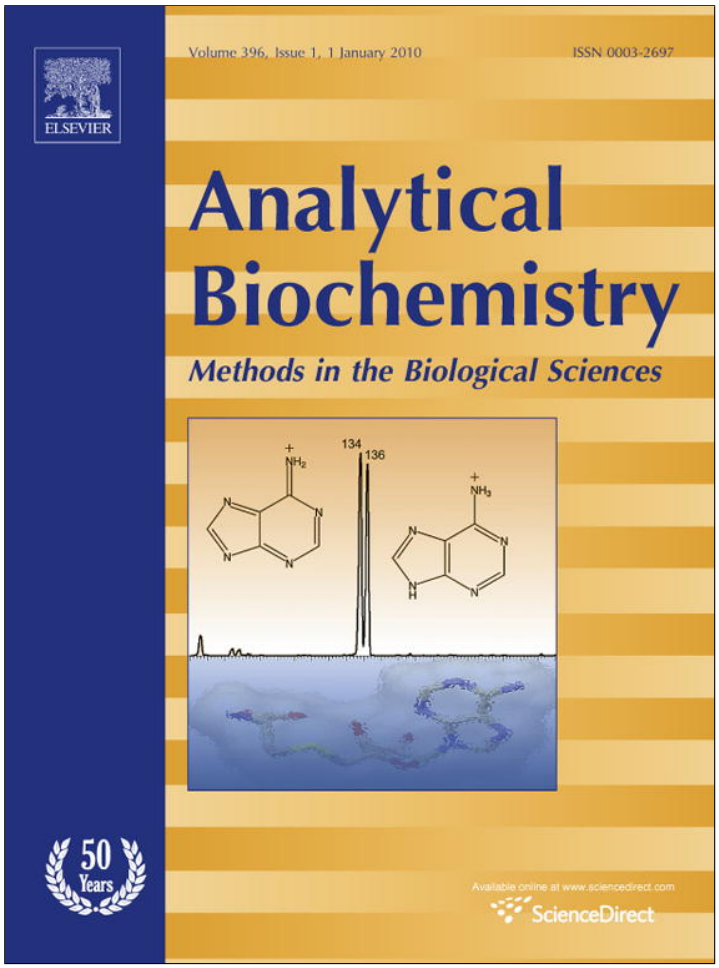

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Metal-enhanced PicoGreen fluorescence: Application for double-stranded DNA quantification

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ABSTRACT

PicoGreen (PG) is a fluorescent probe for both double-stranded DNA (dsDNA) detection and quantification based on its ability to form a luminescent complex with dsDNA as compared with the free dye in solution. To expand the sensitivity of PG detection, we have studied the spectral properties of PG, both free and in complex with DNA in solution, when the fluorophore is in proximity to silver nanoparticles. We show that for a broad range of PG concentrations (20 pM–3.5 μM), it does not form dimers/oligomers and it exists in a monomeric state. On binding to DNA in the absence of silver, PG fluorescence increases approximately 1100-fold. Deposition of PG/DNA complex onto silver island films (SiFs) increases fluorescence approximately 7-fold due to the metal-enhanced fluorescence (MEF) effect, yielding fluorescence enhancement of 7700-fold as compared with the free dye on glass. In contrast to PG in complex with DNA, the free dye on SiFs demonstrates a decrease in brightness approximately 5-fold. Therefore, the total enhancement of PG on binding to DNA on silver reaches a value of approximately 38,000 as compared with free PG on SiFs. Consequently, the metal-enhanced detection of PG fluorescence is likely to find important utility for amplified dsDNA quantification.

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Fluorescence spectroscopy has changed significantly during recent years due to the discovery and implementation of metal-enhanced fluorescence (MEF),[1] a metal–fluorophore near-field interaction that greatly increases both the fluorophore emission intensity and photostability. During recent years, our laboratories have both studied and demonstrated many applications of MEF [1–3]. These have included the increased detectability and photostability of fluorophores [1], improved DNA and protein detection [2,4], amplified directional emission [5], enhanced wavelength ratiometric sensing [6], and application of metallic surfaces to amplified immunoassay detection [7], to name but just a few. Our laboratories' current interpretation of MEF has been underpinned by a model whereby nonradiative energy transfer occurs from excited distal fluorophores to the surface plasmon electrons in noncontinuous films—in essence a fluorophore-induced mirror dipole in the metal. The surface plasmons, in turn, radiate the emission (quanta) of the coupled fluorophores [8].

In this study, we have analyzed the spectral properties of the PicoGreen (PG) fluorophore and its ability to enhance fluorescence in proximity to silver nanoparticles (NPs). It is known that the binding of PG to highly polymeric DNAs can result in an increase in fluorescence greater than 1000-fold, where the binding does not depend on DNA sequences (AT/GC content) [9]. The actual mode of binding of PG to DNA is unclear because there are no structural data of the PG/DNA complex, but it is widely thought that PG may intercalate between the DNA base pairs. Subsequently, the highly luminescent complex, as compared with the free dye in solution, makes PG an excellent choice for both double-stranded DNA (dsDNA) detection and quantification. As with many fluorescence-based assays, there is a continuing need to improve sensitivity; hence, we systematically studied PG and PG/DNA with metallic NPs (i.e., MEF). Our results show an approximately 38,000-fold increase in the emission of PG/DNA from silver surfaces as compared with the dye in the absence of DNA.

Materials and methods

PG dye, commonly used in dsDNA quantitation assays, was purchased from Invitrogen. The concentration of PG was determined by measuring the optical density of the solutions using an extinction coefficient of $E_{500} = 70{,}000\ M^{-1}\ cm^{-1}$ [9]. The structure of PG is shown in the Fig. 1A inset as determined by Zipper and coworkers [10]. The International Union of Pure and Applied Chemistry (IUPAC) name is 2-(*N*-bis-(3-dimethylaminopropyl)-amino)-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-*yl*)-methylidene)-1-phenyl-quinolinium (MW = 552.5 Da).

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[1] *Abbreviations used:* MEF, metal-enhanced fluorescence; PG, PicoGreen fluorescent dye; NP, nanoparticle; dsDNA, double-stranded DNA; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SiF, silver island film; CW, continuous wave; EF, enhancement of PG fluorescence.

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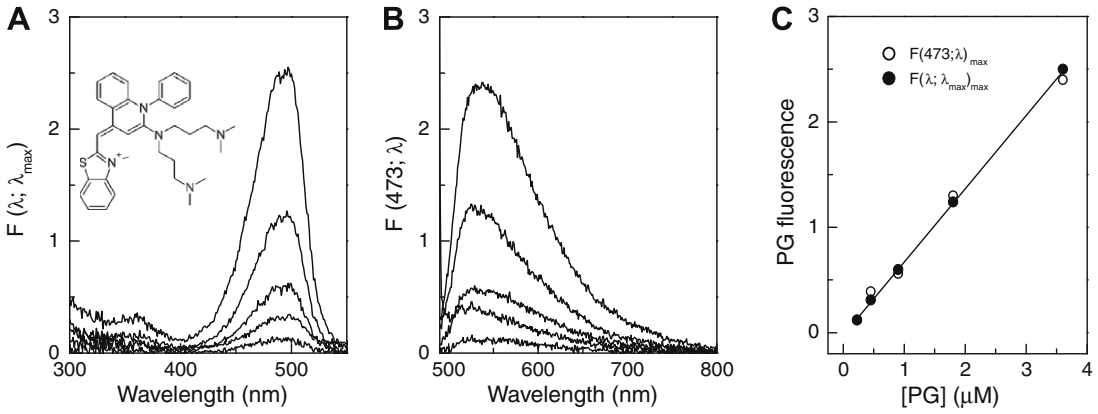

Fig. 1. Changes of the PG excitation (A) and fluorescence (B) spectra versus concentration. The inset in panel A shows the molecular structure of PG. (C) The dependence of PG emission intensity versus concentration is linear in the range of 200 pM to 3.5 μM.

Complementary 16-base oligonucleotides (5′-AGAGCGATATCG CGTG-3′ and 5′-CACGCGATATCGCTCT-3′) were purchased from Integrated DNA Technologies and also purified by anion exchange fast protein liquid chromatography (FPLC) on a Mono-Q column using a linear gradient (0.1–1.0 M NaCl) in 10 mM Tris–HCl buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 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) [11]. 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 was 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, and ammonium hydroxide (30%) were obtained from Sigma. Silver island films (SiFs) were prepared as described previously [12]. In short, amine-coated glass microscope slides (Sigma) are immersed in Tollen's reagent until the electrical resistance approaches 3–10 Ω cm^{-1} (measured using a GDT-11 digital voltmeter).

Wet PG/DNA/SiF and PG/SiF samples were prepared by loading a solution (water, pH 5.5) of the PG or PG/DNA complex at a concentration of 3.6 μM on SiFs and glass slides. The incubation times of the PG and PG/DNA solutions on slides were varied from 1 to 30 min. Before measurements, wet samples were covered by a glass microscope cover slide.

Fluorescence intensity values and spectra of the PG deposited on glass and SiF slides were measured using an HD2000 spectrometer (Ocean Optics). Excitation of the PG emission on slides was performed using a 473-nm continuous wave (CW) laser (Lasermate Group).

Fluorescence and excitation spectra of solutions of free PG and PG in complex with the DNA in a quartz cuvette were measured on a Cary Eclipse spectrofluorometer (Varian) at room temperature. PG was excited at 485 nm, and the fluorescence was monitored over the wavelength range of 490–800 nm. A 0.2-cm pathlength Suprasil quartz cell (Hellma USA) was used.

The fluorescence excited state lifetime of free PG and the PG in complex with the DNA ([PG] = 3.6 μM and [DNA] = 34 μM [base pair]) were measured using two different techniques: a multifrequency fluorometer MF^2 with a 473-nm modulated light source (Horiba Jobin Yvon) and a TemPro fluorescence lifetime system (Horiba Jobin Yvon). In the case of frequency domain measurements, the reference cell (zero lifetime) contained a colloidal silica, Ludox SM-30 solution. Measurements were performed at room temperature.

Results and discussion

Characterization of PG free in solution and in complex with DNA

Oligomerization state of PG in low-ionic-strength solution

In this study, we used solutions of low ionic strength (10 mM Tris buffer, pH 7.0). It was originally thought that low-salt conditions could provoke nonspecific chromophore dimerization/oligomerization, in particular, due to the hydrophobic attraction between the molecules. Intermolecular associations formed in ground and/or excited states can cause perturbation of the corresponding chromophore spectra—excitation and/or fluorescence spectra. To characterize the ability of the PG chromophore to form nonspecific oligomers, we subsequently studied the PG spectral parameters over a broad range of concentrations. Fig. 1A and B show the excitation and fluorescence spectra of PG over the chromophore concentration range of 200 pM to 3.5 μM. Both the excitation and fluorescence spectra did not change. Moreover, their fluorescence intensities depend linearly on PG concentration in solution (Fig. 1C), suggesting that PG exists in a monomeric state up to 3 μM.

Enhancement of PG fluorescence on binding to short linear DNA

Fig. 2 shows the results of titration of PG into buffered solution containing 16 bp DNA. The concentration of PG changes from 20 to 300 pM with a constant DNA concentration of 34 μM (bp). When DNA is in excess, not more than one molecule of PG is bound to the 16 bp DNA. This is thought to prevent complex formation from the effects of dye cooperation on binding and the influence of energy migration between the dyes that would invariably result in a homogeneous mode of interaction and formation of PG/DNA complex. The shape and position of the PG/DNA fluorescence spectra are constant for all PG concentrations (Fig. 1A). The results show that the dependence of PG fluorescence intensity on concentration, in buffer and in the presence of DNA, is linear (Fig. 2C). Analysis of the data reveals that enhancement of PG fluorescence (EF) is approximately 1100.

For free space fluorescence interactions, it is well known that the quantum yield is proportional to the change in chromophore excited state lifetime (τ). We determined the excited state lifetime of free PG and PG in a 1:1 complex with short 16 bp DNA ($\tau^{PG/DNA}$) using a multifrequency domain (phase and modulation) MF^2 fluorometer. The data and the results of the analysis are shown in Fig. 3. The lifetime of free PG is, however, too short for direct measurement; therefore, we assume that it is less than 200 ps (i.e., below the time resolution of the instrument). In contrast, the excited state lifetime of PG in complex with DNA was readily measured

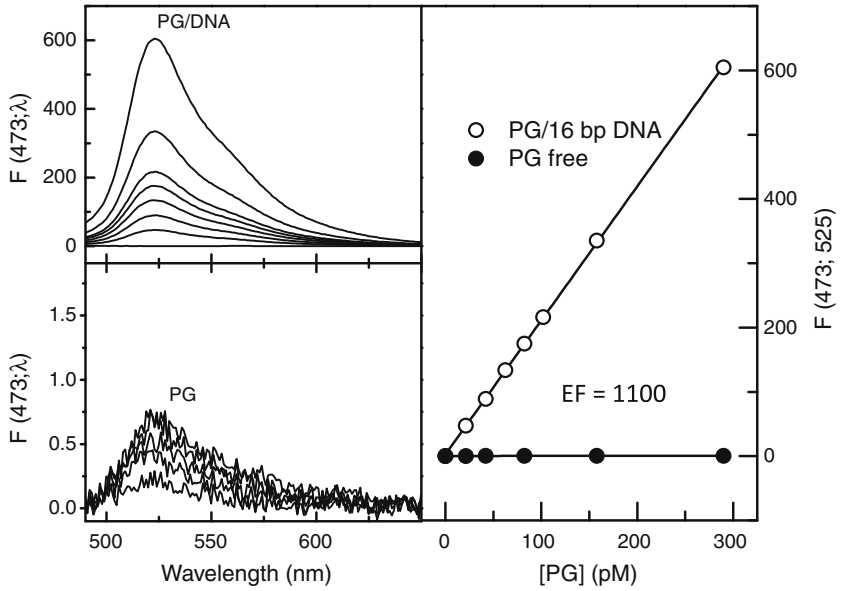

Fig. 2. Fluorescence spectra of PG in complex with short 16 bp DNA (A) and free in solution (B) determined in the concentration range of 20–300 pM. (C) Dependence of PG fluorescence intensity, excited at 473 nm and measured at 525 nm, on PG concentration in buffer and in solution containing 16 bp DNA. Concentration of the 16 bp DNA = 34 μM (bp). Buffer: 10 mM Na phosphate (pH 6.5). Enhancement of PG fluorescence (EF), calculated from the data shown in panel C, is approximately 1100.

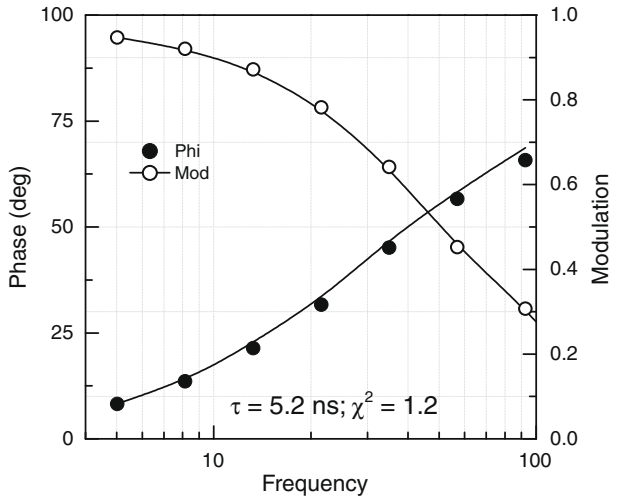

Fig. 3. The excited state lifetime of PG in a 1:1 complex with short 16 bp DNA, measured using a multifrequency domain (phase and modulation) fluorometer (MF^2), is $\tau^{PG/DNA}$ = 5.2 ns. Concentration of [DNA] = 34 μM (bp); [PG] = 3.6 μM.

with a value of $\tau^{PG/DNA}$ = 5.2 ns (χ^2 = 1.2). The same measurements were also performed using a TemPro fluorescence lifetime system; the excited state lifetime of PG in complex with DNA was 4.4 ± 0.01 ns (χ^2 = 1.004), and for free PG τ = 3.9 ± 3.0 ps (χ^2 = 1.02). The measured value of PG lifetime in complex with DNA is similar to the previously published value [13] (i.e., monoexponential decay time). This can be explained simply by the conditions we used in this study, which allowed the preferred formation of one type of PG/DNA = 1:1 complex. Assuming that lifetime (τ) is proportional to quantum yield (Q) of a chromophore, one can theoretically estimate the PG excited state lifetime free in solution as

$$\tau^{PG} = \tau^{PG/DNA}(Q^{PG}/Q^{PG/DNA}) \sim 5 \text{ ps}. \quad (1)$$

The result of this estimation is in proximity to the experimentally measured value (~4 ps), suggesting intrinsic dynamic quenching of free chromophore fluorescence.

MEF of the PG

Deposition of PG/DNA solution on SiFs significantly enhances the emission of the dye (EF = 7) in comparison with a control sample (i.e., emission from glass slides containing no silver) (Fig. 4). This result is consistent with our previous data and the general concept of the MEF phenomenon [2,3,12]. Accordingly, enhancement of the PG/DNA fluorescence occurs in close proximity to the silver NPs, less than 20–50 Å, due to PG/NP–plasmon coupling.

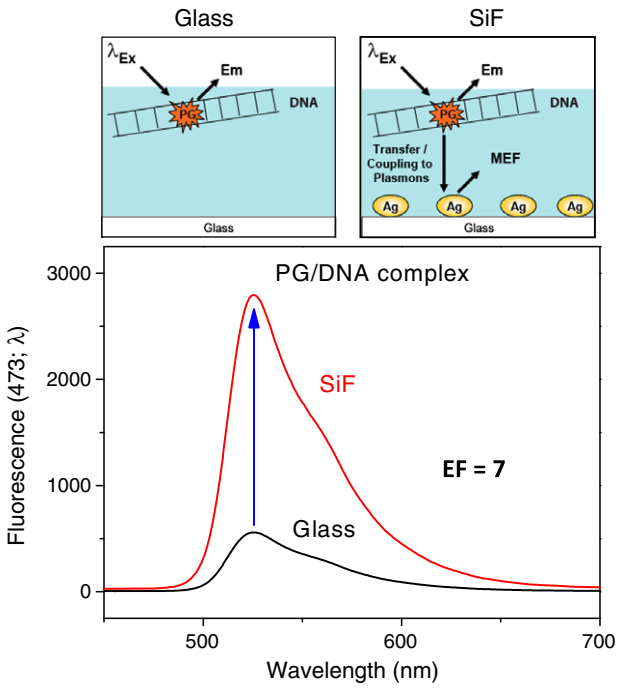

Fig. 4. PG/DNA fluorescence on SiFs and on a glass surface (control sample). The relative enhancement factor of PG fluorescence is EF = 7. The top panels show a graphical representation of the current interpretation of MEF effects obtained in the study.

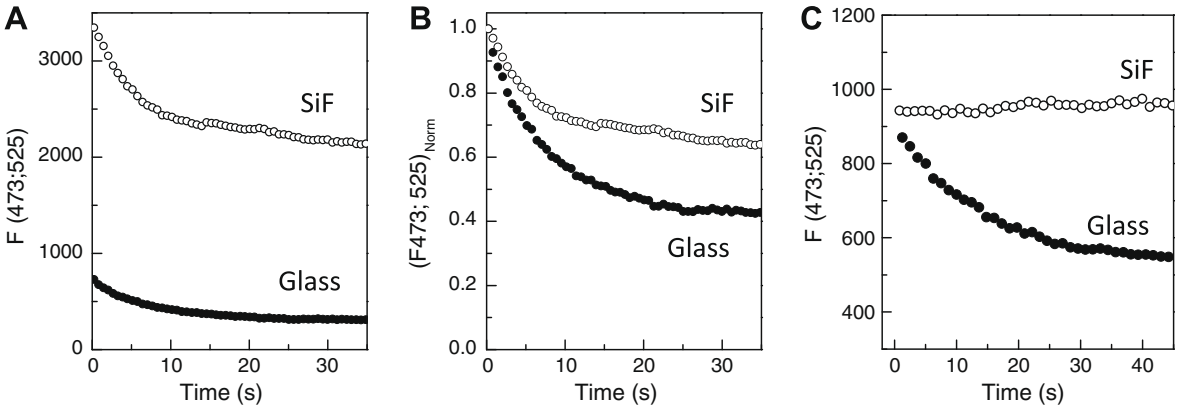

Fig. 5. (A) Photobleaching of PG fluorescence in 1:1 complex with 16 bp DNA deposited on SiF and glass slides. (B) Data in panel A normalized to 1.0 at zero time ($T = 0$ s). (C) Normalized photobleaching curves. The fluorescence of the PG/DNA complex on SiFs slide, at zero exposure time ($t = 0$ s), was attenuated to the value of the initial intensity of the complex on glass using excitation neutral density filters. Irradiation was undertaken using a 473-nm CW laser (125 mW). Concentrations of components in solution: [PG] = 3.6 μM; [DNA] = 34 μM (bp).

Interaction between the PG chromophore and NP plasmons occurs during the lifetime of the fluorophore's excited state, which is approximately 5 ns for PG in complex with DNA, resulting in enhanced brightness and a decreased total lifetime of the coupled emission. (Graphical representations of the current interpretation of MEF effects, discussed above, are shown at the top of Fig. 4.) In addition to its direct fluorescence enhancement, it is also informative to analyze the PG/DNA fluorescence intensity decay curves (i.e., photobleaching curves) (Fig. 5). The rates of fluorescence intensity decay of the samples deposited on SiFs are notably lower than the rates for the samples deposited on glass slides (i.e., control samples containing no silver). This difference becomes more pronounced in normalized photobleaching curves, where the fluorescence of the PG/DNA complex on the SiFs slide at zero exposure time ($t = 0$ s) was attenuated to the value of the intensity of the complex on the glass slide by using excitation neutral density filters. This result clearly shows the difference in photostability between samples deposited on SiFs and on glass. As discussed earlier, close proximity (~10 nm) of the fluorophore to the SiFs decreases the system radiative lifetime in comparison with the bulk solution [8,14]. The important consequence of the system (fluorophore/NP) lifetime decrease is a decrease in photobleaching, in particular, due to a reduced excited state lifetime, where the fluorophore is less prone to photooxidation or other excited state processes that influence the fluorophore intensity.

In contrast to the PG/DNA complex, free uncomplexed PG does not exhibit fluorescence enhancement when deposited on SiFs, showing an EF of approximately 0.2 (i.e., a reduced intensity). This observation is unusual in MEF studies and is the first fluorophore/luminophore reported to date not to show an effect of MEF on SiFs.

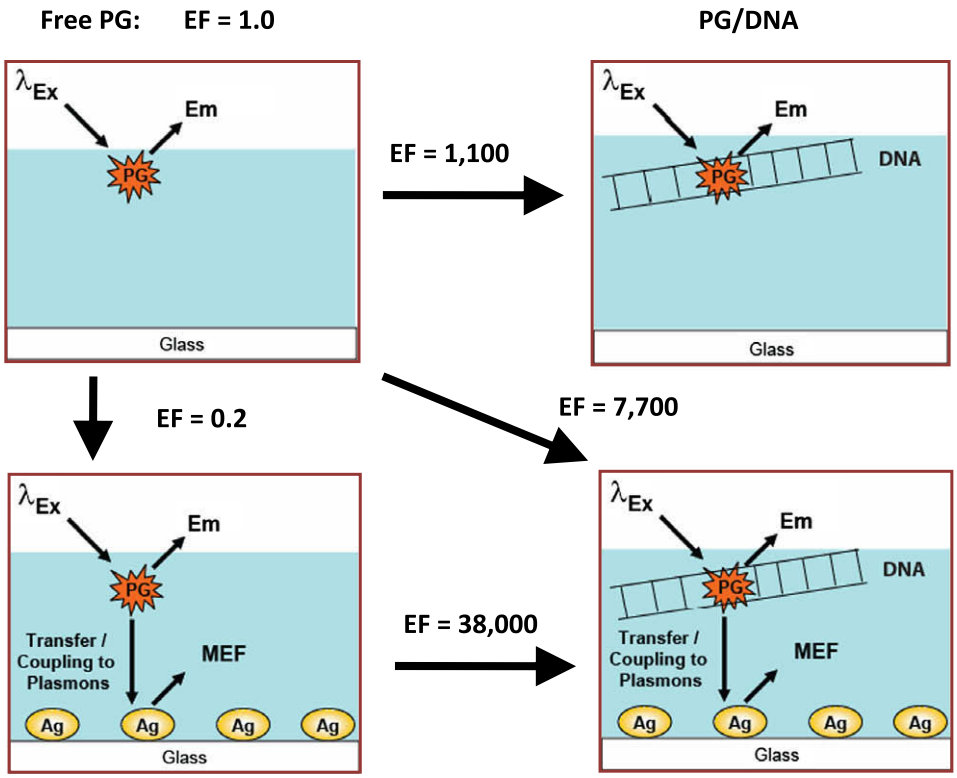

Fig. 6. Graphical interpretations of the MEF effect obtained for the free PG fluorophore and PG complex with DNA.

We speculate that the very short lifetime of PG free dye (estimated to be <5 ps) may be too short for excited state plasmon coupling and that, therefore, MEF is not observed. Further studies are under way in our laboratory and will be reported in due course in this regard.

Conclusions

Free PG in solution is weakly fluorescent with an extremely short excited state lifetime (~5 ps). We assume that these unique spectral properties are not a consequence of intermolecular quenching (because, as we have shown, PG exists in a monomeric state in solution) but rather have an intramolecular origin.

On binding to short 16 bp DNA in solution, PG significantly increases its fluorescence yield approximately 1100-fold. Additional increases of PG brightness (~7-fold) occur in the presence of surface-deposited silver NPs, yielding a total emission enhancement of approximately 7700-fold as compared with the free dye fluorescence on glass (Fig. 6). Interestingly, with an approximate 1-mm solution depth in the wells (silver NPs are on the well bottom) and an interaction distance (fluorophore–plasmon coupling) of less than 10 nm, the 7700-fold enhancement occurs from less than 1/1000 of 1% of the sample, implying near-field enhancements much greater than 1 million-fold (i.e., $\sim 7.7 \times 10^8$).

In contrast to PG in complex with DNA, the free dye on a silver NP surface reveals a decreased brightness (EF ~ 0.2) as compared with emission from glass. We assume that in the case of free PG, the MEF phenomenon is not observed due to the extremely short excited state lifetime, which may be too short for excited state plasmon coupling. Taking into account the low EF of PG on the silver surface, the calculated value of enhancement of the PG/DNA fluorescence is approximately 38,000 (Fig. 6). If we again normalize for sample volume, the enhancement factor approaches a staggering 4×10^9-fold as compared with the free dye in solution. Work is currently under way in our laboratories to measure these near-field enhancements using femtoliter volumes of PG/DNA complexes bound to silver NPs and will be reported in due course.

Proximity to silver NPs also results in a notable increase in the PG/DNA photostability. Fluorophore photostability is often a primary concern in microscopy, flow cytometry, and sensing platforms, where laser dwell times are long. However, the enhanced PG/DNA brightness in combination with an increased photostability in the presence of silver NPs makes this system attractive for highly sensitive approaches to DNA detection, quantification, and imaging.

Acknowledgments

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