Part-B

*Physicochemical parameters of selected nanocapsule preparations*
Part - B

4.1 Preparation of alginate-MTX at 100:1 polymer-drug ratio

1.5 mg drug (MTX) dissolved in organic solvent (10 ml ethyl acetate containing 0.5 ml butyl amine) was emulsified into aqueous polymer solution of 0.15 gm of sodium alginate in 30 ml of demineralized water using Tween 80 as emulsifier and the coemulsifier used was glycerol for microemulsion formation. The microemulsion formed was sprayed through in-house developed pneumatic nebuliser into different concentrations of CaCl₂ solutions. The nanocapsules formed were separated by ultracentrifugation, at 30,000 rpm. for 30 minutes. They were then resuspended in 10 ml of water, redispersed, recentrifuged and dried up to a constant percent water content under vacuum at 4°C.

A generalized polymer to drug ratio was used in all these formulation studies. Attempts to produce 10:1 alginate:drug products were grossly unsuccessful mostly because a thinner polymer film often collapsed producing aggregates rather than nanocapsules as visible in TEM micrographs. Batches of alginate : drug 50:1 were also not very successful as percent drug load was very poor and TEM (Hitachi H-600) micrograph showing many nonuniform nanocapsules. Nanocapsules in 100:1 formulations however were uniform and spherical and was studied further in detail.
4.2 *Factorial Design Studies*\(^{15}\)

In order to study and optimize different parameters involved in alginate nanocapsulation, a factorial design study was designed for alginate - MTX nanocapsules. The effect of two variables of i) CaCl\(_2\) concentration and ii) emulsifier, tween 80 concentrations on the properties of nanocapsules were investigated in factorial design studies. Suitable high (2M) and low (1M) molar concentrations of calcium chloride solutions were chosen and studied against 0.34% w/w (high) and 0.17% w/w (low) concentration incorporation of surfactant tween 80. Three sets of four formulations each of the combinations (table-4) were prepared following the generalized procedure for further investigation.

**Table 4: Parameter variations in factorial design studies**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>CaCl(_2) concentration</th>
<th>Emulsifier concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>High (2 molar)</td>
<td>Low (0.17 % w/w)</td>
</tr>
<tr>
<td>II</td>
<td>High (2 molar)</td>
<td>High (0.34 % w/w)</td>
</tr>
<tr>
<td>III</td>
<td>Low (1 molar)</td>
<td>High (0.34 % w/w)</td>
</tr>
<tr>
<td>IV</td>
<td>Low (1 molar)</td>
<td>Low (0.17% w/w)</td>
</tr>
</tbody>
</table>
4.3 *Preparation of BSA-MTX at 50:1 polymer-drug ratio*

3.0 mg of methotrexate was dissolved in 10 ml of ethyl acetate containing 0.5 ml of butyl amine. This solution was emulsified into 30 ml of 0.5% aqueous solution of BSA using 0.4 ml tween 80 as emulsifier. Microemulsion was formed by slow addition of 18ml. co-surfactant, propylene glycol. This was then atomised into the coaservate acetone media using a pneumatic nebuliser (section 2.1.3). Nanocapsules were then separated out by ultracentrifugation at 30,000 rpm. 0°C for 45 minutes, washed in small volume of cold acetone, recentrifuged and dried under reduced pressure.

4.4 *Preparation of BSA-MTX at 50:1 polymer-drug ratio in large scale*

Similar protocol as in 4.3 was used for the preparation of BSA-MTX at 50:1 polymer drug ratio in three times larger scale to verify the adaptability of the developed technology on larger batch size operations.

9.0 mg of methotrexate was thus dissolved in 30 ml of ethyl acetate containing 1.5 ml of butyl amine. This solution was emulsified in 90 ml of 0.5% aqueous solution of BSA under continuous stirring at 150 rpm. 1.2 ml Tween 80 was then similarly added for emulsification. Drop wise addition of 54 ml co-surfactant propylene glycol provided the microemulsion. This was atomized through the in-house developed pneumatic nebuliser (2.1.3) into 3 litres coaservate media of acetone. Nanocapsules were then separated out by ultracentrifugation at 30,000 rpm 0°C for 45 minutes. The separated nanocapsules were redispersed in cold acetone, washed, dried and recentrifuged to harvest the nanocapsules prepared.
5. **Evaluation Results of selected nanocapsules**

5.1 **Drug entrapment efficiency**

The drug (MTX) entrapment efficiency was studied as described in 3.1.1 and 3.1.2, taking 10 mg of aliquate from each batch alginate nanocapsules prepared. Methotrexate standard graph (fig.7) was used as usual for calculation of percent drug loading and the results were tabulated in table 5. Similar procedure was followed for BSA-MTX nanocapsules, taking 10 mg samples of preparation 4.3 and 4.4 and analyzed in HPLC. Percentage drug load observed were (57.01 ± 0.82) % and (52.9 ±2.21) % respectively, thus concluding that batch size variations are unlikely to affect the nanocapsular drug pay load.

**Table 5 : Drug loading efficiency of Alginate-MTX nanocapsules (100:1)**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>CaCl₂ concentration</th>
<th>Emulsifier concentration</th>
<th>Drug loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>High (2 molar)</td>
<td>Lcw (0.17 % w/w)</td>
<td>(27.60 ±0.26)%</td>
</tr>
<tr>
<td>II</td>
<td>High (2 molar)</td>
<td>High (0.34 % w/w)</td>
<td>(32.06 ±0.34)%</td>
</tr>
<tr>
<td>III</td>
<td>Low (1 molar)</td>
<td>High (0.34 % w/w)</td>
<td>(33.58 ±0.96)%</td>
</tr>
<tr>
<td>IV</td>
<td>Low (1 molar)</td>
<td>Low (0.17% w/w)</td>
<td>(25.13 ±0.47)%</td>
</tr>
</tbody>
</table>
It was evidenced that lower concentration (1 molar) of calcium chloride solution and higher concentration (0.34%) of emulsifier, tween 80 provided good drug pay load. Thus the formulation III was therefore considered for further physicochemical evaluations.

Calcium chloride concentrations were though considered crucial for drug loading and drug release of alginate nanocapsules\textsuperscript{16}, it was apparent that an insufficient calcium chloride concentration in the polymer cross linking media can significantly affect the drug load or release profile. Such effect was however absent when the alginate polymer was completely cross linked.

5.2 \textit{Determination of Ca-content in the nanocapsules}

The Ca content of the nanocapsules were determined following the method as described by Takka and Acarturk\textsuperscript{17}. 50 mg of alginate nanocapsules were dissolved in 1 ml conc. nitric acid and diluted to 10 ml with double distilled water. The calcium content was then measured by atomic absorption spectroscopy (AAS) using 2 ppm to 5 ppm calcium salt solutions as reference standards. For each set of formulation four samples were taken calcium content was estimated averaged and the results were tabulated in Table-6.

In order to estimate the degree of cross linking, the sodium ion concentration in the nanocapsulate was also determined in AAS from the same samples. Standard sodium salt solutions of 1 ppm. to 5 ppm. were used and sodium concentration measured. No sodium ion was found to be present in the prepared nanocapsules, thus concluding complete replacement of sodium ions in the alginate nanocapsules.
### Table 6: Estimation of calcium content in Ca-alginate nanocapsules

<table>
<thead>
<tr>
<th>Formulation</th>
<th>CaCl₂ concentration</th>
<th>Emulsifier concentration</th>
<th>Calcium Content Per 10 mg of nanocapsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>High (2 molar)</td>
<td>High (0.34 % w/w)</td>
<td>1.37 ± 0.04 mg.</td>
</tr>
<tr>
<td>III</td>
<td>Low (1 molar)</td>
<td>High (0.34 % w/w)</td>
<td>1.112 ± 0.06 mg.</td>
</tr>
</tbody>
</table>

5.3 **Nanocapsule size distribution studies**

5.3.1. **Photon correlation spectroscopy (PCS) Studies**

For PCS studies\(^{18,19}\), 1 ml of sample suspension of nanocapsules prepared, was added with 0.5 ml of isopropanol for soaking and was subsequently diluted to 10 ml with HPLC grade water. It was then subjected to PCS studies in Malvern zetasizer 1000HS. PCS size distributions was recorded as volume average diameter. Four batches were measured and averaged for size distribution studies. Size distribution for alginate-MTX nanocapsules 100:1, formulation III preparation 4.1 and BSA-MTX nanocapsules, 50:1, preparation 4.3, were represented in fig 30 and 31 respectively.

5.3.2 **Transmission electron microscopy (TEM) Studies**

A generalized protocol was used for all batches of nanocapsules produced. A drop of water suspension of the nanocapsules was placed on C-grid, air
Fig. 30: PCS studies on alginate-MTX (100:1) nanocapsules, Preparation 4.1
Fig. 31: PCS studies on BSA-MTX (50:1) nanocapsules, Preparation 4.3
dried and the particles were stained with 1% phospho tungstanc acid solution (PTA). In order to optimize the TEM visualization, PTA exposure times were studied. Different C-grids were prepared and exposed to PTA solution for 15sec., 30sec., 45sec. and 1min. The carbon grids containing PTA stained nanocapsules were placed in transmission electron microscope for TEM micrography. Prepared nanocapsule samples were visualized and the C- grids exposed for 30sec., provided good resolution and contrast for photography. Nanocapsule size distribution for preparation 4.1 (formulation III) and preparation 4.3 were represented in fig 32.

5.4 Coating Thickness

The wall thickness of the nanocapsules for each formulation were calculated using the equation as was developed by Luu:

\[ h = \frac{r(1-p)d_1}{[pd_2 + (1-p)d_1]} \]

where, \( h \) is coating thickness; \( r \), particle radius; \( p \), proportion of the drug in the nanocapsules; \( d_1 \), density of the core material; \( d_2 \), density of the coating material.

The densities of the dried methotrexate and polymer substances were measured by liquid displacement method and the mean particle radius (\( r \)) was recorded from TEM studies.
Fig. 32: TEM studies on Methotrexate nanocapsules

- Alginate - MTX (100:1) nanocapsules; Preparation 4.1
- BSA-MTX (50:1) nanocapsules; Preparation 4.3
Nanocapsules wall thickness, calculated were tabulated in table 7. Single BSA-MTX nanocapsule from preparation batch 4.3 was studied in TEM micrography (Fig. 33), showing a continuous coating layer and coating thickness observed corroborated with earlier observations.

5.5 *Karl Fischer-Moisture analysis*

Minimal water content seems crucial for nanocapsular stability as that in itself is one of the major factor of storagability of nanocapsules.

For determination of small amount of water Karl Fischer titration was followed\(^2\) using Karl Fisher reagents. Reaction end point was determined electrometrically using the dead-stop end point procedure.

The average moisture content of alginate-MTX nanocapsules [preparation 4.1, formulation III] and BSA-MTX nanocapsules [preparation 4.3] were determined and tabulated in table 7.

5.6 *Analysis for Residual solvents in nanocapsules harvested*

10 Mg. of nanocapsule prepared was dissolved by gentle shaking in either a 5 ml. (N) NaOH for BSA-MTX preparation or in 5 ml (N) NaOH solution containing 5mg sodium EDTA for alginate-MTX preparation. It was subsequently extracted in three successive quantities of 2 ml chloroform. Ten μl of this chloroform layer was injected into gas chromatograph using N\(_2\) as mobile phase, flow rate 5 ml/min. Jenson DB1 column was used at column temperature of 80°C, flame ionization detector (FID) set
Fig. 33: Transmission Electron Micrograph of a single BSA-MTX(50:1) nanocapsules at 1,70,000 X 2 at 75 KV.
at 150°C. Chromatograph thus produced was compared with that of standard preparations of glycerin, chloroform solution of ethyl acetate and butyl amine.

Prepared and harvested nanocapsules showed no residual solvent peak for butyl amine, ethyl acetate or glycerin.

Table – 7: Physicochemical properties of selected nanocapsules

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Coating thickness</th>
<th>%Moisture content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Alginate – MTX</td>
<td>14.56nm</td>
<td>26.799±2.024%</td>
</tr>
<tr>
<td>Preparation 4.1,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Formulation III)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA-MTX</td>
<td>11.10 nm</td>
<td>5.818±0.181%</td>
</tr>
<tr>
<td>Preparation 4.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.7 I. R. Spectroscopy Studies

Drug entrapment was confirmed in FTIR studies of nanocapsules prepared. Separate FTIR spectrums were taken for Methotrexate, and polymer nanocapsules similarly prepared with and without the drug pay load for comparison. FTIR spectrum of BSA- MTX nanocapsules, showed characteristic additional peak at 830 cm⁻¹ for p-disubstituted benzene of MTX. The spectrum recorded indicate, that no drug - protein binding has appeared, as there is no shift of amide peaks of BSA in prepared nanocapsules. FTIR observation was however difficult to be recorded for alginate-
MTX nanocapsules even at 512 scan because of larger water content and lower drug payload. Characteristic additional peak at 830 cm⁻¹ was however observed for MTX in alginate-MTX nanocapsules. Fig. 34, 35 were the FTIR spectrum of pure Methotrexate and alginate without the drug payload. Fig. 36 provided FTIR spectrum for alginate-MTX nanocapsules [preparation 4.1, formulation III]. Fig. 37 and fig. 38 were similarly the FTIR scans for the BSA without the drug payload and BSA-MTX nanocapsules [preparation 4.3] respectively.

5.8 Release Kinetic studies

In vitro drug release studies were carried out at 37°C, pH 7.4 phosphate buffer 100mM with continuous stirring of 120 rpm. The cumulative drug release was measured under sink conditions using 30 ml solution in dissolution test apparatus. 2 ml samples were withdrawn at fixed time intervals. Samples were centrifuged in a centrifuge YM 50 and the drug content was measured as described earlier in 3.3 by injecting 20μl of the filtrate in HPLC using UV detector set at 257 nm. Each time 2 ml of phosphate buffer was added to compensate for the sample withdrawn and to maintain sink conditions.

The cumulative drug release pattern from the Alginate-MTX and BSA-MTX nanocapsules were represented in fig. 39 and fig. 40 respectively.
Fig. 34: FT-IR spectrum of Methotrexate
Fig. 35: FT-IR spectrum of Alginate

Fig. 36: FT-IR spectrum of Alginate-Methotrexate nanocapsules (100:1); Preparation 4.1
Fig. 37: FT-IR spectrum of BSA

Fig. 38: FT-IR spectrum of BSA-Methotrexate nanocapsules (50:1); Preparation 4.3
Fig. 39: Drug release kinetics of Alginate-MTX nanocapules
[Preparation 4.1 (Formulation III)]
Fig. 40: Drug release kinetics of BSA–MTX nanocapsules [Preparation 4.3]
5.9 Evaluation of mechanism of drug release

Release mechanism of Methotrexate from the nanocapsules prepared were studied primarily using the semiempirical equation\textsuperscript{22}

\[ \frac{M_t}{M_a} = k \, t^n \]

Where, \( \frac{M_t}{M_a} \) is the fraction of drug released up to time \( t \); \( k \) is kinetic constant, \( n \) is release exponent related to the release mechanism.

Initial portion of the release curve \( \frac{M_t}{M_a} < 0.8 \) was analyzed and the values of \( n \) and \( k \) were determined using Sigma Plot curve fit\textsuperscript{13}, 100 iterations. The results were tabulated in table 8. When the value of \( n \) is 0.43 and less, the drug release follows a Fickian diffusion, for \( n \) in between 0.43 and 0.85 the drug release mechanism is non Fickian transport controlled by diffusion and relaxation of the polymer coating due mainly to swelling and when \( n > 0.85 \), release is a case II transport phenomena\textsuperscript{23}.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Polymer:Drug(w/w)</th>
<th>( n )</th>
<th>( k )</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate-MTX</td>
<td>100:1</td>
<td>0.487 ± 0.065</td>
<td>7.04 ± 0.017</td>
<td>0.958</td>
</tr>
<tr>
<td>preparation (Formulation III)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA-MXT</td>
<td>50:1</td>
<td>0.716 ± 0.008</td>
<td>17.06 ± 1.14</td>
<td>0.978</td>
</tr>
<tr>
<td>preparation 4.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( k \), kinetic const; \( n \), release component; \( r^2 \), correlation coefficient;

104