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Enhancement of Molecular Fluorescence near the Surface of Colloidal Metal Films

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Fluorescence enhancement was studied on silver colloidal metal films (CMFs) using two systems: (1) Langmuir−Blodgett monolayers of fluorescein-labeled phospholipids separated from the surface of the films by spacer layers of octadecanoic acid and (2) biotin−fluorescein conjugates captured by avidin molecules adsorbed on top of a multilayer structure formed by alternating layers of bovine serum albumin−biotin conjugate (BSA−biotin) and avidin. The dependence of fluorescence intensity on the number of lipid or protein spacer layers deposited on the surface of the CMF was investigated. The results demonstrate the requirement for adsorbate location within the region between Ag particles for maximal enhancement. The density of avidin molecules on the surface of the BSA−biotin/avidin multilayers adsorbed on the CMF was also determined. A procedure for forming a rigid, uniform silica layer around the Ag particles on the CMF is described. The layer protects the particles from undesirable chemical reactions such as etching by halide ions, for example, and provides the requisite stability for bioanalytical applications. Colloidal films composed of Ag particles covered by ∼10-nm-thick silica layers were tested for fluorescence enhancement using goat immunoglobulin and a conjugate of rabbit anti-goat immunoglobulin with 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanoate. An enhancement factor of ∼20 was obtained.

Current efforts toward the development of new bioanalytical methods are focused primarily on increasing sensitivity and selectivity of the detection methods, simplifying the procedures by eliminating intermediate washing and sampling steps; and avoiding the use of radioactive probes or other types of hazardous reagents. Among the different approaches that have been evaluated in attempts to fulfill these requirements (e.g., electrochemical-based detection[1] and solid-state devices[2]), those based on optical techniques appear to be the most promising.[3,4]

Of the diverse optically based detection methods, two whose potential for bioanalytical applications has yet to be fully explored are surface-enhanced Raman scattering (SERS) and surface-enhanced fluorescence (SEF). Both processes result from the strong increase of local electromagnetic field in close vicinity to metal surfaces, usually silver or gold, that accompanies excitation of surface plasmon resonances in the metals.[5−7] Specific interactions between the metal surface and nearby molecules can also contribute to the magnitude of the enhancement of Raman scattering.[5−7] The largest enhancement factors have been observed from molecules adsorbed on roughened surfaces comprised of nanosized structural features. The sharp decay of the enhanced local electromagnetic field with distance from the surface provides a unique opportunity for selective detection of molecules located near the metal substrate in preference to solution species. This aspect of the enhancement phenomena is particularly appealing for bioassays because it may obviate the intermediate washing steps.

The considerable potential of SERS for bioanalytical applications has been demonstrated in a variety of quantitative[8,9] and qualitative analyses,[10] whereas only the fundamental aspects of SEF have been extensively studied.[6,7,11−17] In contrast to SERS, maximal enhancement of fluorescence does not occur from molecules adsorbed directly on the metal surface. Emission from this population of adsorbates is quenched due to rapid radiationless energy transfer to the metal. Maximal fluorescence enhancement is observed only from molecules at certain distances from the surface. The fluorescence intensity depends on the surface features, including especially the size of the protuberances that are created in the roughening procedure in the case of electrodes or the size of the metal particles in the case of colloids. The use of SEF as an analytical technique is complicated by the fact that

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the conditions for maximum fluorescence enhancement require control of the distance of fluorophore from the surface. To our knowledge, only one example of the use of SEF for detection of biomolecular interactions exists.[18] In this case, enhancement of fluorescence was observed on thin, "smooth" silver films using attenuated total reflectance. The sensitivity of the technique, termed surface plasmon resonance fluoroimmunoassay (SPRF), was over 6 times greater than that of the conventional total internal reflection fluorescence approach toward detection of human chorionic gonadotrophin (hCG). Based on a number of studies that have shown the superiority of roughened metal surfaces as compared to "smooth" ones in terms of enhancement factors, as well as the greater variety of possible experimental configurations for observation of SEF,[6,7,19] it is highly probable that the sensitivity can be improved significantly.

Our recent research efforts have targeted the application of SEF to bioanalytical problems. For this purpose, we have developed new uniform substrates, termed colloidal metal films (CMFs),[20,21] with well-defined roughness features. These substrates are prepared by adsorbing colloidal metal particles on glass (quartz) slides that have been modified with silane coupling agents containing different functional groups, such as thiol, amino, cyano, or pyridyl groups. Adsorption is accomplished through the formation of a specific bond between the metal and the functional group. This procedure results in well-ordered two-dimensional arrays. The density of the metal particles on the surface can be varied by immersion of the modified substrate into different concentrations of the colloidal suspension. One of the unique properties of a CMF as compared to a colloidal suspension of gold or silver is the possibility to control the aggregation state during an experiment. Because a high particle density can be achieved in a CMF (up to 100 μm^{-2}, which would require colloidal suspension of optical density of $\sim 10^5$ to achieve comparable interparticle spacing), these substrates are expected to be very effective in a variety of analytical applications utilizing optical detection, including, for example, biological chip technologies.[22]

The major requirements for fluorescence enhancement on CMFs are addressed in this study. Two systems were investigated: (1) mixed monolayers of fluorescein-labeled and nonlabeled phospholipids transferred to the colloidal films by the Langmuir−Blodgett (L−B) technique and (2) biotin−fluorescein conjugates (biotin−FITC) captured by avidin molecules adsorbed on the top of a multilayer structure formed by alternating layers of bovine serum albumin−biotin conjugate (BSA−biotin) and avidin. A procedure for the formation of uniform, rigid spacer layers of defined thickness around metal particles on a CMF, together with a method for monitoring the distribution of immobilized biomolecules on the surface of these films, is also described. A goat immunoglobulin adsorbed on the CMF and a rabbit anti-goat immunoglobulin labeled with 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate (NBD) were used to test fluorescence enhancement on colloidal films composed of coated Ag particles.

EXPERIMENTAL SECTION

Preparation of CMF. The films were prepared as described elsewhere.[20] First, thiol functional groups were introduced on the surface of glass slides through a silanization reaction with (3-mercaptopropyl)trimethoxysilane (MPS, Gelest) according to the previously published protocol.[23] One deposition cycle was used instead of the three employed in the previous work.[23] The derivatized slides were immersed in an aqueous colloidal suspensions of Ag particles. The formation of the CMF is usually completed after exposure of the slides to the colloidal suspension overnight. The experiments were performed using a CMF with optical densities ranging from 0.15 to 1.5. Drying of the films in air or exposure to methanol causes aggregation of the particles on the surface.[21] Aggregation is accompanied by a decrease in intensity, broadening of the plasmon resonance band, and an increase in extinction in the red spectral region. Such CMFs are termed aggregated colloidal films.

Monolayer Deposition. Monolayers of octadecanoic acid (ODA, Applied Science) and mixtures of *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, diethylammonium salt (FDPPE, Molecular Probes), with 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, Sigma, 99%) were prepared at the air/water interface in a thermostated Teflon Langmuir trough containing 1 mM $MgSO_4$ as the subphase. Glass slides derivatized with MPS, but not exposed to silver colloidal suspensions, were used as a reference in fluorescence measurements. The transfer ratios for monolayers on the CMF and the reference slides were typically 1.1 ± 0.1. The detailed procedure for the preparation of the monolayers and their transfer onto the solid substrates is described in our earlier work.[20]

Formation of Multilayers of BSA−Biotin/Avidin. The first layer, composed of physisorbed bovine serum albumin−biotin conjugates (BSA−biotin, Sigma), was prepared by exposing the films to a solution containing 100 $\mu g\ mL^{-1}$ of the protein in 0.1 M PBS buffer, pH 7.5, for 1−2 h. Next, the slides were washed for 10 min in the PBS and placed in a 100 $\mu g\ mL^{-1}$ solution of avidin (Sigma) in the same buffer for 30−40 min. For multilayer formation, the above procedure was repeated the requisite number of times using 30−40-min exposure times.

Preparation of Gold Markers Labeled with BSA−Biotin. Conjugates of BSA−biotin with colloidal gold particles were prepared according to the procedure described previously.[24] Sixty-six milliliters of colloidal Au particles[25] with a mean diameter of ~20 nm was mixed with 33 mL of a 35 $\mu g\ mL^{-1}$ solution of BSA−biotin in 60 mM MES buffer, pH 5.4, for about 1 h. Next, 2 mL of 1% poly(ethylene glycol) (MW 20 000; Sigma) and 100 mL of water were added, and the particles were centrifuged at 8500 rpm for 50 min. The supernatant was discarded, and the pellet was resuspended in 50 mL of 20 mM HEPES buffer, pH 7.4, containing 0.2 mg mL^{-1} of the poly(ethylene glycol).

Formation of Uniform Silica Layer around the Silver Particles. In a typical experiment, colloidal films were immersed

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in a solution consisting of 130 mL of 2-propanol, 4.4 mL of 30% ammonium hydroxide, 4.8 mL of solution of colloidal Ag particles with a mean diameter of ~35 nm and optical density about 10, and ~100−200 μL of 10% tetraethyl orthosilicate (TEOS, Gelest). Because the surface area of the CMF is small, colloidal Ag was added to provide additional, well-defined surface area and thereby facilitate control over the thickness of the spacer layer on the CMF surface. The reaction was performed at 40 °C until completion (about 16−18 h). The slides were then washed in 2-propanol. A uniform spacer layer of ~10-nm thickness was formed in the reaction. The thickness of the silica layer was determined from a comparison of the scanning electron microscope images of the CMF with and without the silica spacer layer.

Fluorescence Enhancement Experiments. To determine the enhancement of fluorescence, both colloidal films and reference slides containing the same amount of BSA−biotin/avidin multilayers were immersed in a 20 nM solution of biotin−fluorescein conjugate (Sigma) in 50 mM MES buffer, pH 5.5, for 30 min. Glass slides with the MPS layer, but lacking colloidal particles, were used as a reference. The slides were washed in 0.1 M PBS buffer, pH 7.5, and fluorescence measurements were performed in the PBS.

A 80 nM solution of conjugate of rabbit anti-goat immunoglobulins (RagIgG, Sigma) labeled with NBD was employed when goat immunoglobulins (GIgG, Sigma) were immobilized on a solid support. The conjugates of the immunoglobulins with NBD were prepared according to the well-known protocol for amino-reactive probes[26] using succimidyl 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanoate (Molecular Probes). Glass slides with the MPS layer, but lacking colloidal particles, were used as a reference.

The absolute amount of fluorescein- or NBD-labeled conjugates adsorbed on the CMF and the reference slides was determined from the decrease in the fluorescence intensity of the solutions after removal of the substrates. The fluorescence spectra obtained from colloidal and control slides were normalized to the amount of the captured conjugate. The normalized spectra were used for the determination of the enhancement factors.

Instrumentation. UV−visible spectra were measured using a Lambda 6 (Perkin-Elmer) spectrophotometer. The CMFs were placed in an empty or water-filled 1-cm-path length quartz cuvette. A bare glass slide was used as a reference.

Fluorescence spectra were obtained using a triple spectrometer (Spex, Triplemate 1877) equipped with a liquid nitrogen-cooled CCD detector (LN-1152, Princeton Instruments). The 457.9- and 488.0-nm lines of an Ar^+ laser (Coherent, Innova 200) were used for fluorescence excitation. The emitted light was collected by a camera lens (*f*/1.2) in a backscattering geometry. Indene was used to calibrate the spectra.

Electron microscopy was performed in the reflection mode using a JEOL 1200EX scanning transmission electron microscope. A platinum/palladium (80:20) film of approximately 10-nm thickness was deposited on the specimens using a Polaron E5100 sputter-coater.

RESULTS AND DISCUSSION

The ratio of the fluorescence intensity observed from molecules near a roughened silver surface to that from molecules adsorbed on a reference glass substrate can be described by the product of two terms,[17]

$$Y = |L(\omega_{exc})|^2 Z(\omega_{flu})$$

The term $|L(\omega_{exc})|^2$ is the enhancement of local electromagnetic field intensity near the nanosized metal structures at the excitation frequency ω_{exc}. It is representative of the ability of the metal to concentrate the electromagnetic energy when the excitation wavelength is coincident with the surface plasmon resonance of the metal.

The second factor, $Z(\omega_{flu})$, describes the relative radiative yield of the excited dye molecules on the two substrates. The radiative decay rate of the coupled system consisting of the metal particles and adsorbed dyes is proportional to the square of the local field enhancement factor, $|L(\omega_{flu})|^2$, at the fluorescence wavelength.[6] Because of the coupling between the molecular dipole and the metal, the latter can radiate a photon before the excitation is dissipated by nonradiative pathways within the dye molecule. This effect increases the relative radiative yield because the factor Z can be as large as $1/Q_0$, where Q_0 is the fluorescence quantum yield of the dye.[17] Obviously, the factor Z does not give a significant enhancement for dyes with high quantum yields. A maximum enhancement of $1/Q_0$ can be obtained only when the fluorophore is at an optimum distance from the metal surface, and the value of Z decreases rapidly as the distance is decreased below this value. The quenching is a result of a very strong nonradiative energy transfer from chromophores to the metal substrates. The radiationless transfer is a short-range effect relative to the enhancement of the local electromagnetic field. As a consequence, a maximum of fluorescence intensity is predicted for dye molecules spaced at some optimal distance from the metal surface.

Langmuir−Blodgett Monolayers. Monolayers of ODA were transferred onto the CMF to form an intermediate spacer layer between the silver particles and the final monolayer containing FDPPE (lipid labeled with fluorescein). The deposition of ODA and mixed FDPPE/DPPE monolayers leads to a red shift and a decrease in intensity of the extinction band of the CMF. The changes are reversible and partially disappear with prolonged exposure of the CMF to chloroform because of the high solubility of the monolayers in this solvent. The red shift is attributed to an increase of the local dielectric constant around the silver particles due to physisorption of the lipids. This effect has been noted previously for colloidal metal films.[20] A decrease in extinction results from the overlap of the absorption band of fluorescein and the plasmon resonances of the silver particles of the CMF. A similar behavior was previously observed when an electronic transition in the adsorbate overlapped with the plasmon resonance of silver island films as a result of the partial dissipation of the radiation energy by the adsorbate layer.[27,28]

A number of aggregated and nonaggregated CMFs coated with three spacer layers of ODA and a final mixed FDPPE/DPPE monolayer at mole ratio 1:3 were examined for fluorescence

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enhancement. The fluorescence measurements were performed on air-dried films as well as films immersed in 10 mM HEPES buffer, pH 7.0. In the case of nonaggregated and slightly aggregated CMFs, the enhancement is considerably less for air-dried films as compared to those in buffer solution. The extinction of these films decreases monotonically from a maximum in the blue spectral region, corresponding to that of isolated silver particles, toward the red region of the optical spectrum. When the film is dried, the entire extinction spectrum undergoes a blue shift and decreases in intensity,[20] thereby reducing the overlap of the surface plasmon resonances of the film with the excitation and emission spectra of the fluorescein labeled lipids. Consequently, the local field enhancement factors at the excitation and emission wavelengths decrease as well. The fluorescence enhancement on these films increases monotonically with increasing extinction of the films as the density of silver particles on the surface becomes greater.

The fluorescence behavior is more complicated for highly aggregated colloidal films. The extinction spectrum of multiparticle aggregates in the CMF immersed in aqueous solution is shifted far to the red[29] and broadened. Thus, the plasmon resonances of the aggregated particles do not couple efficiently with either the absorption or emission bands of fluorescein, as these are in the green region of the optical spectrum. The fluorescence enhancement is no longer monotonically dependent on the density of silver particles. If, on the other hand, the film is air-dried, the plasmon resonances of aggregates shift toward the green region. As a result, the enhancement of fluorescein fluorescence on air-dried, aggregated CMFs is not significantly less than, and sometimes even exceeds, that of wet films.

The fluorescence enhancement was measured as a function of the number of ODA spacer layers. Mixed monolayers with molar ratios of 1:3 and 1:24 (fluorescein-labeled phospholipids/unlabeled phospholipids) were used in the study. Dilution of the fluorescein-labeled phospholipids by a factor of ~6 resulted in a decrease of only about 25% in the fluorescence signal of fluorescein monolayers on a bare glass slide. This indicates that self-quenching occurs in monolayers containing a high concentration of the labeled phospholipids. Because self-quenching lowers the effective quantum yield of the dye, higher enhancement factors can be observed, as discussed above. This was, indeed, found to be the case. Greater enhancement factors (up to 2-fold) were consistently observed for mixed monolayers containing a 1:3 molar ratio of FDPPE/DPPE relative to that obtained in the case of 1:24 molar ratio.

The emission spectra obtained using 488.0-nm excitation from slightly aggregated CMFs overcoated with a different number of ODA spacer layers and a final monolayer containing FDPPE/DPEE in a 1:3 molar ratio are shown in Figure 1A. The spectra were measured immediately after the monolayers were transferred on the films. Surprisingly, the strongest enhancement of both Raman scattering and fluorescence of the fluorescein moiety of FDPPE was observed when the monolayer was transferred directly to the CMF (no spacer layers were present). Because the surface of the colloidal films is hydrophilic, the first monolayer was transferred with headgroups oriented toward the surface. The

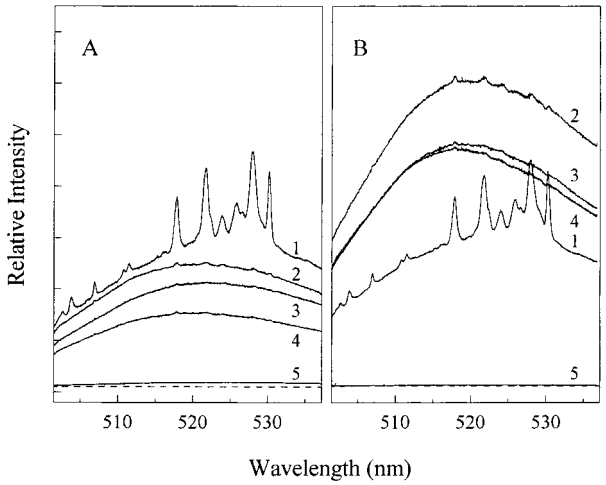

Figure 1. Emission spectra of mixed monolayers of fluorescein-labeled phospholipid (FDPPE)/phospholipid (DPPE) with mole ratio 1:3 for different numbers of octadecanoic acid spacer layers (2.5 nm thick) transferred by the L−B technique to slightly aggregated colloidal films: (1) mixed monolayer directly transferred on a colloidal film; (2) one spacer layer; (3) three spacer layers; (4) five spacer layers; and (5) mixed monolayer with three spacer layers on a bare glass slide. The dashed line represents the instrument background. The spectra were taken with 488.0-nm laser excitation, and the slides were immersed in 10 mM HEPES buffer, pH 7.0, (A) right after the monolayers were transferred on the films and (B) after storage in the HEPES buffer for 4 days. Spectra 1−5 on sections (A) and (B) correspond to the same multilayer structures.

enhancement of Raman scattering is expected to be the greatest for those molecules directly in contact with the silver particles, whereas the fluorescence should be completely quenched. In our previous study, enhancement of Raman scattering was attributed to those chromophores that were directly adsorbed on the Ag aggregates of the CMFs.[21] Enhanced fluorescence from dyes directly deposited on solid substrates with metal roughness features has been reported before.[11,16,20] The fluorescence enhancement was suggested to originate from molecules located in the space between metal particles.[11,20] In this region, the enhancement of the local electromagnetic field is predicted to be the largest and is expected to increase with decreasing distance between the particles.[14,30] The L−B monolayers can bridge the gap between neighboring particles, as shown in Figure 2A, thus positioning labeled phospholipids between the silver particles. The intensity of Raman signal dropped dramatically after deposition of one spacer layer, whereas the fluorescence enhancement decreased monotonically from ~30-fold to ~15-fold as the number of spacer layers was increased from zero to five.

The colloidal films used in the measurements shown in Figure 1A were stored in 10 mM HEPES buffer, pH 7.0, for 4 days, and their emission spectra were again recorded (Figure 1B). The spectra in Figure 1 are displayed on the same scale, so the relative intensities can be directly compared. It is striking that the absolute intensities of the fluorescence emission increased ~2 times for the CMF with one and three spacer layers of ODA and ~3 times in the case of five spacer layers. This is especially surprising as emission spectra characteristic of fluorescein were detected from all of the HEPES buffer solutions in which the slides

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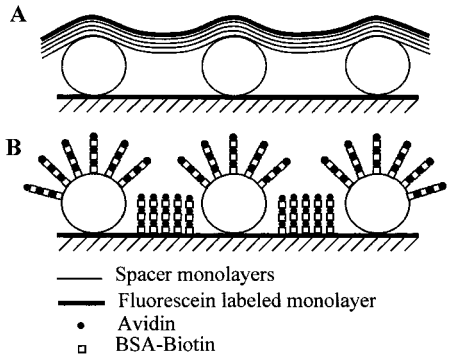

Figure 2. Schematic of the structure of (A) L−B monolayers and (B) BSA−biotin/avidin multilayers on the surface of colloidal metal films.

were stored, including the reference slide, indicating desorption of the fluorescein-labeled phospholipids from the substrates. In fact, the emission spectrum from the bare glass slide decreased almost 4-fold in intensity and is barely discernible in Figure 1B. Based on this decline in the fluorescence intensity, it could be assumed that roughly three-fourths of the monolayer with labeled phospholipids was desorbed during the 4 days of storage. The surface area of a headgroup of a phospholipid is about 40 Å^2, while the surface of the slide is 5 cm^2. Thus, there are 2×10^{-9} mol of phospholipid molecules on one slide. After taking into account the 1:3 molar ratio of labeled/unlabeled phospholipids, the amount of desorbed fluorescein-labeled molecules is $\sim 4 \times 10^{-10}$ mol. The L−B monolayers deposited on the CMFs may be even less stable because they are not in direct contact with the surface in the space between neighboring silver particles (see Figure 2B). A fluorescence enhancement of up to 200-fold was calculated from the data presented in Figure 1B.

No changes in fluorescence intensity were observed within a few hours (1−3 h) after deposition of L−B monolayers on CMFs. The data in Figure 1 represent the characteristic increase in the fluorescence emission which was detected in many other experiments after CMFs with fluorescein-labeled phospholipids were stored in a buffer solution for few days (1−4 days). Although an increase was observed for all CMFs we monitored over a period of time, the time course of the spectral changes was not reproducible from one colloidal film to another. It is important to note that no changes were detected in UV−visible spectra of the CMFs over the same period of time. UV−visible spectroscopy is very sensitive to the morphology of colloidal films (aggregation state, surface density of silver particles, etc.). Consequently, the observed changes in fluorescence intensity can be fully accounted for by the structural changes within the L−B monolayers deposited on the surface of CMFs. This observation cannot be explained by migration of the fluorescein-labeled phospholipids from one layer to another within the multilayers, because in this case the intensity of the emission spectrum would lie within the range of intensities of the spectra displayed in Figure 1A. Neither can the observed increase in fluorescence emission be accounted for by a decrease in the mean distance between the fluorophores due to possible movement of the labeled phospholipids about the surface. When the dye molecules are in closer proximity to one another, the intensity of fluorescence emission declines as a result of self-quenching in the monolayers. To account for the observed increase in absolute intensity of fluorescence signal with time, it is proposed that desorption of part of the lipids results in the loss of integrity of the multilayer structures and its subsequent collapse within the space between closely spaced silver particles of the CMF. As noted above, the local electromagnetic field is expected to be strongest in this region, and dye molecules located therein should experience the strongest fluorescence enhancement.

From the above results, it can be concluded that a quantitative relationship between the fluorescence enhancement and the distance of a chromophore from the CMF surface cannot be determined reliably from lipid multilayers prepared by the Langmuir−Blodgett technique. Desorption of the lipids with time leads to instability of the multilayers and uncertainties as to the concentration of the dye molecules and their exact location within the cavities between the metal particles. A superior model system should provide the capability of monitoring the fluorescence intensity as a function of the elevation of the labeled reporter molecules relative to the surface of the CMF, as well as within the region between silver particles, as shown in Figure 2B.

BSA−Biotin/Avidin Multilayers. It was recently shown that uniform multilayers of macromolecules can be formed on a solid substrate by adsorbing alternate layers of avidin and a biotin-labeled globular protein.[31] This approach was chosen to monotonically increase the spacing of the fluorescein-labeled molecules from the surface of colloidal films (see Figure 2B). A BSA−biotin conjugate and avidin were used to form the intermediate spacer layers, and FITC-labeled biotin was captured by the final layer of avidin.

Conjugates of BSA−biotin with colloidal gold particles (BSA−biotin/gold conjugates) were used to monitor the distribution of avidin on the surface of CMF by employing electron microscopy as the method of detection. Avidin is known to bind nonspecifically to a variety of surfaces. For example, it was shown that avidin forms a uniform dense monolayer on surfaces of platinum, gold, and silver.[31,32] However, a completely different behavior was observed in the case of the CMF. Simple physisorption of avidin molecules on the surface of colloidal films did not result in a uniform dense monolayer of the protein (Figure 3, top left). In fact, no significant difference was noted in the interaction of BSA−biotin/gold conjugates with the CMF that had been exposed to a solution of avidin as compared to control films that had not (Figure 3, top right). The same result was observed when physisorption of avidin was attempted using 0.1 M PBS, pH 7.5, or 20 mM CAPS, pH 10.0, buffer solutions. However, these findings were in sharp contrast to the results obtained when BSA−biotin conjugates were first deposited on the CMF, followed by exposure of the film to an avidin solution. As can be seen in Figure 3, bottom left a highly dense and fairly uniform layer of avidin molecules is formed. The same density was observed for multilayers consisting of two, four, and six layers of alternating BSA−biotin and avidin monolayers, with an avidin layer adsorbed on the top. No adsorption of the conjugates occurred after albumin molecules were deposited on top of the multilayer structure (Figure 3, bottom right). These results provide evidence for the formation of the multilayer structures on CMF.

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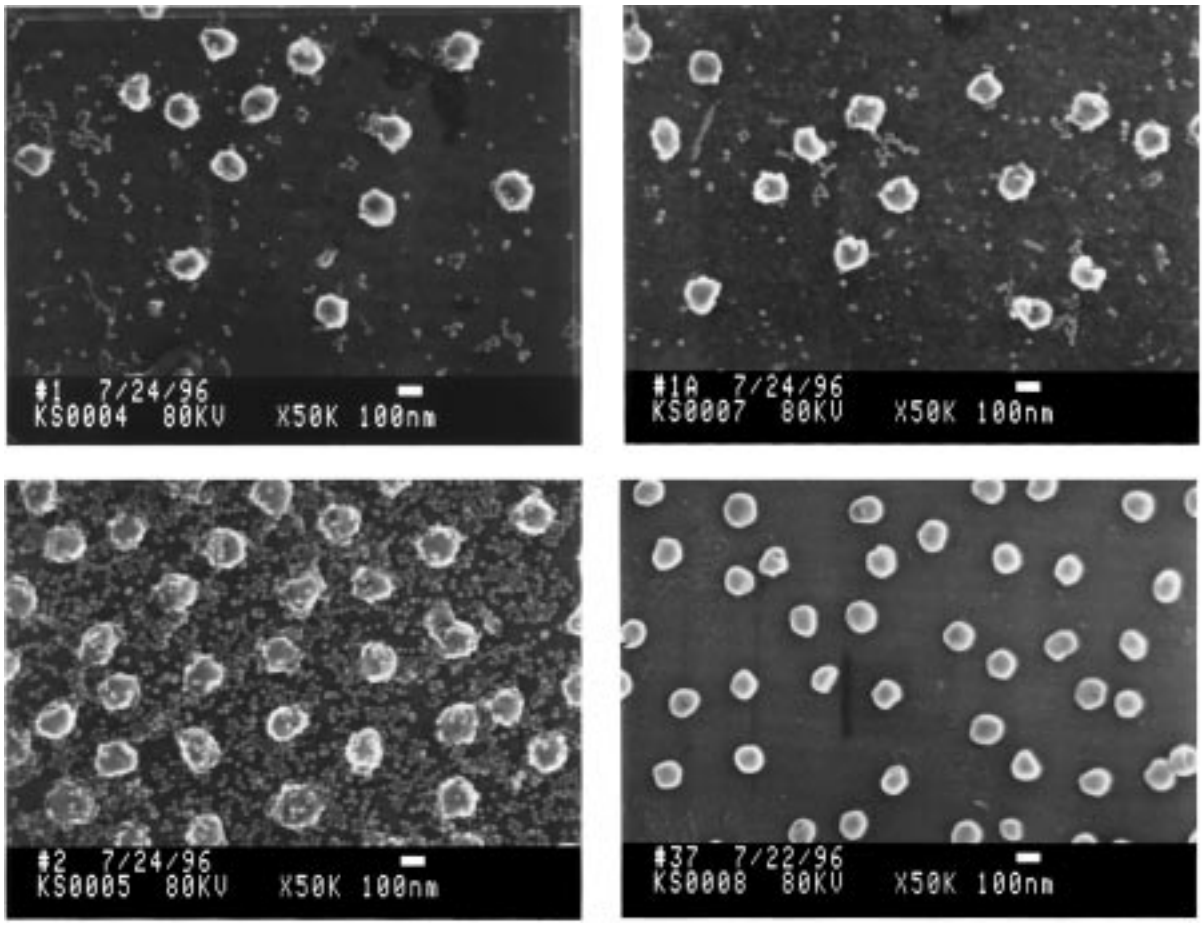

Figure 3. Scanning electron micrographs of colloidal metal films with (top left) a layer of avidin molecules directly physisorbed on the surface of the film; (top right) no protein layer; (bottom left) BSA−biotin/avidin multilayers with the first layer formed by BSA−biotin and an avidin layer on the top of the structure; (bottom right) the multilayers with BSA−biotin on the top after exposure to BSA−biotin/gold conjugates. The large spherical features on the micrographs correspond to Ag particles on the surface of the films, and the small ones are gold conjugates.

X-ray diffraction data show that albumin is a triangular molecule with equilateral 80-Å sides and a 29-Å average thickness.[33] It was shown that, on a solid substrate, BSA molecules tend to lie with their short axes perpendicular to the surface.[34] On most surfaces, the amount of albumin that occupies a monolayer is near 0.15 μg, or 2.2 pmol/cm^2.[35] On the MPS-derivatized surface, we estimated that ~2.2 pmol of biotin−FITC molecules were captured by the first layer of avidin deposited on top of the BSA−biotin layer directly bound to the surface. It is reasonable to propose that, of the four biotin-binding sites per each avidin molecule, two were involved in the interaction with the BSA−biotin layer, and the other two were available for binding of biotin−FITC. Assuming a 1:1 molar ratio between BSA−biotin and avidin, the amount of albumin molecules adsorbed on the MPS-derivatized surface was calculated to be 1.1 pmol, or half of a monolayer.

Both nonaggregated colloidal metal films and reference glass slides containing the same amount of BSA−biotin/avidin multilayers were exposed to a solution of biotin−FITC conjugates. Glass slides with an MPS layer, but lacking colloidal particles, were used as a reference. Only slight variations were observed in the amount of biotin−FITC captured by a given layer (the first, second, and third layers) of avidin. The corresponding emission spectra of the biotin−FITC molecules obtained from the multilayers deposited on colloidal metal films and a reference glass slide are shown in Figure 4. In contrast to the results obtained for L−B monolayers, the enhancement increases with the number of spacer layers. Apparently, the molecules adsorbed on top of colloidal metal particles do not play the determining role in the enhancement of fluorescence. If this were the case, a decrease in the fluorescence intensity with an increase in the thickness of the spacer layer would be observed as in the case of L−B monolayers (compare schematics A and B in Figure 2). Thus, the fluorescein-labeled molecules located within the space between the silver particles (the cavities) determine the enhancement of fluorescence on CMFs. The fact that the enhancement becomes greater with the number of protein spacer layers is consistent with the fact that the electromagnetic field is strongest in the region where the interparticle distance is the smallest. The region of the strongest electromagnetic field is lifted from the glass substrate by an amount equal to the radius of the metal particle. Adding more spacer layers also lifts the flurophore molecule closer to this region, resulting in stronger flourescence intensity. It should be noted that no enhancement (fluorescence or SERRS) was obtained for biotin−FITC molecules directly physisorbed on the

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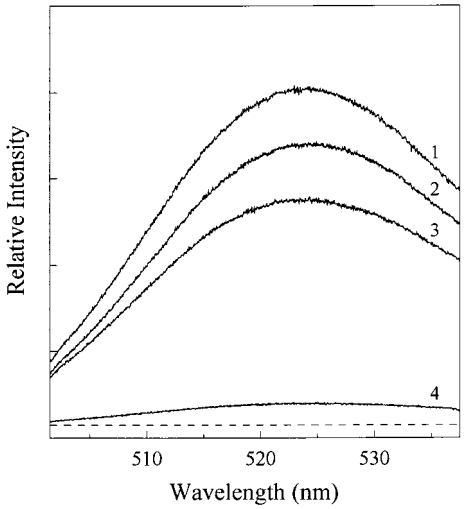

Figure 4. Emission spectra of biotin−FITC molecules captured by avidin on top of (1) six, (2) four, and (3) two monolayers formed by alternating layers of BSA−biotin and avidin on the surface of nonaggregated colloidal metal films. Curve 4 shows the characteristic emission spectrum obtained from the multilayers adsorbed on bare glass slides. The dashed line is the instrument background.

surface of colloidal films (i.e., no spacer layers). This result is consistent with our former observation that no SERS spectra were detected from the underlying MPS layer of the CMF.[20] Under these conditions, the chromophores are not within the interparticle cavities. An 8-fold fluorescence enhancement occurred when avidin molecules were directly deposited on the CMF (one spacer layer), and it increased slowly to ~12−15 times when the number of spacer layers was increased to six. These results indicate that the local field enhancement within the space between the silver particles of CMF decays very sharply outside the cavity but is rather uniform within this region.

The experiments with both the L−B monolayers and the BSA−biotin/avidin multilayers demonstrate that the highest enhancement of fluorescence occurs for the fluorescein molecules that are located within the space between the metal particles of the CMF.

Immunoglobulins. The stability and reproducibility of colloidal metal films are two important properties with respect to their potential applications in analytical chemistry. From this perspective, the most crucial functions of the spacer layer around the silver particles of CMF are protection of the particles from undesirable reactions, such as oxidation or etching, for example, by halide ions, etc., and the prevention of possible degradation reactions of organic or biological molecules on the metal surface. Physisorbed proteins or self-assembled monolayers of organothiols do not provide a sufficiently rigid or chemically stable layer for protection of the silver particles from degradation in 0.14 M solutions of chloride ions, the common constituent of all physiological media. It was recently shown that a rigid silica layer can be formed around particles of iron.[36] The silica layer effectively protected the material from oxidation. We employed slightly modified reaction conditions to coat the silver particles of the colloidal metal film with silica. As can be seen from the electron micrographs displayed in Figure 5, a uniform silica layer was

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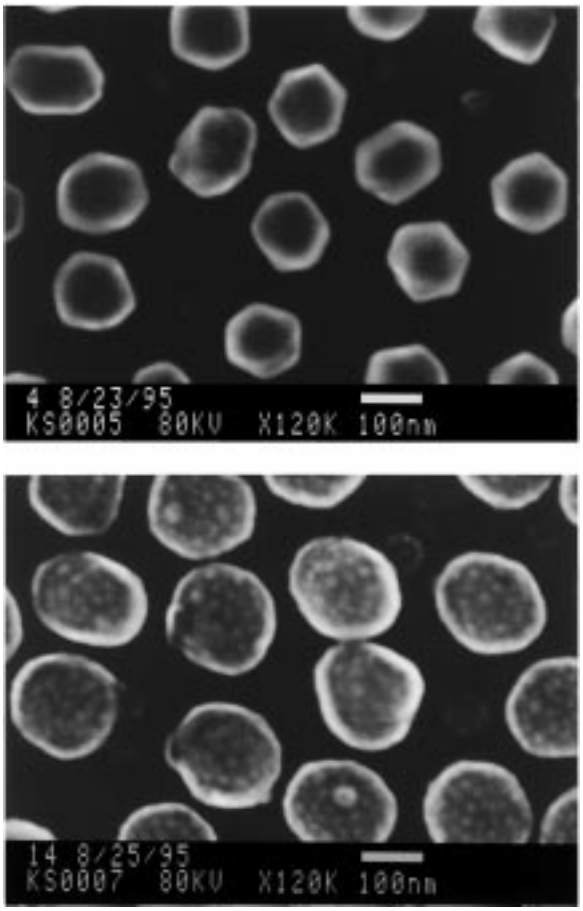

Figure 5. Scanning electron micrographs of colloidal films with (top) unprotected Ag particles and (bottom) Ag particles covered by a silica layer.

formed around silver particles. Similar results were obtained for colloidal suspensions of the particles (not shown). The thickness of the layer can be readily controlled by changing the concentration of TEOS in the reaction mixture. The stability of the silica-coated particles was substantially improved. No degradation of the particles was detected after storage of the films in 0.14 M chloride solution for weeks. Analysis of the silica-coated films by scanning force microscopy revealed that the silica layer was formed only around the silver particles and not on the surface between them.[37]

The silica-coated CMFs were tested for enhancement of fluorescence by physisorbing goat immunoglobulins on the surface and exposing the films to a solution of rabbit anti-goat immunoglobulin−NBD conjugates in 10 mM PBS, pH 7.5, and 0.14 M NaCl. Following a washing step, the emission spectra were measured using 488.0-nm excitation. NBD was used as the label because it has a lower quantum yield than fluorescein and its excitation and emission spectra overlap well with the plasmon resonances of colloidal films. An enhancement factor of ~20-fold was obtained in these experiments.

CONCLUSIONS

To the best of our knowledge, this study presents the first example of the use of surface-enhanced fluorescence on a

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roughened metal surface for improving the sensitivity in the detection of biospecific interactions. This approach should provide a new route for the development of sensitive bioanalytical procedures that will not require intermediate processing steps. Further optimization of the fluorescence enhancement on CMFs is currently in progress. The surface density and size of the colloidal metal particles, the spectral characteristics of the label, the polarization and geometry of excitation, and the immobilization procedures, as well as the format and configuration of tests, are the main parameters that must be optimized.

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