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Research Paper

Entrapment of 5-fluorouracil into PLGA matrices using supercritical antisolvent processes

Pardis Kalantarian[a], Ismaeil Haririan[a], Abdolhossein Rouholamini Najafabadi[a], Mohammad Ali Shokrgozar[b] and Alireza Vatanara[a]

[a]Department of Pharmaceutics, School of Pharmacy, Tehran University of Medical Sciences and [b]National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran

Abstract

Objectives Two different supercritical antisolvent processes were performed to co-precipitate 5-fluorouracil (5-FU) and poly(lactide-co-glycolide) simultaneously. 5-FU is a hydrophilic antitumor agent, and is more effective when administered at a lower dose for a longer period of time.

Methods Controlled-release polymeric systems of 5-FU were produced, and morphology, thermal behavior, in-vitro release and cytotoxicity of microparticles were analysed.

Key findings Dissolution studies showed that 33% of drug was released in 21 days, which represents a long-lasting profile. To evaluate the efficacy of the released drug on cancer cells, the MTT assay cytotoxicity test was performed using human lung carcinoma A549 cell lines. There was no significant difference between the cell inhibition rates of the released drug and unprocessed 5-FU at the same drug concentration level. IC50 values were 69.12 mg/ml for unprocessed 5-FU and 68.71 mg/ml for the released drug.

Conclusions Application of supercritical processing for co-precipitation of 5-FU and PLGA provided mild and non-aqueous conditions, so the hydrophilic drug incorporated in the polymer had good stability during the process.

Keywords 5-fluorouracil; antitumor; cytotoxicity assay; GAS; SAS; supercritical antisolvent

Introduction

As one of the oldest antitumor agents, 5-fluorouracil (5-FU) has been used in the treatment of a wide variety of cancers for several years. Unpredictable and incomplete absorption of 5-FU after ingestion and a marked bio-inactivation of drug in the liver[1] and mucosal membrane of the gastrointestinal tract cause highly variable oral bioavailability and short half-life of drug in the serum.[2,3] In addition, intravenous administration of 5-FU results in a broad systemic distribution with a small fraction of the dose reaching the site of action.[4] Therefore, there is a need for a method to increase the therapeutic effects of 5-FU while limiting its adverse effects. Among all methods, locally sustained-release chemotherapy has proved to have an immediate benefit. To transfer the drug in this method, polymer systems can be used to physically trap the antitumor agent and release it in a sustained form at the tumor site.[5]

Local chemotherapy, implantation of biodegradable polymeric devices directly in the resection cavity and injection in the tumor are advantageous approaches. The formulation of these products comprises the drug and a matrix capable of releasing the active drug.[6] Polylactic acid and poly(lactide-co-glycolide) (PLGA) have been the most commonly used polymers in sustained-release drug delivery, as they degrade by simple hydrolysis of the ester bonds into the natural metabolites, glycolic and lactic acids. These are removed from the body by normal metabolic pathways and therefore do not require surgical removal after completion of drug release. PLGAs may be prepared with any monomer composition, since characteristics of copolymers can be controlled through adjustment of the molecular weight and the molar ratio of lactic to glycolic acids.[7]

Supercritical carbon dioxide ($SC\text{-}CO_2$) technologies are becoming popular around the world, and have been receiving increased attention in various applications.[8] At present, the main pharmaceutical applications of $SC\text{-}CO_2$ developed at the industrial scale are related to

Correspondence: Alireza Vatanara, Department of Pharmaceutics, School of Pharmacy, Tehran University of Medical Sciences, Keshavarz Blvd., Tehran 14176, Iran.
E-mail: vatanara@sina.tums.ac.ir

the extraction and modification of pharmacologically active compounds. However, strong and fast developments are in progress in the field of drug particle design and drug delivery systems at the industrial scale.[9,10] For these applications, the main goal is the generation of drug particles with tailored properties and the incorporation of drugs within polymer or lipid particles to achieve well-defined functions. Among the potential applications of SC-CO_2-based processes in the field of drug delivery systems, the production of drug-loaded composite particles for controlled-, modified- or sustained-release of drugs was the first application reported in the literature.[8,10]

There are several SC-CO_2 processes for particle design, which can be classified into four major categories: rapid expansion of supercritical solution, particles from gas-saturated solution, gas antisolvent (GAS) and the supercritical antisolvent process (SAS) and its various modifications, depending on whether the SC-CO_2 is used as a solvent or an antisolvent.[11]

In this study, SC-CO_2 was applied in the GAS and SAS methods to prepare sustained-release matrices of 5-FU (as a small and hydrophilic molecule) in PLGA 50 : 50 (as a biodegradable polymer). The different parameters which are effective in the process were studied and different matrices were prepared. Furthermore, related characteristics such as encapsulation efficiency and in-vitro release profiles were evaluated. Cytotoxicity tests were accomplished on the released drug, and the dose at which 50% of maximal proliferation was inhibited (IC50) was also calculated.

Materials

5-FU (USP) was purchased from Alpha Aesar (Germany). PLGA (50 : 50) having inherent viscosity of 0.67 and 0.38 dl/g in chloroform at 25°C (Resomer® RG 505 and Resomer® RG 503, respectively) were purchased from Boehringer Ingelheim (Germany). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Biosera (UK), MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), was also purchased from Sigma-Aldrich Co. (Germany). CO_2 (99.99%) was provided from Sabalan Co. (Iran). Solvents including methanol, acetone, isopropanol and dichloromethane were obtained from Merck Chemical Co. (Germany) and used as received.

Methods

Preparation of 5-FU polymeric matrices using supercritical antisolvent

SAS process

The technical details of the apparatus used in this study have been presented elsewhere.[12] The experiments began by delivering CO_2, after passing through a cooling device, to the precipitator (vessel with 400 ml internal volume) by a syringe pump until the desired pressure was achieved (Table 1). The precipitator was placed in an oven equipped for precise temperature regulation. The gas entered the precipitation vessel through a co-centric nozzle placed in the upper side of the precipitator until the pressure reached the predetermined point.

At this time, organic solutions of 5-FU and PLGA were added simultaneously with SC-CO_2 into the precipitation vessel through the nozzle. During this process, particles were precipitated on a filter at the bottom of vessel. This stage took about 60 min to allow the collection of sufficient yield of at least about 100 mg of solid product. The experiment was considered complete when delivery of the drug solution to the chamber ended. Meanwhile, SC-CO_2 flow was continued for 20 min to wash the chamber of the residual organic solvent. When the washing was completed, the CO_2 flow was stopped and the chamber was depressurized down to atmospheric pressure.

GAS process

GAS co-precipitation of 5-FU and polymer was performed by preparing a predetermined volume of 5-FU/PLGA solution (30 ml) for the given operating temperature (Table 1), and loading it into the 400 ml precipitation vessel. The stirrer of the high-pressure vessel was turned on and set to 60 rpm.

When the system was stabilized and equilibrated thermally, the pressurization was initiated by injection of CO_2. A controlled CO_2 flow rate (about 20 ml/min) was maintained until the full liquid volumetric expansion was achieved at 100 bar. Then the CO_2 supply was stopped while stirring was continued for 60 min. A rinsing step was performed by flushing the expanded liquid phase with CO_2 at a constant flow rate, for a minimum period of 1 h. Finally, the precipitation vessel was depressurized by venting the entire fluid mixture of the vessel, and the dry solid powder was collected for analysis.

Table 1 Summary of operational parameters of SAS and GAS processes

Run	Process	T (°C)	P (bar)	Solvent composition	Conc. 5-FU	Conc. PLGA	Polymer
S_1	SAS	36	110	Aceton	3	3	Resomer® RG 505
S_2		36	110	Me : DCM 1 : 2	3	3	Resomer® RG 505
S_3		45	110	Me : DCM 1 : 2	3	3	Resomer® RG 505
S_4		36	110	Me : DCM 1 : 2	3	27	Resomer® RG 505
S_5		36	110	Me : DCM 1 : 2	3	3	Resomer® RG 503
G_1	GAS	40	120	Me : DCM 1 : 2	3	27	Resomer® RG 505
G_2		40	120	Me : DCM 1 : 2	1.5	28.5	Resomer® RG 505

T, temperature; P, pressure; Conc., concentration; Me, methanol; DCM, dichloromethane.

Physical characterization of particles

The morphology of particles was qualitatively assessed using a CamScan MV 2300 scanning electron microscope (Cambridge, UK). Samples were coated with gold–palladium at room temperature before examination. The accelerator voltage for scanning was 25.0–30.0 kV.

Differential scanning calorimetery (DSC) was accomplished using a DSC-60 (Shimadzu, Japan). Approximately 6 mg of the materials was placed in an aluminum pan and analysed under dry nitrogen purge. The temperature ranged between 0°C and 320°C.

Determination of 5-FU content

The actual drug content of particles of each batch was determined by the following procedure: 25 mg of recovered samples was dissolved in dichloromethane to prepare a 10 ml solution. The 5-FU was extracted three times from dichloromethane using 25 ml of phosphate buffer. The mixture was shaken for 15 min and then was left still to equilibrate for 10 min. The absorbance of each aqueous extract was measured at 265 nm using a UV/VIS spectrophotometer (Jasco V-530, Japan) against a blank of phosphate buffer.

In-vitro drug release studies

A USP dissolution apparatus I rotating at 50 rpm was used to obtain the release profile. A dialysis bag containing matrix samples and 5 ml of phosphate buffer saline (PBS) was placed into 250 ml of release medium (PBS, pH 7.4 BP). The 5-FU concentration in the release solution was monitored by UV–VIS spectrophotometry at 265 nm according to the calibration curve of 5-FU in the same buffer.

Cell line

Human lung carcinoma A549 cell line was provided by the national cell bank of Iran. The cells were grown in DMEM medium with 10% (v/v) FBS. The cells were cultured in a 95% air 5% CO_2 atmosphere at 37°C in a humidified incubator, and were dissociated with 0.05% trypsin-EDTA in case of transferring or dispensing before experiment.

Cytotoxicity tests and IC50 value evaluations

The antitumor activity of 5-FU-polymeric systems was evaluated by the MTT method. Samples of polymeric matrices of drug were incubated in PBS (pH 7.4) as regular release medium for 3 weeks at 37°C. At predetermined periods, the released solutions were sampled and diluted by cell culture medium to the desired 5-FU concentrations. An equal amount of 5-FU powder was directly dissolved in cell culture medium as control.

To evaluate the number of live and dead cells, the cells were stained with trypan blue and counted using a hemocytometer. To determine the growth inhibitory activity of the test compounds, 1×10^4 cells were plated into each well of 96-well plates in 100 µl of growth medium incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 h, 100 µl of each solution, including positive control solution (5-FU, 600 µg/ml), negative control solution (DMEM, pH 7.4), released sample solutions (5-FU; 0.3, 3, 30 and 300 µg/ml) and unprocessed 5-FU solution in cell culture medium (5-FU; 0.3, 3, 30 and 300 µg/ml) were, respectively, added into the tumor-cell-cultured well and incubated for another 48 h. Following these procedures, 100 µl MTT solutions (0.5 mg/ml) were added to each well and kept for 5 h in incubation. Finally, the solution in the wells was deserted completely and 100 µl isopropanol was added to each of the wells to dissolve the residue. The optical densities of the solutions were determined by a Microplate Reader (Bio-RAD 550, USA) at 545 nm and a reference wavelength of 690 nm and the cell inhibitions were calculated.

The percentage viability of 5-FU matrices was compared with that of the control and free drug at different concentrations using an in-vitro cytotoxicity test. IC50 values (µg/ml) were calculated by SPSS software.

Statistical analysis

The data obtained were expressed as mean ± SD and analysed statistically by the one-way ANOVA method. Differences in groups were evaluated using Student's *t*-test.

Results and Discussion

The first factor which should be considered in selecting a supercritical process for particle design is the solubility of the solutes in the supercritical fluid.[13] There is a report about the solubility of 5-FU in SC-CO_2, which was performed at pressures and temperatures ranging from 100–220 bar and 35–55°C measured by dynamic methods.[14] The results indicated that SC-CO_2 may be successfully employed as an antisolvent agent for particle formation of 5-FU.

Preparation of 5-FU polymeric matrices using supercritical antisolvent

Successful production of particles of pure biodegradable polymers has increased the interest in generation of polymeric particles containing active ingredients that can be used for controlled-release applications.[15] The supercritical-fluid-based particle formation techniques used for the production of composite polymer particles employ either co-precipitation or particle coating as the basic methodology.

In the supercritical antisolvent processes, SC-CO_2 acts as an antisolvent, which is dissolved in the organic solvent and reduces the solvent strength significantly. This leads to a high degree of supersaturation and nucleation of the solute.[16,17] As long as CO_2 is the fluid of choice, more polymers can be processed by using CO_2 as an antisolvent rather than using it as a solvent because most of the polymers have very limited or nearly zero solubility in carbon dioxide.[15]

Co-precipitation of 5-FU and PLGA using SC-CO_2 as antisolvent was carried out to assess the ability of the technique to produce sustained-release formulations of 5-FU as a hydrophilic drug. Preparation was performed with two processing methods, SAS and GAS, and the effects of some operational parameters were evaluated.

SAS process

The experimental conditions of SAS are summarized in Table 1. 5-FU is a polar compound with low solubility in SC-CO_2 and organic solvents. Among the organic solvents,

Table 2 Summary of physicochemical properties of 5-FU/PLGA matrices produced by supercritical antisolvent

Run	Loading	Observation
S_1	–	Film on filter
S_2	43%	Agglomerated particles
S_3	–	Film on the bottom of vessel
S_4	–	Film on the bottom of vessel
S_5	45%	Particles
G_1	9.76%	Particles
G_2	4.9%	Particles

methanol is the proper solvent for 5-FU. Conversely, PLGA is freely soluble in dichloromethane. In order to study the effect of the solvent, two different solvent systems have been used. In run S_2 a mixture of solvents, methanol/dichloromethane in a ratio of 1 : 2, was used. In this case, methanol acts as an antisolvent for the polymer and hence 5-FU precipitates quickly and PLGA, which is supersaturated, precipitates immediately and forms particles. However, employing acetone, which is a milder solvent for both materials and has lower vapor pressure than dichloromethane, leads to the formation of a film on the filter and no particles are formed. Polar organic solvents and solvents possessing low vapor pressures usually do not expand rapidly and/or completely in SC-CO_2.[18]

Surveying the influence of temperature on polymeric particle formation, run S_3 was performed at 45°C. A thin layer film was observed in the bottom of the vessel (Table 2). Since the glass transition temperature of PLGA is reduced under supercritical conditions, the mobility of PLGA chains is increased and the PLGA particles merge to form a continuous, swollen polymer phase under SC-CO_2 and no individual particles are therefore observed.[19]

To assess the effect of polymer concentration, low and high concentrations of PLGA at 3 and 27 mg/ml were prepared. The product from run S_4 was recovered as a film that adhered firmly to the vessel walls and to the filter. SC-CO_2 is completely miscible with the solvent, while the polymer and SC-CO_2 are partially miscible. When the composition of the polymer-rich phase reaches the gelation point, the morphology of the polymer phase is 'frozen'; SC-CO_2 cannot penetrate well in this gel mass, and as a result cannot lead to complete expansion.[16,20]

In order to analyse the effect of molecular weight of polymer on the particles, run S_2 and S_5 were performed. Resomer® RG 505 and Resomer® RG 503 with higher and lower molecular weights, respectively, were used in these experiments. The results show that the particles formed by Resomer® RG 505 are agglomerates of smaller particles (Figure 1a and 1b). Polymers with higher molecular weights show higher solubilities in SC-CO_2. Supersaturation therefore happens faster, and smaller particles may be formed.[21] However, since these particles do not dry immediately they aggregate and form larger particles.

GAS process

Two experiments were performed by GAS operation at 40°C and 120 bar with two different ratios of 5-FU to PLGA, namely 5 and 10% (Table 1).

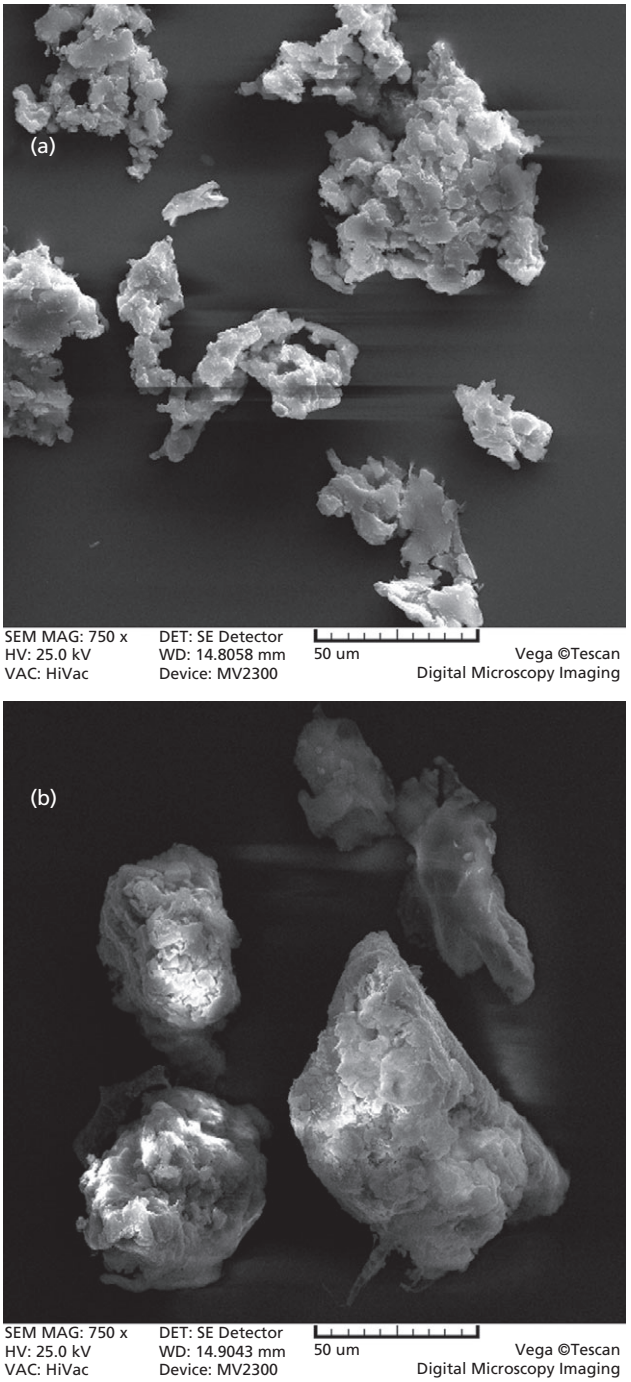

Figure 1 SEM images of SAS runs in the formation of 5-FU loaded matrices: (a) run S_2 (high molecular weight polymer), (b) run S_5 (low molecular weight polymer).

The solute concentration showed a great effect on particle size in GAS methods. At higher concentrations of solute, precipitation of the solute occurs earlier during the expansion process, resulting in increased time for crystal growth.[17] In this study, in order to ignore the effect of concentration of solute on particle size, the solution of 5-FU:PLGA was picked up at a constant concentration of 30 mg/ml in GAS runs.

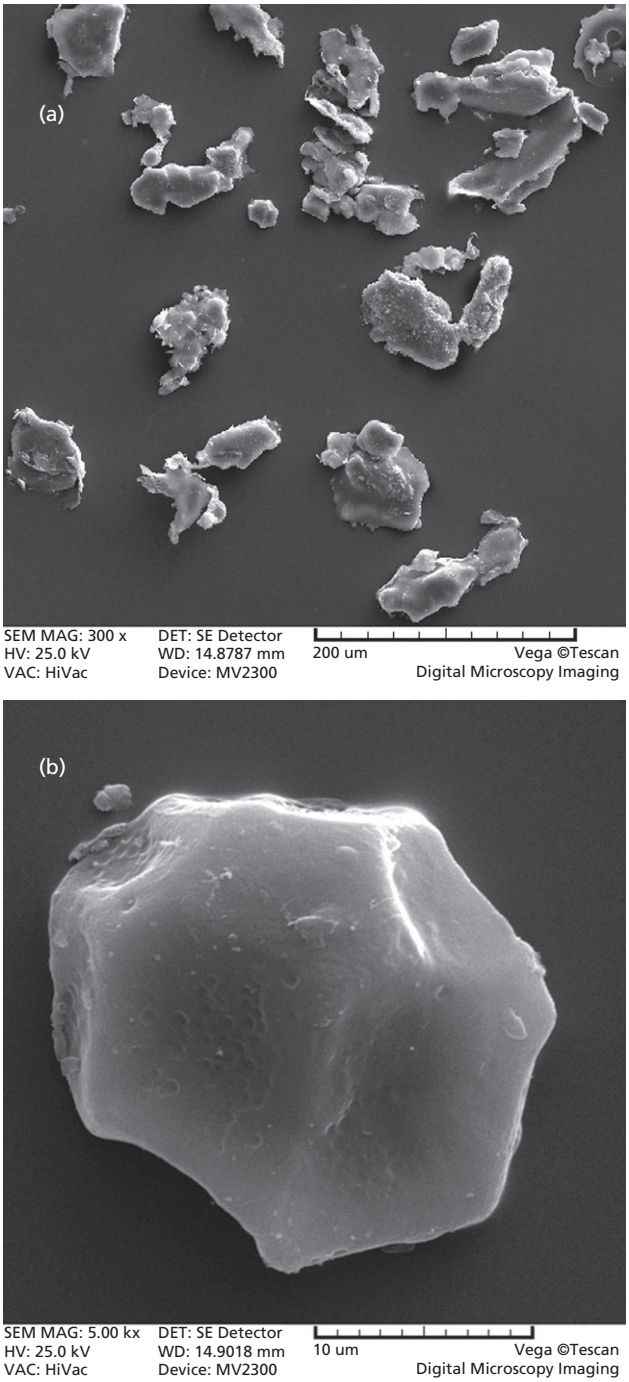

Figure 2 SEM images of GAS runs: (a) run G_1 (9.76% 5-FU in matrices), (b) run G_2 (4.9% 5-FU in matrices).

Since agitation rate has a strong influence on the particle-size distribution, the maximum agitation rate was employed in order to achieve improved results.

In both experimental conditions, precipitates were collected as non-agglomerated particles. The morphology was assessed through SEM pictures, as reported in Figure 2. The particles were not spherical, having oval or triangular shapes. No population of uncoated 5-FU was seen in either experimental condition. The co-precipitation of 5-FU and PLGA

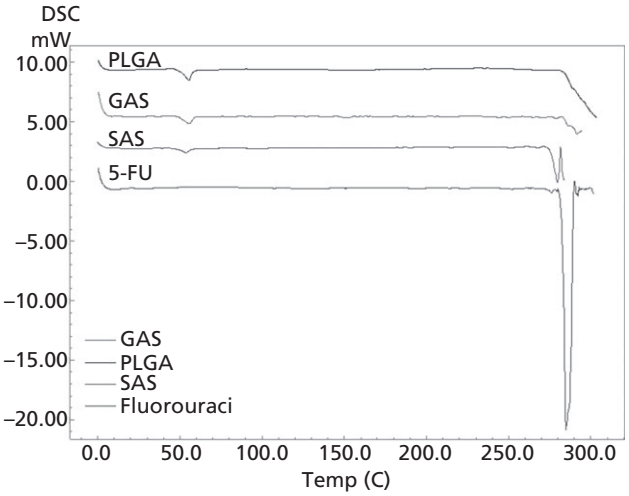

Figure 3 DSC thermograms of PLGA, GAS processed matrices, SAS processed matrices, and 5-FU.

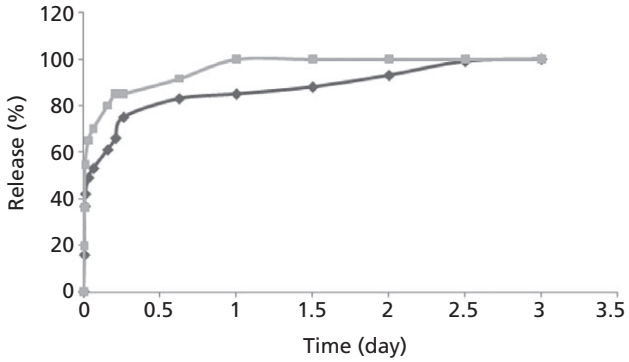

Figure 4 In-vitro release of SAS processed matrices run S_2 (◆) and run S_5 (■) in PBS pH: 7.4.

was successfully completed by the GAS precipitation method. The DSC curve of the particles showed both characteristic peaks of 5-FU and PLGA. In Figure 3, the same melting peaks of 5-FU were found for the 5-FU-loaded particles and unprocessed 5-FU powder. Nonetheless, the crystallinity of these polymeric matrices remained the same after the GAS process as that of the raw material of PLGA.

In-vitro drug release studies

The in-vitro release profiles of 5-FU from particles with different loadings and polymer molecular weights, which were carried out over 3 and 21 days, are shown in Figures 4 and 5, respectively. All release profiles showed a burst release in the first day, followed by a sustained-release phase. The particles with the highest loading (45%) and lower polymer molecular weight (S_5) gave the highest burst release and fastest drug release throughout the release period (85% of 5-FU release occurred in 3 h), while those with a loading of 4.9% (G_2) showed 30% release in 21 days.

Acidic substances, such as 5-FU, are released faster from PLGA matrices because of an acid-catalysed acceleration of polymer degradation.[22] The comparison of the release rates

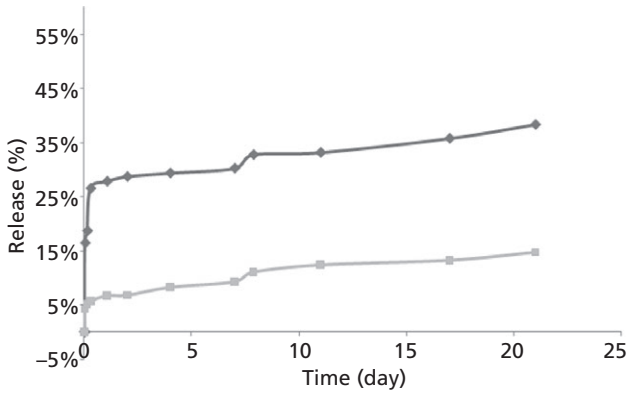

Figure 5 In-vitro release of GAS processed matrices G_1 (◆) and G_2 (■) in PBS pH: 7.4.

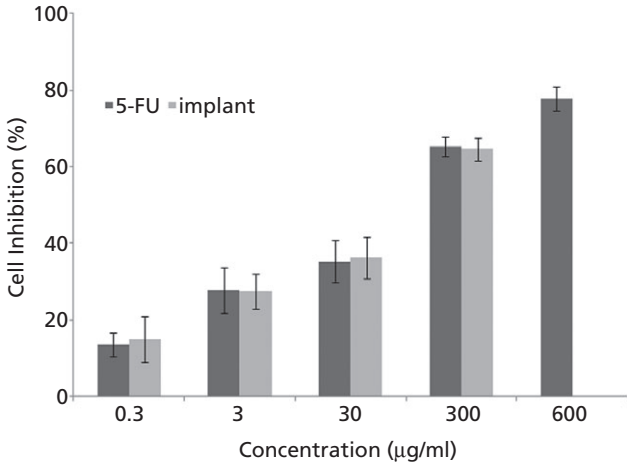

Figure 6 In-vitro cytotoxicity of incubated solution of 5-FU loaded matrices in A549 cells after 48 h.

of 43% (S_2), 9.76% (G_1) and 4.9% (G_2) 5-FU in Resomer® RG 505 showed that more 5-FU in the matrix will result in faster and greater release. This is due to two facts: first, the acidity of 5-FU, which will accelerate the release, and second, higher drug loading. The reason proposed for higher drug loading giving a more rapid release is that a high density of interconnecting channels increases polymer permeability in the presence of drug and as a result increases PLGA degradation and erosion.[22] These results are comparable with the results of another study reported by Gao *et al*. In their work, 5-FU release from fibers with drug loading of 5.6% was about 25% in 21 days and the release from fibers with 33.3% was almost 95% in 1 day.[23]

In-vitro cytotoxicity tests

Human lung carcinoma A549 cell line was used to evaluate the antitumor activity of 5-FU. To exclude the effects of sample pollution, the microparticles were sterilized by UV for 30 min. As shown in the Figure 6, at the same concentration of 5-FU, the incubated solution of polymeric microparticles exhibits the same inhibition effect as unprocessed 5-FU does. The cell inhibition rates were about 67, 34, 27 and 14%, with respect to concentrations of 5-FU at 300, 30, 3 and 0.3 μg/ml. *t*-test analysis also showed that there is no significant difference between the cell inhibition rates of the incubated solution of matrices and free 5-FU at the same drug concentration level ($P > 0.05$). The IC50s of untreated 5-FU and 5-FU:PLGA microparticles were 69.12 and 68.71 μg/ml at 48 h exposure, respectively.

The results of one-way ANOVA indicates that the cell inhibition rates of concentrations of 3, 30 and 300 μg/ml of 5-FU and the incubated solution of polymeric microparticles were significantly different to that of negative control group ($P < 0.001$). A concentration of 0.3 μg/ml with $P < 0.01$ also has a significant difference to the negative control group. The blank PLGA showed little cytotoxicity to the cells and the inhibition rate was less than 2%, and with $P > 0.05$ has no significant difference with negative control group.

As seen in Figure 6, the inhibition of human lung carcinoma cells A549 increases with increase of the drug concentration as well as the incubation time. The results indicate that the activity of 5-FU is not weakened during the process and the supercritical antisolvent processes could be a mild processing technique for maintaining drug activity.

Conclusion

Considering the advantages of supercritical carbon dioxide for industrial processing of anticancer drugs, we used GAS and SAS methods to co-precipitate 5-FU as a hydrophilic antitumor drug and PLGA as a biodegradable polymer with the aim of forming sustained-release matrices. The process conditions were mild and non-aqueous, so the hydrophilic drug incorporated in the PLGA had good stability during the process. More importantly, the matrices could achieve long-term release profiles with partial burst effect. With more 5-FU loading in the produced microparticles, drug release was faster and the amount of burst release was greater. The rapid release probably represented the release of drug-loaded microparticles with a high ratio of 5-FU and lower polymer molecular weight. Particles with 4.9% 5-FU and PLGA with high molecular weight showed the slowest drug release.

The MTT test was performed on human lung carcinoma cell lines (A549), in order to assess the activity of 5-FU, and the cell inhibition and IC50 were determined. There was no significant difference between the cell inhibition rates of the released drug and unprocessed 5-FU at the same concentration level.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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