Immobilization and plasmon-enhanced fluorescence of EGFP on Al nanohole arrays

V E Bochenkov1*, A M Shakhov2, A A Astafiev1,2 and V A Timoshenko1

1 Department of Chemistry, Lomonosov Moscow State University, Leninskiye Gory 1-3, Moscow, 119991, Russia
2 Semenov Institute of Chemical Physics of RAS, Kosygina st. 4, Moscow 119991, Russia
E-mail: boch@kinet.chem.msu.ru

Abstract. Enhanced Green Fluorescent Protein has been covalently immobilized on thin perforated Al films with short-order nanohole arrays using (3-Glycidyloxypropyl)trimethoxysilane and native aluminum oxide layer. The nanoholes’ diameter varied in range 60–120 nm, allowing to tune the surface plasmon resonance frequency. The fluorescence intensity enhancement up to factor 2.75 for the nanohole arrays with the largest overlap between EGFP fluorescence band and surface plasmon resonance.

1. Introduction
Fluorescent microscopy has proven to be an extremely useful technique for microbiology, since it enables studying the microstructure and processes inside living cells, tracing the proteins, nucleic acids, etc [1]. The discovery of a family of fluorescent proteins, which can be genetically encoded for in vivo protein tagging inside the cell, has expanded the possibilities of this technique even further. Nowadays, a number of single-molecule fluorescent microscopy techniques are being developed to increase the resolution and sensitivity of the method. The use of fluorescent proteins for single-molecule techniques requires high fluorescence intensities, which can be achieved by using plasmon-enhanced fluorescence (PEF) [2]. Typically, noble metals like Au and Ag are used for plasmonic applications. For PEF, a spacer layer between metal and dye molecules is needed to avoid fluorescence quenching, which is efficient below approx. 5 nm [3].

Alternative plasmonic materials, such as Al or Cu are of high practical interest due to the lower production cost. Recently, Al nanohole arrays have been used for PEF of DBMBF2 molecules dispersed in a polymer matrix [4]. Here we develop a strategy for covalent immobilization of fluorescent proteins on the native aluminum oxide layer for PEF applications.

2. Experiment
2.1. Nanofabrication
Aluminum nanostructures have been fabricated using the sparse colloidal lithography approach, described in detail elsewhere [5]. In brief, round glass slides (0.2 mm thick, 25 mm in diameter) were cleaned in acetone, ethanol and Milli-Q water (by 10 min with ultrasonication) followed by drying in nitrogen flow and 30 min of UV/Ozone oxidation. Then, triple polyelectrolyte layer was deposited by applying aqueous solutions of Poly(diallyldimethylammonium chloride
(2% wt, 30 sec), Poly(sodium 4-styrenesulfonate) (2% wt, 30 sec) and Polyaluminium chloride (5 % wt, 30 sec). Then, sulfate latex microspheres (Invitrogen, USA) have been deposited from 0.2% wt solution in Milli-Q water. After rinsing with Milli-Q water and drying in nitrogen flow, the samples have been treated for 3 min by UV/ozone to remove polyelectrolyte layers and improve adhesion. Aluminum layer of 20 nm thickness has been deposited on top of the glass slides with polystyrene particles using physical vapor deposition in vacuum at normal incidence. Each nanosphere served as a lithographic mask by shadowing a round region on the substrate under it. The particles have been removed by tape stripping. Samples with average diameter of nanoholes of 60, 80 and 120 nm have been fabricated using this approach. All samples have been treated for 3 min by UV/Ozone to remove the polyelectrolyte residues and to facilitate Al surface oxidation.

2.2. Protein immobilization
Protein immobilization has been carried out using the process depicted in Fig. 1. First, surface oxide has been activated using (3-Glycidyloxypropyl)trimethoxysilane (GLYMO) as a binding agent. The samples have been placed in a desiccator above a small beaker with 0.2 mL of GLYMO and evacuated down to residual pressure of about 0.01 mbar. This way resulted in the most uniform surface coverage. After 1 hour, the samples have been taken out to air. The increased water contact angle (from < 10° to 60°) proved the formation of a hydrophobic layer on the surface. In this way the perforated and non-perforated Al films as well as clean glass slides have been activated for protein binding.

The protein binding step has been carried out right before fluorescence studies. Covalent binding of EGFP has been achieved via the reaction of GLYMO epoxy-groups with one of the protein’s surface amine groups. For each sample, a 20 µL droplet of 0.25 M EGFP solution in phosphate buffered saline (PBS) has been placed on top of the GLYMO-modified surface and covered by a microscope slide to prevent water evaporation. After 20 min, the top cover slide has been removed and the sample has been rinsed by PBS.

2.3. Spectroscopic measurements
Extinction (1-T) spectra of the perforated Al films have been recorded in range of 300–900 nm using UV-vis-NIR spectrometer (Jasco V-770) with a film holder accessory. Spectra have been registered in two-beam mode with a clean glass slide as a reference.
Fluorescence emission spectra have been recorded using a custom built optical setup. Femtosecond laser pulses with a 80 MHz repetition rate, 950 nm central wavelength, and energy up to 25 nJ were generated by a Titanium-Sapphire oscillator (Tsunami, Spectra-Physics). Frequency-doubled pulses at 475 nm were focused by a 40x 0.75NA objective lens (UPlanFLN, Olympus) on the sample’s bottom surface. Fluorescence excited by two-photon absorption of laser pulses was collected by the same objective, filtered with 500 nm long-pass filter and coupled to an Acton SP300i monochromator and then to a PI-MAX 2 CCD camera (Princeton Instruments) which recorded fluorescence emission spectra.

**3. Results and discussion**

The extinction spectra of the perforated Al films in air are presented in Fig. 2. One can see that only the samples with 120 nm holes have good overlap between LSPR and the EGFP emission peak.

To estimate the effect of plasmon resonance on fluorescence intensity, the measurements have been carried out on EGFP, immobilized on perforated as well as on non-perforated Al films and glass without Al. Obtained fluorescence intensities have been normalized by the emission at maximum (510 nm) observed on glass. Then, the intensities have been divided by the transmission at the excitation wavelength (475 nm) to account for different transparency of the samples. The resulted fluorescent spectra are presented in Fig. 3.

As it can be seen from the spectra, the normalized fluorescence intensity obtained on non-perforated Al films is almost the same as on glass, that suggests that 1) there is approximately the same amount of protein immobilized on Al compared to glass and 2) there is no fluorescence quenching observed on Al.

For the perforated Al films, the highest fluorescence intensity is observed for 120 nm holes, about 2.75 times brighter than for EGFP on glass. This result is in a good agreement with the spectral overlap between LSPR band of Al nanoholes and the emission peak of EGFP.
4. Conclusions
We presented a successful strategy for immobilization of fluorescent proteins on surface-oxidized Al nanostructures. The aluminum oxide layer together with the GLYMO linker molecules serve as an efficient spacer to minimize fluorescence quenching.

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