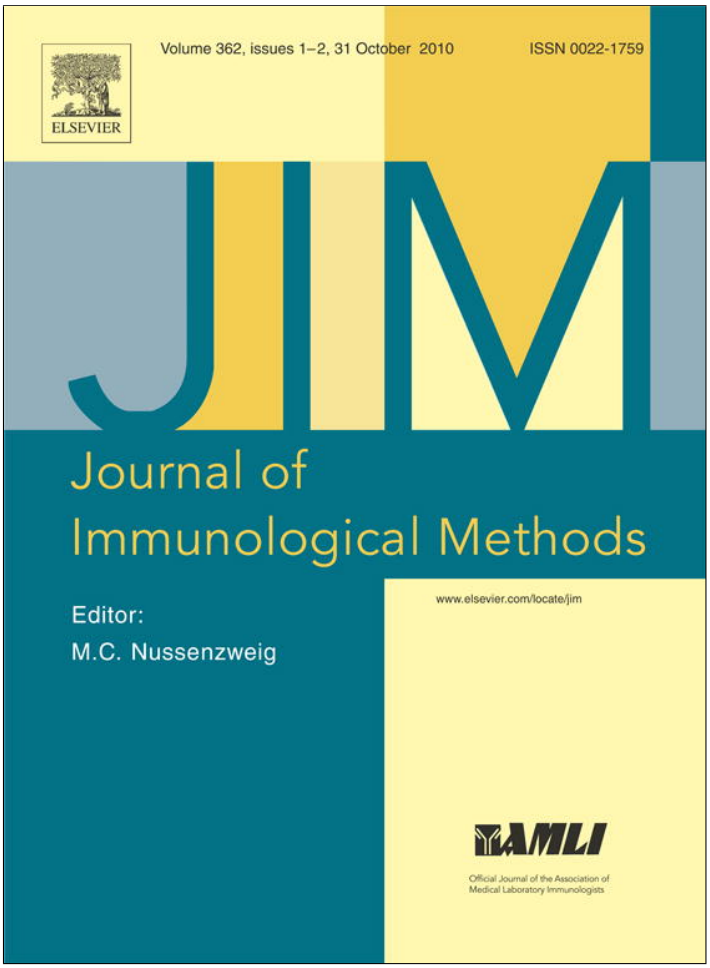

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Research paper

Metal-enhanced PicoGreen® fluorescence: Application to fast and ultra-sensitive pg/ml DNA quantitation

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ABSTRACT

In this paper we provide both a theoretical and experimental analysis of the sensitivity of a DNA quantitation assay using a fluorescent chromophore which non-covalently binds dsDNA. It is well-known that the range of DNA concentrations available for fluorescence quantitation depends on the concentration of the chromophore, its affinity for nucleic acids, the binding site size on DNA and the ratio between the fluorescence intensity of the chromophore when bound to DNA compared to free chromophore in solution. We present experimental data obtained for a PicoGreen® (PG)/DNA quantitation assay, which is in complete agreement with the results of our theoretical analysis. Experimentally measured PG-fluorescence intensity vs DNA concentration functions were fitted by a derived analytical expression, in which parameters of PG binding to DNA and chromophore fluorescence properties were included. We show that silver nanoparticles significantly increase the ratio between the fluorescence of PG bound to DNA and free PG, due to the metal-enhanced fluorescence effect (MEF), which enhances the lower limit of detectability of DNA concentrations by several orders of magnitude. An additional order of magnitude increase of PG/DNA assay sensitivity (~1 pg/ml) can be achieved by decreasing the PG concentration. We show herein that the use of MEF substrates in surface assays has a profound effect on assay sensitivity.

1. Introduction

Detection of DNA in solution is an important problem in a large variety of biochemical assays. The most popular agents for DNA quantitation are fluorescent dyes that strongly interact with nucleic acids and significantly increase their emission intensity in the DNA complex. Fluorescent dyes are used in real-time PCR, DNA-based cell quantitation, gel staining, chromatin and other DNA-based approaches (Glazer and Rye, 1992; Jing et al., 2003; Lakowicz, 2006; Le Pecq and Paoletti, 1967; Lim et al., 1997; Szpechcinski et al., 2008). For example, amongst the fluorescent DNA-binding dyes, ethidium bromide (EB) and Hoechst 33258 have been traditionally used in many applications where detection of dsDNA is involved (Haq et al., 1997; Le Pecq and Paoletti, 1967; McMurray et al., 1991; Morgan et al., 1979; Olmsted and Kearns, 1977; Pjura et al., 1987; Sivolob et al., 1999; Utsuno et al., 1999). The energetics of binding to double stranded DNA, their quantum yields and excited state lifetimes in both the free state and in complex with DNA, the deformation of DNA upon binding and the type of interaction with dsDNA have been well studied for both EB and Hoechst 33258 (Haq et al., 1997; Le Pecq and Paoletti, 1967; Pjura et al., 1987; Utsuno et al., 1999). It is known that EB intercalates between DNA base pairs, has no sequence specificity and, upon DNA binding, enhances its fluorescence >10-fold. Hoechst 33258 binds to the minor groove of DNA, has sequence specificity to AT-rich sites, and significantly (>20-fold) increases its quantum yield

Abbreviations: PG, PicoGreen® dsDNA intercalating fluorescent probe; MEF, Metal Enhanced Fluorescence; SiFs, Silver Island Films; NP, silver nanoparticles; CW, Continuous Wave; LoD, Limit of Detection.

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in a DNA complex, relative to the free state. Detailed knowledge of spectral and binding parameters make these dyes predictable in the design of different DNA-detection assays, fluorescence platforms and methods of DNA investigation in DNA-protein complexes. However, one particular disadvantage of these dyes in DNA detection applications concerns their relatively low fluorescence response on binding to DNA and their relatively low affinity for dsDNA ($K_a = 10^6$–10^7 M^{-1}) (Haq et al., 1997; Le Pecq and Paoletti, 1967; Utsuno et al., 1999).

Relatively new DNA binding dyes, which were introduced about a decade ago, include PicoGreen® (PG) and structurally similar SYBR Green I (SG). These dyes have both extremely high fluorescence response (>1000-fold) upon association with DNA and strong DNA-binding affinity >10^8 M^{-1} (Dragan et al., 2010a; Dragan et al., 2010b; Zipper et al., 2004). These unique properties of PG and SG have made them popular in many DNA detection assays, e.g. in PCR, despite insufficient knowledge of their binding parameters.

Indeed, DNA quantitation assays that use non-covalently bound chemical ligands, such as DNA-binding fluorophores, are sensitive to the parameters of the ligand, i.e. association constant, binding site size, ionic strength dependence of the dyes affinity to DNA, etc. Another notable parameter which has significant impact on the sensitivity of DNA detection assays is the chromophore fluorescence response upon binding, which can be determined by measuring the ratio of dye fluorescence intensity bound to DNA as compared to the free dye in solution, represented as $R_{B/F} = F_{bound}/F_{free}$. Recently, we have studied the spectral properties of PG and the energetics of its binding to dsDNA, including the origin of PG fluorescence quenching in the free state and enhancement when in complex with DNA, PG/DNA binding constants and the size of PG binding site on DNA (Dragan et al., 2010b). Prior knowledge of PG DNA-binding parameters has given us an opportunity to undertake a theoretical analysis of PG fluorescence sensitivity to dsDNA (DNA-quantitation assay) and, also, to investigate its fluorescence response in a broad range of conditions.

The sensitivity of fluorophore/DNA quantitation assays critically depends on the magnitude of the optical (fluorescence) response on binding to DNA, i.e. the $R_{B/F}$ parameter above. An increase in the magnitude of $R_{B/F}$ ultimately leads to improved assay sensitivity. Our recent studies of the metal-enhanced fluorescence (MEF) effect for PG/DNA complexes and free dye, have shown that, in the presence of silver nanoparticles, total fluorescence enhancement of PG upon binding to DNA dramatically increases, with a $R_{B/F}$>30,000 fold (Dragan et al., 2010a). In this study, we have investigated both the theoretical and experimental effect of PG MEF on DNA detection in solution and show that the use of MEF effect increases dsDNA detectability ≈1000-fold as compared to an otherwise identical assay without silver. Further, the simplicity of coating wells with silver nanoparticles for MEF can similarly be used to enhance the LOD of the biochemical assays as well.

2. Materials and methods

Calf thymus DNA was purchased from Sigma. PicoGreen® (PG) dye was purchased from Invitrogen. The concentration of PG was determined by measuring the optical density of the solutions using an extinction coefficient $E_{500} = 70{,}000$ M^{-1} cm^{-1} (Singer et al., 1997).

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 (Geddes and Lakowicz, 2002a).

PG/DNA samples were prepared in TE buffer, pH 7.6 by mixing a solution of PG into a solution of DNA of different concentrations. The final concentration of PG in PG/DNA samples was constant (10 nM or 100 nM). Samples were incubated for 30 min at room temperature before loading into wells. Wells for PG/DNA fluorescence measurements were made by using silicone isolators (press-to-seal) adhesive to standard silane-prep™ glass slides or SiF glass slides for MEF experiments. The diameter of the wells was 9 mm, thickness ≈2 mm. The volume of PG/DNA samples deposited onto glass or SiF bottom wells was 80 μL.

Fluorescence intensity values and spectra of the PG/DNA samples, deposited on glass and SiF wells, were measured using a HD2000 spectrometer, Ocean Optics, FL. Excitation of the PG emission in wells was performed using a 473 nm CW laser (Lasermate Group, Inc.).

Generation of dye/DNA sensitivity functions and fitting experimental data were undertaken using the Origin 8.0 program.

3. Results and discussion

3.1. Metal-enhanced fluorescence (MEF) of PG bound to highly polymeric DNA

Recently we have shown that PG in complex with a short 16 bp DNA fragment has a significant increase (≈7-fold) in fluorescence in the presence of silver nanoparticles, while in the free solution state, its fluorescence is decreased ≈5-fold (Dragan et al., 2010a). Subsequently, the total fluorescence enhancement effect of PG upon binding to DNA, in the presence of silver nanoparticles, is >30,000-fold (Dragan et al., 2010a). MEF of chromophores, including PG in complex with DNA, typically occurs in close proximity (10–50 nm) to silver nanoparticles and has been explained by specific near-field effects, i.e. the coupling of a chromophore's electronic system to induced oscillating surface plasmons of silver nanoparticles (Geddes and Lakowicz, 2002a; Geddes et al., 2003a,b, 2005). Our current graphical interpretation of the MEF effect is shown in Fig. 1 (right). A short 16 bp DNA, which has been used in our previous studies, has a length of about 5 nm. In this case, the PG/DNA complex can be considered to behave as a single "fluorophore", influenced by the nanoparticle near-field, leading to the MEF effect and an enhanced PG emission.

In this study, we have used highly polymeric calf thymus DNA, which is commonly used as a control in different DNA quantitation assays. Commercially available highly polymeric calf thymus DNA has a molecular weight of (10–100) $*10^6$ Da, which corresponds to a molecular length of 15–150 kbp. DNA molecules of this size cannot be considered straight or indeed linear, but, due to the inner flexibility of a polymer (persistent length of dsDNA is about 50 nm (Hagerman, 1988)), folds into a randomly coiled globule with a large diameter. In this work,

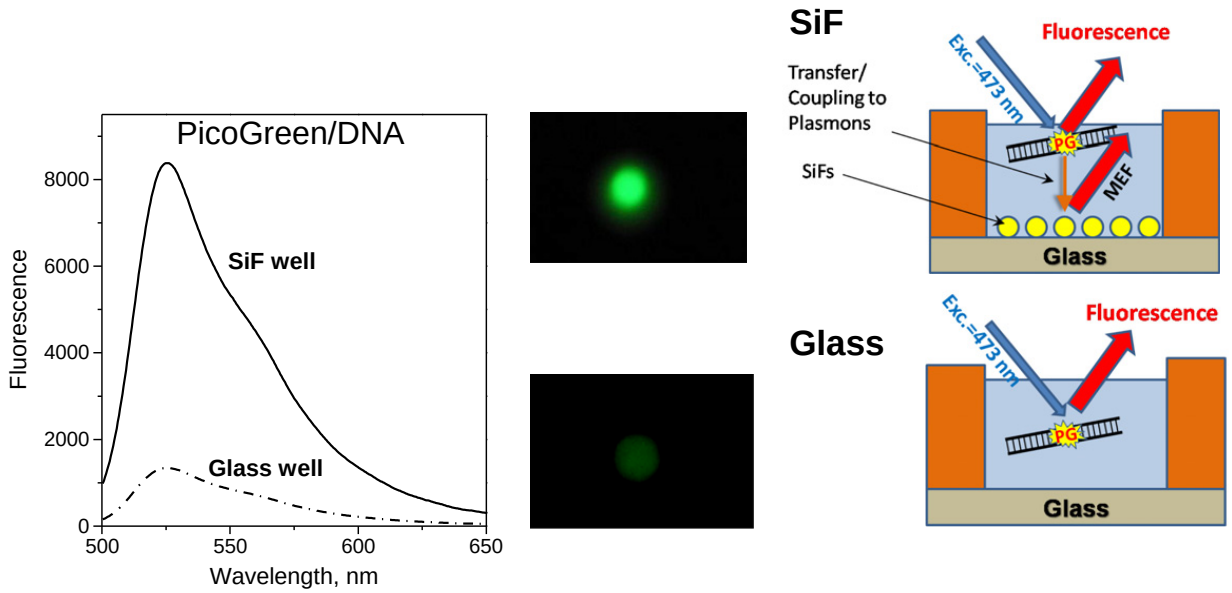

Fig. 1. (Left) Fluorescence spectra of PG in complex with DNA loaded in a glass bottom well and a SiF coated well. (Center) Color photographs of the fluorescence of PG/DNA solution loaded in a glass well and on SiF, excited with a 473 nm laser line. Scattered excitation light was cut off using a 473 nm Notch filter. (Right) Current graphical interpretation of the MEF effect on silver (top) and conventional (far-field) fluorescence from a glass well (bottom).

we have investigated whether the size of DNA in fact influences PG fluorescence enhancement on a SiFs, given that MEF is distance dependent. We have compared the enhancement of PG in complex with DNA with that of a short 16 bp DNA. Fig. 1 (left) shows the fluorescence spectra of PG in complex with DNA loaded on glass and on SiF-coated glass. The fluorescence intensity of the PG/DNA sample deposited onto a SiF surface, is about 5-fold greater than the same sample on a glass bottomed wells. This difference in brightness of PG in complex with DNA can be clearly seen in the color photographs, which are shown in Fig. 1 (center). It is notable that despite a great difference in length between highly polymeric calf thymus DNA and the 16 bp DNA, which has been used in our previous study (Dragan et al., 2010a), the value of PG/DNA MEF effect is similar to that measured for a short DNA, suggesting that DNA size does not greatly influence luminescent enhancement.

The observed >30,000-fold increase of PG fluorescence upon binding to DNA in the presence of silver nanoparticles (SiFs) is expected to have a great impact on the sensitivity of PG/DNA quantitation assays. Below we present both a theoretical analysis as well as experimental data regarding DNA detectability, and compare the sensitivity of the assay from both glass and SiF platforms.

3.2. Theoretical analysis of the sensitivity of DNA quantitation assays based on PG fluorescence

To describe the dependence of PG fluorescence upon concentration of DNA at different constant concentrations of PG, we have used a simplified model for dye-DNA binding:

$$\mathrm{PG} + \mathrm{DNA} \Leftrightarrow \mathrm{Complex} \tag{1}$$

For this equilibrium, the concentration of binding sites [D] on DNA molecules, determined by their size, can be written as:

$$[\mathrm{D}] = [\mathrm{DNA, bp}] / n \tag{2}$$

where n is a binding site size in base pairs, (bp) and the concentration of DNA is in bp. The association constant of PG to DNA can be expressed by the following equation:

$$K_a = \frac{\nu}{(1-\nu) \times ([D]-(\nu \times [PG]))} \tag{3}$$

where ν is the fraction of PG bound to DNA and K_a is the association constant of PG to DNA.

Solving Eq. (3) for the fraction of bound PG, we can generate the analytical expression:

$$\mathfrak{M} = 1 + \left(\frac{[D]}{[PG]}\right) + \left(\frac{1}{[PG] \times K_a}\right)$$
$$\nu = \left\{0.5 \times \mathfrak{M} - \sqrt{0.25 \times \mathfrak{M}^2 - \left(\frac{[D]}{[PG]}\right)}\right\} \tag{4}$$

Fig. 2 (insert) shows the typical binding functions for PG upon concentration of DNA in solution. To calculate these functions, we have used the following parameters of PG/DNA binding measured in TE buffer, pH 7.6: association constant $K_a = 2 \times 10^8\ \mathrm{M}^{-1}$ and binding site size $n = 4$ bp (Dragan et al., 2010b). The functions displayed in Fig. 2 were generated for 1, 10, 100 and 1000 nM PG. In logarithmic coordinates, the fraction of bound PG linearly increases with the concentration of DNA and then approaches saturation. The saturation point depends on the concentration of PG (dye concentration is different but constant). Interestingly, a decrease of PG concentration, at certain constant concentrations of DNA, results in an increase of the fraction of bound chromophore.

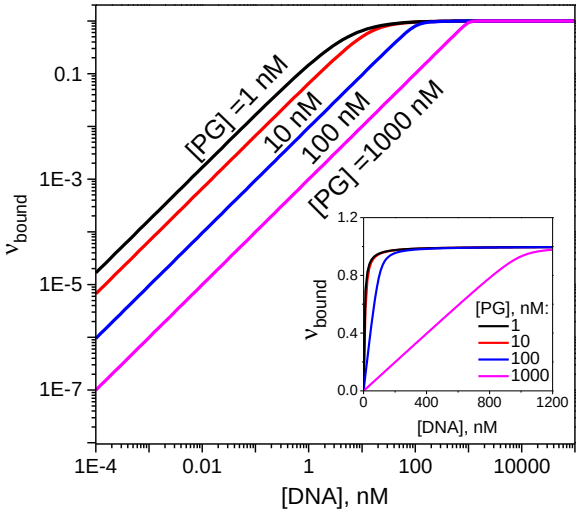

Fig. 2. Dependences of the fraction of bound PG (n_{bound}) upon DNA concentration are shown in logarithmic coordinates. The function plots, for PG concentrations of 1, 10, 100 and 1000 nM, were generated using Eq. (4). Insert: the same function plots in linear coordinates. PG – PicoGreen®.

At first glance this result seems unexpected. However, the trends become clear if one takes into account that the fraction of bound ligand, ν, is a ratio of the concentration of a bound ligand to the total concentration of ligand, [PG], in solution, i.e. there is a reverse proportionality between ν vs [PG].

The functions plotted in Fig. 2 were re-calculated into fluorescence intensity functions plotted against concentration of DNA (Fig. 3). These functions can be considered as PG sensitivity curves in a DNA quantitation assay. If we assume that the fluorescence of bound PG >> fluorescence of free dye, then the observed fluorescence (F_{obs}) can be expressed as:

$$F_{obs} = F_{bound} \times \nu \quad (5)$$

Where F_{bound} is the fluorescence intensity of 100% bound PG.

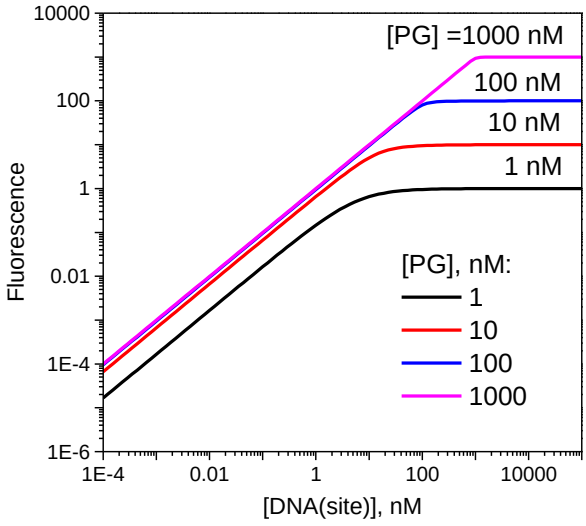

Fig. 3. Theoretical plots generated for PG fluorescence intensity dependence upon concentration of DNA and PG in solution. Fluorescence intensity at [PG] = 1 nM was taken equal to 1.0. The following parameters of PG binding to DNA were used: binding site size – $n = 4$ bp; PG/DNA dissociation constant of $K_d = 5$ nM in TE buffer. Contribution of free PG fluorescence to the total fluorescence intensity has been neglected.

To generate these functions, we have assumed that at [PG] = 1 nM, the fluorescence intensity of 100% bound dye (saturation) is 1.0. It then follows that the value of F_{bound} will be proportional to the concentration of PG: F_{bound} = [PG]/([PG] = 1 nM). Fig. 3 shows functions plotted for four different concentrations of PG, that match those used in Fig. 2. It is noted that the generated curves for [PG] = 100 nM and 1000 nM coincide over a broad range of DNA concentrations and start to deviate when [PG] < 10 nM. This suggests that when the concentration of PG approaches, or becomes less than the value of the dissociation constant, $K_d = 5$ nM (Dragan et al., 2010b), the equilibrium PG/DNA ⟺ PG + DNA shifts to the right, towards dissociation of PG from DNA, resulting in a decrease in the observed fluorescence of PG.

The dynamic range of PG sensitivity to DNA is thus limited by the concentration of PG. As can be seen from Fig. 3, the upper limit of the PG dynamic sensitivity range, D_u, increases from [DNA, bp] = 4 nM to about 4 μM when the concentration of PG is raised increases from 1 nM to 1000 nM. In fact, the D_u value reflects full PG binding to DNA and depends on the concentration of PG, DNA and the PG binding site size. Therefore, to measure high DNA concentrations by means of a PG/DNA quantitation assay, greater concentrations of PG should be used.

To estimate the lower limit of PG sensitivity to DNA, D_L, and its dependence upon the parameters of PG fluorescence and binding to DNA, we have introduced into the binding equation, Eq. (5), an additional term that allows for the contribution of *free PG* to the total observed fluorescence. The PG binding equation (Eq. (5)) can be rewritten as:

$$F_{obs} = F_{bound} \times \nu + F_{free} \times (1-\nu) \quad (6)$$

where ν and $(1-\nu)$ are the fractions of DNA bound PG and free PG in solution; and F_{bound} and F_{free} are the fluorescence of 100% bound and 100% free PG, respectively. Simulations and plots of PG/DNA sensitivity functions undertaken using Eqs. (6) and (4), are shown in Fig. 4. The functions (1) and (2) represent theoretical PG/DNA sensitivity curves

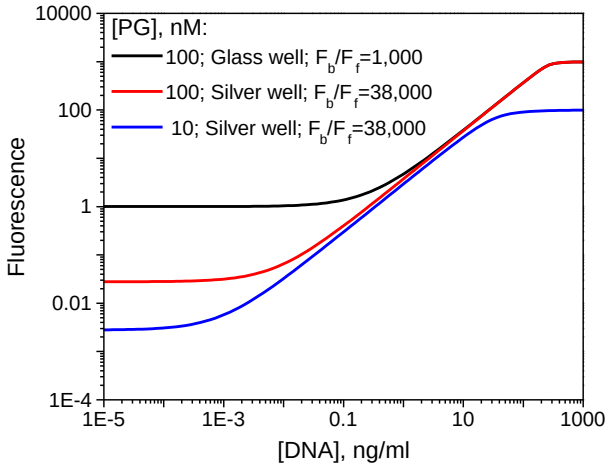

Fig. 4. Theoretical PG/DNA sensitivity curves generated using Eqs. (6) and (4). Fluorescence of 100% bound PG was taken equal to $F_b = 1000$ a.u., for [PG] = 100 nM, and 10-fold lower for [PG] = 10 nM, i.e. $F_b = 100$ a.u.

generated for two practical models: fluorescence of PG/DNA complex determined from a glass bottomed well (or in a cuvette) and for SiF coated wells, respectively. The main difference between these two models is a dramatically increased PG fluorescence enhancement upon binding to DNA, which occurs in the presence of the SiFs, but not from glass. To simulate these "sensitivity" curves, we have used known parameters for PG binding to DNA (the same for both models) and two different values of PG fluorescence enhancement upon interaction with DNA: $R_{B/F}=F_{bound}/F_{free}=1{,}100$, on glass and $R_{B/F}=F_{bound}/F_{free}=38{,}000$ on SiF bottom wells (Dragan et al., 2010a). For both simulated models, the concentration of PG was constant, [PG] = 100 nM. Fluorescence intensity, at high concentration of DNA (saturation), was normalized to 1000. In logarithmic coordinates both functions show linearity for a broad range of DNA concentrations and approach a constant level at low, but significantly different concentrations of DNA. Concentrations of DNA at which the simulated functions become flat represent the lower limit of PG-to-DNA sensitivity, D_L, i.e. classical LOD. Comparison of functions (1) and (2) demonstrates strong dependence of PG/DNA detection sensitivity on the $R_{B/F}$—parameter.

We have also studied the influence of PG concentration on the lower limit of DNA detection. Function (3) in Fig. 4 was generated using the same parameters as before, except that the PG concentration was 10-fold less, [PG] = 10 nM. Of particular note, a 10-fold decrease in PG concentration *significantly increases* the sensitivity of PG to DNA with the lower limit of DNA detectability approaching $D_L \approx 1$ pg/ml.

3.3. Experimental measurements of PG sensitivity to DNA deposited in glass and SiF wells

Fig. 5 shows the dependence of PG fluorescence upon concentration of DNA experimentally measured in glass and SiF wells, when the concentration of PG was fixed at 100 nM. In both cases, the fluorescence intensities, which were taken at the maximum DNA concentration, were normalized, as was done in Fig. 4. Experimental data were fitted using Eqs. (6) and (4). The results of fitting show an impressive increase in PG/DNA sensitivity on SiFs, when compared to glass wells, fundamentally caused only by a change in the $R_{B/F}$-parameter, i.e. by the MEF of PG upon binding to DNA in the presence of silver nanoparticles. Consequently, at a PG concentration of 100 nM, the lower limit of DNA detection (D_L) on SiF bottom wells decreases several orders, when compared to a glass bottom well, where $D_L = 10$ pg/ml.

We have also experimentally studied the influence of PG concentration in solution on the D_L of the PG/DNA quantitation assay. Fig. 6 presents PG/DNA sensitivity curves measured for DNA loaded onto SiF wells and their respective fitted functions, with PG concentrations – 100 nM and 10 nM. As it can be seen from Fig. 6, for [PG] = 10 nM, the lower limit of DNA detectability by PG is significantly extended and reaches an ultra-sensitive level of $D_L = 1$ pg/ml, corresponding to the lower limit of detection estimated by the theoretical simulations performed at the same concentration of PG. Hence, the theoretical model describes very well the experimental assay data.

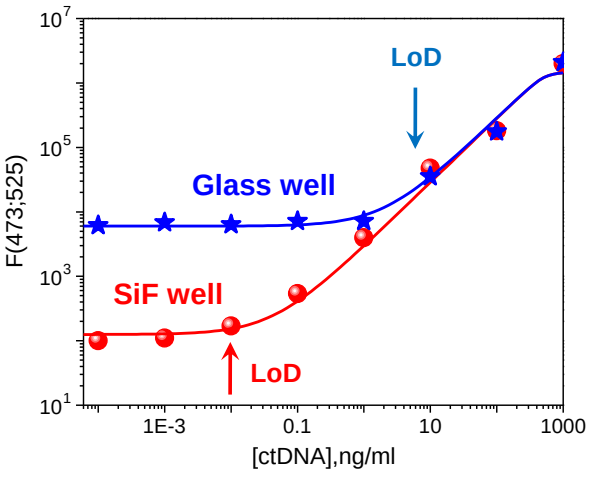

Fig. 5. Experimental data: normalized PG/DNA sensitivity curves, PG fluorescence vs concentration of DNA, measured on SiFs (silver island films) and from a glass bottomed well. Normalization was undertaken at high concentration of DNA. The data was fitted using Eqs. (6) and (4), and the resulting fitting functions are shown in the red solid line (SiFs well) and in blue (glass well). The circles and stars represent experimental data. Concentration of [PG] = 100 nM in TE, pH 7.4. DNA – calf thymus DNA. LoD – Limit of Detection.

4. Conclusions

PicoGreen® fluorescence is enhanced about 1000-fold when the chromophore interacts with double stranded DNA, resulting in high sensitivity DNA detection in solution. In our recent publication (Dragan et al., 2010a) we have shown that in the presence of silver nanoparticles, the enhancement further increases by greater than 30,000-fold. This additional enhancement is due to the MEF effect, which occurs for chromophores in the near-field (Geddes and Lakowicz, 2002b; Geddes et al., 2003a). Remarkably, we have also observed that PG shows similar fluorescence enhancement in complex with short 16 bp DNA and with highly polymeric DNA. This suggests that PG dye in combination with silver nanoparticles may be utilized for the universal ultra-sensitive detection of DNA.

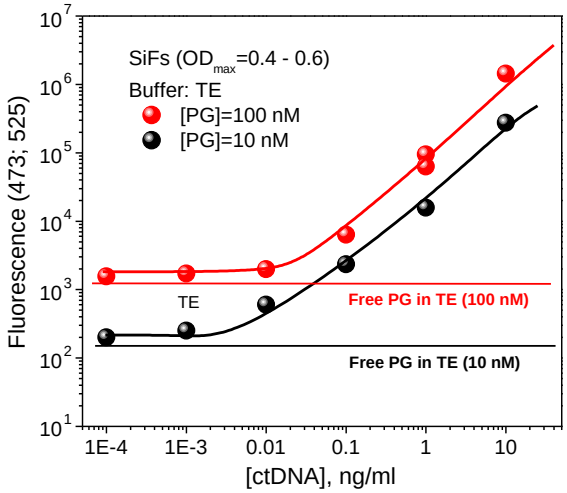

Fig. 6. PG/DNA "sensitivity" curves measured for calf thymus DNA on SiFs at two different concentrations of PG: 100 and 10 nM.

Theoretical analysis of the PG/DNA quantitation assay, which is based on PG non-covalently binding to dsDNA and the equilibrium between the bound and free states, has shown that the range of DNA concentrations available for fluorescence quantitation depends a number of factors. These include the concentration of dye, its affinity to nucleic acid molecules, chromophore binding site size on DNA, and the ratio between fluorescence intensity of the dye in bound to DNA state to that free in solution. An analytical expression has been derived in this work for the analysis of PG sensitivity to DNA and is broadly applicable to other fluorescent dyes that interact non-covalently with DNA. Experimental data obtained for our PicoGreen® (PG)/DNA assay is in very good agreement with the predictions made theoretically.

Finally, we have shown, both theoretically and experimentally, that DNA assays based on the MEF of PG demonstrate sensitivity to DNA concentration of $\approx$1 pg/ml, which is several orders of magnitude more sensitive than without the silver nanoparticles, suggesting the broader practical use of this approach for the ultra-sensitive detection of double stranded nucleic acids.

Acknowledgments

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