ROS-Responsive Chitosan Coated Magnetic Iron Oxide Nanoparticles as Potential Vehicles for Targeted Drug Delivery in Cancer Therapy

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Background and Objective: Cancer cells accumulate high concentrations of reactive oxygen species as a result of their faster and uninhibited metabolic activity. Cancer chemotherapeutic agents release an excess of severe adverse reactions as a result of targeting normal cells. This demands an improvement in targeted drug-delivery systems to selectively discharge anticancer drugs in the vicinity of such highly metabolically and mitotically active cells.

Materials and Methods: Here, magnetic nanoparticles were synthesized by a traditional co-precipitation technique. Fe₃O₄@OA-CS-5-FLU-NPs were synthesized by an easy and rapid in situ loading method. The proposed Fe₃O₄@OA-CS-5-FLU-NPs were productively prepared as well as characterized by various spectroscopic and microscopic studies.

Results: The targeted drug release profile of the Fe₃O₄@OA-CS-5-FLU-NPs was studied in the presence of ROS including H₂O₂ and pH induction. The released product, Fe₃O₄@OA-CS-5-FLU-NP, exhibited desirable levels of cytotoxicity and demonstrated morphological changes and inhibition of colony formation for A549 and HeLa S3 cancer cells. The IC50 values at 24 hours were 12.9 and 23 μg/mL, respectively.

Conclusion: In summary, results from the MTT assay, fluorescence staining as well as colony formation assays, revealed that the Fe₃O₄@OA-CS-5-FLU-NPs were active and safe for anticancer biomedical applications. In summary, the present investigation provides a powerful nano-structured based system for improved cancer theranostics that should be further studied.

Keywords: magnetic iron oxide nanoparticles, oleic acid, chitosan, 5-fluorouracil, cytotoxicity, targeted drug delivery

Introduction

Successful cancer therapy depends on the outcome of surgical resection of the affected tissue in combination with chemotherapy or radiation therapy. Although anticancer drugs are efficient at destroying tumor cells, they are potentially harmful as a result of their non-selectivity, which has limited their therapeutic use. In the last few decades, there have been more and more cases of nanoparticles (NPs), such as Fe₃O₄NPs, which have played a pivotal role in drug delivery specifically targeting cancer cells. Fe₃O₄NPs have gained great attention in various biomedical applications such as targeted delivery, localized hyperthermia treatment, as well as contrast agents for magnetic resonance imaging (MRI). As the Fe₃O₄NPs are unstable in aqueous media, they cannot be used as drug carriers alone. In order to overcome this drawback, a coating is applied to employ lipids, proteins, carbohydrates, liposomes, synthetic and bio-degradable polymers.
Chitosan has been proposed as a potential carrier for drugs and biological macromolecules\(^{15}\) with potential biomedical and pharmacological applications because of several attractive features such as low cost, safety, biodegradability and biocompatibility.\(^{16-18}\) Chitosan and its derivatives have many desirable biological and biochemical properties which could be attributed to reactive functional groups namely hydroxyl (-OH) and amino (-NH\(_2\)) groups. On the other hand, chitosan is not suitable for binding to Fe\(_3\)O\(_4\)NP as the resulting magnetic compounds tend to form aggregates of polymer cross-linked complexes and polymer adsorbates.\(^{19,20}\)

Many investigators have attempted to functionalize CS in order to bind onto Fe\(_3\)O\(_4\)NP. Several reports highlight the internalization of some Fe\(_3\)O\(_4\)NP with an enhanced generation of reactive oxygen species (ROS). Enhanced ROS levels have demonstrated synergistic antitumor efficacy by stimulating mitochondria-mediated apoptosis.\(^{21-24}\)

Fe\(_3\)O\(_4\)NP have been reported to up-regulate ROS including H\(_2\)O\(_2\) which potentially play a key role in biomedical applications especially for targeted drug delivery.\(^{25}\) The assessment of anticancer drug-loaded nanoparticles and their effect on the induction of cell apoptosis, and the extent of the Fe\(_3\)O\(_4\)NP activated ROS-mediated mitochondrial apoptosis pathway, have not been experimentally investigated. 5-FLU is an anti-metabolite of the pyrimidine class, with a broad spectrum of activity and is quite effective in the chemotherapy of solid malignant tumors, such as that of the abdominal cavity, ovary, liver, brain, breast, etc., either alone or in combination.\(^{26,27}\) 5-FLU affects nucleoside uptake and can be incorporated into RNA as well as DNA, leading to cytotoxicity and cell damage. Oleic acid (OA) is a commonly used surfactant to stabilize magnetic nanoparticles synthesized by traditional co-precipitation methods, and some studies have proved that a strong chemical bond is formed between carboxylic acid and amorphous iron and amorphous iron oxide nanoparticles. OA possesses a non-polar hydrocarbon tail and a polar carboxylic acid head group. Carboxylate anions are known to coordinate with the surface of magnetite, presumably through a coordination of iron atoms with both of the carboxylate oxygens. Oleic acid and chitosan-based magnetic aggregates were investigated here as a controlled drug-delivery system capable of pH alteration and ROS release.\(^{28,29}\) They also exhibit enhanced levels of biocompatibility as well as desirable drug release characteristics.\(^{30}\)

Herein, we present the synthesis, characterization and evaluation of Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NPs as a combination drug carrier for the pH-responsive release of 5-FLU delivery in A549 and HeLa S3 cell cultures and the resultant synergistic cytotoxicity of 5-FLU and ROS.

**Materials and Methods**

**Chemicals**

Iron (II) tetrahydrate (FeCl\(_2\)-4H\(_2\)O), iron (III) chloride hexahydrate (FeCl\(_3\)-6H\(_2\)O), sodium hydroxide and oleic acid (OA) were purchased from Merck (India). Chitosan (CS) and 5-fluorouracil (5-FLU) were procured from Sigma-Aldrich. Milli-Q water and organic solvents of analytical grade were used in this study.

**Preparation of Fe\(_3\)O\(_4\)@OA NPs**

Fe\(_3\)O\(_4\) magnetic nanoparticles were synthesized by a chemical co-precipitation process. Concisely, 1.0 g of FeCl\(_3\)-6H\(_2\)O and 0.5 g of FeCl\(_2\)-4H\(_2\)O were dissolved in 50 mL of double-distilled water through a dynamic stirring process and the solution was allowed to reflux at 80 °C. Then, the precipitant of the ammonia solution in 10 mL (NH\(_4\)OH, 25 wt %) was slowly dropped into the above mixture under vigorous stirring for 15 min,\(^{31}\) the reason for using an ammonia solution was to increase the number and size of the nanoparticles. Ammonia could play a role not only in increasing the reaction rate but also in retarding the growth of the particles when used in different concentration ranges. Finally, the drop-wise addition of oleic acid (3 mL) into the above suspension led to an alteration at the surface of the Fe\(_3\)O\(_4\)NP.\(^ {27}\) This mixture was refluxed at 80 °C for 30 min to complete the formation of the Fe\(_3\)O\(_4\) NP. Subsequently, the suspension was cooled down to room temperature and separated by a magnet, cleaned with distilled water and ethanol to remove the reactants and impurities. The final product of Fe\(_3\)O\(_4\)@OA NP (oil-base surface) was desiccated at room temperature under a vacuum at 60 °C for 12 h.

**Preparation of Fe\(_3\)O\(_4\)@OA-CS-NPs**

Chitosan modified Fe\(_3\)O\(_4\)NPs were synthesized by the co-precipitation of Fe\(^{2+}\) and Fe\(^{3+}\) salts in the presence of chitosan.\(^ {32}\) Chitosan (0.125 g) was solidified in 30 mL of 1% acetic acid and the pH was tuned to 4.8 by adding 10 M NaOH. The Fe\(_3\)O\(_4\)@OA NPs (1g) were added to 30 mL of a chitosan solution while stirring under an inert atmosphere at 50 °C. Then, 30 mL of an aqueous ammonia solution (25%) was gently added to the above mixture to yield small nanoparticles. The resulting mixture was further stirred for
30 min. The colloidal Fe$_3$O$_4$@OA-CS-NP was continuously washed with distilled water and separated by magnetic decantation repeatedly.

**Preparation of Fe$_3$O$_4$@OA-CS-5-FLU-NPs**

Fe$_3$O$_4$@OA-CS-5-FLU-NPs were prepared based on an in situ filling process as shown in Figure 1. For the loading method, Fe$_3$O$_4$@OA-CS-NSPs (100 mg) were mixed with 5-FLU and stirred at room temperature for 48 h. The negatively charged 5-FLU molecules electrostatically interacted with positively charged CS molecules for the formation of Fe$_3$O$_4$@OA-CS-5-FLU-NPs. Chitosan is a hydrophilic cationic polymer used to prepare nanoparticles by means of electrostatic interactions. 5-fluorouracil (5-FU) is a hydrophilic drug. Two ionic interactions exist in the reaction process: an intense electrostatic interaction between the positively charged amino group of CS and the negatively charged 5-FU solution. The suspension was carefully collected using a magnet and washed with an abundant amount of water and ethanol to remove the unreacted particles. Finally, the Fe$_3$O$_4$@OA-CS-5-FLU-NP powder was obtained after drying under vacuum pressure.

**Characterization of Fe$_3$O$_4$@OA-CS-5-FLU-NPs**

Powder X-ray diffraction (XRD) of Fe$_3$O$_4$@OA-CS-5-FLU-NP was analyzed by a X’Perto Pro, PANalytical X-ray diffractometer using a CuK$\alpha$ energy source ($\lambda$=1.5406 Å). The Fe$_3$O$_4$@OA-CS-5-FLU-NPs were further characterized using a Perkin Elmer Fourier transform-infra red (FT-IR) spectrophotometer. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) micrographs of the Fe$_3$O$_4$@OA-CS-5-FLU-NP were collected using a JSM-6390LVJEOL (Japan) to analyze surface morphology. The magnetic properties of the Fe$_3$O$_4$@OA-CS-5-FLU-NPs were studied using a Lakeshore 7404-vibrating sample magnetometer (VSM) at room temperature. Thermogravimetric analysis (TGA) was done on a SDT Q600 (USA) TA instrument.

**Estimation of Drug Encapsulation Efficiency of Fe$_3$O$_4$@OA-CS-5-FLU-NPs**

The concentration of the Fe$_3$O$_4$@OA-CS-5-FLU-NP was estimated by UV-Vis spectrophotometry. 10 milligrams of Fe$_3$O$_4$@OA-CS-5-FLU-NP dissolved in 1 mL of DCM (dichloromethane, to allow for a more extensive and increased drug-loading into the interior of nanoparticle) was taken in a closed glass tube and 10 mL of phosphate buffer (pH 7.4) was added to it (the total volume of the releasing medium was maintained as constant by adding an equal volume of fresh buffer solution). The mixture was stirred for 2h at room temperature. The mixture was centrifuged at 6000 rpm for 10 min after the slow evaporation of DCM. The buffer solution was separated and the amount of 5-FU in the buffer solution was estimated by spectrophotometry at 270 nm. The following equations were used to determine drug content and encapsulation efficiency:

\[
\text{Drug content} = \frac{\text{Weight of encapsulated drug in nanoparticles}}{\text{Weight of nanoparticles}} \times 100
\]

\[
\text{Encapsulation efficiency} = \frac{\text{Weight of encapsulated drug in nanoparticles}}{\text{Weight of initial loading of drug}} \times 100\%
\]

**In vitro Drug Release Studies**

The drug release characteristics of the arranged Fe$_3$O$_4$@OA-CS-5-FLU-NP was determined as follows: 100 mg of the each Fe$_3$O$_4$@OA-CS-5-FLU-NP were placed in four individual glass beakers in 5 mL each containing phosphate buffer solutions of pH 7.4 and 5.2, respectively, along with 133 μM H$_2$O$_2$ placed on a mechanical shaker. Samples were collected (1 mL) at regular time intervals, and the concentration of 5-FU released from the Fe$_3$O$_4$@OA-CS-5-FLU-NP was determined by measuring the absorbance at 270 nm. The drug release efficiency of Fe$_3$O$_4$@OA-CS-5-FLU-NP was calculated using the following equation:

\[
\text{Drug releasing efficiency (\%)} = \frac{C_t}{C_0 - C_t} \times 100
\]

where $C_t$ is the concentration of 5-FU released from Fe$_3$O$_4$@OA-CS-5-FLU-NP at time “t” and $C_0$ is the initial concentration of the Fe$_3$O$_4$@OA-CS-5-FLU-NP.

**In vitro Cytotoxicity Studies**

**MTT Cytotoxicity Assay**

A549, HeLa S3, MCF-7 and IMR-90 cells were purchased from ATCC, Manassas, VA, USA. A549, HeLa S3, MCF-7 and IMR-90 cell lines were cultured in DMEM medium (Dulbecco’s Modified Eagle’s Medium) and incubated at 37°C with 5% CO$_2$. Cells were seeded overnight onto 96-well plates at a density of 20,000 cells per well. After
completion of the incubation period, the fresh medium containing blank nanoparticles or drug encapsulated nanoparticles were added to each well. Over a period of time, 10 µL of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT solution (5 mg mL\(^{-1}\)) was added to each well, and then plates were further incubated for 4 h. Then, 100 µL of a formazan lysis solution (10% SDS in 0.1N HCl) was added to each well. The absorbance was measured at a wavelength of 570 nm using a microplate reader (BIO-RAD).

AO/EB Staining Assay

AO/EB fluorescence staining was performed to detect the morphological changes in apoptosis in A549 and HeLa S3 cells. Cells (1 × 10^5) maintained in DMEM for 24 h were exposed to 15 µg/mL (Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NP) at 37 °C and 5% CO\(_2\). Then, 10 µL of a AO/EB dye was added to each well and further incubated for 15 min under dark conditions. The cells under fluorescence images were captured on a Biorevo, BZ-9000, Keyence (20x) fluorescence microscope system.

Colony Formation Assay

A549 and HeLa S3 (5×10^3 cells/well) cell lines were kept at 37°C in a humidified atmosphere containing 5% CO\(_2\). Cells were cultured in DMEM medium and the A549 and HeLa S3 cells were washed twice with PBS buffer and incubated with various concentrations of MMS. After a period of time (10 days), the wells were washed with deionized water and stained with a crystal violet solution for 15 min. The obtained results were calculated by ImageJ software using colony area.

Statistical Analysis

Tests were completed at least three times each and the data were characterized as the average and standard deviation (mean ± SD) of three independent experiments. One-way ANOVA analysis was used for comparing between groups and P <0.05 was considered a statistically significant difference.

Results and Discussion

Morphology and FTIR Analyses of Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NPs

The surface morphology of the Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NPs was analyzed through scanning electron micrographs (SEM). As depicted in Figure 2A, the nanoparticles appeared to be spherically-shaped with an average size of 1.5 µm and a smooth outer surface. Under an external magnetic field, Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NP with a diameter of 50 nm can be selectively targeted to affected tissues.

The corresponding energy dispersive X-ray spectrum (EDX) of Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NP is shown in Figure 2B. The characteristic peaks of carbon, nitrogen, oxygen and iron are presented by CS and 5-FLU. Furthermore, the metallic iron peaks show the existence of Fe\(_3\)O\(_4\) NPs in the Fe\(_3\)O\(_4\)@OA-CS-5-FLU-NPs.
Due to the drying effects, aggregated SEM images for Fe$_3$O$_4$@OA-CS-5-FLU-NPs were achieved. EDX results clearly showed that CS and 5-FLU were loaded on the surface of the Fe$_3$O$_4$NPs. This is only supporting evidence. Meanwhile, the presence of CS and 5-FLU was further confirmed by FT-IR analysis (Figure 3). The FT-IR spectrum of Fe$_3$O$_4$ NPs showed a band at 580 cm$^{-1}$ corresponding to Fe-O stretching. The characteristic O-H stretching of the hydroxyl group present in the oleic acid was observed at 3405 cm$^{-1}$. The peaks observed at 1395, 1612 and 2920 cm$^{-1}$ corresponded to C-O, C=O and C-H stretching vibrations, respectively. The FT-IR spectrum of CS showed a band at 3430 cm$^{-1}$ for the O-H stretching vibration. Further, the peaks corresponding to 1660 cm$^{-1}$ (C=O at amide I), 1600 cm$^{-1}$ (N-H at amide II) and 885 cm$^{-1}$ (C-N at amide III) correspond to $\text{--NH}$ bending vibrations. 5-FLU...
displayed a broad vibrational band which appeared around 1659 cm\(^{-1}\) for the overlapping of peaks corresponding to the C=C, C=N, and C=O groups. The strong absorption bands observed at 1250 as well as 1429–1455 cm\(^{-1}\) can be assigned to the vibration of the multi-substituted pyrimidine compound and C-O, respectively. The FT-IR spectrum of \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NP}\) presented all the characteristic peaks of its components, such as \(\text{Fe}_3\text{O}_4@\text{OA NP}\), CS, and 5-FLU and proved the successful formation of \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NP}\).\(^{35}\)

**Morphology and TEM Analyses of \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NPs}\)**

The morphology of the synthesized \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NPs}\) was characterized at 37°C using TEM analysis. The TEM image shown in **Figure 4** for \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NPs}\) revealed that the magnetic nanoparticles agglomerated, and the average particle size was about 20–35 nm as measured by DLS. Thus, the polymer on the nanoparticles did not lead to aggregation between the particles.

**X-Ray Diffraction Analysis of \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NP}\)**

The crystalline nature of the \(\text{Fe}_3\text{O}_4\) NPs, \(\text{Fe}_3\text{O}_4@\text{OA NPs}\) and \(\text{Fe}_3\text{O}_4@\text{OA-CS-5-FLU-NPs}\) was analyzed by X-ray diffraction (**Figure 5**). Diffraction peaks were observed at 20 values of 31.25°, 33.46°, 52.14°, 61.75°, 75.42° and 86.15° due to its crystalline nature. The \(\text{Fe}_3\text{O}_4@\text{OA}\) XRD pattern contained a slight semi-crystalline peak due to the surface attached oleate moiety. The respective peaks of the planes (311), (400), and
(511) were suppressed in Fe₃O₄@OA-CS-5-FLU due to the surface decorated OA, CS and 5-FLU. The crystalline nature of Fe₃O₄NP was retained even after loading of OA on the surface of the Fe₃O₄ NPs. It is suggested that the Fe₃O₄NPs in situ formed between the CS-5-FLU and the peak at the range of 2θ = 21.33–24.27° was assigned as the semi-crystalline response of CS-5-FLU.³⁶,³⁷ The decoration of CS and 5-FLU on the surface of Fe₃O₄@OA NPs increased in intensity for the semi-crystalline peaks and this confirmed the successful formation of the Fe₃O₄@OA-CS-5-FLU-NPs.

**Thermal Stability**

TGA analysis of the Fe₃O₄@OA-CS-5-FLU-NPs was performed under inert conditions. Figure 6 shows the TGA curves of the Fe₃O₄@OA NPs and Fe₃O₄@OA-CS-5-FLU-NPs. The primary weight loss up to 100 °C might be the adsorbed water in those test mixtures. The TGA data showed the weight loss in the Fe₃O₄@OA NPs and a considerable decomposition was observed around 200–350 °C due to the loss of surface water. The obtained decomposition ~370–450°C matches the charged anticancer drug 5-FLU. The TGA of Fe₃O₄@OA had a weight loss curve in the range of 410–520 °C due to the decomposition of the coated OA.³⁸ These results indicated the reducing thermal strength of Fe₃O₄@OA NPs after the addition of the desired compositions of CS and 5-FLU in the Fe₃O₄@OA-CS-5-FLU-NPs.

**Magnetic Characteristic Analysis of Fe₃O₄@OA-CS-5-FLU-NPs**

The magnetic properties of the Fe₃O₄@OA NPs and Fe₃O₄@OA-CS-5-FLU-NPs were studied by VSM at room temperature and the data is presented in Figure 7A. The higher saturation magnetization value of 73.25 emu/g was obtained.

**Figure 6** TGA curves of the Fe₃O₄@OA NP and Fe₃O₄@OA-CS-5-FLU-NP.

**Figure 7** (A) Room temperature hysteresis curves of magnetite with Fe₃O₄@OA NP and Fe₃O₄@OA-CS-5-FLU-NP and (B) Magnetic field responsive behavior of Fe₃O₄@OA-CS-5-FLU-NP.
for the Fe₃O₄@OA NPs. However, a lower saturation magnetization of 30.21 emu/g was found for the Fe₃O₄@OA-CS-5-FLU-NPs. Hence, the gained magnetic targeted drug-delivery for these NPs has potential for broad biomedical applications. The lower value of the saturation magnetization is due to the smaller size of the nanoparticles and the low saturation magnetization of the Fe₃O₄@OA-CS-5-FLU-NP composite may be attributed to the presence of a thick polymeric layer of CS and 5-FLU on the Fe₃O₄@OA NPs.³⁹ The Fe₃O₄@OA-CS-5-FLU-NPs also retained superior paramagnetic properties at room temperature which is useful in a drug-releasing system. As they do not retain magnetization before and after exposure to an external localized magnetic field, this reduces the probability of particle aggregation due to magnetic dipole attraction. The higher magnetization of the Fe₃O₄@OA-CS-5-FLU-NPs is useful for magnetically targeted drug-delivery systems. Hence, the magnetic performances of Fe₃O₄@OA-CS-5-FLU-NPs (Figure 7B) afford good targeted ability for therapeutic applications.

In vitro Drug Release Studies
Accordingly, these nanoparticles might exhibit cytotoxic activity as carrier molecules consisting of OA along with the drug which could be absorbed by simple diffusion. The in vitro release of 5-FLU from the Fe₃O₄@OA-CS-5-FLU-NP aggregates was studied in PBS at different pH values as illustrated in Figure 8A. At the physiological pH of 7.4, 5-FLU uptake (as determined was adsorbed on the Fe₃O₄@OA-CS-5-FLU-NPs) was released very slowly and the amount of the released 5-FLU presented as a weight

Figure 8 (A) Release curves at pH 7.4 and pH 5.2 in PBS containing Fe₃O₄@OA-CS-5-FLU-NPs. (B) 5-FLU release profile at pH 7.4 (H₂O₂) and pH 5.2 (H₂O₂) in PBS containing Fe₃O₄@OA-CS-5-FLU-NP.
percentage of the total 5-FLU adsorbed, was only about 62% over a period of 500 min. In contrast, in PBS at pH 5.2, to mimic the intracellular conditions of cancer cells, the release rate of 5-FLU from the Fe$_3$O$_4$@OA-CS-5-FLU-NP was much faster and the highest release of 5-FLU was over 73% within 500 min. Furthermore, in acidic conditions, the 5-FLU released in a biologically active manner and as an anti-metabolite of the pyrimidine analogue. In acidic medium (pH 5.2), -NH$_2$ groups in the CS polymer matrix stayed simply protonated into –NH$_3^+$ groups, which can weaken hydrogen bonding interactions between the CS polymer which was dissolved in a buffer solution, and released over the diffusion process. It is well known that chitosan is soluble in water in acidic media (pH = 2–6). At this pH, chitosan swells and its chains undergo deployment due to the electrostatic repulsion of positively charged –NH$_3^+$ groups. These results indicated that the release of 5-FLU from the NPs can be measured by changing the pH, which may facilitate its drug delivery and controlled discharge into cancer cells since the microenvironment of the extracellular tissues of tumors and intracellular lysosomes and endosomes are acidic.

In additional analysis, for the site-specific drug delivery of Fe$_3$O$_4$@OA-CS-5-FLU-NP in cancer, the 5-FLU release behavior from Fe$_3$O$_4$@OA-CS-5-FLU-NPs was carried out at pH 5.2 in the presence of H$_2$O$_2$. The 5-FLU release rate from Fe$_3$O$_4$@OA-CS-5-FLU-NP at pH 7.4 was very low, while at pH 5.2, there was a high...
rate of 5-FLU released in a controlled fashion, simplifying the release of 5-FLU in carcinogenic cells as biologically active. The experimental results exposed that the pH-dependent 5-FLU release from the Fe₃O₄@OA-CS-5-FLU-NPs also led to a high releasing proficiency in the presence of ROS (H₂O₂). The rate of 5-FLU release in the presence of H₂O₂ was higher than in the absence of H₂O₂ at pH 5.2; almost 90% of the 5-FLU was released after 450 min (Figure 8B), although only 70% of the 5-FLU was released at pH 5.2 in the absence of H₂O₂.

It is assumed that only the CS layer degraded at pH 5.2 which led to the release of 5-FLU, but in the presence of H₂O₂, the OA layer also degraded (at the same time breaking of bonds amongst the hydrophilic as well as hydrophobic part of OA) which facilitated a high release of 5-FLU from the Fe₃O₄@OA-CS-5-FLU-NPs; OA acts as a modifier in nanoparticle synthesis. OA showed good dispersion ability, and which could be ascribed to the great reduction in high surface energy and dipolar attraction of the nanoparticles. These results clearly exposed that the Fe₃O₄@OA-CS-5-FLU-NP had favorable 5-FLU release in the presence of ROS (Figure 9), and that the Fe₃O₄@OA-CS-5-FLU-NP are suitable carriers to deliver 5-FLU at targeted cancerous cell sites without worrying about primary release in the circulation. Also, the 5-FLU releasing efficiency from the Fe₃O₄@OA-CS-5-FLU-NPs increased with an increase of the mixed concentrations of CS. The increase in polymer concentration led to an increase in both particle size and encapsulation efficiency of the prepared NPs. This was attributed to the increased viscosity that helped to enlarge the size and maximize encapsulation efficiency.

The 5-FLU release from the Fe₃O₄@OA-CS-5-FLU-NP at pH 5.2 (H₂O₂ in PBS) demonstrated a high amount of 5-FLU released in a controlled manner, as shown in Figure 10. The release of 5-FLU in cancerous cells was also biologically active under acidic conditions. Due to its

Table 1 IC₅₀ Values of the Synthesized NPs Against Human A549, HeLa S3, MCF-7 and IMR-90 Cells

| Complex              | A549 (µg/mL) | HeLa S3 (µg/mL) | MCF-7 (µg/mL) | IMR-90 (µg/mL) |
|----------------------|--------------|-----------------|---------------|----------------|
| 5-FLU                | >50          | >50             | >50           | NT             |
| Fe₃O₄@OA-CS-NP       | >50          | >50             | >50           | NT             |
| Fe₃O₄@OA-CS-5-FLU-NP | 12.9         | 23              | >50           | >50            |

Abbreviation: NT, not tested.
structure, 5-FLU hinders nucleoside metabolism and can be combined into RNA and DNA, leading to cytotoxicity and cell death. Moreover, powerful scavengers of free-radical oxidants result via H-atom donation as well as electron transfer, exerting their antioxidant activity. \(^{48-50}\) However, the \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) exhibited 5-FLU release at pH 5.2, indicating the nature of the loaded 5-FLU suggesting that the \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) are proper carriers to deliver 5-FLU to targeted cancerous cell sites without early release.

**In vitro Anticancer Studies**

To further investigate the cell-toxic properties of the nanoparticles, including 5-FLU and \(\text{Fe}_3\text{O}_4@OA-CS-NPs\) and \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\), to A549, HeLa S3 and MCF-7 cells, MTT assays using different concentrations (1.5–50 µg/mL) for 24 h at 37°C were conducted. As depicted in Figure 11 and Table 1, the viability of the A549, HeLa S3 and MCF-7 cells in the presence of \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) displayed higher cytotoxicity against A549 and HeLa S3 cells with IC50 values after 24 h of 12.9 and 23 µg/mL, respectively. In addition, A549, HeLa S3 and MCF-7 cell viability did not change by \(\text{Fe}_3\text{O}_4@OA-CS-\text{NP}\) exposure. Further, toxicity studies on 5-FLU and \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NP}\) for human normal lung IMR-90 cells were conducted by MTT assays. Remarkably, both 5-FLU and \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) did not show toxicity up to >50 µg/mL for IMR-90 cells.\(^{51}\) The \(\text{Fe}_3\text{O}_4\) nanoparticle aggregates of different sizes were characterized for their uptake and toxicity in three different cell lines. While the aggregation did not elicit a unique toxic response, the uptake patterns were different between single and aggregated nanoparticles. There was a decrease in uptake of aggregated nanoparticles by HeLa S3 and A549 cells in comparison to single and monodispersed nanoparticles.

In addition, the cytotoxic study revealed cell morphological modifications and nucleus fragmentation using a co-staining process with acridine orange (AO) and ethidium bromide (EB) of A549 and HeLa S3 cells. Here, AO was used as a staining reagent of live cells and EB was used as an indicator for dead cells. A549 and HeLa S3 cells were examined in the presence of \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) (15 and 40 µg/mL) for 24 h and pictures were captured with the help of AO/EB under fluorescence microscopy. As presented in Figure 12, in the presence of \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\), cell death of A549 and HeLa S3 cells gradually increased as specified by the red color which co-stained EB.\(^{52}\)

Moreover, this study further investigated whether the \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\) had an ability to control A549 and HeLa S3 cell colonization. As shown in Figure 13, complete inhibition of colony development was demonstrated in the presence of \(\text{Fe}_3\text{O}_4@OA-CS-5-\text{FLU-NPs}\).
against A549 and HeLa S3 cells at 15 and 40µg/mL, respectively. However, Fe₃O₄@OA-CS-5-FLU-NPs did not affect cell colony formation at a lower concentration of 20µg/mL for the HeLa S3 cells. Finally, we conclude here that the Fe₃O₄@OA-CS-5-FLU-NPs not only showed cytotoxicity (including morphological changes) but also showed a capability to reduce the colony formation of A549 and HeLa S3 cells.

**Conclusion**

In summary, Fe₃O₄@OA-CS-5-FLU-NPs were synthesized by an easy and quick in situ loading method. The proposed Fe₃O₄@OA-CS-5-FLU-NPs were successfully prepared as well as characterized by several spectroscopic and microscopic studies. They showed significant ROS reactive drug-releasing properties. Further, the Fe₃O₄@OA-CS-5-FLU-NPs showed promising magnetic properties that could be used for magnetic targeted and pH-responsive drug-delivery systems. The pH triggered drug release of 5-FLU from Fe₃O₄@OA-CS-5-FLU-NPs includes the ROS-responsive polymeric nanocarriers under marginally acidic conditions of pH 5.2 and pH of 7.4. Meanwhile, the MTT assay, fluorescence staining as well as colony formation assay results, revealed that the Fe₃O₄@OA-CS-5-FLU-NPs are active and safe for anticancer biomedical applications, and thus should be further studied.

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Disclosure
The authors have no conflicts of interest to declare.

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