

# Antibody-mediated targeting of liposomes to red cells in vivo

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Covalent attachment of anti-rat erythrocyte  $F(ab')_2$  to liposomes specifically enhanced their binding to rat erythrocytes in vivo and reduced their uptake by the liver. Furthermore, at least 20–30% of the cell-bound liposomes delivered their contents to the cells. Besides, the liposome binding did not affect the survival time of the target cells at least up to 3 h in the blood circulation. These results demonstrate for the first time that liposomes can be successfully targeted to cells other than liver cells in vivo.

*Drug targeting    Liposome    Cell-specific antibody     $F(ab')_2$  fragment    (Erythrocyte)*

## 1. INTRODUCTION

Liposomes coated with cell-specific ligands may prove highly useful as carriers for site-specific delivery of drugs and enzymes in the biophase [1,2]. Several studies have been carried out on the interactions of such liposomes with specific cells in vitro [3–9], but only few attempts have been made to examine the suitability of this method for drug/enzyme delivery to the selected cells in vivo [10–12]. Here, we describe the results of our studies on targeting of liposomes to rat red cells in vivo.

## 2. MATERIALS AND METHODS

$Na^{125}I$  (carrier-free), sodium  $[^{51}Cr]$ chromate and  $[^{14}C]$ sucrose were obtained from Bhabha Atomic Research Centre, Trombay, India.  $[^3H]$ Inulin was from Amersham, England. Egg  $[^{14}C]$ PC was prepared as in [13].

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*Abbreviation:* PC, phosphatidylcholine

### 2.1. Anti-rat erythrocyte $F(ab')_2$

Anti-rat erythrocyte  $F(ab')_2$  were prepared and purified as in [9]. Radiolabelling of the antibody was carried out according to [14].

### 2.2. Liposomes

Liposomes were prepared from egg PC (20  $\mu$ mol), cholesterol (20  $\mu$ mol), gangliosides (4  $\mu$ mol) and traces of egg  $[^{14}C]$ PC (about 20  $\mu$ Ci) or  $[^{14}C]$ sucrose (about 250  $\mu$ Ci) or  $[^3H]$ inulin (about 500  $\mu$ Ci) in 0.8 ml borate-buffered saline (10 mM borate containing 60 mM NaCl, pH 8.4) by sonication [15], and fractionated by centrifugation [7]. Free  $[^{14}C]$ sucrose (or  $[^3H]$ inulin) from the liposomised sucrose (or inulin) was removed by gel filtration over Sephadex G-50 as in [13]. The mean outer diameter of these liposomes, as determined by molecular sieve column chromatography [15], was about 45 nm.

### 2.3. Anti-rat erythrocyte $F(ab')_2$ bearing liposomes

$^{125}I$ -labelled (or unlabelled) anti-rat erythrocyte  $F(ab')_2$  were covalently attached to liposomes as in [9]. The protein-to-lipid ratio in the liposomes was about 70–100  $\mu$ g protein/ $\mu$ mol lipid P.

#### 2.4. *Fab'* fragments of anti-anti-rat erythrocyte *F(ab')*<sub>2</sub> antibody

Anti-anti-rat erythrocyte *F(ab')*<sub>2</sub> antibody was raised in sheep, and isolated from antiserum by the ammonium sulphate precipitation method [16]. *Fab'* fragments of this antibody were prepared essentially as in [17].

#### 2.5. *Tissue distribution of liposomes*

Liposomes (0.8–1.2  $\mu$ mol lipid P, 400  $\mu$ l) were injected into the tail vein of male albino rats (Charles Foster; average weight about 100 g). Blood (about 20  $\mu$ l) was drawn immediately ( $\leq 2$  min) after injecting the liposomes, by retro-orbital puncture in heparinized capillary pipettes, and transferred to preweighed scintillation vials. The vials were reweighed and counted for radioactivity [9]. The <sup>14</sup>C and <sup>3</sup>H counts were corrected for quenching by haemoglobin. Total radioactivity injected in each animal was calculated from the 2 min value by assuming total blood volume as 8% of the body weight. The egg PC-cholesterol-ganglioside liposomes that were not coupled to anti-erythrocyte *F(ab')*<sub>2</sub> were used as controls throughout this study. For determining the tissue distribution of liposomes *in vivo*, the animals were killed immediately after drawing about 2 ml blood at 15 min after the injection, and the various organs (liver, spleen, lung, kidney and heart) removed, washed, blotted and weighed. A 10% (w/v) homogenate of these organs was prepared in sucrose-supplemented Tris-buffered saline (10 mM Tris containing 150 mM NaCl, 44 mM sucrose and 5 mM EDTA, pH 7.4). A measured aliquot of each homogenate was analysed for radioactivity. Distribution of radioactivity in blood was determined as follows: the blood (1 ml) drawn at 15 min was centrifuged. Plasma was carefully aspirated off and transferred to a glass tube. A fixed aliquot of plasma was analysed for radioactivity. The cell pellet remaining after plasma removal was diluted to 50% haematocrit with sucrose-supplemented Tris-buffered saline, and the erythrocytes separated from the white blood cells by using a Ficoll-Conray gradient [9]. The white and red blood cells were washed, and separately suspended in buffer. A measured aliquot containing a known number of cells was analysed for radioactivity. The total amount of

radioactivity associated with red cells and plasma *in vivo* was calculated by determining the number of red cells/ml blood ( $6.2 \pm 0.5 \times 10^9$  cells; 10 animals) and the packed cell volume/100 ml blood ( $38 \pm 2$  ml; 10 animals), respectively. The total blood volume was assumed as 8% of the body weight. Both <sup>125</sup>I and <sup>14</sup>C (or <sup>3</sup>H) counts in the total leukocyte pellet were close to the background levels (about  $3 \times 10^{-4}\%$  and  $2 \times 10^{-5}\%$  of the injected dose of <sup>125</sup>I and <sup>14</sup>C, respectively), and hence the amount of leukocyte-associated liposomes could not be determined accurately.

#### 2.6. <sup>51</sup>Cr-labelled liposome-bound erythrocytes

Liposomes (2  $\mu$ mol lipid P) containing egg [<sup>14</sup>C]PC were incubated with rat erythrocytes (about  $5 \times 10^9$  cells) in buffer (1 ml) at 37°C for 15 min. The cells were harvested by centrifugation, and washed several times till no radioactivity was detected in the washings. The amounts of red cell-associated anti-rat erythrocyte *F(ab')*<sub>2</sub> bearing liposomes and control liposomes were about 6%/10<sup>9</sup> cells and 0.2%/10<sup>9</sup> cells, respectively. These liposome-bound cells were labelled with <sup>51</sup>Cr according to [18]. In identical conditions, fresh red cells isolated from rat blood were also labelled with <sup>51</sup>Cr.

#### 2.7. *Liposome-bound erythrocyte's survival in blood circulation*

<sup>51</sup>Cr-labelled liposome-untreated, control liposome-treated and antibody bearing liposome-treated erythrocytes (about  $5 \times 10^8$  cells/animal) were separately administered intravenously to rats (average weight 100 g). The total amount of radioactivity injected in each animal was calculated from a 2 min post-injection blood sample, as described above. The amount of radioactivity remaining in blood at various periods of time was determined as follows: blood (about 20  $\mu$ l) was drawn in heparinized capillary pipettes, and transferred to preweighed scintillation vials. The vials were reweighed and counted for radioactivity. The <sup>14</sup>C counts were corrected for quenching by haemoglobin. At 3 h after the injection, the animals were killed, and the various organs were taken out and processed as described in section 2.4.

### 3. RESULTS AND DISCUSSION

Unilamellar liposomes were prepared from egg PC, cholesterol and gangliosides by sonication, and fractionated by centrifugation, as described in section 2. These liposomes were covalently coupled to  $^{125}\text{I}$ -labelled anti-rat erythrocyte  $\text{F(ab')}_2$ , and their tissue distribution determined in rats. The liposomes that were not attached to the antibody, rather than normal rabbit IgG  $\text{F(ab')}_2$  bearing liposomes [9], were used as controls throughout this study. These liposomes were selected since the use of rabbit IgG  $\text{F(ab')}_2$  bearing liposomes as controls would not allow us to determine the effect, if any, of the protein coupling on the blood clearance rate of antibody bearing liposomes from the circulation [12].

Fig.1 shows that tissue distribution of liposomes is significantly modified upon attaching anti-rat erythrocyte  $\text{F(ab')}_2$  to their surface. A considerably larger (12–19%) proportion of liposomes became associated with the erythrocytes whereas there was an appreciable decrease in the amounts of liposomes that localized in liver, spleen and plasma. However, only negligible amounts (<0.005%) of liposomes were found to be associated with the white blood cells, and these amounts did not increase by coupling the liposomes to the antibody. Besides, similar fractions of antibody bearing liposomal  $^{14}\text{C}$  (or  $^3\text{H}$ ) and  $^{125}\text{I}$  were associated with the various tissues (fig.1), indicating that the structural integrity of these liposomes was not greatly affected in blood circulation [19]. These findings demonstrate that covalent attachment of anti-rat erythrocyte  $\text{F(ab')}_2$  to the liposomes specifically enhances their binding to rat erythrocytes *in vivo*, and reduces their uptake by the liver and spleen.

To ascertain whether the liposome binding to red cells is time-dependent, we measured the amount of red cell-bound radioactivity at various periods of time after injecting liposomes in rats. Fig.2A shows that the liposome binding to these cells does not increase with time, and that the extent of this binding at 3 h is identical to that observed at 15 min. This demonstrates that the binding between erythrocytes and liposomes is a very fast process, which is consistent with the earlier studies [3,4,9]. However, the liposomal radioactivity in plasma decreased with time

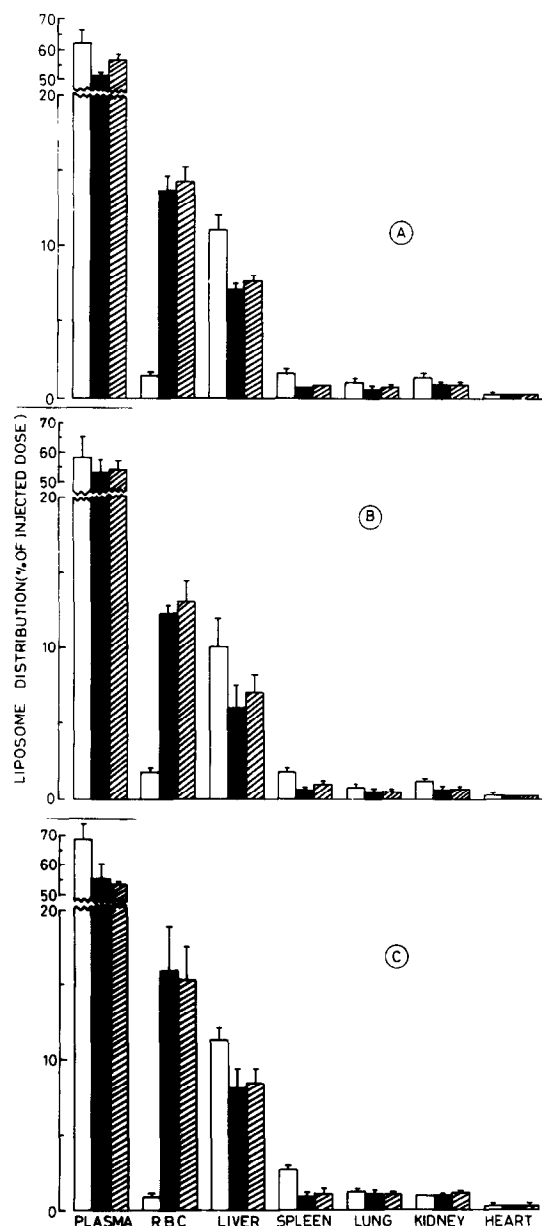


Fig.1. Distribution of liposomes in various organs/tissues. Values shown are mean of 4–6 animals  $\pm$  SD. (A) Liposomes containing egg [ $^{14}\text{C}$ ]PC, before ( $^{14}\text{C}$ , open bars) and after coupling to  $^{125}\text{I}$ -labelled anti-rat erythrocyte  $\text{F(ab')}_2$  ( $^{14}\text{C}$ , solid bars;  $^{125}\text{I}$ , shaded bars). (B) Liposomes containing [ $^{14}\text{C}$ ]sucrose, before ( $^{14}\text{C}$ , open bars) and after coupling to  $^{125}\text{I}$ -labelled anti-rat erythrocyte  $\text{F(ab')}_2$  ( $^{14}\text{C}$ , solid bars;  $^{125}\text{I}$ , shaded bars). (C) Liposomes containing [ $^3\text{H}$ ]inulin, before ( $^3\text{H}$ , open bars) and after coupling to  $^{125}\text{I}$ -labelled anti-rat erythrocyte  $\text{F(ab')}_2$  ( $^3\text{H}$ , solid bars;  $^{125}\text{I}$ , shaded bars). RBC, red blood cells.

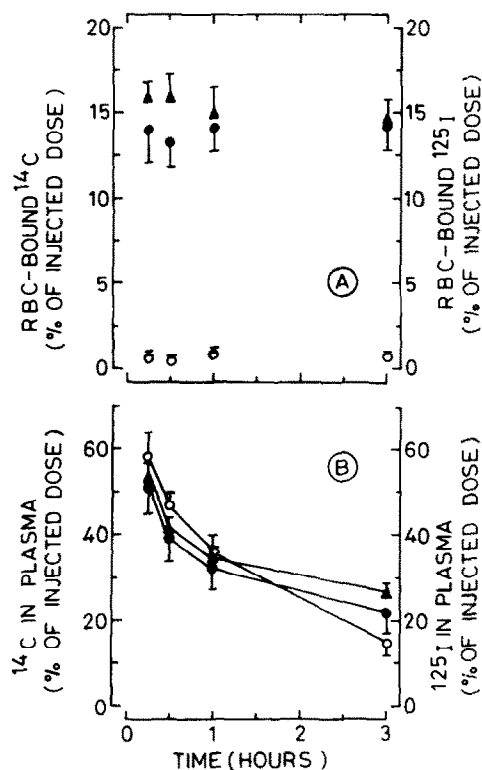


Fig.2. Liposomes containing egg [ $^{14}\text{C}$ ]PC were injected in rats (average weight 220 g) and the amount of radioactivity associated with erythrocytes (A) and the remaining radioactivity in plasma (B) at various periods of time determined, before (open circle, liposomal  $^{14}\text{C}$ ) and after coupling them to  $^{125}\text{I}$ -labelled anti-rat erythrocyte F(ab') $_2$  (solid circles, liposomal  $^{14}\text{C}$ ; solid triangles, liposomal  $^{125}\text{I}$ ), as in fig.1, except that 300  $\mu\text{l}$  blood was drawn at each time point. Values shown are mean of 6 animals  $\pm$  SD.

(fig.2B). This decrease in case of the control liposomes was faster than that observed for the antibody bearing liposomes, suggesting that the blood clearance rate of the liposomes is reduced by coupling them to the antibody. This is quite consistent with our finding that the difference between the liver-associated amounts of control and antibody bearing liposomes was greater at 3 h (fig.3), as compared to that at 15 min (fig.1A).

Further experiments were done to investigate the effects of the injected dose of liposomes on the extent of their binding to the target cells, and also on their distribution patterns in the various organs. Both the extent of liposome binding to

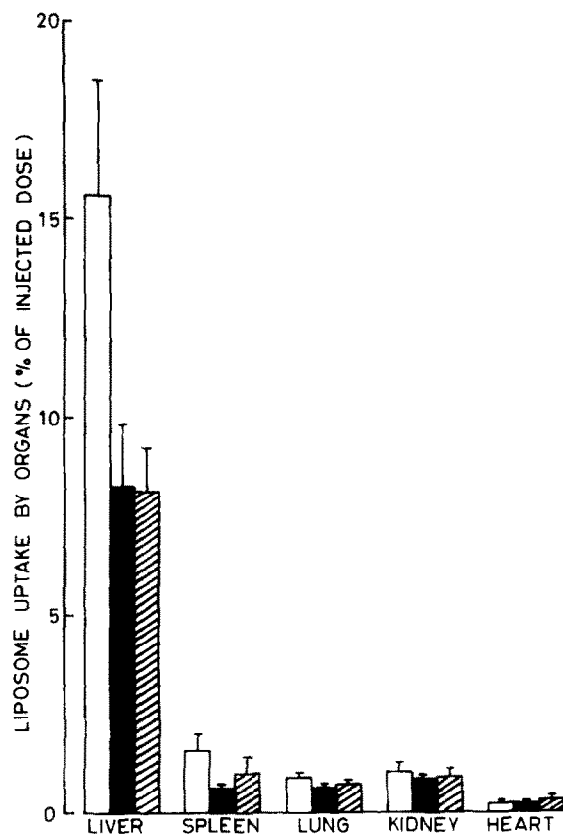


Fig.3. Distribution of egg [ $^{14}\text{C}$ ]PC containing liposomes in various organs at 3 h after the injection, before (open bars,  $^{14}\text{C}$ ) and after coupling them to  $^{125}\text{I}$ -labelled anti-rat erythrocyte F(ab') $_2$  (solid bars,  $^{14}\text{C}$ ; shaded bars,  $^{125}\text{I}$ ). Values shown are mean of 6 animals  $\pm$  SD.

erythrocytes and the organ distribution patterns remained unchanged by varying the injected dose between 0.6 and 2  $\mu\text{mol}$  lipid P/animal (not shown).

Since delivery of the liposomised solutes to the target cells will be ensured only if the cell-bound liposomes either fuse with the cell membrane or become internalised via endocytosis [20], we analysed the mode of interaction between erythrocytes and antibody bearing liposomes in the liposome-bound cells obtained from the 15 min blood samples of the injected animals. Firstly, we estimated the amounts of liposomes that were adsorbed on the cell surface. The surface-adsorbed liposomes were eluted by washing (5-7 times) the liposome-bound cells (0.2-0.3 ml packed volume)

with a solution (7 mg protein/ml, 3 ml each time) of Fab' fragments of anti-anti-rat erythrocyte F(ab')<sub>2</sub> antibody. Only 25–30% of the cell-bound liposomes could be eluted by this method. The maximum amount of liposomes was eluted in the first and second washings whereas the last washing was completely free of radioactivity. Secondly, we attempted to measure the amount of free [<sup>14</sup>C]sucrose (or [<sup>3</sup>H]inulin) delivered to the cells by the liposomes, as it should allow us to ascertain the degree to which liposomes fuse with the cells [20]. The Fab'-washed cells (about 0.2 ml packed volume) were transferred to a 3 ml centrifuge tube, and lysed at 0°C by adding 9 vols precooled distilled water. It was centrifuged at 25000 × g for 30 min at 4°C. The supernatant was carefully transferred to a glass tube. The pellet was washed with water (2 ml), and the washing was mixed with the first supernatant. The washed pellet was diluted with an equal volume of water and measured aliquots of both the supernatant and the pellet were analysed for radioactivity. The liposomal membrane radioactive markers (viz. <sup>125</sup>I-labelled F(ab')<sub>2</sub> and egg [<sup>14</sup>C]PC) were exclusively (>98%) associated with the cell pellet whereas a considerable proportion (23–38%) of the entrapped markers (viz. [<sup>14</sup>C]sucrose and [<sup>3</sup>H]inulin) was also present in the supernatant. These amounts of the liposomal solutes in the cytosol were not due to leakage of the entrapped solutes from the cell-bound liposomes, as 100-fold dilution with distilled water was found not to induce any leakage (<1%) from the liposomes that were not bound to the cells. Moreover, no [<sup>14</sup>C]sucrose (or [<sup>3</sup>H]inulin) could be detected in the supernatant even upon overnight storage of the Fab'-washed liposome-bound cells in buffer at 4°C. From these findings, we infer that of the erythrocyte-bound liposomes, at least 25–30% interact with the cells via adsorption, 20–30% probably via membrane-membrane fusion, and the rest may have interacted with the cells via some other mechanisms [20,21].

Interactions of red cells with liposomes are known to alter their membrane deformability [22], which in turn could accelerate their destruction by the spleen [23]. Therefore, to examine whether the liposome binding has influenced the survival times of erythrocytes in the circulation, we measured the <sup>51</sup>Cr levels in the circulation after injecting <sup>51</sup>Cr-

labelled liposome-bound erythrocytes in rats, as described in section 2. The normal rat erythrocytes which did not receive any treatment with liposomes were used as controls in these experiments. Fig.4 shows that the amounts of the control liposome- and antibody bearing liposome-treated cells in the circulation are similar to that of the control cells. Also, the fractions of the liposome-treated cells associated with the various organs (fig.5) were almost identical to those of the liposome untreated erythrocytes. These results strongly indicate that the interaction of the red cells with liposomes does not alter their stability or survival time at least up to 3 h in the circulation. Further, it may be noted

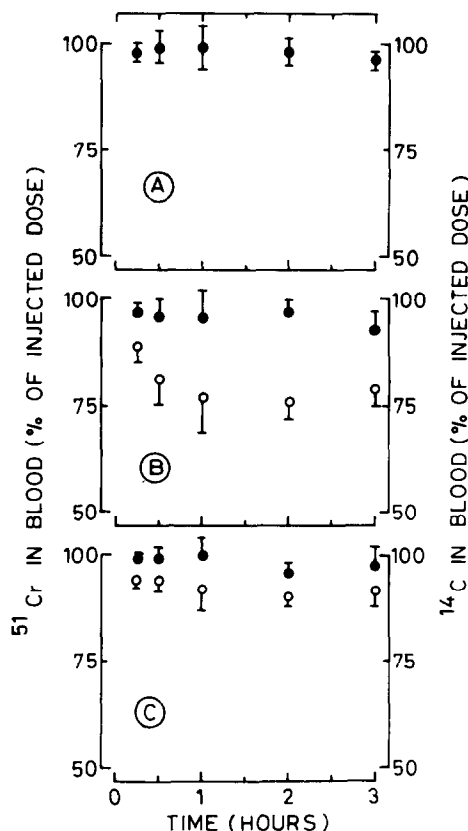


Fig.4. Survival of liposome-treated red cells in circulation of rats. (A) Cells not treated with liposomes (solid circles, <sup>51</sup>Cr); (B) cells treated with egg [<sup>14</sup>C]PC-containing control liposomes (solid circles, <sup>51</sup>Cr; open circles, <sup>14</sup>C); (C) cells treated with anti-rat erythrocyte F(ab')<sub>2</sub> bearing liposomes containing egg [<sup>14</sup>C]PC (solid circles, <sup>51</sup>Cr; open circles, <sup>14</sup>C). Values are mean of 4 animals ± SD.

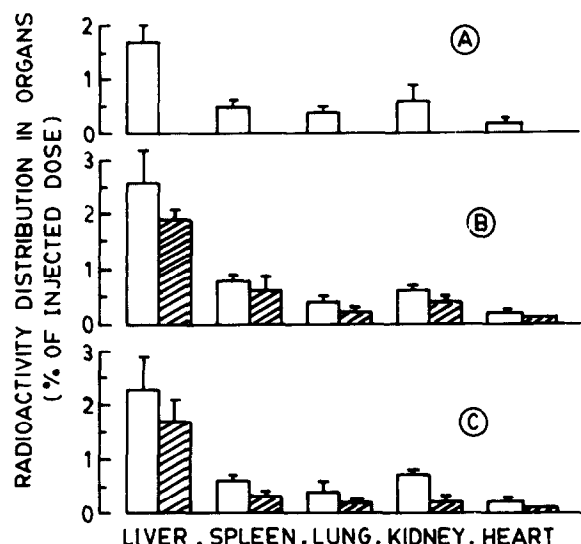


Fig.5. Distribution of radioactivity in various organs at 3 h after injecting  $^{51}\text{Cr}$ -labelled liposome-treated (or untreated) red cells in rats. (A) Cells not treated with liposomes (open bars,  $^{51}\text{Cr}$ ); (B) cells treated with egg [ $^{14}\text{C}$ ]PC-containing control liposomes (open bars,  $^{51}\text{Cr}$ ; shaded bars,  $^{14}\text{C}$ ); (C) cells treated with anti-rat erythrocyte  $\text{F(ab')}_2$  bearing liposomes containing egg [ $^{14}\text{C}$ ]PC (open bars,  $^{51}\text{Cr}$ ; shaded bars,  $^{14}\text{C}$ ). Values are mean of 4 animals  $\pm$  SD.

in fig.4 that about 20% of the control liposomal  $^{14}\text{C}$  and about 10% of the antibody bearing liposomal  $^{14}\text{C}$  disappeared from the circulation. These fractions may represent those liposomes which were loosely bound to the cells, and readily eluted from the cell surface by the plasma proteins in blood circulation.

This study clearly shows that covalent attachment of cell-specific antibody to liposomes specifically enhances their binding to the target cells in vivo. Also, it demonstrates that a significant fraction of the cell-bound liposomes can deliver their contents even to an inert cell like the erythrocyte. We, therefore, conclude that liposomes coupled to cell-specific antibody may prove quite suitable for drug/enzyme delivery to specific cells in the biophase.

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