WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 5, 1995-2009. Research Article ISSN 2277– 7105

BLEND MICROSPHERS OF SUCCINYL CHITOSAN AND GELATIN FOR THE CONTROLLED RELEASE OF 5-FLUOROURACIL

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Article Received on
16 March 2020,
Revised on 05 April 2020,
Accepted on 26 April 2020,
DOI: 10.20959/wjpr20205-17481

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ABSTRACT

The Present study involves the Preparation and in vitro evaluation of targeted microspheres containing 5- Fluorouracil for the treatment of cancer. They were formulated using N-succinyl chitosan (NSCS) blended with Gelatin (GE) to prepare microspheres by w/o emulsion cross-linking in the presence of glutaraldehyde (GA), which acted as a cross-linker. 5-Fluorouracil (5-FU) was encapsulated to investigate its controlled release (CR) characteristics in pH 7.4 buffer media. The microspheres were characterized by FT-IR, DSC and SEM. The microspheres were also evaluated as to encapsulation efficiency, swelling and in vitro drug release. The microspheres which formed were spherical in nature, with smooth surfaces, as concluded by the scanning electron microscopy (SEM). Fourier transform infrared spectroscopy (FTIR) confirmed the Succinylation of CS and the chemical stability of 5-FU in the formulations. Differential scanning calorimetry (DSC) confirmed the physical state and molecular level dispersion of 5-FU. Equilibrium swelling of microspheres was performed in water, in order to understand the water uptake properties.

KEYWORDS: Succinyl chitosan, gelatin, controlled release, 5-FU, blend microsphere.

INTRODUCTION

The aim of any Sustained drug delivery system is to provide a therapeutic amount of active substance to the target site in the body and maintain the desired drug concentration. After administration of a conventional dosage drugs freely move entire the body interacting not only with the target cells but also with the normal healthy cells which often results in toxic

effects. To obtain maximum therapeutic efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount in the right period of time thereby causing little toxicity and minimal side effects.[1-3] In such situations, controlled release (CR) formulations, prepared from biodegradable polymers, offer advantages over conventional dosage forms. Biopolymeric devices not only protect the drug from systemic metabolism but also shield the tissue from direct exposure of the drug. Such biocompatible CR formulations maintain the constant therapeutic concentration of a drug for a long period of time at the site of action, thus improving the drug efficacy.[4] Therefore, it is important to regulate the release of a drug by developing a formulation prepared by dispersing the drug in an inert polymer matrix.[5, 6] There are various systems, formulations prepared from polymeric hydrogels are known to be effective oral CR systems as these can be developed in the form of microspheres, in which a drug can be loaded using biocompatible and biodegradable polymers to offer optimal CR characteristics for controlling the release of a drug over an extended time.[7-9] Biopolymers that are the natural products of living organisms or plants are relatively inexpensive and capable of a multitude of chemical modifications.[10 -11]

Chitosan(CS), a natural linear polycationic, biocompatible, biodegradable and mucoadhesive polymer derived from chitin. CS is insoluble in water and most organic solvents, although it is soluble in aqueous dilute acid, and its poor solubility is a major limiting factor for developing its CR formulation. If a water-soluble CS derivative i.e N- Succinyl chitosan can be prepared by simple chemical reactions, then the resulting derivative could be a potential candidate for developing formulations.[12-13]

Gelatin is obtained from collagen and it is a biocompatible, biodegradable and nontoxic natural polymer. Gelatin is edible and soluble at body temperature, but undergoes gelation at temperatures just below ambient. Due to this it is used in the preparation of microspheres for the pharmaceutical investigations.[14-15]

5-Fluorouracil (5-FU) is an acidic, water soluble, hydrophilic drug and it is has been used for the treatment of different solid tumors types such as cancer of the stomach, liver, intestine and so on. However, like the other drugs which are used for chemotherapy, it affects the growth of normal body cells and often causes side effects, such as hair loss, fatigue, birth defects, mouth sores, and a temporary drop in bone marrow function. To avoid this kind of side effects, it is essential to develop CR devices for such drugs.

The aim of the study was to develop and evaluate N-Succinyl chitosan blended with gelatin microsphere loaded with 5-Fluorouracil for improved physicochemical properties of formulation to facilitate developed capsule dosage form and better patient compliance.

EXPERIMENTAL

MATERIALS AND METHOD

Chitosan (CS), Gelatin, 5-Fluorouracil (5-FU), monochoroaceticacid, analytical reagent grade glutaraldehyde (GA) solution 25% (v/v), n-hexane, and light liquid paraffin were all bought from s.d. Fine Chemicals, Maharashtra, India. Span-80 was purchased from Loba Chemicals, Maharashtra, India. Remaining reagents were used without further purification.

Synthesis of N- succinyl Chitosan (SCS)

N-succinyl chitosan was synthesized with slight modification following the already reported method.[16] In this method 0.5 g chitosan was suspended in 50mL aqueous solution of 5% acetic acid. This mixture was placed at hot plate at 50°C and stirred gently for 30 minutes. After that, dilution of the solution was done by adding 50mL methanol. Furthermore, 1.5 g succinic anhydride was dissolved in 40 mL acetone and added into the chitosan solution drop wise under stirring for the succinylation of chitosan. The stirring was continued at 50°C for 24 hours. After that, mixture was further diluted with excess 1M NaOH solution to raise the pH of reaction mixture and in order to obtain clear solution. The stirring of this clear solution was continued for further 3 hours at the same temperature. Afterward, ethanol was added to form precipitates followed by filtration to separate the precipitates. These precipitates were dispersed in ethanol for 24 hours. Then, dispersed precipitated product was washed with acetone and ethanol to remove impurities and unreacted reactants and dried using freeze-dryer in vacuum oven for 3 hours at 50°C. The obtained product was succinylated chitosan, which was characterized by using FTIR.The reaction is shown in **Figure: 1**.

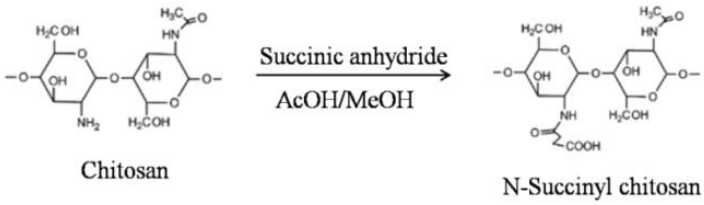

Fig: 1. Synthesis of N-Succunyl chitosan.

Preparation of Hydrogel Microspheres

Blend microspheres of SCS and GE were prepared by water-in-oil (w/o) emulsion cross-linking method, as suggested earlier.[13] A homogenous 20 mL of 14% (w/v) polymer solution was prepared in deionized water using GE and SCS, at room temperature. A required amount of 5-FU (5, 10, or 15 wt %) was dissolved in the polymer mixture, which was slowly added to 100 mL of 2% (w/v) paraffin oil, which was stirred using a stirrer for 10 min. The calculated amount of cross linker i.e., GA (2.5, 5 or 7.5 mL), was added to the above mixture and stirred continuously, until the microspheres were obtained. These were separated by filtration and washed with n-hexane to remove the oil, before finally being washed with 50 mL of 0.1 M glycine solution to mask the unreacted GA. The microspheres were air-dried at 40^0C for 24 h and stored.

Table: 1. The assigned codes for the different formulations are listed in.

Formulation Codes	% SCS in microspheres	% GE in microspheres	Amount of GA added (mL)	% 5-FU added	Encapsulation efficiency±SD (%)	% Equilibrium swelling
GE	0	100	5	5	78 ± 0.8	320
SCS-GE-1	25	75	5	5	70 ± 1.1	342
SCS-GE-2	50	50	5	5	66 ± 0.9	352
SCS-GE-3	75	25	5	5	60 ± 1.1	386
SCS	100	0	5	5	45 ± 0.8	402
SCS-GE-4	50	50	2.5	5	52 ± 1.1	390
SCS-GE-5	50	50	7.5	5	68 ± 0.9	320
SCS-GE-6	50	50	5	10	68 ± 0.6	352
SCS-GE-7	50	50	5	15	72 ± 0.6	398

CHARACTERIZATION OF THE PREPARED MICROSPHERES

Fourier transforms infrared spectroscopy (FT-IR)

FT-IR studies were carried out using a Schimadzu FTIR 8400S. The pellets were prepared by pressing the sample (2mg) with KBr. The positions of the FT-IR bands of important functional groups of the drug and polymer were identified and were crosschecked withthe FT-IR spectra of the drug loaded formulation.

Differential scanning calorimeter (DSC)

DSC thermograms of the pure drug and of the prepared microspheres were obtained using a Schimadzu thermal analyzer DSC-60, Japan, at a scanning rate of 10ºC/min, over the temperature range 30-300ºC in liquid nitrogen environment (flow rate10mL/min).

Scanning electron microscopy (SEM)

The morphology and surface characteristics of the prepared pH sensitive microspheres were determined using SEM (LEO, CFTRI Mysore). The hotographs were observed for the morphological characteristics of the microspheres.

Estimation of Drug Loading and Encapsulation Efficiency

The drug concentration in each formulation was estimated by the method previously reported[17] About 10 mg of the drug-loaded microspheres were placed in 10mL of buffer solution and stirred vigorously for 48 h to extract the drug from the microspheres. The solution was filtered and assayed by UV spectrophotometer (Lab India, Mumbai, India) at fixed λ max value of 266 nm. The results of % drug loading and encapsulation efficiency were calculated, respectively using **Eqn: 1 and 2**.

$$\% \text{ 5-FU loading} = \frac{Weight\ of\ 5{-}FU\ in\ microsphers}{Weight\ of\ microsphers} \text{ X } 100 \quad \text{----- (1)}$$

$$\% \text{ Encapsulation efficiency} = \frac{Actuval\ 5{-}FU\ loading}{Theoritical 5{-}FU\ loading} \text{ X } 100 \quad \text{----- (2)}$$

Equilibrium Swelling (ES) Study

The % equilibrium swelling of the different concentrations of cross-linker as well as three different drug loadings blend hydrogel microspheres was gravimetrically monitored in water at 37^0C. The microspheres were kept in water for 24 h to enable them to reach complete equilibrium. Excess liquid droplets were removed by tissue papers, and the swollen microspheres were weighed and dried in an oven at 40^0C for 5 h, until no change in the dry mass of the samples was observed. The weight % water uptake was calculated using **Eqn: 3**

$$\% \text{ Equilibrium swelling} = \frac{Wt.of\ Ms - Wt.of\ Md}{Wt.of\ Md} \text{ X } 100 \quad \text{..... (3)}$$

RESULTS AND DISCUSSION

FTIR Analysis

From the FTIR spectra of CS and SCS presented in **Fig: 2.** the CS shows O-H and N-H stretching frequency at 3411 cm^{-1} for CS, whereas the peaks at 1652 cm^{-1}, 1553 cm^{-1} and 1424 cm^{-1} correspond to C=O (amide-I), N-H (amide-II) and C-N (amide-III) stretching frequencies, respectively. The bridged-O stretching is observed at 1153 cm^{-1}, whereas C-O-C vibration frequency appeared at 1052 cm^{-1}. However, primary O-H group attached -CH_2OH shows the C-O stretching vibration at 1076 cm^{-1}. In the case of SCS, a new peak at 1717 cm^{-1} corresponding to ester C=O stretching vibrations confirms succinylation at the primary O-H

group of CS, as confirmed by a peak at 1076 cm^{-1}, whose intensity decreased compared to that of CS. Increase in the intensity of amide-I (C=O) and amide-III (C-N) peaks and a decrease in the intensity of amide-II (N-H) peak confirms the succinylation reaction on $–NH_2$ group of CS. Thus, the reactive primary O-H and $–NH_2$ groups of CS are the succinylated sites.

FTIR spectra of (A) 5 – FU, (B) 5 – FU loaded microspheres and (C) placebo microspheres presented in **Fig: 3.** confirm the chemical stability of 5 – FU in the hydrogel microspheres. In case of 5 – FU, a band at 3334 cm^{-1} is due to N–H stretching vibrations. Bands at 3068, 2932, 2887 and 2826 cm^{-1} are attributed to both aromatic and aliphatic C–H stretching vibrations. A peak at 1680 cm^{-1} represents C=O stretching vibrations, whereas the characteristic peak of pyridine is observed at 1624 cm^{-1}. The $-NO_2$ stretching vibrations are observed at 1530 cm^{-1} and N–H bending as well as C-N stretching vibrations are observed at 1497 cm^{-1} and 1227 cm^{-1}, respectively. In case of drug-loaded microspheres, along with the peaks corresponding to placebo microspheres, characteristic peaks of 5-FU at 1680 cm^{-1}, 1631 cm^{-1} and 1531 cm^{-1} are observed. Presence of unmodified functional groups of 5-FU in 5-FU-loaded microspheres indicates the chemical stability of -FU after encapsulation into the polymer matrix.[6]

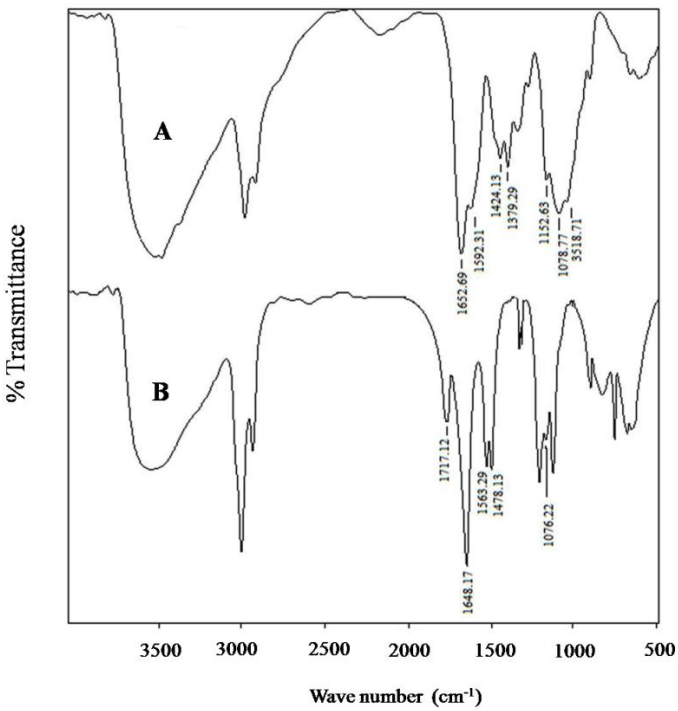

Fig: 2. FTIR spectra of chitosan (CS) and succinyl chitosan (SCS).

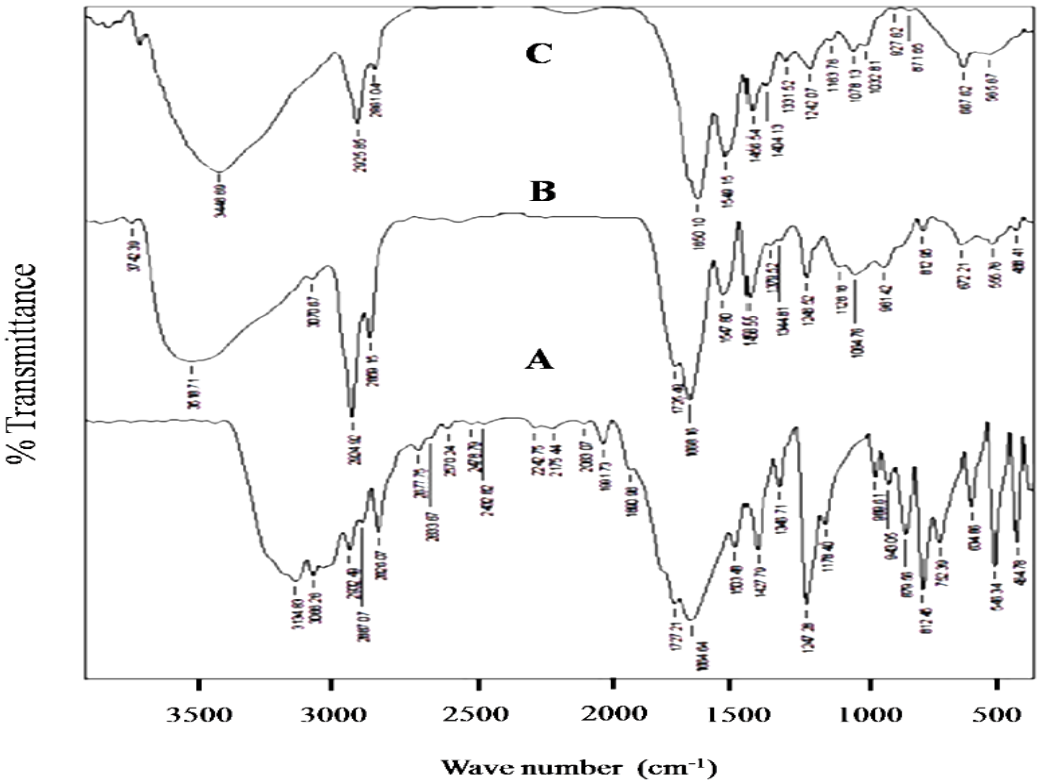

Fig: 3. FTIR spectra of (A) 5-FU, (B) 5-FU-laded blend microspheres, and (C) placebo blend hydrogel microspheres.

Differential Scanning Calorimetry (DSC)

The DSC thermograms of (A) placebo blend hydrogel microspheres, (B) 5-FU-loaded blend hydrogel microspheres, and (C) Pure 5-FU displayed in **Fig: 4,** show a sharp peak at 284 ^{0}C, which corresponds to its melting point. The blend microspheres underwent three endothermic transitions, and a broad endothermic peak at 104 ^{0}C is due to the loss of moisture the peak at 322 ^{0}C suggests the degradation of GE. The phase transition occurring at 211 ^{0}C might be due to interactions between the polymeric chains. The thermogram of 5-FU-loaded blend microspheres shows a new peak at 274 ^{0}C, along with those that are present in the thermograms of placebo blend microspheres, with a slight shift, this indicates the crystalline dispersion of 5-FU in the polymer matrix.

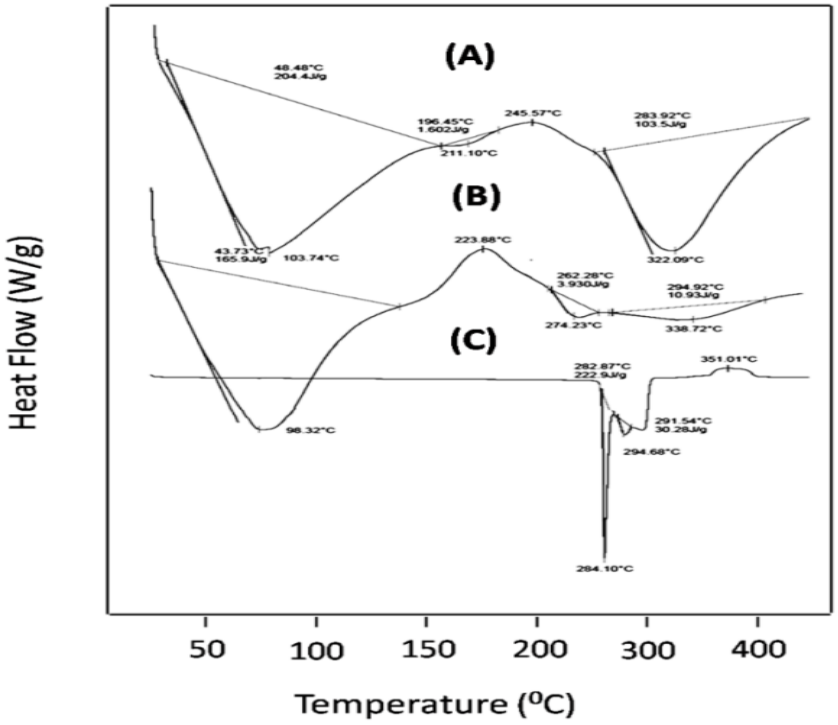

Fig: 4. DSC thermograms of (A) placebo blend hydrogel microspheres, (B) 5-FU-loaded blend hydrogel microspheres and (C) 5-FU.

Scanning electron microscopy

To assess the shape and surface morphology of the hydrogel microspheres, SEM images of 5-FU-loaded blend microspheres at 500X and 1000X magnifications shown in **Fig:** 5, confirm the spherical nature of the microspheres with smooth surfaces. The hydrophilic parts of the blend microspheres shrink a little due to the loss of water during the drying process. The average ranges of the particles are around 20 µm.

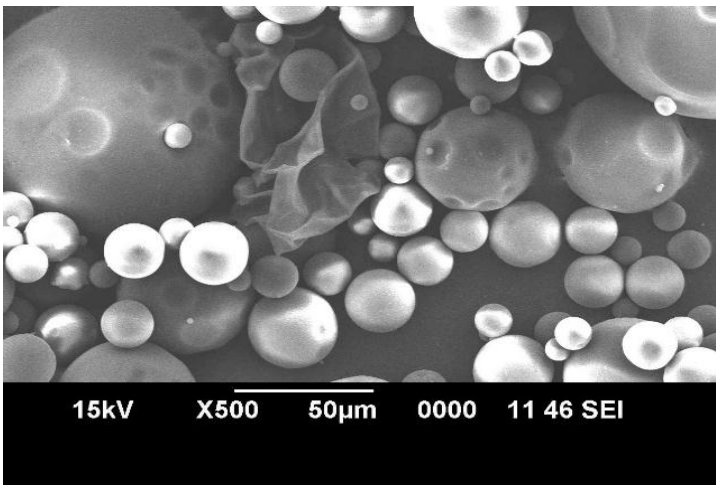

Fig: 5. SEM micrographs of 5-FU-loaded blend hydrogel microspheres.

Encapsulation Efficiency

Results of encapsulation efficiency for different formulations as a function of extents of drug lodging, cross linking and blend composition are including in **Table: 1**. The plain GE microspheres showed 78% EE, whereas plain SCS microspheres encapsulated only 45% of the 5-FU. In the case of blend hydrogel microspheres, the % EE values ranged from 60% to 70%, i.e., blend microspheres prepared with 25 (SCS-GE-2), 50 (SCS-GE-3), and 75% (SCS-GE-4) (w/w) of SCS, showed 70%, 66%, and 60% EE respectively. This is due to the presence of SCS in the matrix, since the presence of SCS results in a loose network formation, which fails to encapsulate more of the drug molecules during the formulation.

For microspheres cross linked with 2.5 (SCS-GE-4), 5 (SCS-GE-2), and 7.5 mL (SCS-GE-5) of GA, encapsulation efficiencies respectively, 52%, 66%, and 68%. This is because the cross-linker increased the cross-link density of the matrix, so that the matrix became rigid, reducing the leaching of 5-FU from the matrix. Microspheres loaded with 5 (SCS-GE-2), 10 (SCS-GE-6), and 15% (w/w) (SCS-GE-7) of 5-FU, have shown 66%, 68%, and 72% EE respectively. This indicate the higher EE values at a higher loading of 5-FU, due to more molecules can be entrapped in the matrix.

Equilibrium Swelling (ES) Study

In microspheres, extent of cross linking depends upon the amount of cross linking agent used. In the present study, different amounts of GA were added as the cross linking agent to the blend microspheres of CS-GA containing 5 wt. % of 5-FU and these data are also included in **Table: 1.** Extent of cross linking is dependent upon equilibrium swelling. For instance, % equilibrium swelling decreased from 390 to 320 with increasing amount of GA from 2.5 to 7.5 mL. This is due to increased crosslink density and decreased pore volume of the blend matrix with increasing amount of GA in the matrix. In the case of the blend formulation SCS-GE-1 containing 25% (w/w) SCS, the % ES is 342%, whereas for the formulation SCS-GE-3 that contains 75% (w/w) SCS, the % ES is 386%. On the other hand, the 50% (w/w) SCS - containing formulation (SCS-GE-2), showed an intermediately value (352%) of ES. Overall, the results of ES indicate that the formulations which contain a higher amount of SCS, exhibit a higher level of swelling than those containing a smaller amount of SCS this could be due to the hydrophilic nature of SCS. On the other hand, the formulation prepared.

***IN VITRO* RELEASE STUDY**

To understand the in vitro release profiles of the 5-FU-loaded blend hydrogel microspheres of SCS and GE, intestinal pH media are used to perform the release experiments. The cumulative % release vs. the time for the 5-FU-loaded microsphere formulation of variation of blend composition polymers, effect of Cross-linking agent and effect of % of drug loading.

Effect of Blend Composition

To understand the drug release behavior of SCS, GE and their blend hydrogel microspheres, *in vitro* release experiments were performed at 37°C in pH 7.4 conditions. The % cumulative release vs. time plots for 5FU-loaded SCS-GE-1, SCS-GE-2, and SCS-GE-3 microspheres are compared in **Fig: 6.** to investigate the effect of blend composition. The % cumulative release is higher for The pristine SCS microspheres released the entire encapsulated drug (100%) within 8 h, but plain GE microspheres released only 60% of 5-FU even after 12 h of dissolution.

In the case of blend formulations viz., SCS-GE-1, SCS-GE-2, and SCS-GE-3, containing 25%, 50%, and 75% (w/w) of SCS, released 72%, 84%, and 91% of 5-FU, respectively, suggesting that the blend hydrogel microspheres with a higher amount of SCS released the encapsulated drug faster, due to the hydrophilic nature of the SCS moiety of the blend. Since drug release from the hydrogel matrix depends on their swelling characteristics under pH conditions, the equilibrium swelling of SCS hydrogel microspheres is higher than that of GE hydrogel microspheres, but the blend hydrogel microspheres showed the intermediary values of swelling. Thus, increased water uptake capacity of the microspheres consequently increases the matrix swelling, which would influence the drug release characteristics of the hydrogel microspheres.

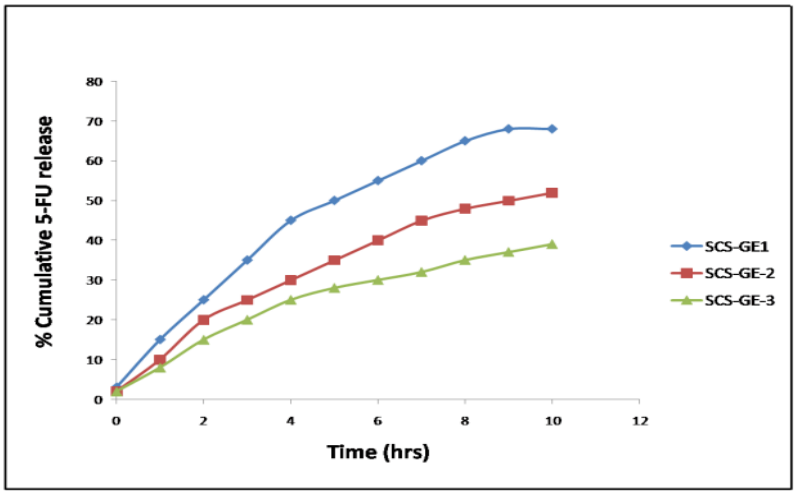

Fig: 6. Effect of blend composition on *in vitro* release of 5-FU from hydrogel microspheres in intestinal (pH 7.4) conditions at 37°C.

Effect of Cross-linking

The % cumulative release data vs. time plots with varying amounts of GA, i.e., 2.5 (SCS-GE-4), 5 (SCS-GE-2), and 7.5 mL (SCS-GE-5) at a fixed amount of 5-FU (5%) are displayed in **Fig: 7**. The % cumulative release is quite fast and large at lower amount of GA (i.e., 2.5 mL), whereas release is quite slower at higher amount of GA (i.e., 7.5 mL). Also, the formulation prepared with a higher amount of GA exhibits a lower water uptake capacity, indicating that high cross-linked matrices are more rigid. Therefore, the slow release of 5-FU from the rigid microspheres is due to the slow penetration of release media into the rigid matrix, due to hindered transport into the matrix.

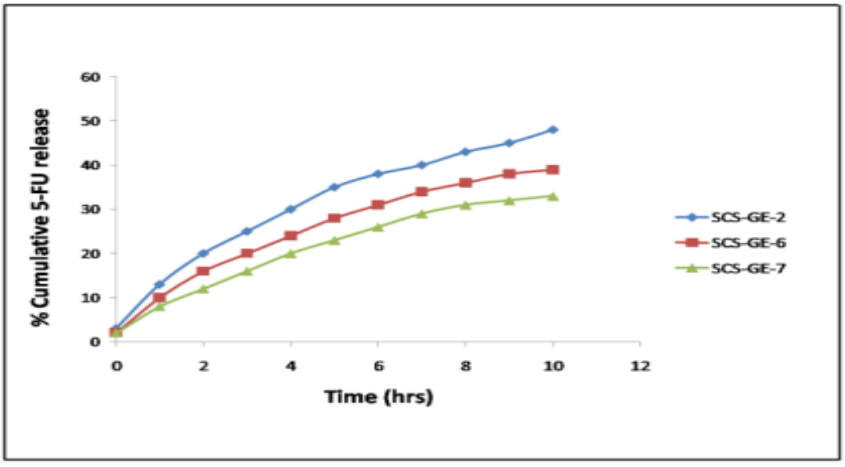

Fig: 7. Effect of cross-linking on *in vitro* release of 5-FU from hydrogel microspheres intestinal (pH 7.4) conditions at 37°C.

Effect of percent drug loading

Fig: 8. shows the effect of drug loading on in vitro release rates, we have used the formulations of SCS-GE-2, SCS-GE-6,SCES-GE-7 blend microspheres at different amount of drug loadings (i.e 5mg, 10mg and 15mg of 5-FU, respectively). Release data showed that formulations containing the highest amount of drug (15%) displayed fast and higher release rates than those formulations containing a small amount of drug (5%). A prolonged release was observed for the formulation containing lower amount of 5-FU. Due to the availability of more free void spaces through which lesser number of drug molecules will transport. Thus, at a higher amount of drug loading, due to the high concentration of the drug on the surface of the microspheres, a burst release occurs, producing a high % cumulative release.

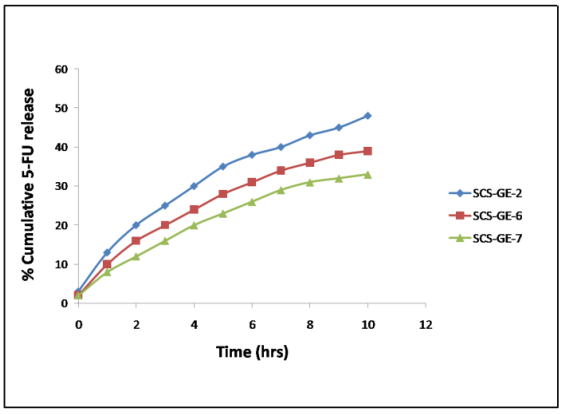

Fig: 8. Effect of Drug Concentration on *in vitro* drug release from SCS-GE-2, SCS-GE-6, SCS-GE-7 hydrogel microspheres at 37°C.

Drug release kinetics

Drug release kinetics is analyzed by plotting the cumulative release data *vs* time and by fitting these data to the exponential equations of the type.[18-20]

$(M_t / M_\infty) = kt^n$ --- (4)

Here, M_t / M_∞ represents the fractional drug release at time t; k is a constant characteristic of the drug-polymer system and 'n' is an empirical parameter characterizing the release mechanism. Using the least square procedure, the estimated values of 'n' and k for all the formulation are given in **Table: 2.** If n = 0.5, then drug diffuses and releases from the polymer matrix following a Fickian diffusion. For n>0.5, an anomalous or non-fickian type drug diffusion occurs. If n = 1, a completely nonFickian of case II release kinetics is operative. The intermediary values ranging between 0.5 and 1 are attributed to the anomalous type of transport. The values of k increased with increasing % of loading of 5-FU in the

microspheres, but 'n' values decreased with decrease in % of loading of 5-FU. This indicates that the interaction between the microspheres and drug are in similar lines studied from the release kinetics Eqn (4) proposed by Ritger and Peppas. The values of exponent 'n' ranges between 0.48 and 0.60 as calculated from empirical, which indicated that drug release followed by an anomalous nontransport occurs. The correlation coefficient values are in the range of 0.910 to 0.958, suggesting a good fit experimental release data**.**

Table 2: Release Kinetics Parameters of Different Formulations.

Formulation code	*k*	*n*	Correlation coefficient, *r*
GE	0.1359	0.60	0.990
SCS-GE-1	0.2346	062	0.995
SCS-GE-2	0.3113	065	0.991
SCS-GE-3	0.1547	056	0.993
SCS	0.1861	0.49	0.992
SCS-GE-4	0.1961	0.50	0.995
SCS-GE-5	0.1921	65	0.998
SCS-GE-6	0.8312	0.54	0.990
SCS-GE-7	0.2174	048	0.993

CONCLUSION

Succinyl chitosan was successfully synthesized and its blend hydrogel microspheres were prepared with Gelatin to investigate the CR of 5-FU, an anticancer drug. About 92% of 5-FU was encapsulated into the hydrogel microspheres prepared by emulsion cross-linking method using GA as a cross-linker. Succinylation of chitosan and chemical stability of 5-FU within the blend matrix was confirmed by FTIR. DSC analyses revealed the presence of 5-FU crystals within the blend microspheres. The microspheres were characterized for their surface morphology, shape and size using SEM. The % equilibrium swelling and *in vitro* release study performed in pH 7.4 buffer media showed a dependence on blend composition, concentration of cross-linking agent and variation of drug. *In vitro* release kinetics of 5-FU was described by empirical equations, which suggest that hydrogel microspheres released 5-FU in a swelling controlled manner, whereas all the other formulations followed the anomalous trend i.e., 5-FUrelease from the hydrogel microspheres followed swelling as well as diffusion controlled mechanisms.

ACKNOWLEDGEMENTS

The author would like thankful to UGC –south Eastern Regional Office, Hyderabad, for providing financial facilities for this Minor research project work. MRP -7087/16 (SERO/UGC) 18 MAR 2018.

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