Real-time fluorescence sensing of single photoactive proteins using silver nanowires

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Abstract

We demonstrate that single functionalized silver nanowires form a geometric platform suitable for efficient real-time detection of single photoactive proteins. By collecting series of images using wide-field fluorescence microscopy, events of single protein attachment can be distinguished with the signal to noise ratio further improved by fluorescence enhancement due to plasmon excitations in the nanowires. The enhancement is evidenced by strong shortening of the fluorescence decay of single photoactive proteins conjugated to the silver nanowires.

Efficient and specific sensing of molecules, proteins, and viruses requires combination of biorecognition protocol aimed at capturing desired species and detecting them with appropriately chosen method \cite{1,2}. The protocol typically relies on specifically binding pairs, such as streptavidin-biotin \cite{3-6}, virus-antibody \cite{7-9}, or Ni-complexation combined with His-tag \cite{10,11}. While proper functionalization of an active sensor area is a prerequisite for selectivity of detection, it is also important to assure that the number of functional groups is maximized in order to enhance the probability of binding events. Upon such a specific attachment, depending on the actual sensor design, changes in either electrical or optical characteristics \cite{1,2} can be monitored, and directly linked to detected species. Particularly attractive are schemes where such changes can be monitored in real-time, as it speeds up the detection process, what can be important in screening tests.

Among structures with high potential in sensing, metallic nanostructures featuring plasmon excitations, have been tested in many contexts, including, monitoring changes in the plasmon frequency upon attachment of molecules \cite{12,13} or proteins \cite{14,15}, emergence of aggregation-driven bands upon introducing viruses \cite{16} or proteins \cite{17}, as well as strong enhancement of Raman scattering signal \cite{18-20}. Nevertheless, the most pronounced effect associated with plasmonically active metallic nanostructures is metal-enhanced fluorescence (MEF), which in connection with highly sensitive detectors has been exploited for detecting fluorescent species \cite{6,21-23}. In contrast to sensing based on Raman scattering, where the distance between molecule and metallic surface should be minimal, in the case of MEF the separation should be in the range of at least a few nanometers, to inhibit the non-radiative energy transfer, which leads to emission quenching \cite{24}.

For plasmonic nanostructures deposited on a surface, it is critical either to assure geometric pattern thereof, which should enable direct read-out of the optical response \cite{21}, or to use nanostructures that can be directly visualized with an optical system. Silver nanowires have emerged as attractive example of the second variant: while featuring strong plasmon resonance \cite{25} in the wavelength range from 400 nm to 1000 nm, their positions can be determined in an optical microscope even under moderate magnifications. It is due to their lengths that can easily reach 100 microns. On the other hand, the diameters of about 100 nm, which are responsible for the plasmonic properties, yield also large surface which, upon
appropriate functionalization, can be the active area of the nanowire-based sensor. As a result, silver nanowires can be considered as generic building-blocks of fluorescence-based sensoric platforms.

In this work we demonstrate that combining wide-field fluorescence microscopy with proper surface functionalization of silver nanowires enables efficient detection of single photoactive proteins in real time. Protein-containing solution is dropped directly on a glass substrate with sparsely placed and randomly oriented silver nanowires. For a low concentration of protein we observed—within approximately 10 s—well-resolved, both temporally and spatially, events of single protein attachment. The fluorescence emission of these proteins is enhanced via interaction with plasmon excitations, as evidenced by shortening of fluorescence decay, thus substantially improving the signal to noise ratio. This result opens yet unexplored avenues of applying elongated metallic nanostructures as active elements for efficient real-time sensing. This rather universal approach can be extrapolated towards detecting variety of species, defined by the functionalization and pattern of the nanowires. Furthermore it differs qualitatively from previous attempts employing either single-molecule detection via remote excitation switching fluorescence microscopy [8], or advanced photoactivation localization microscopy [26], where in addition to complex experimental design, it was also necessary to use long incubation times of nanowires with targeted proteins.

Synthesis of silver nanowires in an aqueous solution was described previously [25]. Their diameters and lengths are around 100 nm and 40 microns, respectively. As-synthesized nanowires are coated with PVP polymer, which was removed and exchanged with cysteamine for further binding of biotin via amide formation [3]. The experiments were also carried out for as-synthesized nanowires, as a reference.

As a photoactive protein we used peridinin-chlorophyll-protein (PCP) photosynthetic complex described in detail previously [27, 28]. Relatively simple structure, excellent optical properties, and solubility in water render this protein a model system for studying interactions in hybrid nanostructures [4, 27]. In order to enable conjugation with biotin-functionalized silver nanowires, we used streptavidin-labeled PCP (BD-Biosciences).

The extinction spectrum of silver nanowires and absorption spectrum of PCP in solution were measured using Varian Cary 50 spectrophotometer. Fluorescence spectrum of PCP in solutions was measured with Jobin-Yvon Fluorolog 3 spectrofluorometer. Absorption spectrum of PCP (figure S1 in supplementary information is available online at stacks.iop.org/MAF/8/045004/mmedia) features a broad band from 350 nm to 550 nm, associated primarily with peridinin absorption with contributions from chlorophyll molecules at 400 nm and 650 nm [28]. The PCP shows emission at 673 nm (figure S1 in supplementary information). The extinction spectrum of silver nanowires (figure S2 in supplementary information) exhibits a broad peak with the maximum at around 390 nm.

The sensing characteristics of silver nanowires was studied with real-time fluorescence imaging using a wide-field fluorescence microscope. The sample was prepared by first spin-coating 15 μl of nanowire suspension (1:100 stock suspension) on a glass coverslip. In the next step, with a transmission mode of the fluorescence microscope, a region (90 × 90 microns) with several well-defined isolated silver nanowires was selected. After initiating acquisition of a sequence of fluorescence images from this region, 2 μl of PCP solution was dropped on the glass coverslip. The movies were collected for 3 min with each frame corresponding to 0.1 s. For excitation of fluorescence we used 100 μW LED illumination (Prizmatix Ltd, Israel) at the wavelength of 485 nm. The excitation beam was reflected by a dichroic mirror (T650 LPXR, Chroma) to an oil-immersion objective (Nikon Plan Apo 100x, NA 1.4). The emission of PCP was detected using iXon3 EMCCD camera (Andor, Ireland) after being filtered using edge filter FELH650 (Thorlabs, Newton, USA) and bandpass filter HQ 670-10 (Thorlabs, Newton, USA). Electron Multiplication gains between 100 and 800 were applied, depending on the concentration of PCP. Experiments for both non-functionalized and functionalized silver nanowires were performed with identical acquisition parameters.

Time- and spectrally-resolved fluorescence measurements of PCP complexes conjugated with silver nanowires were carried out using home-built scanning confocal fluorescence microscope. The emission was excited at the wavelength of 485 nm, which corresponds to the maximum PCP absorption. The laser repetition rate was 20 MHz and power was 16 μW. The excitation beam was focused on the surface glass coverslip using a high numerical aperture objective (LP Plan Apo VC, 60 ×, NA = 1.4, Nikon, Tokyo, Japan). In all experiments the emission signal was directed through a confocal aperture and a long pass filter HQ655LP (Chroma). For measuring fluorescence decay curves and fluorescence intensity maps we used additional bandpass filter ET675/20 (Chroma). Fluorescence maps were measured using a piezo-electric stage with signal readout from SPCM-AQRH-16 avalanche photodiode (PerkinElmer, Waltham, MA, USA). For measuring decay curves in selected place we used time-correlated single photon counting module (SPC-150 Becker & Hickl, Berlin, Germany) with a fast avalanche photodiode (idQuantique id100-50, idQuartique, Geneva, Switzerland).

The results obtained for three experimental configurations are summarized in figure 1, where transmission data is shown together with fluorescence images acquired for the same region at indicated times. Full movies are included in supplementary information. In the case of concentrated PCP solution
(0.2 μg ml⁻¹) deposited on biotin-functionalized silver nanowires (upper row) we observe fast and efficient brightening of elongated shapes in the fluorescence images, with those shapes perfectly correlated with the positions of silver nanowires seen in transmission mode. This result indicates that proteins in the droplet attach to silver nanowires within just a few seconds. Moreover, the fluorescence intensity of PCP attached to silver nanowires is significantly higher than of those in the background, which may suggest plasmon enhancement of the PCP emission. Importantly, with time the emission from the PCP-nanowire conjugate becomes more uniform, which results from sequential attachment of PCP and may indicate also high density of biotin functionalization of silver nanowires.

This aspect is particularly critical when carrying out the experiment for highly diluted PCP solution (0.3 ng ml⁻¹). The result of such a measurement is shown in figure 1 (middle row). In this case the events of PCP attachment to silver nanowires are sporadic, therefore at the beginning the fluorescence pattern cannot be directly linked to the positions of the nanowires seen in transmission image. After first 100 s we find just a few emission spots that can be presumably attributed to single PCP conjugated to silver nanowires. The number of such spots increases with time. Clearly, knowing the positions of the nanowires prior to collecting the movie is critical for precise assessment of conjugation for such highly diluted protein solution.

The importance of surface functionalization of silver nanowires for detection of photoactive proteins in solution is evident when comparing the results obtained for functionalized silver nanowires with those without biotin functionalization (lower row in figure 1). Despite using concentrated PCP (0.2 μg ml⁻¹) solution and applying identical parameters for image acquisition, we find no increase of fluorescence intensity at positions of the nanowires placed on the substrate.

In figure 2 we analyze the temporal behavior of fluorescence intensity measured for highly diluted PCP solution. We plot fluorescence intensity of PCP along a silver nanowire as a function of time. First events of PCP attachment to the silver nanowire can be seen after approximately 10 s, which indicates that the conjugation—even at such a low concentration—can be used for fast detection of molecules. With time the number of attached single PCP complexes increases, as shown by comparing the cross-sections extracted at times of 60 and 180 s (figures 2(b), (c)). In the first case only one well-resolved peak is observed, while after 180 s at least four of them can be distinguished. On the other hand, cross-sections along the temporal axis enable to extract fluorescence timetraces of PCP conjugated to the silver nanowire. Two examples are displayed in figures 2(d), (e). We can determine in this way a moment of attachment of PCP to the nanowire and then monitor the evolution of its fluorescence intensity. In the case of the timetrace shown in figure 2(d) it seems that few PCPs might attach to the nanowire in this particular location. In contrast, the behavior found for the fluorescence intensity displayed in figure 2(e) is more similar to a two-step photobleaching observed previously for individual PCP complexes [28].

The results of fluorescence imaging indicate that silver nanowires are suitable for detecting single photoactive proteins, even at relatively low protein
concentrations. Such a possibility has been postulated in our recent work [4], but experimental demonstration thereof, including verification that indeed the fluorescence signal originates from single proteins, as reported here, lifts the concept of using metallic nanowires for sensing to the next level. By taking into account the weight of the PCP with streptavidin wires for sensing to the next level. By taking into account the weight of the PCP with streptavidin (92 kDa) it can be estimated that the number of proteins in a droplet of diluted solution is of the order of 10^6. If the area of the droplet is about 1 mm^2, then the region probed in the experiment (90 × 90 microns) contains approximately 10^4 PCPs. On the other hand, estimated surface coverage of the silver nanowires (figure 1) is a fraction of percent, which roughly translates to the experimental observation of individual PCP attachment to silver nanowires. In addition to providing some rough estimation, this discussion also points out the approaches to improve the limit of detection. First of all, the number of silver nanowires has to be much higher within the imaging area of the substrate. In addition, it would also be necessary to confine the protein solution to the area imaged within the optical detection system.

The experiment carried out on highly diluted PCP helps resolving the question about plasmonic enhancement of fluorescence of PCP coupled to silver nanowires. The emergence of plasmon coupling in such structures has been assumed recently [4], but unambiguous confirmation requires measurement of emission dynamics. In figure 3(a) we compare fluorescence decay curves measured for PCP conjugated to silver nanowires, both for highly concentrated (blue) protein solution and for single PCP complexes (red) protein solutions, with the reference (black). Importantly, for both conjugates we measure strong shortening of fluorescence decay for PCP complexes coupled to silver nanowires, moreover, the decays are almost monoexponential, indicating rather uniform coupling with plasmon excitations in silver nanowires. The latter is indeed expected for such types of structures [29]. The results shown in figure 3(a) demonstrate also that the shortening of fluorescence decay is the same for an ensemble of PCP conjugated to silver nanowires and for single PCP complexes. As measuring the decays of fluorescence emission gives a direct measure of the plasmonic enhancement, we conclude that the coupling between electronic states in the photoprotein and plasmonic excitations in silver nanowires yields the enhancement which is of the order of 10. In addition, by analyzing emission spots similar to those observed in figure 1, which originate from single PCP emission, we can directly compare their intensities with those measured for single PCP on glass substrate. In figure 3(b) we show such a comparison for around 120 single PCPs on silver nanowires (red) and 50 on glass (black). The average respective intensities being equal to 5700 counts and 2300 cps, yield the average enhancement factor of 2.5, with maximum enhancements reaching a factor of 8. Although experimentally determined values and distributions of fluorescence intensities of single PCP complexes coupled with silver nanowires is partially a direct consequence of a shadow effect, which would cause that the emission intensity measured for a protein attached to the top of the nanowire would be minimal, as can be seen in figure 1, even such a moderate effective enhancement factor is sufficient to provide very high contrast for fluorescence imaging experiment carried out for individual photoproteins.

In summary, combination of functionalization that leads to biorecognition, high density of functional groups on silver nanowires, and geometry constrain provided by silver nanowires, offers sensing efficiency down to single photoproteins in a real-time fluorescence imaging experiment. The optical contrast
is further improved by enhancement of fluorescence attributed to coupling between electronic states of the pigments within the protein and plasmon excitations in silver nanowires.

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References

[1] Borisov S M and Wolbeis O S 2008 Optical Biosensors Chem. Rev. 108 423–61
[2] Fan X, White I M, Shopova S I, Zhu H, Suter J D and Sun Y 2008 Sensitive optical biosensors for unlabeled targets: a review Anal. Chim. Acta 620 8–26
[3] Acuna G P, Moller F M, Holzmeister P, Beater S, Lalkens B and Tannefeld P 2012 Fluorescence enhancement at docking sites of DNA-directed self-assembled nanoantennas Science 338 506–10
[4] Szalkowski M, Sulowska K, Grzelak J, Niedziółka-Jönsson J, Rożniecka E, Kowalska D and Mackowski S 2018 Wide-Field Fluorescence Microscopy of Real-Time Bioconjugation Sensing Sensors 18 290
[5] Zhang C, Chen Y, Liang X, Zhang G, Ma H, Nie L and Wang Y 2017 Detection of hepatitis B virus M204I Mutation by quantum dot-labeled DNA probe Sensors 17 961
[6] Su L et al 2015 Visualization of molecular fluorescence point spread functions via remote excitation switching: fluorescence microscopy Nat. Commun. 6 6287
[7] Holford T R J, Davis F and Higson S P J 2012 Recent trends in antibody based sensors Biosens. Bioelectron. 34 12–24
[8] Grzelak J, Sulowska K, Leśniewski A, Rożniecka E, Janczuk-Richter M, Richter Ł, Loś M, Jönsson-Niedziółka M, Mackowski S and Niedziółka-Jönsson J 2018 Capturing fluorescing viruses with silver nanowires Sensors Actuators B 273 689–95
[9] Lee J-H, Kim B-C, Oh B-K and Choi J-W 2013 Highly sensitive localized surface plasmon resonance immunosensor for label-free detection of HIV-1 Nanomed. Nanotechnol. Biol. Med. 9 1018–26
[10] Yao H, Zhang Y, Xiao F, Xia Z and Rao J 2007 Quantum dot/bioluminescence resonance energy transfer based highly sensitive detection of proteases Angew. Chem. Int. Ed. 46 4346–9
[11] Bolduc O R et al 2011 Modified peptide monolayer binding His-tagged biomolecules for small ligand screening with SPR biosensors Analyst 136 3142
[12] Haes A J, Zou S, Schatz G C and Van Duyne R P 2004 Nanoscale optical biosensor: short range distance dependence of the localized surface plasmon resonance of noble metal nanoparticles The Journal of Physical Chemistry B 108 6961–8

Figure 3. (A) Fluorescence decay curves measured for single PCP complexes conjugated with silver nanowires (red), conjugates with high protein concentration (blue) and the reference (black). (B) Fluorescence intensities of single PCP complexes attached to silver nanowires (red) and deposited on a glass substrate (black).
[13] Murphy C J, Gole A M, Hunyadi S E, Stone J W, Sisco P N, Alkilany A, Kinard B E and Hankins P 2008 Chemical sensing and imaging with metallic nanorods Chem. Commun. 544–57

[14] Gonzalez-Gonzalez M, Jara-Acevedo R, Matarraz S, Jara-Acevedo M, Paradinas S, Sayagües J M, Orfao A and Fuentes M 2012 Nanotechniques in proteomics: protein microarrays and novel detection platforms Eur. J. Pharm. Sci. 45 499–506

[15] Unser S, Bruzas I, He J and Sagle L 2015 Localized surface plasmon resonance biosensing: current challenges and approaches Sensors 15 15684–716

[16] Kannan P, Los M, Los J M and Niedziolka-Jonsson J 2014 T7 bacteriophage induced changes of gold nanoparticle morphology: biopolymer capped gold nanoparticles as versatile probes for sensitive plasmonic biosensors Analyst 139 3563–71

[17] Dominguez-Melina S et al 2016 Adsorption and unfolding of a single protein triggers nanoparticle aggregation ACS Nano 10 2103–12

[18] Kneipp K, Wang Y, Kneipp H, Perelman I T, Itzkan I, Dasari R R and Feld M S 1997 Single molecule detection using surface-enhanced raman scattering (SERS) Phys. Rev. Lett. 78 1667–70

[19] Kowalska A A, Kaminska A, Adamkiewicz W, Witkowska E and Tkacz M 2015 Novel highly sensitive Cu-based SERS platforms for biosensing applications: highly sensitive SERS platforms for biosensing applications J. Raman Spectrosc. 46 428–33

[20] Akil-Jradi S, Jradi S, Plain J, Adam P-M, Bijeon J-L, Royer P and Bachelot R 2012 Micro/nanoporous polymer chips as templates for highly sensitive SERS sensors RSC Adv. 27837

[21] Kinkhabwala A, Yu Z, Fan S, Avoliovich Y, Müller K and Moerner W E 2009 Large single-molecule fluorescence enhancements produced by a bowtie nanoantenna Nat. Photonics 3 654–7

[22] Wientjes E, Renger J, Curto A G, Cogdell R and van Hulst N F 2014 Strong antenna-enhanced fluorescence of a single light-harvesting complex shows photon antibunching Nature Comm. 5 4236

[23] Lakowicz J R 2001 Radiative decay engineering: biophysical and biomedical applications Anal. Biochem. 298 1–24

[24] Anger P, Bhardwaj P and Novotny I 2006 Enhancement and quenching of single-molecule fluorescence Phys. Rev. Lett. 96 113002

[25] Szalkowski M, Janna Olmos J D, Buczyńska D, Maćkowski S, Kowalska D and Kargul J 2017 Plasmon-induced absorption of blind chlorophylls in photosynthetic proteins assembled on silver nanowires Nanoscale 9 10475–86

[26] Lin H, Centeno S P, Su L, Kenens B, Rocha S, Stawa M, Hofkens J and Uji-i H 2012 Mapping of surface-enhanced fluorescence on metal nanoparticles using super-resolution photoactivation localization microscopy Chem. Phys. Chem. 13 973–81

[27] Hofmann E, Wrench P M, Sharples F P, Hiller R G, Welte W and Diederichs K 1996 Structural basis of light harvesting by carotenoids: peridinin-chlorophyll–protein from amphidinium carterae Science 272 1788–91

[28] Wörnke S, Mackowski S, Brotossudarmo T H P, Jung C, Zumbusch A, Ehrl M, Scheer H, Hofmann E, Hiller R G and Brauchle C 2007 Monitoring fluorescence of individual chromophores in peridinin–chlorophyll–protein complex using single molecule spectroscopy Biochimica et Biophysica Acta (BBA)—Bioenergetics 1767 956–64

[29] Niedziolka-Jonsson J and Mackowski S 2019 Plasmonics with metallic nanowires Materials 12 1418