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ORIGINAL ARTICLE

Evaluation of Surfactants-Assisted Folic Acid-Loaded Pectin Submicrospheres: Characterization and Hemocompatibility Assay

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Abstract Folic acid is used for preventing and treating multiple diseases and disorders, administered in the form of oral supplements. The present research work was aimed to study the influence of two non-ionic surfactants Poloxamer and Tween 80 (Polysorbate 80) on pectin submicrospheres formulations. Typical natural polymer pectin was used to encapsulate folic acid by cross linking method. The resultant submicrospheres contributed to improve the aqueous solubility to enhance the bioavailability of folic acid. During investigation, it was observed that pectin polymers influenced kinetics of the rate of reaction more intensively than the surfactants. The physical phenomenon caused the change in their size, shape and chemistry of pectin polymers transforming into submicrospheres in aqueous condition. The characteristic differences of submicrospheres were assessed by scanning electron microscopy, differential scanning calorimetry and Fourier-transform infrared spectroscopy. The average diameters of the submicrospheres ranged between 250 and 500 nm. The encapsulation efficiency of submicrospheres ranged between 80 and 96 %. The characteristic swelling behavior of lyophilized submicrospheres was influenced by the ratio of pectin polymers and folic acid used in the formulations. The submicrospheres systems exhibited controlled release of folic acid due to the pH-dependent solubility of pectin polymers in aqueous medium. The submicrospheres showed good haemocompatibility suggesting them to be promising candidates for oral delivery.

Keywords Pectin · Folicacid · Surfactants · Submicrosphere · Hemocompatibility

Introduction

Folic acid (pteroyl-L-glutamic acid) is a water-soluble vitamin (vitamin B_9) found to be essential for numerous bodily functions [1]. As such, folic acid is not biologically active but the biological properties of the molecule relay upon its reduction to tetrahydrofolate and other related forms. It is evident from earlier reports that tetrahydrofolate is converted into dihydrofolic acid in the liver [2]. Both adults and children need folic acid to make normal red blood cells and prevent anemia. Children and adults both require folic acid to produce healthy red blood cells and prevent anemia [3, 4]. Thus folic acid deficiency hinders DNA synthesis and cell division. Because RNA and protein synthesis are not hindered, large red blood cells called megaloblasts are produced, resulting in megaloblastic anemia [5, 6]. Folic acid also helps prevent changes to DNA that may lead to cancer. Also, one of the most extensively studied small molecule targeting moieties for drug delivery is folic acid (folate) [7, 8]. The high-affinity vitamin is a commonly used ligand for cancer targeting because folate receptors are frequently overexpressed in a range of tumor cells [9–12].

It is evident that poor solubility of drugs adversely affects on the bioavailability after oral administration. As the use of microparticles for oral administration is documented and an attempt to enhance the bioavailability of poorly water-soluble drugs has been well thought of in

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order to increase the intestinal absorption of the drug [13–15]. Pectin is a natural, non-toxic, anionic, soluble and heterogeneous complex polysaccharide composed of linear α-D-galacturonic acid with 1,4-glycosidic linkages [16–19]. In recent days, pectin has also received significant attention as high fibre diet which is beneficial to health. Another important aspect is that it can reduce cholesterol levels, serum glucose levels and may also have anticancer activities [20, 21]. Pectin and pectin oligosaccharides have shown to induce apoptosis in human adenocarcinoma cells [22]. In food industry, pectin is used as a stabilizer for acidified milk drinks and yoghurts [23]. In addition, pectin is capable of reducing the interfacial tension between an oil phase and a water phase, which can be effective in the preparation of emulsions [24, 25].

In comparison, pectin is less susceptible to degradation in the gastrointestinal tract than alginate. The degradation of pectin occurs mainly in the colon by pectinolytic enzymes secreted by microorganisms. As a result pectin has increasingly gained acceptance as the carrier polymer for sustained release and site specific delivery dosage forms, such as beads, pellets, tablets, and films [26, 27]. Upon addition, pectin forms rigid gels with calcium, which cross-links the galacturonic acid chains [28]. Several researchers have successfully incorporated protein or peptide into calcium pectinate beads for a colonic delivery system [29–33]. Cheng et al. [34] recently formulated insulin-loaded calcium pectinate nanoparticles using an ionotropic gelation or cross linking method reported for the manufacture of calcium pectinate beads. This method is advantageous over others because it's short-cut method which does not involve organic solvent, harsh processing conditions and expensive equipment.

In the present study, we have developed a delivery system for folic acid encapsulated by pectin polymers using non-ionic surfactants Poloxamer/Tween 80, as organic templates. The resultant submicrospheres have been characterized for their physical and morphological properties and encapsulation efficiencies. Further, their toxicity on human red blood cells was also evaluated.

Materials and Methods

Materials

Pectin pure (Himedia Laboratories Pvt. Ltd., Mumbai.), folic acid (Loba Chemical Pvt Ltd), Calcium chloride dihydrate AR grade, Sodium hydroxide pellets pure AR, Sodium chloride, EDTA, Gluteraldehyde (S D fine Chemical Limited, Mumbai), Poloxamer/Tween 80 (Himedia, Mumbai). All the above chemicals were used without further purification.

Preparation of Pectin Submicrospheres

Pectin solution (0.5 %) was prepared by dissolving the high methoxy pectin in distilled water under gentle agitation using magetic stirrer as described by the Atmaram P Pawer [16] with slight modification. Folic acid (5 mg) was disperssed in distilled water (10 ml) under constant stirring for uniform distribution using magnetic stirrer. Then calcium chloride solution (0.5 %) was extruded drop wise through a 1.2 mm diameter needle into pectin solution and added with surfactant (Poloxamer/Tween 80, 1 ml) at room temperature with constant stirring. The addition flow rate (4 ml/min) was maintained and then submicrospheres formed were allowed to remain on the stirrer for 10 min. The compositions are shown in Table 1. The submicrospheres formed were collected, freeze dried overnight and stored in airtight container until further use.

Standardization of Folic Acid

Stock solution was prepared by dissolving folic acid (10 mg) in 0.1 N NaOH (10 ml). Working standard (0.01 N NaOH, 1/10) was prepared from the stock solution (0.1 N NaOH) after appropriate dilution. Working standard solution (0.01 N NaOH, 100–600 µl range) was taken into a series of the test tubes and volumes were adjusted to 3 ml. The absorbance was read at 366 nm using UV–VIS spectrophotometer (Optizen 2120UV Plus, Mecasys, Korea) and a calibration curve was constructed against reagent blank.

Estimation of Encapsulation Efficiency

Efficiency of the encapsulation was assayed by the spectrophotometer method as described in our previous studies [35]. A known amount of pectin submicrospheres was taken to measure the free folic acid content of in the supernatant by dissolving in PBS. The pectin submicrospheres were subjected to centrifugation at 3500 rpm for 20 min. Then 1 ml of the supernatant was taken and read the absorbance of folic acid present in the supernatant using UV–VIS spectrophotometer (Optizen 2120UV Plus, Mecasys co., Ltd) at 366 nm. The amount of folic acid incorporated into the pectin submicrospheres was calculated by subtracting the free drug present in the supernatant from the total drug added before the preparation. The following formula was used for the calculation.

$$EE(\%) = \frac{\text{Total amount of the drug} - \text{free drug in the supernatant}}{\text{Total amount of rug}} \times 100$$

Table 1 Compositions and % encapsulation efficiency (EE) of folic acid-free and folic acid-loaded pectin submicrospheres formulations (PE1–PE6)

Formulations	Formulation ingredients	Batch code	Conc. of folic acid (mg)	Conc. of pectin (mg)	Ratio of folic acid and pectin	% EE of submicrospheres with drug at pH 7.0	% EE of submicrospheres with drug at pH 6.8	% EE of submicrospheres with drug at pH 7.4
Folic acid-free pectin submicrospheres with/without surfactants	Pectin-alone	PE1	5	20	1:4	–	–	–
	Pectin-alone + tween 80	PE3	5	20	1:4	–	–	–
	Pectin-alone + poloxamer	PE5	5	20	1:4	–	–	–
Folic acid -loaded pectin submicrospheres with/without surfactants	Pectin + folic acid	PE2	5	20	1:4	84.38 ± 0.93	96.28 ± 0.92	96.20 ± 0.19
	Pectin + folic acid + tween 80	PE4	5	20	1:4	93.52 ± 0.63	96.00 ± 0.36	95.34 ± 0.45
	Pectin + folic acid + poloxamer	PE6	5	20	1:4	92.72 ± 0.85	95.88 ± 0.62	95.99 ± 0.21

Swelling Capacity

Into a pre-weighed centrifuge tube the freeze dried submicrospheres (100 mg) was taken and resuspended with PBS (20 ml, pH 7.4 and 6.8) and incubated for 12 h at 37 °C. The suspension was centrifuged (1000×*g*, 20 min) at 37 °C to separate the swollen spheres, decanted the supernatant and then excess buffer remained with spheres removed carefully using the blotting paper at the edge. The centrifuge tube with swollen spheres was cooled to room temperature and re-weighed immediately. The swelling capacity (%) was calculated using the following equation:

$$Swelling(\%) = \frac{\mathrm{Mw} - \mathrm{M1}}{\mathrm{M1}} x100$$

where, Mw and M1 are the weight of the submicrospheres taken in into centrifuge tube before and after the centrifugation.

Scanning Electron Microscopy (SEM)

An aliquot of freeze dried submicrospheres (2 mg) were used for shape and size analysis by scanning electron microscopy (JNCASR, Bangalore, India.). The observation was performed using a 20 kV, LaB6 (or tungsten filament) scanning electron microscope equipped with an Everhart–Thornley secondary electron detector and a Cambridge four quadrant backscatter detector LEO 1530 (LEO 1455VP Cambridge, England) operated using 0.5 mA filament current. The submicrosphere size in the dry state was determined by averaging the size of 35 submicrospheres. Sample was prepared as aqueous dispersion of submicrospheres were finely spread over a glass slide and dried under vacuum. The dried slide was placed onto carbon conductive double-side tape (Euromedex, France) and dried further at room temperature. The processed submicrospheres were coated with gold (2 nm) and placed inside the vacuum column of the microscope after pumping the air out of the chamber.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is needed to understand the folic acid-pectin polymer interaction in order to determine any chemical interaction between them. It becomes crucial information because of the interactions that affect on the in vitro release behavior and predominant feature to assess its release from the folic acid submicrospheres [36]. The submicrospheres from pectin-alone and folic acid-loaded pectin, and folic acid-alone were allowed to form pellets at pressure of 10.3×10^4 Pa. The FTIR spectrum of the samples was recorded with Nicolet IR 200 (Thermo Electron Corporation, USA). FTIR spectra of the samples were taken in the wavelength region 4000–400 cm^{-1} at room temperature using potassium bromide pellets (Merck, IR grade).

Differential Scanning Calorimetry (DSC)

In order to evaluate the crystalline state of folic acid in the pectin encapsulated polymeric nanoparticles Differential Scanning Calorimetry is used. The freeze-dried submicrospheres samples (3–6 mg) were weighed into an aluminium pan and their thermal behaviour was characterized by DSC (Mettler-Toledo DSC). Initially, the pectin was heated to 30 °C and reached gradually to 360 °C at a heating rate of 10 °C/min per cycle. Inert atmosphere was maintained throughout by purging nitrogen at the flow rate of 100 ml/min.

In Vitro Release Studies

In vitro release studies were performed by dialysis method [37] at 37 ± 0.5 °C and folic acid release was monitored spectrophotometrically. Folic acid loaded pectin submicrospheres samples (3 mg) were dispersed in 0.1 N NaOH (3 ml) and placed in the dialysis membrane bag (Mw cut-off 10,000; Sigma, USA). The bag tied at both ends by using cotton thread and immersed in phosphate buffer saline (pH 7.4, 25 ml) containing vessel. The dialysis set was placed in a shaking water bath (50 rev min^{-1}) and a constant temperature was maintained at 37 °C throughout the dialysis. An aliquot of folic acid-loaded pectin submicrospheres (0.5 ml) was withdrawn from the dialysate at predetermined time intervals (1, 2, 4, 6, 12 and 24 h) and immediately equal volume of PBS was replaced. The samples (0.5 ml) withdrawn were analyzed for folic acid release by measuring the absorbance at 366 nm using UV–VIS spectrophotometer (Optizen 2120UV Plus, Mecasys, Korea).

Hemocompatibility Study

Fresh blood (20 ml) from a healthy volunteer was withdrawn into centrifuge tubes containing EDTA (2 mg). The tube was centrifuged (117 g, 20 min) in a REMI 24-C centrifuge to separate the erythrocytes. Buffy coat was removed and the packed cells were washed thrice with normal saline (0.9 % NaCl). Equal amount of 0.9 % NaCl was added to the erythrocytes and centrifuged (117 g, 10 min). The supernatant was discarded and the process was repeated thrice to obtain washed erythrocytes. Normal saline was added to the erythrocytes to obtain 50 % hematocrit. The final concentration of erythrocytes was arrived to contain around 2×10^8 cells per ml in normal saline, as counted by hemocytometer. Haemolysis experiments were in accordance with a method used previously in our laboratory with slight modifications [38]. A 0.1 ml samples solution (15 mg/ml) in normal saline was then added to 0.1 ml of erythrocyte solution and the final volume was made 3 ml with normal saline. The contents were incubated at 37 °C for 1 h in a water bath (ILE instrument, Bangalore), at that time blood cells and sample precipitates were sedimented in a centrifuge (10 min at 11,752 g). The reaction was terminated using 50 µl of 2.5 % gluteraldehyde. The experiment was carried out in the triplicate. The samples were then centrifuged (117 g, 10 min) and release of hemoglobin from the erythrocyte cells was measured spectrophotometrically at 540 nm using UV–VIS spectrophotometer Optizen 2120UV Plus, Mecasys co., Ltd, Korea. A spontaneous hemolysis control group was determined by incubating erythrocytes in normal saline at the concentration 1×10^8 cells ml^{-1}.

$$Hemolysis(\%) = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the positive control}} \times 100$$

Statistical Analysis

All the experiments were conducted in triplicate. The Statistical analysis of experimental data utilized the Student's *t* test, and the results were given as mean ± standard deviation (SD). Differences were considered significant at a level of $p < 0.05$ (Fig. 1).

Result and Discussion

The folic acid and pectin submicrospheres formulations prepared in the presence or absence of surfactants is presented in Table 1. The morphological examinations by SEM of dried folic acid-loaded pectin submicrospheres revealed less clumping and spherical in nature ranging between 250 and 500 nm (Fig. 2PE1–PE6). The use of calcium chloride to assist in cross-link of pectin polymers the addition of either Tween 80 or Poloxamer did not adversely affected on spheres showing uniform size distributions. Upon careful observation, PE2 and PE4 spheres revealed more spherical form, smoother surface and discrete with less clumping than other spheres. Addition of either Tween 80 or Poloxamer did not influence on size and spherical nature of any category of submicrospheres, but kept the spheres away from clumping.

The FTIR spectra taken at the room temperature indicated there was no interaction between the pectin and folic acid (Fig. 3). Many peaks of pectin and folic acid were observed which shows a broad-OH stretching absorption band at 3534.1 cm^{-1} in folic acid. Another major absorption band at 3417.38 cm^{-1} corresponded to free amino group ($–NH_2$) a major peak present in pectin. Generally, the spectral analysis measures the selective absorption of light by the vibration modes of specific chemical bonds in

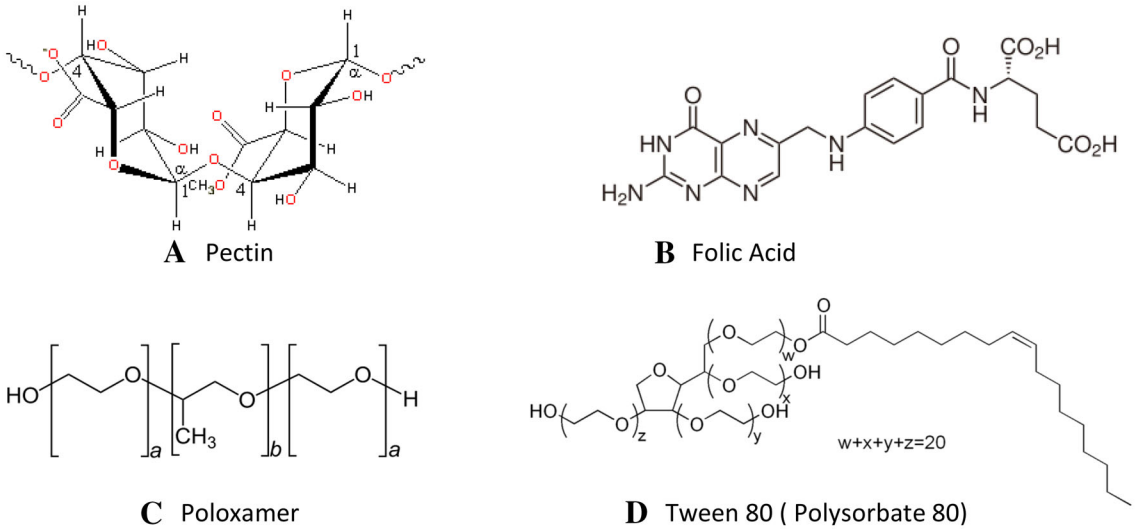

Fig. 1 General structures of **a** pectin, **b** folic acid, **c** poloxamer and **d** Tween 80 (*Source* Wikipedia)

the compounds interacting [39]. While evaluating the experiment, any interaction occurring between the folic acid and pectin polymer would produce altered vibrations of their atoms involved in the interaction. Normally, the change in the frequency and intensity of interacted molecules and uninterested (stand-alone) complexes were compared with their respective standard compounds in free form (here folic acid-alone and pectin-alone) [40]. Figure 3 exhibited FT-IR spectra of folic acid-alone (F) and formulations containing folic acid free-pectin submicrospheres with/without surfactants (PE1, PE3 and PE5) and formulations of folic acid-loaded pectin submicrospheres with/without surfactants (PE2, PE4 and PE6), respectively. The pure folic acid compound (F) gave the peaks contributed by an intense and broad band in the region characteristic peaks at 3534.1 cm^{-1} to the proton vibrations in a medium short hydrogen bond formed between O–H group and the O-atom of the carboxylate group. One sharp peak at 3417.38 cm^{-1} corresponded to N–H bending. The spectra for pectin of different formulations gave the peaks at 1752 cm^{-1} which is its characteristic peak due to typical C=O (carbonyl) group, exhibited C–O stretching at 1610 cm^{-1}. Physically, pectin in its free form whether surfactants present or not formulations showed peaks resulting from simple super position of their separated components in the infrared spectra. In the case of folic acid-loaded submicrospheres, the peak of folic acid showing very less intensity as the folic acid concentration in the submicrospheres was low. It is also observed that there were neither shifting of peaks nor disappearance of functional peaks in the pectin and folic acid- loaded pectin formulations. The spectral analysis folic acid and pectin polymer indicated that the specific functional groups such as O–H, C–O, C=O, N–H and others of polymeric material in the submicrospheres surface have almost the same chemical characteristics of the pure polymer and the folic acid entrapped exhibited their main characteristics peaks. The study concluded that there was no occurrence of molecular interactions which may likely to cause altered structure of both folic and pectin in the various submicrospheres formulations.

Differential scanning calorimetry (DSC) is a thermal analytical technique which provides information about the physical properties of products, i.e. about the crystalline or amorphous nature of the formulations prepared. To determine the state of folic acid in nanoparticle, the samples were subjected to DSC.

By virtue of their molecular structure, folic acid exhibits two endothermic peaks (138.0 and 201.3 °C) indicating a wide range of crystallinity of the formulations prepared. Whereas, in polymeric formulations like folic acid–loaded pectin system, both encapsulated molecules like folic acid and encapsulating polymer pectin would never counter influence on each other. Therefore, release kinetics of folic acid molecules located at the core in any given polymeric formulations, will not be affected when they exist in crystalline form [36]. In Fig. 4F, PE1–PE6 the DSC thermograms of submicrospheres of pure folic acid (F) and Pectin either individually or loaded with folic acid in the presence or absence of surfactants depicted sharp endothermic peaks at different melting points (Table 2). Figure 4F, PE1, PE3 and PE5 exhibited the DSC thermograms of folic acid-alone (F) exhibited two different melting peak at 138.0 and 201.3 °C, whereas the

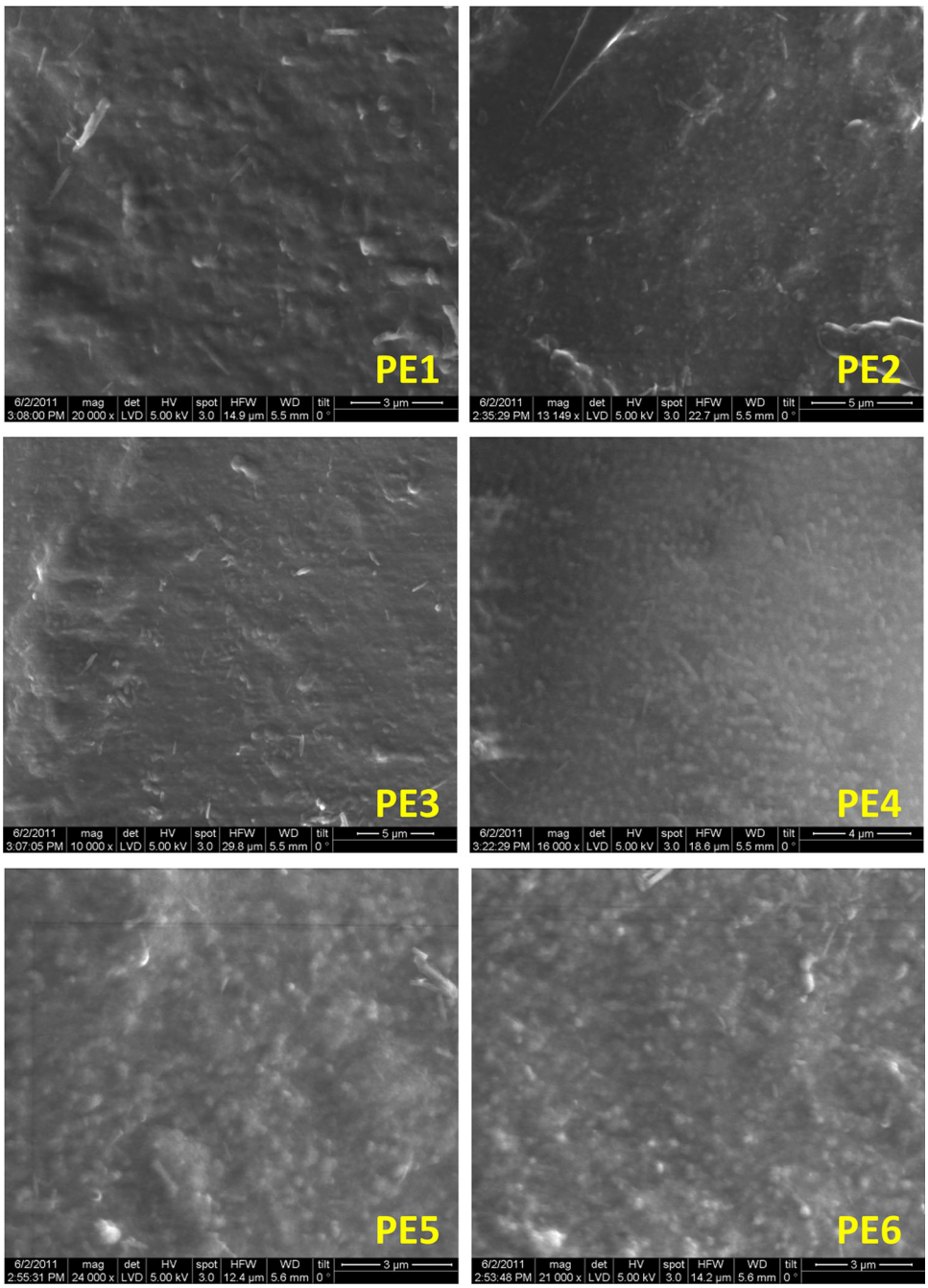

Fig. 2 Scanning electron micrographs of folic acid-free and folic acid-loaded pectin submicrospheres formulations (PE1–PE6)

formulations of spheres containing pectin-alone submicrospheres with/without surfactants, respectively showed nearly the same thermal behavior as the individual components, indicating that there was no interaction between the folic acid and the pectin polymer in the crystalline state (Table 2). Similarly, Fig. 4PE2, PE4 and PE6 were the formulations of folic acid-loaded pectin submicrospheres with/without surfactants, respectively, interesting those exhibited identical peaks resembled the same and appeared to be crystalline (Table 2).

Swelling Test

Pectin appeared as key formulation parameters in submicrospheres formulation, as it controlled microsphere swelling and degradation with time and pH. In the results a pectin presenting lower swelling properties as the increased amount of drug in the formulations. It exhibited in the range of 44–52 % swelling. It would permit to enhance the pectin mass fraction in the formulations, and thus final macroporosity of the formulations, but would also result in

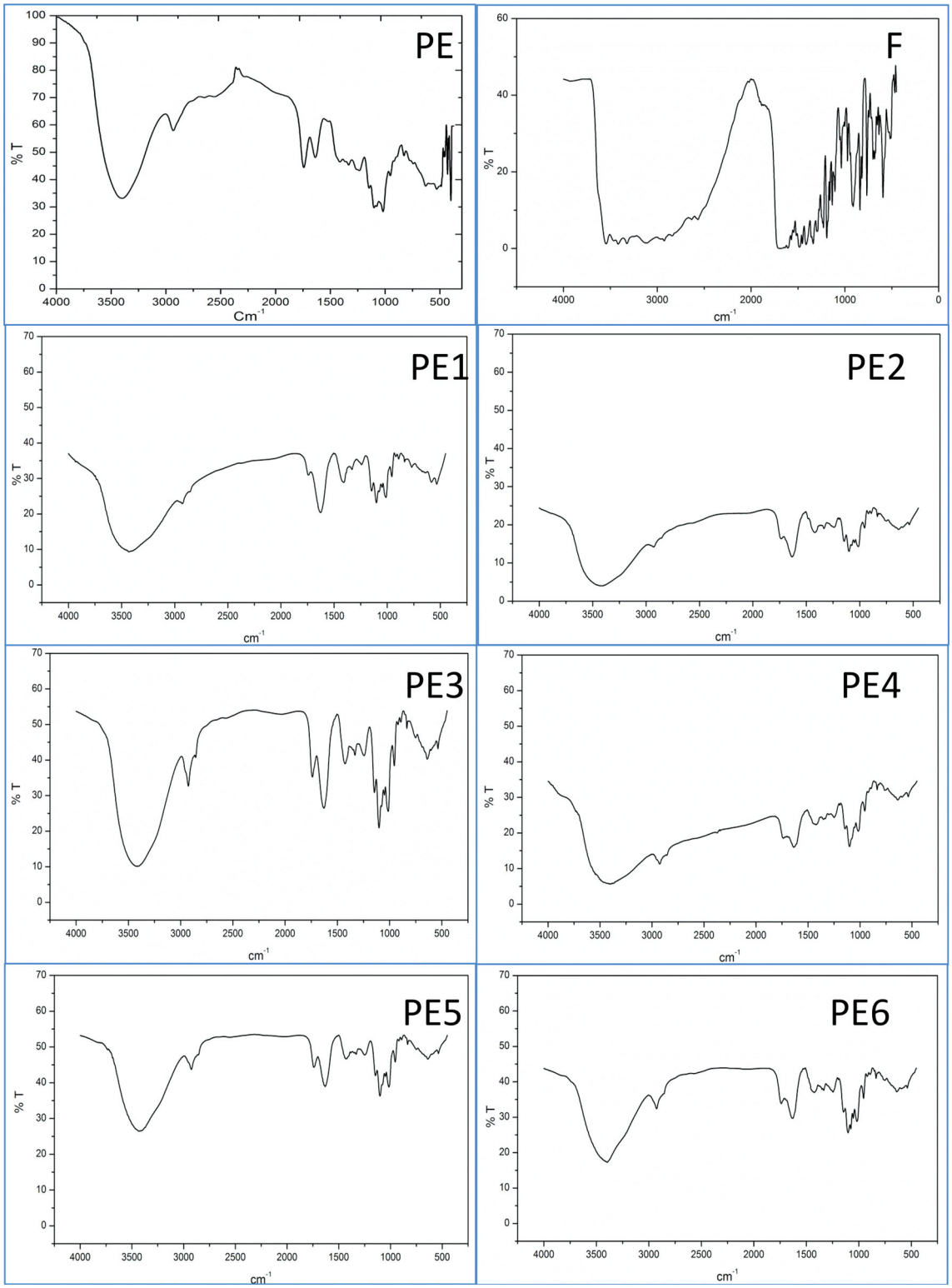

Fig. 3 FTIR spectra of folic acid and pectin formulations. PE: pectin blank; F: folic acid-alone; PE1, PE3 and PE5 are the formulations containing folic acid free-pectin submicrospheres with/without surfactants formulations; and folic acid-loaded pectin submicrospheres with/without surfactants formulations (PE2, PE4 and PE6)

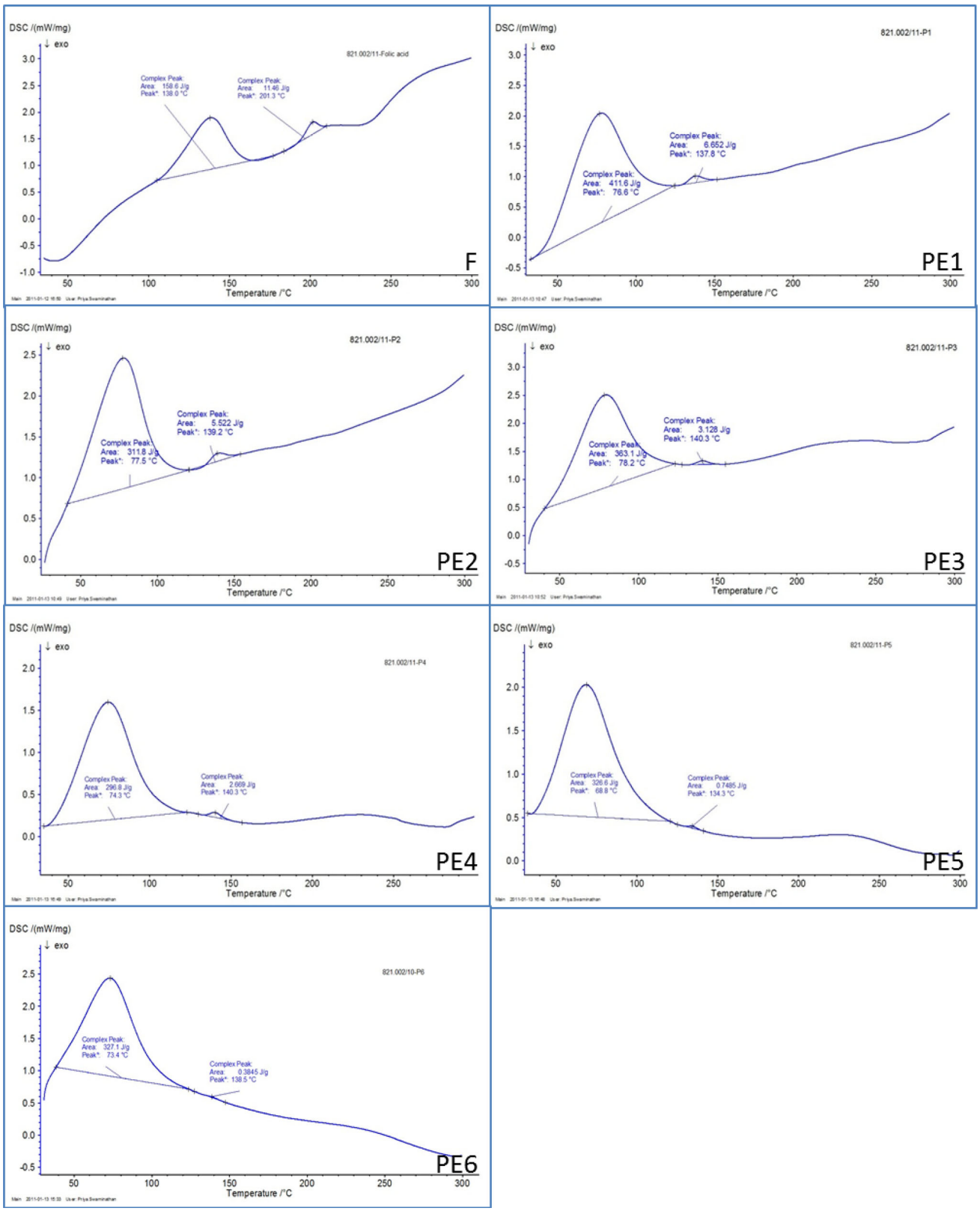

Fig. 4 DSC thermograms of folic acid-alone (F), pectin submicrospheres containing folic acid (PE2, PE4 and PE6) and pectin submicrospheres without folic acid (PE1, PE3 and PE5) prepared in presence or absence of surfactants

Table 2 Points of melting in folic acid-alone, pectin-alone and folic acid-loaded pectin submicrospheres

Formulations	Initial melting point (°C)	Final melting point (°C)
F	138.0	201.3
PE1	76.6	137.8
PE2	77.5	139.2
PE3	78.2	140.3
PE4	74.3	140.3
PE5	68.8	134.3
PE6	73.4	138.5

reduced drug release ability and longer microsphere degradation time. The Table 3 showed the formulations with percentage of swelling in folic acid free-pectin submicrospheres with/without surfactants (PE1, PE3 and PE5) and formulations of folic acid-loaded pectin submicrospheres with/without surfactants (PE2, PE4 and PE6), respectively. A compromise must be found to adjust on-demand formulation properties in terms of delivery and final macroporosity.

Finally, it was observed that submicrospheres formulation of free-pectin submicrospheres with/without surfactants (PE1, PE3 and PE5) and formulations of folic acid-loaded pectin submicrospheres with/without surfactants (PE1, PE3 and PE5) showed moderate swelling ability. Pectin submicrospheres behave as hydrophilic matrices whose release ability is currently related to their swelling ability in dissolution media. This result considered of good prognostic in obtaining further sustained drug delivery and controlled erosion/degradation with time and pH [41].

Encapsulation Efficiency

The spectrophotometric method was used for determination of the encapsulation efficiency of folic acid in pectin submicrospheres. The folic acid-loaded pectin submicrospheres were separated from the aqueous suspension medium by centrifugation at 3500 rpm for 20 min. The amount of free folic acid was measured in the clear supernatant by UV at a wavelength of 366 nm. The folic acid encapsulation efficiency (EE) of the pectin submicrosphere and their % encapsulation efficiency (% EE) were calculated. The amount of the unincorporated folic acid present in the supernatant is presented in Table 4. Submicro-encapsulation efficiency of PE2, PE4 and PE6 formulations was 85.03, 90.35 and 96.11 %, respectively.

In Vitro Release of Folic Acid from Pectin Submicrospheres

In vitro studies by dialysis were carried out in the phosphate buffer solution at two different pH 7.4 and pH 6.8, at 37 °C for PE2, PE4 and PE6 folic acid-loaded pectin submicrospheres with/without surfactants. The folic acid release may vary from formulation to formulation depending upon the pectin concentration. From Table 5, PE2 formulation showed initial folic acid release of 30.55 % at pH 6.8, and slightly decreased release 22.43 % at pH 7.4 within 30 min this may be due to initial burst effect which is important also to the pharmacological point of view. Whereas PE4 and PE6 formulations showed similar initial burst of folic acid 26.3 and 25.6 % at pH 6.8, and slightly decreased release 21.89 and 24.22 % at pH 7.4

Table 3 Percentage swelling of folic acid-free and folic acid-loaded pectin submicrospheres

Polymer	Code	Folic acidconc. (mg)	Pectin conc. (%)	Percentage of swelling
Pectin	PE1	–	0.5	53.33 ± 1.53
Pectin	PE2	5	0.5	49.33 ± 2.52
Pectin	PE3	–	0.5	44.67 ± 2.52
Pectin	PE4	5	0.5	44.67 ± 3.06
Pectin	PE5	–	0.5	42.33 ± 2.52
Pectin	PE6	5	0.5	40.33 ± 3.51

Table 4 Encapsulation efficiency of folic acid-loaded pectin submicrospheres

Pectin polymer	Code	Conc. of folic acid (mg)	Conc. of pectin (mg)	Ratio of the folic acid:pectin	Encapsulation efficiency (folic acid content)
Pectin	PE1	–	20	–	–
Pectin	PE2	5	20	1:4	85.03 ± 0.64 %
Pectin	PE3	–	20	–	–
Pectin	PE4	5	20	1:4	90.35 ± 0.62 %
Pectin	PE5	–	20	–	–
Pectin	PE6	5	20	1:4	96.11 ± 0.63 %

Table 5 Percentage of folic acid release in different time intervals at pH 6.8 and pH 7.4 from folic acid-loaded pectin submicrospheres

Time interval (h)	Folic acid-loaded pectin submicrospheres folic acid release (%) at pH 6.8			Folic acid-loaded pectin submicrospheres folic acid release (%) at pH 7.4		
	PE2	PE4	PE6	PE2	PE4	PE6
0.5	30.55 ± 1.99	26.30 ± 3.62	25.60 ± 2.66	22.43 ± 1.28	21.89 ± 1.10	24.22 ± 1.12
1.0	41.07 ± 3.51	34.67 ± 3.99	34.46 ± 2.51	24.28 ± 1.14	23.26 ± 1.12	25.68 ± 1.18
2.0	49.37 ± 4.48	46.23 ± 2.48	45.91 ± 2.13	27.46 ± 1.43	26.26 ± 1.23	26.42 ± 1.39
3.0	53.26 ± 4.90	52.48 ± 2.07	51.70 ± 2.84	29.34 ± 1.46	28.35 ± 1.17	29.53 ± 1.39
4.0	52.93 ± 2.50	58.11 ± 2.34	53.90 ± 2.62	30.70 ± 1.29	29.66 ± 1.43	31.36 ± 1.23
5.0	55.40 ± 3.08	60.50 ± 2.08	56.59 ± 2.85	32.65 ± 1.43	31.53 ± 1.34	34.69 ± 1.41
6.0	57.87 ± 3.09	61.44 ± 1.18	59.57 ± 4.50	33.14 ± 1.05	33.19 ± 1.03	38.64 ± 1.38
8.0	63.08 ± 2.53	67.45 ± 1.05	66.43 ± 5.51	44.40 ± 1.18	46.35 ± 1.22	46.23 ± 1.04
12.0	78.13 ± 5.31	79.57 ± 1.51	80.30 ± 1.06	57.43 ± 1.25	58.69 ± 1.27	59.32 ± 1.14

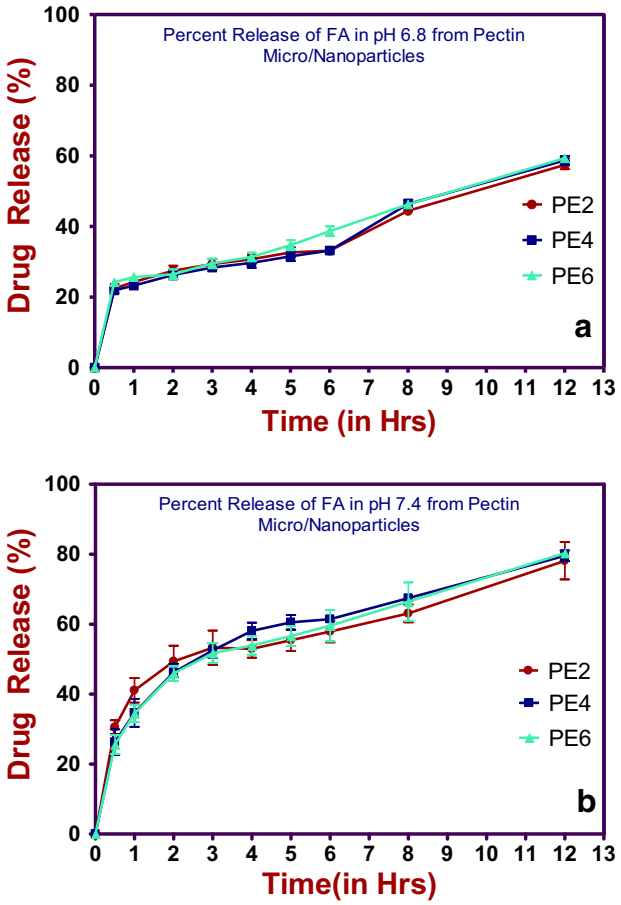

Fig. 5 In vitro release folic acid (%) with the passage of time at **a** pH 6.8 and **b** pH 7.4 from folic acid-loaded pectin submicrospheres (PE2, PE4, PE6)

respectively, within 30 min. Interestingly, the PE2, PE4 and PE6 submicrospheres formulations showed the significant increase in folic acid release 80.30 % the most and 78.13 % the least at pH 6.8 after 12 h gave the release pattern in controlled manner, but surprisingly the same formulations showed a significant decrease in folic acid release 59.32 % the most and 57.43 % the least at pH 7.4 after 12 h. The trend continued to follow the same in pH 6.8 from first hour of release and onwards indicating slow and sustained release of folic acid. On comparison of the release profile of the three (PE2, PE4 and PE6) formulations, it was observed that the release pattern had marginal difference between the PE2, PE4 and PE6 submicrospheres formulations, but pH 6.8 was found to be the preferred condition than that of pH 7.4. Release percentage of folic acid within 48 h (80.30 % in pH 6.8 and 59.32 % in pH 7.4), clearly showing that there is no interaction between the polymer pectin and the drug folic acid. The data reveals that slightly acidic pH was appropriate than that of slightly basic pH 7.4, which favored the release with respect to percent encapsulation efficiency (%EE) and bettered folic acid diffusion out of the dialysis bag will fall in the order PE6 > PE4 > PE2 submicrospheres formulations. Such sustained release can be explained by folic acid-loaded pectin submicrospheres sensitivity to ionic conditions. They behave as hydrophilic matrices whose release ability is currently related to their swelling ability and the degree of porosity of the formulations in dissolution media (Fig. 5).

Hemocompatibility Study

The hemolytic potential of any administered pharmaceuticals must be evaluated to avoid the possibility of red blood cells toxicity. In vitro haemolysis test of folic acid-alone and folic acid-loaded pectin submicrospheres were carried out (Fig. 6). From study, it was evident that folic acid-alone and pectin-alone did not cause any hemolysis at 0.5, 1 and 1.5 mg/ml, whereas folic acid-loaded pectin submicrospheres also showed negligible amount of the hemolysis at different concentrations.

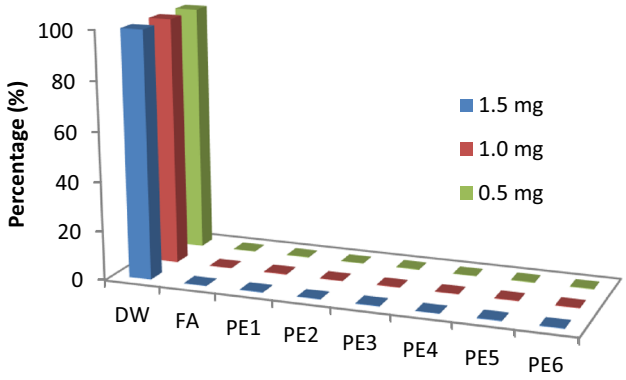

Fig. 6 Hemolysis of folic acid-alone, pectin-alone and folic acid-loaded pectin submicrospheres (PE1–PE6)

Conclusion

In the present investigation, for the first time folic acid-loaded pectin submicrospheres have been shown to have a novel controlled folic acid delivery which offer several potential benefits. Folic acid-loaded pectin submicrospheres have shown an excellent capacity for their association. It is an anti-anemic drug and was selected as candidate in the present study in order to explore the possibility of whether the polymer pectin could show better encapsulation? If so, does it release the same in a slow and sustained manner that is needed to establish rapid onset and relatively short duration of action? Average submicron size diameter particles, drug-polymer interaction and percentage encapsulation efficiency were found to be the criteria for optimal formulations.

The folic acid-loaded pectin submicrospheres formulations were evaluated with respect to their size, shape, encapsulation efficiency and other physical characteristics. The DSC analysis measurement had suggested that the folic acid in the folic acid-loaded pectin submicrospheres formulations was found in the crystalline form. FT-IR showed that there is no interaction between the folic acid and the pectin polymer. The encapsulation efficiency evaluated by spectrophotometric method exhibited better encapsulation efficiency. The in vitro release profile of folic acid from folic acid-loaded pectin submicrosphere exhibited a typical biphasic release phenomenon, showing initial burst release within 30 min and followed by slow and sustained release in an incremental form until 12 h. The in vitro release also indicated that the release property of folic acid from folic acid-loaded pectin submicrospheres not only depended on adsorption of the folic acid but also on diffusion through the pectin matrix. Folic acid-loaded pectin submicrospheres and pectin-alone submicrospheres show no hemolysis.

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