Cancer Nanotechnology

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Targeted magnetochemotherapy modified by 5-Fu-loaded thermally on/off switching nanoheaters for the eradication of CT26 murine colon cancer by inducing apoptotic and autophagic cell death

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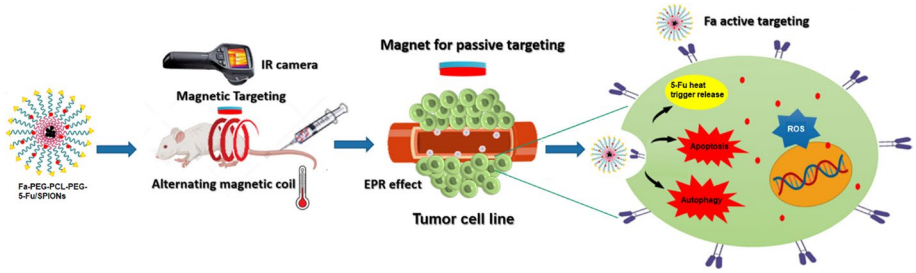
Abstract

Despite significant breakthroughs in diagnosis and treatment of colorectal cancer (CRC), the extent of morbidity and mortality secondary to CRC is still concerning. In this study, we evaluated the efficacy of our new tumor-selective nanoplatforms at induction of apoptosis and autophagy, which was tested using active 5-fluorouracil (5-Fu)-based targeting of tumor cells in a BALB/c murine model of CRC combined with magnetic thermal therapy. Nanoparticles were synthesized and characterized by zeta sizer, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). The cytotoxicity and tissue uptake of 5-Fu-loaded folic acid (Fa)-modified magnetic nanoparticles (5-Fu/MNPs-Fa) was assessed using MTT, ICP-OES, and HPLC. The rate of apoptosis and autophagy, as two major indicators of antitumor activity, was measured based on protein expression of Bax, Bcl2, Caspase 3, mTOR, P-mTOR, Beclin-1, and LC3B in CT-26 murine CRC, along with tumor volume and survival time. The spherical 5-Fu/MNPs-Fa exhibited sustained thermal on/off switching drug release and higher therapeutic index compared to free 5-Fu. Our de novo synthetized magnetic nanoheaters successfully delivered the therapeutic agent to the tumor site, enhanced the conversion of radio frequency energy to heat in tumor cells, exhibited higher antitumor efficiency based on Bax/Bcl2 ratio and overexpression of Beclin-1 and LC3B, increased the survival time, and decreased the tumor volume ($P < 0.05$). Our findings indicated that magnetochemotherapy (MHC) was substantially more effective than hyperthermia and/or chemotherapy alone. From a translational standpoint, the 5-Fu/MNPs-Fa would be a promising candidate sustained drug targeting system that could improve cancer cell therapy via inducing apoptosis and autophagy.

Keywords: Magnetochemotherapy, Colon cancer, Tumor-selective drug delivery, Thermally on/off switching, Autophagy

Graphical Abstract



Introduction

Known to be associated with high levels of morbidity and mortality, colorectal cancer (CRC) is the second most commonly occurring malignancy of epithelial origin in the world (Clay et al. 2022), which is often treated with chemotherapeutic regimens as of today (You et al. 2016). However, chemotherapy has long been identified to be at a certain disadvantage as a result of non-specific delivery and uneven biodistribution of chemotherapeutic agents at the tumor site. As such, delivery of chemotherapeutic agents at effective concentrations to the tumor microenvironment (TME) mandates systemic administration of large doses of the drug, which would result in the emergence of certain adverse effects due to the unwanted precipitation of such drugs at non-target organs (Shirvalilou et al. 2020). The 5-fluorouracil (5-Fu) is currently recognized as the first choice for colorectal cancer chemotherapy, which interferes with DNA replication and RNA generation by inhibiting thymidylate synthesis (Keyvan Rad et al. 2019; Li et al. 2010). However, the efficacy of 5-Fu might be significantly perturbed as a result of systemic biodistribution and multi-drug resistance (MDR), along with the short half-life of the drug itself in the circulation (Demeckova et al. 2022; Longley et al. 2003). To overcome these issues, a great number of clinical attempts have been made at site-specific delivery of 5-Fu by means of nanomaterial-based strategies, such as encapsulation of drug for subsequent sustained and remote-controlled release of 5-Fu at TME (Li and Chen 2022). A good example of such nanomaterials is amphiphilic copolymers, which are composed of hydrophobic and hydrophilic components. The dual nature of these nanoparticles has rendered them particularly useful vehicles for drug delivery, as they can be assembled into drug-encapsulating hydrophobic micelles with a hydrophilic shell that would increase their half-life in the plasma (Bodratti and Alexandridis 2018). Conjugation of these nanoparticles with certain ligands known to be highly expressed in TME is an effective strategy to enhance drug delivery by active targeting of tumor cells (Hu et al. 2022; Sargazi et al. 2022). One of the most widely used ligands in drug delivery is folic acid (Fa), a hydrophilic vitamin with low molecular weight, since folate receptors are over-expressed in the membrane of most cancer cells (Kularatne and Low 2010).

Today, iron oxide nanoparticles are used for concomitant therapies, such as chemo-thermotherapy (Mohammadi Gazestani et al. 2018). Superparamagnetic nanoparticles

Table 1 Physico-chemical properties of nanoparticles, (n = 3, mean ± SD)

Nanoparticles	Particle size (nm)	PDI	ζ potential (mV)	DLC%	EE%
MNPs-Fa	36.86 ± 0.63	0.11	− 32.52 ± 0.06	–	–
5-Fu/MNPs-Fa	71.81 ± 2.04	0.22	− 26.97 ± 0.02	4.35	43.5

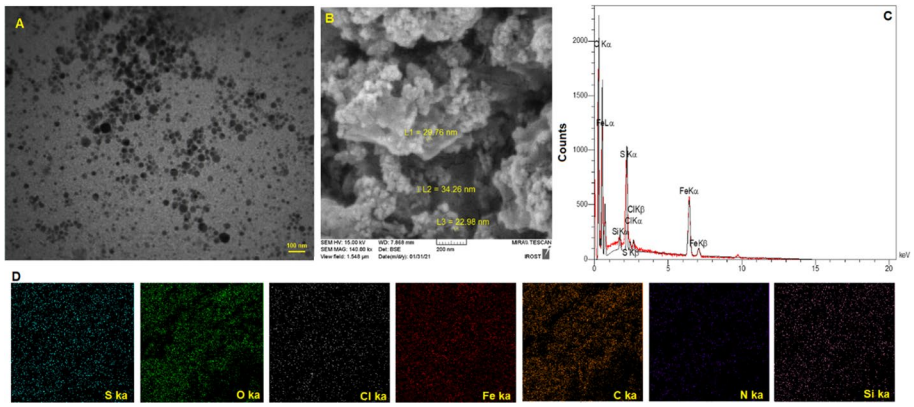


Fig. 1 Structure and composition characterization of 5-Fu loaded (5-Fu/MNPs-Fa). **A** TEM image of 5-Fu/MNPs-Fa, **B** SEM image of 5-Fu/MNPs-Fa, **C** X-ray energy dispersive spectroscopy (EDS) of 5-Fu/MNPs-Fa, **D** SEM–EDS elemental mapping of 5-Fu/MNPs-Fa

(SPIONs) are one of the most well-known nanosystems for the initiation of magnetic hyperthermia (Salunkhe et al. 2014; Shirvalilou et al. 2021), as they are capable of locally increasing the temperature of TME by converting alternating magnetic field energy into heat, hence the consequent death of tumor cells (Afzalipour et al. 2019; Irajirad et al. 2019). In this regard, we designed a new type of copolymer micellar nanoparticles to function as magnetic nanoheaters for induction of apoptosis and autophagy in CRC. We then evaluated the efficacy of these nanoparticles based on their size, shape, toxicity, cellular uptake, and antitumor activity in a BALB/c murine model of CRC. As 5-Fu therapy is known to promote apoptosis in vitro (Mirzaghavami et al. 2021), we measured apoptosis through Western blotting method based on the expression of the Bax and Bcl-2. As for autophagy, the expression levels of mTOR, ph-mTOR, Beclin-1, and LC3B were determined as well. While mTOR and Bcl2 proteins are important inhibitors of autophagy and apoptosis; LC3-II, Beclin-1, and Bax proteins are pro-autophagy and apoptosis in cells, respectively (Adornetto et al. 2021; Ghaznavi et al. 2021). Therefore, an increase in Bax/Bcl-2 expression ratio or a decrease in mTOR expression indicates an increased induction of apoptotic and autophagic cell death (You et al. 2018; Zhang et al. 2019).

Results

Size, surface charge, shape, and in vitro drug release

As reported in our previous study (Mirzaghavami et al. 2021), the hydrodynamic size of MNPs-Fa nanoparticles with and without 5-Fu was measured via DLS and the average diameter was 71.81 nm and 36.86 nm, respectively (Table 1). TEM imaging of the 5-Fu/

MNPs-Fa showed that the nanoparticles had a spherical shape (Fig. 1A), with an average size of about 50 nm (Fig. 1B). X-ray energy dispersive spectroscopy (EDS) of NPs confirmed the presence of the main elements of nanoparticles (Fig. 1C and 1D). Other characteristics of nanoparticles, such as surface charge, DLC%, and EE%, are summarized in Table 1.

The 5-Fu release profiles of thermosensitive MNPs-Fa at 37 and 43 °C are illustrated in Fig. 2A. The release graph shows a biphasic drug release pattern consisting of an initial release burst, followed by a continuous drug release for both 37 and 43 °C (Fig. 2A). However, their significant difference is the amount of 5-Fu released in the first 100 h. The results show that heat accelerated drug release, indicating the thermal sensitivity of nanoparticles. In addition, a considerable increase in drug release under an AMF demonstrated the "on/off" behavior of nanoparticles and their remote-controlled capability by an external magnetic field for both targeting and therapy cases (Fig. 2B).

Biosafety assessment of nanoparticles

MTT toxicity assay

To investigate the cytotoxic effects of 5-Fu, MNPs-Fa, and 5-Fu/MNPs-Fa based on the MTT assay kit, CT26 cells were treated with different drug concentrations and equivalent concentrations of nanoparticles (Fig. 2C and D). These findings indicate that 5-Fu/MNPs-Fa treatment significantly decreased cell viability compared to free 5-Fu and non-loaded nanoparticles (Fig. 2C and D). The reason for this phenomenon could be the presence of folic acid ligand that can facilitate the entry of 5-Fu/MNPs-Fa into folate

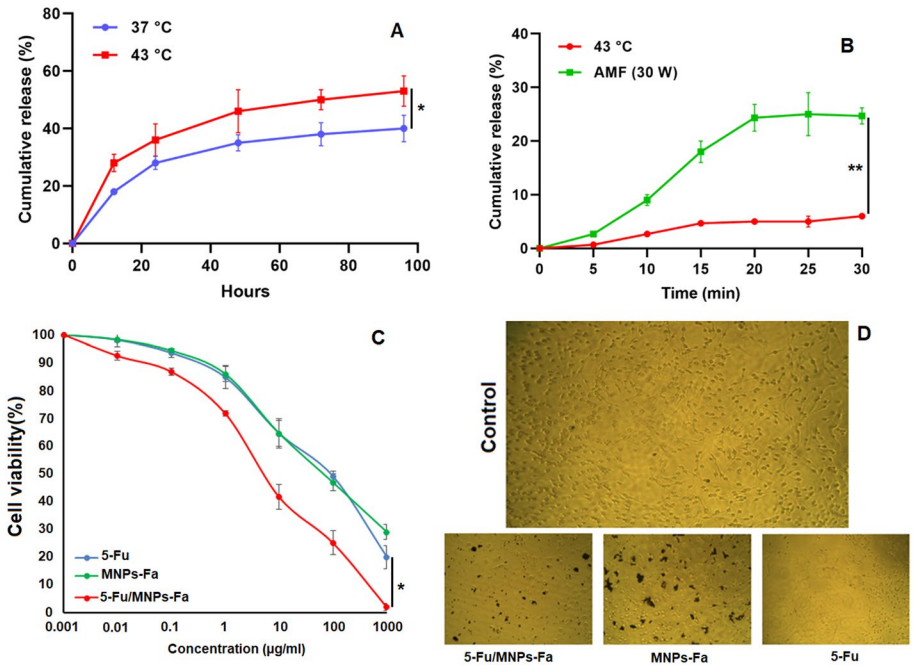


Fig. 2 **A** In vitro 5-Fu release in PBS of MNPs-Fa at different temperature 37 °C and 43 °C, **B** In vitro 5-Fu release in PBS of MNPs-Fa at 43 °C and under AMF (20 min, 30 W), **C** In vitro cell viability assessment using MTT assay after 24 h incubation of CT26 cells; **D** Optical microscopy images of cells after treatment with various concentrations of 5-Fu, MNPs-Fa, and 5-Fu/MNPs-Fa for 24 h

receptor-expressing cancer cells through receptor-mediated endocytosis, which is highly plausible considering the already well-established expression of folate receptor in colon cancer cells (Didion and Henne 2020; Soe et al. 2019).

Hemolysis test

To evaluate the effects of synthesized MNPs-Fa and 5-Fu/MNPs-Fa on blood cells, murine RBCs were incubated with different concentrations of nanoparticles for 2 h. While a low rate of hemolysis was observed with both formulations of nanoparticles (MNPs-Fa and 5-Fu/MNPs-Fa) at a concentration of below 3 mg/mL, increased concentrations of nanoparticles were found to aggravate hemolysis (Fig. 3A). According to ASTM E2524-08 (ASTM), hemolysis rate higher than 5% can damage RBCs and prompt cytolysis.

In vivo cellular uptake of MNPs-Fa

Enhancement of nanoparticle uptake in tumor tissues (Fig. 3C) was confirmed by ICP-OES (by measuring iron concentration) once tumor cells were treated for 2, 6, 12, and 24 h with 15 mg/kg MNPs-Fa using two mechanisms of active (folic acid ligand/receptor system) and passive targeting (external permanent magnetic field). Based on the obtained data, the average iron content during the first 12 h showed a significant

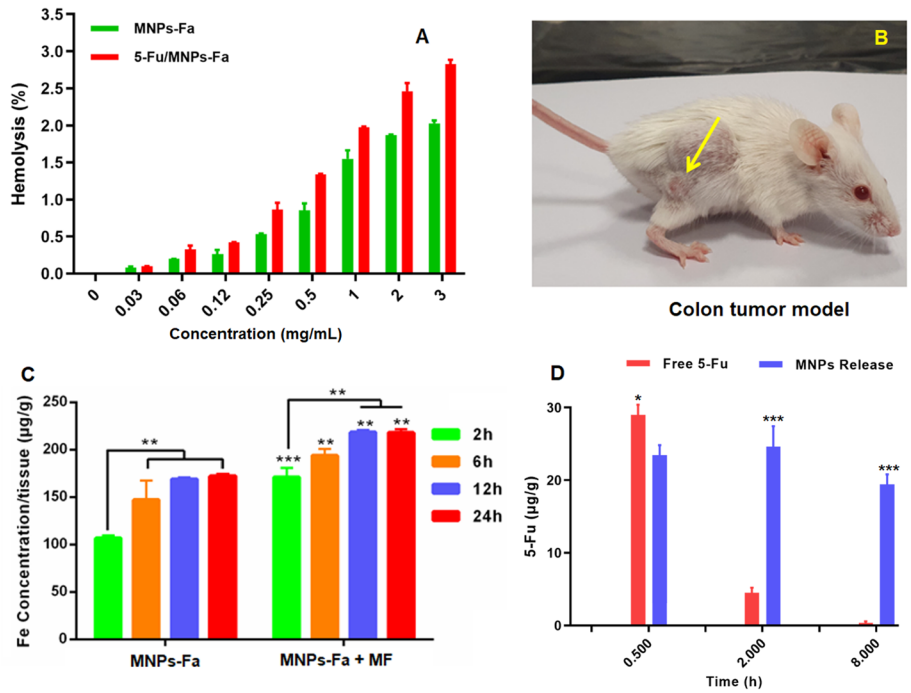


Fig. 3 **A** Ex vivo hemolysis results of the different concentrations of MNPs-Fa with/without loading 5-Fu after incubation (2 h) with diluted red blood cells of mice, **B** Colon mice tumor model, **C** Iron concentrations of 5-Fu/MNPs with/without magnetic field at different time after injection of nanoparticle, **D** The 5-Fu tumor tissue concentrations following i.v. injection of free 5-Fu or 5-Fu/MNPs-Fa at a dose of 5 mg/kg 5-Fu in mice

increase in both targeted and untargeted transport mechanisms ($P < 0.01$, Fig. 3C). There was no significant difference in the quantity of uptaken nanoparticles between treatment periods of 12 and 24 h ($P > 0.05$). However, the comparison of the mechanism of targeted transfer with simple systemic transfer indicated the significant superiority of targeted delivery, confirming the effectiveness of simultaneous active and passive dual transfer.

Uptake of 5-Fu in tumor tissue

To evaluate the effectiveness of drug loading in MNPs-Fa with the purpose of sustain release, we injected free 5-Fu or 5-Fu/MNPs-Fa nanoparticles into mice. To evaluate the effectiveness of drug loading in MNPs-Fa with the aim of sustained release, we injected free 5-Fu or 5-Fu/MNPs-Fa nanoparticles into mice, and then the tumor tissue was extracted at different times and the amount of drug absorbed in the tissue was measured by the method obtained by HPLC. Figure 3D shows that free 5-Fu only has a rapid uptake, but using drug-loaded MNPs-Fa system, 5-Fu was detected in the tumor tissue for a long time. The significant difference in the amount of 5-Fu in the tumor tissue when using MNPs-Fa can be due to effective drug delivery through targeted delivery (Fa ligand + MF) and increased blood circulation time of nanoparticles. According to the data, loading the drug on nanoparticles could increase the half-life of the drug in the tissue and cause the drug to be released slowly into the tumor tissue.

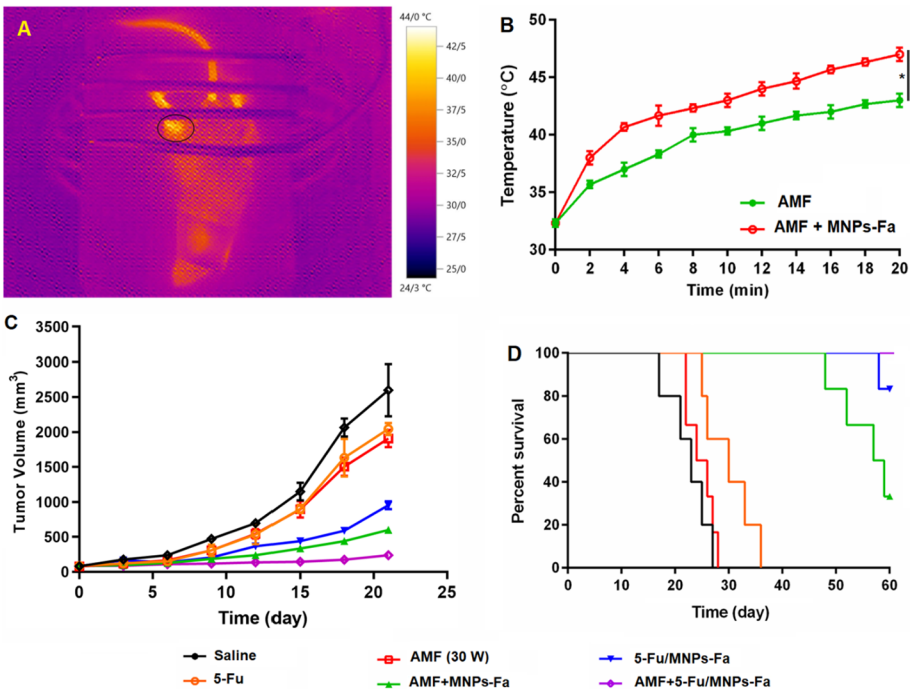


Fig. 4 MNPs as an effective in vivo heat mediator under AMF. **A** Infrared camera images of the colon tumor mice during 20 min of Oncothermia, **B** The profile of temperature variations the CT26 tumor-bearing mice treated with intravenous injection of 600 mg/kg MNPs in the presence or absence of AMF (13.56 MHz, 40 A/m, 20 min) as a function of time. In the presence of a magnetic field, the antitumor efficacy of free 5-Fu and 5-Fu/MNPs was determined in the CT26 colorectal tumor model, **C** Tumor volume in different treatment groups, **D** Kaplan–Meier plot

Table 2 Thermal dose values for colon-bearing tumor mice for AMF (13.56 MHz, 30 W) with or without 5-Fu/MNPs-Fa at various times

Type of therapy	CEM 43 °C for 2 min	CEM 43 °C for 9 min	CEM 43 °C for 20 min
AMF	0.00006	0.073 ± 0.0002	35.975 ± 1.4
AMF + 5-Fu/MNPs-Fa	0.00024	14.93 ± 0.84	686 ± 7.4

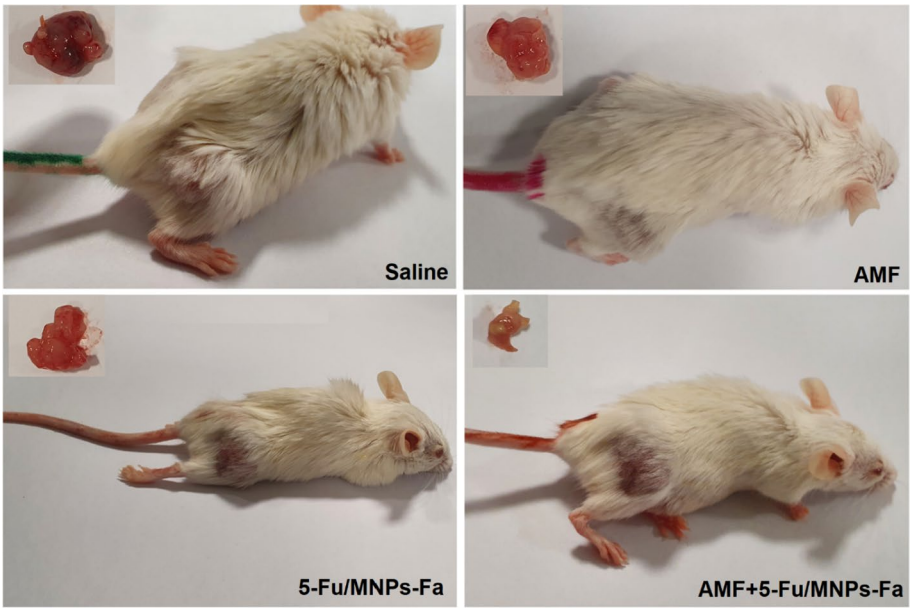


Fig. 5 Photographs of colon tumor-bearing mice and tumor volume on the 21st day after the onset of treatments

Thermal dose calculation

For magnetic hyperthermia treatment, after intravenous injection of nanoparticles, mice with colon tumor were placed by the femoral area under an alternating magnetic field and the temperature changes of the tumor area were recorded with an IR camera (Fig. 4A). The curve of temperature changes is drawn in Fig. 4B. The results showed that magnetic nanoparticles caused a significant increase in temperature compared to the group who had only been exposed to AMF ($P < 0.05$). Nanoparticles caused a rapid increase in temperature, which was more pronounced in the femoral area. To compare the thermal dose received by mice with and without magnetic nanoparticles, CEM of 43 °C was calculated for different times (Table 2). CEM of 43 °C for rats treated with magnetic hyperthermia (686) showed a significant increase compared to AMF alone (35.975) after 20 min ($P < 0.001$).

In vivo antitumor effects of magnetochemotherapy

The antitumor effects of 5-Fu, AMF, MNPs-Fa + AMF, and 5-Fu/MNPs-Fa + AMF were investigated on CRC mice by measuring the tumor volume and follow-up of the survival rate (Figs. 4 C, D and 5). Figure 5 shows the images of mice with colon tumors and the tumor volume 21 days after the onset of treatment. Tumor volume in the saline

Table 3 Tumor growth inhibition and survival time data of CT-26 colon-bearing BALB/c mice ($n=6$) after different treatment modalities

Treatment groups	Tumor growth inhibition		
	Median tumor volume (mm^3)	Mean growth Inhibition rate (%)*	*P*-value
Saline	2845.7	–	–
MNPs-Fa	2795.5	1.76	NS
5-Fu	2143.09	24.69	NS
5-Fu/MNPs-Fa	1366.41	51.98	< 0.01
AMF	1987.65	30.15	NS
AMF + MNPs-Fa	648.42	77.21	< 0.001
AMF + 5-Fu/MNPs-Fa	198.38	93.02	< 0.001

* Mean growth inhibition rate (%) was calculated as [(C-T)/C] × 100, where T is mean median tumor volume of treated mice and C is median tumor volume of control mice. NS = not significant

Table 4 Effects of combination therapy based on tumor volume

Treatment modality	[*A*]	[*B*]	[*A* + *B*]	[*A*] × [*B*]/100	Effect*
A = 5-Fu/MNPs-Fa *B* = AMF	1366.41	1987.65	198.38	27,159.44	198.38 < 27,159.44 ≡ Synergistic

* If [*A* + *B**] < [*A*] × [*B*]/100, the effect is synergistic

group (control) showed a fivefold increase on the 21st day after the onset of treatment (Fig. 4C; Table 3). The results confirmed that free 5-Fu injection or AFM exposure alone would not lead to tumor suppression, as the tumor tended to increase in size over time. Compared to the saline and AMF group, the tumor size decreased gradually following treatment with 5-Fu/MNPs-Fa alone, but after 9 days of therapy, the tumor volume increased once again (Fig. 4C). MNPs-Fa or 5-Fu/MNPs-Fa in the presence of AMF increased survival time up to 60 days ($P<0.05$) and significantly reduced tumor size (~ fourfold) compared to control on day 21 (Fig. 4C; Table 3). Table 3 lists the mean rate of growth inhibition in different treatment groups, highlighting the significant role of combined chemotherapy and hyperthermia treatment at inhibition of tumor growth in mice ($P<0.001$). The efficacy of combination therapy was determined using a set of equations presented by Ito et al. (Ito et al. 2007) and Hauser et al. (Hauser et al. 2016). Based on the findings presented in Table 4, the combination of targeted chemotherapy (5-Fu/MNPs-Fa) and hyperthermia (AMF) resulted in a synergistic effect, as it was able to significantly reduce tumor growth and increase the survival of mice with colon tumor. In general, there was no significant difference between MNPs-Fa + AMF and 5-Fu/MNPs-Fa + AMF groups ($P>0.05$), which indicates the effective role of MNPs magnetic nanoheaters under AMF, which leads to a significant enhancement in treatment efficacy ($P<0.001$).

Protein expression study

In this study, the levels of Bax and Bcl2, and mTOR, P-mTOR, Beclin-1, and LC3B were used to compare the pro-apoptotic and pro-autophagic effects of free 5-Fu, 5-Fu/MNPs-Fa, MH, and magnetochemotherapy (5-Fu/MNPs-Fa plus AMF) among different groups. As shown in Fig. 5A, B, consistent with the results of survival rate and

tumor volume changes, combination treatment protocols (MNPs-Fa ± 5-Fu plus AMF) increased Bax expression ($P < 0.05$), while the change in Bax protein after 5-Fu or AMF single treatment was negligible. Changes in Bcl-2 protein levels were insignificant in all groups. Bax/Bcl-2 ratio was remarkably higher in magnetochemotherapy group (5-Fu/MNPs-Fa plus AMF) compared to control cells ($P < 0.01$). Furthermore, a decrease in P-mTOR protein expression and an increase in Beclin-1 and LC3B proteins in the MHC group suggested autophagic death (Burada et al. 2015; Kim and Guan 2015). Results of the Western blotting analysis showed changes in protein expression of apoptosis and the autophagic pathway that was in agreement with our observations regarding survival time of CRC mice (Figs. 4, 5).

Discussion

Despite the use of the chemotherapeutic drug 5-Fu for a wide range of cancers, including breast and colorectal cancer (De Angelis et al. 2006), critical limitations, such as its short half-life in plasma as well as extensive systemic distribution, have largely overshadowed its efficacy (de Mattos et al. 2016). Among the suggested solutions to overcome this problem is the encapsulation of 5-Fu into polymeric nanoparticles to enhance its blood circulation and facilitate sustained drug release (Elsabahy and Wooley 2012; Sargazi et al. 2020). In the present study, loading the drug into the self-assembled magnetite/PEG-PCL-PEG triblock copolymer nanoplatforms was performed (Fig. 1). Then, the thermal control of 5-Fu release by an "on/off" switching system was investigated (Fig. 2). Various studies have previously confirmed the superiority of PCL-based MNPs over PLGA-based MNPs in sustained release applications (Ashour et al. 2019; Wang et al. 2001). Apart from the challenge of maintaining the drug in the body, another important challenge is the effective transfer of the drug to the tumor tissue. One of the best ligands for active targeting is folic acid, which is highly expressed in tumor cells (Zwicke et al. 2012). Consistently, ICP-OES (Fig. 3C) and HPLC (Fig. 3D) both clearly showed the effective role of folic acid in targeted delivery to tumor tissue. On the other hand, the high toxicity of nanoparticles-conjugated folic acid ligand on CT26 cells is proof that the receptor/ligand transfer system can be effective (Fig. 2C). The presence of a folic acid ligand and the application of a magnetic field, in the current study, caused more nanoparticles to enter the tumor tissue and prompted more drug to be released in the tissue over time compared with that of the free drug (Fig. 3D).

Another capability of the synthesized 5-Fu/MNPs-Fa nanoparticles was the convenience of delivery under remote controlling through the application of an alternating magnetic field. These nanoparticles were able to act as magnetic nanoheaters under the AMF and release the drug at the right time and place (Fig. 2 and 3). The results of the HPLC test (Fig. 3D), on one hand, confirms the presence of the drug in the tissue about 20 h after the injection of the nanoparticles, which shows the ability of the nanoparticles to properly deliver the drug, and on the other hand, the calculation of the thermal dose indicates the high capability of the nanoparticles as a magnetic nanoheaters (Table 2).

Nanoparticle toxicity poses a major barrier to biomedical utility and clinical translation of nanocarriers. Therefore, a comprehensive evaluation of the toxicity and biosafety of nanoparticle platforms is an essential step. With regard to our previous

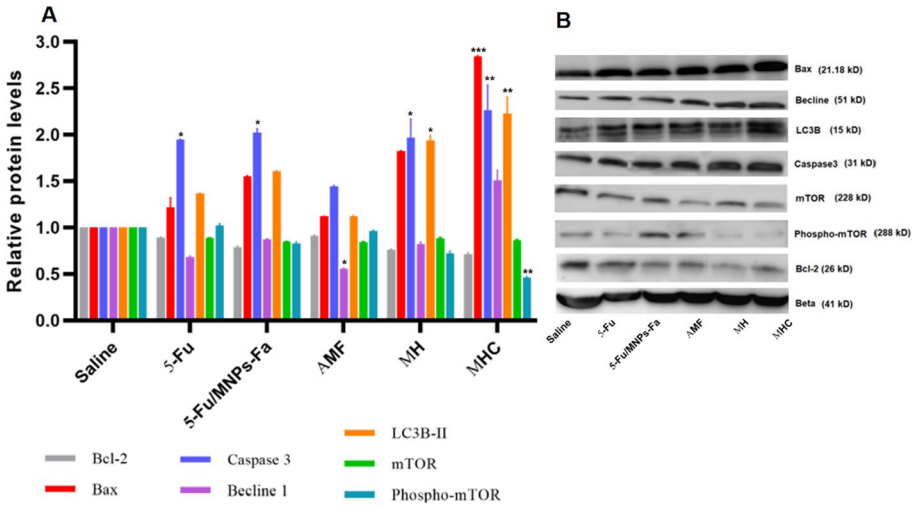


Fig. 6 **A** Effects of blank MNPs-Fa (), free 5-Fu (200 mg/kg/day), 5-Fu/MNPs-Fa (1 mg/kg/day), AMF (13.56 MHz, 40 A/m, 20 min), MH (MNPs-Fa + AMF), and MHC (5-Fu/MNPs-Fa + AMF) on relative protein expression of Bax and Bcl-2, Caspase 3, mTOR, P-mTOR, Beclin-1, and LC3B, **B** Protein expression detected by Western blot

work, in which we proposed nanoparticles were safe as in vitro drug nanocarriers (Mirzaghavami et al. 2022), herein we report our findings on the potential ex vivo biosafety of MNPs-Fa nanoparticles toward erythrocytes (3A) and the therapeutic potential of 5-Fu/MNPs-Fa as an effective type of hybrid nanocarriers/nanoheaters for delivery of chemo-thermal agents in an established cancer model (Fig. 6).

For this purpose, the antitumor effect of 5-Fu, AMF, MNPs-Fa, and 5-Fu/MNPs-Fa ± AMF in the treatment of CRC in mice was evaluated by different tests. Examining the results of changes in tumor volume and the survival rate of mice confirmed the tumor suppressive effect of magnetochemotherapy. Western blot results also showed the activation of apoptotic death pathways with increased Bax protein expression, as well as autophagic death with increased Beclin-1 and LC3B protein expression and decreased P-mTOR expression in groups with combined treatment. In accordance with these results, several studies have shown that 5-Fu induces apoptosis and autophagy through the production of ROS (Burada et al. 2015; Focaccetti et al. 2015; Lamberti et al. 2012). We have also shown the effective role of drug and nanoparticles in ROS production in a previous study (Mirzaghavami et al. 2022). Therefore, among the cases of activating the mechanism of apoptotic death and autophagy can be the production of ROS by drugs and magnetic nanoparticles (Ghaznavi et al. 2022; Kma and Baruah 2022). The results obtained from the treatment of tumor-bearing mice (Fig. 4) showed that treatments with free 5-Fu and AMF alone had no significant effect in inhibiting tumor growth or increasing survival time ($P > 0.05$). In contrast, combined treatments (MNPs-Fa ± 5-Fu plus AMF) can have significant effects in increasing the survival of mice and temporarily inhibiting the tumor. As a final finding, the combination of magnetic hyperthermia and chemotherapy (magnetochemotherapy) showed the most effective therapeutic efficiency in this group. Survival time

increased by fivefold compared to the control group, tumor volume decreased, and Bax/Bcl-2 increased ($P < 0.01$).

According to the obtained results, the synthesized nanoparticles, due to their significant therapeutic potential, may solve the issue regarding the short half-life of the drug in the blood and the systemic toxicity of the drug by serving as both targeted nanocarriers and nanoheaters, which appear to be quite promising as adjuvant treatment for deeply located tumors, especially colon cancer.

Conclusion

In the current study, folic acid-conjugated PEG-PCL-PEG triblock copolymer magnetic nanoheaters were synthesized and subsequently loaded with 5-Fu and magnetite nanoparticles to constitute 5-Fu/MNPs nanoparticles. All results showed that the combination therapy of hyperthermia and 5-Fu magnetic nanoparticles was significantly more effective than the single treatment with either hyperthermia or chemotherapy. These studies showed that PEG-PCL-PEG magnetic nanoparticles played a key role in drug delivery to the tumor, the conversion of radio frequency energy to heat in the cell, and increasing apoptotic and autophagic death. In addition, the combination of these nanoparticles with AMF hyperthermia can be introduced as a new and effective treatment for colon cancer.

Materials and methods

Materials

Cell culture medium (RPMI) was purchased from Gibco (Invitrogen, USA). Fetal Bovine Serum (FBS) and penicillin–streptomycin were purchased from Biowest. Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Merck Chemical Company (Darmstadt, Germany). 5-fluorouracil (5-fu), folic acid, N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypsin, and EDTA were purchased from Sigma Chemical Company (St. Louis, MO, USA). The CT26 cells procured from Pasteur Institute Cell Bank, Tehran, Iran BALB/c mice with a weight of about 20–25 g were procured from Pasteur Institute of Tehran, Iran. BALB/c Mice.

Methods

Synthesis of drug-loaded Fa-conjugated magnetic nanoheaters

We synthesized our nanoparticles based on a modified W1/O/W2 double emulsion solvent evaporation method described by Mirzaghavami et al. in 2021(Mirzaghavami et al. 2021). Here, the improved synthesis method of magnetic nanoheaters is briefly mentioned.

Synthesis of Fa-conjugated PEG-PCL-PEG triblock copolymer

First, poly(ε-caprolactone) (PCL) was synthesized and then functionalized with adipoyl chloride. For this purpose, ε-caprolactone (3 ml, 27 mmol) was dissolved in DMF (5 mL), and ethylene glycol (5 µL, 1 mmol) was added to this solution under a nitrogen atmosphere. Then, the temperature was gradually raised to 80 °C, and stannous octoate [$Sn(Oct)_2$] was added to reaction medium, which was followed by another surge in

temperature up to 120 °C. After an overnight incubation, the polymer was precipitated in water at 0 °C and then dried at 40 °C. 82%. The polymer was dissolved in DMF and precipitated in water to produce pure PCL with 82% yield. Then, PCL (1 g, 0.2 mmol) was dissolved in DMF, and adipoyl chloride (146 µL, 0.001 mol) and triethylamine as catalyst were mixed with the solution at 80 °C. The obtained product was settled in water and then dried in a vacuum oven at 40 °C. Finally, DCC (206 mg) and DMAP (122 mg) were gradually added to the functionalized PCL solution in DMSO. After 1 h, PEG (2 gr) was added to the solution and placed on a stirrer for 24 h at room temperature. The resulting copolymer was precipitated in diethyl ether (DE) and dried by a vacuum oven at 40 °C. For repurification, dissolution in DMSO and precipitation in DE were repeated. At this step, 220 mg of folic acid, 103 mg of DCC and 61 mg of DMAP were dissolved in 10 mL of DMSO. To activate the acidic group of folic acid, the reaction was left to proceed overnight. The product was participated in DE and dried at 40 °C. Finally, the folic acid-conjugated polymer was dissolved in DMSO and precipitated in DE for purification. The product was obtained with a yield of 82%.

Synthesis of 5-Fu-loaded copolymer-coated magnetite nanoparticles (MNPs-Fa)

For preparation of 5-Fu-loaded Fa/copolymer-coated magnetite nanoparticles, free 5-Fu (10 mg) was dissolved in 1.5 mL distilled water containing Tween 60 (10 mg). Fe_3O_4 (45 mg) was dispersed in DCM (7 mL) under an ultrasonic probe (30 s). Then the synthetized Fa-conjugated copolymer (50 mg) and Span 60 (200 mg) were added to the oil phase (O). Next, the drug solution was added to the magnetic nanoparticles suspension under ultrasonication (30 s in an ice bath). After that, the mixture of distilled water (DW) (8 mL) and glycerin (8 mL) was mixed with Tween 60 (100 mg). The double emulsion product was diluted in 30 mL of an aqueous solution consisting of 15 mL of distilled water and 15 mL of glycerol with mechanical stirring for 3 h at room temperature and DCM was removed by solvent evaporation. The resulting 5-Fu/MNPs-Fa were washed twice with DW and then collected with a magnet. Finally, the 5-Fu-loaded magnetite nanoparticles coated with Fa-conjugated copolymer were freeze-dried and stored at 4 °C.

Characterization of nanoparticles

The synthesized nanoparticles were analyzed by dynamic light scattering (DLS), zeta size (Nanoflex, Germany), transmission electron microscope (TEM, Zeiss LEO906, Germany), and scanning electron microscope (SEM, FESEM; Zeiss SuprATM 55, Germany) to measure the hydrodynamic size, surface charge, and shape, respectively. The drug loading capacity (DLC) and encapsulation efficiency (EE) of 5-Fu/MNPs-Fa were determined. Additionally, the in vitro drug release profile of 5-Fu was determined based on the equilibrium dialysis bag diffusion method at pH ~ 7.4 at two different temperatures of 37 and 43 °C by incubating in a thermostatic bath. The nanoparticles were placed in the alternating magnetic coil (AMC) to evaluate RF-triggered 5-Fu release. After collecting the samples, the absorption of released 5-Fu was measured using an UV spectrophotometer at the absorption wavelength of the drug (265 nm).

MTT assay an in vitro study

Cytotoxicity of free drug and synthesized MNPs-Fa with and without 5-Fu was evaluated using MTT assay. The CT26 cells were cultured as a monolayer in a 96-well plate at a density of 104 cells per well, containing complete RPMI medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 mg/mL). After an overnight incubation, the cultured cells were incubated with varying concentrations of free 5-Fu, blank, and 5-Fu/MNPs-Fa (with equivalent concentrations of 5-Fu) for 24 h. Then, treated cells were washed with PBS buffer, followed by the addition of 100 μL of MTT solution (5 mg/mL) to each well. The plates were incubated at 37 °C for 4 h. Next, the MTT solution was removed and DMSO (200 μL) was added to the wells, and the plate was placed on a shaker for 15 min. Finally, the absorbance was measured at 570 nm using a microplate reader to determine cell viability.

Hemolysis assay an ex vivo study

The biocompatibility of 5-Fu/MNPs-Fa on murine red blood cells (RBCs) was evaluated based on hemolysis assay. Mice blood samples were collected and centrifuged; then RBCs were extracted and diluted by being mixed with PBS at a ratio of 1:4. Then 5-Fu/MNPs-Fa suspension was prepared in different concentrations and added to the RBCs samples. PBS and deionized water (DW) were used as negative and positive controls, respectively. All samples were incubated at 37 °C for 2 h, and then the supernatant of the samples was collected by centrifugation and transferred to a 96-well plate. The absorbance of the samples was measured by a UV–visible spectrophotometer (BioTek, Winooski, USA) at a wavelength of 577 nm.

CT26 mouse colon tumor model

To create a tumor model, BALB/c mice with a weight of about 20–25 g were kept in the animal research center of Iran University of Medical Sciences, Tehran, Iran, under standard Helsinki conditions for the experiment. To establish a colon tumor model, 2 million CT26 cells in RPMI medium (100 μL) were injected subcutaneously into the right leg of the test mice. The subjects with implanted cancer cells were followed up daily to develop an appropriate tumor volume (50 to 100 mm^3).

In vivo cellular uptake of nanoparticles

Investigation of dual drug targeting using 5-Fu/MNPs-Fa under permanent magnetic field (MF) in colon tumor-bearing mice was performed by injecting nanoparticles (containing 2 mg/kg Fe) into the tail vein of mice 12 days after tumor implantation under permanent magnetic fields (0.2 Tesla). To passively target MNPs, a magnet was placed on the tumor area in the femoral region of the mice for 1 h. Then, the subjects were sacrificed, with their tumors being extracted for prospective ICP-OES testing. To prepare the samples, tissues were digested by adding HNO3/HCl solution (1:3) for 1 h on a heater stirrer at 150 °C. Ultimately, ICP-OES (ICP spectrometer, Varian) was performed to determine iron concentration in different tissues samples.

Tissue uptake of 5-Fu

High-Performance Liquid Chromatography (HPLC) was evaluated to analyze the amount of drug absorbed at different times (0.5, 2, and 8 h) in the tumor site. Free 5-Fu or 5-Fu/MNPs-Fa were injected intravenously into tumoral mice as a single dose (5 mg/kg), then the mice were sacrificed after 0.5, 2, and 8 h. After the injection, the mice were subjected to constant magnetic field for 30 min to 2 h depending on their group. After extracting the tumor tissues, the tissues were weighed. Then 10 mg of the tissue was homogenized with an equal volume of water and mixed with 20 µl of HCl (0.1 M) and 70 µl of saturated $(NH_4)2SO_4$ solution and vortexed. Then the samples were centrifuged at 5000 × g for 10 min and the supernatant was collected. The obtained suspension was diluted with the mobile phase before injection into the HPLC device (Young Lin Gradient pump SP930D; Young Lin detector UV730D). A reverse ACE5-C18 (250 × 4.6 mm) column was used with a mobile phase of methanol/water (10:90) and a flow rate of 0.8 ml/min. The UV detector was set at 265 nm and the HPLC column temperature was maintained at 30 °C with a heater and cooler (Jin et al. 2011).

In vivo antitumor effects of magnetochemotherapy

To investigate the efficacy of antitumor treatment, after 12 days of having cancer cells implanted in the mice, when the size of the tumor reached about 50–100 mm^3, the mice were randomly divided into 6 interventional groups of 8 subjects, including G1) normal saline (control), G2) Free 5-Fu, G3) 5-Fu/MNPs-Fa, G4) Alternating magnetic field (AMF, 13.56 MHz, 40 A/m), G5) Magnetic hyperthermia (AMF + MNPs-Fa), and G6) Magnetochemotherapy (AMF + 5-Fu/MNPs-Fa). The treatment protocol applied in different groups is as follows:

a. CT26 tumor-bearing mice received 1 mg/kg of free 5-Fu, and 200 mg/kg of MNPs with or without drug by tail vein on the 12th day of the experiment for 3 consecutive days.
b. Mice in the treatment groups that were injected with nanoparticles were exposed to an MF for one hour.
c. Based on camera-assisted temperature monitoring, mice in the AMF group were kept in the coil for 20 min and in the AMF + MNPs-Fa or AMF + 5-Fu/MNPs-Fa group for 9 min. Hyperthermia was applied for 3 consecutive (12–14) days.

After applying the treatment protocols, tumor size was measured daily and tumor volume was calculated with $V = \left[\text{width}^2/2\right] \times length$. Five mice from each group were followed for 2 months in terms of lifespan and changes in tumor volume.

Heating profile and thermal dose calculation

For thermal treatment, the time–temperature curve was drawn to calculate the time required for the mice to be inside the coil to reach an average temperature of 43 °C. For this purpose, the mice were placed by their femoral area at the center of the coil and exposed to AMF (13.56 MHz, 50 W), with the temperature being monitored by an IR

camera. The cumulative equivalent minute thermal dose at 43 °C (CEM43) was estimated using Eq. 1:

$$\mathrm{CEM43\,^{\circ}C} = \sum_{i=1}^{n} t(i) R^{[43-T(i)]}. \quad (1)$$

In this equation, t (i) is the time interval of the (i)th sample, and T (i) is the average temperature during the specified heating interval at the (i)th minute. The R constant is 1/4 and 1/2 for temperatures below and above 43 °C, respectively.

Protein expression study using Western blotting analysis

To study alterations in the levels of the apoptotic proteins Bax, Caspase 3, and Bcl2, and the autophagic proteins mTOR, P-mTOR, Beclin-1, and LC3B, tissues were washed with cold PBS and lysed in RIPA buffer. After storing the lysed tissues in ice for 30 min, they were centrifuged with a speed of 12,000 rpm at 4 °C for 20 min. The supernatant containing the total protein was collected and the protein content of the samples was measured using the Bradford method. Protein samples were separated by gel electrophoresis and transferred to a nitrocellulose membrane. Then, the membranes were incubated overnight at 4 °C in the solution containing primary antibodies. The primary primers used were Caspase 3 (1:500), mTOR (1:500), ph-mTOR (1:500), Beclin-1 (1:500), LC3B (1:500), Bax (1:500), Bcl-2 (1:1000), and β-actin (1:500). Cellulose membranes incubated with primary antibodies were rinsed with a mixture of Tris-buffered saline and tween 20 (TBST) and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Protein bands were visualized by the chemiluminescence (enhanced chemiluminescence) reagent kit. Protein bands quantification was performed by ImageJ software and then data normalized to the intensity of β-actin.

Statistical analysis

The results of the experiments were statistically analyzed based on one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad 6, and the data are presented as mean ± standard deviation. A p-value ≤ 0.05 was considered statistically significant.

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Author contributions
All the authors contributed to the study conception and design. Prof. SK, MRK, and Dr. MS. contributed to material preparation. ES, Dr. SS., and Prof. SKhoei conducted the experiments and collected the data. The first draft of the manuscript was written by Dr. SSh. and Prof. SKh. All the authors reviewed the manuscript. All aspects of the study were supervised by Prof. SKh and Dr. SSh.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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