CHAPTER VI

Development of Lignosulfonicacid and Gelatin Interpenetrating Network microspheres for Controlled Release of Pyronaridine.
VI.1. INTRODUCTION

Encapsulation of biologically active agents in particulate carriers as a method of controlled delivery of molecules has been studied extensively. In recent years, a number of different particulate systems, such as hydrogels, beads, microcapsules, micro beads and microspheres (MSs), have been proposed and used in topical formulations as drug carrier vehicles. MSs can function as cell micro carriers [01–05], delivery vehicles for drugs [06–12], growth factors [13–18] and injectable scaffolds as well [19, 20]. Colloidal carriers in the form of microspheres and nanoparticles are being investigated as potential drug delivery systems [21-23]. These systems involve microspheres in diameters ranging from below 1µm to over 100µm.

Gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the skin, boiled crushed horn, hoof and bones, connective tissues and organs. Food-grade gelatin is produced mainly from two raw materials i.e., beef skin and pig hides. Gelatin is an animal protein unlike many other gelling agents used by the food industry. Gelatin is used in pharmaceutics due to its biocompatibility and biodegradability properties. It can be utilized for the preparation of oral as well as injectable microspheres. Aldehydic derivatives such as formaldehyde, glutaraldehyde or other bifunctional reactants have been used to produce insoluble biodegradable gelatin MSs. Glutaraldehyde is used as a cross-linking agent to obtain rigid MSs. Glutaraldehyde produces cross-linking between gelatin molecules and thus reduces the rate of drug release from the microspheres. Gelatin MSs are generally prepared by solvent evaporation method[24-26] and crosslinking method [27-32]. In the present study we have prepared GT/LSA microspheres by ‘desolvation’ method using glutaraldehyde (GA) as crosslinker.

Lignin based polymers present two-fold advantage. One is that they are abundantly available as tons of lignin is thrown off as a waste product in pulp and paper industries. Secondly, lignin is completely biodegradable slowly. Sodium lignosulfonates (lignosulfonicacid, sodium salt) are used in the food industry as a de-foaming agent for paper production and in adhesives for items that come in contact with food. It has antimicrobial and preservative properties, and is used as an ingredient in animal feeds.
Lignin is a macromolecular compound more chemically active than cellulose or other natural polymers, due to the functional groups contained in its macromolecule, being considered the main aromatic component of plant tissues. Globally, lignin is regarded as a raw material with a high recovery potential, accessible from renewable sources, with low costs and a negligible pollution degree [33-39]. Information on the synthesis of lignin-based nanoparticles is relatively limited and covered by patents [40]. Lignin becomes sulfonated and as such is soluble in water and under a range of aqueous solution conditions. As the lignosulfonate macromolecule is water soluble, this class of polymer shows great promises in future Nano technological and surface chemistry applications beyond those where it is already finding use.

Pyronaridine is a benzonaphthyridine derivative first synthesized in 1970 at the Institute of Chinese Parasitic Disease, Chinese Academy of Preventative Medicine [41-43]. The drug is formulated as pyronaridine tetraphosphate, a yellow, odorless powder with a bitter taste [44]. As the use of pyronaridine for the treatment of malaria has been limited to China over the last 30 years, it is expected that resistance will be slow to develop across other malarial regions of the world.

Pyronaridine Tetrakis Phosphate

The gelatin and lignosulfonic acid blend (GT and LSA) microspheres were developed by crosslinking with glutaraldehyde (GA). Pyronaridine an antimalarial agent was loaded into these microspheres. Various formulations were prepared by varying ratios of GT/LSA, GA and % Pyronaridine loading. Microspheres were characterized by Fourier transforms infrared
spectroscopy (FTIR), differential scanning calorimetric (DSC), X-ray diffraction (X-RD) and Scanning electron microscopy (SEM). FTIR spectroscopy confirmed the crosslinking and presence of drug in the GT/LSA microspheres. X-RD studies were performed to understand the crystalline nature of drug after encapsulation into IPN microspheres. SEM images gave the beads with smooth surface. Drug release profiles of the IPN microspheres at pH 1.2 and 7.4 confirmed that the microspheres formed are pH-sensitive, resulting in controlled release of drug during in vitro dissolution experiments. It has been analyzed with an empirical equation to understand the diffusion nature of drug through the GT/LSA microspheres. Both encapsulation efficiency and release patterns are found to be dependent on the nature of the cross-linking agent as well as amount of drug loading and % of GT/LSA microspheres. In vitro release studies indicated that the microspheres enhance the release rates of Pyronaridine drug up to 10 hours.

VI.2.1. EXPERIMENTAL

The details of the materials and the experimental procedures adopted in the present study have been explained in chapter II under experimental section.

VI.2.2. The details of experimental procedures adopted in the present study have been explained in chapter II.3.D.1. Under experimental section.

VI.3. RESULTS AND DISCUSSIONS

VI.3.1. Fourier transforms infrared spectroscopy (FTIR) analysis

The crosslinking was confirmed by comparing the FTIR spectrum of LSA with GT/LSA, which was polymerized with GT in presence of GA as a crosslinker. Figure VI.1. (a.) reveals the FTIR spectrum of LSA; the peak at 3404 cm\(^{-1}\) is due to the \(-\text{OH}, \text{N-H and } \text{–SO}_3\text{H}\) stretching vibrations. Bands around 833 cm\(^{-1}\), 755 cm\(^{-1}\) and 691 cm\(^{-1}\) can be described as the out-of-plane vibrations of substituted benzene rings in the structure of LSA. The band at 1623-1628 cm\(^{-1}\) indicated the carbonyl stretching conjugated with aromatic ring. The absorption peaks at 1185 cm\(^{-1}\) and 1049 cm\(^{-1}\) attributed the vibrations of sulphonic group and the asymmetric \(-\text{C-H}\) deformations of aromatic rings indicated at 1464 cm\(^{-1}\).
Figure VI.1: FTIR Spectra of (a) Lignosulphonic acid, (b) Gelatin and (C) GT/LSA microspheres.

FTIR spectrum of GTfigure VI.1.(b.) depicted the presence of characteristic functional group of amine bands at 1536 cm\(^{-1}\) and 1641 cm\(^{-1}\) are typical for the N-H bending and C-N stretching vibrations; 1670 cm\(^{-1}\) indicated the C=O stretching of amide group of GT. GT has a positive charge at acidic pH due to presence of more amino groups in it. The peaks of free amino groups that are present in GT were disappeared in GT/LSAMSs in Figure VI.1.(c). A new peak was observed in the Figure VI.1.(c) for amide in the region 1530-1650 cm\(^{-1}\) which confirmed the formation of microspheres due to reaction between sulphonic group of LSA and amino group of GT crosslinked with GA.
VI.2. FTIR Spectra of (a) Pyronaridine, (b) GT/LSA MSs and (c) drug loaded GT/LSA microspheres.

Figure VI.2 (a-c) revealed that FTIR spectrum of pure drug, drug loaded GT/LSAMSs and plain GT/LSA MSs showed characteristic peaks. Pure pyronaridine showed an absorption peak at 1028 cm\(^{-1}\) for –C-Cl and at 1387 cm\(^{-1}\) for phenolic C-O, and an up-shift of C-H band occurred from 2926 cm\(^{-1}\) to 2936 cm\(^{-1}\) due to intramelecular vibrations of hydrogen bonding. These are evidences the intact nature of pyronaridine in GT/LSA MSs.

VI.3.2. X-ray Diffraction Studies

X-RD analysis can helps to find the crystallinity of drug in crosslinked matrix of GT/LSA MSs. Dried and drug loaded MSs of uniform size were mounted on a Sample holder and X-RD patterns were recorded. Figure VI.3. show (a) pure drug, (b) plain MSs and (c) drug loaded MSs. Pure drug pyronaridine shows high intense peak at \(2\theta = 24^\circ\) and \(11^\circ, 18^\circ,\) and \(21^\circ\)suggesting its crystalline nature whereas these peaks are not observed in plain MSs and drug loaded GT/LSA MSs [Figure VI(3.b.) and (3.c.)]. These evidences fulfilled that strong interaction has occurred between drug and crosslinked GT/LSAMSs which suggest the amorphous nature of drug present in the MSs.
Figure VI.3: X-RD Spectra of (a) Pyronaridine, (b) GT/LSA microspheres and (c) drug loaded GT/LSA microspheres.

VI.3.3. Scanning Electron Microscopy Studies

Figure VI.4: Scanning Electron Micrographs of GT/LSA MSs for different magnifications (a-d).
Figure VI.4.(A-D) optical micrographs shows SEM micrographs of pyronaridine loaded crosslinked GT/LSAMs figure 4(A) displays the average size of spheres is around 200µm and figure VI. 4(B) &4(C) shows average size of spheres are 200 and 300µm respectively measured as different magnifications from SEM images. MSs of this study were almost spherical with smooth surfaces figure VI.4 (D) showed no phase separation and all formulations are almost spherical and spherical-shaped with smooth surfaces. Optical microscopy gave particle size of the micro beads for all formulations and the same results.

VI.3.4. Microscopic images of LSA- GT Microspheres

Figure 5: Optical microscopic images of GT/LSA microspheres of different particle sizes.
VI.3.5. Encapsulation Efficiency

Effects of GT, LSA and crosslinker contents on encapsulation efficiency of drug loaded microspheres are given in Table VI.1. Encapsulation efficiency of pyronaridine increases with increasing amount of LSA. This can be recognized that at higher concentrations, viscosities leading to a less diffuse matrix structure that obstruct drug departure from the GT/LSAMSs during the microsphere formation. GA also affects the encapsulation efficiency of pyronaridine into the MSs. The enhancement of GA in the feed formation of GT/LSAMSs decreased tendency in encapsulation efficiency. This is due to amplification in crosslinking density of GT/LSAMSs will become more rigid thereby reducing the free level spaces within the polymer matrix. Microspheres were loaded with pyronaridine and encapsulation efficiency is found to be around 61% has shown in the Table.VI.2.

VI.3.6. In-vitro release studies

VI.3.6. 1. Effect of Cross-linking agent on cumulative release

![Graph showing cumulative release](image)

Figure VI. 6: Effect of Cross linker on cumulative release of Pyronaridine; GT/LSA-5 (0.4 mL), GT/LSA-6 (0.5 mL) and GT/LSA-7 (0.6 mL).
To understand the drug release from pyronaridine loaded crosslinked LSA-co-GT microspheres, *in vitro* release experiments were carried out at different time intervals in phosphate buffer media. The effect of crosslinker (GA) in the compositions of GT/LSA-5 (0.4 mL), GT/LSA-6 (0.5 mL) and GT/LSA-7 (0.6 mL) are presented in Figure VI.6. The cumulative release is higher at lower amount of GA (GT/LSA-5), due to the increased amount of GA during the crosslinking; the porosity of the microspheres was decreased through amide linkage formation indicating the slower release of pyronaridine at GT/LSA-7. The % of cumulative release is incredibly fast and large at lower amount of GA (i.e., 0.4 mL), whereas the release is slower at higher amount of GA (i.e., 0.6 mL). The cumulative release is lesser when lower amount of GA was used possibly because at higher concentration of GA, polymeric chains of MSs becomes rigid, thus decreasing % of cumulative release of pyronaridine through the GT/LSA MSs. As expected, the release becomes slower at higher amount of GA, but becomes faster at lower amount of GA.

**VI.3.6. 2: Effect of gelatin content on cumulative release**

![Graph showing cumulative release of pyronaridine through GT/LSA MSs containing different amounts of polymer (GT)](image)

*Figure VI. 7:* Percentage cumulative release of pyronaridine through GT/LSA MSs containing different amounts of polymer (GT); GT/LSA-1 (2 g), GT/LSA-4 (3 g), GT/LSA-5 (4 g).
Effect of GT content on cumulative release of pyronaridine from drug loaded MSs was investigated and showed in figure. 7. GT rapidly dissolves in an aqueous atmosphere at body temperature, and exhibits uncontrolled, fast release kinetics of growth factors. Drug release rate from GT/LSAMSs can be determined by formulations (GT/LSA-1, GT/LSA-4 and GT/LSA-5) having various amount of GT. This can be explained on the basis of higher degree of swelling due to ionization of carboxylic groups in the polymeric networks of MSs. The release profile data revealed that cumulative release was increased with increasing of GT content in the formation of MSs. This may be attributed to the GT and LSA crosslinked with GA; both are having hydrophilic nature and interact through H-bonding.

VI.3.6.3. Effect of drug content on cumulative release

![Graph showing release profile](image)

Figure VI.8:Percentage cumulative release of pyronaridine through GT/LSA MSs containing different amounts of drug GT/LSA-1 (5 %), GT/LSA-8 (10%), and GT/LSA-9 (15 %).
The effect of drug content on drug loading and % cumulative release profile was showed in Figure VI.8. The pyronaridine loaded GT/LSAMSs at different amount of drug loading formulations were demonstrated in Table VI.1. Drug release rate from GT/LSAMSs can be determined by formulations (GT/LSA-1, GT/LSA-8 and GT/LSA-9) containing the highest amount of drug (15 %) displayed fast and higher release rates than those formulations containing a small amount of pyronaridine. An extended release was monitored for the formulation GT/LSA-1 containing lower amount of drug. In other words, with a lower amount of drug in GT/LSA-1 exhibits less than that of GT/LSA-8, and it is noticed that cumulative release was increased with increasing into the polymeric microspheres. This is due to the convenience of more free void spaces through which lesser number of drug molecules will transport.

**Table VI.1: Results of % of encapsulation efficiencies for different formulations.**

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Gelatin (gm)</th>
<th>LSA (gm)</th>
<th>GA (ml)</th>
<th>% Drug</th>
<th>% Encapsulation efficiency ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA-GT-1</td>
<td>2</td>
<td>0.3</td>
<td>0.4</td>
<td>5</td>
<td>54.5 ± 0.7</td>
</tr>
<tr>
<td>LSA-GT-2</td>
<td>2</td>
<td>0.4</td>
<td>0.4</td>
<td>5</td>
<td>52.1 ± 1.3</td>
</tr>
<tr>
<td>LSA-GT-3</td>
<td>2</td>
<td>0.5</td>
<td>0.4</td>
<td>5</td>
<td>51.6 ± 0.8</td>
</tr>
<tr>
<td>LSA-GT-4</td>
<td>3</td>
<td>0.3</td>
<td>0.4</td>
<td>5</td>
<td>53.8 ± 1.7</td>
</tr>
<tr>
<td>LSA-GT-5</td>
<td>4</td>
<td>0.3</td>
<td>0.4</td>
<td>5</td>
<td>54.7 ± 0.8</td>
</tr>
<tr>
<td>LSA-GT-6</td>
<td>2</td>
<td>0.3</td>
<td>0.5</td>
<td>5</td>
<td>48.5 ± 1.2</td>
</tr>
<tr>
<td>LSA-GT-7</td>
<td>2</td>
<td>0.3</td>
<td>0.6</td>
<td>5</td>
<td>47.4 ± 0.1</td>
</tr>
<tr>
<td>LSA-GT-8</td>
<td>2</td>
<td>0.3</td>
<td>0.4</td>
<td>10</td>
<td>58.6 ± 0.4</td>
</tr>
<tr>
<td>LSA-GT-9</td>
<td>2</td>
<td>0.3</td>
<td>0.4</td>
<td>15</td>
<td>61.09±1.0</td>
</tr>
</tbody>
</table>

SD-standard deviation, calculated 95% accurately.

**VI.3.7. Drug release kinetics**

Drug release kinetics was analysed by plotting the cumulative release *versus* time and by fitting these data to the exponential equation [45].
Here, $M_t/M_\infty$ represents the fractional drug released at time $t$, $k$ is a constant characteristic of the pyronaridine-polymer matrix, and $n$ is an empirical consideration characterizing the release mechanism, it was the slope of the plot of log (t) versus In ($M_t/M_\infty$). Using the least squares procedure, we have to estimate the values of $n$ and $k$ for all the nine formulations at 37 °C and these values are given in Table 1. If $n$ value is 0.5 represents that Fickian diffusion is anomalous or non-Fickian type drug diffusion occurs (case I release). Otherwise if $n > 0.5$, an anomalous or non-Fickian type drug diffusion occurs. If $n = 1$, it is completely non-Fickian or more commonly called case II release kinetics is operative. The middle values ranging between 0.5-1.0 are attributed to the anomalous type release.

The in vitro release mechanism was depending upon of $k$ and $n$, and had shown a dependence on the amount of crosslinking, drug loading percentage and as well as polymer content of the matrix. Values of $n$ for microspheres are prepared by variable amount of gelatin in the polymeric microspheres 2 gms, 3 gms and 4 gms respectively by keeping crosslinker (GA= 0.4 ml) and drug content pyronaridine (5%) constant, ranging between 0.662 to 0.719 leading to a release of non Fickian or anomalous type. The pyronaridine loaded microspheres have the $n$ values in between 0.633 to 0.719 table VI. 2., signifies that the released pattern was non Fickian or anomalous type throughout the experiment for all formulations. This could be probably due to a reduction in the regions of low micro viscosity and closure of micro cavities in the swollen state of the microspheres. Comparable results have been observed elsewhere, in which the effect of different polymer, monomer volumes on dissolution kinetics was studied. And also dictated the values of $k$ are quite different for the pyronaridine-loaded microspheres, suggesting their lesser interactions compared to microspheres containing varying amounts of gelatin and LSA.

VI.3.8. Conclusions

This paper described the possibility of the preparation of novel biopolymeric MSs. The advantage of the present study lies in using of LSA as cheap and low cost material. It was also seen that LSA so obtained could be successfully used for preparation of biopolymeric MSs. An
anti-malarial drug pyronaridine loaded GT/LSAMs were prepared by using GA as a crosslinker; X-RD studies confirmed the molecular level dispersion of drug in the MSs. SEM pictures have shown the good compatibility of GT and LSA compositions present in the MSs with smooth surface. The encapsulation efficiency was found to vary between 47.4 % and 61.09 % depending upon the blend composition, cross-linking and the amount of drug loading. In vitro release profile of pyronaridine implied decreased drug release rate with increased GA.

Table VI.2. Release kinetics parameters for different formulations at pH-7.4

<table>
<thead>
<tr>
<th>Sample code</th>
<th>% Encapsulation efficiency ± S.D.</th>
<th>n</th>
<th>k</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT/LSA-1</td>
<td>54.5 ± 0.7</td>
<td>0.662</td>
<td>0.045</td>
<td>0.9951</td>
</tr>
<tr>
<td>GT/LSA-2</td>
<td>52.1 ± 1.3</td>
<td>0.718</td>
<td>0.039</td>
<td>0.9979</td>
</tr>
<tr>
<td>GT/LSA-3</td>
<td>51.6 ± 0.8</td>
<td>0.684</td>
<td>0.164</td>
<td>0.9901</td>
</tr>
<tr>
<td>GT/LSA-4</td>
<td>53.8 ± 1.7</td>
<td>0.719</td>
<td>0.125</td>
<td>0.9983</td>
</tr>
<tr>
<td>GT/LSA-5</td>
<td>54.7 ± 0.7</td>
<td>0.714</td>
<td>0.091</td>
<td>0.9867</td>
</tr>
<tr>
<td>GT/LSA-6</td>
<td>48.5 ± 1.2</td>
<td>0.669</td>
<td>0.218</td>
<td>0.9862</td>
</tr>
<tr>
<td>GT/LSA-7</td>
<td>47.4 ± 0.1</td>
<td>0.683</td>
<td>0.180</td>
<td>0.9930</td>
</tr>
<tr>
<td>GT/LSA-8</td>
<td>58.6 ± 0.4</td>
<td>0.633</td>
<td>0.351</td>
<td>0.995</td>
</tr>
<tr>
<td>GT/LSA-9</td>
<td>61.09 ± 1.0</td>
<td>0.672</td>
<td>0.173</td>
<td>0.998</td>
</tr>
</tbody>
</table>

SD-standard deviation, calculated 95% accurately.
References


