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ORIGINAL PAPER

Voltage-Gated Metal-Enhanced Fluorescence II: Effects of Fluorophore Concentration on the Magnitude of the Gated-Current

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Abstract In this letter we report further findings on the ability of an applied direct current to modulate Metal-Enhanced Fluorescence (MEF). Fluorophores in close-proximity to *just-continuous* silver films (JCS) show significantly enhanced fluorescence intensities. However, when a current is applied to the films, the enhanced fluorescence can be gated in a manner that depends on both the fluorophore concentration, the magnitude of the applied current and the extent of the protein mono to multi-layer surface coverage. Our results are consistent and indeed further support our previous hypothesis and model that fluorophore-metal near-field interactions can be influenced by an applied direct current.

Keywords Metal-Enhanced fluorescence · Surface-Enhanced fluorescence · Radiative decay engineering · Plasmon controlled fluorescence · Surface plasmons · Mirror dipole · Near-Field effects · Voltage-gated fluorescence

Abbreviations

MEF	Metal-Enhance Fluorescence
JCS	Just Continuous Silver Island Films
VGE	Voltage-Gated Effect
HSA-FITC	Human Serum Albumin labeled with Fluorescein

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Introduction

Over the last 7 years our laboratory has described the favorable interactions of fluorophores in close-proximity (< 10 nm) to plasmon resonant particles. These interactions include, enhanced fluorescence intensities [1–3], enhanced fluorophore photostabilities [4–6] and enhanced triplet yields [7, 8], to name but just a few. While the interactions of fluorophores with metallic nanoparticles has been known for over 30 years [9, 10], the exact mechanism for enhanced fluorescence is still of some debate [11, 12]. Some workers have described the interaction as due to a modification in the radiative decay rate of a fluorophore [13, 14], while Metal-Enhanced Fluorescence (MEF), described by Geddes in 2002 [1], has rationaled the effect as due to a near-field coupling between an excited state dipole and induced surface plasmons, the surfaces plasmons in turn radiating the coupled quanta [1], Fig. 1-Top. Subsequently, the notion of "radiating plasmons" has catalyzed several works [15] and supports current metal-fluorophore observations to date [16].

In a recent paper, we have shown that an applied direct current to low resistance just-continuous silver-island films (JCS), can gate the Metal-Enhanced Fluorescence of fluorophores in the near-field, i.e. ~ 10 nm [17], Fig. 1—Middle. This observation is not only helpful in the design of gated-fluorescence technologies, such as for Immuno-assays, but the interpretation and observations of the effect further support the notion of a radiating plasmon model [18, 19]. In this letter, we subsequently build upon our previous observations and further show that the enhanced fluorescence can be gated in a manner that depends on i) the fluorophore concentration, ii) the magnitude of the applied current and iii) the extent of the protein mono to multi-layer surface coverage, not only further supporting the radiating

plasmon model [18, 19], but indeed showing that protein-fluorescence based systems can be utilized with JCS, opening up the possibilities for future voltage gated immunoassays, Fig. 1—bottom.

Experimental

Materials

Fluorescein labeled human serum albumin (HSA-FITC), premium quality silane-prep™ glass slides, silver nitrate, ammonium hydroxide (30%) were obtained from Sigma.

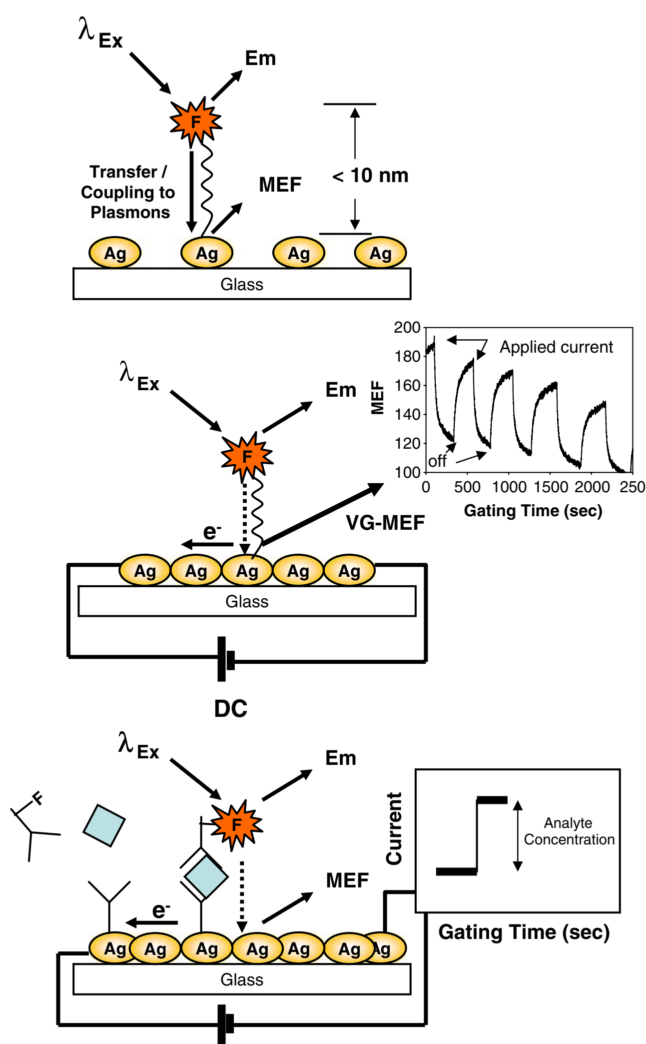

Fig. 1 Graphical representation of the current interpretation of Metal-Enhanced Fluorescence (**Top**), Metal-Enhanced Fluorescence perturbed with applied Voltage/Current (**Middle**), and a graphical representation of the possibility of Metal-Enhanced Digital Immunoassays (MEDIA). (**Bottom**). **F**—Fluorophore, **MEF**—Metal-Enhanced Fluorescence, **Ag**—Silver nanoparticles

Methods

Just Continuous Silver Island Films (JCS) were prepared as we have previously described [17]. In short, amine-coated glass microscope slides (3*1 Sigma) are immersed in Tollen's reagent until the electrical resistance approaches 1 Ohm cm^{-1} measured using a GDT-11 digital voltmeter. Laser excitation was made using a 473 nm solid state blue laser (500 mW), with a 488 notch filter (Semrock), the emission measured using a HD2000 spectrometer, Ocean Optics, FL.

For the preparation of dried samples, a protein solution in PBS-buffer, pH 7.4 was loaded onto the JCS at concentrations of 0.03, 0.3 and 3 μM. After incubation for 30 min (in another case for 3 h), the slides were washed with water and dried under a stream of dry nitrogen gas.

Wet samples were prepared in a similar manner as compared to the dry ones, with the exception of the drying stage. After incubation of the protein on JCS for 30 min, the slides were subsequently washed and then soaked with buffer (Gly-HCL, pH 3; PBS-buffer, pH 7.4 or just water, pH 5.5). Before measurements, wet samples were covered by a glass microscope cover slide.

Results and discussions

In a recent paper we have demonstrated the voltage gated phenomena with dry fluorophore samples absorbed onto JCS [17]. In an attempt to test whether the effect could be useful with biologicals, we have subsequently used fluorescein labeled HSA (HSA-FITC), which is known to passively absorb to several surfaces [18].

To characterize the extent of protein absorption to the JCS we studied fluorescence from the protein covered slides, prepared using a broad range of protein concentrations, and compared the rates of photobleaching under constant laser irradiation. Figure 2a shows the effect of the concentration of deposited HSA-FITC protein on sample fluorescence. At low protein concentrations (0.03 and 0.3 μM) the magnitude of the fluorescence signal from the samples is almost constant (Fig. 2a). The magnitude of the fluorescence from both samples was also significantly enhanced, about 5 times (Fig. 2a, insert), in comparison to the control samples prepared on glass slides, i.e. no silver. This is a clear indication of the metal-enhanced fluorescence (MEF) effect, i.e. the effect that occurs only for fluorophores in close proximity to metal nanoparticles. This suggests that under these conditions JCS slides are fully covered by the protein, which forms a single layer on the metal surface. A further increase of the protein concentration to 3 μM, results in a significant increase of sample fluorescence, almost 7 times (Fig. 2a). Such an increase of

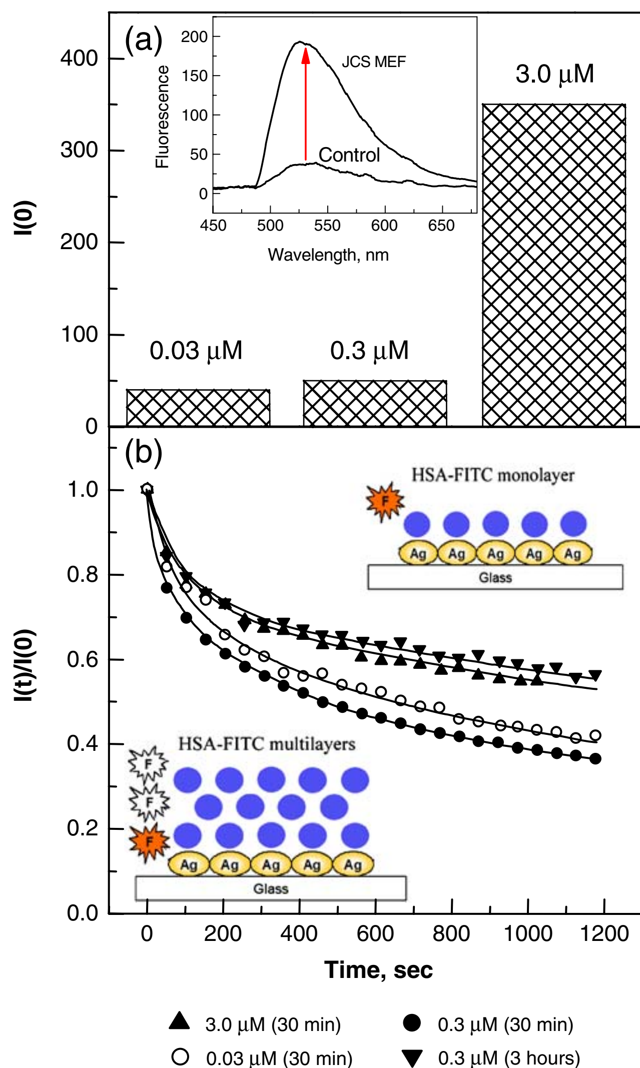

Fig. 2 **a** Intensities of fluorescence of HSA-FITC JCS films at the beginning, I(t=0 sec), of laser irradiation. **b** Normalized fluorescence decay curves, I(t)/I(0 sec), obtained at different concentrations of the deposited protein and for deposition times of 30 min and 3 h. **Insert:** Graphical representation of interpretation of the results of the photobleaching experiment. **Insert Top**, HSA-FITC monolayer that demonstrates a low rate of photobleaching caused by close proximity of the dye to the silver nanoparticles. **Insert Bottom**, formation of HSA-FITC multilayers results in enhanced rates of the photobleaching caused by moving the fluorophore away from JCS film, i.e. no MEF. The HSA is shown by blue circles; **F**—Fluorophore; Photo-stable and unstable states of fluorophore are shown by orange and open stars, respectively; **Ag**—Silver nanoparticles

fluorescence at high HSA-FITC concentration could only be explained by additional absorption of considerable amounts of the protein on the JCS, i.e. multilayer coverage. Additional insight comes from the analysis of the fluorescence intensity decay curves, i.e. photobleaching curves (Fig. 2b). The rate of fluorescence intensity decay of the samples obtained at low concentrations (0.03 and 0.3 μM) is notably lower than the rates for the samples at high concentration, i.e. 3 μM. It is interesting to comment on the basis of this difference in photobleaching efficiency. It is known that close proximity (~10 nm) of the fluorophore to the SiFs significantly decreases the radiative lifetime in comparison to the bulk solution [1, 16]. The important consequence of the fluorescence 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. In our case, the chromophore is attached to the large protein, Mw 66 kDa, with a size of about 7 nm. Therefore, the thickness of the protein monolayer on JCS determines the effective distance between the fluorophore and metal surface. This distance, in essence, modulates the extent of photobleaching. At high concentration, the protein forms multilayers which inadvertently moves the fluorophore away from the JCS surface, which results in a higher rate of photobleaching.

An increase in incubation time of the protein (0.3 μM) on the JCS from 30 min to 3 h also shows a similar trend, where both the fluorescence of the sample and photobleaching

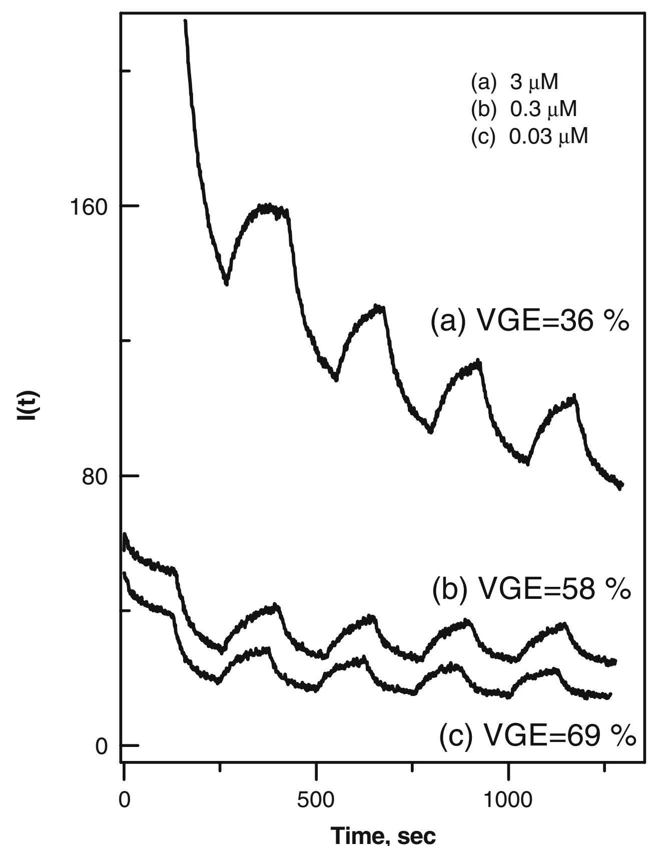

Fig. 3 Voltage gated effect (VGE) for HSA-FITC on JCS slides. The time of FITC-labeled protein deposition on silver coated slides was 30 min. The concentration of deposited protein in solution was varied from 0.03 to 3 μM. Electrical current through the silver was constant, 1.6 Amp. Time interval between voltage pulses was 2 min. Excitation at 473 nm; Emission collected at 530 nm

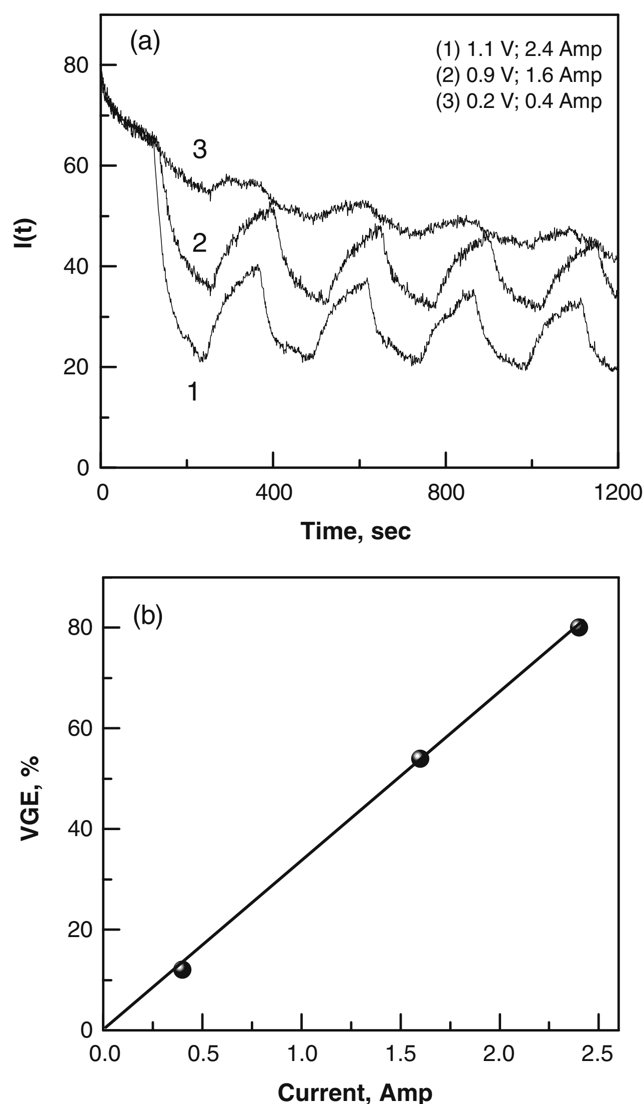

Fig. 4 **a** Voltage gated effect (VGE) for HSA-FITC on JCS slides for different applied currents. **b** The dependence of VGE (%) upon electrical current through the just-continuous silver film. VGE is a ratio of the fluorescence intensities, VGE=I(On)/I(Off) ×100%, On and Off are designations of states when the voltage is turned On/Off. Protein deposited on the JCS at a concentration of 0.3 μM. Excitation at 473 nm and fluorescence was collected at 530 nm

rate increase. These observations undoubtedly reveal a time-dependent non-specific increase of the protein absorption to JCS and the formation of HSA-FITC multilayers on the slide. Subsequently, we used HSA-FITC monolayer slides prepared for 30 min at low protein concentration (0.03 and 0.3 μM), for the remainder of this work, where a *photostable monolayer* is thought to form.

Figure 3 shows the effect of applied voltage on HSA-FITC covered JCS films, where the electric current through the just-continuous silver surface was 1.6 Amp. The time interval between the voltage pulses was 2 min. The results shown in Fig. 3 display modulation of the MEF (Metal-Enhance Fluorescence), induced by switching On/Off the electric current passage through the JCS, with constant CW irradiation (473 nm). The depth of modulation is expressed by the extent of the fluorescence decrease upon applying voltage to the system, i.e. the voltage gated effect (VGE). Interesting the VGE for protein monolayer samples (0.03 and 0.3 μM) is approximately the same. In contrast, the VGE for the multilayer samples (3.0 μM) is significantly lower. Thus, the VGE appears to depend on the properties of the protein coverage, i.e. mono- vs multi-layer. As was mentioned above, fluorophore protein *monolayers* demonstrate pronounced MEF, i.e. plasmon-fluorophore coupling [1, 18]. The coupling effect decreases for multilayers, due to the increased distance of the fluorophores from the metal, recalling that MEF is a close-proximity near-field interaction [1, 18]. Therefore, if we assume that a current flow perturbs (partially turn off) plasmon coupling to the near-field fluorophores, the depth of VGE could in essence reflect the distribution of chromophores relative to the surface.

The magnitude of the VGE depends linearly upon the strength of the electric current through the JCS (Fig. 4a, b). At a current of 2.5 Amp the VGE reaches a maximum value

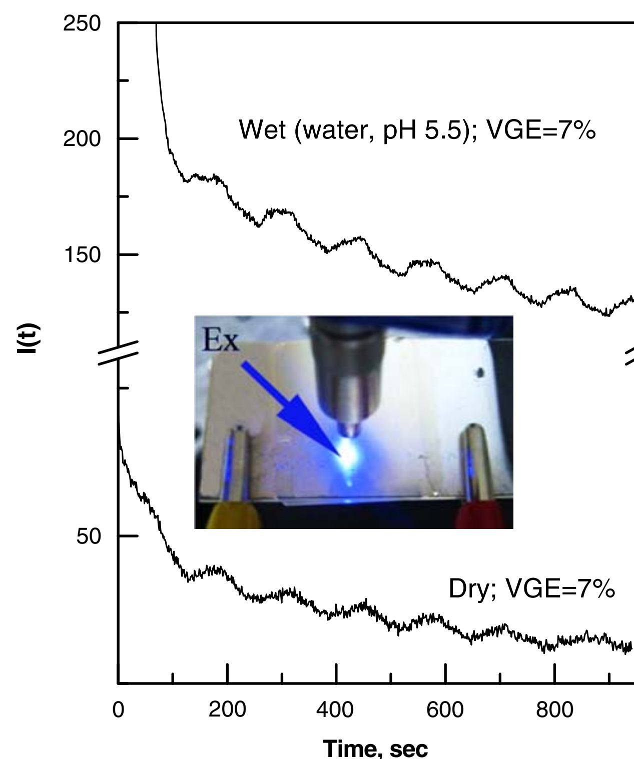

Fig. 5 Voltage gated effect (VGE) for wet and dry protein films deposited on JCS. Protein film was obtained by incubation of HSA-FITC (0.3 mM) on JCS for 30 min. To prepare wet samples, protein films on JCS-slides were soaked with water, pH 5.5, and covered by a glass cover slip. **Insert**: photograph of voltage gated experimental set-up for wet protein samples

of about 80% (fluorescence reduction of ≈ 5 times). If we take into account that MEF cause 5 times enhancement of fluorophore emission then a value of VGE=80% suggests that the MEF has fully diminished.

Pulses of applied current directly coincide with the observed changes in HSA-FITC fluorescence. It should be noted that in comparison to a sharp change in applied current (switching On/Off) the change in observed fluorescence signal is bi-exponential with the slowest decay time of about 1–2 min. As it was discussed in our previous paper [17], such slow kinetics of the fluorescence change could be due to a heating effect (although unlikely) in the JCS. However an increase in temperature, would not account for a decrease in gated fluorescence, it only offset its magnitude.

Application of the gated effects described here to bioassays requires the use of wet samples. Subsequently, we have also examined gating effects for JCS slides covered by (wet/moist) protein solutions. The experimental set-up for wet protein samples is shown in Fig. 5 (insert). Figure 5 shows two traces of VGE obtained in both dry and wet samples. It is notable that the magnitude of the VGE for the samples is the same, ≈ 7 %. The obtained result was expected, because in the studied samples, both wet and dry, the current passes mainly through low resistance JCS ($R < 7\ \Omega/cm$) (water has $R=18\ M\Omega/cm$) and induces perturbation of the coupled fluorescent system in a similar way. This result is a strong indication that the VGE is a direct result of the applied current.

We have also considered the possibility that the fluorescein dye protonation state is a factor which can influence the magnitude of the VGE. It is well known that fluorescein has a pK_a of 6.4. Therefore, at alkaline pH it is a doubly charged anion and becomes protonated at acidic pH. Analysis of electric current effects in wet samples at both pH 7.4 and pH 3.0 reveal (data not shown) *the absence* of a pH-dependence of the VGE, i.e. observed effect was the same as at pH 5.5 (Fig. 5), suggesting that the *charge of the FITC* plays *no role* in the effects reported here.

Conclusions

In this paper we have shown that Metal-Enhanced Fluorescence can be modulated by the passage of an applied current through just continuous silver island films (JCS). The results show that the effect is more significant for monolayer protein coverage as compared to multilayer coverage, which reflects the inability of distal protein anchored fluorophores to interact and couple to surface plasmons, i.e. MEF. The applied current is also directly proportion to the depth in the modulation of the MEF effect for both wet and dry samples, which opens up the possibility of new detection and sensing strategies based on gated amplified fluorescence, such as digital immunoassays as depicted by Fig. 1—Bottom. Further studies are underway in our laboratory and will be reported in due course.

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