was again incubated for 1 h with gentle agitation. A final rinsing with deionised water was performed before the gel was scanned using the Odyssey™ CLx infrared system. Alternatively, the proteins in the gel were blotted onto nitrocellulose membranes and analyzed by Western blot, as described above.

References

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