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# Drug targeting to inflammation: Studies on antioxidant surface loaded diclofenac liposomes

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ABSTRACT

Inflammation is associated with enhanced vascular permeability, production of inflammatory markers and over production of reactive oxygen species (ROS) with depletion of endogenous antioxidants. Several drug targeting approaches to inflammation taking clues from these events have been evolved. Surprisingly, a drug targeting approach utilizing abundant oxidative stress at inflammatory site has not been followed. Antioxidant surface loaded liposomes might preferentially localize at inflammatory sites via redox interaction where at high level of ROS exist. The present study was focused to investigate the role of antioxidant as a targeting ligand on the surface of liposome employing rat granuloma air pouch model of inflammation. We developed conventional and antioxidant loaded diclofenac (DFS) liposomes (co-enzyme Q10 and ascorbyl palmitate) for i.v. administration and characterized for vesicle size, zeta potential and percent entrapment. In vivo drug targeting studies showed an increase in AUC, therapeutic availability of DFS in air pouch fluid (APF) and APF/serum DFS concentration ratios from antioxidant loaded liposomes compared to conventional liposomes and drug solution. The promising results suggest the role of antioxidant as a possible ligand in drug targeting to a site where at abundant ROS exist.

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## 1. Introduction

Successful treatment of life-threatening and chronic diseases by intravenous administration of therapeutic agents often involves relatively high and frequent dosing. Due to rapid elimination or a large volume of distribution, many drugs poorly accumulate at target sites while large amounts are wasted or unintendedly localize at healthy tissue sites. As a consequence, a systemic treatment approach is frequently limited by toxicity and therefore characterized by a low benefit/risk ratio. For decades research has been focusing on the possibility of encapsulating drugs in carrier vehicles that take their drug load specifically to target sites in the body, meanwhile protecting it against rapid degradation and/or elimination and preventing undesired localization in non-diseased organs.

At inflammatory sites, due to leaky vasculature a passive targeting of colloidal particles was observed, which was attributed to enhanced permeation and retention effect (EPR) (Yuan et al., 1994; Hashizume et al., 2000; Hobbs et al., 1998). The passive targeting is based on leaky endothelium which is due to the oxidative damage of vascular cells whereas for active targeting various surface epitopes on inflammatory cells have been tried which is more complex. Literature evidence strongly suggests that apart from many biological mediators, the overproduction of free radicals has been implicated in many of the inflammatory disorders and cancer (Beckman and Ames, 1998). Hitherto reports do not take into consideration the quenching of oxidative stress in the inflamed areas which interferes with the normal functioning of the cell. It is not only worthwhile to selectively target the anti-inflammatory drug to the inflamed site rather it will be beneficial if we can load an antioxidant in the carrier along with the drug.

In the recent past, in our laboratory ascorbyl palmitate vesicles (aspasomes) were prepared and studied for permeation enhancing properties (Gopinath et al., 2004) and also evaluated their potential for drug targeting to brain in mice (unpublished data). Recently, D'Souza et al. (2008) reported that surface modified liposomes with ascorbyl residues enhanced the effectiveness of encapsulated paclitaxel compared to paclitaxel encapsulated in 'plain' liposomes. Therefore, it appears promising from therapeutic point of view, that in inflammatory disorders it is worthwhile designing a liposomal drug delivery system containing therapeutic agent with an antioxidant on it as surface ligand.

Antioxidant liposomes besides carrier function can facilitate preferential localization of therapeutic agent via their ability to neutralize ROS at inflammatory sites. Further such an interaction might enhance the retention of drug carrier at the inflammatory site thereby enhancing drug availability in that area. These antiox-

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idant liposomal carriers can in turn act to be bi-functional as they facilitate drug targeting and bring down the oxidative stress in the affected area.

Since the 6-day-old air pouch model of inflammation in rats closely simulates many aspects of joint inflammation and would seem to be a better model for studying the anti-inflammatory effects of steroidal (SAID) and non-steroidal drugs (NSAID) (Edwards et al., 1981; Sedgwick and Lees, 1986), we have employed this model of inflammation and tested the targeting potential of diclofenac sodium loaded antioxidant liposomes. Broadly, the study encompasses the development of conventional and antioxidant surface loaded diclofenac liposomes for improved delivery of DFS to the inflammatory site. The extent of localization and targeting potential was assessed in rats by monitoring DFS content and lipid peroxide levels in both serum and the exudates formed at the air pouch inflammation.

## 2. Materials and methods

### 2.1. Materials

Epikuron 200SH (highly purified hydrogenated soy phosphatidylcholine, 98% purity, HSPC) was obtained from Lucas Meyer, Germany. Ascorbic acid-6-palmitate (>99%, ASP), cholesterol (≥99%, CHOL), dicetyl phosphate (>98%, DCP) and tetra methoxy propane (>99%, TMP) were purchased from Sigma, St. Louis, MO, USA. Co-enzyme Q10 (>99%) was generously supplied by Biocon India Ltd (Bangalore, India). 2-Thiobarbituric acid (A.R.) and carrageenan were purchased from Himedia (Mumbai, India). Diclofenac sodium (DFS) and chlorzoxazone were kindly provided by Matrix Laboratories (Hyderabad, India). Trichloroacetic acid (G.R.) was obtained from Merck India Ltd (Mumbai, India). All other chemicals used were of analytical grade and solvents were of HPLC grade.

### 2.2. Preparation of liposomes

The conventional liposomes (DL) were prepared by using film hydration method (Sinico et al., 2005). Briefly, 200 μM of lipid mixture containing HSPC, cholesterol, dicetyl phosphate in molar ratio of 45:45:10 respectively were dissolved in chloroform and methanol mixture (9:1) and evaporated under reduced pressure in a rotary evaporator (Laborota 4000, Heidolph, Germany) at 50 °C (Gopinath et al., 2004). The dried thin lipid film was hydrated with 10 mL of DFS solution (5 mg/mL in phosphate buffered saline, pH 7.4 maintained at the same temperature) filtered through a 0.22 μm filter. The formed vesicles were sonicated (Vibra Cell, Sonics, USA) for 2 min maintaining the same temperature and stored in nitrogen-purged vials. Antioxidant containing liposomes were prepared by adding ascorbyl palmitate (DLA) and co-enzyme Q10 (DLQ) at 10 mol% of total lipid in organic solvent mixture and processed similarly as mentioned above. Both the liposomal formulations were then passed through a 0.22 μm polycarbonate filter (Millipore, Massachusetts, USA) before characterization.

### 2.3. Characterization of liposomes

#### 2.3.1. Liposome shape and morphology

The shape and morphology of the liposomes was examined by optical microscopy. A drop of unsonicated liposomes was placed on a glass slide and observed at a magnification of 450× through an epi-fluorescent microscope (Eclipse E 600, Nikon, Japan) and microphotograph was taken.

#### 2.3.2. Size and zeta potential measurements

The mean size and polydispersity index of the size distribution of liposomes was determined by photon correlation spectroscopy using zetasizer 3000 HSA (Malvern Instruments, Malvern, UK). Each sample was diluted to a suitable concentration (1 in 100) with filtered phosphate buffered saline (pH 7.4) and analysis was performed at 25 °C with an angle of detection of 90 °C. For zeta potential measurement, the sample was suitably diluted with filtered phosphate buffered saline (pH 7.4) and the zeta potential (ZP) was determined using zetasizer 3000 HSA (Malvern Instruments, Malvern, UK). The measurements were taken in triplicate.

#### 2.3.3. Entrapment efficiency

The entrapment efficiency of the liposomal formulation was determined by measuring the concentration of free drug in the dispersion medium using ultra filtration (Gopinath et al., 2004). In brief, ultra-filtration was carried out using Centrisart I (20,000 MWCO, Sartorius AG, Goettingen, Germany) filters at a centrifugation speed of 3500 rpm for 15 min. The amount of DFS in the aqueous phase at the base of sample recovery chamber was estimated by high performance liquid chromatography. The experiment was performed in triplicate and percentage entrapment of DFS in liposomes was calculated from the following equation: % drug entrapment = (total amount of drug added − unentraped drug)/total amount of drug added × 100.

#### 2.3.4. Differential scanning calorimetry

The thermotropic properties and phase transition behavior of liposome dispersions was studied by using differential scanning calorimeter (Mettler DSC 821e, Mettler-Toledo, Switzerland). Average sample weight of 5 ± 2 mg were heated in hermitically sealed aluminum pan over a temperature range of 20–300 °C under a constant nitrogen gas flow of 30 mL/min at a heating rate of 10 °C/min.

### 2.4. Pharmacokinetic study

Healthy male wistar rats (180–200 g) were used for inducing air pouch to evaluate the formulations for drug targeting potential. The animals were given a diet advised by National Institute of Nutrition, Hyderabad, India and had free access to water. The studies were conducted with prior approval of Institutional animal ethics committee of University College of Pharmaceutical Sciences, Kakatiya University. Euthanasia and disposal of carcass was in accordance of the guidelines.

Air pouches were produced on the dorsal surface of rats using a modified method (Edwards et al., 1981). In brief, on day zero the rats were lightly anesthetized with diethylether and 20 mL of sterile air passed through a 0.22 μm filter (Millipore, Germany) was injected subcutaneously on the dorsal surface. On day 3, air pouches were reinflated with 10 mL of sterile air. On day 6, the inflammogen carrageenan (2 mL; 1%, w/v, in sterile phosphate-buffered saline, pH 7.4) was injected into the pouch. The inflammatory effects of carrageenan were allowed to develop for 24 h and the animals were divided into four groups (six each) and were randomly administered with each treatment. Required volume of liposome formulations or drug solution containing DFS (10 mg/kg body weight) was administered as i.v. bolus injection via caudal vein under light ether anesthesia. At predetermined time points (0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0 h) blood was collected from retro-orbital venous plexus puncture and simultaneously air pouch fluid (APF) samples from the air pouch under light ether anesthesia. Serum separated from the blood and APF samples were stored under frozen condition until analysis.

*2.4.1. HPLC analysis of DFS and malondialdehyde (MDA)*

Diclofenac was quantitatively determined in serum and APF by HPLC using 60:10:30 (v/v) methanol, acetonitrile mixed with 100 mM ammonium acetate at pH 4.2, respectively as mobile phase at a flow rate of 1.0 mL/min equipped with LC-10 AT solvent delivery unit (Shimadzu, Japan). An octadecylsilane ($C_{18}$) reverse phase stainless steel analytical column (250 mm × 4.6 mm) with 5 μm particle size was employed for chromatographic separation (Lichrospher, Merck, Germany). The column eluent was monitored at a wavelength of 280 nm using an SPD-10 AVP ultraviolet detector and the sensitivity was set at 0.005 AUFS at ambient temperature.

Malondialdehyde is a β-scission product formed from the peroxidation of certain polyunsaturated fatty acids and is considered an index of lipid peroxidation and its measurement serves as an indicator of free radical damage. The formation of highly colored product between malondialdehyde and 2 molecules of thiobarbituric acid (TBA) is the basis for most frequently used technique for analysis of MDA levels. Assay for detection of MDA was performed according to a modification of the high pressure liquid chromatographic technique (Richard et al., 1992). The instrumentation and analytical conditions were similar as described above except mobile phase comprised of 40:60 (v/v) methanol mixed with water containing 80 nM of sodium hydroxide with 250 μL of orthophosphoric acid.

*2.4.2. Sample treatment for analysis of DFS and MDA*

The serum sample was processed for quantification of DFS by using reported method with slight modification (Mohamed et al., 1994). To 200 μL of serum sample, 100 μL of methanol, 100 μL of 2 M HCl and 100 μL of internal standard (5 μg/mL of chlorzoxazone in methanol) were added. The mixture was extracted with 3 mL of chloroform and centrifuged. The separated organic layer was dried under vacuum. The residue was reconstituted with 50 μL of methanol and 20 μL was injected onto the HPLC. In our preliminary experiments, the DFS concentration in APF was less and the DFS peaks were insignificant at few sample time points. Because of this reason we decided to increase volume of the APF sample to 500 μL so as to obtain significant peak heights and processed similarly as serum samples. The concentration peak height ratio plots were linear ($r^2 > 0.998$) over the concentration range of interest and the DFS content in samples was determined using this plot.

To 200 μL of serum/APF samples, 500 μL of 30% (w/v) aqueous trichloroacetic acid solution was added to precipitate the proteins and the mixture was vortexed and centrifuged. The clear supernatant separated was treated with 100 μL of 1% (w/v) thiobarbituric acid and heated (at 95 °C for 1 h in a water bath), cooled in ice and 20 μL was injected onto the HPLC (Richard et al., 1992). A calibration plot was made using 1,1,3,3-tetra methoxy propane (TMP) as a standard substance over the concentration range of interest 0.25–16 nM/mL. The MDA levels in samples were interpolated from the calibration plot.

*2.4.3. Pharmacokinetic parameters*

Serum concentration versus time data for DFS in individual rats was analyzed by non-compartment estimations using WinNonlin (V.1.1.; Pharsight Corporation, USA) software. The DFS availability ($AUC_{0-t}$) in air pouch was determined by trapezoidal rule from APF drug concentration versus time data (Madhusudhan et al., 2007). The mean residence time (MRT) was calculated by dividing the $AUMC_{0-8}$ with $AUC_{0-8}$. The overestimated clearance was determined by dividing the dose with $AUC_{0-8}$ (Mosqueira et al., 2001). The $C_{max}$ and $t_{max}$ were obtained from the APF concentration versus time data. Two parameters were used to assess the targeting potential of liposomes. One is APF/serum drug concentration (A/S) ratio (Brewster et al., 1991) and the other is therapeutic availability (TA)

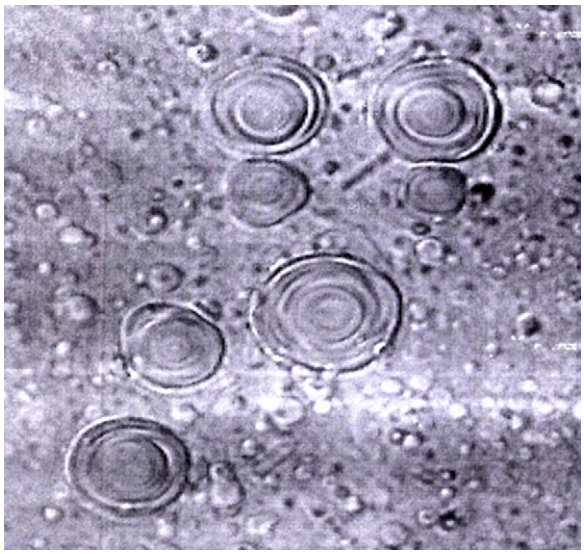

**Fig. 1.** Optical microphotograph of diclofenac liposomes at 450× magnification.

(Hunt et al., 1986). The percent reduction of MDA levels in serum and APF was calculated as follows:

$$\%\ \text{reduction} = \frac{\text{MDA levels at time 't' after treatment} - \text{MDA levels at zero 't'}}{\text{MDA levels at zero 't'}} \times 100.$$

*2.5. Statistical treatment of data*

Statistical analysis of the data obtained was performed using one-way ANOVA followed by Neuman Keuls post test with GraphPad Prism software (version 4.0; GraphPad Software, San Diego, CA). The level of statistical significance was chosen as less than $P < 0.05$.

## 3. Results

*3.1. Characterization of liposomes*

Presence of vesicles in the unsonicated dispersion was confirmed by viewing the system using an optical microscope. The vesicles were spherical and majority of them were multilamellar and also a few unilamellar vesicles were seen (Fig. 1). The mean vesicle size of conventional liposomes (DL) was 135 ± 2.3 nm and was increased to 174.4 ± 1.0 and 186 ± 2.6 with the inclusion of ascorbyl palmitate and coenzyme Q10, respectively; such an increase was found to be significant ($p < 0.05$). The zeta potential of the DL formulation was −34.1 ± 2.6 mV. However, the intensity of the surface charge was increased significantly to −42.9 ± 3.2 and −46.9 ± 2.7 mV in case of DLA and DLQ formulations, respectively ($p < 0.01$). The DFS could be entrapped into liposomes by film hydration method with the entrapment levels of 29.13% for DL formulation and 33.21 and 34.72% for DLA and DLQ formulations, respectively, which is significantly higher compared to DL ($p < 0.05$) (Table 1). Differential scanning calorimetric data of anhydrous physical mixtures of lipids and liposomal formulations were obtained to study thermotropic properties and phase transition behavior. The transition temperature and enthalpy of all the samples are recorded in Table 2. In all the cases, the enthalpy and phase transition temperature were lower for liposomal formulations compared to their respective physical mixture samples which denotes the formation of vesicular arrangement.

**Table 1**
Mean size, zeta potential and percent entrapment of DFS liposomal formulations.

| Formulation code | Size (nm) | P.I. | Zeta potential (mV) | % Entrapment |
|---|---|---|---|---|
| DL | 135.0 ± 2.3 | 0.211 ± 0.03 | −34.1 ± 2.6 | 29.13 ± 1.2 |
| DLA | 174.4 ± 1.0* | 0.156 ± 0.02 | −42.9 ± 3.2** | 33.21 ± 0.9* |
| DLQ | 186.0 ± 2.6* | 0.127 ± 0.04 | −46.2 ± 2.7** | 34.72 ± 1.4* |

Each point is mean ± SD ($n = 3$); P.I. indicates polydispersity index; DL: conventional diclofenac liposomes; DLA and DLQ: liposomes loaded with ascorbyl palmitate and co-enzyme Q10, respectively.
* Significant at $p < 0.05$ vs. DL.
** Significant at $p < 0.01$ vs. DL.

**Table 2**
Transition temperatures and enthalpy of different liposomal formulations and their respective physical mixtures.

| Formulation | Physical mixtures | | Liposome dispersions | |
|---|---|---|---|---|
| | Main transition peak (°C) | Heat (J/g) | Main transition peak (°C) | Heat (J/g) |
| DL | 45.66 | 32.51 | 41.34 | 21.91 |
| DLA | 66.58 | 53.02 | 45.42 | 37.48 |
| DLQ | 54.32 | 42.63 | 43.31 | 38.08 |

DL: conventional diclofenac liposomes; DLA and DLQ: liposomes loaded with ascorbyl palmitate and co-enzyme Q10, respectively.

### 3.2. Pharmacokinetic study

The decrease in serum DFS concentration with time for liposomes and drug solution followed a bi-exponential clearance model. Following i.v. bolus administration of liposome formulations to air pouch induced rats, a rapid fall in serum DFS levels is seen up to 1 h and there after fall in levels is slow up to 8 h. The serum DFS concentrations following treatment with DL, DLA and DLQ formulations were significantly lower at all time points than DS. Thus liposomes cleared relatively rapid than DFS solution (Fig. 2). Pertinent pharmacokinetic data of DFS in serum following i.v. bolus administration of various formulations are listed in Table 3. In general, with liposomal formulations $t_{1/2}$ decreased, clearance increased, as a result a substantial decrease is observed in the area under the curve ($AUC_{0-\infty}$) with respect to drug solution. Among the formulations tested, the half life was lower for antioxidant loaded liposomes compared to conventional liposomes and drug solution. The area under the curve for DL and DS was 21.48 and 56.76 μg h/mL, respectively and the same was significantly lower for DLA and DLQ formulations was 14.06 and 18.49 μg h/mL, respectively. The steady state volume of distribution ($Vd_{ss}$) was significantly higher for DLA and DLQ formulations compared to DS. Based on DFS serum availability ($AUC_{0-\infty}$) the liposomes can be ranked in the following order DLA < DLQ < DL. The $C_{max}$ in air pouch fluid was significantly higher for antioxidant loaded formulations, i.e. DLA and DLQ compared to DL and DS systems (Fig. 3). The DFS clearance in APF was significantly lower in case of DLA and DLQ in comparison with DL and DS (Table 4). Normally the system which shows rapid clearance in serum should allow higher extra vascular distribution and hence higher DFS levels in APF for DLA and DLQ formulations.

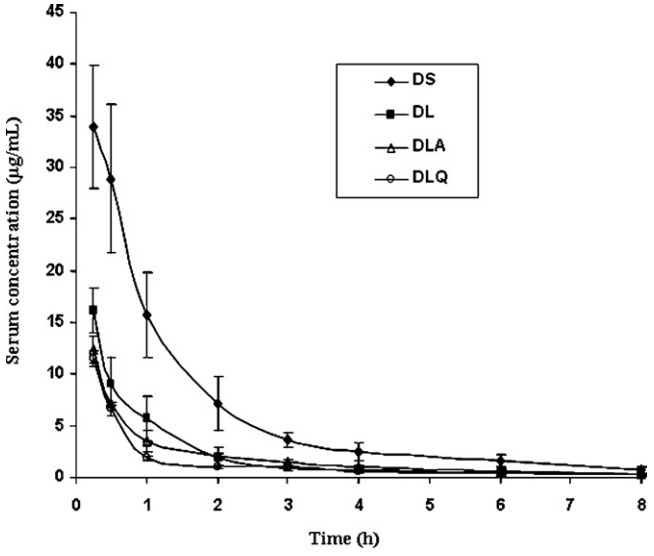


**Fig. 2.** Mean serum concentration of DFS from different formulations following i.v. bolus administration to air pouch induced rats ($n = 6$).

Tissue to serum concentration ratio can be commonly employed as an index of targeting. If this ratio is more than one indicates drug targeting to that tissue (Brewster et al., 1991). We found that the APF to serum ratio was more than one for all the liposomal formulations. Interestingly, the presence of antioxidant in the bilayer (DLA and DLQ) led to an increase in the ratios greatly compared to DL and DS systems (Fig. 4). The total drug reaching the inflamed site

**Table 3**
Pharmacokinetic parameters in serum following i.v. bolus administration of diclofenac sodium solution and liposomal formulations in air pouch induced rats ($n = 6$).

| Pharmacokinetic parameters | DS | DL | DLA | DLQ |
|---|---|---|---|---|
| $C_0$ (μg/mL) | 40.04 ± 4.54 | 24.90 ± 4.04 | 20.30 ± 3.84 | 21.75 ± 4.33 |
| Half-life (h) | 2.16 ± 0.22 | 1.61 ± 0.18$^{c1}$ | 1.90 ± 0.09$^{a1,2}$ | 1.84 ± 0.08$^{a1}$ |
| Clearance (L/h/kg) | 0.18 ± 0.04 | 0.48 ± 0.09$^{c1}$ | 0.71 ± 0.05$^{c1,2b3}$ | 0.55 ± 0.08$^{c1}$ |
| $Vd_{ss}$ (L/kg) | 0.36 ± 0.11 | 0.68 ± 0.18$^{a1}$ | 1.32 ± 0.13$^{c1,2}$ | 1.12 ± 0.17$^{c1,2}$ |
| $AUC_{0-\infty}$ (μg h/mL) | 56.76 ± 5.69 | 21.48 ± 4.18$^{c1}$ | 14.06 ± 1.14$^{c1,a2}$ | 18.49 ± 2.55$^{c1}$ |
| MRT (h) | 1.97 ± 0.24 | 1.47 ± 0.27$^{a1}$ | 1.87 ± 0.29$^{a2}$ | 2.05 ± 0.11$^{b2}$ |

Data are presented as mean ± SD; a, b, and c represent $p < 0.05$, 0.01, and 0.001, respectively; 1, 2, and 3 represent comparison against formulation DS, DL and DLA, respectively. DS: drug solution; DL, DLA and DLQ: conventional diclofenac liposomes, liposomes loaded with ascorbyl palmitate and co-enzyme Q10, respectively; $C_0$: initial concentration; Cl: clearance; $Vd_{ss}$: steady state volume of distribution; AUC: area under the curve; MRT: mean residence time.

**Table 4**
Pharmacokinetic parameters in APF following i.v. bolus administration of diclofenac sodium solution and liposomal formulations in air pouch induced rats ($n = 6$).

| Pharmacokinetic parameters | DS | DL | DLA | DLQ |
|---|---|---|---|---|
| $C_{max}$ (μg/mL) | 4.20 ± 0.78 | 4.16 ± 0.25 | 8.51 ± 1.44 | 7.25 ± 1.66 |
| $t_{max}$ (h) | 2.00 ± 0.00 | 2.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| Clearance (L/h/kg) | 1.05 ± 0.28 | 0.89 ± 0.17 | 0.25 ± 0.04$^{c1,2}$ | 0.41 ± 0.10$^{c1,b2}$ |
| $AUC_{0-8}$ (μg h/mL) | 10.05 ± 2.60 | 11.48 ± 2.00 | 40.83 ± 5.92$^{c1,2,4}$ | 25.49 ± 6.39$^{c1,2}$ |
| MRT (h) | 2.09 ± 0.19 | 2.33 ± 0.30 | 2.43 ± 0.09 | 2.04 ± 0.18 |
| TA | 1.00 ± 0.00 | 1.23 ± 0.49 | 4.39 ± 0.77$^{c1,2a4}$ | 2.81 ± 1.45$^{a1,2}$ |

Data are presented as mean ± SD; a, b, and c represent $p < 0.05$, 0.01, and 0.001, respectively; 1, 2, 3, and 4 represent comparison against formulation DS, DL, DLA and DLQ, respectively. DS: drug solution; DL, DLA and DLQ: conventional diclofenac liposomes, liposomes loaded with ascorbyl palmitate and co-enzyme Q10, respectively; $C_{max}$: peak concentration; Cl: clearance; AUC: area under the curve; MRT: mean residence time; TA: therapeutic availability.

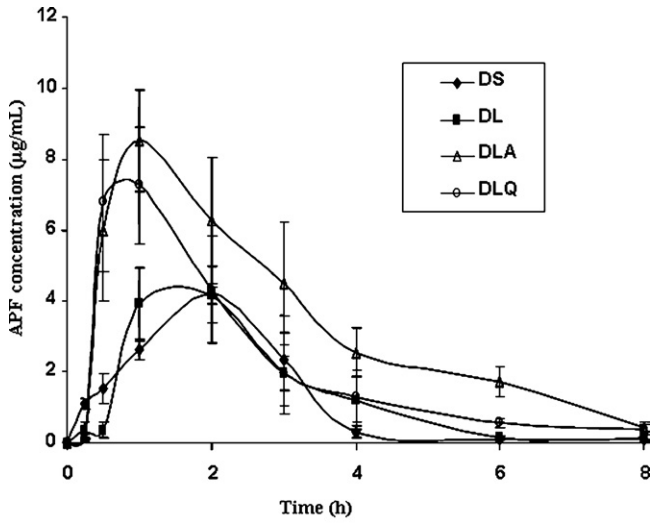


**Fig. 3.** Mean air pouch fluid concentration of DFS from different formulations following i.v. bolus administration to air pouch induced rats ($n = 6$).

assessed by calculating the $AUC_{0-8}$ in APF was significantly higher for DLA and DLQ than DL and DS formulations (Fig. 5). The therapeutic availability (TA) was also used as a parameter to assess the drug targeting potential of drug carrier system which indicates how many times a drug carrier system compared to drug alone can allow the localization of a drug in a tissue (Madhusudhan et al., 2007). The TA in APF was 3–4 times higher for DLA and DLQ formulations compared to DL and DS. Based on extent of accumulation ($AUC_{0-8}$), degree of localization and therapeutic availability (TA) of DFS to the target site, i.e. APF, the formulations can be ranked in the following order DLA > DLQ > DL > DS. On the basis of these parameters, DLA and DLQ formulations was found to have more potential in allowing DFS to the target site.

The variation of MDA levels in the serum and air pouch fluid was followed by measuring TBARS levels at each time point. The MDA levels in serum and APF were reduced initially within 2 h following treatment with all the formulations and DS and thereafter no significant change was observed. However, the percent reduction of MDA levels in serum and APF with DLA and DLQ formulations was significantly higher compared to DL and DS (Fig. 6).

## 4. Discussion

Among many animal models of inflammation (sponge implant, paw edema, peritoneal, pleurisy, and air pouch) the air pouch model has the added advantage of not involving internal organs which can be damaged or perforated during sampling (Sedgwick and Lees, 1986). Henceforth we used the same model to assess the potential of antioxidant liposomal carrier in localization of the drug to the inflammatory site.

In accordance with previous report (Stevens et al., 1995), our serum concentration versus time profiles for DFS could best be described by bi-exponential equation. Further, according to this report the steady state volume of distribution ($Vd_{ss}$) of DFS in rats bearing air pouches was 0.19 ± 0.08 L/kg. However our $Vd_{ss}$ value 0.36 ± 0.11 L/kg which is higher than the one reported might be due to the change in degree of severity and intensity of the inflammation. Peris-Ribera et al. (1991) obtained a systemic clear-

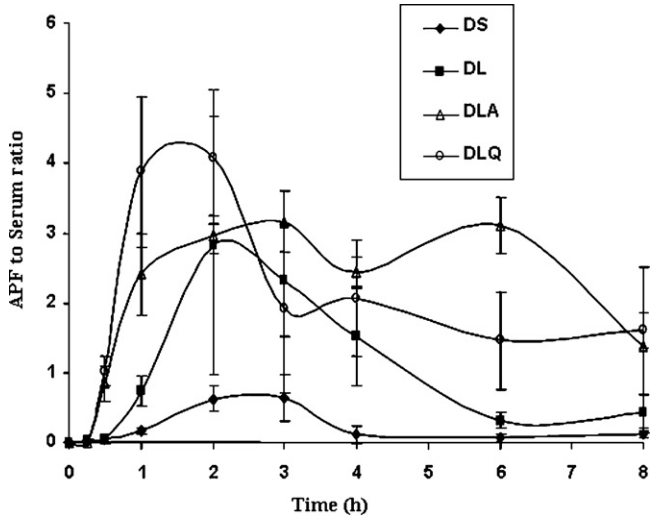


**Fig. 4.** Mean APF to serum ratio of DFS from different formulations following i.v. bolus administration to air pouch induced rats ($n = 6$).

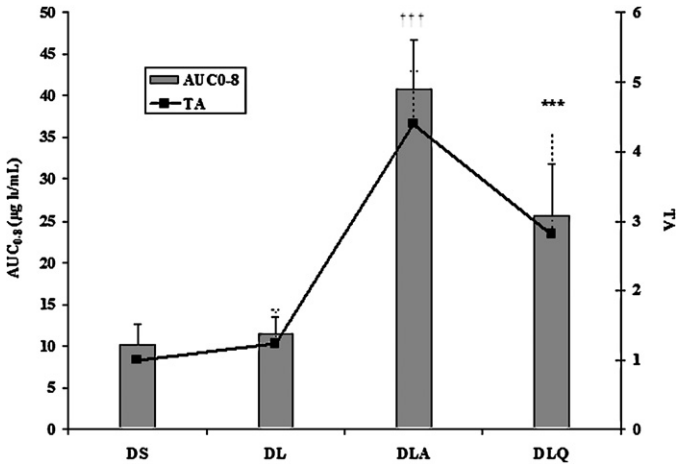


**Fig. 5.** Mean AUC and therapeutic availability (TA) of DFS in air pouch fluid from different formulations following i.v. bolus administration to air pouch induced rats ($n = 6$). †††Significant difference at $p < 0.001$ against DS, DL and DLQ formulations; ***significant difference at $p < 0.001$ against DS and DL formulations.

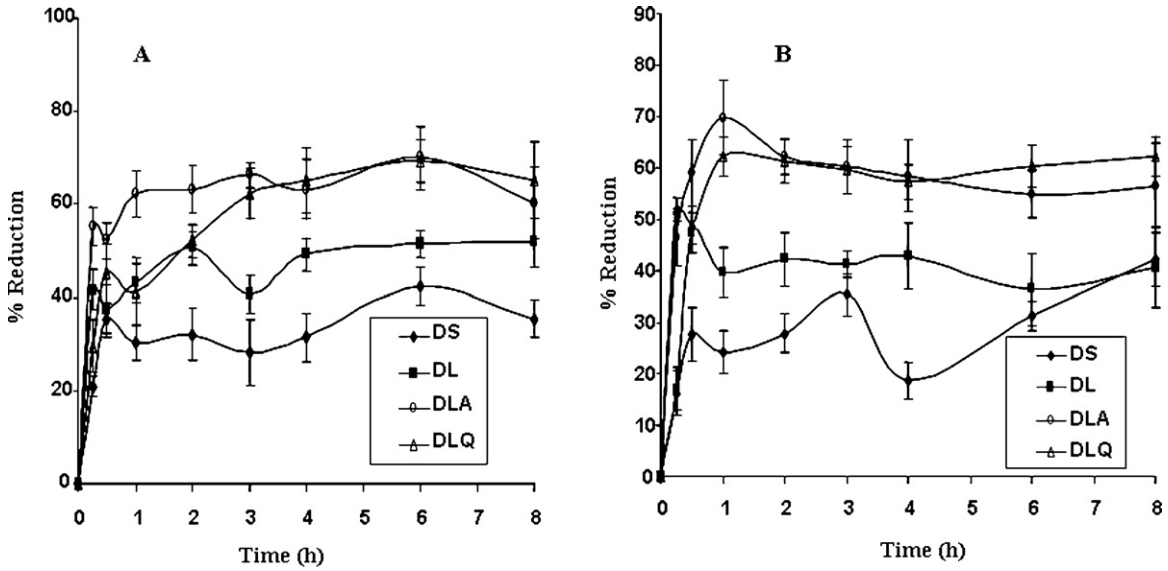


**Fig. 6.** % Reduction in malondialdehyde levels of (A) serum and (B) air pouch fluid from different formulations following i.v. bolus administration to air pouch induced rats ($n = 6$). *Note*: Percent protection offered by the formulations.

ance of $0.25 \pm 0.05$ L/h which compares favorably with our value of $0.18 \pm 0.04$ L/h.

The blood clearance of sonicated large multilamellar liposomes of heterogeneous size is compatible with a bi-exponential clearance rate (Harashima and Kiwada, 1996). Furthermore the clearance of negative small liposomes appears to be bi-phasic (Palatini et al., 1991). The radioactivity is detectable in synovial fluid within 10 min after the intravenous injection of Tc-99m labeled negatively charged unilamellar liposomes and the difference is clear between normal individuals and patients with rheumatoid arthritis (Williams et al., 1987). In accordance with these reports, our liposomal DFS formulations surface decorated with ASP and Co-$Q_{10}$ (DLA and DLQ, respectively) with high negative charge cleared rapidly from serum and such a clearance was bi-exponential. We observed quantitative evidence that charge density is directly related to rate of clearance; higher the negative charge density rapid is clearance irrespective of size. The sustained DFS levels in serum for drug solution could be due to the high affinity of the DFS to the serum proteins which result in protein binding (>99% in bound form) (Willis et al., 1979).

All liposomal formulations which disappeared rapidly from blood with almost equal rate of clearance showed differential distribution behavior towards tissue sites. In general, liposomes show a preferential distribution to reticuloendothelial system (RES) via opsonization thereby accumulating in liver, spleen etc. However because of EPR effect at the inflammation and surface decorated antioxidant, our liposomes showed higher accumulation at the inflammatory site and were retained there for a long time. A rapid uptake of control liposome (DL) into liver and spleen appears to be more likely than its uptake at inflammatory site. This can be deduced based on the fact that these liposomes showed a lag time in uptake into inflammatory site. Further such an uptake is significantly slower and lesser than the liposomes containing antioxidant surface ligands (DLA and DLQ). Since we have not made an effort to estimate free drug and bound drug in the APF obviously the higher DFS levels with DLA and DLQ reflect the accumulation of liposome associated drug.

In the recent past the mechanisms involved in the manifestation of Enhanced Permeation and Retention (EPR) effect of macromolecules and lipids (liposomes) in solid tumors and inflammatory sites are investigated to a large extent (Schiffelers et al., 2000). In particular the enhanced permeation effect has been attributed to the excessive production of superoxide (SO) and nitric oxide (NO) levels at the inflammatory sites which are produced by infiltrated leukocytes. We therefore presume that the rapid and higher accumulation of liposomes at inflammatory site could be due to extravasation of the liposomes via leaky vascular endothelium. Our results envisage the higher degree of localization of antioxidant liposomes (DLA and DLQ) compared to DL which clearly suggests the preferential uptake of antioxidant liposomes at inflammatory site. The mechanism and biochemical interactions are not clearly understood for selective uptake and accumulation and might be due to firm adhesion of antioxidant surface loaded intact liposomes at the site of inflammation via redox interactions (antioxidant and free radical interaction). The higher % reduction of MDA levels in serum and APF for DLA and DLQ can be attributed to their higher degree of affinity to ROS due to redox interactions. The MDA levels have also come down following treatment with conventional liposomes which is due to the antioxidant functionality of hydrogenated phosphatidylcholine used in the formulations as a vesicle former (Shchipunov, 2002).

Abundant similarities between inflammation and cancer are reported (Maeda et al., 2000). Cancer cells are usually equipped with a means to counter the oxidative stress, such as superoxide and NO as well as their metabolites, which are generated by infiltrating leukocytes as a part of the host defense system against tumor cells. Heme oxygenase I is induced markedly in tumor cells which is responsible for production of antioxidant biliverdin/bilirubin. Inhibition of this enzyme reduces the production of antioxidant; as a result the tumor growth is suppressed effectively. This clearly indicates the significant role of ROS which are produced predominantly by leukocytes in triggering vascular permeability and hence tumor growth and even promote cancer metastasis. However when this endogenous antioxidant mediated mechanism fails or homeostasis is disturbed, the administration of exogenous antioxidants is advocated to quench the free radicals thereby arresting the tumor cell growth. Thus we presume the antioxidant loaded liposomes as potential carriers for NSAIDs and anticancer agents because of their bi-functional character such as targeting and quenching of free radicals.

All our liposomal formulations contain HSPC and cholesterol in 1:1 ratio since earlier reports suggest that stable vesicles with good entrapment efficiency can be obtained at equimolar ratio of HSPC and cholesterol (Puglisi et al., 1992; Betagiri, 1993). The formula-

tions above 10 mol% of antioxidant showed crystallization and the instability of the liposomes with increasing proportion of antioxidants might be due to the limited accommodation of antioxidant in the bilayer; hence the concentration of antioxidant was fixed to 10 mol% of total lipid. The mean size of all the formulations is within the range of 130–190 nm. The substantial increase in size of DLA and DLQ liposomal formulations suggest the possible orientation of antioxidant on the surface of bilayer thus altering the characteristics of bilayer with net increase in the surface charge of the liposomes. The higher surface charge might have increased swelling of lipid, which in turn resulted in higher uptake of aqueous portion inside the bilayer thus accommodating higher DFS content. The phase transition temperature of vesicle dispersions (Ishigami and Machida, 1989) and aspasomes (ascorbyl palmitate vesicles) was lower than their corresponding anhydrous lipid mixtures (Gopinath et al., 2004). The decrease in phase transition temperature of liposomal formulations can be owed to the formation of vesicles. Further, the presence of antioxidant in the bilayer might have resulted in a less ordered structure of lipids lowering the phase transition temperature and that can be assigned to the colloidal dimensions of the particles in particular to their large surface to volume ratio (Siekmann and Westesen, 1994).

## 5. Conclusions

The potential therapeutic use of antioxidant liposomes is a rapidly evolving area of medical research that has not been extensively attempted. Surface loaded antioxidant liposomes appear to be potential carriers for targeting inflammatory sites and cancerous tissue wherein abundant reactive oxygen species are produced. While the precise mechanism underlying these observations still requires investigation, these data suggest that biochemical factors in the inflammatory disorders and similarly in tumor could be expected to favor the association of antioxidant liposomes within the target site.

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