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HIGH-CONDUCTIVE NANOSTRUCTURES IN BIOCHEMICAL STUDIES: FLUORESCENCE ENHANCING

V. I. CHEGEL[1], *A. M. LOPATYNSKYI*[1], *V. K. LYTVYN*[1], *O. M. NAUM*[1], *V. I. NAZARENKO*[2], *A. P. DEMCHENKO*[2]

[1]Lashkaryov Institute of Semiconductor Physics of the National Academy of Sciences of Ukraine, Kyiv

[2]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv

E-mail: Nazarenko@biochem.kiev.ua

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This paper presents the results of experimental and theoretical studies of quenching and enhancement of fluorescence by colloidal solutions of nanoparticles and arrays of nanostructures on solid substrates — nanochips.

The literature data and the results of authors' own studies on the possibility of fluorescence signal manipulation in the presence of gold and silver nanostructures were shown. Mathematical modeling and comparative investigation of the samples with high-conductive metal nanostructures as active elements for the regulation of fluorescence signal were also performed. Nanochips samples were fabricated by thermal annealing of highly conductive gold and silver island films. Using developed novel laser-based fluorometer FluorotestNano it was shown that fluorescence intensity of Rhodamine 6G dye can be enhanced up to 23 times near gold nanostructures by spacing the dye from the nanoparticle at the distance of 20 nm using SiO_2 coating. Using high-conductive metal nanostructures to adjust the fluorescence signal opens promising new directions in biochemical studies, such as increasing the sensitivity of fluorescence methods, development of new biosensors, fluorescence microscopy techniques and medical diagnostics.

Key words: nanostructure arrays, gold and silver nanoparticles, surface-enhanced fluorescence, polymer matrix.

Fluorescence techniques are widely used in biomedical research due to their high sensitivity, multiplex sensing possibility, compatibility with living organisms and high response speed [1–3]. It is possible to study living cells and even whole organisms by using fluorescence spectroscopy [2, 4, 5]. Fluorescence is a rapid and sensitive method for studying the structure, kinetics and functions of biological macromolecules such as nucleic acids and proteins [6–9].

Recently, nanoscale fluorescent emitters has attracted special attention for application in biological studies [10, 11]. In a short time, a number of applications has been found for these objects in clinical diagnostics [12], environmental monitoring [13], food quality control [14], biological weapons detection [15] and in various related fields. This diversity of applications is provided by bionanointeractions that allow delivery of nanoparticles to the biologically important systems to obtain information about them and even to change their properties [16–18]. Significant progress has been achieved in the application of high-conductive plasmonic nanomaterials for biochemical and diagnostic applications [19]. This review describes existing approaches for the development of sensors and biodiagnostic instruments based on surface-enhanced fluorescence (SEF) [20, 21] as well as the developed method and the device for its application.

Fluorescence enhancement by using high-conductive nanostructures

Studying the possibilities of using high-conductive metal nanoparticles (MNP) has attracted great interest. This is caused by the prospects of the MNP applications in biophysics and biochemistry, materials science

and fluorescent spectroscopy based on the surface enhancement methods [22]. Optical properties of MNP are mostly determined by the surface plasmons, which are inherent collective electron oscillations in the metal. Localized surface plasmon resonance (LSPR) that occurs in metal nanostructures is widely used in highly sensitive sensors and optical devices [23, 24]. To obtain enhancement of the fluorescence, silver and gold nanoparticles that have unique optical, electronic and catalytic properties are usually exploited. This is caused by their attractive properties, which include not only the simple fabrication, but also such features as the intense plasmon resonance band in the visible spectral region and significant extinction at the LSPR wavelength [25].

Enhancement of fluorescence of the dyes by using silver and gold nanostructures significantly depends on the resonance energy transfer from MNP generating the plasmon field to the dye molecule, which is located near MNP [26–28], shape and size of MNP [29], distance between the dye molecule and the MNP [23, 24, 26], as well as the characteristics of dye molecule such as intrinsic quantum yield and excited state lifetime [26, 27, 30].

In general, the fluorescence emission enhancement — is caused by the resonance energy transfer from plasmon-generating nanostructures to the dye molecule and subsequent change in the quantum yield of the "plasmon nanostructure-dye molecule" system and can be described by the equation [31]:

$$\Phi = \left(\frac{E_p}{E_0}\right)^2 \frac{q}{q^0},$$

where E_p is the plasmon electric field strength in the observation point that is generated by the incident light E_0; q is the quantum yield of the dye molecule induced by the plasmon field E_p; q^0 is the intrinsic quantum yield of the dye molecule.

Optimal distance to observe fluorescence enhancement

Optimal distance between the fluorophore and the metal surface is a very important factor in the mechanism of surface-enhanced fluorescence. This optimal distance depends on the immanent properties of the molecule relevant to energy exchange between the molecule and the surface plasmon. There are three cases for the location of dye molecule near the surface of nanoparticles. First of them is very close to the metal surface when the quenching of fluorescence is observed; second is an optimal distance resulting in the most significant enhancement of fluorescence; and the third is much greater than the distance at which the enhancement occurs [32].

The optimal distance can be determined both theoretically and experimentally [30]. For example, the emission simulation for a layer of fluorescent dye molecules with the thickness of 5 nm placed on the 80 nm spherical gold nanoparticle has shown that the optimal distance between the fluorophores and the metal surface to observe fluorescence enhancement was about 20 nm; in other cases there were weak enhancement or quenching [30]. In [33], the experimental studies of the dye fluorescence emission near the silver and gold nanostructures were carried out. It was found that the enhancement of fluorescence was observed when the distance between dye molecules and metal surface was 24–25 nm and the quenching was observed at 15 nm. If we consider the model system "fluorescent sphere-silver nanoparticle" [34], it turns out that when the distance between them is equal to 90 nm the emission quenching by 30% is observed. When the distance is decreased to some optimal value, 1.5-fold enhancement occurs. And if the second silver particle with the same properties is added to the system, 2.7-fold enhancement of fluorescence emission of the fluorescent sphere will be produced.

Methods for fluorescence enhancement using spacer layers

Consideration of the influence of the distance between the fluorophore and the surface of the metal nanostructures has led to the development of the methods enabling the fluorescence enhancement observation. The most effective technique for distance-controlled surface enhancement is to use the artificially created spacer layer between the fluorescent molecule and the silver or gold nanostructure, which prevents the emission quenching effect [23, 28, 33]. The function of spacers could be provided by organic (lipid or protein) [28, 35] (Fig. 1) or inorganic (silicon dioxide) separation layers.

For fluorescence enhancement studies organic "sandwich" structures are often used [28], which are dye-labeled protein molecules located directly on the surface of the high-conductive nanostructures. Here, protein molecules themselves act as the separation layers between the nanostructure and fluorophore. Such structures allow obtaining up to 18-times enhanced emission of dye molecules. In some experiments, the distance

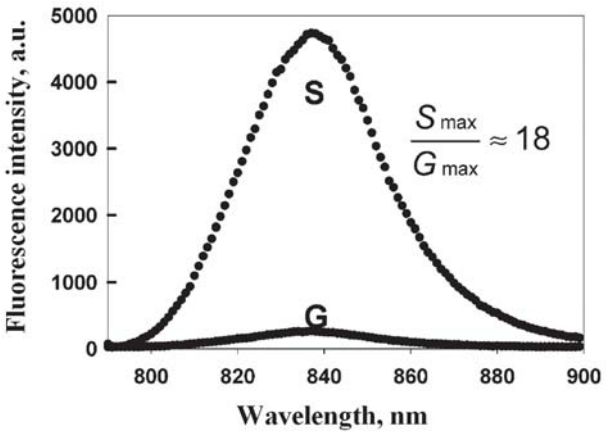

Fig. 1. **Fluorescence intensity of HSA-ICG-coated glass:**
without (curve G) and in the presence (curve S) of silver nanoparticles on the glass substrate [28]

between the nanoparticle and the dye molecule was adjusted by a double-stranded DNA molecule [26] or its replicating form [36]. Another interesting option is to exploit spacer layers fabricated from silicon dioxide. This coating deposited on nanostructures provides strength, chemical inertness and versatility for binding to biomolecules or any hydrophobic fluorophores, and also allows adjusting the distance between the fluorophores and the nanostructures [37]. For precise spacing of fluorophore molecules from nanostructured surface, the LbL (layer-by-layer) technology is often used [38] to produce oppositely charged polymer "sandwich" that enables fluorescence signal enhancement.

Influence of the size, position and shape of nanostructures on the enhancement effect

As mentioned above, the fluorescence enhancement using high-conductive nanostructures depends on their size and shape. In [39], the comparative analysis of fluorescent enhancement of the dye using colloidal gold nanoparticles of different diameters with protein-modified surface was carried out. It was found that nanoparticles with a 40 nm diameters allow obtaining 1.5-fold enhancement of fluorescence signal and nanoparticles of 200 nm diameters provide 2-fold enhancement, respectively. 40 nm cubic and spherical silver nanoparticles were used as the plasmon-generating nanostructures for fluorescence enhancement [40]. The authors experimentally found that the cubic silver nanoparticles provide higher fluorescence enhancement in comparison with the spherical silver nanoparticles of the same size. Effect of the size and the shape of nanoparticles on the enhancement value can be considered through the spectral overlap of the absorption spectra of nanostructure and the emission spectra of the fluorophore [41], because there is a direct relationship of the wavelength position and the half-width of the light extinction spectra with the geometrical parameters of the nanoparticles.

Another important fact is that the emission intensity of the dye placed near the nanostructure exhibits nonlinear dependence on their relative position [36, 37, 42]. In [23, 42–44], it was found that the largest enhancement of fluorescence was observed in the gap between closely spaced nanoparticles, where the intensity of the electric field near their surface is greater than at the surface of individual nanostructures. The study [45] showed that the enhancement of fluorescence of the dye placed near a plasmon-generating separate silver nanoparticle is about two times smaller than in the case of placing the dye molecule between two silver nanoparticles.

It should be noted that the fluorescence emission enhancement of the dye molecule located between two silver or gold nanoparticles depends on the geometry of this "sandwich" structure [47]. Namely, when the fluorescent sphere (FS) is sandwiched in the hot spot region right between the two gold nanoparticles on the axis connecting their centers, it leads to a strong fluorescence enhancement; in the other case the FS is not in the hot spot, and consequently, the fluorescence enhancement is less pronounced (Fig. 2).

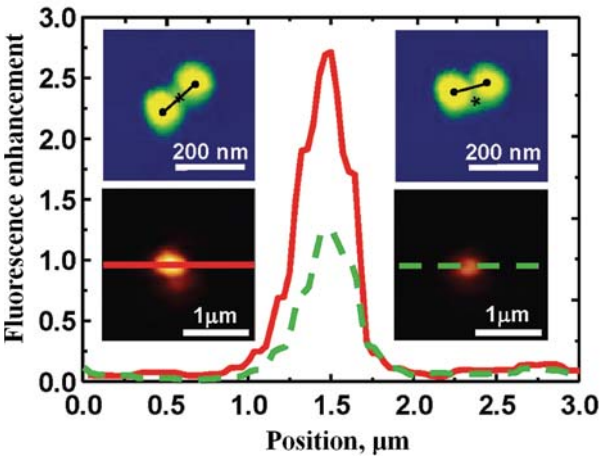

Fig. 2. **Dependence of the fluorescence intensities obtained with two different fluorescent sphere (FS) positions:**
solid red line — FS is sandwiched in the hot spot right between the two gold nanoparticles; dashed green line — the FS is not in the hot spot [47]

Mechanisms of surface enhancement

The interaction between the metal surface and fluorescent molecule provides such effects as increasing the radiative or non-radiative decay rates of fluorescent molecule and, accordingly, increasing or decreasing the quantum yield [30]. Enhancement of fluorescence depends on the plasmon resonance energy transfer from the nanostructured metal surface to the dye molecules located near the surface [26, 28, 46–48]. This transfer is determined by two mechanisms: first of them is the plasmon field generated around the nanoparticle by the incident light that, depending on wavelength, can enhance the excitation of the fluorophore, which, in turn, determines the level of fluorescence emission. The second one is the nanoparticle-fluorophore interaction that reduces the ratio of radiative to non-radiative decay rate and, depending on the presence of the dielectric layer, influences the quantum yield of the fluorophore, resulting in fluorescence quenching [31]. It should be noted that the enhancement of fluorescent emission depends not only on the properties of metal nanoparticle, but also on the properties of the fluorescent molecule. These properties are the quantum yield and the excitation relaxation time, which depend on the probability of quantum transitions involved in the radiative processes. Accordingly, the probability of quantum transitions under the certain conditions increases when fluorescent molecule is located near the metal surface. Quantum yield, which is defined as the ratio of the number of emitted to absorbed photons of the molecule, determines the efficiency of the emission, which, in turn, depends on the distance between the fluorophore and the surface of nanostructures [30, 44]. In [30], it was theoretically and experimentally shown that the quantum yield of a molecule decreases when the distance between the fluorophore and the surface of metal nanostructure is too small, even in the case of the sample excitation increase, and results in fluorescence quenching.

Studies of fluorescence of the dye solutions containing the high-conductive nanoparticles established that the quantum yield also depends on the pH. In [49], scientists found that the high pH of the dye solution has no effect on the enhancement; but when pH reduces – the fluorescence enhancement occurs, which is caused by increasing overlap of emission spectrum of the dye with the plasmon resonance band of the metal nanoparticles.

The presence of the nanostructured metal surface near the dye molecule affects not only the quantum yield and fluorescence enhancement, but also the radiative lifetime of the excited molecule [30, 49]. For example, 13-fold emission enhancement of the fluorophore is accompanied by decrease of the fluorescence lifetime by a factor of 22 [50].

An orientation of the dipole moment of the dye molecules and the polarization of excitation light has a considerable effect on the fluorescence enhancement [30, 44]. In the study [47] the fluorescence intensity of the sandwich structure "Au nanoparticle-FS-Au nanoparticle" is enhanced when the laser light is polarized parallel to the axis of the sandwich, whereas the fluorescence is decreased when the laser is polarized perpendicular to it (Fig. 3). The gray horizontal line shows the fluorescence level of the fluorescent sphere before the Au nanoparticles have been approached (using unpolarized excitation).

Theoretical calculations showed [30] that the maximum enhancement was observed in the case of perpendicular orientation of the dipole moment of the molecules to the nanostructured surface and when the distance between the

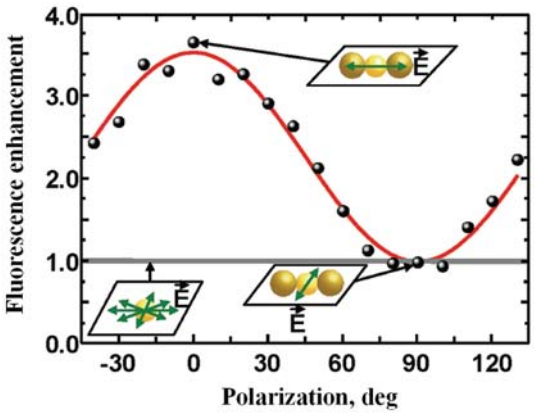

Fig. 3. **The fluorescence enhancement dependence on the polarization angle [47]**

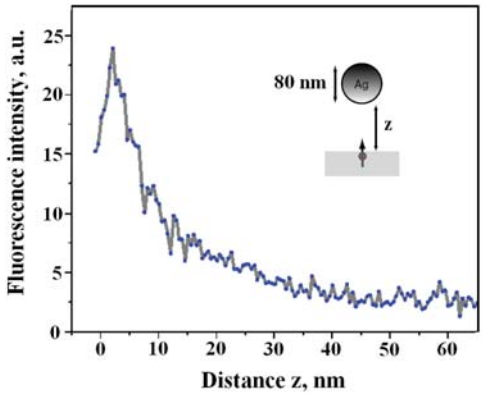

Fig. 4. **Single molecule fluorescence rate dependence on the particle-surface distance for an 80 nm silver nanoparticle [48]**

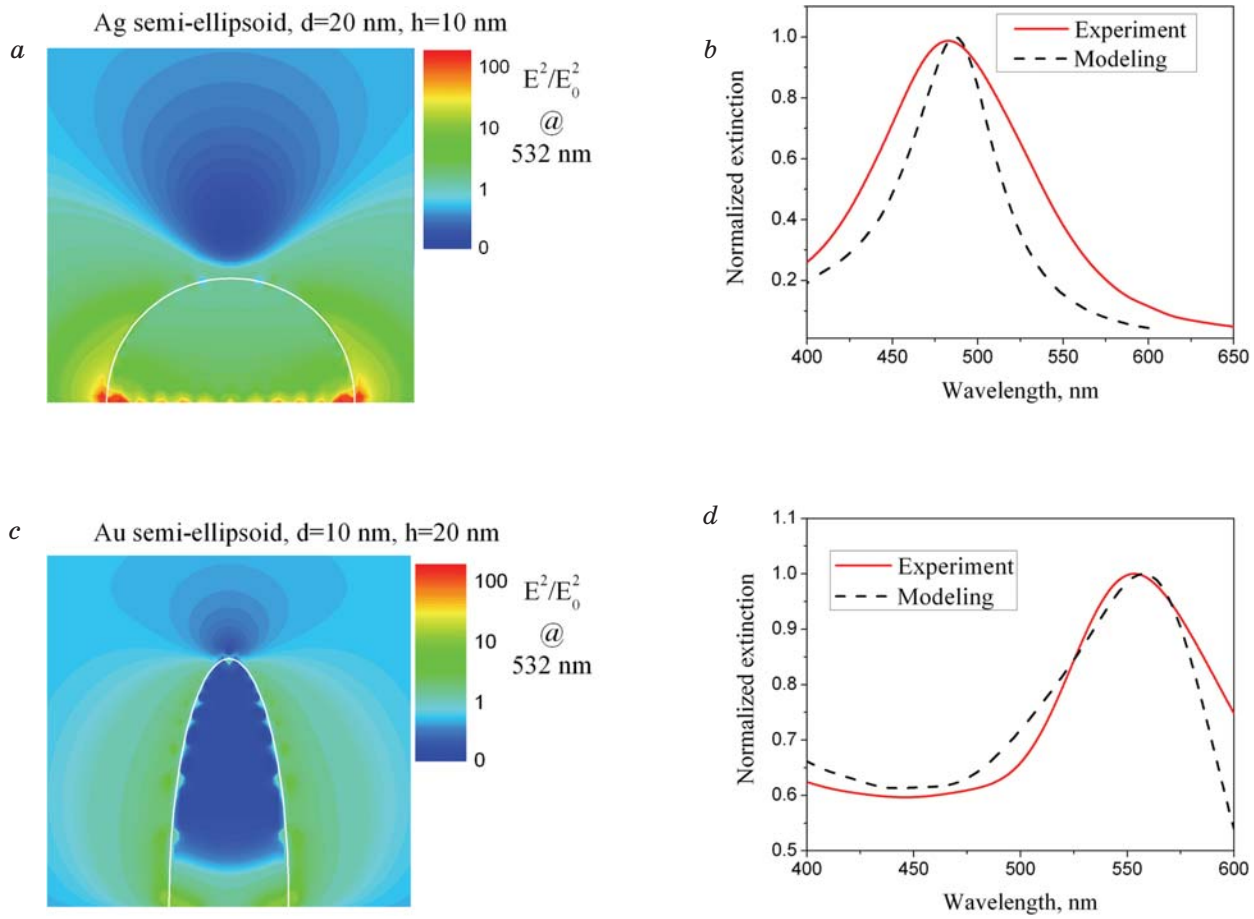

Fig. 5. **Simulated profiles of electric field intensity distributions for Ag (*a*) and Au (*c*) semi-ellipsoids on glass substrate; extinction spectra of unordered silver (*b*) and gold (*d*) nanostructure arrays**

molecule and the metal surface was optimal. Fig. 4 shows a single molecule fluorescence rate as a function of the particle-sample distance for an 80 nm silver particle in the case of perpendicular orientation of the dipole moment of the molecule [48]. At a short distance, the fluorescence enhancement drops due to the increased non-radiative decay rate (energy transfer from the excited molecule to the particle).

Experimentally, it is difficult to identify molecules with such orientation of the dipole moment, but the results of performed studies indicate that even at the angle of the dipole moment to the normal of the plane in which the nanostructures are placed equal to 15 degrees, 19-times enhancement of fluorescence can be observed [51, 52].

Modeling and comparative analysis of gold and silver nanostructures as the fluorescent signal amplifiers

Random silver and gold nanostructure arrays (NA) were obtained by thermal annealing of silver (mass thickness 8 nm, 250 °C, 1 hour) and gold (mass thickness 10 nm, 450 °C, 2 hours) island films [53]. Light extinction spectra of random silver and gold NA were measured using the LSPR biosensor [54]. Silver and gold NA exhibited the excitation of localized surface plasmon at 480 nm (Fig. 5, *b*) and at 555 nm (Fig. 5, *d*), respectively.

The average sizes of nanostructures forming the NA were calculated by matching the wavelength peaks positions in the spectra obtained experimentally and theoretically. Thus, for silver nanostructures the equivalent base diameter was found to be equal to 20 nm and height to 10 nm, and for gold nanostructures the equivalent base diameter is 10 nm and height is 20 nm.

For determined sizes of the gold and silver nanostructures, the profiles of electric field intensity enhancement around the nanoparticles were calculated (Fig. 5, *a, c*). These enhancement values correlate with the increase of the fluorescence excitation rate [27]. The calculation was performed at the 532 nm, which is an inherent excitation wavelength for the organic

dye Rhodamine 6G (R6G). The maximum electric field intensity enhancement value calculated on the 30×30 nm^2 area outside the nanostructure in the plane in which the propagation vector and polarization vector of incident light wave lie was about 100 times for silver nanostructure and about 30 times for gold nanostructure. The broadening in the extinction spectra of silver NA, observed in the experiment, compared with the simulation results (Fig. 5, *b*), can be explained by the growing mismatch between the model semi-ellipsoid and experimental nanostructure shapes and variety of sizes of nanostructures in the NA. Thus, silver nanostructures produced by thermal annealing of island films are potentially more promising in creating the nanochip. However, the gold nanostructures are mostly used in most scientific researches at the present time due to their higher chemical stability.

Experimental studies of fluorescence enhancement of Rhodamine 6G by using gold nanostructure arrays (GNA)

In this work, using the developed method of surface-enhanced fluorometry, based on the phenomenon of localized surface plasmon resonance in unordered GNA, the fluorescence measurements of R6G were carried out on the developed and patented novel laser-based fluorometer FluoroTest^{Nano}-2S (Fig. 6). The dye was placed on the plasmon-generating GNA with various thicknesses of the dielectric SiO_2 coating. SiO_2 layer deposited on the GNA provides strength, chemical inertness, versatility required to compound the biomolecule or any hydrophobic fluorophore, and also allows adjusting the distance between the fluorophore and the nanoparticle.

The samples of GNA with the thicknesses of dielectric spacer equal to 10, 15, 20 and 25 nm coated with a polymer composite, consisting of an aqueous solution of R6G and polyacrylic acid (PAA), were studied. R6G concentration in the polymer composite was equal to 10^{-5} mol/l. Fluorescence measurements of R6G on GNA were carried out with the 532-nm green laser used as an excitation light source. For all samples the enhancement of R6G fluorescence near the GNA was observed compared with the signal on the sample without GNA (Fig. 7). The intensity of fluorescent signal dependence on the SiO_2 coating was non-linear with an expressed peak. Maximum enhancement of R6G fluorescence was obtained for the sample with 20 nm thickness of SiO_2. Fig. 8 shows the enhancement factor dependence on the thickness of dielectric SiO_2 coating. Enhancement factor is defined as a

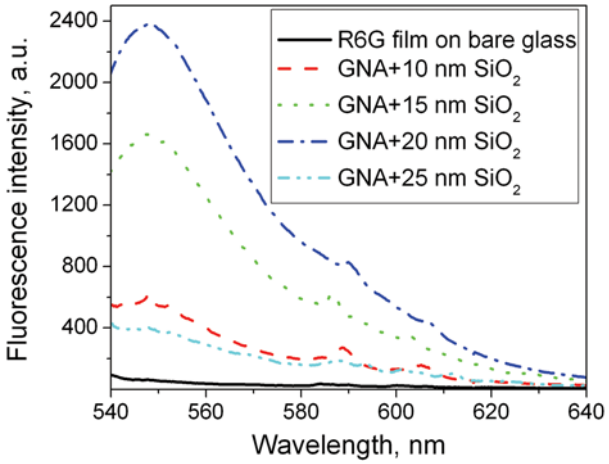

Fig. 7. **Fluorescence spectra of R6G dye for various thicknesses of the dielectric SiO_2 coating placed on the gold nanostructure arrays**

Fig. 6. **Portable laser-based fluorometer FluoroTestNano-2S**

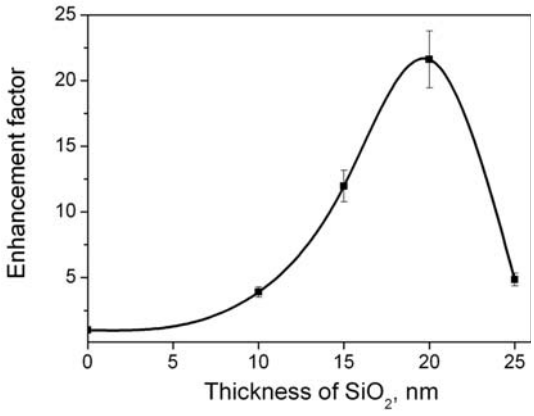

Fig. 8. **Enhancement factor of R6G fluorescence for different thicknesses of SiO_2 coating**

ratio of R6G fluorescence intensities for GNA with SiO_2 shell and bare glass substrate at the maximum emission wavelength.

The experimental results of R6G fluorescence enhancement (about 23 times) obtained by using nanochips are in agreement with calculated value of the electric field enhancement on the gold nanostructures. The disagreement between the calculated and experimental enhancement values is due to the influence of the dielectric substrate and the spread of the size and shape of nanoparticles on the real nanochip.

Plasmon-generating nanochips for plasmon-enhanced fluorescence based on the gold nanostructures arrays with dielectric SiO_2 coating of different thickness were developed and fabricated. Experimental studies of R6G fluorescence by using nanochips were carried out on the developed laser-based fluorometer FluoroTest^{Nano}-2S. The device enables registration of plasmon-enhanced fluorescence by using nanochips at visible wavelengths. It has been shown that the system "MNP-dielectric coating-dye", in which plasmon resonance appears, can be used to obtain the enhancement of the dye molecule. For gold nanostructure arrays with defined dimensions, the optimal thickness of the SiO_2 dielectric coating that provides enhancement of Rhodamine 6G was found to be 20 nm. This confirms the possibility of using the method of plasmon-enhanced fluorescence for detection of low-intensity fluorescence signals in biochemical research. The results are important for improving the fluorescence analysis technique and methods of fluorescent signals registration based on the phenomenon of surface enhancement by using high-conductive nanoparticles, development of the nanoscale SEF-nanochips and optoelectronic LSPR sensors with high sensitivity.

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ВИСОКОПРОВІДНІ НАНОСТРУКТУРИ У БІОХІМІЧНИХ ДОСЛІДЖЕННЯХ: ПІДСИЛЕННЯ ФЛУОРЕСЦЕНЦІЇ

В. І. Чегель[1]
А. М. Лопатинський[1]
В. К. Литвин[1]
О. М. Наум[1]
В. І. Назаренко[2]
О. П. Демченко[2]

[1]Інститут фізики напівпровідників ім. В. Є. Лашкарьова НАН України, Київ
[2]Інститут біохімії ім. О. В. Палладіна НАН України, Київ

E-mail: Nazarenko@biochem.kiev.ua

Описано теоретичні та експериментальні дослідження затухання і підсилення флуоресценції високопровідними наночастинками та масивами наноструктур на твердотільних носіях — наночипах.

Наведено дані літератури та власних досліджень авторів щодо можливості зміни сигналу флуоресценції наноструктурами золота і срібла. Проведено моделювання й порівняльне дослідження зразків з наноструктурами високопровідних металів як активних елементів для регулювання сигналу флуоресценції. Методом термічного відпалу високопровідних острівцевих плівок золота і срібла виготовлено експериментальні зразки наночипів. Із використанням розробленого авторами лазерного флуориметра Fluorotest^{Nano} показано, що інтенсивність флуоресценції барвника родаміну 6Ж може бути підсилено не менш як у 20–25 разів поблизу наноструктур золота шляхом віддалення барвника на відстань 20 нм за допомогою шару SiO_2. Застосування наноструктур високопровідних металів для регулювання сигналу флуоресценції барвників відкриває перспективні напрями досліджень, зокрема підвищення чутливості флуоресцентних методів, флуоресцентної мікроскопії та медичної діагностики, а також розроблення нових біосенсорів.

Ключові слова: острівцеві плівки, наночастинки золота і срібла, поверхнево-підсилена флуоресценція, полімерна матриця.

ВЫСОКОПРОВОДЯЩИЕ НАНОСТРУКТУРЫ В БИОХИМИЧЕСКИХ ИССЛЕДОВАНИЯХ: УСИЛЕНИЕ ФЛУОРЕСЦЕНЦИИ

В. И. Чегель[1]
А. Н. Лопатинский[1]
В. К. Литвин[1]
О. М. Наум[1]
В. И. Назаренко[2]
А. П. Демченко[2]

[1]Институт физики полупроводников им. В. Е. Лашкарева НАН Украины, Киев
[2]Институт биохимии им. А. В. Палладина НАН Украины, Киев

E-mail: Nazarenko@biochem.kiev.ua

Описаны экспериментальные и теоретические исследования затухания и усиления флуоресценции высокопроводимыми наночастицами и массивами наноструктур на твердотельных носителях (наночипах).

Приведены данные литературы и собственных исследований авторов относительно возможности изменения сигнала флуоресценции наноструктурами золота и серебра. Осуществлено моделирование и сравнительное исследование образцов с наноструктурами высокопроводящих металлов как активных элементов для регулирования сигнала флуоресценции. Методом термического отжига высокопроводящих островковых пленок золота и серебра изготовлены экспериментальные образцы наночипов. С использованием разработанного авторами лазерного флуориметра Fluorotest^{Nano} показано, что интенсивность флуоресценции красителя Р6Ж может быть усилена не менее чем в 20–25 раз вблизи наноструктур золота путем удаления красителя на расстояние 20 нм с помощью диэлектрического покрытия SiO_2. Применение наноструктур высокопроводящих металлов для регулирования сигнала флуоресценции открывает перспективные направления исследований, в частности увеличение чувствительности флуоресцентных методов, флуоресцентной микроскопии и медицинской диагностики, а также разработка новых биосенсоров.

Ключевые слова: островковые пленки, наночастицы золота и серебра, поверхностно-усиленная флуоресценция, полимерная матрица.