

## **Evaluation of the clearance characteristics of liposomes in the human nose by gamma-scintigraphy**

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### **Abstract**

The nasal cavity possesses many advantages as a site for drug delivery, such as, ease of administration, applicability for long term treatments and a large surface area for absorption. One important limiting factor for nasal drug delivery is the limited time available for absorption within the nasal cavity due to mucociliary clearance. Several drug delivery systems including different kinds of microspheres and liposomes have been tried for encapsulation of drugs and increasing the residence time in nasal cavity. In this study the clearance rate of three kinds of liposomes: neutral [phosphatidylcholin (PC) and cholesterol (Chol)], cationic (PC, Chol and stearylamine) and fusogenic (PC, Chol, dioleoylphosphatidylethanolamine) was determined by gamma scintigraphy with lactose powder being used as negative control.

Liposomes were prepared by dehydration-rehydration method. <sup>99m</sup>Tc labeled liposomes were prepared using technetium pertechnetate in the presence of a potent reducing agent, stannous chloride. The labeling procedure was set in a manner that each 150 µl of liposome suspensions contained 2 MBq of radioactivity. Labeling efficiency was calculated by paper chromatography using acetone as mobile phase. Each delivery system containing 2 MBq of activity was sprayed into right nostril of four healthy volunteers and one-minute static views were repeated each half hour until 4 hours. Clearance rates were compared using two Regions of Interest (ROIs); the initial site of deposition of particles, and all of nasopharynx region. The clearance rate of each one of liposomes was calculated after applying the physical decay corrections.

The mean labeling efficiencies for neutral, cationic and fusogenic liposomes were calculated as 91%, 20% and 69%, respectively. The cleared percent of preparations from nasopharynx region after 4 hours was determined as follows: neutral liposomes 18±2.9%; fusogenic liposomes 53.5±1.2%; cationic liposomes 69.7±4.2%; lactose powder 74.5±4.9%. Neutral liposomes showed the lowest clearance rate compared to lactose powder (P<0.0001), followed by fusogenic liposomes (P<0.01) and cationic liposomes (P<0.05). The clearance profiles of formulations from deposition ROI and nasopharynx ROI were identical.

This study shows the neutral liposomes have the highest mucoadhesion properties and are suitable nasal delivery systems. Furthermore, this study proves that limiting step for the nasal clearance of nasally administered particulate systems is their dislocation from the initial site of deposition, and their following interactions with mucus layer in the rest of nasal passage does not significantly affect the clearance time.

**Keywords:** Mucociliary clearance; Liposomes; Gamma scintigraphy; Nasal delivery.

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## Introduction

Liposomes are microscopic vesicles consisting of phospholipid bilayers which enclose aqueous compartments and are utilized as a delivery systems for drugs, both water and lipid soluble, peptides, proteins and DNA (1). The physico-chemical properties of liposomes can influence their utility as a delivery system and vaccine adjuvant. Of variables considered, could be referred to the liquid-crystalline phase-transition temperature ( $T_c$ ) of the lipids, the charge of the lipids, inclusion of cholesterol and liposomes size (2-4).

Neutral liposomes can be prepared by uncharged phospholipids, such as, phosphatidylcholin (PC), and in most cases cholesterol is include to increase the stability of phospholipid bilayers and decrease the leakage of liposomes (3). Cationic liposomes can be prepared by addition of one cationic lipid, such as stearylamine, to the above formula (5, 6). Fusogenic liposomes can introduce their contents into the cytoplasm by fusing with cellular membrane and endosomal membrane. These liposomes are of great importance as delivery systems of membrane-impermeable molecules with biological activities, such as proteins, genes and oligonucleotides. Fusogenic liposomes can be prepared using lipids capable of undergoing a bilayer-to-hexagonal II transition, such as dioleoylphosphatidylethanolamine (DOPE) (7).

In the last few years, different kinds of liposomes have been evaluated as potential intranasal drug delivery systems, but little or no study has yet described the nasal clearance characteristics of these delivery systems in human *in vivo*.

The non-invasive imaging technique of gamma scintigraphy was developed originally for use in diagnostic tests in nuclear medicine (8). Specific radiopharmaceuticals which are localized in different organs and are visualized by gamma camera are used to provide vital information about the structure and function of various body systems. Since about 1980 the technique has been extended to the evaluation of pharmaceutical dosage forms delivered by the oral (9), rectal, pulmonary (10), nasal (11, 12, 13, 14), ophthalmic and vaginal (15) routes.

When used in this manner, the rationale of gamma scintigraphy is that the drug formulation is radiolabelled with a small quantity of an appropriate gamma-ray-emitting radiotracer, and a gamma camera, coupled to a sophisticated data processing system, is used to quantify the behavior of the formulation *in vivo*. This method enables direct visualization and quantification of where the formulation has been delivered, what it is doing, and whether or not it is behaving according to its proposed rationale (8). According to a recent comprehensive review of the subject (16): "Gamma scintigraphy has become the method of choice for investigating the fate of pharmaceutical [dosage] forms in the body". When gamma scintigraphy is used in the assessment of nasal drug delivery, the formulation is usually labeled with the gamma-ray emitting radionuclide  $^{99m}\text{Tc}$ , which has an ideal radiation energy (140 keV) for use with a gamma camera (8). The short half-life of  $^{99m}\text{Tc}$  (6 h), coupled with a very 'clean' radiation emission profile which contains few beta-particles, results in very low radiation doses, so that satisfactory scintigraphic data can be obtained using only a fraction of the radiation dose required for diagnostic X-ray procedures (8).

The primary aim of this study was to investigate the clearance characteristics of different liposomes from the human nasal mucosa. This paper describes the characterization and radiolabeling of three bioadhesive nasal delivery systems: neutral, cationic, and fusogenic liposomes. The clearance characteristics of these liposomes after nasal administration to human volunteers were investigated using the technique of gamma scintigraphy. In this study lactose powder was used as negative control.

## Experimental

### Materials

Stearylamine (SA) and phosphatidylcholin (PC) were purchased from Fluka (Buchs, Switzerland). Dioleoylphosphatidylethanolamine (DOPE) was purchased from Sigma (USA). Cholesterol (Chol) was purchased from Merck (Darmschadt, Germany).  $^{99m}\text{Tc}$ -pertechnetate was provided by AEOI (Atomic Energy Organization of Iran).

### Preparation of liposomes

Liposomes were prepared as dehydration-rehydration vesicles (DRV) (17) with following lipid compositions: PC/Chol (16.5  $\mu$ mole from each one, neutral liposomes), PC/Chol/SA in a molar ratio of 7:7:1 (cationic liposomes) and PC/Chol/DOPE in a molar ratio of 7:7:1 (fusogenic liposomes). Briefly, the lipid phase was dissolved in chloroform:methanol; 2:1, v/v in a round-bottom flask. The solvent was removed by rotary evaporation resulting in the deposition of a thin lipid film on the walls. This lipid film was then freeze-dried (Heto Drywinner, DW3, Heto-Halter, Allerod, Denmark) overnight to ensure total removal of the solvent. The lipid film was hydrated with distilled water at 45°C and vortexed for 30 min. The resulting multilamellar vesicles (MLVs) were converted to small unilamellar vesicles (SUVs) using probe-type sonicator (Soniprep-150, MSE, Sussex, UK). The resulting SUVs flush was frozen in liquid nitrogen and freeze-dried overnight. The dried broken liposome powder was rehydrated at 45°C for 30 min with distilled water, using a volume equivalent to one-tenth of the total SUV used. Rehydration was aided by gentle vortexing. The liposomes were then diluted with PBS (phosphate buffered saline).

### Size analysis of liposomes

The volume mean diameters of liposomes were determined by a laser diffraction size analyzer (Zetasizer 2000, Malvern, UK).

### Radiolabeling procedure of liposomes

The radiolabeling procedure was carried out in the presence of powerful reducing agent, stannous chloride. The stannous ion reduces  $^{99m}$ technetium from the +7 oxidation state to the more reactive +5 oxidation state to promote binding. The electron donating functional groups, for example the hydroxyl groups of phospholipids, may accept the technetium (11).

The radiolabeling method for liposomes (neutral, cationic and fusogenic) was adopted from procedure described by Saari et al. (10). Briefly, 1.25 ml of the liposome suspension was mixed with 0.6 ml of 3 mM SnCl<sub>2</sub> solution. Then 1.25 ml of technetium pertechnetate in sterile saline was added; the mixture was shaken

vigorously for 1 min and left to react at room temperature for 30 min. The activities were calculated such that 100  $\mu$ l of each liposome suspension would have 2 MBq of activity at the time of administration.

The lactose powder was labeled and used in *in vivo* studies as a negative control. Fifty mg lactose powder was desolvated in labeling media containing 1.5 ml of normal saline, 1 ml 5mg/ml SnCl<sub>2</sub>.2H<sub>2</sub>O and 0.7 ml technetium-99m pertechnetate eluate containing about 6 MBq of activity and incubated for 10 min, followed by addition of 10 ml acetone. The labeled lactose was desolvated and precipitated in presence of acetone. Supernatant was decanted and powder was washed with acetone and dried in 60 °C for 30 min (14).

### Determination of labeling efficiency of liposomes

The labeling efficiency was determined by paper chromatography using acetone as mobile phase. After labeling process of liposomes and before washing step, liposomes suspension samples were placed on chromatograph paper. In this system, free pertechnetate migrates to the top of the paper, while liposome attached material remains at the application point. The labeling yield was expressed as a percentage of the total amount of radioactivity applied in the testing system (10, 14).

### In vivo nasal clearance studies

About 5 mg labeled lactose powder or 100  $\mu$ l of liposomes suspensions, containing 2 MBq of radioactivity was administered into the right nostril of 4 healthy human volunteers (male, 20-30 years of age, mean weight of 65 Kg). Volunteers completed a questionnaire about their health and excluded if they smoked, had a history of respiratory allergic conditions or taken any nasal medication within the last month. The study was approved by the regional ethical committee.

The powders were administered intra-nasally using polyethylene tubes, filled with 5 mg of powders. The powders were released from the tubes using a syringe containing 5 ml of compressed air. The suspension samples were sprayed using a Miacalcic® mechanical sprayer.

**Table 1.** Mean diameters ( $\pm$ SD, n = 3) and labeling efficiencies of liposomes

Preparations	Liposomes		
	Neutral	Cationic	Fusogenic
Mean diameter ( $\mu$ m)	2.3 $\pm$ 0.6	3.4 $\pm$ 0.6	4.3 $\pm$ 0.2
Labeling efficiency	91%	20%	69%

The volunteers were trained to abstain from sneezing and blowing their nose (14). Data obtained from volunteers that sneezed during the studies were discarded.

The deposition, distribution and subsequent clearance of liposomes and lactose powder was followed by gamma scintigraphy, using a SMV Sofa Gamma Camera (General Electric) fitted with a low energy collimator. Static right lateral views (60 s duration) of the head were recorded in 30 min intervals for 4 hours. The position of the head of the volunteer was fixed on the collimator of the gamma camera using a specially designed template. The camera-to-patient distance was standardized by placing the collimator close to the head of volunteers (14).

Quantification of the data from the volunteers involved defining regions of interest around the desirable areas. Two region of interests (ROIs) were drawn, the first one around the initial site of deposition of the particles in the nasal cavity and the second one around all nasopharynx region to throat. The count rate from each region of interest (ROI), corrected for radioactive decay and background, was then expressed as a proportion of the highest 1 min count rate, typically the image recorded in the nasal cavity ROI immediately after dosing. The highest count rate was assigned a 100% value, which was then used to calculate the percentage remaining for the other time points. In this way the clearance of the formulations from the nasal cavity was evaluated as a decrease in percentage activity against time for each volunteer (14).

#### *Statistical analysis*

Statistical analysis of the results was carried out using unpaired Student t-test.

## **Results and discussion**

#### *Size analysis of liposomes*

All three kinds of liposomes were heterogeneous in size, ranging from 0.5 to 5.0  $\mu$ m

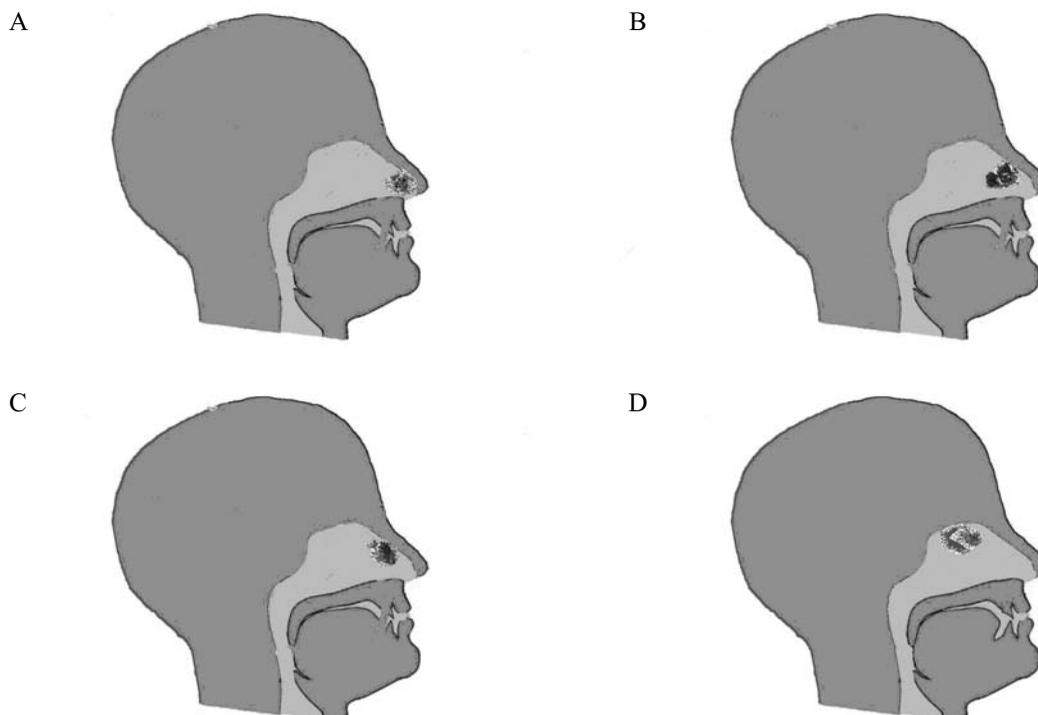
**Table 2.** Percent of preparations remained in the nasopharynx and deposition ROIs after 4 h ( $\pm$ SE, n = 4)

Preparations	Liposomes			Control
	Neutral	Cationic	Fusogenic	Lactose powder
Nasopharynx ROI	72.0 $\pm$ 2.9	30.3 $\pm$ 4.2	46.5 $\pm$ 1.2	25.5 $\pm$ 4.8
Deposition ROI	62.6 $\pm$ 4.3	23.6 $\pm$ 3.8	35.7 $\pm$ 1.6	11.5 $\pm$ 4.6

with volume mean diameters of 2.3 $\pm$ 0.6, 3.4 $\pm$ 0.6 and 4.3 $\pm$ 0.2 for neutral, cationic and fusogenic liposomes, respectively (Table 1). Regards to the differences between liposome average size, there was no significant ( $p > 0.05$ ) difference between neutral and cationic liposomes. However, the average size of fusogenic liposomes was a little bit more than neutral and cationic liposomes. The reason for this could be due to fusogenic potential of these liposomes which could fuse to each other and make bigger liposomes. In general, liposomes prepared by DRV method are multilamellar vesicles and heterogeneous in size with average of around 2  $\mu$ m (17). In this study, even though the average liposome sizes were a little bit different, however the size ranges were almost the same and liposomes with similar pattern of size ranges were used. As a nasal drug and antigen delivery system, one of the most important characteristics of liposomes is their particle size. Despite an expanding body of information concerning the uptake and distribution of microparticulate materials following peroral administration, comparatively little is known about the fate of nasally delivered particulates (18). Some studies have demonstrated that nasally applied latex microspheres could rapidly enter the blood circulation, and hence access systemic immunoresponsive tissues in the spleen, indicating that microparticulates are translocated through the nasal epithelium (19). Particles larger than 3  $\mu$ m in diameter have been shown in humans to be retained in the nasal cavity when inhaled (20) and it has been observed in calves that tonsils could absorb resin particles of 1-5  $\mu$ m in diameter (21).

#### *Labeling efficiency of liposomes*

The labeling efficiency of liposomes was determined by paper chromatography using acetone as mobile phase (Table 1). Labeling efficiencies listed in table 1 are lower than the labeling efficiencies reported by other groups (mostly  $>90\%$ ). The possible reason for these



**Figure 1.** The deposition sites of liposomes (neutral liposomes (A), cationic liposomes (B), fusogenic liposomes (C)) and lactose powders (D) in the nasal cavity of volunteers. Each image represents the pictures of deposited particles in the nasal cavity of four volunteers, which are over-layered together and superimposed on a schematic diagram.

low labeling efficiencies could be the generator of technetium which was old. The meta-stable radioactive technetium ( $^{99m}\text{Tc}$ ) is decayed to stable non-radioactive form ( $^{99}\text{Tc}$ ). This stable form can also react with electron donating groups and compete with radioactive technetium; therefore, high concentration of non-radioactive technetium in old generators eluates can decrease the binding potential of radioactive technetium.

The very low labeling efficiency (20-25%) of positively charged (cationic) liposomes can be attributed to the charge repulsion of  $-\text{NH}_3^+$  groups of stearylamine molecules for technetium for efficient interaction with liposomal surface electron donating groups.

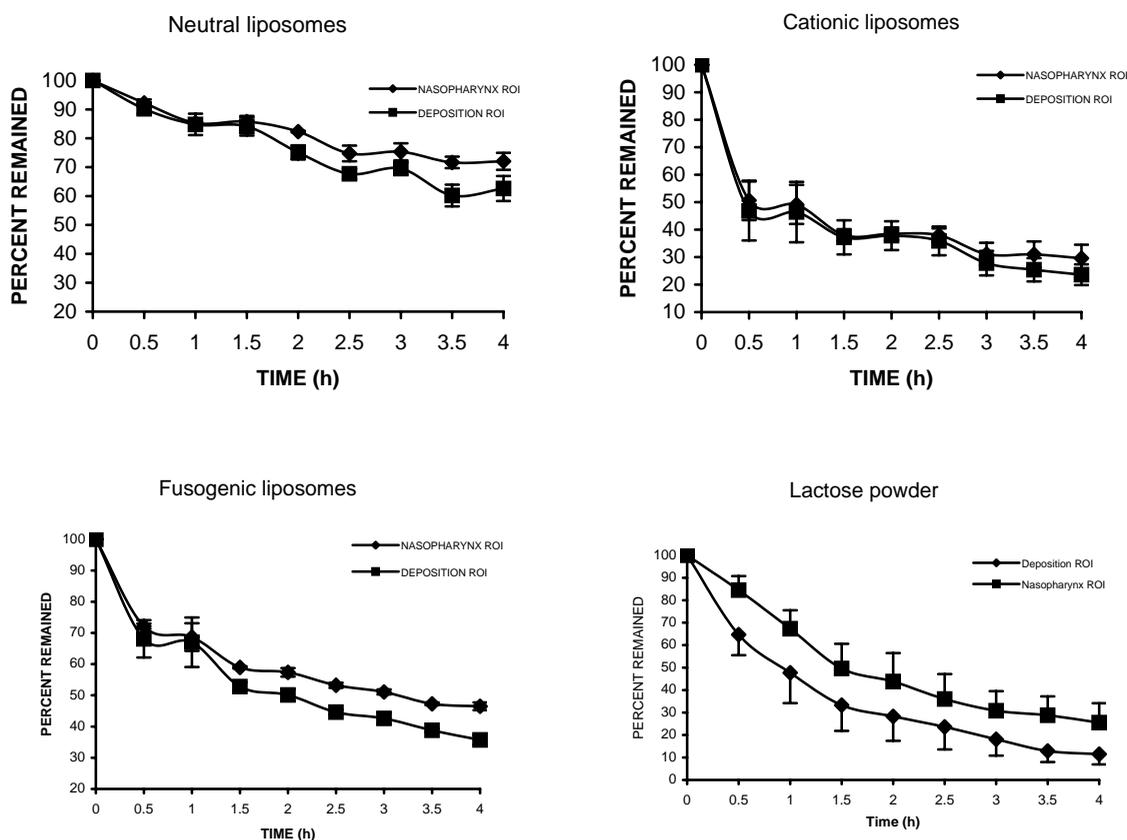
#### **In vivo nasal clearance studies**

The nasal clearance characteristics of four liposome drug delivery systems, potentially applicable as nasal drug and antigen delivery systems were studied. Lactose powder was used as the negative control. The deposition sites of liposomes and lactose powders in the nasal cavity of volunteers was visualized by superimposing of deposition ROIs of volunteers on each other

and on a schematic diagram of skull (Fig. 1). The averaged clearance data for each formulation from the nasopharynx and deposition ROIs can be seen in figure 2. The percent of the formulations cleared from the nasopharynx and deposition ROIs, in the time course of study (4 h) was shown in table 2. This data shows that the control lactose powder was cleared rapidly (half-life of nasopharynx clearance was 1.5 h), whereas the liposomes were retained within the nasal cavity for extended periods of time (half-lives of nasopharynx clearance were  $>3$  h, except cationic liposomes).

It has been reported that the normal half-life of nasal clearance in man is about 20 min (22). The nasal clearance half-lives of liposomes and lactose powder were extremely higher than normal clearance half-life of human nose (at least four-fold higher), which is representative of high mucoadhesive strength of these particulate systems.

In previous studies, the clearance of technetium labeled liposomes in mucosal surfaces proved to be strikingly slow (23-25). Vidgren et al. (25) monitored the clearance of



**Figure 2.** The clearance characteristics of radiolabelled formulations from nasopharynx region and initial region of deposition of particles in human nose. Error bars represent the SE (n = 4).

<sup>99m</sup>Tc-labelled Bec-DLPC liposomes in healthy volunteers in whom 93% of the original dose was still detected in the lungs after 3 h. In a similar study, Farr et al. (23) measured the deposition and clearance of DPPC liposome aerosol after inhalation by normal volunteers. Subjects were monitored for 6 h after inhalation; 88% of the inhaled radioactivity was still present in the lungs. Saari et al. (10) have reported that the clearance of liposome-bound <sup>99m</sup>Tc was strikingly slow and the clearance kinetics was similar in both groups of liposome formulations.

The bioadhesion of multilamellar liposomes bearing nifedipine with different charged component was studied by Vyas et al. (6). An in situ bioadhesion study using the incised nasal cavity of rat was used for evaluation of bioadhesion of neutral, cationic and anionic liposomes. In this study maximum bioadhesion was shown by cationic liposomes followed by neutral liposomes while no bioadhesion was seen by anionic liposomes.

In our study the least clearance rate from

nasopharynx ROI was shown by neutral and followed by fusogenic and cationic liposomes (Figure 3).

The clearance of inhaled materials from the nasal cavity of man has been shown to follow a biphasic pattern (26-28). This biphasic pattern is the result of an initial fast rate of clearance of material from the ciliated regions of the nose, followed by a comparatively slow second phase of clearance associated with material deposited on the non-ciliated anterior region of the nose (13).

Referring to clearance from deposition ROI, the neutral liposomes, which had the least clearance rate, showed a monophasic pattern of clearance and other kinds of liposomes have a biphasic pattern. Regards to figure 1, the deposition area of neutral liposomes is more anteriorly than other liposomes. Among the biphasic cleared liposomes, the highest first-phase cleared one is cationic liposomes. Therefore it seems that in the present study the clearance rate of liposomes from deposition

ROI is mainly affected by deposition region and charge interaction between liposomes and mucus layer doesn't have a determinantal role.

It has been shown that the nasal clearance rate of preparations is affected by their deposition site in nasal cavity (29, 30). A drug deposited in the nose posteriorly is cleared more rapidly from the nasal cavity to the nasopharynx than a drug deposited anteriorly, because the mucociliary clearance is slower in the anterior part of the nose than the more ciliated posterior part (22).

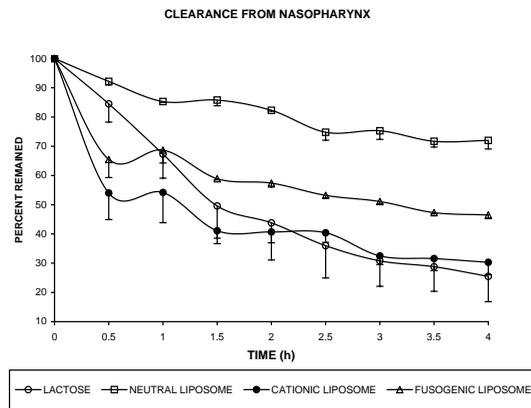
It has been reported that the particle size distribution of droplets or powders administered to the nasal cavity will affect deposition in and hence clearance from the nasal cavity (26, 31, 13). It has been suggested that 4  $\mu\text{m}$  is a sufficient particle size for intranasally administered drugs (32). On the other hand, Illum et al. (29) have considered that 10  $\mu\text{m}$  is the most suitable particle size for nasal administration. Particles smaller than 1  $\mu\text{m}$  pass the nasal cavities with the inspired air, whereas particles larger than 10  $\mu\text{m}$  deposit at the anterior parts of the nose and thus avoid ciliated absorption areas (29, 33).

In the present study, all of particulate systems had mean diameters of 2.3-4.3  $\mu\text{m}$ . As it is shown in figure 1, except neutral liposomes, which have been deposited in more anterior parts of the nasal cavity, other preparations had nearly the same deposition areas.

Factors such as type of formulation (solution vs. powder), administration device and aerodynamic properties of the liquid droplets or powders can affect insufflation and deposition patterns, and ultimately mucociliary clearance, especially with the presence of mucoadhesive polymers (30). The site of drug deposition in the nose is also highly dependent on the dosage form. Nasal sprays deposit drugs more anteriorly, resulting in a slower clearance of sprays than drops (26).

In this study, two different types of formulations (suspensions and dry powders) and two different devices (pressurized air for powders and mechanical sprayer for suspensions) were used, but resulting deposition areas for both of formulations and administration devices were nearly the same.

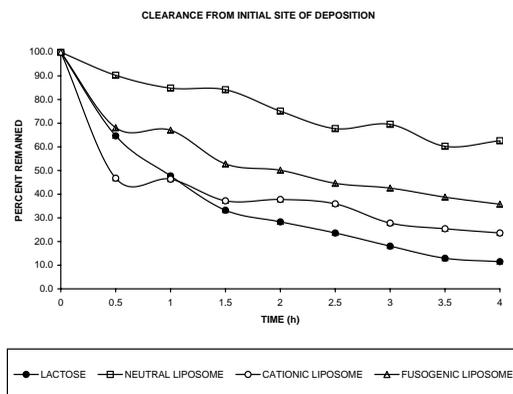
In every study a certain amount of the preparations was not cleared within the study period. This was probably the quantity deposited



**Figure 3.** The clearance characteristics of radiolabelled liposomes (neutral, cationic and fusogenic) from the human nasopharynx region as compared to lactose powders as control. Error bars represent the SE (n = 4).

at the less or not ciliated anterior part of the nasal cavity. As a result, its clearance was very slow. Such regional differences in the clearance rate of intranasally administered particles have previously been noted (27, 30). Referring to the remaining percent of formulations in the deposition ROI (table 2), the most remaining percent belongs to the neutral liposomes ( $62.6 \pm 4.3$ ) that have been deposited most anteriorly in the nasal cavity, compare to other formulations.

Two ROIs was drawn around the initial site of deposition of preparations and the whole nasopharynx region. Comparing the clearance profiles from the two ROIs could result in some ideas about the rate limiting step in nasal clearance of preparations. As it has been shown in figure



**Figure 4.** The clearance characteristics of radiolabelled liposomes (neutral, cationic and fusogenic) from their initial region of deposition in human nose as compared to lactose powders as control. Error bars represent the SE (n = 4).

2, the clearance patterns of studied particulate systems from both ROIs were nearly identical, indicating that the main time consuming step in the clearance of these particulate systems is their displacement from initial deposition site. As soon as the particles are dislocated from their deposition area, they are passing the remainder of the nose passage very soon. Therefore, it could be realized that rate limiting step for the nasal clearance of nasally administered particulate systems is their dislocation from the initial site of deposition and their following interactions with mucus layer in the rest of nasal passage does not significantly affect the clearance time. This interesting observation was not affected by dosage form, administration device or even initial site of deposition.

It can be concluded that all of particulate delivery systems showed high mucoadhesion strength compared to the normal clearance time. Powders and suspensions were administered using different devices but deposition areas of both kinds of dosage forms and devices were nearly identical. Among preparations, the least clearance rate from nasopharynx region was shown by neutral liposomes. The highest clearance rate was shown by cationic liposomes followed by fusogenic, and neutral liposomes. The clearance profiles of preparations from initial site of deposition and all nasopharynx region, disregard from dosage form and administration device, were identical. Therefore, it could be concluded that as soon as deposited particles are dislocated from their deposition area, they rapidly cleared and their following interactions with mucus layer in the rest of nasal cavity doesn't have a significantly role in total clearance time.

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