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INTERACTION OF LIPOSOMAL DRUG DELIVERY SYSTEMS WITH CELLS AND TISSUES: MICROSCOPIC STUDIES

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Abstract

Liposomes, as drug carriers, can be administered into the body by several routes e.g. intravenously, intraperitoneally, intramuscularly, intratracheally and topically among others. Radiolabelled markers are suitable to monitor the distribution and elimination of liposomes, but the tissue deposition of intact liposomes, the mode and sites of drug release from the liposomes and liposome-cell interactions cannot be investigated morphologically. Microscopic techniques could provide information regarding the intact state of liposomes and possibly the dynamics of liposomes in tissues provided that they can be identified with certainty in vivo. This is a formidable problem and in spite of several attempts, there is still a lot of work and new ideas needed to overcome this problem.

This paper gives a detailed review of liposome markers used in light and electron microscopy. The use of markers or the technique involved in the identification of liposomes in cells or tissues is discussed.

The feasibility of using colloidal iron, a new electron dense marker, as a marker for intravenously injected liposomes was investigated in mice. Intact multilamellar vesicles containing colloidal iron were identified in the liver, spleen and lung of mice injected with liposomes. The liver and the spleen are organs for the storage of iron containing proteins (ferritin, hemosiderin), therefore studying the disposition of colloidal iron from the liposomes was not possible. However, in organs not containing iron, e.g. lung, the presence of colloidal iron can easily be recognized. The colloidal iron marker may be suitable to label liposomes targeted to the brain, heart or certain tumors.

KEY WORDS: Liposomes, electron microscopy, liposome markers, liposome-cell interactions, drug delivery, light microscopy, colloidal iron.

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Introduction

Liposomes are microscopic vesicles comprising of phospholipid bilayers which enclose aqueous spaces. Multilamellar vesicles or MLV (0.5-20 µm) are concentric membranes with numerous enclosed aqueous compartments (Fig.1). Large and small unilamellar vesicles (LUV [100-500 nm] and SUV [25-100 nm], respectively) consist of one single bilayer and one enclosed aqueous compartment. The structure of the liposome bilayer is similar to cellular membranes, appearing on electron micrographs as characteristic unit membrane or "railroad-track".

Liposomes were discovered in the 1960s by Bangham (Bangham et al., 1965). Since then, they were utilized as model membranes to study transport of molecules across bilayers, lipid-protein interactions and physicochemical properties of amphipatic molecules. Liposomes are also used as potential drug delivery systems to increase the efficacy and specificity of drugs.

Indeed, liposome-mediated drug delivery to selected tissues or cells is a very promising way of improving controlled or selective drug treatment of various diseases. Interaction of liposomes with tissues at the cellular level is a highly complex phenomenon that is not fully understood. The main interaction processes are believed to be endocytosis, fusion, adsorption and lipid transfer (Pagano and Weinstein, 1978; Poste, 1980; Pagano et al., 1981; Schroit et al., 1986; Foldvari et al., 1991) (Fig. 2)

Liposomes can adhere to the cell surface by specific or non-specific interactions. Depending on the type of binding, the tightly bound liposomes can become internalized by an endocytotic process. Specific or receptor-mediated endocytosis occurs when a liposome-component (protein or glycolipid) is recognized by a cell surface receptor and the receptor-ligand binding triggers the internalization of the liposome into coated pits. Non-specific (electrostatic or hydrophobic) binding of liposomes to the cell surface might also promote adsorptive endocytosis or "piggy-back" endocytosis (Lloyd,1986). The latter refers to the uptake of a liposome along with a specifically adsorbed molecule.
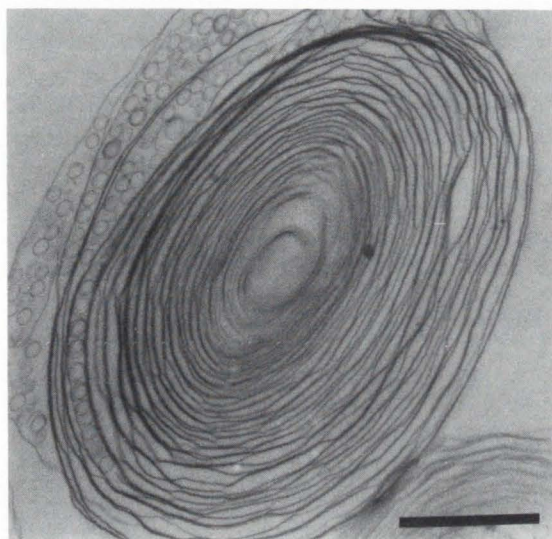
Fig. 1. Thin section of a multilamellar liposome. Liposomes (dipalmitoylphosphatidylcholine - bovine brain phosphatidylethanolamine-cholesterol 1:1:1 m.r.) were fixed with glutaraldehyde and osmium tetroxide, embedded in epoxy resin and sectioned. Bar 1μm.

Both large and small liposomes can be adsorbed to cell membranes (Pagano and Takeichi, 1977; Szoka et al.,1980). A stable adsorption can, but not necessarily lead to endocytosis or fusion. Exchange or transfer of lipids between vesicles and cells has been demonstrated. The various liposome components (phospholipids or cholesterol) can be exchanged with cells at different rates (Pagano and Huang, 1975) and the main characteristic of the process is that the liposome content does not come into contact with the cell interior.

Literature data from the 1970s indicated great possibilities of liposome fusion (Grant and McConnell,1973; Magee et al., 1974; Papahadjopoulos et al., 1974; Huang and Pagano, 1975; Batzri and Korn, 1975; Martin and MacDonald, 1976; Weissmann et al., 1977; Huang et al., 1978), but the results from the employed techniques, used for the demonstration of the process, were not completely conclusive. The high incidence of other types of liposome-cell interaction processes sometimes could be confused with fusion without a reliable marker. Evidence for fusion can be obtained from morphological observations of the fusion event at the ultrastructural level, and from measurements of the incorporation of liposomal lipid into the cell membrane with the concomitant uptake of drug into the cytoplasm (Pagano and Weinstein, 1978). However, it is very difficult to distinguish between incorporation of the liposomal lipid into the cell membrane by fusion and the rapid lipid redistribution after endocytosis of liposomes or lipid-exchange/transfer. Also the detection of the liposome-entrapped substance in the cytoplasm cannot exclude the possibility of the leakage of the compound from the liposomes and the uptake of the material by other mechanisms than fusion. Liposome-cell fusion is still

a debatable pathway with regard to the frequency of the event, the possibility to control its occurrence and the efficiency of drug transfer from the liposomes to the cells.

In order to characterize the drug carrier properties of liposomes, it is important to know the fate of the liposomes, i.e., what organ, tissue or cell type do they associate with in the body after administration and what type of interaction process takes place. A common method to determine this is to study the biodisposition of liposomes by using radioactive labelled lipids and/or encapsulated drugs (e.g. Ellens et al.1981; Abraham et al.1984; Hwang, 1987). These types of studies reveal the pharmacokinetic fate of the liposomes and the encapsulated drugs, but do not provide accurate information related to the in vivo deposition of the intact liposomes, the mechanism and the sites of drug release from the lipid vesicles, and the liposome-cell interactions. While the vesicular configuration and the biophysical and biochemical similarities of liposomes to cellular membranes are advantageous properties as potential drug carriers, it poses a very difficult task to identify them in the biological environment. Since liposomes became the focus of many investigative studies, various approaches have been tried to label the liposomes so as to identify accurately their presence under different experimental conditions. These methods include the use of an electron dense material encapsulated into liposomes, e.g. colloidal gold, ferritin, uranyl acetate, percoll, potassium dichromate or stains which can be converted into electron dense products by a chemical reaction such as horseradish peroxidase (HRP) and nitroblue tetrazolium (NBT) (see Table 1).

The nomenclature of liposome-cell interactions is sometimes ambiguous, therefore the terms used in this paper are defined as follows. "Association" and "uptake" are used interchangeably when a liposome-cell complex forms regardless of the interaction types. They both represent the sum of all interactions. "Adsorption" means the attachment of liposomes to the cell surface by a nonspecific interaction as opposed to "binding" which defines the specific attachment of liposomes to the cell. "Lipid transfer" refers to the exchange of the lipid components between liposomes and cells. "Internalization" is the general term for the uptake of liposomes inside the cell by "endocytosis" (into the lysosomal compartment) or "fusion" (membranes merge and drug is expelled into the cytoplasm). Within the "fusion" term in addition to the real merging of membranes, drug delivery to the cytoplasm may occur without the merging of the cell and liposome membrane by "kissing" fusion or juxtapositional transfer.

Overview of currently used liposome markers

Gold

Colloidal gold is an inorganic, hydrophobic colloid which can be used as a marker in a variety of techniques including immunoelectron microscopy. It
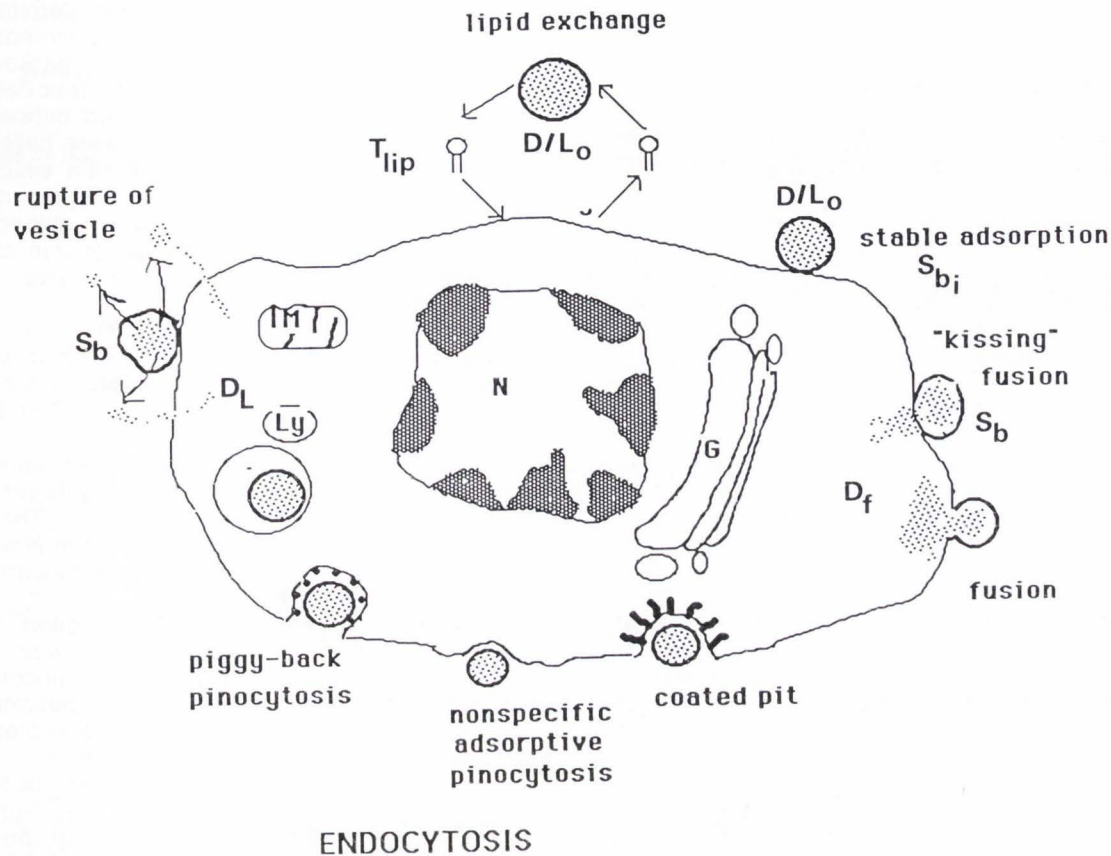
Fig. 2. Liposome-cell interaction pathways. Any combination of the above interactions can occur simultaneously during incubation of cells with liposomes.
T_{lip}, lipid transferred from liposomes to cells; S_b, liposomes bound to the cell surface; S_{bi}, intact lipo-somes bound to the cell surface; D/L, drug to lipid ratio in liposomes; D/L_o, drug to lipid ratio in liposomes originally; D_f, drug internalized by fusion or juxtapo-sitional transfer; D_L, drug internalized in free form by leakage of liposomes; M, mitochondrion; N, nucleus; Ly, lysosome, G, Golgi apparatus.

has also been used to label liposomes following encapsulation of the gold particle. The colloidal gold particles are prepared from the auro-complex $H[AuCl_2]$, which dissociates into H^+ and $AuCl_2^-$ on the particle surface. As a result of this dissociation the particles carry a net negative charge (Pauli, 1949).

There are a number of advantages of the colloidal gold marker. The preparation of various sizes of gold particles is fairly easy. Gold particles are very electron dense therefore easily recognizable in electron microscopy (EM). Colloidal gold can also be stained with silver providing an extremely sensitive technique for light microscopy and immunoblotting (Holgate et al., 1983). In this immunogold-silver staining reaction the metallic silver forming from silver lactate catalysed by the metallic gold is depositing on the gold probe thereby increasing the visibility of the marker.

The colloidal gold sol is prepared by the reduction of tetrachloroauric acid with various reducing agents such as white phosphorus, citrate-tannic acid or sodium borohydride (Horisberger, 1981; Henegouwen and Leunissen, 1986; Lucocq and Roth, 1985). The size of gold particles is dependent on the reducing agent. The prepared colloidal gold sol usually contains gold particles of uniform size. This marker would appear to be a good candidate as a liposome marker. Hong et al. (1983) developed a method where the gold chloride/citrate solution encapsulated in liposomes was converted to colloidal gold inside the liposomes by incubating at 37°C for 0.5-1h. With this procedure, one or two colloidal gold particles can be formed inside the liposomes. The number of particles per liposome is dependent on the initial concentration of gold chloride. Debs et al. (1987) utilized gold labelling technique for the identification of aerosolized liposomes in the lungs of mice. Vesicles encapsulating two gold particles were found in the lung tissue. Stern et al. (1987) used gold label to follow the fate of liposomes in the aphakic

vitrectomized rabbit eye. Liposomes with encapsulated gold particles were identified in the epiretinal cells.

The colloidal gold marker itself cannot be encapsulated efficiently into liposomes because at the high concentration necessary for liposome encapsulation this marker tends to precipitate. It is very difficult to prepare liposomes sufficiently labelled with gold, because the preparation of small size gold particles ($\leq$ 5 nm) is not easy and encapsulating more than 2-3 particles in one liposome is almost impossible (Hong et al.1983; Straubinger et al., 1983). The few particles may not distinguish clearly enough the liposomes in biological environment because a gold particle released from a disrupted liposome during interaction with tissues or cells could be spontaneously entrapped into a membrane fragment of cellular origin. Consequently the gold marker would no longer represent the liposomes.

To improve the efficiency of gold entrapment, Gao and Huang (1987a, b) developed agarose-gelatin microspherules into which a larger number of colloidal gold particles of 10-14nm size were introduced. Then the gold-bearing microspherules were encapsulated into liposomes. This method intends to improve not only the number of gold particles encapsulated but also to increase the stability of gold containing liposomes. It was previously demonstrated that liposomes containing gold particles are not very stable due to the heavy density of colloidal gold (Hong et al., 1983). The limitation of the technique by Gao and Huang (1987 a, b) is that only larger liposomes (> 1μm) can be labelled this way and the lipid composition determines the success of liposome formation around the microspherules.

Ferritin

Ferritin is a 500,000 molecular weight metalloprotein. The iron core which is concentrated into a cubical region of 5-6 nm contains about 5000 iron atoms (Fischbach and Anderegg, 1965) surrounded by a protein coat measuring approximately 10-11 nm (Easterbrook, 1970). Ferritin can be encapsulated into MLV or LUV. Although this marker is somewhat less electron dense then colloidal gold it can be easily recognized because of its characteristic structure. The ferritin-labelled liposomes are prepared essentially by the same encapsulation methods used for other drugs. Ferritin is included in the aqueous phase of the liposomes. This marker was used for in vitro (Petty and McConnell, 1983; Vargas et al., 1990) and in vivo (Cudd et al., 1984) identification of liposomes. Ferritin can also be conjugated to antibodies which can recognize (protein) antigens in liposome membranes (Martin and MacDonald, 1976; Poste et al, 1980). Poste et al. (1980) for example used this method to demonstrate the fusion of liposomes containing ortho- and paramyxovirus envelope glycoproteins with mouse 3T3 cells. After incubation of these proteoliposomes with the cells, the ferritin labelled antibodies recognized the liposomal protein in the cell membrane. In a previous work (Ladhoff et al.1984)

the incorporation of a hydrophobic derivative of ferritin into LUVs was attempted to increase the encapsulation efficiency of this marker into liposomes. Ladhoff et al. (1984) reported that electron dense particles accumulated between the liposome bilayers However, these were believed to be iron particles derived from ferritin which was destroyed during the chemical reaction preparing the "hydrophobic" ferritin with stearoyl chloride. As opposed to ferritin, encapsulation of iron clusters appears to be facilitated by their smaller size.

Colloidal silver

Colloidal silver is a cationic stain. It can be prepared by reduction of silver chloride with aldehydes. The metallic silver is precipitated in the form of fine granules of 3-50 nm size (Sternberger, 1979).

Torchilin et al. (1988) used this particulate electron dense marker, AgCl aggregates, to investigate liposome-cell interactions. They also demonstrated that the marker did not interfere with the interaction process between liposomes and cells.

Lanthanum nitrate

Lanthanum nitrate, an EM stain often used in tracer investigations (Hayat, 1981), was encapsulated into intravenously injected liposomes to demonstrate the presence of liposomes in intravascular macrophages in alveolar wall capillaries of sheep Miyamoto et al. (1988). Liposomes appeared as electron dense "bodies" on micrographs. Lanthanum (La^{3+}) has numerous disadvantages including toxicity and ability to penetrate membranes and organelles. Furthermore the stain can precipitate out in tissues during fixation and washing (Hayat, 1981).

Percoll

Percoll was used by Cudd and Nicolau (1986) as a marker for MLV or LUV. Percoll is polyvinyl-pyrrolidone coated silica particles in colloidal solution. It is a non-toxic marker, and cannot adsorb to or penetrate membranes. The size of percoll is 15-30 nm, therefore it is not suitable for SUV. Percoll particles are inherently electron dense and not easily confused with natural tissue components. Disadvantages of the marker are that it decreases liposome stability and it can be released from the liposomes during incubation or storage.
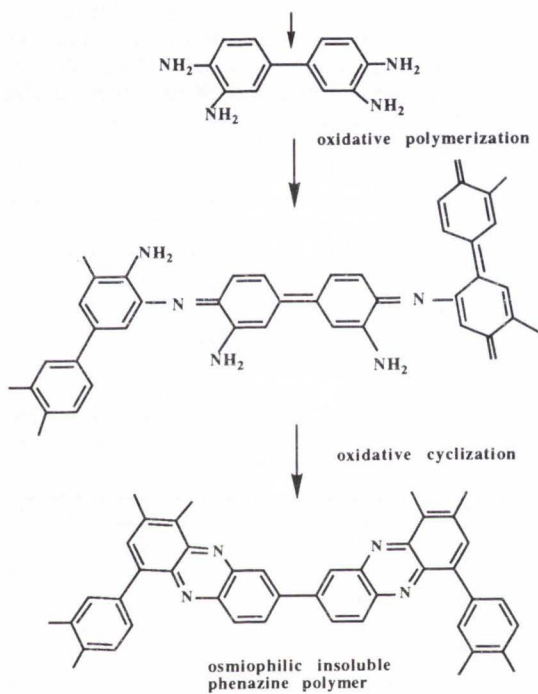
Horseradish peroxidase (HRP)

Horseradish peroxidase (HRP) was the first marker to attempt intracellular localization of liposome contents after incubation with HeLa cells (Magee et al., 1974). Since then, HRP was used by several research groups as a liposomal marker (Table 1). The usual procedure involves fixation of cells and tissues with buffered glutaraldehyde then incubating with 3, 3-diaminobenzidine (DAB) in buffer containing 10% dimethylsulfoxide (to increase permeability of membranes to DAB) and 1 mM imidazole (to increase the specificity of reaction by scavanging free radicals). Hydrogen peroxide is then added. The identification of HRP in the liposomes is based on its enzymatical cleavage of H_2O_2. Oxygen radicals generated react with DAB which is converted into an insoluble polymer.
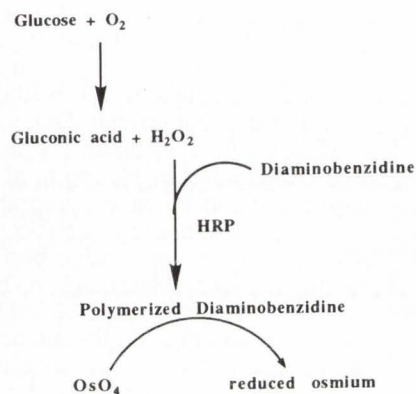
a) Reaction scheme for horseradish peroxidase (HRP)

oxidation of DAB (Sternberger, 1979)

$HRP + H_2O_2 \rightarrow H_2O + 1/2\ O_2$

oxidative polymerization

oxidative cyclization

osmiophilic insoluble
phenazine polymer

b) HRP + GO method (Bugelski et al., 1989)

Glucose + O_2

Gluconic acid + H_2O_2

Diaminobenzidine

HRP

Polymerized Diaminobenzidine

OsO_4 → reduced osmium

c) Tetrazolium salts (Lewis, 1977)

NBT: X =

TCNBT: X = —⟨⟩—CS-NH₂

DSNBT: X = —CH=CH—⟨⟩

$Y-C$ ... $+ 2H \longrightarrow$ ... $+ H^+$

tetrazolium salt   diformazan
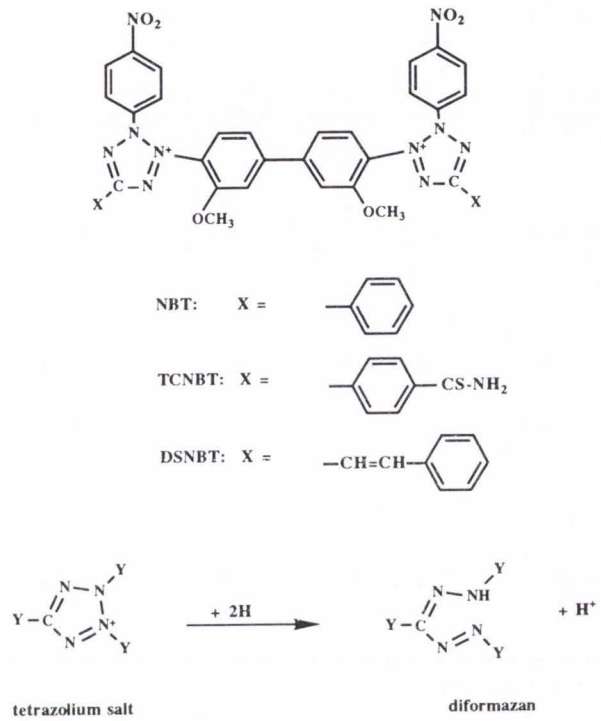
d) Fluorescent markers (Allen, 1984; Weinstein et al., 1984)

$R = CH_2NH^+$

Carboxyfluorescein   Calcein (at pH=7)

Fluorescein isothiocyanate

Fig. 3. Summary of chemical structures and reactions involved in the identification of liposome markers.

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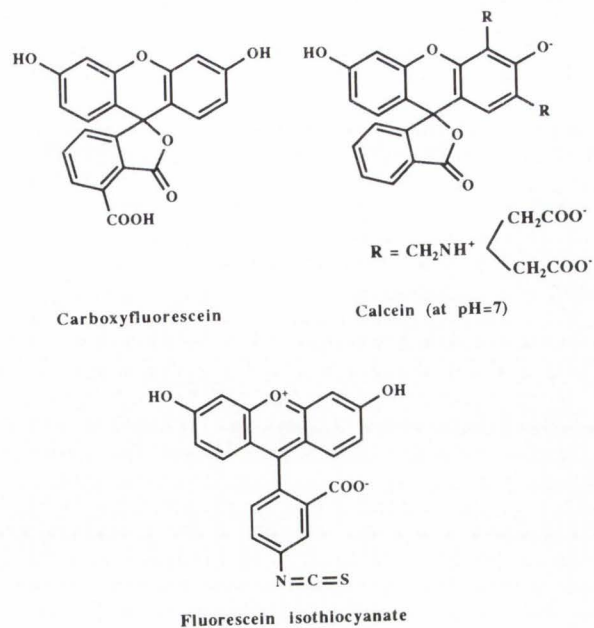The polymerized DAB is rendered electron dense with osmium tetroxide (Fig. 3a). The polymerized form of DAB is highly insoluble making its diffusion from the site of formation unlikely (Sternberger, 1979).

The HRP method was criticized by several authors as being fairly unspecific. Endogeneous peroxidase activity often can obscure results. Bugelski et al. (1989) developed a modified version of the HRP technique to increase both the sensitivity and specificity of the method which facilitates the detection of intact liposomes. In this case the authors coencapsulated glucose oxidase (GO) and HRP in liposomes (Fig. 3b). When fixed tissues are incubated with glucose and DAB, glucose oxidase produces H_2O_2 which is then reduced by HRP. Theoretically only sites where GO and HRP are co-localized will stain. However false positives are still possible in several cell types (e.g. phagocytes) that can enzymatically generate H_2O_2, or where cells endogeneously contain large amounts of enzymes which can oxidize DAB (e.g. myeloperoxidase, catalase, cytochrome C reductase) (Bugelski et al., 1989). It should also be taken into consideration that small unilamellar vesicles (25-100 nm) may not contain large enough quantities of HRP necessary for positive identification.

Uranyl acetate

Cudd et al. (1984) employed liposome-encapsulated uranyl acetate to follow the fate of i.v. injected liposomes in mice. The liver of animals was inspected for the presence of liposomes. Oligolamellar vesicles with electron dense content were identified in association with liver mitochondria. The observations were made on sections which had not been en bloc poststained with uranyl acetate. Cudd et al. (1984) used PBS (pH 7.4) for the preparation of liposomes. At this pH the uranyl acetate is probably present as polyuranates. Unfortunately the authors did not comment on this aspect of the marker. One has to keep in mind that the presence of "electron dense bodies" in osmium-fixed material is not uncommon, therefore caution has to be exercised when interpreting results.

Nitroblue tetrazolium

Nitroblue tetrazolium (NBT) was used as a liposome marker by Segal et al. (1974) for the cellular and subcellular identification of liposomes in rat liver, spleen and kidney after intravenous injection of liposome-entrapped NBT or diformazan in the light or electron microscope, respectively. NBT is a pale yellow substance and not electron dense. But diformazan, its reaction product with phenazine methosulphate (a reducing agent) is an electron dense, although a relatively low contrast material (Fig. 3c). The diformazan deposits in sections can be obscured by the usual poststaining procedure with uranyl acetate and lead citrate. It appears that other derivatives of NBT (Fig. 3c) such as TCNBT (thiocarbanyl-NBT) or DSNBT (distyryl-NBT) could be a better choice because these are osmiophilic and therefore could provide a better contrast. DSNBT is fairly cheap and stable but TCNBT is unstable, expensive and light sensitive (Lewis, 1977).

Potassium dichromate

Heath et al. (1984) used potassium dichromate as a marker for liposomes in vivo. The heavy metal chromium is not very electron dense and unsuitable for routine EM identification but in conjunction with X-ray microanalysis it can be very efficient. In this way, Heath et al. (1984) identified the chromium in liposomes which were taken up into parasitophorus vacuoles of mouse liver cells infected with *Leishmania donovani*. After visual identification of liposome configurations in the cells, the presence of liposomes was confirmed with X-ray microanalysis. Chromium (X-ray emission spectrum 5.4 keV) could be resolved fairly easily from background peaks. In addition, Heath et al. (1984) targeted sodium antimony gluconate by liposomes to the intracellular parasite of the liver. Antimony has a peak in the X-ray emission spectrum at 3.75 keV, and therefore difficult to separate from other background peaks in the cell, especially calcium (3.8 keV). In this case the drug itself could not be utilized for EM detection with the X-ray microanalytical technique.

Autoradiography

Autoradiography is a somewhat different method for localization of liposomes or liposome-encapsulated drugs (Huang and Pagano, 1975; Batzri and Korn, 1975; Huang et al., 1978; Niggemann et al., 1984; Cudd and Nicolau, 1985; Mueller and Munz, 1988; Singh et al., 1988, Foldvari et al., 1990). Either radioactive lipids or drugs are used for the labelling of liposomes. After treatment with liposomes, the cells or tissues are fixed and embedded into resin as usual. The thin sections are transferred to formvar coated grids which in turn are secured to glass slides and covered with an appropriate photographic emulsion in the dark. After drying, the slides are stored in lightproof boxes for a predetermined time interval. Then the slides are developed and the sections are inspected for the presence of silver grains, indicating the location of the labelled compound. Autoradiography can be used for cells incubated in vitro with liposomes or for certain tissues of animals injected with liposomes. Whole body autoradiography of animals can also be carried out (Niggemann et al., 1984; Komatsu et al., 1986).

The interaction of liposomes composed of dimiristoyl lecithin (DML), dipalmitoyl lecithin (DPL) ("solid vesicles") and dioleoyl lecithin (DOL) and egg yolk lecithin (EYL) ("fluid vesicles") with mouse thymocytes was investigated by Huang et al. (1978). These investigators found by autoradiography and transmission electron microscopy techniques, that DML and DPL liposomes were adsorbed on the surface of the thymocytes. Metabolic inhibitors (a combination of 5 mM Na-azide and 50 mM deoxy-glucose) did not have an effect on the association of these liposomes with cells, but trypsinization released about 70% of liposomes from cells incubated with these "solid vesicles" at 2°C and 20 - 40% at 37°C. These results (Huang et al., 1978) indicated that DML and DPL vesicles were mostly adsorbed to the cells in contrast to the "fluid vesicles" (DOL and EYL), which were less affected by the trypsin treatment and metabolic inhibitors, but highly

decreased when the cells were pretreated with 2% glutaraldehyde to cross link the cell surface proteins. Huang et al. (1978) concluded that the major interaction of DOL and EYL liposomes with the mouse thymocytes was fusion, which was also confirmed by the diffuse cytoplasmic staining of cells incubated with the above liposomes containing 6-carboxyfluorescein.

Several investigators, however, caution about the reliability of these types of experiments. In in vitro experiments the extraction and intercellular and intracellular redistribution of radiolabelled lipids can give false results. A label originally concentrated in a particular organelle or region of the cell would be distributed to other regions of the cell during processing of the sample. This was demonstrated by Poste et al. (1978). By mixing glutaraldehyde-fixed mouse L1210 cells which had been previously incubated with sonicated lipid vesicles containing ^{3}H-dipalmitoylphosphatidylcholine with an indicator cell population (fixed avian erythrocytes) with no associated radioactive material, both cell types contained silver grains upon examination in EM. Presently available newer techniques such as freeze substitution, decrease the possibility of autoradiographic artifacts (Harvey, 1982).

Fluorescence markers

Fluorescence markers are used for the qualitative and quantitative identification of liposomes at the light microscopic level (Fig. 3d). A self-quenching fluorophore, 5(6)-carboxyfluorescein (CF, excitation maximum 492 nm, emission maximum 520 nm [green region]) was used by numerous investigators (Weinstein et al., 1977, 1978; Szoka et al., 1979; Blumenthal et al., 1982; Straubinger et al., 1983; Chander et al., 1983; Stevenson et al., 1984; Lelkes and Friedman, 1985; Abraham and Downing, 1990). Only the purified form of carboxyfluorescein is recommended since the commercial preparation contains organic impurities which will lead to excessive leakage from the liposomes (Ralston et al., 1981). In dilute solutions of CF the fluorescence is proportional to the number of dye molecules present, but at higher concentrations (about 10-15 mM) the fluorescence is negligible because of the interaction between the fluorophore molecules. Thus, a suspension of vesicles containing 100-200 mM CF fluoresces only slightly. Fluorescence increases more than 30-fold when the dye is released and diluted into the solution or the cell cytoplasm.

This marker can be employed for the clarification of the interaction event of liposomes with cells. Vesicles adhering to the cell membrane would show very low fluorescence, but when their content is released into the cell cytoplasm or medium, the fluorescence intensity increases proportionally. Disruption of liposome containing CF by detergent will give the maximum fluorescence intensity (Weinstein et al., 1984). The released (leaked) CF from liposomes however could be confused with fluorescence due to cellular delivery. Another important property of CF is its pH sensitivity. Its fluorescence decreases as the acidic groups are protonated. As a result of this, fluorescence efficiencies are decreased at low pH, e.g. in the lysosomes.

The quantitation of uptake of CF-labelled liposomes can be carried out by fluorescence activated cell sorter (FACS) technique (Blumenthal et al., 1982; Stevenson et al., 1984; Lelkes and Friedman, 1985).

Calcein is an impermeant, aqueous-soluble, self-quenching marker used by several research groups to replace CF (Straubinger et al., 1983; Connor and Huang, 1985; Ho et al., 1986). The excitation maximum (490-500 nm) and emission maximum (511-520 nm) for calcein is similar to CF (Allen, 1984). The advantage of calcein over CF is its pH insensitivity in the physiological pH range. At pH=7 calcein carries a net negative charge of -3.

Fluoresceinisothiocyanate - conjugated dextran (FITC-dextran) (4-70 kDa) has also been used as a water-soluble fluorescent marker (Straubinger et al., 1985; Mochizuki et al., 1986; Rosenberg et al., 1987). FITC-dextran is a high molecular weight compound, therefore not easily released from the liposomes. By itself it will not penetrate cell membranes, therefore interpretation of liposomal delivery is easier.

Fluorescent derivatives of lipids such as NBD-PE (N-(7-nitro-2, 1, 3-benzoxadiazole-4-yl phosphatidyl-ethanolamine), diI-DOPC (dihexadecylindocarbocyanide iodide dioleoyl-phosphatidylcholine) also can be used for microscopic visualization of liposomes taken up by cells (Szoka et al., 1980; Blumenthal et al., 1982). Szoka et al. (1980) demonstrated that liposomes were most likely adsorbed to the cell surface without internalization, since there was no recovery of fluorescence at the photobleached spot determined by the fluorescence recovery after photobleaching method (FRAP technique). In this technique the fluorescence intensity on the cell surface is detected by the measuring beam of the fluorescence microscope. Photobleaching in a designated area on the cell membrane will extinguish fluorescence. Fluorescence will reappear in the bleached spot if the fluorescence lipids are freely moving in the membrane. This only happens when liposomes fuse with the cell membrane. If only surface adhesion to the cell occurs no fluorescence recovery can be observed.

Other techniques

Some research groups rely on the "characteristic" vesicular or multilamellar appearance of liposomes to identify them in tissues (Poste et al., 1982; Debs et al., 1987). This sometimes can give indication, however it is not advisable to be used as conclusive evidence of liposomes. Myelin-like figures and endogenous vesicles in the tissues can be easily confused with liposomes.

Quantitation of liposome uptake by cells and tissues can be carried out by scintillation counting of radiolabelled compounds (Papahadjopoulos at el., 1974; Pagano and Huang, 1975; Poste and Papahadjopoulos, 1976; Kayawake and Kako, 1982; Richardson et al., 1982; Foldvari et al., 1991). To determine the association of liposomes with subcellular

organs cell fractionation can be carried out (Mayhew et al., 1980; Cudd and Nicolau, 1985).

Scanning EM (Fraley et al., 1981) and freeze fracture EM (Laudonio et al., 1990) are alternative techniques to investigate liposome-cell interactions. Laudonio et al. (1990) investigated liposome-cell interactions in human M14 melanoma cell line to determine the mechanism of cell damage by heat after liposome pretreatment. Laudonio et al. (1990) used freeze fracture technique to observe the cell surface. The presence of numerous vesicles associated with the cell membrane indicated adsorption or fusion of liposomes with the cells. The liposomes were identified as vesicles without intramembraneous particles (proteins).

The topical application of liposomes is a focus of attention of an increasing number of scientists. After extensive experimentation to establish the carrier potential of liposomes for the skin, the mechanism of drug delivery by liposomes on the ultrastructural level started to become an area of interest. Although CF appeared to be a suitable marker for SUV to study interaction between isolated corneocytes and liposomes (Abraham and Downing,1990), the majority of studies with skin in vitro or in vivo were using freeze fracture EM. For example, Hofland et al. (1991) investigated the interaction of non-ionic surfactant vesicles (NSV) with skin. After exposing to NSV for 48h, the skin was freeze-fractured. Distinct ultrastructural changes were noted compared to the untreated skin. Hofland et al. (1991) argued that the vesicular structures appearing between the corneocytes have a very regular construction resembling the original NSV and furthermore such structures are not present in the untreated skin. The freeze-fracture method is uniquely suitable to reveal membraneous structures, however, in an environment which is rich in lamellar bodies and sheets (such as the skin), it is usually necessary to support the data with alternative techniques.

Colloidal iron as a new marker

Colloidal iron as a stain was first used by Hale (1946) on the light microscopic level. After iron incubation negatively charged groups of the tissue bind to the positively charged colloidal iron solution at pH 2. The bound colloidal iron was then precipitated by potassium hexacyanoferrate (II) to form Prussian blue (Mowry, 1958). Hale (1946) and subsequently other workers (Gasic and Berwick, 1963; Gasic et al., 1968; Weiss and Subjeck, 1974; Mareel et al., 1976) adopted the colloidal iron for electron microscopy by omitting the Prussian blue reaction, and utilizing the electron dense, particulate (3-9 nm) nature of the colloidal iron for staining acidic mucopolysaccharides and sulfate, sialic acid and hyaluronic acid containing mucins of cell surfaces on thin sections.

The preparation of colloidal iron solution involves the hydrolysis of ferrous chloride by adding it (as a concentrated solution) dropwise to boiling distilled water. During this reaction, a brownish-red colloid solution forms which probably contains several forms of oxidized iron ($Fe(OH)Cl_2$, $Fe(OH)^{2+}$, $Fe(OH)_2^+$, $FeCl_2^+$, $FeCl^{2+}$, $FeCl_6^{3-}$, $Fe(OH)_3$, $Fe(OH)_2^{4+}$) in form of colloidal iron particles (Lillie et al., 1973). The colloidal iron particles have an insoluble $Fe(OH)_3$ core carrying ionizing solubilizer molecules of FeOCl on its surface. The molecular architecture of the particle can be seen on Fig 4, redrawn from Pauli (1928). The particles have a net positive charge because they generally contain 20-50 times more Fe atoms as Cl atoms (Pauli, 1928).

Previous investigations indicated that colloidal iron is a potential electron dense marker for liposomes since it could be prepared in small particle size and encapsulated into liposomes at high concentrations (Foldvari et al., 1988).

The electron microscopic investigation of the colloidal iron solution showed colloidal iron particles of 1 - 11 nm size. Fig. 5A shows the appearance of colloidal iron solution prepared for encapsulation purposes in the electron microscope without any staining. The majority of the colloidal iron grains (76%) measured less than 5 nm as was determined by statistical analysis of 150 randomly selected particles. The colloidal iron particles are distinctly electron dense (although less so than colloidal gold) (Foldvari et al., 1988).

The applicability of colloidal iron liposomes in cell culture (Fig 5 C) and on the skin (Fig 5D) was also demonstrated before (Foldvari et al., 1988 and 1990). The results indicated that the colloidal iron is a suitable marker for liposomes of different sizes.The encapsulation of the marker is fairly easy and the colloidal iron containing liposomes can be identified with considerable certainty. It also appears that the presence of marker does not influence the interaction process between liposomes and cells, since the
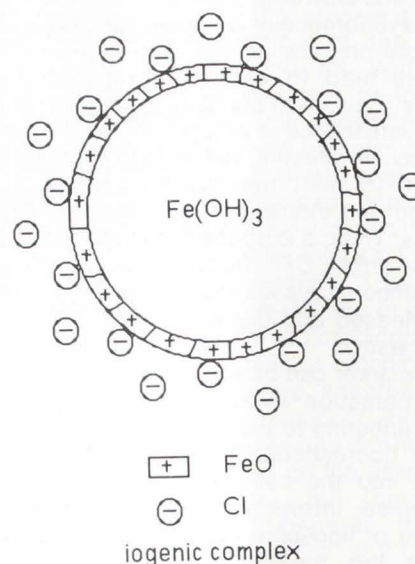

$Fe(OH)_3$

| + | FeO |
| ⊖ | Cl |

iogenic complex

Fig. 4. Schematic picture of the colloidal iron particle. Redrawn from Pauli (1928).
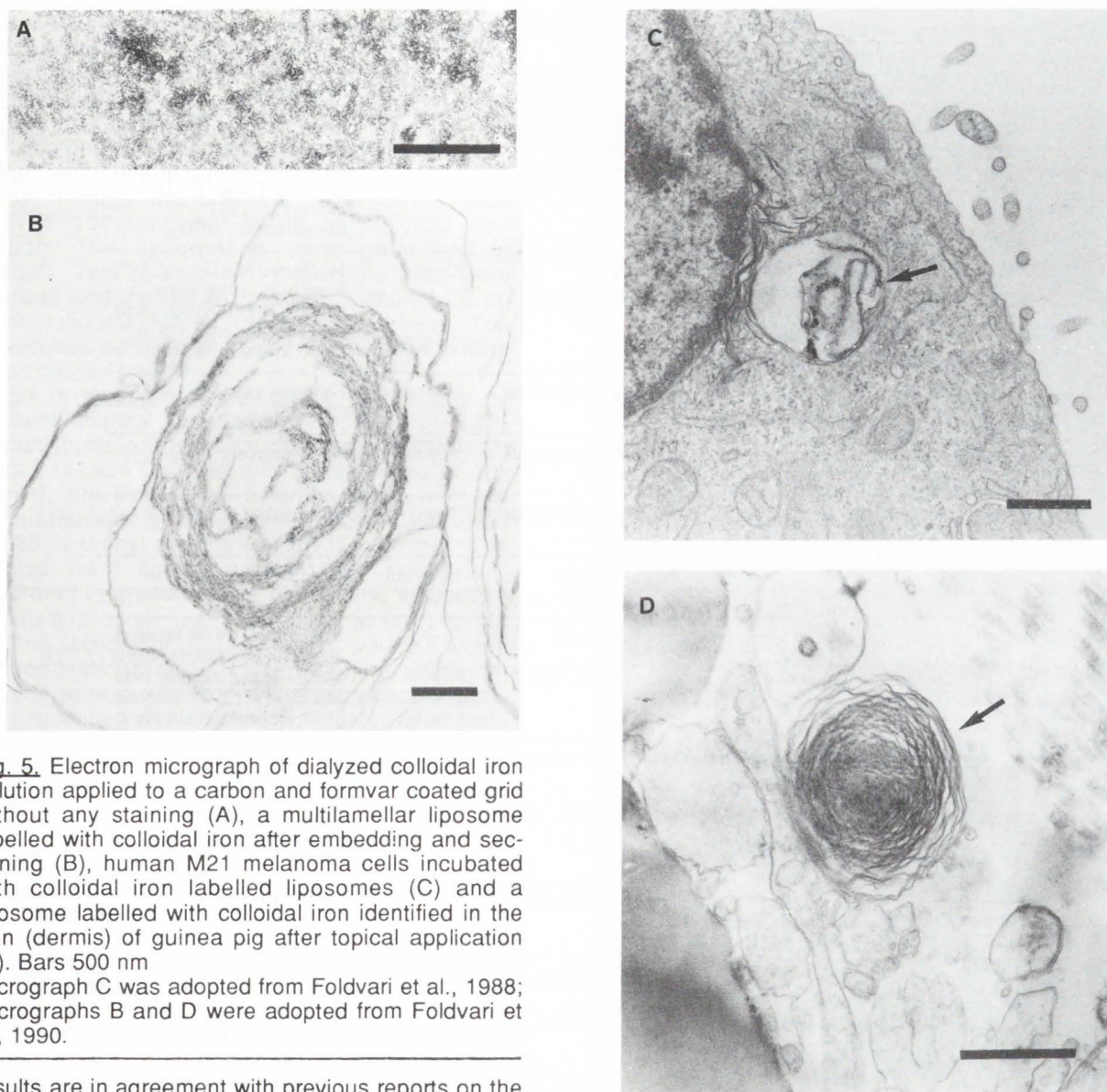
Fig. 5. Electron micrograph of dialyzed colloidal iron solution applied to a carbon and formvar coated grid without any staining (A), a multilamellar liposome labelled with colloidal iron after embedding and sectioning (B), human M21 melanoma cells incubated with colloidal iron labelled liposomes (C) and a liposome labelled with colloidal iron identified in the skin (dermis) of guinea pig after topical application (D). Bars 500 nm

Micrograph C was adopted from Foldvari et al., 1988; micrographs B and D were adopted from Foldvari et al., 1990.

results are in agreement with previous reports on the endocytotic uptake of neutral liposomes. The initial liposome - cell interaction experiments indicated that colloidal iron is a promising tool to aid the clarification of several important aspects of liposome-cell fusion and other type of interactions (Foldvari et al., 1988).

The colloidal iron marker was found to be useful in studying liposome-skin interactions. Liposomes containing colloidal iron were applied topically on guinea pig skin. After multiple dose treatment, the excised skin was analyzed by electron microscopy. The presence of unilamellar and multilamellar liposomes in the dermis could be demonstrated on micrographs (Fig 5B). The characteristic density of the iron grains in the liposomes represented the intact state of the vesicles, which could be assessed on the micrographs (Foldvari et al., 1990).

The main objective of the present investigation was to test whether colloidal iron is a suitable marker for liposomes in vivo.

Materials and Methods

Preparation of colloidal iron liposomes

Colloidal iron was encapsulated into MLV prepared from either dipalmitoyl-phosphatidylcholine (DPPC; Sigma Chemical Company, St Louis, MO) or soya phosphatidyl-choline (SPL; American Lecithin Company, Atlanta GA) and cholesterol (Sigma Chemical) 2:1 molar ratio (m.r.) as described by Foldvari et al. (1988). Briefly, the lipids were dissolved in chloroform-methanol 2:1 (v/v). After the evaporation of the organic solvents, the thin, even lipid film deposited on the surface of glass beads and the wall of the flask was hydrated with a diluted colloidal iron solution (the stock solution, diluted with 0.01M PBS [7.53 mM Na_2HPO_4; 2.5 mM NaH_2PO_4; 145 mM NaCl, ph 7.1] 1:1 v/v). The colloidal iron stock

Table 1. Markers used to identify liposomes in biological environment

Marker	Method	Material to be detected	Size of label	References
Colloidal gold	TEM encapsulated colloidal gold, gold-conjugated antibody	colloidal gold particles	3-150 nm	Hong et al., 1983 Straubinger et al., 1983 Debs et al., 1987 Gao and Huang, 1987a, b Stern et al., 1987 Childers et al., 1990
Ferritin	TEM encapsulated ferritin, ferritin-conjugated antibody	iron core	10 nm	Martin and MacDonald, 1976; Poste et al., 1980; Petty and McConnell, 1983; Cudd et al., 1984; Ladhoff et al., 1984; Vargas et al., 1990
Colloidal iron	TEM, encapsulated colloidal iron	colloidal iron particles	1-10 nm	Foldvari et al., 1988, 1990
Colloidal silver	TEM, encapsulated colloidal silver	colloidal silver granules	3-50 nm	Torchilin et al., 1988
Lanthanum nitrate	TEM, encapsulated lanthanum nitrate	heavy metal lanthanum	[2 nm]	Miyamoto et al., 1988
Percoll	TEM, encapsulated percoll	percoll particles	15-30 nm	Cudd and Nicolau, 1986
Horseradish peroxidase	TEM encapsulated HRP → DAB reaction	osmicated DAB	---	Magee et al., 1974; Wisse et al., 1976; Finkelstein, 1981; Morimoto and Adachi, 1982 Machy et al., 1987; Derksen, 1987; Bugelski et al., 1989
Uranyl acetate	TEM, encapsulated uranyl acetate	heavy metal uranium	[2 nm]	Cudd et al., 1984
Nitroblue tetrazolium	TEM encapsulated NBT → reduction reaction	diformazan	---	Segal et al., 1974
Potassium dichromate	TEM + X-ray microanalysis encapsulated K-dichromate	heavy metal chromium	---	Heath et al., 1984
Radioactive material	TEM or light microscopic autoradiography encapsulated lipids or drugs	silver grains	---	Huang and Pagano, 1975; Batzri and Korn, 1975; Huang et al., 1978; Poste et al., 1978; Niggemann et al., 1984; Cudd and Nicolau, 1985; Mueller and Munz, 1988; Singh et al., 1988; Knoll et al., 1988; Foldvari et al, 1990
Fluorescent material	Fluorescent microscopy encapsulated lipids or drugs	fluorescence	---	Weinstein et al., 1977, 1978; Szoka et al., 1979, 1980; Blumenthal et al., 1982; Straubinger et al., 1983,1985; Chander et al., 1983; Stevenson et al., 1984; Lelkes and Friedman, 1985 Connor and Huang, 1985 Ho et al., 1986; Mochizuki et al., 1986; Rosenberg et al., 1987; Caramelli et al., 1989; Abraham and Downing, 1990
No marker	TEM, SEM, Freeze fracture	cell surface morphology	---	Fraley et al., 1981; Poste et al., 1982; Debs et al., 1987; Knoll et al., 1988; Schreier, 1989; Laudonio et al., 1990

solution was prepared by the condensation method as described previously (Foldvari et al., 1988). The liposomes were shaken for 20 min at 45°C.

Intravenous administration of colloidal iron containing liposomes

Colloidal iron containing liposome preparation (100μl, 4mg lipid/mouse) was injected into one mouse through the tail vein. The control mice received 100μl normal saline or colloidal iron solution. The experiments were performed in duplicats. Two hours after injection the mice were sacrificed and the following organs sampled : liver, spleen, lung, fat, kidney, brain, skin and heart. The tissue cubes were cut into small pieces in the fixative and processed for EM.

Tissue processing for electron microscopy

Small pieces of tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 for 2 hrs at 4°C . After rinsing with the buffer (3 x 10 min), the tissues were further fixed with 1% osmium tetroxide in buffer for 2 hrs at 4°C, followed by 0.25 % uranyl acetate treatment overnight. The samples were rinsed with distilled water and dehydrated in graded series of acetone, embedded in Taab 812 epoxy resin and cured at 60°C for 48 hrs. Thin sections of about 70-80 nm were cut with a diamond knife on an LKB - Huxley ultramicrotome. Sections were collected on 400 mesh copper grids and poststained with 2% uranyl acetate. Lead stain was completely avoided to prevent the masking effect of lead on the iron particles. The sections were viewed in a Philips EM 200 electron microscope.

Results and Discussion

Physical and chemical stability of colloidal iron containing liposomes

A high concentration of colloidal iron solution can be encapsulated into MLV, LUV, and SUV as was demonstrated before. (Foldvari et al. 1988). Virtually all liposomes contained large number (> 100) of colloidal iron particles which are usually non-uniform in size. The typical granularity of the packed colloidal iron particles appearing between the lamellae of multilamellar liposomes provides a basis for a fairly certain identification when compared to "empty" lipid vesicles (compare Fig. 1 and 5 B). The physical stability i.e. the ability of liposomes to retain the encapsulated colloidal iron particles was investigated after 1 week, 1 and 2 month storage intervals by electron microscopy. These studies revealed that the colloidal iron was still retained in the liposomes after 2 months of storage in the multilamellar liposomes.

Thin layer chromatography (TLC) to identify degradation products of lipids in colloidal iron containing liposomes due to peroxidation demonstrated that up to 1 month the liposome preparations (DPPC-Chol 2:1m. r. or SPL-Chol 2:1 m.r.) showed identical spots on the TLC plates to corresponding lipid standards, indicating that the peroxidation of lipids in the liposomes is not facilitated by the presence of colloidal iron (results not shown). This in in agrement with the report which showed that ferritin and haemosiderin in liposomes

can induce peroxidation only if the iron is mobilized in the form of Fe^{2+} (by e.g. the presence of ascorbate) (O'Connel et al.,1985).

Fine structure of liposomes after intravenous administration

In this study, the applicability of the colloidal iron as a marker was tested for the identification of liposomes in tissues after i.v. injection into mice. It was initially believed that the presence of iron in these organs might obscure the colloidal iron derived from liposomes, however, the characteristic multilamellar liposome structure with the colloidal iron particles between the lamellae might be still recognizable.

Electron microscopic investigations of tissue sections from the liver and spleen confirmed our concerns about the difficulty distinguishing the endogenous iron particles from liposome derived colloidal iron. The liver and the spleen are organs for the storage of iron containing proteins (ferritin, hemosiderin), therefore studying the disposition of colloidal iron from the liposomes was not possible. The heavy deposition of iron (Fig. 6 A-C small arrows) even in the spleen sections of untreated (Fig. 6 A) or colloidal iron solution treated (Fig. 6 B) mice would not allow identification of iron grains derived from liposomes after their destruction in the tissue. Multilamellar myelin-like whorls, which can easily be confused with liposomes when they are not labelled, are also present in the control (untreated) sections (Fig. 6 A, large arrow). These whorls can form as a result of degradation of red blood cell membranes (Simon, 1980) or also could be fixation artifacts.

It appears, however, that intact multilamellar liposomes containing the colloidal iron marker are recognizable in the spleen (Fig. 6 D, arrow). The size of the colloidal iron liposome is about 250 nm, and its structure is fairly distorted. The spleen of mice injected with free colloidal iron solution contained colloidal iron particles in single walled membraneous structures (Fig. 6 B, arrow). The origin of these particles is not certain, but most likely part of the endogenous iron deposits in the spleen.

Similar difficulties to those in the spleen were encountered in the liver (Fig. 7 A-D) regarding the identification of the liposome-released colloidal iron and unilamellar vesicles containing iron particles. These membrane bound colloidal iron particles were identified in colloidal iron liposome injected mouse liver (Fig. 7 D, arrows), but most likely they are endogenous storage vesicles not liposomes, because similar structures can be observed in untreated liver (Fig. 7 A , arrow) and colloidal iron solution injected mouse liver as well (Fig. 7 B, arrow). However, multilamellar liposomes of about 500 - 600 nm size containing colloidal iron could be identified in hepatocytes (Fig 7 C, large arrow). It is noteworthy that numerous MLV containing the colloidal iron marker were observed to be associated with red blood cells in the hepatocytes.

Thin sections from the lungs of mice injected with colloidal iron containig liposomes or colloidal iron solution were also examined. Colloidal iron containing multilamellar vesicles (Fig. 8 C, large arrow) and colloidal iron particles could be identified
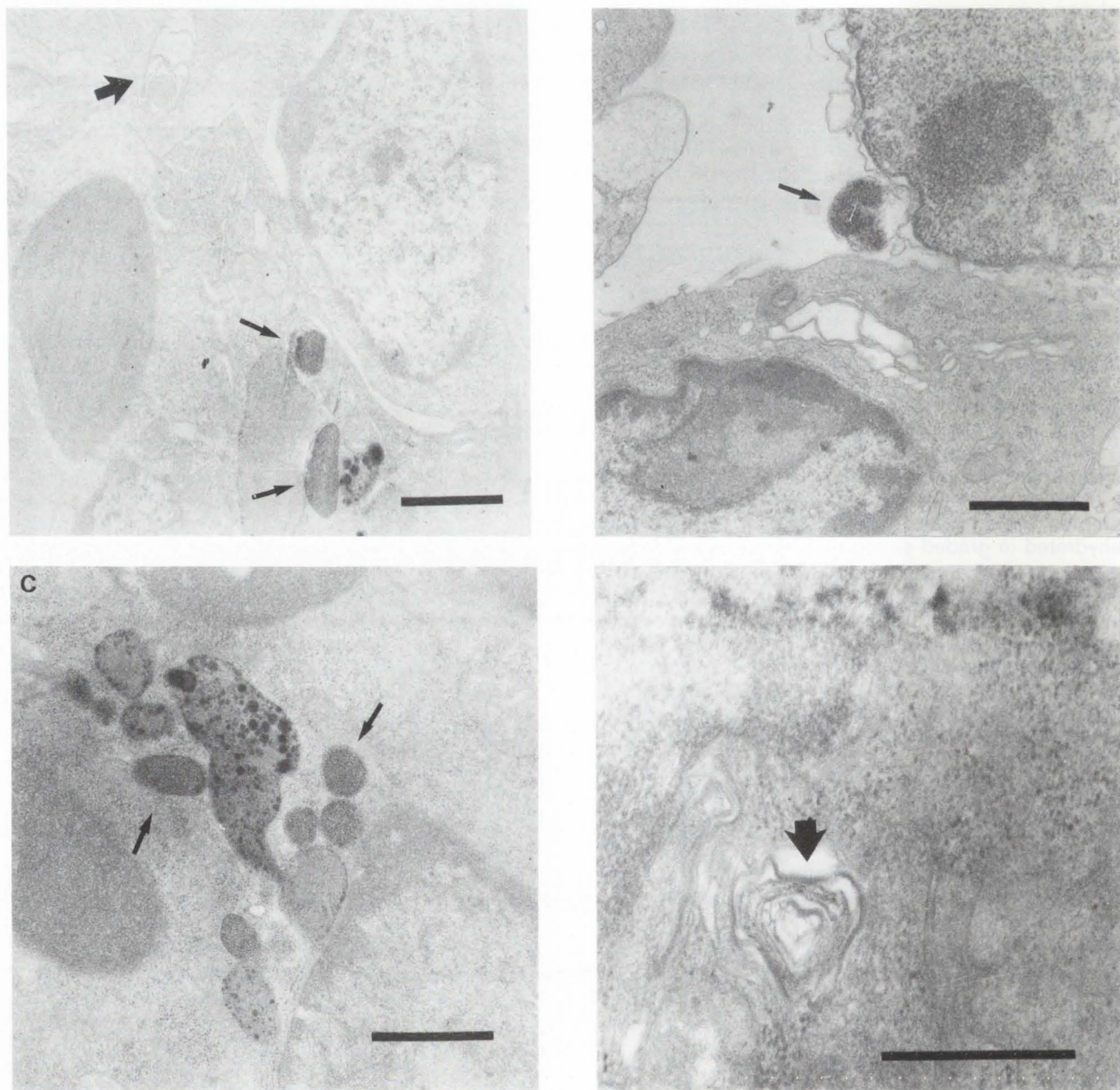
Fig. 6. Fine structure of liposomes containing colloidal iron in the spleen after intravenous administration into mice. Bars 1µm (A-C), 0.5 µm (D).
Micrograph A: untreated; B: mice injected with colloidal iron solution; C and D: mice injected with liposomes containing colloidal iron. Small arrows point to iron deposits on all micrographs, large arrow shows a multilamellar whorl on micrograph A and a multilamellar liposome containing colloidal iron on micrograph D.

in the liposome injected mouse (Fig. 8 D, arrowheads). No colloidal iron was visible in lung sections of controlled animals injected with saline (Fig. 8 A) or colloidal iron solution (Fig. 8 B). Fig. 8 A shows an area where numerous endogenous multilamellar structures are visible in the control lung section. Due to the presence of encapsulated colloidal iron, the liposomes can be distinguished from these endogenous vesicles (Fig. 8 C, large arrow).

A number of reseach groups showed that after intravenous injection the liposomes are cleared from the circulation mainly by the liver and the spleen (Gregoriadis and Ryman, 1972; Hinkle et al., 1978; Roedrink et al., 1981; Beaumier and Hwang, 1983). The lungs can also trap a portion of the injected liposomes possibly in the capillaries (Abra et al., 1984; Harmsen et al., 1985). But in general, the other organs take up negligible amounts of liposomes.
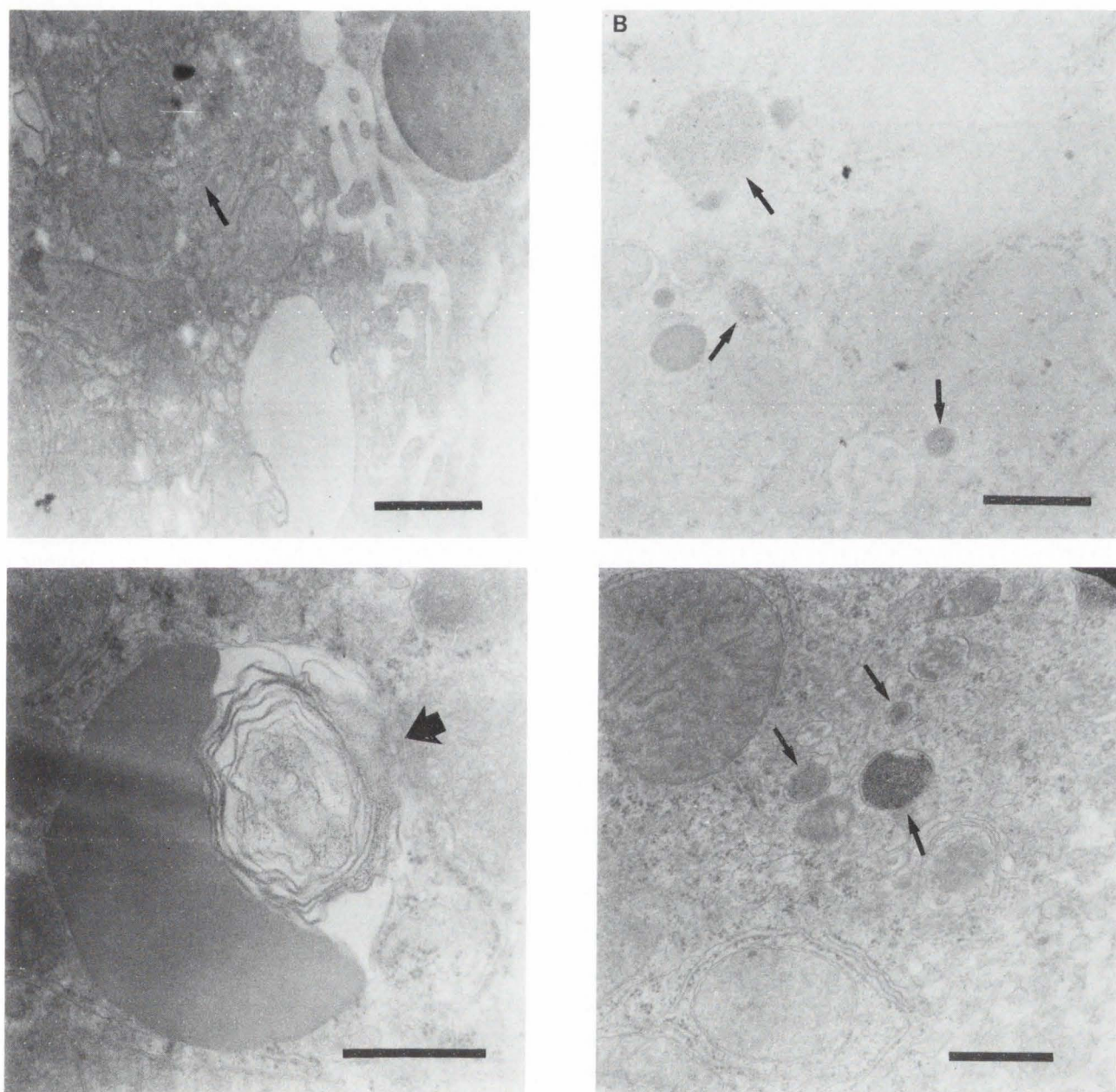
Fig. 7. Fine structure of liposomes containing colloidal iron in the liver after intravenous administration into mice. Bars 500 nm.
Micrograph A: untreated; B: mice injected with colloidal iron solution; C and D: mice injected with liposomes containing colloidal iron. Small arrows point to iron in vesicular structures on all micrographs, large arrow shows a multilamellar liposome containing colloidal iron on micrograph C.

Cudd et al. (1984) investigated the fate of intravenously injected liposomes composed of egg yolk phosphatidylcholine, ox brain phoshatidylserine and cholesterol and in certain cases lactosylceramide and encapsulating either uranyl acetate or ferritin as electron dense marker. It was found by Cudd et al. (1984), that liposomes or liposome - encapsulated DNA associated with mitochondria of Kupffer cells, hepatocytes and endothelial microvilli. Our results are in agreement with other studies where the preferential uptake of small liposomes by the liver hepatocytes was found (Scherphof et al., 1983; Roedrink et al., 1984). In the present studies the liposomes which were identified in the liver had about 500 nm diameter size and were located in the hepatocytes, although no preferential association of liposomes with hepatocyte mito-chondria could be confirmed.
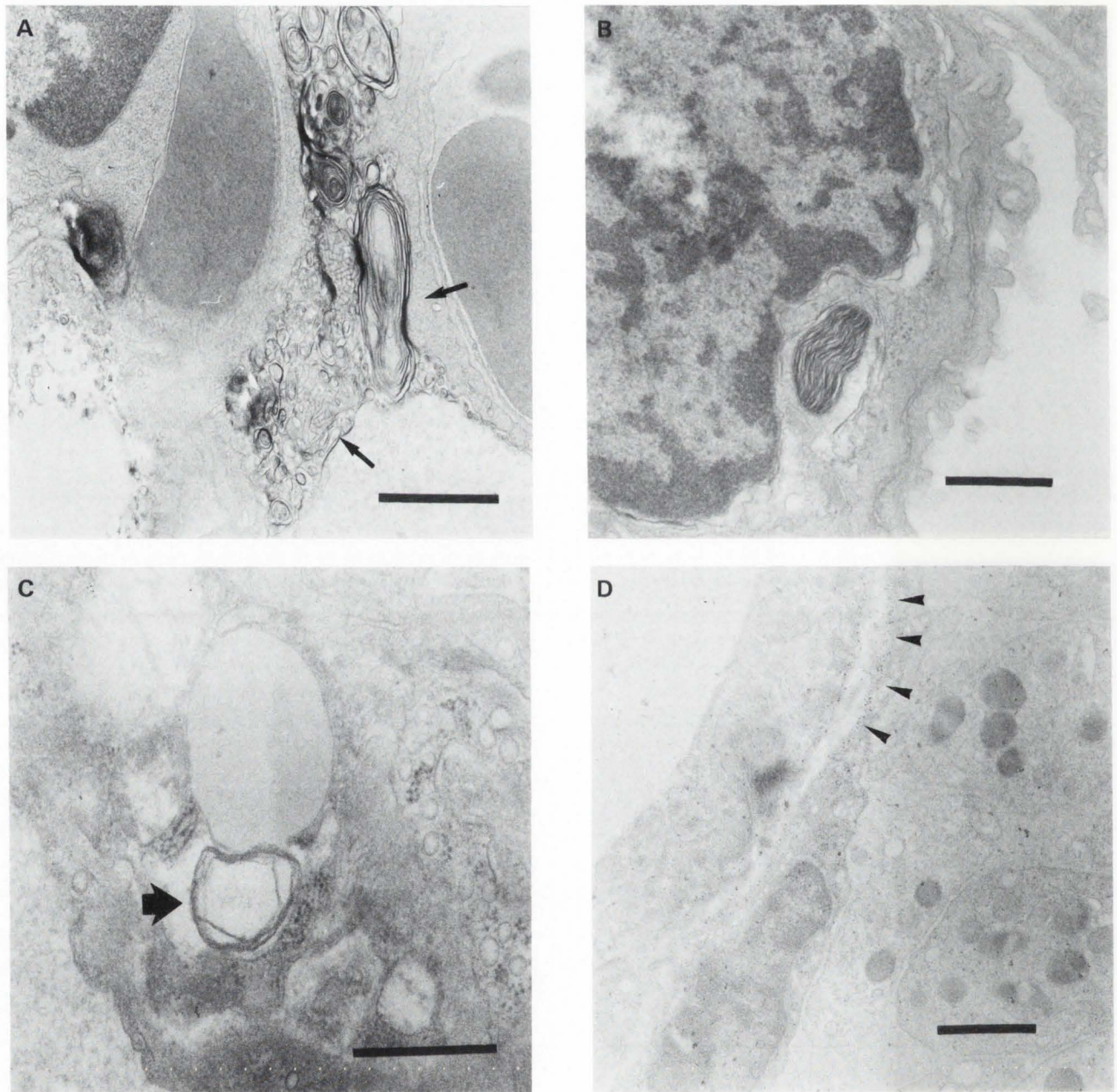
Fig. 8. Fine structure of liposomes containing colloidal iron in the lung after intravenous administration into mice. Bars 0.5μm (B, C), 1μm (A,D).
Micrograph A: untreated; B: mice injected with colloidal iron solution; C and D: mice injected with liposomes containing colloidal iron. Small arrows point to multilamellar structures on micrograph A, large arrow shows a multilamellar liposome containing colloidal iron on micrograph C and arrowheads show fine iron deposit on micrograph D.

However, the present findings are also in agreement with those previous results, which found that liposomes up to 0.5 μm could pass the fenestrated lining of sinusoids (Roedrink et al., 1981; Rahman et al., 1982) and taken up into the hepatocytes.

Conclusions

Colloidal iron proved to be a suitable marker in cell culture and in the skin after topical application of liposomes to visualize intact liposomes and possible

drug release pathways from liposomes (Foldvari et al., 1990). The colloidal iron labelled liposomes were easily identified in cells by routine electron microscopic procedures. Liposomes could be identified in lysosomes or endosomes of human M21 melanoma cells after incubation of cells with colloidal iron containing liposomes (Foldvari et al., 1988). Intact, as well as partially degraded liposomes were present after two hours of incubation.

The present experiments indicated that colloidal iron might also be useful to follow the fate of intravenously injected liposomes in tissues which do not contain endogenous iron deposits unlike the liver and the spleen.

In the last few years the major interest and effort was devoted to direct liposomes away from the reticuloendothelial system (RES) and to manipulate the lipid composition of liposomes to increase the liposome uptake into the brain (Yagi and Naoi, 1986), ischemic heart (Mueller et al., 1981) and lungs (Sunamoto, 1986). To follow the fate of these modified liposomes in the mentioned organs the colloidal iron could serve as a useful marker. Future investigations could aim the encapsulation of colloidal iron into liposomes bearing specific carbohydrate residues or antibodies which direct liposomes specifically to selected organs or tumors. The drug release from the liposomes could also be modelled by the colloidal iron due to its high concentration in the liposomes. This aspect of the study however should extend the attention to the behaviour of the colloidal iron itself in the biological milieu.

Acknowledgement

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DISCUSSION WITH REVIEWERS

P.J. Bugelski: Have you tried to encapsulate colloidal iron into liposomes made of various lipids including charged lipids?
Authors: Yes, we have used a few different lipid species in the liposomes including dipalmitoyl-phosphatidylcholine (DPPC) and phosphatidyl-ethanolamine in combination with DPPC and cholesterol and soya phosphatidylcholine. So far we have not tested charged lipids.

P.J. Bugelski: What about using colloidal iron dextran?
Authors: We have been using the colloidal iron solution, prepared in our lab by the condensation method from ferrous chloride. The colloidal iron solution is easy to make, reproducible, inexpensive and stable for several months.

H.E. Junginger: What is the proposed route of penetration of intact liposomes through the stratum corneum and the epidermis? In addition to the dermis, did you find intact liposomes in the epidermis?
Authors: It is still debated how exactly liposomes cross the stratum corneum and viable epidermis if at all. In our studies, intact liposomes containing the colloidal iron particles were identified in the dermis after multiple application of liposomes on guinea pig skin _in vivo_. Since large numbers (>100) of colloidal iron particles were present in these liposomes, similarly to the original preparation, it is unlikely that these liposomes reformed after being disrupted on the skin surface or during transit through the epidermis. It is still not clear what percentage of liposomes can penetrate in intact state and what other mechanisms are playing a role in the liposome-skin interaction process. We have only found distorted liposome-like structures associated with colloidal iron in the epidermis. We are actively investigating the mechanism of liposomal drug delivery into and through the skin.

H. Schreier: Please comment on the validity of electron microscopy to demonstrate partition of liposomes/niosomes into various skin layers upon topical administration.
Authors: Electron microscopy as a single technique can give visual evidence for penetration of liposomes/niosomes into the skin but usually has to be used together with another technique (e.g. radiotracer technique where both the liposomal lipid and drug are labelled and the ratio of isotopes can be monitored) for more conclusive evidence.

C. W. M. Grant: Please comment on the quantitative aspect of electron microscopy in liposome visualization.
Authors: It is very difficult if not impossible to quantitate the number of liposomes interacting with cells and tissues. It is usually very time consuming to look at large number of cells in the electron microscope to statistically determine the frequency of a certain event. And even then one only looked at a very small fraction of the tissue or cell culture involved in the experiment. One can get a feel for the frequency of a certain interaction process since if it is of frequent occurrence it will be more obvious after investigating fewer sections.