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DESIGN AND DEVELOPMENT OF BENDAMUSTINE LOADED CHITOSAN NANOPARTICLE IN-*VITRO* CYTOTOXICITY

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Keywords:

Nanoparticles, Passive targeting, Ionotropic gelation, Cytotoxicity, HeLa cell lines

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ABSTRACT: Nanoparticles represent one of the alternatives in the treatment of cancer chemotherapy. In the present study, the prime objective was to formulate anti-cancer drug, bendamustine into polymeric chitosan nanocarrier system for its sustained release and passive targeting mechanism. Based on particle size, zeta potential and polydispersity index, F3 was selected as the optimized formulation. The SEM images revealed nanostructure with linear/rod shape enabling better internalization. The X-ray powder diffraction analysis clearly indicates reduction in crystallinity of bendamustine in nanoparticles. The nanoparticle formulation showed good results in terms of assayed drug content and encapsulation efficiency. The *in-vitro* permeation studies using egg membrane revealed that F3 formulation showed a sustained drug release for 28 h with an initial burst release within 1 h.

INTRODUCTION: Nanoparticles are solid colloidal particles composed of natural, synthetic or semi-synthetic polymers. The size ranges from 1 nm to 1000 nm. The drugs or other molecules may be dissolved in the nanoparticles, entrapped, encapsulated and/or adsorbed or attached [1]. Applications of nanoparticle drug delivery system: Tumor targeting using nanoparticulate delivery systems, long-circulating nanoparticles, reversion of multidrug resistance in tumor cells, nanoparticles for oral delivery of peptides and proteins, nanoparticles for gene delivery, nanoparticles for drug delivery into the brain.

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Cancer is a major human health problem and the leading cause of death worldwide. It was estimated that every year about 7.6 million people are dying due to cancer and about 70% death in developing counties are because of cancer. Cancer develops due to deregulated cell division of an abnormal cell or from the changes that cause normal cells to acquire abnormal functions.

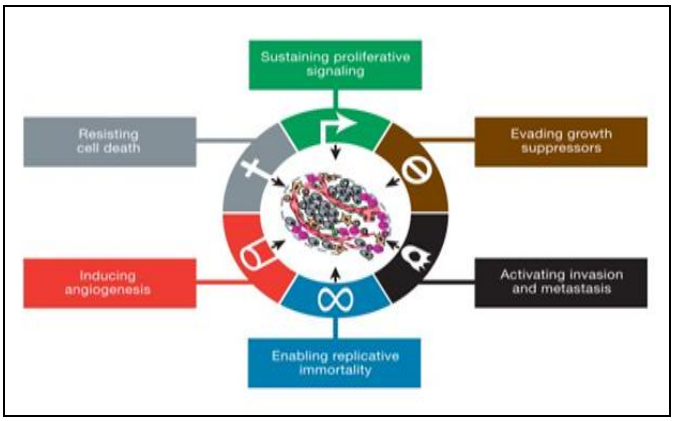


FIG. 1: HALLMARKS OF CANCER

The use of nanoparticles as drug delivery vehicles for anticancer therapeutics has great potential to revolutionize the future of cancer therapy. The primary drawback of conventional chemotherapy using cytotoxic drugs is the bystander toxicity on normal healthy cells. In order to prevent unwanted effects on normal healthy dividing cells, it would be desirable to target these cytotoxic drugs to tumor cells as it possible through nanoparticle drug delivery system [2].

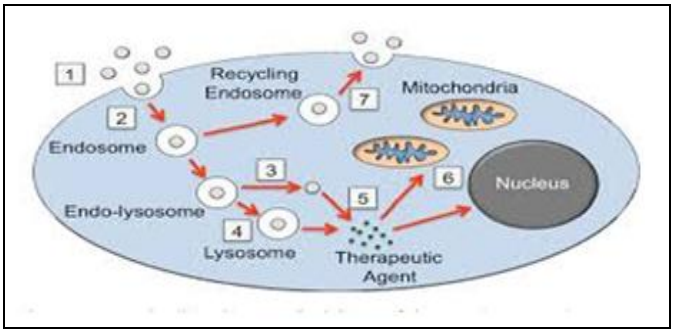


FIG. 2: STEPS DETAILING THE CYTOSOLIC DELIVERY OF THERAPEUTIC AGENTS *VIA* NANOPARTICLE CARRIERS

Two basic requirements should be realized in the design of nanocarriers to achieve effective drug delivery. First, drugs should be able to reach the desired tumor sites after administration with minimal loss to their volume and activity in blood circulation. Second, drugs should only kill tumor cells without harmful effects on healthy tissue. These requirements may be enabled using two strategies: passive and active targeting of drugs.

Importance of nanomaterials as carriers for cancer medicine is the ability to control their size, shape, surface charge and surface chemistry, *etc*. to improve the pharmacokinetics of the chemo drugs and enhance their specificity by way of conjugating with cancer-specific ligands. Nanoparticles made from natural hydrophilic polymers have been proved efficient in terms of better drug-loading capacity, biocompatibility and possibly less opsonization by reticuloendothelial system (RES) through an aqueous steric barrier [3].

Chitosan as biodegradable polymer [4], Chitosan has emerged as a useful drug delivery carrier owing to its polycationic nature, biodegradability, biocompatibility, better stability, low toxicity, simple and mild preparation method, versatile routes of administration and its mucoadhesive properties. Its ease of physical and chemical modification further enhances its properties.

Ionotropic gelation is the most convenient method that involves the electrostatic interaction between the chitosan chain and the negatively charged group of a polyanion like Tripolyphosphate (TPP).

Bendamustine is used to treat chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma and may be used for other types of cancer. The drug has also demonstrated clinical activity in breast cancer [5] and small-cell lung cancer. Bendamustine hydrochloride has unfavorable physicochemical and pharmaceutical properties, translating into unfavorable pharmacokinetic behaviour [6].

The present thesis involves incorporation of anti-cancer drug, bendamustine into the nanocarrier chitosan for its sustained delivery. The surface charge of chitosan and bendamustine is the same, *i.e.*, positive charge, encapsulation of the drug in chitosan-based nanocarrier would be difficult. This problem can be overcome by addition of cross-linker like SDS at 0.05% which even enhance the encapsulation efficiency. Also, addition of an amphiphilic anionic surfactant like SDS to bendamustine is found to increase the aqueous stability in plasma. So, different batches of bendamustine encapsulated chitosan nanoparticles were prepared by ionotropic gelation method with the addition of SDS and further evaluated.

MATERIALS AND METHODS: Bendamustine hydrochloride, Chitosan, Sodium TPP, Tween 80, Sodium Dodecyl Sulphate, Glacial acetic acid.

Experimental Methods:

Pre-Formulation Studies:

a. Characterization of Polymers: Physical Characterisation - Physical appearance, Degree of deacetylation & viscosity.

b. Characterization of API: physical characterization - Physical Appearance, Melting Point, melting point apparatus (Equiptronics, Sigma). Identification of API –FTIR, FTIR Spectroscopy/ 4100 (Jasco, South Africa) range of 400-4000 cm^{-1}.

c. Determination of λ_{max} for bendamustine - UV-Visible spectrophotometer, UV-Visible Spectro-

photometer (Shimadzu, Shimadzu Corporation, Japan) by scanning from 200-400 nm.

Drug Excipients Compatibility Study: FTIR range of 400-4000 cm^{-1}.

Pre-Optimisation Studies: The main objective of the pre-optimization study was to obtain smaller particle size as much as possible since particle size can influence the oral bioavailability. To achieve this objective, at first, chitosan nanoparticles were prepared by changing chitosan: TPP weight ratio as 5:1, 3.5:1 and 2:1. Then, the effect of stirring time (1 h and 4 h) on particle size distribution was also examined. Finally, all the prepared nanoparticles were investigated in terms of particle size and the optimum condition were selected as the criteria for chitosan nanoparticle production.

Formulation of Blank Nanoparticles: The chitosan nanoparticles were produced based on ionic gelation of TPP with chitosan with certain modifications. Chitosan solution (5mg/ml) was prepared by dissolving chitosan in 1% acetic acid solution under overnight stirring at room temperature. The prepared chitosan solution was further diluted to obtain 0.25, 0.5, 0.75, 1, 1.25 (mg/ml). Tween 80 (0.5% v/v), as the re-suspending agent was added to the chitosan solution in order to prevent aggregation. TPP solution was dissolved in distilled water to maintain concentrations of 0.125, 0.25, 0.375, 0.5 and 0.625 (mg/ml).

The prepared TPP solution was added drop-wise to chitosan solution under magnetic stirring, Magnetic stirrer (Remi, Mumbai) (600 rpm) for 4 h at room temperature to obtain blank nanoparticle. Nano-particles were further centrifuged, Centrifuge apparatus (Remi, Mumbai) re-suspended in distilled water and then freeze-dried, Lyophilizer (Lyodel, Chennai) for subsequent analysis.

Formulation of Bendamustine loaded Chitosan Nanoparticles using Pre-Optimised Results: Chitosan solution (5 mg/ml) was prepared by dissolving chitosan in 1% acetic acid solution under overnight stirring at room temperature. The prepared chitosan solution was further diluted to obtain 0.25, 0.5, 0.75, 1, 1.25 (mg/ml). Bendamustine drug solution was prepared by dissolving the drug in SDS – mannitol mixture to increase the stability in aqueous medium 70. For the preparation of Bendamustine loaded nano-particle, the drug solution was added slowly to chitosan solution with magnetic stirring at 600 rpm. TPP solution was dissolved in distilled water to maintain concentrations of 0.125, 0.25, 0.375, 0.5 and 0.625 (mg/ml). After addition of drug solution, TPP solution was added drop-wise to drug - chitosan solution under magnetic stirring (600 rpm) for 4 h at room temperature. The drug-loaded nanoparticles were recovered by centrifugation, re-suspended in distilled water and further lyophilized.

Selection of Optimised Formulation based on Particle Size, Zeta Potential & Poly Dispersity Index: [7] Positive zeta potential measured by zeta Sizer (Malvern, UK) is an important criterion for effective electrostatic interaction with tumor surface. The tumor vasculature permeability was both temporally and spatially heterogeneous, the pore size ranged from 200-600 nm. The particle size distribution is reported as a PDI. The range for the PDI is from 0 to 1; values close to zero indicate a homogeneous dispersion and those greater than 0.5 indicate high heterogeneity.

Evaluation of Drug-Free Chitosan Nanoparticle for:

Bio-Adhesion Study: [8] The study involves determination of mucoadhesive property of blank chitosan nanoparticles by measuring the weight fraction required to detach the nanoparticles adhered to intestinal mucosa. For the study, chick intestine was used as model biological tissue and was obtained from slaughterhouse. A modified double-sided balance was devised to measure the minimum detachment force **Fig. 3A**. The formulation is placed between the two layers of mucosal membrane of which one was tied on to left-hand sidearm of the balance and the other was placed in a petri dish containing defined volume of phosphate buffer pH 6.6 **Fig. 3B**. Initially 5g weight was added to right side arm of the balance, for the purpose of stabilization which was maintained for a period of 5 min. After the specified time, weights were added on to the left-hand sidearm. The weight required to completely detach the nanoparticle from mucosal surface was noted as the bioadhesive strength.

FIG. 3: A. MODIFIED DOUBLE SIDED BALANCE FOR MUCOADHESION STUDY, B. NANOPARTICLE ASSEMBLY BETWEEN MUCOSAL LAYER

pH Sensitivity: [9] The blank chitosan nanoparticles were soaked in phosphate buffer solutions at room temperature with different pH values of 5, 6.6, and 7.4 in order to investigate pH, digital pH meter (EUTECH Instruments) responsive behaviour of nanoparticles. Chitosan nanoparticles were incubated for 1 and 3 h ensuring for swelling equilibrium. Data of particle size distribution obtained in different pH solution after 1 and 3 h incubation period was measured by Zeta Sizer Nano ZS 90 (Malvern, UK).

Evaluation of the Optimized Nanoparticle for:

Entrapment Efficiency: [10] Prepared nanoparticle suspension was centrifuged at 2000 rpm for 30 min. The supernatant was collected and the particles were washed with water and then subjected to another cycle of centrifugation. The amount of free drug in the supernatants was determined by the UV-Visible Spectrophotometer at 231 nm.

Entrapment efficiency % = (Amount of bendamustine added – Amount of free bendamustine) × 100 / Amount of bendamustine added

Characterization by Scanning Electron Microscopy (SEM): [11] The formulation was kept on an SEM (JOEL, Japan) stub using double-sided adhesive tape at 50 mA for 6 min through a sputter. A scanning electron microscope with a secondary electron detector was used to obtain digital images of the nanoparticle.

Characterization by X-ray Diffraction Study (XRD): [12] X-ray diffraction analysis by XRD Diffractometer (Shimadzu, Japan) is used to detect the crystallinity of the pure drug and the formulation. The powder was placed in an aluminum sample holder. Cu radiation was generated at 30 mA and 40kV. Samples were scanned at a range of 10º to 90º with scan speed of 10º min^{-1}.

Determination of Drug Content: [10] 25 mg of the prepared nanoparticles were weighed and dissolved in 5 ml of glacial acetic acid and made up to 25 ml with phosphate buffer pH 6.6. From the aliquots, 1 ml was taken and diluted to 10 ml with the buffer and absorbance was taken in UV-Visible Spectroscopyat 231 nm. From the absorbance value, the drug content was calculated.

***In-vitro* Diffusion Studies using Natural Membrane:** [13] Permeation study was conducted by using egg membrane. The eggshell was kept in concentrated HCl for 2 h. The separated membrane was attached to diffusion cell. An amount equivalent to 5 mg of the drug was placed in the diffusion cell with 10 ml of phosphate buffer pH 6.6. 100 ml of phosphate buffer pH 6.6 was placed in the receptor compartment in a 250 ml beaker. The assembly was then attached to the magnetic stirrer. Samples were withdrawn at specific time interval for 28 h and analyzed by using UV-Visible spectrophotometer at 231 nm.

Drug Release Kinetic Data Analysis: [14] In order to investigate the mechanism of drug release from chitosan nanoparticles, *in-vitro* release data were analyzed using various kinetic models. The zero-order kinetic model (Equation 1) describes the release from the system where the release rate is independent of its concentration of the dissolved substance.

$$Ct = C0 + K0t, \text{ ---------- (1)}$$

Where $C0$ = initial amount of drug, Ct = cumulative amount of drug release at time "t", $K0$ = zero-order release constant expressed in units of conc./time.

Higuchi's model (Equation 2) described the release of drugs from the insoluble matrix as a square root of time-dependent process based on the Fickian diffusion as the cumulative amount of drug released *vs.* square root of time:

$$Qt = Kt1/2, \text{ ---------- (2)}$$

Modeling was performed using the parameters that provide the closest fit between experimental observations and the nonlinear function. The model that best fits the release data was selected based on the correlation coefficient ($R2$) in models described above. The model, which gives highest $R2$ value, is considered as the best fit of

Mechanism of Drug Release: [15] To evaluate the mechanism of drug release for Bendamustine nanosuspension, data of drug release were plotted in Korsmeyer *et al.,*'s equation (Equation 3) as log cumulative percentage of drug released vs log time, and the exponent n and was calculated through the slope of the straight line.

$$Mt\text{-}M\infty = Ktn \text{ ---------- (3)}$$

Where $Mt/M\infty$ is the fractional solute release, t is the release time, K is a kinetic constant characteristic of the drug/ polymer system, n is an exponent that characterizes the mechanism of release of tracers.

If the exponent n = 0.45, then the drug release mechanism is Fickian diffusion, and if $0.45 \geq n \geq 0.89$, then it is non-Fickian or anomalous diffusion. An exponent value of 0.89 is indicative of case-II or typical zero-order release.

***In-vitro* Cytotoxicity:** [16, 17]

Cell Line: The human cervical cancer cell line (HeLa) grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS) The monolayer cells were detached with trypsin-ethylenediaminetetra acetic acid (EDTA) to make single-cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1×10^5 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 ºC, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples.

Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of the medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated. The medium containing without samples were served as control and triplicate were maintained for all concentrations.

MTT Assay: After 48 h of incubation, 15µl of MTT (5 mg/ml) in phosphate-buffered saline (PBS) was added to each well and incubated at 37 ºC for 4 h. The medium with MIT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using microplate reader. The percentage of cell viability was then calculated with respect to control as follows,

$$\% \text{ Cell viability} = [A] \text{ Test} / [A] \text{ control} \times 100\text{---------- (4)}$$

Assessment of Nanoparticles Stability: [18] Stability studies were conducted by storing the nanoparticles at 40 ºC ± 2 ºC, 70% RH ± 5% for 45 days. The samples were withdrawn at initial, 30th & 45th day and analyzed suitably for the physical characteristics, drug content and cumulative drug release by Stability Chamber (Technico, Mumbai).

RESULTS AND DISCUSSION:

Preformulation Study:

Characterization of Polymer:

Physical Characterisation: Physical Appearance - The chitosan was found to have a yellowish-white appearance, Degree of deacetylation & viscosity – 96%, Viscosity = 64.0 cP.

Characterization of API:

Physical Characterisation: Physical Appearance - The drug was pure white and crystalline in appearance. Melting Point was 152-156 ºC.

Drug Excipients Compatibility Study: The IR spectra of pure drug, Bendamustine hydrochloride was analyzed and compared with IR spectra's obtained for drug and excipients. The results showed that the drug and excipients in the formulation was not having any interaction.

Pre-Optimisation Studies:

Pre-Optimisation of Nanoparticle-based on Chitosan:

TPP Weight Ratio and Stirring Time: Size and size distribution of the chitosan nanoparticles depend largely on the concentration of chitosan and TPP solutions. For the success of chitosan with nanosized scale, the concentration of chitosan and TPP should be controlled at a suitable range. The mean size and size distribution of each batch of chitosan nanoparticle suspension were analyzed using the Zetasizer analysis. Effect of stirring time on particle size was also analysed. With the increase in stirring time from 1 h to 4 h, particle size was found to decrease substantially. Hence, 2:1 chitosan-TPP wt ratio and 4 h stirring time were selected as optimum criteria for preparation of blank and drug-loaded nanoparticles **Fig. 4**.

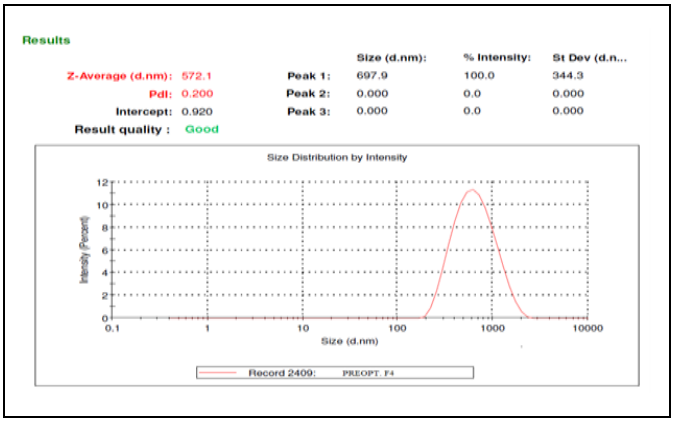


FIG. 4: PARTICLE SIZE ANALYSIS OF FORMULATION WITH 2:1 WT RATIO & 1 h STIRRING TIME

Selection of Optimised Formulation based on Particle Size, Zeta Potential & Poly Dispersity Index: Out of the 5 formulations, F3 formulation has been selected as optimized formulation as the particle size, zeta potential & polydispersity index are all favorable **Table 1**. It possesses a positive zeta potential and hence can easily interact with the negatively charged cancer cell surface. The PDI value indicates it to be a homogeneous dispersion.

TABLE 1: PARTICLE SIZE, ZETA POTENTIAL & POLY DISPERSITY INDEX OF DRUG LOADED NANOPARTICLE

Formulation	Chitosan Conc. (mg/ml)	TPP Conc. (mg/ml)	Drug Conc. (mg/ml)	Particle Size(nm)	Zeta potential	PDI
F1	0.25	0.125	0.325	351.6	-7.1	0.219
F2	0.5	0.25	0.325	398.3	-5.8	0.397
F3	0.75	0.375	0.325	422.1	14.5	0.406
F4	1.0	0.5	0.325	477.6	19.8	0.517
F5	1.25	0.625	0.325	572.1	22.1	0.523

Evaluation of Drug-Free Chitosan Nanoparticle for:

Bio-Adhesion Study: The result shows that as chitosan concentration increases the mucoadhesive property also increases. Hence, the positively charged chitosan nanoparticle helps in passive targeting as the cationic group of chitosan may be attracted electrostatically to the negatively charged phospholipid head group expressed on tumor endothelial cells. This study proves the advantage of using chitosan as the polymer for nanoparticle preparation.

pH Sensitivity: The pH sensitivity of chitosan nanoparticles were investigated and shown in **Table 2**.

TABLE 2: pH RESPONSIVENESS OF CHITOSAN NANOPARTICLE

Particle size of blank nanoparticle	Particle size (nm) at different pH					
175	pH 5		pH 6.6		pH 7.4	
	1 h	3 h	1 h	3 h	1 h	3 h
	378	380	392	397	160	166

Results concluded that particle size is very sensitive to the changing pH values of the residing aqueous environment and chitosan-based nano-particle helps in selective targeting to the tumor environment.

Evaluation of the Optimized Nanoparticle for: Entrapment Efficiency (%) - 87%, pH was found to be 6.54 which was identical to pH of blood.

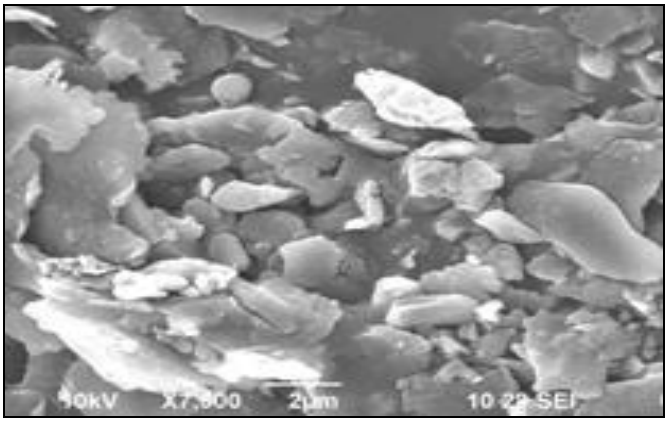


FIG. 5: SEM IMAGE OF OPTIMIZED NANO-PARTICLE (F3)

Characterization by SEM: The SEM images showed that each particle unit exhibited nano-structure and was found to be linear/rod-shaped **Fig. 5**. Linear polymeric carriers can easily penetrate into tissues better than the more structured spherical carriers because of its flexible random coil structure suggesting linear polymers are efficient in passive targeting.

Characterization by XRD: XRD pattern of pure drug and optimized nanoparticle are shown in **Fig. 6** and **Fig. 7** respectively. The nanoformulation was characterized by less intensity of the diffraction peak when compared to Bendamustine hydrochloride. This clearly indicates the reduction in crystallinity of Bendamustine in nanoparticles.

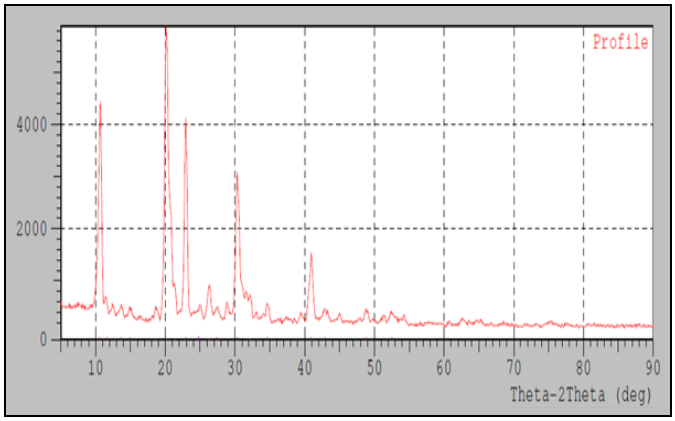


FIG. 6: XRD PATTERN OF BENDAMUSTINE HYDROCHLORIDE

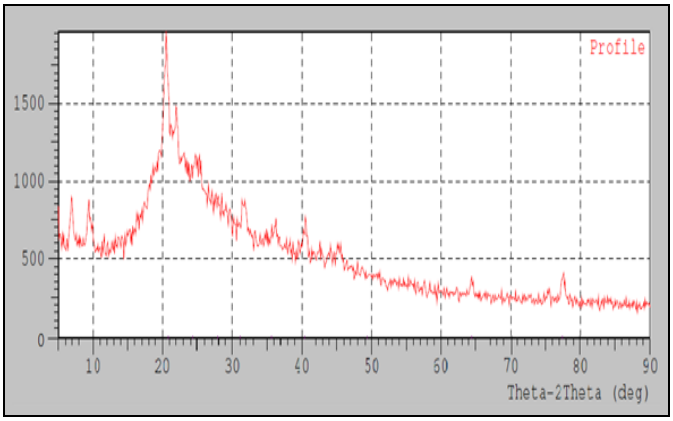


FIG. 7: XRD PATTERN OF OPTIMIZED NANOPARTICLE (F3)

Drug Content: The drug content of F3 formulation was determined by measuring the absorbance at 231 nm and was found to be 85.4%.

***In-vitro* Drug Diffusion using Natural Membrane:** The data obtained for F3 formulation showed 3 phases:

1. A first initial burst release of 44% within 1hr, due to the drug dispersed close to particles surface, which easily diffuse out.

2. A plateau up to 10 h, resulting from diffusion of the drug dispersed in the polymer matrix.

3. A constant sustained release of the drug, resulting from swelling of polymer matrix releasing 89% of drug at the end of 28 h.

Drug Release Kinetic Data Analysis: The release data of nanoparticle formulation were fitted into various mathematical models (Zero order, First order, Higuchi model and Korsmeyer Peppas model) to evaluate the kinetics and mechanism of drug release from the nanoparticles. The results were explained in **Fig. 8** and **Fig. 9**.

***In-vitro* Cytotoxicity by MTT Assay:** The cytotoxicity of optimized formulation, F3 was determined by MTT assay using HeLa cell lines was shown in **Fig. 11**. At low conc., the nano-particle failed to produce any toxicity to viable cells and exhibited viability of 101.26%. This may be because Bendamustine is more sensitive to hematologic malignancies than cell lines derived from a solid tumour.

However, the viability of the HeLa cell lines showed a decline gradually with an increase in the drug concentration as shown in **Fig. 10**.

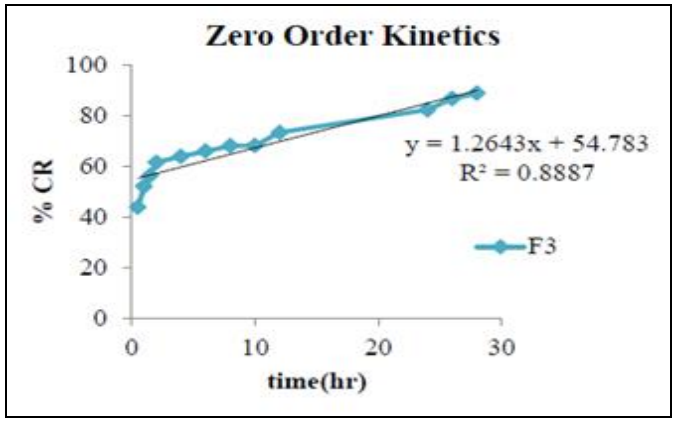


FIG. 8: ZERO ORDER PLOT OF F3

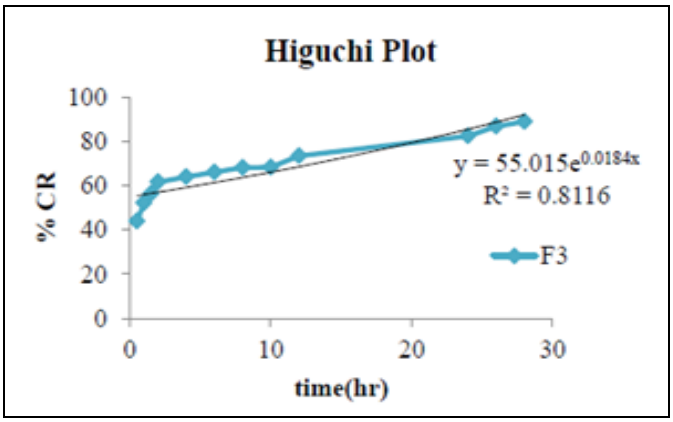


FIG. 9: HIGUCHI PLOT OF F3

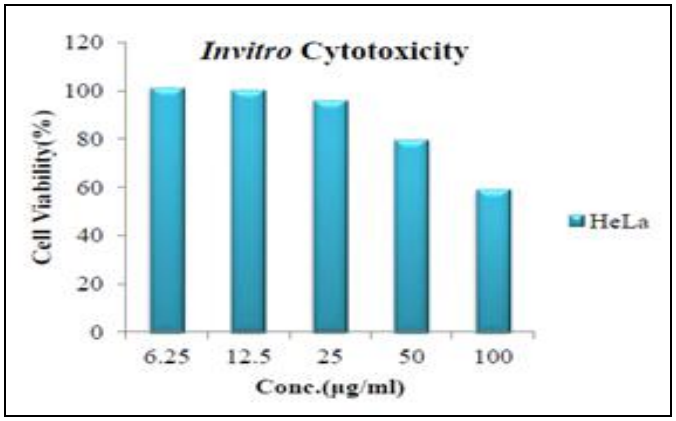
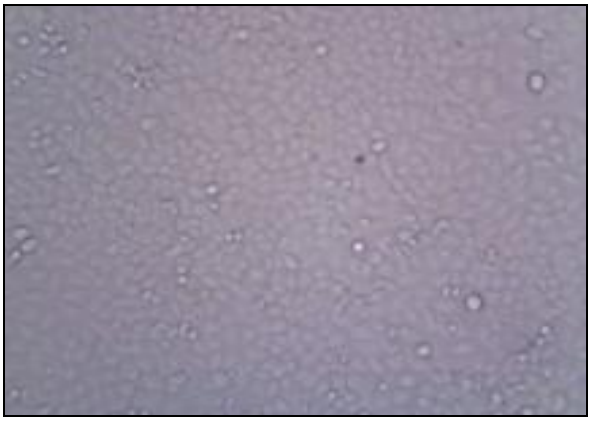
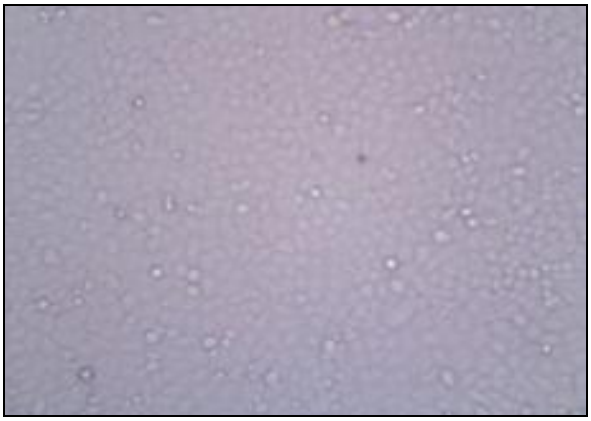


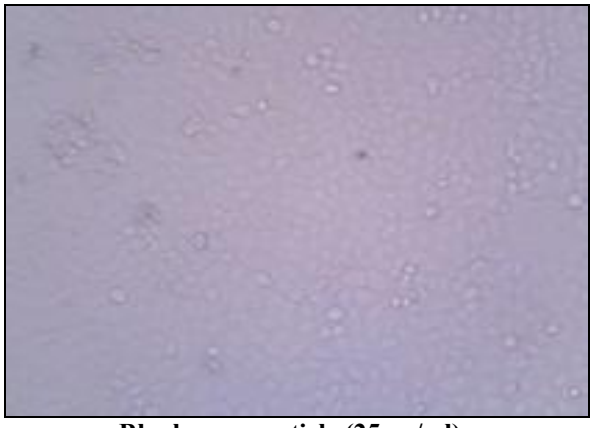
FIG. 10: *IN-VITRO* CYTOTOXICITY STUDIES USING MTT ASSAY



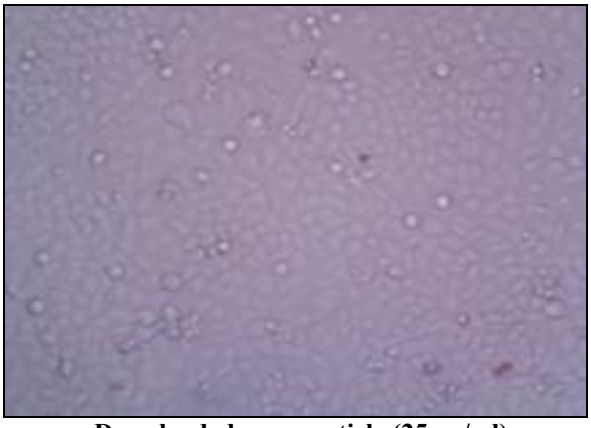
Blank nanoparticle (6.25 µg/ml)



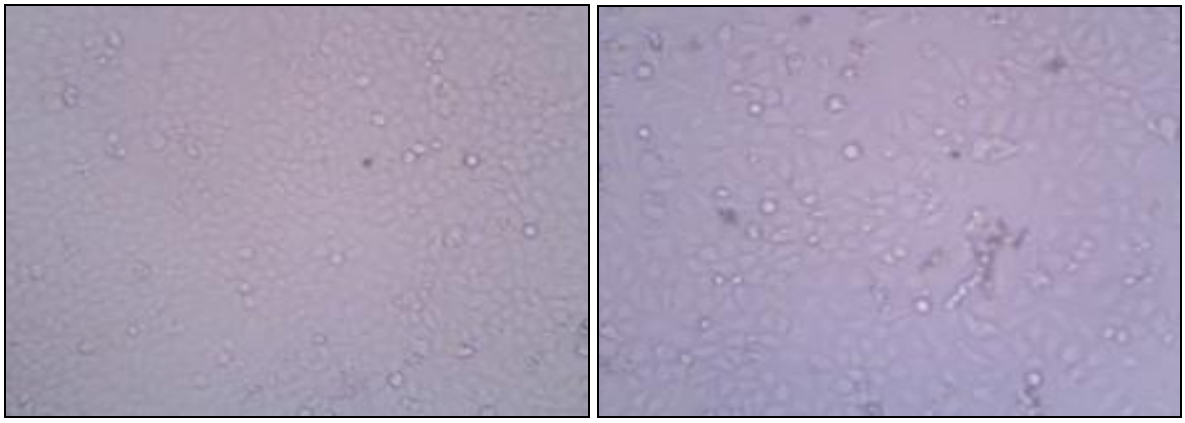
Drug loaded nanoparticle (6.25 µg/ml)



Blank nanoparticle (25 µg/ml)



Drug loaded nanoparticle (25 µg/ml)



Blank nanoparticle (100 µg/ml) **Drug loaded nanoparticle (100 µg/ml)**

FIG. 11: MICROSCOPIC IMAGES (10X) OF HELA CELL LINES TREATED WITH VARIOUS CONCENTRATION OF BLANK AND DRUG LOADED NANOPARTICLE

Stability Studies: Stability studies of the best formulation was carried out at Accelerated temperature at 40° ± 2° / RH 70% ± 5% in humidity control oven for 45 days. After 45 days the sample was evaluated for the physical appearance, drug content, and *in-vitro* drug release studies. The values are shown in **Table 3**.

TABLE 3: STABILITY STUDIES OF OPTIMIZED FORMULATION (F3)

S. no.	Parameters	Accelerated temperature at 40° ± 2°/ RH 70% ± 5%		
		Initial	30th day	45th day
1	Physical Appearance	Opalescent	Opalescent	Opalescent
2	Drug Content (%)	85.4	8.3	83.8
3	% Cumulative release at 28th h	89.0	88.6	88.3

CONCLUSION: The primary objective of the present investigation was to explore biodegradable chitosan as a polymeric material for formulating Bendamustine nanoparticles to be used as a delivery system for cancer treatment. Bendamustine, a class of nitrogen mustard used in the treatment of leukemia, certain lymphomas, and breast cancer has limited chemical stability in plasma, thereby requiring high or repeated doses in order to achieve a therapeutic effect.

In the present study, attempts have been made to increase the stability of Bendamustine by complexing such molecule with polymeric materials. *In-vitro* release studies showed a sustained release characteristic for 28hrs after a burst release at the beginning of 1hr. *In vitro* cytotoxicity studies were carried out on human cervical cancer cell line (HeLa). The current study clearly indicates the potential of chitosan nanoparticles of Bendamustine in development of nanoparticulate based drug delivery system for cancer chemotherapy with better patient compliance. Further studies using animal model will throw more light on the effectiveness of the formulation *in-vivo*.

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