Journal of Nanomedicine & Nanotechnology - Open Access

Research Article
OPEN ACCESS Freely available online
doi:10.4172/2157-7439.1000103

# Fabrication of Lomustine Loaded Chitosan Nanoparticles by Spray Drying and in Vitro Cytostatic Activity on Human Lung Cancer Cell Line L132

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## Abstract

This study was aimed to develop lomustine loaded chitosan nanoparticles using a homogenization and spray drying technique. Effect of crosslinking agents (sodium tripolyphosphate (TPP), and sodium hexametaphosphate (HMP)) were studied on the leaching of drug, water uptake of hydrogels, drug release from matrix and its mechanism. **N**anoparticles were obtained in the average size range of 111±16.2 to 942±11.7 nm with polydispersity index (PDI) from 0.116±0.039 to 0.517±0.037. Zeta potential of nanoparticles was ranged from 29.0±1.1 to 56.0±1.1 mV. The % encapsulation efficiency of nanoparticles ranged between 58±0.88% and 96±0.51%.nanoparticles were coated with PEG 6000 to modulate drug release. 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-HMP and chitosan-HMP-PEG nanoparticles. Drug release was sustained and diffusion controlled. Optimized formulation was tested for anticancer activity and drug retention study. Cytotoxicity on human lung cancer cell line L132 was studied by trypan blue dye exclusion test. Drug loaded nanoparticles killed L132 cells more efficiently than the corresponding drug alone ($p < 0.05$). Due to the increased surface area lomustine loaded TPP and HMP crosslinked chitosan nanoparticles showed better anticancer activity.

**Keywords:** Spray drying method; Lomustine; Chitosan nanoparticles; Human lung cancer cell line

## Background

Nanoparticle suspensions very often present a physicochemical instability during their storage. In order to overcome this lack of stability and facilitate the handling of these colloidal systems, the water elimination from the aqueous dispersions to obtain a dry solid form appears as the most promising strategy [1]. Small size of nanoparticles leads to particle–particle aggregation, making physical handling of nanoparticles difficult in liquid and dry powder forms. To solve these limitations, the production of spray-dried powders containing nanoparticles, Which are localized in the lungs as polymeric nanoparticles of sufficiently small dimensions avoid mucociliary and phagocytic clearance until the particles have delivered their therapeutic payload, have been reported [2-6].

Reduction of particle size to the nanometer scale can be achieved by precipitation or by milling. The latter requires a special technique such as high-pressure homogenization [10]. In order to obtain a dry form, further pharmaceutical operations are required (e.g. lyophilisation or spray-drying). Spray drying is known to produce amorphous material due to rapid solvent evaporation [11]. Spray-drying technique has wide applications in the preparation of pharmaceutical powders with specific characteristics such as particle size and shape. It is an established method that is initiated by atomising suspensions or solutions into fine droplets followed by a drying process, resulting in solid particles [7]. The process allows production of fine, dust-free powders as well as agglomerated ones to precise specifications [8]. Although most often considered as a dehydration process, spray drying can also be used as an encapsulation method when it entraps active materials within a protective matrix, which is essentially inert to the material being encapsulated [9]. Spray drying is the most commonly used in the drug or food industry due to its continuous production and easiness of industrialization [12]. The retention of drug loaded nanoparticles and their potential as better therapeutic option in the treatment of intractable disease are the subject of a number of research articles.

## 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 (LMT) of Indian Pharmacopoeias grade was obtained from Fujian Provincial Medicines and Health Products, Xiamen Import and Export Corporation (China). Sodium hexametaphosphate (HMP) of analytical grade was procured from Qualigens Fine Chemicals, India. All other chemicals were of analytical grade and used as received. Distilled water was used throughout the study. Homogenizer (Ika Laborthechnik) and spray dryer (Bauchi B-191 mini spray dryer) were used for the respective operations.

### Preparation of nanoparticles

The components of the different batches of nanoparticles loaded with LMT are shown in Table 1. Chitosan in 1% v/v acetic acid and then 1% w/v aqueous solution of the cross linking agent was added. Afterwards LMT was added to it gradually with homogenization at 24,000 rpm which was continued for 15 min. In formulations A7 to A12 the PEG 6000 1%w/v aqueous solution was added to a solution

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**Received** October 02, 2010; **Accepted** October 25, 2010; **Published** October 27, 2010

**Citation:** Mehrotra A, Nagarwal RC, Pandit JK (2010) Fabrication of Lomustine Loaded Chitosan Nanoparticles by Spray Drying and in Vitro Cytostatic Activity on Human Lung Cancer Cell Line L132. J Nanomedic Nanotechnolo 1: 103. doi:10.4172/2157-7439.1000103

of chitosan and crosslinking agent in 1% v/v acetic acid and then LMT was added to this solution. These solutions were then spray dried. The conditions optimized for spray drying were: inlet temperature 165°C, liquid flow rate 1.5 ml/min, compressed air flow 70 psi and nozzle size 0.5mm.

## Nanoparticles characterization

**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 in water of dried 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. For SEM analysis, a thin film of aqueous dispersion of nanoparticles was applied on double stick tape over an aluminum 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.

## Differential scanning calorimetry (DSC)

DSC thermogram was recorded on a differential scanning calorimeter equipped with a thermal analysis data system (Perkin-Elmer DSC-7).

## Encapsulation efficiency

Accurately weighted quantity of spray dried nanoparticles was digested in 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 230 nm spectrophotometrically (Shimadzu 1601, Japan). The encapsulation efficiency was calculated using equation:

$$\%\ \text{Encapsulation efficiency (EE)} = \frac{\text{Total mass of drug in nanoparticles}}{\text{Mass of drug used in the formulation}} \times 100$$

## 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 predetermined time period (0.5, 1, 2, 3, 4, 5 and 6 h).

The percentage swelling was calculated by using equation: $S_{sw} = W_t\text{-}W_o/W_o$ X 100

Where, $S_{sw}$ is the percentage swelling, $W_t$ is weight of the nanoparticles at time t, $W_o$ is the initial weight of nanoparticles.

## In vitro drug release studies

Lomustine release from different DNPs was determined using dialysis bag under magnetic stirring. 25 mg lomustine loaded nanoparticles were redispersed in 3ml phosphate buffer (pH 7.4) and placed in a dialysis membrane bag with a molecular cut-off of 5 kDa, tied and placed into 150 ml PBS solution in a beaker. The entire system was kept at 37°C with continuous magnetic stirring. At appropriate time intervals, 3 ml of release medium was removed and replenish with fresh PBS solution. The amount of lomustine in the release medium was evaluated by dilution with ethanol 95%w/v by UV spectrophotometer (Shimadzu 1601) at $\lambda_{max}$ 230 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 cell line was cultured in RPMI-1640 supplemented 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/well in 180 µL growth medium RPMI-1640 and incubated for 24 h, then the cells were immediately treated with various doses of DNPs for another 48 h. Viable cells were identified and counted using the trypan blue dye exclusion test in which 1:1 dilution of cell suspension using a 0.4% trypan blue solution was prepared. The counting chambers of a hemocytometer was loaded with the dilution and left for 1-2 minutes. The number of stained cells and total number of cells were counted. The calculated percentage of stained cells will represent the percentage of cells killed.

## Statistical analysis

Results are given as mean ± SD. Mean values of nanoparticle size, polydispersity index, zeta potential and encapsulation efficiency were compared using the Student's t-test. Differences are considered significant at a level of $p<0.05$. In vitro data was analyzed with a one-

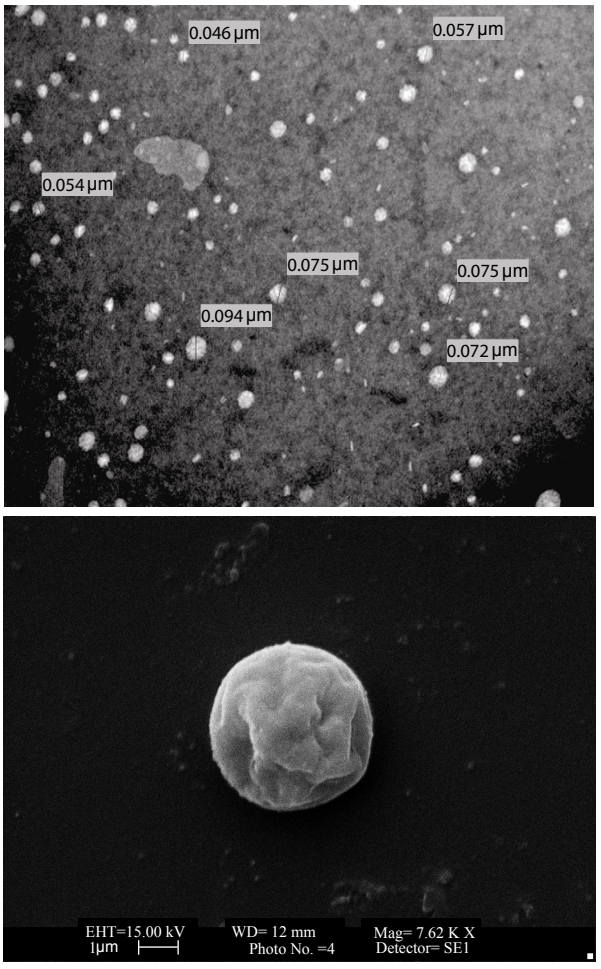


**Figure 1:** Lomustine loaded chitosan nanoparticles.

| S.no | Batch code | CH % w/v | Cross linking agent | LMT % | PEG (ml) | Particle size (nm) Mean±SD* | PDI Mean±SD* | ZP (mV) MeanSD* | ZP (mV) in 0.1 mol/l KCl | % Swelling Mean±SD* | %EE Mean±SD* | Release kinetics Higuchi | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | $R^2$ | n-value |
| 1. | A1 | 0.8 | 10 ml TPP | - | - | 111±16.2 | 0.231±0.049 | 38±1.2 | -2.76±0.21 | 428±12.6 | - | - | - |
| 2. | A2 | 0.8 | 10 ml HMP | - | - | 136±11.4 | 0.116±0.039 | 32±1.7 | -2.19±0.16 | 465±12.7 | - | - | - |
| 3. | A3 | 0.8 | 10 ml TPP | 1 | - | 478±13.1 | 0.172±0.032 | 56±1.1 | -3.09±0.1 | 437±13.4 | 58±0.88 | 0.9924 | 0.49 |
| 4. | A4 | 0.8 | 10 ml HMP | 1 | - | 510±14.2 | 0.318±0.027 | 50.8±2.3 | -1.80±0.07 | 482±14.2 | 64±0.60 | 0.9976 | 0.52 |
| 5. | A5 | 0.8 | 15 ml TPP | 1 | - | 249±15.1 | 0.223±0.014 | 43±1.2 | -2.15±0.14 | 369±11.4 | 81±0.67 | 0.9984 | 0.59 |
| 6. | A6 | 0.8 | 15 ml HMP | 1 | - | 458±13.9 | 0.199±0.059 | 46±1.5 | -1.05±0.19 | 375±10.4 | 87±0.85 | 0.9939 | 0.57 |
| 7. | A7 | 0.8 | 10 ml TPP | - | 5 | 265±12.8 | 0.517±0.037 | 36±1.0 | -1.86±0.09 | 350±13.2 | - | - | - |
| 8. | A8 | 0.8 | 10 ml HMP | - | 5 | 388±17.6 | 0.263±0.022 | 29±1.1 | -1.32±0.05 | 395±14.4 | - | - | - |
| 9. | A9 | 0.8 | 10 ml TPP | 1 | 5 | 717±19.3 | 0.121±0.036 | 46±0.96 | -3.47±0.17 | 455±15.3 | 76±0.90 | 0.9899 | 0.50 |
| 10. | A10 | 0.8 | 10 ml HMP | 1 | 5 | 942±11.7 | 0.276±0.031 | 42±1.4 | -2.76±0.2 | 478±15.2 | 79±0.48 | 0.9913 | 0.53 |
| 11. | A11 | 0.8 | 15 ml TPP | 1 | 5 | 571±17.8 | 0.224±0.026 | 37±1.7 | -3.85±0.06 | 347±14.8 | 85±0.82 | 0.9932 | 0.55 |
| 12. | A12 | 0.8 | 15 ml HMP | 1 | 5 | 648±16.3 | 0.142±0.046 | 35±2.2 | -2.94±0.07 | 392±13.7 | 96±0.51 | 0.9963 | 0.58 |

PEG: Polyethylene glycol 6000, TPP: Sodium tripolyphosphate, HMP: Sodium hexametaphosphate, LMT: Lomustine, CH: Chitosan, PDI: Polydispersity index; SD: Standard deviation for three determinations.
*****n = 3. The experiments were repeated twice.

**Table 1:** Formulation composition and characterization of DNPs and PEG coated DNPs.

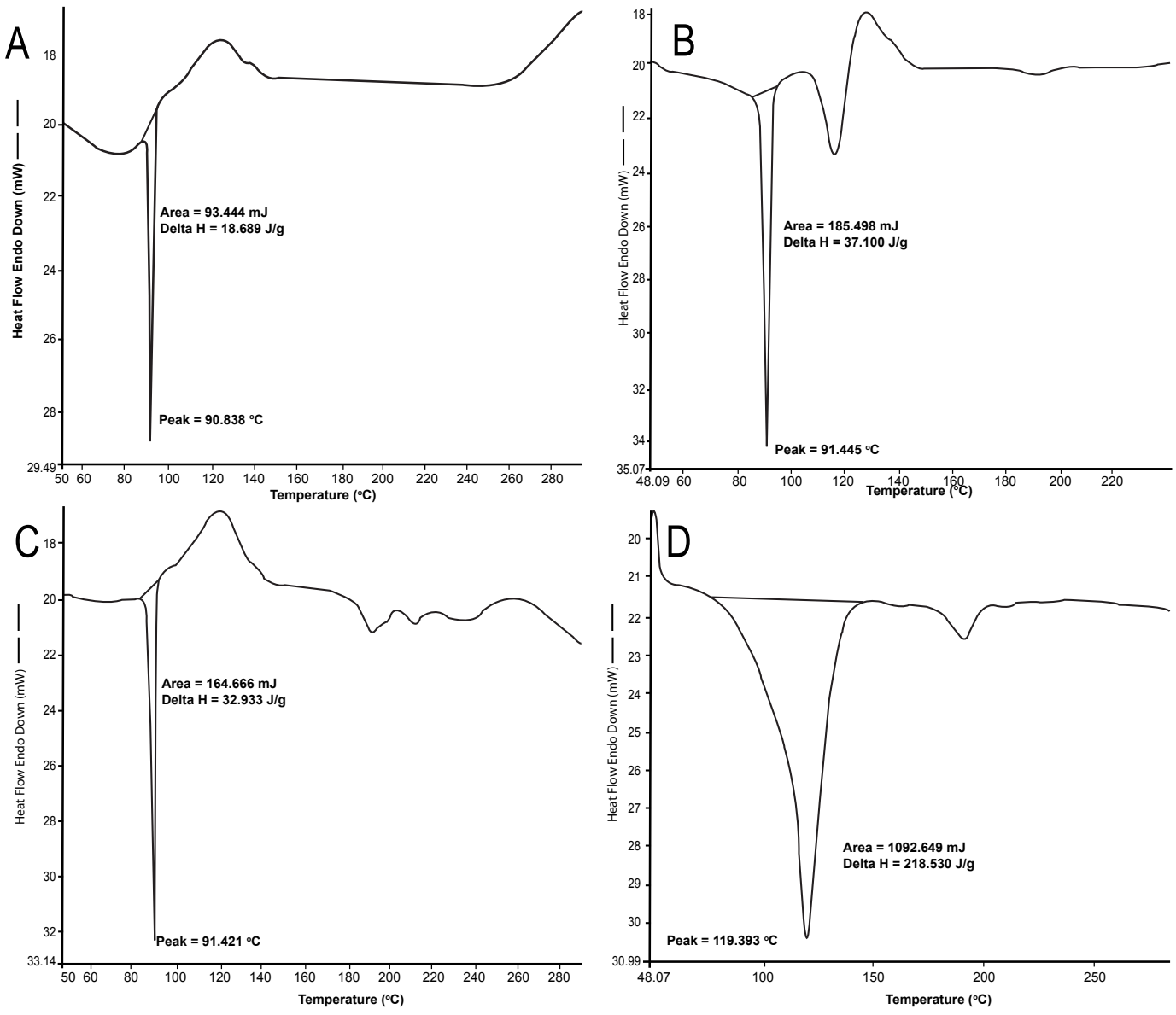


**Figure 2:** (2A)- lomustine loaded chitosan-HMP nanoparticles (A3), (2B)-lomustine loaded chitosan-TPP nanoparticles (A4), (2C)-lomustine loaded PEG coated chitosan-HMP nanoparticles (A9), (2D)-lomustine loaded PEG coated chitosan-TPP nanoparticles (A10).

way ANOVA and swelling index data was analyzed with a two-way ANOVA. Critical value of F was considered at 5% level of significance.

## Results

### Nanoparticle characterization

Chitosan nanoparticles were obtained in the average size range of 111±16.2 to 942±11.7 nm with PDI from 0.116±0.039 to 0.517±0.037 (Table 1). LMT loading (Batch A3 and A4) resulted in increased particle size significantly ($p<0.05$) as compared to placebo nanoparticles (Batch A1 and A2). Further, increase in volume of crosslinking agent decreased particle size to about 50% (Batch A5) and 10% (BatchA6) with TPP and HMP respectively. Similarly, after PEG coating particle size increased to 265±12.8 and 388±17.6 for TPP and HMP respectively. Further increase in concentration of crosslinking agent (Batch A11, A12) showed about 20% and 30% particle size reduction as compared to batches A9 and A10 respectively. Zeta potential of nanoparticles ranged from 29.0±1.1 to 56.0±1.1 mV (Table 1). Zeta potential was increased significantly ($p<0.05$) on incorporation of drug into nanoparticles (56±1.1 for batch A3 and 50.8±2.3 for batch A4), which decreased slightly on increasing the volume of crosslinking agent (batch A5 and A6) as compared to batch A3 and A4. PEG coating of DNPs resulted in decreased zeta potential. According to method used by Pan et al [13] we diluted samples with 0.1mol/l KCl for zeta potential analysis and found that nanoparticles had a negative surface charge (Table 1). When we prepared the samples for zeta potential analysis in distilled water only, it showed positive charge (Table 1) % EE of nanoparticles ranged between 58±0.88% and 96±0.51% (Table 1). It was slightly higher when HMP crosslinking agent used and increased significantly ($p<0.05$) when 15 ml of 1% HMP was used (Batch A12). PEG coating produced DNPs with increased encapsulation efficiency, which was also increased on higher concentration of crosslinking agent.

TEM images of CH-HMP and CH-TPP nanoparticles (Figures 1a) showed solid dense spherical structure. The SEM images of LMT loaded CH-TPP and CH-HMP nanoparticles showed well-formed spherical shape with smooth surface (Figure 1b).

### Drug interaction study by DSC

The DSC curve of lomustine revealed an endothermic melting peak (max 91.79°C) [14]. In DSC thermogram of nanoparticles endotherms were observed which showed change in melting peaks of lomustine at 90.83°C in lomustine loaded CH-HMP nanoparticles (Figure 2A), 91.44°C in LMT loaded CH-TPP nanoparticles (Figure 2B), 119..3°C in LMT loaded PEG coated CH-HMP nanoparticles (Figure 2C), and 91.42°C in LMT loaded PEG coated CH-TPP nanoparticles (Figure 2D). Thermograms confirmed presence of drug to polymer interactions and showing stability of drug in the nanoparticles. The shift of endothermic peak of lomustine in DNPs, indicated that the drug was molecularly dispersed inside the matrix of chitosan as a solid solution. By a shift of endotherm in lomustine loaded PEG coated chitosan-HMP and chitosan-TPP nanoparticles, it could be concluded that lomustine exists in the PEG coated chitosan nanoparticles in the crystalline state. This observation confirms the presence of drug to polymer interaction but shows stability of drug in the nanoparticles.

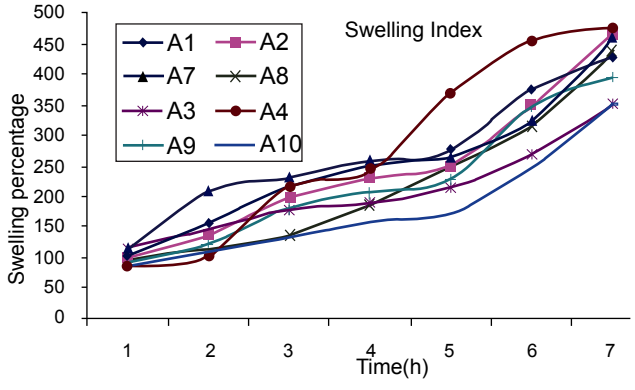


**Figure 3:** Swelling behavior of different batches of chitosan nanoparticle formulations.

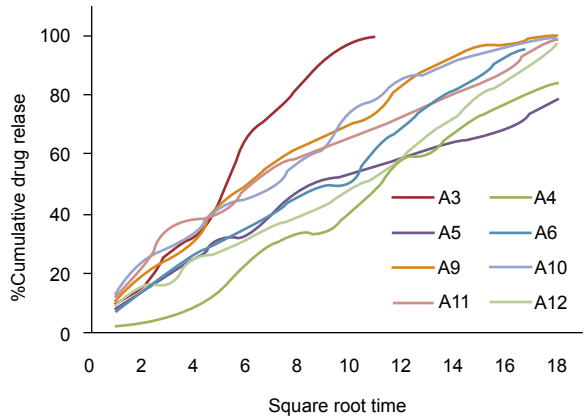


**Figure 4:** Percent drug release vs. square root time plots of in vitro dissolution studies of different. nanoparticle formulations.

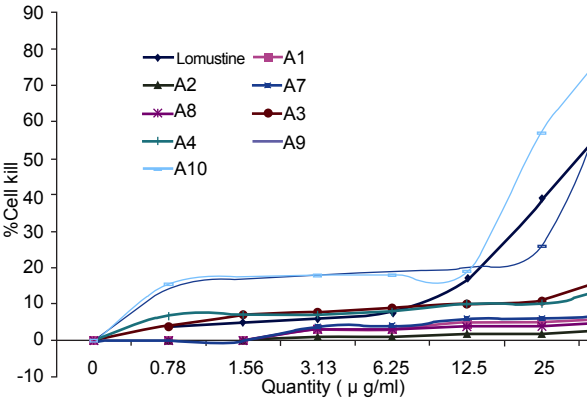


**Figure 5:** Comparison of cell toxicity of different nanoparticles formulations in L132 human lung cancer cells.

### Swelling behavior

Swelling studies (Table 1, Figure 3) of nanoparticles of all formulations were performed in PBS pH 7.4. It was observed that swelling indices of CH-TPP and CH-TPP-PEG nanoparticles were about 428±12.6% and 350±13.2% over the 4 h and it was more about 465±12.7% and 395±14.4% for CH-HMP and CH-HMP-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 was more than the critical value of F. Hence there was significant difference in the % swelling of the formulations at different time intervals, showing that the type of cross linker and PEG coating affected the swelling behavior of the nanoparticles.

### In-vitro drug release

The *in vitro* drug release studies (Figure 4) were performed in

phosphate buffered saline (pH 7.4). The results revealed that the type and concentration of crosslinking agent affected 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 was more than the critical value of F at 5% level of significance and the null hypothesis was not accepted. Hence there was significant difference in the percentage of drug release by different samples. 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 LMT from chitosan nanoparticles decreased. To study the mechanism of LMT release from nanoparticles, the dissolution data was plotted as % drug release against the square root of time. By applying least-squares method to the release data at 95% confidence level, values of *n* were estimated. These data along with correlation coefficients, $r^2$ are given in Table 1. The values of *n* increased with increasing crosslink density. These values ranged between 0.49 and 0.59, indicating that drug release deviates from the Fickian trend.

### In-Vitro anticancer activity

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 graded of cytotoxicity in proportion to the applied drug concentration (Figure 5). Drug loaded nanoparticles killed cells more efficiently than the corresponding drug alone ($p < 0.05$).

## Discussion

The present study demonstrates the preparation of TPP and HMP crosslinked chitosan nanoparticles by homogenization followed by spray drying. Nanoparticles were crosslinked with chemical crosslinking agents and coated with PEG to control the release of drug from the particulate system and to study zeta potential changes during spray drying [15].

Nanoparticle size was observed to increase with increase in chitosan concentration. This is due to the greater amount of chitosan contained in the same volume of a liquid droplet [16-17] So the chitosan concentration was optimized at 0.8 %w/v solution to strike a balance between the spray nozzle size, liquid flow rate and drying temperature for obtaining nanoparticles of well within the nanoscale range. The particle size was decreased with increasing amount of crosslinking agent because crosslinking agent hardens the chitosan nanoparticles leading to decreased water absorption. TPP crosslinked chitosan nanoparticles were smaller in size as compared to HMP crosslinked chitosan nanoparticles, which showed a strong rigid matrix formation in TPP crosslinked nanoparticles. With increasing extent of crosslinking, a slight reduction in particle size was possible due to the formation of a rigid matrix. Loose matrix structure of PEG coated DNPs which was a molecular rearrangement of chitosan and glycol groups was markedly converted to rigid structure by crosslinking with high volume of HMP and TPP crosslinkers.

The positive zeta potential values of the DNPs showed that the surface of the nanoparticles was more composed of chitosan than lomustine. This is an important aspect of the positively charged nanoparticles that can easily interact with negatively charged cell membrane and drug can be released easily into the cytoplasm of the cell [18]. It should be noted that the zeta potential was strongly dependent on the media in which it was measured. In an aqueous environment, the electrolyte dissociates into ions. The ions, especially the counterions, which have a charge opposite to that of the nanoparticles, will be attracted to the charged particles by an electrostatic attraction. According to the DLVO theory and Schultz–Hardy rule, this effect is inversely proportional to the sixth power of the valence of the counterions [19]. The influence of the mono-valent anions, such as potassium and chloride on the zeta potential of the positively charged chitosan nanoparticles was much greater than that of tri-valent TPP and hexameta-phosphate anions. Therefore, in subsequent studies, distilled water was used as a neutral medium (pH 7) to measure zeta potentials, unless specified. The positive zeta potential of chitosan decreased with an increase in the amount of the crosslinking agent. The zeta potential of chitosan nanoparticles decreased rapidly with increasing phosphate concentration.

Encapsulation efficiency of CH-TPP and CH-HMP nanoparticles showed satisfactory results by 0.8%w/v concentration of chitosan. This may be explained on the basis that relatively lower viscosity of chitosan with lower concentration promotes the encapsulation of drug and gelation between chitosan and crosslinking agent. This is in agreement with the results of Vandenberg et al [20] that highly viscous nature of gelation medium hinders the encapsulation of drug in the study of chitosan microspheres. Encapsulation efficiency of nanoparticles crosslinked with HMP (Batches A4, A6, A10 and A12) was slightly higher than TPP crosslinked nanoparticles (Batches A3, A5, A9, A11) that might be due to homogeneously distributed matrix structure formation. Nanoparticles with a high crosslinking extent (Batches A5, A6 and A11, A12) showed a slight increase in the encapsulation efficiency. This could be due to an increase in crosslink density, which might prevent the leaching of drug particles during the formation of nanoparticles. PEG coating increased encapsulation efficiency (A9, A10, A11, and A12). This may be due to increased capture of free dissolved drug within the matrix of nanoparticles associated to PEG 6000.

In SEM image the surface of lomustine loaded chitosan-HMP nanoparticles showed vacuoles because of dehydration of surface of nanoparticles. Crcarevska et al. [22] observed nearly the same morphology and described that generally spherical morphology with presence of spherical disks with a collapsed centre could be noticed. Similar result were described by Schmid[23] for spray dried calcium pantothenate nanoparticles showing mainly particles that were damaged during preparation of the SEM sample either by the high vaccum of the SEM or by the heat created during the sputter coating process. Most of the spray dried calcium pantothenate particles were collapsed due to instability of previously formed thin walled spheres under the high vaccum of the SEM.

The type of crosslinking agent did not affect the shape, but PEG coating slightly affected the shape because PEG coated lomustine loaded chitosan HMP nanoparticles were egg shaped. This may be due to increased shear and stress on the particles and increased cohesiveness between particles. PEG coated lomustine loaded chitosan STPP nanoparticles were solid spheres showing sufficient strength and hardening. The PEG coated lomustine loaded TPP crosslinked chitosan nanoparticles were solid spheres somewhat transparent in appearance which was opposite to the findings of Sacchetti et al [21] that the use of PEG 6000, in a range 0.5–5% as alternative component in mannitol/caffeine mixtures, produced aggregation of individual microparticles giving rise to an irregular shape when they used PEG 6000 as a shaper.

After preliminary trials, the homogenization speed that produced low-particle size (24,000 rpm) was considered for the study. Increase

in size of all PEG coated nanoparticles can be attributed to particle coagulation as a result of exposure of the nanoparticles to greater cavitation forces for a longer time and resultant increased kinetic energy of the system.

In this study the compressed air flow rate of 70 psi, inlet temperature of 165°C, liquid flow rate 1.5 ml/min and nozzle size 0.5 mm. were applied for all batches, As below these conditions, the solvent in the droplets could not be fully evaporated.

In the present study, the swelling capacity of spray dried chitosan nanoparticles crosslinked with TPP and HMP were increased with time. As the volume of TPP and HMP solution was increased, the swelling capacity decreased considerably apparently because the 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 was loose and had high hydrodynamic free volume to accommodate more solvent molecules thereby inducing chitosan-TPP and chitosan-HMP 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 [24]. Higher water uptake values at lower levels of crosslinking and vice versa observed in the study confirmed the formation of rigid chitosan-TPP networks. The higher concentration of crosslinking agent increased the rigidity of the polymer surface and also decreased the hydrophilic groups available in the chitosan molecule responsible for swelling. Swelling ability of TPP crosslinked nanoparticles was found less pronounced than HMP crosslinked 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 [15]. Volume increase ratio for placebo chitosan nanoparticles and lomustine-loaded nanoparticles in phosphate buffer was higher as compared with PEG coated unloaded chitosan nanoparticles and PEG coated DNPs for 24 h. The swelling behavior could be well justified by the fact that nanoparticles tend to absorb water (free or bulk water) in order to fill the void regions of the polymer network within the nanoparticles that remain dehydrated until they reach the equilibrium state [22]. This phenomenon may be attributed to protonation of primary amino groups ($–NH3^+$) on chitosan and thus creating an expulsive force within the test hydrogel.

Initially lomustine was rapidly released from the nanoparticles followed by slow release. The initial fast release phase could be due to the rapid dissolution of surface adhered/entrapped drug. In the later phase, the release medium penetrates into the nanoparticles and dissolves the entrapped drug. It was observed that release was slower in those formulations in which a higher amount of TPP and HMP were used. This was due to the fact that at higher crosslinking, free volume of the matrix was small, thereby hindering the easy transport of drug molecules through the matrix. This also reduced the rate of swelling as well as rate of drug release from the matrix. The decrease in lomustine release with increasing crosslinking agent was the reason that the release rate of drugs from chitosan particulate carriers depended on the density of crosslinked chitosan matrix. The density of the chitosan crosslinked matrix increases with increasing volume of crosslinking agent [25].

In the present study, the greater zeta potential of chitosan nanoparticles showed stronger interactions with tumor cells leading to higher anticancer property. As a kind of cationic polymer, the surface charge of nanoparticles was 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 lomustine loaded TPP and HMP crosslinked nanoparticles than PEG coated nanoparticles helped in their higher anticancer activity. The effect of TPP and HMP crosslinked chitosan nanoparticles can be explained from several aspects. Since parts of the primary amino groups in chitosan molecules were substituted by phosphoric anions of STPP and SHMP, positive charge density and the toxicity exhibited by the primary amines decreased overall. Polymers with relatively rigid structures and branches were more cytocompatible. The introduction of large hydrophilic moieties, like PEG, not only enhanced the rigidity but also showed branching with the chitosan chains, thus prevented strong binding to the cell membrane and neutralized the cell surface charge more effectively, which might have improved the cytocompatibility of chitosan nanoparticles. The phosphoric groups of TPP and HMP on the chitosan chains also acted to shield a proportion of the positive charges and increased the space between reactive amine groups present on it, reducing the cytotoxicity caused by charge density and the active groups [26-27].

## Conclusion

Lomustine, loaded chitosan nanoparticles were successfully fabricated as chitosan nanoparticles by high-speed homogenization and spray drying technique and obtained nanoparticle in the size range of $111 \pm 16.2$ to $942 \pm 11.7$ nm. Crosslinking agents were significantly changed the particle size and encapsulation efficiency. Drug loaded nanoparticles killed L132 cells more efficiently and showed better anticancer activity than the corresponding drug alone ($p < 0.05$) due to the increased surface area of lomustine loaded chitosan nanoparticles cross linked with TPP and HMP.

### Acknowledgements

We acknowledge to Dr. Amit Mishra (Scientist, CDRI, Lucknow), Dr.C.K. Kataria, Dr. Anil Kanaujia, Ranbaxy Reasearch Laboratories), and Prof. Dr. V.K.Lal (Scientific Member Indian Pharmacopoeia Commission 2007) for continuous support during study.

### Financial and Competing Interests Disclosure

The authors have no financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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