Research Article

Preparation and evaluation of chitosan based *in-situ* gelling system for delivery of Amphotericin B

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Abstract

Objective: Objective of present work was to develop and characterizes chitosan based in-situ gelling system for delivery of Amphotericin B. **Material and methods**: The chitosan based *in situ* gel was developed in which liposomal Amphotericin B was incorporated. **Results and conclusion:** The evaluation showed that in comparison to pure drug incorporated *in situ* gel, the liposomal Amphotericin B incorporated *in situ* gel was more effectively forming gel and the gelation time was also quick. The best formulation among liposomal Amphotericin B incorporated *in situ* gel was G2 which gel formed at 36°C and the gelation time of G2 formulation was found to be quicker than the rest of the formulations i.e. 41sec. The viscosity was found to be adequate in case of G2 formulation i.e. 877.67cps and 10132.00cps. The *In vitro* drug release studies showed that the pure Amphotericin B incorporated *in situ* gel gave less release as compared to the liposomal Amphotericin B incorporated *in situ* gel.

*Keywords***:** Chitosan based *in situ* gel, liposome, microsphere, amphotericin B

Introduction

Human fungal infections have increased dramatically in incidence and severity in recent years, owing mainly to advances in surgery, cancer treatment, and critical care accompanied by increases in the use of broad spectrum antimicrobials and HIV epidemic. These changes have resulted in increased numbers of patients at risk for fungal infections.

Amphotericin A and B are antifungal antibiotics produced by *Streptomyces nodosus*. Amphotericin A is not in clinical use (Altmannsberger et al., 2004; Anderson, 1995; Antony et al., 2003). Amphotericin B is selective in its fungicidal effect because it exploits the difference in lipid composition of fungal and mammalian cell membranes. Resistance to amphotericin B occurs if ergosterol binding is impaired, either by decreasing the membrane concentration of ergosterol or by modifying the sterol target molecule to reduce its affinity for the drug (Bagnis and Deray, 2002; Barcia, 1998).

Owing to its broad spectrum of activity and fungicidal action,

amphotericin B remains a useful agent for nearly all life threatening mycotic infections, although newer less toxic agents have begun to replace amphotericin B for many conditions (Brajtburg et al., 1996; Burke et al., 2006; Cagatay et al., 2007).

Amongst the extensive research has been carried in designing of polymeric drug delivery systems. The development of *in situ* gel systems has received considerable attention over the past few years. *In situ* gel formulations offers an interesting alternative for achieving systemic drug effects of parenteral routes, which can be inconvenient or oral route, which can result in unacceptably low bioavailability and passes the hepatic first-pass metabolism, in particular of proteins and peptides (Sarasija and Shyamala, 2005; Wataru et al., 2004; Garg et al., 2019a; Garg et al., 2019b; Marsha and Philip, 2002; Peppas et al., 2000). This novel drug delivery system promotes the importantly ease and convenience of administration, deliverance of accurate dose as well as to prolong residence time of drug in contact with mucosa, that problems generally encountered in semisolid dosage forms. *In situ* gel formation occurs due to one or combination of different stimuli like pH change, temperature modulation and solvent exchange. Smart

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polymeric systems represent promising means of delivering the drugs; these polymers undergo sol-gel transition, once administered.

Materials and methods

Amphotericin B was generously supplied as a gift samples by Evonik Pvt.Lmtd., Mumbai. Chitosan, Cholesterol and Phospholipid were purchased from Lobachem Laboratories (India). All other chemicals and reagents were used of analytical grade.

Preparation of Amphotericin B based liposomes

Accurately weighed 125mg cholesterol and 250mg phospholipid was taken in a round bottom flask. In the same round bottom flask, 4.5ml of chloroform and 4.5ml of methanol was added and properly mixed. Now accurately weighed 50mg Amphotericin B was added to the above mixture. This mixture was shaken manually till the complete solvent evaporation. Later, the film formed inside the round bottom flask was hydrated with 25ml of 7.4pH phosphate buffer. There were other two batches of liposomes prepared in which the amount of cholesterol was varied as mentioned, 100mg cholesterol and 200 mg cholesterol (Table 1).

Characterization of prepared Amphotericin B loaded liposomes

Zeta Potential

Zeta potential of the liposomes prepared from hand shaking method was determined using Zeta Sizer 300HSA.

Percent drug entrapment

PDE was determined by mini-column centrifugation method. In brief, Sephadex® G50 solution (10 %, w/v) was prepared in water and was kept aside for 48 hours for complete swelling. To prepare mini-column, Whatman filter pad was inserted in 1mL syringe and swollen Sephadex was added carefully to it to avoid air entrapment in the column. Excessive amount of water was removed by spinning the column at 2000 rpm for 3 min using centrifuge. Amphotericin B liposomes suspension (100 μL) were slowly added on prepared column and centrifuged at 5000 rpm for 3 min, and then the same procedure was repeated by adding 100 μL of water. The remaining free drug bound to the gel, while liposomes passed through the gel and were collected from the first and second stage of centrifugation. The eluted liposomes obtained were ruptured using ethanol and percent encapsulation was calculated from total amount of liposomes present in 100 μL of liposomes by UV-Visible spectrophotometer (slope) using the given equation, the method is validated using free drug instead of liposomal dispersion. The free drug was analyzed by UV-Visible spectrophotometer.

$$Encapsulation\ efficiency\ (EE) = (Qe/Qt) \times 100$$

Where,

Qe is the amount of encapsulated Amphotericin B and Qt is the amount of Amphotericin B in 100μL of liposomes suspension.

Solubility study of prepared Amphotericin B liposome dispersion

Solubility was determined by adding excess of Amphotericin B dispersion in to the water in sealed glass containers at room temperature. The liquid was agitated for 24h on rotator shaker then centrifuged for 15min at 5000 rpm to remove excessive Amphotericin B. The supernatant was filtered through membrane filter then filtrate was diluted with distilled water to prepare dilutions. Absorbance of these samples was measured by using UV

Table 1. Composition of Amphotericin B loaded liposomes

Formulation Code	Cholesterol	Phospholipid	Amphotericin B	Chloroform	Methanol
F1	100mg	250mg	50mg	4.5ml	4.5ml
F2	125mg	250mg	50mg	4.5ml	4.5ml
F3	200mg	250mg	50mg	4.5ml	4.5ml

Table 2. Formulation composition of liposomal Amphotericin B loaded *in situ* gel

Formulation	% of chitosan	Amount of liposomal dispersion
G1	6%	20ml
G2	8%	20ml
G3	10%	20ml

Table 3. Formulation composition of pure drug loaded *in situ* gel

Formulation	% of chitosan	Amount of Phosphate buffer
G4	6%	20ml
G5	8%	20ml
G6	10%	20ml

spectrophotometer and the concentration was calculated by using calibration curve.

Preparation of liposomal Amphotericin B loaded *in situ* gel

In three batches of liposomal dispersion 20ml, 6%, 8% and 10% chitosan was added and kept overnight under refrigeration. Next day, the chitosan was dissolved completely and a solution was formed (Table 2). There were other three batches in which pure drug along with 20ml phosphate buffer was used and to this, 6%, 8% and 10% was added and kept overnight under refrigeration (Table 3).

Evaluation of liposomal Amphotericin B loaded *in situ* gel

Determination of Drug Content

1 ml of each formulation of *in situ* gel was dissolved in methanol and the volume was made to 100 ml. Uniformity of the drug content was evaluated by measuring the absorbance at 379nm in UV spectrophotometer (Shimadzu-1800) after suitable dilution. Drug content was also evaluated for pure drug loaded *in situ* gel.

Gelation Temperature

2ml of the *in situ* gel was heated in a test tube which was placed on a thermostatically controlled water bath. The water was set to heat at a rate of 2°C/5 min constantly. Gelation was considered complete, when the gel in the test tube did not flow when overturned.

Gelation Time

It was done by tube inverting method. The temperature of water bath was set at gelation temperature and allowed to maintain for 10 min. A thin walled test tube containing 2 ml of *in situ* gel was placed in water bath. The *in situ* gel was observed for gelation by inverting the test tube at periodic intervals. The gelation time was noted when there was no-flow when the test tube was inverted.

Determination of viscosity

The viscosity studies of all the formulations were measured by using Brookfield viscometer (Model-DV II+ pro, USA) using spindle no. 5 at 30 rpm (as shown in Fig4.1). Viscosity was measured (n=3) at two different temperatures viz. 8±1°C and 37±1°C.

In vitro **drug release studies**

In vitro drug release study was carried out using open (diffusion) tube apparatus. The semi- permeable cellophane membrane, presoaked overnight in the freshly prepared phosphate buffer (7.4), was tied to one end of an open tube, acted as donor compartment. 1 ml of *in situ* gel was placed inside the donor compartment in contact with the cellophane membrane. The tube was vertically held by a stand and suspended in 100 ml of simulated phosphate buffer maintained at 37±1°C touching the surface of receptor medium. The receptor medium was stirred at 100 rpm using magnetic stirrer. The aliquots of 3 ml were withdrawn at regular intervals and replaced by an equal volume of warm receptor medium every time. The amount of pilocarpine released was analyzed spectrophotometrically at 215.5 nm (Shimadzu UV-1800, Japan).

The drug release profiles obtained were fit into various mathematical models to determine the mechanism of drug release and release kinetics.

Hemolytic Activity

The human blood samples were obtained from healthy volunteers. The blood was centrifuged at 5,000 rpm for five minutes. 2 % erythrocyte suspension was prepared in sterile phosphate buffer saline for hemolytic study. The hemolytic activity of the liposomes was tested under *in vitro* conditions, for each liposomal suspension, various concentrations (50–500 µg/ml) of extracts were added to 0.85% NaCl solution and then received a 2% suspension of human erythrocytes.

After 30-min incubation at room temperature, cells were

centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. Two controls were prepared without liposomes; negative control received sterile phosphate buffer saline, while positive control received 0.1% Triton X-100.The average value was calculated from triplicate assays. Hemolysis percentage for each sample was calculated by dividing sample's absorbance on positive control absorbance (complete hemolysis) multiplied by 100.

Results and discussion

Characterization of Amphotericin B loaded liposomes

Zeta Potential

The value of zeta potential of F1, F2 and F3 was found to be -40mV, -30mV and -10mV respectively. According to previous studies, -30mV was proved to be more stable range than others. Therefore, this suggests the stability of F2 formulation.

Percent drug entrapment

The percent drug entrapment in liposomes was determined

by minicolumn method and found to be 70%, 86%, and 68% for F1, F2 and F3 respectively. The drug entrapment in case of F2 was high and therefore, this was optimized for further study.

Solubility study of prepared Amphotericin B liposome dispersion

The solubility of prepared Amphotericin B liposome dispersion was done and it was found that F2 gave significantly more solubility than others. Though the solubility was increased in all the liposomal formulations as compared to pure drug, but F2 was more soluble than others. Therefore, this F2 formulation was carried forward for further studies.

Evaluation of liposomal Amphotericin B loaded *in situ* gel

Determination of Drug Content

The Amphotericin B loaded *in situ* gel was subjected to determine the drug content. The % drug content was found to be 85%, 92.04% and 79.01% in case of G1, G2 and G3. Whereas for pure drug loaded *in situ* gel i.e. G4, G5 and G6, the % drug content was found to be 65%, 67% and 71%. As the result indicates that the liposomal formulations showed higher % drug entrapment as compared to pure drug loaded *in situ* gel, yet among G1, G2 and G3, G2 showed higher drug content. Therefore, G2 had the highest drug content among all the other formulations.

Gelation Temperature

An ideal *in situ* preparation should possess a gelation temperature between 30-37°C. The gelling temperature of liposomal Amphotericin B loaded *in situ* gel i.e. G1, G2 and G3 was found to be 37 C, 36 C and 39 C whereas in case of pure drug loaded *in situ* gel i.e. G4, G5 and G6, the gelation temperature was more than the liposomal formulations i.e. 48°C, 50°C and 52°C. In this study, G1 and G2 showed characteristic property of an ideal gel.

Gelation Time

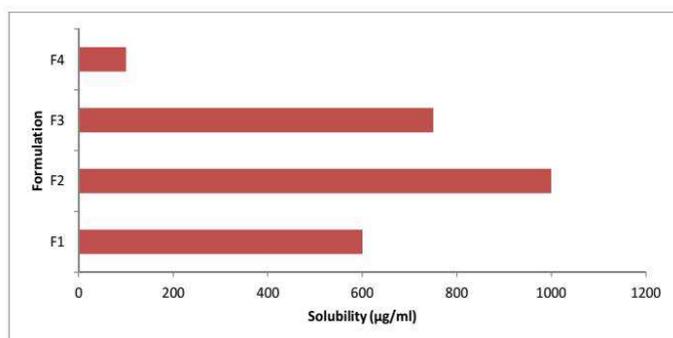The gelling time of the liposomal Amphotericin B loaded *in situ*

Figure 1. Solubility study of Amphotericin B loaded liposomes

gel i.e. G1, G2 and G3 was found to be 45, 41 and 48 sec respectively. In case of G4, G5 and G6, the gelation time was 57sec, 60sec and 1min 59sec respectively. The optimum result in this study was given by G2.

Determination of viscosity

The viscosity of liposomal Amphotericin B loaded *in situ* gel gels were evaluated at two temperatures, 8°C and 37°C representing their storage and body temperature. Viscosity was low at storage temperature (8°C), but their viscosities increased at body temperature studied (37°C) in lieu of sol-gel transition. Formulation G1, G2 and G3 exhibited the highest viscosity values of 733.4cps and 899.59cps, 877.67cps and 10132.00cps and, 978.89cps and 11457.97cps at 8°C and 37°C respectively. The viscosity of pure drug *in situ* gel formulations G4, G5 and G6 was 134.22cps and 154.96cps, 145.53cps and 456cps and, 200.02cps and 221.23cps at 8°C and 37°C respectively. The optimum viscosity in case of storage as well as body temperature was exhibited by G2.

In vitro drug release studies

In vitro release data provides information about the efficiency of a delivery system under test conditions. The proposed model needs to be similar with regard to in vivo conditions. The study helps in predicting the residence time, drug release, bioavailability and related parameters from the study. The best *In vitro* drug release was exhibited by G2 formulation as compared to G1 and G3.

Hemolytic Activity

The results showed that there was a remarkable hemolytic activity in case of pure drug in situ gel whereas in case of liposomal Amphotericin B incorporated in situ gel, there was very little hemolytic activity found in human erythrocytes. Hemolytic activity was compared between in
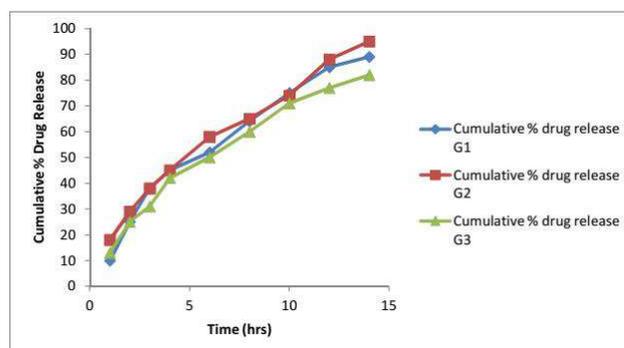

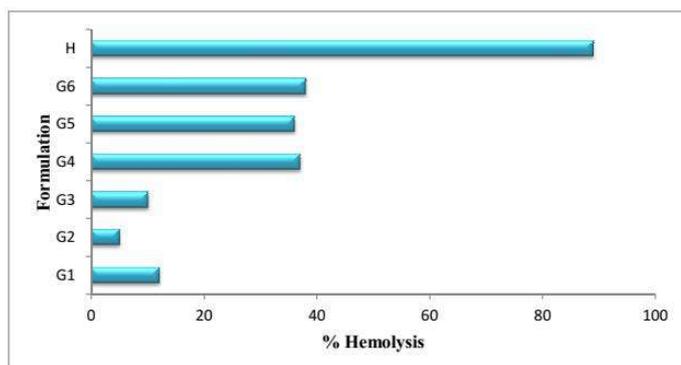**Figure 2.** *In vitro* drug release profile of Amphotericin B incorporated gel

Figure 3. Hemolysis of human RBC's by various Amphotericin B formulations

situ gels (both the batches i.e. liposomal Amphotericin B loaded in situ gel namely G1, G2 and G3 as well as pure drug loaded in situ gel namely G4, G5 and G6) and pure drug. The results showed that the pure drug (i.e. Formulation H) exhibited higher percentage of hemolysis as compared to in situ gel. The least hemolytic activity was found in G2 formulation (Figure 3).

Conclusion

The introduction of *in situ* gelling systems has further strengthened the link between therapeutic need and drug delivery. A lot of research is ongoing in various laboratories to explore *in situ* gel as drug delivery systems for better patient care. The utility of *in situ* gelling system in drug delivery and biomedical application is immense. Also liposomes play an important role to overcome solubility related problems. Thus, future studies can be conducted to investigate this novel manufacturing process to various facts of drug delivery.

Acknowledgment

Conflict of Interests

No conflict

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