Journal of Pharmacreations

PharmaCreations

ISSN: 2348-6295

Pharmacreations | Vol.2 | Issue 4 | Oct - Dec - 2015

Journal Home page: www.pharmacreations.com

Research article ***Open Access***

NIOSOMES (NON IONIC SURFACTANT VESICLES) PREPARATION AND STABILITY IN BIOLOGICAL ENVIRONMENT

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ABSTRACT

Niosomes are nanometric in size use for drug targeting at a rate directed by the needs of body during tratment. Niosomes are non-ionic surfactant vesicles obtained on hydration of synthetic non ionic surfactant wth or without incorporation of cholestrol or either lipids and can be used as carrier of amphiphilic and lipophilic.

Niosomes (non-ionic surfactant vesicles) were biodegradable, biocompatible, and non- immunogenic in nature and having flexibility in structure & storage. These are chemicaly stable, ecently many researchers works on niosomes by oral drug delivery to provide better bioavailability to drug. Niosomes provides better encapsulation in biological membrain and maintain stability.

Drug entraped vallues were measured by using flurosant markers like 5-6-Carboxyfluroscein and drug release rate is evaluated in biolgical media that is (serum & plasma) as a function of surfectant composition and in the presence or absence of cholesterol. Surfactant charge measurment is done by zeta potential as a function of pH , gel electrophoresis and immunoblotting were used to know the compatability study between biological fluid componant and prepared vesicles. It was found that all the vesicle carries negative charge & rapidly bound to the plasma protein which incluid albumin & imunoglobulin-G that affects the latency of entraped marker.

Uptake & degradation of niosomes in a living unicellular, eukaryotic micro-oraganism was also investigated. In this work the well chrecterise liposomes were compared with niosoms.

Keywords: Niosomes, Biological membrain , Zeta potential, Stability, 5-6-Carboxyfluroscein, Tetrahymenaelliotti strain.

INTRODUCTION:

Most common routes of administration include the preferred non-invasive per oral, topical, transmucosal (nasal, buckle /sublingual, vaginal, ocular and rectal) and inhalation. Many medications such as peptide and protein, antibody, vaccine and gene based drugs, in general may not be delivered using these routes because they might be susceptible to enzymatic degradation or cannot be absorbed into the systemic circulation efficiently due to molecular size and

charge issues to be therapeutically effective. For this reason many protein and peptide drugs have to be delivered by injection or a nanoneedle array.

Current efforts in the area of drug delivery include the development of targeted delivery in which the drug is only active in the target area of the body (for example, in cancerous tissues) and sustained release formulations in which the drug is released over a period of time in a controlled manner from a formulation. In order to achieve efficient targeted delivery, the designed system must avoid the host's defense mechanisms and circulate to its intended site of action. Types of sustained release formulations include liposomes, drug loaded biodegradable microspheres and drug polymer conjugates[1].

Drug delivery technologies modify drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance. Drug release is from, diffusion, degradation, swelling, and affinity-based mechanisms [2].

Drug carriers: Are the substances that serve as mechanisms to improve the delivery and the effectiveness of drugs. Drug carriers are used in sundry drug delivery systems such as:

- controlled-release technology to prolong *in vivo* drug actions;
- decrease drug metabolism, and
- reduce drug toxicity

Carriers are also used in designs to increase the effectiveness of drug delivery to the target sites of pharmacological actions. To be efficient, the drug-carrier should fulfill several criteria;

1. The carrier should be pharmacologically inactive and the drug must be released in an active form after interaction of the carrier with the target cells.
2. The drug carrier complex must be stable in plasma and extracellular spaces.
3. The carrier should have the ability to take the drug through those anatomical barriers which separate the site of administration from the target; it must also be specifically recognized by receptors or antigens present on the outer membrane of the target cells.
4. The carrier should be non-toxic, non-immunogenic and biodegradable to avoid cellular overload during long term repetitive treatment.
5. Production of the drug loaded carrier in the amounts and conditions required for clinical use, for example, sterility, apyrogenicity, should be easily achieved and pharmaceutically acceptable in terms of stability and reproducibility. Several systems for achieving some of these goals have been proposed. Liposomes[3], resealed erythrocytes[4], microparticles[5], nanoparticles [6] and albumin microspheres are examples of particulate colloid carriers which are used as targetable drug delivery systems.

Biological strategy: Several drug delivery approaches make use of biological entities, such as antibodies, red cells, and liposomes, as drug carriers. The biologically based delivery systems share some of the characteristics of polymeric and prodrug systems and can be used for similar purposes. Implicit in the work on biological carriers is the goal of using highly specific physiological recognition mechanisms as the basis of targeted delivery. Carriers such as liposomes and red cells can be used to achieve sustained and controlled drug administration. Drug loaded liposomes[7] and erythrocytes[8] can be introduced directly into the systemic vasculature or for localized action into the appropriate organ or body cavity.

Niosomes: Potential Drug Carriers

The discovery that non-ionic surfactant molecules, such as surfactant I, as shown in figure 1, are capable of forming vesicles, niosomes, entrapping an aqueous solution, was a lead to their potential use as drug carriers[9].

A system which could combine the advantages of liposomes with the ability to increase membrane permeability displayed by the non-ionic surfactants would be of great interest. An investigation to compare and contrast some of their relevant properties with the apparently similar and well characterized liposome system is vital to the development of a niosomal drug carrier system. A major prerequisite to the use of niosomes and other vesicles as drug carriers is their integrity in biological fluids. These are for example, interstitial fluid (sub-cutaneous administration), synovial fluid (intra – articular injection), contents of the stomach and intestine (oral route) and peritoneal cavity (intra-peritoneal administration). In a carrier role, niosomes must be able to both circulate in the body and retain drugs for significant periods of time to optimize access to, and interaction with, target tissue and in appropriate circumstances delivery of their contents to the interior of cells.

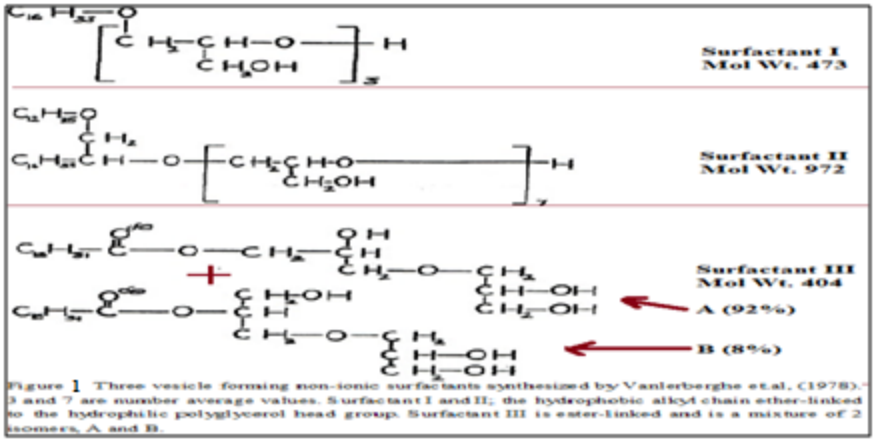


Figure 1 Three vesicle forming non-ionic surfactants synthesized by Vanlerberghe et.al, (1978).- 3 and 7 are number average values. Surfactant I and II; the hydrophobic alkyl chain ether-linked to the hydrophilic polyglycerol head group. Surfactant III is ester-linked and is a mixture of 2 isomers, A and B.

Intravenous injection of hand shaken niosomes containing doxorubicin into mice[10] showed no apparent liver or spleen loading with drug but some evidence of accumulation of doxorubicin in the lungs. These results may be a direct consequence of the size distribution of the vesicles used, although the lung-loaded perhaps indicates intravenous aggregation of the niosomes. Increased anti-leishmanial activity after passive targeting of sodium stibogluconate to the liver using niosomally-entrapped drug [11] is further evidence of the potential of drug-carrier role for niosomes. As part of an approach to the optimization of this drug-carrying potential of niosomes, it is important to characterize their stability in terms of release of entrapped solute. The result of a series of *in vitro* experiments, simulating physiological conditions *in vivo*, are presented here to define the effects of blood proteins in niosome stability and in addition the influence of pH and temperature on the integrity of various types of non-ionic vesicle.

MATERIALS & METHODS:

The Non-ionic surfactants, I, II and III, were a gift from L'Oreal, India, Chakan Pune. 5, 6-carboxyfluorscein (Sigmaaldrich, Powai, Mumbai.) was partially purified over activated charcoal before use.

4-chloro-l-napthol , dicetylphosphate (DCP), Acrylamide, ammonium persulphate (APS), amidoblack (napthol blue black), calcein, carboxylicester hydrolase, cholesterol, dipalmitoylphosphatidylcholine (DPPC), dimyristalphosphatidylcholine(DMPC), glutaraldehyde, glycerol, glycine, horse radish peroxidase, mercaptoethanol, phospholipase A2, sodium dodecyl sulphate (SDS), stearylamine, thrombin and trypsin were purchased from Hi Media Laboratories Pvt Ltd, Ghatkopar (West).

EDTA was a gift sample from Bombay lubricants Oil Co. (Mumbai). & N, N, N', N''-tetramethylethylenediamine (TEMED) and urea were purchased from Alpha Chemika (Gujarat). Plasma, pooled citrated human, was obtained from the Shree Swaminarayan Charitable Hospital Bhuj. Serum was prepared from citrated plasma by the addition of thrombin and heat- inactivated. Proteose peptone and liver extract were purchased from titan biotech ltd. Snake venom from Surya SurgiPharma,(Nashik, Maharashtra). All other reagents were of analytical lab grade.

Production of Vesicles

Various methods are reported for the preparation of niosomes such as:

a) Ether injection method
b) Hand shaking method (Thin film hydration technique)
c) Sonication method
d) Reverse phase evaporation technique (REV)
e) Micro fluidization
f) Multiple membrane extrusion method
g) Trans membrane pH gradient (inside acidic) drug uptake process (remote loading)
h) Bubble method
i) Formation of niosomes from proniosomes.
j) Purification of CF

Here preparation of the niosomes was done by Hand shaking method.

Hand shaking method (Thin film hydration technique)

The mixture of vesicles forming ingredients like surfactant and cholesterol (1.50 X 10^{-4} M) are dissolved in a volatile organic solvent (diethyl ether,

chloroform or methanol) in a round bottom flask. The organic solvent is removed at room temperature (20^0C) using rotary evaporator leaving a thin layer of solid mixture deposited on the wall of the flask. The dried surfactant film can be rehydrated with aqueous phase at 0-60°C with gentle agitation[12].

Purification of CF

CF was purified by modifying the method of[13]Commercially available CF (25g) was treated with activated charcoal (10g) in boiling ethanol (300ml) contained in a round-bottomed flask (1 liter). After refluxing for 30 minutes the mixture was cooled and filtered through Whatman No. 50 filter paper. Cold distilled water (600ml) was added & solids allowed precipitating overnight at 0^0CT. he water was removed by filtration and the orange-colored precipitate was washed thoroughly (4 times, 50ml distilled water), dried at 50^0C in an airtight container and stored in the dark at room temperature.

CF Solution: Aliquots of this orange powder (0.2M) were weighed, made up to volume with the medium (distilled water or PBS) and adjusted to pH 7.4 with sodium hydroxide (NaOH, 4N).

Buffered CF: CF is usually made up in distilled water to the required molarity at pH 7.4 by adding sodium hydroxide solution (NaOH, 4N). In buffered CF, the distilled water was replaced with phosphate buffered saline (PBS) and the requisite amount of NaOH, to the final pH 7.4.

Separation of Free and Entrapped CF: Volumes of aqueous surfactant or phospholipid dispersions (5ml) from ether injection or hand shaken techniques were exhaustively dialyzed against PBS, (1.30×10^{-3} M, 0.9%w/v NaCl solution, pH 7.4). In experiments with T. elliotti, NaCl solution was omitted from the buffer.

Stability of Vesicles:

Leakage of CF: The CF entrapped within the vesicles is self-quenched at the working concentration of 0.2M at the wavelengths of measurement. Leakage and ensuing dilution into the extra-vesicular bulk volume, increases the fluorescence of CF markedly, which was measured (486 nm excitation, 514 nm analyzer wavelengths) using a spectrofluorimeter.

Samples (2.5×10^{-2} ml) were added to the test media at "time zero" to give a final volume of 5ml. The fluorescence measured at these times was taken as zero percent, although in practice 15 seconds elapsed before these readings could be recorded. This amounted to 2-6% intensity of the total fluorescence. The samples were gently agitated throughout the experiments and further readings obtained at various time intervals. Maximum fluorescence (100%) for all niosomes and liposome suspensions, F*tot*, was measured after vesicle disruption by addition of propan-l-ol (0.1ml), or Triton X-100 (0.1ml). The leakage of CF was corrected for background fluorescence at "time zero", F_o. The percentage of CF released in each sample was calculated as

$$\% \, \boldsymbol{Realese} = \frac{\mathbf{100}(\boldsymbol{Ft} - \boldsymbol{Fo})}{\boldsymbol{Ftot}}$$

Where Ft - intensity at time 't'.

Effect of pH: The leakage of CF was measured as described above, by challenging the vesicle suspensions (2.5×10^{-2}ml) with a variety of different buffers (citric phosphate buffer, pH 2.0 to 8.0) and incubating at 37^0C. All the solutions used were of equal ionic strength (1.24) to that of the CF solution within the vesicle, thus preventing osmotically driven leakage of the CF solution from the vesicles.

Effect of temperature: The efflux of CF from the vesicles was measured after incubation of a suspension (2.5×10^{-2}ml) in buffer (5ml, PBS at pH 7.4) at various temperatures (4^0C, 22^0C, 37^0C and 50^0C for various time intervals.

Preparation of Human Serum: Serum was prepared from plasma by treatment with thrombin (20 NIH units ml^{-1}) at 37^0C for 10 minutes with gentle stirring, after which period the clot formed was removed. Serum was heat- inactivated by incubation at 51^0C for 30 minutes. Any dilutions of plasma or serum were made by addition of PBS (pH 7.4) to the correct volume.

Effect of Plasma: Leakage of CF from the vesicles was measured as above after incubation of a suspension (2.5×10^{-2}ml) of vesicles in human plasma (100%) at 37^0C. Total fluorescence (100%) was evaluated by disrupting all the vesicles using Triton X-100 (0.1ml).

Effect of 10% BSA: BSA (400mg) was dissolved in PBS (100ml, pH 7.4) at 37^0C. Leakage of vesicles was measured as above.

Measurement of Surface Potential:

The electrophoretic mobility (μ) was measured as a function of pH in a laterally placed flat cell micro-electrophoresis apparatus with an optical assembly and constant temperature bath at 25^0 C. The mobility was determined by measuring the time taken in seconds for the vesicles to travel a pre-determined distance (usually 2cm) under the influence of a constant known electric field (80 Volts). The vesicles were suspended in solution (NaCl, $2X\ 10^{-3}$M), the pH of which was varied by the addition of dilute

hydrochloric acid (HCI) or sodium hydroxide (NaOH). The mobilities of at least 40 vesicles were measured at each pH and an average mobility obtained.

To measure the effect of plasma (human, 50%) on electrophoretic mobility, vesicles were prepared entrapping NaCl (2 X 10^{-3} M) in glucose solution (0.2M). These vesicles were incubated in plasma for 2 hours, centrifuged at 210g for 5 minutes, washed twice and their electrophoretic mobility measured as described above. These measurements were used to calculate surface potentials.

***In Vivo* Uptake and Degradation Bycells:**

The ciliate, T. elliotti strain 1630/IC, was cultured axenically in proteose peptone (2% w/v) and liver extract (0.1% w/v) at room temperature (22^0C). The cells were harvested from these cultures after 4 days growth by centrifugation at 110g for 15 minutes and the cell pellet was resuspended in phosphate buffer (100ml, pH 7.4). The cells were resedimented at 110g for 15 minutes and the supernatant decanted. The pellet was made up to volume (100ml) and left to recover from centrifugation at room temperature for 24 hours. Samples (0.1ml) of niosome or liposome suspension were mixed with this cell suspension (1ml) at time zero. At suitable intervals, volumes (1.5 X 10^{-2}ml) of this mixture were fixed in glutaraldehyde (2.5 X 10^{-2}ml, 0.1% solution) and examined by epi- fluorescence microscopy. Further evidence of vesicular integrity was obtained using a camera mounted on the microscope and time-sequence photography. At time intervals, the total fluorescence in each of 100 T. elliotti cells was measured using a spectrophotometer and an average percent of total fluorescence obtained. Photographs at these various time intervals (0, 1, 2,3,4,5 and 6 hours) were taken and vacuole counts were recorded for over 40 cells at each interval and vesicle type.

Effect of Enzymes on Vesicles (*in vitro*)

Solutions of phospholipase A_2, snake venom and carboxylic ester hydrolase were all freshly prepared before use. Phospholipase A_2 activity has an absolute requirement for calcium ions (Ca^{2+}) and stock solutions were prepared by dissolving pure phospholipase (10mg) or crude venom (12.5 mg) in Tris-HCI buffer (2.0 X 10^{-1}M, 100ml, pH 7.4), containing $CaCI_2.2H_2O$ (3.2g). Carboxylic ester hydrolase (5mg) was reconstituted before use by dissolving in Tris-HCI buffer (2.0 X 10^{-2} M, 1000ml, pH 7.4). The leakage of CF (0.2M) from vesicles varying in their compositions was measured, as described, and compared, when challenged *in vitro* with the above enzyme solutions (2.5 X 10^{-2} ml). Leakage was measured over a time period of 6 hours. These values were corrected for CF leakage into buffer, as follows:

$$\% \boldsymbol{Reslease} = \frac{\boldsymbol{Fe - Fb}}{\boldsymbol{Ftot}}$$

Where F_e = fluorescence intensity in enzyme solution,

F_b = intensity in buffer, both at time T.

F_{tot} = maximum intensity of fluorescence.

RESULT

Production of Niosomes

Vesicles of various compositions (table 1) were prepared for use in this study using hand-shaken method (Bangham et.al, 1965). The former method results, as reported for liposomes, in the formation of large unilamellar vesicles, with good entrapment efficiencies and greater stability in terms of leakage (graph 1,). However, since not all the components required for vesicle production were soluble in diethyl ether, the hand-shaken method was favored for this present study. This method produced multilamellar vesicles with comparable entrapment efficiencies (table 2) but higher rates of leakage (Graph 1).

Table No. 1: Formulation of vesicles of various compositions.

100% Surfactant I	I 100
50% Surfactant I + 50% Cholesterol	I 50 : CHOL 50
60% Surfactant I +30% Cholesterol +10% DCP	I 60 : CHOL 30: DCP 10
68%SurfactantI+30Cholesterol +2% Stearylamine	I 68 : CHOL 30: SA 2
50% Surfactant II + 50% Cholesterol	II 50 : CHOL 50
60% Surfactant II +30% Cholesterol +10% DCP	II 60 : CHOL 30: DCP 10
100% Surfactant III	III 100
50% Surfactant III + 50% Cholesterol	III 50 : CHOL 50
70% Surfactant III +20% Cholesterol +10% DCP	III 70:CHOL 30: DCP 10
100 DPPC	DPPC 100

50 DPPC+ 50 Cholesterol	DPPC 50: CHOL 50
70% DPPC +20% Cholesterol +10% DCP	DPPC70:CHOL 30: DCP 10
50 %Egg PC + 50 Cholesterol	Egg PC 50 + CHOL 50
50 %DMPC + 50 Cholesterol	DMPC 50 + CHOL 50

Quantification of Entrapment

The entrapment values reported here (Table 2,) were expressed in volume (ml) of CF per mole of surfactant, or lipid mixture.

Vesicle type	Hand Shaken			Either injection		
	Entrapment	Efficiency		Entrapment	Efficiency	
	(ml. mol $^{-1}$)	(%)	(n*)	(ml. mol $^{-1}$)	(%)	(n*)
I100	1670± 255	6	6	1111± 200	4.2	3
I50:CHOL50	760±100	3	6	763±100	3	6
I60:CHOL30:DCP10	700±99	3	6	601±108	2.3	3
II50:CHOL50	979±124	4	4	1568±120	5.9	6
II60:CHOL30:DCP10	720±86	3	6	1212±99	4.5	3
III100	1580± 60	6	6	1480±200	5.6	4
III50:CHOL50	650±50	3	6	503±80	1.9	4
II70:CHOL20:DCP10	320±68	1	6	355±45	1.3	3
DPPC100	2300±320	9	4	NOT DETERMINED		
DPPC50:CHOL50	1140±90	5	2	NOT DETERMINED		

Tabel No 2: Method of preparation
(n*) = is number of Determinations

Occasionally it was necessary to centrifuge the vesicles at low speeds (<1000g) in the preparation of samples for polyacrylamide slab gel electrophoresis, but here the leakage properties of the vesicles were not of prime concern.

CF, obtained commercially, is a mixture of the 5- and 6- isomers of carboxylic acid in unequal proportions. It also contains several impurities which can affect;

A. leakage of CF from the vesicles,

B. the stability of the vesicles.

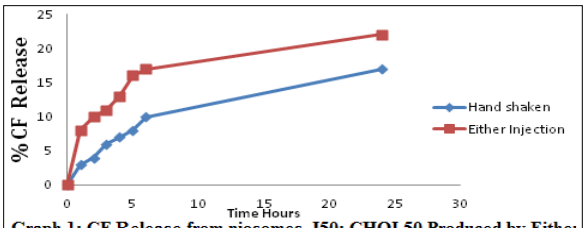
The most effective method of purifying CF is already described (Ralston et.al, 1981) but alternative short cuts are also employed. In the experiments described in this work, only two batches were purchased. In order to obtain at least 80% yields, the CF was partially purified over activated charcoal and recristallized as described in the experimental. The CF was not purified completely since column chromatography gave very poor yields (40%). Therefore, the CF used in these experiments may contain a small number of impurities which can affect its leakage through membranes. This may explain any discrepancies in the results when compared with the literature values for liposomes. However, the same material was used in all vesicles studied, & a comparison was possible since it was assumed that the impurities affect all types of vesicles in a similar manner. Ideally purified CF should be checked by HPLC or by TLC for other products.

To determine the interference by Triton X-100, propan-l-ol or the vesicles on the fluorescence of CF, various concentrations of each (0.5%-5.0%) were added to CF solutions. The mixtures were incubated for 90 minutes at 37^0C and monitored spectrofluorometrically. The detergent, alcohol or vesicles had no adverse effect on the fluorescence of CF.

Leakage of CF

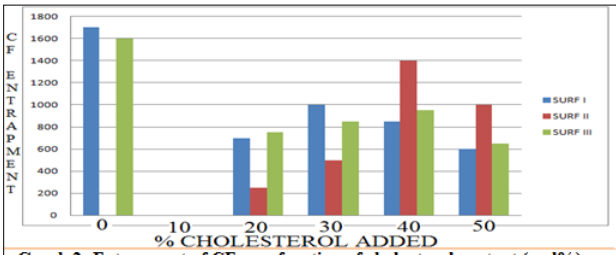
Buffer

The rates of leakage from vesicles is dependent on the method of production, the nature of the entrapped substance, external factors such as, temperature and pH, osmotic pressure (Baillie et.al, 1985) and their composition. Graph 1 illustrates the different rates of leakage from I50: CHOL50 vesicles (a representative vesicle preparation) produced by the E. I. technique and the hand-shaken (H. S.) method.

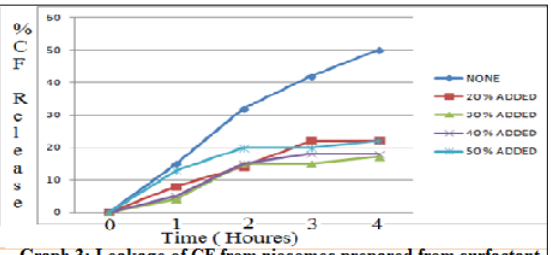


Graph 1: CF Release from niosomes, I50: CHOL50 Produced by Either injection and hand shaken methods at 22°C.

The leakage rate of niosomes were produced containing various percentages of cholesterol (0-50 mol %) and their entrapment values measured, as in graph 2. For all surfactants, II and III, optimal CF entrapment was found with the inclusion of 40 mot% cholesterol. Addition of DCP (10 mol %) significantly reduced entrapment values. The results indicate, for surfactant I niosomes, that leakage of CF is also greatly reduced with inclusion of 30-40 mot% cholesterol, as in graph 3.

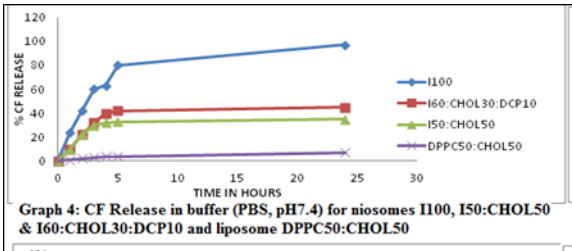


Graph 2: Entrapment of CF as a function of cholesterol content (mol%) for niosome from surfactant I, II & III(the entrapped marker was CF at 200 mM & entrapment is expressed in liters/mol)

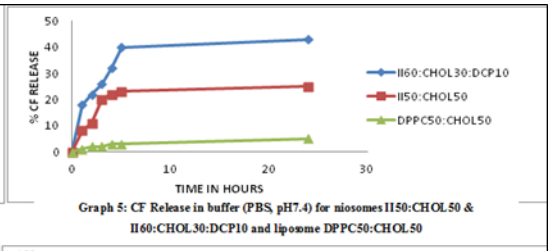


Graph 3: Leakage of CF from niosomes prepared from surfactant. I containing varying amount of cholesterol (in mol%).

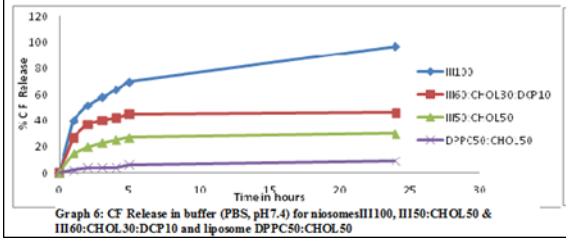
Vesicles in buffer (PBS) leak their contents gradually with time & as stated earlier, the stability increases with the inclusion of cholesterol and decreases with the inclusion of DCP for every surfactant investigated, as in graph 4, 5, and 6. No significant difference was found in the leakage from niosomes encapsulating "buffered" or "non-buffered" CF (graph 7,) suggesting that buffered CF solution is not essential for entrapment.



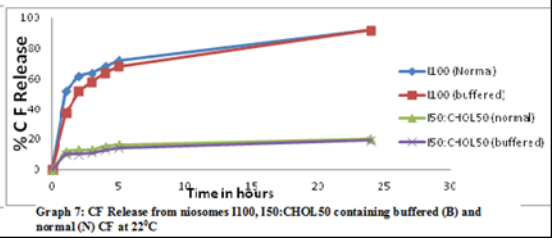
Graph 4: CF Release in buffer (PBS, pH7.4) for niosomes I100, I50:CHOL50 & I60:CHOL30:DCP10 and liposome DPPC50:CHOL50



Graph 5: CF Release in buffer (PBS, pH7.4) for niosomes II50:CHOL50 & II60:CHOL30:DCP10 and liposome DPPC50:CHOL50



Graph 6: CF Release in buffer (PBS, pH7.4) for niosomesIII100, III50:CHOL50 & III60:CHOL30:DCP10 and liposome DPPC50:CHOL50



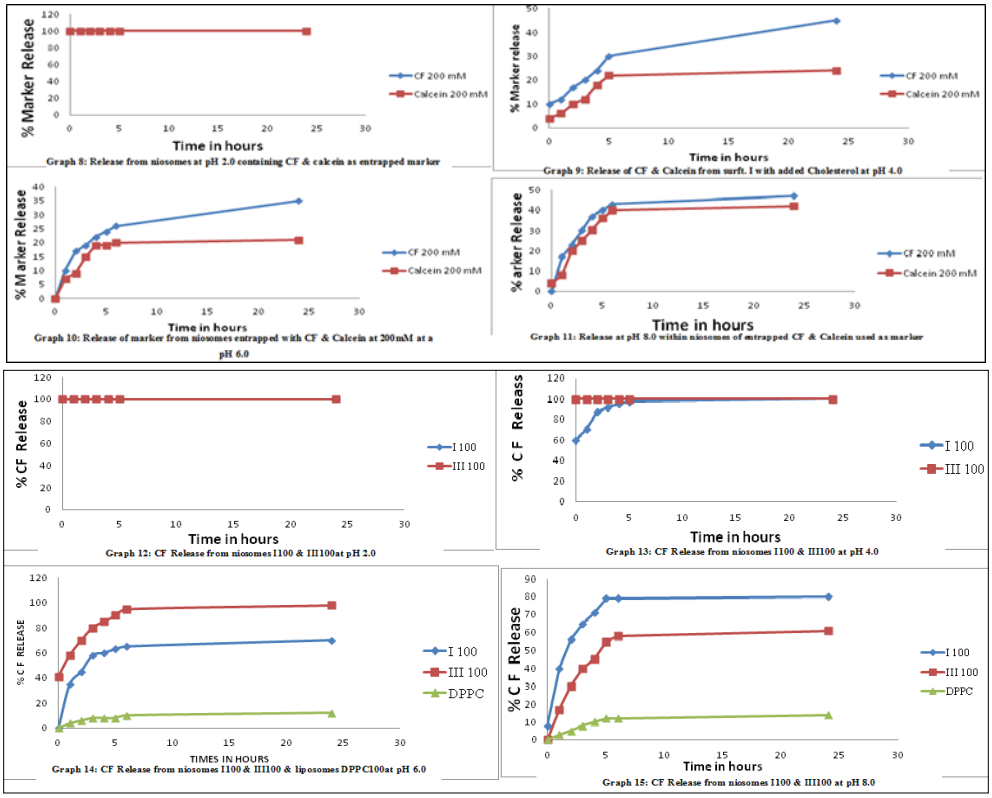
Graph 7: CF Release from niosomes I100, I50:CHOL50 containing buffered (B) and normal (N) CF at 22°C

pH Effects

CF is a trivalent anion at neutral pH which becomes electrically neutral at acidic pH; pKa's at 6.7, 4.4 and 3.5 (Heiple and Taylor, 1982). Calcein is more strongly charged than CF as a result of two methyliminodiacetic acid residues; carboxyl pKa<4.0; methyliminopKa 10-12. Calcein has been reported to be more resistant to changes in pH especially over the range pH 6 to 8. Niosomes, I50: CHOL50, were produced entrapping 200 mM CF or 200 mMcalcein and their efflux as a function of external pH shown in graph 8, 9, 10 & 11. Little

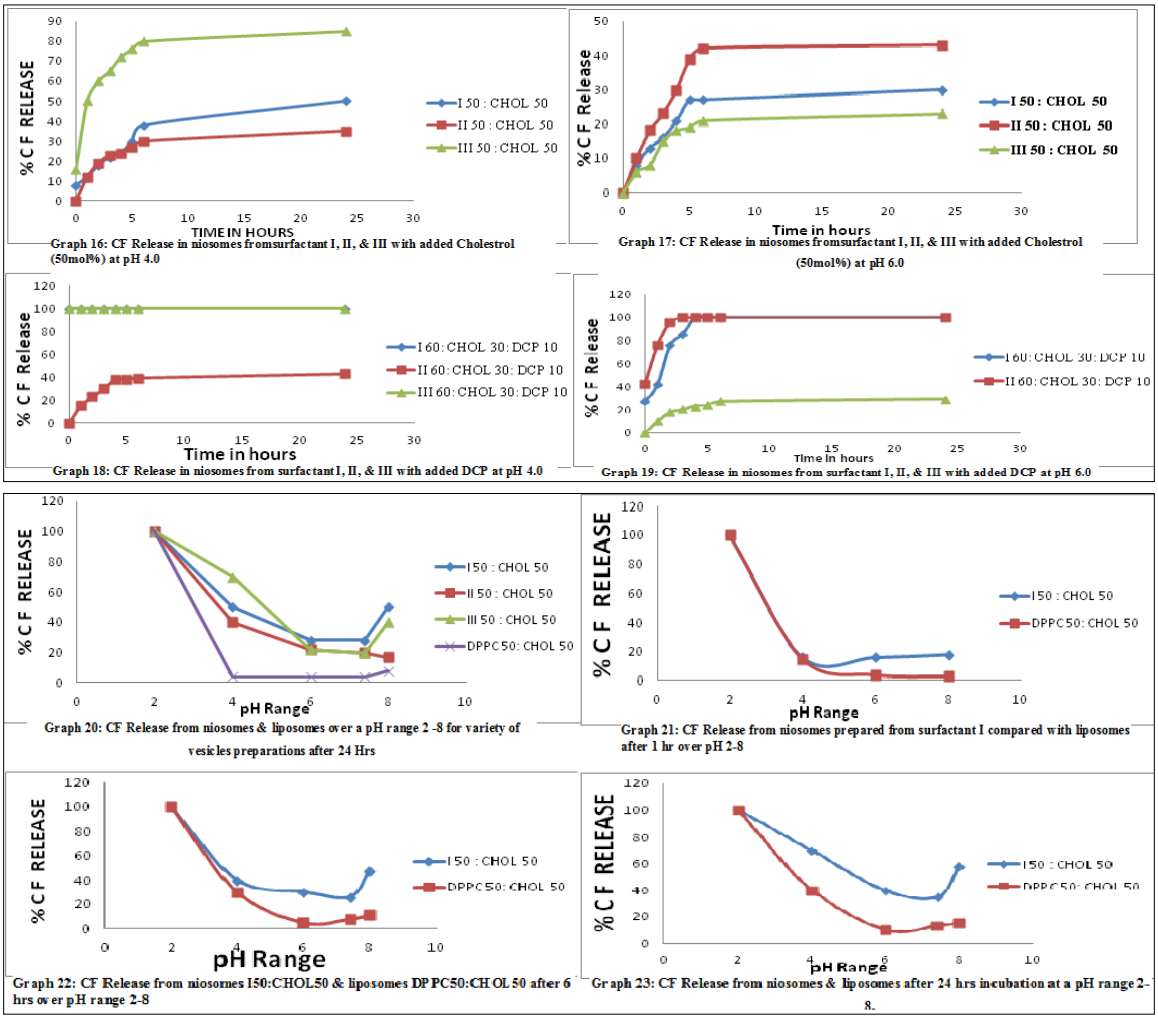
significant difference in the profile of leakage between these two markers was observed over the pH range 2.0-8.0. Therefore, CF was considered to be an adequate marker for these experiments.

The effect of pH on CF leakage from 100% surfactant vesicles of all types is shown in figure graph 12, 13, 14 & 15. For all vesicles examined, CF efflux was most rapid at pH 4.0 and below. Rapid efflux at low pH (4.0 and below) is probably a result of



protonation of the carboxyl moiety of CF (at high H" concentration) which enhances CF diffusion across the bilayer to the external media. However, inclusion of cholesterol in the bilayer appeared to confer a stabilizing effect on the vesicle at pH 4.0 and 6.0, as in graph 16, & 17 and inclusion of DCP increased CF efflux, as in graph 18, & 19. At pH values between 6 and 8, vesicles made from the dialkyl surfactant II showed a greater latency of CF than those prepared with the monoalkyl surfactant III which in turn displayed a higher retention than vesicles composed of the other single chain surfactant I, as in graph 20. This presumably is a consequence of membrane structure and the dialkyl surfactant II molecule might be expected to pack more tightly resulting in an increased barrier to CF efflux. Since 100% surfactant II vesicles could not be prepared, this comparison was only made with vesicles containing 50 mol% cholesterol, as shown in graph 20 legend. However, the highest latency for all vesicles studied was found near pH 74, minima of all graphs in graph 20. The pH of the CF marker at the beginning of the experiment was measured and set at 7.4 and it is envisaged that when the pH of the external media is at 7.4, equilibrium exists reducing loss of CF from within the vesicles. The CF exists as the anion at this pH and charged particles are most probably retained within the vesicle by weak bonding interaction with the membrane.

To study the time course of the pH effect, small known volumes of vesicle suspension were incubated in an appropriate buffer for 1, 6 and 24 hours. As expected the leakage at the lower pH's, 2.0-4.0, was much greater (maxima % CF release in graph 21, 22, 23) than that at pH 6.0-8.0 for all the incubation periods investigated (the minima in graph 21, 22, 23). After 6 hours incubation, graph 22, leakage at all pH's was greater (approximately 30%, as in graph 21 v/s graph 22) and this was more evident after 24 hours, as in graph 23.



Graph 16: CF Release in niosomes fromsurfactant I, II, & III with added Cholestrol (50mol%) at pH 4.0

Graph 17: CF Release in niosomes fromsurfactant I, II, & III with added Cholestrol (50mol%) at pH 6.0

Graph 18: CF Release in niosomes from surfactant I, II, & III with added DCP at pH 4.0

Graph 19: CF Release in niosomes from surfactant I, II, & III with added DCP at pH 6.0

Graph 20: CF Release from niosomes & liposomes over a pH range 2 -8 for variety of vesicles preparations after 24 Hrs

Graph 21: CF Release from niosomes prepared from surfactant I compared with liposomes after 1 hr over pH 2-8

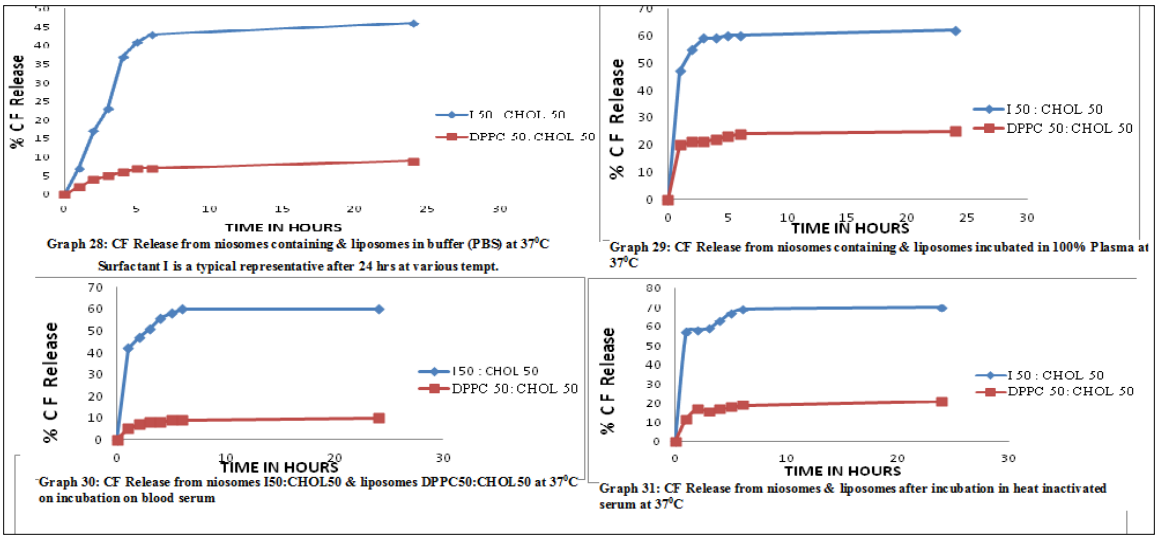
Graph 22: CF Release from niosomes I50:CHOL50 & liposomes DPPC50:CHOL 50 after 6 hrs over pH range 2-8

Graph 23: CF Release from niosomes & liposomes after 24 hrs incubation at a pH range 2-8.

Liposomes follow a similar trend but are more stable, as seen in graph 21, 22, 23. These above experiments were all performed at 37^0C and were measured at appropriate time intervals together with a control for CF to account for any changes in the fluorescence characteristics. The retention of entrapped CF, for all compositions investigated, were significantly less for niosomes than for liposomes.

Effect of Plasma

To optimize the potential of niosomes as drug carriers, it is important to correlate their stability in terms of release of their contents, *in vitro*, with physiological conditions in vivo. Dye release was non-linear with time, when plotted on Cartesian coordinates, graph 28, 29, 30, 31, displaying a substantial rapid component occurring during the first 30-60 minutes of incubation. This initial high leakage rate induced by plasma may be due to disturbances in the bilayers and breakdown of the east stable vesicles. Subsequent recovery of the barrier function may result from formation of a protective outer coat by plasma protein bound to the outermost membrane Similar reports on liposomes [14] suggest a rapid efflux of CF in the presence of plasma for the first 30 minutes, until a plateau is reached. The leakage of CF from vesicles challenged by buffer, 100% human plasma, serum and heat- inactivated serum are compared in graph 28, 29, 30, 31. In these environments, niosomes, I50: CHOL50 and liposomes, DPPC50: CHOL50 (suitable representatives), are distinctly different.



Graph 28: CF Release from niosomes containing & liposomes in buffer (PBS) at 37°C
Surfactant I is a typical representative after 24 hrs at various tempt.

Graph 29: CF Release from niosomes containing & liposomes incubated in 100% Plasma at 37°C

Graph 30: CF Release from niosomes I50:CHOL50 & liposomes DPPC50:CHOL50 at 37°C on incubation on blood serum

Graph 31: CF Release from niosomes & liposomes after incubation in heat inactivated serum at 37°C

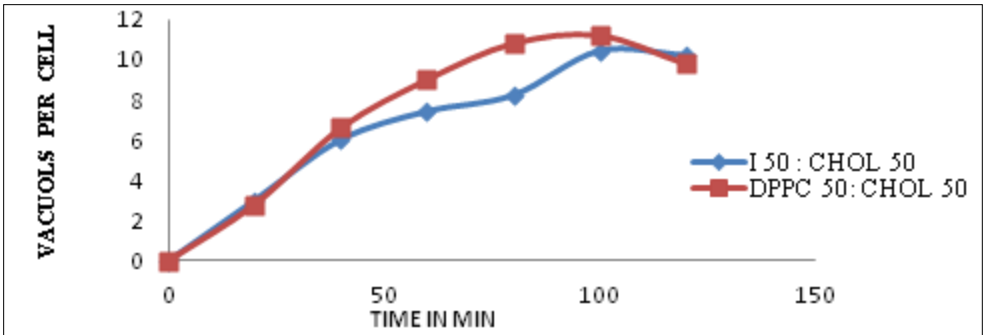
When challenged with serum (graph 29 & 30), a different response was observed. Liposomes, DPPC50: CHOL50 displayed similar leakage kinetics to that in buffer, as in graph 28, when challenged with both serum and heat- inactivated serum and no increase in CF efflux was detected. This indicates that, for liposomes, the major destabilizing factor(s) is present in plasma (100%), as in graph 29. With niosomes, I50: CHOL50, However, even after 24 hours, not all the CF within these niosomes had migrated to the extra-vesicular bulk volume (plateau region of graphs 28, 29, 30, & 31),

Studies with TetrahymenaElliotti Cells

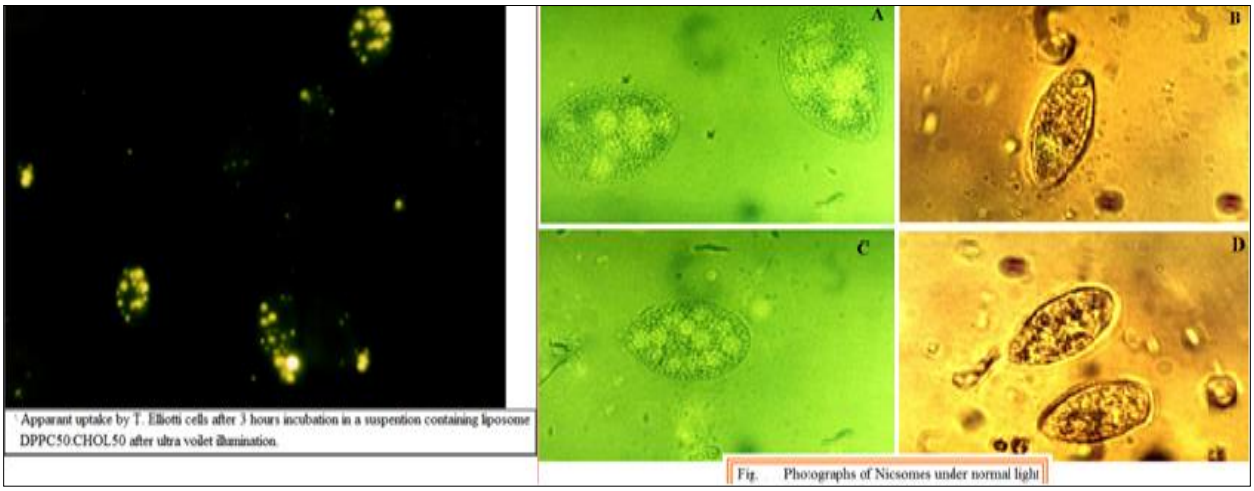
The rate of uptake and subsequent breakdown of niosomes containing surfactants I, II and III and liposomes by the micro-organism T. elliotti were measured. The rate of uptake is expressed as an average number of food vacuoles formed per T. elliotti cell in a given time period. Although there are wide variations in the numbers of ingested vesicles in the food vacuoles between individual cells, the results show a trend and conform to a general pattern. T. elliotti cells used in this study remained fully motile and active in the vesicle suspensions with little or no signs of toxicity after 24 hours. This lack of toxicity was repeatedly noted with every preparation.

The results showed little variation between the rates of uptake for the niosomes of all types and liposomes. This was expected since T elliotti ingests most materials at a steady rate. The vesicles were broken down within the food vacuoles resulting in the appearance of a diffuse intracellular fluorescence, as in graph 32. This occured more readily for liposomes and niosomes prepared from surfactant III, that is, **III100,III50:CHOL50**, and **III70:CHOL20:DCP10**, than for vesicles with surfactants I, for example, **I100, I50:CHOL50** and **I60:CHOL30:DCP10**, or surfactant type II, such as. **II50: CHOL50** and **II60:CHOL30:DCP10**. An explanation for this observation is that Surfactant III is more susceptible to intracellular degradation than the ether linked surfactants I and III. The efflux of vesicular CF into the cytoplasmic space of the T. elliotti cell may also be a function of the low pH within the food vacuole which has been approximated as pH 4.0 to 5.0 (Nuccitelli and Deamer, 1982). Alternatively, intracellular degradation above pH 4.0 may be caused by enzymes, normally used to digest matter within the food vacuoles. These enzymes may be responsible for the apparent vesicle degradation observed *in vivo*, or the CF leakage is a product of the two processes simultaneously.

The uptake of vesicles and eventual release of entrapped CF from vesicles was examined using epifluorescence microscopy. The uptake and breakdown of vesicles was recorded by delayed time sequence photography and was seen clearly, using the self-quenching properties of CF.



Graph 32: Apparent uptake of Niosomes & liposomes by T. elliotti cells over a given time period.



CONCLUSION:

A study of niosomes, prepared from three different non-ionic surfactants, has been compared to liposomes. The niosomes have been fully characterized in terms of their composition, stability and behavior in a variety of systems, by analogy to the well-documented liposome delivery system. The ability of these bodies to interact with biological fluids has been studied thoroughly.

The rate of survival of these vesicles in a living, eukaryotic cell, such as T.elliotti, was a new approach to evaluate their stability within an in vitro system. This organism, which served as a simple model, has a number of lytic enzymes within its digestive tract (food vacuoles) and the observation that niosomes were more stable than liposomes in this environment was encouraging for future development work for specific targeting related to the present model.

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