Reversible Switching between Nonquenched and Quenched States in Nanoscale Linear Arrays of Plant Light-Harvesting Antenna Complexes

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ABSTRACT: A simple and robust nanolithographic method that allows sub-100 nm chemical patterning on a range of oxide surfaces was developed in order to fabricate nanoarrays of plant light-harvesting LHCII complexes. The site-specific immobilization and the preserved functionality of the LHCII complexes were confirmed by fluorescence emission spectroscopy. Nanopatterned LHCII trimers could be reversibly switched between fluorescent and quenched states by controlling the detergent concentration in the imaging buffer. A 3-fold quenching of the average fluorescence intensity was accompanied by a decrease in the average (amplitude-weighted) fluorescence lifetime from approximately 2.24 ns to approximately 0.4 ns, attributed to the intrinsic ability of LHCII to switch between fluorescent and quenched states upon changes in its conformational state. The nanopatterning methodology was extended by immobilizing a second protein, the enhanced green fluorescent protein (EGFP), onto LHCII-free areas of the chemically patterned surfaces. This very simple surface chemistry, which allows simultaneous selective immobilization and therefore sorting of the two types of protein molecules on the surface, is a key underpinning step toward the integration of LHCII into switchable biohybrid antenna constructs.

INTRODUCTION

All photosynthetic systems in living organisms contain light-harvesting pigment–protein complexes (LHCs) that enhance photosynthetic efficiency by capturing and concentrating light energy for the reaction center complexes (RCs), where the primary conversion of light energy into electrochemical potential takes place. There have been several recent examples of immobilization of RCs on various artificial support materials in order to study their ability to generate electric current in the substrate in response to light.1−4 Several reports also demonstrate that is possible to immobilize LHCs onto artificial surfaces, where they retain their functional properties.5−7 The advent of new lithographic techniques, such as those based on light8 or nanoimprinting,9−11 have opened up the possibilities of controlling the surface arrangements of groups of LHC molecules in order to examine their collective properties for energy propagation.12 The major target for such nanopatterning work has been the light-harvesting 2 (LH2) complex from the photosynthetic bacterium Rhodobacter sphaeroides, both as the normal, wild-type complex and in the form of site-directed mutants with genetically introduced tags such as cysteine residues that allow coupling to gold and other substrates.13,14 An understanding of how we can manipulate the functional properties of nanoscale arrays of LHCs immobilized on surfaces is the first step toward generating effective artificial systems that convert light energy into usable electrical current.

The major trimeric chlorophyll a/b-binding light-harvesting complex of plants (LHCII), which serves both photosystem I (PSI) and photosystem II (PSII) and is probably the most abundant membrane protein on earth,15−17 is an excellent test case for construction of nanoscale arrays. LHCII absorbs light over much of the visible spectral range and is able to transfer excitation energy rapidly (within a few picoseconds) and at high quantum efficiency to neighboring light-harvesting complexes and then toward the PSII or PSI RCs. Single-molecule and time-resolved fluorescence studies on LHCII have shown that the complex can be readily and reversibly switched between two conformational states, one (highly fluorescent) with a long fluorescence lifetime of ∼4 ns and the other (weakly fluorescent) with a much shorter lifetime of ∼0.3 ns, by controlling the environmental conditions such as detergent and pH.18−25 These experiments reflect the intrinsic ability of LHCII to switch between highly and weakly...
fluorescent states, which is believed to play an important photoprotective role in controlling energy input into the RCs by nonphotochemical quenching of chlorophyll fluorescence (NPQ)26–30. The fact that this property of LHCCI can be triggered in vitro by altering the environment of this membrane protein makes the LHCCI complex, or an engineered variant, a possible candidate as a component in future biohybrid optoelectronic devices. The fabrication of nanoarrays of LHCCI is therefore a key underpinning step toward the integration of LHCCI into biohybrid antenna constructs.

Nanoimprint lithography (NIL)31–36 is one of the most widely used technologies for high-throughput nanofabrication and nanoscale patterning and has the capability to produce sub-100 nm features.36,37 In particular, linear patterns of functional integration of LHCII into biohybrid antenna constructs. LHCII is therefore a key underpinning step toward the optoelectronic devices. The fabrication of nanoarrays of possible candidate as a component in future biohybrid protein makes the LHCII complex, or an engineered variant, a

**EXPERIMENTAL SECTION**

**Protein Expression and Purification.** LHCCI. Trimeric LHCCI from spinach was isolated as previously described by Ruban et al.40 Xanthophyll/chlorophyll composition was determined as previously reported for violaxanthin-enriched LHCCI complexes in Kruger et al.41

**SATP-Modified EGFP.** Introducing the combined F64L, S65T, V68L, S72A, M153T, V163A, S175G, and A206K mutations into the gene sequence of yellow fluorescent protein (YFP) [amplicified by polymerase chain reaction (PCR) from pCS2-Venus vector] resulted in enhanced green fluorescent protein (EGFP) gene.42 The resulting NdeI/BamHI fragment was cloned into a PET11b expression vector (Novagen). EGFP proteins were produced by heterologous expression in *Escherichia coli* (BL21); cells were grown to an OD600 of 0.6 at 37 °C and then induced by use of isopropyl β-D-1-thiogalactoside (IPTG; 35 μM) at 3000 rpm, resulting in ~300 nm resist layer. The EBL exposure dose ranged from 200 μC·cm−2 (for larger structures) to 1700 μC·cm−2 for the narrower lines. Then, the resist was developed with n-amyl acetate. Next, a 20 nm thickness of Al was deposited in an electron beam thermal deposition system and used as an etch mask for the Si wafer. The final etching of the template into the Si wafer was performed in a plasma-assisted etcher in CF4/Ar atmosphere with the plasma power set to 100 W. The process was optimized to obtain an etch depth of 80–100 nm. The final process step is a soak in piranha solution to remove the aluminum etch mask.

**Chemical Patterning of Surfaces.** Poly styrene (PS) (*M*<sub>n</sub> = 234 kDa, Polymer Source) was dissolved in toluene (HPLC-grade, Fisher Scientific) to a concentration of 55 mg·mL<sup>−1</sup>. The master template with the lithographically formed ridges was cleaned in piranha solution, washed copiously with ultrapure deionized (DI) water, and blown dry with a nitrogen stream. Then it was spin-coated with the polystyrene solution at 2000 rpm for 30 s, resulting in a layer of polystyrene approximately 180 nm thick [the thickness was measured by atomic force microscopy (AFM) over a scratch in the PS film, data not shown]. The thickness of the PS layer was controlled by the spinning speed and solution concentration, and it is important that the PS film is thick enough to completely cover the features on the master mold. Then, after the PS layer was carefully scratched off the edges of the master template, the coated surface was immersed into a clean Petri dish filled with approximately 40 mL of ultrapure DI water at a shallow angle (10°–15°), letting the water wet the hydrophilic master mold surface and lift the hydrophobic PS film onto the water surface by means of the surface tension forces. The result is a free-standing PS film, the topography of which is a negative replica of the master mold surface. At this point the free-standing PS film can be picked up with a wire loop, inverted, and deposited onto a flat Si substrate for inspection. Subsequently, the free-standing PS film can be transferred (floated on) to a clean flat substrate (either a piranha-cleaned glass coverslip or Si substrate) in the same orientation as it was released from the master mold. In doing so, the relief of the polymer film would make contact with the flat substrate only with its protruding parts. After the edges of the PS film were trimmed (in order to ensure that the channels formed between the PS film relief and the substrate are open to the atmosphere), the substrate with the masking polymer layer was dried under vacuum for 16 h in order to remove any residual water trapped between the substrate and the PS film. During the next step, an organosilane self-assembled monolayer (SAM) was created by a chemical vapor deposition (CVD) process. The substrate with the masking PS layer on top was placed into a 0.6 L desiccator and was purged with dry nitrogen for 10 min. Then 30 μL of 3-mercaptopropyltrimethoxysilane (MPTMS), placed in a cap from a microcentrifuge tube, was introduced into the desiccator. After the sample was purged with dry nitrogen for another 10 min, the desiccator was sealed, pumped down to a pressure of approximately 20

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mbar, and left under vacuum for 16 h to allow formation of a SAM on the exposed parts of the substrate. After the organosilane deposition was completed, the protein was then incubated with the substrate followed by a second CVD step in order to assemble a contrasting 1H,1H,2H,2H-perfluorooctyltriethoxysilane (fluorosilane) SAM on the newly exposed (clean) parts of the substrate. Alternatively, the second deposition step can be conducted in solution (e.g., 5 mM fluorosilane dissolved in dry toluene) under protective nitrogen atmosphere.

The chemically patterned surfaces used for the simultaneous immobilization of two proteins were prepared following the same procedure with two differences: first, a master template with lithographically formed trenches was used to produce the PS replica; second, during the first CVD step a 3-aminopropyltriethoxysilane (APTMS) SAM was assembled onto the exposed areas of a glass substrate followed by a second CVD step filling in the gaps with a MPTMS contrasting monolayer.

**Protein Immobilization.** *LHClI-Only Nanoarrays.* The MPTMS/fluorosilane nanopatterned surfaces were incubated with a 1 mM solution of sulfosuccinimidyl 4-(N,N'-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) in PBS, pH 7.4, for 40 min, leading to a coupling reaction between the maleimide groups of the sulfo-SMCC and the sulphydryl groups on the patterned surfaces and leaving the active N-hydroxysuccinimide ester (NHS ester) groups of the cross-linker molecule exposed. After extensive washing with PBS buffer, the modified surfaces were incubated with a 5 nM solution of LHCI in nitrogen-sparged buffer [PBS, pH 7.4, with 0.03% n-dodecyl β-D-maltoside (β-DDM)] for 5 min at room temperature in the dark. After another extensive wash step with nitrogen-sparged buffer, the samples were mounted for either AFM or fluorescence microscopy imaging.

**Mixed LHCl/EGFP Nanoarrays.** The APTES/MPTMS nanopatterned surfaces were incubated with a 1 mM solution of sulfosuccinimidyl 4-(N,N'-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) in PBS, pH 7.4, for 40 min converting the sulhydryl groups of the MPTMS regions on the surface into active N-hydroxysuccinimide ester (NHS ester) groups and, at the same time, converting the amine functional groups of the APTES regions to maleimide groups. After a wash with PBS buffer, the modified surfaces were incubated with mixed LHClI/EGFP solution in nitrogen-sparged buffer [PBS, pH 7.4, with 0.03% β-DDM]. The total protein concentration was approximately 25 nM (LHClI:EGFP ratio of 10:1) and the incubation time was 40 min at room temperature in the dark. After an extensive wash with nitrogen-sparged buffer, the samples were mounted for fluorescence microscopy imaging.

**Atomic Force Microscopy Characterization.** The AFM data were collected on a Multimode 8 instrument equipped with a 15 μm scanner (E-scan) coupled to a NanoScope V controller (Bruker). NanoScope software (v8.15, Bruker) was used for data collection, and Gwyddion (v2.32, open source software covered by GNU general public license, www.gwyddion.net) and OriginPro (v8.5.1, OriginLab Corp.) software packages were used for data processing and analysis. The measurements of the patterned SAMs were performed in tapping mode in air at ambient conditions by use of AC160TS probes (Olympus) with a nominal spring constant of approximately 40 N·m⁻¹ and a nominal resonant frequency of around 300 kHz.

The chemically patterned surfaces with the immobilized protein molecules on them were imaged in peak force tapping mode at nearly physiological conditions in buffer (PBS, pH 7.4), at room temperature by use of BL-AC40TS probes (Olympus). In this case, the Z-modulation amplitude was adjusted to values in the range 20–24 nm, while the Z-modulation frequency was 2 kHz and the contact tip-sample force was kept in the range 80–100 pN.

**Fluorescence Measurements.** The glass substrate (coverslip) with the protein nanoarray was mounted on a standard microscope slide, nanopatterned side facing the slide, with a droplet (20 μL) of nitrogen-sparged buffer (PBS, pH 7.4, with or without 0.03% β-DDM) and was sealed with DPX mountant (Sigma–Aldrich).

Fluorescence emission properties of the LHClI nanoarrays were measured on a home-built inverted optical microscope (based on AxioObserver A1m, Zeiss) equipped with a spectrometer (Acton 150, Princeton Instruments) and an electron-multiplying charge-coupled device (EMCCD) camera (ProEM S12, Princeton Instruments). Excitation source was from a collimated light-emitting diode (LED) light source (M470L2, Thorlabs), and the resulting fluorescence emission was detected through the spectrometer onto the EMCCD camera.

During fluorescence imaging and spectral measurements, the excitation light was filtered by a 470/40 nm bandpass filter, then reflected by either 605 or 488 nm dichroic beam splitter to the sample, and the fluorescence emission from the sample was filtered by either 593 or 500 nm long-pass filters. The spectra were captured with a slit width of 800 μm and a 150 line-mm⁻¹ grating at a central wavelength of either 680 or 540 nm in the spectrometer. Each fluorescence image and the spectra were average of 10 frames with 0.1 s exposure time with an electron multiplication gain of 90.

Time-lapsed fluorescence measurements were conducted in a home-built flow cell (with a volume of approximately 100 μL) made of commercial optical adhesive (NOA 81, Norland Corp.), which was mounted on a standard microscope slide. The glass coverslip with linear LHClI nanopatterns was attached and sealed within the flow cell by using DPX microscopy resin (Sigma–Aldrich). Initially, the flow cell was flushed with imaging buffer supplied with 0.03% β-DDM, and the data acquisition started at a rate of approximately 0.2 image-s⁻¹ (each image was an average of 8 frames with 0.08 s exposure). Then the flow cell was flushed with approximately 500 μL of imaging buffer without detergent at a flow rate of 1.5 mL-min⁻¹. Finally, another 500 μL of imaging buffer with detergent was injected into the flow cell while data were continuously acquired. The average fluorescence intensity of the LHClI complexes for each frame was calculated as an average from 10 pixels (each pixel belonging to a line of LHClI). The intensity of the same set of pixels was measured for each one of 12 images acquired in a time sequence, and the variation of the average fluorescence intensity was plotted against the time (or consecutive frame number).

**Time-Resolved Measurements.** For time-resolved measurements, a supercontinuum white light laser, (SC 480-10, Fianium) with a repetition rate of 80 MHz was used as a light source and the excitation light was cleaned up by additional 470/40 nm band-pass filter. The laser beam was focused on the sample surface illuminating a diffraction limited spot. The illumination of the laser was synchronized with a time-correlated single-photon counting (TCSPC) module (SPC-150, Becker & Hickl) for fluorescence lifetime measurements. Fluorescence lifetimes were recorded by parking the focused laser spot over one of the LHClI nanolines and selecting a central wavelength by use of the monochromator. Then the signal from the illuminated spot on the sample surface was sent to a photomultiplier tube (PMT) detector. The secondary slit in front of the PMT allows further spectral narrowing of the measured signal; typically we were able to select ±15 nm around the central wavelength of 680 nm selected by the monochromator. SPCM software (Becker & Hickl) was used for data acquisition, and OriginPro was used for data analysis and fitting.

During the time-resolved measurements, the pulse energy was approximately 0.05 pJ resulting in approximately 14 photons-pulse⁻¹-time⁻¹.

**Time-Resolved Measurements on Homogeneous LHClI Monolayers.** Time-correlated single-photon counting measurements were performed by use of a FluoroTime 200 ps fluorometer (PicoQuant). Fluorescence lifetime decay kinetics were measured on LHClI monolayers with excitation provided by a 470 nm laser diode at a 10 MHz repetition rate. These settings were carefully chosen to be far below the onset of singlet–singlet excited annihilation (≈0.1 fs). Fluorescence was detected at 680 nm, using a 1 pm slit width. The instrument response function was ~50 ps.

**RESULTS AND DISCUSSION**

**Nanolithography and Self-Assembly of Organosilane Molecules on Oxide Surfaces.** The simple lithography process, schematically represented in Figure 1, eliminates the
need for high temperature and high pressure during the replication step as well as the residual-layer removal step (breakthrough reactive ion etching) in order to expose the clean substrate under the masking polymer layer (required steps in all NIL variants).43−47 Two different types of master templates were used in this work, one with protruding ridges and one with sunken trenches, produced by a standard electron beam lithographic process onto silicon wafers (see Experimental Section).

After spin-coating (Figure 1B) a thin polystyrene (PS) layer onto either of the master templates (shown in Figure 2A,B), the PS film was floated off in a water bath, (Figure 1D), resulting in a free-standing polymer film with a relief replica of the master template features.

Inversion of this film (Figure 1E) exposes the surface previously in contact with the master template for inspection by atomic force microscopy (AFM) (Figure 2C,D). The AFM images in Figure 2 show that the PS films replicate the master template features, resulting in trenches with an average width of 82 nm with a 4 μm period (Figure 2C) or 350 nm wide ridges with a 2 μm period (Figure 2D) depending on the master templates used. The high fidelity of the imprinting procedure
replicates the variable width of the channels together with some larger defects (Figure 2C). The free-standing PS film can be transferred (floated on) to a clean flat substrate such as a piranha-cleaned glass coverslip or a silicon (Si) wafer in the same orientation that was released from the master template (Figure 1F). In making contact with the flat substrate (Figure
respectively. Error bars represent the standard deviation from the average of 10 data points. Photoactive biological systems are typically studied by optical techniques, making opaque substrates such as Si wafers problematic because of their inability to transmit light and their tendency to quench the fluorescence of the sample. Glass is a much more convenient substrate for investigation of biological systems, and for that reason we prepared chemically nanopatterned glass substrates using the method described above. Figure 4A shows an AFM topographic image of 70 nm wide lines of MPTMS formed onto a glass substrate with a height of approximately 7 Å, corresponding to the thickness of a monolayer. Next, a contrasting 1H,1H,2H,2H-perfluoroctyltrimethoxysilane (fluorosilane) SAM was assembled in order to fill in the gaps between the MPTMS nanolines. Then a small cross-linker molecule, sulfo succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), was used to covalently link the lysine residues of LHCII, which are particularly enriched on the N-terminal (chloroplast stroma-facing) side of the complex, to the sulfhydryl groups of the linear nanopattern (see Experimental Section). The resulting linear protein nanoarrays of LHCII were characterized in situ by AFM and by time-resolved fluorescence microscopy.

The AFM topographs revealed that the LHCII proteins are immobilized directly over the MPTMS monolayer with very little nonspecific attachment to the fluorosilane areas of the surfaces. The average height of the protein lines was approximately 5.8 nm, in good agreement with the 6 nm height of the LHCII trimers. The average width of the protein lines is approximately 70 nm with a line width down to 58 nm at full width at half-maximum (fwhm) measured across the straight defect-free parts of the linear pattern (Figure 4B,D). The AFM data also suggest very close packing of the LHCII trimers in the linear arrays since the height of the lines is very uniform with no observable gaps or interruptions.

In order to confirm the localization and the preserved functionality of the LHCII complexes attached along the nanopatterned lines, the samples were characterized in a home-built fluorescence microscope capable of spectral and time-resolved data acquisition. The ability of LHCII to switch between highly and weakly fluorescent states allows control of

**Figure 5.** LHCII linear nanopattern on a glass substrate imaged in the presence of (A, C) 0.03% β-DDM and (B) in its absence. The corresponding intensity profiles, in panel D, were obtained along the dashed lines in panels A and B. The images were acquired at the same camera settings and are represented with identical brightness and contrast settings. (E) Average fluorescence intensity dependence on time while detergent concentration was varied with time. The time intervals when detergent was present (I and III) or absent (II) in the flow cell are shown in green and pink, respectively. Error bars represent the standard deviation from the average of 10 data points.
energy transfer to the RCs where photochemistry takes place. It has been shown that this switching can be replicated with bulk LHCII complexes in vitro by manipulating detergent concentration and pH.\textsuperscript{18−21} We were able to observe reversible changes in the fluorescent emission of the immobilized LHCII complexes in real time by mounting the sample with the nanopatterned LHCII in a home-built flow cell (see Experimental Section) and acquiring time-lapsed fluorescence data, shown in Figure 5, while varying the concentration of \textit{n}-dodecyl \(\beta\)-D-maltoside (\(\beta\)-DDM) detergent in the imaging buffer (as described in the Experimental Section). The emission intensity of the LHCII nanolines in the presence of \(\beta\)-DDM detergent (Figure 5D, maroon trace) is approximately 2.9 times higher compared to the emission intensity of the LHCII nanoline in the absence of \(\beta\)-DDM (Figure 5D, green trace). The fwhm of the peaks in both cases is approximately 265 nm (diffraction-limited). Figure 5E shows the variation of average intensity from the LHCII complexes depending on the detergent concentration (with time) in the imaging buffer.

When detergent-containing buffer was flushed out of the flow cell and replaced by detergent-free buffer, a significant, \(~\)3-fold drop decrease (from 1300 to approximately 400 au) of the average fluorescence intensity of the LHCII complexes was observed. When the detergent concentration in the imaging buffer was restored, the average fluorescence intensity increased to approximately 1200 au. It is worth noting that the bright and dark regions along the lines of LHCII, clearly visible in the fluorescence images, are the consequence of the nonuniform width of the LHCII lines: the wider part of the lines appear brighter due to the larger number of light-emitting LHCII molecules “per unit length” of the nanoline.

In order to further investigate the transition between the highly fluorescent and the weakly fluorescent state of the immobilized molecules, we recorded the emission spectra and the fluorescence lifetime of 70 nm lines of LHCII complexes either in the presence of 0.03\% \(\beta\)-DDM detergent or in its absence. Figure 6A shows fluorescence image acquired on a sample of LHCII immobilized on nanopatterned glass substrate with line widths of approximately 70 nm. A region of interest (ROI) on the sample was defined by closing the entrance slit on the monochromator and binning the CCD detector rows accordingly (Figure 6A). The signal was sent from the defined ROI to a diffraction grating within the monochromator and spread around a chosen central wavelength onto the CCD detector, thus allowing the acquisition of an emission spectrum (Figure 6D).

The spectrum acquired over one of the 70 nm lines has a maximum at 682 nm and also displays a shoulder at around 730 nm, consistent with the spectrum of LHCII in aqueous solution (shown for comparison in Figure 6B). In addition, 470 nm excitation light predominantly excites chlorophyll \(b\) and carotenoids (chlorophyll \(a\) absorbance maximum is at 430 nm), while the observed fluorescence emission maximum is at 682 nm (chlorophyll \(a\) emission), which is evidence for internal energy transfer from chlorophyll \(b\) to chlorophyll \(a\). This is a clear indicator that immobilizing LHCII complexes on the substrate preserves their structural and functional integrity. The emission spectrum of a control ROI, defined within the gap between two LHCII lines, showed only the background baseline with no prominent peaks.

Figure 6C shows fluorescence lifetime decays recorded over one of the LHCII lines in imaging buffer supplied with detergent (0.03\% \(\beta\)-DDM; orange data), and in imaging buffer...
without detergent (dark cyan data). The best fit of the decay curves recorded in the presence of detergent identified two exponential components with lifetimes of 3.2 ns (64%) and 0.52 ns (36%), giving an average (amplitude-weighted) lifetime of approximately 2.24 ns. The absence of detergent resulted in much faster fluorescence decay with an average (amplitude-weighted) lifetime of approximately 0.4 ns (99% for the 0.38 ns component and 1.1% for the 2.1 ns component). This dramatic reduction of the lifetime is consistent with previous studies, which indicated that detergent removal shifts the equilibrium between the number of strong and weak emitters, leading to a decrease in the fluorescence emission and fluorescence lifetime of the population of LHCII complexes.48–51 Monolayers of LHCII complexes, prepared by protein immobilization onto nonpatterned MPTMS SAMs (on glass surfaces), were used for comparative fluorescence lifetime measurements (again with and without detergent present in the buffer) in a commercial instrument (Figure 6D). The best fit of the data gave average (amplitude-weighted) lifetime values of approximately 2.23 ns in the presence of detergent and 0.35 ns for the quenched state (no detergent present), which are in very good agreement with the values obtained for the nanopatterned LHCII samples. In summary, both the internal energy transfer from chlorophyll \( b \) to chlorophyll \( a \) and the capacity of the LHCII complex to reversibly switch between highly and weakly fluorescent states have been retained following nanopatterning and immobilization on the glass substrate. Given that the LHCII complexes are covalently attached, and therefore immobilized, to the glass substrate, we can discount the possibility that this switchable quenching behavior is a consequence of altering the aggregation state of the complexes.

**Simultaneous Immobilization of Two Photoactive Proteins.** Integration of the LHCII into biohybrid light-harvesting constructs requires retention of the ability of these antenna complexes to transfer absorbed excitation energy to their neighbors and, most importantly, to photochemical RCs. This, in turn, requires patterned multicomponent LHC-RC nanoarrays.

In order to demonstrate the versatility of the nanopatterning method proposed here and its usefulness in sorting and controlling the nanoscale positioning of two different proteins, we simultaneously immobilized two different photoactive proteins, LHCII and an enhanced green fluorescent protein (EGFP), onto a chemically patterned glass surface. Only a few patterning methods, mainly based on the click-chemistry approach, are suitable for the realization of multicomponent patterns.49–51 To fabricate the two-protein samples, the nanopatterned glass surfaces were prepared in a slightly different way: a PS replica with 350–380 nm wide trenches (Figure 2D), produced with the master template in Figure 2B, was used to pattern either fluorosilane or 3-aminopropyltriethoxysilane (APTES) SAMs during the first functionalization step (Figure 1H). In the second CVD step (Figure 1K), these patterns were complemented with a MPTMS monolayer, resulting in alternating linear arrays of either fluorinated (broad) lines and sulphydryl (narrow) lines or amine (broad) lines and sulphydryl (narrow) lines. Then the nanopatterned surfaces were incubated with sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) for 40 min at pH 7.4. This additional functionalization step converts the sulphydryl groups into active N-hydroxysuccinimide ester ( NHS ester) groups and, on the second type of sample, simultaneously converts the amine functional groups to maleimide groups. Both maleimide–sulphydryl and NHS ester–amine reactions are well understood and have attractive characteristics such as high selectivity, high yield, fast reaction in aqueous phase at room temperature, and biocompatibility.52 Moreover, the two reactions are highly orthogonal to each other and can be carried out simultaneously with a minimum amount of nonspecific immobilization. In the last step, the first type of nanopatterned glass surface was incubated with LHCII solution (see Experimental Section) in order to produce LHCII-only nanopattern; the second type of patterned surface was incubated with a mixed solution of LHCII and N-succinimidyl S-acetyliopropionate (SATP) -functionalized EGFP (see Experimental Section) in order to produce a mixed LHCII/EGFP nanopattern.

Figure 7 panels A and B show fluorescence images acquired on LHCII-only and mixed LHCII/EGFP samples, respectively. The results clearly show that the bifunctionalized nanopatterned surface sorted the mixture of two proteins according to their functional groups: the LHCII complexes with available lysine residues bound predominantly to the NHS ester regions on the surface, while the SATP-functionalized EGFP bound predominantly to the maleimide regions. The selectivity of the surface immobilization was confirmed by fluorescence spectroscopy (Figure 7C). Emission spectra recorded over the LHCII and EGFP domains of the patterns confirmed the site.
specificity of the immobilization process and show that this method for patterning two types of protein has further potential. One application includes fabricating combinations of antenna and RCs for investigating energy migration and trapping in novel 2D arrangements not found in native or engineered photosynthetic organisms.

**CONCLUSION**

In conclusion, LHCII was successfully immobilized onto a chemically patterned glass surface. Site-specific immobilization was confirmed by fluorescence emission spectroscopy, and detergent-induced switching between fluorescent and quenched states verified the functionality of these immobilized antenna complexes. In addition a second protein, EGFP, was immobilized onto LHCII-free areas of the chemically patterned surfaces by very simple surface chemistry that allows simultaneous selective immobilization and therefore sorting of the two types of protein molecules on the surface. During the one-pot functionalization, both surface groups recognized their respective functionalities on the different protein molecules, and thus the surface was selectively tagget by the proteins according to the predesigned chemical pattern.

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**Notes**

The authors declare no competing financial interest.

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