

## Transnasal nimodipine-loaded mucoadhesive nanoliposomes: Preparation and *in-vitro* evaluation

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### Abstract

**Background and objective:** Nimodipine is effective in the treatment of various cerebrovascular impairment, but its clinical potential is limited due to several undesirable characteristics such as low bioavailability which caused by first pass effect in the liver and low aqueous solubility. The main purpose of this study was to prepare nimodipine-loaded nanoliposomes for intranasal delivery and performing *in-vitro* studies.

**Methods:** The nimodipine loaded nanoliposome formula was prepared by ethanol-based proliposome method and characterized in term of particle size and size distribution, zeta potential, entrapment efficiency, and permeability studies. In addition, mucoadhesive nanoliposomes loaded nimodipine was prepared using chitosan as a mucoadhesive agent.

**Results:** The *in-vitro* studies explored particle size increased ( $122.48\text{nm} \pm 0.002$ ) for chitosan coated formulation after sonication in comparison to non-coated formulations ( $114.09\text{ nm} \pm 0.025$ ). Also, the zeta potential was positive for the sonicated chitosan-coated formulation ( $5.286\text{ mV} \pm 0.341$ ) while for non-coated formulations with chitosan were found to be negative ( $-1.317\text{ mV} \pm 0.153$ ). The entrapment efficiency ( $76.033\% \pm 0.094\%$ ) and drug permeability ( $2.53\text{ }\mu\text{g/ml}$  in the duration of 240 min) were significant for chitosan-coated liposome compared to other formulations.

**Conclusion:** This study concludes that chitosan glutamate (PROTASAN<sup>®</sup> UPG213) coated nimodipine-loaded liposomes can be considered as a promising novel formulation for an efficient intranasal delivery of nimodipine. Additionally, changes in the size of liposomes and zeta potential confirmed the existence of a coating layer on the surface of liposome pellets. Chitosan was found to significantly enhance the drug entrapment and could also be considered as a permeability enhancer.

**Keywords:** Mucoadhesive; Nasal; Nimodipine; Nanoliposome.

### Introduction

Nasal drug delivery will eventually grow and emerge as a potential alternative to various routes of administration of numerous drugs. The use of nasal route is highly advantageous for the delivery of drugs in the treatment of local and systemic diseases.<sup>1</sup> It is a non-invasive route which is considered as an alternative, especially for the systemic drugs which have problems such as low bioavailability or enzymatic degradation when administered through the oral route.<sup>2</sup> The administered drugs through nasal route have the ability to reach the central nervous system and

bypassing the blood brain barrier.<sup>1,3</sup> Using suitable pharmaceutical carriers such as micelles, nanoparticles, microspheres, or liposomes are necessary to achieve good bioavailability. Among them, liposomes have shown high potential for intranasal administration of drugs.<sup>3-5</sup> Liposomes are spherical vesicles and have the tendency of entrapping peptide, protein, and various drug molecules. The limitation of liposome is easily removed by mucociliary clearance after nasal delivery.<sup>4</sup> The mucociliary clearance can be however slowed by the use of polysaccharides which have the ability to prolong the contact time between

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drug and nasal mucosa, thus promoting drug absorption.<sup>6,7</sup> Among the various polysaccharides, chitosan, and its derivatives are widely used as a mucoadhesive agent,<sup>8</sup> due to their capability of providing strong mucoadhesion via binding with sialic acidic residues of mucin molecules.<sup>9,10</sup> Upadhyay et al. developed a formulation of Quetiapine Fumarate loaded nanoliposomes for better diffusion and administration through nasal route to the brain. *In vivo* studies were carried out in mice demonstrated intranasally administered Quetiapine Fumarate loaded nanoliposomes resulted in higher brain level of the drug compared to simple dispersion.<sup>3</sup> Wang and coworker in 2006 showed nimodipine loaded liposome produces higher and more stable plasma and cerebral drug concentrations compared with nimodipine solution after intravenous administration.<sup>11</sup> Nimodipine is a 1, 4-dihydropyridine calcium antagonist which mainly dilates the cerebral arterioles and thus increases cerebral blood flow in animals and humans.<sup>11,12</sup> It has high potential role in the treatment of various cerebrovascular disorders such as migraine, cerebrovascular spasm, and stroke.<sup>13</sup> Clinical studies of Nimodipine has shown low oral bioavailability of about 4-13% in healthy subjects due to the poor aqueous solubility of the drug (3.86 µg/ml) and high first-pass effect in liver.<sup>14,15</sup> This study aimed to design a novel formulation of nimodipine loaded nanoliposomes using ethanol-based proliposome method. Parameters involved in the preparation were investigated to optimize the loading efficiency of nimodipine in nanoliposomes using two different concentrations of lipid and to establish an optimum formulation for nasal administration. Furthermore, the investigations were carried out for another batch, which included the mucoadhesive polymer(chitosan glutamate) to study its influence on the liposome formulation.

## Methods

### Materials

Nimodipine was purchased from Apollo healthcare, Singapore. Soya phosphatidylcholine (SPC) was obtained from Lipoid, Switzerland. Cholesterol (analytical grade) was purchased from Sigma-Aldrich, UK. All HPLC grade solvents (i.e., water, methanol, 96% ethanol and acetonitrile) were purchased from Fisher Scientific Ltd., UK. Chitosan glutamate (PROTASAN<sup>®</sup> UPG213) was obtained from Novamatrix, FMC BioPolymers, Norway. Ultra-thin silicone membrane was purchased from J-flex, UK.

### Methods

#### Preparation of nimodipine-loaded liposomes

The method of ethanol-based proliposome was modified from that of Elhissi et al. (2006) by using 4:1 w/w of lipid to cholesterol ratio, and increasing the amount of ethanol to lipid ratio in order to enhance the dissolution of lipid. Liposomes containing nimodipine were prepared by incorporating two different lipid concentrations (low lipid and high lipid concentration). For low lipid formulation, pellets of liposome were prepared by adding 228 µl absolute ethanol into a 15 ml glass vial containing 120 mg lipid of Soya phosphatidylcholine (SPC) at 70°C to enhance dissolution of cholesterol (Table 1), SPC and cholesterol (4:1 w/w) were used in both low and high lipid formulation. Cholesterol was added to increase vesicle membrane rigidity and to prevent drug leakage. While for high lipid formulation, 240 mg of lipid (SPC) was dissolved in 456 µl of ethanol. Nimodipine (30 mg) was then added to the lipid solution at 70°C. Drug lipid solutions then added to the vial which containing cholesterol (30 mg for low lipid and 60 mg for high lipid formulation), and the temperature was kept at 70°C using a water bath. To this drug lipid solution, 1.5 ml of HPLC water was added (primary hydration step) and agitated for 2 minutes

using a WhirlMixer TM (Fisher Scientific, UK) to achieve concentrated suspension of liposomes. Liposomes were produced by adding the rest of the aqueous phase, which was 11.5 ml of water plus 2 ml of phosphate buffer called secondary hydration step and vortexed for a further 2 minutes. The generated liposomal pellets were kept for 1 hour at 25°C to anneal before using for subsequent studies.<sup>16</sup> Chitosan coated nimodipine-loaded liposome formulation was prepared by including chitosan glutamate (PROTASAN® UPG213) to liposomes generated from ethanol-based proliposomes. The mucoadhesive solution was prepared by dissolving 30mg of chitosan glutamate (PROTASAN® UPG213) in 11.5 ml of HPLC water. The chitosan solution was used in the secondary step of hydration to generate mucoadhesive liposomes. The formulation was left for 1hour at 25°C to anneal before using. A blank liposome formulation (drug-free liposomes) was also prepared for comparison.

#### Size reduction of liposomes

Size reduction of liposomes was performed by probe-sonication (Sonics Vibra-cell-CV33, USA). The probe sonicator was placed in the center of the beaker containing 30 mL of liposomal dispersion. The beaker containing liposomal dispersion was kept in an ice bath to prevent heat damage, which is generated during size reduction. The reduction size of particles run at 30% amplitude for 10 minutes.<sup>17</sup>

#### Determination of particle size

The particle size distribution of liposomes was measured using laser diffraction

(Malvern Mastersizer 2000, Malvern Instruments Ltd., UK) and also photon correlation spectroscopy (Zetasizer nano, Malvern Instruments Ltd., UK). The average value of the pellet size was expressed as the mean volume diameter (D0.5). The polydispersity was expressed as a Span factor, and a high Span value indicates a wide size distribution of particles.

$$\text{Span} = (D0.9 - D0.1) / D0.5 \dots\dots\dots (\text{Eq. 1})$$

Where D0.9; 90% undersized, D0.5; 50% undersized, and D0.1; 10% undersized.<sup>18</sup>

#### Determination of zeta potential

The zeta potential of the prepared formulations was measured using a zetasizer nano instrument (Malvern Instruments Ltd., UK). The phosphate buffer solution was used to hydrate proliposomes and then appropriately diluted with distilled water. A zetasizer cell filled by 0.8 ml of the liposomal dispersion and surface charge of the pellets was measured using Malvern zetasizer. The results are expressed as mean  $\pm$  standard deviation for at least three different batches of each liposome formulation.<sup>19</sup>

#### Determination of drug entrapment efficiency

The entrapment efficiency was measured by passing 1 ml of the liposome dispersion through syringe filters (0.22  $\mu\text{m}$ ) using a 5 ml syringe. Then, the filter was washed out by passing 2 ml of water. To this aqueous dispersion of passed liposomes, 7 ml of methanol was added, followed by shaking. The obtained solution was quantified by HPLC and considered as the entrapped

**Table 1:** Liposome formulations.

Formula	Lipid SPC (mg)	Cholesterol (mg)	Ethanol ( $\mu\text{l}$ )	Nimodipine (mg)	Chitosan (mg)
Low lipid formulation	120	30	228	30	-
High lipid formulation	240	60	456	30	-
Chitosan coated Nimodipine-loaded liposomes	240	60	456	30	30
Drug-free liposomes	240	60	456	-	-

fraction. Simultaneously, the filter was dipped in 4 ml of methanol, and then the fraction which did not pass through the filter was pushed and pulled out through syringe. The resultant of the methanolic solution was then similarly analyzed by HPLC (Agilent 1200, USA) and was regarded as un-entrapped fraction.<sup>20</sup> The entrapment efficiency (EE) was finally calculated as:

$$EE \% = \frac{\text{Entrapped fraction}}{\text{Entrapped fraction} + \text{Un-entrapped fraction}} \times 100 \dots (\text{Eq. 2})$$

### Permeability measurement

Permeability of the liposome dispersion was measured using ultra-thin silicone membrane (J-flex Rubber Products, UK) which was pre-dipped in phosphate buffer saline (pH 6.5). Two Franz cells were taken and filled with 3.5 ml of phosphate buffer saline (pH 6.5). A small magnetic stirrer was placed at the bottom of the Franz cell. Ultra-thin silicone membrane was placed between the donor (cap), and acceptor of the Franz cell then fixed with a clip. These cells were then placed on a magnetic plate water bath (JB Aqua 26 Plus, Grant Instruments, UK) at  $37^{\circ}\text{C} \pm 1$  and motor was allowed to rotate the magnetic bar at 600 rpm. One ml of sonicated nimodipine-loaded nanoliposome (without chitosan) added to one Franz cell, and one ml of sonicated chitosan coated nimodipine-loaded nanoliposome dispersion added to the other Franz cell. 0.2 ml of the samples from each cell were collected at time intervals of 1, 2, 5, 10, 30, 60, 90, 120, 150, 180, 210 and 240 min using HPLC syringe and placed them into HPLC vials.<sup>21</sup> To each of the samples, 0.8 ml of methanol was added and quantified using HPLC at a wavelength 237 nm.<sup>22</sup>

### Statistical analysis

Statistical analysis was performed using the statistical package for the social sciences statistics software (version 21). All experiments were carried out in triplicate, and the results were reported as mean

$\pm$ SD. Analysis of variance (ANOVA) and Student's t-tests (paired t-tests) were used to determine the difference between samples. The variation was considered statistically significant when the obtained *P* value was less than 0.05.

## Results

### Particle size and size distribution of nimodipine loaded liposomes

The effect of lipid concentrations, loading drug and inclusion of mucoadhesive agents into liposome formulation on the size and size distribution of liposomes generated from soya phosphatidylcholine and cholesterol were measured both before and after probe sonication as shown in Table 2. Probe sonication decreased the size and size distribution of pellets significantly ( $P < 0.05$ ), since the size of liposomes reduced from micro size range to the nano size range except for size distribution of liposome free drug formulation, was not changed significantly ( $P = 0.592$ ) (Table 2).

### Zeta potential

Table 3 demonstrated the zeta potential of liposome formulations was measured for all the formulations before and after probe sonication. The zeta potential for drug loaded liposome in both low and high lipid concentrations was in negative level, and there were not significantly different. Whilst in chitosan coated liposome formulation, the zeta potential was positive ( $13.803 \pm 0.195\text{mV}$ ) and significantly increased in the surface charge of pellets compared to the non-coated chitosan formulation of high lipid content ( $P = 0.001$ ) as shown in Table 3.

### Entrapment efficiency of nimodipine

The drug entrapment efficiency of nimodipine loaded in liposomes before and after probe sonication was measured using high performance liquid chromatography (HPLC) following the separation of the non-entrapped drug from liposome entrapped fraction of the drug (Table 4). The loading drug efficiency was between  $63.313 \pm 1.077\%$  to  $76.033 \pm 0.094\%$  for nimodipine

loaded liposomes with low lipid content, and chitosan coated nimodipine-loaded liposomes respectively. Inclusion of chitosan to the liposome formulation

significantly increased in loading drug efficiency ( $P = 0.001$ ). The loading drug efficiency reduced significantly after probe sonication ( $P < 0.05$ ) (Table 4).

**Table 2:** Particle size and size distribution of the liposome formulations.

Liposome formulations	Entrapment efficiency Before probe sonication (%)	Entrapment efficiency after probe sonication (%)	<i>P</i> value
Nimodipine-loaded liposomes with low lipid content	63.313 ± 1.077	52.423±1.195	<0.001
Nimodipine-loaded liposomes with high lipid content	66.957 ± 3.641	57.461±1.287	0.013
Chitosan coated Nimodipine-loaded liposomes	76.033 ± 0.094	69.762±0.216	<0.001

**Table 3:** Zeta potential of the liposome formulations.

Liposome formulation	Zeta Potential (mV)		<i>P</i> value
	Before probe-sonication Mean SD	After probe-sonication Mean + SD	
Drug free liposomes	-4.21 ± 1.256	-1.655 ± 0.629	0.401
Nimodipine-loaded liposomes with low lipid content	-3.21 ± 1.003	-2.02 ± 0.649	0.160
Nimodipine-loaded liposomes with high lipid content	-1.960 ± 1.446	-1.317 ± 0.153	0.486
Chitosan coated Nimodipine-loaded liposomes	13.803 ± 0.195	5.286 ± 0.341	<0.001

**Table 4:** Entrapment efficiency of drug loaded liposome formulations.

Liposome formulation	Volume mean diameter (VMD)			Polydispersity of particles		
	Before probe-sonication (µm)	After probe-sonication (nm)	<i>P</i> value	Before probe-sonication (Span)	After probe-sonication (PDI)	<i>P</i> value
Drug free liposomes	2.5450 ± 0.056	122.40 ± 0.015	<0.001	1.7040 ± 0.042	2.0000 ± 0.070	0.592
Nimodipine-loaded liposomes with low lipid content	6.3070 ± 0.123	119.41 ± 0.007	<0.001	6.2066 ± 1.071	0.4223 ± 0.066	0.008
Nimodipine-loaded liposomes with higher lipid content	3.6066 ± 0.513	114.09 ± 0.025	<0.001	7.5960 ± 2.086	0.3387 ± 0.075	0.003
Chitosan coated Nimodipine-loaded liposomes	2.9290 ± 0.076	122.48 ± 0.002	<0.001	5.9706 ± 0.111	0.4586 ± 0.026	<0.001

**Permeability studies of nimodipine**

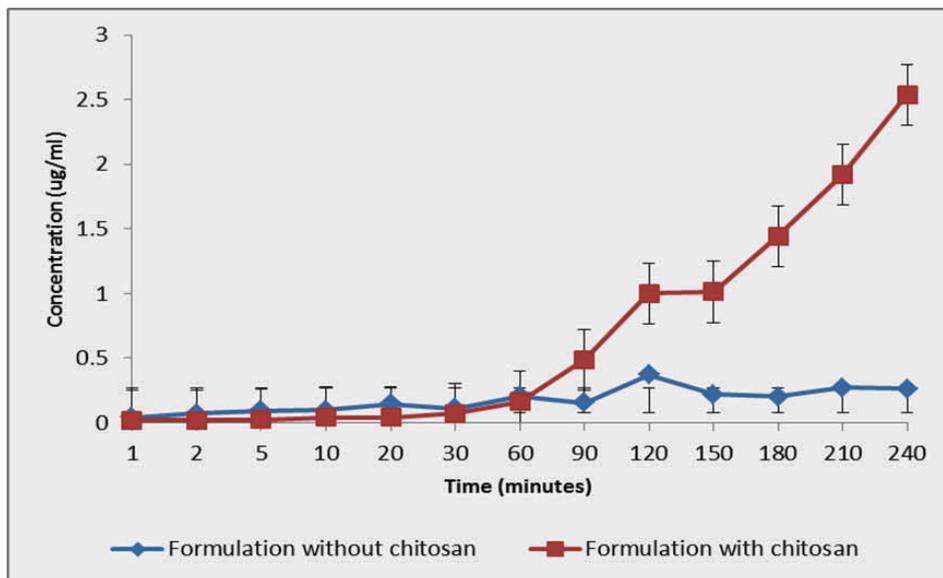
The permeability study in the present research was performed using ultra-thin silicone membrane and was determined by obtaining the drug release profile of both drug loaded liposome and chitosan coated drug-loaded liposome formulation, as shown in Figure 1. The permeability of the drug through the membrane improved with chitosan coated liposome formulations, and drug permeability significantly increased at 240 min compared to non-coated formulation ( $P = 0.0361$ ).

**Discussion**

**Particle size and size distribution of liposomes**

The particle size of the liposomes has been considered an important factor for delivery of drugs. The particle sizes of liposome formulations and size distribution before and after probe sonication are shown in Table 2. Probe sonication resulted in a significant decrease in particle size ( $P < 0.05$ ) for all the liposome formulations (Table 2) and varied in the range of 114nm -122.4nm. The volume mean diameter (VMD) of nimodipine-loaded liposomes with low lipid content ( $119.41 \text{ nm} \pm 0.007$ ) and

with higher lipid content ( $114.09 \text{ nm} \pm 0.025$ ) were almost in similar range ( $P = 0.3577$ ), however these were found to slightly lower than the VMD of drug free liposomes ( $122.40 \text{ nm} \pm 0.015$ ). On the other hand, the formulation with chitosan coating exhibited VMD of ( $122.48 \text{ } \mu\text{m} \pm 0.002$ ) which was higher than the VMD of formulations with varying lipid concentrations ( $P < 0.05$ ). However, it was found to be in equal range compare to blank liposomes ( $P > 0.05$ ). This suggests that chitosan coating increased the size of liposomes marginally and it is supported by studies performed by Guo co-workers, 2003, who studied the effect of chitosan concentration and lipid type on the chitosan coated liposome.<sup>23,24</sup> Polydispersity of drug loaded liposome pellets (low lipid, high lipid, and chitosan coated liposome formulation) before probe sonication was significant ( $P < 0.05$ ) compare to the drug free liposome formulation. In similarity to particle size, probe-sonication resulted in higher PDI for the chitosan coated nimodipine-loaded liposomes ( $0.4586 \pm 0.026$ ) in comparison to the other drug loaded liposome formulations (Table 2). However, the difference was not found to be



**Figure 1:** *In vitro* penetration-time profile of chitosan coated and non-chitosan coated liposome formulation ( $P = 0.0361$  at 240 min)

statistically significant ( $P = 0.9412$ ). In accordance with the preliminary literature findings, this difference in PDI might be attributed to the increased concentration of the cholesterol in the formulation which would have enhanced stability to the bilayers in comparison to other formulations thereby avoiding disruption of the vesicles.<sup>25,26</sup>

### Zeta potential

Zeta potential is the difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. It provides an indication related to the potential stability of the respective colloidal system.<sup>24</sup> Zeta potential values of more than + 30mV or lower than- 30mV suggest stronger repulsion between the particles and thus fewer tendencies for the particles to adhere together.<sup>27</sup> The zeta potential of all the formulations except the chitosan coated formulation was found to be in the slight negative range (Table 3) both before and after probe-sonication. The zeta potential of liposomes coated by chitosan changed significantly ( $P = 0.001$ ) from slightly negative to positive values ( $13.803 \text{ mV} \pm 0.195$ ) by the inclusion of chitosan into liposome formulation. The positively charged surface of chitosan coated liposomes contributed to the highly positive charge carried by chitosan, which favors adhesion to the cells membranes.<sup>24,28</sup> Furthermore, the zeta potential was found to be reduced (less negative value) on increasing the concentration of the lipid. However, there was no significant difference observed among both formulations with varying lipid concentrations ( $P = 0.286$ ). This finding is in agreement with the studies carried out by Szczes, 2013.<sup>29</sup> Overall, the zeta potential was not changed significantly among the formulations with varying lipid concentrations ( $P > 0.05$ ). The differences in zeta potential among all the liposome formulations before probe-sonication compared to after probe-sonication were found to be statistically significant ( $P < 0.05$ ).

### Entrapment efficiency

Nimodipine is a hydrophobic drug, and it is distributed in the phospholipid bilayers rather than the aqueous core of liposomes. Table 4 clearly shows the chitosan coated formulation with maximum nimodipine entrapment ( $76.033\% \pm 0.094$ ), which was found to be significantly higher among all three formulations ( $P < 0.05$ ). The result in accordance with a study conducted by Singla et al., 2001, who demonstrated increased entrapment efficiency of nifedipine microspheres when coated with chitosan.<sup>30</sup> Increased drug entrapment can be explained on the basis that incorporation of chitosan into formulation enhances its viscosity, which further prevents the escape of the drug molecules from the droplets.<sup>31</sup> Lowest entrapment efficiency was given by the formulation with lower lipid content ( $63.313\% \pm 1.077$ ), and the loading efficiency of nimodipine increased when the lipid concentration increased. The obtained result was in agreement with studies conducted by Wang, et al. 2006, who explored increased drug entrapment with increasing lipid concentration to the formulation.<sup>11</sup> Probe sonication caused significantly ( $P < 0.05$ ) decreased drug entrapment in all formulations (Table 4). The decreasing of drug encapsulation may be caused by possible degradation of phospholipids and leakage of the drug during size reduction.<sup>32</sup>

### Permeability study

The permeability of chitosan coated nimodipine-loaded liposomes was much higher than the liposomes without chitosan, as shown in Figure 1. This difference between the two formulations was found to be statistically significant ( $P < 0.05$ ) after 240 min ( $P = 0.0361$ ). Chitosan has been considered as permeability enhancing agent on the basis of ability to open tight junctions of the membrane.<sup>33,34</sup> The opening of tight junctions was allowed due to the interaction of negatively charged sites of cell membranes with the positively charged amino group attached on the

C-2 position of chitosan.<sup>34</sup> The maximum drug permeation through the membrane shown by the chitosan coated nimodipine-loaded liposomes was 2.53 µg/ml in the duration of four hours. The permeation of the drug molecules for the formulation without coating through the silicone membrane was almost similar throughout four hours' time-period.

### Conclusion

Liposomes have been suggested as one of the novel and efficient drug delivery systems through various research studies. This study also contributed to it by preparing and characterizing nimodipine-loaded liposomes. The present study reflected the suitability and efficiency of ethanol based proliposome method to prepare the nimodipine-loaded liposomes. High lipid contained formulation provided maximum entrapment efficiency of the drug. Reduction of particle size using probe sonication showed a dramatic effect on the drug loading efficiency. This study explored that liposomes with higher lipid concentration and chitosan coating can be considered as efficient carriers for the nose to brain delivery of nimodipine. Additionally, chitosan can be regarded as drug entrapment and permeability enhancer.

### Competing interests

The authors declare that they have no competing interests.

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