



State-of-the-art plasmonic crystals for molecules fluorescence detection

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Abstract: We propose a method of measuring low concentrations of fluorescent molecules located in a small volume of a liquid solvent (about 5 μ l) based on the Ebbesen effect of the extraordinary transmission (EOT) of light through a state-of-the-art plasmonic crystal formed by a nanohole array perforated in the ultra-high-quality Ag film. In the method, the EOT effect is realized at the fluorescence wavelength of the detected molecules with a low transmission of light at the absorption wavelength. This approach enables the realization of high level sensor sensitivity approaching a sensitivity level of single molecules counting sensors, owing to the suppression of the sensor substrate's inevitable parasitic luminescence. The proposed method was successfully demonstrated by detection an ultra-low concentration of Cy-5 fluorescent markers in a dimethyl sulfoxide solution corresponding to less than 1000 molecules in the sensor detection volume.

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1. Introduction

The detection of small concentration is extremely important in fundamental research and various applications. The ability to detect a single atom or molecule is a key aspect of investigating single quantum object properties and QED effects [1–5]. The detection of ultra-low concentration at the level of single molecules has practical importance with regard to the detection of clinically relevant biomarkers in blood serum [6–10].

The most developed methods of single molecule detection are based on measuring the fluorescence of these molecules [3, 4, 6–8, 11]. However, as a rule, the practical implementation of single-molecule sensitivity is limited by the background parasitic signal [3, 4]. This background signal has different origins, such as the scattering of laser light and the luminescence of various materials (substrates, solvents). However, the luminescence of media surrounding the detected molecule is practically undistinguished from the molecule fluorescence and, as a rule, exceeds it since it is determined by a much larger volume of media. This leads to a known trade-off between the volume of molecule excitation (a large volume is needed to perform fast measurements) and the sensitivity of the corresponding single molecule counting (SMC) sensors: a use of small excitation volume reduces parasitic luminescence and helps realize single molecule detection but in this case a lot of time is needed to count enough number of molecules to measure molecules' concentration [6].

In this paper, we propose a new molecule detection method based on the EOT light transmission through an array of nanoholes perforated in a metal film (plasmonic crystal, PC), which allows the suppression of various types of parasitic luminescence. We developed a new type of sensor based on this method which has a sensitivity that approaches the sensitivity of the SMC sensors. The sensor is based on the optical excitation of molecules, their subsequent fluorescence detection,

and the Ebbesen effect of the extraordinary transmission of light (EOT) to detect fluorescence of a small number of molecules.

The EOT effect is already being used in sensorics [12, 13]. It has been shown that small variations in the refractive index (RI) of the media adjacent to a nanohole array (plasmonic crystal) lead to changes in the dispersion of excited plasmonic waves, which in turn results in the shifting of the EOT effect's resonant wavelength measured through the optical transmission of the nanohole array [13–19]. But they cannot reach the sensitivity of the SMC sensors because the RI change caused by the presence of a single molecule is negligible in comparison with the RI changes caused by temperature or pressure instabilities. The smallest detected RI change corresponds to 10^8 molecules introduced into the sensor detection region [20].

2. Experimental section

2.1 Methods

In this study, we utilized the EOT effect using a completely new approach to perform effective fluorescence measurements with a sensitivity approaching the sensitivity of the SMC sensors. In our approach, we used the EOT effect to create an optical filter, and this resulted in a significant increase in the signal-to-noise ratio, owing to a significant decrease in the parasitic substrate luminescence. The spectral transmission of the PC (nanohole array) was chosen to achieve the high transmission of light (EOT effect) at the wavelength of molecule fluorescence, and low light transmission at the laser wavelength. This allows us to significantly suppress the parasitic luminescence of the substrate caused by the exciting laser light, which is typically the main limitation factor of sensor sensitivity based on the fluorescence measurements of large analyte volumes; that is, volumes larger than 1 nl. Moreover, the sensitive fluorescence measurement of such large volumes is a key factor in achieving fast measurement with SMC sensors [6].

Furthermore, plasmonic sensor based on Ebbesen effect allows a further increase of sensitivity due to reducing background signal associated with a surface imperfections (corrugations, scratches). The large background signal is formed by the laser scattering on the quartz surface and the luminescence of the particles on the surface. These surface imperfections have different origins, such as surface polishing defects and the adsorption of particles from the air. The detection of molecule fluorescence through a high quality PC has next advantages to prevent the surface imperfections impact on sensing. First, the luminescence from such particles does not enter the fluorescence detection zone because the microparticles block the passage of light through the nanohole. Secondly, most particles are located between the nanoholes, and their luminescence does not reach the detection zone.

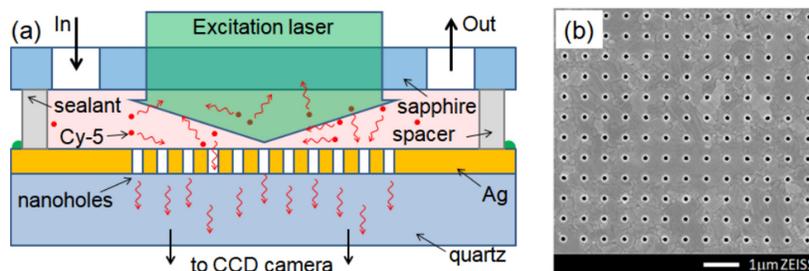


Fig. 1. Plasmonic sensor based on Ebbesen effect: (a) schematic diagram of sensor; (b) electron microscope image of PC formed by a matrix of nanoholes perforated in 100 nm thick silver film.

The EOT transmission through a PC formed by nanoholes perforated in metal film consists of the enhanced incident light wave's energy transfer to the other side through the PC via surface

plasmons [14, 21–23]. The light transmission through the PC can be much higher than the geometric transmission determined by the total area of all of the PC's holes. At the normal illumination of a PC, the wave vector of the excited plasmon wave is determined as follows [21]:

$$\vec{k}_{spp} = \left(\frac{2n_x\pi}{\Lambda} \right) \vec{i} + \left(\frac{2n_y\pi}{\Lambda} \right) \vec{j}$$

where Λ is the pitch of the nanohole array, and n_x and n_y are integers. Thus, the spectral regions of the EOT effect and the low light transmittance of a PC are realized at the wavelengths determined by the period of the nanohole array [21, 22].

2.2 Sample design and fabrication

Figure 1(a) illustrates the design of a plasmonic sensor. The sensor is built such that it can effectively measure the fluorescence of a single drop of Cy-5 molecules in a solution. The sensor uses an ultra-high quality PC [24–27], formed by nanoholes with a diameter of 175 nm perforated in large grain silver film with a thickness of 100 nm [28, 29] on a quartz substrate. (Figure 1(b)) Plasmonic properties of Ag film were characterized with the use of plasmonic waves optical microscopy. [30, 31] The size of the nanohole array is 1×1 mm. In the sensor, we used an analyte solution (Cy-5 dye molecule in dimethyl sulfoxide, DMSO) placed between two surfaces, namely, a PC surface and a YAG substrate surface with low surface roughness (Shinkosha Ltd.). In the YAG substrate, two holes with a diameter of 2 mm were formed for the input and output of the analyte. Between these surfaces, quartz stripes were arranged to create a gap with a thickness of $100 \mu\text{m}$. The listed components of the plasmonic sensor were glued together, and this provided the sensor with mechanical stability, tightness, and ease of use.

Fluorescence measurements The volume of the plasmonic sensor filled by analyte was equal to $5 \mu\text{L}$. A drop of analyte solution was introduced into the sensor through one of the holes made in the YAG substrate using a pipetator. Further, this drop was dragged into the sensor by the forces of surface tension, and spread evenly without bubbles over the entire PC volume.

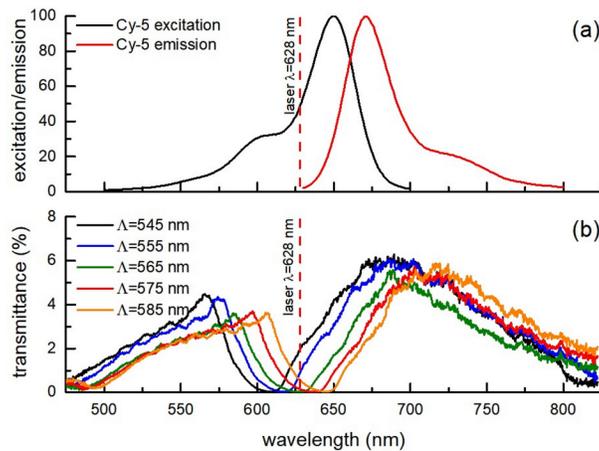


Fig. 2. Spectral selectivity of plasmonic sensor based on Ebbesen effect: (a) excitation spectra of Cy-5; (b) transmission spectra of nanohole arrays with different pitch (Λ). Dashed line indicates laser wavelength $\lambda = 628$ nm.

A detailed theoretical analysis of the plasmonic sensor under investigation is a rather difficult problem because it requires the consideration of various effects such as: the excitation of spatial hybrid “photon + plasmon” modes [24]; changes in the refractive index of DMSO, when Cy-5 molecules are introduced into it [17]; the adsorption of some Cy-5 molecules on the surface [32];

the change in the transmission of light through the nanoholes, when the Cy-5 molecules are close to the nanohole [33]; the QED effects in the molecule fluorescence near the nanohole [34].

The plasmonic sensor was located in the object plane of a Nikon Eclipse/Ti-U microscope. The sensor was illuminated by laser radiation at a wavelength of $\lambda = 628$ nm, with a power of 10 mW from a diode laser. The laser radiation illuminated the surface of the PC normally to the Ag film. The size of the laser spot on the sample was equal to $20 \mu\text{m}$.

The emission of the Cy-5 molecules excited by the laser radiation passed through the nanohole array and was collected with a $\times 10$ microscope objective on an EM CCD camera (Princeton Instruments). The CCD camera was installed next to a bandpass filter with a bandwidth of 100 nm, which corresponded to the Cy-5 dyes center of fluorescence wavelength (Figure 2(a)).

Figure 2 (b) shows the measured transmittance spectra of the sensors formed by PCs with different nanohole array pitches (Λ): 545 nm, 555 nm, 565 nm, 575 nm, and 585 nm. The figure clearly shows the spectral windows of the light's high and low transmission, owing to the EOT effect. During these measurements, the sensors were filled with a pure DMSO solution without Cy-5 molecules. As can be seen in the figure, the transmission spectra were characterized by a region of high and low transmittance. The spectral position of these regions depends on the pitch of the nanohole arrays (Λ).

The smallest transmission through a PC was realized at the wavelength of laser light ($\lambda = 628$ nm) for a nanohole array pitch of $\Lambda = 565$ nm. The measured transmittance was equal to $T_{exc} = 0.03\%$. Such a strong laser radiation attenuation by a PC makes it possible to significantly weaken the luminescence of the quartz substrate excited by laser radiation.

The highest transmission of a PC with $\Lambda = 565$ nm was realized at the wavelength of the Cy-5 molecules and was equal to $T_{Cy-5} = 5\%$ (PC transmittance at wavelength of Cy-5 molecule luminescence). Thereby, the figure of merit (FOM) characterizing the sensitivity increase realized by the PC such that $FOM = T_{Cy-5}/T_{exc} \approx 166$. Note that such a high FOM value results from the high quality of the PC formed by silver film with minimal losses for plasmon waves, nanoholes with identical size and shape, and the identity of the distance between nanoholes throughout the entire PC area. It is important to note that the use of Cy-5 molecules in concentrations of less than 1 pg/ml does not change the spectral transmission of the plasmonic sensors, or its FOM. We would like to note that the filter possesses a high spectral selectivity (small spectral shift between reflection and EOT transmission windows, presented on Fig. 2 (b)) and characterized by a spectrally wide (about 40 nm) transmission window. This feature is important to realize effective spectral filter for the plasmonic sensors compared to Fabry-Perot type filters.

3. Results and discussion

Figure 3 presents the results of detection of Cy-5 molecules in DMSO solution with plasmonic sensor. Figure 3(a) shows an optical image of the PC surface on a CCD camera in the case where a pure DMSO solution (without Cy-5 molecules) was introduced into the sensor. The optical image shows a white spot corresponding to the luminescence of the quartz substrate, which was used to build the sensor. The green curve in the graph corresponds to the image cut of the spot along the horizontal axis. The amplitude of this parasitic luminescence determines the minimum concentration of dye molecules that are detectable by the sensor.

When a solution of Cy-5 dye molecules with a concentration of 40 pg/ml was introduced into the sensor, a noticeable increase in the recorded signal occurred in the image of the CCD camera (Figure 3(b)). In comparison with the images shown in Figures 3(a) (the sensor without dye molecules) and 3(b) (the sensor contains dye molecules), the signal exhibited a three-fold increase when a solution with dye molecules was introduced.

Figure 3(c) shows the detection of various Cy-5 molecule concentrations in DMSO solution. In this figure, it can be seen that the measured fluorescence signal depended directly on the the Cy-5 molecule concentration in the solution, while the plasmonic sensor allowed us to confidently

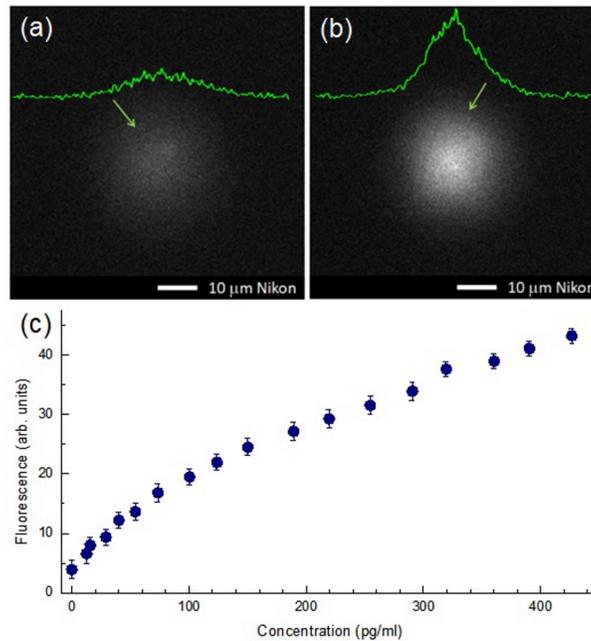


Fig. 3. Detection of Cy-5 molecules in DMSO solution with plasmonic sensor: (a) measured by 2D CCD camera luminescence of a pure DMSO solution without Cy-5 molecules in plasmonic sensor; (b) measured luminescence of Cy-5 molecule solution with a concentration of $n = 40$ pg/ml in plasmonic sensor; (c) amplitudes of luminescence with different concentrations of Cy-5 molecule solution introduced in plasmonic sensor, as measured by 2D CCD camera. Light-green curves in (a) and (b) indicate optical image sections.

register the dye molecules in the range from 20 pg/ml to 400 pg/ml.

The minimum recorded dye molecule concentration was 20 pg/ml (3 ppt). At this concentration, the detected fluorescence signal from the molecules was twice as high as the level of parasitic luminescence. At such a low concentration of Cy-5, the distance between the molecules was approximately $3.5 \mu\text{m}$. With the chosen laser beam sizes, this concentration corresponded to a detection of less than 10^3 Cy-5 molecules in the entire volume of the plasmonic sensor. This corresponds to the detection of a total dye molecule mass of less than 1 attogram, which is a new record with regard to plasmonic sensor sensitivity [20, 35]. Note that the sensitivity is approaching the detection limit of the SMC sensors because, for SMC sensors, more than 10^3 molecules must be detected to exceed a shot noise, owing to the Poisson molecule sampling [6]. The SMC sensors require hours to detect statistically reliable data [6]. In such cases, it is therefore necessary to concentrate the analytes into small volumes before interrogating them with a laser in the SMC sensors. In comparison, our approach helps in detecting the same number of molecules much faster, that is, in approximately one minute (CCD exposition time).

In the investigated range of dye molecule concentrations, the detected fluorescence signal should be proportional to the number of molecules. However, as can be seen in Figure 3(c), the dependence of the Cy-5 molecules' measured fluorescence is much more complex. We attribute this distinction to the quenching of fluorescence, when the molecules form agglomerates, because the number of the agglomerated molecules depends directly on their concentration [36].

The Cy-5 dye molecules used in the plasmonic sensor are known as fluorescent biomarkers [37]. Through appropriate antibodies, these molecules can be attached to various biomolecules serving as markers of various processes in the human body [38]. Thus, the proposed plasmonic sensor

can potentially become a hardware platform for medical applications using a small volume of blood plasma, which has to be analyzed at the highest sensitivity level, comparable to that of SMC sensors.

In summary, we developed a new type of plasmonic sensor based on the use of the Ebbesen effect of the extraordinary transmission (EOT) of light. A record level of sensitivity in plasmonics was demonstrated compared with the best known results [20, 35], paving a way to ultrasensitive sensors for biomarkers molecules using plasmonic structures.

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