Spectral Properties of Fluorescein Molecules in Water with the Addition of a Colloidal Suspension of Silver

Przemysław Siejak and Danuta Frąckowiak*

Institute of Physics, Poznań University of Technology, Nieszawska 13A, 60-965 Poznań, Poland

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The absorption, fluorescence excitation, and fluorescence emission spectra of water solutions of fluorescein dye with the addition of various amounts of a colloidal silver suspension have been measured in order to check if in such systems it is possible to distinguish the change in photonic mode density due to the metal presence from the other effects such as the influences of the microemulsion system on the spectral properties of the dye. It has been found that the presence of the silver colloid changes the concentrations of the various ionic forms of fluorescein, characterized by different yields of fluorescence. This effect is partially responsible for the change in the yield of the fluorescence emission observed at certain concentrations of the dye and the colloids. But even at the same concentration of various ionic forms of fluorescein (at the same pH of the dye solution and the dye−colloid mixture), at certain concentrations of fluorescein and the colloid, the yield of the dye fluorescence increases, which must be due to the interaction between the dye and the silver colloid. Because of the superposition of several processes influencing the dye yield of fluorescence, it is necessary to carefully establish the properties of the dye in a given environment, before considering its practical application as a marker of the metal presence. It is not excluded that similar complex effects could also occur in biological samples containing natural pigments and colloids of metals. Investigations of other dyes with other forms of metallic samples are in progress.

Introduction

Enhancement of the fluorescence of natural, weakly fluorescent components of biological cells due to the presence of metal in their close proximity can be applied in biological investigations.[1−5] Fluorescein is a dye used in several biological and medical applications. In most solvents, fluorescein exhibits a very high quantum yield of fluorescence, usually higher than 0.9.[6−9] The enhancement of the emission intensity of this dye due to the presence of metal could be superimposed with other effects generated by the interaction of the dye with the microemulsion medium, which can take place even in metal-free systems.[10] Such effects could also occur in colloid suspensions containing metal. In biological systems, metals can exist in colloidal forms.

Recently, much attention has been paid to changes in the fluorescence yield of synthetic dyes or natural pigments related to the presence of metal molecules in close proximity to their molecules, caused by changes in the photonic mode density (PMD).[1−5] The PMD is theoretically explained by the generation of surface plasmons.[11,12] PMD effects should also modify the radiative decay rates.

In the absence of metal, the lifetime of a fluorofore is

$$\tau_0 = (\Gamma + k_{nr})^{-1} \tag{1}$$

where Γ is the radiative deactivation rate and k_{nr} the rate of nonradiative deactivation.

When the metal is in the proximity of the dye, τ_m, the lifetime of fluorecence of a similar molecule, is

$$\tau_m = (\Gamma_m + k_{nm})^{-1} \tag{2}$$

The nonradiative deactivation rate in the presence of metal is the same as that in the sample without metal, so $k_{nm} = k_{nr}$. The symbol Γ_m stands for the radiative rate in the presence of metal, defined as $\Gamma_m = \gamma\Gamma$, which means that it is γ times higher than the Γ observed for the dye molecule in the absence of metal.

The quantum yield of emission in the absence of metal is

$$Q_0 = \Gamma/(\Gamma + k_{nr}) \tag{3}$$

whereas in the presence of metal this yield increases to

$$Q_m = \gamma\Gamma/(\gamma\Gamma + k_{nr}) \tag{4}$$

As follows from eqs 1−4, the fluorofores in the proximity of metal exhibit unusual behavior; their emission lifetime decreases, and the quantum yield of their fluorescence increases.

The fluorescein dye emission can also be changed by the dye linking to polymer molecules, by the dye being located in micellar or microemulsion media, even those which do not contain metal components,[10] or by changes in the amounts of various ionic forms of dye exhibiting different yields of emission. Optical properties of metal−dye nanoparticles differ strongly for the same dye in solution.[13−15] The fluorescein is usually a neutral or anionic dye, but as a result of the interaction with the medium, its different ionic forms, characterized by different positions of the absorption and fluorescence bands as well as by the different yields of fluorescence, can be cre-

* Corresponding author. E-mail: frackow@phys.put.poznan.pl. Phone: +48 61 665 31 80. Fax: +48 61 665 31 78.

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ated.[10,15,16] In microemulsion systems, the average pH of the sample can be different from that in close proximity to the suspended particles. The dye molecules can tend to be nonuniformly distributed in the sample; its concentration can be higher in the neighborhood of the emulsion particles than those at large distances from these particles.

The change in the spectral properties of natural pigments or synthetic dyes due to the presence of metal molecules in their close proximity enables using them for the detection of harmful metals in the environment[5] or for the investigation of processes occurring in biological molecules.[1−3] Biological molecules that need to be protected against metal poisoning are very complex; therefore, it is not easy to predict their properties and interactions with their surroundings on the basis of quantum mechanical calculations. Thus, it is necessary to establish their properties on the grounds of experimental study carried out, at first, on simple model systems.[2,5] This paper presents the results of investigations of changes in the yield of the fluorescence of fluorescein as a result of interactions of this dye with colloidal particles of silver in a water medium of various pH values. Fluorescein is a very well-known and strongly fluorescent pigment.[7,8,17,18] The changes in the lifetimes of fluorescence of the same systems will be a subject of a separate work.

The yield of fluorescein emission in water, as in most fluid solvents, is very high—higher than 0.9.[8,9,17] The PMD effect changes only radiative deactivation efficiency; therefore, it can be expected that for a highly fluorescent dye this effect would not be very strong. For such systems, the effects of the microemulsion medium on the dye's spectral properties could be comparable with the effects due to the presence of metal. Different types of effects can be distinguished by their different dependencies on such parameters as pH of the medium, concentrations of various ionic forms of the dye, and concentrations of the dye and the colloid.

In a microemulsion medium, fluorescein absorption peaks at 455 and 480 nm are observed.[10] In a water−methanol solvent, the absorption coefficient increases with increasing water content and both peaks are partially superimposed.[10] This shows that several effects are responsible for the shapes and intensities of fluorescein spectra.

The aim of this study is to establish the effect of the dye's surroundings on its absorption and fluorescence spectra as well as on the yield of its fluorescence. The results are expected to verify whether the dye can be used as a synthetic marker indicating the content of metals in biological or medical samples.

Materials and Methods

Fluorescein was purchased from Sigma-Aldrich Chemie, Germany, and used without further purification. The literature reports methods for the production of nanometric metal particles,[19] but in this study we applied commercially available colloidal silver particles produced by Aurum Health Products Ltd, Great Britain.

The concentration of silver in 1 mL of the initial sample of the silver colloid was about 10 mg/mL; therefore, in the undiluted sample, the average silver concentration, c_{Ag}, was about 9.3×10^{-5} M. The silver colloid suspension in water has pH = 6. The absorption spectra of the samples investigated were taken using a Cary 4000 (Varian), whereas the fluorescence excitation and emission spectra were taken by means of the Fluorescence Spectrophotometer F4500 (Tokio, Hitachi, Japan). The front side configuration was applied in the fluorescence emission measurement. The exciting light beam was incident at an angle of 32° to the cuvette wall, and the observation was made at an angle of 58°. In this configuration, the influence of reabsorption and secondary fluorescence is relatively low. The decrease in the emission of the dye due to absorption and scattering of light by metal colloids was evaluated to be low. It was shown that the corrections for the silver colloid absorption cause smaller changes in the fluorescence yields than the error introduced by spectral measurements.

Taking into account the facts that the dye and dye−colloid mixture absorption coefficients for the wavelengths of the exciting beam were similar, the coefficients of absorption of the fluorescence light were practically the same for both types of samples, and the geometry of the measurements was the same, the relative yield of fluorescein emission for the sample with metal colloid, Φ, to that without colloids, Φ_0, was obtained using formula 5 (ref 6, p 52):

$$\Phi/\Phi_0 = (F/F_0)(\mathrm{OD}_0/\mathrm{OD})(n^2/n_0^2) \qquad (5)$$

where F and F_0 are the integrated fluorescence intensities, OD and OD_0 the optical densities, and n and n_0 the refractive indexes for fluorescein in a water−colloid solution and for the sample of the same dye concentration but without the colloid, respectively.

The emission of silver particles was neglected, because in the spectral region of the fluorescein $Q(0,0)$ bands of all the neutral and ionic fluorescein forms occurring in the samples this emission is low. On the basis of this condition, the emissions of the colloid−fluorescein samples can be treated as those of a dye placed in a weakly absorbing medium.

The shapes and intensities of the absorption, fluorescence excitation, and fluorescence emission spectra of the dye solutions with and without the presence of the colloid particles were established at various dye and colloid concentrations and at several pH values. The experimentally measured pH values of the colloid−dye mixtures were different; therefore, for final PMD measurements, samples in buffers with the same pH values as those of the dye solutions and the dye−colloid mixtures were used. The measurements of pH give only average values of the pH of the mixtures. When the dye molecules gather in close proximity to colloidal particles, dye can be located in some regions with pH values different from the average pH value; therefore, the shapes of the absorption, fluorescence excitation, and fluorescence emission spectra produce more accurate information about the presence of acidic or alkaline forms of dye.

Results

Absorption, Fluorescence Emission, and Fluorescence Excitation Spectra. The shape of the absorption spectrum of the colloid sample (not shown), exhibiting maxima at 463 and 744 nm, suggests (on the basis of the literature data[1]) that the colloid particles have the shape of elongated ellipsoids.[1] The samples studied were obtained by mixing a known volume of the colloid−water suspension with a proper amount of water−dye solution. Colloid absorption was many times lower than that of the dye, even at the lowest dye concentration used, but silver colloid presence has a strong influence on the dye spectra predominantly because of its influence on the pH of the sample. The pH of the dye and of the dye−colloid solutions was regulated by HCl or NaOH addition and experimentally established. Solutions of dye alone and of the dye−colloid mixture in buffer having the same values of pH were used for the final pH measurements. In such samples, the concentrations of various ionic forms of dye should be at least similar. The

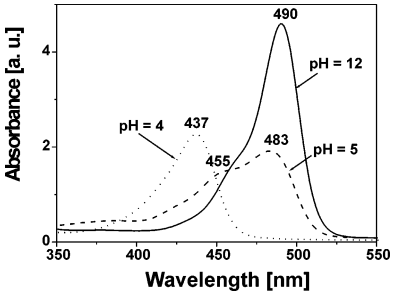

Figure 1. Absorption spectra of a water solution of fluorescein at various pH values (concentration = 5×10^{-5} M).

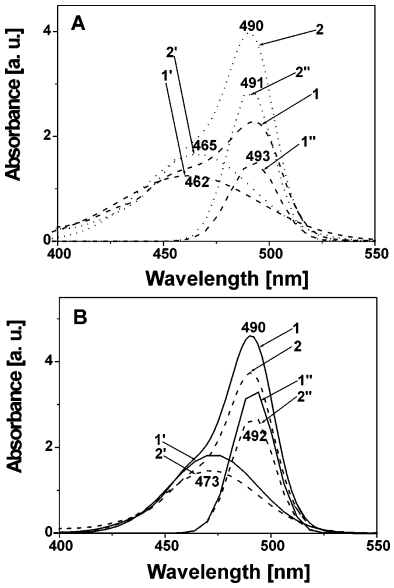

Figure 2. Gaussian analysis of the absorption spectra of dye with colloid mixtures in buffer at various pH values. Silver concentration: (1) $c_{Ag1} = 4.6 \times 10^{-5}$ M; (2) $c_{Ag2} = 8.3 \times 10^{-5}$ M. (**A**) (1) pH = 5.0; (2) pH = 4.0. (**B**) (1) pH = 8.0; (2) pH = 10.0. Gaussian components of given absorption spectra are marked as prime (′) and double prime (″). The dye concentration in all samples is 5×10^{-5} M.

final concentrations of the dye and the colloid in the samples were calculated from sample dilution as well as from the absorption spectra. The spectra of the samples with various ratios of the dye and colloid concentrations at different pH values were measured.

Figure 1 shows the absorption spectra of water−fluorescein solutions at various pH values. All samples have the same (5×10^{-5} M) dye concentration. From the literature,[6,8,10] it is know that in acidic media the maximum fluorescein absorption is at 437 nm but in alkaline solvents it is at about 490 nm and it is also known that the absorption coefficient of the alkaline form is usually higher than that of the acidic form.

In the pH range used in our experiments (from pH = 4 to pH = 8), the contributions from both forms are superimposed. Figure 2 shows the Gaussian analysis of the absorption spectra of fluorescein with colloid mixture solutions. For the sample at lower values of pH (4 and 5) (Figure 2A), two Gaussian components, the first one narrower with a maximum in the region 490−493 nm and the second much broader with a maximum at about 460 nm, are seen. At higher values of pH (8 and 10) (Figure 2B), two components of different half widths are also obtained with maxima in the region 490−492 nm and at about 473 nm. For the dye−water solution without the colloid having a pH = 8, two components of different half widths and with slightly different locations of maxima are also observed (not shown). For the samples with the colloid, the exact positions of the components depend on the amount of the colloid added (not shown).

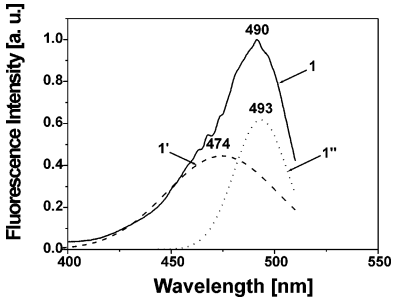

Figure 3. Gausian analysis of the fluorescence excitation spectrum of a water solution of fluorescein (dye concentration = 5×10^{-5} M, pH = 8.0). The wavelength of observation of emission is 520 nm. Gaussian components of given absorption spectra are marked as prime (′) and double prime (″).

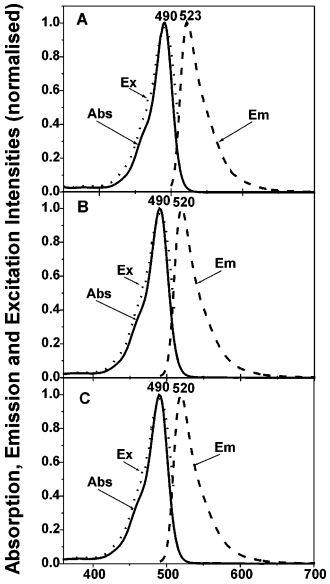

Figure 4. Normalized absorption (Abs), fluorescence excitation (Ex), and fluorescence emission (Em) spectra at pH = 8.0. (A) Water solution of fluorescein; (B and C) mixtures of fluorescein−silver colloid. Dye concentration: $c_d = 5 \times 10^{-5}$ M. Silver concentration: (B) $c_{Ag1} = 4.6 \times 10^{-5}$ M; (C) $c_{Ag2} = 8.3 \times 10^{-5}$ M. The fluorescence excitation (Ex) wavelength is 490 nm; in Ex, the observation wavelength λ_{obs} = 520 mn.

Figure 3 presents the Gaussian analysis of the fluorescence excitation spectrum of fluorescein solution at pH = 8. The concentration of the dye is the same, $c_d = 5 \times 10^{-5}$ M, for all samples with spectra shown in Figures 1−3. As follows from a comparison of Figures 2B and 3, the positions of the components in the fluorescence excitation spectra and in the absorption spectra are similar, which means that both absorption components are contributing to emission. The shapes of the excitation spectra of the alkaline samples (Figure 3) suggest, similarly as analysis of the absorption spectra (Figure 2), the occurrence of two forms of the dye: one with greater half widths of the band with a maximum at 474 nm and the second with smaller half widths and a higher maximum at 493 nm. The colloid addition to acidic fluorescein solution causes an increase in the amount of the alkaline forms because of the diminishing amount of the form occurring in acidic media (not shown).

Figure 4 shows the normalized absorption, fluorescence excitation, and fluorescence emission spectra for a water solution of fluorescein (Figure 4A) and for fluorescein−colloid mixtures (Figure 4B and C). The dye concentration was the same for all

TABLE 1: Yields of Fluorescence of the Fluorescein−Water Solution in the Samples with Silver Colloid Addition (ϕ) relative to that without Colloid (ϕ_0) for Various Concentrations of Dye (C_d) and Silver Colloid (C_{Ag}) and pH Values[a]

λ_{exc} (nm)	pH	C_d (μM)	C_{Ag} (μM)	V_{Ag}/V_d	C_{Ag}/C_d	ϕ/ϕ_0[b]
490	4.0	500	46	1:1	0.09	0.23
		100	83	9:1	0.83	0.29
		50	46	1:1	0.92	0.21
			83	9:1	1.66	0.92
			92	99:1	1.84	0.93
		10	83	9:1	8.30	**1.16**
490	8.0	100	83	9:1	0.83	0.85
		50	46	1:1	0.92	**1.42**
			83	9:1	1.66	**1.39**
		10	46	1:1	4.60	**1.19**
			83	9:1	8.30	**1.15**
		5	46	1:1	9.20	0.85
			83	9:1	16.60	0.90
			92	99:1	18.40	0.85
458	8.0	100	83	9:1	0.83	0.85
		50	46	1:1	0.92	**1.51**
			83	9:1	1.66	**1.46**
		10	46	1:1	4.60	**1.24**
			83	9:1	8.30	**1.10**
		5	46	1:1	9.20	0.69
			83	9:1	16.60	0.82
			92	99:1	18.40	0.71

[a] V_{Ag}/V_d is the ratio of the volume of colloid to the volume of dye solution. C_{Ag}/C_d is the ratio of the concentration of colloid to the concentration of dye−water solution. λ_{exc} is the excitation wavelength. [b] The accuracy of ϕ/ϕ_0 is 0.05.

spectra; the pH value of the samples was also the same and equal to 8, the same as that in the fluorescence yield measurements (Table 1). The fluorescence excitation spectra were for all samples similar to the corresponding absorption spectra. The spectra of fluorescein alone and fluorescein−colloid mixtures (Figures 1−4) were similar but not identical even at the same pH values of both types of samples, which suggests the influence of the colloid on the fluorescein spectra. The colloid addition causes an increase in the intensity of the fluorescence bands (effect not shown in the normalized spectra in Figure 4). This increase could be due, at least partially, to an increase in the local pH value. The average pH value of the mixture depends, of course, on the amount of the colloid added. At a pH of the mixture equal to or higher than 5, the alkaline forms of the dye are dominant. All spectra presented in Figure 4 were measured at pH = 8. The observed increase in the fluorescence intensity can be due to the PMD effect. The small differences between the spectra of the dye alone (Figure 4A) and with the colloid (Figure 4B and C) can be partly related to the change in the pH of the medium, because even at the same average values of pH this value can be different in close proximity to colloidal particles where the dye molecules could be gathered and where the alkaline forms can be created. This effect seems probable because further addition of the colloid has little influence on the shapes of the spectra, which are similar to those observed at a much higher pH = 11.9 (not shown).

The spectra of the samples with colloids for lower dye concentration are similar to the spectra for higher dye concentrations (not shown). This suggests that in all samples the monomeric alkaline fluorescein forms are predominant. In all cases, the main absorption maximum is at about 490 nm. The aggregation of fluorescein has no influence on the effects observed. The shapes of the normalized absorption spectra measured at a given pH are independent of the dye concentrations in the concentration range used (not shown). The same conclusion follows from Figure 5, presenting the dependence

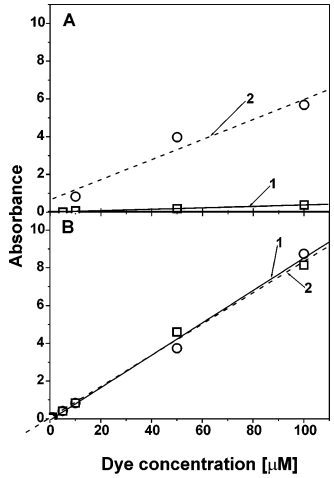

Figure 5. Absorbance at 490 nm of water solutions of (1) fluorescein and (2) fluorescein−silver colloid mixtures vs dye concentration: (A) pH = 3.0; (B) pH = 11.9. The silver concentration is 8.3×10^{-5} M.

of absorption at 490 nm for different dye concentrations. At pH = 4 in a water solution (Figure 5A, curve 1), the absorption in this region is low because the main absorption band is at 443 nm, but after the colloid addition (curve 2), it increases and grows linearly with the dye concentration. At higher pH values (Figure 5B), the increase in absorption with increasing dye concentration is practically the same for the samples with and without the colloid. This shows again that the contributions of the silver colloid to the absorption can be neglected.

At basic pH, the intensities of emissions depend, of course, on the value of absorption at the wavelength of excitation, but the emission band is always located at about 520 nm. This shows that there is practically only one alkaline fluorescent form of the dye or that other forms are transferring excitation to this long wavelength form. The fluorescence bands after normalization at the maximum have identical shapes, showing that in the range of dye concentrations studied the aggregation of fluorescein can be neglected and also that the absorption of samples linearly increases with dye concentration (Figure 5).

Fluorescence Yields. The quantum yields of fluorescence of the dye with the colloid (ϕ) relative to that of the sample without the colloid (ϕ_0), calculated on the basis of formula 5, are given in Table 1. The PMD effect should give higher values of ϕ than that of ϕ_0, so that $\phi/\phi_0 > 1$.

The relative yields depend on the concentrations of the dye, the amount of the colloid, the pH of the sample, and the wavelengths of fluorescence excitation. For alkaline samples exhibiting similar shapes of spectra for samples with and without colloid, the relative quantum yield for some concentrations of dye and colloid is higher than unity, suggesting the occurrence of the PMD effects. The data shown in Table 1 are obtained for the same pH values of the mixture and of the dye alone solutions.

As follows from Table 1, the relative yield of the dye fluorescence increases predominantly at basic pH values, at which practically the same ionic forms of the dye are present in the samples with and without colloids. In such cases, the fluorescence yield increase should be due to PMD effects. For acidic samples (pH = 4), an increase in the relative yield of fluorescence is observed only at higher amounts of the colloid and a lower dye concentration. The decrease in the yield of the dye emission at high dye concentrations and low amount of the colloid can be due to the fluorescence light scattering by colloidal particles. At pH = 4, the acidic form of fluorescein,

exhibiting a lower yield of fluorescence, is present in the sample. When the amount of the colloid is higher, the local pH in the neighborhood of the colloid could be higher and stronger fluorescent forms can be created.

The increase in the yield observed is different on excitation with radiation of different wavelengths, showing that the PMD effect is different for particular ionic forms of the dye at pH = 8. The PMD effect depends on the amounts of the dye and the colloid in the mixture. When the concentration of the dye is too high, this effect is lowered or even disappears. In such cases, probably only part of the dye molecules are located in close proximity to the colloid particles and exhibit PMD effects. When all sites close to the colloid molecules are occupied, other dye molecules are located at a greater distance from the metal and, therefore, do not exhibit an increase in the fluorescence yield. The average increase of the fluorescence yield of such a sample is lower than the yield observed for the sample in which most of the dye molecules are located near colloid particles. Only the dye molecules located near the colloid molecules can strongly interact with metal. It is not clear why at very low amounts of the dye the PMD effect is not observed.

Discussion and Conclusions

The influence of micellar and microemulsion systems on the spectral properties of fluorescein was investigated by Biswas et al.[10] They performed measurements for alcohol with water addition in alkaline solvents in which predominantly neutral and anionic forms of fluorescein occur, showing absorption in the range 440−454 nm and fluorescence in the range 510−517 nm. An increase in the water amount shifts the absorption and fluorescence bands toward longer wavelengths, and their intensities increase. These effects are explained as a result of the change in the interactions of the dye with the environment.[10] In the spectrum of the dye in the medium with micellar systems, the maximum of the absorption band is shifted to 494 nm and a new band at 500 nm appears.[10] Fluorescence intensity also increases but without a shift in the band's position. It is a complicated system, but it can be compared with the sample presently investigated. A comparison of the effect caused by changes in different parameters of the samples investigated could give an opportunity to separate the effect caused by colloidal−solvent interaction from the very specific effects generated by the dye interaction with metal molecules due to the surface plasmons. Such effects occur in our samples, but they are not very strong. It is not easy to separate the effects due to microemulsion presence and related to the shift of the equilibrium between various ionic forms from PMD effects, but an increase in the relative fluorescence yield for the samples exhibiting similar shapes of all spectra strongly suggests the participation of PMD effects. The PMD related increase in the fluorescence yield is much lower for fluorescein with silver colloids than that which is observed for other dye−metal systems.[1−4]

Because of the superposition of various effects, fluorescein can be used as a marker of the presence of colloid metals in biological samples only after very systematic investigations of this dye's properties in specific types of samples have been conducted. After such investigations, fluorescein can be used not only as a synthetic marker for the establishment of the presence and the concentration of colloidal metal in various media but also for investigations of other properties of samples because the yield of fluorescein emission depends not only on the concentrations of dye and metal molecules but also on the pH of the dye environment.

At some pH values and ratios of the dye to colloid concentrations, the silver colloid particles cause an increase in the fluorescence yield of this dye but this increase is due to the superposition of several effects. The fluorescein is not an optimal marker of the presence of metal in colloid form because the photonic mode density effects can be masked by other superimposed effects. Therefore, investigations of the interactions of other dyes with metal colloids are currently underway.

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