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# FORMULATION AND EVALUATION OF DOXORUBICIN CONTAINING NANOGELS FOR DELIVERY TO CANCER CELLS

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## ABSTRACT

The objective of this study is to prepare nanogels were prepared *via* charged gellan gum. It was prepared by *in situ* cross linking reaction between two oppositely charged materials by green method without use of chemical cross linking agents. The prepared nanogels were characterized by dynamic light scattering, scanning electron microscopy, differential scanning calorimetry and X-Ray diffractometry. The prepared formulation had average particle size of 226 nm with polydispersity index of 0.3. The doxorubicin loaded nanogel demonstrated sustained release for 20 h. The prepared nanogels were hemocompatible and cyctocompatible as revealed by hemocompatibility and MTT assay respectively. All results confirmed that these nanogels can be used for cancer treatment.

**Keywords**: Nanogel, Chitosan, Gellan gum, Doxorubicin, Cancer.

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## INTRODUCTION

Cancer is a prospective incurable illness, characterize by unwanted proliferation and spread of normal cells [1]. In spite of the increase of many anticancer drugs, there are still challenges in employing them in cancer therapy because of their poor bioavailability, water insolubility, high toxicity and nonspecific targeting which result in damaging the healthy cells. Doxorubicin (DOX) is one of the most effective anticancer drugs, with a wide scope of activity in human cancers, including acute lymphoblastic leukemia, breast carcinoma, ovarian carcinoma, and hepatocellular carcinoma [2]. However, its clinical application is limited by its harmful side effects, the most significant of which is its cardio toxicity that can lead to cardio myopathy and congestive heart failure[3].

Nanogels are defined as clear, thermodynamically stable, isotropic mixture of oil, water and surfactant frequently in combination with a co surfactant [4]. The concept of nanogels was first introduced by Hoar and Schulman in 1942 and the term nanogels was first coined by Schulman and coworkers in 1959 to describe the clear fluid systems obtained by titration to the point of clarity of an ordinary milky gel (macro emulsion) by the addition of a medium chain alcohol such as pentanol and hexanol. The droplet size (100-600 nm) was much smaller than that of milky white ordinary emulsion; hence their transparent appearance and the adoption of the term nanogels (Schulman et al., 1959) [5, 6].

Thus it decided, in this study that formulation of doxorubicin containing nanogel and evaluated for anti-cancer activity.

## MATERIALS AND METHODS

### Materials

Chitosan (degree of deacetylation ~ 79%, Low mol wt ,Viscosity 200-800 cP, 1 wt. % in 1% acetic acid) from Sigma Aldrich, India, Gellan gum (Low acyl content, mol. wt > 70,000 Daltons) obtained as gift sample from Burzin and Leons, CP Kelco division of the Monsanto Company, USA. Doxorubicin (95% purity, Product no. 0000155108) purchased from Hi Media Lab. Pvt. Ltd, Mumbai, India.

### Method

### Preparation of chitosan- gellan gum nanogel

Chitosan 0.85% w/v solution was prepared in acetic acid and 0.04% w/v gellan gum solution was prepared in distilled water. Both the solutions were filtrated by vaccum filter using 0.22 µm filter paper. Chitosan solution added slowly to gellan gum under agitation at room temperature for 3h. The pH of the resultant solution was about 4.3 and was adjusted to pH 5.4. The mixture was heated at 80˚C for 20 min to produce homogeneously dispersed nanogel, and then the nanogels were separated by centrifugation at 45,000 rpm for 35 minutes (Beckman Coulter, ultracentrifuge) [7].

### Preparation of Doxorubicin loaded chitosan- gellan gum nanogel

Doxorubicin was loaded into the nanogel along with chitosan solution and the same procedure was followed as described for chitosan – gellan gum nanogel. The doxorubicin loaded nanogels were separated by centrifugation at 45,000 rpm for 35 minutes (Beckman Coulter ultracentrifuge). Deposited particles were re dispersed in distilled water containing 5% mannitol as a cryoprotectant. The resultant dispersion was subjected to lyophilisation at -75°C and vacuum was maintained at 76 m Torr (Vertis Lyophilizer) [8].

## Characterization of nanogel

The average particle size and polydispersity index was determined by photon correlation spectroscopy and Zeta potential of system was measured using Zeta sizer, (Nano ZS 90, Malvern Instrument., UK). The surface morphology of nanogel was examined by scanning electron® microscopy (JSM 6390, Japan). To demonstrate physical state of doxorubicin within nanogels, differential scanning calorimetry (DSC, Mettler-Toledo, Zurich, Switzerland), and X-ray diffractometry (XRD, Brucker Axs, D8 Advance; Germany) analysis were performed [9].

### Entrapment Efficiency, Loading Efficiency and *In Vitro* Drug Release Studies of Doxorubicin Loaded Nanogel

Doxorubicin entrapment in chitosan-gellan gum nanogel formulation was determined by triturating lyophilized powder with DMSO and methanol in mortar pestle and diluted up to 10 ml in volumetric flask. The resulting solution was centrifuged at 30,000 rpm for 20 min and the supernatant was collected and analyzed spectrophotometrically (UV 1700, Shimadzu, Japan) at 423 nm. Drug content was estimated using equation (y = 0.08x - 0.010) generated as result of plot of concentration *vs* absorbance that obeys Beer's and Lambert's law in concentration range 2 to 12µg/mL. Loading efficiency was calculated in relation to yield of nanogel obtained after lyophilisation (equation- 1).

$$\%\ \text{Loading efficiency} \frac{\text{Actual amount of curcumin in nanogel}}{\text{Total yield of curcumin loaded nanogel}} \times 100$$

Entrapment efficiency was calculated on the basis of total amount of doxorubicin in nanogel to total amount of doxorubicin used during drug loading.10 (equation 2)

$$\%\ \text{Entrapment efficiency} = \frac{\text{Total amount of curcumin in nanogel}}{\text{Total amount of curcumin}} \times 100$$

*In vitro* doxorubicin release from nanogel formulation was carried out by using Franz type diffusion cell of 25 ml capacity. Dialysis membrane (Himedia, Avg mol. weight cut off range 12000 – 14000 kDa) was used as diffusion membrane. Dialysis membrane was soaked in phosphate buffer pH 7.4 for 24 h prior to experiment. Diffusion cell was filled with buffer solution and dialysis membrane was mounted on cell. The temperature was maintained at 37$^{o}$C ± 0.5$^{o}$C. After a pre-incubation time of 20 minutes, the lyophilized nanogel was placed in the donor chamber. Samples were periodically withdrawn from the receptor compartment and replaced with the same amount of fresh buffer solution, and assayed by a UV spectrophotometer at 423 nm [10].

To evaluate the mechanism of drug release from the nanogel, data for the drug release was plotted in Korsmeyer-Peppas equation. The release exponent n and K value was calculated through the slope of the straight line. [3]

$$\frac{Mt}{M\infty} = Kt^{n}$$

### Hemolysis Assay

The blood compatibility of the chitosan –gellan gum nanogel by hemolysis assay using human blood obtained from blood bank. In different concentrations of nanogel (0.2, 0.4, 0.6, 0.8,1 mg/ml), 0.1 ml of anti coagulated fresh human blood was added. Positive and negative control selected for this study were PBS (0% hemolysis) and 0.1% of $Na_2Co_3$ (100% Hemolysis). After incubation at 37 for 90 min sample were centrifuge at 4500 rpm for 5 min. Absorbance of supernant was analyzed spectrophotometrically against PBS at 545 nm [11].

The percentage of haemolysis was calculated by the following equation 4.

$$\%\ \text{Haemolysis} = \frac{\text{Abs of sample} - \text{Abs of negative control}}{\text{Abs of negative control}} \times 100$$

### Cell culture protocol

Astrocytoma-glioblastoma cell line (U373MG) was used for this study .The cells were seeded into 96-well plates at a density of 1.0 × 104 cells per well and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, 200 µg/mL streptomycin, 2 mM L-glutamine; and culture was maintained in a humidified atmosphere with 5% $CO_2$.The cells were divided into two treatment groups, including: (a) doxorubicin sus-

pension and (b) freeze dried doxorubicin loaded nanogel and % growth inhibition was determined by MTT assay based on mitochondrial reduction of yellow MTT tetrazolium dye to highly colored blue formazan product. The plates were incubated for 48h at 37 ± 0.5°C in RPMI/DMEM/MEM with 10% FBS medium. Then the above media was replaced with 90µL of fresh serum free media and 10 µL of MTT reagent (5mg/mL) and plates were incubated at 37 ± 0.5°C for 4 h, there after the above media was replaced with 200µL of DMSO and incubated at 37±0.5°C for 10 min. Further studies were carried in accordance with our previous protocol [12].

## RESULTS AND DISCUSSION

### Preparation and characterization of doxorubicin loaded chitosan- gellan gum nanogel

Doxorubicin loaded chitosan- gellan gum nanogel was formed immediately as result of cross-linking between positively charged chitosan and negatively charged gellan gum by co- acervation reaction due to electrostatic attraction between two opposite charged polymers. However, the networks thus obtained by *in situ* cross-linking are reversible and unable to retain integrity of nanogel system. Heating at 80ºC for 20 min leads to formation of nanogel; these nanogels have good stability and do not require any stabilizer. Doxorubicin loaded nanogel was obtained by addition of doxorubicin to chitosan solution prior to cross-linking with gellan gum.

### Characterization of Nanogel

The average particle size for chitosan- gellan gum nanogel was found to be 201 nm and 226 nm for doxorubicin loaded nanogel with polydispersity index value of 0.31 and 0.39 respectively, demonstrating uniform distribution of nanogel population. Such nano range size distribution crucial for passive tumor targeting due to enhanced permeation and retention effect as majority of solid tumors exhibit a vascular pore cut-offs between 380 and 780 nm.[13] From SEM image of nanogel it is clear that the freeze dried nanogel have smooth surface with porous nature. Zeta potential of freeze dried doxorubicin loaded nanogel was found to be 5.52. The Zeta potential represents the electrical charge to the nanogel surface suggest that chitosan molecules are enriched on the particle surface. During thermal analysis doxorubicin showed strong endothermic peak at a temperature around 180 ºC, due to its crystalline nature (Figure 2A) but nanogel formulation displayed absence of peak in this region (Figure 2B). Also in X-ray diffractometry pattern numerous crystalline peaks were observed in the 2θ range of 15- 30º demonstrate crystalline nature of doxorubicin (Figure 3A) where as there were decrease in peak intensity in nanogel formulation confirms the amorphous state of doxorubicin in nanogel (Figure 3B).

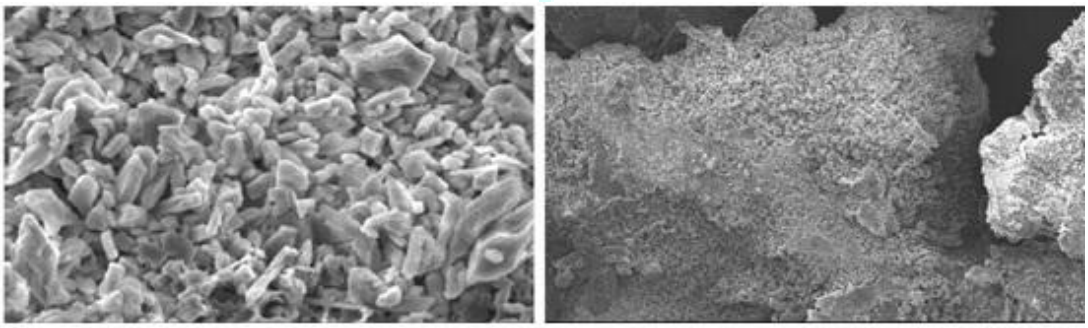

**Figure 1: SEM image of freeze dried Doxorubicin loaded nanogel**

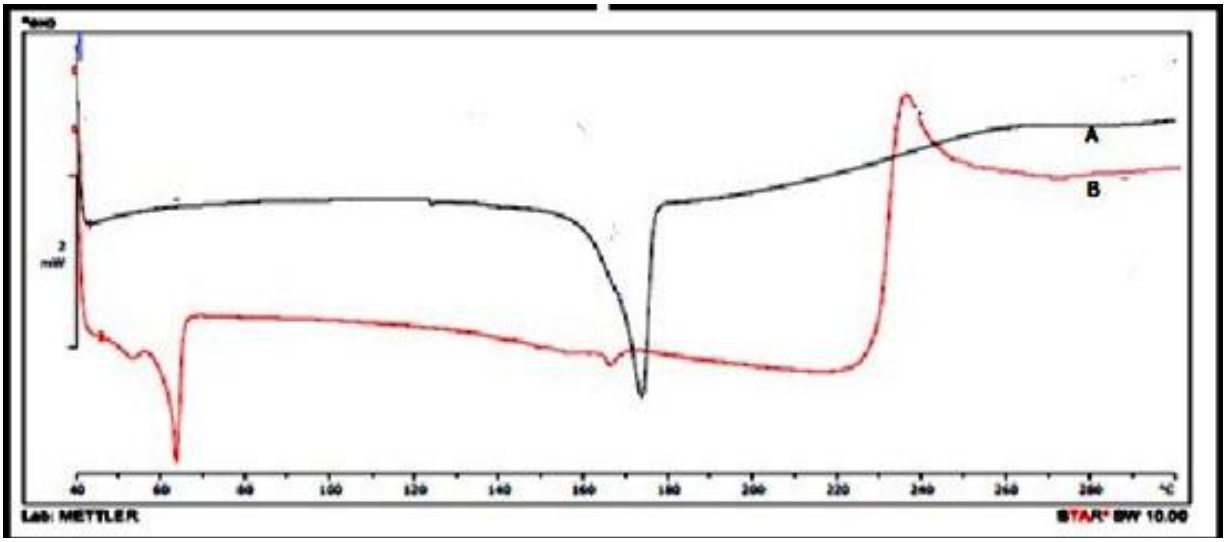


**Figure 2: DSC thermaogram of Doxorubicin (A) and nanogel (B)**

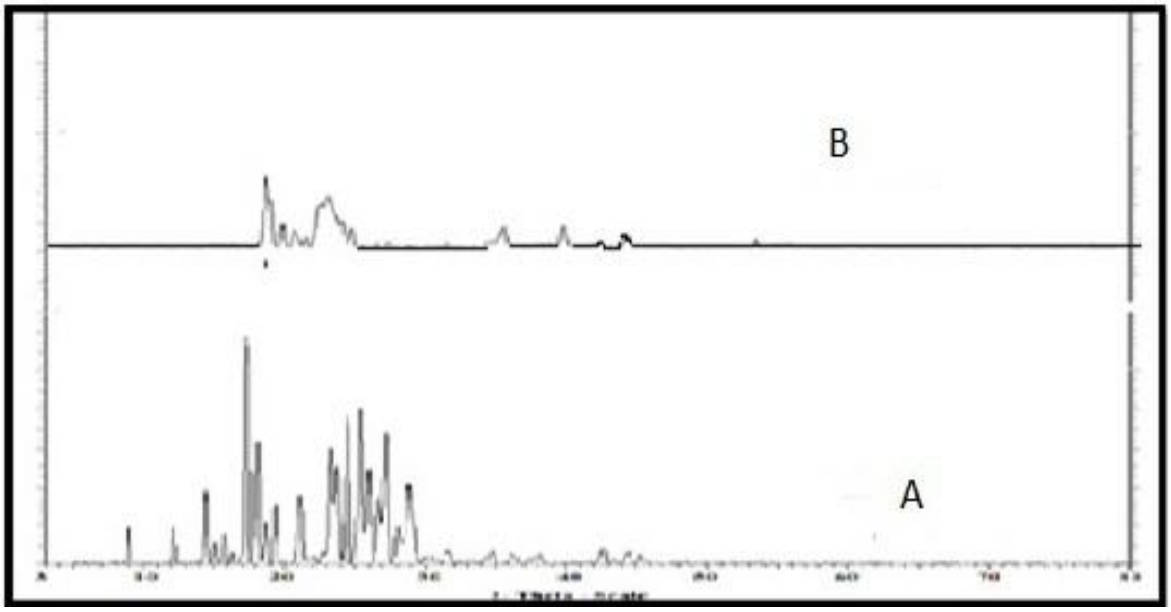


**Figure 3: X- Ray Diffractogram of Doxorubicin (A) and nanogel (B)**

**Entrapment efficiency, Loading efficiency and drug release studies**

The loading and entrapment efficiency of doxorubicin loaded nanogel system was found to be 5.66 ± 0.78% and 96.60± 1.28% respectively. The higher entrapment of doxorubicin in nanogel structure is attributed to in situ cross linking between two polymers and addition of doxorubicin prior to completion of interaction. To establish the suitability of chitosan – gellan gum nanogel as delivery system for doxorubicin, the *in vitro* release was studied. The release profile of doxorubicin -loaded nanogel was studied using dialysis membrane. Sustained release of doxorubicin from nanogel was observed (Figure 4a). The corresponding plot of Korsmeyer - Peppa's equation (Figure 4b) indicated a good linearity of regression coefficient (R2) 0.913. The release exponent (n) of Korsmeyer-Peppa's equation was found to be 0.91 and K value 0.869. The n value was greater than 0.5 and less than 1 indicating anomalous (non-fickian) transport mechanism suggesting doxorubicin release due to diffusion through swell chitosan- gellan gum matrix.

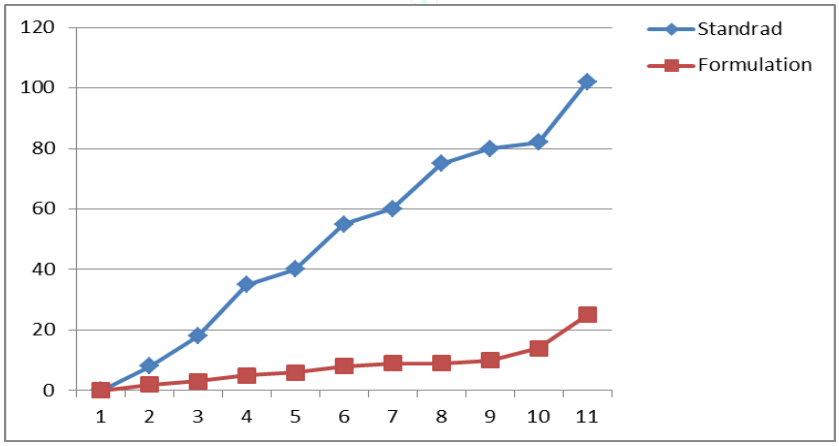


**Figure 4: Doxorubicin release profile (A) and release mechanism (B)**

**Haemolysis Assay**

Haemocompatibility of the nanogels was essential as nanogels are designed for drug delivery purpose that interacts with a large fraction of blood component in the blood before distribution to individual tissue. For drug delivery purpose nanogels must show minimum interaction with components in the blood stream and should possess long-term stability. On analysis of haemolytic potential of five different concentrations of nanogels it was observed that increase in nanogel concentration leads to increase in hemolytic ratio (Figure 5). It is about to 2.5% i.e. below 5 %, the critical safe hemolytic ratio for bio-materials, indicated that these chitosan-gellan gum nanogel samples are compatible with blood components.

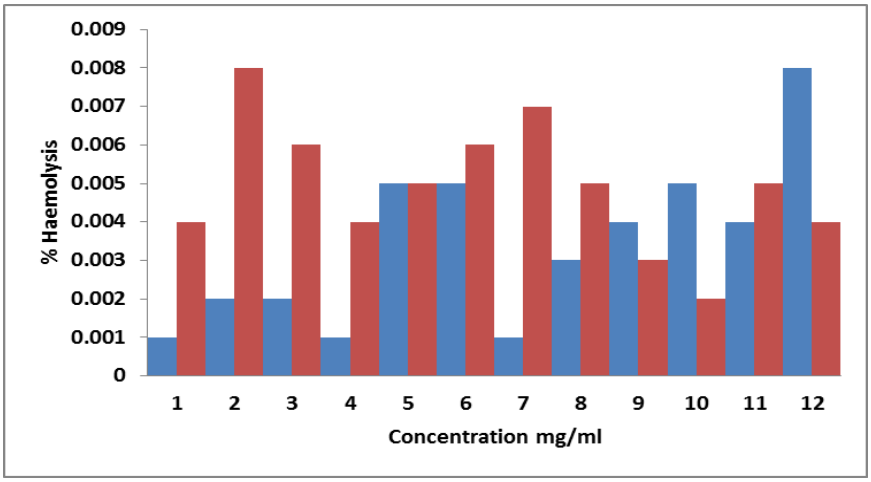


**Figure 5: Hemocompatibility of nanogels**

### Cytotoxicity Studies

Cytotoxicity assay of doxorubicin loaded nanogel was performed and compared with standard (doxorubicin) by cell viability testing. Figure 6 shows plot of % inhibition of astrocytoma-glioblastoma cell line (U373MG) incubated with the doxorubicin loaded nanogel vs doxorubicin. The $IC_{50}$ values were found out to be 9.8 and 13.6 ng/ml for doxorubicin loaded nanogel and doxorubicin suspension respectively. Thus, doxorubicin loaded nanogel showed lower cellular viability demonstrating increased cytotoxicity of formulation towards cells.

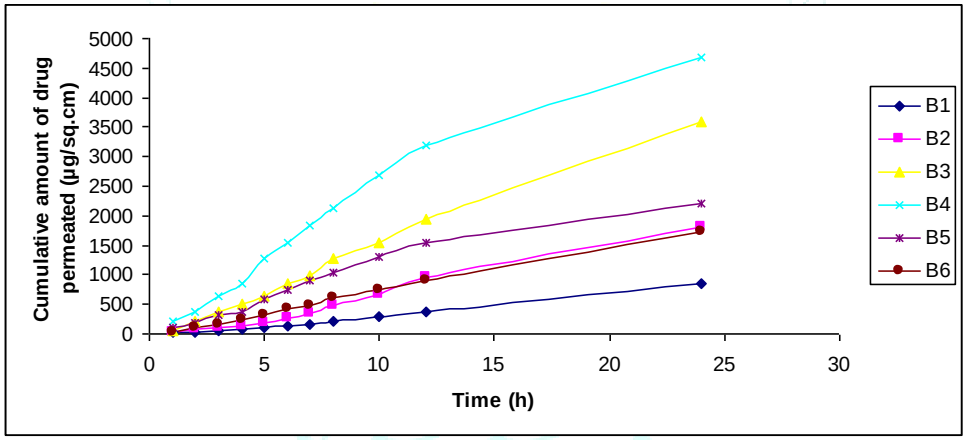


**Figure 6: *In vitro* cytotoxicity assay**

## CONCLUSION

Doxorubicin loaded chitosan- gellan gum nanogels were prepared by *in situ* cross-linking between two oppositely charged polymers. The prepared nanogels subjected good stability devoid of use of external chemical cross-linkers. Prepared nanogels were characterized for particle size, zeta potential, and surface morphology. The result of particle size analysis reveals there suitability for effective tumor targeting. The prepared nanogel showed porous nature due to lyophilisation with positive zeta potential confirming dominance of chitosan on surface. The doxorubicin loaded nanogel showed good entrapment efficiency and sustained drug release. *In vitro* cytotoxicity and hemocompatability assay proved the nontoxic and hemocompatible nature of the nano-gels. These results indicated that chitosan- gellan gum nanogel could be suitable carrier for doxorubicin in cancer treatment.

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