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Liposomes sequestered in chitosan gel as a delivery device for dapsone

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Liposomes containing oleic and stearic acids were prepared and sequestered in chitosan gel. Dapsone and bromothymol blue were entrapped in the liposomes. The carboxyl group of sequestered liposomes were subsequently coupled to the amino group of chitosan by carbodiimide. In vitro release studies of the drug from liposomes and liposomes sequestered in chitosan gel in phosphate buffer and mice plasma were carried out.

INTRODUCTION

Liposomes are one among the several drug carrier systems which have been intensively studied in the last few decades (De La Maza *et al.*, 1992; Taylor & Farr, 1993; Lasch *et al.*, 1991; Allen *et al.*, 1992; Viani *et al.*, 1993). Structural versatility is the most prominent among the advantages that liposomes offer as a drug delivery system. The correct choice of lipid composition, size, surface charge and surface groups can contribute towards the control of behaviour of the carrier as well as ultimate fate. This in turn will help to achieve optimization of the action of transported drugs. The stability of liposomes in blood plasma is posing a major problem in the controlled delivery of drugs. Various modifications were sought in the method of preparation of liposomes which are stable in plasma (Viani *et al.*, 1993; Maruyama *et al.*, 1992; Alamelu & Panduranga Rao, 1989; Panduranga Rao & Alamelu, 1992; Cho & Chung, 1988; Liu & Huang, 1989; Weissig *et al.*, 1989). In the present study, a system having a combination of two carriers has been investigated to achieve both stability and slow release of drugs. The liposomes containing oleic and stearic acids were prepared and subsequently sequestered in chitosan gels. Dapsone (DDS) was chosen as a model lipophilic drug for encapsulation in liposomes. The stability and release studies were carried out with the above liposomes sequestered in chitosan gel.

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MATERIALS

Oleic acid and stearic acid (Wilson Laboratories, Bombay, India), dapsone (a gift sample from Central Leprosy Research Institute, Chingelput, India), phosphatidyl choline and 1-ethyl 3-(3-dimethyl amino propyl) carbodiimide hydrochloride (Sigma Chemical Co., USA), bromothymol blue (BDH, UK) and chitosan (a gift sample from Central Institute of Fisheries Technology, Cochin, India) were used as obtained. All other reagents used were of analytical grade.

EXPERIMENTAL

Preparation of fatty acid containing liposomes

Oleic acid (OA) and stearic acid (SA) containing phosphatidyl choline (PC) liposomes were used for entrapping both hydrophilic marker bromothymol blue (BTB) and lipophilic drug dapsone (DDS). These two types of liposomes (PCOA and PCSA) were subsequently sequestered in natural polymeric gel of chitosan (Ch). Bromothymol blue gives a blue colour at pH 7·4 and above. Hence it was used as a marker at pH 7·4.

Preparation of oleic acid containing liposomes

PC and OA in 10:1 mole ratio, in chloroform were mixed well. The organic phase was evaporated and the dried sample was thoroughly mixed with 25 ml phosphate buffer (0·01 M phosphate buffer of pH 7·4 is used

throughout the experiment). The liposome suspension was then sonicated intermittently for 40 min at a frequency of 32 kHz.

For entrapment of DDS, 5 mg DDS in 2·5 ml methanol was added to the chloroform solution of PC and OA. In the case of BTB, 8·0 mg BTB was dissolved in 40 ml phosphate buffer and this was directly used for the preparation of liposomes instead of phosphate buffer. The sonicated unilamellar liposomes were dialysed at 25°C for 72 h to remove the unentrapped BTB and DDS. The dialysate was changed every 24 h. The purified liposomes were stored in the refrigerator until further use.

Preparation of stearic acid containing liposomes

PC and SA in 10:1 mole ratio, in chloroform were mixed well and the organic phase was evaporated. The dried sample was thoroughly mixed with 25 ml phosphate buffer (pH 7·4). The liposome suspension was then sonicated and dialysed as in PCOA liposome preparation.

Bromothymol blue and dapsone entrapment in the liposomes

The amount of BTB and DDS entrapped in the liposomes was calculated by an indirect method. The dialysates (for BTB, water is used as the dialysate and for DDS 0·02 N HCl is used as the dialysate. Water was brought to pH 7·4 before assay of BTB) were collected and assayed for the unentrapped bromothymol blue and dapsone. The entrapped material was then calculated by subtracting the amount of unentrapped material from the total amount of material added for entrapment in the liposomes.

Sequestration of liposomes in chitosan gel

Two millilitres of 1·5% chitosan (in 2% acetic acid), 0·5 ml each of phosphate buffer (pH 7·4) and liposome suspension were mixed well. Two percent sodium hydroxide (0·5 ml) was added in drops to the above liposomes–chitosan mixture (alkaline pH around 7·6) while stirring. It was gelled at 37°C for 30 min.

Coupling of liposomes to chitosan gel

Liposomes were coupled to chitosan gel during sequestration of carbodiimide (EDAC) as previously reported (Panduranga Rao & Alamelu, 1992).

The procedure is the same as sequestration of liposomes in chitosan gel except that phosphate buffer contains EDAC for coupling. Two millilitres of 1·5% chitosan, 0·5 ml each of liposome suspension and phosphate buffer containing EDAC (of varying concentrations) were mixed well. Two percent sodium hydroxide of 0·5 ml was added dropwise while stirring the liposome mixture and gelled at 37°C for 30 min.

TRANSMISSION ELECTRON MICROSCOPY

Chitosan was dissolved in 2% acetic acid so that the final concentration was 15 mg/ml. 0·5 ml of liposome suspension was added to this chitosan solution. The solution was rapidly precipitated by the addition of 0·5 ml of 2% sodium hydroxide. A drop of the suspension was placed on a carbon coated grid and stained with 2% uranyl acetate. The grids were washed with 50% ethanol followed by double-distilled water and air-dried. The grid was placed on the cavity of a glass slide and by its side a pellet of sodium hydroxide was kept to absorb atmospheric carbon dioxide. The grid was stained again with 1% lead citrate (for 15 min) and washed with 50% ethanol followed by double distilled water. The air-dried grid was then examined in a Philips CM 12 transmission electron microscope at an accelerating voltage of 60 kV.

In vitro release studies of bromothymol blue and dapsone

All in vitro release experiments were carried out in phosphate buffer (pH 7·4) and 1% mice plasma at 37°C.

Release of bromothymol blue in phosphate buffer
The liposome-sequestered chitosan gel was taken in a dialysis tube (obtained from Sigma Chem. Co., USA: cellulose tube, average flat width 25 mm) and placed in 100 ml of phosphate buffer. The whole solution was incubated at 37°C. At appropriate time intervals, 1·0 ml aliquots of incubated suspension were withdrawn, diluted to 3·0 ml with phosphate buffer and assayed spectrophotometrically at 615 nm.

Release of bromothymol blue in 1·0% mice plasma
The liposome-sequestered gel containing BTB was taken in a dialysis tube and placed in 100 ml of 1·0% mice plasma. The release studies were carried out at 37°C and the procedure was the same as in phosphate buffer.

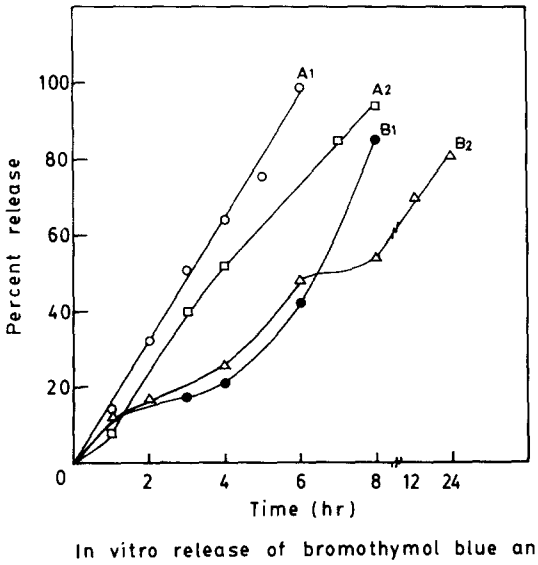
Release of dapsone in phosphate buffer
The liposome-sequestered chitosan gel was taken in a dialysis tube and placed in 100 ml of phosphate buffer. The whole solution was incubated at 37°C. At appropriate time intervals 1·0 ml aliquots of incubated suspension were withdrawn and diluted to 3·0 ml with 2N HCl and assayed spectrophotometrically at 550 nm (Ellard *et al.*, 1974).

Release of dapsone in 1·0% mice plasma
The liposome-sequestered chitosan gel containing DDS was taken in a dialysis tube and placed in 100 ml of

1·0% mice plasma. The release studies were carried out at 37°C in the same way as in phosphate buffer.

RESULTS AND DISCUSSION

Liposomes will find wider applications if only they were made more targetable. Sequestering liposomes in gel matrix is one of the ways of stabilizing them (Weiner *et al.*, 1985) and these systems form non-toxic, slow release drug carriers. Keeping this in view, an attempt was made to sequester liposomes in a gel matrix system. Oleic acid and stearic acid containing liposomes were sequestered in chitosan gel. From the oleic and stearic acid containing liposomes, the release of bromothymol blue and dapsone was faster both in phosphate buffer (Fig. 1a) and plasma (Fig. 1b) compared with the release of entrapped materials from the sequestered liposomes irrespective of the medium (phosphate buffer or plasma). In PCOA and PCSA there exists the possibility of liposome–liposome interactions due to surface charge; this might lead to membrane destabilization. This can be avoided by sequestering liposomes in gel matrices. The liposome-sequestered gels were subsequently coupled using the carboxyl functionality of the liposomes with the amino groups present in chitosan gels via difunctional carbodiimide.



In vitro release of bromothymol blue and dapsone from liposomes (not sequestered) containing oleic (PCOA) and stearic acids (PCSA) in phosphate buffer

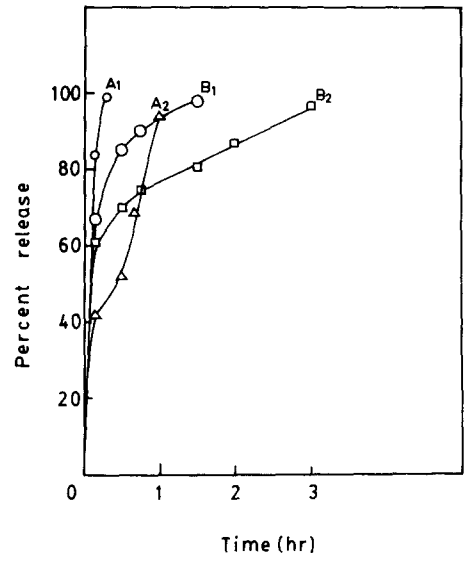
BTB release from (A_1) PCOA

BTB (A_2) PCSA

DDS release from (B_1) PCOA

DDS (B_2) PCSA

Fig. 1a. In vitro release of BTB and DDS from liposomes (not sequestered) containing oleic (PCOA) and stearic acids (PCSA) in phosphate buffer; BTB release from (A1) PCOA; (A2) PCSA, DDS release from (B1) PCOA; (B2) PCSA.



In vitro release of bromothymol blue and dapsone from liposomes (not sequestered) containing oleic (PCOA) and stearic acids (PCSA) in 1% mice plasma

BTB release from (A_1) PCOA

(A_2) PCSA

DDS release from (B_1) PCOA

(B_2) PCSA

Fig. 1b. In vitro release of BTB and DDS from liposomes (not sequestered) containing oleic (PCOA) and stearic acids (PCSA) in 1% mice plasma; BTB release from (A1) PCOA; (A2) PCSA, DDS release from (B1) PCOA; (B2) PCSA.

BTB and DDS were entrapped in the liposomes and their entrapped amounts are given in Table 1. After entrapment, these liposomes were sequestered in chitosan gels.

DDS is one of the most frequently used antileprosy drug. Even though it is cheaper and less toxic its constant use as a single and irregular dose regime makes the patient complain and resistant to the drug. Hence DDS was chosen to encapsulate in the liposomes. Its amino functional groups and lipophilicity is also one of the reasons for choosing this particular drug.

Table 1. Percent entrapment of bromothymol blue (BTB) and dapsone (DDS)

Liposome type	% Entrapment	
	BTB	DDS
PCOA*	78·5	16·0
PCSA†	89·9	16·3

*PCOA—Phosphatidyl choline: oleic acid.
†PCSA—Phosphatidyl choline: stearic acid.

Transmission electron microscopy

Transmission electron micrographs of chitosan gel matrix are shown in Figs 2 and 3, respectively. Figure 2 shows the granular structure of chitosan gel matrix. Figure 3 shows the well defined spherical vesicles sequestered in the granular structure of chitosan.

Effect of the difunctional crosslinking agent carbodiimide on the rate of release of BTB from PCOA liposomes sequestered in chitosan gel matrix

The effect of a difunctional crosslinking agent EDAC (of varying concentrations of EDAC 5 mg–30 mg) on the release of BTB from PCOA liposomes sequestered in chitosan gel was previously reported in detail (Panduranga Rao & Alamelu, 1992). The results prove that crosslinking concentration affects the stability and release of the incorporated bioactive agent. Since it was found that 30 mg EDAC gave highest stability and delayed release of BTB, this concentration of EDAC was chosen for the preparation of liposomes in the rest of the experiments.

***In vitro* release of bromothymol blue from liposomes sequestered in chitosan gel**

Studies on BTB release from liposomes (PCOA & PCSA) sequestered in chitosan gel—both coupled (PCOAcCh, PCSAcCh) and uncoupled (PCOACh, PCSACh) forms were carried out in phosphate buffer, pH 7·4 and 1% mice plasma.

Release in phosphate buffer

Figure 4 shows the release profiles of BTB from liposomes sequestered in chitosan gel in both the coupled and uncoupled forms.

The release of BTB from the sequestered liposomes followed the pattern given below:

In the first hour

PCOACh	released	25·2% of the entrapped BTB
PCSAcCh	released	19·0% of the entrapped BTB
PCSACh	released	9·0% of the entrapped BTB
PCOAcCh	released	3·0% of the entrapped BTB

When compared to the uncoupled system, the coupled gel systems released the BTB marker at much slower rate as: PCOACh took 40 h to release 99% BTB; PCSACh took 24 h to release 86% BTB and PCSAcCh took 72 h to release 96% BTB. PCSAcCh followed a bimodal release profile. Initially, there was a boost release of 19% BTB in the first 1 h; after that the release rate slowed down to a steady state. After 20 h (the terminal phase) the release of BTB was faster and attained 96% release by 72 h.

Release in 1% mice plasma

The release profiles of BTB from the liposomes sequestered in chitosan gels are shown in Fig. 5. The release pattern in mice plasma followed the trend as in the case of phosphate buffer. In the same systems without any coupling of liposomes to the gel matrix, PCOACh released 97% BTB within 30 min and PCSACh liposomes released 96% BTB in 5 h. Conversely, in the liposomes coupled to chitosan gel

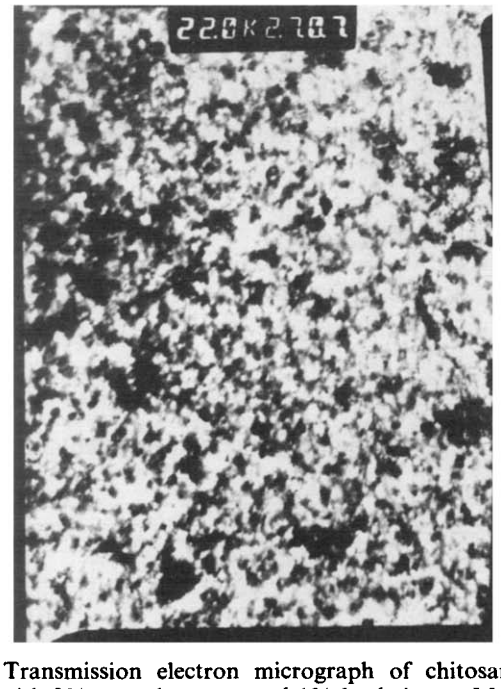


Fig. 2. Transmission electron micrograph of chitosan gel; stained with 2% uranyl acetate and 1% lead citrate. Magnification: 22 000×.

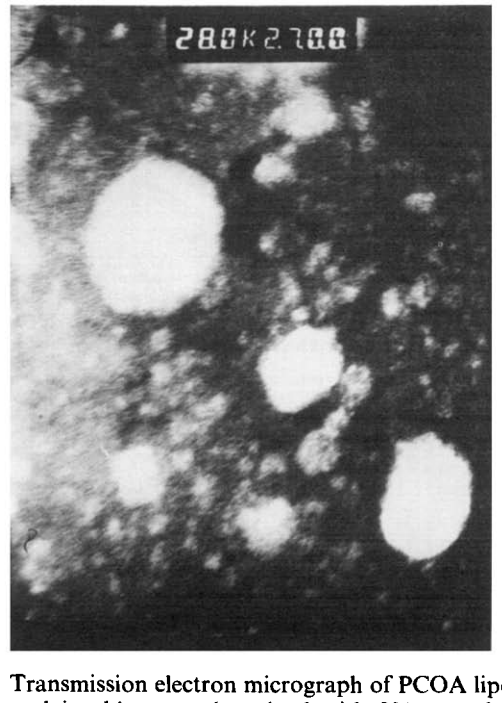
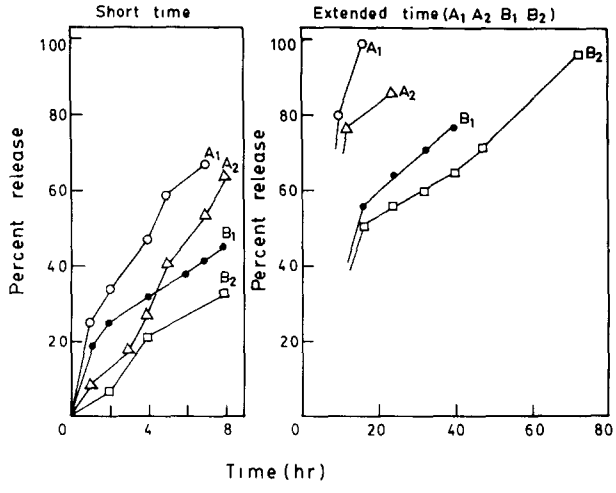


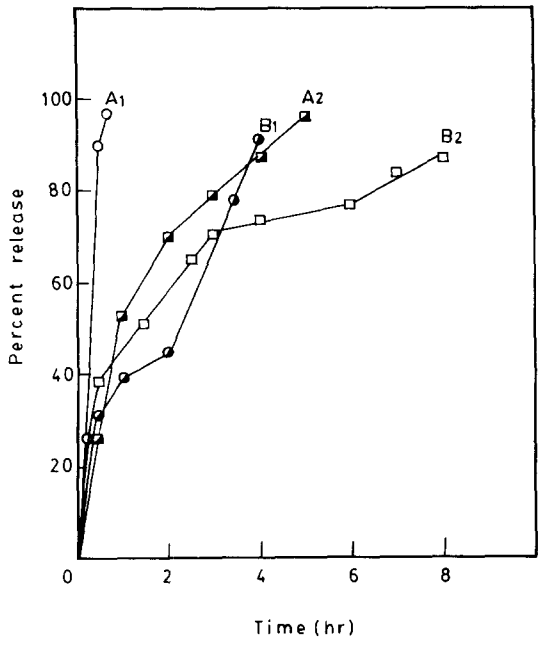
Fig. 3. Transmission electron micrograph of PCOA liposomes sequestered in chitosan gel; stained with 2% uranyl acetate and 1% lead citrate. Magnification: 28 000×.



In vitro release of BTB in phosphate buffer from liposomes (A_1) PCOACh (A_2) PCOAcCh (B_1) PCSACh (B_2) PCSAcCh

Fig. 4. In vitro release of BTB from liposomes in phosphate buffer, pH 7.4.

matrix, both PCOAcCh and PCSAcCh released the same amount of BTB (70%) in 3 h. After 4 h PCOAcCh released 97% BTB whereas PCSAcCh required 8 h to release 96% BTB. The release profile of PCSAcCh was characterized by a rapid rate of release of 70% by 3 h, followed by a steady rate of release (up to 85%) in 6 h and a second mode of fast release of BTB (from 85 to 95%) at the terminal phase. The release curve followed the same trend as in the case of PCSAcCh in phosphate buffer.



In vitro release of BTB in mice plasma from liposomes (A_1) PCOACh (A_2) PCOAcCh, (B_1) PCSACh, (B_2) PCSAcCh

Fig. 5. In vitro release of BTB from liposomes in 1% mice plasma.

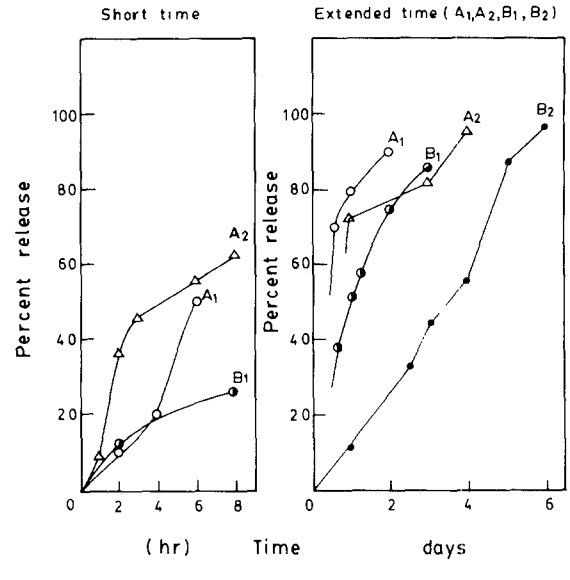
Release of DDS from liposomes sequestered in chitosan gels

Release in phosphate buffer, pH 7·4

The cumulative release profiles of DDS from liposomes sequestered in chitosan gels in phosphate buffer are shown in Fig. 6. Oleic acid containing liposomes sequestered in chitosan (PCOACh) released 80% DDS in one day while the liposomes crosslinked to chitosan (PCOAcCh) took three days to release the same amount. Stearic acid containing liposomes sequestered in chitosan (PCSACh), released 12% DDS in the first 1 h and it took three days to release about 85% DDS. In the case of crosslinked liposomes (PCSAcCh), the release of DDS was slow and linear. On the first day, the release of DDS was about 11% and it took six days to release 96% of DDS. As in the case of BTB release, liposomes coupled to chitosan gel displayed much slower release of DDS than uncoupled liposomes.

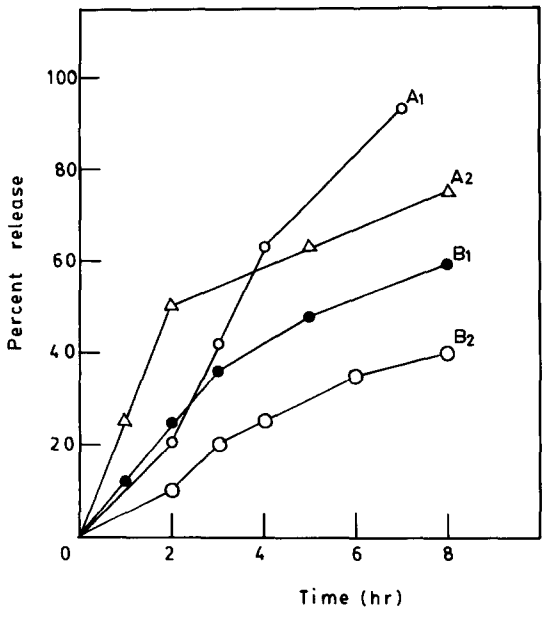
Release in 1% mice plasma

The cumulative release profiles of DDS from liposomes sequestered in chitosan gels in mice plasma are shown in Fig. 7. The release data indicated that in the first 2 h, about 25% DDS was released from PCOACh while in the case of PCOAcCh about 38% DDS was



In vitro release of DDS in phosphate buffer from liposomes (A_1) PCOACh (A_2) PCOAcCh (B_1) PCSACh (B_2) PCSAcCh

Fig. 6. In vitro release of DDS from liposomes sequestered in chitosan gel (in phosphate buffer, pH 7.4).



In vitro release of DDS in mice plasma from liposomes: (A_1)PCOACh (A_2) PCOAcCh (B_1)PCSACh (B_2)PCSAcCh

Fig. 7. In vitro release of DDS in 1% mice plasma from liposomes sequestered in chitosan gel.

released during the same period. But after the second hour DDS released rate from PCOAcCh slowed down and it took about 8 h for the release of 75% DDS. In the case of PCOACh the rate of DDS released followed a zero order pattern after 2 h and 99% of the drug was released by 7 h. On the other hand the DDS release from PCSACh and PCSAcCh was considerably slower than from the OA containing liposomes. PCSACh released 22% DDS in 2 h whereas PCSAcCh released only 10% DDS in the same period. After 8 h, 59% DDS was released from PCSACh while 40% DDS was released from PCSAcCh during the same period. These results indicated that the release of DDS was retarded due to the coupling of liposomes to the chitosan gel matrix. Further PCSA liposomes sequestered in chitosan gel were less permeable to DDS as compared with PCOA liposomes sequestered in the gel. This may be attributed to the absence of unsaturation in the PCSA liposomes sequestered in the gel matrix.

The lipophilic drug DDS took a longer time for release from liposome–chitosan gel matrix as compared to the aqueous marker BTB. This is in accordance with the reports given in literature (Sasaki *et al.*, 1986). The difference in the release of bromothymol blue and dapsone from chitosan gel matrix may be explained due to their viscosity and the permeability of the drugs. The gel matrix provides a stable slow release system for the controlled delivery of lipophilic drugs such as dapsone.

In phosphate buffer, the lipophilic drug DDS was released much slower than the aqueous marker BTB irrespective of the liposomes—unsaturated oleic acid or saturated stearic acid containing liposomes (PCOA and PCSA)—sequestered in the gel. In mice plasma, the release of DDS and BTB followed the same trend as in phosphate buffer. Improved stability of liposomal systems affected the release of both BTB and DDS. The release was slower in the case of liposomes sequestered in gel matrix and subsequently coupled with difunctional EDAC as compared with uncoupled systems. Here again the release of BTB was faster than that of DDS. These results clearly indicated that using the combination of two carriers, i.e. liposomes and gel matrices, it is possible to improve the stability of liposomes in blood plasma. Further one can manipulate the rate of release of the drugs favourably by appropriate crosslinking of the liposomes to the gel matrices.

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