Model Membranes

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Synthesis and Functional Reconstitution of Light-Harvesting Complex II into Polymeric Membrane Architectures

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Abstract: *One of most important processes in nature is the harvesting and dissipation of solar energy with the help of light-harvesting complex II (LHCII). This protein, along with its associated pigments, is the main solar-energy collector in higher plants. We aimed to generate stable, highly controllable, and sustainable polymer-based membrane systems containing LHCII–pigment complexes ready for light harvesting. LHCII was produced by cell-free protein synthesis based on wheat-germ extract, and the successful integration of LHCII and its pigments into different membrane architectures was monitored. The unidirectionality of LHCII insertion was investigated by protease digestion assays. Fluorescence measurements indicated chlorophyll integration in the presence of LHCII in spherical as well as planar bilayer architectures. Surface plasmon enhanced fluorescence spectroscopy (SPFS) was used to reveal energy transfer from chlorophyll b to chlorophyll a, which indicates native folding of the LHCII proteins.*

Light-harvesting complex II (LHCII) of higher plants is one of the most abundant membrane proteins in the world. One LHCII protein can bind 14 chlorophyll molecules (eight *Chl a*, six *Chl b*) and 4 carotenoids.[1] LHCII serves as an antenna complex and is one of the few membrane proteins that can be spontaneously refolded in vitro, however, it tends to insert into membrane architectures with random orientation.[2–4] LHCII is an attractive choice for use in membrane protein research, and it has potential biotechnological importance as a pigment "organizer" in the context of sustainable, robust, and efficient solar cells.[5] So far, LHCII research has been dependent on reconstitution of the purified protein in detergents, amphiphiles, and lipid membranes.[6,7]

Cell-free expression in systems supplied with artificial membranes, also referred to as in vitro membrane-assisted protein synthesis (iMAPS), offers a robust and reliable technique for the de novo synthesis of membrane proteins in artificial membrane supports, as has been shown for GPCRs,[8] claudin-2,[9] and others.[10,11] Recent attempts to replace lipid membranes by polymeric membrane mimics offer an interesting alternative since polymeric membranes produce reproducible and robust alternative membrane architectures.[12] We selected polymeric membrane mimics from amphiphilic diblock copolymers, which assemble into bilayered membrane structures in aqueous environments.[12–14] The polymer membrane architectures are easily tunable in terms of thickness, permeability, and rigidity through the selection of different polymers.[14] The use of cell-free protein production allows de novo synthesis of membrane proteins in membranes, while bypassing potentially limiting cellular regulatory mechanisms and the bottleneck of purification through detergent-induced denaturation.[9,11,15,16]

Herein, we show the functional and unidirectional insertion of LHCII and LHCII–pigment complexes into spherical polymersomes and planar polymer bilayers. Cell-free protein synthesis provides co-translational, unidirectional membrane protein insertion into polymeric membrane structures, as we have described previously.[9] We monitored the self-assembly of thiolated lipoic acid tethered polymeric membranes on planar gold substrates, followed by integration of LHCII produced by iMAPS from wheat-germ-based cell-free lysates. Surface plasmon resonance (SPR) spectroscopy was used to monitor membrane formation, whereas energy transfer from *Chl b* to *Chl a* was observed by surface plasmon enhanced fluorescence spectroscopy (SPFS). Successful energy transfer following *Chl b* excitation by the evanescent plasmon field indicates native folding of LHCII moieties in the polymeric membranes and functional presentation of the chlorophyll molecules.

The synthesis of LHCII apoprotein and its integration into polymeric membranes was achieved in wheat-germ-based cell-free lysate (Promega, L4140) as a suitable cell lysate system for plant proteins. The influence of polymersome concentration on the protein yield of N- and C-terminal VSVG-tagged LHCII was assessed by using SDS-PAGE followed by Western blotting (Figure 1 A). We observed stable LHCII expression levels up to a concentration of 0.5 $\mu g\,\mu l^{-1}$ polymer material (Figure 1 B). We hypothesize that the optimal polymersome versus cell lysate ratio can be

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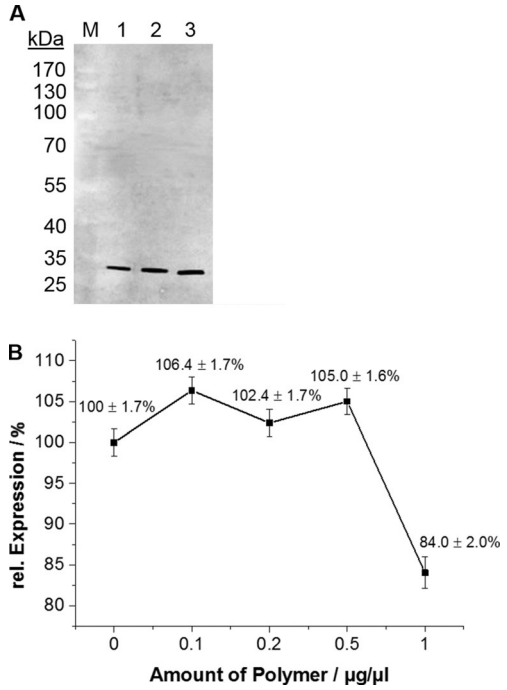

Figure 1. Expression of LHCII in the presence of polymersomes. A) Western blot of LHCII in the presence and absence of polymersomes. Lane 1 shows the expression of N-VSVG-tagged LHCII alone. Lane 2 shows the synthesis reaction for N-VSVG-tagged LHCII with 0.2 µg µL^{-1} polymersomesand Lane 3 shows the equivalent synthesis with C-VSVG-tagged LHCII. B) The dependence of relative protein expression (as represented by signal intensity ($n=3$)) on polymer concentration.

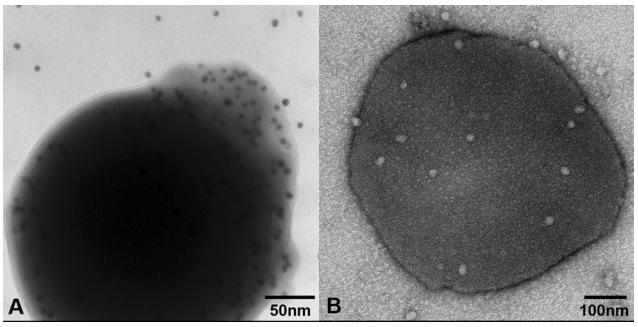

Figure 2. Transmission electron micrographs of LHCII-PPs purified by centrifugal ultrafiltration. A) Spherical polymersomes containing immunogold-labelled LHCII. B) A polymersome after treatment with OsO_4: the white marks indicate unstained LHCII protein molecules.

interpreted as being the result of molecular crowding, which is known to increase the robustness of cell-free gene expression.[17] The surface-to-volume ratio also plays a role, as we have demonstrated in a microfluidic system.[18] However, at high polymersome concentrations, the protein yield decreases, likely as a result of reduced molecular mobility, which negatively influences the synthesis efficiency of LHCII.

Transmission electron microscopy (TEM) was performed to visualize the presence, organization, number, and localization of the LHCII protein moieties. Polymersomes with incorporated LHCII, so called Proteopolymersomes (PPs), were purified as described by Nallani et al.[9] The resulting PPs were stained by two different methods: 1) Membrane and protein staining with 1% Uranyl acetate and subsequently with 1% OsO_4 (Figure 2A), as well as immunostaining with gold-labeled anti-LHCII antibodies, and 2) staining with 1% OsO_4, thereby rendering the LHCII protein moieties as light spots (see Figure 2B). For both preparation methods, the polymersomes tended to deform slightly during sample preparation. To minimize this effect, we loaded the polymersomes with 0.4 M trehalose, which is an established method for increasing stability.[19]

Integration of LHCII into the polymersomes was confirmed by using purification with a combination of immunoprecipitation[20] and sodium carbonate extraction as described by Fujiki et al.,[21] with minor adaptations and modifications (see the Supporting Information). According to this method, a highly ionic buffer disrupts simple adsorption of proteins to the polymersome membrane surface and thus extracts partially embedded proteins. However proteins that are properly integrated into the membrane plane would be resistant to extraction. Our polymersomes were purified by using an anti-polyethylene glycol (α-PEG) antibody immobilized onto silica nanoparticles (SiNP) as described by our group,[20] which allows integrated LHCII to be separated from components of the cell lysate. This is possible because the α-PEG antibody is able to bind to the poly(ethylene oxide) part of the polymer. Figure 3 shows an example result, where the sodium carbonate treated samples indicate that iMAPS-generate LHCII is fully incorporated into the polymeric membrane. The specific signal indicates the presence of LHCII as an integral membrane protein. Loss of signal strength is caused by the additional carbonate treatment. The antibody-based SiNP purification procedure allowed harvesting of the membrane protein of interest even from 1000-fold dilutions.

A suitable and well established method to confirm the orientation of LHCII integrated into polymeric membranes is trypsin assay. Transmembrane domains and intracellular parts are protected from digestion by the membrane structure. Since the VSVG-tag, which is used for antibody–based recognition, has two trypsin cleavage sites, it represents a valid target sequence for digestion. LHCII presents a total of 21 trypsin cleavage sites.

We analyzed the resulting fragment pattern in a western blot experiment with the software PeptideCutter by Expasy (Figure 4B). The digestion pattern in Figure 4A revealed that LHCII was unidirectionally integrated into the polymeric membrane plane with the N terminus exposed. LHCII with a VSVG-tag on either the N or C terminus was used. Within a cor-

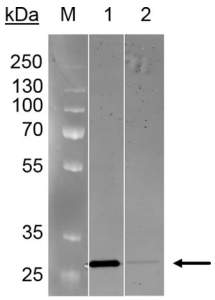

Figure 3. Western blot of SiNP purification after sodium carbonate extraction of LHCII-PPs. Similar amounts of reaction mixture were loaded on each lane. Lane 1 shows α-PEG SiNP-purified PPs after cell-free protein synthesis. Lane 2 shows purified PPs after sodium carbonate extraction.

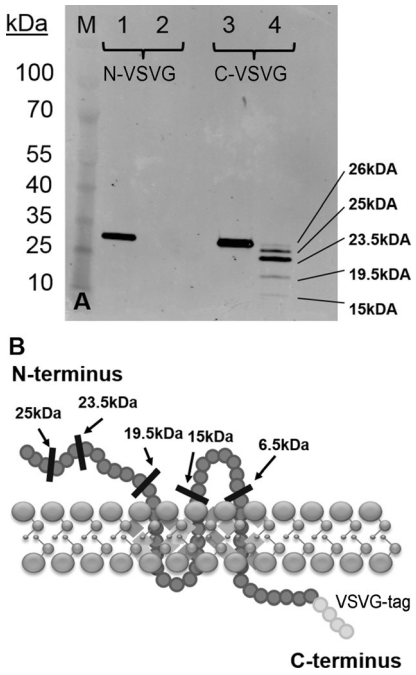

Figure 4. A) Prolonged digestion for 30 min of purified samples. Lanes 1 and 4 show untreated PPs while lanes 2 and 5 show samples digested with trypsin. Prolonged treatment with trypsin resulted in a distinct digestion pattern for C-VSVG LHCII. B) An overview of the LHCII orientation of C-VSVG-labeled LHCII and relevant cleavage sites.

rectly folded and integrated LHCII only one terminus was found to be exposed to the trypsin, thus resulting in distinct digestion patterns owing to protection of transmembrane and inner parts from the protease. Western blotting experiments of trypsin-treated samples revealed a protected C terminus with a digestion pattern corresponding to correctly inserted LHCII proteins. These findings suggest directed LHCII insertion during cell-free protein synthesis. The addition of full pigment extract from pea leaves to the PPs resulted in no change in digestion protection although chlorophylls and carotenoids are essential for complete folding of LHCII in vivo and in vitro.[22]

Although the data suggest that LHCII was successfully inserted into the polymersome membrane, the question arose as to whether the protein is able to successfully bind chlorophylls and carotenoids as it does in the native setting of the thylakoid membrane. Owing to the intrinsic hydrophobic character of chlorophylls, a detectable amount is integrated into the polymersomal membrane even in the absence of LHCII. However the presence of LHCII increases the local accumulation of chlorophylls, with the LHCII moieties gathering the chlorophylls from the hydrophobic core of the polymeric membrane. Chlorophylls as well as carotenoids were extracted from pea leaves and were subsequently added to the iMAPS of LHCII. The samples were probed for fluorescence emission specific to *Chl a*. Comparative measurements showed increased characteristic chlorophyll fluorescence emission, thus suggesting LHCII-mediated orientation of pigments that matches the physiological state in the thylakoid system.

In view of the successful assembly of LHCII light-harvesting complexes in the polymersomes, we transformed the spherical architecture of the PPs to planar polymeric membranes, organized on solid-supported surfaces. For efficient and strong polymer binding to the gold-coated SPR surface, the polymer material was modified with thiolated lipoic acid,[23] enabling layer formation through binding of the sulfur groups to the gold surface. Protein synthesis and pigment incubation were separated into two steps. Protein synthesis was performed for 4 h at room temperature in situ. As a negative control, iMAPS was performed in the absence of any cDNA. Layer formation and subsequent modification was recorded as a function of optical thickness by using a self-made SPR setup (data not shown). Notably, a smaller increase in optical thickness was recorded in the case of LHCII expression, thus suggesting that LHCII integration reduces the surface area available for nonspecific adsorption.

The surface plasmons generated by the HeNe Laser (632.8 nm) are able to excite *Chl b*. Since chlorophylls *a* and *b* are oriented by LHCII, the energy from *Chl b* should be transferred to a neighboring *Chl a*, from which it is emitted in the native thylakoid context as a photon with a wavelength of 670 nm. This specific excitation transfer phenomenon was observed in our experimental setting based on iMAPS-functionalized polymeric membranes (Figure 5).

To evaluate the specific effect of LHCII presence in the membrane, we employed an alternative membrane protein claudin2 (Cldn2) as a reference protein of human origin with no known affinity for chlorophyll. In the presence of LHCII, the *Chl a* fluorescence significantly increases upon *Chl b* excitation, while with Cldn2, the fluorescence emission of the membrane surfaces decreases significantly over several independent measurements (Figure 6). This finding indicates that the increase in *Chl a* fluorescence is related to proper arrangement of the pigments in the presence of LHCII. Reversibility of fluorescence emission after photobleaching was achieved through pigment exchange, thus demonstrating reusability of the polymeric membrane surface as a novel property compared to the standard lipid- or detergent-based LHC-containing arrays.

In conclusion, we have demonstrated the suitability of polymersomes for the directed integration of LHCII into a robust polymeric membrane, in both spherical and planar configurations. The chlorophyll–binding capacity of LHCII was shown through fluorescence emission in an SPFS configuration. The results suggest that in vitro synthesized LHCII is functionally embedded in a robust and reproducible polymeric membrane with the ability to exchange chlorophylls after photobleaching. Moreover iMAPS-derived LHCII is able to bind pigments in a similar way to that found in the chloroplasts of living cells. These findings represent a further step towards the aim of utilizing solar energy by using bioinspired recyclable materials from sustainable sources.

Experimental Section

Polybutadiene$_{1200}$-polyethylene oxide$_{600}$ (PolymerSource) was dissolved alone or together with pigment extract in chloroform (≥ 99%, Roth) and prepared by film rehydration[24] and subsequent

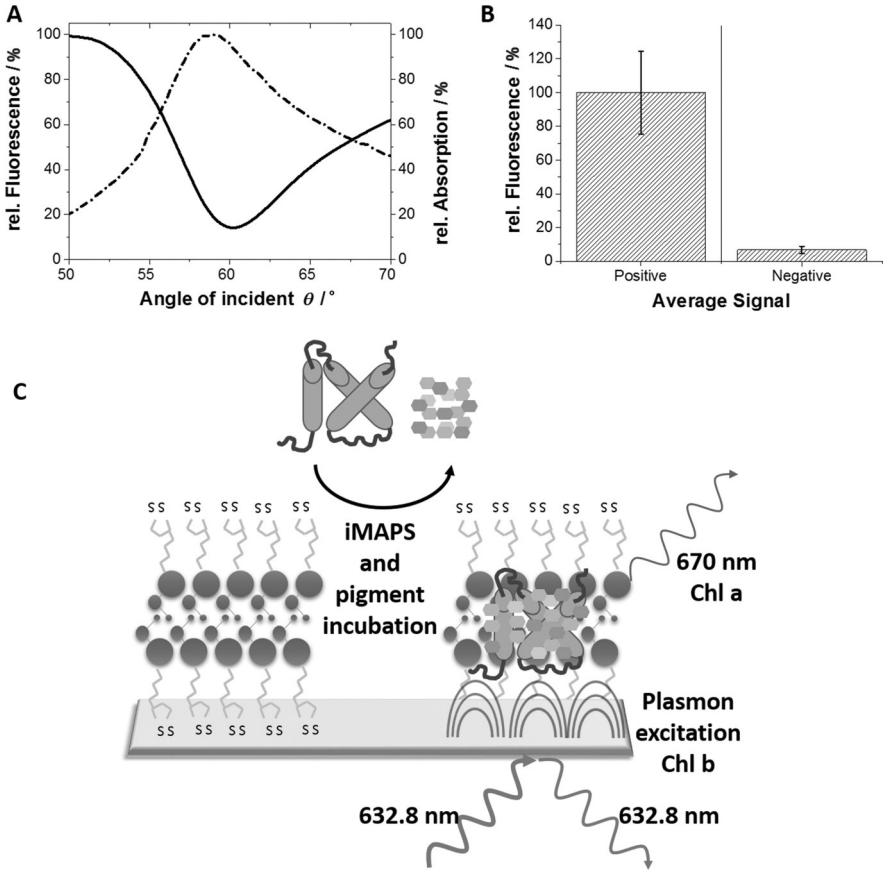

Figure 5. Surface-plasmon-induced energy transfer from *Chl b* to *Chl a*. A) A representative example of energy transfer within the polymer bilayer in the presence of LHCII. B) A detailed comparison of the average measured *Chl a* fluorescence upon *Chl b* excitation. ($n=4$) C) Representative fluorescence spectra in accordance with the surface modification.

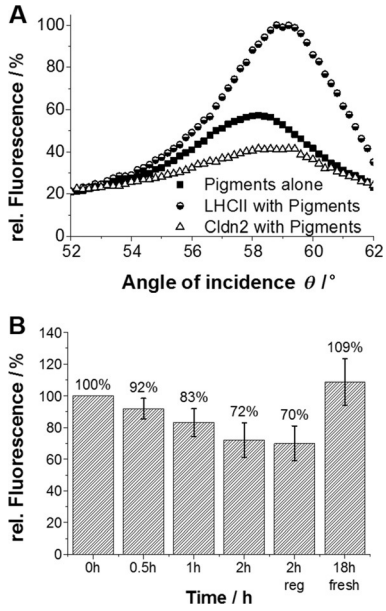

Figure 6. A) Comparative analysis of LHCII and Cldn2 shows the LHCII dependent rearrangement of chlorophyll moelcules to facilitate energy transfer from *Chl b* to *Chl a* ($n=3$). B) Reversibility of fluorescence emission after photobleaching was performed through incubation with fresh pigment solution. ($n=4$).

extrusion. Pigments were extracted as described by Paulsen et al.[6] Polymer modification with lipoic acid for the formation of tethered bilayers was done as described by Belegrinou et al.[23]

Proteins and PPs were prepared using the TNT-coupled transcription–translation wheat germ extract system (Promega, L4140). Protein was expressed in the presence or absence of pigment extract. For digestion assays, trypsin (0.5% Trypsin EDTA, Gibco) was used. Monoclonal mouse anti-VSVG antibody (Sigma–Aldrich, VSVG = vesicular stomatitis virus glycoprotein) was used as the primary antibody and goat anti-mouse IgG labelled with an infrared fluorescence dye (IRDye 800CW, Licor) was used as the secondary antibody. The antibody detection was achieved by using the Odyssey infrared imaging system (Licor).

For TEM analysis, rabbit anti-LHCII primary antibody (1 $\mu g\,mL^{-1}$) and immunogold-labelled goat anti-rabbit antibody (5 nm colloidal gold, Sigma) secondary antibody, as well as Uranyl acetate and OsO_4 membrane staining, were used. Another approach was to simply stain purified polymersomic membrane with OsO_4, leaving proteins unstained.

PP purification was achieved by microfiltration with Amicon filters (Ultrafree-MC-VV, Durapore PVDF 0.1 μm)[9] and SiNP immunoprecipitation as described elsewhere.[20] In case of tethered polymer bilayers, gold-coated (50 nm) glass surfaces were used and SPR/SPFS experiments were performed within a self-built set-up. Fluorescence measurements were made using a luminescence spectrometer (LS 55, PerkinElmer Instr.).

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