Preparation and evaluation of lipid vesicles of camptothecin as targeted drug delivery system

Prabhu Prabhakara[1]*, Teles Zenia[2], Koland Marina[2], Khandige Prasanna Shama[2], Shetty Nisha Girish[2] and Nairy Harish Matapady[2]

[1]Shree Devi College of Pharmacy, Rajiv Gandhi University of Health Sciences, Airport Road, Kenjar, Mangalore, Karnataka, India
[2]Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences, Nitte University, Mangalore, Karnataka, India

Abstract: Site-specific delivery of anticancer based therapy of human cancers has led to several remarkable outcomes, particularly in the therapy of breast cancer and lymphoma. Camptothecin, a plant secondary metabolite is widely used in the treatment of metastatic breast cancer and lymphoma. However its side effect profile often results in cessation of therapy. In this study the principle of both active as well as passive targeting using camptothecin loaded stealth liposomes as per the magic gun approach was followed. Stealth liposomes of camtothecin were prepared by thin film hydration method using a PEGylated phospholipid like DSPE-MPEG 2000. Similarly conventional liposomes were prepared using phospholipids like DPPC, DSPC. Conventional liposomes were coated with a hydrophilic biocompatible polymer like chitosan. It was found that chitosan coating of the conventional liposomes increased the physical stability of the liposomal suspension. Further, chitosan coated conventional liposomes and the PEGylated liposomes released the drug for a prolonged period of time, compared to the uncoated conventional liposomes. *In vivo* screening of the formulations for their antitumor efficacy was carried out in rats. Breast cancer was induced in female Sprague–Dawley rats using an indirectly acting chemical carcinogen DMBA (7, 12 dimethyl benz(a)anthracene). It was found that there was significant decrease (P<0.01) in tumor volume in the rat group treated with test 2 formulation and test 1 formulation compared to standard free CPT. However the chitosan coated liposomal formulation showed a better antitumor efficacy than that of the PEGylated liposomal formulation.

Keywords: Camptothecin, stealth liposomes, conventional liposomes, chitosan coating, site-specific delivery, antitumor efficacy.

INTRODUCTION

Liposomes are the promising drug carriers because of their increased therapeutic efficacy and decreased toxicity compared with the drug prototype. Drug delivery systems offer the potential to enhance the therapeutic index of anticancer agents, either by increasing the drug concentration in tumor cells and/or by decreasing the exposure in normal host tissues (Chang *et al.,* 2008; Martins *et al.,* 2007). However, the *in vitro* and *in vivo* stability problem of liposomes, limits their application and development as liposomes tend to degrade or aggregate and fuse, which leads to the leakage of entrapped drug during storage (Pajean *et al*., 1991) and rapid clearance from the circulation system after intravenous injection. Many studies have found that, among all the factors affecting the stability of liposomes, surface characteristics of the carriers, such as hydrophobicity (Senior 1987; Storm *et al*., 1995; Torchilin & Trubetskoy 1995) charge, (Chonn *et al*., 1991; Juliano & Stamp 1975) and fluidity (Patel 1992), are of great importance. The mechanisms explaining their effect are associated with the inter-molecular interaction and the interaction with various plasma components (Chonn *et al.,* 1992). Therefore, the simple surface modification of the carriers using polymers with specific properties may be an easy approach to modulating their *in vitro* and *in vivo* stability.

Solid tumours such as breast cancer have historically provided many challenges to systemic therapy. Theoretical barriers to drug penetration in solid tumors include heterogeneous vascular supply and high interstitial pressures within tumor tissue, particularly in necrotic zones.

Delivery systems can even exacerbate these problems due to the slow diffusion of macromolecular agents through tumor tissue. Long circulating macromolecular carriers such as liposomes (lipid vesicles) can exploit the ‘enhanced permeability and retention’ effect for preferential extravasation from tumor vessels. Liposomal anthracyclines have achieved highly efficient drug encapsulation, resulting in significant anticancer activity. PEGylated liposomal doxorubicin has shown substantial efficacy in breast cancer treatment both as monotherapy and in combination with other chemotherapeutics. Human breast tumors often exist in an acidic and hypoxic microenvironment, which can promote resistance to radiation and chemotherapies. A tumor-selective pH gradient arises in these tumors which favors uptake and retention of drugs like camptothecin that are weak acids

**Corresponding author:* e-mail: prabhuprashu@rediffmail.com

(Park 2002). Wu *et al*. (2007) prepared and characterized cationic liposomes containing 20 mol % cationic lipid dimethyl dioctadecyl ammonium bromide (DDAB) and loaded with doxorubicin (DOX), as cationic liposomes have been shown to selectively target tumor endothelial cells, thus can potentially be used as a carrier for chemotherapy agents. The cationic liposomal DOX showed 10.8 and 9.1 times greater cytotoxicity than control PEGylated liposomal DOX in KB oral carcinoma and L1210 murine lymphocytic leukemia cells, and 7.7- and 6.8-fold greater cytotoxicity compared to control neutral non-PEGylated liposomal DOX, respectively, in these two cell lines. Although cationic liposomal DOX had higher tumor accumulation at 30 min after intravenous administration compared to control liposomes ($p < 0.05$), DOX uptake of these liposomes at 24 h post-injection was similar to that of PEGylated liposomal DOX ($p > 0.05$) and approximately twice the levels of the free drug and non- PEGylated liposomes.

Drugs used in the treatment of diseases like cancer usually have a narrow therapeutic index (TI) and can be highly toxic to normal tissues. The toxicity of these drugs may be minimized by decreasing delivery to critical normal organs. It has been shown that even a small reduction in distribution of the drug to critical organs by encapsulation in lipid vesicles can significantly reduce the drug toxicity. Lipid vesicles are taken up poorly by tissues such as heart, kidney, and GI tract, which are major sites for toxic side effects of a variety of antineoplastic drugs. Thus lipid vesicle formulations may improve the TI by altering the bio-distribution of drugs away from drug sensitive normal tissues (Sharma *et al*., 1997). The drug under study camptothecin (CPT), a potent anticancer agent is found to be active against colon, breast, ovarian and lung cancers. CPTs usually administered by continuous infusions or injections, showed a number of practical disadvantages like low patient comfort and compliance, low water solubility and toxic effects (Sugarman 1996). The purpose of this study was to address these problems by formulating and evaluating CPT in a safe nanocarrier system that can increase drug solubility and stability. Early studies of CPT and lipid based formulations demonstrated that the insoluble parent compound CPT, was readily soluble in various lipids while maintaining its biologic activity (Burke 1992; Burke & Mishra 1993). These findings suggest that liposomes or emulsions may be an effective delivery vehicle for these drugs. It has been demonstrated that camptothecin binds to membranes by intercalating between the acyl chains of the phospholipid membrane while at the same time it remains stable (Burke & Mishra 1993). Thus an attempt was made to prepare PEGylated and non-PEGylated liposomes of camptothecin, and coat the formed liposomes with polymers such as chitosan and evaluate them for various parameters such as size distribution, stability study, percentage drug entrapment, *in vitro* drug release and *in vivo* studies. *In vivo* studies of CPT formulations for anticancer activity was performed by inducing breast cancer in Sprague–Dawley rats using dimethyl benz(a)anthracene (DMBA), as a chemical carcinogen, and evaluated the antitumour efficacy by measuring the tumour volume and tumour growth rate.

Studies on chitosan-coated liposomes as antitumor drug carriers for injection use are rare. In addition, most papers focused only on the *in vitro* properties of chitosan-coated liposomes, with few dealing with the systemic evaluation of them. In this paper, we tried to give an overall investigation on the *in vitro* and *in vivo* stability of chitosan-coated liposomes.

MATERIALS AND METHODS

Materials

Camptothecin was obtained from Sigma Aldrich. DSPE-MPEG 2000 (1,2-Distearoyl phosphatidylethanolamine-methyl-polyethyleneglycol conjugate 2000) was a gift from Lipoid GmBH, Ludwigshafen, Germany. DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine) and DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine) were also gifted as samples from Genzyme Pharmaceuticals, Liestal, Switzerland. Cholesterol was obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade.

Methods

Preparation of vesicles

In the present study, vesicles (liposomes) of CPT were prepared by film hydration method (Bangham 1965) All the lipid components of formulation as per the formula (table 1) were taken in round bottom flask and dissolved into sufficient quantity (10 ml) of organic solvent (chloroform). Organic solvent was evaporated using rotary flash evaporator under reduced pressure at a temperature about 60 °C till the lipid film was formed inside the round bottom flask. Dried lipid film obtained was hydrated with aqueous phase of phosphate buffer (pH 7.4, 10 ml) containing drug. The flask was shaken for 1 h to get lipid vesicle formulations. It was allowed to equilibrate at room temperature. The stable colloidal suspension was then sonicated for 2 cycles.

During the first cycle, the suspension was sonicated at 80% amplitude with a pulse of 0.5 cycles per second for a period of 3 min, followed by 3 min rest (excess heat may be generated during probe sonication, which may damage the lipids). After 3 min, second cycle was processed for 3 min at 80% amplitude with 0.5 cycles per sec pulse for another 3 min. The size of the vesicles was analyzed by Malvern Zeta sizer (Zetasizer 3000 HAS; Malvern Instruments Ltd., Worcestershire, UK).

Different molar ratios of lipids were used to formulate the

lipid vesicles DSPC/DPCC: cholesterol, 10:1, 10:2 for conventional lipid vesicles and for stealth lipid vesicles DSPC/DPCC: MPEG-DSPE: cholesterol, 10:0.1:2; 10:0.2:2; 10:0.4:2; 10:0.5:2 etc.) and drug-to-lipid ratio were tested (D/L; 0.2, 0.25, 0.3, 0.35, 0.4, 0.5 etc).

Table 1: Composition of formulations and their characterization

Formulation code	Content of liposomes with ratio
F 1 (Conventionalliposomes)	DSPC: CH 10: 2.
F 2 (Conventionalliposomes)	DPPC: CH 10: 2
F3 (Stealth Liposomes)	DSPC: DSPE –MPEG 2000: CH 10: 0.2: 2.
F4 (Stealth Liposomes)	DPPC: DSPE–MPEG 2000: CH 10: 0.2: 2.
F5 (Coated conventional liposomes)	DSPC: CH 10: 2.
F6 (Coated conventional liposomes)	DPPC: CH 10: 2

DSPC: 1,2-Distearoyl-sn-glycero-3-phosphocholine, DPPC: 1,2-Dipalmitoyl-sn-glycero-3 phosphocholine, DSPE-MPEG 2000 (1,2-Distearoyl-phosphatidylethanolamine-methyl polyethyleneglycol conjugate-2000, CH: Cholesterol.

Coating of the liposomes with a cationic hydrophilic polymer chitosan

Coating of MLVs (multilamellar vesicles) was done by mixing equal volume of an aliquot of the vesicular suspension with the chitosan solution in 0.5% v/v of glacial acetic acid. Chitosan solution (containing 0.1% w/v, 0.2% w/v, 0.4% w/v and 0.6% w/v) was added dropwise into the respective vesicular suspension placed on the magnetic stirrer under controlled stirring rate of 50 rpm at room temperature (Gonazalez-Rodriguez *et al.,* 2007). After the coating of vesicles, incubated at 10°C in the refrigerator for 1 h in a 50 ml beaker. The dropping rate of coating solution was optimized. This was sonicated at 80% amplitude, 0.5 cycles per sec pulse for 3 min with a rest period of 3 min, followed by sonication for further 3 min.

Determination of size and shape

Particle size, polydispersity index (PDI) of the vesicles were determined using Malvern Zeta sizer (Zetasizer 3000 HAS; Malvern Instruments Ltd, Worcestershire, UK).

Entrapment efficiency

Entrapment efficiency of CPT in the vesicles was determined as follows: After sonication, 1 ml of the vesicle suspension was taken in a 1 ml micro centrifuge tube, centrifuged at 80,000 rpm for 1 h at 4ºC in a cold centrifuge to get a white pellet. To the pellet, 500 µL of 0.1 N NaOH, was added and vortexed thoroughly for 3 min to get a white suspension. To this 5 ml of Triton X-100, was added to get a clear solution, this was further vortexed for 2 min such that to ensure that the vesicles are lysed completely to release the drug. This solution (1 ml) was further diluted and the absorbance was determined using a Shimadzu UV/VIS spectrophotometer (Shimadzu-1700, Kyoto, Japan), at λ_{max} of 369 nm.

Coating efficiency of chitosan

Aliquots of chitosan-coated vesicular suspensions (0.4 ml) were centrifuged at 12,000 rpm for 5 min in an ultimate filtration tube (molecular weight cut 10,000). The non-adhered chitosan at the bottom of the tube was determined by a colorimetric method described by Gao *et al.,* 2003; with a slight modification. A solution of Alizarin Red S was prepared in deionized water, at a concentration of 1.5 mg/ml. To prepare the standard curve, a stock solution of chitosan was prepared at a concentration of 1 mg/ml. Different volumes of the stock solution were transferred into volumetric flasks. Aliquots of the dye solution (0.8 ml) were added to each flask, after which the volume in each flask was filled to 10 ml with sodium acetate buffer solution (pH of 5.0). The UV adsorption was detected at 580 nm with an UV-Visible Spectrophotometer (Shimadzu-1700, Kyoto, Japan) within 2 h.

In vitro desorption of chitosan from liposomes

To protect the lipid vesicles from deteriorating by the plasma components, chitosan should not be removed immediately on dilution after entering the systemic circulation. In this experiment, 0.4% chitosan solution was first diluted with sodium acetate buffer solution (pH 6.0) to half of the original concentration and used as control and chitosan (0.4%)-coated liposomes without drug were first prepared according to the method described earlier. Thereafter, 4 ml of the chitosan-coated liposomes or 4 ml of chitosan solution was respectively placed in a cellulose membrane tubing (molecular weight cut-off was 10,000, membrane pore size 10 nm). Then the tubing was tightened and soaked in 40 ml of phosphate buffer solution (pH 7.4) as dissolution medium. At predetermined intervals, 0.5 ml of the dissolution medium was withdrawn and replaced with the same amount of prewarmed dissolution medium. The samples of the replicates were pipetted directly into disposable cuvettes and analyzed for chitosan content as described in “Coating efficiency of chitosan.” Statistical analysis was performed using Student’s t-test with $P<0.05$ as the minimal level of significance on the raw data of the release rate of chitosan solution and adsorbed chitosan from liposomes at each time point.

In vitro drug release study

The lipid vesicles (1 ml) of SUVs (small unilamellar vesicles) placed on one side of the sigma dialysis membrane in a vertical Franz diffusion cell. Other side of the membrane was in contact with the dissolution medium. Entire dissolution assembly was placed on a

magnetic stirrer at temperature of 37°C. Dissolution medium was 50 ml of PBS pH 7.4. Aliquots (5 ml) of dissolution medium was withdrawn at different time intervals- 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 2 h, 4 h, 8 h, 12 h and 24 h. Drug concentrations in the dissolution medium were determined by UV spectro-photometric method. All the experiments were carried out in triplicates.

Stability study

Stability study was carried out for the sonicated liposomal suspension of SUVs at two different temperatures i.e., refrigeration temperature (4±2°C) and room temperature (27±2°C) for 4 weeks. Sampling was done, suitable dilutions were made with PBS pH 7.4 and UV absorbance was determined. The entrapment efficiency was calculated from the regression equation Y=0.0051x obtained from the standard plot of CPT in PBS pH 7.4 at 369 nm.

In vivo studies

Antitumor efficacy studies

The protocol of the experiment was approved by the Institutional Animal Ethics Committee (K.S Hegde Medical Academy, Reg No.115/1999/CPCSEA). Sprague-Dawley female rats of 4-7 week-old were used in the study. They were divided into 4 groups consisting of six animals in each group as standard, breast cancer control (BCC), test 1 and test 2. They were maintained at 28±1°C, with relative humidity of 60°C (12-h light and 12-h dark cycle), and provided with standard food pellets (diet composition, wheat broken-moisture 9.0%, crude protein 11.5%, crude fat 1.9%, crude fibre 4.0%, ash 0.2 %, nitrogen-free extract 73.4 %) and tap water. For the induction of tumors, rats were administered with single s.c. injection of DMBA (7,12-dimethylbenz (a) anthracene) in 1 ml sesame oil at a dose of 25 mg to the breast by air pouch technique. Tumor yield and size were stabilized 90 days after induction and these served as breast cancer control (BCC) animals without any treatment. The standard group of rats received CPT injection i.p. (0.5 mg/kg), the test groups of rats, test 1 received the PEGylated lipid vesicular suspension F3 (DSPC: MPEG-DSPE: CH-10: 0.2:2 at a dose of 0.5 mg/kg CPT) and the test 2 received the chitosan coated conventional lipid vesicles (F5, DSPC: CH -10:2 at a dose of 0.5 mg/kg-CPT) in the breast tissue by the subcutaneous route. Animals were palpated for tumor weekly and the location and size of tumor were recorded. After 110 days all the animals were starved overnight and sacrificed by cervical decapitation. The breast tumor was surgically dissected out; tumor volume (mm in diameter) of the breast cancer controls, as well as the experimental groups were measured using vernier calipers. The antitumor efficacy of the liposomal formulations – test 1 (PEGylated liposomal formulation), test 2 (chitosan coated conventional liposomal formulation) and standard (free CPT) was evaluated on the basis of the changes in tumor volume (mm in diameter) and the % tumor inhibiting activity (Samy *et al.,* 2006; Gupta 2009).

Histopathological analysis

Histopathological studies were carried out on all the groups of animals to study the type, extent of the tumor formed and the effectiveness of the treatment given.

STATISTICAL ANALYSIS

The data were presented as Mean ± SEM. One way analysis of variance (ANOVA) followed by post Dunnet multiple comparison tests to compare the efficacy of the formulations by using graph pad prism version 4.03354 software.

RESULTS

The morphological appearance of chitosan-coated liposomes and uncoated liposomes was visualized using TEM. It was found that both types of liposomes presented a spherical morphology, with no significant morphological differences between liposomes with or without chitosan coating were observed. Particle size analysis of the sonicated liposomes-SUVs was determined using a Malvern zeta sizer instrument. The results obtained by vesicular size analysis indicated that the size of liposomes prepared without sonication was greater compared to the size of liposomes prepared with sonication. It was found that the average particle size of conventional unsonicated uncoated liposomes (F1-DSPC: CH:10:2) was found to be 994.6 nm, where as the average particle size of conventional sonicated coated liposomes (F5) was found to be 784.3 nm. Zeta potential of the sonicated liposomes–SUVs was determined by Malvern nano zeta sizer instrument**.** It was found that

Coating efficiency of chitosan

The maximum adsorption of chitosan to the vesicles was achieved at a polymer concentration of 0.4%. The coating efficiency at maximum adsorption was estimated by measuring the concentration of free chitosan after centrifugation. The coating efficiency at maximum adsorption is 76% and when calculated on a weight basis, the weight ratio of chitosan to phospholipids is 1/13.16 at a polymer concentration of 0.4%.

Table 2: Zeta potential of chitosan coated lipid vesicles

S. No.	Concentration of Chitosan (% w/v)	Zeta Potential
1.	0.0	- 3.45
2.	0.1	+10.12
3.	0.2	+17.67
4.	0.4	+22.4
5.	0.6	+23.3

Table 3: *In vitro* release profile of liposomes

Time (min)	Percentage Drug Release*					
	Formulation F1	Formulation F2	Formulation F3	Formulation F4	Formulation F5 Coated	Formulation F6 Coated
0	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00
5	14.62±0.64	16.68±0.54	10.96±0.65	11.12±0.50	11.51±0.56	11.34±0.54
10	16.84±0.56	18.75±0.43	13.15±0.67	13.24±0.55	14.76±0.57	14.56±0.58
15	20.20±0.67	22.37±0.37	16.01±0.63	18.13±0.53	17.52±0.54	18.54±0.51
30	25.72±0.43	26.09±0.56	21.70±0.68	22.17±0.52	22.22±0.53	23.45±0.61
45	27.14±0.54	29.26±0.55	24.96±0.69	26.96±0.60	26.70±0.52	27.34±0.62
60	29.83±0.65	31.76±0.67	25.13±0.56	26.99±0.65	27.94±0.60	30.67±0.57
120	37.48±0.86	40.46±0.76	26.52±0.77	35.77±0.63	32.46±0.64	34.34±0.46
240	49.41±0.96	52.34±0.75	45.89±0.75	49.99±0.67	48.32±0.62	50.67±0.44
480	64.53±0.53	67.67±0.78	68.54±0.62	67.56±0.61	59.14±0.61	63.98±0.47
720	85.35±0.34	89.32±0.80	88.41±0.60	90.45±0.58	65.10±0.70	67.29±0.55
1440	90.56±0.12	95.42±0.92	78.40±0.52	75.09±0.70	70.63±0.75	75.67±0.52

*The values are expressed as Mean ± SEM, n=3

In vitro desorption of chitosan

Both adsorbed chitosan and chitosan solution released 27% of their total amount within the first 5 min. Maximum release of chitosan was obtained both at 8 h with a release of 70% of adsorbed chitosan from liposomes in contrast to 100% of chitosan solution.

The *In vitro* release profile of formulation F3, released 78% of the drug in 24 h, where as formulation F4 released 75 % of the drug in 24 h. Formulation F5 coated (conventional coated) released 70% of the drug in 24 h, where as formulation F6 coated (conventional coated) released 75% of the drug in 24 h.

Anti tumor efficacy

It was found that there was a significant reduction in tumor volume (in mm^2) in the test 2 group of rats (table 4) followed by the test 1 group and the standard group compared to that of the BCC control group. Test 2 formulations showed a very good tumor inhibiting activity (88.75%, P<0.01) compared to the test 1 formulation (50.01 %, P<0.01).

Histopathological analysis

BCC

As shown in the fig. 2, section showed breast tissue with a tumour tissue made up of masses, sheets and lobules of pleomorphic cells having hyperchromatic nuclei. Many

Table 4: Average tumor volume and tumor inhibition in different animal groups

S. No.	Animal groups	Average tumor volume (mm^2)	% Tumor inhibition
1.	Breast Cancer Control (BCC)	80.00±1.72	0
2.	BCC+standard (free CPT)	68.27±1.37	20.33
3.	BCC+test1 (PEGylated Liposomes)	40.31±0.78**	50.01**
4.	BCC+test2 (conventional chitosancoated liposomes)	09.23±1.04**	88.75**

The values are expressed as Mean ± SEM, n=6 rats in one group. **P<0.01 highly significant when compared with BCC.

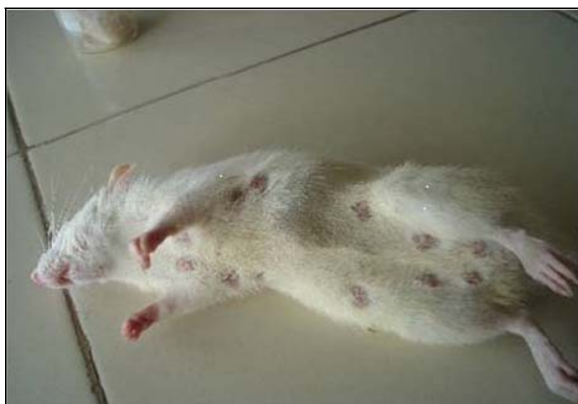

Fig. 1: Tumor induced rat.

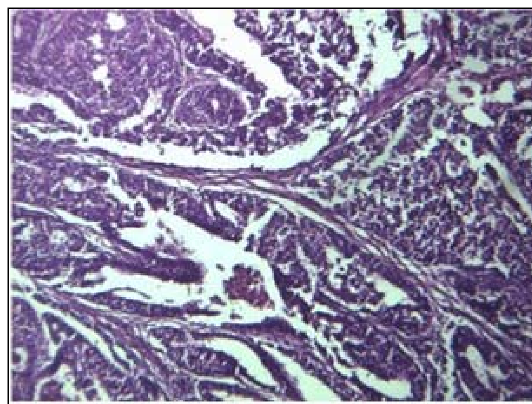

Fig. 2: Breast Cancer Control

acinar and tubular structures are seen with necrotic material indicative of duct carcinoma of the breast.

It was found that there was a significant reduction in tumor infiltration and proliferation in the test 2 group of rats followed by the test 1 group and the standard group compared to that of the BCC control group.

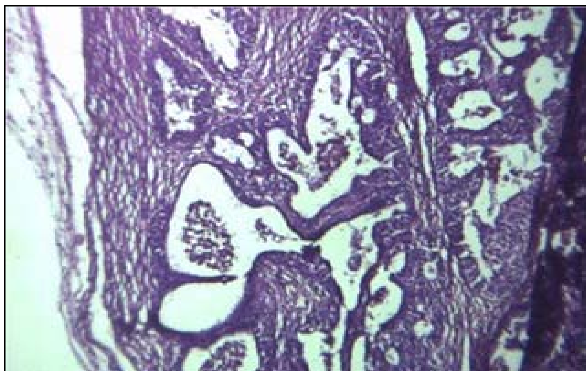

Fig. 3: Tumor induced rat treated with standard free CPT

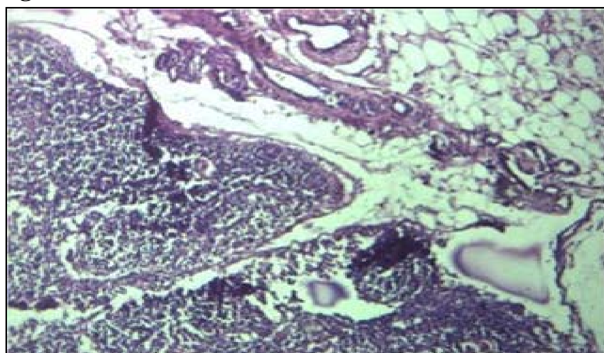

Fig. 4: Tumor induced rat treated with test 1 lipid vesicular formulation (test 1).

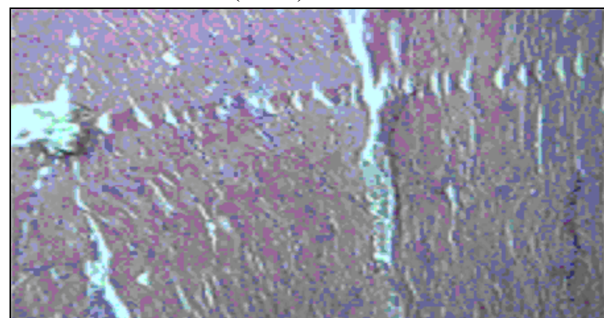

Fig. 5: Tumor induced rat treated with test 2 lipid vesicular formulation (test 2)

Stability study

The stability study indicated that formulations stored at refrigeration temperature showed higher drug content (75-95% of initial drug content) when compared to the formulations stored at room temperature, after a period of 4 weeks. Stealth lipid vesicles (F3 and F4) were found to be more stable (86-91% of initial drug content) than the conventional lipid vesicles (F1 and F2, 75-80% of initial drug content)) and showed much lesser extent of drug leakage compared to the conventional liposomes.

DISCUSSION

Lipid vesicles were prepared by the thin-film hydration method as per the method described by Bangham *et al*, 1965. Drug to lipid ratio was optimized as drug:lipid ratio is 1:5. Hydration volume was experimented as: 2, 2.5, 3, 3.5, 4, 5, and 6 ml was optimized to 4.86 mg CPT dissolved in 6 ml PBS 7.4. Hence the optimized ratios of stealth lipid vesicles are: F3:DSPC:MPEG-DSPE:CH10: 0.2:2 and F4:DPPC:MPEG-DSPE:CH-10:0.2:2 whereas for conventional lipid vesicles: F5 chitosan coated: DSPC:CH-10:2 and F6 chitosan-coated: DPPC: CH-10:2, whereas F1 and F2 are conventional lipid vesicular formulations without chitosan coating.

The zeta potential of the chitosan coated formulation – F5 – DSPC : CH – 10 : 2 was positive (+ 22.4 mv) indicate the successful coating of the formulation with chitosan. The uncoated liposome had a marked negative charge (-3.45 mv) which, after the incubation with chitosan at various concentrations (0.1% w/v, 0.2% w/v, 0.4% w/v and 0.6% w/v), was inverted to positive values (Table 2). This inversion of the surface charge of the liposomes confirmed the presence of chitosan coating on the surface of the liposomes. Therefore, the main interaction between liposomes and chitosan was electrostatic attraction. The zeta potential reached a relatively constant value as the chitosan concentration increased to 0.4%. Table 2 indicating the saturated adsorption of chitosan to liposomes. A similar finding was reported by other researchers, where the saturation of chitosan coating was found at 0.3% (Fang, *et al.,* 2001). This is probably due to the strong adsorption between the polymer and the liposomal bilayer, resulting in a flat configuration and making it difficult to observe the polymer at the liposome surface. Similar, result has been reported by others (Blakmeer *et al.,* 1990; Henriksen *et al.,* 1994). The PDI remained in the range of 0.2–0.3, indicating good dispersion of uniformly sized lipid vesicles. The entrapment efficiency study revealed that a drug: lipid ratio (1:5) gave the best entrapment efficiency.

The release rate of adsorbed chitosan from vesicles was found to be significantly slower than that of chitosan solution ($P<0.05$). It is postulated that chitosan combines with the phospholipid bilayers mainly through electrostatic interactions. In addition, chitosan's hydrophobic side chains can also exert hydrophobic interaction with the phospholipid bilayers (Fang *et al.,* 2001). The release of adsorbed chitosan was mostly retarded over the period due to the effect of two kinds of interactions. Therefore, it is possible that chitosan can still protect the liposomes after entering the systemic circulation, and can not be easily removed through dilution. The initial rapid release of adsorbed chitosan in the first 5 minutes was probably due to the free chitosan molecules adsorbed on the surface of liposomes.

The *in vitro* release profile of the drug is given in table 3. Initial burst release was observed for conventional formulations, owing to their surface hydrophilicity. PEGylated and chitosan coated formulations showed a slower initial release compared to the conventional (F1 & F2) formulations. This burst release was observed due to the presence of drug on the surface in the adsorbed form. All the formulations released the drug for a period of 24 h. These results indicate that the release of CPT followed a slow release status, which suggests that it takes time for CPT to be released once encapsulated in the liposomes because the lipid bilayers are stabilized by cholesterol, due to the rigidity of the bilayer (lipid interactions like hydrogen bonding, hydrophobic effect and the Van der Waals' interactions between chains) in presence of small amount of cholesterol (Boggs 1987). In the present study, different molar ratios of lipids were used to formulate the lipid vesicles DSPC/DPCC: cholesterol, 10:1, 10:2 for conventional lipid vesicles and for stealth lipid vesicles, and found that the ratio of 10:2, optimized ratio considering the structural integrity of the vesicles. Thus a depot effect could be achieved using liposomal vesicles.
The antitumor efficacy of chitosan coated liposomes may be due to:

1) Chitosan being a hydrophilic polysaccharide increases the surface hydrophilicity of the liposomal vesicles, providing a steric protection, thus avoiding the adsorption of plasma proteins opsonins, preventing the uptake by the phagocytic (mononuclear phagocytic system/ reticuloendothelial system), prolonging the circulation time in blood, hence leading to enhanced extravasation into the tumor interstitium. Since there is poor lymphatic drainage in tumor tissue there is increased retention of the liposomes (EPR–Enhanced permeation and retention effect.
2) The phospholipid used DSPC which is having a high phase transition temperature may release the drug only at the tumor site where there is hyperthermia.
3) Chitosan coated cationic liposomes can increase the binding of the liposomes to the negatively charged endothelial cell surface of the angiogenic tumor blood vessels.
4) The coating of chitosan which is soluble only at acidic pH may lead to the release of the drug selectively at the tumor site.

In animals treated with standard free drug formulation, little or no improvement was seen with the tumor infiltrating in the adjacent breast tissue (fig. 3). Acinar and tubular structures were seen filled with necrotic material. Features suggest infiltration of the ducts indicating duct carcinoma of the breast. The animals treated with test 1 lipid vesicular formulation, breast tissue showing many tubules and acini lined by cuboidal cells. Few slightly dilated ducts were seen with a mild degree of adenosis. Occasional dilated ducts filled with necrotic material was seen. Features are suggestive of a benign proliferative epithelial lesion of the breast (Fibrocystic disease). As a result no malignancy is seen in fig. 4. In fig. 5 (the animals treated with test 2 lipid vesicular formulation), sections showed only tiny focus of normal breast tissue with underlying muscle tissue. This suggested the absence of malignancy. Hence, the present investigation on lipid vesiclular formulations has revealed that how effectively the formulation under investigation can be effectively targeted to the site and there by therapeutic efficiency of the drug molecule can be improved along with reduction in its adverse effects.

The stability of the liposomes is a major consideration in all steps of their production and administration: from process steps to storage to delivery. A stable dosage form maintains its physical integrity and does not adversely influence the chemical integrity of the active ingredient during its life on the shelf. As the liposomes are thermodynamically unstable systems, they tend to fuse, grow into bigger vesicles resulting in breakage of the liposomes on storage which poses a problem of drug leakage from the vesicles. Unsaturated phospholipids undergo oxidation easily. Hence, in the present work only saturated phospholipids like DPPC, DSPC, and MPEG-DSPE were used to formulate the liposomes, to avoid oxidation .Therefore no antioxidant like α-tocopherol was used. Since the saturated phospholipids have a high T_m they showed a good physical stability. Stability of the vesicles is the major determinant for the stability of the formulations, hence, study was carried to evaluate drug entrapment at room temperature (27±2°C) and refrigeration temperature (4±2°C). Stability could not be carried out at higher temperature (>room temperature) because phospholipid was used as the component for liposomes and gets deteriorated at higher temperature. chitosan coated lipid vesicles (F5 coated and F6 coated) was found to be highly stable (92-95% of initial drug content) compared to the stealth and conventional liposomes. Chitosan coated liposomes also showed a significantly lesser drug leakage when compared to that of the stealth and conventional liposomes. This may be attributed to the steric repulsion created by the surface positive charge on the liposomes leading to steric stabilization of the colloidal suspension.

CONCLUSION

Site-specific delivery of drugs can significantly reduce the drug toxicity and increase the therapeutic effect. CPT has been used in the treatment of breast cancer and its side effect profile often results in cessation of therapy. It was found that chitosan coating of the conventional liposomes increased the physical stability of the liposomal suspension as well as its entrapment efficiency. Further chitosan coated conventional liposomes and the PEGylated liposomes released the drug initially at slower rate, compared to the uncoated conventional liposomes. *In vivo* screening of the formulations for their anti tumor activity revealed that there was a significant reduction in edema volume and tumor volume in the rat group

administered with the test stealth liposomal formulation (PEGylated and chitosan coated conventional) compared to that of the control and standard (administered with free CPT) group of rats. Hence, the lipid vesiclular formulations can be effectively targeted to the site and there by therapeutic efficiency of the drug molecule can be improved along with reduction in its adverse effects.

ACKNOWLEDGEMENT

The authors wish to acknowledge Nitte University, Mangalore (Karnataka) India, for providing the necessary facilities and financial support to carry out this project. Authors are also thankful to Manipal College of Pharmaceutical Sciences, Manipal, for providing the necessary facilities to carry out some of the investigations of the project. Further, the authors are grateful to Lipoid GmbH, Germany for providing free samples of phospholipids (DSPC, DPCC, MPEG-DSPE), to carry out this project successfully.

REFERENCES

Bangham AD (1965). Diffusion of univalent ions across unilamellar of swollen phospholipids. *J. Mol. Biol*., **13**: 238-252.

Blakmeer J, Behmer MR, Cohen Stuart MA and Fleer GJ (1990). Adsorption of weak polyele ctrolytes on highly charged surfaces: polyfacrylic acid on olystyrene latex with strong cationic groups. *Macromol.,* **23**(8): 2301-2309.

Boggs M (1987). Lipid intermolecular hydrogen bonding: Influence on structural organiztion and membrane function. *Biochimica et Biophysica Acta,* **906**(3): 353-404.

Burke TG (1992). Liposomal stabilization of camptothecin's lactone ring. *J. Am. Chem. Soc.,* **114**: 8318-8319.

Burke TG and Mishra AK (1993). Lipid bilayer partitioning and stability of camptothecin drugs. *Biochem.,* **32**: 5352-5364.

Chang CC, Liu DZ, Lin SY, Liang HJ, Hou WC, Huang WJ, Chang CH, Ho FM and Liang YC (2008). Liposome encapsulation reduces cantharidin toxicity. *Food. Chem.,* **46**(9): 3116-3121.

Chonn A, Cullis PR and Devine DV (1991). The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol*., **46**(12): 4234-4241.

Chonn A, Semple SC and Cullis PR (1992). Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. *J. Biol. Chem*., **267**(26): 18759-18765.

Fang N, Chan V, Mao HQ and Leong KW (2001). Interactions of phospholipid bilayer with chitosan: effect of molecular weight and pH. *Biomacromol*., **2**(4): 1161-1168.

Gao GZ, Jiao QC, Ding YL and Chen L (2003). Study on quantitative assay of chitosan in compound sample with a spectrophotometric method. *Food and Ferm. Ind*., **29**(5): 49-52.

Gonzalez-Rodriguez ML, Barros LB, Palma J, Gonzalez-Rodriguez PL and Rabasco AM (2007). Application of statistical experimental design to study the formulation variables influencing the coating process of lidocaine lipid vesicles. *Int. J. Pharm*., **337**(1): 336-345.

Gupta SK (2009). Anticancer Agents. *In*: Drug Screening Methods (Preclinical Evaluation of New Drugs). 2nd ed. JAYPEE Brothers Medical Publishers (Pvt.), Ltd., New Delhi, p.175.

Henriksen I, Smistad G and Karlsen (1994). Interactions between liposomes and chitosan. *Int. J. Pharm*., **101**(3): 227-236.

Juliano RL and Stamp D (1975). The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs. *Biochem. Biophys. Res. Commun*., **63**(3): 651-658.

Martins S, Sarmento B, Ferreira DC and Souto EB (2007). Lipid-based colloidal carriers for peptide and protein delivery-liposomes versus lipid nanoparticles. *Int. J. Nanomed*., **2**(4): 595-607.

Pajean M, Huc A and Herbage D (1991). Stabilization of liposomes with collagen. *Int. J. Pharm*., **77**(1): 31-40.

Park JW (2002). Liposome-based drug delivery in breast cancer treatment. *Breast Cancer Res.,***4**: 95-99.

Patel HM (1992). Serum opsonins and liposomes: their interaction and opsonophagocytosis. *Crit. Rev. Ther. Drug Carrier Syst*., **9**(1): 39-90.

Samy RP, Gopalkrishnakone P and Ignacimuthu S (2006). Anti-tumor promoting potential of luteolin against. 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. *Chemico-Biological. Interactions,* **164**: 1-14.

Senior JH (1987) Fate and behavior of liposomes *in vivo*: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst*., **3**(2): 123-193.

Sharma A and Sharma US (1997). Review-Lipid vesicles in drug delivery: Progress and limitations. *Int. J. Pharm*., **154**: 123-140.

Storm G, Belliot SO, Daemeh T and Lasic DD (1995). Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv. Drug Deliv. Rev.,* **17**(1): 31-48.

Sugarman SM (1996). Lipid-complexed camptothecin: formulation and initial biodistri bution and antitumor activity studies.*Cancer Chemother.Pharmacol.,***37**: 531-538.

Torchilin V and Trubetskoy VS (1995). Which polymers can make nanoparticulate drug carriers long-circulating. *Adv. Drug Deliv. Rev*., **16**(23): 141-155.

Wu J, Lee A, Lu Y and Lee RJ (2007). Pharmaceutical Nanotechnology: Vascular targeting of doxorubicin using cationic liposomes. *Int. J. Pharm*., **337**: 329-335.