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Solid core liposomes with encapsulated colloidal gold particles

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Solid core liposomes with encapsulated colloidal gold particles were prepared through four major steps: (1) Preparation of prevesicles with encapsulated solid cores of agarose-gelatin by emulsification of agarose-gelatin sol in organic solvent containing emulsifiers followed by cooling. (2) Extraction of lipophilic components from prevesicles to obtain microspherules of agarose-gelatin. (3) Introducing colloidal gold particles into microspherules and coating with protein molecules. (4) Encapsulation of colloidal gold-bearing microspherules with the modified organic solvent spherule evaporation method for preparation of liposomes (Kim et al. (1983) Biochim. Biophys. Acta 728, 339–348 and Kim et al. (1984) Biochim. Biophys. Acta 812, 793–801). Electron micrographs showed that if liposomes were prepared by using a lipid mixture containing dioleoylphosphatidylcholine / cholesterol / dioleoylphosphatidylglycerol / triolein (molar ratio 4.5:4.5:1:1), there was only a single continuous bilayer membrane for each solid core liposome. However, if no triolein was added to the lipid mixture, it would cause the formation of multilamellar liposomes. In both cases, there were hundreds to thousands of colloidal gold particles within each solid core liposome.

Introduction

In recent years, efforts have been made to use liposomes as drug carrier to deliver drugs and other biologically active materials into cells [1,2]. It becomes an urgent problem to study the interaction between cells and liposomes, as well as the fate of internalized liposomes within cells. Unfortunately, there exist many natural vesicle-like structures in cells, which cause difficulty for the identification of liposomes when they get into cells. To solve the problem, Hong et al. (1983) [3] developed a method with which liposomes were labeled with colloidal gold, but very few gold particles were entrapped. We have tried several methods of preparing liposomes with a higher loading capacity for colloidal gold particles. We and others [3] found that colloidal gold particles are not stably entrapped in the liposomes due to their heavy density. An effort was then made to embed the gold particles in a gel matrix, i.e. a solid core with impregnated colloidal gold particles [4]. A large number of gold particles of uniform size could be introduced into the matrix of the agarose-gelatin solid core [4]. We now report the method with which one can entrap the gold-labeled solid cores into liposomes. A pre-

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Abbreviations: Au, colloidal gold (particles); AuSC, colloidal gold-labeled solid core(s) of agarose-gelatin; AuSCL, solid core liposome(s) with encapsulated colloidal gold; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; OSSE method, organic solvent spherule evaporation method for the preparation of liposomes.

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liminary report of a portion of this work has been presented [5].

Materials and Methods

Preparation of colloidal gold labeled solid core

This was done by the method of Gao and Huang [4]. Briefly, solid cores of agarose-gelatin were prepared by emulsification of a warm (approx. 70°C) aqueous mixture (200 μl 5.33% agarose and 2.67% gelatin, 80 μl 1 M *n*-octyl glucoside, 80 μl 50 mM Hepes buffer (pH 7.4) with 5 mM EGTA and 750 mM NaCl, 40 μl 50 mM calcein and 0.6 g glass beads) in an organic phase (3880 μl cyclohexane with 53.3 μmol egg phosphatidylcholine and 10 μmol Span 80 as emulsifiers and 120 μl absolute ethanol as a co-surfactant). The emulsification procedures were performed in warm conditions by vortexing and sonication. The emulsion was then cooled down to obtain solidified microspherules of agarose-gelatin (solid cores) dispersed in organic solution. The lipophilic components were extracted with several washes of the solid cores in cyclohexane, then in ether and finally in water. Next, colloidal gold particles were introduced into the solid cores according to Gao and Chen [6]. Briefly, solid cores were first dispersed in 100-fold (v/v) 0.05 M sodium hydrogen maleate buffer (pH 6.0) containing 0.06% tannic acid. About 15 min later, an equal volume of 0.12% gold chloride was added while the mixture was sonicated in a bath type sonicator. The reaction between tannic acid and gold chloride predominantly took place within the solid cores with most of the colloidal gold particles formed in the outer zone of every solid core. Agarose-gelatin solid cores containing colloidal gold particles (AuSC) were washed several times in water.

Preparation of AuSCL

Liposomes with encapsulated agarose-gelatin solid core containing colloidal gold particles (AuSCL) were prepared according to the organic solvent spherule evaporation (OSSE) method [7,8] with some modifications. Two different procedures for preparation of AuSCL were used:

Procedure A. 50 μl of pelleted AuSC (average diameter around 0.5 μm, but larger ones could also be used) were washed twice and resuspended by sonication in 100 volumes of 0.02% bovine serum albumin and 0.02% NaN_3 and then collected by centrifugation at 8000 rpm for 15 min. The protein-treated AuSC were washed with 10 ml of water and 10 ml of 0.15 M sucrose and collected by centrifugation at 8000 rpm for 15 min. Then, 1 ml of 0.15 M sucrose was added to make a suspension by sonication. The concentration of AuSC in sucrose solution was about 5%. The suspension was transferred to an organic phase containing DOPC/cholesterol/DOPG/triolein (4.5, 4.5, 1, 1 μmol, respectively, in 1 ml of chloroform/diethyl ether (1 : 1, v/v)). The mixture readily formed a water-in-oil emulsion by hand shaking. The emulsified aqueous particles in suspension were further reduced in size by strong vortex for 10 min. The water-in-oil emulsion was then divided into two portions, and each portion (1 ml) was transferred to another tube containing 2.5 ml of 0.2 M sucrose. The water-in-oil emulsion was secondarily emulsified by applying the mixture to a vortex-genie (Scientific Industries, Inc., Bohemia, NY) at speed setting 6–7 for 2 × 30 s to make a water-in-oil-in-water multiple emulsion. The final water-in-oil-in-water emulsion collected from both tubes was combined into a flask and the organic solvent was evaporated at 35–37°C under a stream of nitrogen with gentle swirling. AuSCL was formed after the solvent had completely evaporated. Then 4.5 ml of 5% dextrose were added to the AuSCL suspension. Liposomes were collected by centrifugation at 600 rpm for 5 min and the pellets were resuspended in 2 ml of 5% dextrose.

Procedure B. 10 μl of (bovine serum albumin coated and pelleted) AuSC were dispersed in 1 ml of chloroform containing 20 μmol dipalmitoylphosphatidylcholine and 10 μmol of cholesterol. AuSC was then collected by centrifugation at 8000 rpm for 20 min at 4°C, and resuspended in DOPC/cholesterol/DOPG (9, 9, and 1 μmol, respectively) in 3 ml of Freon 114/diethyl ether (1 : 1, v/v), at 4°C. After sonication for 1 min, the suspension was added dropwise to 0.15 M sucrose and 1/4 strength L buffer (pH 7.4) *.

* L buffer: 10 mM Hepes (pH 7.4), 1 mM EGTA and 150 mM NaCl.

This mixture was vortexed for 1 min, and evaporated with a stream of nitrogen at 4°C for 10 min and then continued at room temperature until all organic solvent was removed. The resulting AuSCL was dialysed overnight at room temperature against 1 liter L buffer (pH 7.4) to remove residual organic solvent.

Electron microscopy

AuSCL obtained from procedure B were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) containing 0.2 M sucrose for 1 h; collected by centrifugation at 500 rpm for 5 min; post fixed in 2% OsO_4 in 0.05 M phosphate buffer (pH 7.4) for 30 min; washed with water overnight; pre-embedded in 3% agarose and en bloc stained in uranyl acetate overnight; dehydrated through graded series of dioxane and embedded in LX-112 resin (Ladd Research Industries, Inc. Burlington, VT). AuSCL obtained from procedure A basically went through the same procedures except for being post fixed in 1% OsO_4 in L buffer (pH 7.4) and dehydrated with acetone. Ultrathin sections were cut with a diamond knife on a Reichert Om U3 ultramicrotome (Vienna) and collected on Formvar-coated grids. Samples were examined in a Hitachi H-600 electron microscope.

Results and Discussion

As indicated in the previous work [4] microspherules (now called solid cores) of agarose-gelatin could be obtained by emulsification of agarose-gelatin sol in organic solvent containing egg phosphatidylcholine and other surfactants and fractionated by centrifugation. The lipophilic components were extracted by organic solvent; Au particles could be introduced into the matrix of the solid cores [6]. We are reporting the encapsulation of the solid cores in liposomes with modified OSSE methods [7,8]. Fig. 1 shows the the basic process of preparing AuSCL. Fig. 2 is a phase contrast micrograph of AuSC. Most of the solid cores were 0.2–0.6 μm in diameter and were monodispersed. Fig. 3–7 indicate the different stages of AuSCL preparation according to procedure A. Figs. 3 and 4 show water-in-oil emulsions before and after vortexing, respectively. As can be seen, many purple-red AuSC were trapped in the emulsions. The size of the emulsified vesicles was significantly reduced from 5–50 μm to 1–5 μm by vigorous vortexing. Fig. 5 shows the water-in-oil-in-water multiple emulsion before evaporation of organic solvents with nitrogen gas. Each multiple emulsion droplet contained several water-in-oil droplets within which the dark-reddish AuSC could be seen. Fig. 6 shows the formation of AuSCL after the evaporation of organic solvent. The green background is due to a green filter inserted in the phase contrast microscope. Fig. 7 shows the same sample as Fig. 6 in a different viewing area but without the green filter. Notice the dark-reddish AuSC particles inside the liposomes. The size of liposomes varied from 0.5 to 3

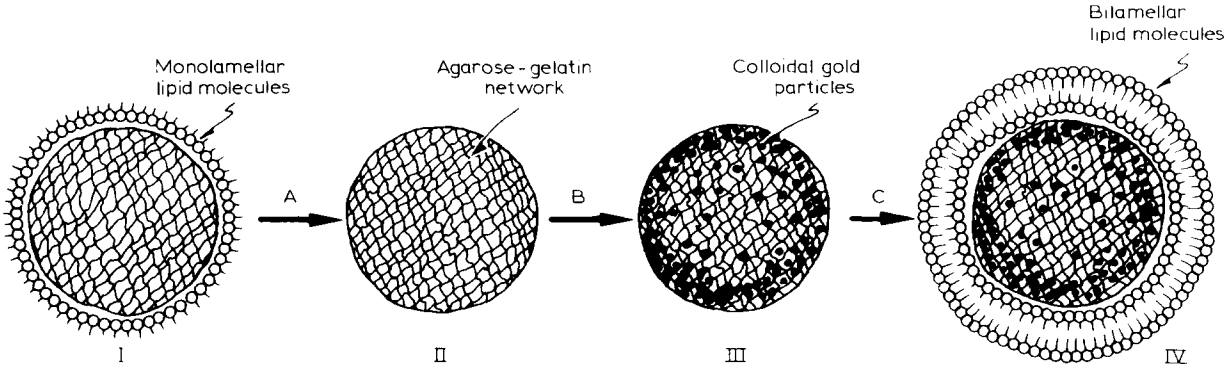


Fig. 1.Diagrammatic explanation of the main steps in making a solid core liposome encapsulated with colloidal gold particles. (A) Extract lipid with organic solvent. (B) Tannic acid and gold chloride interaction to form colloidal gold particles. (C) Add lipid layers to the solid core containing colloidal gold particles. I, prevesicle with a solid core of agarose-gelatin network; II, agarose-gelatin network (bare core); III, colloidal gold particles embedded in agarose-gelatin network; IV, solid core liposome encapsulated with colloidal gold particles.

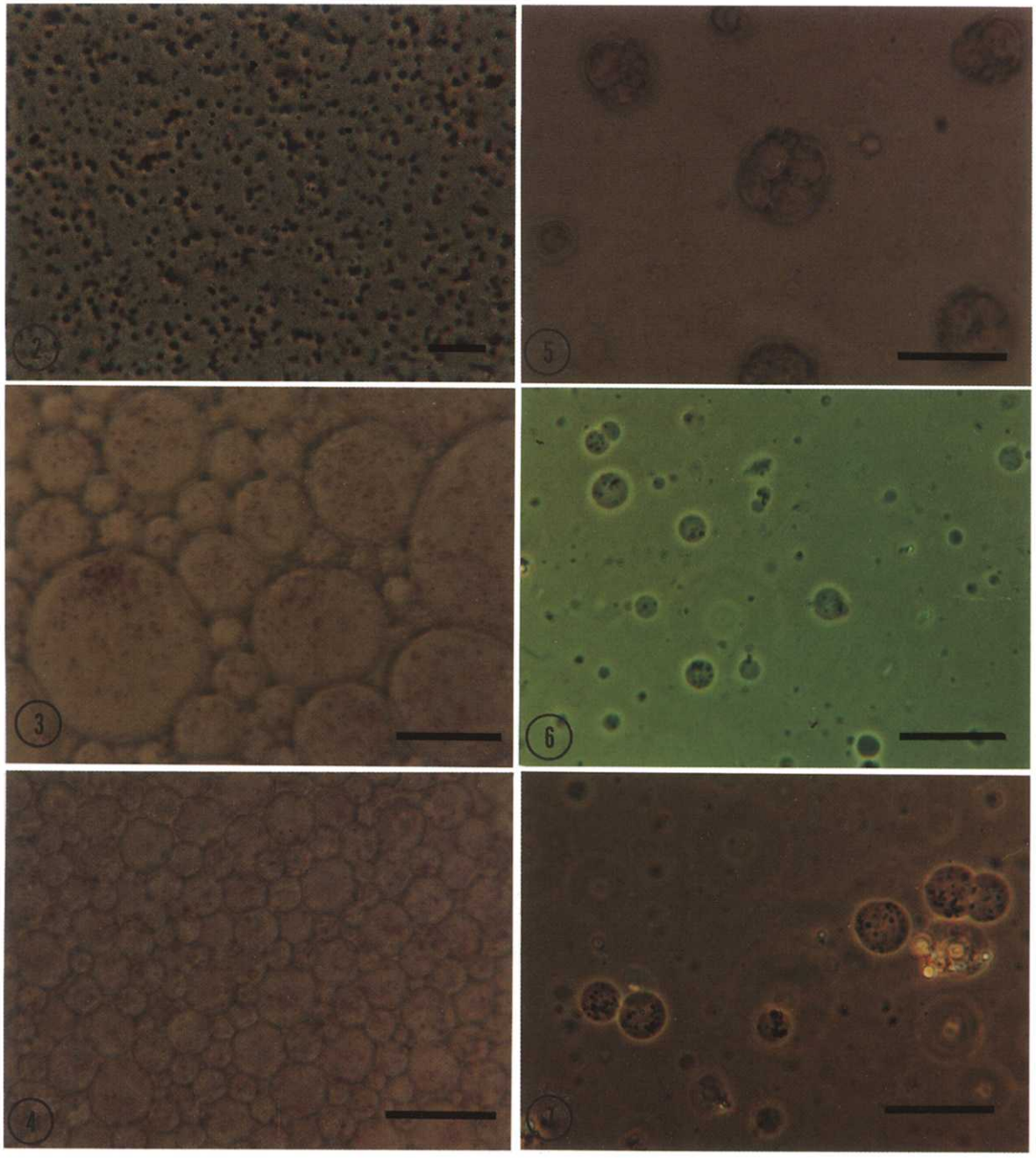


Fig. 2. Phase contrast micrograph of AuSC. Bar: 2 μm.

Figs. 3–7. Phase contrast micrographs of water-in-oil emulsions before (3) and after (4) vortex; water-in-oil-in-water multiple emulsion (5) and AuSCL with green filter (6) and without filter (7). Bar: 10 μm.

μm in diameter (Figs. 6, 7).

Since Au is electron dense, both AuSC (Fig. 8) and AuSCL (Figs. 9a–c, and Figs. 10 a, b) can be seen under the electron microscope. The major difference between procedures A and B seems to be that when no triolein was added to the lipid

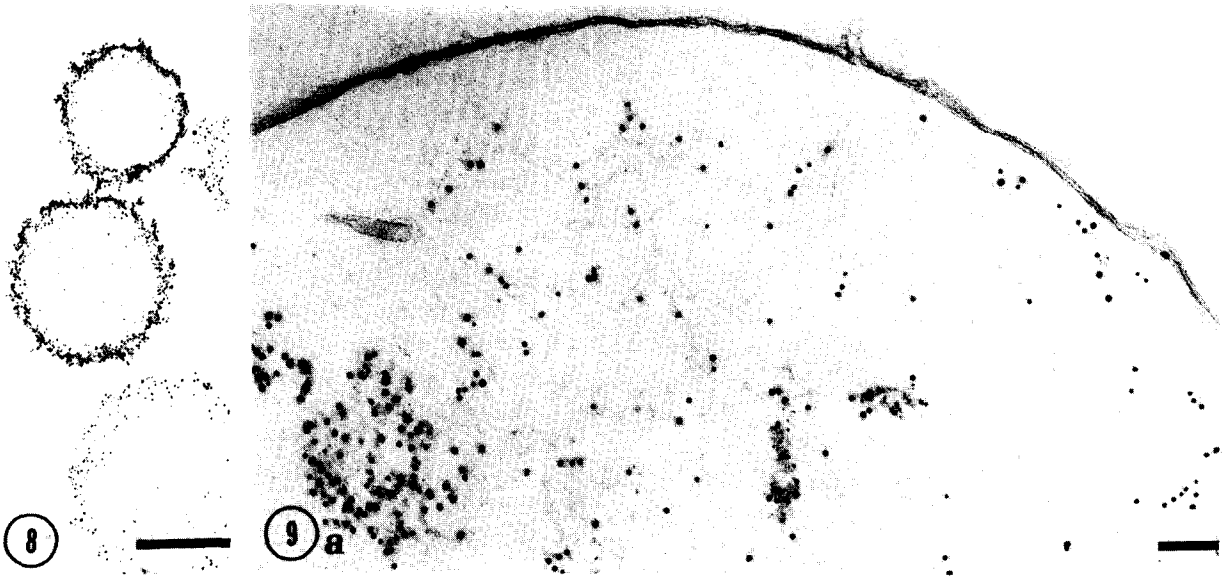
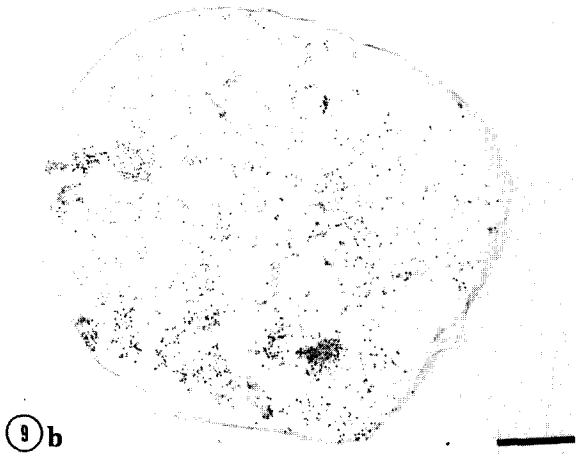


Fig. 8. Thin-section electron micrograph of AuSC. Bar: 1 μm.



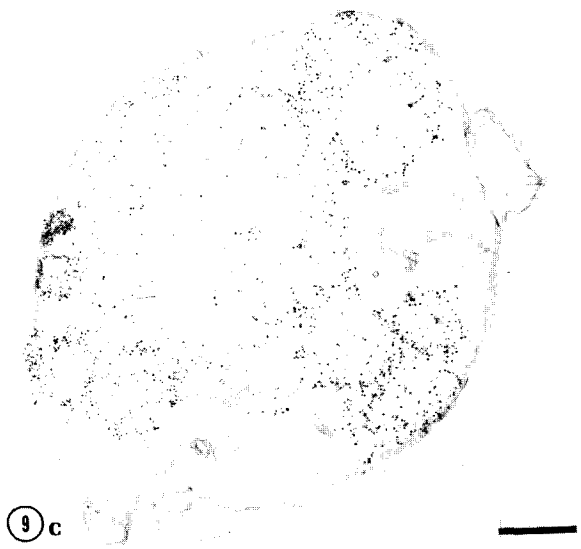


Fig. 9. Thin-section electron micrographs of AuSCL prepared according to procedure A, showing a typical bilayer membrane of liposome (a) (bar: 0.1 μm) and two whole AuSCL (b, c). There are some rod-like structures as well as some fiberous structures in the liposomes. Bar: 0.5 μm.

mixture, the liposomes had multilamellar structures and some of the AuSC were not enclosed by lipid membranes (Figs. 10a, b). AuSCL prepared with procedure A had a typical appearance of a bileaflet lipid membrane. The membrane in Fig. 9a looks very smooth and somewhat similar to the membrane of the red blood cell. Although there is only one bilayer membrane in every AuSCL shown in Figs. 9 b and c, these membranes were continuous with no apparent holes. Colloidal gold particles were abundant in every AuSCL. Some fiberous material (probably the poorly stained agarose-gelatin network) as well as some rod-shaped membraneous structures could be seen in the interior of some AuSCL (Fig. 9b). It is apparent that multiple solid cores were entrapped in each AuSCL. The number of the solid cores per liposomes depended on the diameter of the liposome. For example, about 5–6 cores were found in liposomes of 2–3 μm in diameter; about 10–20 cores were found in liposomes of approx. 5 μm in diameter (Figs. 6, 7).

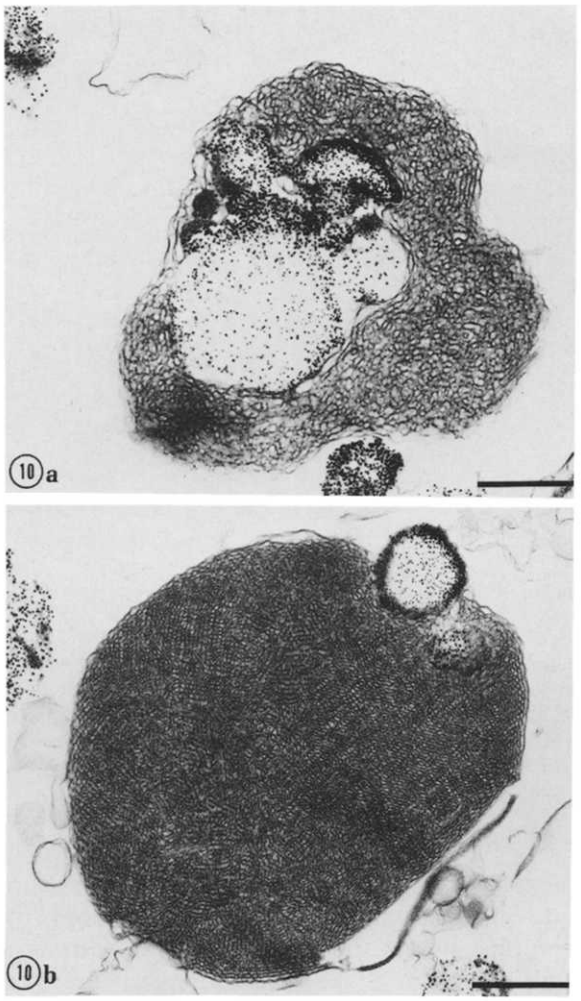


Fig. 10. Thin-section electron micrographs of AuSCL prepared with procedure B. It is evident that both multilamellar liposomes with multi-AuSC (a) and AuSC covered only by one lipid bilayer as part of a multilamellar liposome (b) as well as unencapsulated AuSC (a and b) are seen in the preparation. Bar: 0.5 μm.

From the results obtained, we conclude that procedure A is superior to procedure B, because the AuSCL prepared with procedure A are generally unilamellar with a relatively large volume of internal aqueous space. Furthermore, trapping the solid cores in liposomes with procedure A seems to be a random event. For liposomes of average diameter 2 μm and AuSC of diameter 0.4 μm, the volume ratio of liposome to AuSC is 125. Since AuSC were suspended in the aqueous solution at a concentration of 5% (v/v) during the formation of water-in-oil-in-water multiple emulsion, one would predict that there are approximately 6 AuSC particles trapped in each liposome. This is in reasonable agreement with our result.

The water-in-oil-in-water multiple emulsion is an unstable system [9,10]; thus, the lipid composition used for producing the water-in-oil-in-water system should have a suitable hydrophilic and lipophilic balance (HLB) [11]. In the preliminary experiment, we found DOPG seemed to flexibly form both concave and convex curvatures in a multiple emulsion system. Thus, DOPG is suitable for the formation of the water-in-oil-in-water system. Since this is a negatively charged lipid, it may also prevent aggregation of individual water-in-oil emulsion vesicles. The role triolein plays may be similar to that of DOPG, i.e. it makes the membrane more flexible and more fluid such that the lipid molecules may be favorably arranged into both concave and convex curvatures. However, lipids with suitable HLB could also suffice.

Although the formula of lipids we used for procedure A was derived empirically, it works very well. The solid core liposomes have provided a new opportunity of using colloidal gold to label liposomes which can either be examined under the light microscope or detected by electron microscopy. Future studies of liposome–cell interactions should be easier. Since the solid core liposomes could be prepared without the colloidal gold labeling, the gold-free liposomes may be useful in improving the drug delivery potential of liposomes, particularly for the topical or localized administration of liposomes as a sustained release drug carrier [12,13]. The polymeric matrix of the solid core may retard the release of macromolecules such as enzymes and peptides. Colloidal gold particles are very adsorptive for proteins and peptides. Thus AuSCL may also be suitable for the sustained release of these macromolecules. The internal solid core may also increase the mechanical stability of the liposomes. These potential applications are currently under investigation.

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