Confocal microscopy of plasmonic hybrid nanostructures

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Abstract—We describe the results of confocal microscopy imaging of a hybrid nanostructure composed of elongated gold nanoparticles and light-harvesting complexes LH2 from purple bacteria. The images obtained for the hybrid nanostructure feature strong inhomogeneity of fluorescence intensity as well as its higher average value in comparison with the reference. Both are the result of plasmon excitations generated in metallic nanoparticles. We observe that excitation into longitudinal plasmon resonance of the nanorods (808nm) yields larger fluorescence enhancement than for excitation of the transverse mode (556nm).

Plasmon excitations characteristic of free electrons confined to metallic nanoparticles have been shown to enable efficient manipulation of electromagnetic fields at the nanoscale [1]. One of the most spectacular effects is the enhancement of fluorescence emission of molecules placed in the vicinity of a metallic nanoparticle. Such phenomena have been observed for organic molecules [2], semiconductor nanocrystals [3], and, recently, for multichromophoric biomolecules, such as light-harvesting or photosynthetic complexes [4-6]. Importantly, extending concepts and methods that have been developed for describing the coupling of single organic chromophores with plasmon excitations in metallic nanoparticles to multi-chromophoric biological systems are not straightforward both from theoretical and experimental points of view. On the one hand, organic molecules or semiconductor quantum dots are much more robust than pigment-protein complexes, therefore, the sample preparation requires more careful treatment in the latter case. Furthermore, photosynthetic complexes degrade relatively quickly upon laser illumination, which places additional constraints upon the conditions of the experiment. Importantly, preserving protein structure is essential as it implies proper functionality of the complex with all the energy transfer pathways between various chromophores preserved. On the other hand, from the theory standpoint, biomolecules, and in particular light-harvesting complexes, render themselves a real challenging system to model due to a multitude of interactions between chromophores such as chlorophylls and carotenoids, which results in many energy transfer pathways, formation of strongly coupled excitonic systems, and conformational changes of the protein itself.

Nevertheless, driven by the continuous development of optical spectroscopy and microscopy techniques, significant progress has been achieved in understanding interactions and functions of light-harvesting complexes. These efforts have been helped by high-resolution crystal structures of the light-harvesting complexes [7] that provided solid foundation for interpreting the results of optical spectroscopy experiments using actual structure of the protein itself and spatial arrangement of the pigments in these systems.

In this contribution we describe the results of confocal microscopy imaging of hybrid nanostructures composed of light-harvesting complex LH2 from the purple bacteria and gold nanorods. The fluorescence maps obtained for this system feature strong inhomogeneity of the intensity which is due to plasmon interaction between metallic nanoparticles and biomolecules. We find that the excitation of high-energy plasmon mode of the nanorods (556nm) results in a weaker increase of the fluorescence intensity as compared to the excitation of low-energy plasmon mode (808nm). This is in agreement with a larger value of the dipole moment for plasmons parallel to the symmetry axis of the nanorods. The results demonstrate the power of fluorescence imaging of hybrid plasmonic nanostructures, as well as provide valuable information about spectral dependence of the plasmon induced enhancement of the optical properties of biomolecules.

The structure of the pigment-protein complex LH2 studied in this work is shown in Fig. 1. This protein is placed in the thylakoid membrane in order to harnesses sunlight energy and transfers it efficiently to reaction centers in purple bacteria *Rhodopseudomonas palustris*. The X-ray crystallography studies of the LH2 complex have shown that out of the 27 Bacteriochlorophyll (BChl) molecules 18 form a strongly coupled ring with average distances between the molecules less than 1 nm. This excitonically coupled ring of BChls is responsible for the absorption band at 850nm. The remaining 9 BChl molecules form a ring of weakly coupled BChls as they are spaced by more than 2nm. They are responsible for the absorption at 800nm. All pigments, BChls and

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carotenoids, are embedded in a hydrophobic protein (not shown in Fig. 1). The fluorescence emission of the LH2 complex is centered at 870nm.

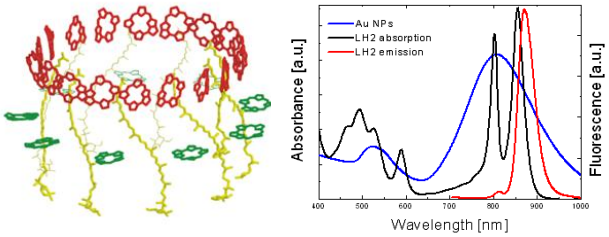

Fig. 1. (Left) Pigment structure of the LH2 complex: red and green molecules are Bacteriochlorophylls of the B850 and B800 rings, respectively, carotenoids are marked in yellow. Protein is omitted for clarity. (Right) Absorption (black) and fluorescence (red) spectrum of the LH2 complex. Blue curve corresponds to the absorption spectrum of the gold nanorods used in the experiment.

Recently it has been shown [4] that the absorption of the LH2 complex in the visible range can be significantly enhanced upon coupling with spherical gold nanoparticles. However, the presence of strong absorption bands and fluorescence emission in the infrared spectral range implies application of metallic nanoparticles that feature plasmon resonances in the near infrared in order to influence the optical properties of the LH2 complex. Spectral properties of gold nanorods fulfill these criteria, as the plasmon resonance of these nanostructures can be tuned from approxiamtely 600nm to over 1200nm [8].

The synthesis of Au nanorods (NRs) was based on seed-mediated growth in water solution [9]. All chemicals (HAuCl4×3H2O (99.9%), NaBH4 (99%), L-Ascorbic Acid (99+%), hexadecyltrimethylammoniumbromide (CTAB) (99%), and AgNO3 (99+ %)) were purchased from Aldrich and used without further purification. Deionized water (Fluka) was used in all experiments. In order to prepare Au seeds CTAB solution (4.7ml, 0.1M) was mixed with 25µl of 0.05 M HAuCl4. To the stirred solution, 0.3ml of 0.01M NaBH4 was added, which resulted in the formation of brownish yellow solution. Seeds solution was kept at room temperature until further used. For the synthesis we use Au seeds prepared beforehand. The "seed-mediated" method was developed previously; it is carried out in aqueous solution at atmospheric pressure and near room temperature. Appropriate quantities and molarities of CTAB (150ml, 0.1M), HAuCl4 (1.5ml, 0.05M), L-Ascorbic acid (1.2ml, 0.1M), 0.01M AgNO3 (1.6ml, 1.8ml, 2ml) and seed (360µl) water solutions were added one by one in a flask, followed by a gentle mixing. Addition of ascorbic acid, as a mild reduction agent, triggered a mixture color change from dark yellow to colorless. After addition of the seed solution, the mixtures was put into a water bath and kept at a constant temperature of 28ºC for 2 hours. Obtained products were separated from unreacted substrate and spherical particles by centrifugation at 9000rpm for 60 minutes. The supernatant was removed using a pipette and the precipitate was redissolved in pure water. The nanorods were examined using scanning electron microscopy in order to determine their size, and with absorption spectroscopy to assign the energies of plasmon resonances. Au nanorods were of 15nm in diameter and 60nm in length. The absorption spectrum of the Au nanorod solution is shown in Fig. 1, where we compare it with the absorption of the LH2 complex. It can be seen that the plasmon resonances of the nanorods match very well the absorption bands of the LH2 complex. The transverse plasmon band appears around 560nm while the longitudinal band is at 800nm. The absorption of the Au NR layer is similar to that of Au aqueous solution.

The sample studied in this work was obtained by spin-coating an LH2 solution (OD 25 at 850nm) in PVA on a glass cover slip, on which gold nanorods were deposited beforehand. We believe the nanords form a submonolayer on a glass surface. Although this method gives no control over the separation between the light-harvesting complexes and metallic nanoparticles, it can provide valuable information about the coupling in such a hybrid nanostructure. As a reference, the LH2 complexes with equal concentration were spin-coated on a glass cover slip.

In order to image fluorescence of light-harvesting complexes coupled to metallic nanoparticles we constructed a confocal fluorescence microscope based on Olympus infinity-corrected microscope objective LMPlan 50x, characterized with a numerical aperture of 0.5 and working distance of 6mm [10]. The resulting laser spot size is about 1µm for the excitation laser of 485nm. The sample is placed on a XYZ piezoelectric stage (Physik Instrumente) with 1nm nominal resolution of a single step, which enables us to raster-scan the sample surface in order to collect fluorescence maps. They are formed by combining fluorescence intensity measurements with the motion of the XY translation stage. Fluorescence intensity maps are collected with an avalanche photodiode (PerkinElmer SPCM-AQRH-14) with a dark count rate of about 80cps. For excitation of fluorescence, we use two lasers: 556nm, which corresponds to the excitation of the high-energy plasmon resonance in the nanorods, and 808nm, which corresponds to the excitation of the low-energy plasmon resonance in the nanorods. The excitation power was for both wavelengths approximately 20-40µW. Such low excitation powers supress photobleaching effect and thus allow for longer acquisition times during the experiment. The fluorescence is detected in a back-scattering geometry and focused on a confocal pinhole (150µm) in order to reduce stray light coming out of the focal plane. The emission of LH2 complexes was

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extracted using a combination of a longpass filter HQ850LP and a bandpass filter D880/40m (Chroma).

In Fig. 2 we show the result of fluorescence imaging experiment carried out on LH2 complexes deposited directly on gold nanorods with plasmon resonances at 560nm and 800nm. The maxima of the resonances match ideally with absorption bands of the LH2 complex, attributed to carotenoids and bacteriochlorophylls, respectively. In the experiment we probe the fluorescence enhancement for these two excitation wavelengths.

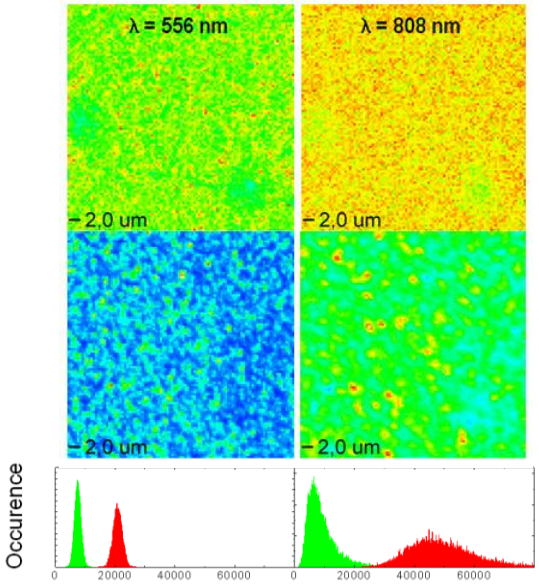

Fig. 2. 50 x 50 micron- large fluorescence images of LH2 complexes deposited on glass substrate (upper row) and Au nanorods (lower row) excited with a 556nm and 808nm laser. Below intensity histograms extracted from the maps are shown.

In order to assess the influence of the plasmon resonance upon the optical properties of the LH2 complex it is necessary to probe the same area for the two excitation energies that correspond to both plasmon resonances. The maps obtained for LH2-only sample show that this can indeed be achieved: areas with low fluorescence intensity are clearly correlated. In the case of LH2 complexes on glass substrate fluorescence maps acquired for both excitation wavelengths are very uniform, as shown with intensity histograms. In both cases the histograms feature Gaussian shape with maxima at 7500 and 21000cps for 556 and 808nm excitation, respectively. The picture changes qualitatively for the LH2 complexes deposited on gold nanorods. The most pronouncing effect is much larger inhomogeneity of the fluorescence maps. There are clear regions of a few micron size that feature much stronger emission intensity. We can attribute them to either favorable separation between LH2 and Au nanorods or orientation/geometry of gold nanorods that would lead to formation of hot-spots of strongly localized electromagnetic field. Interestingly, the average intensity measured for the 808nm excitation increases 2.5 times upon coupling with gold nanorods, while for the 556nm excitation interaction with plasmon excitation in metallic nanoparticles results mainly in a redistribution of intensity towards higher values.

In addition to highly homogeneous fluorescence images, there are also significant differences of the distribution of fluorescence intensity, in spite of using the same excitation powers for a given laser wavelength. Indeed, the maximum of fluorescence intensity measured for 556nm appears roughly at the same value as for the reference sample, but the histogram features substantial high-intensity tail of intensities, which is due to plasmon-induced enhancement in the hybrid nanostructure. Conversely, for the excitation of 808nm we also observe a broad tail towards higher intensities, but in this case the average intensity is over twice the average intensity measured for the reference sample. These preliminary results demonstrate that by using gold nanorods we are able to modulate the optical properties of multi-chromophoric systems such as light-harvesting complexes, which absorb in the infrared spectral range. Further work is required to coherently describe the complexity of plasmon interactions in this system.

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