

Article

***In Vitro* and *In Vivo* Evaluation of a Folate-Targeted Copolymeric Submicrohydrogel Based on N-Isopropylacrylamide as 5-Fluorouracil Delivery System**

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Abstract: Folate-targeted poly[(*p*-nitrophenyl acrylate)-*co*-(N-isopropylacrylamide)] nanohydrogel (F-SubMG) was loaded with 5-fluorouracil (5-FU) to obtain low ($16.3 \pm 1.9 \mu\text{g}$ 5-FU/mg F-SubMG) and high ($46.8 \pm 3.8 \mu\text{g}$ 5-FU/mg F-SubMG) load 5-FU-loaded F-SubMGs. The complete *in vitro* drug release took place in 8 h. The cytotoxicity of unloaded F-SubMGs in MCF7 and HeLa cells was low; although it increased for high F-SubMG concentration. The administration of $10 \mu\text{M}$ 5-FU by 5-FU-loaded F-SubMGs was effective on both cellular types. Cell uptake of F-SubMGs took place in both cell types, but it was higher in HeLa cells because they are folate receptor positive. After subcutaneous administration (28 mg 5-FU/kg b.w.) in Wistar rats, F-SubMGs were detected at the site of injection under the skin. Histological studies indicated that the

F-SubMGs were surrounded by connective tissue, without any signs of rejections, even 60 days after injection. Pharmacokinetic study showed an increase in MRT (mean residence time) of 5-FU when the drug was administered by drug-loaded F-SubMGs.

Keywords: drug delivery; folate-conjugate nanogel; 5-fluorouracil; culture cells; pharmacokinetic

1. Introduction

5-Fluorouracil (5-FU) is one of the chemotherapeutic compounds most used for the treatment of a great variety of tumors [1], such as colorectal cancer [2-4], breast cancer [5], pancreatic cancer [6], or gastric cancer [7]. It is an antimetabolite of pyrimidine analogue type that acts mainly through the inhibition of synthesis of DNA and RNA during the S-phase of the cell cycle. It is metabolized rapidly in the body which may affect the therapeutic response of the drug [8-10]. Moreover, due to its short plasma half-life and poor oral absorption, 5-fluorouracil has been chosen to be included in different controlled release systems [8-13]. In this type of system, the drug is protected from degradation following administration, the delivery system can be administered close to the tumoral cells, the drug is released with a specific patron and the action of the drug on tumoral cells can be direct. Among them, many nano-size drug delivery systems (liposomes, liquid-core nanocapsules, dendrimers, polymer-drug conjugates, nanoparticles, polymeric micelles and polymeric nanogels) have been developed [14,15] for the treatment of pathologies as cancer [16,17]. Due to their size, nanocarriers can penetrate within even small capillaries, and they can be taken up, in many cases, very efficiently by cells, internalized and stored into cytoplasm or different organelles [18]. Nanocarrier uptake into a cell depends on the cell-type, since some cells are more susceptible to include nonfunctionalized systems via their design. An active targeting strategy can improve the efficacy of the therapy and diminish side effects associated with drugs, since not all nanocarriers can overcome the cell membrane barrier without a targeting motif. To increase the delivery of a given drug to a specific target site, targeting ligands are conjugated to carriers [14]. Folate has been extensively investigated for targeting various tumor cells overexpressing folate receptors [14,19]. The folate in target drug delivery systems is primarily due to convenient and easy conjugation step and to the high affinity for the folate receptor after conjugation [20] which enables transportation of the conjugate via receptor mediated endocytosis.

Folate-targeted nanoparticles have shown to be effective in a number of tumors using folic acid-polymer conjugates [21] and liposomes [22]. Among nanocarriers, nanogels are promising vehicles for drug delivery. A hydrogel is a three-dimensional cross-linked network capable of swelling in biological fluids and retaining a large amount of fluids in the swollen state [23]. Nanogel particles are gels with dimensions in the colloidal range (10–1,000 nm). These nanosystems can be synthesized using very different monomers, which make them capable of responding to external stimuli such as pH or temperature [24,25]. Furthermore, the presence of reactive pendant groups in nanogels eases their vectorization forward specific cell motif by binding ligands as folate molecules.

In this work, folate-conjugate submicrogels (F-SubMGs), synthesized by Katime's research group [26], have been evaluated for antitumor therapy by loading them with 5-fluorouracil. Colloidal

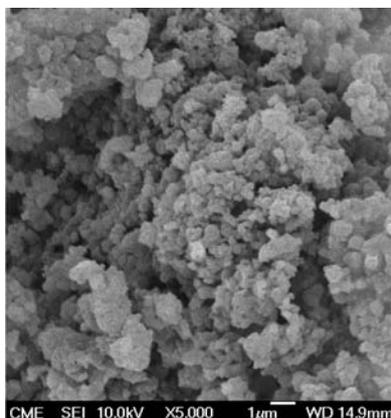
nanosize folate-conjugate hydrogels were prepared via a post-synthetic pathway starting with poly(*p*-nitrophenyl acrylate-*co*-*N*-isopropylacrylamide) [25,26]. A specific submicrogels composition with a folic acid concentration of 1.3 ± 0.8 mmol/g gel and an average size of 601 ± 28 nm was employed as a 5-fluorouracil delivery system *in vitro*, in the cellular lines MCF7 of breast cancer and HeLa of cervical cancer, and *in vivo* in female Wistar rats. Thus, administration of 5-FU by drug-loaded folate-conjugate submicrogels can be an approach to achieve high concentrations of the antineoplastic drug near to the target site for a given period of time, increasing its therapeutic effect.

2. Results and Discussion

2.1. In Vitro Studies

The size of F-SubMGs employed in this work was 601 ± 28 nm, which was determined in water by quasielastic light scattering [26]. 5-FU is not soluble in organic solvent, slightly soluble in metanol (5 mg/mL) [8], but its solubility increases to 13 mg/mL in aqueous medium [27]. Thus, load of F-SubMGs with the drug was carried out by swelling of nanocarriers in 5-FU solution in phosphate buffer (1 mM, pH 7.4) using two solutions of different concentrations, 0.4 mg 5-FU/mL and 7 mg 5-FU/mL. 5-FU loads were 16.3 ± 1.9 μ g 5-FU/mg F-SubMG (named low load 5-FU-loaded F-SubMGs) and 46.8 ± 3.8 μ g 5-FU/mg F-SubMG (named high load 5-FU-loaded F-SubMGs), respectively. SEM micrographs of 5-FU-loaded F-SubMGs showed a spherical morphology (Figure 1).

Figure 1. SEM micrograph of 5-FU-loaded folate-conjugate nanogel (F-SubMGs) after freeze-drying.

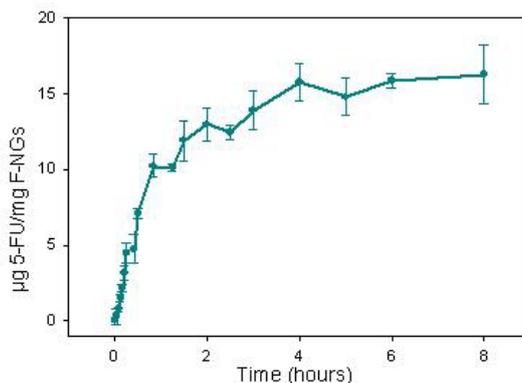


The *in vitro* release of drug from low load 5-FU-loaded F-SubMGs is shown in Figure 2. The release of the drug was complete in 8 h, and two 5-FU release stages with different rates were determined. A quick release of the most external 5-FU from F-SubMGs were observed during the first 50 min, which took place at a rate of 12.6 μ g 5-FU/h per mg F-SubMGs ($r^2 = 0.97$). Afterward, a slower rate of 0.85 μ g 5-FU/h per mg F-SubMGs ($r^2 = 0.90$) from 50 min to 8 h was determined.

In vitro experimental conditions employed in this work were based on previous studies, in which swelling of these submicrogels was determined to be pH-, ionic strength- and temperature-sensitive [25,26]. Taking into account that nanogel swelling increases with acid pH (pH 5.2), high ionic strength (more than 0.02 M) and temperature lower than 33 °C, 5-FU release from drug loaded F-SubMGs was carried

out at 37 °C in phosphate buffer 1 mM and pH 7.4, similar to biological fluids conditions, in which drug solubility was favorable.

Figure 2. Cumulative amount of 5-fluorouracil (5-FU) released from low load 5-FU-loaded F-SubMGs.



2.2. Cell Culture Studies

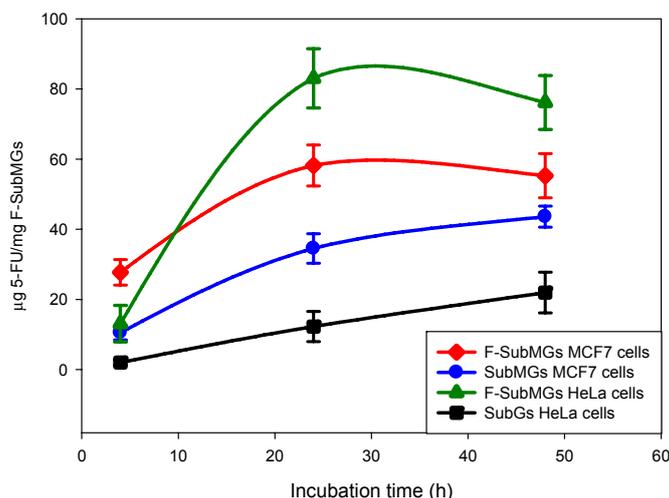
The over expression of folate receptors (FR) in many cancer cells [14,19] has been considered to target anticancer drug-loaded nanocarriers by binding folate molecules in the surface of such nanosystems, which can be transported via receptor mediated endocytosis. One of the main advantages of these controlled drug delivery systems is that the folate conjugate submicrogels could be included mainly in tumor cells, avoiding toxic effects in normal cells.

In this work, antitumoral efficiency of 5-fluorouracil loaded folate-conjugate submicrogels (5-FU-loaded F-SubMGs) has been evaluated in two cellular lines, MCF7 of breast cancer, and HeLa of cervical cancer, which are folate receptor negative (FR(-)) and folate receptor positive (FR(+)), respectively. Thus, on one hand, cellular uptake of folate conjugate submicrogels and submicrogels without folate were studied to evaluate the effect of folic acid on intracellular transport of nanocarriers. On the other hand, the viability of those cells lines was evaluated in the presence of unloaded, 5-FU-loaded F-SubMGs and 5-FU in solution.

Fluorescence markers are frequently used in cellular uptake studies [28,29]. In our case, in order to measure cell uptake, folate-conjugate submicrogels (F-SubMGs) as well as control submicrogels (SubMGs) were loaded with Alexa Fluor[®] 488.

The presence of submicrogels was confirmed in both cell types, which was easily observed by contrast microscopy and by fluorescence microscopy since Alexa Fluor[®] 488 has green fluorescence. Nevertheless, these methods do not show the amount of submicrogels inside the cell and to allow evaluation of the role of folic molecule in their vectorization. Therefore, studies with flow cytometry were carried out. Figure 3 shows plots of total nanogel-associated events as a function of incubation time, obtained from the corresponding histogram. The cells grown in the presence of Alexa Fluor[®] 488 were used as a control.

Figure 3. Uptake of Alexa Fluor[®] 488-loaded folate-conjugated submicrogels (A448 F-SubMGs) and submicrogels without folate (A448 SubMGs). Quantitative comparison of total submicrogels associated events as a function of incubation time (4, 24 and 48 h) for MCF7 and HeLa cells obtained from the corresponding histogram of flow cytometry assay: A488 F-SubMGs in MCF 7 cells (♦); A488 SubMGs in MCF 7 cells (●); A488 F-SubMGs in HeLa cells (▲); A488 SubMGs in HeLa cells (■).



Cell uptake of control submicrogels (SubMGs) was observed in both cell types, and was larger for MCF7 cells at all incubation times (Figure 3). So that, after 4 h, 24 h and 48 h of incubation, penetration of SubMGs into cells was 10.51%, 34.52%, and 43.59%, respectively in MCF7 cells, and it was 2%, 12.26% and 21.94%, respectively in HeLa cells. Thus, the uptake of these SubMGs took place by unspecific mechanisms, and was more favorable in MCF7 cells.

Cell uptake of folate-submicrogels (F-SubMGs) was larger for MCF7 cells at 4 h of incubation (25%), than in HeLa cells (15%). However, the presence of folic acid on the nanogel surface caused a significant improvement of the submicrogels uptake in HeLa cells compared to MCF7 cells after 24 h of incubation (80.04% for HeLa; 58.18% for MCF7) whose level was maintained at 48 h (Figure 3).

These results can be related to the expression of folate receptor (FR) in one of two cellular lines studied. The carried out studies have shown that the FR type α levels in the tumors are variable and are generally relatively low in estrogen receptor positive (ER(+)) tumors [30]. It has been described that HeLa cells are FR(+), but MCF7 cells are FR(−) and ER(+) [31]. Therefore, the uptake of F-SubMGs in MCF7 is mainly by unspecific interactions, although the presence of folic acid seemed to improve the uptake when compared with that of control sample. In this way, Lee and co-workers [32] observed in MCF7 cells that the introduction of folate into adriamycin-loaded polyHis/PEG-folate micelles and PLLA/PEG-folate micelles enhanced the cell killing effect by active internalization with respect to the same micelles without folate.

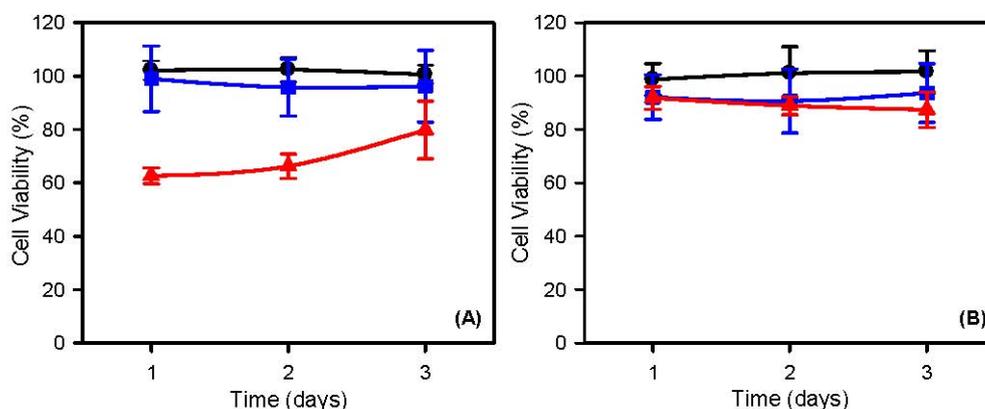
Our studies on cell uptake of F-SubMGs showed that these submicrogels were internalized into both types of cells, independently of the presence of FR on their surface, although the percentage of HeLa cells that internalized the F-SubMGs was higher. In this way, studies carried out by Sonvico and co-workers [31] with folate-conjugated iron oxide nanoparticles showed that these nanoparticles were taken up by HeLa as well as MCF7 cells in a similar proportion after 30 min of incubation with the

nanoparticles. Although in our studies, the percentage of cell uptake of folate conjugated submicrogels in HeLa cells was slightly larger than MCF7 cells. Therefore, the largest fluorescence signal in HeLa cells, which are FR(+), seemed to indicate that they are able to internalize more of this nanocarrier, thereby producing the entry of the functionalized submicrogels mainly by a receptor-mediated endocytosis.

Cell viability was evaluated by the methylthiazolotetrazolium (MTT) method to study the cytocompatibility of unloaded F-SubMGs and antitumoral availability of low load 5-FU-loaded F-SubMGs.

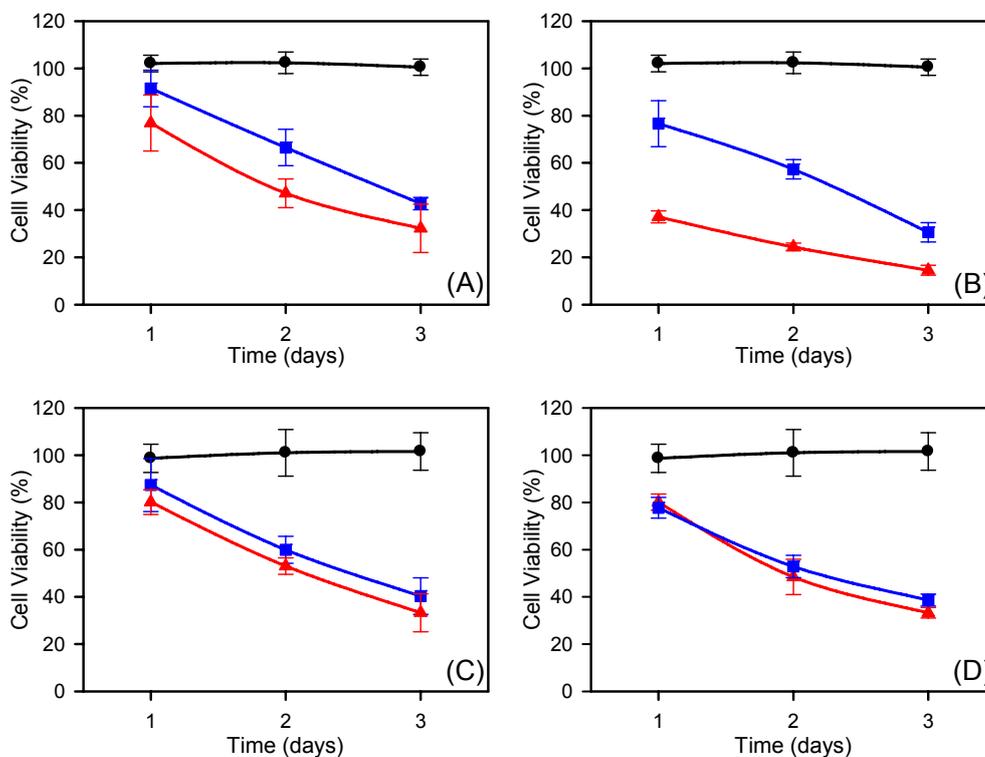
The range of cell survival, in the presence of low concentrations of unloaded F-SubMGs (80 $\mu\text{g/mL}$), was between $99 \pm 8\%$ and $96 \pm 13\%$ for MCF7 (Figure 4(A)), and between $94 \pm 11\%$ and $91 \pm 12\%$ for HeLa cells (Figure 4(B)) during 3 days of incubation. Thus, these F-SubMGs had good biocompatibility and produced minimal cytotoxicity. However, as the concentration of unloaded F-SubMGs increased up 400 $\mu\text{g/mL}$, a cytotoxicity effect was observed, specially in MCF7, whose range of cell survival was between $63 \pm 3\%$ and $80 \pm 11\%$, whereas in HeLa cells was between $92 \pm 4\%$ and $87 \pm 7\%$. Therefore, since MCF7 are FR(-), the greater sensitivity observed in these breast cancer cells may be related to nonspecific interactions, rather than a receptor-mediated endocytosis as was indicated above in studies of cellular uptake.

Figure 4. Cytotoxicity of unloaded folate-conjugate submicrogels (F-SubMGs). Cell viability of (A) MCF7 and (B) HeLa. Without F-SubMGs (●) or with unloaded F-SubMGs at a concentration 80 $\mu\text{g/mL}$ (■); 400 $\mu\text{g/mL}$ (▲); Concentration of F-SubMGs was in accordance with data of Table 2. Data were shown as mean \pm SD ($n = 15$).



On the other hand, two concentrations of 5-FU (10 and 50 μM) were administered to MCF7 and HeLa cells by drug solution and by 5-FU-loaded F-SubMGs (Figure 5). The concentration range of free 5-FU was selected based on preliminary experiments, in which increasing concentrations of 5-FU from 0.01 μM to 1 mM were tested in culture. Those drug concentrations were in accordance with data reported in the literature [33-35]. The administration of 5-FU 10 μM in solution produced a decrease of the percentage of viability in MCF7 cells, which was between $91 \pm 8\%$ and $43 \pm 3\%$ after 3 days of incubation.

Figure 5. Cytotoxicity of 5-fluorouracil-loaded folate-conjugate submicrogels (F-SubMGs). Cell viability of MCF7 (A,B) and HeLa (C,D): in the presence of 10 μ M (A,C) and 50 μ M (B,D) of 5-fluorouracil (5-FU): Without drug (\bullet); 5-FU in solution (\blacksquare); 5-FU-loaded F-SubMGs (\blacktriangle). Data were shown as mean \pm SD ($n = 15$).



When the drug was administered by 5-FU-loaded F-SubMGs at the same drug concentration, the viability was lower (between $77 \pm 12\%$ and $32 \pm 10\%$) (Figure 5(A)). Since this F-SubMGs concentration (80 μ g/mL) did not cause cytotoxic effect on MCF7 cells (Figure 4(A)), the efficacy of 5-FU administered by 5-FU-loaded F-SubMGs was evident. When 5-FU concentration was larger (50 μ M), a decrease in viability was also observed in the presence of the drug in solution ($77 \pm 10\%$ – $31 \pm 4\%$) as well as 5-FU-loaded F-SubMGs ($37 \pm 2\%$ – $14 \pm 2\%$). In this case, the decrease in cell viability is due to the drug administered by 5-FU-loaded F-SubMGs, but a percentage of the observed decrease must be attributed to the high F-SubMGs concentration (Figure 4(A)).

The survival of HeLa cells was not modified by increasing 5-FU concentration (Figure 5(C,D)), which indicated a lower sensitivity to this drug than MCF7 cells. The administration of 5-FU in solution at 10 and 50 μ M caused a similar decrease in HeLa viability ($87 \pm 11\%$ – $40 \pm 8\%$ at 10 μ M; $78 \pm 4\%$ – $39 \pm 2\%$ at 50 μ M). The administration of the drug by 5-FU-loaded F-NGs produced the same effect as the drug in solution at the beginning of the experiment, which was in accordance with the low percentage of F-NGs uptake at short time (Figure 4) and the kinetic release of the drug (Figure 2) from F-NGs.

2.3. In Vivo Studies: Biocompatibility and Pharmacokinetic

The *in vitro* 5-FU release, as well as the effect of 5-FU-loaded F-SubMGs on viability of MCF7 and HeLa cells, inform on the possible use of this system for targeted drug administration as potential

antitumoral treatment. However, it is necessary to know the behavior of 5-FU-loaded F-SubMGs *in vivo*, because the presence of physiological substances, such as enzymes, ions, or cells, may influence the drug release. Moreover, the interaction between the 5-FU-loaded F-SubMGs and the living tissues can be harmless for the organism. So that, 5-FU-loaded F-SubMGs were subcutaneously administrated to rats in order to evaluate their biocompatibility and the plasma levels of the drug in blood stream. The small size of submicrogels allowed their subcutaneous implantation in the back of rats by injection using a conventional syringe. The amount of unloaded and drug-loaded F-SubMGs injected was 133 mg, which corresponds with the selected dose of 5-FU (28 mg 5-FU/kg b.w) taking into account the 5-FU load of the high load formulation ($46.8 \pm 3.8 \mu\text{g}$ 5-FU/mg F-SubMGs).

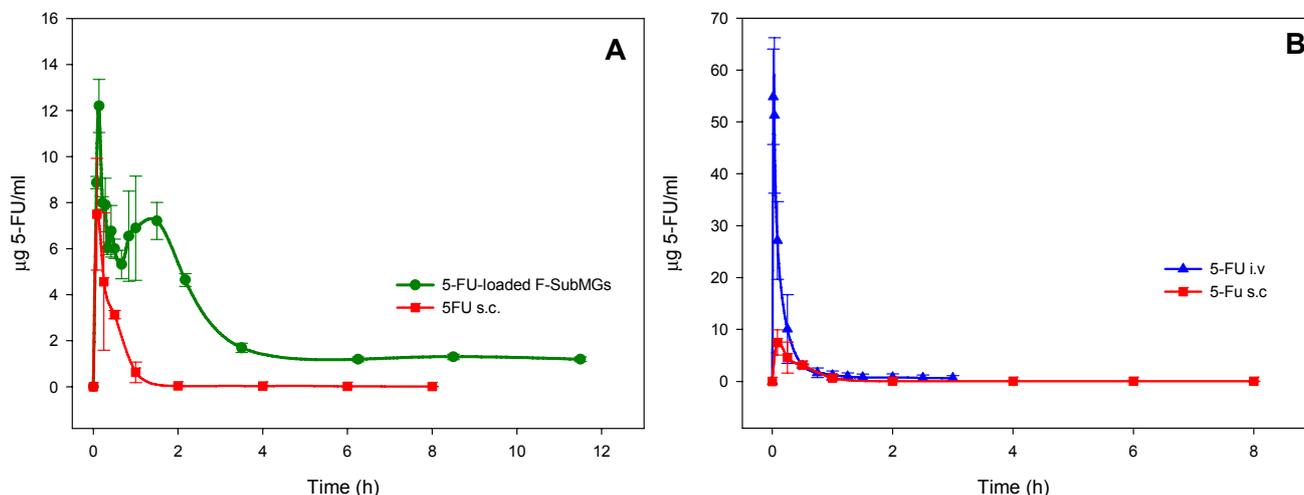
The maximum tolerated dose of 5-FU in rats is 100 mg/kg of body weight [36], and doses of 20 mg/kg of 5-FU has also been administered by intraperitoneal route in rats for seven days [37]. Furthermore, 35 and 50 mg/Kg b.w. have been subcutaneously administered in rats by polyester microspheres [38]. Hence, the dose utilized in our experiment was in accordance with above mentioned doses.

After administration of 5-FU loaded F-SubMGs (Figure 6(A)), the drug was detected in the blood stream at 5 min, and the maximum drug concentration ($12.2 \pm 1.1 \mu\text{g/mL}$) was reached at 8 min. Then, the drug concentration in plasma decreased at 40 min, to increase again reaching a second maximum at 90 min ($7.2 \pm 0.8 \mu\text{g/mL}$). From then, a decrease of 5-FU plasma concentration was observed up to 3.5 h, and 5-FU concentration was almost constant ($\approx 1.2 \mu\text{g/mL}$) up to 11.5 h. The plasma concentration of 5-FU obtained during the first stage of release from F-SubMGs can be considered the result of the quick release of the drug observed *in vitro*. Whereas, the following lower levels of the drug *in vivo* seemed to be caused by the slower second stage of *in vitro* 5-FU release from nanocarriers.

When 5-FU was intravenously administered at the same dose in solution, the maximum drug concentration was $55 \pm 9 \mu\text{g/mL}$ and it was not detected starting at 3 h (Figure 6(B)). The subcutaneous injection of 5-FU in solution resulted in a maximum concentration of $7.2 \pm 2 \mu\text{g/mL}$, 5 min after administration, with the drug being detected in plasma for 8 h (Figure 6(B)).

Differences observed in residence time and in plasma concentration of 5-FU showed the effect of the administration route as well as the different formulation of the drug used. When a drug is given in different dosage forms or by different routes of administration, it will yield varying amounts of absorbed drug and, hence, differences in onset, intensity, and duration of the pharmacologic or clinical effect [39]. After its administration, the drug has to be released from the dosage form and then be absorbed into the systemic circulation by passing through various membranes, except when the drug is administered intravenously in the form of a solution [39]. A polymer drug delivery system exerts a protection on the loaded drug against its degradation or metabolism. In the case of 5-FU, its pharmacokinetic is characterized by a rapid distribution and elimination phases resulting in a short plasma half-life (10–20 min) [40].

Figure 6. Plasma concentration of 5-fluorouracil (5-FU) after: (A) subcutaneous injection of 5-FU-loaded folate-conjugate submicrogels and subcutaneous (s.c.) injection of 5-FU; (B) subcutaneous and intravenous injection of an aqueous solution of 5-FU; Drug dose: 28 mg 5-FU/kg b.w.



After administration, 5-FU is metabolized by extracellular catabolic or intracellular anabolic pathways. More than 85% of an administered dose of this anticancer agent is degraded through the catabolic pathway in the liver by dihydropyrimidine dehydrogenase (DPD) [1,41], and only a reduced part of 5-FU is available for bioactivation. DPD is the rate-controlling enzyme of endogenous pyrimidine and fluoropyrimidine catabolism [1]. This enzyme is present in most of the tissues including liver and peripheral blood mononuclear cells, although the DPD activity is much greater in liver than in any other tissue as gastrointestinal tract, kidney and spleen [40]. DPD activity is highly variable in the population [1]. In this way, a considerable variability (interpatient and inpatient) in pharmacokinetic studies of both bolus and continuous infusion therapy of 5-FU is thought to be due mainly to variability in DPD activity and consequent variability in the rate of catabolism. Moreover, DPD also displays a circadian variation [40].

Thus, the drug absorption from polymer drug delivery systems after administration depends on the release of the drug from the carrier, the permeability across the tissue barriers, the dissolution of the drug under physiological conditions, and finally, the drug metabolism.

As mentioned above, the administration route and the formulation of 5-FU determined pharmacokinetics and responses to the drug. The values of pharmacokinetic parameters of 5-FU are summarized in Table 1. MRT and AUC reflect the residence time and amount of free drug respectively, in systemic circulation. AUC values allow to calculate the absolute [AB (%) = $(\text{AUC}_0^{\infty}\text{s.c.}/\text{AUC}_0^{\infty}\text{i.v.}) \times (\text{i.v. dose/s.c. dose}) \times 100$] and relative bioavailability [42] [RB (%) = $(\text{AUC}_0^{\infty}\text{nanogel s.c.}/\text{AUC}_0^{\infty}\text{solution s.c.}) \times 100$] of different formulations of 5-FU. The absolute bioavailability of 5-FU was 33% when the drug in solution was subcutaneously administered, whereas the relative bioavailability of 5-FU was 983% when 5-FU-loaded F-SubMGs was subcutaneously administered. On the other hand, the mean residence time (MRT) showed a larger value (4.4 and 7.2 times larger) when 5-FU was subcutaneously administered by F-SubMGs, in comparison with the subcutaneous and intravenous administered of 5-FU in solution (Table 1).

Therefore, the more prolonged presence of 5-FU in plasma after its administration by F-SubMGs, can be considered an advantage for drugs with short half-life, due to a protection on the drug loaded into polymeric systems against its degradation or metabolism. Moreover, side effects of 5-FU on whole body, could be minimized by maximizing the drug concentration in the target tissues due to a possible uptake into a cell via receptor mediated endocytosis as active targeting of 5-FU-loaded F-SubMGs.

Table 1. Pharmacokinetic parameters of 5-Fluorouracil (5-FU) after subcutaneous injection of the 5-FU-loaded folate-conjugate submicrogels (F-SubMGs), subcutaneous (s.c.) injection of 5-FU, intravenous (i.v.) injection of 5-FU in solution.

	5-FU-loaded F-SubMGs	5-FU s.c.	5-FU i.v.
AUC_0^∞ ($\mu\text{g}\cdot\text{h}/\text{mL}$)	36.4 ± 2.3^a	3.7 ± 1.1^b	11.2 ± 3.9
$AUMC_0^\infty$ ($\mu\text{g}\cdot\text{h}^2/\text{mL}$)	233.4 ± 9.1^a	4.1 ± 0.7	10.4 ± 5.9
MRT (h)	6.5 ± 0.1^a	1.2 ± 0.4	0.9 ± 0.3

Data are presented as mean \pm standard deviation (n = 6). Dose of 5-FU: 28 mg 5-FU/kg body weight; i.v.: intravenous administration; s.c.: subcutaneous administration. AUC: area under the plasma concentration *versus* times curve; AUMC: area under the concentration times time *versus* time curve; MRT: mean residence time; a: significant difference with regard to 5-FU s.c. y 5-FU i.v. injection groups, $p \leq 0.05$. b: significant difference with regard to 5-FU i.v. injection group, $p \leq 0.05$.

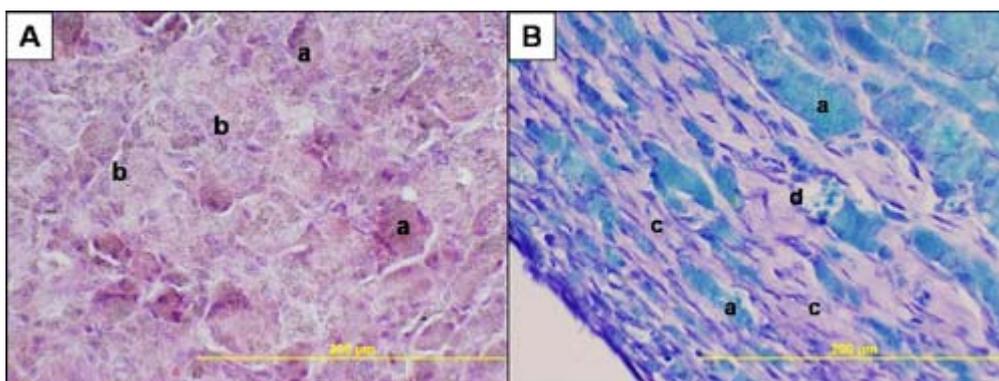
To evaluate the biocompatibility of F-SubMGs and follow the morphological changes of subcutaneously administered submicrogels, animals were sacrificed 15, 30 and 60 days after injection, and the tissue of the injection site was removed. A fibrous capsule, around the injected nanocarriers under the skin, was observed in every moment of study. This fact can be considered in accordance with the normal body reaction to a biocompatible material, which consists in walling it off in an avascular, collagenous bag. This event was also observed after the injection of unloaded and 5-FU-loaded PLA and PLGA microspheres [38].

Fifteen days after the nanogel injection, histological studies showed groups of nanogel surrounded by connective tissue inside of the capsule (Figure 7(A)). Lymphocytes or mast cells were not observed in any case. After thirty days of submicrogels injection, the histological study did not show differences with regard to the studies carried out at earlier times. However, at day sixty, more disperse and smaller groups of submicrogels, as well as a significant amount of collagen fibers between them, were observed inside of the capsule and there were blood vessels close to submicrogels groups (Figure 7(B)), whose size was reduced. Differences between animals injected with 5-FU-loaded F-SubMGs and animals injected with unloaded F-SubMGs were not observed in any case or at any time.

The implantation of biocompatible material induces the activation of humoral and cellular mechanisms to produce inflammatory and healing responses of the material. Polymorphonuclear leukocytes, monocytes and lymphocytes are the cell types associated with this event [38]. Accumulation of extravessel liquids in the implant area was not detected, which was indicative that there was not an acute inflammatory response when the animals were sacrificed. Thus, unloaded and 5-FU-loaded F-SubMGs did not cause immunological processes when they were subcutaneously injected, since lymphocytes or mast cells were not observed in the implants, and signs of rejection of the implanted submicrogels did not exist in any case. Therefore, histological studies carried out indicate that folate

conjugate submicrogels analyzed in this work present a high biocompatibility with tissue where they are placed.

Figure 7. Photomicrograph of a transversal cut of the removed capsule from the injection site after 15 (A) and 60 days (B). a: groupings of F-SubMGs; b: connective tissue; c: collagen fibers; d: blood vessels. Staining method: Hematoxylin-Eosin (A) and Toluidine Blue (B).



3. Experimental Section

3.1. Materials

5-Fluorouracil (Sigma-Aldrich, Barcelona, Spain), NaOH, KCl, NaCl, Na₂HPO₄, KH₂PO₄, K₂HPO₄ (Panreac, Barcelona, Spain) and Isoflurane (Isoba[®] vet, Schering-Plough, S.A., Madrid, Spain) were used as received. All the chemicals used were of the highest commercially quality available. Milli-Q[®] water (Millipore, Madrid, Spain) was used.

3.2. Preparation of 5-Fluorouracil-Loaded Folate-Conjugate Submicrogels (F-SubMGs)

Sub-microgel sample NPA35EA4AMP0.5FOL [26], whose composition is 4-nitrophenyl acrylate (NPA) 30 mol%, 4-methylpyridine (4-MP) 14 mol%, ethylamine (EA) 16 mol%, N-isopropylacrylamide (NIPAM) 70 mol% and folic acid concentration 1.3 ± 0.8 mmol/g sub-microgel, was used to obtain 5-Fluorouracil-loaded sub-microgels. Briefly, poly[*p*-nitrophenyl acrylate-*co*-(N-isopropylacrylamide)] [p(NPA-*co*-NIPAM)] precursor microgels were synthesized and characterized [25]. The *p*-nitrophenyl carboxylate groups in the initial microgels ensured an easy chemical functionalization by the nucleophilic attack of free amines. Furthermore, these modification reactions can be monitored by UV spectroscopy because *p*-nitrophenol, the leaving group during nucleophilic substitution at the carboxylate group, shows a characteristic absorbance peak at 400 nm. These reactive sub-microgels were specifically and partially functionalized with 4-MP in a first functionalization sequence, by the reaction with 4-aminomethylpyridine. The second step of the synthesis strategy was the introduction of EA groups, which allows the subsequent coupling of folic acid with the primary amine group. The functionalization with EA was carried out by the nucleophilic attack with previously synthesized N-ter-butoxycarbonylethylenediamine.

Folate-conjugate submicrogels (F-SubMGs) were loaded with 5-FU by immersion in solutions of the drug with two solutions with different concentrations (0.4 mg/mL and 7 mg/mL) in phosphate buffer (1 mM, pH 7.4) at room temperature for 72 h. The suspension was centrifuged (41,000 rpm, 15 min, Beckman Coulter OptimaL-100XP Ultracentrifuge), and then, the pellet was freeze-dried (Heto PowerDry LL 1500 Freeze Dryer, Thermo Electron Corporation).

Morphology of 5-FU-loaded F-SubMGs was determined by SEM (Jeol JSM-6400 Electron Microscope, 150 s, 20 mA, 2 kV). The samples were fixed to an adhesive sheet on a rigid support and coated with gold for their later visualization.

3.3. Estimation of 5-Fluorouracil Content

To determine the amount of 5-FU included in the F-SubMGs, 10 mg of the drug-loaded submicrogels were swollen in 10 mL of phosphate buffer (1 mM, pH 7.4) at 25 °C (since LCST of PNIPAA is 32 °C and NIPAA gels are swollen at 25 °C) in orbital shaking (100 rpm) for 72 h. At intervals, the suspension was vigorously shaken in vortex and centrifuged (41,000 rpm, 15 min). Then, 100 µL of supernatant was collected, and used to determine the amount of 5-FU by HPLC (Spectra-Physics SP8800 HPLC pump, SP 100 UV absorbance detector and SP 4400 computing integrator) [38]. The stationary phase was Spherisorb ODS2, C₁₈, 5 µm (25 × 0.46 cm; Waters), and the mobile phase was KH₂PO₄ 0.01 M, pH 4. The flow rate was set at 1 mL/min and the detector wavelength was 266 nm. For calibration curve, solutions of 5-FU in phosphate buffer (1 mM, pH 7.4) between 0.025–100 µg/mL were used, and a good linear correlation ($r^2 = 0.99$) was obtained. The validity of the HPLC method was demonstrated from the precision of the assay calculating the coefficient of variation (CV) [43]. The CV for intra-run of 5-FU at 0.025, 0.1, 5 and 75 µg/mL were 4.1%, 3.1%, 2.1% and 1.4% respectively; and 7.7%, 6.5%, 3.7% and 2.4% for inter-run at the same concentrations. The 5-FU retention time was 6.81 ± 0.15 min. Stability of 5-FU was also studied by UV/Vis Spectra (188–600 nm) (UNICAM 8700 Spectrophotometer). The experiment was carried out in triplicate.

3.4. In Vitro Release of 5-Fluorouracil from Folate-Conjugate Submicrogels (F-SubMGs)

For drug release studies, 10 mg of 5-FU-loaded F-SubMGs were added to 2 mL phosphate buffer (1 mM, pH 7.4) contained in a dialysis bag (6-8000, Serva 20/32) which was placed in an erlenmeyer containing 10 mL of the same phosphate buffer at 37 °C and at constant shake (100 rpm) in an orbital incubator (Ecotron[®] Inforts AG CH-4103). At intervals, 100 µL of samples were withdrawn from the solution in order to follow the change in 5-FU concentration by HPLC method described above. The release medium removed from the vessel was replaced with the same volume of phosphate buffer. In drug release experiments, 5-FU concentrations were always very much lower than its solubility; thus, sink conditions were maintained. The experiments were carried out in triplicate. In order to determine the time for the drug to reach dialysis equilibrium, a solution of 5-FU (5 mL of 0.56 mg/mL) in phosphate buffer (1 mM, pH 7.4) were dialysed. The time at which the drug in solution needed to reach dialysis equilibrium was 1 h.

3.5. In Vitro Cell Culture Studies

3.5.1. Cell Culture Maintained

Human breast adenocarcinoma (MCF7) cells were obtained from von Kobbe and human cervical cancer (HeLa) cells were obtained from Dr. Tierrez. Cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) + GlutaMax-I supplemented with 10% heat inactivated fetal bovine serum, penicillin (50 U/mL) (GIBCO[®], Invitrogen Life Technologies, Grand Island, NY, USA), streptomycin (50 µg/mL) (Invitrogen Life Technologies) and gentamicin (50 µg/mL) (Sigma-Aldrich Company, UK) in a humidified incubator at 37 °C and 5% CO₂ atmosphere (HERA cell, Sorvall Heraeus, Kendro Laboratory Products GmbH, Hanau, Germany). Cells were plated in 75-cm² flask (Sarstedt Ag and Co., Barcelona, Spain) and were passaged when reaching 95% confluence, by gentle trypsinization (0.05% trypsin/0.53 mM EDTA; Invitrogen Life Technologies).

3.5.2. Cellular Uptake of Submicrogels

Cellular uptake of submicrogels was investigated with a mono-culture of MCF7 and HeLa cells. Submicrogels (1 mg) were loaded with Alexa Fluor[®] 488 (A488; Molecular Probes, Eugene, OR) by immersion in a solution of A488 in DMSO (20 µg A488/100 µL DMSO) at 4 °C in dark for 48 h under orbital shaking (100 rpm). Then, submicrogels were freeze-drying at −110 °C. In order to establish a comparison between the cell uptake of folate-conjugate submicrogels (F-SubMGs) and submicrogels without folate, control submicrogels (SubMGs) of equivalent composition (sample R35EA4AMP0.5) [26] were used.

Cells were seeded in 24-well flat-bottom plates at 50,000 cells/well for MCF7 and 25,000 cells/well for HeLa. Twenty-four hours later, the medium was replaced with 1 mL medium with 1% FBS containing 377 µg of Alexa Fluor[®] 488-loaded submicrogels per mL of growth medium, which is very similar to the maximum concentration of submicrogels used in viability studies. As a control, cells were grown separately with 4 µL of Alexa Fluor[®] 488 per well in the absence of submicrogels. Cells were incubated during 24 h for microscopy studies and 4, 24 and 48 h for flow cytometry studies. Afterward, cells were washed three times with phosphate buffer saline (PBS) to remove submicrogels not internalized by the cells.

After final washing, PBS was replaced with 1 mL of growth medium in order to carry out microscopy studies. Localization of fluorescent dye was examined by light fluorescence microscopy (Leica DMIL microscope, Leica Microsystems, Switzerland). Cells cultures were photographed with a Leica DFC 300FX digital camera and used Leica Application Suite software for processing the pictures (Leica Microsystems, Switzerland).

Additionally, internalization of submicrogels into MCF7 and HeLa cells was evaluated using flow cytometry. Cells were harvested, washed with PBS, resuspended in PBS and analyzed in a FACScan flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA, USA). Cells were analyzed by gating based on forward and side scatter. Results are expressed as percent positive cells for uptake of submicrogels referred to auto-fluorescence of cells.

3.5.3. Cytotoxicity Assay

Cell viability was evaluated by using the methylthiazolotetrazolium (MTT; Sigma-Aldrich) method. All experimental conditions were performed in quintuplicate. Each experiment was carried out in triplicate. In preliminary experiments, increasing concentrations of 5-FU from 0.01 μM to 1 mM were tested in culture. The selected drug concentrations were 10 and 50 μM . Cells were seeded in 96-well flat-bottom plates at 5,000 cells/well in the case of MCF7 and at 2500 cells/well in the case of HeLa. Twenty-four hours later, the medium was replaced with 100 μL medium with 1% fetal bovine serum (FBS; Invitrogen Life Technologies) containing F-SubMGs, 5-FU-loaded F-SubMGs or the drug in solution. The amount of F-SubMGs was between 80 $\mu\text{g}/\text{mL}$ and 400 $\mu\text{g}/\text{mL}$ (Table 2), which was in accordance with the concentration of 5-FU used in the experiment, considering the low load 5-FU-loaded F-SubMGs. After 1, 2 and 3 days, each well was added with 10 μL MTT solution (5 mg/mL). After 2 h incubation at 37 $^{\circ}\text{C}$, 5% CO_2 , each well was replaced with 100 μL DMSO [44]. The cell viability was determined by measuring the absorbance at 570 nm using a spectrophotometer (Varioskan, Thermo Fisher Scientific, Barcelona, Spain). Results are presented as the percentage survival in relation to untreated control cells.

Table 2. Concentration of F-SubMGs needed to obtain 10 μM and 50 μM of 5-FU in the cell culture medium.

Formulation	5-FU load (μg 5-FU/mg F-SubMGs)	Concentration of F-SubMGs ($\mu\text{g}/\text{mL}$) to obtain	
		10 μM of 5-FU	50 μM of 5-FU
Low load 5-FU-loaded F-SubMGs	16.2 \pm 1.9	80	400

3.4. In Vivo 5-FU Administration

The study protocol was approved by the Animal Experimentation Ethics Committee of the Universidad Complutense de Madrid. Female Wistar rats, weighing 222 \pm 4 g, were obtained from the Animalario of the Universidad Complutense de Madrid (Spain) which operates according to the requirements relating to animal experimentation regulations (DC 86/609/CEE; RD 223/1988; OM 13/X/1989, RD 1201/2005). Guidelines contained in NIH publication on the Principles of Laboratory Animal Care 85-23 revised in 1985 were followed throughout. The animals were housed in cages under environmentally controlled conditions of light (12:12 h light:dark cycle), temperature (22 \pm 2 $^{\circ}\text{C}$) and humidity (65–70%). They were fed standard rat food and water *ad libitum*. The surgical material used in the experiment was previously autoclaved. Just before injection, the dissolution solvent was put under ultraviolet light (Ecogen Lamp, Viber Lourmat, Intensity 7 mW/cm^2) at 254 nm for 4 s because of the germicidal action of this wavelength.

Different groups of animals were established using six animals per group. Group 1: Animals injected with 133 mg of high load 5-FU-loaded F-SubMGs. Group 2: Animal injected with 133 mg of F-SubMGs without drug. Submicrogels were dispersed in 1 mL of saline solution (0.9% NaCl) and subcutaneously injected in the upper part of the back of the rat using a sterile syringe with a 1.2 \times 40 mm nozzle (Microlance 3). Group 3: Animals subcutaneously injected with 1 mL of a saline solution of

5-FU. Group 4: Animals intravenously injected with 1 mL of a saline solution of 5-FU by femoral vein cannulation with polyethylene tubing. The dose of 5-FU used in those experiments was 28 mg/kg body weight (b.w.), and the amount of drug administered with submicrogels and in solution was 6.3 ± 0.1 mg/mL. The animals were anaesthetized with an equipment of isoflurane (Burtons, Series 5 T.C.V. Kent, UK).

3.5. Determination of 5-Fluorouracil in Plasma

At predetermined times after the injection of 5-FU-loaded F-SubMGs and the 5-FU solutions, animals were anaesthetized with isoflurane. Blood (0.3 mL) was collected by puncturing the jugular vein in heparinized (75 units = 15 μ L) polypropylene tubes. Blood samples were taken from rats subcutaneously injected with 5-FU-loaded F-SubMGs at 1.5, 2, 3.5, 8.5, 11.5, 32, and 50 h after the injection and at 24 h intervals thereafter. Animals which were injected with 5-FU in solution, blood samples were taken 5, 15, 30 min, 1, 2, 4, 6, 8, 27, 48, 58, 72 h after subcutaneous administration; and 1, 2, 5, 15, 30, 45 min, 1, 1.25, 1.5, 2, 2.5, 3 and 4.5 h after intravenous administration. The heparinized blood was centrifuged at 10,000 rpm for 10 min in a Sigma 202 M centrifuge immediately after collection so as to obtain plasma. Plasma samples were then stored at -20 °C.

5-FU was extracted from plasma samples according to a modification of the method proposed by Buckpitt and Boyd [45]. 7.5 μ L of trichloroacetic acid 2 M was added to 150 μ L of plasma. After shaking and centrifugation (11,000 rpm, 10 min), supernatant was collected and placed into eppendorf tubes. 30 μ L of phosphate buffer (0.5 M, pH 8) and 1.8 mL of ethyl acetate were added. After vigorous shaking for 10 min and centrifugation (11,000 rpm, 10 min), the organic phase was collected and evaporated at 55 °C. The samples were reconstituted with 50 μ L of KH_2PO_4 0.01 M, pH 4, and 5-FU concentration was determined by the HPLC system [38]. For calibration, drug-free plasma pooled with known amounts of 5-FU, to obtain a 5-FU concentration between 0.01 μ g/mL and 100 μ g/mL, was used after undergoing the same extraction procedure. 5-FU standards were run for external standardization and a linear curve with a correlation coefficient of 0.99 was generated from the area under the peak measurements. The validity of the method was investigated by the determination of precision of the assay, which was demonstrated by the coefficients of variations (CV). For concentrations of 0.5, 10, 50 and 100 μ g 5-FU/mL, CV for intra-run were 5.8, 5.5, 6.0 and 1.0%, respectively; and CV for inter-run were 4.6, 9.4, 6.3 and 3.2%, respectively. The 5-FU retention time was 6.3 ± 0.2 min.

3.6. Pharmacokinetic Parameters

Non-compartmental analysis was performed. The basic calculation are based on the area under the plasma concentration *versus* times curve (AUC) (zero moment) and the first moment curve (AUCM). The AUC can be calculated by the trapezoidal rule. The AUCM is the area under the concentration times time *versus* time curve, and it can be also calculated by the trapezoidal rule. From the AUC and AUCM values, the mean residence time (MRT) was calculated ($\text{MRT} = \text{AUCM}/\text{AUC}$) [46]. Data analysis of the pharmacokinetic parameters was performed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

3.7. Biocompatibility Studies

Animals were sacrificed in CO₂ atmosphere 15, 30 and 60 days after the subcutaneous injection of F-SubMGs. An incision was made on the rat back to remove the tissue around the place of the injection. A piece of the removed tissue, fixed with formol (10% v/v), was immersed in paraffin. Cuts (10 µm) were carried out with a paraffin microtome (Minot type). Samples were dyed using the alcian blue hemalum picro-indigo, the toluidine blue, and the hematoxylin-eosin methods [47] and histological studies were carried out.

4. Conclusions

We have evaluated folate-conjugate poly[(*p*-nitrophenyl acrylate)-*co*-(N-isopropylacrylamide)] submicrogels (F-SubMGs) as 5-Fluorouracil (5-FU) delivery systems. Submicrogels can be successfully loaded by swelling in 5-FU solutions in phosphate buffer. The complete *in vitro* drug release took place in 8 h with two stage drug release and different rates. Unloaded F-SubMGs are not toxic to MCF7 and HeLa cells *in vitro* up to high concentrations, and 5-FU-loaded F-SubMGs present effective elimination of carcinoma cells. The presence of folate in the submicrogels enhances their internalization in HeLa cells, which are receptor folate positive. In the case of MCF7 line, it is mainly produced by a nonspecific mechanism. Moreover, subcutaneous injection of F-SubMGs does not cause an acute inflammatory response or rejection signs. Subcutaneous drug administration by 5-FU-loaded F-SubMG increases the mean residence time of drug. Therefore, the developed non-toxic folate-conjugate submicrogels have high potential to control the release of 5-FU, which may lead to a new option for treating several cancer malignances.

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