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Development of Gelatin-Lignosulfonic acid Blend Microspheres for Controlled Release of an Anti-Malarial Drug (Pyronaridine)

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ABSTRACT

In the present work gelatin (GT) and lignosulfonic acid (LSA) blend 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 (FT-IR) spectroscopy, differential scanning calorimetric, X-ray diffraction (X-RD) and scanning electron microscopy (SEM). FT-IR 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 interpenetrating polymer network (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 percentage of GT/LSA microspheres. In vitro release studies indicated that the microspheres enhance the release rates of pyronaridine drug up to 10 h.

Key words: *Gelatin, Lignosulfonic acid, Interpenetrating polymer network microspheres, Pyronaridine and in-vitro release studies.*

1. INTRODUCTION

Synthetic polymers pose one of the greatest threats to ecology today. A possible solution to the problem is the prospect of biodegradable polymers. Biodegradable polymers offer the following advantages. Firstly, they are made from renewable resources and thus do not face the problem of exhaustion. Secondly, they are biodegradable, which implies that the polymer after use will ultimately turn into compost. Encapsulation of drug molecules 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 microcapsules, micro beads and microspheres, have been proposed and used in topical formulations as drug carrier vehicles. Microspheres can function as cell micro carriers [1-5], delivery vehicles for drugs [6-12], growth factors [13-18] and injectable scaffolds as well [19,20]. Microspheres gained a lot of attention among drug delivery systems. 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 1 μm to over 100 μm.

Gelatin (GT) is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the skin, boiled the crushed horn, hoof and bones, connective tissues and organs. Food-grade GT is produced mainly from two raw materials, i.e., beef skin and pig hides. GT is an animal protein unlike many other gelling agents used by the food industry. GT 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 (GA) or other bifunctional reactants have been used to produce insoluble biodegradable GT

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microspheres. GA is used as a cross-linking agent to obtain rigid microspheres. GA produces cross-linking between GT molecules and thus reduces the rate of drug release from the microspheres. GT microspheres are generally prepared by solvent evaporation method [24-26] and crosslinking method [27-32]. In the present study, we have prepared GT/lignosulfonic acid (LSA) microspheres by "desolvation" method using GA as crosslinker.

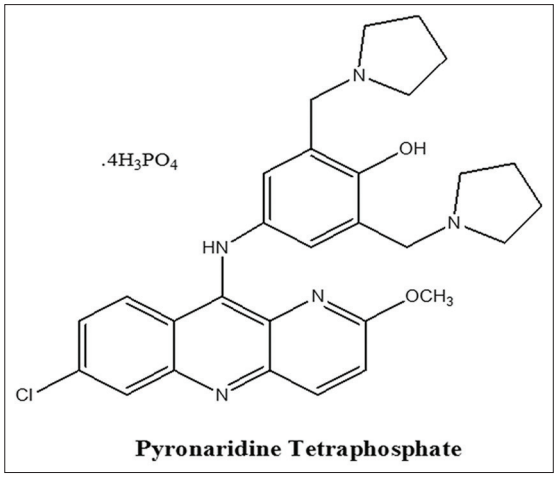
Lignin based polymers present two-fold advantage. One is that they are abundantly available as tons of lignin is thrown off as a waste product in pulp and paper industries. Secondly, lignin is completely biodegradable, though slowly. Thus, it is a prospective biodegradable polymer. Sodium lignosulfonates (LSA, 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 nanotechnological 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.

2. MATERIALS AND METHODS

2.1. Materials

Analytical Reagent grade sample of GT is purchased from Fisher Scientific Mumbai, India; LSA and pyronaridine tetraphosphate from Sigma Aldrich, USA; Acetone was purchased from Merck Specialties Private Limited, Mumbai, India; GA (25% aqueous solution) was purchased from S.D. Fine-Chem Limited Mumbai, India; All Chemicals were used without further purification and double distilled water was used throughout the experiment.



Pyronaridine Tetraphosphate

2.2. GT/LSA Microspheres Desolvation Method

A total of 2.0 g of GT was dissolved in 20 mL of distilled water under gentle heating. First desolvation step is carried out by the addition of 20 mL acetone. After sedimentation of precipitated GT fraction for a certain time, the supernatant consisting of some desolvated GT as well as GT in solution has to be discarded. Now, the sediment is getting dissolved again by the addition of 20 mL water. *In situ*, GT microspheres are formed during the second desolvation step by dropwise addition of 30 mL acetone under constant stirring. After 10 min, 400 µL of GA (25%) is added to the reaction mixture to crosslink the GT microspheres. Finally, after stirring 30 min, 0.1 g of LSA is added to the above mixture and after 30 min LSA-co-GT microspheres were formed and redispersed in acetone/water (30/70).

2.3. Estimation of Drug Loading and Encapsulation Efficiency

The drug loaded microspheres (10 mg) were pulverized and incubated in 10 mL of 0.02 M phosphate buffer (pH=7.4) at room temperature for 24 h. The suspension was agitated with agitate mortar and filtered through the filter paper. The drug solution was assayed spectrophotometrically for pyronaridine content at the wavelength of 270 nm. The results of percentage of drug loading and encapsulation efficiency were calculated using following equations.

$$\%\ \text{Drug loading} = \left(\frac{\text{Amount of drug in microspheres}}{\text{Amount of microspheres}}\right)\times 100 \quad (2)$$

$$\%\ \text{Encapsulation efficiency} = \left(\frac{\text{Actual loading}}{\text{Theoretical loading}}\right)\times 100 \quad (3)$$

2.4. In-vitro Release Studies

Dissolution was carried out using tablet dissolution tester (Lab India, Mumbai, India) equipped with eight baskets. Dissolution rates were measured at 37±0.5°C

at constant speed of 100 rpm. Drug release from the microspheres was studied in 1.2 and 7.4 pH phosphate buffer solutions. At regular intervals of time, sample aliquots were withdrawn and analyzed using ultraviolet spectrophotometer (Lab India, Mumbai, India) at the fixed λ_{max} value of 204 nm. After each sample collection, the same amount of fresh release medium at the same temperature was added to the release medium to maintain the sink condition and values were plotted with standard deviation errors.

2.5. Fourier Transforms Infrared (FT-IR) Spectroscopy

Fourier transforms infrared spectroscopy (Perkin Elmer Spectrum Two, UK) analysis was performed to identify the chemical structure and to crosslink in GT-LSA matrix. Semi-interpenetrating polymer network (IPN) microspheres were finely ground with spectroscopic grade KBr to prepare pellets using a hydraulic pressure of 600 kg/cm^2 to scan the spectra between 4000 and 400 cm^{-1}.

2.6. X-ray Diffraction Studies (X-RD)

X-RD study helps to find the crystallinity of drug in the IPN microspheres. The X-RD measurements of plain drug, drug-loaded microspheres and plain microspheres were recorded with a Rigaku Geiger flex diffractometer (Tokyo, Japan) equipped with Ni-filled Cu K α radiation (λ=10518Å). The dried particulates of uniform thickness were mounted on a sample holder, and the patterns were recorded in the range 0 to 50°.

2.7. Scanning Electron Microscopy (SEM)

To determine the particle size and size distribution, drug loaded microspheres were taken on a glass slide and their sizes were measured using an optical microscope under regular polarized light. SEM images of microspheres were recorded using a JSM 6400 SEM (JEOL Ltd., Akishima, Tokyo, Japan) at ×50 and ×500 magnifications. Working distance of 8.5-9.5 mm was maintained and the acceleration voltage used was 10 kV, with a secondary electron image as a detector.

3. RESULTS AND DISCUSSIONS

3.1. FT-IR Spectroscopy Analysis

The copolymerization was confirmed by comparing the FT-IR spectrum of LSA with LSA-co-GT, which was polymerized with GT in presence of GA as a crosslinker. Figure 1a reveals the FT-IR spectrum of LSA; the peak at 3404 cm^{-1} is due to the –OH, N-H and –SO3H 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 and 1049 cm^{-1} attributed the vibrations of sulfonic group and the asymmetric C-H deformations of aromatic rings indicated at 1464 cm^{-1}.

FT-IR spectrum of GT Figure 1b 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 the presence of more amino groups in it. The peaks of free amino groups that are present in GT were disappeared in LSA/GT microspheres in Figure 1c. A new peak was observed in the Figure 1c for amide in the region 1530-1650 cm^{-1} that confirmed the formation of microspheres due to the reaction between sulfonic group of LSA and the amino group of GT crosslinked with GA.

Figure 2a-c revealed that FT-IR spectrum of pure drug, drug loaded LSA/GT microspheres and plain LSA/GT microspheres showed characteristic peaks. 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

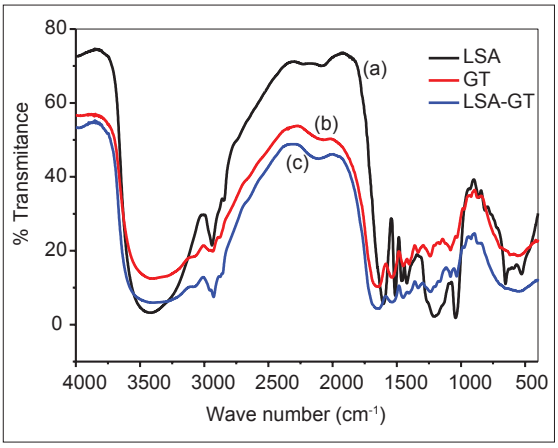


Figure 1: Fourier transforms-infrared spectra of (a) Lignosulfonic acid (LSA), (b) gelatin (GT), and (c) LSA/GT microspheres.

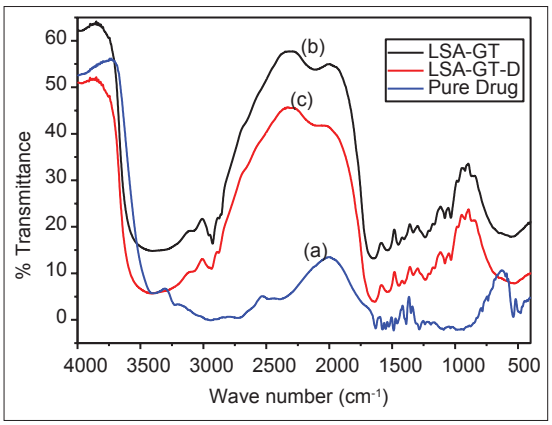


Figure 2: Fourier transforms-infrared spectra of (a) Pyronaridine, (b) lignosulfonic acid/gelatin (LSA/GT) microspheres, and (c) drug-loaded LSA/GT microspheres.

2926 cm^{-1} to 2936 cm^{-1} due to intramelecular vibrations of hydrogen bonding. These are evidences the intact nature of pyronaridine in LSA/GT microspheres.

3.2. X-RD Studies

X-RD analysis helps to find the crystallinity of drug in crosslinked interpenetrating polymer networks of LSA/GT microspheres. Dried and drug loaded microspheres of uniform size were mounted on a sample holder and X-RD patterns were recorded. Figure 3 show, (a) pure drug, (b) plain LSA/GT microspheres and (c) drug loaded LSA/GT microspheres. Pure drug pyronaridine shows high intense peak at 2θ=24°; and 11°, 18°, and 21° suggesting its crystalline nature, whereas these peaks are not observed in plain LSA/GT microspheres and drug loaded LSA/GT microspheres (Figure 2b and c). These evidences fulfilled that a strong interaction has occurred between drug and crosslinked LSA/GT microspheres which suggested the amorphous nature of drug present in the microspheres.

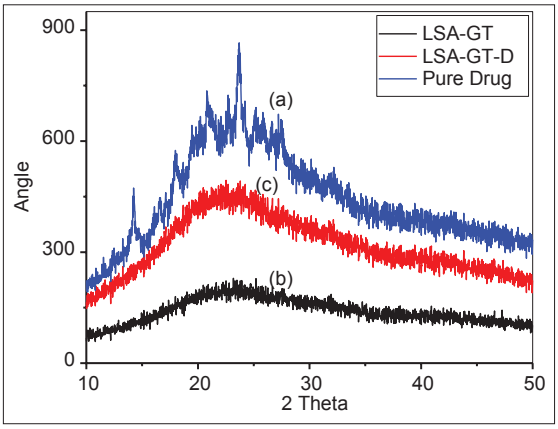


Figure 3: X-ray diffraction spectra of (a) Pyronaridine, (b) lignosulfonic acid/gelatin (LSA/GT) microspheres, and (c) drug-loaded LSA/GT microspheres.

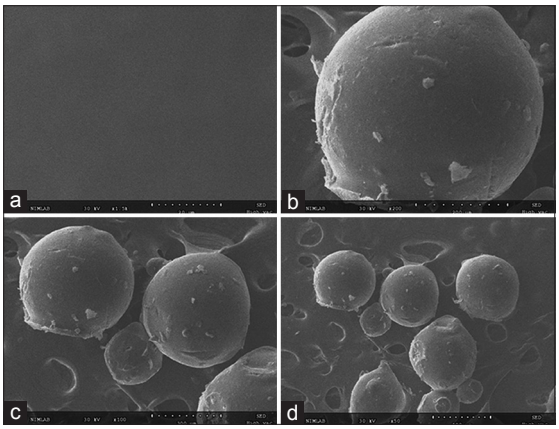


Figure 4: Scanning electron micrographs of LSA/GT microspheres for different magnifications (a-d).

3.3. SEM Studies

Figure 4a-d shows SEM micrographs of pyronaridine loaded crosslinked LSA-GT microspheres Figure 4a displays the average size of spheres is around 20 µm and Figure 4b and c shows average size of spheres are 130 and 160 µm respectively measured as different magnifications from SEM images. Microspheres of this study were almost spherical with smooth surfaces Figure 4d shows no phase separation and all formulations are almost spherical and spherical-shaped with smooth surfaces. Optical microscopies gave particle size of the micro beads for all formulations and the same results and are presented in Table 2.

3.4. Microscopic Images of LSA-GT Microspheres

Images of lignosulfonicacid/gelatin microspheres of different particle sizes were measured by optical microscope. These images are captured at different magnifications and different sizes for all formulations. All the formulation composition and codes are presented in Table 1 and their mean particle sizes [(µm) ±SD] are placed in Table 2. Figure 5 provides the optical images of LSA/GT microspheres for different magnifications.

3.5. Encapsulation Efficiency

Effects of polymer, monomer and crosslinker contents on encapsulation efficiency of drug loaded microspheres are given in Table 1. Encapsulation efficiency of pyronaridine increases with increasing amount of polymer and also monomer. This can be recognized that at higher concentrations, viscosities leading to a less diffuse matrix structure that obstruct the drug departure from the LSA/GT microspheres during the microsphere formation. GA also affects the encapsulation efficiency of pyronaridine into the microsphere. The enhancement of GA in the feed formation of LSA/GT microspheres decreased tendency in encapsulation efficiency. This is due to amplification in crosslinking density of LSA/GT microspheres 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 2. These values confirm that the microspheres do not modify the loading properties of gel based spheres.

3.6. In-vitro Release Studies

3.6.1. Effect of cross-linking agent on cumulative release

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 LSA/GT-5 (0.4 mL), LSA/GT-6 (0.5 mL) and LSA/GT-7 (0.6 mL) are presented in Figure 6. The cumulative release is

higher at lower amount of GA (LSA/GT-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 LSA/GT-7. The percentage 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 chain microspheres becomes rigid, thus decreasing percentage of cumulative release of pyronaridine through the LSA/GT microspheres. As expected, the release becomes slower at higher amount of GA, but becomes faster at lower amount of GA.

Table 1: Results of percentage of encapsulation efficiencies for different formulations.

Sample code	Gelatin (g)	LSA (g)	GA (mL)	% Drug	% Encapsulation efficiency±SD
LSA/GT-1	2	0.3	0.4	5	54.5±0.7
LSA/GT-2	2	0.4	0.4	5	52.1±1.3
LSA/GT-3	2	0.5	0.4	5	51.6±0.8
LSA/GT-4	3	0.3	0.4	5	53.8±1.7
LSA/GT-5	4	0.3	0.4	5	54.7±0.8
LSA/GT-6	2	0.3	0.5	5	48.5±1.2
LSA/GT-7	2	0.3	0.6	5	47.4±0.1
LSA/GT-8	2	0.3	0.4	10	58.6±0.4
LSA/GT-9	2	0.3	0.4	15	61.09±1.0

SD = Standard deviation, calculated 95% accurately.
LSA/GT = Lignosulfonic acid/gelatin; GA = Glutaraldehyde

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

Sample code	n	k	Correlation coefficient, r	Mean particle size (µm)±SD
LSA/GT-1	0.662	0.045	0.9951	124±5
LSA/GT-2	0.718	0.039	0.9979	136±4
LSA/GT-3	0.684	0.164	0.9901	167±9
LSA/GT-4	0.719	0.125	0.9983	133±4
LSA/GT-5	0.714	0.091	0.9867	145±6
LSA/GT-6	0.669	0.218	0.9862	124±8
LSA/GT-7	0.683	0.180	0.9930	134±5
LSA/GT-8	0.633	0.351	0.995	170±3
LSA/GT-9	0.672	0.173	0.998	133±9

SD = Standard deviation; LSA/GT = Lignosulfonic acid/gelatin; GA = Glutaraldehyde

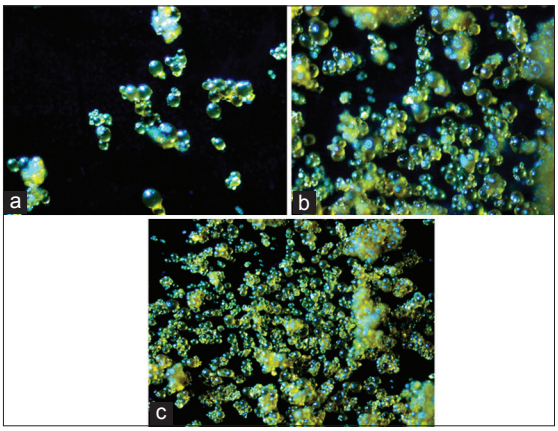


Figure 5: Optical microscopic images of lignosulfonic acid/gelatin microspheres of different particle sizes.

3.6.2. *Effect of GT content on cumulative release*

Effect of GT content on cumulative release of pyronaridine from drug loaded microspheres was investigated and shown 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 LSA/GT microspheres can be determined by formulations (LSA/GT-1, LSA/GT-4 and LSA/GT-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 microspheres. The release profile data revealed that the cumulative release was increased with increasing of GT content in the formation of microspheres. This may be attributed to the GT and LSA crosslinked with GA; both are having hydrophilic nature and interact through H-bonding.

3.6.3. *Effect of drug content on cumulative release*

The effect of drug content on drug loading and percentage of cumulative release profile was shown in Figure 8. The pyronaridine loaded LSA/GT microspheres at different amount of drug loading formulations were demonstrated in Table 1. Drug release rate from LSA/GT microspheres can be determined by formulations

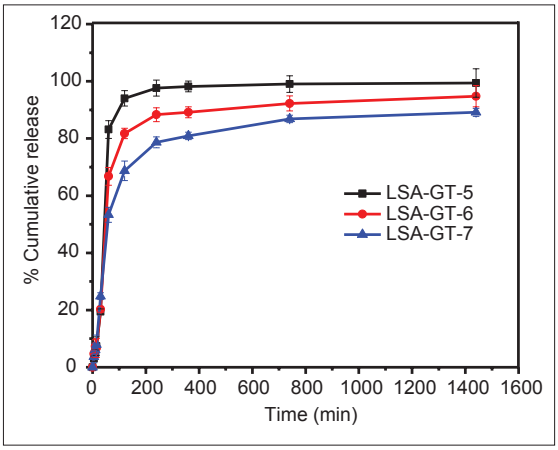


Figure 6: Effect of cross linker on cumulative release of pyronaridine; lignosulfonic acid/gelatin (LSA/GT) - 5 (0.4 mL), LSA/GT - 6 (0.5 mL), and LSA/GT - 7 (0.6 mL).

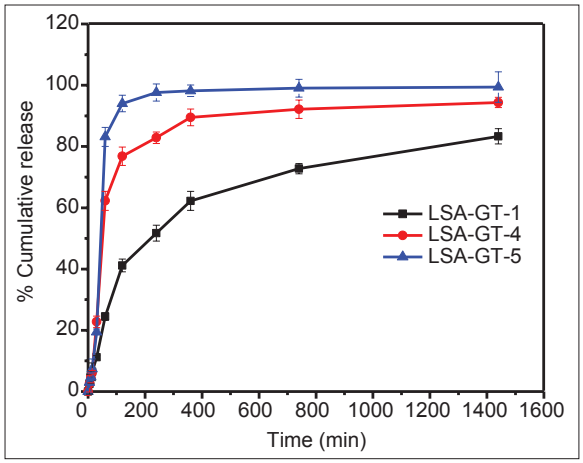


Figure 7: Percentage cumulative release of pyronaridine through lignosulfonic acid/gelatin (LSA/GT) microspheres containing different amounts of polymer (GT); LSA/GT - 1 (2 g), LSA/GT - 4 (3 g), LSA/GT - 5 (4 g).

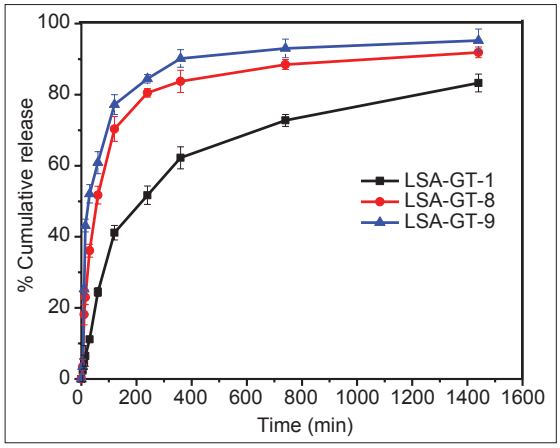


Figure 8: Percentage cumulative release of pyronaridine through lignosulfonic acid/gelatin (LSA/GT) microspheres containing different amounts of drug LSA/GT-1 (5%), LSA/GT - 8 (10%), LSA/GT - 9 (15%).

(LSA/GT-1, LSA/GT-8 and LSA/GT-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 LSA/GT-1 containing the lower amount of drug. In other words, with a lower amount of drug in LSA/GT-1 exhibits less than that of LSA/GT-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.

3.7. Drug Release Kinetics

Drug release kinetics was analyzed by plotting the cumulative release versus time and by fitting these data to an exponential equation [45].

$$M_t/M\alpha=kt^n \quad (4)$$

Here, M_t/M_∞ 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_∞). 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 and 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 GT in the polymeric microspheres 2 g, 3 g and 4 g respectively by keeping crosslinker (GA=0.4 mL) and drug content pyronaridine (5%) constant, ranging between 0.662 and 0.719 leading to a release of non-Fickian or anomalous type. The pyronaridine loaded microspheres have the n values in between 0.633 and 0.719 Table 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 microviscosity 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 GT and LSA.

4. CONCLUSIONS

In this paper, we had explored the possibility of the preparation of novel biopolymeric microspheres. And the novelty of the present study lies in using of lignosulfonic acid as chief and low cast material. It was also seen that LSA so obtained could be successfully used for preparation of biopolymeric microspheres. An anti-malarial drug pyronaridine loaded LSA-co-GT microspheres were prepared by using GA as a crosslinker; X-RD studies confirmed the molecular level dispersion of drug in the microspheres. SEM pictures have shown the good compatibility of GT and LSA compositions present in the microspheres 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.

5. ACKNOWLEDGEMENTS

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