Poly (methacrylic acid)-based molecularly imprinted polymer nanoparticles containing 5-fluourouracil used in colon cancer therapy potentially

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The objective of this work was to synthesize molecularly imprinted polymer (MIP) nanoparticles based on methacrylic acid (MAA) monomer with a high selectivity against an anti-cancer drug, 5-fluorouracil (5-FU), as a template. In this case, the nanoparticles were prepared via precipitation polymerization in the presence of ethylene glycol dimethacrylate as cross-linker and azobisisobutyronitrile as initiator. Besides, 3 independent variables including MAA: 5-FU molar ratio (X1), temperature (X2), and time (X3) were investigated utilizing response surface methodology. The scanning electron microscopy and dynamic light scattering resulted the average diameter of approximately 65 nm, and the MIP nanoparticle sample with the imprinting factor of 1.57 was polymerized in optimized conditions as follows: X1 = 6:1, X2 = 60°C, and X3 = 3 days in acetonitrile as porogenic solvent. Also, Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis confirmed the formation of MAA/5-FU complex and lower thermal stability of the washed MIP sample than the unwashed MIP and non-imprinted polymer (NIP) samples, respectively. Moreover, the optimized MIP nanoparticles have more controlled release of 5-FU rather than the NIP sample. Finally, the flow cytometry showed that 5-FU-loaded MIP sample has the highest apoptosis of human colon cancer cell line, HCT-116, after 3 days compared with NIP sample and also the exclusive use of drug.

KEYWORDS
5-fluourouracil, cancer, drug delivery systems, molecular imprinting, nanoparticles

1 | INTRODUCTION

In recent years, researchers have clearly shown that nanoscale medical devices are capable of performing very complex tasks, ie, enhanced drug delivery within biological tissues owing to their unique properties.1,2 Many studies have been conducted to synthesize and develop nanomaterials used for advanced drug delivery systems, especially hydrophobic medicines which cannot be prescribed without a controlled dose program.3,4 In this regard, the application of polymer materials is strongly recommended due to their versatility, suitable physical chemistry characteristics, and ability to conjugate with drug molecules as well as acceptable rate of degradation under appropriate biological conditions,5 preserving the concentration of drugs in their therapeutic window.6-8

Amid the extensive polymer carriers, molecularly imprinted polymers (MIPs) are promising candidates for biomedical and pharmaceutical applications3,9-12 owing to a series of exceptional properties.13-15 They are extremely networked polymer materials and are characterized by their high selectivity against a specific drug molecule because of the presence of specific recognition sites within their cavities.3,7 Briefly, the MIP synthesis is made by the following procedure: (1) the formation of a complex between a functional monomer and template (drug molecule) by means of non-covalent interactions, which are often hydrogen bonds, (2) reacting the provided complex through a radical polymerization by using a suitable crosslinking agent,13,14,16 and (3) the template removal to form specific binding sites corresponding to the size, shape, and orientation of the targeted drug molecule.10,17,18
In the past half century, 5-fluorouracil (5-FU) has found a distinct place for chemotherapy in treatment of different cancer cells occurred at liver, chest, neck and colon.\textsuperscript{19-22} The action mechanism of this drug includes the inhibition of thymidylate synthase enzyme which interferes with the synthesis of nucleic acid and deoxyribonucleic acid; consequently, it can prevent the growth of the cancer cells.\textsuperscript{19,23} Recent studies showed that the drug response rate particularly on advanced colon cancer cells was less than 15\%\textsuperscript{19} in spite of dissolving the drug in both aquatic and blood fluids. The most important reason for this low therapeutic level is the rapid metabolism of 5-FU while passing through the gastrointestinal track and its short half-life in the body (10-20 min), limiting its availability in the human metabolism system.\textsuperscript{3,15,24} Moreover, the continuous use of 5-FU to compensate the concentration fluctuations and to achieve the effective therapeutic doses can have chronic toxic effects on gastrointestinal pathway, bone marrow, and nervous system as well as skin reactions during the course of the therapy. Therefore, the drug concentration not only must be in its therapeutic dosage, but it should be also below the toxicity threshold.\textsuperscript{5,21,22,24} By considering these shortcomings, it seems that choosing an appropriate polymer carrier for transferring 5-FU will be an inspiring solution for reducing the systemic adverse side effects and achieving safer treatment processes.\textsuperscript{23,25-29}

Tummala et al\textsuperscript{30} synthesized chitosan nanoparticles to minimize the adverse side effects of 5-FU. Their results exhibited that the drug molecule was able to accumulate in intestine along with a sustained release overnight. In addition, Sutar and co-workers\textsuperscript{22} prepared poly(lactic-glycolic) acid nanoparticles containing 5-FU using emulsion droplet coalescence method in the presence of eudragit s-100. The in vitro results exhibited that 5-FU nanoparticles had an acceptable potential for treating colon cancer and could kill approximately 80\% of the cancer cell line, HT-29. Hosseinifar et al\textsuperscript{32} reported a newly published article on the preparation of pressure sensitive 5-FU-loaded nanogels based on alginate modified with β-cyclodextrin as cross-linker through emulsification method. Their obtained results showed that the nanogels containing the drug could be employed as an excellent candidate to overcome the inefficiency of the drug using exclusively in cancer therapy. Recently, Raza et al\textsuperscript{33} synthesized chondroitin sulphate-poly (vinyl alcohol) cross-linked microcapsules (miCAPs) using emulsion procedure for controlled delivery of 5-FU. Their results showed that the loading capacity of the drug was 75.3\% and the drug release mechanism followed Fickian diffusion. In another work, Jalalvandi et al\textsuperscript{24} designed dextran/polyhydrazide-based hydrogel network samples to investigate their capability for 5-FU release. The obtained outcomes revealed that these samples enabled to prolong the drug release for almost 2 days.

By following up the literature, rare reports have been found on the MIPs containing 5-FU. Instantly, Puoci and co-workers\textsuperscript{34} synthesized methacrylic acid (MAA)-based MIP nanoparticles containing 5-FU using ethylene glycol dimethacrylate (EGDMA) as cross-linker to investigate controlled release of the drug in a biological fluid. On one hand, their observations resulted in that the drug adsorption capacity of the MIP sample was higher than the non-imprinted polymer (NIP) ones. On the other hand, the MIPs gradually released 5-FU during 30 hours. By recapitulating this unique work, lack of information about the effect of polymerization parameters on the size of nanoparticles, binding experiment between the MIPs and NIPs, the drug release kinetic, and 5-FU-loaded MIP nanoparticles performance on programmed cancer cell death is obvious.

This current work is aimed to synthesize MAA-based MIP nanoparticles containing 5-FU by using precipitation polymerization. A series of main independent variables of polymerization process such as MAA:5-FU molar ratio, temperature, and time are optimized using D-optimal design method to attain the sample with a minimum size of diameter. Afterward, the optimized MIP and NIP samples are characterized in viewpoint of morphology, binding capacity, the drug release along with its kinetic, and induction of colon cancer cell line apoptosis.

2 EXPERIMENTAL PROCEDURE

2.1 Materials

Methacrylic acid (MAA, functional monomer, synthesis grade, purity of 98.5\%), azobisisobutyronitrile (AIBN, thermal initiator, 98\% purity), and EGDMA (crosslinking agent, technical grade) were purchased from Merck Co. (Darmstadt, Germany). Prior to use MAA, inhibitors like hydroquinone were removed via vacuum distillation. In addition, AIBN has been re-crystallized by ethanol before use. 5-Fluorouracil [5-FU (C$_4$H$_7$FN$_2$O$_2$)] with molecular weight of 130.077 g/mol, anti-cancer drug, white, odourless and crystalline powder, and melting point of 282°C was provided from Merck Co. (Darmstadt, Germany). The cell line related to human colon cancer, HCT-116, was obtained from the cell bank of Pasteur Institute of Iran (Tehran, Iran). The other chemicals were technical reagent grades and used without further purification.

2.2 Synthesis of 5-FU template-based MIP and NIP nanoparticle samples

The overall schematic of 5-FU template-based MIP nanoparticles synthesis via precipitation polymerization is shown in Figure 1. In this line, 5-FU drug as template, MAA as functional monomer, EGDMA as crosslinking agent, and acetonitrile as porogenic solvent were used. In brief, the polymerization process for the formation of MIP nanoparticle samples based on 5-FU template was as follows: first, due to MAA/5-FU complex formation, the predetermined amount ratios of MAA to 5-FU (2, 4, and 6) were dissolved in 8 mL of acetonitrile in 100 mL of glass vessels and were then placed into an ultrasonic bath with 80-MHz frequency for 6 minutes. In this step, owing to providing more stable hydrogen bonds between the monomer and template groups, the resulting solutions were chilled in an ice and water bath and stirred for 6 hours at 30 rpm. Subsequently, EGDMA and AIBN and the remaining solvent were added to the mixtures with the amounts of 16 mmol, 0.019 g, and 32 mL, respectively, and then they were placed again into the ultrasonic bath for 6 minutes. Thereafter, the vessels were degassed by purging nitrogen gas for 20 minutes to ensure the oxygen molecules removal from the polymerization media. After carefully sealing the vessels, the prepared samples were slowly stirred at 20 rpm in an oil bath at 60°C until the polymerization reaction was started. It is worth noting that the polymerization
temperature was slowly increased over a period of 2 hours starting from ambient to the desired temperature. After the polymerization reaction was accomplished, the synthesized MIP nanoparticle samples were washed with 100 mL of distilled water and 100 mL of warm methanol. The final step, the removal of the template molecules from the polymeric networks, was carried out using a Soxhlet apparatus containing the washing mixture of methanol:acetic acid (9:1 v/v) during 24 hours, and then pure methanol. Accordingly, a series of active sites through the MIP cavities were created. To ensure the template removal completely, the concentration of 5-FU in the supernatant was measured using an ultraviolet-visible (UV-vis) spectrophotometer (2800, UNICO, United States) at maximum wavelength of 266 nm. Eventually, to eliminate the remaining solvent and unreacted monomer, the samples were washed with distilled water several times. In order to dry the samples, they were washed with acetone and placed into a vacuum chamber at temperature of 40°C for 24 hours. Furthermore, the NIP samples were prepared similar to the protocol for MIPs synthesis, exceptionally without 5-FU as template followed by complex formation step.

2.3 D-optimal design

D-optimal design is a subset of response surface methodology and has received a great deal of attention to optimize the process conditions with different independent variables. Herein, by the use of this method, 3 numerical factors in 3 levels including MAA:5-FU (X₁), polymerization temperature (X₂), and polymerization time (X₃) were selected. As is seen in Table 1, the ranges of X₁, X₂, and X₃ were considered 2 to 6, 60°C to 76°C, and 1 to 3 days. Table 2 shows the total runs of 14 recommended by D-optimal design method, thereby providing the optimum condition for the minimal size of MIPs as a response (Y). It should be noted that by varying the amounts of X₁ to X₃ the amounts of other components including porogenic solvent, initiator, and cross-linker were kept constant.
2.4 Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy (model Spectrum Two, PerkinElmer Inc., United States) was utilized to evaluate the formation of complex between the monomer and template. The wavenumber range was considered 400 to 4000 cm\(^{-1}\) at resolution of 4 cm\(^{-1}\).

2.5 Thermogravimetric analysis

The thermal behavior of the samples including washed and unwashed MIPs as well as NIP sample was assessed using thermogravimetric analysis (TGA, model Q50, TA Instrument, United States). By considering initial weight around 5 mg for the 3 samples, the weight loss percentage was studied. This experiment was carried out in temperature ranging from 25°C to 600°C at the rate of 20°C/min under argon atmosphere with flow rate of 10 mL/min.

2.6 Scanning electron microscopy and dynamic light scattering

The morphology observation of PMAA-based MIP nanoparticles was evaluated using a scanning electron microscopy (SEM, model AIS 2100, Seron Technology, South Korea) at 30,000 x magnification. The dynamic light scattering (DLS, model ZEN 3600, Malvern Instruments, Worcestershire, UK) was used to evaluate the average diameter and the size distribution of the nanoparticles.

2.7 Binding capacity experiments

The absorption efficiency of 5-FU drug in the polymer matrix and drug loading on MIP and NIP nanoparticles dispersed into acetonitrile were investigated at pH of 7.4 and ambient temperature. In summary, 100 mg of the MIP and NIP nanoparticles was weighed and suspended in 30 mL of acetonitrile containing 5-FU with concentration of 25 μg/mL. The mixture was stirred at 300 rpm for 6 hours in an ice and water bath, and then it was centrifuged at 12,000 rpm for 30 minutes. Consequently, the absorption amount of 5-FU in the supernatant was measured using the UV-vis spectrophotometer at 266 nm, and its concentration was calculated using the calibration curve (Equation (1)).

\[
A = 0.0887 \times C, \quad \text{(1)}
\]

where “A” and “C” are the absorbance intensity and concentration of the 5-FU in distilled water, respectively.

The amount of 5-FU bound to the polymer matrix was obtained by comparing the concentration of drug in MIP and NIP samples. The capacity of absorption (Q) as the amount of bonded template (mg) to the amount of dried nanoparticles (mg) was obtained according to Equation (2).

\[
Q = \frac{(C_0-C) \times V}{W}, \quad \text{(2)}
\]

where “C\(_0\)” is the initial drug concentration, “C” is the concentration of drug in the supernatant, “V” (mL) is the volume of adsorption medium, and “W” (mg) is the mass of the nanoparticles. Moreover, imprinting factor (IF) as the main parameter in the molecular imprinted technique was calculated according to Equation (3).

\[
IF = \frac{Q_{\text{MIP}}}{Q_{\text{NIP}}}, \quad \text{(3)}
\]

2.8 In vitro drug release

To evaluate the 5-FU release and its trend from the MIP and NIP nanoparticles samples, 50 mg of each sample containing the drug was dispersed in 5 mL of phosphate buffer solution (PBS, pH 7.4) and put into a dialysis bag (molecular weight cut off 12 kD, Sigma-Aldrich Co., Pilsburg, The Netherlands) sealed and soaked in 120 mL of PBS stirring at 50 rpm at 37°C. The samplings with volume of 3 mL were withdrawn from the medium at predetermined time intervals; instead, an equivalent volume of the fresh PBS was added to keep the volume of the medium at 120 mL. This trend was done up to 96 hours, and the drug concentration in the supernatant was measured by the UV-vis spectrophotometer at 266 nm. Finally, the cumulative release profile of 5-FU was plotted for MIP and NIP samples as a function of time. Moreover, to analyze the data on drug release from the samples, the release kinetic was studied using Korsmeyer-Peppas equation (Equation (4)).

\[
\frac{M_t}{M_0} = k t^n \left( M_t/M_0 < 0.6 \right), \quad \text{(4)}
\]

where “M\(_t\)” is the concentration of drug at time “t”, and “M\(_0\)” is the total concentration of drug loaded. Also, “k” is a constant coefficient of the drug-polymer system, which depends on the release of the drug from the polymeric network. The propagation power or characteristic of the discharge mechanism is showed by “n”, which illuminates the mechanism of its release process. For further information, when “n” is less than 0.5, the release of the drug in the system follows a Fickian diffusion or propagation phenomenon. In the case where 0.5 < n < 1, its release mechanism is in accordance with non-Fickian diffusion. If “n” is 1, the release mechanism is time independent and the model is governed by zero-order equation.

2.9 MTT assay

The cell cytotoxicity (ISO 10993-12) of the MIP nanoparticles containing different concentrations of 5-FU (10, 25, and 50 μg/mL) was characterized at overnight by using 3-[4-dimethyl thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, thiazolyl blue (MTT) according to the following steps: HCT-116 cells were cultivated at a density of 1 \times 10^4 cells/well onto 96-well plates (Nunc, Denmark) in Roswell Park Memorial Institute medium containing 10% fetal bovine serum and 1% antibiotics (penicillin/amphotericin) for 24 hours in the incubator (in moist atmosphere with 5% CO\(_2\) at 37°C). On the other hand, the MIPs were put in an autoclave (15 minutes at pressure of 1.5 bar and temperature of 121°C), then relocated in extraction medium (a Roswell Park Memorial Institute medium with 10% fetal bovine serum) for 5 days. The extract was collected and added to the full-growth media of confluent HCT-116 cells to obtain the predetermined drug concentrations and incubated for 24 hours, then
100-μL PBS containing 0.5 mg mL\(^{-1}\) MTT was added to each cell, and cells were once more incubated for 4 hours at 37°C in CO\(_2\) atmosphere. Then, the resultant formazan crystals were suspended into solubilization buffer. The optical density of the solution was measured at a wavelength of 545 nm by ELISA reader device.

2.10 | Flow cytometry analysis

Apoptosis (programmed cell death) illustrates a chief causative parameter for the anticancer activity of a drug.\(^{35,36}\) Flow cytometry analysis is a powerful tool to investigate whether apoptosis can result in the cell death induced by drug-loaded system.

In this work, human colon cancer cell line, HCT-116, was pre-treated with 5-FU-loaded MIP, 5-FU-loaded NIP, washed MIP, and 5-FU drug. In order to determine the percentage of apoptosis and necrosis, the cells were stained with Annexin V/PI (eBioscience detection kit FITC). Then, they were cultured in a 6-well plate and allowed to reach 50% confluence before incubation with the nanoparticles samples to induce cell death. After 72 hours, first, the cells were removed from the plate using trypsin, and the precipitates were collected by the use of centrifuge at 3500 rpm. The precipitated cells with approximately 1 to 1.5 × 10\(^6\) cells were suspended in 200 μL of binding buffer. Then, the cells contained with Annexin-FITC dye (5 μL) and PI (5 μL) were incubated at suitable time and analyzed using a flow cytometer device. The data analysis was performed using FlowJo software. The determination of cell population and distribution was performed using 2-dimensional plots. The areas of the 4 regions (Q1-Q4) were determined using the software which are defined as the percentage of necrotic cells, old apoptotic cells, young apoptotic cells, and natural cell, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Formation of the complex between the MAA and 5-FU

Supplementary 1 (S. 1) shows FTIR spectra of the functional groups between MAA and 5-FU before and after pre-polymerization to study the formation of complex. As is seen from S. 1, the locations of characteristic peaks were identical before and after the formation of complex. After complex formation, 4 characteristic peaks could be observed at locations of 1100 cm\(^{-1}\), 1451 cm\(^{-1}\), 1650 cm\(^{-1}\), and 1718 cm\(^{-1}\). These characteristic peaks in the order mentioned above were attributed to the stretching vibration of C–O, bending motion of –CH\(_2\)–, N–H bond, and vibration of –C=O. Furthermore, the presence of H–F bond through the complex formation could not be seen owing to its wavenumber around 4200 cm\(^{-1}\).\(^{37}\) Although, O–H bond was observed around 3500 cm\(^{-1}\) before complex formation, its intensity was increased after complex happened due to strong interactions between MAA and 5-FU functional groups. Therefore, the formation of complex between the functional groups on the structures of MAA (monomer) and 5-FU (template) was the fundamental procedure to create active sites in the cavities of the MIP samples.

3.2 | Optimizing the different variables of polymerization

According to the Table 2, D-optimal design gave 14 runs to evaluate and optimize the involved 3 variables (X1-X3) as a function of average diameter of nanoparticles (Y). The obtained experimental data were evaluated by analysis of variance (ANOVA) and a generalized 2-factor interaction (2FI) equation was estimated using the software as follows:

\[
Y = 151.7 + 23.7X_1 - 0.23X_2 - 96.7X_3 - 0.45X_1X_2 + 0.1X_1X_3 + 1.3X_2X_3.
\]

In Equation (5), in order to get the minimum diameter for MAA-based MIP sample which was approximately 65 nm, the optimized conditions for X1, X2, and X3 parameters were as follows: MAA:5-FU (X1) = 6, the polymerization temperature (X2) = 60°C, and the polymerization time (X3) = 3 days. The regression coefficient (r\(^2\)) for the Equation (5) was calculated 0.92.

Generally, every parameter eliminated in this equation was because of their unimportant effects on the answer, had a P-value > 0.05.

Supplementary 2 (a-f) [S. 2 (a-f)] depicts the 3-dimensional (3-D) and interactions of 3 parameters on the answer in the optimum conditions. As is seen in S. 2 (a-c), it was clear that by increasing the MAA:5-FU ratio and time and decreasing the temperature, the average diameter of the MIP sample was decreased. Also, the interaction between the time and temperature has an antagonistic property on the answer [S. 2 (f)]. Moreover, as is evident from S. 2 (d), the interaction of 2 parameters namely MAA: 5-FU and temperature has a synergistic property, thereby, increasing the temperature led to decrease the average diameter of MIP nanoparticles. At last, S. 2 (e) revealed that the interaction of MAA:5-FU and time has no effect on each other. It could be concluded that amid the 3 variables, temperature has more effect on the average diameter of nanoparticles sample compared with the other parameters. Regarding the monomer concentration, it is worth noting that in higher monomeric ranges, due to the dimerization of the MAA, the size of the nanoparticles has increased, in which the MAA:5-FU ratios higher than 6 have not been considered.

3.3 | Morphology and size distribution of the optimized MIP nanoparticles sample

According to Figure 2A.B, it is clear that the MIP nanoparticles synthesized in optimal conditions have a uniform shape with narrow size distribution. Besides the effective parameters, in general, the choice of suitable solvent, plays a significant role in the size of synthesized MIP nanoparticles and their distribution as well. Therefore, MIP synthesis in the presence of acetonitrile, the solvent used in this work, seemed more effective. Because acetonitrile is subset of aprotic polar solvents, it has good compatibility with all the components involved in the polymerization process, leading to faster deposition of polymerized precipitates, as a result, the size of the MAA-based MIP nanoparticles underwent smaller diameter. By considering the obtained results from SEM, the average diameter for optimized MIP sample was around 65 nm. Nevertheless, the DLS outcome showed greater average diameter size for the sample approximately 100 nm because of
3.4 | Thermal behaviors of the optimized MIP and NIP nanoparticles samples

Figure 3 illustrates the thermal behaviors of the unwashed MIP, washed MIP, and NIP samples. In this test, the variation of weight loss of the samples as a function of temperature was studied. From these TGA curves, 2 notifications could be considered: firstly, the thermal stability of the samples, in which the initial weight loss of the washed MIP sample was occurred at around 200°C, while this trend was happened for unwashed MIP and NIP samples at around 340°C. It was due to the recognition active sites in the cavities of washed MIP sample which were highly sensitive against higher temperatures whereas in the unwashed MIP sample the presence of hydrogen bonds led to higher thermal stability for those provided sites. Also, in the NIP sample, there was no active sites because of the lack of

5-FU as template. Secondly, the second weight loss was observed at temperature of around 320°C to 340°C, which was related to the degradation of the polymeric network. In addition, by comparing the thermal curves of the NIP and unwashed MIP samples, they indicated that the stability of the unwashed MIP sample was a little higher than the NIP sample due to the network structure in MIPs. Eventually, all the polymer nanoparticles were completely decomposed at 470°C and converted to ashes.

3.5 | Binding capacity and the drug loading experiments

In general, the potential of polymer matrices (MIP and NIP) for the detection and absorption of drug is influenced by various factors such as pH, monomer to drug ratio, type of absorption medium, and drug concentration, etc. According to the results of the previous study, the absorption of drug in acidic and non-acidic environments was not clear very well, but at pH 7.4, there was the greatest difference between MIP and NIP samples, because the concentration of OH− and H+ was low, and the drug and monomer were in the best condition for the formation of a bond. In the acidic pH, both the drug and the monomer were protonated, and the monomer was ionized and degraded in the basic pH; consequently, there was no possibility of effective hydrogen bonding between the monomer and the drug. For this reason; herein, the adsorption test has been investigated at pH 7.4. Therefore, by adjusting the conditions for obtaining the binding capacity (Q) of the optimized MIP sample and NIP ones, this value was calculated 21.4 and 13.6, respectively. In conclusion, by dividing these values, the IF was obtained 1.57 which showed the remarkable efficiency of the optimized MIP nanoparticles sample to select the 5-FU molecules as template.

3.6 | In vitro drug release of the optimized MIP and NIP nanoparticles samples

5-FU release studies related to the optimal MIP and NIP samples were simultaneously performed in PBS at pH 7.4 and 37°C. Figure 4 clearly shows that the drug release from NIP sample was significantly faster than MIP ones. In other words, because of the presence of special
recognition sites through the MIP nanoparticles structure, they were able to control the release of 5-FU rather than NIP sample. Furthermore, the initial burst release for both samples was occurred before 10 hours which was associated with the adsorption and poor non-specific interactions between the polymer structure and template. After 10 hours, it could be seen that the MIP sample has a gradually release profile with a gentle slope, thereby diffusing out 80% of the drug released until 96 hours. To compare the obtained results with those reported by Puoci and coworkers, it is worth noting that their MIP samples based on PMAA could diffuse out 5-FU during 30 hours, and the accumulative release was calculated around 60%. Whereas, in the similar conditions mentioned in this work, the prepared nanoparticles showed higher extent of release time (96 hours) with the total accumulative drug release of 80%.

In order to analyze the release kinetic of 5-FU from the samples, the well-known Korsmeyer-Peppas equation was fitted on the experimental data and exponent value of “n” was calculated approximately 0.43. As mentioned before, this value revealed that the main mechanism of the drug release was governed by Fick’s law.

3.7 Cell cytotoxicity of the optimized MIP nanoparticles sample containing 5-FU

Cytotoxicity of 5-FU-loaded MIP nanoparticles sample on HCT-116 cells was studied using MTT assay [Supplementary 3 (S. 3)]. As is shown, a high viability (80%-95% as compared with control) of HCT-116 cells was remarkable when exposed to 10, 25, and 50 μg mL$^{-1}$ of the sample. These results exhibited that 5-FU-loaded MIP sample in this concentration range has a non-toxic effect on HCT-116 cells; as a result, it could be considered biocompatible.

3.8 Induction of cell apoptosis for the optimized MIP and NIP samples

Figure 5A-D demonstrates the cell apoptosis patterns for the samples including washed MIP, NIP, 5-FU-loaded MIP and exclusive
use of 5-FU (control) after 3 days. By comparing Figure 5A-D, it could be observed that the exclusive use of 5-FU has the lowest cell death (23.92%) (Figure 5A) compared with NIP (39.22%) (Figure 5B), washed MIP (51.39%) (Figure 5C) and 5-FU-loaded MIP (89.64%) (Figure 5D) samples. It could be referred to the short half-life this drug (10-20 min) which significantly reduced its efficiency. Regarding the cell apoptosis window related to washed MIP, the programmed cell line death was also minimal because of MAA in the MIP sample in the absence of drug (owing to washing) did not have any influence on cancer cell line apoptosis. However, the amount of cell apoptosis of washed MIP was higher than the exclusive use of 5-FU, because of the residual drug might be remained in the MIP’s cavities during the washing. Interesting results were considered in the NIP and 5-FU-loaded MIP samples for the induction of HCT-116 cell death (Figure 5B,D). As is evident, the cell apoptosis of 5-FU-loaded MIP sample was higher than NIP ones because of sustained control release of the drug from MIP nanoparticles. However, the NIP results on this test were considerable as well owing to the presence of the drug molecules adsorbed onto the surface of the sample.

4 | CONCLUSIONS

To synthesize MIP based on MAA for higher efficiency to select 5-FU molecules and having great binding capacity along with minimum average diameter of the nanoparticles, a series of formulations were designed using D-optimal method and were then evaluated. By considering and optimizing 3 independent parameters including monomer:template molar ratio of 6, polymerization temperature of 60°C, and polymerization time of 3 days, the minimum size of nanoparticles sample was obtained around 65 nm in the presence of acetonitrile as porogenic solvent. To study the thermal stability of the samples before and after washing and also NIP sample, they showed that active sites thermal sensitivity through the sample removed the template. On the other hand, the MIP based on MAA monomer had a controlled release of 5-FU compared with NIP sample due to the recognition sites formed in the cavities. Also, the release kinetic followed Fickian diffusion theory in the PBS. Finally, the MIP sample showed a high cytocompatibility and could effectively improve cell line (HCT-116) apoptosis. In summary, the different experiments confirmed that the MIP based on MAA was the promising candidate for adsorption and controlled release of 5-FU for using in colon cancer therapy potentially.

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SUPPORTING INFORMATION
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