

# Effect of cross-linking agent on the characteristics of celecoxib loaded chitosan microspheres

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The purpose of the present investigation was to compare the characteristics of the microspheres of chitosan prepared using two different cross-linking agents: viz. formaldehyde and glutaraldehyde and by simple heat treatment. Chitosan microspheres were prepared by emulsification cross-linking method. Microspheres were characterized for entrapment efficiency, particle size, *in-vitro* drug release and surface morphology was studied by scanning electron microscopy. The entrapment efficiency of the glutaraldehyde and formaldehyde cross-linked microspheres was significantly higher ( $P < 0.05$ ) than the heat-cross-linked microspheres. *In-vitro* drug release studies indicated that the microspheres cross-linked using glutaraldehyde showed slower release rate than those cross-linked with formaldehyde while the heat cross-linked microspheres showed the fastest release.

**Key words:** Celecoxib, chitosan, cross-linking, encapsulation, microspheres

## INTRODUCTION

Chitin is a naturally occurring and the second most abundant organic material next to cellulose. Chitin is relatively inert material. Deacetylation of chitin yields chitosan, which is relatively reactive and can be produced in numerous forms such as powder, paste, film, fiber.<sup>[1]</sup> Due to its bio-compatibility, there have been a substantial number of studies on the biomedical use of chitosan as a drug carrier. As a drug carrier, chitosan has been formulated into granules,<sup>[2]</sup> membranes,<sup>[3]</sup> tablets,<sup>[4]</sup> and microspheres.<sup>[5-9]</sup> Chitosan has advantages over other polysaccharides due to its non-toxicity and bio-degradability as it is broken down in the human body to harmless products (amino sugars) that can be easily absorbed.<sup>[10]</sup>

Chitosan can be cross-linked to various degrees to modulate drug diffusion in their matrix and hence to achieve a sustained release of drugs. There are numerous reports on the use of glutaraldehyde as a cross-linking agent in the preparation of microspheres.<sup>[11-15]</sup> Very few reports are available where an alternative cross-linking agent/method is described out of which

use of formaldehyde or heat<sup>[10,16-18]</sup> for cross-linking chitosan microspheres are prominent. Heat treatment on hydrophilic polymers such as chitosan induces the formation of cross-links between the polymeric molecules and/or formation of crystallites increasing the water resistance of the materials.<sup>[17]</sup> In the present investigation, an attempt has been made to study the effect of the different cross-linking methods on the characteristics of the celecoxib loaded chitosan microspheres.

Celecoxib is a newer anti-arthritis drug which is selective cyclo-oxygenase-II (COX-II) inhibitor. But, since COX-II is constitutively present in some organs and can be induced in other organs, selective COX-II inhibitors are not devoid of side effects. The side effects can be avoided by targeting the drug to the arthritic joints. One way of achieving higher concentrations in the joint for prolonged period of time is to inject the drug intra-articularly. In our previous report,<sup>[19]</sup> we have shown that post intra-articular injection, celecoxib incorporated chitosan microspheres are able to maintain significantly higher concentrations of the drug in the arthritic joint than the celecoxib solution. Thus, the present investigation was aimed to prepare an optimized formulation for use as an intra-articular injection. The effect of the different cross-linking agents on the characteristics of the microspheres was studied and a cross-linking agent which gave the desirable properties

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of the microspheres intended for intra-articular injection was chosen for *in-vivo* studies as reported earlier.<sup>[19]</sup>

## EXPERIMENTAL

### Materials and methods

The drug Celecoxib was gifted by Sun Pharmaceutical Advanced Research Centre, Vadodara, India. Chitosan was kindly gifted by Central Fisheries Technology Limited, Cochin, India. Glutaraldehyde (25%w/v aqueous solution) was purchased from E. Merck (India) Limited, formaldehyde (37%w/v aqueous solution) was purchased from Qualigens fine chemicals limited, India. Tween-80 was purchased from S.D. fine chem. Ltd, Mumbai. All other chemicals and solvents were of analytical grade and used without further purification.

### Preparation of chitosan microspheres

Chitosan microspheres were prepared using emulsion cross linking technique.<sup>[20]</sup>

Weighed amount of chitosan was dissolved in 2%w/v acetic acid to give a 3%w/w solution. Tween-80 was added at a concentration of 2%w/w. Celecoxib was finely triturated in a mortar and was sieved through sieve no. 400. 10 mg of finely powdered celecoxib was added to the above solution and sonicated to obtain a uniform dispersion. 1 ml of this dispersion was injected to a mixture of 20 ml heavy liquid paraffin and 1.0 ml of span-85 while stirring at 2500 RPM. Stirring was continued for 10 min to obtain a w/o emulsion. The chitosan in the internal phase of the w/o emulsion was cross-linked using either i) chemical treatment or by ii) Heat treatment as explained below:

1. To produce chemically cross-linked microspheres glutaraldehyde or formaldehyde were used. Cross linking of the chitosan present in the internal phase of the w/o emulsion was done by adding a designated volume (0.5 ml or 1.0 ml) of glutaraldehyde (25%w/w aqueous solution) or formaldehyde (37%w/w aqueous solution). Stirring was continued for a definite interval of time (1 h or 3 h) and then the microspheres formed were separated by centrifugation and washed with petroleum ether to remove the liquid paraffin. The microspheres were then suspended in 5%w/v sodium bisulphite solution and stirred on a magnetic stirrer for 10 min followed by washing with water to render them free from residual glutaraldehyde. Finally, the microspheres were dried at room temperature and stored in a desiccator till further use.
2. To produce heat cross-linked microspheres, the temperature of the emulsion was raised gradually while stirring at 2500 RPM to either 70°C or 90°C. Stirring was continued till the emulsion attained room temperature. Microspheres formed were then separated by centrifugation, washed with petroleum ether to remove the liquid paraffin and air dried.

### Characterization of microspheres

#### *Entrapment efficiency*

Weighed amount of microspheres were suspended in 0.1 N hydrochloric acid and allowed to stand for 24 h. The dispersion was then shaken with methylene chloride to extract celecoxib. The organic extract was then evaporated to dryness and the residue dissolved in methanol. The absorbance of the resulting solution was measured at 250 nm on Shimadzu 1601 UV-Vis Spectrophotometer to determine the amount of celecoxib present in the microspheres.

#### *Particle size*

The particle size distribution of the microspheres was determined by laser light scattering on a Malvern Particle Size Analyzer (Malvern Master Sizer 2000, SM, UK). The microspheres were added to the sample dispersion unit containing the stirrer and stirred to reduce the aggregation between the microspheres and laser obscuration range was maintained between 15 and 20%. The average volume-mean particle size was measured after performing the experiment in triplicate.

#### *In-vitro drug release*

Drug release from the microspheres was determined using phosphate buffer pH 7.4 containing 2%w/w tween-80 as the release medium. Microspheres were suspended in 50 ml of the dissolution medium in a 100 ml glass vials and stirred on a magnetic stirrer at 50 rpm in a thermo stated bath at 37°C. 2 ml samples were withdrawn at appropriate time intervals and centrifuged at 5000 rpm. Supernatants were diluted suitably and absorbance of the resulting solution was measured at 250 nm using the dissolution medium as blank. The residue was redispersed in 2 ml of the fresh dissolution medium and replaced back into the vial.

#### *Surface morphology of microspheres*

Scanning electron microscopy of the chitosan microspheres was carried out to examine the surface morphology. The microspheres were mounted on metal stubs and then coated with a 150 Å layer of gold. Photographs were taken using Jeol Scanning Electron Microscope (Jeol. JSM-5610LV SEM).

#### *Analysis of data*

Using Graphpad Instat 3 (USA), data comparison was accomplished using Student's *t*-test. At 95% confidence interval, 2 tailed *P*-values less than 0.05 were considered to be statistically significant.

## RESULTS

The effect of the various cross-linking agents on the characteristics of the microspheres prepared was studied. The microspheres were characterized for entrapment efficiency, particle size, *in-vitro* drug release, and surface morphology.

**Table 1: Effect of glutaraldehyde concentration and duration of cross-linking on the entrapment efficiency and particle size**

Batch code	Glutaraldehyde concentration	Duration of cross-linking	% entrapment efficiency	Particle size GMD ( $\mu\text{m}$ )
A	0.5 ml	1 h	89.16 $\pm$ 1.32	10.53 $\pm$ 0.77
B	0.5 ml	3 h	88.54 $\pm$ 1.65	9.13 $\pm$ 0.37
C	1.0 ml	1 h	89.75 $\pm$ 1.75	10.55 $\pm$ 1.30
D	1.0 ml	3 h	90.14 $\pm$ 1.60	8.65 $\pm$ 1.59

All results are expressed as mean $\pm$ SD (n=3)

**Table 2: Effect of formaldehyde concentration and duration of cross-linking on the entrapment efficiency and particle size**

Batch code	Volume of formaldehyde solution (37%w/v)	Duration of cross-linking	% entrapment efficiency	Particle size GMD ( $\mu\text{m}$ )
E	0.5 ml	1 h	88.72 $\pm$ 1.71	14.68 $\pm$ 0.98
F	0.5 ml	3 h	90.15 $\pm$ 1.59	12.60 $\pm$ 1.18
G	1.0 ml	1 h	91.64 $\pm$ 3.69	11.62 $\pm$ 1.12
H	1.0 ml	3 h	90.53 $\pm$ 1.69	12.75 $\pm$ 1.08

All results are expressed as mean $\pm$ SD

**Table 3: Effect of temperature on entrapment efficiency and particle size of the heat cross-linked microspheres**

Batch code	Temperature	% entrapment efficiency	Particle size GMD ( $\mu\text{m}$ )
I	70°C	24.56 $\pm$ 0.78	9.62 $\pm$ 1.22
J	90°C	20.91 $\pm$ 2.50	6.48 $\pm$ 0.67

All results are expressed as mean $\pm$ SD

The glutaraldehyde cross-linked microspheres were yellowish brown in color and were obtained as a fine powder.

As shown in Table 1, the glutaraldehyde volume used for cross-linking and the duration of cross-linking does not have significant influence ( $P>0.05$ ) on the entrapment efficiency and the particle size of the microspheres. The microspheres cross-linked using glutaraldehyde shows high entrapment efficiency of around 90%.

The formaldehyde cross-linked microspheres were obtained as a white fine powder.

As shown in Table 2, the volume of formaldehyde and the duration of cross-linking do not have a significant influence ( $P>0.05$ ) on the entrapment efficiency and the particle size of the microspheres. The particle size of the microspheres prepared using formaldehyde is significantly different ( $P<0.05$ ) from those prepared using glutaraldehyde. The geometric mean diameters of the formaldehyde cross-linked microspheres ranged from 11.62 to 14.68  $\mu\text{m}$  while the particle size of the glutaraldehyde cross-linked microspheres ranged from 8.65 to 10.55  $\mu\text{m}$ .

The heat cross-linked microspheres shows significantly lower ( $P<0.05$ ) entrapment efficiency than the chemically cross-linked microspheres. No microspheres were formed at a temperature of 60°C or less.

As shown in Table 3, both batches I and J show the entrapment

efficiency of only 20-25%. The microspheres were obtained as a fine white powder. The geometric mean diameter of the heat cross-linked microspheres decreased from 9.62 to 6.48 as the temperature was increased from 70°C to 90°C.

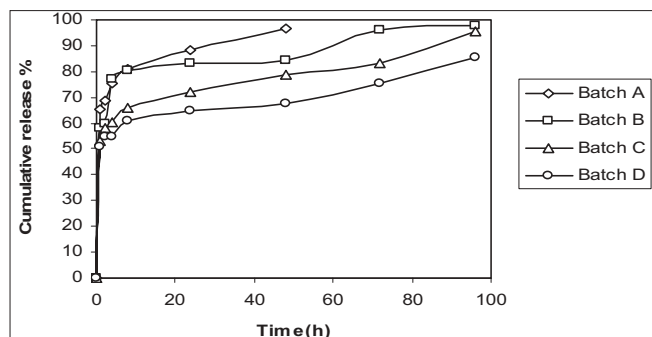
As shown in Figure 1, an increase in the concentration of glutaraldehyde led to a decrease in the rate of drug release. Microspheres prepared using 1.0 ml of glutaraldehyde releases the drug slowly compared to the microspheres in which 0.5 ml of glutaraldehyde is used. However, a burst effect was observed in all the formulations. In general, around 50% of the drug is released in the first hour, followed by slower release for a period of 96 h.

Same results were obtained for formaldehyde cross-linked microspheres. Increasing the formaldehyde volume and duration of cross-linking leads to a decrease in the drug release rate. Microspheres prepared using 1.0 ml formaldehyde releases the drug slowly compared to the microspheres prepared using 0.5 ml formaldehyde. However, a burst effect was observed in all the formaldehyde cross-linked microspheres. About 60% of the drug is released in the first hour followed by a controlled release for a period of 72 h. There is a significant difference ( $P<0.05$ ) in the drug release rates of the formaldehyde cross-linked microspheres and the glutaraldehyde cross-linked microspheres.

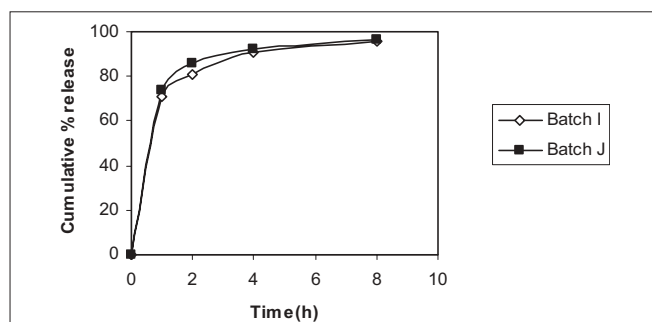
The duration of cross-linking also had an effect on the drug release.

An increase in the duration of cross-linking from 1 to 3 h led to a decrease in the drug release as shown in Figures 1 and 2, respectively for glutaraldehyde and formaldehyde cross-linked microspheres [Figure 3].

The heat cross-linked microspheres shows the fastest drug release. About 70% of the drug is released in the first hour



**Figure 1:** Effect of volume of glutaraldehyde (25%w/v) and duration of cross-linking on the drug release



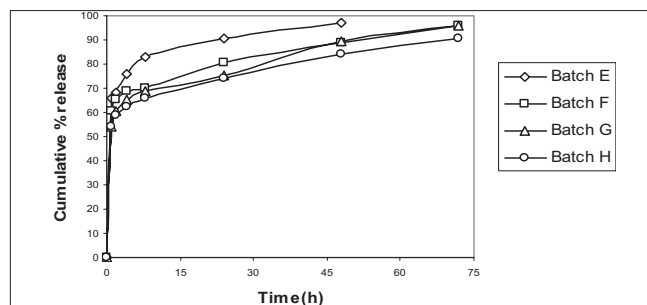
**Figure 3:** Drug release profile of heat cross-linked microspheres

and 95% of the drug is released in 8 h.

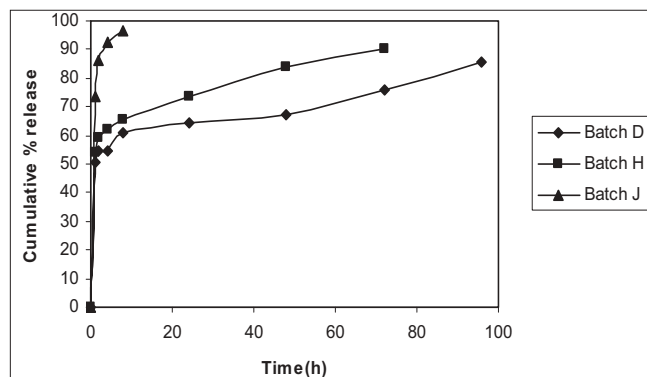
## DISCUSSION

The microspheres were prepared by emulsion polymerization technique. In this method, the drug is dispersed in the chitosan solution and then emulsified in the oily phase in presence of an emulsifier. Then the chitosan present in the internal phase is cross-linked either by chemical or thermal treatment. Since celecoxib is not soluble in the external phase of the emulsion and in the cross-linking agents glutaraldehyde or formaldehyde at the conditions used for preparation of microspheres, higher entrapment efficiency was obtained for chemically cross-linked microspheres. In case of heat cross-linking, the higher temperature used for the preparation of the microspheres solubilizes the drug in the external phase of the emulsion. Thus, very less entrapment efficiency of around 20-25% was obtained in case of heat cross-linked microspheres. This was confirmed by the presence of the solubilized celecoxib in the external phase of the emulsion.

The particle size of the microspheres was affected by the cross-linking agent used. The formaldehyde cross-linked microspheres shows a larger particle size than the glutaraldehyde cross-linked microspheres and the lowest particle size was obtained for the heat cross-linked microspheres. Cross-linking with glutaraldehyde is reported to produce greater number and more stable cross-links than with formaldehyde.<sup>[21]</sup> This may be the reason of the smaller



**Figure 2:** Effect of volume of formaldehyde (37%w/v) and duration of cross-linking on the drug release

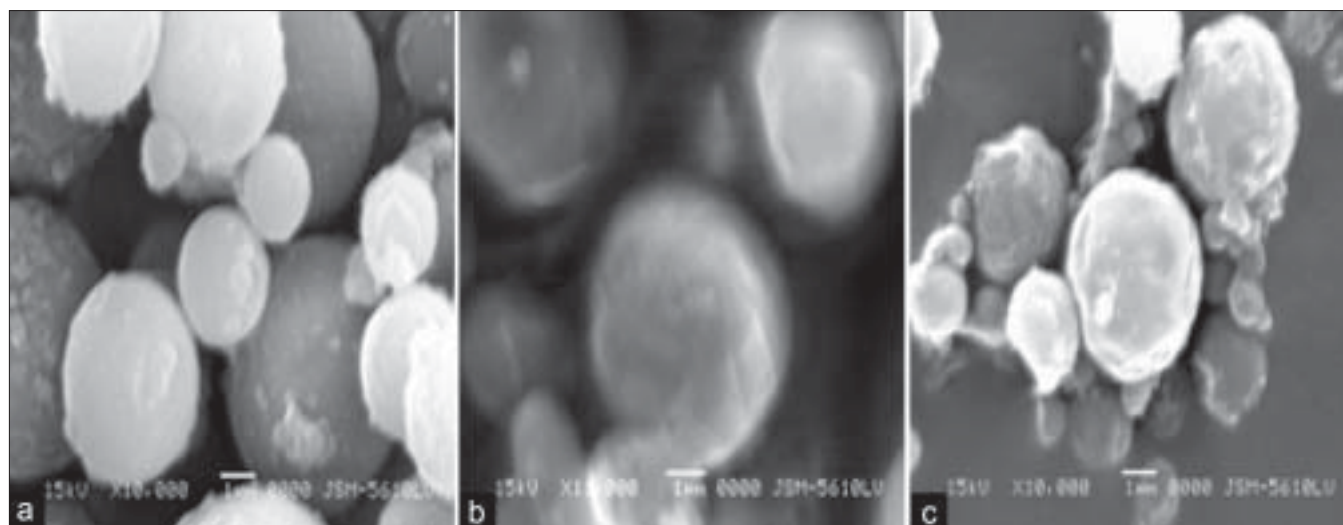


**Figure 4:** Comparative drug release profiles of glutaraldehyde, formaldehyde, and heat cross-linked microspheres

particle size obtained for the glutaraldehyde cross-linked microspheres. This is also reflected in the *in-vitro* drug release study which shows significantly slower drug release rates from microspheres cross-linked with glutaraldehyde [Figure 4].

The temperature at which the heat cross-linked microspheres are prepared has an influence on the entrapment efficiency and the particle size. No microspheres were formed when the temperature of the emulsion was raised to 60°C. A temperature of 60°C may not be sufficient for complete evaporation of water from the internal phase of the emulsion. Thus, a jelly like material was obtained instead of microspheres. Microspheres prepared at 70°C shows higher entrapment efficiency than the microspheres prepared at 90°C. The reason behind this finding is that the solubility of celecoxib in the external phase of the w/o emulsion increases with an increase in the temperature. But, in both the cases, the entrapment efficiency is only between 20 and 25%. The particle size of the heat cross-linked microsphere is significantly smaller than the chemically cross-linked microspheres ( $P < 0.05$ ). Similar results were obtained by previous workers.<sup>[17,10]</sup> This may be because of the fact, that there is complete dehydration of the microspheres resulting in the shrinkage at the temperature at which the microspheres are prepared.

As shown in Figure 5, the drug release rate from the heat cross-linked microspheres is significantly higher ( $P < 0.05$ ) than the chemically cross-linked microspheres. The results



**Figure 5:** (a) Formaldehyde cross-linked microspheres, (b) Glutaraldehyde cross-linked microspheres, (c) Heat cross-linked microspheres

are in concurrence with the earlier reports.<sup>[10]</sup> This may be because; there is an actual chemical reaction of chitosan with formaldehyde or glutaraldehyde leading to a stronger and a more rigid matrix than the heat cross-linked microspheres. No significant difference was observed between the release rates of microspheres prepared at 70°C or 90°C. However, a burst effect which may be attributed to the drug present on the surface of the microspheres is observed in all the formulations.

The scanning electron microscopy study reveals that the surface of the microspheres is rough which may be because of the drug present on the surface.

## CONCLUSIONS

Chitosan Microspheres with high entrapment efficiency were prepared using chemical cross-linking method. Heat cross-linking gives the microspheres with low entrapment efficiency and low particle size. The glutaraldehyde cross-linked microspheres releases the drug slowly compared to the formaldehyde cross-linked microspheres while fastest drug release was observed in heat cross-linked microspheres.

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