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Metal-enhanced fluorescence of tryptophan residues in proteins: Application toward label-free bioassays

Henryk Szmacinski *, Krishanu Ray, Joseph R. Lakowicz

Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland Baltimore, Baltimore, MD 21201, USA

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ABSTRACT

The detection of submonolayers of proteins based on native fluorescence is a potentially valuable approach for label-free detection. We have examined the possibility of using silver nanostructures to increase the emission of tryptophan residues in proteins. Fluorescence spectra, intensities, and lifetimes of multilayers and submonolayers of proteins deposited on the surfaces of silver island films were measured. Increased fluorescence intensities from two- to three-fold and similar decreases in lifetimes were observed in the presence of the silver nanoparticles compared with the proteins on the surface of the bare quartz. The observed spectral effects of silver nanoparticles on tryptophan fluorescence indicates the possibility for the design of analytical tools for the detection of proteins without traditional labeling by extrinsic fluorophores.

Fluorescence detection is a central technology in clinical chemistry, drug discovery, proteomics, genomics, and cell biology. Almost without exception, detection is accomplished using extrinsic fluorophores that are used to label the biomolecules. The need for labeling of the biomolecules with extrinsic fluorophores results in increased costs and complexity. Because of this added complexity, there is a rapidly growing interest in methods that provide label-free detection. A wide variety of surface-based methods are being tested for label-free detection of binding. These methods include surface plasmon resonance (SPR)[1] [1,2], surface-enhanced Raman scattering [3,4], electrochemical approaches [5,6], nanowires [7], optical microcavities [8], optical transmission [9,10], reflectivity [11], interferometry [12], and photonic crystals [13]. The most widely used label-free detection method is SPR, which is being extended to high-throughput capabilities by the use of imaging modality [14–16]. The sensing mechanism of SPR is based on high-sensitivity of reflected light at a specific SPR angle. The system uses a thin continuous metal film ($\sim$ 40–50 nm) of gold or silver to which molecules bind or dissociate, causing detectable changes in the refractive index in the interface between metallic film and bulk solution. The quantitative interpretation of SPR signal to adsorbed biomolecules and estimated detection sensitivity of approximately 1 to 10 pg/mm^2 have been described elsewhere [17,18]. Another approach for construction of label-free detection has exploited the localized surface plasmon resonance (LSPR) of metallic nanostructures. Transmission surface plasmon resonance (T-SPR) was introduced for measurement changes in extinction peak of plasmon spectra of discontinuous gold films in the visible and near-infrared wavelength range [19]. Wavelength shifts of extinction spectra of metallic nanoparticles on dielectric substrates and nanoholes in gold film have also been demonstrated for optical biosensors [20,21].

In this report, we describe the use of intrinsic fluorescence of proteins for detection of binding reactions on surfaces. Proteins possess three intrinsic fluorophores: phenylalanine, tyrosine, and tryptophan. The emission from proteins is dominated by tryptophan because of its longer excitation and emission wavelengths, good quantum yield, and fluorescence resonance energy transfer (FRET) from tyrosine to the tryptophan residues [22]. On average, tryptophan is present at 1.3% of the amino acid residues, resulting in many proteins containing multiple tryptophan residues. For example, a typical protein with a molecular weight of 50 kDa will contain approximately 400 amino acid residues and, thus, an average of 5.2 tryptophan residues. The abundance of tryptophan residues in proteins is both an advantage and a disadvantage for fluorescence label-free detection. The abundance is an advantage because most target proteins will contain this intrinsic label and is a disadvantage because all of the other proteins in the sample will also contain tryptophan and be fluorescent. For this reason, tryptophan fluorescence has not been used in analytical applications. However, there have been several recent reports on using ultraviolet (UV) fluorescence for detection and quantification of protein interactions [23–27]. Relatively high detection sensitivities

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* Corresponding author. Fax: +1 410 706 8408.
E-mail address: henry@cfs.umbi.umd.edu (H. Szmacinski).

[1] *Abbreviations used:* SPR, surface plasmon resonance; LSPR, localized surface plasmon resonance; T-SPR, transmission surface plasmon resonance; FRET, fluorescence resonance energy transfer; UV, ultraviolet; MEF, metal-enhanced fluorescence; SIF, silver island film; BSA-Bt, biotinylated bovine serum albumin; IgG, immunoglobulin; CCD, charge-coupled device; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin.

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were reported using frequency tripled output of Ti:sapphire lasers, 1 to 5 ng per spot on two-dimensional gel electrophoresis for several proteins [23], and better than 10 pM of antibody BP53-12 bound to immobilized p53 on nitrocellulose membrane [26].

We believe that recent progress in metal-enhanced fluorescence (MEF) can be used for high-sensitivity detection of target proteins in samples that contain other tryptophan-containing proteins. The MEF approach is based on short-range interactions of fluorophores with metallic nanostructures that, depending on the metal geometry, occur at distances from 5 to 100 nm. The emission can be modified by the presence of a nearby metal nanostructured surface. The metal nanostructure can result in more rapid emission of the fluorophore or may change the normally isotropic emission into directional emission. For fluorophores, which absorb and emit at visible wavelengths, it has been shown in many experiments that proximity to silver and/or gold particles results in increases of intensity, radiative decay rate, and photostability [28,29]. Observations of MEF due to silver and aluminum nanostructures have been also reported for the UV wavelength range [30–34].

Materials and methods

Preparation of silver island films and surface deposition of proteins

The wet chemical deposition method was used to coat the quartz substrate with the silver island films (SIFs). The procedure of deposition of SIFs on glass and quartz substrates has been described elsewhere [29,35]. The wet chemical deposition technique results in variability of the particle sizes and shapes, as has been shown previously using atomic force microscopy with particle sizes up to 500 nm and thicknesses of 50 to 100 nm [29]. The absorption spectrum maximum of the fabricated SIFs is near 450 nm with an optical density of approximately 1.15, indicating that the particles were of subwavelength size. We used high-density silver islands to limit the void areas between particles where the proteins can also adsorb and tryptophan residues would not effectively interact with particle plasmon resonance.

The proteins used were biotinylated bovine serum albumin (BSA-Bt) from Sigma–Aldrich (St. Louis, MO, USA), avidin from Pierce Biotechnology (Rockford, IL, USA), and immunoglobulins (IgG_3 and anti-IgG_3) from Southern Biotech (Birmingham, AL, USA). For deposition of proteins on the SIFs and bare quartz substrates, we used direct deposition of proteins by spotting from aqueous solution and by noncovalent electrostatic immobilization. Because the fluorescence of tryptophan molecule is relatively weak in comparison with organic fluorophores, due mostly to its low extinction coefficient (5500 M^{-1} cm^{-1} at 280 nm) and UV spectral range where autofluorescence can usually be high, we performed initial experiments using avidin. This protein contains 16 tryptophan residues and is relatively bright compared with other proteins. First, avidin was deposited through spotting a water solution on the SIF surface that allowed an increase in protein concentration on the surface and facilitated easy measurements of tryptophan fluorescence. Second, a layer-by-layer method was used to deposit layers of BSA-Bt and avidin. The initial BSA-Bt layer was electrostatically immobilized on the SIFs and quartz through incubation of a solution of 20 μg/ml in phosphate buffer at pH 7.4 for 1 h. After washing out unbound BSA-Bt, the avidin solution of 10 μg/ml was incubated for 1 h, creating one layer of BSA-Bt–avidin. The procedure was repeated to construct up to four BSA-Bt–avidin layers [$(\text{BSA-Bt–avidin})_n$, $n = 1, 2, 3, \text{or } 4$]. Finally, a model immunoassay was performed with a monolayer of BSA-Bt adsorbed on the substrate with SIFs (capture bioactive layer) and various concentrations of avidin (binding analyte) demonstrating detection of submonolayer of avidin. In addition, an antibody system using IgG_3 and anti-IgG_3 was evaluated in the presence of blocking solution. Schematics of experimental configurations are shown in Fig. 1.

Spectroscopic measurements

The extinction spectra of SIFs were measured using a single beam spectrophotometer (Hewlett–Packard model 8453). The emission spectra of avidin were measured using Varian Cary Eclipse fluorometer with excitation of 280 nm.

Fluorescence lifetimes were measured using a frequency domain fluorometer (K2, ISS, Champaign, IL, USA). The excitation source was frequency-doubled output from rhodamine 6G dye laser pumped with mode-locked argon ion laser. The excitation was 280 nm, and the emission observed through the bandpass filter was 320 to 360 nm. We are aware that the tryptophan fluorescence properties, quantum yield, and lifetime depend strongly on the environment [36]; thus, experiments were performed at identical conditions for samples on bare quartz and SIFs.

The intensity decays were analyzed using the multiexponential model

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i), \tag{1}$$

where α_i is the amplitude ($\sum_i \alpha_i = 1.0$) and τ_i is the decay time. The intensity decay parameters (α_i and τ_i) were determined using nonlinear least-squares fitting. Two average lifetimes were calculated, amplitude ($\langle\tau\rangle$) and intensity (τ_M), weighted for comparison between values on bare quartz and SIFs:

$$\langle\tau\rangle = \sum_{i=1}^{n} \alpha_i \tau_i, \quad \tau_M = \sum_{i=1}^{n} f_i \tau_i, \tag{2}$$

where the fractional contribution of each component (f_i) to the steady-state intensity is defined as

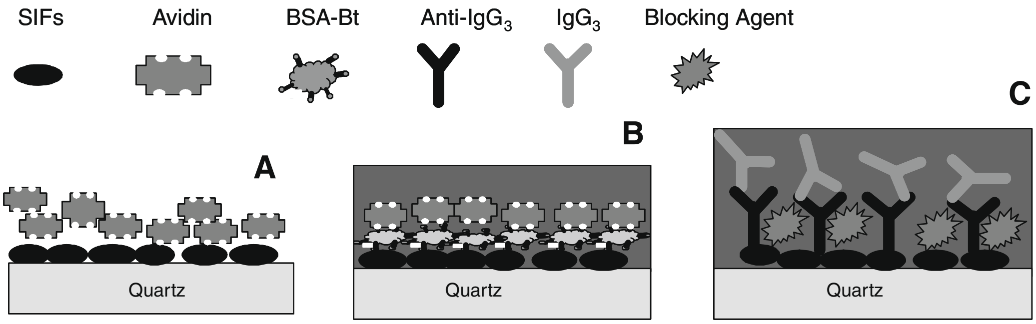

Fig. 1. Schematics of experimental configurations of protein layers on SIFs: (A) avidin deposited from aqueous solution; (B) binding of avidin to the BSA-Bt functionalized surface; (C) binding of anti-IgG_3 to functionalized surface with capture IgG_3 and blocked with blocking agent.