Original Research Article

Development and in vitro characterization of 5-flurouracil-loaded, colon-targeted drug delivery system

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Abstract

Purpose: To prepare chondroitin sulphate–polyvinyl alcohol cross-linked microcapsules (miCAPs) for controlled delivery of 5-flurouracil (5-FU) in cancer patients.

Method: Nine different miCAP formulations were prepared using emulsion cross-linking procedure. The formulations were evaluated for their physicochemical properties, complex formation, stability at variable temperatures, safety, as well as drug-loading and drug-release characteristics. The effects of glutaraldehyde (GA), polymer concentration and stirring speed on 5-FU release at various pH were also assessed.

Results: One of the miCAP formulations (miCAP-1) was adjudged the most suitable based on its particle size, high drug loading (75.3 %, p = 0.034), and high entrapment efficiency (85.2 %, p = 0.031). Best-fit drug release model was Higuchi model based on regression coefficient value (R2) while drug release mechanism was Fickian.

Conclusion: Highly stable, crosslinked, amorphous and drug delivery system has been successfully developed. The delivery system is potentially suitable for acid-sensitive therapeutic moieties and where controlled release is desired.

Keywords: Emulsion cross-linking, Colon-specific delivery, 5-Flurouracil, Glutaraldehyde, Kinetic models

INTRODUCTION

Microcapsules (miCAPs) refer to tiny, non-toxic particles of size below 200 micrometers, which are used as high-capacity carriers for several drug molecules. They are compatible with biological systems, and are characterized by their efficient capacities for targeted drug delivery, thereby ensuring attainment of desired clinical outcomes [1].

Efforts aimed at development of miCAP-based targeted drug delivery have continued to engage the attention researchers. These studies usually focus more on polymers of natural origin, rather than their synthetic counterparts, due to the fact

that the former are non-toxic, non-immunogenic, bio-compatible, and easily biodegraded by human gut microflora [2].

Colon-based targeted systems have the advantage of consistently sustaining high therapeutic drug levels while protecting it from the low pH of gastro-intestinal tract. Natural polymers and polymers of synthetic origin have been employed in the design of colon-based targeted drug delivery applications. Other types of targeted drug delivery systems include osmotically-controlled devices, pro-drug systems, pH-dependent devices, and systems in which the carrier is degraded enzymatically[3,4].

Chondroitin sulphate, a natural, biocompatible and water-soluble polymer which consists of repeating units of D-glucuronic acid and *N*-acetyl galactosamine, is biodegradable by gram negative anaerobic bacteria in colonic segment of the large intestine [5]. It has been utilized in fabrication of a number of polymeric systems like hydrogels, miCAPs, microgels and nanogels. Many researchers have also utilized chondroitin sulphate in developing colon-targeted drug delivery systems [6]. Hydrolysis of polyvinyl acetate yields polyvinyl alcohol (PVA) which serves as a potential drug carrier for delayed-release drug delivery systems [7].

Intracellular metabolism of 5-FU converts it to its active form, fluoro-deoxyuridine which is employed in treatment of cancers of the pancreas, colon, breast and stomach. Due to its short *in –vivo* half-life (less than 30min) and variable bioavailability, 5-FU is clinically dispensed in oral dosage formulations [8]. To overcome the constraints of short half-life, 5-FU should be administered through an efficient, effective and sustained site-specific delivery, especially for etiologies of colonic origin. Site-specific delivery is of advantage in promoting drug availability where it is required, thereby eliminating the need for repeated administration of the drug while minimizing unwanted adverse effects [7]. Various formulations are used for controlled delivery of 5-FU. These include miCAPs of chondroitin sulphate/chitosan, and microspheres of poly (lactide-co-glycolide), sodium alginate and pectin [9,10].

In the present study, chondroitin sulphate was successfully grafted with PVA by using glutaraldehyde as a potential cross-linker for site-specific delivery of 5-FU. This approach reduced dosing frequency, side effects and cost of therapy associated with frequent dosing.

EXPERIMENTAL

Materials

The drug, 5-FU was a generous gift from Pharmedic Laboratories (Pvt) Ltd (Lahore, Pakistan). Chondroitin sulfate-A sodium, PVA, sodium metabisulphite, grade-1 GA solution and Tween-80 were products of Sigma Aldrich (Germany). Food-grade paraffin oil was purchased from a local market at Multan, Pakistan. Magnesium stearate was product of Fluka (Italy). All chemicals and reagents used were of analytical grade except paraffin oil.

Preparation of miCAPs

The miCAPs were prepared from PVA and chondroitin sulphate using a modified emulsion cross-linking method incorporating GA as cross-linker (Table 1). Glutaraldehyde (GA) was selected as cross-linker due to its low price, availability and high reactivity, i.e., it readily produces aldehyde groups as shown in Table 1. The exact quantity of chondroitin sulphate was taken and dissolved in distilled water (15mL) maintained at 60 °C on a magnetic hot plate (Stuart series 600, Germany) at 100 rpm until clear solution was formed. Exactly 200 mg of 5-FU was added to the clear solution of chondroitin sulphate. This was labelled solution A. Polyvinyl alcohol (PVA) was dispersed, with stirring, in 10 ml distilled water maintained at 60 °C. Stirring was continued for 30 min until a clear solution was formed. The PVA solution was labelled solution B. Solution A and solution B were mixed using a magnetic stirrer at 100 rpm to produce the aqueous phase.

The external phase was prepared by stirring a mixture of 100 mL paraffin oil, 5 % v/v tween-80 and 0.3 % w/v magnesium stearate at 600 rpm for 15 min. The aqueous phase was then added drop-wise, with continuous stirring, from a micropipette, over a period of 1 h. Stirring was thereafter continued for 30min, resulting in a water-in-oil emulsion. Finally, 1.5 ml of 25% GA solution containing 100 – 200 µl of concentrated sulphuric acid was added drop-wise, with continuous stirring for the next 2h, leading to acetal ring formation due to interaction of aldehyde groups with hydroxyl groups of PVA and CS. The resultant suspension was transferred into paraffin oil and retained for 1h. The supernatant portion was removed by decantation to reveal the formed miCAPs which were rinsed many times in acetone to eliminate oil traces, and then in sodium metabisulphite for complete removal of unreacted GA. The pure

miCAPs were vacuum-dried and kept in glass vials prior to further investigations [11].

Determination of 5- FU levels

The levels of 5-FU within each prepared miCAP were determined spectrophotometrically at 266nm, coupled with standard regression as outlined previously. Each miCAP formulation was stirred for 5min at 200 rpm in 50 mL phosphate buffer, pH 7.4 at 60 $^{\circ}$C, and thereafter allowed to stand for 24h before filtration. After dilution, the absorbance of the filtrate was read at 266nm. The concentration of 5-FU was then determined through a standard regression procedure [12].

Table 1: Composition of chondroitin sulphate-grafted PVA microcapsules

Formulation code	5-Fu (mg)	Chondroitin sulphate (g)	Polyvinyl Alcohol (g)	Glutaraldehyde (ml)
CP-1	200	0.1	0.1	1.5
CP-2	200	0.7	0.3	1.5
CP-3	200	0.8	0.2	1.5
CP-4	200	1	1	1.5
CP-5	200	1.4	0.7	1.5
CP-6	200	1.6	0.8	1.5
CP-7	200	1.5	1.5	1.5
CP-8	200	2.1	0.9	1.5
CP-9	200	2.4	0.6	1.5

Evaluation of drug-loading and encapsulation efficiency

Encapsulation efficiency, which is an index of percentage of drug loaded, was determined. Extraction method was used to calculate entrapment efficiency and product yield. Prepared microcapsules were poured into phosphate buffer (50 ml) to facilitate 5-FU release. 5-FU content was determined spectrophotometrically at 266 nm. Encapsulation efficiency and product yield as previously described [13].

Determination of micromeritic properties

All prepared formulations were subjected to micromeritic evaluation. The indices determined included bulk density, angle of repose, tapped density, Hausner ratio and Carr's compressibility index[19].

Angle of repose

Angle of repose (Θ) was calculated by the funnel method as in Eq 1. [14]:

$$Tan\theta = \frac{h}{r} \quad \text{.................. (1)}$$

where h = height of cone and r = radius of cone. Angle of repose < 30° indicates free flow of powder blend.

Bulk density

Powder blend was transferred into a measuring cylinder and initial volume was noted. After this weight of the powder was determined and bulk density (ρb) was calculated by using following equation [14]:

$$Bulk\ density\ (\rho b) = \frac{M}{vb} \quad \text{................. (2)}$$

Tapped density

Measuring cylinder containing powder mixture was subjected to tapping for specified number of tapings. Tapped volume (V_t) was noted and tapped density was calculated using Eq 2 [14].

$$Tapped\ density\ (\rho t) = \frac{M}{vt} \quad \text{................. (2)}$$

Carr's compressibility index

Compressibility index (I) of the powder blend was calculated as in Eq 3 [14].

$$(I) = \frac{vt - vb}{vb} \ x\ 100 \quad \text{.................. (3)}$$

Where, Vb and Vt are bulk and tapped volume respectively. Carr's index value between 13 -19 % confirms good flow and if it is more than 21 % it presents poor flow of powder.

Hausner ratio

It was computed as the ratio of tapped density to bulk density [14]. A value < 1.25 is an indication of good flow of powder while > 1.25 indicates poor flow.

Fourier transform infrared spectroscopy (FTIR)

Drug-polymer interaction was investigated by ATR-Fourier transform infrared spectroscopy (Tensor 27). The IR spectra were obtained for 5-FU, chondroitin sulphate and PVA; and for physical mixtures of drug and polymers, unloaded chondroitin sulphate-grafted PVA miCAPs, and the 5-FUmiCAPs using ATR-FTIR (Tensor 27). The range of wavelengths used for the scan was 500 − 4000 cm^{-1} at a resolution of 4 cm^{-1} [15].

GC-MS analysis

Gas chromatographic system (Chemitto GC8610) consisting of 30m capillary helium gas column (db-5) with long narrow bore, was used to estimate GA residues in the miCAPs. The flow rate of the gas was kept at 1ml/min, and the sample size injected was 1 µl. Pure GA solution (1 µl) was injected into the column, and the chromatogram was recorded.

Each MiCAP (1g) was accurately weighed, poured in 25ml of water and shaken mechanically for 24h in a screw capped vial on thermos-shaker incubator about 37°C. The supernatant was collected using insulin syringe after centrifugation at 6000rpm for 10min and it was filtered through 0.45 µm syringe filter (Sartorius). After appropriate dilution, each sample was injected into chromatographic system for estimation of GA content [16].

Thermal characterization of miCAPs and raw materials

Chondroitin sulphate, 5-FU, polymer-drug mixtures, unloaded miCAPs and PVA were analyzed for their phase transition characteristics using TA device. Prior to sample application, the pans were subjected to tarring. With the reference pan empty, the sample pan was half-filled, and heat was applied to the furnace, raising the temperature to 450 °C at a flow rate of 15°C/min in a nitrogen medium [15]. The temperature at which melting of the raw materials and miCAPs occurred as well as the temperature for entire combustion was calculated to assess the stability of the miCAPs.

Assessment of effect of miCAP on crystallinity of 5-FU and raw materials

Powder x-ray diffraction XRD was employed to assess the effect of miCAP formation on 5-FU crystallinity, using X-ray diffractor (model; JDX 3532, JEOL, Japan). The powder x-ray diffraction patterns of raw materials and triturated miCAPs were obtained at 60 kV and 50 mA using CuKα radiation, with a scanning rate of 1°/min over a diffraction angle (2q) range of 10° to 40° [17].

Confirmation of elemental composition of miCAPs

The elementatal composition of the miCAPs was confirmed using energy dispersive spectroscopy (Inca 200, Oxford UK). This technique also serves to confirm sucessfull loading of active moeity within the carrier system [15].

Scanning electron microscopy (SEM)

The surface and internal morphologies of the miCAP formulations were determined by electron microscope scans. For external morphology, the scanning electron microscope (SEM) image of each powdered miCAP was captured in vacuum at 20kV after fixing it to a support and coating it with gold by means of a SPI sputter module.

In the assessment of internal morphology, the miCAPs were embedded in acrylate glue, dried and cut into slices which were placed on scanning stubs to obtain the internal SEM images [15].

Determination of particle sizes of miCAPs

Particle size analyzer (Zeta-sizer Nano-series ZEN3600, Malvern Instruments Ltd. with software DTS-nano, Kingdom) was used for the estimation of the particle sizes of the miCAPs [15].

Assessment of drug release *in vitro*

Drug release from the miCAPs was investigated by applying the paddle procedure which involves USP dissolution apparatus-II. Accurately weighed miCAPs (equivalent to 50 mg of 5-FU) was placed in a dialysis bag. The bag was attached to a paddle via a thread, and the ensemble was placed separately in 900mL of three media of different pH: HCl (pH 1.2), phosphate buffer (pH 6.8) and phosphate buffer (pH 7.4), each for 12h at a rotation speed of 100rpm.

Triplicate portions (3 mL each) of the medium (3 mL) were removed at specific time intervals and analyzed for drug levels by UV spectrophotometry at 266 nm after prior filtration using a micro-pore membrane. Each time a sample was taken, it was replaced with an equal volume of medium.

Release kinetic studies

In order to ascertain the underlying mechanism that governed the release of 5-FU from the miCAPS, different kinetic models were used to analyze the results from assessment of *in-vitro* drug release. The models tested zero order, first order, Higuchi and Korsmeyer-Peppas. Regression coefficient (r^2) values were calculated using DD solver Microsoft Excel adds in program, V 11.83, UK was utilized and regression coefficient (r^2) values were calculated [18].

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was carried out using SPSS 18[®]. Comparison among formulations was made by applying one–way analysis of variance (ANOVA). Values of $p < 0.05$ were taken as indicative of statistical significance.

RESULTS

Encapsulation efficiency

Values of encapsulation efficiency and drug loading were highest in miCAP-1 which contained 1% polymer and equal amounts of PVA and chondroitin sulphate, and decreased with increases in polymer level. When the polymer level was 1%, the diameter of the inner cavities of the miCAPs was 100 μm, and the cavity linings were porous. However, when the level of polymer was raised to 2 - 3 %, the resultant miCAPs had significantly ($p = 0.037$) decreased pore size, and the pore linings became rigid.

Rheological properties

The bulk and tapped densities of all miCAPs were computed and applied in the determination of Hausner ratio and Carr's index. The results obtained revealed that the compactness of the miCAPs was directly proportional to polymer content in all the formulations. The values of bulk density, tapped density, angle of repose, Carr's index and Hausner's ratio were in the ranges of 0.2– 0.3 g/ml, 0.3– 0.3 g/ml, 23.21 to 28.21°, 10.2 - 30.7% and 1.11 – 1.44, respectively. These results indicate that the miCAPs had good flow properties.

Effect of miCAP formation on the Fourier infrared spectra of 5-FU

The formation of miCAPs did not bring about any alterations in the Fourier infrared spectrum of 5-FU, which indicates absence of chemical reaction between the drug and the polymeric component (Figure1). Glutaraldehyde (GA) exposure leads to contact dermatitis, bronchial asthma, and eye irritation [24]. The chromatograms of pure GA and the prepared miCAPs were recorded and compared, to ensure absence of unreacted GA. Sharp intense peaks were present at different retention times while these peaks were completely absent in chromatogram of the miCAP formulations, indicating the absence of unreacted GA. This is in agreement with results obtained in previous studies in which the absence of unreacted GA was confirmed by gas chromatography [21,25].
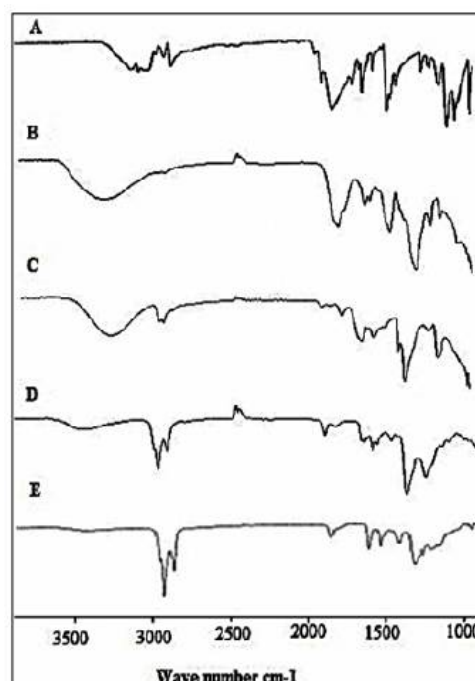

Figure 1: FTIR spectra (A) 5-FU (B) chondroitin sulphate (C) PVA (D) unloaded microcapsules (E) 5-FU-loaded microcapsules
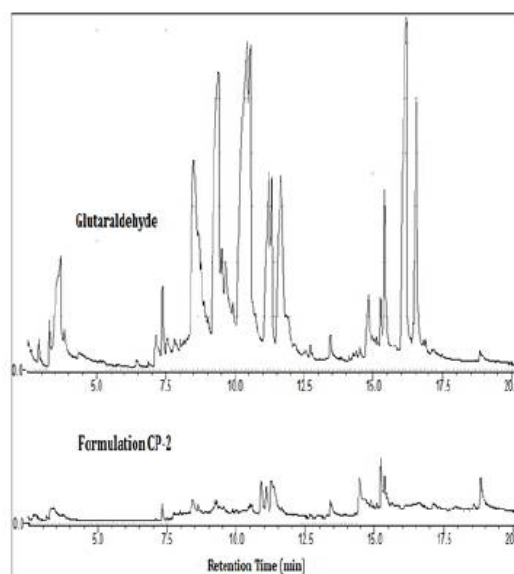

Figure 2: GC-MS chromatograms of glutaraldehyde and 5-FU formulation

Thermograms of 5-FU, chondroitin sulphate and 5-FU-loaded miCAPs

The thermogram of 5-FU showed a sharp endothermic peak at 281 °C which reflected its melting point, while another peak was seen at 200 °C which corresponded to the melting temperature of PVA. A broad peak was seen at 123.8 °C for chondroitin sulphate as a result of

its amorphous characteristics (Figure 3a and Figure 4a). Furthermore, drug-loaded miCAPs did not exhibit the characteristic peak of 5-FU, thereby indicating successful entrapment of the drug within the miCAPs. Absence of characteristic drug peaks after loading has also been reported in previous studies [19].
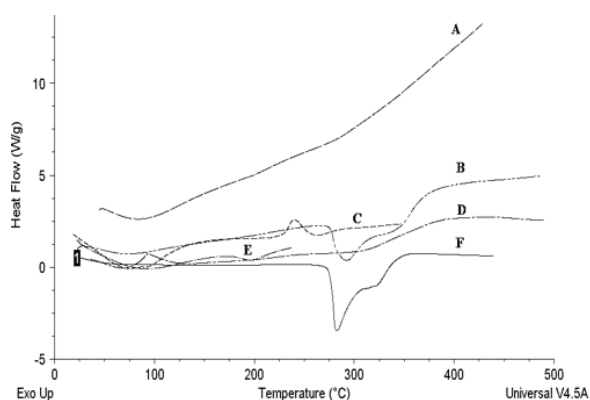

Figure 3: DSC thermograms of (A) unloaded microcapsules (B) physical mixture (C) Chondroitin sulphate (D) 5-FU loaded microcapsules (E) PVA (F) 5-FU
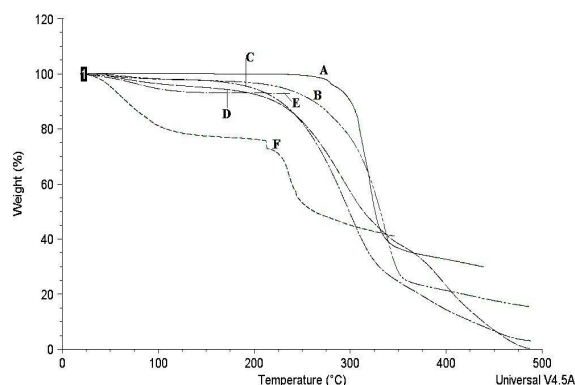

Figure 4: TGA curves of (A) 5-FU (B) physical mixture (C) 5-FU loaded microcapsules (D) Unloaded microcapsules (E) PVA(F) Chondroitin sulphate

PXRD patterns of 5-FU, PVA, chondroitin sulphate and 5-FU-loaded miCAPs

The PXRD patterns of 5-FU, PVA, chondroitin sulphate, PVA, physical mixture, unloaded miCAPs and 5-FU-loaded miCAPs are shown in Figure 5. Sharp and intense peaks were produced by 5-FU at 2θ ranging from 15.9° to 32.1°, which revealed that its structure was crystalline. This was in contrast to the broad peak exhibited by the unloaded miCAPs at 2θ ranging from 10° to 25°, which revealed that the PVA complex was less crystalline in nature.
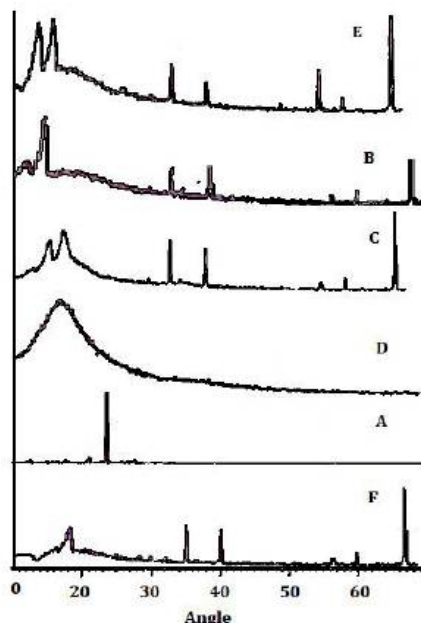

Figure 5: XRD diffractograms (A) 5-FU (B) chondroitin sulphate (C) PVA (D) unloaded microcapsules (E) 5-FU loaded microcapsules (F) Physical mixture

Elemental composition

EDX spectra showing the elements present in 5-FU, unloaded and 5-FU-loaded miCAPs are presented in Figure 6. The carbon contents of 5-FU, unloaded miCAPs and loaded miCAPs were 73.29, 62.49 and 68.21, respectively, while their oxygen compositions were 45.45, 23.20 and 41.82, respectively.
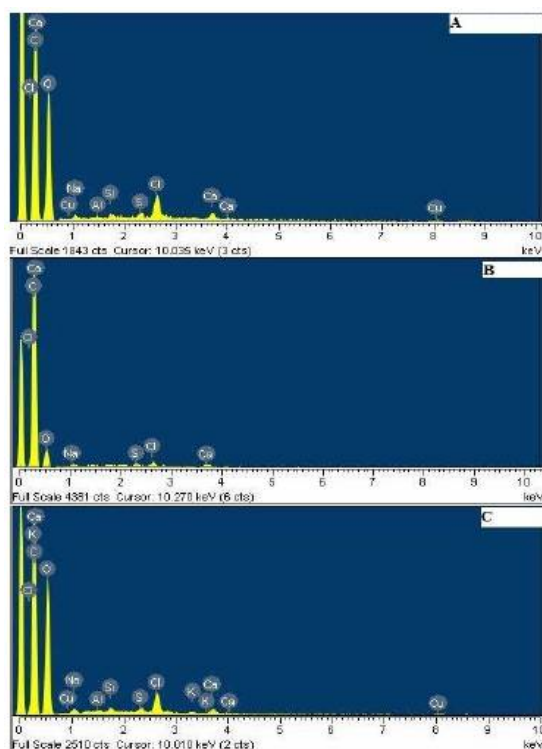

Figure 6: Elemental -ray profile of (A) 5-FU (B) unloaded microcapsules (C) 5-FU loaded miCAPs

Morphology of loaded and unloaded miCAPs

The unloaded miCAPs had smooth surfaces and spherical morphologies; while the 5-FU-loaded miCAPs had slightly rough surfaces (Figure 7).A rough surface facilitates penetration of solvent and drug diffusion from miCAPs [15,20]. It was evident that miCAPs containing 1 % polymer (Figure 7, P1) were more spherical in shape, and exhibited much smoother surfaces and more spherical shapes than those containing 2 – 3 % polymer components (Figure 7, P2 and P3).

Spherical, smooth, non-porous surface and rough miCAPs were obtained due to effects of polymer concentration, stirring speed, stirring time and GA concentration. Microcapsules with 1% polymer load exhibited thin-walled, smooth surfaces and larger inner cavity cross sections (Figure 7, P1 and C1). On the other hand, miCAPs with 2 and 3 % polymeric loads had thick-walled, rigid cracked surfaces, and reduced inner cavities due to coagulation of polymer and rapid solidification. Stirring speeds below 500rpm produced agglomerates (Figure 7, S1) having irregular morphologies, while stirring speedshigher than 600 rpm produced rigid miCAPs with compact surfaces (Figure 7, S2). Smooth-surface, spherical and regular miCAPs were generated at stirring speed of 600 rpm (Figure 7, S3).
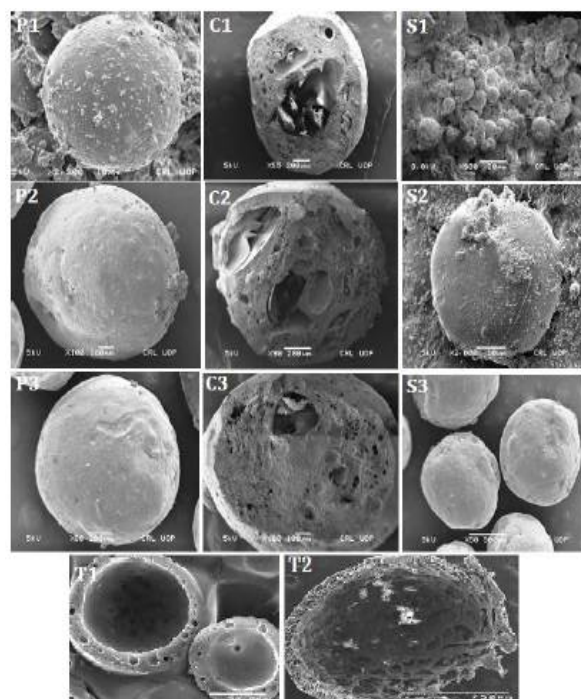

Figure 7: SEM micrographs **(P1, P2, P3)** Microcapsules having 1, 2 and 3 % polymer load: **(C1, C2, C3).**Different cavity size with respect to polymer load: **(S1, S2, S3);** microcapsules prepared at 500 and 600rpm, and spherical particles: **(T1, T2)** Thickness of microcapsules

Increases in polymer concentrations resulted in higher sizes of particles (Table 2).

Table 2: Particle size, drug loading and entrapment efficiency of the 5-FU microcapsules (mean ± SD, n = 3)

Formula tion code	Mean particle size(μm)	Drug loading (%)	Entrapme nt efficiency (%)
CP-1	30.44±2.499	75.31±1.13	85.21±0.14
CP-2	30.00±1.025	73.20±0.739	84.08±0.78
CP-3	35.21±1.559	70.92±1.108	80.12±0.610
CP-4	60.76±2.055	65.05±0.652	72.93±1.05
CP-5	58.77±1.615	69.88±0.762	75.12±0.932
CP-6	78.60±1.025	66.83±1.021	70.14±0.996
CP-7	90.77±1.115	64.11±1.137	66.21±1.06
CP-8	100.01±2.00	62.38±0.739	68.01±1.48
CP-9	140.08±2.27	60.32±1.108	65.11±1.55

Drug release from miCAP formulations

The amount of 5-FU released from all formulations was below 20 % at pH 1.2, but at pH 7, the release of 5-FU reached a maximum level of 82 % (Figure 8).
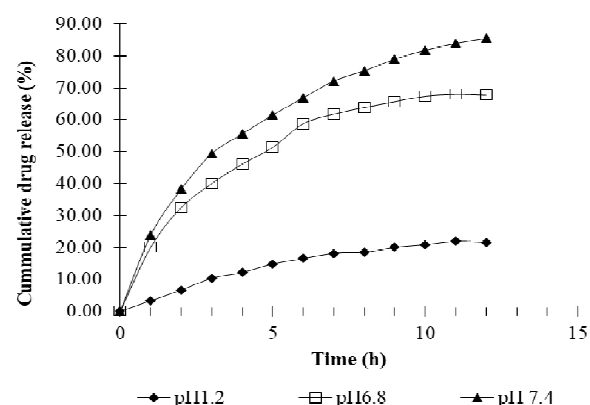

Figure 8: Release profiles of 5-FU from CP-1 at different pH

When the drug release data were subjected to kinetic analysis, it was found that in zero order kinetics, drug release rate was constant and was not a function of drug concentration. On the other hand, drug release rate in first order varied with the concentration of 5-FU, while in the Higuchi model, it varied with $\sqrt{time}$. Korsmeyer-Peppas is used to determine the mechanism involved in the release of drugs from systems. The most suitable model was selected on the basis of best

fit of data. Values of regression coefficient (r^2) and release constant (k) were determined for the various models. It was revealed that drug release from PVA/chondroitin sulphate miCAPs was consistent with the Higuchi model, as was evident in the relatively high r^2 value (Table 3). This shows that the release of drug from these miCAPs was diffusion-dependent. This conclusion was confirmed from the 'n' value range of 0.27 – 0.5 obtained from Korsmeyer–Peppas equation by fitting data from 90% drug release.

Table 3: Release kinetic data for 5-FU formulations

Formulation code	Zero order	First order	Higuchi	Korsemeyer-Peppas	
	r^2	r^2	r^2	r^2	n
CP- 1	0.8826	0.9163	0.9919	0.9600	0.451
CP- 2	0.8778	0.9124	0.9965	0.9646	0.416
CP- 3	0.8459	0.9018	0.9809	0.9638	0.425
CP-4	0.8815	0.9155	0.9900	0959 5	0.406
CP-5	0.8131	0.9030	0.9711	0.9468	0.442
CP-6	0.8060	0.9004	0.9826	0.9489	0.410
CP-7	0.8111	0.9105	0.9818	0.9471	0.402
CP-8	0.8975	0.9031	0.9932	0.9516	0.425
CP-9	0.8367	0.9181	0.9757	0.9494	0.471

DISCUSSION

Decreases in entrapment efficiency and drug loading have been attributed to increases in thickness of wall material and reduction of the diameter of inner miCAP cavity. In the present study, there were reductions in inner cavity diameter with increasing polymer load. This is in agreement with the findings of Huang *et al,* who demonstrated that 5-FU release in miCAPs formulated from chondroitin sulphate and chitosan was decreased by increasing polymer concentration [21].

Flow properties are important markers for granules, and they influence the flowability and compactness of powders. All the formulations had excellent flowability. Differential Scanning Calorimetery and Thermal Gravimetric analysis are very useful tools in the investigation of the thermal properties of miCAPs, and they provide useful information regarding drug-polymer interaction. The presence of an identical thermogram for 5-FU in the physical mixture is

proof that there was no reaction between 5-FU and the polymer. The cross-linked polymeric complexes (miCAPs) exhibited increased thermal stability as demonstrated by decomposition temperature which was higher than 300 °C.

The nature of 5-FU miCAPs (crystalline or amorphous) was confirmed from PXRD studies. The PXRD pattern of the 5-FU loaded miCAPs did not show any characteristic peak, which is proof of a change from crystalline nature to amorphous nature post-encapsulation. The absence of the characteristic drug peaks after loading into miCAPs has also been reported in previous studies [27,28]. The carbon and oxygen contents of the 5-FU-loaded miCAPs were increased due to successful drug entrapment. Similar reductions in carbon and oxygen levels have been reported by Rodzinski *et al* [22].

Increases in polymer levels resulted in higher medium and increased particle size at constant stirring speed: smaller fine droplets were not produced even at increased polymer load due to high viscosity of medium. Similarly, Motlekar *et al* observed increases in miCAP particle sizes, which were attributed to accentuated viscosity [23].

The lower release of 5-FU at pH 1.2 could be explained by the fact that at acidic pH, cross-linking hinders free access of water to the polymeric network, resulting in reduced water diffusion within the miCAP, and hence low polymer chain mobility. Drug release was maximal at pH 7.4 due to the fact that the acetal linkage of GA undergoes hydrolysis at alkaline pH, so that 5-FU diffuses more readily and the dissolution media penetrates more freely. This finding is in agreement with reports in previous studies [25]. The results from kinetic modeling of drug release studies are consistent with those reported by Ciftci *et al* [24].

CONCLUSION

Thermally-stable 5-FU-loadedmiCAPs have been successfully prepared by a slightly-modified chemical emulsion cross-linking method. This approach may also be feasible for formulation of targeted delivery systems for other chemotherapeutic agents.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest associated with this work.

Contributions of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dr. Asif Mahmood and Dr. Rai Muhammad Sarfraz proposed this work and prepared draft. Dr. Hina Raza and Zermina Rashid had conducted all experimental work. Shazia Ghuman has read and approved the draft. Dr. Farooq Azam did the interpretation of results. Mehvish Ansari and Rahat Shamim have supported in characterization of prepared product.

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