Stability Study of Stavudine-Loaded O-Palmitoyl-Anchored Carbohydrate-Coated Liposomes

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ABSTRACT

The purpose of this study was to evaluate the physicochemical stability of carbohydrate-anchored liposomes. In the present study, carbohydrate (galactose, fucose, and mannose) was palmitoylated and anchored on the surface of positively charged liposomes (PL). The stabilities of plain neutral liposomes (NL), PL, and O-palmitoyl carbohydrate-anchored liposomes were determined. The effects of storage conditions (4°C ± 2°C, 25°C ± 2°C/60% ± 5% relative humidity [RH], or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days) were observed on the vesicle size, shape, zeta potential, drug content, and in vitro ligand agglutination assay by keeping the liposomal formulations in sealed amber-colored vials (10-mL capacity) after flushing with nitrogen. The stability of liposomal formulations was found to be temperature dependent. All the liposomal formulations were found to be stable at 4°C ± 2°C up to 1 month. Storage at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH adversely affected uncoated liposomal formulations. Carbohydrate coating of the liposomes could enhance the stability of liposomes at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH.

KEYWORDS: Carbohydrate-coated liposomes, stavudine, stability, oxidation index, thiobarbituric acid-reactive species.

INTRODUCTION

In the field of drug targeting, delivery systems are often poorly defined in physicochemical terms and little attention is paid to issues related to long-term stability and reproducibility in preparation and performance. This often makes results difficult to reproduce. These issues are critical to the success of these new delivery systems.

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The stability of liposomal formulations on storage is of great concern, as it is the major hindrance to the development of marketed preparations. Liposomes are self-assemblages of amphiphiles into closed bilayer structures. Hydrated bilayer vesicles, however, have been deemed thermodynamically unstable and are thought to represent a metastable state in that the vesicles possess excess energy. Liposomal phospholipids can undergo chemical degradation such as oxidation and hydrolysis. As a result of either these changes or other factors, liposomes maintained in aqueous dispersion may aggregate/fuse and dump their contents.

Carbohydrate-anchored liposomes have been designed that differ from conventional liposomes in that their surfaces have been altered through the use of natural or synthesized components such as glycolipids.[1,2] Polysaccharides are also attractive for liposome coating because of their protein-rejecting ability, biodegradability, low toxicity, and cell targetability through specific moieties.[3-5] An increase in structural stability, long stability (up to 30 days), and membrane integrity was recorded in lipid membranes anchored with hydrophobized polysaccharides.[6]

In the present study, the purpose of using carbohydrate to coat liposomes was not only to improve stability but also to target the coated liposomes to the cells (HIV reservoirs) that have lectin receptors specific for these carbohydrates. Because of the presence of lectin, liposomes could release stavudine (d4T) in the vicinity of the target site. In addition to improved stability, these carbohydrate-coated liposomes have targeting potential. The carbohydrate prevents liposomal lipids from getting oxidized by providing a layer of protection. The glycocalyx, a saccharide-rich zone on the cell surface, creates a thick hydrated barrier that protects the cell. This hydrated saccharide layer inhibits nonspecific protein adsorption and improves specificity toward desired sites. A similar mechanism applies to carbohydrate-coated liposomes. The carbohydrates preserve the structural integrity of the liposome.

Aggregation and fusion, which lead to changes in particle size and particle size distribution, are the main results of the physical instability of phospholipid vesicles. Such processes occur to a significant extent over long periods of storage. Chemically, phospholipids are susceptible to oxidation and hydrolysis. Phospholipids that contain unsaturated acyl chains

may be oxidized in the absence of specific oxidants by a free radical chain mechanism. Oxidation and oxidative effects can be minimized by storing the lipids at a low temperature and in an inert atmosphere and through careful handling.[7-9] The effects produced by both these instabilities can influence the in vivo behavior of liposomes (targeting, cell uptake, and clearance). Therefore, extensive studies are required before a liposomal formulation is used for pharmacological therapy.[10]

In this study, d4T-loaded liposomes (as described by Garg et al[11]) were used for stability studies. The stability of uncoated and carbohydrate-coated liposomes was determined. We observed the effects of storage conditions (4°C ± 2°C, 25°C ± 2°C/60% ± 5% relative humidity [RH], 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days) on the vesicle size, shape, zeta potential, drug content, and in vitro ligand agglutination assay[12] by keeping the liposomes in sealed amber-colored vials (10-mL capacity) after flushing with nitrogen. For initial stability parameter determination formulations, the vials were stored at 4°C ± 2°C.

Materials and Methods

Materials

Stavudine (d4T) was received as a gift sample from M/s Hetero Drugs (Hyderabad, India). Egg phosphatidylcholine (PC), cholesterol (CH), phosphatidylethanolamine (PE), D-mannose, Sephadex G-50, butylhydroxytoluene, *Ricinus communis* lectin, concanavalin A (con A), and *Ulex europaeus* were purchased from Sigma Chemical Co (St Louis, MO). Thiobarbituric acid and palmitoyl chloride were purchased from Fluka (Buchs, Switzerland). Dimethylformamide and pyridine were obtained from Central Drug House (P) Ltd (New Delhi, India), as was trichloroacetic acid. All the other solvents and reagents were of analytical grade and used as received. All other chemicals used were obtained from Merck (Mumbai, India).

Synthesis of O-palmitoylcarbohydrate

O-palmitoylgalactose (OPG) was synthesized by esterification of galactose by the reaction of palmitoyl chloride in dimethylformamide (DMF) under anhydrous catalytic conditions following the method described earlier.[11]

Two grams of galactose was dissolved in dry DMF (100 mL) at 70°C. To the resulting solution, 2 mL of dry pyridine, and 0.2 g of palmitoyl chloride dissolved in 0.5 mL dry DMF, were added. The mixture was stirred using a magnetic stirrer (Expo India Ltd, Mumbai, India) for 3 hours at 60°C followed by 2 hours at room temperature. The mixture obtained was then slowly poured into 100 mL of absolute ethanol with stirring. The precipitate so formed was collected and washed 3 times with 120 mL of absolute ethanol and 80 mL of dry diethyl ether. The white solid material obtained was dried in a vacuum at 50°C for 2 hours.

OPF (O-palmitoylfucose)-anchored and OPM (O-palmitoylmannose)-anchored liposomal formulations were prepared in a similar manner.

Preparation and Development of Liposomal Formulations

Liposomes were prepared by reverse-phase evaporation. Egg PC, CH, and PE were taken in different molar ratios (Table 1) and dissolved in 5 mL of diethylether, to which 2 mL of aqueous phase, that is, phosphate-buffered saline (PBS pH 7.4), containing 2 mg d4T was added. The mixture was sonicated (titanium probe ultrasonicator; Imeco Ultrasonics, Mumbai, India) for 10 minutes. A thick emulsion was formed, which was then vortexed (Superfit, Mumbai, India) to remove any residual ether. To this emulsion, 3 mL of PBS (pH 7.4) was added to hydrate the vesicles. Liposomes were then extruded through polycarbonate membranes (Millipore, Bedford, MA) of 200-nm pore size. Vesicle size was evaluated by dynamic light scattering using a Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics, Hialeah, FL). Entrapment efficiency was determined after separation of unentrapped drug by the Sephadex G-50 minicolumn using the centrifugation technique. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton X-100 (Merck, Mumbai, India), and the liberated drug was determined in a UV spectrophotometer (Shimadzu 1601, Kyoto, Japan) at 266 nm.

Coating of Liposomes

For coating, 2 mL of uncoated liposomal formulation was incubated with OPM, OPG, or OPF solution (in PBS, pH 7.4) and was then stirred gently at room temperature. After completion of coating, the excessive unbound OPG, OPF, or OPM was removed by passing the resultant suspension through the Sephadex G-50 column at 2000 rpm for 10 minutes.

The percentage of carbohydrate coating was determined spectrophotometrically by treating the liposomal dispersion with Fehling's reagent.

Vesicle Size and Shape

All formulations (uncoated and carbohydrate-coated liposomal formulations) were stored in amber-colored glass bottles at 4°C ± 2°C, 25°C ± 2°C/60% ± 5% RH, or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days.

Table 1. Composition and Characterization of NL and PL*

Formulation Code	Molar Lipid Ratios (PC:CH)/(PC:CH:PE)	Particle Size d_{50} (nm)†	% Entrapment Efficiency†	Zeta Potential (mV)
d4T NL1	9:1	145.00 ± 2.36	32.40 ± 0.02	–2.41 ± 0.63
d4T NL2	8:2	130.10 ± 1.43	37.60 ± 1.05	–5.94 ± 0.23
d4T NL3	7:3	120.13 ± 0.16	49.13 ± 1.26	–6.57 ± 0.21
d4T NL4	6:4	164.41 ± 4.58	21.03 ± 2.36	–9.35 ± 0.29
d4T NL5	5:5	181.42 ± 6.06	18.51 ± 2.61	–10.44 ± 0.28
d4T PL1	9:0.5:0.5	156.40 ± 2.13	28.16 ± 1.23	5.62 ± 2.39
d4T PL2	8:1:1	144.16 ± 2.76	34.27 ± 1.57	9.12 ± 0.36
d4T PL3	8:1.5:0.5	132.13 ± 3.13	41.51 ± 0.86	1.26 ± 0.25
d4T PL4	7:2:1	120.00 ± 1.52	49.60 ± 1.23	8.21 ± 0.15
d4T PL5	7:2.5:0.5	159.06 ± 4.17	40.47 ± 1.32	0.20 ± 0.06
d4T PL6	6:3:1	169.30 ± 9.60	26.40 ± 1.01	0.03 ± 0.01
d4T PL7	6:3.5:0.5	174.41 ± 5.21	19.51 ± 0.43	–0.28 ± 0.03
d4T PL8	5:4:1	192.30 ± 6.50	14.36 ± 1.22	–0.30 ± 0.21
d4T PL9	5:4.5:0.5	195.36 ± 8.13	11.43 ± 0.13	–0.32 ± 0.09

*PC indicates egg phosphatidylcholine; CH, cholesterol; PE, phosphatidylethanolamine; d4T, stavudine; NL1-NL5, formulation codes for neutral liposomes; PL1-PL9, formulation codes for positively charged liposomes.
†All values are expressed as mean ± SD (n = 6).

As above, vesicle size was evaluated by dynamic light scattering using a Coulter N4 MD Submicron Particle Size Analyzer. Vesicular shape was determined after 30 days of storage by a transmission electron microscope (Philips, Tokyo, Japan).

Zeta Potential

The zeta potential of the liposomal formulations was determined using 0.1 M KCl buffer in demineralized water at 4°C ± 2°C, 25°C ± 2°C/60% ± 5% RH, or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days (Zetasizer 3000 HS, Malvern Instruments Co, Worchestershire, UK).

Drug Content

Uncoated and carbohydrate-coated liposomal formulations were stored in amber-colored glass bottles at 4°C ± 2°C, 25°C ± 2°C/60% ± 5% RH, or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days. The initial drug content was considered to be 100% for each formulation.

The drug content was determined after separation of unentrapped drug by a Sephadex G-50 minicolumn using the centrifugation technique.[13,14]

Sephadex G-50 (1 g) was allowed to swell in 0.9% NaCl at room temperature, with occasional shaking, for at least 5 hours, after which the gel was formed and stored at 4ºC.

Minicolumns were prepared by placing Whatman paper pads at the bottom of the barrels of 1.0 mL syringes that were filled with the gel. Excess water was removed by centrifugation (Remi, Mumbai, India, centrifuge) at 3000 rev min^{-1} for 3 minutes.

Liposomes (200 μL) were applied dropwise to the center of the column. When a saturated drug solution was used instead of the liposome suspensions, the entire drug remained bound to the gel. This confirmed that there would be no free drug present after recovering the vesicles. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton X-100, filtering, and then determining the amount of liberated d4T using a UV spectrophotometer (Shimadzu 1601) at 266 nm.

In Vitro Ligand Agglutination Assay

All formulations (uncoated and carbohydrate-coated liposomal formulations) were stored in amber-colored glass bottles at 4°C ± 2°C, 25°C ± 2°C/60% ± 5% RH, or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days. The galactose, mannose, and fucose-coated liposomal systems (1 mL, 100 μL, and 1 mL, respectively) were assessed for in vitro ligand-specific activity by *R communis* lectin (30 μg/mL), con A (500 μg/mL), and *U europaeus* (60 μg/mL) agglutination assay.

The galactose-coated liposomal system was assessed for in vitro ligand-specific activity by *R communis* lectin agglutination assay, with slight modification.[15] One mL of original liposomal formulation (galactosylated) was incubated with increasing concentrations (5, 10, 20, 30, 50, and 60 μg/mL) of *R communis* agglutinin in a cuvette containing 1 mL of PBS (pH 7.4) at 25°C. A time-dependent (0-60 minutes) increase in turbidity at 360 nm was monitored turbidimetrically (Bioscreen Analysis Labs Systems, Helsinki, Finland).

Mannosylated liposomes were assessed for in vitro ligand-specific activity by mannose-binding con A as reported by Copland et al[16] with slight modification. A 100-μL sample

of the original mannose-coated liposomal dispersion was diluted 10 times with PBS (pH 7.4), and 1 mL of varying concentrations of con A (100-700 μg/mL in PBS containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) was added to it at 25°C. Turbidity at 550 nm was monitored turbidimetrically for 4 hours.

One mL each of fucosylated liposomal formulations was incubated with increasing concentrations (5, 10, 40, 50, 60, and 80 μg/mL) of fucose-specific lectin *U europaeus* agglutinin (UEA I) in a cuvette containing 1 mL PBS (pH 7.4) at 25°C. The time-dependent (0-90 minutes) increase in turbidity at 660 nm was monitored turbidimetrically.[17]

Percent agglutination after storage was calculated using the following equations:

Galactosylated liposomes:

$$\text{Agglutination}(\%) = \frac{\text{Abs of RGL complex after 45 min at 360 nm (day 10, 20, or 30)}}{\text{Abs of RGL complex after 45 min at 360 nm (day 0)}} \times 100 \quad (1)$$

Mannosylated liposomes:

$$\text{Agglutination}(\%) = \frac{\text{Abs of CML complex after 120 min at 550 nm (day 10, 20, or 30)}}{\text{Abs of CML complex after 120 min at 550 nm (day 0)}} \times 100 \quad (2)$$

Fucosylated liposomes:

$$\text{Agglutination}(\%) = \frac{\text{Abs of UFL complex after 60 min at 660 nm (day 10, 20, or 30)}}{\text{Abs of UFL complex after 60 min at 660 nm (day 0)}} \times 100 \quad (3)$$

where abs is absorbance, RGL is *R communis* lectin–galactosylated liposome complex, CML is con A–mannosylated liposome complex, and UFL is *U europaeus*–fucosylated liposome complex.

Oxidation Index

The amount of conjugated dienes at a definite time was estimated as follows: liposome aliquots (1 mL) were dissolved in 3 mL of anhydrous methanol, and optical densities at 233 and 205 nm were measured in a UV spectrophotometer (Shimadzu 1601). Oxidation indexes were calculated as optical density $(OD)_{233\ nm}/OD_{205\ nm}$. The oxidation index of liposomal formulations with nitrogen atmosphere was determined at 4°C ± 2°C, 25°C ± 2°C/60% ± 5% RH, or 40°C ± 2°C/75% ± 5% RH for a period of 10, 20, and 30 days.

Thiobarbituric Acid–Reactive Species Determination

Thiobarbituric acid–reactive species (TBARS) were measured following the method of Buege and Aust.[18] Aliquots (0.5 mL) of liposome suspensions were put into test tubes and mixed with 0.5 mL of water and 2 mL of TBA reagent (3.75 g/L TBA; 150 g/L trichloroacetic acid; HCl 0.25 mol/L; 0.1 g/L butyl-hydroxytoluene). The tightly closed tubes were heated in a boiling bath for 15 minutes, then immediately cooled and centrifuged (1500*g*; 10 minutes). The absorbance at 532 nm of the supernatant was read against a blank. TBARS were expressed as absorbance at 532 nm per mg of phospholipid (A532/mg phospholipid).[19]

Uncoated (d4T NL and d4T PL) and galactose-coated liposomes (d4T OPG liposomes) were used for this study. Furthermore, liposomes with coatings of different carbohydrates showed a similar oxidation index profile, so only galactose-coated liposomes were studied.

Results and Discussion

The reverse-phase evaporation method for the preparation of liposomes is reported to encapsulate large hydrophilic molecules with high entrapment efficiency. It is clear from Table 1 that as the lipid ratio used in the preparation of liposomes varies, encapsulation efficiency and particle size change significantly ($P < .001$). Formulation d4T PL4 was found to have the highest (49.60 ± 1.23%) entrapment efficiency and the lowest particle size (120.00 ± 1.52 nm). Therefore, formulation d4T PL4 was chosen from among the others for galactose coating of the liposomes.

The percent coating of carbohydrate varied from 0.1% to 0.4%. It was observed that the maximum stability was attained at a carbohydrate coating of 0.3%. The stability data presented here are for this optimized carbohydrate coating (0.3%).

The change in entrapment efficiency after coating was insignificant ($P > .05$), as compared with the uncoated formulation, indicating no loss of drug after OPM, OPG, and OPF coating. After extrusion through the polycarbonate membranes, a significant ($P < .05$) increase in vesicle size was observed for OPM-, OPF-, and OPG-coated liposomes (Table 2) compared with uncoated liposomes (120 ± 1.52 nm).

The stability of liposomes is dependent on the lipid content and the buffer system. Liposomal formulations are reported to be stable at 4°C ± 2°C. Therefore, all the formulations were stored at 4°C ± 2°C to compare their stability profile (*AHFS Drug Information*)[20] with that of the formulations stored at higher temperatures. Higher temperatures increase the propensity of lipids to undergo a transition to a nonbilayer phase. As membrane fusion is believed to proceed through nonbilayer intermediates, higher temperatures are expected to facilitate fusion.[21] It is known that the oxidative deterioration of

Table 2. Composition and Characterization of O-Palmitoyl Carbohydrate-Anchored Liposomal Formulations*

Formulation Code	Particle Size d_{50} (nm)†	% Entrapment Efficiency†	Zeta Potential (mV)
d4T OPG L	143.11 ± 1.46	48.73 ± 1.53	2.50 ± 0.50
d4T OPM L	140.00 ± 2.25	47.16 ± 2.25	2.40 ± 0.60
d4T OPF L	143.20 ± 3.17	47.21 ± 1.53	2.64 ± 0.40

*d4T indicates stavudine; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively.
†All values are expressed as mean ± SD (n = 6).

the liposome's phospholipid constituents plays a fundamental role in its stability. The main factors that are deleterious to liposome integrity are the presence of aldehydes produced in the peroxidation process and the splitting of the phospholipid into an acyl chain and the respective lysophospholipid. Both processes occur simultaneously during liposome storage. To evaluate the oxidative stress, conjugated dienes and TBARS were measured.[22]

Vesicle Size and Shape

An insignificant ($P > .05$) difference was found in the vesicle size of all liposomal formulations stored at 4°C ± 2°C for 30 days. But a significant ($P < .05$) increase in the vesicular size was observed when the uncoated liposomal formulations were stored at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH up to 30 days. The increase in the size could be due to the fusion of liposomes. A significant increase in the vesicle size of carbohydrate-coated formulations was observed at 40°C ± 2°C/75% ± 5% RH after 30 days. These results indicate that fusogenicity is temperature dependent. No significant ($P > .05$) change in the vesicle size of carbohydrate-coated liposomal formulations was observed when they were stored at 25°C ± 2°C/60% ± 5% RH up to 30 days (Figure 1). The results are in good agreement with Yohannes et al.[7] The tendency of the liposomes to aggregate was observed for uncoated liposomal formulations. This fusion of vesicle is shown in the transmission electron microscopy photomicrograph of the liposomal formulation (Figure 2).

Zeta Potential

The membrane surface potential plays an important role in the rate of aggregation and fusion of vesicles and hence in the physical stability of liposomes. As far as zeta potential is concerned, liposomes have mostly negative electrophoretic mobilities. NL showed only a slight negative potential, because of the ionization of the phosphate and choline groups. Although PE is a zwitterionic molecule at the pH of the measurements (7.4), its presence in the liposome can modify

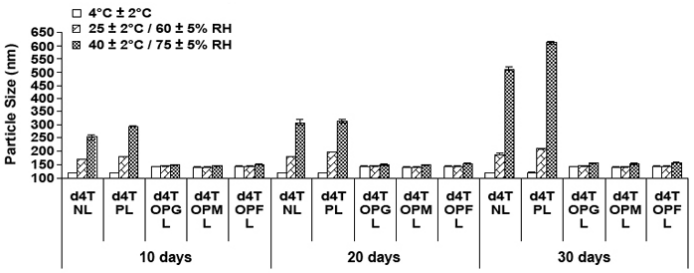

Figure 1. Effect of storage conditions on particle size of d4T-loaded uncoated liposomal formulations and O-palmitoyl carbohydrate-anchored liposomal formulations (mean ± SD; n = 3). RH indicates relative humidity; d4T, stavudine; NL, neutral liposomes; PL, positively charged liposomes; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively.

the negative charge of the phosphate group. The effect on incubation was negligible in all of the carbohydrate-coated preparations, and the zeta potentials maintained their values.

No significant ($P > .05$) change in the zeta potential of the liposomal formulations was observed when they were stored at 4°C ± 2°C for 30 days. Uncoated liposomes exhibited a significant ($P < .05$) difference in their surface charge when stored at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH for 10 days. The drop in zeta potential was found to be a function of time up to 30 days (Figure 3). The drop in the zeta potential's negativity in the case of liposomal formulations composed of phosphatidylcholine and cholesterol may be due to the fusion of vesicles at 25°C ± 2°C, which could cause loss of CH and PC from the bilayer. In the case of carbohydrate-coated liposomal formulations the increase in zeta potential was found to be insignificant ($P > .05$) when they were kept at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH for 30 days. The very slight increase may be due to externalization of the PE molecules, which may be attributed to the protective carbohydrate coating on their surface, which was responsible for the slight initial positive zeta potential.

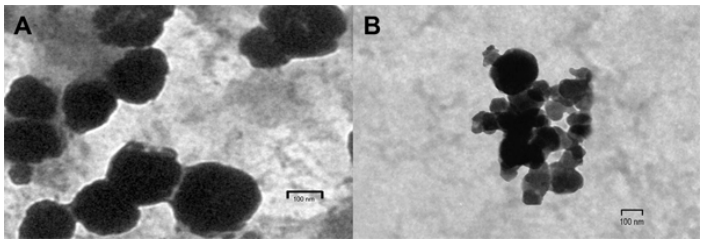

Figure 2. Transmission electron microscopy photomicrograph of uncoated liposomal formulation at (A) 25°C ± 2°C/60% ± 5% RH; and (B) 40°C ± 2°C/75% ± 5% RH after 30 days of storage (× 80; k 50 kV).

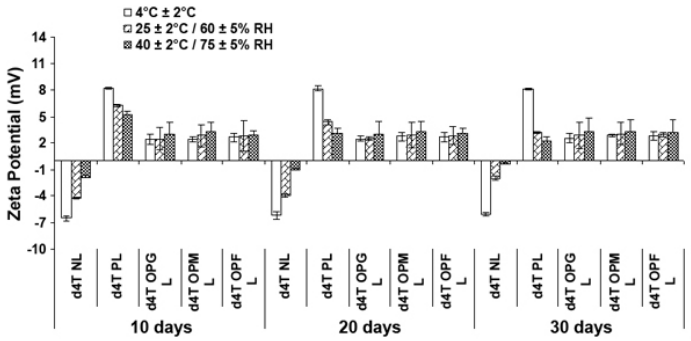

Figure 3. Effect of storage conditions on zeta potential of d4T-loaded uncoated liposomal formulations and O-palmitoyl carbohydrate-anchored liposomal formulations (mean ± SD; n = 3). RH indicates relative humidity; d4T, stavudine; NL, neutral liposomes; PL, positively charged liposomes; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively.

Residual Drug Content

More than 99% (<100%) of the drug was retained for 30 days in all the liposomal formulations during storage at 4°C ± 2°C. While 74% to 80% of the drug was found to be entrapped in uncoated liposomal formulations after 10 days, 50% to 60% of the initial drug was present after 30 days of storage at 25°C ± 2°C/60% ± 5% RH. The liposomal formulations with a covering of carbohydrate (galactose, mannose, or fucose) on their surface could retain up to 80% to 85% of their encapsulated drug, which is in good agreement with the earlier reported results.[23] However, at 40°C ± 2°C/75% ± 5% RH, uncoated liposomes could retain ~8% to 10% of their initial drug, while ~80% of the initial drug was retained by coated formulations after 30 days (Figure 4).

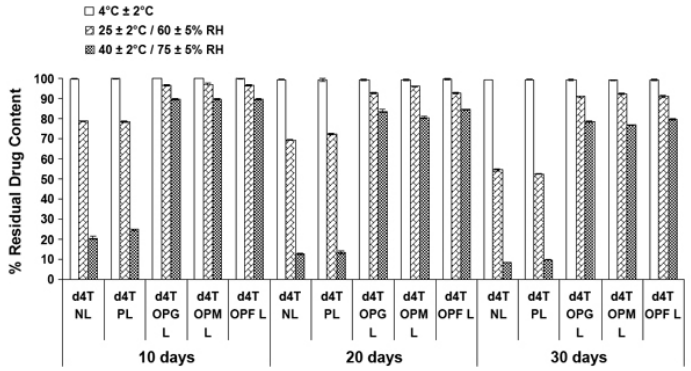

Figure 4. Effect of storage conditions on the percentage of residual drug content of d4T-loaded uncoated liposomal formulations and O-palmitoyl carbohydrate-anchored liposomal formulations (mean ± SD; n = 3). RH indicates relative humidity; d4T, stavudine; NL, neutral liposomes; PL, positively charged liposomes; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively.

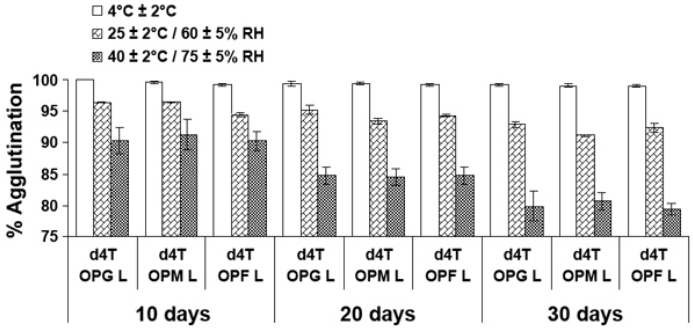

Figure 5. Effect of storage conditions on percent agglutination of drug-loaded O-palmitoyl carbohydrate-anchored liposomal formulations (mean ± SD; n = 3). RH indicates relative humidity; d4T, stavudine; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively.

Drug Leaching

The fact that the total percentage of residual drug and drug leached out from the vesicular carrier system was almost 100%, which indicates practically no drop in potency of the encapsulated drug, rules out any loss in potency of the drug encapsulated in the system. Hence, drug decomposition in the system is ruled out. The extent of drug leaching was found to be higher at accelerated conditions (40°C ± 2°C/75% ± 5% RH), probably due to the expansion of aqueous channels and the increase in pore dimensions. Results also suggest that apart from playing the role of steric stabilizer, the carbohydrate prevents drug leaching even at elevated temperature conditions by masking the aqueous channels.

In Vitro Ligand Agglutination Assay

More than 99% of the carbohydrate was present on the carbohydrate-coated liposomal systems stored at 4°C ± 2°C

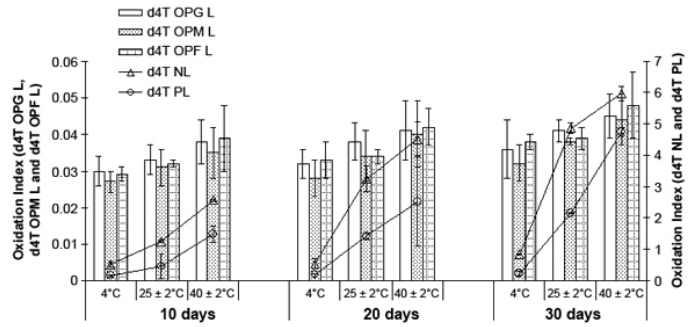

Figure 6. Effect of storage conditions on the oxidation index of uncoated liposomal formulations and O-palmitoyl carbohydrate-anchored liposomal formulations stored in a nitrogen atmosphere (mean ± SD; n = 3). d4T, stavudine; OPG L, OPM L, and OPF L are the formulation codes for liposomes prepared using O-palmitoylgalactose, O-palmitoylmannose, and O-palmitoylfucose coating, respectively; NL, neutral liposomes; PL, positively charged liposomes.

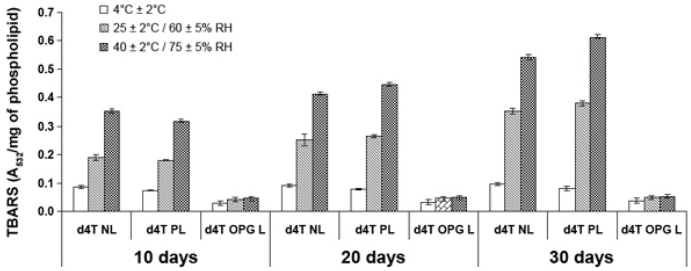

Figure 7. Effect of storage conditions on TBARS formed in liposomal formulations stored in a nitrogen atmosphere (mean ± SD; n = 3). RH indicates relative humidity; TBARS, thiobarbituric acid–reactive species; d4T, stavudine; NL, neutral liposomes; PL, positively charged liposomes; OPG L, formulation codes for liposomes prepared using O-palmitoylgalactose.

up to 30 days, as indicated by their agglutination assay. Storage of these carrier systems at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH up to 30 days reduced their agglutination nearly to 90% and 80% of initial stability, respectively, indicating the presence of a carbohydrate protective layer on their surface (Figure 5).

Oxidation Index

During the incubation time, liposomes suffered an autoxidation process, producing a variety of products such as conjugated dienes and lipid hydroperoxides.[24-27] The mechanisms of lipid oxidative degradation involve a first step of autoxidative reactions followed by secondary reactions, which can be either oxidative or not and take place via a free radical mechanism. All these processes lead to the formation of hydro and cyclic peroxides and finally malondialdehyde (MDA), short-chain aldehydes, esters, ketoacids, and so forth.[28-30]

The oxidation index (OI) was significantly ($P < .05$) higher for uncoated liposomes as compared with liposomes having a carbohydrate coat on their surface when stored in a nitrogen atmosphere. OI increased significantly ($P < .05$) when the uncoated liposomal formulations were stored, irrespective of their storage temperature. This may be due to the exposure of phospholipids to the atmosphere. No significant difference in OI of liposomes having a layer of carbohydrate on their surface was observed at any temperature (Figure 6).

TBARS Determination

Lipid peroxidation was assessed by TBARS production. This assay is based upon the formation of a red adduct (absorption maximum 532 nm) between TBA and MDA, a colorless end product of lipid peroxide decomposition.[31]

TBARS production was significantly ($P < .05$) higher for uncoated liposomes stored at 25°C ± 2°C/60% ± 5% RH, but insignificant changes in TBARS values were observed in these formulations stored at 4°C ± 2°C. Temperature had an insignificant ($P > .05$) effect on TBARS production by galactose-coated liposomes (Figure 7).

Lipid peroxidation processes start with the hemolytic fission of the phospholipid acyl chains or as a consequence of the attack of reactive oxygen species, such as superoxide anion, hydrogen hydroperoxide, and hydroxyl radical, which are able to abstract a hydrogen from an acyl chain, leading to acyl radical formation. All these chemical processes require energy, which can be supplied by electromagnetic radiation, heat, redox reactions, and so on. The first acyl radicals formed react with oxygen, producing acyl peroxyl radicals, which react with other acyl chains. These mechanisms are involved in the propagation phase of lipid peroxidation, leading to the formation of hydro and cyclic peroxides and later MDA, short-chain aldehydes, esters, ketoacids, and so on. TBA reacts with carbonyl substances (aldehydes, ketones).[22]

CONCLUSION

It can be concluded that the stability of liposomal formulations is temperature dependent. All the liposomal formulations were found to be stable at 4°C ± 2°C up to 1 month, and hence this temperature can be recommended for their storage. Storage at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH adversely affected uncoated liposomal formulations. Carbohydrate coating of the liposomes could enhance the stability of liposomes at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH.

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