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SAND97–8283 • UC–408
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LDRD Final Report on Microencapsulated Immunoreagents for Development on One-Step ELISA

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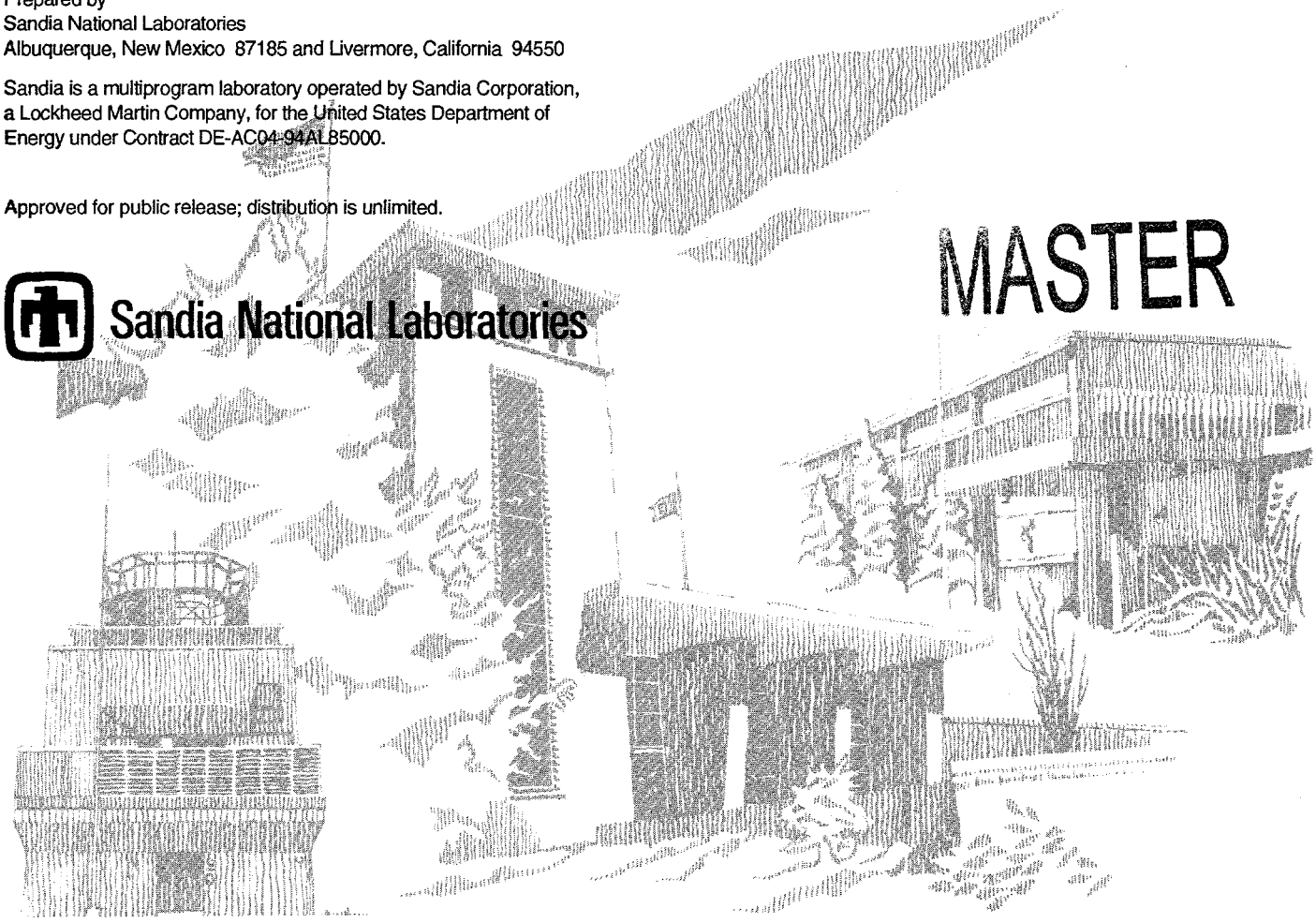

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LDRD FINAL REPORT ON MICROENCAPSULATED IMMUNOREAGENTS FOR DEVELOPMENT OF ONE-STEP ELISA

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ABSTRACT

Microencapsulation of biological macromolecules was investigated as a method for incorporating the necessary immunoreagents into an improved enzyme-linked immunosorbant assay (ELISA) package that would self-develop. This self-contained ELISA package would eliminate the need for a trained technician to perform multiple additions of immunoreagent to the assay. Microencapsulation by in-solution drying was selected from the many available microencapsulation methods, and two satisfactory procedures for microencapsulation of proteins were established. The stability and potential for rapid release of protein from these microencapsulates was then evaluated. The results suggest that the chosen method for protein entrapment produces microcapsules with a considerable amount of protein in the walls making these particular microcapsules unsuitable for their intended use.

ACKNOWLEDGMENTS

The authors wish to acknowledge the efforts of numerous colleagues for their support during the course of this work. In particular, we thank Joseph Schoeniger (8120), Professor Rueben Carbonell (North Carolina State University), and Pin Kao (8120) for helpful discussions related to this project.

Sandia National Laboratories is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin company, for the U. S. Department of Energy. This work was supported by the Laboratory Directed Research and Development Program under contract DE-AC04-94AL85000.

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I. INTRODUCTION

The work described in this report was performed as part of the Laboratory Directed Research and Development (LDRD) program in the area of biochemistry sponsored by the Department of Energy under contract DE-AC04-94AL85000. The purpose of the work was to select and demonstrate a suitable method for microencapsulating immunoreagents and then evaluate the potential for rapid release of the encapsulated protein from the microcapsules for use in an improved enzyme-linked immunosorbant assay (ELISA).

General ELISA Process

ELISA is a very powerful assay technique for the detection of harmful antigens in the body, e.g. herpes simplex virus or rubella IgG. The general procedure for the ELISA technique is shown in Figure 1. An antibody which binds strongly to the antigen in question is adsorbed or, less often, covalently attached to the surface of a well that subsequently acts as the reaction chamber for the rest of the assay. A sample that potentially includes the antigen is introduced to the well and antibody-antigen binding takes place if the targeted antigen is present. The well is rinsed several times with pure water and then treated with an aqueous solution of the same antibody with an enzyme covalently linked to it. If the antigen-antibody complex is already present, the enzyme-linked antibody will bind to the antigen as well to produce a "ELISA sandwich" complex that is rinsed with pure water and then detected by addition of a subtrate. The added substrate undergoes a chemical change, usually colorimetric, due to the enzyme, but only if the antigen was present in the sample and the antibody-antigen-antibody with enzyme complex was formed. The colorimetric change can often be quantified to give a measure of the amount of antigen present in the original sample.

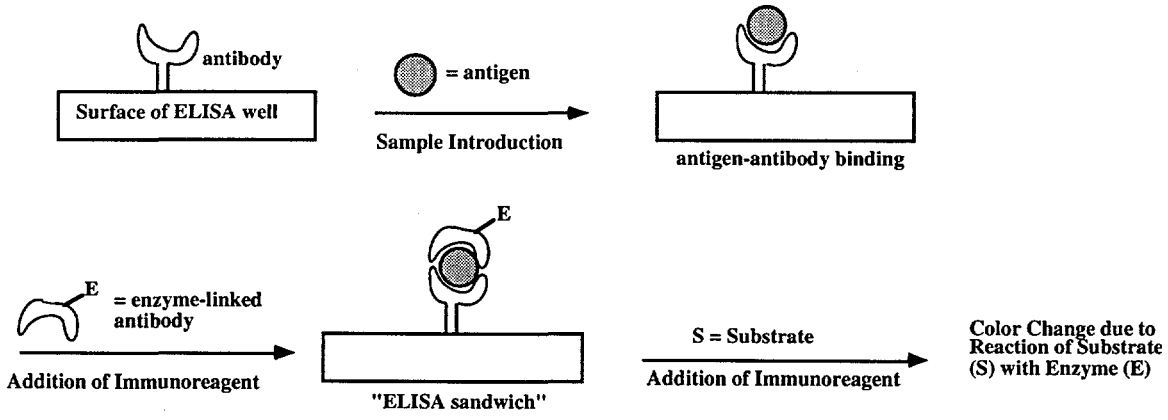

Figure 1: General scheme for enzyme-linked immunosorbant assays (ELISA).

The enzyme (E) linked to the antibody is often alkaline phosphotase (ALP) which catalyzes the decomposition of a substrate (S), *p*-nitrophenyl phosphate, to the yellow-colored product, *p*-nitrophenol.

Design of a "One-Step" ELISA Process

The ELISA process is fairly complex in practice requiring two additions of reagent, intermediate aqueous rinses, and a skilled operator. The process can be automated using accurate dispensing and autowashing equipment, but this is expensive and not very practical for antigen screening in the field. An analogy is photo film processing, where rapid, automated film developing is available for commercial photolabs but is not easily transportable to the field. The development of Polaroid™ film many years ago, however, does allow easy, rapid photo development in the field by non-skilled operators. Packaging the necessary immunoreagents for ELISA into a format analogous to Polaroid™ film would offer the prospect of a "one-step ELISA" process that could be performed in the field by a non-skilled operator.

One approach to such a one-step ELISA process involves the use of microencapsulated immunoreagents and the ability to release the reagents from the capsules in a rapid, but controlled fashion. A microencapsulated immunoreagent would be in the form of small polymeric spheres (10-100 μm diameter) that have an aqueous core with dissolved immunoreagent (Figure 2). Physical crushing of the microsphere at the proper time would then allow the released immunoreagent to interact at the surface of the ELISA well. The key element of most carbonless paper technology involves a similar crushing of microcapsules coated on the back of the paper. The main focus of this work therefore was to investigate reliable methods for microencapsulating aqueous solutions of proteins into inert polymer capsules and determine their potential use in a one-step ELISA device. The following section briefly describes an simple ELISA processing unit that was proposed to take advantage of microencapsulated immunoreagents.

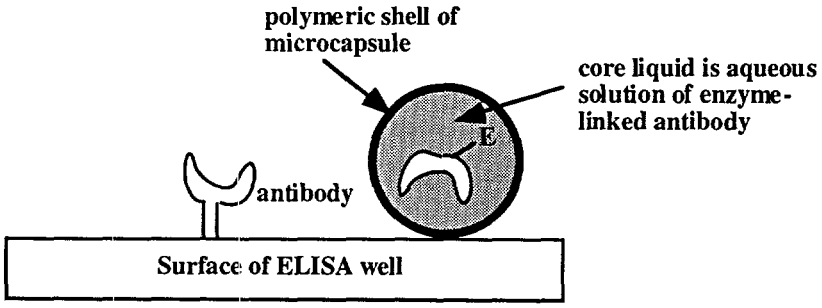

Figure 2: Proposed ELISA surface with microencapsulated reagents present.

One possible concept design for a one-step ELISA device is shown in Figures 3 and 4. The proposed reaction chamber shown in Figure 3 consists of a small threaded quartz reaction chamber precoated with the necessary antibody. The non-threaded end of the chamber has a glass frit and an empty flexible plastic tube for collecting aqueous waste. The other piece of the reaction chamber is a threaded flexible plastic tube containing buffer solution and the microencapsulated antibody-enzyme and substrate. The threaded connection on the supply tube also has a glass frit cap. The test sample would be placed in the quartz reaction well by pipette and the two tubes would then be screwed together. Next the final assembly would be inserted into the hand-held ELISA processing unit outlines in Figure 4.

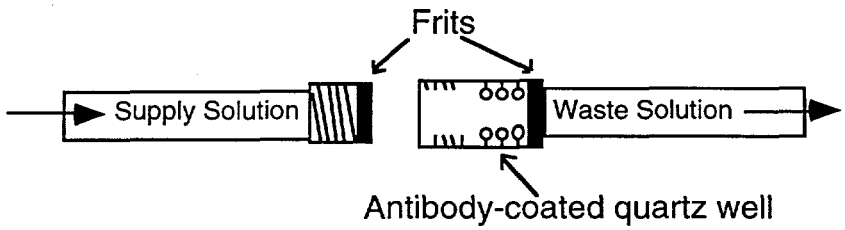

Figure 3: Proposed reaction chamber for one-step ELISA

A series of prealigned rollers in the developer unit pinch the supply tube in the closed position (Figure 4) separating the buffer solution into discreet portions as shown. The rollers are similar to a peristalic pump and would force the aqueous solutions through the reaction

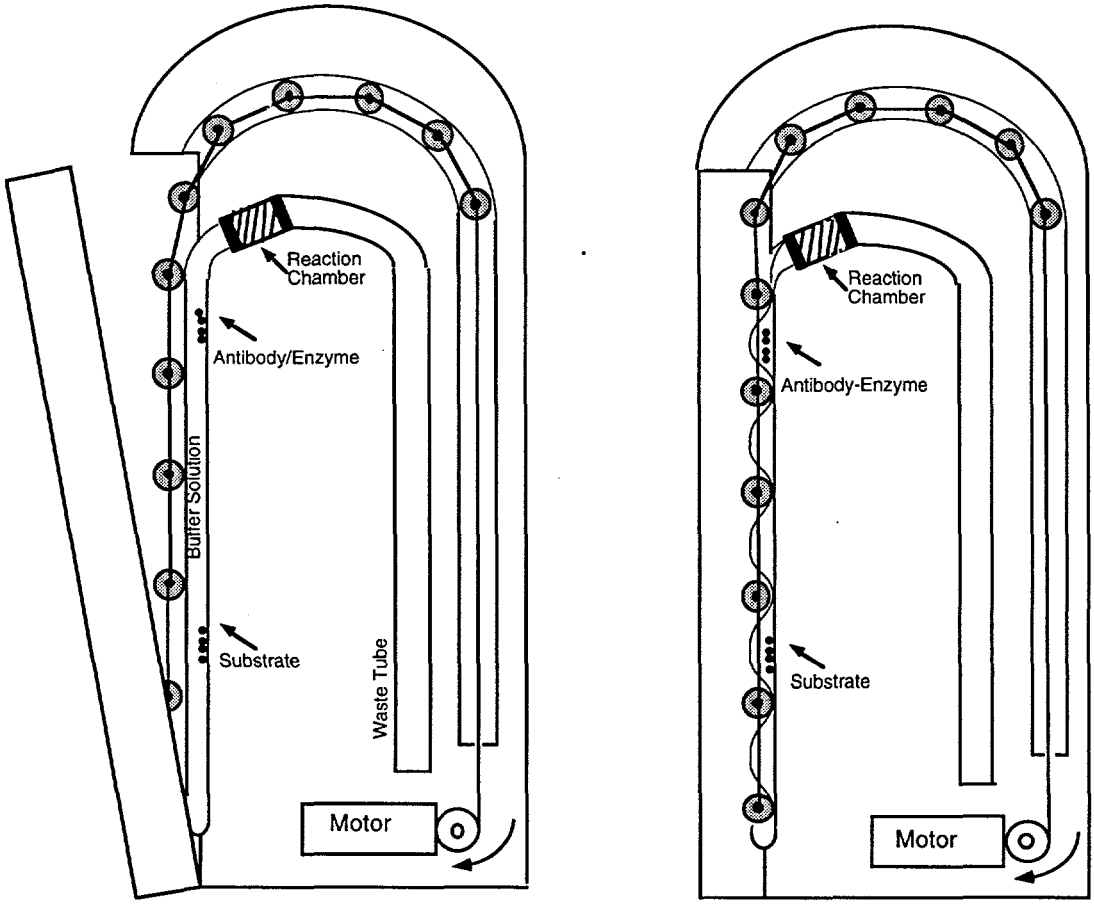

Figure 4: Simplified conceptual design for hand-held one-step ELISA developing unit (opened and closed positions).

chamber as the rollers are pulled through the device by an electric motor. In theory, the rollers would also crush the microencapsulated reagents, releasing the immunoreagents that are contained in the capsules into the localized portion of buffer solution. In this fashion, the resulting pockets of aqueous rinse solution, antibody-enzyme solution, aqueous rinse solution, and substrate solution would be pushed through the reaction chamber in a timed sequence, allowing for incubation times for the antibody-antigen binding to take place. A color change in the reaction chamber would then indicate the presence of the antigen in the original sample. An

advanced version of the device could include the photonics necessary to quantify the color change and the amount of antigen present.

Microencapsulation of aqueous solutions

Microencapsulation of a small droplet of any solution involves forming a polymeric shell completely around the liquid core material. Various techniques (1) have been developed for accomplishing this task as well as microencapsulation of small, solid particles. Many of these common technologies are most useful for the microencapsulation of an oil solution, but a few have been developed for microencapsulation of aqueous solutions.

Microencapsulation of solutions generally fall into two categories: polymer formation from monomers by chemical reaction at the oil-water interface of an emulsion or polymer deposition at the emulsion interface by physicochemical processes. Microencapsulation by chemical processes, such as inter- or intrafacial polymerization of monomers, was not selected for this project due to concerns about the stability of antibodies in the presence of a large excess of reactive monomer. Physiochemical processes, however, usually rely on phase separation of a polymer at an emulsion interface due to removal of solvent or changes in pH. An example (2,3) of the latter is the complex coacervation technique where a gelatin-gum arabic polymer is formed at the interface of an oil-in-water upon changes in pH. The gelatin and gum arabic are dissolved in the continuous aqueous phase of the emulsion and phase separate as a polymer around oil droplets in the emulsion as the pH lowered. Complex coacervation is therefore most commonly used for microencapsulation of oil solutions, not aqueous solutions.

The "in-solution drying" method (Figure 5) is a physicochemical process designed for the microencapsulation of aqueous solutions. (4-6) In this method, the aqueous solution to be encapsulated is first dispersed as the discontinuous phase in an organic (oil) solution containing a few weight percent of a linear polymer. This primary emulsion is then quickly dispersed into an aqueous solution containing a colloid such as gelatin or polyvinylalcohol to form a complex "water-in-oil-in-water" emulsion that is stabilized by the colloid. The polymer shell then is formed by slowly removing (drying) the organic solvent from the complex emulsion through evaporation as shown in Figure 5.

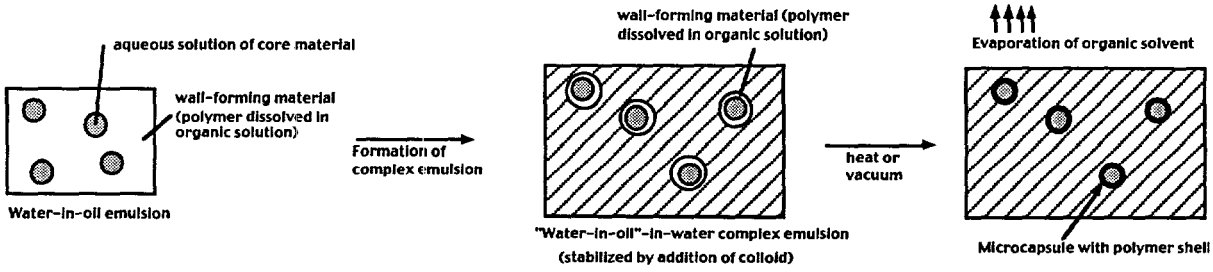

Figure 5: "In-solution drying" method of microencapsulation of aqueous solutions

The "in-solution drying" method, also known as the complex emulsion process, does not involve any chemical reactions, so this process seemed quite suitable for microencapsulation of active biological macromolecules such as antibodies. The process (4) has been used elsewhere to encapsulate several robust enzymes that were then used to catalyze substrate reactions while remaining encapsulated. The polymer walls of the microcapsules are often found to be semi-permeable, allowing substrates to diffuse into the interior while the larger, encapsulated enzymes are unable to diffuse out of the capsule. Following the substrate reaction, the encapsulated enzymes are easily filtered out of solution to purify the product. Microencapsulation of vaccines in a biodegradable polymer shell (6) have also been performed

using the in-solution drying method to produce capsules that have been evaluated as oral vaccines, slowing dissolving in the body and releasing the vaccine over a period of weeks.

II. EXPERIMENTAL

Microencapsulation Methods using In-Solution Drying

Two methods for the production of microencapsulated proteins using the in-solution drying process were established during the course of this work based on modifications of literature work (1):

Method A:

A 5 wt. % solution of polystyrene (Aldrich, M_W 45,000) is prepared by dissolving 5 g of the polystyrene in 95 g of benzene. An aqueous solution of protein (e.g. enzyme or antibody) is also prepared by dissolving 40-50 mg of the protein to be microencapsulated in 3.0 mL of distilled water. The aqueous protein solution is then added to 15 mL of the benzene solution of polystyrene. This mixture is emulsified using a high speed homogenizer with a stator-rotor mixing head operating at the top speed of 25,000 rpm for 30 seconds. The primary emulsion that results is quickly poured into a glass beaker containing 300 mL of an 1% aqueous solution of polyvinylalcohol (PVA) that is being rapidly stirred with an overhead mechanical stirrer. The milky-white complex emulsion (water-in-oil-in-water) is stirred in a fume hood for 4 hours during which the benzene slowly evaporates from the liquid mixture. The stirrer is then stopped, and the polystyrene microcapsules are filtered from the solution using a coarse fritted glass funnel. The microcapules are washed with distilled water several times (6 x 10 mL) and collected by centrifuge. The white powder material that is recovered is dried in a vacuum dessicator overnight. Weight of microcapsules recovered in this fashion averages 0.4 - 0.5 g. The remaining balance of polystyrene (0.15 - 0.25 g) is typically lost on the stirring blade and the filter.

The above procedure can be modified to include the addition of a few drops of a surfactant, Tween 80 or another non-ionic surfactant, from a medicine dropper to the primary emulsion to stabilize this emulsion before addition to the PVA colloid suspension. The primary emulsion is now stable for several minutes and can be added slowly to the PVP colloid suspension.

Method B:

An aqueous solution (0.5 mL) of protein (40 mg) is added to 5 mL of a 5 wt% solution of polystyrene (Aldrich M_w 45,000) in methylene chloride. The mixture is emulsified using a high speed homogenizer with a stator-rotor mixing head operating at medium speed (approximately 15,000 rpm) for 30 seconds. The resulting emulsion is quickly added to a scintillation vial containing 10 mL of a 1 wt. % solution of polyvinylalcohol in distilled water, and the new mixture is emulsified for 20 seconds on a vortex mixer (Vortex Genie) at the highest setting. The milky complex emulsion, which is stable for minutes, is then diluted by pouring it into a more dilute 0.1 wt. % solution of PVA (500 mL) which is rapidly stirred with a magnetic stir bar. The methylene chloride is allowed to evaporate over a 3-4 hour period stirring in the fume hood. The microcapsules are collected by centrifuge (50 mL aliquots) and then washed multiple times with distilled water (6 x 10 mL). After the final centrifuge operation, the water is carefully removed and the plastic centrifuge tube is cut, separating the good microcapsules at the bottom of the tube from the small amount of polystyrene debris that floats in water. The wet microcapsules are then dried in a vacuum dessicator overnight to give

a white powder. The weight of recovered microcapsules averages approximately 0.3 g of material using this method. Very little polystyrene debris is produced in this method.

Protein Analysis of Microcapsules

Bicinchoninic acid (BCA), in the form of its water-soluble sodium salt, is a sensitive and highly specific reagent for Cu1+. The presence of peptide bonds and amino acid side chains such as cysteine, tryptophan and tyrosine enables a protein to reduce soluble Cu2+ to Cu1+. Two molecules of BCA then complex with one cuprous ion (Cu1+) to give a purple product, concentration of which can be monitored at 562 nm to determine the amount of protein present in the sample. A microBCA kit was obtained from the Pierce Chemical Company and the following protocol was used:

1) Prepare working reagent by combining 2 parts of reagent C, 48 parts of reagent B and 50 parts of reagent A.
2) Prepare a set of protein standards by diluting the stock BSA solution in phosphate buffer.
3) Pipet 100 µl of each standard, blank or unknown sample into microtiter plate wells.
4) Add 100 µl of working reagent to each well.
5) Incubate the plate at 60^0C for an hour while shaking on a plateshaker.
6) Read absorbance at 562 nm with a microtiter plate reader.
7) Prepare a calibration curve by plotting the absorbance (blank corrected) of standards vs. protein concentration.

Synthesis and Analysis of HRP-FITC Complex

Materials: Horseradish Peroxidase (HRP) from Sigma Chemical Company, fluorescein isothiocyanate(FITC) from Molecular Probes. Buffer used- 0.05 M borax/borate, pH 9.0.

Protocol was as follows:

1) Dissolve 20 mg FITC in 500 µl DMF to make a 40 mg/ml solution
2) Dissolve 10 mg HRP in 3 ml borax/borate, pH 9.0
3) Add 50-fold molar excess of fluorescent dye to enzyme solution. 121.7 µl of FITC was added to HRP solution, total volume = 3.122 ml.
4) React for 4 hours at room temperature with gentle stirring in dark.
5) Apply the reaction mixture to an ultrafree-CL centrifuge tube (Millipore Corp.) and centrifuge for one hour at 4500xg to reduce the reaction volume to 1.5 ml by slow filtration.
6) Apply the concentrated reaction mixture to a size exclusion column packed with Bio-gel P-2 media (Bio-Rad Laboratories, Hercules, CA) equlibrated with phosphate buffer saline. Fractions are collected and analyzed by measuring absorbance at 405 nm and 495 nm.
7) Samples with highest absorbance at 405 nm are pooled and stored at 4^0C.

Spectroscopic characterization of enzyme-fluor conjugate:

Concentration of fluorescein (molar) = Absorbance at 495nm/ 68,000

Concentration of HRP (molar) = (Abs @ 405 - Abs @ 495 x 0.072) x 1.4×10^{-5}

Enzymatic Analysis of Microencapsulated Products

Activity measurements are performed in 50 mM citrate buffer using 2,2' azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and H_2O_2 as substrates at 25^oC. 3.2 ml of substrate (2 mM ABTS and 2.75 mM H_2O_2) are pipetted into sample and reference cuvettes. Ten µl of enzyme solution are added to the sample, blanked against buffer and the change in absorbance is recorded at 410 nm with a Cary 3E UV-visible spectrophotometer (Varian Instruments).

III. Results and Discussion

Two variations of the "in-solution" drying method, Method A and Method B, were developed for the microencapsulation of proteins and are described above. A majority of the process development work was done using bovine serum albumin (BSA) as the target protein to be encapsulated.

Microencapsulation of Immunoreagents

Of the two methods, Method A proved to be the inferior process for microencapsulation of BSA. In the first trials, this general method produced very large spherical capsules with diameters as large as 1 μm, which were mixed among a considerable amount of broken capsules, non-spherical capsules, and other debris. Variations in the shear mixing speed, volume ratios, and emulsification process all led to significant improvements in the encapsulation process, but the level of debris was still fairly high as shown in Figure 6 below. These BSA-polystyrene (BSA/PS) capsules were mostly in the 10-100 μm diameter range, although some larger capsules and debris were evident.

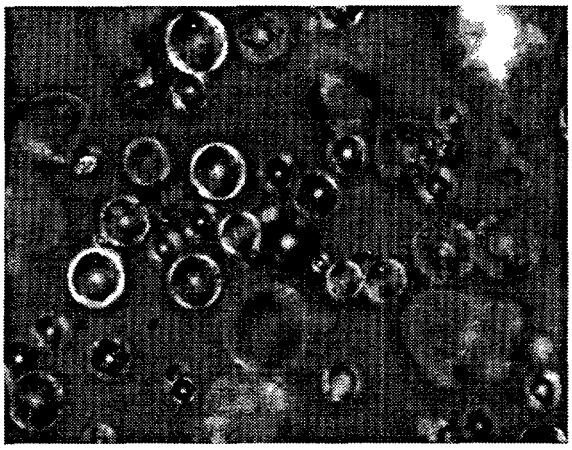

Figure 6: Optical micrograph of BSA/PS microcapsules prepared by Method A.

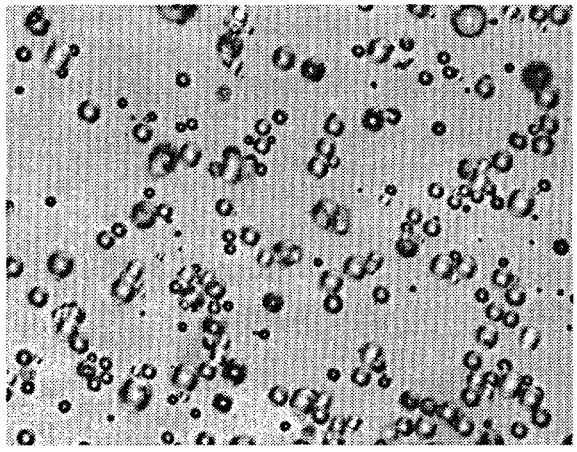

Figure 7: Filtered BSA/PS microcapsules analyzed for protein content.

The BSA/PS microcapsules produced by method A were sieved through a 100 μm mesh to remove the small portion of larger capsules and most of the debris. The resulting white powder was more uniform in appearance (Figure 7) and contained mostly intact BSA microcapsules. This "purified" material was analyzed for protein content using a microBCA analysis described above. Initial protein assay results indicated that the BSA/PS microcapsules prepared by Method A contained approximately 1 ug of protein per mg of microcapsule, which represented a modest 10-15% microencapsulation efficiency. However, in this type of protein assay, the assumption is made that the copper indicating reagent is able to diffuse through the semipermeable walls of the polystyrene microcapsule during the incubation time of the assay. If this diffusion does not take place completely, the BCA results will give a low estimate of the protein content of the capsule. Thus, the conclusion is that these polystyrene microcapsules shown in Figure 6 and 7 contain at least 1 ug of BSA protein per mg of capsules.

Method A was also used to encapsulate an enzyme, horseradish perioxidase (HRP), that had been labeled with a fluorescent group, fluorescein thioisocyanate (FTIC) as described above. The HRP molecule has an absorbance at 405 nm while the FTIC group absorbs at 495 nm, making it possible to determine spectroscopically the ratio of FTIC groups to HRP molecules in the complex. The ratio was found experimentally to be approximately 1.6 FTIC groups per

HRP molecule. The absorbances of the HRP group also afford an additional method for the determination of mg of protein/mg of microcapsules. A sample of HRP-FTIC/PS microcapsules was dissolved in methylene chloride, and the enzyme complex was extracted into water via five separate washings. A spectroscopic analysis of the combined extracts showed that the total amount of enzyme in the microencapsulated product was 2.51 mg, or 18% of the 14.0 mg HRP-FITC that was in the aqueous solution to be encapsulated. This alternative method of measuring the microencapaulation efficiency for Method A agrees fairly well with the earlier estimate from the microBCA analysis above. Thus, microBCA analysis was used throughout the rest of this work as a simple, accurate method for determining the protein content of microcapsules.

The microcapsules of fluorescent HRP-FITC were also useful for characterizing the internal structure of microcapsules produced by Method A. Using a fluorescent microscope it was straightforward to determine that the HRP-FITC microcapsules were a combination of mostly spherical multi-nuclear with some irregularly shaped multi-nuclear (Figure 8). The microscope was not equipped at the time with its CCD camera, so the fluorescent image of the HRP-FITC/PS microcapsules could not be recorded. The fluorescence, however, seemed to originate from within the walls and clearly indicated that the capsules were virtually all multinuclear.

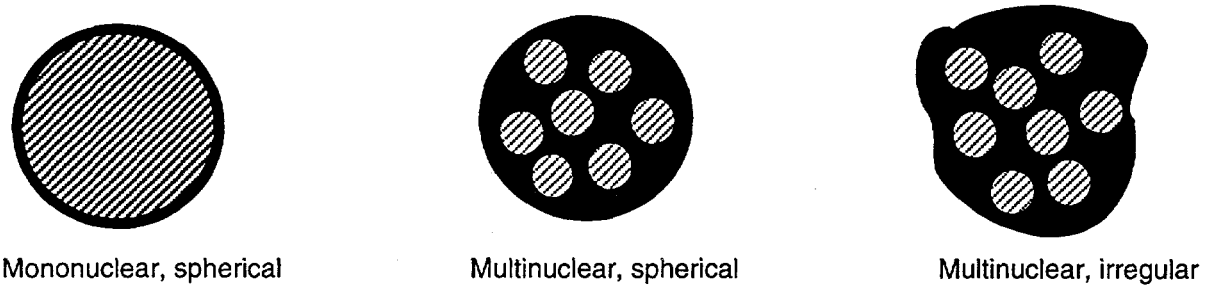

Figure 8: Internal structures of microspheres.

The multinuclear structure of the Method A microcapsules was undesirable because it would probably be very difficult to release all of the entrapped material during the proposed crushing operation. It was also a concern that the fluorescence appeared to originate from within the walls of the microcapsules, because protein in the walls could only be released through dissolution of the microcapsule. Changes to Method A were investigated to eliminate these two concerns.

The small inner cores of the multinuclear microcapsules from Method A are probably formed by the very high speed shear mixing speed using to form the primary emulsion. The higher speeds were originally used to establish an emulsion that would be stable long enough to form the subsequent complex emulsion. These extremely small aqueous droplets encased in benzene then aggregate in the complex emulsion to form multinuclear capsules upon removal of the benzene solvent. After considerable experimentation with mixing methods and speeds, it was determined that the production of aggregates of small aqueous droplets in the complex emulsion could be avoided by reducing the mixing speed of the homogenizer used to make the primary emulsion and by using a new method of forming the complex emulsion in the second step.

In Method B, the complex emulsion is formed by pouring the primary water-in-oil emulsion into twice the volume of 1 wt. % aqueous PVA and establishing the complex emulsion by violently mixing the solutions on the a vortex mixer for 20 seconds. Any aggregates that are formed in the primary emulsion appear to be broken up in the vortex mixing step. The resulting complex emulsion is evenly milky-white in appearance and is stable for several minutes without stirring. This emulsion is then diluted in a large volume of 0.1 wt. % PVA, and the organic solvent is removed by evaporation. The resulting microcapsules recovered from Method B are very spherical and are mononuclear. In Figure 9, BSA/PS microspheres produced by Method B are shown. The average diameter is in the range of 1-10 μm, much smaller and uniform than the microcapsules produced by Method A. A fluorescent image of a HRP-FITC microcapsule produced by Method A was taken with a CCD camera and is shown in Figure 10. The capsules are clearly mononuclear in this case, but the fluorescence (dark areas in Figure 10) still appears to come from the walls of the sphere. Thus, it was concluded that the changes made to the microencapsulation method solved the multinuclear problem, but that the potential problem of wall entrapment remained.

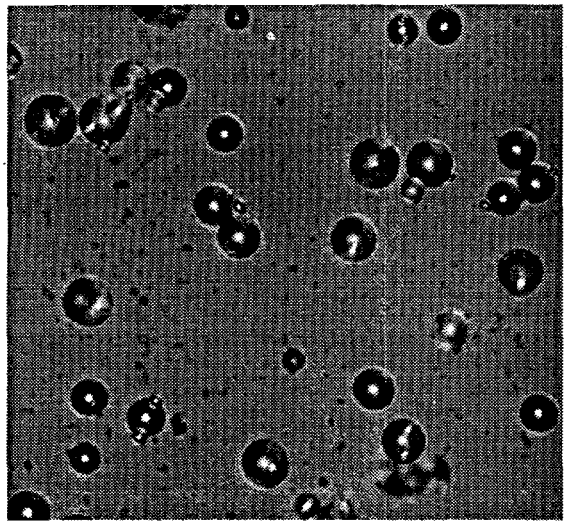

Figure 9: Optical micrograph of BSA/PS microcapsules prepared by Method B.

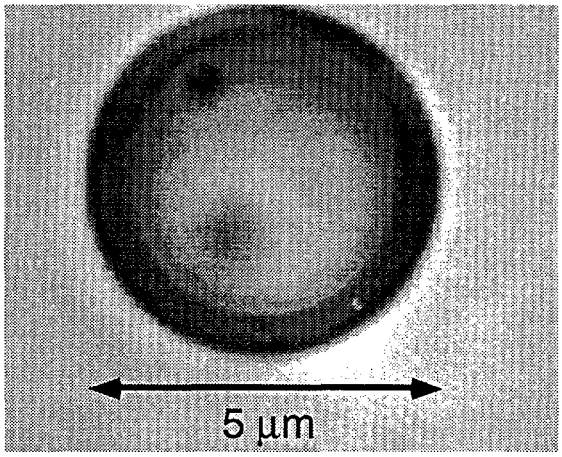

Figure 10: Fluorescent micrograph of HRP-FITC/PS microcapsule prepared by Method B.

A series of microBCA analyses were done on BSA/PS microcapsules to determine the location of the protein entrapped in the microcapsule. BSA (40 mg) and polystyrene (340 mg) was used in Method B to produce 287 mg of dried microcapsules. Four 5.0 mL aqueous samples were prepared and the amount of protein in each determined by microBCA analysis:

Sample 1: intact — 20 mg of dried sample suspended in 5.0 mL of phosphate buffered saline (PBS) solution

Sample 2: crushed/washed — 20 mg of dried sample was crushed with rounded glass rod at the bottom of a centrifuge tube. The material was washed with 1.0 mL of PBS buffer five times to give a combined washing of 5.0 mL. The crushed polystyrene was removed by centrifuge for each washing.

Sample 3: crushed/dissolved — the 20 mg of crushed, washed polystyrene from Sample 2 was dissolved in 1.0 mL of methylene chloride which was extracted with 3 x 1.5 mL of PBS buffer. Total volume was brought up to 5.0 mL with PBS buffer.

Sample 4: dissolved — 20 mg of dried sample was dissolved in 1.0 mL of methylene chloride which was extracted with 3 x 1.5 mL of PBS buffer. Total volume was brought up to 5.0 mL with PBS buffer.

Sample 1 (intact) should give the total amount of protein encapsulated in 20 mg of the BSA/PS product. Sample 4 (dissolved) should also give the total amount encapsulated in 20 mg provided that the extraction step removes all of the protein from the organic solvent used to dissolve the capsules. Sample 2 (crushed) should give the amount of protein entrapped in the interior of the capsule, and Sample 3 should indicate the amount of protein contained in the walls of the capsule. The results are shown in Table 1:

Sample Number	Description	BCA analysis (mg/mL solution)	BSA in sample (mg protein/ 20 mg sample)	Microencapsulation Efficiency (%)
1	Intact	0.356	1.78	64
2	Crushed/Washed	0.081	0.41	-
3	Crushed/Dissolved	0.254	1.27	-
4	Dissolved	0.330	1.65	59

Table 1: MicroBCA analysis of protein content of BSA/PS microcapsules

The results in Table 1 give rise to two important conclusions. First, the microencapsulation efficiency of Method B, measured as the actual amount of protein in recoverable microcapsules compared to total possible amount of protein (40 mg), is about 60%, which is much higher than Method A (10-15%). The results also support the earlier conclusion from the HRP-FITC fluorescence imaging experiment that the majority of the protein is probably trapped in the walls. Dissolving crushed polystryene capsules (Sample 3) gave about 3 times as much protein as simply washing the crushed spheres with buffer. The conclusion, therefore, is that the polystyrene microcapsules produced by the "in-solution drying" method described here are not suitable for the intended purpose of a one-step ELISA device with microencapsulated reagents.

Wall materials other than polystyrene are possible which may not result in the protein being trapped in the walls. Polystryene is hydrophobic, and hydrophobic BSA may prefer to deposit in the PS walls rather than remain in the aqueous core material. Changing the wall material to the more polar polymethylmethacrylate (PMMA) was suggested as a way to exclude the BSA from the wall region. Method B was used to encapsulate BSA in PMMA; the optical micrograph of the resulting microcapsules is shown in Figure 11. Unlike the PS microcapsules, the PMMA capsules appear multinuclear in the optical micrograph. The

capsules are also noticeably more fragile than the PS microcapsules. Unfortunately, this LDRD program ended before the PMMA capsules could be fully characterized and the effect of the PMMA wall material determined. In principle, almost any linear polymer can be used to make microcapsules using the "in-solution" drying method, and preventing the protein from depositing in the wall should be possible. Further work in the microencapsulation of immunoreagents or biological macromolecules may take place at a later time, perhaps at the Chemical and Radiation Detection Laboratory (CRDL) at Sandia/CA. Any further work will also have to address two other critical issues: the stability of the microcapsules in aqueous solution and the remaining biological activity of macromolecules released from a microcapsule. Some preliminary data for each of these issues follows in the next two sections.

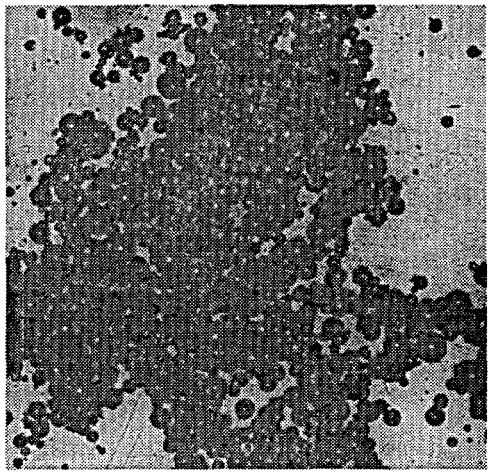

Figure 11: Optical micrograph of BSA/PPMA microcapsules from Method B.

Stability of HRP-FITC/PS Microcapsules in Aqueous Solution

The HRP-FITC microcapsules produced by Method B were washed four times with distilled water and the fluorescent absorptions of the washes were checked at 405 nm and 495 nm for the presence of the HRP-FITC complex. After the fourth wash the enzyme complex could no longer be detected spectroscopically. The microcapsules were then stored in an aqueous PBS buffer solution for over two weeks at 4 °C. The solution's absorbances at 405 and 495 nm were checked periodically to determine the concentration of HRP-FITC in the solution. The results shown in Figure 12 clearly indicate that the amount of HRP-FITC in the solution is increasing with time, suggesting that the enzyme is slowly leaking out of the polystryene. This apparent problem could also be solved by using a different wall material instead of PS or PMMA, but further research will be needed to produce stable capsules that can be stored wet.

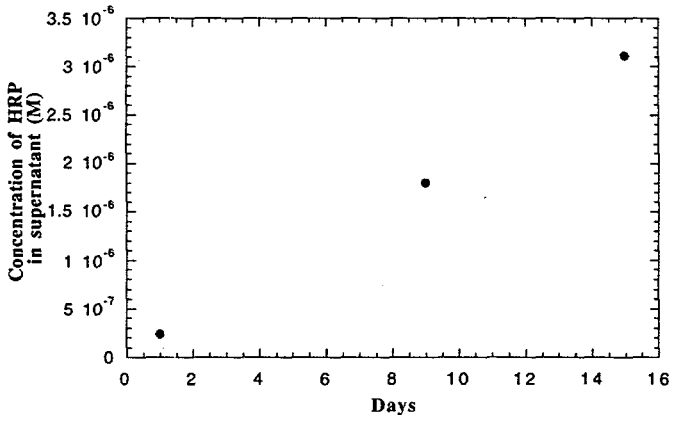

Figure 12: Concentration of HRP-FITC in the solution of HRP-FITC/PS microcapsules stored wet in aqueous solution versus storage time.

Enzymatic Activity of HRP Released from Polystyrene Microcapsules

Horseradish peroxidase was released from PS microcapsules by dissolving the capsules in methylene chloride and extracting the enzyme with distilled water. The enzymatic activity was then measured (see Experimental Section) and compared to the HRP sample before encapsulation. The enzyme released from the PS microcapsules was found to have only 15% of the specific activity of the original material. The severe reduction in enzymatic activity could be due to the microencapsulation process or the extraction process using methylene chloride which has been known to denature some enzymes. In some cases of microencapsulated enzymes reported in the literature (1,4), the enzyme retains most of its enzymatic activity in the encapsulated state or if it is released very slowly through hydrolysis of a biodegradable shell. These results suggest that the loss in enzymatic activity is probably due to the methylene chloride extraction that was used. Further work in this area is needed to establish the biological activity of microencapsulated immunoreagents released by rupture of the capsule.

Conclusions and Recommendations

Two variations of the "in-solution drying" method were used to microencapsulate aqueous solutions of proteins in polystyrene. Characterization of the microcapsules from the first method showed them to be multinuclear and probably unsuitable for the intended use in a "one-step" ELISA device. The second method produced the desired mononuclear microcapsules, but the experimental evidence indicated that a majority of the enzyme was entrapped in the walls of the sphere, again making them unsuitable for the intended use. The stability of the microcapsules in aqueous solution and the loss of enzymatic activity for released enzymes also would need to be improved significantly before these types of microcapsules could be used as immunoreagents in an ELISA device.

There are several possible avenues to improving the suitability of the encapsulated immunoreagents for ELISA. As mentioned above, the use of a more polar polymer for the shell wall may greatly decrease the amount of BSA protein material that ends up in the wall. BSA/PMMA capsules were made but not characterized during this program. Future research should include improving the microencapsulation by tailoring the wall material to prevent deposition of the core material in the walls.

A drawback to the "in-solution drying" microencapsulation is that the shell wall is formed slowly over several hours. This allows time for the protein to be partitioned into the polymer material as it starts to form around the aqueous droplet. Forming the polymer shell quickly in a few seconds may eliminate this problem entirely. A microencapsulation process capable of this type of rapid shell formation is known as the "in-solution curing" method or the "orifice process." The general scheme is shown in Figure 13 below.

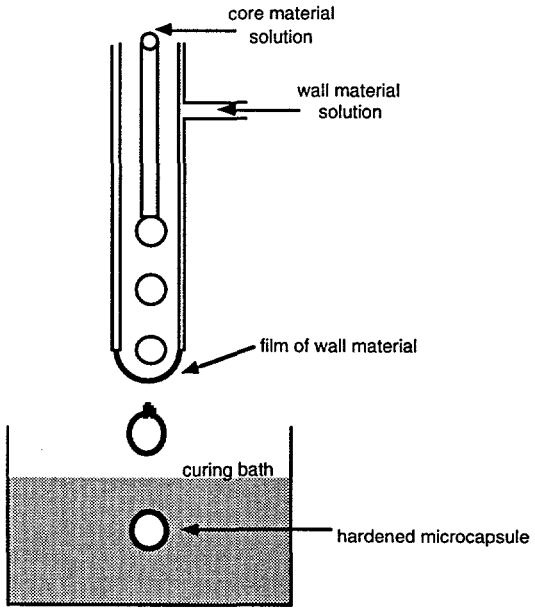

Figure 13: The "in-solution curing" or "orifice" method of microencapsulation.

In this method, interfacial precipitation (curing) of a polymer takes place in the following fashion using a concentric double orifice. A solution of the core material is delivered from the inner orifice of the double needle with a solution of the wall material coming from the outer needle in the concentric arrangement. Each inner droplet is coated with outer material as it

passes through the end of the double orifice and falls into the curing bath. In the curing bath, the polymer is quickly precipitated or cured at the interface. For example, if the core solution is an aqueous protein solution and the coating solution is a polyethylene glycol (PEG) solution of a methacrylic polymer, the polymer will be precipitated rapidly as the drop falls through an aqueous bath. The PEG solvent dissolves into the water, leaving solid polymer to encapsulate the core aqueous solution. Alternatively, the core solution can be an oil solution and the wall solution an aqueous solution of sodium alginate polymer. The curing bath then is calcium chloride which rapidly "cures" the alginate polymer by forming a shell of water-insoluble calcium alginate around the oil core. Although this type of microencapsulation is extremely slow to produce large amount of materials and generally produces very large capsules (1 mm in diameter), the method would probably help prevent the core material from ending up in the wall of the microcapsule. Further research into microencapsulated immunoreagents for ELISA should investigate the possibility of using the orifice method for making the microcaspules.

During the course of this work, an interesting method for the production of "microtubes" of aqueous solutions was published in the literature (7). In this method, an array of polypyrrole microtubes are formed by electrochemically coating polypyrrole on the inside walls of the open channels in a microporous polycarbonate filtration membrane. Polypyrrole is simultaneously coated along the bottoms of the membrane channels to form the bottom end caps. The microtubes are then filled with an aqueous solution of an enzyme using vacuum infusion, and the tops of the microtubes are then sealed shut with an epoxy cap. The microtubes, which have diameters of only 0.4 μm, are released by dissolving the templating membrane. Adaptation of this process to the production of larger microtubes could lead to useful reservoirs of immunoreagents for ELISA devices.

Finally, the option of encapsulating the immunoreagent into one large, single reservoir should also be part of future research in this area. For example, a very, thin polymer tube could be filled with an aqueous solution of the immunoreagent by capillary action, and the ends could then be closed by photopolymerization of a capping material. The length and diameter of the tube could be controlled to ensure the proper amount of reagent. Rupture of a single tube of material in a device similar to the one proposed in Figure 4 then would deliver the proper amount of immunnoreagent at the proper time. It could be more practical to have just one capsule that needs to be ruptured in comparison to many microcapsules. Microcapsules of biological macromolecules, however, may find other uses in sensor applications or other research programs at Sandia.

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