**.Novel grafted copolymer self-assembled micelles as nanocarrier for Amphotericin B**

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## Introduction

The continuous search for new drug delivery system is driven by the ardour to maximize therapeutic activity while minimizing negative side effects. Since Barder et al. [1984] proposed polymeric micelles as drug carriers in which they have proposed it has most promising modalities as drug carriers. Particularly, much attention now is being paid to amphiphilic polymeric micelles as nanocarrier for drug delivery systems. Grafted copolymer micelles as a drug carriers are able to produce highly desirable advantages as the hydrophobic core has a large capacity to accommodate hydrophobic drugs , their relatively small size (typically between 10 to 90 nm) and hydrophilic surface allow polymeric micelles prolonged circulation in the bloodstream after intravenous administration. The micellar encapsulation of bioactive materials into hydrophobic core of grafted copolymer have a great potential to allocate the drug in their nanoreservoir and have features to impart biological stability. We have synthesized water-soluble grafted copolymers by coupling Pluronic F-68 onto chitosan using 1-ethyl-3-(3-dim-ethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents. An attempt was made to develop a novel formulation of Amphotericin B (AmB) by providing a hydrophobic core using novel grafted copolymer to enhance its solubility profile and stability in systemic circulation.

## Material and methods

Chitosan (percentage of deacetylation degree: 85%) was purchased from Fluka (Fluka chemika, USA). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and succinic anhydride were purchased from Aldrich (Sigma– Aldrich, USA). Pluronic F-68 and 4-dimethylaminopyridine (DMAP) (BASF, Korea) and Sigma (Sigma–Aldrich, USA). Amphotericin (AmB) was procured from SPARC, Vadodara, India. Dialysis membranes (Mwt:12-14000 dalton) were purchased from Spectra/Por (Houston,TX, USA). The water used throughout the experiment was purified with a Milli Q system from Millipore Co., USA.

## Synthesis of pluronic grafted chitosan and loading of Amphotericin B (AmB) in polymeric micelles

Pluronic F-68 was carboxylated with succinic anhydride to produce monocarboxy Pluronic (MP). Then, MP was coupled with chitosan by EDC/NHS at R.T. for 24 h . The product was dialyzed against distilled water using a membrane (Molecular Weight Cut Off: 12,000) for 2 days and finally lyophilized. Then 50 mg polymer was dissolved in 10.0 ml of dimethylformamide (DMF) with the aid of warming (40$^{o}$C, 5 min). AmB (20 mg) was added to the solution and dissolved by stirring. The solution was dialyzed three times for 48 hr against 2 L of distilled water, pH (11.2-11.5) (Spectrapor, MWCO 12,000– 14,000 Dalton). The solution in the dialysis bag was obtained, filtered through a 0.22 mm filter and freeze- dried.





## Characterization

Infrared (IR) spectra of Chitosan, pluronic and grafted copolymer were measured with Perkin Elmer on carefully dried samples embedded in KBr pellets. $^{1}$H NMR (Bruker 300 Hz) spectroscopy measurements were carried out with to confirm the change of chemical structure of chitosan–Pluronic grafted polymer. Differential Scanning Calorimetry (DSC) curves of chitosan, pluronic and grafted copolymer were collected with a thermo analyzer (Diamond DSC, Perkin Elmer, Germany) within a temperature range of 30–300 °C and with the rate of increasing temperature of 5 °C /minutes to ensure the interaction between pluronic and chitosan (Fig.1).

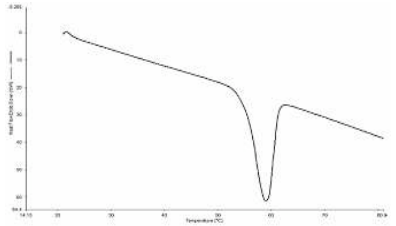

Fig.1. DSC of pluronic F-68

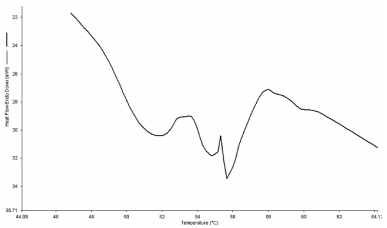

Fig. 2 DSC of grafted copolymer

Micellar preparation and encapsulation of AmB was determine by the zetapotential and mean particle size in milli Q water by using Zetasizer Nano ZS (Malvern Zetasizer, 3000 HS). The zetapotential value was the average of five successive measurements that varies from -6 to -13mv and +5 to +14mv in case of loaded and unloaded micelles respectively.

## Result and Discussion

A novel encapsulation strategy for hydrophobic drug (AmB) inside the core of micelles was selected as tool using chitosan-pluronic grafted polymers. The FT-IR spectrum of chitosan–Pluronic indicated that peaks appeared at 1635 cm$^{-1}$, 1530 cm$^{-1}$ could be assigned to carbonyl stretching vibration (amide I), and N–H bending vibration (amide II) of a primary amino group, characteristic peaks of amide II was observed when it compares with chitosan itself. This result suggests that the amide bonds between carboxylic groups of monocarboxy pluronic and the amine ones of chitosan are formed (Fig. 2.).

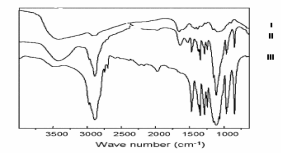


**Fig.2. FT-IR spectra of (I) chitosan, (II) chitosan–Pluronic-68, and (III) Pluronic-68**

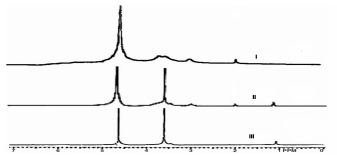


**Fig. 3. 1H NMR spectra of (I) chitosan, (II) chitosan–Pluronic-68, and (III Pluronic-68**

In $^{1}$H NMR results, the spectrum of chitosan (Fig.3) exhibits the typical peaks including the proton on the anomeric carbon (at 4.80 ppm), the methyl protons from partially acetylated chitosan (at 2.10 ppm), and the proton on the carbon bearing amino (partially acetamido) groups (at 3.10 ppm). The spectrum of pluronic (Fig.4) exhibits a peak at 3.60 ppm (PEOs methylene protons) and a weak peak at 1.12 ppm due to the protons of PPOs methyl pendant groups. The spectrum of chitosan– pluronic (Fig.3) is similar to that of pluronic but it shows a weak peak at 2.10 ppm and a proton peak at 3.11 ppm from the newly formed amide bond by the EDC/NHS chemistry. DSC curve of grafted chitosan at 55$^{o}$C distinguished the pluronic F-68.Comparitively study of polymer drug ratio ,drug incorporatd drug wt particle size and zeta potential reveals that incorporation efficiency decreases as the drug proportion increases.Micelle hydrodynamic diameters with or without drug were measured using dynamic light scattering(DLS).The mean particle diameters were obtained in between 90 to 100 nm with the micelles of loaded AmB (Fig.5) and 60 to70 nm (Fig.4).observed in case of unloaded micelles which illustrated that the size increases as the drug polymer ratio increases. Zeta potential of micelles shows that formulations have surface charge.that varies from -6 to -13mv and +5 to +14mv in case of loaded and unloaded micelles respectively.

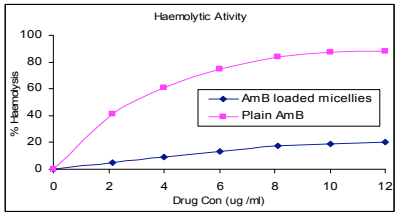


**Fig.6. Haemolysis of RBC at varied level of AmB and AmB loaded micelles**

| Polymer | Polymer :AmB | Drug incorporated % ±SE | Drug weight in micelles | Micelle size (nm) | Zeta potential (mV) |
|---|---|---|---|---|---|
| Grafted polymer | 10:1 | 3.86±0.68 | .17±.03 | 89.5 | -12.8±1.2 |
| | 10:2 | 2.49±.29 | .23±.02 | 93.0 | -9.54±1.4 |
| | 10:4 | 1.93±.10 | .32±.01 | 110.4 | -13.0±1.2 |

**Table 1. Optimization parameters for formulation development**

The loading of AmB into micelles drastically lowers the haemolytic activity of AmB, even at an AmB level of 10 mg/ ml (Fig.6.).Completely solubilizes AmB is very haemolytic, reaching a 80% haemolysis at a level of 10.0 µg/ml. where as AmB loaded polymeric micelles cause 20% haemolysis at the same level for a period of 5.5 h instead of 30 min, indicating that the release of AmB from micelles is slow. A lack of haemolytic activity is evident for AmB loaded micelles. The slow release of AmB from micelles may be due to the solid-like nature of their cores.

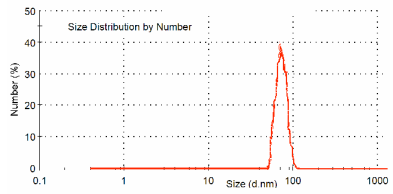


**Fig.4.Mean Particle size of micelles (without AmB).**

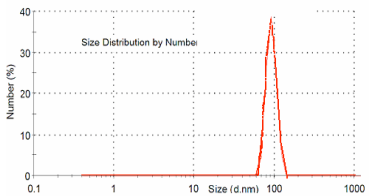


**Fig .5. Mean Particle size of micelles (AmB loaded).**





Loading efficiency of AmB in polymeric micelles.were found to be near 20 to 24 % that was determine by dissolving (1.0 mg) freeze dried sample in 0.20 ml of distilled water and DMF (10 ml) was added ,stirred for 30 min to ensure break down of the micelles and release of AmB. The level of AmB in this solution was measured by UV/VIS spectroscopy at 388 nm. (UV 1700-Pharma Spec SHIMADZU).

## Conclusions

Amphiphilic grafted copolymer self aggregates spontaneously into nanosized micelles in aqueous solution. The nanocarrier prepared from the functionalized triblock copolymer and chitosan had a very low CAC indicates a good stability of the micelles. The drug entrapment efficiency of the micelles was satisfying and enhanced greatly with the inclusion of pluronic as the solubility of AmB was increased to 150 to 1400 fold compared to its water solubility. The small size of polymeric micelles and the absence of toxic effects represent promising characteristic for the development of a novel polymeric drug carrier. The result suggest that chitosan- pluronic micelles present an excellent candidate for a drug delivery system In this research it has been discussed that chitosan-pluronic micelles are a feasible choice to enhance the solubility of hydrophobic drugs and hence can be a new approach in the struggle to find better carriers for drugs that are virtually insoluble in water .Further studies will determine if the micelles are able to provide sustained drug release and whether they can be in drug targeting.

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