

Application of Antibody and Fluorophore-Derivatized Liposomes to Heterogeneous Immunoassays for D-dimer

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Small unilamellar liposomes comprised of cholesterol and phospholipids, in which one of the lipids is labeled with a fluorophore, have been covalently functionalized with antibodies. The liposomes were conjugated with thousands of fluorescein molecules and 10–20 monoclonal antibodies per liposome. These bifunctional liposomes were used in a direct (sandwich-type) immunoassay for the detection of thromboembolic disorders by assaying for d-dimer. D-dimer is the final and the smallest proteolytic product in the degradation of cross-linked fibrin by the plasma protein plasmin. The immunoassay using liposomes was compared to a conventional immunoassay that uses a fluor-antibody conjugate. The liposomes, by virtue of having thousands of fluorophores coupled to one liposome in contrast to one or a few reporter molecules in the conventional fluor-antibody conjugate, performed better on two counts: (1) they lowered the detection limit by a factor of 120 and (2) they provided a 1 order of magnitude amplification in signal. The minimum detectable concentration (MDC) of d-dimer was 5.6 ng/mL with the liposomal assay as compared to an MDC of 674 ng/mL with conventional fluor-antibody conjugate. The results of fluorescence assays were also compared with the results obtained by Singh et al. (*Biotechnol. Prog.* 1995, 11, 333-341) in an enzyme immunoassay developed using liposomes. These results demonstrate the potential of liposomes in lowering detection limits and increasing the sensitivity of immunoassays.

Introduction

Immunoassays have found widespread use in detection and measurement of a near limitless range of analytes in clinical, food, and environmental applications. The immunoassays take advantage of the specific recognition and binding of a biological ligand to another molecule, the prominent example being binding of an antibody to an antigen. The generality of immunoassays stems from the fact that most of the analytes are either antigens or antibodies or are molecules against which an antibody can be generated by utilizing the immune system of a host animal. To obtain a signal, one member of the recognition pair needs to be labeled with a signal-generating molecule such as an enzyme, a radioisotope, or a fluorophore. Fluorescent labels have been used quite extensively as they do not pose the health hazard associated with radiolabels and are more stable than enzymes. Also, since the fluorophores are much smaller than enzymes, the activity of the protein to which they are conjugated is not reduced drastically. One drawback associated with fluorescent labels is limited sensitivity due to high background fluorescence caused by proteins and other molecules in serum. This can be overcome by using a heterogeneous assay format where most of the serum components are washed away prior to fluorescence signal measurement.

In an immunoassay with labeled antibody, at most 1–10 signal molecules can be conjugated to one antibody without reducing the binding affinity. Hence, there are 1–10 signal molecules for each antigen-antibody binding event. If the number of signal molecules corresponding to an antigen-antibody binding event can be increased, the amplification in the signal can result in a lower

detection limit. Liposomes, spherical bilayer structures (≈ 100 nm in diameter) in water made up of lipids, have been employed as one such signal enhancement agent in immunoassays (Singh and Carbonell, 1995b). Liposomes provide a large intrinsic surface area per unit volume together with a large internal volume. In the internal aqueous core a large number of fluorophores or enzymes can be entrapped. On the outside, taking advantage of the reactive head groups of phospholipids, both reporter molecules and antibodies can be covalently attached. Such immunogenic liposomes bearing numerous reporter molecules can be used in immunoassays to improve the sensitivity. Most of the effort has been concentrated on developing homogeneous assays employing liposomes carrying entrapped marker molecules (Ho and Huang, 1988). Recently, a few heterogeneous assays using liposomes carrying encapsulated fluorophores were developed. Rongen et al. (1994) developed a sandwich assay for interferon- γ (IFN γ) using biotinylated liposomes containing carboxyfluorescein. Wells of a microtiter plate were coated with anti-IFN γ antibody and incubated with sample containing IFN γ . After washing, the wells were incubated successively with second anti-IFN γ conjugated with biotin, avidin, and liposomes. The authors reported that the detection limit of the liposomal assay was comparable to that of the colorimetric ELISA for IFN γ . O'Connell et al. (1985) developed a competitive assay for digoxin using liposomes containing entrapped sulforhodamine-B and bearing digoxigenin on the surface. The assay was carried out in propylene test tubes coated with rabbit anti-digoxin serum. Locasio-Brown et al. (1990, 1993) and Yap et al. (1991) used liposomes containing encapsulated carboxyfluorescein to develop a flow-injection immunoassay for the detection of theophylline. Choquette et al. (1992) used a similar formulation of liposomes to develop a planar waveguide sensor and

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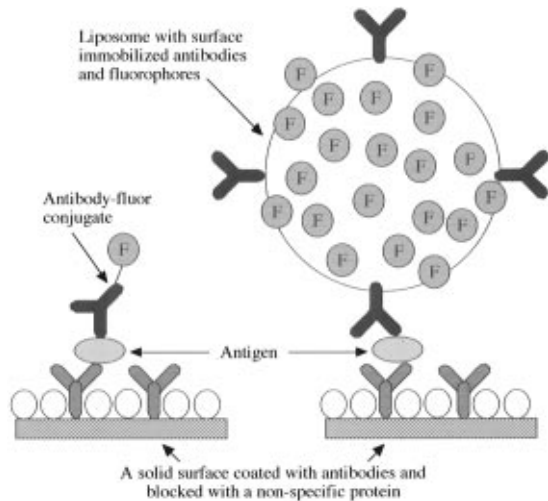


Figure 1. How liposomes with fluors and antibodies on the surface can enhance the signal in an immunoassay compared to an antibody labeled with a fluor. With liposomes we can get thousands of fluors corresponding to a single antigen compared to few (1–10) fluors in the case of fluor–antibody conjugate.

reported that liposomes amplified the signal by 1 order of magnitude over that of a theophylline–fluorescein conjugate.

All the examples of liposome immunoassays mentioned above involved use of liposomes carrying entrapped fluorophores or enzymes in the core and having a few receptor molecules (antigen or antibody) attached on the outside. The problem with entrapment is that in the case of large signal molecules like enzymes, only a few (<10) can be entrapped in a unilamellar liposome. In the case of smaller molecules like fluorophores, a huge number can be entrapped, but leakage due to diffusion and nonspecific lysis results in loss of signal molecules (Wagner and Baffi, 1987; Fiechtner et al., 1989; Katoh et al., 1993). In addition, the liposomes containing encapsulated marker must be maintained in aqueous solution, thereby rendering long-time storage very expensive. In an order to get around these problems an alternative arrangement has been suggested (Jones et al., 1993b; Singh et al., 1995a) as illustrated in Figure 1. Here, a large number of signal molecules are covalently attached to the outer liposome surface, together with few recognition molecules (e.g., antibody). This not only provides a large number of signal molecules per liposome but also eliminates the problem of leakage. These liposomes can also be lyophilized and resuspended without any loss of marker molecules. Such liposomes with enzyme (horseradish peroxidase) and antibody conjugated to the outer surface have been applied in a sandwich assay to detect d-dimer in serum (Singh et al., 1995a). In the present work, the preparation, characterization, and application of liposomes with fluorophores and antibodies on the surface to the detection of d-dimer is examined. This offers the opportunity to compare the performance of enzyme and fluorescent liposome assisted immunoassays on the same system.

Thromboembolic diseases, such as arterial thrombosis which leads to myocardial infarction, are a leading cause of mortality all over the world. The mechanism of blood coagulation involving fibrin formation and polymerization has been studied extensively (Doolittle, 1981; Hermans and McDonagh, 1982). The plasma protein fibrinogen is converted into fibrin by the enzyme thrombin. Monomeric fibrin polymerizes into protofibrils which covalently cross-link laterally with other protofibrils to form a network. These huge networks are insoluble and clot

the blood. This mechanism comes into effect in case of a bleeding lesion or wound to avoid excessive blood loss. A blood clot is a transitory device and is soon degraded and solubilized by another plasma protein, plasmin. The need for solubilization of a clot arises from the fact that a fraction of a clot or an embolus present in the circulatory system can block a small blood vessel. In certain disease processes such as atherosclerosis, a thrombus or blood clot is formed inside a vessel even when there is no injury, and this can lead to heart attack or stroke. It is imperative that assays be developed to detect the formation of a thrombus or an embolus at an early stage of a thromboembolic disease, when it is still curable. One method of detection is to look for fibrin degradation products in the blood (Dale et al., 1994). D-dimer is the final proteolytic product formed by the action of plasmin on cross-linked fibrin (Francis and Marder, 1982). Monoclonal antibodies have been developed against d-dimer, thus allowing for development of an immunoassay. It should be noted that these antibodies also recognize a broad range of intermediate degradation products that carry the d-dimer configuration.

The liposomes used in this study were formed by sonication or extrusion using phosphatidylethanolamine (PE) labeled with fluorescein in conjunction with unlabeled PE, phosphatidylcholine (PC), and cholesterol. An antibody against d-dimer was radiolabeled with ^{14}C using reductive methylation. Up to 20 antibody molecules were linked to the liposomes utilizing periodate coupling chemistry. The liposomes were characterized by measuring their size using quasi-elastic light scattering (QLS), determining their concentration using a phosphate assay, and estimating the number of antibodies per liposome by radioactivity measurements. The d-dimer assay conditions were optimized with respect to incubation duration, sample and liposome dilutions, blocking protein, concentration of coating antibody, and concentration of surfactant for lysis. The liposomes were used in a sandwich assay performed in a 96-well microtiter plate. The assay using fluorescent liposomes was compared against an assay that uses a conventional antibody–fluor conjugate, as well as to a liposome-based assay using enzyme-conjugated liposomes (Singh et al., 1995a).

Materials and Methods

Materials. *N*-(5-Fluoresceinthiocarbonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (fluorescein-DPPE) was obtained from Molecular Probes, Eugene, OR. *N*-(Caproylamino)-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DM cap-PE) was purchased from Avanti Polar Lipids, Alabaster, AL. Dimyristoylphosphatidylethanolamine (DMPE), distearoylphosphatidylcholine (DSPC), cholesterol (Chol), fluorescein 5-isothiocyanate (isomer I), bovine serum albumin (BSA) fraction V (RIA grade), phosphate buffered saline (PBS), and Sepharose CL-6B were obtained from Sigma Chemical Co., St. Louis, MO. The nonionic surfactant pentakis(ethylene glycol) mono-*n*-dodecyl ether (C_{12}E_5) was obtained from Nikko Chemicals, Japan. Polystyrene 96-well high-binding, flat-bottom microtiter plates were obtained from Costar Corp., Cambridge, MA. [^{14}C]Formaldehyde was obtained from Amersham, Arlington Heights, IL. D-dimer and two anti-d-dimer monoclonal mouse antibodies, designated 5-4-C and 8-8-G, respectively, were kindly provided by Organon Teknika Corp., Treyburn, NC. All other chemicals were from either Fisher Scientific or Sigma Chemicals and of reagent grade or better.

Methods. Liposome Preparation. Small unilamellar liposomes were prepared with compositions of DSPC:

Chol:DMPE:fluorescein-DPPE (40:40:5:15 mol %) using sonication (Szoka and Papahadjopoulos, 1980) or extrusion (Hope et al., 1993). Mixtures of various lipids weighing 30 mg total were dissolved in 10 mL of chloroform/methanol (9:1) and dried in a rotary evaporator to form a thin lipid film on the inside wall of the flask. The lipids were then hydrated in 10 mL of 50 mM citrate buffer (pH 6.0) at 65 °C to form multilamellar vesicles (MLV). Unilamellar liposomes were formed by either sonication or extrusion.

Sonication. The MLV solution was placed in a clean glass vial and sonicated with a titanium-tipped probe sonicator (Model W-385, Heat Systems Ultrasonics, Farmingdale, NY) at 70–80 °C. The energy input was intermittent, with 3 s of sonication followed by 2 s of pause. The total sonication time was 1 h. In few cases, the liposomes appeared cloudy after sonication and were sonicated for an additional 30 min.

Extrusion. The MLV solution was sonicated in a bath sonicator for 10 min to reduce the average size of the liposomes. This solution was then injected into an extruder assembled in our laboratory. The filter holder of the extruder contained two stacked 50 nm polycarbonate membranes (Nucleopore). The liposomes were extruded nine times at 65 °C using pressurized nitrogen at 400 psi.

Aggregated lipids, residual multilamellar vesicles, and titanium particles (in sonicated vesicles) were removed by centrifugation at 3000 rpm on a fixed rotor table-top centrifuge (model Centra-4, International Equipment Co., Needham Heights, MA) for 20 min and filtering the solution through a 0.2 μ m Acrodisc filter (Gelman Sciences, Ann Arbor, MI).

Radiolabeling of Antibody. The monoclonal anti-d-dimer IgG was labeled with [¹⁴C]formaldehyde by reductive methylation (Jentoft and Dearborn, 1983). [¹⁴C]Formaldehyde (10 μ Ci) and 50 μ L of 0.1 M sodium cyanoborohydride (NaCNBH₃) were added to 1 mg of antibody in 0.5 mL of PBS (phosphate buffer saline) buffer. The reaction was carried out at room temperature for 2 h. Unreacted formaldehyde and excess reducing agent were removed by size exclusion on a 10 cm Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Richmond, CA) connected to an online Econo UV monitor (Bio-Rad). The specific radioactivity of radiolabeled antibody was in the range (0.1–1) $\times 10^6$ dpm/mg of antibody.

Immobilization of Antibody on Liposomes. The radiolabeled monoclonal antibody (5-4-C) was covalently linked to liposomes using the periodate oxidation method (Heath et al., 1980; Nakane and Kawaoi, 1974). The sugar moieties in 1 mg of antibody (1–2 mg/mL) in 0.05M citrate buffer (CB) (pH 6.0) were oxidized with 0.1 M sodium periodate for 30 min at 25 °C in dark with gentle stirring. Excess periodate was neutralized by 0.32 M ethylene glycol for 1 h at 25 °C. Unreacted reagents were removed by size exclusion chromatography on a Bio-gel P-6 column, and the activated antibody was added to 1 mL of 3 mg of lipid/mL of liposomes. The reaction was carried out at room temperature for 2 h, and the pH was maintained at 8.4 by adding 0.1 N NaOH. 100 μ L of 20 mg/mL sodium cyanoborohydride (NaCNBH₃) was added, and the solution was left for 16–18 h at 4 °C. The sample was applied to a 100 \times 1.5 cm size exclusion column packed with Sepharose CL-6B and equilibrated with 0.05 M borax/borate buffer at pH 8.4. The flow rate was maintained at 0.5 mL/min using a peristaltic pump, and 1 mL fractions were collected on a Gilson FC 204 automatic fraction collector. The fractions were analyzed by measuring radioactivity in a liquid scintillation counter

and visually monitoring the presence of orange color (due to the presence of fluorescein-PE) in the samples.

Characterization of Liposomes with Immobilized Antibody. The concentration of phospholipid in a liposome sample, and ultimately the liposome concentration, was determined by a phosphate assay using the method of Chen et al. (1956). The detailed protocol is described elsewhere (Singh et al., 1995a). The hydrodynamic radii of the liposomes before and after immobilization were determined using quasi-elastic light scattering (QLS). The liposome solutions were centrifuged at 3000 rpm for 15 min to remove dust particles. Liposome solution (1–2 mL) was pipetted into a clean borosilicate glass tube and placed in a temperature-controlled decaline bath situated in a BI-200 (Brookhaven) goniometer. Measurements were performed at a 90° scattering angle using a Coherent Innova 70-3 argon-ion laser in conjunction with an EMI 9863/350 photomultiplier tube. The QLS data were analyzed by a BI-9000AT digital correlator having 164 real-time data channels. A size distribution characterized by a mean diameter and a variance was obtained using the constrained regularization method (CONTIN) of Provencher (1982a,b).

Estimation of the Number of Antibody Molecules per Liposome. The number of antibody molecules immobilized per liposome could be estimated knowing the number of lipids per liposome, the lipid concentration and the antibody concentration in the liposome solution. The lipid concentration was ascertained by phosphate assay as mentioned earlier. The antibody immobilized on the liposome surface was radiolabeled, and hence, the antibody concentration was determined by measuring the radioactivity (DPM/mL) of the liposome sample. The number of lipids per liposome for spherical unilamellar liposomes of radius R with bilayer thickness t and an average area per lipid molecule A , is given by

$$\frac{\text{lipids}}{\text{liposome}} = \frac{4\pi}{A} [R^2 + (R - t)^2] \quad (1)$$

The liposome radius R was determined by QLS. The bilayer thickness, t , was assumed to be 40 Å (Israelchivili and Mitchell, 1975). The average area per lipid molecule was calculated using values of 71 Å², 41 Å², and 19 Å² for phosphatidylcholine, phosphatidylethanolamine, and cholesterol projected head areas, respectively (Israelchivili and Mitchell, 1975). The average value of area per lipid obtained for these liposomes was 44.2 Å²/lipid.

Preparation of Fluor–Antibody Conjugate. Isothiocyanate derivative of fluorescein readily reacts with the amines in an antibody at an alkaline pH (>9) to form thiourea bonds which are moderately stable (Goding, 1976; The and Feltkamp, 1970). Monoclonal IgG 5-4-C (1 mg) was reacted with FITC in molar fluor/protein ratios varying from 6 to 40. Dry FITC was first dissolved in dimethylformamide (DMF) to obtain a concentration of 2 mg/mL. The required volume of this solution was added dropwise to the protein solution containing 1 mg of antibody in 0.5 mL of carbonate/bicarbonate buffer (pH 9.6), taking care that the final DMF concentration did not exceed 15% by volume. The pH was adjusted to 9.4–9.5 using 0.1 M NaOH, and the reaction was carried out in an amber vial with gentle stirring for 2 h at room temperature. The separation of excess FITC was done in a desalting column equilibrated with 0.05 M borax/borate buffer at pH 8.5. The fractions were monitored using an online UV detector by measuring the absorbance at 280 nm. The purified conjugate was stored at 4 °C in the dark. The number of fluors attached to one antibody was calculated using the following relations (Molecular

Probes, FITC labeling kit):

$$\text{fluors per Ab} = \frac{[\text{FITC}]}{[\text{antibody}]}, \text{ where} \quad (2)$$

$$[\text{FITC}] = A_{495}/68\,000 \quad (3)$$

$$[\text{antibody}] = \frac{A_{280} - 0.3A_{495}}{1.4 \times 150\,000} \quad (4)$$

Protocol for Microtiter Plate Preparation. The inner 60 wells of a 96-well costar microtiter plate were coated with monoclonal antibody 8-8-G. The outer wells exhibited high well-to-well variation, possibly due to uneven temperature distribution. Coating solution (150 μL) containing 40 $\mu\text{g/mL}$ antibody in 50 mM carbonate/bicarbonate buffer (pH 9.6) was dispensed in each well. After covering with a plate sealer, the plate was incubated at 4 $^{\circ}\text{C}$ on a plate-shaker at 500 rpm for 18 h. The coating solution was aspirated and the wells were blocked with 300 μL /well of 1 wt % BSA for 3 h at 4 $^{\circ}\text{C}$. The wells were aspirated dry and then washed two times with PBS.

Protocol for the Sandwich Fluorescence Immunoassay with Liposomes. The stock solution of d-dimer antigen was serially diluted in 1 wt % BSA in PBS (pH 7.4). Either diluted samples or control (100 μL) were applied to the coated wells. The plate was covered with a plate sealer and incubated at 37 $^{\circ}\text{C}$ for 1 h in a mechanical convection incubator. After washing the plate four times with PBS, 100 μL /well of antibody-fluorescein-liposomes were added and the plate was incubated at 37 $^{\circ}\text{C}$ for 1 h. The wells were washed six times with PBS to remove the unbound and nonspecifically bound liposomes. Lysis buffer (100 μL /well) containing 1 mM of the nonionic surfactant C_{12}E_5 in borax/borate (pH 8.5) was added, and the plate was incubated at room temperature for 30 min in a reciprocating shaker at 120 rpm. The fluorescence signal was read in a fluorescence plate-reader (Cytofluor 2350, Millipore, Bedford, MA) using an excitation filter of 485 nm (bandwidth 20 nm) and emission filter of 530 nm (bandwidth 25 nm). The sensitivity level was adjusted to maximize the specific signal (total signal – background due to polystyrene wells).

Protocol for the Sandwich Fluorescence Assay with FITC–Antibody Conjugate. The assay was performed exactly the same way as described in the previous section for liposomes except with a modification in the lysis step. When FITC-antibody binds to d-dimer, which in turn is bound to the antibody immobilized on the polystyrene surface, its fluorescence is considerably quenched. Several alkaline denaturants such as 6 M guanidine hydrochloride (pH 8.5), 0.1 M NaOH, 0.2 M NH_4OH , and 10% SDS (pH 8.5) were used in an attempt to disrupt the antigen–antibody complex allowing FITC–antibody conjugate to return to the bulk solution. In the assay protocol, after washing to get rid of nonspecifically bound FITC–Ab conjugate, the plate was incubated with 100 μL of a denaturant for 30 min at room temperature with gentle shaking. The fluorescence signal was then read in the fluorescence plate-reader.

Results and Discussion

Liposome Preparation and Characterization. Unilamellar liposomes were prepared with one of the constituents being phospholipids (DPPE) conjugated with the fluorescent marker fluorescein. The liposomes contained 15 mol % fluorescein–DPPE (Fl–DPPE) and, depending on their size, 7000–20 000 fluors per liposome.

In order to provide the reactive amines to which antibodies can be conjugated, 5 mol % of DMPE was also incorporated into the liposomes. The presence of fluorescein (MW 332) makes the head groups of DPPE bulkier relative to unmodified DMPE head groups and hence the terminal amines on the DMPE might be partially or completely blocked sterically. This was circumvented by using a caproylamine–DMPE consisting of a six-carbon spacer between the phosphate and the amine group, thus extending the amine away from the liposome surface. The total PE concentration in the liposomes, i.e. sum of Fl–DPPE and DMPE, was limited to 20 mol %. Theoretically, it is possible to obtain a higher number of fluors per liposome by using a higher percentage of PE, but a reduction in the concentration of either PC or cholesterol tends to destabilize the liposomes as inferred from an increase in size upon storage.

The presence of fluorescein in the liposomes interfered with the size measurement by QLS. The argon-ion laser used for QLS has an emission wavelength of 514.5 nm and is close to the maximum excitation wavelength of fluorescein at 495 nm. Potentially, some of the incident intensity could be used for exciting the fluor and thereby decreasing the intensity available for scattering. Fluorescein has an emission maximum at 520 nm. The signal received by the photomultiplier tube would then be a combination of two components: scattered intensity due to liposomes and emitted intensity due to fluors. The interference due to fluorescence will be proportional to the concentration of fluors in the sample, assuming the fluors are not concentration quenched. In the size measurement of fluorescein–liposomes, the interference was minimized by using the lowest concentration of liposomes required to give an adequate signal-to-noise (S/N) ratio. The extent of interference was estimated by measuring the size of monodisperse latex particles of diameter 40 nm in the presence of various concentrations of fluorescein isothiocyanate. A typical fluorescein concentration in liposomes would be in the 1–10 mM range, and at this concentration, the error in the mean diameter measurement was estimated at 10–25% on the low side of actual diameter.

Concentration Quenching of Fluors in Liposomes. The fluorescein molecules in a liposome are in very close proximity to each other and undergo concentration quenching. Fluorescein, like other xanthene dyes, dimerizes and the dimer is nonfluorescent. There have been three mechanisms postulated for concentration quenching: (1) dimerization of dye, (2) energy transfer from monomers to dimer, and (3) collisional quenching. With fluorescein–PE, the collisional quenching can be ruled out as the fluors are too far apart to collide within a fluorescence lifetime of 4.6 ns (MacDonald, 1990). Therefore, formation of nonfluorescent dimer and energy transfer without emission to the dimers account for the concentration quenching of fluorescein in liposomes (Chen and Knutson, 1988; MacDonald, 1990). The extent of quenching was determined by dissolving (and hence diluting) the liposomes with the nonionic surfactant C_{12}E_5 (pentakis(ethylene glycol) mono-*n*-dodecyl ether). The liposome concentration was maintained constant at 20 μM total lipids, and the surfactant concentration was increased from 0 to 1 mM. The results are presented in Figure 2. The intact liposomes yielded a signal six times lower than the case in which liposomes were completely disrupted, indicating that fluorescein is quenched by 84% in an intact liposome. Hence, the liposomes, when used in the immunoassay, need to be disrupted to maximize the fluorescence signal. Addition of surfactant progres-

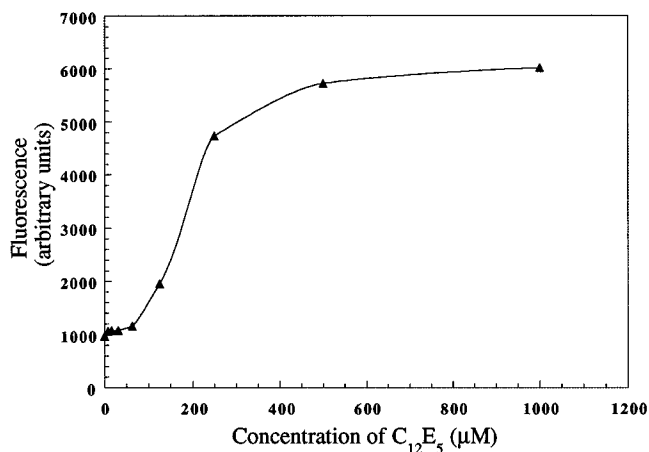


Figure 2. Dequenching of the fluorescence signal of liposomes on addition of a nonionic surfactant C₁₂E₅ (critical micellar concentration of C₁₂E₅ = 65 μM).

sively caused fluorescence dequenching as illustrated in Figure 2. Initially the increase in fluorescence upon surfactant addition is gradual until the critical micellar concentration (CMC) of C₁₂E₅ (65 μM) is reached. Dequenching in this region results from intercalation of C₁₂E₅ monomers into the liposomes which increase the separation between the fluorescein-PE. As the concentration of C₁₂E₅ is increased beyond the CMC, liposomes begin to solubilize into micelles leading to a rapid dilution of fluorescein-PE. This region is marked by a rapid increase in the fluorescence signal. Once the surfactant concentration is large enough to solubilize the bulk of liposomes, increase in fluorescence upon surfactant addition is marginal. Finally, a saturation point is reached at a C₁₂E₅ concentration of 1 mM, at which the fluors are completely dequenched. On the basis of these findings, 1 mM C₁₂E₅ was used to disrupt the liposomes prior to fluorescence measurement in the sandwich immunoassay.

Attachment of Monoclonal IgG to Liposomes. Monoclonal antibody against d-dimer (5-4-C) was immobilized on the preformed fluorescein-liposomes using periodate chemistry. Periodate coupling is a relatively inefficient method of conjugation with an efficiency of about 10%; however, the conjugated antibody molecules are least affected in terms of antigen-binding capacity compared to some other chemistries. The conjugation takes place by forming a secondary amine bond by reacting the amine of a PE to a carbohydrate moiety of an IgG molecule. The carbohydrate groups are located solely in the F_c fragment of the IgG, and thus, the binding does not affect the F_{ab} fragments. In most other conjugation techniques, amines in the antibody molecule are used. Since there are lysine residues located in the F_{ab} fragment that can participate in conjugation, the antigen-binding activity can be reduced to some extent. Periodate coupling also yields immobilized antibody molecules with F_{ab} regions extending away from the liposome surface. This minimizes any steric hindrance in binding to an antigen. After immobilization the antibody-fluorescein-liposomes were isolated on a size exclusion column where they eluted in the void volume. Table 1 shows the antibody reaction conditions and the results of three different immobilization reactions. The reaction conditions were optimized to obtain 10–20 antibodies immobilized per liposome. In order to bind efficiently to the antigen in a solid-phase immunoassay, 8–10 antibodies per liposomes are sufficient, as reported earlier (Singh et al., 1995a). The liposomes exhibited a size increase upon antibody conjugation. The end-to-end

Table 1. Reaction Conditions and Results of Antibody Immobilization on Liposomes

	batch		
	1	2	3
	Reaction Conditions		
mg/mL IgG	0.22	0.22	0.43
vesicle concn (mM lipids)	0.33	0.33	0.65
vesicle size (Å)	603 ± 15	603 ± 15	754 ± 18
	Results		
vesicle size (Å)	445 ± 13	798 ± 12	1013 ± 19
IgG/vesicle	17.3	10.3	18.6
fluor/vesicle	6800	12279	20238

vertical distance between F_{ab} domain and F_c region in an antibody molecule is approximately 100 Å. Assuming that the antibodies are immobilized on liposomes through F_c portion, the increase in the diameter of a liposome after antibody attachment will be 200 Å. This compares reasonably well with the size increase observed in immobilization reactions (Table 1) where liposomes undergo an increase in diameter of 195 and 259 Å for batches 2 and 3, respectively.

Preparation and Characterization of FITC-Antibody Conjugate. A control assay was done using an antibody labeled with a fluor. An antibody can be labeled with a fluor such as FITC to a varying extent depending on the fluor-to-antibody ratio in the reaction mixture. Antibodies are relatively sensitive to substitution as there are reactive amines present in or near the binding sites. Excessive labeling can reduce the ability of an antibody to bind to its antigen. A high degree of labeling also leads to concentration quenching of fluors. Haagland (1992) reported that FITC is about 50–70% quenched on an IgG with a FITC/IgG mole ratio of 5. In the present study the effect of fluor/protein (F/P) ratio in the reaction mixture on the degree of labeling was examined. The results are shown in Figure 3. At an initial F/P ratio of 6, an average of only 1.3 fluors per antibody were obtained in the product. The F/P ratio in the reaction mixture was increased gradually to 40, resulting in as many as eight fluors on one antibody. At this ratio, antibody also began to reach labeling saturation as reflected by the flattening of the curve in Figure 3. The conjugates with different degrees of labeling were used in an immunoassay for detection of d-dimer (Figure 4). The conjugates with only 1.3 and 1.8 fluors per antibody did not produce adequate signal, indicating that the number of fluors per antibody was too small. The signal increased, as the number of FITC conjugated to an antibody was increased reaching a maximum at an FITC/antibody ratio of 8.

The last step in the sandwich assay with labeled antibody was binding of labeled antibody to the antigen. The antigen molecules, in turn, were bound to the antibody immobilized on the plate. Hence, the environment around the bound fluor-conjugated antibody would be comprised mostly of the blocking protein, bovine serum albumin (BSA). This may alter the pH and ionic strength very significantly in the close proximity of the fluor compared to the bulk solution. Fluorescein is a very pH sensitive fluorophore and its fluorescence is maximized in basic solutions (pH > 8). Hence, fluorescence was measured in the presence of borax/borate buffer at pH 8.5. At this pH, the BSA (pI = 4.9) will be predominantly negatively charged thus attracting the cations from bulk solution. The accumulation of cations leads to a local reduction in pH in the vicinity of antibody molecules, resulting in a considerable reduction in fluorescence. In fact, this unique property of fluorescein to undergo quenching or dequenching in response to pH changes in

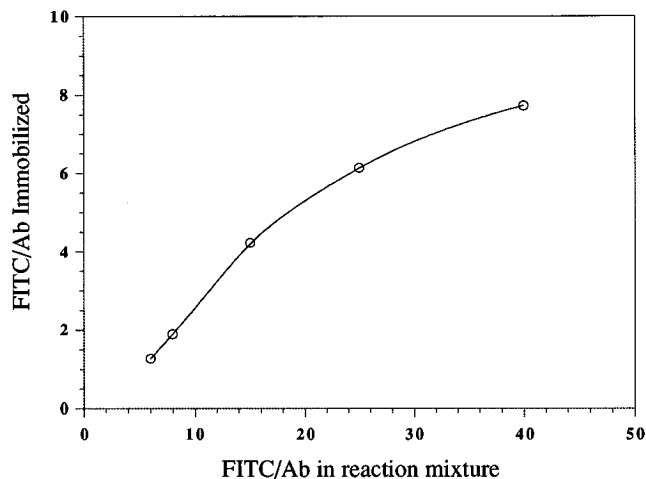


Figure 3. Effect of FITC/antibody molar ratio in the reaction mixture on the degree of labeling in preparation of the FITC-antibody conjugate.

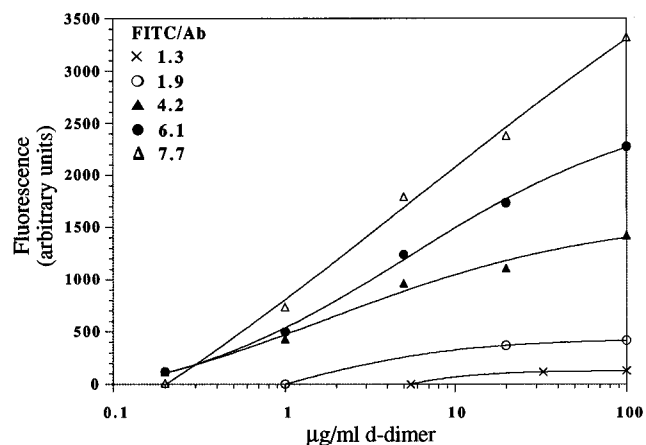


Figure 4. Effect of the degree of labeling of the antibody on the fluorescence signal in a sandwich assay. The conjugates with 1.3 and 1.9 FITC per antibody do not yield adequate signal.

microenvironment has been exploited to develop biosensors (Lee et al., 1991) and to monitor intracellular pH (Thomas et al., 1986). In the assay using FITC-antibody conjugate, the fluors were quenched by 70–75% if the labeled antibody remained attached to the surface. A variety of chaotropic agents were used to disrupt the antigen-antibody bond, thus allowing the fluor-antibody conjugate to return to the bulk environment. As the bulk solution has a more favorable pH and ionic strength conditions, the fluorescence increases upon antibody-antigen bond disruption. Figure 5 shows the effect of adding the chaotropic agents on the signal generated during the immunoassay. All the chaotropic agents used were at alkaline conditions to maximize the emission signal. The fluorescence signal was the least in the presence of plain buffer (borax/borate, pH 8.5) as fluor-Ab conjugates remained bound to the antigen on the surface. When denaturants were added, the fluor-Ab conjugate came off the surface and the signal increased. SDS (10%) worked the best, followed by 6 M guanidine hydrochloride, 0.2 M NH_4OH , and 0.1 M NaOH.

Fluorescence Immunoassays with Liposomes and FITC-Ab Conjugate. The antibody-fluorescein-liposomes and the FITC-antibody conjugate were used in heterogeneous sandwich assays to detect d-dimer. Both conjugates performed well in the assay, and Figure 6 illustrates a typical plot of fluorescence signal against dilutions of d-dimer (using batch 3 of liposomes and fluor-antibody batch with FITC/Ab = 8). The curves

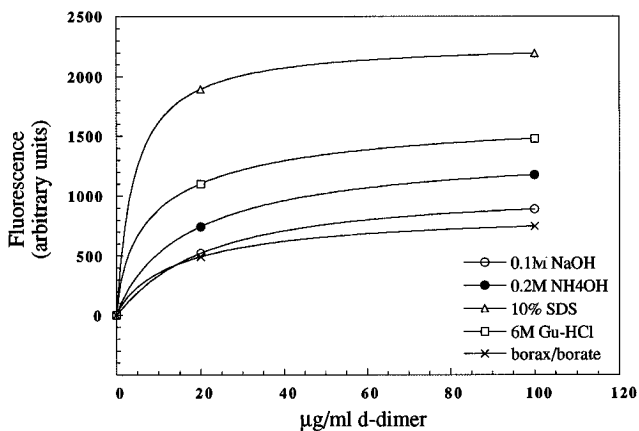


Figure 5. Disruption of antigen-antibody bond with various chaotropic agents as observed by an increase in the fluorescence signal in the sandwich immunoassay for d-dimer. The fluors are 70–75% quenched if fluor-antibody remains bound to the antigen on the plate surface.

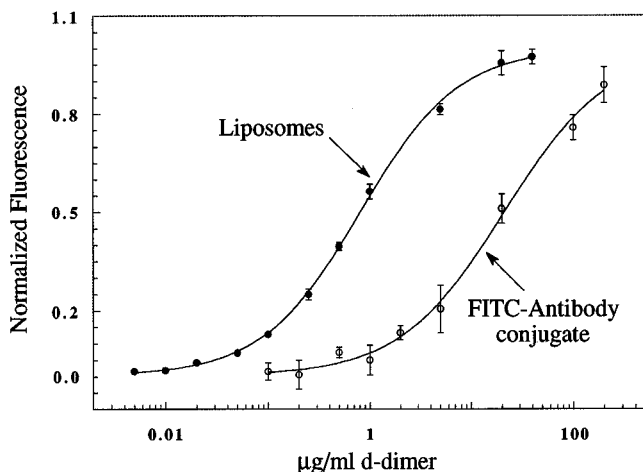


Figure 6. Comparison of the sandwich solid-phase fluorescence immunoassays with fluorescent liposomes (batch 3) and with fluor-antibody conjugates (with FITC/Ab = 8). The filled circles and open circles represent the experimental calibration points for liposome assay and FITC-Ab conjugate assay, respectively. The solid lines represent the curve fits using logistic model. The values of model parameters are listed in Table 2. Signal is normalized by dividing the fluorescence by the maximum fluorescence obtainable. The liposomes yield a detection limit 120-fold lower than the fluor-antibody conjugate.

have a sigmoidal shape as expected with a sandwich immunoassay. The data was fit by a four-parameter logistic model (O'Connell et al., 1993) of the form

$$F = \beta_2 + \frac{\beta_1 - \beta_2}{1 + ([A]/\beta_3)^{\beta_4}} \quad (5)$$

where F and $[A]$ are fluorescence signal and antigen (d-dimer) concentration, respectively. β_1 is the asymptote as the antigen concentration $[A] \rightarrow 0$, β_2 is the asymptote as $[A] \rightarrow \infty$, β_3 is the predicted concentration at the response halfway between the two asymptotes, and β_4 is related to the slope. The logistic model fit the experimental values very well for both the liposome and labeled-antibody assays as indicated by the regression coefficients of 0.9996 and 0.9977 for the liposome and labeled-antibody assays, respectively. The numerical values of the model parameters for the assays depicted in Figure 6 are listed in Table 2. For qualitative comparison of the two assays, the signals were normal-

Table 2. Logistic Model Parameters of Liposomal (Batch 3) and Fluor–Ab Conjugate (Batch with FITC/Ab = 8) assays Shown in Figure 6

	assay with liposomes ^a	assay with FITC–Ab conjugate ^b
β_1	5.5	8.8
β_2	2558	2988
β_3	0.816 $\mu\text{g/mL}$	21.4 $\mu\text{g/mL}$
β_4	0.898	0.848
R^2	0.9996	0.9977

^a The sensitivity level in the fluorescence plate-reader was set at 4. ^b The sensitivity level in the fluorescence plate-reader was set at 6.

ized with respect to the parameter β_2 (the predicted maximum signal) and plotted on the same graph (Figure 6).

For quantitation purposes the minimum detectable concentration (MDC) was defined as the lowest concentration of analyte that results in an expected response (fluorescence signal) that is two standard deviations higher than the response at zero concentration. Figure 7 shows a replot of the calibration curve for liposomal assay where the fluorescence is measured using a higher sensitivity level in the fluorescence plate-reader. By increasing sensitivity, the voltage delivered to the photomultiplier tube is increased. This amplifies the signal significantly in the lower concentration range of the calibration curve. Using the logistic model, the average values of MDC over several assays were 5.6 and 674 ng/mL for liposome and FITC–Ab conjugate, respectively (Table 3). Hence, the use of liposomes leads to detection of d-dimer at a concentration 120 times lower than what could be detected using a conventional fluor–antibody conjugate. The liposomes carrying 10 000–20 000 fluors have 1250–2500 times higher number of fluors than a fluor–antibody conjugate carrying eight fluors per conjugated antibody. Consequently, the improvement in detection limit should be 1250–2500-fold. However, liposomes are large in size and the binding of one liposome to the antigen on the plate surface renders approximately 10 neighboring antigen molecules spatially unavailable for binding. The fluor–antibody conjugate is comparable in size to the antigen and does not lead to this problem. The two factors combined predict that liposomes should perform approximately 125–250-fold better in terms of detection limit compared to the fluor–antibody conjugate. In reality, they performed 120-fold better, which agrees well with the theoretically predicted limits. Liposomes also provide a signal enhancement compared to the fluor–antibody conjugate at any given d-dimer concentration. To get comparable signals in the two assays, fluorescence in the liposome assay was measured at a sensitivity level of 4 while the signal in the fluor–Ab assay was measured at a sensitivity level of 6. An increase in sensitivity level by two steps corresponds to signal enhancement by a factor of 12. Hence, liposomes yielded signals at least 1 order of magnitude higher than the fluor–Ab conjugate. This was also confirmed by measuring signals at the same sensitivity level for both the assays. At the same sensitivity level, liposomes yielded signals 10–20 times higher than fluor–Ab conjugate.

β_3 has been used as a measure of affinity (Azimzadeh and Rongenmortel, 1990) and reciprocal of β_3 was used as the apparent association constant (K_a). The molecular weight of d-dimer was assumed to be 228 kD in the calculations. When polymerized fibrin is degraded, d-dimer is the final and the smallest product but there are intermediate products present too containing multiple

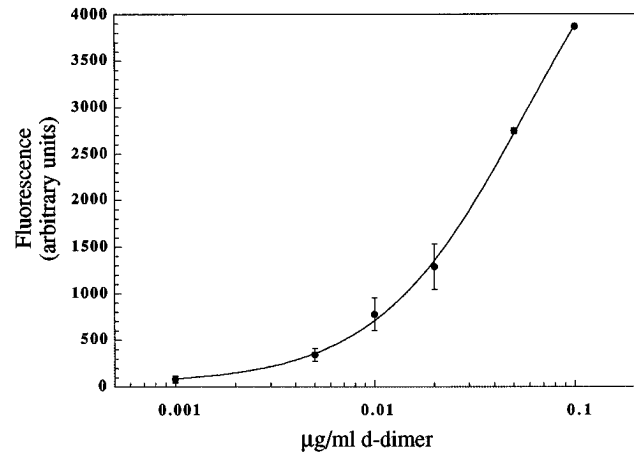


Figure 7. Remeasurement of the fluorescence signal in the lower concentration range of the iposome assay depicted in Figure 6 using a higher sensitivity level (higher PMT voltage) in the fluorescence plate-reader. The average value of MDC is 0.0059 $\mu\text{g/mL}$ of d-dimer.

Table 3. Comparison of Various Immunoassays

	MDC ^a (ng/mL d-dimer)	K_a ^b (M^{-1})	% nonspecific signal
Enzyme			
liposomes	2.0	4.2×10^9	10–33%
enzyme–Ab	20	1.1×10^9	5–14%
Fluorescence			
liposome	5.6	3.98×10^8	2–12%
fluor–Ab	674	1.1×10^7	<10%

^a Minimum detectable concentration—the lowest concentration of analyte that results in an expected response (fluorescence signal) that is two standard deviations higher than the response at zero concentration. ^b Apparent association constant—inverse of parameter β_3 .

number of d-dimer units. It has been assumed that these intermediates are present in small concentration, and hence, the average molecular weight of the antigen is the same as the molecular weight of d-dimer. The average K_a values were 3.98×10^8 and $1.1 \times 10^7 M^{-1}$ for the liposomes and the FITC–antibody, respectively. The 40-fold higher association constant implies that liposomes bind more strongly to the antigen than the fluor–Ab conjugate. This can be attributed to multiple binding of liposomes to the antigen on the plate surface. Liposomes carry 10–20 antibodies on the surface, and hence, a single liposome can bind to more than one antigen at the same time. This phenomenon has also been observed in prior work (Jones et al., 1993a,b; Locasio-Brown et al., 1990).

Earlier, the authors developed an enzyme-based immunoassay using liposomes and enzyme–antibody conjugate for d-dimer (Singh et al., 1995). In Table 3, the results from enzyme-based assays are presented together with the findings of fluorescence-based work, enabling us to compare the two assays on the same antigen–antibody system. The experimental results in all assays were fit by the four-parameter logistic model. The enzyme-based immunoassay using liposomes performed slightly better than the fluorescence-based liposome assay in terms of detection limit. On the other hand, the enzyme–antibody conjugate worked considerably better than the fluor–antibody conjugate. The improvement in detection limit obtained by using liposomes in immunoassays were 120 and 10 times, respectively, for the fluorescence and enzyme cases. Possible explanations for inferior improvement in the case of enzyme–antibody–liposomes are: (1) the ratio (enzymes per enzyme–Ab–liposome)/

(enzymes per enzyme–Ab conjugate) is smaller than the ratio (fluors per fluor–Ab–liposome)/(fluors per fluor–Ab conjugate) and (2) the background or nonspecific signal is considerably higher with enzyme–Ab–liposomes, thus masking the specific signal at lower d-dimer concentrations. The apparent association constants were higher with liposomes compared to marker–antibody conjugates in both enzyme- and fluor-based assays, confirming the multivalent binding of liposomes to the antigen.

Conclusions

Liposomes have been used in immunodiagnosics to provide signal amplification and to lower the detection limit for analyte. Most of the attention has been concentrated on developing homogeneous assays where binding of antigen to an antibody triggers a complement, cytolytic, or phase transition mechanism leading to lysis of liposomes. The liposomes used for this purpose carry entrapped reporter molecules which are released following the lysis, and the signal can be related to the analyte concentration. The advantage of this format is speed and simplicity. The disadvantages are nonspecific lysis of liposomes in the presence of serum, the relative instability of liposomes in serum, and the leakage of marker molecules (especially uncharged small fluors) upon storage. In the present work, a heterogeneous microtiter plate assay was used where liposomes never come in direct contact with the serum. The assay is based on a noncompetitive or sandwich format where all the binding events are allowed to reach equilibrium. This makes the assay more time consuming, but the sensitivity is improved significantly when compared to homogeneous inhibition (or competitive) assays. To avoid the leakage of signal molecules from liposomes, they were attached to the bilayer of the liposomes instead of being entrapped in the aqueous core. The liposomes with entrapped markers have to be maintained in aqueous solution to conserve the structure, making long-term storage expensive. On the other hand, surface attachment of marker molecules makes it possible to lyophilize the liposomes without any loss of markers upon resolubilization. This may increase the shelf-life of liposomes tremendously. The detailed study of liposomal stability was not undertaken in this study, but liposomes did not undergo any significant aggregation (less than 10% lipid loss) for up to 3 months when stored in liquid solution at 4 °C. Liposomes carrying 10 000–20 000 fluor molecules and 10–20 antibody molecules on the surface were successfully prepared. For comparison, an antibody-fluor conjugate was prepared by directly linking 4–8 fluors to the antibody molecule. The fluor chosen was fluorescein due to its high quantum yield and reasonable stability to quenching due to ambient light (Guilbault, 1990). The liposomes performed better than fluor–antibody conjugate in terms of both lowering the detection limit (by 120-fold) and amplifying the signal (by 1 order of magnitude). Liposomes also exhibited approximately 40-fold higher association constant relative to the fluor–Ab conjugate.

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