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Comparing size particle, release study and cytotoxicity activity of PHMB encapsulated in different liposomal formulations: neutral and cationic liposomes

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Abstract

Charges in liposome composition plays a critical role, so it has significant outcome on the manner of liposome in vitro and in vivo. Neutral and cationic liposomes (LPFS and CLPFS) were prepared and characteristics of LPFS and CLPFS were studied after PHMB encapsulation. According to results, CLPFS exhibited higher cytotoxicity activity, while kept similar size distribution, encapsulation efficiency, lower prolonged retention release profile in comparison to LPFS. CLFPS represented more stable release due to its mutual repulsive force. MTT assay of the produced formulation was examined versus normal primary human skin fibroblast cells and free PHMB presented stronger cytotoxic activity when compared to both cationic and neutral liposomes. It was concluded that PHMB are more toxic to the body but encapsulation liposome specially in neutral liposome can reduce the toxic effect of them.

Keywords: PHMB, Neutral and cationic liposomes (LPF_S and $CLPF_S$), cytotoxicity activity, MTT assay.

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1. Introduction

The agglomeration of therapeutic agents at particular disease spots in the body can intercede by liposomal drug delivery systems. Liposomes are miniscule spherical structures that are characterized as phospholipid vesicles comprising of single or multiple concentric lipid bilayers encompassing aqueous spaces inside and between the lipid bilayers. The lipids can be observed in the human body or from commercially existing lipid molecules that are utilized in formulation of liposomes. In contrast to lipid monolayer structures, liposomes are specified by protracted, two-dimensional, and distinctly detached hydrophilic and hydrophobic domains. Liposomes have distinctive capability to catch hydrophobic molecules into the lipid membrane and hydrophilic molecules in the aqueous core. These vesicles can encapsulate a various range of drugs and substances because of similar morphology of them to that of cellular membranes, so they have been known as a principle drug-carrier system [1, 2]. Lipid bilayers of Liposomes comprise of cationic, anionic, or neutral (phospho) lipids and cholesterol, that can be encompassed an aqueous section. Cationic liposomes contain natural neutral phospholipids and positively charged lipids. Liposome with cationic charges are

effective in the intracellular delivery of biomolecules, such as oligonucleotides, nucleic acids, peptides, proteins and drug molecules for therapeutic or diagnostic intentions. They have capability to interact with negatively charged cell surfaces because of their positively charged headgroups [3].

PHMB is a cationic oligomer due to its amine-rich back bone that has an average of 7–11 biguanide groups arranged by flexible hexamethylene sectors. It is associated with antimicrobial activity because of these functional groups, this trigger separation of phospholipid phase and disrupt the cell membrane

and has a high effectiveness against microorganisms [4, 5].

In our study, PHMB as a hydrophilic antibacterial material like Acyclovir [6] was encapsulated in neutral and cationic liposomes to compare the behavior in vitro. The particle size, size distribution, zeta potential, entrapment efficiency, release study and cytotoxicity activity of PHMB compared between neutral and cationic liposomes.

2. Materials and Methods

PHMB was supplied by Lemandou Chemicals, China. Soya phosphatidylcholine (SPC, purity: 98%) was procured from Applichem, Germany. Cholesterol (CHOL, purity > 99%) for preparation of liposome, casein–peptone soymeal–peptone (CASO) agar and stearylamine as cationic surfactant and all other organic chemicals were purchased from Merck, Germany.

Table 1: Particle size and zeta potential and %EE of neutral and cationic liposomes prepared through thin layer hydration methods.

Samples	Particle size ± SD (nm)[a]	PDI[a]	Zeta potential (mV)[a]	%EE
LF	990 ± 0.1	0.830 ± 0.001	40.0 ± 0.1	–
LF$_S$	60.4 ± 0.05	0.253 ± 0.001	39.2 ± 0.1	–
LPF	1002.0± 0.05	0.985 ± 0.002	30.5 ± 0.08	90.0± 0.2
LPF$_S$	67.36± 0.02	0.181 ± 0.001	28.7 ± 0.1	55.4± 0.3
CLF	1018.1 ± 0.2	0.991 ± 0.001	+50.2 ± 0.1	–
CLF$_S$	43.2 ± 0.2	0.140 ± 0.003	+39.9 ± 0.08	–
CLPF	1042.7 ± 0.1	1.000 ± 0.001	+30.1 ± 0.2	71.0± 0.1
CLPF$_S$	56.8 ± 0.1	0.176 ± 0.001	+36.3 ± 0.3	53.6± 0.2

PDI: poly dispersity index; SD: standard deviation.

[a]Data are the mean of three independent analyses ± SD.

2.1. Preparation of cationic and neutral liposomes with or without PHMB (LF, LPF, CLF and CLPF)

According to Ahani et al., 2016 and 2017: Multilamellar vesicles (MLVs) were prepared by lipid film hydration method. Cationic liposomes components are Soya Phosphatidyl Choline [SPC], cholesterol and stearylamine in the molar ratio of 6:1.5:1 were poured in chloroform (20 mL) in a 50 mL round-bottomed flask and vigorously mixed by shaking. The mixture was placed in a rotary vacuum evaporator fitted with an A3S aspirator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) in a circulating bath (Spac- N Service, Kolkata, India) and rotated at 150 rpm and 37 °C to evaporate the solvent. The solvent was removed under vacuum at 20–30 °C and 30-50 rpm with a rotary evaporator. The homogeneous dry and thin lipid film was vacuumed overnight under nitrogen flow to complete removal of chloroform. The dried lecithin film was dispersed in 10 mL distilled water and stirred at room temperature to complete removal of the lipid layer and formation of lipid vesicles. In order to produce cationic liposomes loaded with PHMB, the dried lecithin film was dispersed in 10 mL PHMB (0.4 %) and stirred at room temperature to complete removal of the lipid layer and formation of lipid vesicles. To ensure large LMVs formation, a drop of the liposomes was observed under the microscope. For preparing neutral liposome, all steps are similar to cationic liposome just in components of neutral liposome, there is no any stearylamine.

For preparing of nano cationic and neutral liposome: The cationic and neutral liposome was sonicated with a probe-type sonicator (Ultrasonic UH-600) at 65 W for 30 min to produce uniform nano cationic and neutral liposomes [7, 8].

2.2. Encapsulation efficiency of PHMB

In a typical procedure for calculating PHMB encapsulation efficiency, 2 mL of LPF_S and $CLPF_S$ was poured into a filter tube and centrifuged at 23000 rpm and 4 °C for 1 h. The same procedure was done for LF_S or CLF_S as control samples. The absorbance of the obtained samples was measured at 236 nm and related to concentration using calibration equation according to Ahani et al., 2016 and 2017 [7, 8]. The encapsulation efficiency was calculated based on Eq. (1):

EE%= (The PHMB loaded amount/ Initial added amount) × 100 (1)

2.3. In vitro release study

The release profile of the optimized liposomal formulation (LPF_S and $CLPF_S$) was investigated in vitro in PBS (pH=7.4). Liposomal formulation (1.5mL) was poured and tightly sealed in dialysis tube (12 kDa cut-off) and stirred in 400 mL PBS buffer at 37 °C and 50 r/min in a shaker bath under ideal sink conditions. In predetermined time intervals (0.5, 1, 1:30, 2, 4, 6, 8, 12, 16, and 24h), 1mL of medium was taken out and same volume of fresh media was fulfilled to keep the sink condition. The samples were analyzed by UV–vis spectrophotometry.

2.4. Characterization tests

Dynamic light scattering (DLS) measurements were employed to characterize particle size, poly dispersity index (PDI) and zeta potential (*z*-average) of samples using Malvern Zetasizer (Malvern Instruments, Malvern, U.K.).

2.5. Cytotoxicity assay

Normal primary human skin fibroblast was cultured in growth medium (Dulbecco's Modified Eagle's Medium DMEM) (1X) (Biochrom, Berlin, Germany) + 2 mM GlutMAXTM (GibcoTM) supplemented with 10% fetal calf serum (FCS) and incubated at 37 °C in humidified 5% CO_2/95% air. 96-well plates were used for all the experiments. Cells from passage 3 were seeded in each well and incubated for 2 days. Thereafter, samples including PHMB, nano cationic liposome, and nano cationic liposome loaded with PHMB were added to 2 mL sterile PBS (buffer

phosphate saline) for 24 h at 37 °C. The cultured medium with leaching substance was added to the cultured cells and incubated for 24 h. After incubation, the samples were investigated by optical microscopic examinations. The cells were reincubated for a further 24 h in fresh medium and then tested with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A Tecan SunriseTM microplate reader at 492 nm was used to measure the absorbance. Experiments were performed four times and the obtained results were recorded as percentage absorbance relative to control cells. The cytotoxicity results were used to calculate percentage relative cell viability after incubation with the samples using Eq. (2):

$$\text{Cell Viability \%} = (\text{abs}_{\text{sample}}/\text{abs}_{\text{control}}) \times 100 \quad (2)$$

Where $\text{abs}_{\text{sample}}$ and $\text{abs}_{\text{control}}$ are the absorbance of well containing samples and control.

3. Results and Discussion

3.1. Particle size and zeta potential measurements

The size and distribution of the particles and the surface charge are presented in Table 1. There are two essential factors that have a remarkable effect on the properties of nanoparticles delivery systems is Particle size and zeta potential. It can be considered that factor of surface electrostatic charge of particles is Zeta potential [9].

The particle size distribution was 0.830 ± 0.001 for *LFP* and 0.253±0.001 for LFP_S. The particle size of the final LPF_S and CLPF_S formulations were 67.36 ± 0.02 and 56.8 ± 0.1nm with an acceptable PDI of 0.253 ± 0.001 and 0.176 ± 0.001 respectively. Indeed, cholesterol can favorably intermingle with the core of the membrane, therefore sustaining it and known as a hydrophobic molecule. Also, cholesterol can be utilized to attach PHMB to the nano liposomes as “stealth” drug vector. [10]

3.2. PHMB entrapment and in vitro release of PHMB:

It was revealed that loading method could proficiently entrap PHMB as a hydrophilic polymer into nano neutral and cationic liposomes. According to the experiments, there were no considerable distinction in PHMB entrapment efficiency among the neutral and cationic liposomes. All the entrapment efficiency for both nano neutral and cationic liposomes were 53-54% (Table 1), it was exhibited that the surface charge had insignificant effect on PHMB loading efficacy.

A shown in Figure. 1: it can be seen that free PHMB released completely in 4h but both neutral and cationic liposomes were noticeably released by delaying post 96 h, because entrapping of PHMB in liposome affect the release profile and retain it in their membrane, so it can be release slowly.

A brisk release at 24 h time point can be seen for LFP_S, whereas the release profile of CLFP_S formulation commenced at 48 h point, and reached 75~ 85% accumulated release at 72 h. At first, the gradual abrupt dissolution rate within liposomes can be controlled the drug release behavior that cause the sustained release for an elongated period of time.

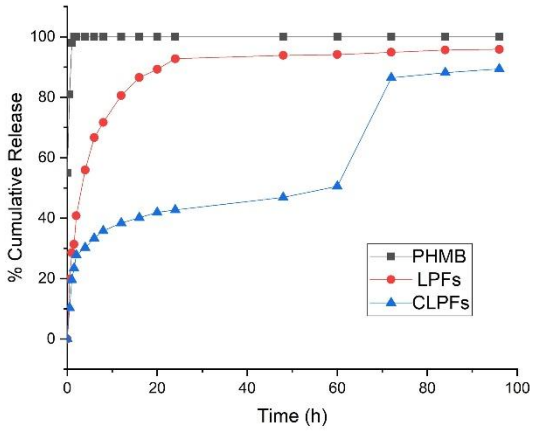

Figure 1. The PHMB release profiles from different neutral and cationic PHMB-loaded liposomes.

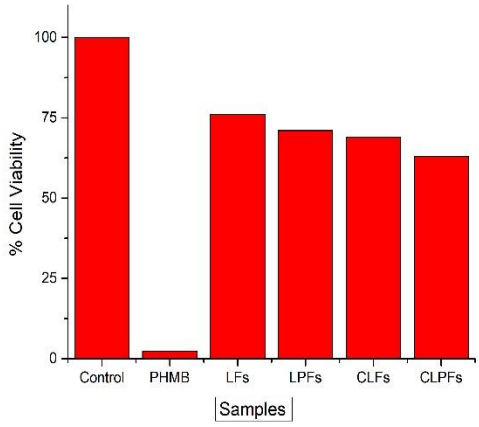

Figure 2. Cell viability percent of different formulations: control, PHMB, LF_S, LPF_S, CLF_S and $CLPF_S$.

Afterwards the dissolved PHMB could diffuse from the lipid membrane. On the other hand, A sharp release can be ascertained from the graph in first 4 h of the study for both cationic and neutral liposomes. The reason for this amount that released sharply in first 4 h is PHMB molecules, that are attracted on the surface phospholipid layer of liposomes which is recognized as burst release. It can be mentioned that is the disengagement of PHMB molecules from the outer lamellae causes initial burst release of PHMB form both cationic and neutral liposomes.

$CLFP_S$ were more stable than neutral LFP_S because of their mutual repulsive force in the suspension system. It could be acknowledged that, because of superior electrostatic interactions on membrane penetrance, $CLFP_S$ might have some structure imperfections after 60 h therefore it led to precipitate dissemination of membrane and resulting in entire PHMB release [11].

3.3. Cytotoxicity

The cytotoxicity of PHMB, LF_S, LPF_S, CLF_S and $CLPF_S$ was assessed toward normal primary human skin fibroblast cells by MTT cytotoxicity test. The cell-viability percentage curve for samples are shown in Figure 2. PHMB demonstrated detrimental toxic outcomes in dermal fibroblast, it implied entire cell destruction in almost all cells. The percentage cell-viability of PHMB is very lower than liposomal formulation and confirms that PHMB is more lethal to the cells than PHMB-loaded nano cationic and neutral liposome. When PHMB encapsulate in both nano cationic and neutral liposome, it causes considerable raise in cell viability percent from 2.4 to 63 and 71 % for $CLPF_S$ and LPF_S respectively. Indeed, nano cationic and neutral liposome are applied as a detoxifying agent to diminish the toxicity of PHMB because they indicated notably lower toxicity than PHMB.

$CLPF_S$ presented notable toxicity in comparison to LF_S, LPF_S and CLF_S in all cells. Also, cell viability percentage of neutral liposome (LF_S:76 and LPF_S:71%) is higher than cationic liposome (CLF_S: 69 and $CLPF_S$ 63%). It can be mentioned that the cytotoxicity of PHMB entrapped in cationic liposome is more than neutral liposome because positively charged liposome due to have positive charge may attack cells and detoxify them than neutral liposome. Also, another reason is that toxicity is related to the high positive zeta potential and higher charge density that cationic liposomes have than neutral liposomes, are mostly more toxic to a kind of cell varieties [12].

4. Conclusion

In our work, PHMB as an antibacterial material was satisfactorily encapsulated into neutral and cationic liposome formulations. It can be mentioned that any unfavorable side issue of the encapsulated PHMB are noticeably decreased in comparison to the free formula. Particle size distribution of the both cationic and neutral liposomes were pleasing. The release profile of cationic and neutral liposome loaded with PHMB illustrated a sustained release of PHMB for both cationic and neutral liposome but LPF_S represented higher prolonged retention release profile in comparison to $CLPF_S$, while $CLPF_S$ were more stable due to its mutual repulsive force. The results of the MTT assay clearly demonstrated that free PHMB represented remarkable toxicity when compared to

liposomal formulation that PHMB encapsulated to them. Also, CLPFs demonstrated notable toxicity in comparison to other liposomal formulation.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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