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Exploration of Various Techniques for Quantitative Detection of Antibodies in Solution

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ABSTRACT

We have continued our development of a biosensor and are developing new methods of antigen detection. A fluorometer was previously constructed and was shown to have the ability of quantitatively detecting the amount of fluorescence of fluorescent beads purchased from Molecular Probes. This ability thus corresponds to an ability to quantitate the amount of fluorescent beads which are in solution. Difficulties arose in attaching protein to these beads by the method given by molecular probes and this problem was examined using UV-Vis spectroscopy. Amongst other proposed approaches to antigen detection, one most attachment of antibody to gold so that antigen could be detected by bridging the gold and antibody covered beads. An alternative to this which is planned to be examined is to use polymer trays with wells from ELISA kits instead of gold substrate.

INTRODUCTION

Uses for bio-sensor technology as well as a pressing need to develop reliable detection methods for antigens and viruses is growing in the current ages of Biotechnology, Bio-terrorism, and quickly spread virus outbreaks. In addition to these "threat orientated" reasons for wishing to develop biosensors, there are other reasons as well. Techniques that could quickly and specify what bacteria and viruses were in an ill, or even healthy, patient's blood would be of particular use. In response to these needs, the researchers at UM-Rolla have begun developing a bio-sensor to test for the presence of an antigen, with the intent of further development and application in detection of potentially harmful biological agents and viruses.

The work utilizes 4μm diameter, yellow-green, Sulfate-modified FluoSphere beads (Molecular Probes product F-8859). These beads, made of polystyrene and are listed by the manufacture as having a 505/515 excitation/emission. The beads are covered with sulfate functionality groups, allowing them to be coated by way of a chemical synthesis to protein.

This property makes it possible to use the beads as a platform upon which an antibody, (a type of protein), can be placed. That antibody, in this case Monoclonal Anti-Human IgG unconjugated from mouse (Sigma product I 5885), will theoretically readily bind to a corresponding antigen. The antigen chosen was IgG from human serum (Sigma product I 8640). Also available for use are OvA and anti-Ova as less costly alternatives.

When excited by 505 nm light the yellow-green FluoSpheres fluoresce as this report shows we have quantitated, emitting light of a higher wavelength. Since this amount of light fluorescence is measurable using a photosensitive detector, differences in the amount of fluorescence between beads that have been coated with antibody (Anti-Human IgG) and those with out can then be quantitatively measured. A silicon photo detector was used to quantitatively measure the amount of fluorescence emitted from the microsphere beads under a variety of conditions.

Due to considerations of cost and the "proof of concept" nature of the experiment, it is not necessary to use IgG. Rather, Oval albumin (OvA) from chicken and anti-OvA may be used. This allows for increased amounts of protein and lowered cost during the initial experimentation.

EXPERIMENTAL

Metal Substrates

Toward the development of gold (or other metal substrate surface) glass slides were covered with each of the following metals: gold, silver, and copper. Subsequently solution containing OvA was applied to the Au plated substrates in the form of concentrated 500 µl drops and left in open air to dry. What was then planned was to rise the remaining OvA off of these substrates, but after 12 hrs in a fume hood drying, the Au plating had shriveled and peeled away from the glass.

A test to ascertain the source of the shriveling was conducted as both the OvA and the solvent evaporation from the surface were suspected sources. Buffer with no OvA and even cold ice which was applied and tested since the OvA was applied cold, fresh from the refrigerator, had no shriveling affect on the substrate. As with Au, similar results were noted with Ag substrates, but with Cu substrates only loss of color was noted.

Dilution of the OvA applied was able to reduce the shriveling effect and let a layer of Au remain on the surface.

Coating of Fluorescent Beads with Proteins

To make sure that the beads are indeed being coated by the OvA a procedure supplied by Ed Leber of Molecular Probes was used. The technique is simple in principle: add beads to a solution in a UV cuvette cell with a quantitatively known amount of protein and monitor the adsorption of OvA; if the beads are centrifuged or allowed to settle after having opportunity to react with the protein in solution, then they would precipitate with the OvA attached and a subsequent reading should theoretically have a lower absorbance.

To best determine what wavelengths would be best for quantitative detection of the protein in solution, a scan of the OvA protein was run from 200 - 400 nm. A subtle peak was noticed at 273 nm (0.2246 abs) and a series of major peaks at 205, 215, 217, and 221 nm. The tallest, 217 nm experienced an absorbance of 2.3522. These peaks however were due to the plastic material of the disposable cuvette cells absorbing, so the peak at 273 nm was referred to in making a standard curve.

The standard curve thus came from the following data:

All samples were prepared in 50 mM PBS pH 7.40, 0.9 NaCl Buffer.

Sample	Abs	Wavelength of greatest Abs
Blank (Buffer only)	0.0000	---
0.328 M OvA	0.2451	278
0.655 M OvA	0.4999	279
1.31 M OvA	1.03	278

This data is very quantitative and shows that the 0.5 M to 1.0 M range is close to ideal for working with UV detection of OvA presence.

To have greater sensitivity may require referencing a lower absorbance value, however as many tests were conducted at the lower range of absorbencies, except quartz cells were used to eliminate the effect of the plastic cell absorbance. Use of these cells allowed for quantitative absorbencies down as low as 205 nm, and perhaps 200 nm, but for our purposes the slightly weaker Abs signals achieved at 205 nm were enough to reach conclusions while avoiding the noise in the signal experienced at 200 nm and below. All measurements after this will refer to 205 nm readings unless otherwise stated.

Further, the cells used were half cells. It was found that they needed only 400 µl of solution to attain a consistent reading with the Beckman DU-600 UV-Vis spectrometer (this reduced the volume of material which had to be used during tests).

Additionally, the beads were known to show a absorbance in this range, so this necessitated that the amount of absorbance due to the beads for each sample be accounted for.

Determination of the concentration of OvA to use was done by dilution of OvA to various concentrations. 28 µg / ml was found to have an absorbance near 0.6, which is in the ideal range for quantitative measurements of absorbance.

To test for the bonding of OvA to the beads the following procedure was used. Disposable centrifuge cells were filled with a volume of 500 µl each under the following procedure:

Sample A: pH 6.5 Phosphate buffer (Blank)
Sample B: 25 µg / ml OvA in pH 6.5 Phosphate buffer
Sample C: 0.05% solids Sulfate modified fluorescent beads (Blank also used for D, to eliminate the effect of the beads)
Sample D: 25 µg / ml OvA in pH 6.5 Phosphate buffer and 0.05% solids Sulfate modified fluorescent beads

Each of the samples was shaken for several minutes by vortex during which time the protein should attach itself to the beads according to the claims made by molecular probes [attachment would be rapid lasting only a few seconds]. Molecular Probes did note that conformational changes of the protein might last several hours, but this was not of concern to us.

After this initial binding period the samples were centrifuged to separated the supernatant (which unless interaction with beads had occurred should still contain OvA protein at a close to 0.6 Abs or slightly less since these samples are 25 mg/ml rather than 28 µg/ml). A micropipette was then used to decant the top 400 µl to Quartz half cells and UV scans of the range 195 to 220 nm were taken, for which the values at 205 nm are reported below:

	Run 1	Run 2	Blank Used
Sample B:	0.52 Abs	0.55 Abs	(if blanked to A)
Sample C:	0.08 Abs	0.08 Abs	(if blanked to A)
Sample D:	0.68 Abs	0.64 Abs	(if blanked to A)
Sample D:	0.58 Abs	0.57 Abs	(if blanked to C)

These data seemed to indicate that there was little or no interaction between the beads and the OvA protein. Surprisingly the absorbance actually increased in the samples which contained beads as though there must be some residual material in the liquid the beads are supplied in which has absorbance similar to the protein under study. Sample D's absorbance of 0.58 (once the effect of the bead "residue" which sample C measures is subtracted) is still more than the OvA protein alone! So even with the residual effect of the beads removed the effect seems to be opposite of the intended and expected result. It should be noted that without centrifuging the absorbance of this concentration of beads is extremely high and noticeable. Due to this fact and used of an LED light which can be used to activate the beads so they are individually visible to the naked eye, we can be certain that the supernatant contained no whole beads and only residuals from the solution or the surface modifiers on the beads which came off during degradation.

It was thought that perhaps a high enough concentration of beads was not used as Molecular Probes recommended that 0.5 to 1.0 % solids be used. Suspected was that the protein had simply be centrifuged out, but this was not a major issue as given that the absorbance read in sample B was close to what would have been expected at the starting absorbance of ~0.6 Abs at 0.28 µg/ml.

In case the concentration of the beads was not great enough relative to the concentraion of the protein to achieve the desired effect of the beads adsorbing all or most of the protein in solution, a similar test with a much higher concentration of beads as in the following preparation procedure:

Samples prepared in 500 µl centrifuge tubes.

Sample A: 200 µl of pH 6.5 Phosphate buffer
Sample B: 100 µl of buffer, 100 µl of 28 micrograms / ml OvA
Sample C: 100 µl of buffer, 100 µl of 2% Sulfate-modified beads (Note: 2% is the undiluted stock concentration)
Sample D: 100 µl of 28 micrograms / ml OvA, 100 µl of 2% Sulfate-modified beads

All samples subjected to the following treatment: 3 min vortex, 3 min centrifugation, dilution by adding 300 ml buffer to the side of the centrifuge tube, 3 min centrifugation, the upper 400 µl decanted to new centrifuge tubes by use of pipette, centrifuge the new set of centrifuge tubes (A', B', C', D') for 3 min, decant the upper 380 ml to quartz UV half cells, and take UV readings in the 195 - 220 nm range.

The results were as follows for the 205 nm readings:

Sample B' (protein):	0.21 Abs	(blanked to A)
Sapmle C' (beads):	0.74 Abs	(blanked to A)
Sample D' (beads and protein):	1.00 Abs	(blanked to A)
Sample D' (beads and protein):	0.25 Abs	(blanked to C)

Despite the added precautions to remove beads, the effect of the "bead residual" is still very apparent from these measurements. In theory the sample which contains the beads should only have zero absorbance. That it does not is not a problem as the residual from the beads can be blanked out as has been done, but what is very clear is that with the bead residual blanked out the amount of fluorescence left would be expected to be due to protein left in solution. This amount of absorbance left, 0.25 Abs, is very similar to the standard, solution B, which contained no beads and had an even lower absorbance of 0.21! So the effect of the beads in all tests conducted was to increase the absorbance rather than reduce it, which implies that there was no interaction between the beads and the protein.

NOTES

As a side note for application in future research: a complication was experienced when plastic cuvettes made of materials that absorbed under 280 nm were erroneously used. If disposable cuvettes are used, the type which does not absorb until 220 nm should be used.

Another error experience was the incomplete dissolving of the OvA. Even after vigorous shaking and dilutions this was always a problem with the OvA as stringy pieces of material remained in solution.

If concentrations of protein are too high (for which many beads, perhaps even a costly amount, may be needed to have a substantial effect to absorb a measurable about of) what could also be done to reduce the amount of beads needed to be used in each run in addition to use of half cells is use of Bradford dye reagent. This dye could be applied to protein and make small quantities of protein much more easily visible, so less protein would be needed, and hence less beads as well. Of course the main advantage of this would be that the concentration of protein / concentration of beads ratio would be substantially lowered. In fact this was an idea visited with little success in a preliminary experiment, but it could be revisited as the use of centrifugation prior to addition of the dye would be a very helpful revision on the old procedure.

CONCLUSIONS

All of the data produced was indicative of there being no attachment of protein to the beads, which contradicts claims made by molecular probes. It is possible that the beads were expired and no longer able to attach to protein. This is the only logical explanation outside of failure of the protein, the OvA, itself to attach or an unseen error in experimental design (which would have likely been detected from the over 10 attempts and trials of the Molecular Probes procedure).

The first recommendation for future research is thus to purchase more beads from Molecular Probes (or another set of beads from a comparable company). It would still be highly recommended that Sulfate modified beads be used as these beads provide the simplest method for attachment of protein to the beads, however a different size - perhaps smaller - might prove better for the experiment. In fact, in retrospect it has been realized that for the original plan which incorporated the beads in solution to have antigen detection by way of changing the fluorophore environment would have been difficult to achieve since the beads were large and the fluorescent dye was well inside the beads. If such a method were to have a good chance at working, this method should be attempted with the absolute smallest beads possible. With extremely tiny beads, detection could be more feasible, perhaps even by measuring the diffraction using instrumentation that could detect the change in the overall diameter of the beads as they were coated.

A more practical approach, however, would be to focus on the use of these antibody coated beads (large or small) incorporated into the position in an ELISA in the position traditionally occupied by fluorescent dyes or radioactive labels. Here the beads are at their maximum potential for used in detection of small assays - possibly even on the order of one molecule. Such a technique if developed would have great potential for "lab on chip" techniques and could possibly even replace conventional ELISA techniques as an individual bead contains the same fluorescence experienced by numerous dye labeled antibodies; if a single antibody was able to attach link a fluorescent polystyrene bead to a polymer or gold surface, this would provide a means of quantitative single molecule detection.

It might be possible to monitor the degradation of the beads by centrifugation: by examining the supernatant over time to see if there was any 205 nm absorbance, which is known to be present after a year (as revealed in the above experimentation) could possibly explain why the protein does not attach to the beads, particularly in the unlikely, however theoretically possible, case that the modifiers on the surface of the beads which are meant to attach proteins have somehow come off the bead surfaces and remain floating in solution, thus explaining why there would be an increase in absorbance even after the beads have been centrifuged and explaining why the beads no longer are active in the manner Molecular Probes claimed they would be. I originally suspected that this effect was due to the fluorescent dye leaching from the interior of the beads, but this is very unlikely. What is probable is that the sulfate modifiers are leaching from the surface as the beads pass expiration. This could be easily monitored using the centrifugation technique described, but an IR analysis might be more sensitive and definitive as to what groups and chemicals were leaching / present and not. If fluorescent dye was leaching from the interior this could be seen best with a fluorometer, such as the one constructed for this project, or a conventional scanning fluorometer.

Also examination of the beads at 280 nm (or 273 nm), even though this would require increased amounts of protein and potentially beads as well to be used, would be a highly recommended next step. Such an examination should provided better results with fewer side effects due to the "bead residue" phenomena.

As speculation I might suggest that interaction between the residual chemicals from the beads (speculated to exist) and the protein which would be speculated to bind to these chemicals in solution explains the reason why the absorbance could consistently slightly increase; these chemicals are interacting, just not on the bead surface. If there is

interaction between the OvA protein and the sulfate modified groups which it might be presumed are no longer on the bead surface and now suspended in solution, this interaction might be monitorable using IR to look for the formation of new functional groups.

Future work will need to establish a way to attach protein to Sulfate-modified or perhaps another type of bead before proceeding to ELISA technique tests and development.

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