ABSTRACT

A novel optical biosensor matrix has been developed to exploit the native fluorescence of certain proteins. This matrix uses a gold colloid monolayer attached to an end of a fiber as a substrate for protein attachment. The effect of the gold monolayer size has been investigated through the techniques of fluorescence, scanning electron microscopy, and transmission electron microscopy. It has been shown that the size of the gold colloid does produce a marked difference in the fluorescence intensity measured. It is surmised through the use of microscopy techniques that the intensity changes seen in the fluorescence emission are not a result of surface coverage, or availability of sites for protein adsorption, but instead of quenching or enhancement by the gold itself.

Keywords: optodes, gold colloids, biosensor, sensor

1. INTRODUCTION

Sensors developed to measure concentrations of ions and small molecules are of interest for direct analysis outside and inside single cells. Optical sensors have proven invaluable for making these measurements, since it is easy to produce fiber optic tips small enough to be relatively non-invasive even in a cell. Many of these optical sensors make use of an ion-selective molecule trapped inside a polymer matrix. Even though there are many proteins which are also sensitive to ions or small molecules, it is difficult to immobilize them in the same manner as molecular probes. In order to exploit the native fluorescence of certain proteins, gold colloid based fiber optic biosensors have been developed as novel sensor substrates. Sensors of this type have many advantages over previous methods of protein immobilization. For instance, acrylamide matrices tend to photobleach fluorescent proteins during the polymerization process and catalysts necessary for polymerization can denature delicate proteins. Some proteins have been successfully immobilized in this type of matrix, but these proteins (such as Glucose Oxidase) are particularly rugged [1-4]. Sol gel matrices have also been used [5,6], but have proven to be hampered by long response times, due to the amount of time it takes for diffusion through the glassy matrix. The gold colloid substrate, though, does not denature the protein, and, in most cases, does not affect the activity of the protein [7]. There also is no polymer matrix surrounding the sensor, so there is no diffusion to slow the response time of the sensor. Most importantly, perhaps, is the suggestion that the plasmon resonance of the gold colloid can actually enhance the fluorescence of the protein, at certain distances from the gold. This possible enhancement, and the effect of different sizes of gold colloids have been investigated.

The gold colloid substrate design is based on the tailored SERS substrates of Natan [7-14]. These gold colloid monolayers on glass, extensively studied by Natan for Raman enhancement, have been reproduced in this work on the end of an optical fiber. The fluorescent proteins are then easily attached by spontaneous adsorption from solution. The sensors are fairly rugged, since protein attachment takes place through adsorption and/or covalent attachment. The covalent attachment is particularly strong due to cysteine-gold interactions, which take place through a Au-S bond.
In this study, gold colloid based sensors have been studied using three methods: fluorescence, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The information from these three measurements was combined to compare the fluorescence intensity to the gold surface area available for protein attachment. With this knowledge, a possible fluorescence enhancement will be discussed with respect to the size of the gold colloid.

2. EXPERIMENTAL METHODS

Fiber Optic Sensors

Gold Colloid Attachment Sensors were prepared by silanizing a freshly cleaved multimode fiber by immersing the distal end for two hours in 3-(mercatopropyl) trimethoxysilane. The end was then rinsed copiously with methanol, then triply distilled water. The silanized fiber was placed in colloidal gold (used as received) for three hours, then rinsed with water. The sensor can be stored at this point in water or air. For comparison of sizes of gold colloids, six different gold sizes were used: 5, 10, 20, 50, 100 and 250 nm. The intensity of the signal was ratioed to the intensity of the laser light, to account for differences in coupling efficiency.

Sensor Preparation (R-phycoerythrin) Protein solution was prepared by dissolving 0.1% R-phycoerythrin (Molecular Probes, Eugene, OR) in phosphate buffer, pH 6.0. The fiber was then immersed in the protein solution for one hour, rinsed with buffer, then used.

Sensor Preparation (Bovine Serum Albumin/Texas Red) Protein solution was prepared by dissolving 0.1% bovine serum albumin (BSA) in phosphate buffer, pH 6.0. The fiber was then immersed in the protein solution for one hour, rinsed with buffer, then used. After BSA attachment, the fiber tip was placed in Texas Red-X (Molecular Probes, Eugene, OR) solution for one hour. The dye solution was prepared by dissolving 1 mg Texas Red-X in dimethyl sulfoxide (DMSO) then adding 1 M sodium bicarbonate solution (pH 8.3). The sensor was rinsed with water before use.

Optics The complete optical path for the fiber optic sensors included: Ion Laser Technology (Salt Lake City, UT) argon ion laser; 514.5 nm laser band-pass filter (Newport Corp. Irvine, CA); Uniblitz shutter controller (Rochester, NY); fiber coupler (Newport Corp. Irvine, CA); Olympus inverted fluorescence microscope, IMT-II (Lake Success, NY); Nikon 50 mm f/1.8 camera lenses; Acton 150 mm spectrograph (Acton, MA); and, a Princeton Instruments 1024x256 LN2 cooled CCD array (Trenton, NJ).

Glass Macro-Sensors

Gold Colloid Attachment Macro-sensors were prepared on glass microscope slides cleaned by immersion in piranha solution (10:1 sulfuric acid :hydrogen peroxide), copious rinsing in triply distilled water and oven drying. The glass was then silanized in a 10% solution of 3-(mercatopropyl) trimethoxysilane in methanol for 24 hours. The glass was rinsed with methanol, then triply distilled water. The silanized glass was placed in colloidal gold (used as received) for 24 hours.
then rinsed with water. For comparison of sizes of gold colloids, six different gold sizes were used: 5, 10, 20, 50, 100 and 250 nm.

Sensor Preparation (Bovine Serum Albumin/Texas Red) Protein attachment and dye labeling was performed as described above with the fiber optic sensors.

Scanning Electron Microscopy (SEM) SEM samples were prepared by breaking off the tips of the sensors and mounting them on Hitachi SEM stubs with graphite conductive adhesive. The mounted tips were then sputtered with 10 nm of gold and examined in a Hitachi SEM operated at 30kV.

Transmission Electron Microscopy (TEM) Specialty TEM grids were used that consisted of a nickel substrate, formvar layer, and a SiO₂ coating. These grids were used as received. Grid preparation included floating the grid on 3-(mercaptopropyl) trimethoxysilane for two hours, rinsing with methanol, then water. The grids were then floated on a drop of gold colloid solution for three hours. The grids were rinsed with water and dried before bright-field examination in a JEOL 2000FX TEM operated at 200kV.

Pulled Multi Mode Sensors Sub-micron sized fiber optic sensors were prepared by pulling multimode fiber in a home-built puller consisting of a modified pipet-puller heated with a CO₂ laser [15]. The pulled tips were then silanized, coated with aluminum in a home-built evaporator to a thickness of 1500 Å and prepared with gold colloid and protein as above.

3. RESULTS AND DISCUSSION

A dye can be attached to the end of a fiber through a non-fluorescent protein (such as bovine serum albumin) and used as an indicator of the hydrophobicity of differing regions of a cell. All that has to be done is to use a dye which can easily be attached to a protein, and which is sensitive to changes in environment. Attaching dyes to proteins can also be a useful method for measuring analytes. For example, in recent work [17,18] the dye Oregon Green was attached to cytochrome c' or a heme domain of SGC and used to measure NO via an effect of the “spectator dye”. This dye attachment provided a stronger fluorescence signal than the native protein fluorescence and thus better sensor sensitivity.

The effects of changing the size of the gold colloid attached to the surface of the optical fiber have been investigated with a protein representative of two uses of this substrate, namely a natively fluorescent protein and a dye-labeled protein. A natively fluorescent protein, R-phycoerythrin, and a non-fluorescent protein, bovine serum albumin, with a dye label, Texas Red, have been used in these studies due to their strongly fluorescent nature. Gold colloid sizes ranging from very small, 5 nm, to very large, 250 nm, have been immobilized on the end of a fiber to provide a base for protein attachment.

Even though there is only a monolayer of fluorescent material on the surface of the optical fiber, a strong signal was measured. Figures 2 and 3 display representative spectra of both the R-phycoerythrin and the Texas red labeled bovine serum albumin, immobilized onto the end of an optical fiber. Controls consisting of silanized fibers without gold were studied, and fluorescence due to non-specific binding was not observed.
Figure 2: Spectra of gold colloid based sensor prepared on a cleaved optical fiber. BSA labeled with Texas Red has been attached to the gold colloids.

Figure 3: Spectra of gold colloid based sensor prepared on a cleaved optical fiber. Natively fluorescent R-phycoerythrin is attached to the gold colloids.
As can be seen in Figure 4, the size of the gold colloid used in the sensor development plays a role in the amount of fluorescence signal obtained. With only slight variations, both the natively fluorescent protein, R-phycoerythrin, and the Texas Red labeled protein, bovine serum albumin, produced similar responses.

**Figure 4:** Graph of fluorescence intensity vs. size of gold colloid used. R-phycoerythrin is attached to the gold colloid based sensor, and each fluorescence intensity is normalized to the power of the laser coupled into the optical fiber, as measured with a power meter.

**Figure 5:** Graph of fluorescence intensity vs. size of gold colloid used. BSA labeled with Texas Red is attached to the gold colloid based sensor, and each fluorescence intensity is normalized to the power of the laser coupled into the optical fiber, as measured with a power meter.
Since it seemed that the size of the gold colloid used for the monolayer played a significant role in the response of the sensor, the cause of this effect was investigated further. The gold size could have effected the intensity of the signal in a couple of ways. First, it is possible that each size of gold colloid forms a unique monolayer, with gold coverage varying significantly. This would then allow differing amounts of surface area for protein attachment. Another option is that the gold colloid either enhances or quenches the protein fluorescence, depending on the size of the gold that is used. Fluorescence quenching/enhancement has been noted before [19] and it was found that distance from the gold plays a role in enhancing or quenching the fluorescence. In our case, though, the distance from the gold surface does not vary, just the size of the gold colloids used.

To answer these questions, TEM (Figure 6) and SEM (Figure 7) analysis was performed. By thoroughly characterizing the surface of the sensor, it was possible to calculate the gold colloid coverage per $\mu m^2$, and then the surface area of the gold colloid. These two pieces of information could then be combined to get a total possible surface area for protein attachment, with the assumption that all of the gold is available for protein binding. Table 1 shows the results of these calculations. The surface coverage is a count of the number of gold particles per $\mu m^2$, and the gold surface area is the surface area of a gold colloid (estimated as the surface area of a sphere of the same diameter as the colloid) multiplied by the number of gold particles per $\mu m^2$. As can be seen, the gold surface area is much less for large colloid sizes, but the fluorescence intensity is greater. This indicates that surface area, and thus greater protein attachment, is not the driving force in the intensity increase that is seen in the fluorescence measurements. Thus the sensitivity of a sensor can be maximized, by changing the size of the gold used.

![Figure 6: SEM image of 100 nm gold colloids immobilized onto a cleaved optical fiber.](image-url)
Table 1: Correlation of gold colloid size with sensor coverage and fluorescence intensity. Surface coverage was counted by griding off 5 random sections of the electron micrograph for each size and averaging the number of gold particles attached to the surface. Gold surface area takes into account the size of the colloids, assuming that each colloid is fully able to bind proteins. The total surface area combines these two measurements.

In conclusion, the gold colloid substrate provides a convenient method for attachment of proteins to be used for sensors. The size of the gold colloid affects the fluorescence intensity of the attached protein, but this is not explained by the surface area available for attachment alone, since fewer of the larger colloids attach to the surface, rather the fluorescence intensity tends to be greatest from the larger sized colloids. Further work should be done to better elucidate the reasons for the size effects, such as cleaning the gold surface before attaching the proteins (to rule out competitive binding of stabilizers) or attaching tethers in order to change the protein distance from the surface. Regardless, the gold colloid monolayer has proven effective [16], not only in these studies, but in the development of several nitric oxide sensors [17,18].
4. ACKNOWLEDGMENTS
The authors acknowledge support from the U.S. National Institutes of Health (NIH) GM503000-04.

5. REFERENCES


