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THIN-LAYER CHROMATOGRAPHY WITH AGAROSE GELS

A QUICK, SIMPLE METHOD FOR EVALUATING LIPOSOME SIZE

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Summary

Thin-layer gels can be made with agarose and used to assess within a few minutes the efficiency with which multilamellar vesicles are converted to small unilamellar ones by sonication. A fluorescent lipid marker or vesicle-encapsulated solute permits continuous monitoring of the chromatography. Advantages over agarose gel column chromatography include speed of analysis, small sample size, the possibility of running multiple samples simultaneously, and direct accessibility to fluorescence microscopy. This approach should also be useful in the study of liposome-lipoprotein interactions and in affinity chromatography of liposomes.

Small unilamellar vesicles are used in a variety of physico-chemical, cell biological, and medical studies [1—3]. They are usually obtained by sonication of multilamellar lipid dispersions, and it is generally important to know how effective was the sonication in producing small unilamellar vesicles. The answer has most often been obtained by agarose gel chromatography in a column, according to methods developed by Huang [4]. However, column chromatography has several drawbacks for analytical use: first, the reduction in vesicle size is assessed only after all of the lipid has been eluted from the column; second, only one sample at a time can be analyzed per column; third, relatively large sample size is required (an advantage for preparative purposes).

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In this report we describe the development of thin-layer chromatographic methods for quickly assessing the efficiency of liposome* sonication. Other, broader areas of application for this technique will become apparent as well.

Thin-layer chromatography with Sephadex gel was first described by Determann for the determination of protein molecular weight [5]. For our purpose Sepharose 4B (Pharmacia), an agarose gel with the appropriate range of size discrimination, was used instead. Fig. 1A shows the construction of a support for the gel bed, and Fig. 1b shows the position of the support during a chromatographic run. The support was made from thoroughly cleaned glass plates, held together by tape. Sheets of dry Whatman No. 1 filter paper (4.6 × 10 cm) were attached to the bed support with masking tape. With the support horizontal, approximately 3 ml of a slurry of Sepharose 4B (33 mg dry particle weight per ml of the desired elution buffer) was spread onto the bed support using a spoon, starting from the center of the bed and moving toward the filter paper wicks. Additional slurry was used to assure contact between the wicks and gel bed. Uptake of excess buffer by the wicks took 5—10 min. Any disturbance of the gel during this stage resulted in a layer of uneven thickness, but such uneveness had only minor effects on the elution pattern. As soon as the bed had dried to a dull, matted appearance, it could be readied for use by cutting the wicks to 6 cm in length, mounting a glass cover plate, and immersing both wicks in reservoirs containing the eluent. Eluent flows from the upper to the lower reservoir because of capillary and the hydrostatic pressure difference. No special precaution (e.g. a moist chamber) was required to prevent the gel from drying out.

For preparation of test liposomes we used a mixture of 2.5 mg of the fluorescent lipid *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (de-excitation approx. 470; demission approx. 545) and 50 mg of dioleoyl phosphatidylcholine (both from Avanti Biochemicals Inc., Birmingham, AL). The presence

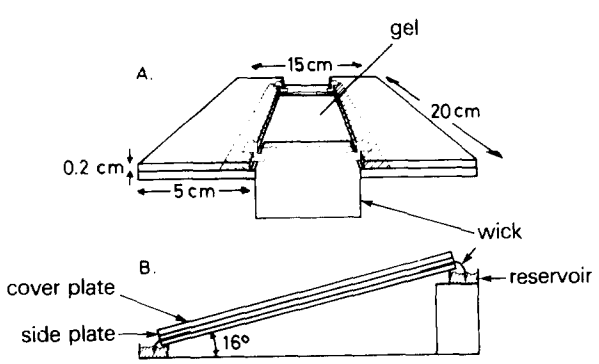

Fig. 1. The gel bed support. A. End-view. The side plates are attached to the base plate by strips of Scotch magic tape (striped areas) folded back on themselves with edges away from the gel; absolute water-tightness of the tape-seal is necessary to prevent leakage of eluent and distortion of the flow pattern. This can be guaranteed by applying silicone grease between side- and base-plate behind the tape. B. Position of the support during chromatography. For the glass plates we used sections of pre-scored 20 × 20 cm chromatography plates (Analtech, Newark, DE), with the silica gel removed. The 16° angle is not critical; a larger angle would still allow a good (and faster) separation.

* In this report the terms 'liposome' and 'vesicle' will be used interchangeably.

of 5 mol% *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine did not affect formation of small unilamellar vesicles or the characteristics (size, stability) of the formed vesicles. Lipid purity was assessed as in previous studies [6]. The mixture was evaporated to dryness from benzene under a stream of argon and lyophilized overnight. The lipid film was dispersed by vortex-mixing in 5 ml of phosphate-buffered saline (pH 7.4). Part of the resulting multilamellar vesicle preparation was sonicated under argon at about 30°C for periods of up to 1 h with a Heat Systems sonicator equipped with a titanium microprobe.

To test the resolution of the Sepharose 4B thin-layer, we mixed vesicles sonicated for 1 h with unsonicated ones, added purified [6] carboxyfluorescein (Eastman Kodak Co., Rochester, NY) to a final concentration of 20 M, and chromatographed the resulting mixture. The separate constituents were run simultaneously on the same plate. After 75 min the chromatogram was photographed under long-wavelength ultraviolet light (see Fig. 2A). There was excellent separation of multilamellar from small unilamellar vesicles, while separation of the latter from carboxyfluorescein was relatively poor. Some of the unsonicated vesicles remained at the origin, in part representing vesicles too large to enter the gel.

Fig. 2B shows the spots obtained with liposome preparations subjected to increasing times of sonication. Samples sonicated for 2, 5, 15, and 60 min, had, respectively, a turbid, heavily opalescent, and clear appearance. Obviously,

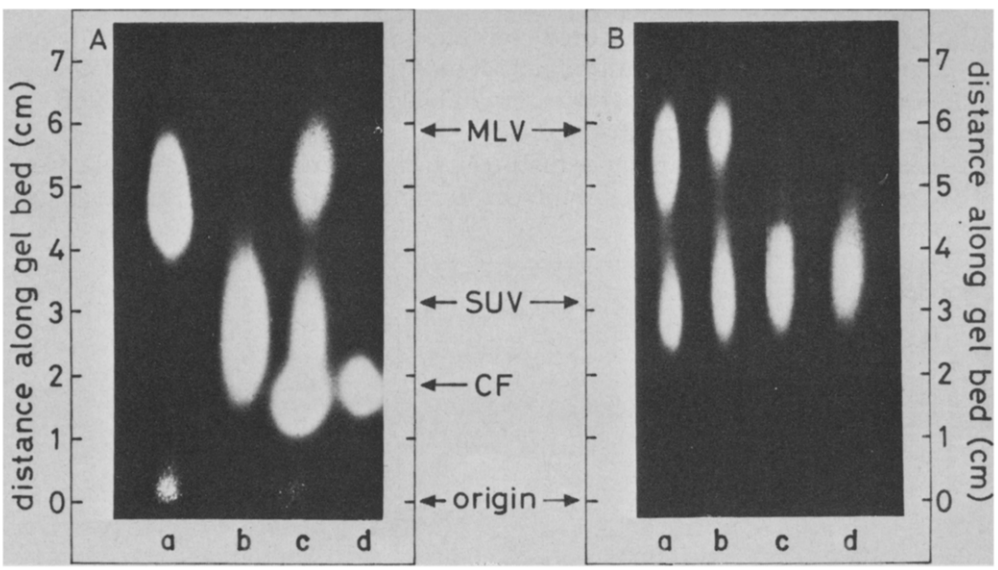

Fig. 2. Thin-layer gel chromatograms of liposome preparations on Sepharose 4B. Liposomes were prepared as described in the text. A. Resolution of multilamellar vesicles (MLV) and free solute. 10 μl samples (100 μg total lipid) of multilamellar (a), small unilamellar vesicles (b), 1:1 (w/w) mixture of multilamellar and small unilamellar vesicles plus 20 μM free carboxyfluorescein (c), and 20 μM free carboxyfluorescein (d) were applied using a 5 μl Oxford micropipette. Careful application of the spot was important to a good separation, and the Oxford pipette was considerably easier to control than others tried. Chromatography was stopped after 69 min by levelling the plate and removing the paper wicks. B. Distribution of liposome sizes after different times of sonication. 10 μl samples of liposome preparation sonicated for 2 min (a), 5 min (b), 15 min (c), and 60 min (d) were applied to the gel. Chromatography was stopped after 73 min. The sharp central streaks are due to scattering from scorings on the glass.

a 15-min sonication was not sufficient to convert all multilamellar vesicles into small unilamellar ones. In contrast to Fig. 2A, no lipid remained at the origin, indicating that sonication for as little as 2 min sufficed to break up very large structures (and to form substantial amounts of small unilamellar vesicle).

For quantitative analysis a mixed vesicle preparation similar to that in Fig. 2A was chromatographed. The thin-layer was then scanned for fluorescence. As shown in Fig. 3, multilamellar vesicles were well separated from small unilamellar ones within a total distance of about 6 cm.

The fluorescent spots on a similar gel were studied in situ with a fluorescence microscope (Fig. 4). At the origin (A) some very large liposomes (arrow) remained, and all of the gel beads had fluorescent rims, probably because of adsorption of vesicle lipid. In the spots containing carboxyfluorescein (B) and small unilamellar vesicles (C), the gel beads appeared uniformly fluorescent, indicating free dye and vesicles, respectively, inside the beads. At the outer edge of the multilamellar vesicle spot (D), by contrast, fluorescence was mainly observed outside the beads, as expected. Some gel beads had brightly fluorescent rims, probably corresponding to adsorbed vesicle lipid. Vesicles also appeared between beads (arrow).

We also chromatographed vesicles with ^{14}C-labelled lipid, but the results were less satisfactory; if the gel were dried for scanning of the tracer, the elution pattern became somewhat blurred, and the gel surface tended to crack. In any case, fluorescent labeling has the advantage over radiotracers that the chromatographic process can be monitored continuously. As an alternative to intrinsic fluorescent dyes (e.g. *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine in our experiments) vesicles could be labeled with lipophilic fluorophore just prior to chromatography.

Advantages of the thin-layer method over conventional column gel chromatography include (1) the possibility of running several samples simultaneously

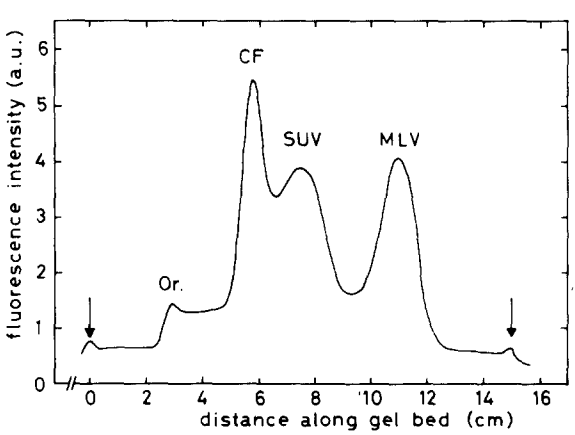

Fig. 3. Fluorescence scan of a thin-layer Sepharose 4B gel chromatogram. The scan was obtained with a fiber-optic thin-layer scanner attached to a fluorimeter (both from American Instrument Co., Silver Spring, MD). Scan-speed was 1.5 cm/min with excitation at 470 nm and emission at 520 nm. 10 μl (100 μg total lipid) of a 1:1 (w/w) MLV/SUV (multilamellar/small unilamellar vesicle) preparation (see text), containing 4 M free carboxyfluorescein was applied to the gel. Chromatography was stopped after 93 min. Arrows indicate ends of the gel bed. Or. Origin; a.u., arbitrary units.

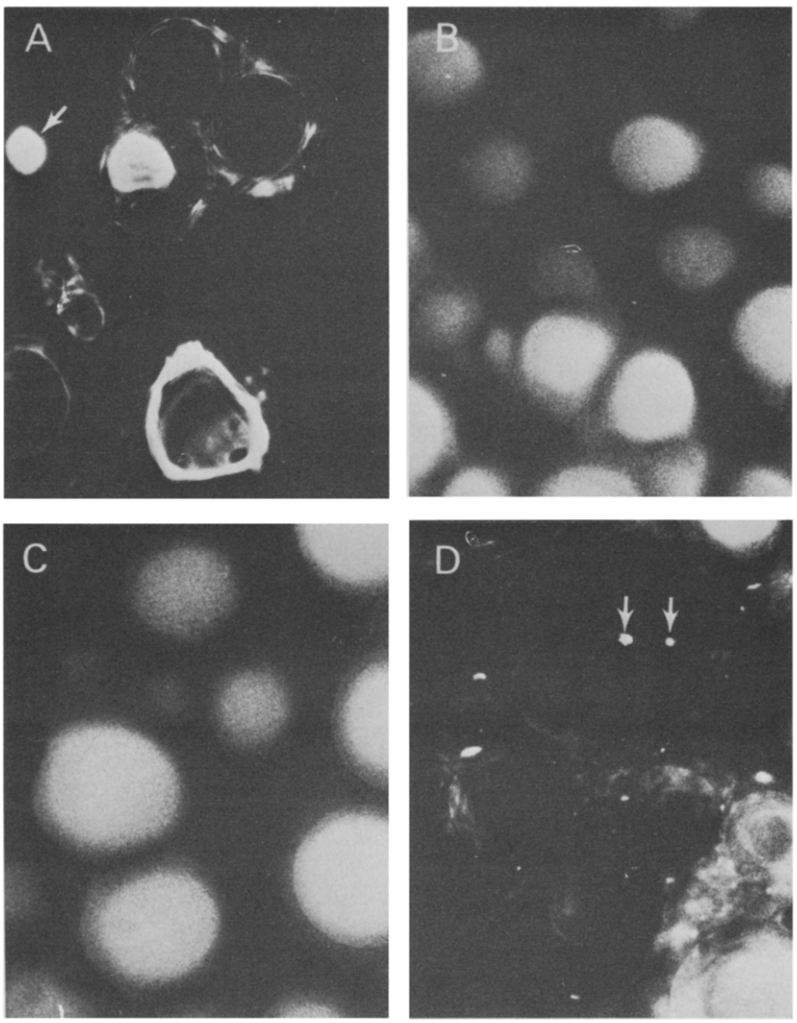

Fig. 4. Fluorescence microscopy of a Sepharose 4B thin-layer gel after chromatography of the mixture used for the scan in Fig. 3. Pictures were taken through a Zeiss Universal fluorescence microscope with filter combination for fluorescein. A, origin; B, carboxyfluorescein spot; C, small unilamellar vesicle spot; D, outer edge of multilamellar vesicle spot. Arrows: see text. Magnification 100 X.

on the same gel; (2) speed. A gross impression of the vesicle size distribution can be obtained after as little as 10 min of elution time; and (3) the very small size of the samples applied. In the experiments reported here we applied 100 μg of vesicle lipid, which gave very bright fluorescent spots (Fig. 2) easily detectible on fluorescence scans (Fig. 3). The data indicate that the sensitivity of the method is 1—10 μg of total lipid, depending on the conditions of the experiment and the fluorescent probe used.

After the experiments reported here, we found that the speed of chromatography could be doubled by substituting a cross-linked agarose (Sepharose CL-4B) for Sepharose 4B. Solvent flow through the bed could also be manipulated by changing the inclination of the plate, by altering reservoir levels, or by

changing the thickness of the wicks The separation of small unilamellar vesicles from low molecular weight solutes such as carboxyfluorescein was improved considerably by combining a dextran stacking gel (e.g. Sephadex G-50) with a Sepharose 4B separation gel. This was done by pouring the two gels simultaneously onto the support, separating them initially with a thin polystyrene blade positioned perpendicular to the gel. After 3 min the blade was carefully withdrawn.

With inclusion of sodium azide (0.1%) and adequate pH buffering in the eluent, the thin layer can be run like a conveyor belt for at least several weeks of routine use; previously applied material simply runs into the lower reservoir. In addition to its use in assessing efficiency of sonication, agarose thin-layer gel chromatography should prove immediately applicable to liposome-lipoprotein interactions [7] and to affinity chromatography of liposomes.

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