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MODELING OF CALMODULIN-MEDIATED PROCESSES IN TISSUES USING CALMODULIN-FUNCTIONALIZED GOLD NANOPARTICLES AND FLUORESCENT DYES

MODELIRANJE INDIREKTNIH PROCESOV KALMODULINA V TKIVU Z UPORABO KALMODULINA, FUNKCIONALIZIRANEGA Z ZLATIMI NANODELCI IN FLUORESCENTNIMI BARVILI

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We prepared and investigated a nanomaterial consisting of calmodulin functionalized with fluorescent dyes and gold nanoparticles and we have shown that calmodulin covalently linked to an excited fluorescent dye and gold nanoparticle can stimulate a surface-plasmon-coupled emission, resulting in a strong fluorescence enhancement. Herewith, calmodulin molecules were functionalized and stabilized with gold nanoparticles and fluorescent dyes in liquid and solid environments. The photo-optical properties of the proposed novel nanomaterial are promising for the development of a simple and effective method for the targeting, labelling and visualization of calmodulin-mediated processes, such as proliferation, inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, etc.

Keywords: nanomaterial, calmodulin, fluorescence dye, gold nanoparticles

Avtorji so pripravili in preiskovali nanomaterial, ki je vseboval kalmodulin (vrsta znotrajcelične beljakovine), funkcionaliziran s fluorescentnimi barvili in zlatimi delci. Raziskave so pokazale, da kovalentno vezan kalmodulin, na vzbujeno fluorescenčno barvilo in nanodelce, lahko stimulira površinsko sklopljeno plazemsko emisijo, ki povzroči močno vzbuditev fluorescence. S tem so z zlatimi delci in fluorescentnimi barvili funkcionalizirali in stabilizirali molekule kalmodulina v tekočem in trdnem okolju. Foto-optične lastnosti predlaganega novega materiala, ponujajo možnost izboljšanja enostavne in učinkovite metode za ciljano označevanje in uravnavanje, vizualizacijo indirektnih procesov kalmodulina, kot so: proliferacija (bujna rast tkiva), vnetja, metabolizem, apoptoze (tip programirane celične smrti), mišične kontrakcije ter intracelično gibanje itd.

Ključne besede: nanomaterial, kalmodulin, fluorescentno barvilo, zlati nanodelci

1 INTRODUCTION

Due to their advantageous physical, chemical and biological properties, nanomaterials hold great potential in the nanomedicine area for applications in biological sensing, biomedical imaging, drug delivery and photo-thermal therapy. The biological activities of these structures are highly influenced by the surrounding environment that has a significant role in the designing of these materials.[1] Nanoparticles can traverse through the vasculature and localize any target organ, leading to novel therapeutic, imaging and biomedical applications.[2] Gold nanoparticles (GNPs) are among the most extensively studied nanomaterials. GNPs are precious metal and they have a high surface area, easy fictionalization, high electric conductivity, high stability and corrosion resistance; their pronounced plasmon-resonance band in the visible range as well as sensitivity to aggregation are among their most attractive features.[3] In addition, combining the properties of GNPs with those of known organic dyes has already led to many interesting applications including sensing of biologically relevant molecules.[4,5] The bioconjugation of different biological structures to nanoparticles happens through the bonding of biomolecules to nanoparticles by chemical or biological means, which render them ideal for clinical applications.

A range of functionalized groups can be attached to nanoparticles including low molecular weight ligands, peptides, proteins, polysaccharides, polyunsaturated and saturated fatty acids, DNA, plasmids, RNA, etc.[6] Of particular interest are the emerging biomedical applications that directly utilize the plasmon-enhanced phenomenon for the targeting and visualization of cell proliferation, controlled by many networks of regulatory proteins. It is known that the action of calmodulin (CaM) and CaM-dependent signalling systems control the vertebrate cell proliferation, programmed cell death and, autophagy.[7] According to the literature, in tumor and

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transformed cells, the mobilization of Ca^{2+} is altered, which has important implications for the tumor development and progression.[8,9] Moreover, many effects of Ca^{2+} in cells are mediated by the binding of Ca^{2+} to CaM, which causes CaM to bind and activate target proteins.[10] Consequently, the targeting and visualization of the regions in biological tissues with an overexpressed concentration of the Ca^{2+}/ CaM complex could be a valid therapeutic approach to the targeting, labelling, and visualization of CaM-mediated processes.

The acquisition of images of biological matter using fluorescent labels and nanomaterials is generally referred to as bioimaging that forms a large field of its own.[11] Actually, CaM does not absorb or emit light in the visible part of the optical spectrum. Therefore, it could be of great interest to use a nanomaterial consisting of CaM conjugated with a fluorescent dye and GNPs as the potential optical indicator used for the labelling of tissues and biological cells including cancer cells. In this work, we prepared and investigated a nanomaterial consisting of CaM functionalized with a fluorescent dye and GNPs and showed that covalent linking of CaM to the excited fluorescent dyes and GNPs can stimulate the surface-plasmon-coupled emission, resulting in a strong fluorescence enhancement.

2 EXPERIMENTAL PART

Colloidal monodispersed GNPs with 50 nm in size, with a concentration of 7.15×10^{10} N/mL and molecular weight of 196.97 g/mol dispersed in an aqueous/citrate buffer (0.02 mg/mL), and a CaM (calmodulin bovine recombinant, expressed in *E. coli*, lyophilized powder, ≥98 %) with a molecular mass of 16.79 kDa, were purchased from Sigma–Aldrich. As the fluorescent dye, we used a Nile blue (Nb), (from "Exiton"). Nb is a cationic dye that is widely used for the labelling of biomolecules. Derivatives of Nb are potential photosensitizers in the photodynamic therapy of malignant tumors. This dye aggregates in the tumor cells, especially in the lipid membranes, and/or are sequestered and concentrated in the subcellular organelles.[12,13] Due to the strong interaction between the Nb molecules and the large localized fields induced by the plasmonic coupling, a highly enhanced fluorescence is produced allowing the localization of GNP aggregates.[14] The absorption and fluorescence spectra of our samples were recorded with a multi-fiber-optic spectrometer (Avaspec-2048, "Avantes"). A photoexcitation of the nanomaterials was performed with a laser light source at λ = 532 nm. Imaging-based techniques, involving a fluorescence microscope (FM) and a scanning electron microscope (SEM), were used to image the prepared nanomaterials and to examine the interaction between the GNP, CaM and Nb constituents. To measure the decay of the fluorescence intensity with time, a Hamamatsu photomultiplier tube coupled to an oscilloscope was used.

3 RESULTS

In the experiments, we used the above-mentioned initial materials to prepare three separate colloidal mixtures embedded into different volumetric vials: one was filled with the Nb fluorescent dye dissolved in double distilled water, with a concentration of 5×10^{-3} mg Nb/1.5 mL water/pH 7.5; the second one was filled with 5×10^{-3} mg Nb/1.5 mL water/1 mg CaM/1 mL water/pH 7.5; the third mixture consisted of 2×10^{-2} mg GNPs/mL/5×10^{-3} mg Nb/1.5 mL water/1 mg CaM/1 mL water/pH 7.5. All the mixtures were stirred for 20 min at room temperature to avoid the aggregation and to obtain homogeneous solutions. The prepared liquid solutions were placed into the cuvettes having a square base (1 × 1 cm) and a height of 3 cm. In particular, one cuvette was filled with 5×10^{-3} mg Nb /1.5 mL water solution and the other two with 5×10^{-3} mg Nb/1.5 mL water/1 mg CaM/1 mL water, and 2×10^{-2} mg GNPs/mL /5×10^{-3} mg Nb/1.5 mL water/1mg CaM/1 mL water solution, respectively. Prepared mixtures were stored overnight at room temperature. In order to investigate the absorption and emission spectra of Nb/water, Nb/CaM / water, and Nb/CaM/GNPs/water solutions, we utilized a spectrometer. In experiments, we found that the doping of CaM and GNPs substances in Nb/water solution significantly changes the absorption and emission spec-

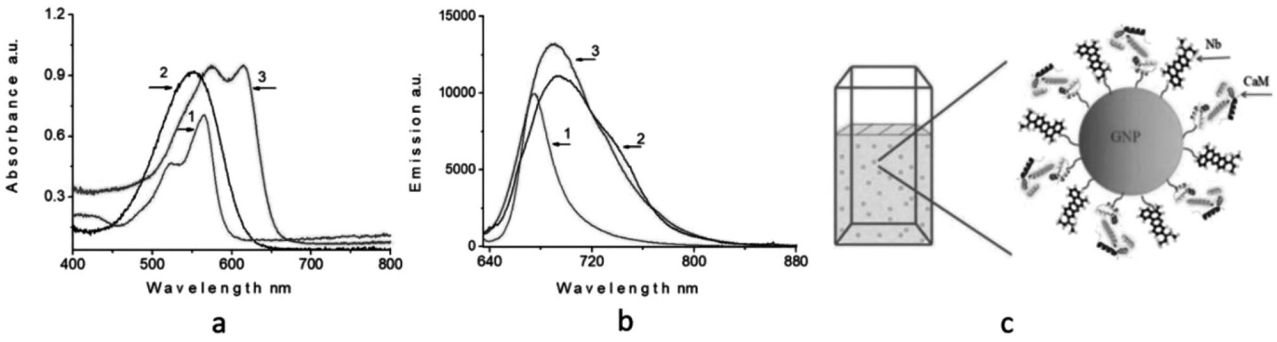

Figure 1: a) Absorption, b) emission, spectra of solutions: 5×10^{-3} mg Nb/1.5 mL water (1); 5×10^{-3} mg Nb/1.5 mL water/1 mg CaM/1 mL water (2); 2×10^{-2} mg GNPs/mL/5×10^{-3} mg Nb/1.5 mL water/1 mg CaM/1 mL water (3) and c) schematic illustration of a cuvette filled with Nb/CaM/GNPs/water suspension and GNPs functionalized with Nb and CaM

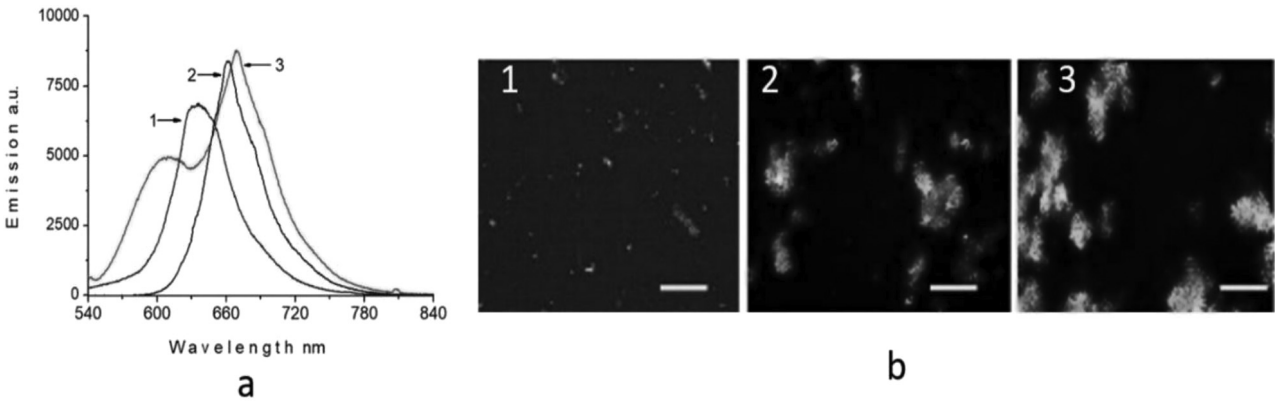

Figure 2: Fluorescence intensities emitted from Nb 1), Nb/CaM 2) and Nb/CaM/GNPs 3) clustered composites excited by the laser light source with λ = 532 nm: a), and FM images of randomly distributed nanoclusters on the glass surfaces b). The scale bar corresponds to 100 μm

tra of the obtained composites. As shown in **Figure 1**, the absorption bands of Nb/CaM/water and Nb/CaM / GNPs/water solutions are significantly broadened compared to the absorption band of the Nb/water solution. Moreover, an absorption peak of Nb/CaM/ GNPs/water solution is red shifted by 18 nm compared to the Nb/water solution. As regards the light emission, a small amount of CaM doped in Nb/water solution leads to the significant enhancement of the fluorescence emission spectrum which becomes even stronger in Nb/CaM/GNPs/water solution.

In the second part of the experiments, each solution (i.e., Nb water, Nb/CaM/water, and Nb/CaM/GNPs/ water was extracted from the corresponding cuvette and deposited by drop-coating onto the glass slides treated with deionized water. The liquid films were stored on the substrates for 24 h at room temperature to let the water evaporate completely. As a result, we obtained aggregations of nanomaterials in the form of clusters on the glass substrates. To investigate the output light intensities, emitted from the Nb, Nb/CaM and Nb/CaM/ GNPs clustered composites, we used an FM equipped with a green laser light source with λ = 532 nm. Fluorescence spectra were recorded using a spectrometer. Similarly to the previously described experiments, compared to the light emission from the Nb aggregations, in the Nb/CaM and Nb/CaM/GNPs composites, an enhancement of the emitted light and a redshift of the fluorescence peaks were observed.

Figure 2a demonstrates the fluorescence emissions from Nb 1), Nb/CaM 2) and Nb/CaM/GNPs 3) clustered composites, and Figure 2b shows the self-assembled clusters with different levels of aggregations, as seen with the FM. These self-assemblies appeared due to the dipole-dipole interactions between the nanomaterials. It should be noted that the Nb dyes are aggregated as small clusters, **Figure 2b** 1), whereas Nb/CaM from **Figure 2b** 2) and the Nb/CaM/GNPs composites from **Figure 2b** 3) are presented as large clusters.

To visualize and identify the spatial distribution of the Nb/CaM/GNPs nanomaterials, we utilized SEM. **Figure 3** shows the location and spatial distribution of the Nb/CaM/GNPs nanomaterials.

It should be noted that the emission spectra of the Nb/CaM and Nb/CaM/GNPs nanocomposites are in the deep-red part of the optical spectrum. Due to the reduced scattering and absorption of deep-red light in biological tissues, the proposed nanomaterial can be highly advantageous for the fluorescence image-guided techniques.

4 DISCUSSION

According to the Mie theory, small colloids up to 40 nm in diameter are expected to quench fluorescence because absorption is the dominant mechanism, while larger colloids, above 40 nm, are expected to enhance fluorescence because scattering becomes the dominant mechanism.[15] Besides, there are at least two factors that alter the fluorescence properties of a fluorescent dye in the presence of nanoparticles. These include the distance between the fluorescent dye and the nanoparticle and the orientation of the molecular dipole of the fluorescent dye in relation to the nanoparticle surface.[16,17] Because of its cationic attribute, Nb acts as a CaM binding molecule.

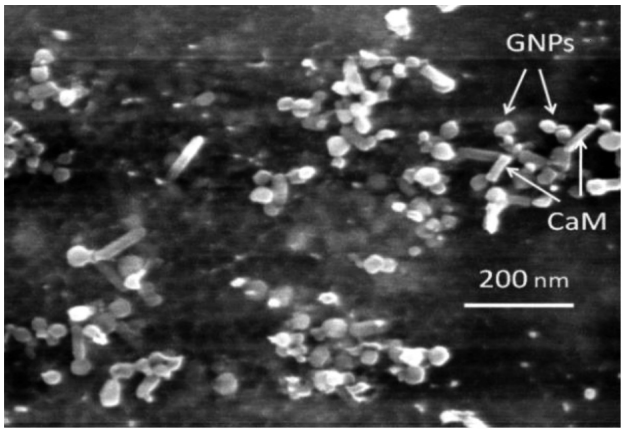

Figure 3: SEM image of Nb/CaM/GNPs nanomaterial, Nb/CaM molecules form the nanoclusters (100–150 nm) and are distributed in the proximity to the GNPs

Conjugation is achieved by forming an electrostatic attraction between the CaM and Nb molecules. Upon the Nb binding, CaM modifies its electrostatic configuration, which results in the modification of the optical properties of Nb molecules. One of the most important parameters that plays a crucial role in the distance-dependent energy transfer between the acceptor and donor is the energy-transfer efficiency, given with Equation (1) $E = 1 - \tau/\tau_0$, where τ and τ_0 are the lifetimes of the donor in the presence and absence of the acceptor, respectively.

To determine the time-resolved fluorescence, the fluorescence lifetimes of the samples were measured as a function of time after being excited by a laser beam. In particular, pulsed Nd:YAG laser light of 5 nanoseconds with a wavelength of 532 nm was used to excite the Nb/CaM (i.e., to determine τ_0), and Nb/CaM/GNPs (i.e., to determine τ) composites. As the detector, we utilized a Hamamatsu photomultiplier coupled to a GHz oscilloscope, which displayed the decay of the fluorescence intensity with respect to time. The estimated times were $\tau_0 \approx 8, 24 \times 10^{-9}$ s, and $\tau \approx 1, 36 \times 10^{-9}$ s, respectively. The induced electric field originating from the charge separation in the nanoparticles during the plasmon-resonance oscillations is very large at very small distances from the surface. The energy-transfer efficiencies from Equation (1) can be rewritten as Equation (2): $E = R_0^6/(R_0^6 + r^6)$. According to the measured data for the energy-transfer efficiencies from Equation (1), we found that $E = 0.84$. Finally, based on the experimental results and the calculated data, we found the distance between the dye molecules (Nb) and GNPs, which are statistically distributed on the surface, to be $R = 8 \pm 0.6$ nm.

5 CONCLUSIONS

We prepared and investigated Nb, CaM and GNPs based nanomaterials and demonstrated that CaM conjugated with the Nb fluorescent dye and GNPs can increase its fluorescence intensity upon the excitation of a pumping laser source. The obtained results can be put forward as a useful modality for the in-vitro representation of nanoparticle-mediated cancer biomarkers, cell labelling and tracking in biological tissues through fluorescence. It opens new possibilities for plasmonic applications in nanobiology and nanomedicine.

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