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LIPOSOMES CONTAINING COLLOIDAL GOLD ARE A USEFUL PROBE OF LIPOSOME-CELL INTERACTIONS

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A method is described for the preparation of liposomes containing colloidal gold as an electron-dense marker to trace liposome-cell interactions. Since gold sols would precipitate at the high concentrations necessary for loading a large proportion of liposomes, gold sols were formed within preformed liposomes which had encapsulated gold chloride. The optimal conditions for encapsulating the marker were ascertained for liposomes prepared by the method of reverse-phase evaporation. Gold sols formed rapidly at ambient temperature and without organic solvent, and produced homogeneous populations of gold granules inside liposomes. Most vesicles contained the marker, allowing us to determine unambiguously the intracellular fate of liposomes and their contents. The in vitro experiments showed that gold-liposomes were internalized by African green monkey kidney cells in a manner similar to receptor-mediated endocytosis of well-characterized ligands. Preliminary in vivo studies also indicated that liposomes were endocytosed by Kupffer cells via the coated vesicle pathway.

Liposomes are useful carriers for intracellular delivery of drugs and macromolecules [1] in vitro, and are the subject of considerable interest as carriers of chemotherapeutic agents in vivo [2,3]. Despite extensive analysis of liposome-cell interaction, the mechanisms for cellular incorporation of liposomes and their contents are still uncertain. Radioactive compounds, fluorescent dyes, RNA and DNA [4–9] have been encapsulated successfully in large liposomes and some of the encapsulated material subsequently has been incorporated into cells. At the ultrastructural level, it has been difficult to trace unambiguously the path of liposome internalization for lack of a suitable marker. Ferritin and horseradish peroxidase have been used to follow liposome fate in vitro, [10–12]. However, the endogenous peroxidase activity of many tissues, as well as the natural occurrence of intracellular ferritin, complicates the use of such markers. Colloidal gold particles are an attractive histological marker, owing to their high electron density, uniform size and shape, and versatility [13–15]. However, colloidal gold is unsuitable for encapsulation in liposomes, as it precipitates at the high concentrations necessary to load a large fraction of a liposome population. In this report, we describe the preparation of liposomes containing gold granules and demonstrate the utility of gold-containing liposomes as histochemical markers of liposome uptake in cells.

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

For each preparation of gold-liposomes, gold chloride/citrate solution was freshly prepared by adding chloroauric acid (12.72 mM $HAuCl_4$ in distilled water) to basic citrate solution (13.6 mM trisodium citrate/3.33 mM K_2CO_3) immediately before the preparation of the liposomes. The final gold chloride/citrate solution consisted of 3.18 mM $HAuCl_4$/2.5 mM K_2CO_3/10.2 mM trisodium citrate, and pH was maintained at 6.0–6.2 to avoid premature nucleation of gold sols during liposome preparation.

Gold chloride/citrate solution was encapsulated in large phospholipid vesicles prepared by the reverse-phase evaporation method [16]. Specifically, 10 μmol phospholipid was dissolved in 1 ml of freshly water-washed diethyl ether. Gold chloride/citrate solution (0.5 ml) was added, and the mixture was emulsified by sonication for 3 min at 25°C in a bath-type sonicator. Diethyl ether was removed under reduced pressure at 30°C. The resulting vesicles were extruded through polycarbonate membranes (Bio-Rad, Richmond, CA) of 0.2 μm pore diameter [17] to ensure a narrow distribution in sizes. Gold sols were formed by incubating the liposome suspension at 37°C for 0.5–1 h.

The unentrapped gold granules, either free or associated with the liposome surface, were removed by passing liposome-gold sol suspension through a Sephacryl S-1000 (Pharmacia, Piscataway, NJ) column (1 cm × 10 cm) which was preequilibrated with buffer (5 mM Hepes/100 mM NaCl (pH 7.0)). To avoid loss of liposomes by adsorption to the Sephacryl gel, the column was presaturated with liposomes prepared as described above, except that column buffer was encapsulated instead of gold chloride/citrate. The liposomes containing gold granules moved down the column as a red band, while the free gold remained at the top of the column as a brown-black band. The red liposome band was collected in a clear polystyrene tube and then stored at 4°C under argon until use. We used a short DEAE-cellulose column (1 × 4 cm) as an alternative method to separate the gold-liposomes from free or adherent gold sols. Since gold sols carry a net negative charge in water, they bind strongly to DEAE-cellulose. The DEAE-cellulose column was pretreated with buffer-loaded liposomes as described above to avoid the binding of anionic phospholipid to DEAE-cellulose. The recovery of liposomes from each column was high; greater than 70% of the phospholipid was recovered from the columns. Phospholipid was quantified using the procedure of Bartlett [18]. Gold sols were quantified by measuring absorbance at 525 nm after gold liposomes had been dissolved in 1% Triton X-100.

To obtain an overview of gold liposomes, the vesicle suspension was stained with 2% ammonium molybdate for 30 s on a newly glow-discharged carbon-coated grid. For thin-section electron microscopy, the liposomes were fixed with 1.5% glutaldehyde (in 0.1 M sodium cacodylate, 1% sucrose, pH 7.4) for 1.5–2 h at room temperature, then postfixed sequentially with 1% osmium tetroxide (in 5% sucrose/0.1 M veronal acetate (pH 7.4), 4°C, 1 h), 1% tannic acid (in 0.05 M sodium cacodylate (pH 7.0), 25°C, 1 h, in the dark) and then stained with 0.5% uranylacetate (in 0.1 M veronal acetate (pH 6.0), 37°C, 1 h). Fig. 1A, B clearly shows that gold sols are uniform size (approx. 15 nm diameter) and shape, and entrapped inside the liposomes. Routinely, 80–90% of the liposomes produced contained gold particles. However, less than 10% of liposomes contained gold when liposomes were prepared in the presence of monodispersed gold colloid [19].

Several lines of evidence suggest that the gold granules are entrapped within the liposomes rather than adsorbed to the outer liposomes surface. First, the column chromatography was effective in removing free gold; no gold colloid was detected in the liposome fraction after the column separation when mixing preformed gold colloid with liposomes. Second, gold liposomes are stable in buffer containing 0.1 M NaCl which floculates free gold sols. Third, when preformed liposomes were mixed with gold chloride/citrate solution used for encapsulation, gold sols that remained with liposome fractions after column chromatography were clustered and not entrapped inside the liposomes (see Fig. 1C). Fourth, column chromatography does not result in a significant loss of gold-containing liposomes, but does retain free gold colloid readily.

African green monkey kidney (AGMK) cells avidly bind liposomes containing negatively-charged phospholipids [20], and some liposome-

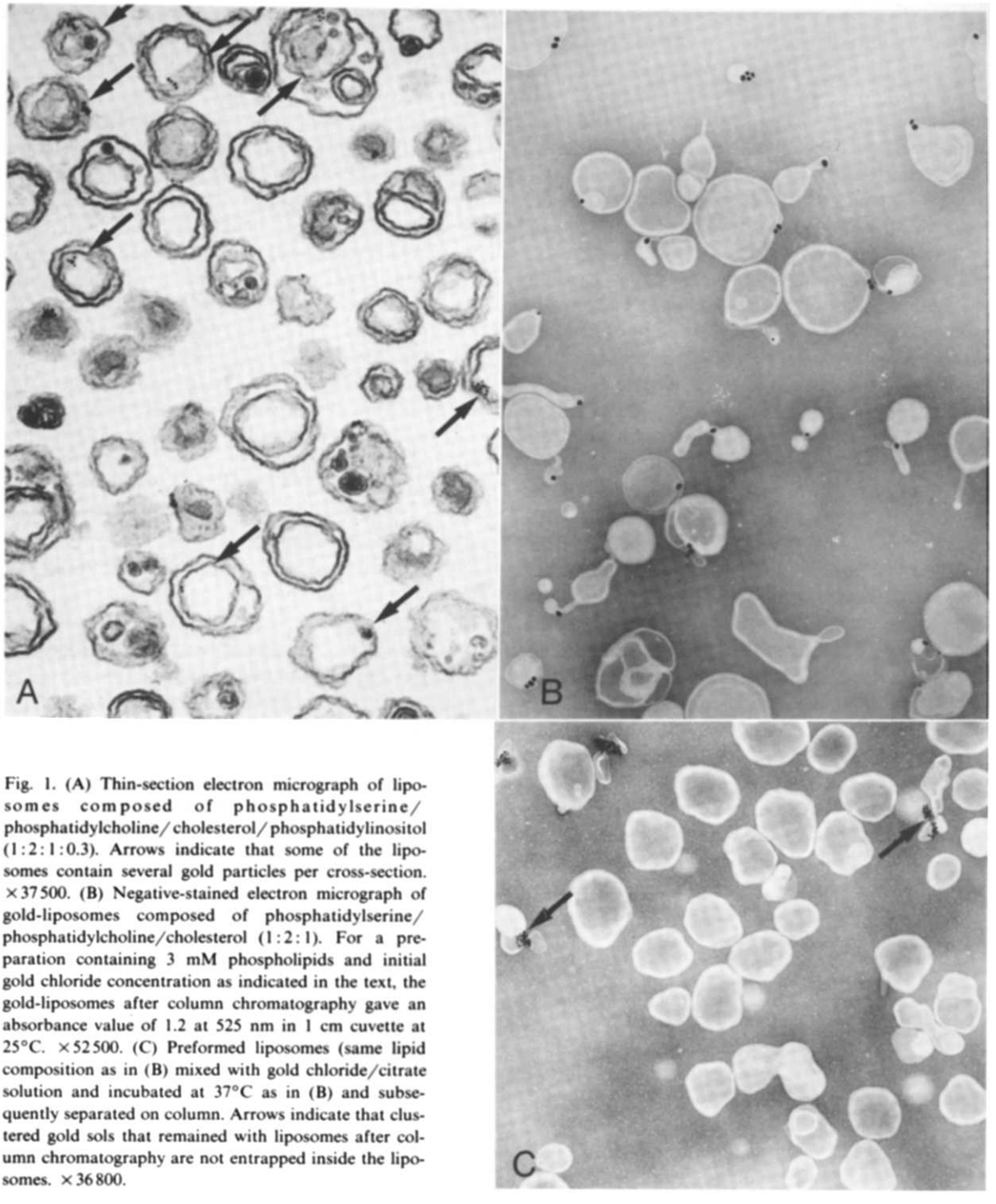

Fig. 1. (A) Thin-section electron micrograph of liposomes composed of phosphatidylserine/phosphatidylcholine/cholesterol/phosphatidylinositol (1:2:1:0.3). Arrows indicate that some of the liposomes contain several gold particles per cross-section. ×37500. (B) Negative-stained electron micrograph of gold-liposomes composed of phosphatidylserine/phosphatidylcholine/cholesterol (1:2:1). For a preparation containing 3 mM phospholipids and initial gold chloride concentration as indicated in the text, the gold-liposomes after column chromatography gave an absorbance value of 1.2 at 525 nm in 1 cm cuvette at 25°C. ×52500. (C) Preformed liposomes (same lipid composition as in (B) mixed with gold chloride/citrate solution and incubated at 37°C as in (B) and subsequently separated on column. Arrows indicate that clustered gold sols that remained with liposomes after column chromatography are not entrapped inside the liposomes. ×36800.

entrapped materials gain access to the cytoplasm. Gold liposomes were used in AGMK cells to examine cellular uptake of liposomes. Thin-section electron microscopy with gold liposomes [21] suggests that liposomes bind to the cell surface, are endocytosed in coated pits, and proceed intracelluarly according to the well-defined coated-vesicle pathway described for many ligands which undergo receptor-mediated endocytosis [22,23]. The preliminary results of hepatic uptake of liposomes in intact rats show that gold-liposomes were identified in coated vesicles, uncoated vesicles and the large endosomal vacuole of Kupffer cells during 2 min circulation of injected gold liposomes.

The conditions described here have been used successfully in making liposomes with several different compositions. With this newly developed gold-liposome preparation, we are presently conducting a detailed study of some important parameters of endocytosis, such as liposome size, lipid composition, and existence of a liposome receptor in cell membranes, in order to find out whether the same internalization mechanism applies to various types of liposomes and various cells.

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