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Metal-enhanced ethidium bromide emission: Application to dsDNA detection

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ABSTRACT

Ethidium bromide (EB) is a commonly used probe for fluorescence detection and quantification of nucleic acids, since EB forms a highly luminescent complex with dsDNA. Typical detection sensitivity of EB-based assays to dsDNA in solution is about 0.1 μg. With the aim to increase the sensitivity of EB assays we have employed the Metal-Enhanced Fluorescence technology. We show that deposition of the EB/DNA complex solutions onto the silver nanoparticle surface additionally increases the far-field observable fluorescence ≈5-fold, yielding a net fluorescence enhancement factor of ≈180-fold, as compared to the free dye in solution. Subsequently, our approach enables the more sensitive detection of dsDNA.

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1. Introduction

The ethidium bromide (EB) dye is a well-known reagent which is routinely used for nucleic acids detection. Upon binding to DNA, EB shows characteristic changes in absorbance, displaying a bathochromic shift and hyperchromicity of its absorption band and fluorescence properties, demonstrating an enhancement in fluorescence intensity by about one order of magnitude, as compared to free dye in solution [1]. The quantum yield of EB fluorescence is low [2]. It has been proposed that in an excited state, the ethidium cation donates a proton to the solvent which significantly increase its nonradiative decay rate [3]. Intercalation into DNA isolates the EB from the solvent and consequently eliminates the proton-transfer mechanism associated with its quenching, i.e. causes an increase in EB brightness. The mechanism of EB interaction with DNA is well understood [4–6] (the structure of EB/DNA complex determined by X-ray crystallography [6,7]), as well as the increase in EB excited-state lifetime upon binding [2,8]. These unique spectral characteristics make EB relatively easy to use for measuring DNA concentrations in solution. However, the sensitivity of the EB to dsDNA is limited to about 0.1 μg nucleic acid in solution [8,9].

In the last decade our laboratory has published numerous reports, where in the presence of silver nanoparticles (NP), the fluorescence intensity of chromophores can be increased 5–100-fold [10,11] with a simultaneous significant increase in chromophore photostability, a near-field technology we have termed Metal-Enhanced Fluorescence (MEF). The MEF phenomenon is becoming widely used to increase the sensitivity of different fluorimetric assays in medicine and biology [10,12–14]. Here, we show that EB in complex with DNA in the presence of silver nanoparticles enhances the far-field fluorescence about 5-fold and becomes dramatically photostable. This allows fluorescence detection and quantitation of DNA molecules with near picogram sensitivity.

2. Materials and methods

Ethidium bromide (EB) was purchased from Invitrogen. The concentration of the EB was determined by measuring the optical density of EB solutions using an extinction coefficient of $E_{480} = 5600\ M^{-1}cm^{-1}$.

Complementary 16 bases oligonucleotides (5′-AGAGCGATATCGCGTG-3′ and 5′-CACGCGATATCGCTCT-3′) were purchased from Integrated DNA Technologies Inc. and additionally purified by anion exchange FPLC on a Mono-Q column, using a linear 0.1–1.0 M NaCl gradient in 10 mM Tris–HCl buffer (pH 7.0), 1 mM EDTA, 20% acetonitrile. The DNA was precipitated with ethanol, pelleted and air-dried. Concentrations of single strands and duplex were determined from the A_{260} of the nucleotides after complete digestion by phosphodiesterase I (Sigma) in 100 mM Tris–HCl (pH 8.0) [15]. The DNA duplex was prepared by mixing the complementary oligonucleotides in equimolar amounts, heating to 70 °C, and then cooling slowly to room temperature. The molecular weight of the 16 bp dsDNA is 9825.4 Da. Solutions of duplex DNA for the experiments were prepared by extensive dialysis against the required buffer.

Premium quality silane-prep™ glass slides, silver nitrate, ammonium hydroxide (30%) were obtained from Sigma. Silver-Island Films (SiF) were prepared as we have previously described [16].

Abbreviations: EB, ethidium bromide; MEF, Metal-Enhanced Fluorescence; SiFs, Silver-Island Films; NP, silver nanoparticles; CW, continuous wave.

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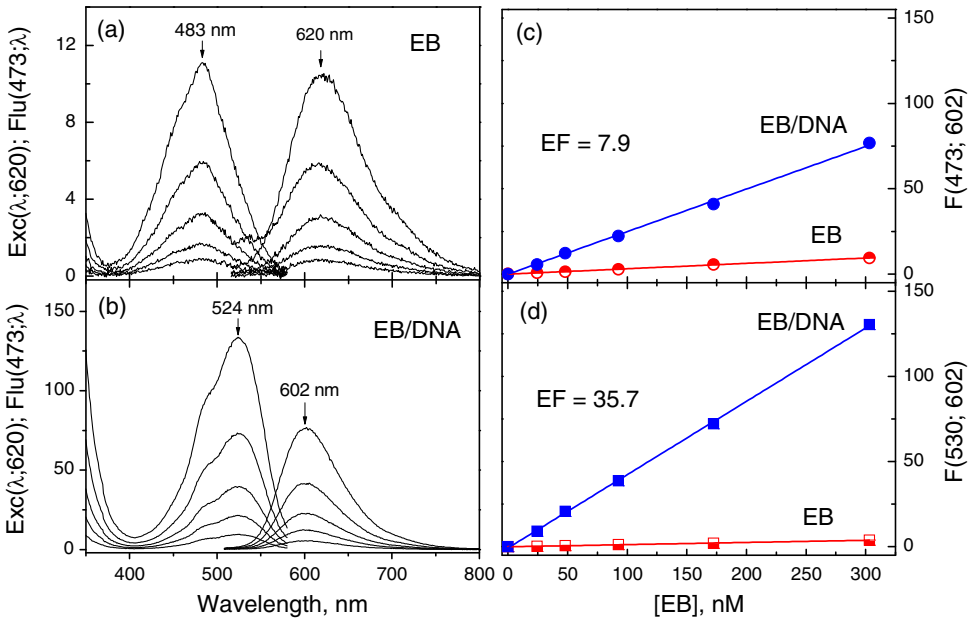

Fig. 1. Fluorescence and excitation spectra of EB free in solution (a) and in complex with short 16 bp DNA (b) collected for fluorophore concentrations of 24–300 nM. Fluorescence was excited at 473 nm; for excitation spectral measurements EB fluorescence was fixed at 602 nm. The dependence of the EB fluorescence intensity, excited at 473 nm (c) and at 530 nm (d), and monitored at 602 nm, for fluorophores in buffer and in solution, containing 16 bp DNA. Concentration of the 16 bp DNA = 34 μM (bp). Buffer: 10 mM Na-phosphate, pH 6.5.

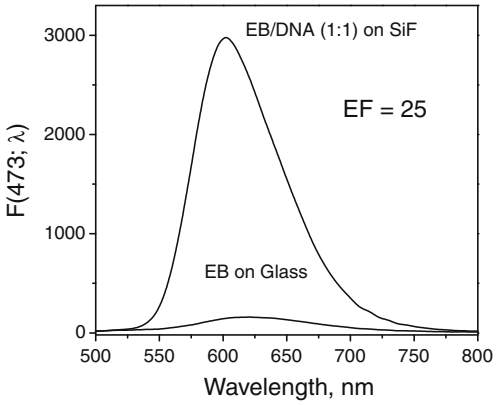

Fig. 2. Enhancement of EB fluorescence in complex with 16 bp DNA, loaded on SiFs, is EF = 25. The EB/DNA spectra were measured immediately after loading on SiFs.

In this study we used standard SiFs fabricated at deposition time 1–2 min. Mean size of silver islands was about 20 nm.

Samples of free EB and EB/DNA were loaded into a 20 μl well covering either glass or SiFs slides. The incubation time of the sample solutions in wells was varied from 1 to 30 min. Before measurements, samples were covered by a glass microscope cover slide to prevent drying.

Fluorescence intensity and spectra of the EB deposited on glass and SiF slides were measured using a HD2000 spectrometer, Ocean Optics, FL. Excitation of the EB emission on slides was performed using 473 nm CW laser.

Fluorescence and excitation spectra of solutions of free EB and EB in complex with the DNA in a quartz cuvette were measured on a Varian spectrofluorimeter at room temperature. The EB was excited at 473 nm and the fluorescence monitored over the wavelength range 500–800 nm. Excitation spectra were recorded be-

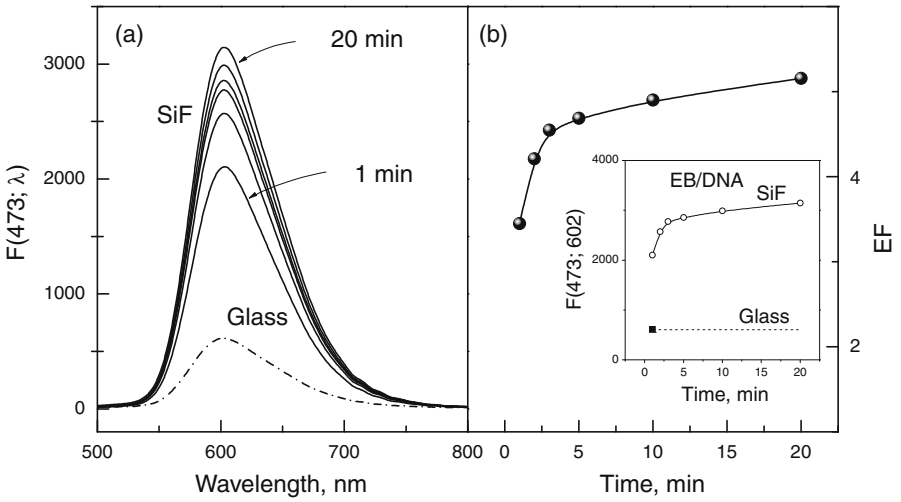

Fig. 3. (a) Fluorescence spectra of EB/DNA complex on SiF, registered at different times, and on a glass; (b) time dependence of the enhancement factor (EF) of EB/DNA fluorescence on SiF, relative to glass, upon the time of incubation on the SiFs surface. Insert: the dependence of EB/DNA complex emission upon incubation time on SiFs.

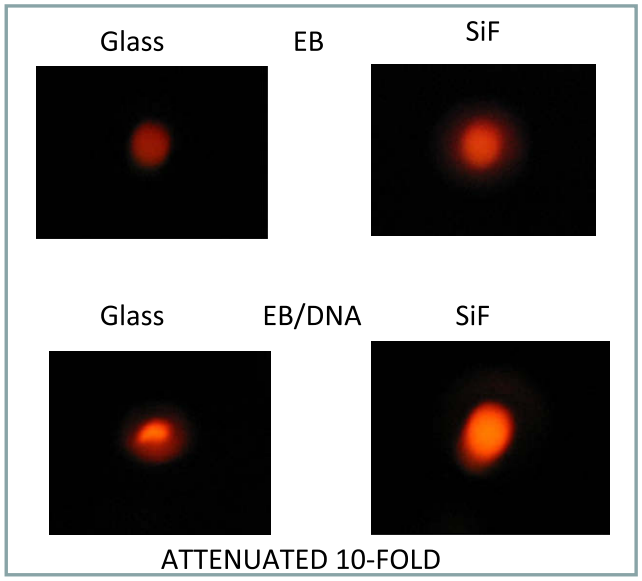

Fig. 4. Digital photographs of the fluorescence spots of free EB and EB in complex with 16 bp DNA, loaded on SiFs and glass slides. Attenuation of EB/DNA emission was undertaken using a neutral density filter for emission (OD = 1.0), attenuation was 10-fold. Power of a 473 nm laser was 500 mW. The ratio of EB/DNA = 1:1, [DNA] = 4 μM.

tween 350 and 580 nm at the maximum of the EB fluorescence spectra. A 0.2 cm path-length quartz Suprasil cell was used.

3. Results and discussion

3.1. Characterization of spectral properties of EB free and in complex with short DNA in solution

Fig. 1a and b shows fluorescence and excitation spectra of free EB and EB in complex with 16 bp DNA. The concentration of EB in solution changed from 24 nM to 300 nM; the concentration of DNA was constant, 3.4 μM, in excess of the dye (EB/DNA ≪ 1). It is notable that for all concentrations, the positions of fluorescence and excitation spectra maximum of EB free and EB/DNA complex were constant: 620 nm and 483 nm for free EB; for EB/DNA - 602 nm and 524 nm, respectively. This implies that there are only two species, free and bound EB. Moreover, bathochromic shifts of 40 nm in the ground state and about 20 nm in the excited state are the result of EB intercalation into DNA.

The fluorescence intensity of free EB linearly depends on concentration (Fig. 1c and d), suggesting that EB exists as a monomer. Similarly, in the presence of 16 bp DNA, the linear dependence of EB fluorescence on its concentration suggests that only bound chromophore contributes to the observed emission. The ratio of the slopes of these linear curves gives the fluorescence enhancement factor (EF) of EB upon interaction with DNA: EF ≈ 8 at 473 nm excitation and increase almost 4-times, EF ≈ 36, when excited at 530 nm. The difference in fluorescence enhancement factor can be explained by the red-shift and hypochromicity of the EB absorption spectra upon intercalation into DNA. It should be noted that the obtained dependence of EF upon excitation wavelength can be potentially used for the fine tuning of the EB/DNA fluorescence enhancement in fluorimetric assays.

3.2. Characterization of spectral properties of EB free and in complex with short DNA in the presence of silver nanoparticles; i.e. MEF

Samples of EB/DNA = 1:1 complex, immediately after loading on silver-island films (SiFs), display a 25-fold enhancement of fluorescence (Fig. 2) as compared to the free EB on glass. Incubation of EB/DNA solution on SiFs results in further time-dependent diffusion-based increases in EB fluorescence (Fig. 3). It should be noted that an increase in fluorescence does not change spectral position, demonstrating that we are observing fluorescence of the DNA-bound EB (Fig. 3a). The change in observed fluorescence upon incubation time occurs up to 5 min and then plateaus, EF ≈ 5 (Fig. 3b) as compared to the complex on a glass surface. As compared to the free dye on glass, the fluorescence enhancement of EB/DNA on SiFs is ≈35-fold at 473 nm and ≈180-fold when excited at 530 nm.

The observed increase of EB/DNA fluorescence on SiF can readily be explained by the Metal-Enhanced Fluorescence (MEF) phenomenon. According to the MEF concept, dramatic increases of fluorescence occur at fluorophore–NP distances <20–50 Å. This effect is due to fluorophore/NP-plasmon coupling, during the EB excited-

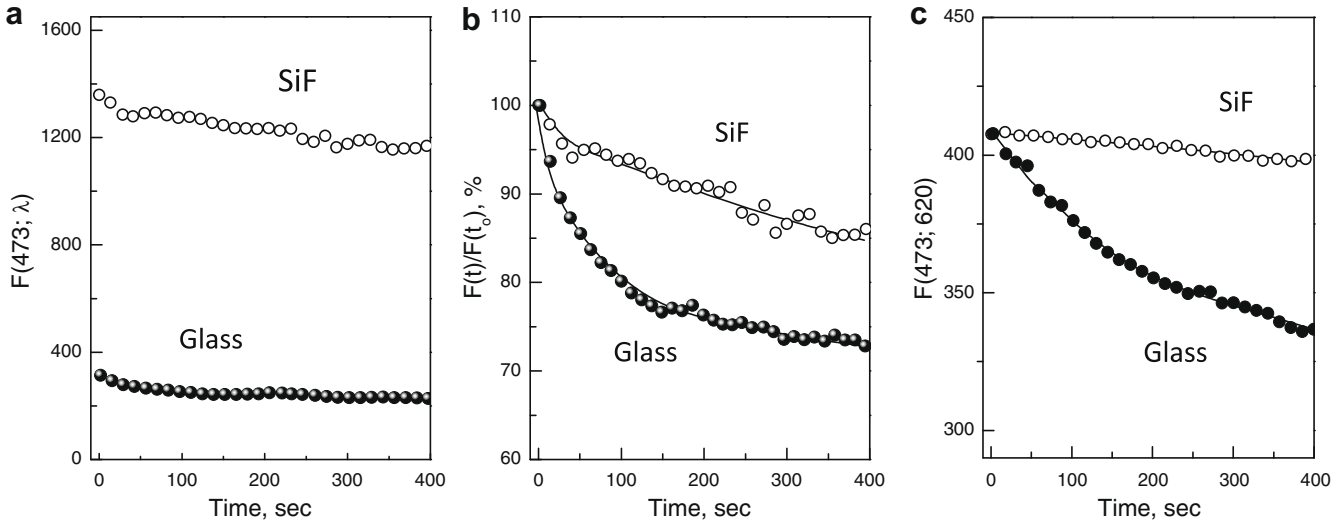

Fig. 5. Photobleaching of EB fluorescence in 1:1 complex with 16 bp DNA, deposited on SiF and on glass slides (a); (b) the data shown in (a) normalized to 1.0 at zero time. (c) Normalized photobleaching curves: fluorescence of the EB/DNA complex on SiF slide, at zero exposure time (t = 0 s), attenuated to the value of the fluorescence intensity of the complex on glass using excitation neutral density filters. Irradiation was undertaken using a 473 nm CW laser. Concentration of components in solution: [EB] = 3.4 μM; [DNA] = 34 μM (bp).

state lifetime. In our experiments we observed a 5-fold increase in EB/DNA fluorescence. The thickness of the sample in the wells is about 1 mm. Therefore, the observed fluorescence intensity contains two components: the near-field coupled emission for thicknesses < 50 Å and a far-field 'normal' component of fluorescence. Interesting, one therefore assumes that a decrease in sample thickness should dramatically increase the EF, as compared to free EB sample (the same thickness) on glass, i.e. selective visualization of the near-field enhanced fluorescence component.

Visual demonstration of the observed MEF effect is shown in Fig. 4. Digital photographs of the fluorescence spots of free EB and EB in complex with 16 bp DNA, loaded on SiFs and glass slides were taken under identical conditions. As one can see, the most intense spot is for EB/DNA loaded on SiFs as compared to the glass control sample where the signal on SiFs was attenuated 10-fold. Free EB on both SiFs and glass sufaces displays a significantly lower brightness.

The photobleaching of EB fluorescence in 1:1 complex with 16 bp DNA, deposited on SiF and on glass slides is shown in Fig. 5a. To compare the rates of photobleaching the data were normalized to 1.0 at zero time (Fig. 5b). One can see that the rate of photobleaching of EB/DNA on SiFs is much lower than on glass. We also characterized the photostability of the EB/DNA by normalizing the photobleaching curves by attenuation of the fluorescence of the EB/DNA complex on SiFs using a neutral density filter (Fig. 5c). Here, the difference in stability becomes even more pronounced. This result is consistent with the MEF phenomena, i.e. an increase in brightness coupled with a simultaneous drop in chromophore excited-state lifetime [10]. Accordingly, chromophores with a reduced excited-state lifetime are more resistant to photo-oxidation or other excited-state processes, which influence the fluorophore stability and observed intensity.

4. Conclusions

In this Letter we have demonstrated the favorable effects of the ethidium bromide chromophore near-to silver nanoparticles. In free solution EB readily intercalates into dsDNA and shows an enhanced luminescence of ≈36-fold. Near to silver, the far-field luminescence is even further enhanced, with an overall enhancement factor of nearly 180-fold as compared to the free EB chromophore. Interestingly, the enhancement factors we observe originates from <<<1% of the sample volume, suggesting that the near-field enhancement factors are considerably larger. Our findings suggest that highly sensitive fluorimetric assays for both dsDNA detection and quantitation can be realized on SiFs, especially at the single molecule/single copy level, where fluorophore brightness, photostability and background luminescence are a primary concern. Further studies are underway and will be reported in due course.

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