Anal. Chem. **2002,** *74,* 1493−1496

A Strip Liposome Immunoassay for Aflatoxin B_1

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A technique has been developed for the preparation of aflatoxin B_1 (AFB_1)-tagged liposomes encapsulating a visible dye. These liposomes have several useful potential analytical applications, one of which is demonstrated. A simple plastic-backed nitrocellulose strip is the basis for an assay for detecting AFB_1. Samples containing aflatoxin B_1 are allowed to migrate by capillary action along the strip into a zone containing immobilized antibodies; then aflatoxin B_1-tagged, dye-containing liposomes are allowed to migrate into the same area, filling any remaining antibody sites. The liposomes that bound to the antibody zone exhibit an intense purplish pink color whose optical density is inversely proportional to the aflatoxin concentration in the sample. The device is capable of detecting aflatoxin B_1 at levels down to 20 ng and could serve as a rapid procedure for visual screening of agricultural and food samples for AFB_1 or, with densitometry, as an inexpensive quantitative assay.

There is a need for rapid and inexpensive field assays for screening food and animal feed samples for mycotoxins. Immunoassays, which offer sensitivity, speed, and simplicity of operation, provide potential solutions for this need. Radioimmunoassays,[1−2] antibody affinity columns,[3] and enzyme-linked immunosorbent assays (ELISA)[4−7] have been developed for aflatoxin B_1 (AFB_1). Some of the immunoassays have been approved and adopted by the AOAC.[8] The Aflatest (Vicam L.P., Watertown, MA) immunoaffinity column is widely used in the USDA and commodities at points of sale. However, the cost for a nonreusable, single affinity column is about $7 U.S.

A liposome immunoassay (LIA), using fluorescent dye-loaded liposomes whose surface has been tagged with the antigen of interest, has been developed for a variety of assays, particularly a flow injection automated procedure.[9−13] As part of a project on the development of a flow injection liposome immunoanalysis (FILIA) system for AFB_1, a procedure for tagging liposomes with AFB_1 using a bridging molecule was developed to overcome hydrophobic disruption of the liposome structure. These liposomes proved useful in a preliminary development of a test strip semiquantitative assay for AFB_1 that has potential as a rapid and inexpensive field test.

EXPERIMENTAL SECTION

Reagents and Materials. All inorganic chemicals and organic solvents used were reagent grade or better. AFB_1, polyclonal antibody against AFB_1, cholesterol, poly(vinylpyrrolidone) (PVP, MW 10 000), Tween-20, gelatin, Sephadex G-50, triethylamine (TEA), Trizma base (tris[hydroxymethyl]aminomethane, Tris), and *n*-octyl β-D-glucopyranoside (OG) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylethanolamine (DPPE) and sulforhodamine B were purchased from Molecular Probes (Eugene, OR). Polycarbonate syringe filters of 3-, 0.4-, and 0.2-μm pore size were purchased from Poretics (Livermore, CA), and plastic-backed nitrocellulose membranes with pore size of >3 μm were from Schleicher and Schuell (Keene, NH). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL). N-Succinimidyl-*S*-acetylthioacetate (SATA), 4(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH), and *N*-ethylmaleimide (NEM) were purchased from Pierce (Rockford, IL). Carnation nonfat dry milk (NDM) powder was obtained locally.

Preparation of Test Strips. A microprocessor-controllcd TLC sample applicator, Linomat IV (CAMAG Scientific Inc., Wrightsville Beach, NC), was used to immobilize AFB_1 antibody on the plastic-backed nitrocellulose membranes. The membrane was cut into 8 × 15.7 cm sheets, which were then mounted on a mobile platform that moved at a constant rate in front of the airbrush used to spray the antibody solution at concentration of 2 mg/mL. If a TLC sample applicator is not available, an alternative procedure may be used in which antibody solution is manually dot-blotted

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10.1021/ac010903q CCC: $22.00
Published on Web 03/06/2002

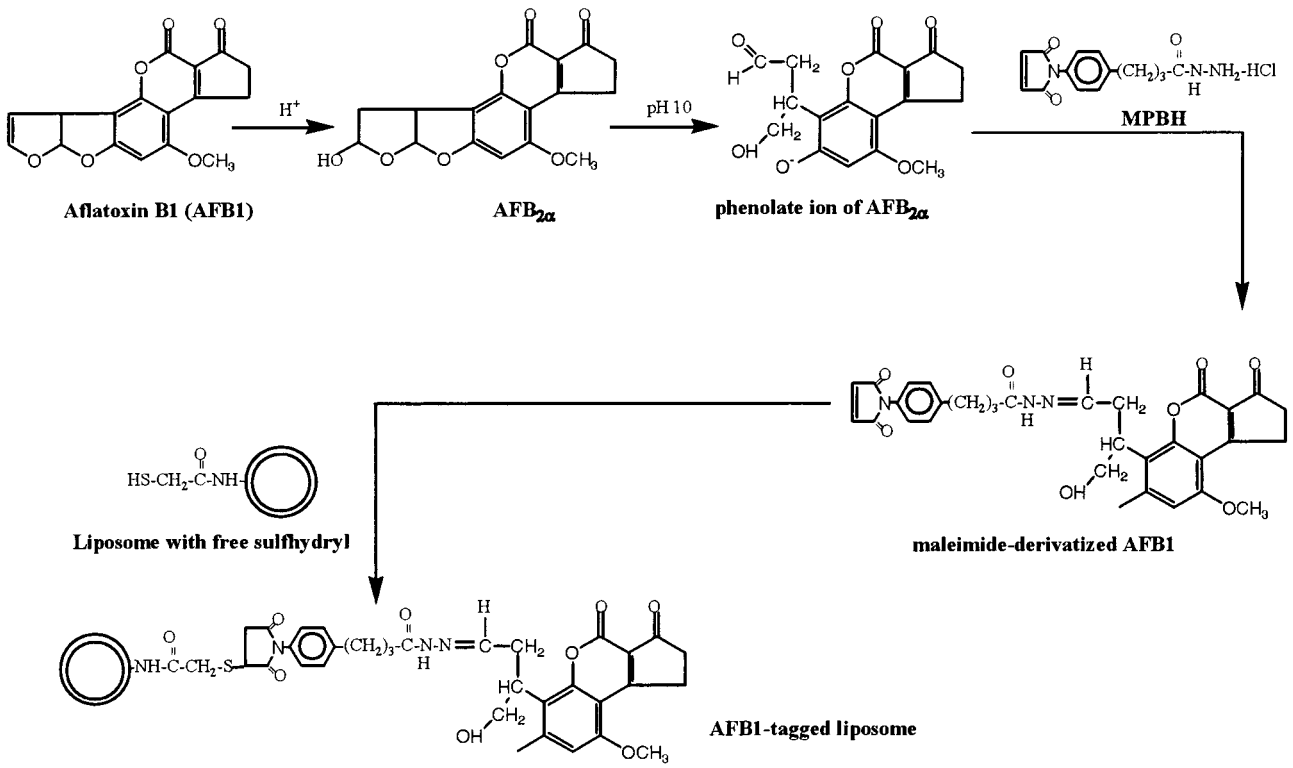

Figure 1. Conjugation of aflatoxin B_1 to liposome (drawing not to scale).

onto the membrane. The antibody coat bands were allowed to air-dry in the hood for 5 min and further dried under vacuum at room temperature for 1.5 h. The coated nitrocellulose sheet was then immersed in a blocking solution consisting of 0.5% poly(vinylpyrrolidone) and 0.07% nonfat dry milk in Tris-buffered saline (TBS) at pH 7.0 for 45 min on a rotating shaker, followed by drying under vacuum for at least 6 h. The prepared sheets were then cut into 0.5 × 8 cm strips using a paper cutter, producing strips with an antibody zone 1.5 cm above the bottom of the strip.

Preparation of AFB_1-Tagged Liposomes Containing Dye: Overcoming the Hydrophobicity of AFB_1. To provide antigenic sites on the surface of the liposomes, it was necessary to conjugate AFB_1 to DPPE, a lipid component of liposomes. The reaction sequence is illustrated in Figure 1. A multistep conjugation method was employed in which SATA was used as the first coupling agent to introduce a sulfhydryl group into DPPE via an amide linkage. The conjugate, DPPE−acetylthioacetate (ATA−DPPE), was then incorporated into the liposomes, providing protected sulfhydryl groups on the surface of the liposomes. The second cross-linker, MPBH, which contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide on the other end, was used to derivatize an activated aldehyde group generated on the AFB_1. AFB_1 was subsequently coupled to the liposome via maleimide−sulfhydryl interaction.

A 5-mg sample of DPPE (7.2 μmol) was suspended in 1 mL of 0.7% triethylamine in chloroform and sonicated for 1 min at 45 °C under nitrogen. Approximately 3.5 mg of SATA (14 μmol) was added to the DPPE suspension and sonicated for 3 min under nitrogen at 45 °C. The reaction flask was capped and placed on a shaker at room temperatue for 20 min or until the solution became clear. Organic solvent was removed by evaporating under nitrogen at 45 °C, followed by three reconstitutions with chloroform and evaporations to make sure no trace of triethylamine remained. Finally, the dry residue was reconstituted with 1 mL of chloroform.

Dye-loaded liposomes were prepared by the reversed-phase evaporation method.[14,15] The lipids mixture consisted of a 5:5:0.5:4 molar ratio of DPPC, cholesterol, DPPG, and DPPE−acetylthioacetate. The lipids were dissolved in 8 mL of a solvent mixture consisting of 6:6:1 volume ratios of chloroform, isopropyl ether, and methanol, followed by a 1-min sonication at 45 °C under nitrogen. Then 1.4 mL of a warmed aqueous solution of 150 mM sulforhodamine B (SRB) in TBS, adjusted to pH 7.0−7.5 with 1 N NaOH, was added. After sonication of the solution for 6 min more, the organic solvent was removed by evaporating under nitrogen at 45 °C, leaving a dark purple, gel-like suspension of liposomes. An additional 2.6 mL of SRB−dye was added, followed by another 5 min of sonication at 45 °C. Liposomes were kept warm before passing through three different pore sizes of prewarmed polycarbonate filters (3.0, 0.4, and 0.2 μm) to produce a homogeneous suspension. The liposomes were separated from unencapsulated SRB and traces of organic solvent by gel filtration on a 1.5 × 25 cm Sephadex G-50 column at room temperature, followed by dialysis (MWCO, 12 000−14 000) in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.0 at 4 °C in the dark.

Since there is no ready-to-use functional group in the AFB_1 molecule for direct conjugation with DPPE, the derivatization of AFB_1 with a maleimide group was necessary for its attachment to the sulfhydryl liposomes. A total of 4.8 mg of AFB_1 (15.4 μmol) was dissolved in 1 mL of acetonitrile and 1 mL of 1.0 M HCl. The mixture was heated to 60 °C for 3 h with occasional mixing and

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then stood overnight at room temperature. The AF hemiacetal ($AFB_{2\alpha}$) formed was extracted into chloroform. The phenolate ion of $AFB_{2\alpha}$ was prepared by evaporating off the chloroform under nitrogen and redissolving the residue in 0.5 mL of 20% MeOH and 0.5 mL of 0.1 M sodium pyrophosphate, pH 10. A total of 8.4 mg of MPBH (23.1 μmol) was dissolved in 55 μL of DMSO, and the resultant mixture was added to the solution of phenolate ion of $AFB_{2\alpha}$. The reaction mixture was then allowed to react on a shaker for 6 h at room temperature in the dark. Concurrently, the acetylthioacetyl liposomes were deprotected by adding hydroxylamine hydrochloride (100 μmol) to obtain free sulfhydryl groups. The maleimide-derivatized AFB_1 was then reacted with sulfhydryl liposomes overnight at 4 °C. Unreacted sulfhydryl groups on the liposome were subsequently capped with *N*-ethylmaleimide. Excess unconjugated AFB_1 molecules were removed by dialysis against TBS (pH 7.0) or by ge1 filtration on a Sephadex G-50 column.

Stability Study and Characterization of Liposomes. Sulforhodamine B is a visible as well as fluorescent dye; thus, the stability (intactness) of lipsomes means the maintenance of their integrity, which can be determined by measuring fluorescence intensity before and after lysis. Almost instantaneous lysis of liposomes was observed at room temperature when a solution of *n*-octyl β-D-glucopyranoside was added; total lysis of the liposomes was achieved by addition of OG to a final concentration of 50 mM. For these fluorescence tests, the SRB dye was excited at 541-nm wavelength, and the fluorescent emmission intensity measurements were made at wavelength 596 nm. Temperature effects on liposome stability were conducted by adding 10 μL of liposome solution to 3 mL of osmotically balanced TBS, pH 7.0, in a test tube preheated to the required temperature in a water bath. The liposomes were incubated for 5 min before measurement of fluorescence intensity.

The diameter of the liposomes was measured with a Coulter LS particle size analyzer (Coulter Corp., Miami, FL) using the manufacturer's method. Liposomes were visualized by transmission electron microscopy of negatively stained preparations to check the integrity and size of the liposomes.

Test Strip Preparation and Procedure. AFB_1 is hydrophobic and can only be dissolved in a relatively nonpolar organic solvents such as chloroform or acetonitrile. However, those solvents were found to cause instability and rupture of liposomes. Thus, a procedure was designed to operate in a stepwise procedure to avoid the contact of liposomes with disruptive solvents. A 75−100-μL sample of AFB_1 sample (in acetonitrile/methanol (7:3, v/v) mixture) solution to be assayed was dispensed into a 10 × 75 mm glass test tube. The 5 × 80 mm antibody-coated test strips were attached at the top to a 3 × 50 mm strip of filter paper and inserted into the tube. The strip was left in the tube long enough (3 min) to allow the AFB_1 solution to migrate into the antibody zone. The test strip was then transferred to another test tube containing 75 μL of diluted liposome solution (the dilution varying according to preparation); the strip was left in the tube until the solution front reached the upper end of the extended filter paper (8−9 min); the strip was then removed and air-dried. The color intensity of the antibody zone was estimated either visually or by scanning densitometry.

Table 1. Characteristics of the Liposomes

mean diameter (nm)	230 nm
volume of liposome (μL)	6.37×10^{-12}
liposome concentration (number/mL)	3.14×10^{12}
SRB concentration (mM)	150
SRB molecules per liposome	5.2×10^5
AFB_1 molecules on the liposome surface	1.5×10^4

Safety Consideration. Aflatoxins are a highly toxic materials that should be handled with care. Crystalline aflatoxin B_1 and organic solvents for use in the modification and production of the conjugated liposomes and performance of the assay were handled in a chemical hood with surgical gloves.

RESULTS AND DISCUSSION

Preparation of AFB_1-Tagged Liposomes. An initial attempt to conjugate AFB_1 to the liposome was made by preparing a DPPE−AFB_1 conjugate and incorporating it into the liposome. However, the liposome produced did not show any affinity to anti-AFB_1 antibody. It is conjectured that the nonpolar AFB_1 tended to fold within the hydrophobic lipid bilayer, rather than pointing outward on the outer surface of the liposomes to provide the antigenic epitopes. To overcome this, a multistep conjugation method as described above was used. The spacer between AFB_1 and DPPE, and the hydrophilic nature of the phenolate ion of $AFB_{2\alpha}$, overcame the problem of AFB_1 folding within the lipid bilayer. The liposomes produced are quite stable. The AFB_1 liposomes are stable at temperatures up to the boiling point in TBS, a result also reported by others.[16,17]

Characteristics of Liposomes. Having a narrow size range for the tagged liposomes was extremely important in order to obtain an even capillary migration on the test strips. Extrusion of the liposome preparations through polycarbonate filters was found to reduce the size heterogeneity. Liposomes passed twice through polycarbonate filters of 3.0, 0.4, and 0.2 μm, arranged in tandem, had a mean diameter of 230 nm with standard deviation of 50 nm. The 230-nm liposomes were used in all subsequent experiments.

The characteristics of liposomes are listed in Table 1. With liposomes of 230-nm diameter, it is possible to calculate that the average volume of a single liposome is 6.37×10^{-12} μL and the volume entrapped (assuming a bilayer thickness of 4 nm) is 5.73×10^{-12} μL. Assuming the dye concentration inside of the liposomes was equal to the original dye solution used, and comparing the fluorescence of lysed liposomes to that of standard SRB solutions, it is possible to calculate that there were $\sim 3.14 \times 10^{12}$ liposomes/mL and that each liposome contained $\sim 5.2 \times 10^5$ molecules of dye. If the average surface area of the DPPC molecules is 71 Å, and that of cholesterol 19 Å,[18] it was estimated that $\sim 1.5 \times 10^4$ molecules of AFB_1 were on the outer surface of each liposome given that 4 mol % of DPPE-sulfhydryl successfully reacted with maleimide-derivatized AFB_1.

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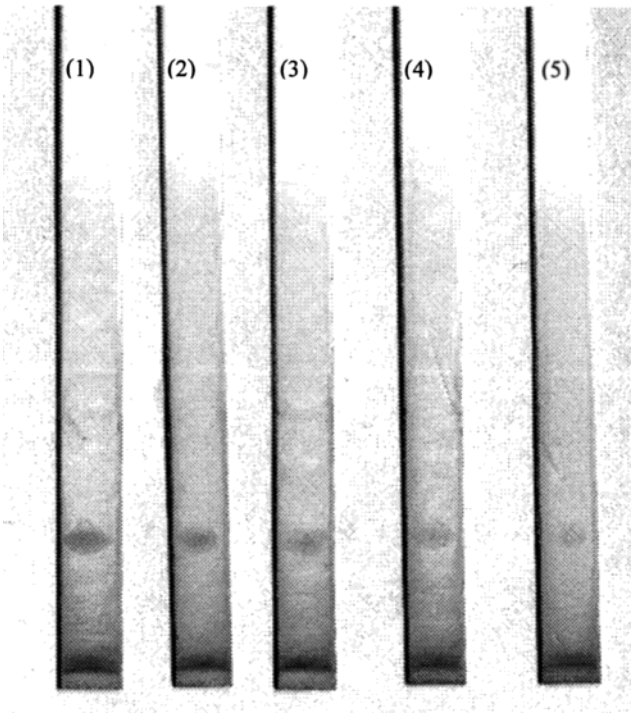

Figure 2. Scanned image of strips showing the competition between a series of AFB_1 standards and constant amount of AFB_1-tagged liposomes. Each strip has 2.0 mg/mL anti-AFB_1 antibody immobilized on it: (1) AFB_1-tagged liposomes only; (2) 10 ng of AFB_1 + AFB_1-tagged liposome; (3) 100 ng of AFB_1 + AFB_1-tagged liposomes; (4) 1 μg of AFB_1 + AFB_1-tagged liposomes; (5) 10 μg of AFB_1 + AFB_1-tagged liposomes.

Assay Performance. The concept of the assay is to have an immobilized antibody zone in the membrane strip that is first exposed to free AFB_1 in a sample solution, and then unbound antibody sites in the zone are filled by a second exposure to a solution of AFB_1-tagged liposomes. Thus, the color exhibited by the bound liposomes on the antibody zone is inversely proportional to AFB_1 present in the sample. Color intensity may be measured semiquantitatively by visual examination (as might be done in the field), but the use of a computer scanner and scan analysis densitometry software (Biosoft, Ferguson, MO), which converts the red coloration into gray-scale readings, can provide more accurate quantitation. Figures 2 and 3 show the results obtained with a series of AFB_1 standards. The dose−response curve exhibits the sigmoidal shape of competitive immunoassays. The limit of detection estimated at 2 SD was determinated to be 18 ng for aflatoxin B_1 with a 95% confidence limit.

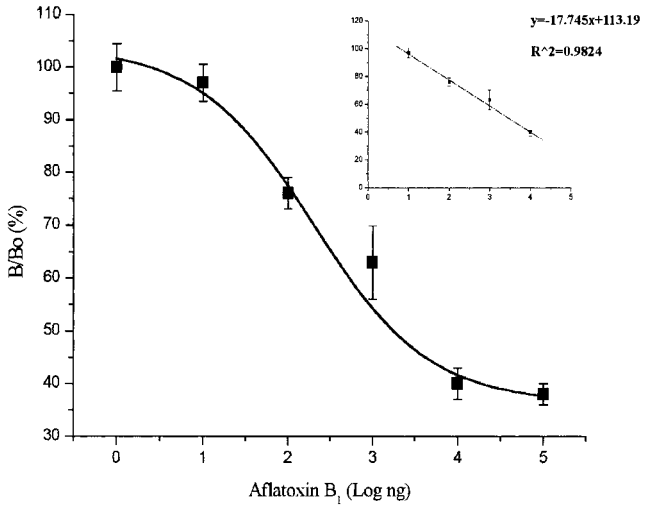

Figure 3. Aflatoxin B_1 dose−response curve. Each point represents the mean of three measurements; error bars represent ±1 SD.

CONCLUSION

The U.S. Food and Drug Administration has set a maximum allowable level of total aflatoxins at 20 parts per billion; commodities used for human and animal consumption must be tested to ensure that aflatoxin levels are below this level. The Aflatest immunoaffinity column is claimed to have a range of quantitation from 1 to 50 ng with lower limit of detection of 1 ng; however, this membrane test strip procedure is able to detect 18 ng of aflatoxin B_1, featuring a rapid and relatively inexpensive monitoring method (∼$1 U.S./assay) combined with instantaneous visualization. It needs to be tested in real sample matrixes and under field conditions to determine its limitations. In our laboratory, the total assay time for a sample was less than 12 min, with the potential of testing multiple samples simultaneously. These results also suggest that AFB_1-tagged, dye-loaded liposomes may be successfully used in a FILIA procedure. Such a technique would provide an automated and still relatively inexpensive technique for Aflatoxin B_1.

ACKNOWLEDGMENT

The authors thank Dr. Richard A. Durst and Sui Ti Siebert of Cornell University for valuable suggestions and assistance. This work was supported by the American Peanut Foundation under Grant 04-81898 and by the National Science Council in Taiwan, ROC, under Grant 89-2113-M-260-010.

Received for review August 13, 2001. Accepted December 21, 2001.

AC010903Q