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Steady-state and time-resolved fluorescence studies on the conjugation of Rose Bengal to gold nanorods

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HIGHLIGHTS

- Examination of fluorescence performance of Rose Bengal conjugated to gold nanorods.
- Emission enhancement controlled by chemical and electromagnetic environment.
- Radiative and non-radiative deactivation revealed by fluorescence lifetime studies.
- Promising applications of Rose Bengal-gold nanorods in imaging and cancer therapy.

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This paper is dedicated to Professor Simion Simon on the occasion of his 65th birthday.

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ABSTRACT

This work examines the fluorescence properties of Rose Bengal (RB) molecules conjugated to cetyltrimethylammonium bromide (CTAB) – coated gold nanorods (GNRs) by performing steady-state and time-resolved fluorescence measurements. We show that the quantum yield and fluorescence lifetime can be significantly modified by the electromagnetic coupling of RB to GNRs but the overall fluorescence signal depends also on the environmental conditions in which RB molecules reside – in solution or on solid substrate. For example, we have observed the increase of fluorescence intensity after binding RB to GNRs (RB@GNR) as result of both non-radiative rate decrease and contribution from the electromagnetic coupling of RB to GNRs. For RB@GNRs conjugates deposited on solid substrate we can provide evidence for a clear metal-enhanced fluorescence (MEF) mechanism by observing the decrease of fluorescence lifetime of RB from an average of 2.1 ± 0.36 ns, when complexed to CTAB solely, to 1.6 ± 0.26 ns, when conjugated to GNRs, together with the increase of fluorescence intensity. Our findings contribute to the development and evaluation of novel fluorescent tags based on plasmonic nanoparticles for biomedical applications.

Introduction

The interaction of fluorescent molecules with plasmonic nanoparticles (NPs) has been intensively addressed over the last decade aiming to design new spectral properties and improved functionalities in medical diagnostics and biotechnology [1–3]. For instance, the coupling of photosensitizing molecules to plasmonic NPs has received a great deal of attention due to the attractive perspectives of performing both fluorescence imaging and photodynamic therapy (PDT) of cancer [4–6]. Specifically, the metallic moiety can provide the enhancement of the fluorescence signal though the phenomenon known as metal-enhanced fluorescence (MEF) while the photosensitizer in close vicinity of NP can offer the basis of performing PDT with enhanced rate of singlet oxygen generation [6]. Additionally, considering photosensitizer-NP conjugates with plasmon resonance band located in the near-infrared "biological window", it is possible to exploit the photo-to-heat conversion effect called plasmon-assisted hyperthermia together with PDT for performing a dual anticancer therapy.

The dianionic xanthene dye Rose Bengal (RB) is known as potent photosensitizer (singlet oxygen quantum yield of nearly 76% under 532 nm light irradiation [7]) but as poor emitter in water (quantum yield of 0.02). Due to their photodynamic activity, RB molecules are applicable in the treatment of skin diseases like psoriasis and atopic dermatitis [8], inhibition of oral cancer DNA polymerases [9] or inactivation of various biological species such as vaccinia virus or *Escherichia coli* [10]. Other exploitation of RB molecules include photo-catalysis [11], photo-activation of

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the fabrication of three-dimensional cross-linked bovine serum albumin microstructures [12] and staining for the diagnosis of eye disease in ophthalmology [13].

Most recently, the detection and therapy of oral cancer by using RB coupled with gold nanorods (GNRs) has been demonstrated [14,15]. GNRs represent a particular class of anisotropic gold NPs with unique optical properties generated by two modes of localized surface plasmon resonance (LSPR), i.e., transversal and longitudinal modes, and the intense electromagnetic field concentrated at their ends and corners [16,17]. GNRs have been demonstrated as versatile plasmonic NPs in photothermal cancer therapy [18,19], detection of biomarkers [20] or imaging contrast agents [21].

As result of chemical synthesis, GNRs exhibit a positively charged bilayer of cetyltrimethylammonium bromide (CTAB) surfactant which enables adsorption and transport of negatively charged photosensitizing drugs as RB molecules. Although both the investigation of fluorescence properties of RB molecules in relation to their photodynamic activity and the demonstration of RB@GNRs conjugates formation [22] have been reported, the investigation of fluorescence properties of RB in interaction with GNRs in solution and solid film have not been addressed from the perspective of extending both therapeutic and fluorescence properties. In this work, we studied the fluorescence properties of free RB and RB in RB@GNRs conjugates both in solution and deposited onto solid substrate through steady-state and time-resolved fluorescence measurements. Specifically, time-resolved fluorescence spectroscopy can provide useful information about the dynamic of molecular excitation and local interaction that is not accessible from steady-state fluorescence data. This technique allows for example discrimination of fluorophore when placed in heterogeneous environments and when the recorded fluorescence spectra look similarly. As the resonant excitation of LSPR promotes the enhancement of RB fluorescence intensity, time-resolved fluorescence measurements are indispensable to elucidate the enhancement mechanism. Moreover, we performed fluorescence lifetime imaging (FLIM) studies on RB@GNRs conjugates deposited onto solid substrate to reveal spatial and temporal response of RB molecules in experimental conditions close to biological samples.

Experimental details

Reagents

Tetrachloroauric acid ($HAuCl_4{\cdot}3H_2O$), cetyltrimethylammonium bromide (CTAB), ascorbic acid and Rose Bengal (RB) were purchased from Aldrich. Sodium borohydride ($NaBH_4$, 99%) and silver nitrate ($AgNO_3$) were obtained from Merck. All reagents were used as received. Ultrapure water (resistivity 18.2 MΩ) was used as solvent in all the experiments.

Samples preparation

CTAB-coated GNRs were synthesized using the seed-mediated growth approach detailed in a previous publication [23]. For the preparation of RB@GNRs conjugates, we incubated for several hours a solution of as-prepared GNRs (concentration of 0.57×10^{-9} M) with RB molecules (the final concentration of RB in the solution was 10^{-6} M) to allow the attachment of negatively charged RB to positively charged CTAB-stabilized GNRs. The interaction was monitored during incubation by absorption spectroscopy. As reference samples for fluorescence measurements we used 10^{-6} M RB in water and 10^{-2} M CTAB aqueous solutions, respectively.

Experimental measurements

The experimental results of this study were obtained by using the following techniques: (i) absorption spectroscopy, which was performed on a Double-beam Jasco V-670 UV–Vis/NIR spectrophotometer with 1 nm spectral resolution, equipped with a deuterium lamp (190–350 nm) and a halogen lamp (330–2700 nm), (ii) steady-state fluorescence spectroscopy by using a Jasco LP-6500 spectrofluorimeter; we have selected the wavelength of 523 nm for excitation and bandwidths of 3 nm in both excitation and emission and (iii) time-resolved fluorescence measurements by employing the MicroTime200 time-resolved confocal fluorescence microscope system, from PicoQuant equipped with a picosecond diode laser head operating at 510 nm and 40 MHz and a 60×/NA = 1.2 water immersion objective; the signal collected through the objective was spatially and spectrally filtered by a 50 μm pinhole and a FF01-519LP (Semrock, USA) emission filter, respectively, before being focused on a PDM Single Photon Avalanche Diode (SPAD) from MPD; the detector signals were processed by the PicoHarp 300 Time-Correlated Single Photon Counting (TCSPC) data acquisition unit; fluorescence lifetime decays in solution were obtained at room temperature after dropping the sample on microscope cover glass while FLIM imaging was performed on samples spin-coated on microscope cover glasses; the fluorescence lifetimes were obtained through reconvolution of the experimental decay curves with the instrument response function (IRF) measured by collecting the back scattered light from the laser; the goodness of the fit was judged by the chi-square values and by inspection of the residuals; time and spectral information from selected points in the FLIM images were simultaneously obtained by using a SR-163 spectrograph equipped with a Newton 970 EMCCD camera from Andor Technology coupled to an exit port of the main optical unit of MicroTime200 through a 50 μm optical fiber; a 50/50 beamsplitter was used to split the signal from the analyzed point towards the spectrograph and TCSPC unit of the MicroTime200 system; the integration time used for the acquisition of the fluorescence spectra was 5 s.

Results and discussion

When dealing with fluorescence properties of molecules in solution it is essential to limit their concentration in order to avoid intermolecular interactions and interference with so called inner filter effects [24]. Specifically, the attenuation of the excitation light or the re-absorption of the emitted light when may distort and alter the real fluorescent spectra of the high concentration samples. Additionally, when exceeding certain concentration the intermolecular interaction cause the formation of molecular dimmers or small aggregates which further influence the spectroscopic signal. Consequently, we first performed some preliminary experiments to calibrate the optimal concentration of RB in solution (data not shown here). Our findings showed that the inner filter effect and formation of dimmers start to become appreciable only at concentrations higher than 10^{-6} M. Therefore to ensure reliable spectroscopic data from free RB in solution and RB@GNRs conjugates we work here with RB concentration of 10^{-6} M.

Fluorescence of RB@GNRs conjugates in solution

The GNRs present two well-defined surface plasmon resonance bands at 520 and 736 nm, originating from the coherent oscillations of electrons perpendicular and parallel to the longitudinal axis of GNRs, respectively, as shown in Fig. 1.

The red spectrum in Fig. 1 provides reliable evidence for RB conjugation to GNRs surface. Indeed, 2 nm red-shifting of the