Research Article

LIPOSOMES: DRUG DELIVERY SYSTEM OR POSSIBLE DOPING AGENT?

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ABSTRACT

The use of liposomes as drug delivery vehicle for treatment of various diseases is well known in medical field but its possible role as a masking agent in sports came into light when Liposom Forte® was found stored together with banned and non-banned drugs during investigations carried out by Italian legal authorities and recent availability of IGF-1 Liposomal Spray on internet. Role of liposomes as masking agent for anabolic steroids in the field of doping has been investigated by Botre et al. The aim of the present work was to study the effect of different parameters like temperature, pH, charge, time, concentration etc. on the interaction of liposomes and doping agents and to identify a possible marker for detection of their abuse in sports. The results showed that out of variety of doping agents, the direct addition of liposomes to urine samples containing anabolic steroids shows strong tendency to interact with the liposomes which results in the reduced concentration of the compound in the sample. However, there was no significant effect of temperature and incubation time on the interaction of liposome and doping agents while other parameters such as charge and concentration of liposome affect the interaction capacity.

Keywords: WADA, Liposomes, Doping

INTRODUCTION:

Liposomes are self-assembled lipoidal vesicles used for encapsulation of drugs in drug delivery system. The hydrophobic drugs intercalate in between lipid bilayer while hydrophilic drugs encapsulated in aqueous core of the liposome. This property of liposomes could be misused by athletes to alter the concentration of drugs in urine sample when liposomes were added externally in urine at the time of sample collection. The concept of interaction between liposome and anabolic steroids has been studied and reported by Botre et al[1, 2]. The present work aimed to ascertain the effect of various parameters on in-vitro interaction of liposomes with various threshold and non-threshold substances prohibited in sports; and secondly to identify a possible marker for the detection of liposome abuse.

MATERIALS AND METHOD:

Reagents and Chemicals

Reference standards were obtained from Sigma-Aldrich, USA, and National Measurement Institute (NMI), Australia. Empty liposomes [Coatsomes EL Series: EL-01-A (Anionic (DPPC: Cholesterol: DPPG=30:40:30 μmol/vial)), EL-01-C (Cationic (DPPC: Cholesterol: Stearylamine=52:40:8 μmol/vial)) and EL-01-N (Neutral(DPPC: Cholesterol: DPPG=54:40:6 μmol/vial))] from Nippon Oil & Fats Co., Ltd (NOF) (Tokyo, Japan), β- Glucuronidase from E. coli (Roche Diagnostics, USA) and other reagents, solvents of HPLC or analytical grade were procured.

Experimental Design

Each vial of freeze dried liposomes was reconstituted as per manufacturer's specification. Interaction of liposomes was studied with approximately 120 WADA Prohibited Drugs. Out of 120 drugs, 5 threshold compounds (19-Norandrosterone, 19-Noretiocholanolone, Morphine, Salbutamol, Carboxy-Tetrahydrocannabinol) and 6 endogenous steroids (Androsterone, Etiocholanolone, Testosterone, Epitestosterone, 5α-androstane-3α,17β-diol (5α-diol), 5β-androstane-3α,17β-diol (5β-diol)) were selected to ascertain effect of various parameters i.e. concentration, pH, time, temperature on the interaction efficiency of liposomes. To study the effect of concentration of liposomes on binding efficiency with various compounds, five different volumes (10-100 µl) of reconstituted liposomes of different types (anionic, cationic and neutral liposomes) were added to urine samples spiked with drugs at threshold level. To determine the effect of pH (pH 5, 7, and 9), time of interaction (24 & 48 hrs) and operating temperature (4 ^{0}C, 37 ^{0}C & room temperature (RT 25±2)), compounds were spiked in urine at their threshold level, 40 µl of reconstituted liposome of different ionic strength was added in each sample. Entire experiments were repeated for five consecutive days. Post reaction samples were screened using in house extraction procedure for anabolic steroids and analysed on Gas Chromatography-Mass Spectrometry (GC-MSD)[3].

Monitoring of Cholesterol

Cholesterol was incorporated in routine method duly validated for quantification. Concentration of cholesterol was measured in 1000 routine samples and samples of liposome interaction experiments.

Data Analysis

With each experiment one spiked sample was prepared and analyzed without addition of liposomes. Recovery of target compounds was calculated by peak area ratios of each analyte to internal standard.

RESULT AND DISCUSSION:

Interaction of Liposomes with drugs:

Out of variety of doping agents, the direct addition of liposomes to urine samples containing anabolic steroids showed strong tendency to interact which results in the reduced concentration of compound in the sample.

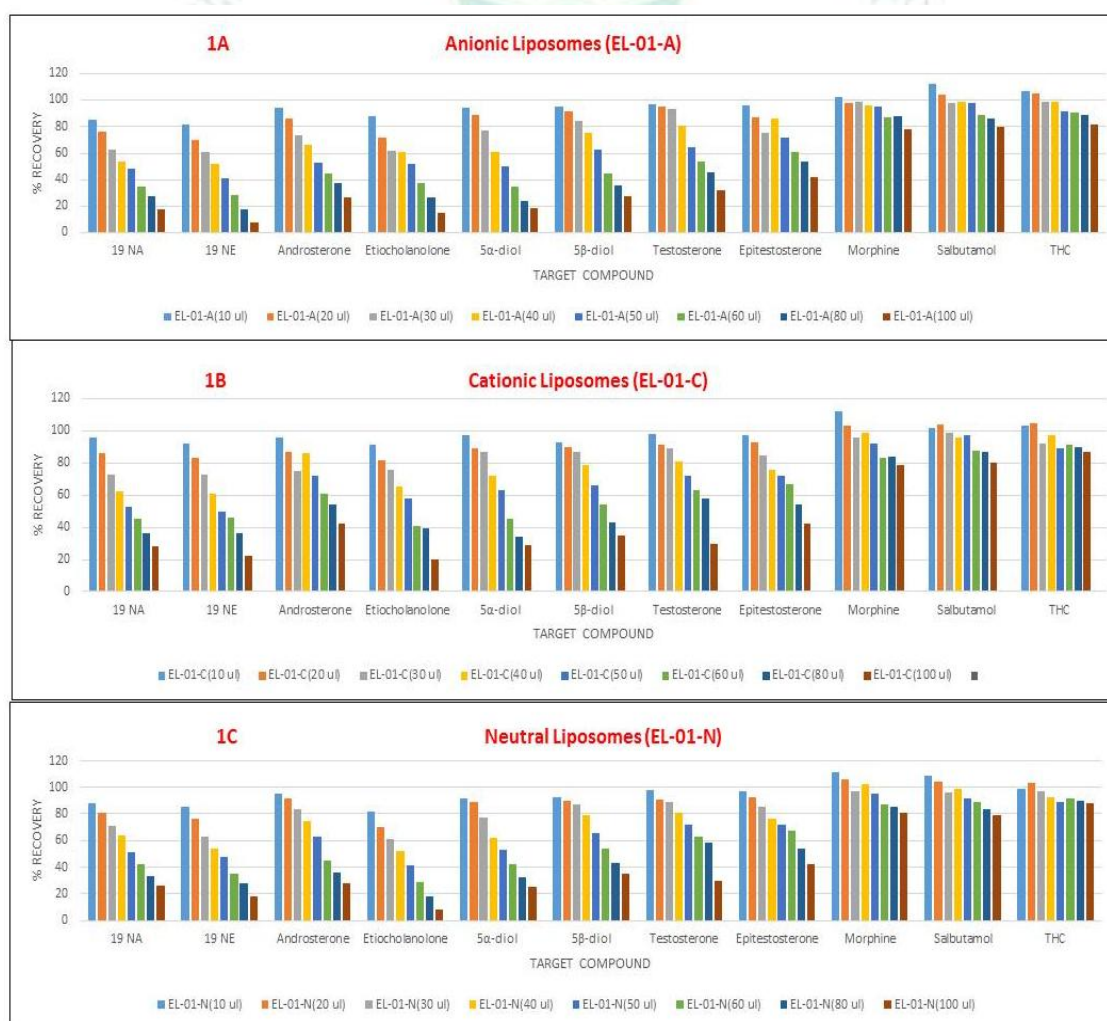### Effect of Concentration of liposomes on binding efficiency:

Figure 1: Effect of increasing concentration of reconstituted liposomes (10-100 µl) (1A-Anionic, 1B-Cationic, 1C-Neutral) on recovery of various drugs

The recovery of various drugs in urine decreased with increasing concentration of liposomes in the urine. Out of three different formulations of liposomes, anionic showed maximum binding capacity with doping agents. However, anabolic steroids showed maximum tendency to interact with liposomes as most of the steroids are derivatives of cholesterol. The interaction of other drugs like morphine, salbutamol and Carboxy-Tetrahydrocannabinol (THC) with liposomes was minimal. (Fig. 1).

Effect of pH on binding efficiency:

Results of urine samples having different pH (5, 7 and 9) spiked with reconstituted liposome (40 µl) and drugs revealed that basic pH facilitated the interaction as recovery of steroids was 5 to 10 % less in basic pH as compared to neutral or acidic pH. Significance of data was calculated by statistical analysis using SPSS 16.0 by applying one-way analysis of variance with post-hoc Bonferroni analysis. P value observed was .001 which is below $P<0.05$, hence considered as significant.

Effect of incubation temperature and time on binding efficiency:

No significant effect of Temperature and time on the binding efficiency of liposomes with doping agents was observed
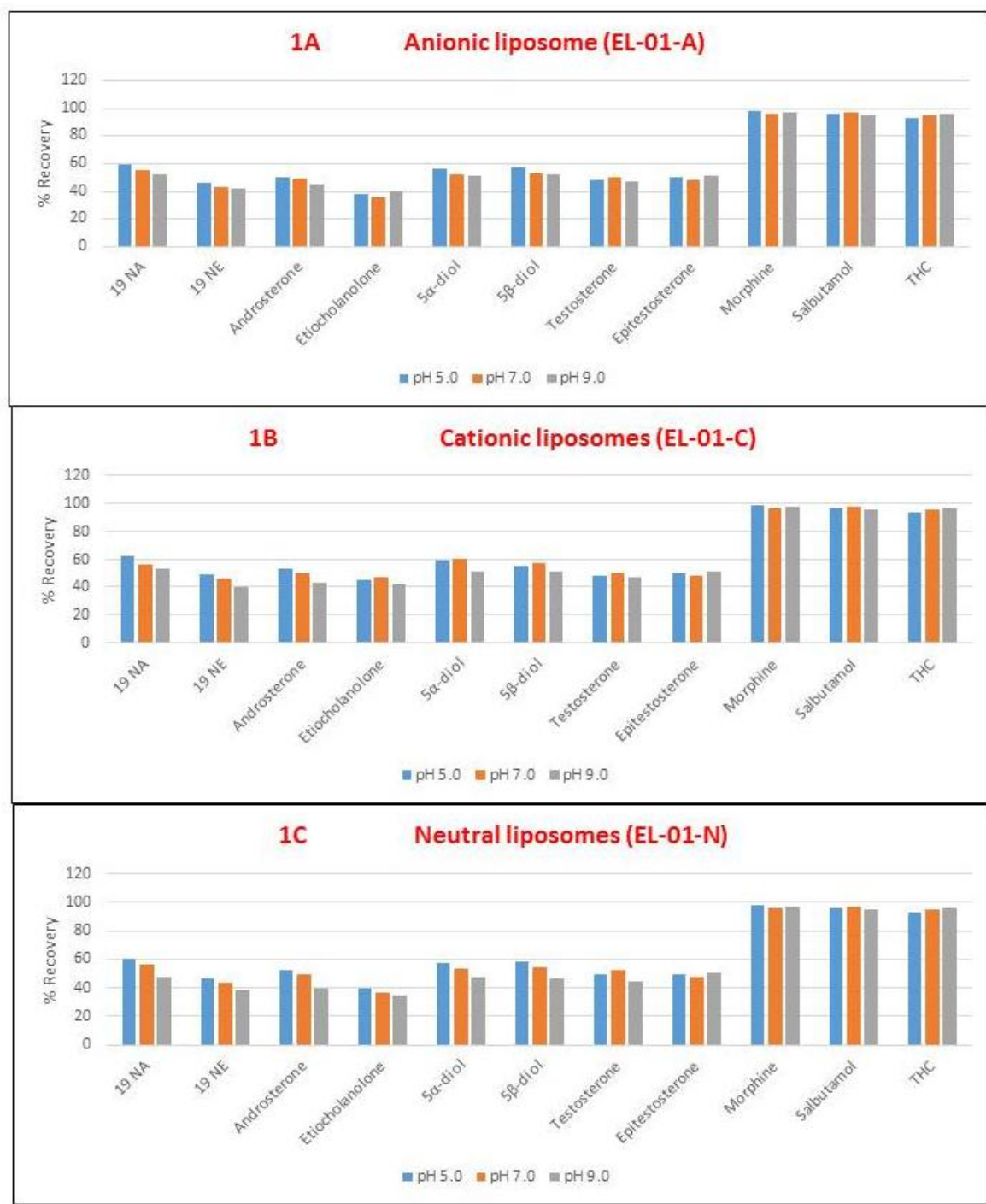

Figure 2(1): Effect of pH on interaction of liposomes (1A-Anionic, 1B-Cationic, 1C-Neutral) with various doping agents
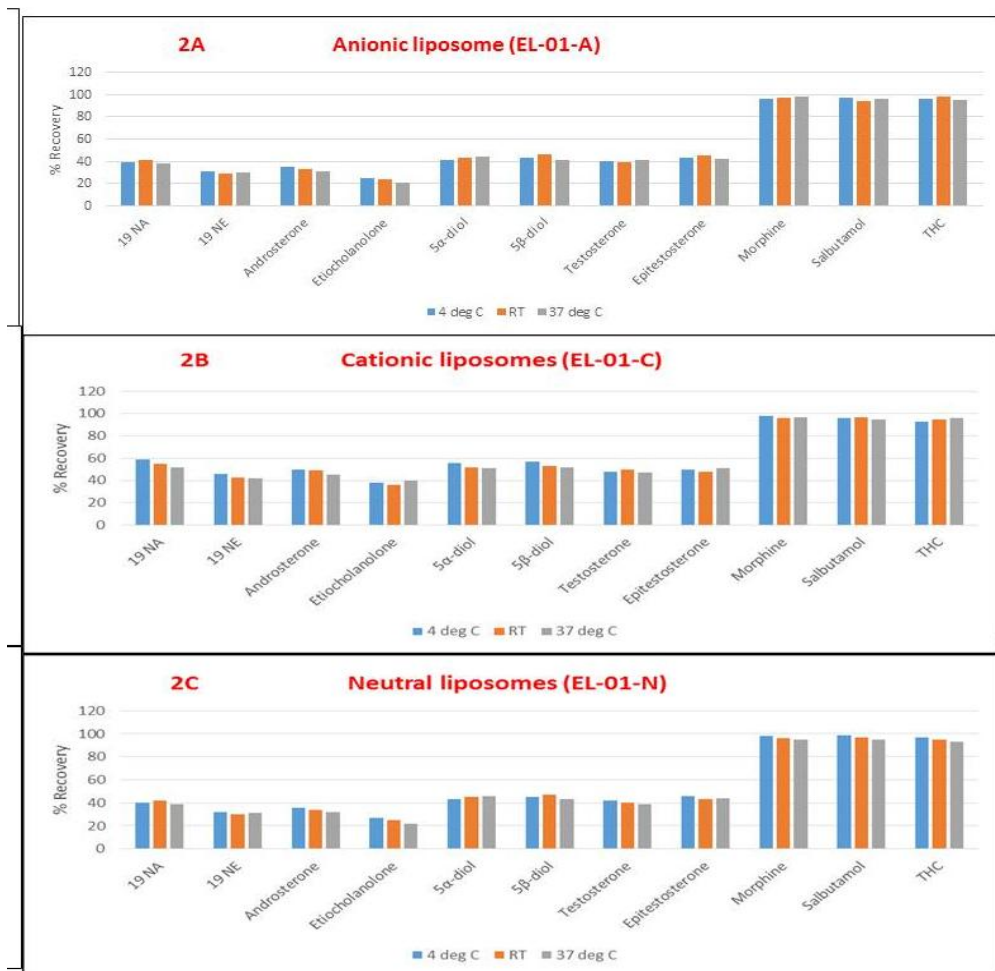
Figure 2(2): Effect of Temperature on interaction of liposomes (2A-Anionic, 2B-Cationic, 2C-Neutral) with various doping agents
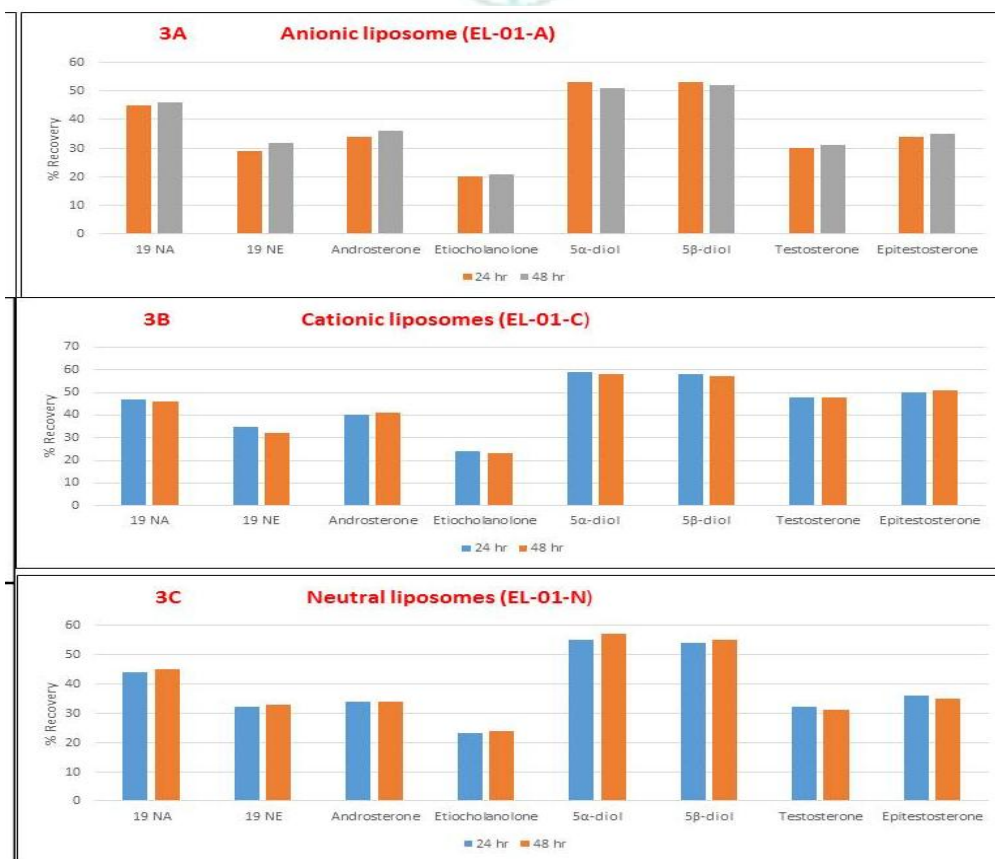

Figure 2(3): Effect of Incubation time on interaction of liposomes (3A-Anionic, 3B-Cationic, 3C-Neutral) with various doping agents

Cholesterol: Possible marker for liposome abuse

It has been reported that cholesterol is main ingredient of liposome. It was assumed that during interaction of liposomes with various drugs, cholesterol should be released. Cholesterol is an endogenous compound and excreted in urine. Its concentration might be increased in various physiological concentration. The concentration of cholesterol was monitored in routine dope samples (n=1000) and was found to be in the range of 45 ng/ml to 376 ng/ml (Fig-3). The concentrations of cholesterol were verified on each vial of liposomes used in the study by direct liposome analysis and during the experiments liposomes were spiked in urine at a concentration of 15µg/ml of cholesterol. Concentration of cholesterol was quantified in blank urine (n=5) and urine spiked with different formulations of liposomes. Results revealed that the concentration of cholesterol increased 4 to 5 folds when same urine samples were spiked with liposome (Fig. 3). The result of this preliminary study postulate that detection of cholesterol in routine doping sample can act as marker for abuse of liposome as masking agent.
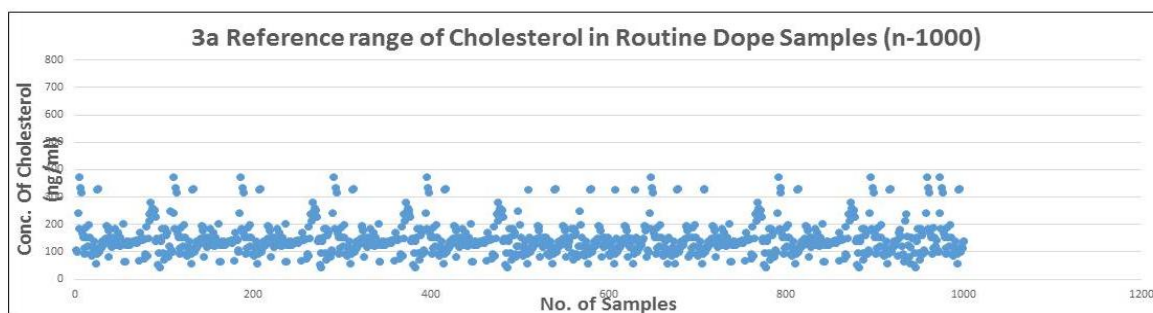

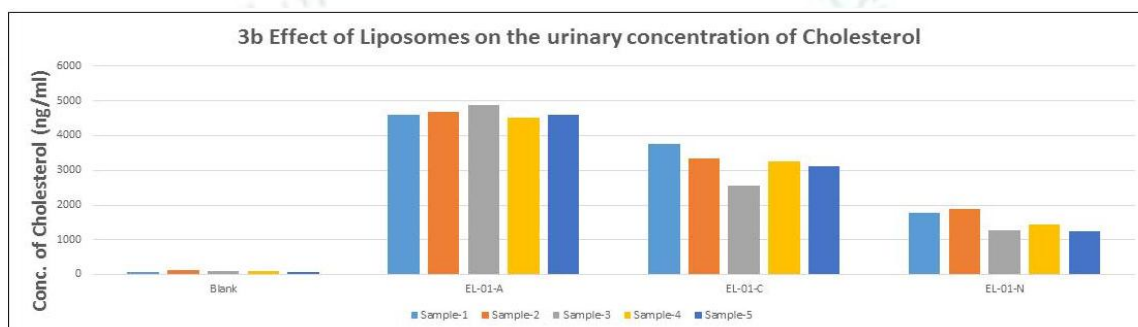**Figure 3a: Concentration of cholesterol in routine dope testing samples (n=1000)**

Figure 3b: Effect of liposomes on the urinary concentration of cholestrol

CONCLUSION:

This study reveals that liposome have the ability to mask different classes of drug in the urine matrix. However, androgenic anabolic steroids showed strong affinity towards liposomes when added in urine. The masking of drugs with liposome is proportional to the concentration of liposome added in the sample. Elevated pH (pH 9) facilitated interaction of liposomes with all classes of drugs studied in the present work.

Cholesterol seems to be a potential marker of liposomes abuse with elevated level recovers in urine spiked with liposomes. However, Cholesterol is endogenously produced and may be a by-product of various physiological conditions therefore an extensive profiling of cholesterol threshold value should be established to be used as direct marker of liposome use.

Acknowledgement

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