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PREPARATION AND PHYSICOCHEMICAL AND *IN-VITRO* EVALUATION OF LOMUSTINE NANOPARTICLES

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Abstract

In the present study nanoparticles of lomustine were fabricated using chitosan polymer crosslinked with different crosslinking agents like sodium tripolyphosphate and sodium hexametaphosphate. Different formulations of nanoparticles were prepared using different concentrations of crosslinking agents and polyethylene glycol 6000. The average particle size ranged between 112 nm to 942 nm. Zeta potential of nanoparticles ranged between 29.0 mV up to 56.0 mV. Encapsulation efficiency was variable from 58%-96%. The nanoparticles were solid spherical. In vitro drug release study was carried out in phosphate buffered saline solution pH 7.4 for 10 h. The analysis of regression values of Higuchi plot suggested diffusional mechanism and follows Fick's law of diffusion. Drug polymer interaction was absent as evidenced by FT-IR spectra and DSC thermograms. With polyethylene glycol inclusion shows interaction between lomustine and PEG. Cell viability assay (MTT Assay) showed that the lomustine nanoparticles were able to reduce the tumour cell proliferation and increased cell viability significantly ($p < 0.05$) as compared to pure drug in L 132 human lung cancer cell line.

Keywords: Nanoparticles, Chitosan, Peg 6000, Lomustine.

INTRODUCTION

Nanoparticles have been reported to improve *in-vitro* dissolution rate of poorly water-soluble drugs [1]. Nanoparticles have all the advantages of liposomes including the size property but unlike liposomes have a long shelf life and can entrap more drugs [2]. The object of the present study is to prepare nanoparticles of lomustine, an anticancer drug and characterization in terms of physicochemical properties and *in vitro* drug release testing. Lomustine is a cell-cycle phase nonspecific antineoplastic drug. It is mainly used in brain tumors, resistant or relapsed Hodgkin's disease, other lymphomas, lung cancer, malignant melanoma and various solid tumors. It is highly toxic with low therapeutic index. About 60% of cyclohexyl moiety of this drug is found to be bound to plasma proteins. Also, it is poorly soluble in aqueous solutions, but soluble in

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organic solvents such as alcohol. When delivered to the lungs, the unique properties of nanoparticles avoiding mucociliary clearance and delivering drug directly to target tissue or target cells might be utilized for therapeutic treatment of lung specific diseases like cancer. The above said factors make lomustine a suitable candidate for nanoparticles as a particulate carrier for lung cancer. The retention of lomustine bioactivity was demonstrated by the *in vitro* cytotoxic activity assay because it is one of the most important systems to observe how cells respond to specific conditions. As compared to *in vivo* test it is advantageous because of continued growth and immortality and it is a study using easy cell differentiation.

MATERIALS AND METHODS

Chitosan (deacetylation degree 85%) was obtained as a gift sample from Marine Chemicals (Cochin, Kerala). Sodium tripolyphosphate (TPP) of analytical grade was obtained as a gift sample from Devdhar chemicals (Pune). Lomustine pharmaceutical grade (as per Indian Pharmacopoeia) was obtained from Fujian Provincial Medicines and Health Products, Xiamen Import and Export Corporation (China). Sodium hexametaphosphate (SHMP) of analytical grade was procured from Qualigens Fine Chemicals. All other chemicals were of analytical grade and used as received. Distilled water was used throughout the study. Homogenizer was used of Ika Laborthechnik and spray dryer was Buchi B-191 mini spray dryer.

Preparation of nanoparticles :

Table 1 shows the components used for the fabrication of the nanoparticles by homogenization method. Nanoparticles were prepared by homogenization at 24,000rpm by first dissolving the chitosan in 1% v/v acetic acid and then added crosslinking agent. Afterwards drug was added to it gradually with homogenization at 24,000 which was continued for 15min. In formulations A7 to A12 the PEG 6000 aqueous solution was added to a solution of chitosan and crosslinking agent in 1% v/v acetic acid and then lomustine was added to this solution. These solutions were then spray dried. The conditions optimized of spray dryer were: inlet temperature 165^{o}, liquid flow rate 1.5 ml/min, compressed air flow 70psi and nozzle size 0.5mm.

Table 1: Formulae used for the fabrication of the nanoparticles.

Formulation code	Chitosan in 1% v/v aqueous acetic acid solution (%W/V)	Crosslinking agent 1% W/V aqueous solution	1%w/v PEG solution (ml)	Lomustine (%w/v)
A1	0.8	10ml TPP	-	-
A2	0.8	10ml SHMP	-	-
A3	0.8	10ml TPP	-	1
A4	0.8	10ml SHMP	-	1
A5	0.8	15ml TPP	-	1
A6	0.8	15ml SHMP	-	1
A7	0.8	10ml TPP 10ml	5	-
A8	0.8	SHMP	5	-
A9	0.8	10ml TPP	5	1
A10	0.8	10ml SHMP	5	1
A11	0.8	15ml TPP	5	1
A12	0.8	15ml SHMP	5	1

Particle size and morphology :

The particle size, size distribution and zeta potential of chitosan nanoparticles were measured in a Zetasizer (Malvern instruments DTS Ver 4.10). Shape and surface morphology were determined by transmission electron microscopy (TEM) using FEI Morgagni 268 D instrument at an accelerating voltage of 120 kV. A drop of aqueous solution of sample was placed on a membrane coated grid surface. A drop of 1% phosphotungstic acid was immediately added to the surface of the grid. After 1 min. excess fluid was removed and the grid surface was air dried at room temperature before being loaded for TEM. A thin film of aqueous dispersion of nanoparticles was applied on double stick tape over an aluminium stub and air dried to get uniform layer of particles. These particles were coated with gold using sputter gold coater, and subjected to SEM on Leo 435 VP, Cambridge, UK.

FTIR analysis and DSC:

Infrared spectra were recorded on Shimadzu 8400S FT-IR spectrophotometer using KBr pellet method. DSC thermogram was recorded on a differential scanning calorimeter equipped with a thermal analysis data system (Perkin-Elmer DSC-7).

Encapsulation efficiency:

Appropriate amount of spray dried nanoparticles were digested with minimum amount of 95%v/v ethanol until no further material was dissolved. The digested homogenates were centrifuged at 15000 rpm for 30 min and the supernatant was analyzed for drug entrapment. The lomustine entrapment was measured at 230nm using Elico SL164 UV/vis spectrophotometer. The encapsulation efficiency was calculated using equation:

$$\% \text{ encapsulation efficiency} = \frac{\text{Total mass of drug in nanoparticles}}{\text{Mass of drug used in the formulation}}$$

Swelling index:

200 mg of spray dried nanoparticles were dispersed in phosphate buffer pH 7.4 for a period of 6 h. The swollen nanoparticles were collected by centrifugation and the wet weight of the swollen nanoparticles was determined by first blotting with filter paper to remove excess water on the surface and then weighing immediately on electronic balance. The weight of swollen nanoparticles was recorded at a predetermined time period (0.5, 1, 2, 3, 4, 5 and 6 h). The percentage swelling was calculated by using equation: Ssw = Wt-Wo/Wo X 100

Where, Ssw is the percentage swelling, Wt is weight of the nanoparticles at time t, Wo is the initial weight of nanoparticles.

***In vitro* drug release studies:**

Lomustine release from different drug loaded nanoparticles DNPs was determined using dialysis bag under magnetic stirring. 25 mg lomustine loaded nanoparticles were redispersed in 3ml phosphate buffer saline solution pH 7.4 and placed in a dialysis membrane bag with a molecular cut-off of 5kDa, tied and placed into 150 ml PBS solution in a beaker. The entire system was kept at 37^{o} with continuous magnetic stirring. At appropriate time intervals, 3ml of release medium was removed and the same volume of fresh PBS solution was added into the system. The amount of lomustine in the release medium was evaluated by dilution with etnaol 95%w/v by UV spectrophotometer (Elico SL164) at λmax 230.4 nm.

***In vitro* cytostatic activity test :**

The antiproliferative action of pure drug and the nanoparticle formulations were tested on human lung cancer cell line L132. The *in vitro* cytotoxicity of nanoparticles with or without drug was assessed by the colorimetric MTT cell viability assay in L132 human lung cancer cell line. The cell line was cultured in RPMI-1640 supplimented with 10% heat inactivated fetal bovine serum. The tumour cells were cultured for 24 h in a 96-well plate at an initial concentration of $5x10^4$ cells, then the cells were immediately treated with various doses of DNPs for another 24 h. Viable cells were identified and counted using the tryphan blue dye exclusion test.

RESULTS AND DISCUSSION

Recently, the use of complexation between oppositely charged macromolecules to prepare chitosan micro/nanoparticles as a controlled release formulation has attracted much attention because of simple and mild process. The present study demonstrates the preparation of TPP and SHMP crosslinked chitosan nanoparticles by homogenization. Nanoparticles were crosslinked with chemical crosslinking agents and PEG to control the release of drug from the particulate system and for targeted delivery. Nanoparticles were cross-linked covalently and ionically with SHMP and TPP to observe the effect of crosslinking mechanism on the various evaluation parameters of formulations. Ionic interaction between the negative charges of crosslinking agent and positively charged groups of chitosan are main interaction inside the network [3].

In the FTIR spectrum of Blank chitosan-SHMP nanoparticles('a' of Fig.1) peaks at 1837cm^{-1}(C=O str.), 1652cm^{-1}(N-H def.), 1383 and 1320cm^{-1}(C-H def.), 1075cm^{-1}(C-Ostr.) were observed.

In the FTIR spectrum of lomustine loaded SHMP crosslinked chitosan nanoparticles ('b'of Fig.1), as compared to spectrum of chitosan-SHMP nanoparticles a peak at 1837cm^{-1} was disappeared, a new peak at 1234cm^{-1}(-C-OH) appeared. The peaks at 1383 and 1320cm^{-1} were shifted to peaks 1443 and 1377cm^{-1}. The characteristic ring absorption of cyclohexyl ring of lomustine was observed at 1558 cm^{-1} and also a peak of N-O-N linkage at 1073 $cm^{-1}$and of C-Cl at 894 cm^{-1} were observed. This result shows the presence lomustine in chitosan-SHMP nanoparticles with electrostatic interactions.

In the FTIR spectrum of lomustine loaded chitosan- TPP nanoparticles ('c' of Fig.1) as compared to spectrum 'b' the peak at 1234cm^{-1} was not seen. It shows that there is interaction at the –C-OH group in chitosan.

In the spectrum of lomustine loaded PEG coated SHMP crosslinked chitosan nanoparticles('d'of Fig.1) as compared to spectrum 'b', new peaks at 1977, 1922 1879 and 1841cm^{-1} (N-Hstr.)were observed.

A peak at 1232 and 1072 cm^{-1} was shifted to 1252 cm^{-1} (C-Ostr. Of CH_2OH) and 1103 (C-Ostr. Of C-O-C) respectively. Also a peak of 890 cm^{-1} (C-Cl str.) disappeared. This shows the interaction of PEG at chitosan amino and at lomustine –C-Cl group. This is partly similar to the result observed by Kim and Lee [4] that incorporation of PEG in the gel system is through intermolecular hydrogen bonding between the electropositive amino hydrogen of chitosan and electronegative oxygen atom of PEG, thus forming a chitosan-PEG semi interpenetrating network.

Fig.1: FTIR spectra of nanoparticles. a- spectrum of chitosan-SHMP nanoparticles, b- spectrum of lomustine loaded chitosan-SHMP nanoparticles, c- spectrum of lomustine loaded chitosan-TPP nanoparticles, d- spectrum of lomustine loaded PEG coated chitosan-SHMP nanoparticles,

In DSC thermogram of nanoparticles endotherms were observed which shown melting peaks at 90.83°C in lomustine loaded chitosan-SHMP nanoparticles (Fig.2a), 91.44°C in lomustine loaded chitosan-STPP nanoparticles (Fig.2b), 119.3°C in lomustine loaded PEG coated chitosan-SHMP nanoparticles (Fig. 2c), and 91.42°C in lomustine loaded PEG coated chitosan-STPP nanoparticles (Fig.2d) confirming presence of drug to polymer interactions but showing stability of drug in the nanoparticles

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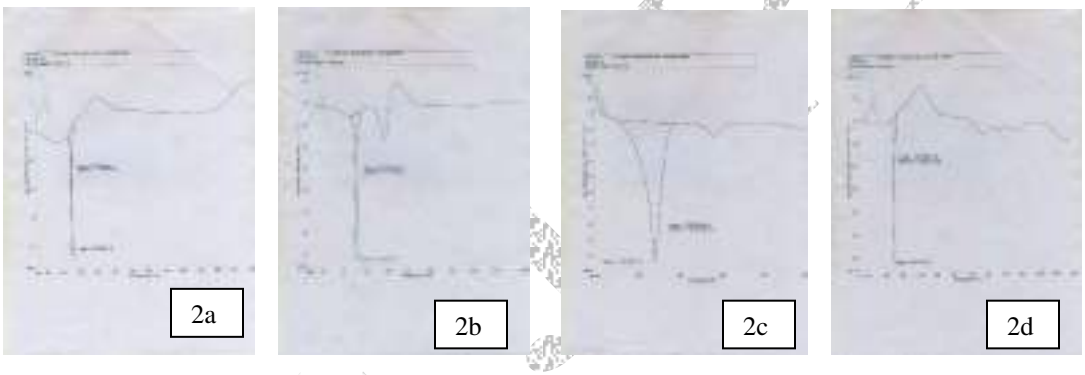

Fig. 2: Differential scanning calorimetry of chitosan nanoparticles (DSC) 2a- lomustine loaded chitosan-SHMP nanoparticles (A3), b-lomustine loaded chitosan-TPP nanoparticles (A4), c-lomustine loaded PEG coated chitosan-SHMP nanoparticles (A9), d-lomustine loaded PEG coated chitosan-STPP nanoparticles (A10).

TEM pictures of chitosan-SHMP and chitosan-TPP nanoparticles (Fig. 3 a and b) showed solid dense spherical structure. The factors like concentration of chitosan, volume of crosslinking agent affect the shape of the nanoparticles. The type of crosslinking agent did not affect the shape, but PEG coating slightly affected the shape because PEG coated lomustine loaded chitosan SHMP nanoparticles (Fig. 3 c) were solid slightly compressed i.e. egg shaped and PEG coated lomustine loaded chitosan TPP nanoparticles (Fig. 3 d) were solid spherical.

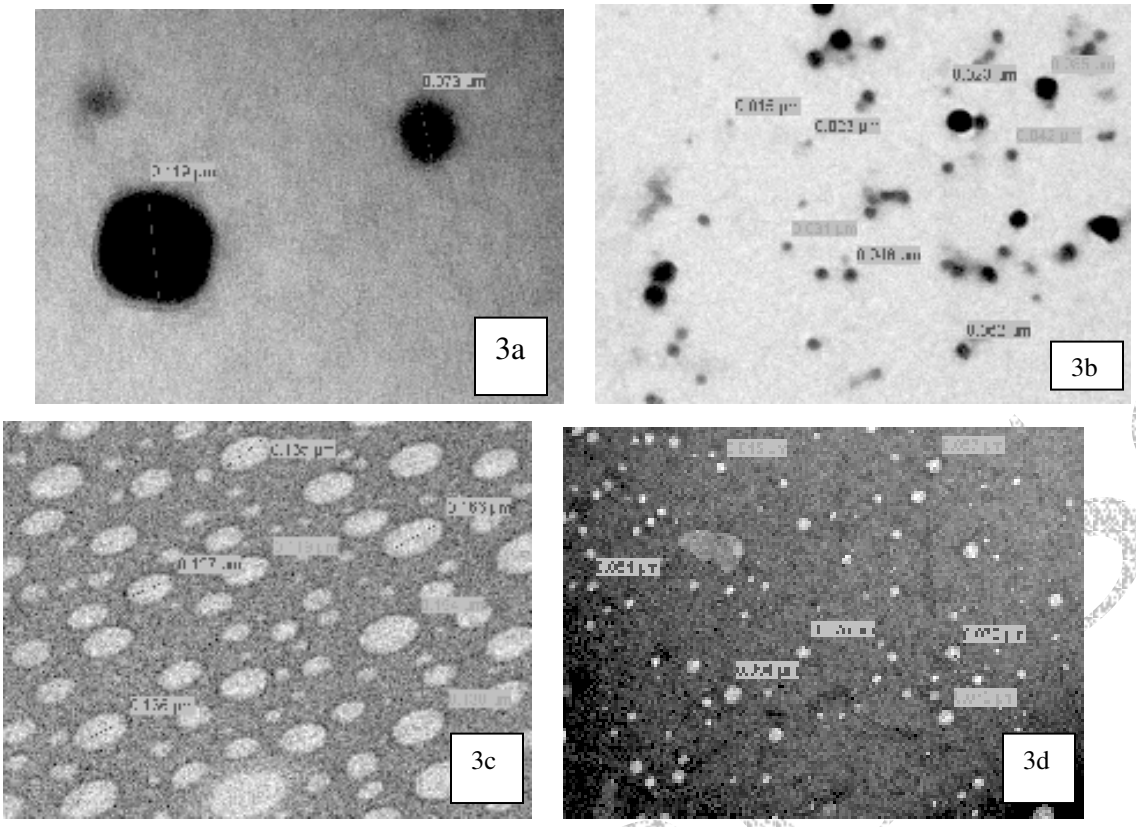

Fig. 3: Transmission electron microscopic photograph (TEM) of chitosan nanoparticles a- TEM of lomustine loaded chitosan-SHMP nanoparticles (A3), b- TEM of lomustine loaded chitosan-TPP nanoparticles (A4), c- TEM of lomustine loaded PEG coated chitosan-SHMP nanoparticles (A9), d- TEM of lomustine loaded PEG coated chitosan-STPP nanoparticles (A10).

The SEM picture of lomustine loaded chitosan-STPP nanoparticles showed well formed spherical particles with smooth surface (Fig. 4 a). In SEM of lomustine loaded chitosan-SHMP nanoparticles surface observed with vacuoles because of dehydration of surface of nanoparticles (Fig. 4 b). In case of drug loaded chitosan-STPP nanoparticles, the formulation parameters like drug loading, concentration of chitosan produced remarkable change in the surface morphology of nanoparticles. However, drug crystallinity decreases the surface smoothness of the spray dried chitosan microspheres [5] which may also be one of the reasons of vacuoles on the surface of nanoparticles. In case of chitosan microspheres [5] concentrated chitosan solution (1% w/v) generated well formed spherical particles with smooth surface when compared to dilute solutions (0.5% w/v) which generated irregularly shaped particles. Roughness of the spray dried nanoparticles of chitosan increased when the drug loading was increased from 1 to 2% w/v because of drug crystallinity. Also the volume of crosslinking agent affects the surface morphology of carrier particles. When 5, 10 ml were used of 1% w/v TPP solution for crosslinking with chitosan. Spray dried nanoparticles were spherical with smooth surface when 1% w/v chitosan solution was crosslinked with 5 and 10 ml TPP solution. When volume of solution was increased to 15 ml it showed roughness on the surface of spray dried chitosan microspheres [5]. Observing all these factors in the present study, the optimized chitosan concentration 0.8% w/v, drug loading 1% w/v, 1% w/v TPP solution 10

ml and 1% w/v SHMP solution 10 ml used for preparation of nanoparticles, showed better results with smooth surface.

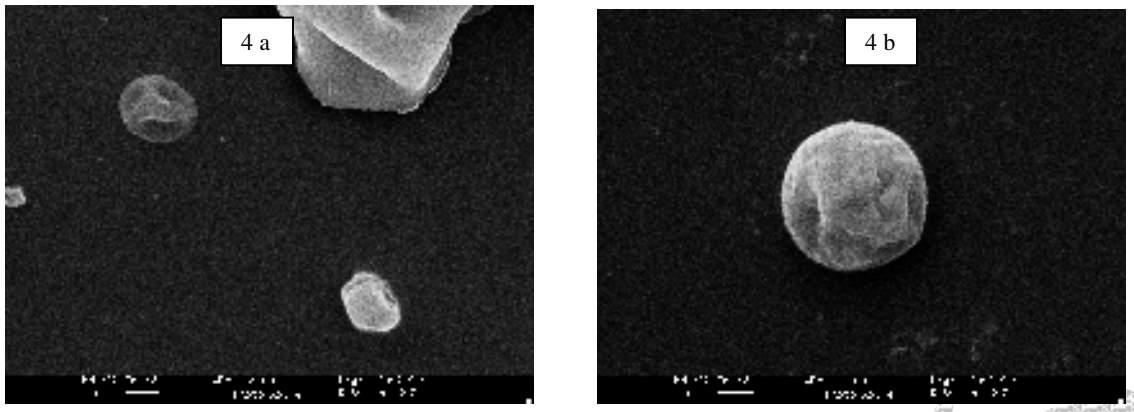

Fig. 4: Scanning electron microscopic photograph (SEM) of chitosan nanoparticles 4a- SEM of lomustine loaded chitosan-TPP nanoparticles, 4b- SEM of lomustine loaded chitosan-SHMP nanoparticles

Chitosan nanoparticles were obtained in the average size range of 112-942nm (Table 2) and with zeta potential range from 29.0 upto 56.0mV (Table 2).The % encapsulation efficiency ranged between 58-96%(Table 2). Factors like nature and type of crosslinking agent, volume of crosslinking agent, chitosan concentration, drug concentration, nozzle size of spray dryer, liquid flow rate in the spray dryer affect properties of chitosan based nanoparticles. Nanoparticle size increases with increase in chitosan concentration. This is due to greater amount of chitosan contained in the same volume of a liquid droplet [6, 7]. So the chitosan concentration was optimized as 1%w/v solution. Also increased viscosity of chitosan solution because of increased concentration, demands for appropriate spray nozzle size and liquid flow rate in spray dryer and ultimately drying temperature. As viscosity increases nozzle size of larger diameter should be chosen which affects particle size.

Table 2: Physical characteristics of lomustine nanoparticles

Formulation batch code	Average particle size (nm)(mean±SD*)	PDI (Mean±SD*)	Encapsulation efficiency (%) (Mean±SD*)	Zeta potential (mV) (Mean±SD*)	% Swelling index (Mean±SD*)
A1	111±16.2	0.231±0.049	-	38±1.2	428±12.6
A2	136±11.4	0.116±0.039	-	32±1.7	465±12.7
A3	478±13.1	0.172±0.032	58±0.88	56±1.1	437±13.4
A4	510±14.2	0.318±0.027	64±0.60	50.8±2.3	482±14.2
A5	249±15.1	0.223±0.014	81±0.67	43±1.2	369±11.4
A6	458±13.9	0.199±0.059	87±0.85	46±1.5	375±10.4
A7	265±12.8	0.517±0.037	-	36±1.0	350±13.2
A8	388±17.6	0.263±0.022	-	29±1.1	395±14.4
A9	717±19.3	0.121±0.036	76±0.90	46±0.96	455±15.3
A10	942±11.7	0.276±0.031	79±0.48	42±1.4	478±15.2
A11	571±17.8	0.224±0.026	85±0.82	37±1.7	347±14.8
A12	648±16.3	0.142±0.046	96±0.51	35±2.2	392±13.7

n=3, SD*= Standard deviation

Similarly, encapsulation efficiency of chitosan-TPP nanoparticles and chitosan-SHMP nanoparticles was affected by chitosan concentration and volume of 1% w/v TPP solution. This may be explained on the basis that

increase in the viscosity of chitosan solution with increase in concentration prevents itself and drug crystals from leaving the droplet [5]. It has been previously reported that highly viscous nature of gelation medium hinders the encapsulation of drug in the study of chitosan microspheres [8]. So relatively lower viscosity of chitosan with lower concentration promotes the encapsulation of drug and gelation between chitosan and crosslinking agent [2]. Encapsulation efficiency of nanoparticles crosslinked with SHMP was slightly higher than TPP crosslinked nanoparticles. Further PEG coating showed better encapsulation efficiency with both the crosslinking agents. This may be due to formation of semi interpenetrating network for drug adsorption.

Swelling studies (Table 2, Fig. 5) of all nanoparticles of all formulations were performed in PBS pH 7.4. It was observed that %swelling index of chitosan-TPP and chitosan-TPP-PEG nanoparticles was about 428% and 350% over the 4 h and it was more about 465% and 395% for chitosan-SHMP and chitosan-SHMP-PEG nanoparticles. All the results were statistically analyzed as per two way ANOVA test. The calculated values of F were 4.13 and 61.23 which is more than critical value of F. Hence there is significant difference in the %swelling by the formulations at different time intervals. The swelling capacity of spray dried chitosan is higher than the pure chitosan [9]. The swelling capacity of spray dried chitosan nanoparticles crosslinked with TPP and SHMP solution were increased with time. As the volume of TPP and SHMP solution added increased the swelling capacity decreased considerably, apparently because more tightly crosslinked chitosan matrix does not swell as much as the loosely crosslinked chitosan matrix. At lower volume of crosslinking agent the chitosan network is loose and has a high hydrodynamic free volume to accommodate more solvent molecules thereby inducing chitosan-TPP and chitosan-SHMP nanoparticles matrix swelling. The water uptake in hydrogels depends upon the extent of hydrodynamic free volume and availability of hydrophilic functional groups for water to establish hydrogen bonds [5]. Higher water uptake values at lower levels of crosslinking and vice versa observed in the study confirm the formation of rigid chitosan-TPP networks. The higher concentration of crosslinking agent increase the rigidity of the polymer surface and also decrease the hydrophilic groups available in the chitosan molecule that are responsible for swelling. Swelling ability of TPP crosslinked nanoparticles was found to be less pronounced than SHMP nanoparticles. This may be explained on the basis that a conformational change of chitosan with TPP leads to the formation of a permanent network due to stronger covalent bond between two reactive groups of TPP and chitosan polymer chains [3].

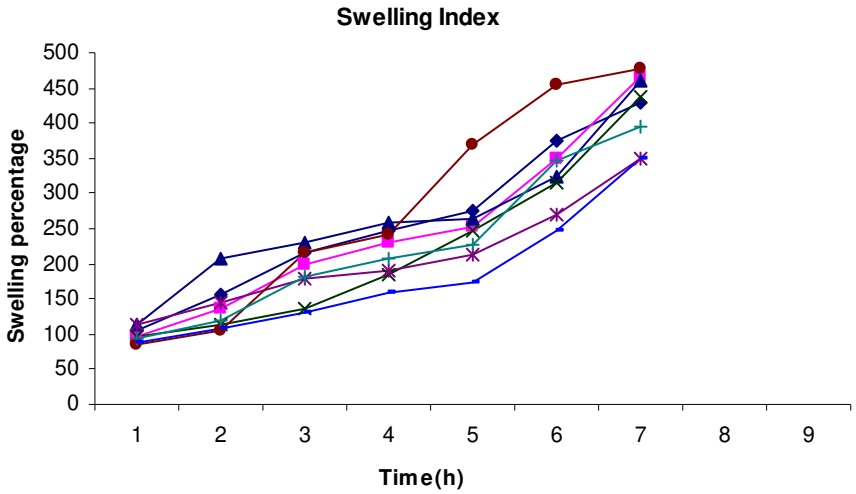

Fig. 5: Swelling behavior of different nanoparticles formulations.The plot shows swelling index of formulations A1, A2, A3, A4, A5, A6, A10

The *in vitro* drug release studies (Fig. 6) were performed in phosphate buffered saline pH 7.4. The results revealed that type of crosslinking agent and concentration of crosslinking agent affects the percentage drug release significantly. All the results were statistically analyzed as per one way ANOVA test. The calculated value of F was 4.0 which is more than the critical value of F at 5% level of significance and the null hypothesis is not accepted. Hence there is significant difference in the percentage of drug release by different samples. The crosslinking density has a remarkable effect on the release of drugs from chitosan based particulate carriers [10, 11].

When the volume of 1% w/v cross linking agent increased from 10ml (A3 and A4) to 15ml (A5 and A6) the release rate of lomustine from chitosan nanoparticles decreased. This is due to that the release rate of drugs from chitosan particulate carriers depends on the density of crosslinked chitosan matrix. The density of the chitosan crosslinked matrix increases with increasing volume of crosslinking agent [12, 13, 14]. Surface morphology plays an important role in the percentage drug release. The nanoparticles were assessed to establish the release kinetics of lomustine. To study the mechanism of lomustine release from nanoparticles, the dissolution data was plotted as % drug release against the square root of time. Linearity of the plots indicated that the release of lomustine from chitosan nanoparticles is by diffusion and followed Fick's law of diffusion and showed root time dependent release.

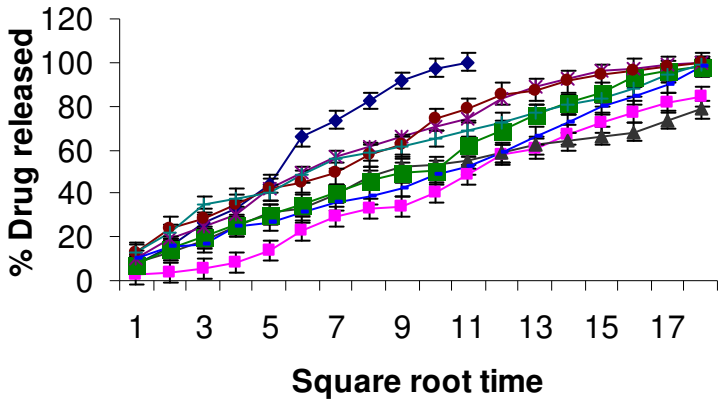

Fig. 6: Percent drug release vs. square root time plots of *in vitro* dissolution studies of different nanoparticle formulations. The plot shows release profile of eight formulations.

The retention of lomustine bioactivity was demonstrated by the *in vitro* cytostasis assay. It was observed that all the nanoparticle formulations were able to slow the tumour cell proliferation. In contrast to the control nanoparticles that induced little cytotoxicity, the drug loaded nanoparticles induced a gradation of cytotoxicity in proportion to the applied drug concentration (fig. 7). Drug loaded nanoparticles could kill cells more efficiently than the corresponding drug alone ($p < 0.05$). Zeta potential of nanoparticles was also an important factor which contributes their interaction *in-vivo* with the tumor cell membrane. Earlier study showed that the tumor cell uptake of nanoparticles can be viewed as a two-step process: first a binding step on the cell membrane and second the internalization step. Electrostatic interactions govern the adsorption of the nanoparticles onto the cell membrane[15]. The greater the zeta potential of chitosan nanoparticles, the stronger the interactions with tumor cell membrane, and leads to higher anticancer property. As a kind of cationic polymer, the surface charge of nanoparticles is the major factor affecting its anticancer activity due to the electrostatic ionic interaction between the negatively charged groups of the tumor cells and the positively charged amino groups of chitosan. The greater zeta potential of nanoparticles (batch A3 and A4, Table 2) than PEG coated nanoparticles (batch A9 and A10, Table 2) helps for their higher anticancer activity as shown in Fig. 7.

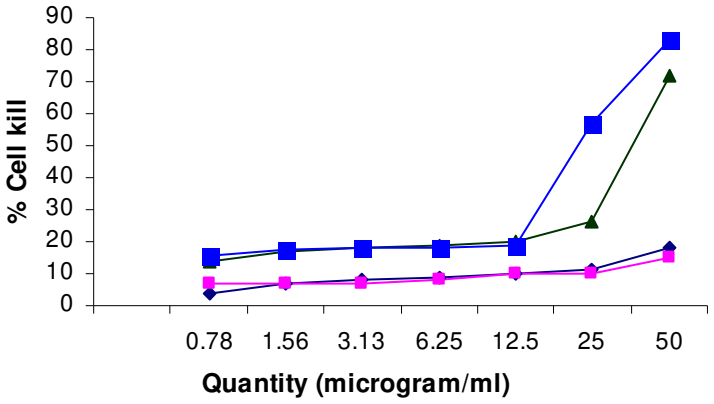

Fig. 7: Comparison of cell toxicity of different nanoparticles formulations in L132 human lung cancer cells. The plot shows % viability of cells after treatment with formulations, , , , .

CONCLUSION

By observing above said parameters of lomustine loaded chitosan TPP and chitosan-SHMP nanoparticles with high encapsulation efficiency, smooth solid spherical surface morphology, extreme positive zeta potential increased surface area, it can be concluded that these nanoparticles can be successfully formulated by homogenization method for increasing therapeutic index and be formulated as intravenous dosage form and also further as a suitable pulmonary device to deposit carrier in the lower alveolar region of lungs for ready internalization in the cells associated with cancerous tumors or targeted epithelial cells.

REFERENCES

1. *Jaihui hu, Johnston KP, Williams RO. Nanoparticle engineering processes for poorly water soluble drugs. Drug Dev Ind Pharm 2004;30:233-45*
2. *Yan W, Yang W, Wang C, Jianhua Hu, Shoukuan Fu. Chitosan nanoparticles as a novel drug delivery system for ammonium glycyrrhizinate. Int J Pharm 2005;295:235-45*
3. *Berger J, Reist M, Mayer JM, Felt O, Peppas NA, Gurny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm 2004;57:19-34*
4. *Kim SS, Lee YM. Synthesis and properties of semi-interpenetrating polymer networks composed of β-chitin and polyethylene glycol. Macromer polym 1995; 36:4497-501*
5. *Desai KG, Park HJ. Preparation and characterization of drug loaded chitosan- tripolyphosphate microspheres by spray drying. Drug Develop Res 2005;64:114-28*
6. *Pavenetto F, Genta I, Giunchedi P, Conti B, Conte U. Spray dried albumin microspheres for the intra-articular delivery of dexamethasone. J Microencapsulation 1994;11:445-54*
7. *Wagennar BW, Muller BW. Piroxicam release from spray dried biodegradable microspheres. Biomaterials 1994;15:49-54*
8. *Vandenberg GW, Drolet C, Scott SL, Noue JD. Factors affecting protein release from alginate- chitosan coacervate microcapsules during production and gastric/intestinal simulation. 2001;77:297-307*
9. *Mi FL, Shyu SS, Kuan CY, Lee ST, Lu KT, Jang SF. Chitosan polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. J Appl Polym Sci 1999;74:1868- 79*
10. *Hejazi R, Amiji M. Chitosan based gastrointestinal delivery systems. J Control Rel 2003;89:151-65*
11. *Sinha VR, Singla AK, Wadhawan S, KaushikR, Kumaria R, Bansal K, et al. Chitosan microspheres as a potential carrier for drugs. Int J Pharm 2004;274:1-33*

12. *Shu XZ, Zhu KJ. A novel approach to prepare tripolyphosphate/chitosan complex beads for controlled drug delivery. Int J Pharm 2000;201:51-8*

13. *Mi FL, Sung HW, Shyu SS, Su CC, Peng CK. Synthesis and characterization of biodegradable TPP/genepin crosslinked chitosan gel beads. Polymer 2003;44:6521-30*

14. *Lim LY, Wan LSC, Thai PY. Chitosan microspheres prepared by emulsification and ionotropic gelation. Drug Develop Ind Pharm 1997;23:981-85*

15. *Qi L, Xu Z, Jiang X, Li Y, wang M. Cytotoxic activities of chitosan nanoparticles and copper loaded nanoparticles. Bioorg. Med. Chem. Lett. 2005;15:1397-1399*

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