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Light microscopic localization of silver-enhanced liposome-entrapped colloidal gold in mouse tissues

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Silver-enhanced liposome-entrapped colloidal gold was developed for light microscopic localization of liposomes. Preparation of colloidal gold entrapped in liposomes was achieved by a modified method of Hong, et al. (1983) Biochim. Biophys. Acta 732, 320–323). In this report, a gold chloride / citrate solution of low pH (3.4) was used to inhibit the formation of gold granules during the liposome preparation. The diameter of most liposomes ranged from 80 to 100 nm. Following liposome preparation, the pH was adjusted to 6, and the temperature increased to 55°C. The majority of the liposomes contained one to three gold particles. Liposomes were injected into mice via tail vein; 24 h later, tissues were collected. Sections were processed for silver enhancement of the gold particles and examined by light microscopy. Silver-enhanced gold particles were clearly observed in both liver and implanted tumor. Localization was confirmed by electron and fluorescence microscopy. Thus, we have shown that silver enhancement of colloidal gold liposomes is a direct and sensitive method for tracing the fate of liposomes in vivo, providing minimal background interference and a good definition of various cell types.

As a modality for drug delivery, liposomes have been shown to increase drug retention, reduce toxicity, and enhance therapeutic efficacy compared with unencapsulated compounds [1,2]. To better understand the mechanism of liposome uptake by various tissues, and to improve therapeutic efficacy, it would be advantageous to know the pathway and the final localization of liposomes in tissues. A variety of markers such as proteins (ferritin) [3], enzymes (horseradish peroxidase) [4], and fluorescent molecules (pyranine and carboxyfluorescein) [5,6] have been encapsulated in liposomes and used to monitor their interaction with cultured cells in vitro; however, it is difficult to use these methods in vivo because of interference of the natural occurrence of these markers or problems of resolution of tissue morphology. At the ultrastructural level, chemicals (5-Br,4-Cl,3-indolylphosphate) [7], paired enzymes (glucose oxidase, horseradish peroxidase) [8], and colloidal gold [9] have been used in vivo as liposome-encapsulated cytochemical or histological markers. Of these methods, colloidal gold entrapped in liposomes can provide an unambiguous image with high electron density, and uniform size and shape. The endocytosis of liposomes by Kupffer cells in the reticuloendothelial system (RES) rich tissue of the liver has been demonstrated by this method [10]. It is still difficult, however, to detect the small gold particles in thin sections of non-RES tissues such as tumor, where liposome distribution is relatively low [11]. Recent pharmacokinetic studies revealed that small liposomes (< 100 nm in diameter) can achieve prolonged circulation time in blood, thus increasing accumulation in tumor of radioactive ^{67}Ga as a liposome marker [11,12].

It has also been established that silver enhancement of immunogold-labeled tissue makes it possible to detect certain molecules by light microscopy [13], probably because the tissue sections used for light microscopy are about 30 times thicker and 30 times larger than those used for electron microscopy. In this report,

Abbreviations: RES, reticuloendothelial system; PC, egg phosphatidylcholine; C, cholesterol; G_{M1}, monosialoganglioside G_{M1}; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RITC-Dex, Rhodamine B isothiocyanate-dextran.

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we present a new application of silver enhancement on colloidal gold entrapped in small liposomes, that enables us to localize liposomes in tissues by light microscopy.

For best results, gold chloride/citrate solution was made immediately before the preparation of liposomes. Fresh citric acid (120 mM)/K_2CO_3 (30 mM) solution was mixed with $HAuCl_4$ (12.72 mM) in a ratio of 1 : 1, and the final pH of the solution was adjusted to 3.4. Low pH aqueous phase is necessary to prevent gold granules from forming and precipitating during liposome preparation.

Liposomes with a desired lipid composition of $PC/C/G_{M1}$ (mol ratio 2 : 1 : 0.2), were prepared by the method of reverse-phase evaporation with gold chloride/citrate in aqueous phase [9,14]. A thin lipid film (10 μmol phospholipid) was dissolved in 1 ml of diethyl ether, and mixed with 0.5 ml gold chloride/citrate solution. The mixture was emulsified by sonication for 3 min, and diethyl ether was removed at room temperature. The liposomes formed by the above method [14] underwent three cycles of freezing (−56°C) and thawing (55°C), and then were extruded through Nuclepore membranes (Pleasanton, CA), twice with pore-size 0.1 μm and five times with pore-size 0.05 μm [15,16]. Immediately after final extrusion, the pH of the liposome suspension was raised to 6 by adding NaOH. Formation of gold particles was initiated by incubating the suspension at 55°C for 30 min. The color of liposome suspension turned pink-purple, which indicated an appropriate size of gold particles. Unencapsulated free gold and excess citrate were removed by passing the liposome suspension through a column (1 × 15 cm) of Sephacryl S-500 (Pharmacia, Piscataway, NJ). Liposome size and clearance of free colloidal gold were examined by electron microscopy of negatively stained (2% ammonium molybdate) preparations.

Colloidal gold-containing liposomes (0.25 ml, 2 μmol phospholipid) were injected into mice (female BALB/c) via the tail vein. The mice were sacrificed 24 h after liposome injection. Tissues were collected following perfusion with heparinized PBS and fixative (1.5% glutaraldehyde, 0.1 M sodium cacodylate, 1% sucrose, pH 7.4). All procedures involving tissue handling were performed at 4°C. Dehydration, infiltration and embedding procedures were done under vacuum (15–20 mmHg). The specimens were fixed for 2–4 h with occasional agitation. After fixation, the specimens were rinsed with PBS and then allowed to set in PBS for from 1 h to overnight. The specimens were dehydrated with 50%, 95% acetone/water and 100% acetone at 30 min sequences. For infiltration, the specimens were incubated in 50% acetone/50% glycol methacrylate monomer for 30 min and then transferred to 100% glycol methacrylate monomer for 4–8 h. The specimens were embedded in a mixture of glycol methacrylate monomer (20 ml), benzol peroxide (0.09 gm), and glycol polyethylene glycol 400 with 0.5 ml *N,N*-dimethylaniline (JB-4, Polysciences, Inc., Warrington, PA) for 12 h [17]. Sections were cut from embedded specimens with a Sorvall JB-4 microtome at a thickness of 2.5 μm.

Reagents A (enhancer) and B (initiator) for silver enhancement were purchased from Amersham (Arlington Heights, IL). Reagent A and B were mixed immediately before using. The sample area on the slide was covered with silver-enhancement mixture for 15 min at 22°C. To avoid the self-nucleation of the silver-enhancement solution, the duration of treatment was controlled 5 min below the safety margin of an acceptable background. The slide was washed thoroughly in distilled water. The thin sections were stained with hematoxylin for 1 min, Eosin Y (1%) for 15 min and Azure II (0.1%) for 30 s, and washed after each interval.

For electron microscopy, colloidal gold liposome injection and tissue collection were performed as described for light microscopy. The specimens were embedded in Epox 812 resin (Ernest F. Fullam, Inc., Latham, NY), and specimens (70 nm thickness) examined with a JEOL 100CX transmission electron microscope operating at 80 kV.

For fluorescence microscopy, $PC/C/G_{M1}$ liposomes were prepared by reverse-phase evaporation method described above, but with aqueous contents of 100 mg/ml Rhodamine B isothiocyanate-dextran (RITC-Dex) (M_r 9000, with excitation and emission wavelengths of 546 nm and 590 nm, respectively) in 10 mM Hepes buffer. The final solution was adjusted to 300 mosmol/kg with NaCl (pH 7.4). Unencapsulated RITC-Dex was removed by the combination of G-150 Sephadex gel filtration and Amicon concentration unit (Amicon, Beverly, MA) with 30000 mol. wt. cut off filter membrane. RITC-Dex encapsulated liposomes (0.25 ml, 2 μmol phospholipid) were injected into the tail vein of mice. The mice were killed after 24 h. Tissues were collected following perfusion with heparinized PBS, and fixed with 4% paraformaldehyde. Frozen-sections (5 μm thickness) of the specimens were examined by fluorescence microscopy.

Negatively stained colloidal gold-containing liposomes are shown in Fig. 1. Most of the liposomes are between 80 and 100 nm in diameter. More than 80% of the liposomes contained one to three gold particles. There are very few free gold grains present. The negative staining process has probably distorted some images of gold localization inside the liposome. Several clear pictures of gold-liposomes in thin section electron microscopy were published in our earlier studies [9,10,18].

The stability of colloidal gold liposomes was investigated both *in vitro* and *in vivo*. During 2 weeks of

storage under argon at 4°C, there was no appearance of precipitation of liposomes or aggregated gold particles separated from liposomes. Moreover, no gold particles were separated from liposomes after 1 min vortexing at room temperature and maximum scale. We have also determined the extent to which gold particles were separated from liposomes during blood circulation. Blood was collected from retroorbital sinuses at 1 h and 24 h after injection, and the plasma was examined by negative staining electron microscopy. By scoring more than 50 liposomes in the size range from 80 to 150 nm, we found that the relative ratios of gold-containing liposomes to the plain ones were almost the same as the ratio before injection in blood. The ratios varied within 5%.

The localization of colloidal gold-containing liposomes in normal liver was examined by light microscopy after silver enhancement. Heavy silver enhancement of colloidal gold was clearly observed almost exclusively within the cytoplasm of Kupffer cells with a clean background of hepatocytes (Fig. 2). Furthermore, silver enhancement of colloidal gold was also found in regions of tumor produced by S.C. implanted colon carcinoma [19] (Fig. 3). Similar silver treatments did not produce any enhanced particles in the sections of either liver or tumor tissues from the mice without colloidal gold liposome injection. The fine resolution of electron micrograph of liver section (Fig. 4) showed gold particles in the intracellular vesicles of Kupffer cells but not in hepatocytes. This confirmed our interpretation that silver-enhancement image was derived from the gold particles either free or still liposome-encapsulated.

The fate of liposome-free gold in circulation was examined by obtaining a similar number of gold particles, which were separated from gold liposomes by high speed centrifugation ($10000 \times g$ for 5 min), into mice. The silver-enhanced gold was seen exclusively in the Kupffer cells, rarely in the tumor region 24 h after injection. This suggests that if any gold particles were separated from liposomes in the circulation, they would probably be removed by Kupffer cells before reaching the tumor. Thus, we consider the silver particles in the tumor region are most likely due to accumulation of gold-liposomes which have reached that region intact.

As an additional control to ensure that the silver enhancement image represents the true liposome localization, we have used a second liposome marker, fluorescent RITC-Dextran, in frozen section fluorescence microscopy. The fluorescence patterns show a striking similarity to the silver enhancement image in the liver (Fig. 5) and the tumor region. No fluorescence could be observed in the liver and tumor region of control mice injected with 10 mg of free RITC-Dex. This indicates that even if the aqueous contents marker (RITC-Dex) leaks out of the liposomes, it does not remain in the tissues, so the fluorescence signal from frozen-sections of the tissues with liposomal RITC-Dex

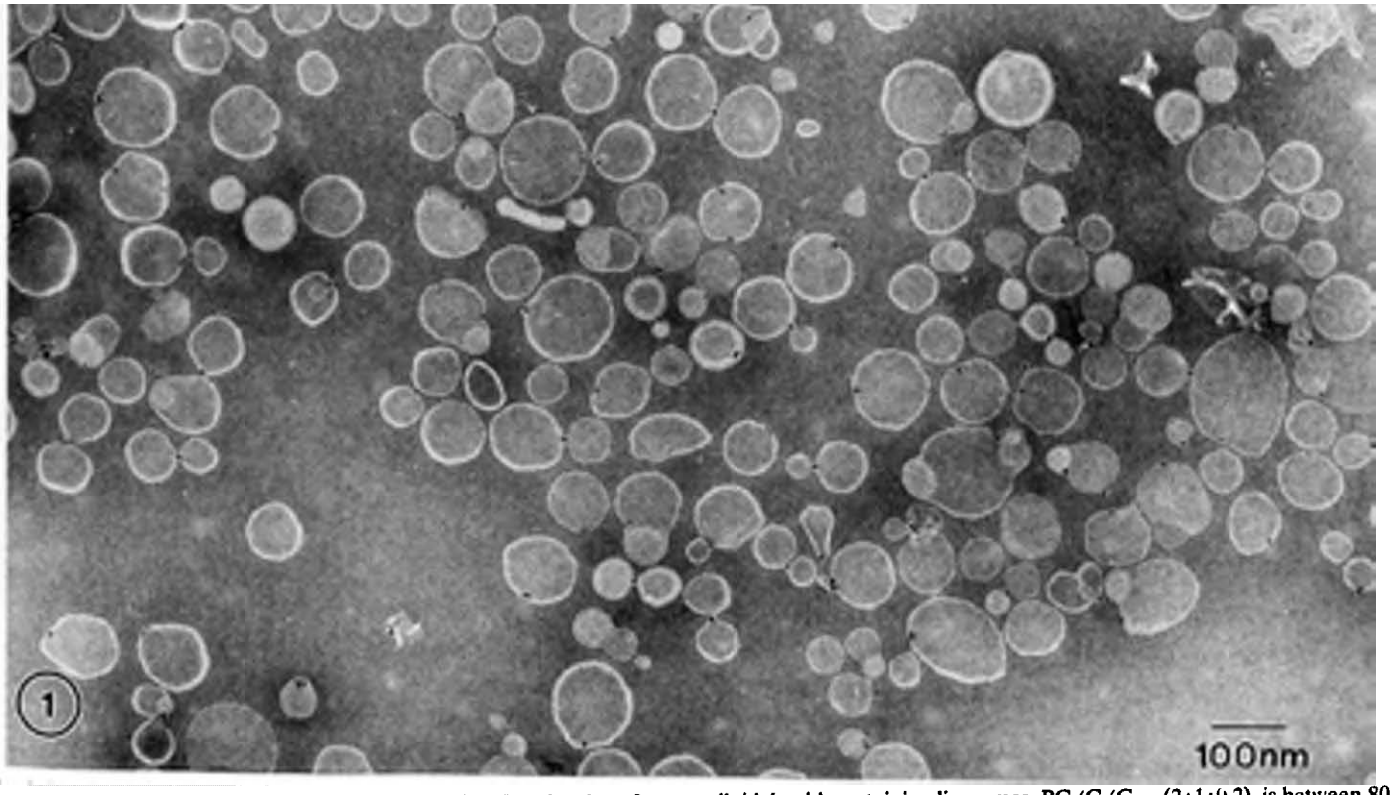

Fig. 1. Negative-stain electron micrograph showing that the size of most colloidal gold-containing liposomes, $PC/C/G_{M1}$ (2:1:0.2), is between 80 and 100 nm in diameter. The majority of them contain one to three gold particles.

is truly reporting the localization of the intact liposomes.

The method presented here indicates that it is possible to localize the presence of liposome contents within tissues such as tumors, where liposomes do not accumulate in large amounts. The ability to detect colloidal gold-containing liposomes by silver enhancement light microscopy is much easier and more sensitive than by electron microscopy. It is also obvious from the results that the resolution of silver enhancement light microscopy is much better than fluorescence microscopy because it allows morphological staining in the same tissue section for positive identification of various cells. However, fluorescence microscopy provides additional control for the presence of intact liposomes irrespective of colloidal gold particles, and might also be useful for quantitation purposes in specific regions of different tissues.

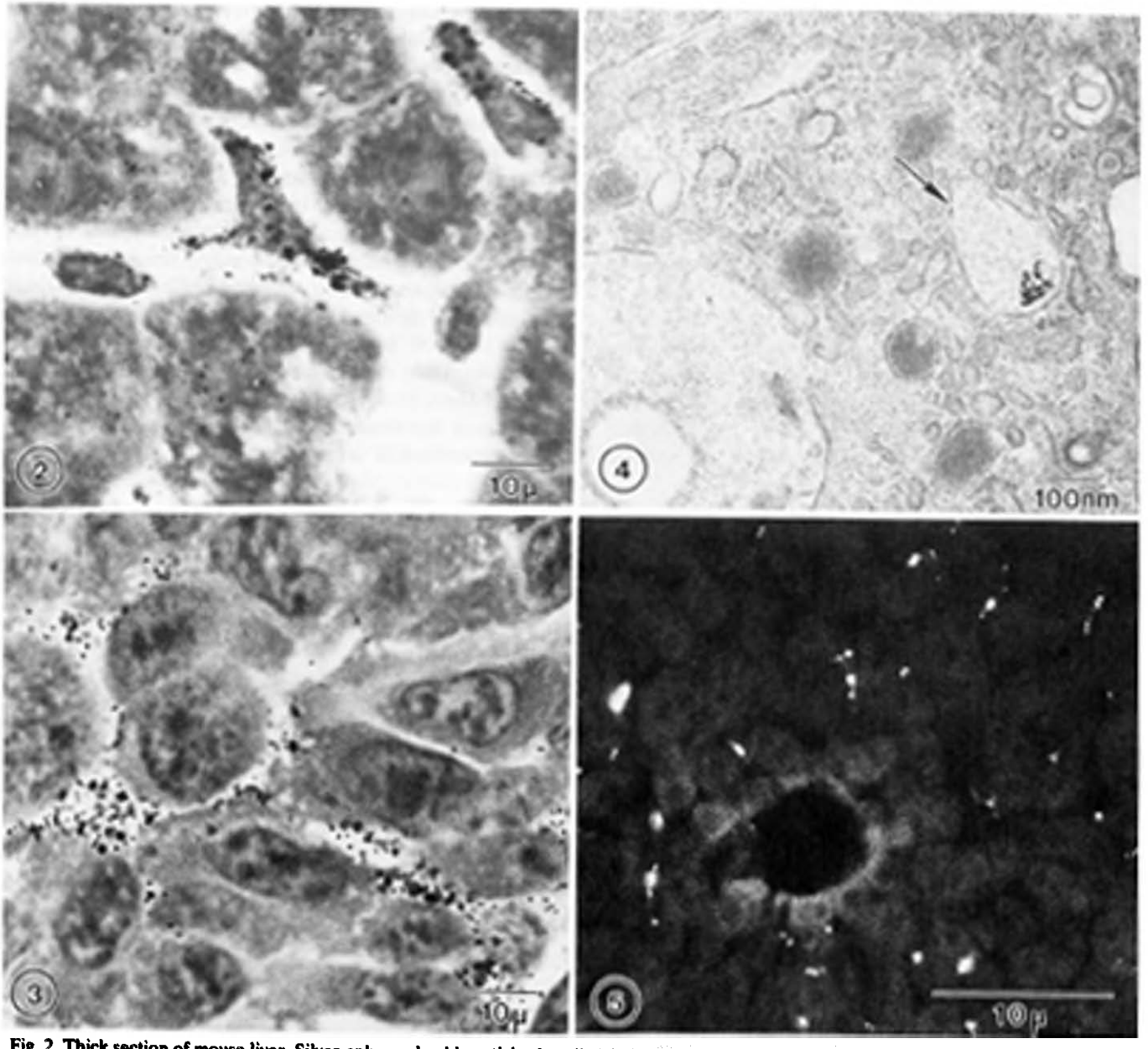

Fig. 2. Thick section of mouse liver. Silver-enhanced gold particles heavily label Kupffer cells, but very few particles can be seen in hepatocytes.

Fig. 3. Thick section of C-26 colon carcinoma implanted S.C. tumor. The silver-enhanced gold particles are seen in the extracellular space between the poorly differentiated tumor cells.

Fig. 4. Electron micrograph of mouse iiver. Colloidal gold particles are observed in an intracellular vesicles of Kupffer cell.

Fig. 5. Fluorescence micrograph of a frozen section of mouse liver. Fluorescence of Rhodamine Dextran (encapsulated in liposomes) can be clearly seen in the small and elongated Kupffer cells residing along the sinusoids.

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