

Loading the Anionic $[\text{Pd}(\text{dipic})_2]^{2-}$ Complex into the Cavities of Magnetic and Non-Magnetic Halloysite Nanotubes Conjugated with Folic Acid and Investigating the *in vitro* Therapeutic Effects

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To overcome the non-specific entry of cisplatin and other transition metal complexes into both normal and cancer cells, we developed folic acid-modified halloysite nanotubes (HNTs-FA) and magnetic halloysite nanotubes (MHNTs-FA) as targeted drug carriers. These carriers were loaded with an anionic palladium complex, $[\text{Pd}(\text{dipic})_2]^{2-}$ (dipic: dipicolinic acid), and tested on colon cancer cells. On folate receptors (FRs)-positive HT29 cells, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ showed significant cytotoxicity, reducing cell viability below 50% at low concentrations. While free $[\text{Pd}(\text{dipic})_2]^{2-}$ and cisplatin were ineffective at these concentrations, MHNTs-FA performed better due to enhanced folate receptor targeting and the presence of Fe_3O_4 nanoparticles. On FR-negative LoVo cells, results suggested the transferrin receptor-1 (TfR1) provided an alternate entry path for MHNTs-FA. Real-time polymerase chain reaction (PCR) confirmed the increased apoptosis via the mitochondrial pathway, with MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ boosting the Bak1/Bclx ratio by 1500-fold and raising Caspase-3 expression. Additionally, it suppressed AKT1 gene expression, lowering drug resistance. Flow cytometric analysis also confirmed the ability of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ to induce apoptosis in HT29 cells. Importantly, modified HNTs did not cause oxidative stress in erythrocytes. These findings highlight MHNTs-FA as an efficient, targeted delivery system that enhances the therapeutic effects of palladium complexes while minimizing harm to healthy cells.

Keywords: colon cancer, oxidative stress, AKT1, caspase-3, Bak1/Bclx ratio, erythrocytes

Introduction

Metal-based complexes, of which *cis*-diammine-dichloridoplatinum(II) or cisplatin is the most famous and potential, are usually used to treat various types of solid cancers.¹ Cisplatin interferes with deoxyribonucleic acid (DNAs), including gDNA (genomic) or mtDNA (mitochondria) and blocks the production of messenger ribonucleic acid (mRNA) and protein, which ultimately stops the proliferation of cancer cells, causes necrosis and apoptosis.² However, the highest potential of cisplatin has not yet been observed because drug resistance may develop or side effects such as hepatotoxicity, renal toxicity, neurotoxicity, etc., may occur.³ Lack of correct targeting, reduction of drug accumulation, side effect

on normal cells, change of apoptosis signaling proteins, tumor plasticity, and increase of DNA repair are among the problems that made researchers synthesize different metal complexes to reduce the clinical limitations of cisplatin.^{4,5}

Due to the similarity of the coordination chemistry of palladium (Pd^{II}) and platinum (Pt^{II}), many researches have been conducted on Pd complexes to be used as anticancer drugs.^{6,7} The advantages of Pd complexes include significant cytotoxic activity of Pd derivatives, fewer side effects than other heavy metal anticancer compounds, faster hydrolysis of Pd complexes (10^5 times faster than Pt analogues), better solubility of Pd complexes compared to Pt, induction of apoptosis in cancer cells, and causing serious damage in the DNA of cancerous tumors.⁸

Of course, it should be noted that the greater reactivity of Pd^{II} complexes compared to Pt^{II} analogs makes them not stable enough in the physiological environment and

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hydrolyzed quickly, and never reach the target DNA.⁹ For this reason, the role of the ligand attached to them is important. Dipicolinic acid (dipic) or pyridine-2,6-dicarboxylic acid is a good chelator that can bind to metal ions through the N atom of the pyridine ring and the O atoms of its carboxylate groups and create monodentate, didentate and tridentate complexes which are easily dissolved in water.¹⁰ Due to the antioxidant property of the hydrophilic ligand, complexes containing dipic act as electron carriers in biological environments and can cause DNA cleavage and nitric oxide (NO) removal.¹¹ For this reason, the synthesized complexes have shown medicinal activity and due to their low toxicity, amphiphilic nature, and extensive biological activity, they have attracted a lot of attention.^{12,13}

One issue with metal complexes is their inability to differentiate between cancer cells and normal cells, meaning they cannot specifically target cancer cells without affecting normal cells.¹⁴ The main structure of the Pd^{II} complex is crucial for its interaction with DNA strands, requiring the use of nanotubes for targeting the cancer cells. Nanotubes can host targeting agents, allow the anionic complex into their pores, and preserve the complex's square planar structure without the need for additional functional groups.

In the past, the anionic complex $[\text{Pd}(\text{dipic})_2]^{2-}$ was synthesized by our team and found to inhibit the growth of breast cancer cells.¹⁵ Several methods such as fluorescence and UV-Vis measurements showed that the complex can bind to circulating tumor DNA (CT-DNA) through H-bonds and van der Waals interactions and show cytotoxic properties due to its negative charge. Therefore, it can be a good candidate for loading in the cavities of halloysite nanotubes (HNTs). HNTs are aluminosilicate clay minerals that have Si–O–Si groups on the external surface and Al–OH groups folded inside. They can be used as a green and environmentally friendly carrier for actively loading compounds with nanometer-appropriate size. At neutral pH, they have a positively charged inner surface that allows for the loading of anionic materials, thereby increasing solubility. This enables the compounds to disperse well in the physiological environment, with release ranging from a few hours to two weeks.¹⁶

Furthermore, the functional group on the external surface of HNTs is suitable for modification with two or more different functional groups.¹⁷ One ligand that can be attached to HNTs to enhance their ability to target tumors and actively identify them, while also reducing side effects, is folic acid (FA).¹⁸ FA targets folate receptors (FRs) that are overexpressed in various human tumors such as ovarian, brain, and colon, allowing for the targeting and entry of

cancer cells using receptor-mediated endocytosis.¹⁹ On the other hand, there is evidence that FA can play a preventive role in colorectal cancer. A deficiency of FA in the diet may alter the expression of genes involved in cell cycle control, cell death, and DNA repair, ultimately increasing the risk of colon cancer.²⁰ Additionally, several studies demonstrate that FA can induce a pro-apoptotic effect by enhancing the expression and/or activation of p53,²¹ and contribute to cancer recovery.

The tumor suppressor p53 gene plays a crucial role in mitochondria by inducing apoptosis and regulating the cell cycle.²² Numerous studies have shown that the decrease in mitochondrial membrane potential triggers the intrinsic cell death pathway, leading to the activation of Bak and the inactivation of Bcl-2.²³ This activation stimulates the mitochondrial apoptosis pathway, allowing cytochrome c to move from the mitochondrial intermembrane space into the cytosol, where it activates effector Caspase-3.²⁴ Caspases can also be activated through the extrinsic pathway of apoptosis, which depends on tumor necrosis factor (TNF) or Fas. Conversely, AKT, a key substrate of Caspase-3, acts as a major inhibitor of cell apoptosis.²⁵ A study suggested that the cleavage and inactivation of AKT is influenced by Caspase-3 activity, indicating that reducing Caspase-3 levels could inhibit apoptosis and improve cancer tumor survival.²⁵ Therefore, we decided to load anionic Pd complex into the cavities of magnetic and non-magnetic halloysite nanotubes modified with folic acid (MHNTs-FA and HNTs-FA, respectively) and investigate their cytotoxic, apoptotic, and inflammatory effects on colon cancer cells. For this study, two colon cancer cell lines were selected: LoVo, lacking the folate receptor, and HT29, with the folate receptor. The goal was to compare the effectiveness of compounds with and without a folic acid targeting agent on these cell lines. On the other hand, both the LoVo and HT29 cell lines overexpress transferrin receptor-1 (TfR1), and the role of magnetic nanoparticles (MNPS) on the surface of HNTs for the entry of Pd complex into cancer cells can be well investigated.

Experimental

Materials and reagents

The content of this article was reviewed by the ethics committee of Zabol University of Medical Sciences and approved with the ethics code IR.ZBMU.REC.1401.122. All materials and solvents required for the synthesis of magnetic halloysite nanotubes modified with folic acid (MHNTs-FA) and non-magnetic halloysite nanotubes modified with folic acid (HNTs-FA) were purchased

from Merck (Darmstadt, Germany) and Sigma-Aldrich Company (Taufkirchen, Germany). HT29 and LoVo cell lines were obtained from the Pasteur Institute (Tehran, Iran). Cell culture essentials such as fetal bovine serum (FBS) was acquired from PAN Biotech (Aidenbach, Germany), penicillin/streptomycin and trypsin were bought from Gibco (Carlsbad, USA), and RPMI (Roswell Park Memorial Institute) cell culture medium was provided by VIVA CELL (Isfahan, Iran). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide), trichloroacetic acid (TCA), and Tris base were received from Merck (Darmstadt, Germany). Sulforhodamine B (SRB) was obtained from Sigma-Aldrich (Taufkirchen, Germany). The cDNA synthesis kit was acquired from The SambioTM (Tehran, Iran) and the SinaPureTM RNA isolation kit was obtained from the SinaClonTM Company (Tehran, Iran). The Annexin V Apoptosis Detection Kit was obtained from Pars Tous (Mashhad, Iran).

Synthesis of HNTs-FA

Modification of halloysite nanotubes with folic acid has been shown to enhance their biocompatibility and targeting ability for drug delivery applications. To achieve this, 300 mg of HNTs were added to 20 mL of phosphate-buffered saline (PBS) and exposed to ultrasonic waves at 100% power for 30 min using a probe sonicator.²⁶ The mixture was then centrifuged at 500 rpm for 10 min, and the resulting sediment was dried at 60 °C for 48 h. Next, 100 mg (0.34 mmol) of shortened HNTs were combined with 10 mL of ethanol in a flat bottom flask. To this mixture, 0.079 mL of 3-aminopropyltriethoxysilane (APTES) (0.34 mmol) and 5 μL of triethylamine (TEA) were added, and the reaction was refluxed for 24 h using a heater stirrer at 80 °C and 500 rpm. Upon completion of the reaction, the HNTs-APTES were washed with ethanol four times, collected using a centrifuge (1000 rpm, 5 min), and dried for 24 h at 60 °C. The yield for this step was calculated to be 86.1%.

To activate the carboxyl group of folic acid (FA), 139.53 mg (0.32 mmol) of FA, 40.2 mg (0.35 mmol) of *N*-hydroxysuccinimide (NHS), 71.74 mg (0.35 mmol) of *N,N'*-dicyclohexyl carbodiimide (DCC), and 500 μL of TEA were dissolved in 10 mL of dimethyl sulfoxide (DMSO).²⁷ The reaction was then carried out on a magnetic stirrer (500 rpm) for 24 h under an argon blanket at room temperature.

After that, all the obtained HNTs-APTES were added to the reaction solution and stirred for another 24 h under the same conditions. Once the reaction was complete, any unreacted NHS and DCC were separated using hexane. The

resulting HNTs-FA were washed with acetone three times, pelleted with the help of a centrifuge (1000 rpm, 5 min), and dried using a rotary vacuum dryer. The conjugation yield for this step was calculated to be 76%.

Synthesis of MHNTs-FA

First, in order to magnetize the HNTs, 20 mg (0.1 mmol) of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 54 mg (0.2 mmol) of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (molar ratio 1:2) were added to a double-necked flat-bottom flask, following the procedures outlined in previous works.^{28,29} The mixture was dissolved in 10 mL of water and refluxed at a temperature of 80 °C under argon gas. Next, 100 mg (0.34 mmol) of shortened HNTs were added to the flask. Subsequently, 1 mL of NH_4OH was added dropwise, and the mixture was stirred on a hot plate stirrer for 30 min. Following this, 20 mg of citric acid dissolved in 1 mL of water were added to the reaction mixture. The reaction temperature was maintained at 95 °C for 90 min to complete the reaction. The reaction mixture was then transferred to a 15 mL Falcon tube and placed inside the Dynamag magnet device to separate the magnetic HNTs (MHNTs) from the solution. The MHNTs were washed three times with ethanol and dried at 60 °C.

In the next step, MHNTs were added to 10 mL of ethanol in a flat-bottom flask. To this mixture, 0.079 mL of APTES (0.34 mmol) and 5 μL of TEA were added, and the reaction was refluxed for 24 h using a heater stirrer at 80 °C and 500 rpm. Upon completion of the reaction, the MHNTs-APTES were collected using the Dynamag device, washed with ethanol four times, and dried for 24 h at 60 °C. Then, 139.53 mg (0.32 mmol) of FA, 40.2 mg (0.35 mmol) of NHS, 71.74 mg (0.35 mmol) of DCC, and 500 μL of TEA were dissolved in 10 mL of DMSO and stirred for 24 h under an argon blanket at room temperature.

After that, all the obtained MHNTs-APTES were added to the reaction solution and stirred for another 24 h under the same conditions. To separate the final products from unreacted NHS and DCC, the mixture was transferred to a 50 mL Falcon tube and placed inside the Dynamag device. The resulting MHNTs-FA were washed with acetone three times and dried using a rotary vacuum dryer. The yield was calculated to be 65%.

Loading of $[\text{Pd}(\text{dipic})_2]^{2-}$ in tubular of HNTs-FA and MHNTs-FA

In two Erlenmeyer flasks, 10 mg of $[\text{Pd}(\text{dipic})_2]^{2-}$ complex were dissolved in 40 mL of distilled water using a stirrer. To determine the initial amount of the complex in the solution, UV-Vis absorption was measured at a

wavelength of 471 nm. Subsequently, 10 mg of HNTs-FA were added to one flask and 10 mg of MHNTs-FA were added to the other. The mixtures were thoroughly stirred using a magnetic stirrer and an ultrasonic probe for 40 min. The Erlenmeyer flasks were then placed in a vacuum tank and subjected to a pressure of -1 bar by a vacuum pump for 30 min to remove air from the halloysites tubes in the form of small bubbles. After releasing the vacuum, the solution was stirred for 35 min with a magnetic stirrer and for 5 min with an ultrasonic probe. This process was repeated three times. Centrifugation (10,000 rpm for 15 min) was used to separate the HNTs-FA containing $[\text{Pd}(\text{dipic})_2]^{2-}$ complex (HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$) from the reaction solution, while Dynamag was used to separate the MHNTs-FA containing $[\text{Pd}(\text{dipic})_2]^{2-}$ complex (MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$). Finally, UV-Vis absorption was measured from the remaining solution at a wavelength of 471 nm to determine the amount of the remaining $[\text{Pd}(\text{dipic})_2]^{2-}$ complex. By using the calibration curve and the absorption values obtained, the amount of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex loaded inside the halloysites holes can be determined.

Characterization of HNTs-FA and MHNTs-FA

The initial identification of modified HNTs was conducted using Fourier transform infrared (FTIR) spectroscopy. To do this, each sample was mixed with moisture-free KBr, turned into a homogeneous powder, and then transformed into a thin disk under high pressure vacuum. The FTIR device used was the Shimadzu IR PRESTIGE 21 spectrophotometer from Tokyo, Japan, capable of scanning the range of 4000 to 400 cm^{-1} to detect the bending and stretching vibrations of molecules.

The field emission scanning electron microscope (FESEM) is equipped with a wide range of detectors suitable for routine morphological characterization and size determination of the synthesized nanotubes. To perform this analysis, HNTs-FA and MHNTs-FA were placed inside the TESCAN MIRA3 XMU FESEM device after applying a gold coating and photographed at 50 k $\times$ magnification.

To investigate the morphological features, size distribution, and surface properties of MHNTs-FA, transmission electron microscopy (TEM) was used with a LEO 906 (Carl Zeiss, Germany) operating at an accelerating voltage of 80 kV.³⁰ Before imaging, the sample was prepared by dispersing approximately 1 mg of MHNTs-FA powder in 1 mL of ethanol. This dispersion was sonicated for 15 min using an ultrasonic bath to ensure uniform suspension and minimize aggregation. The resulting colloidal solution was centrifuged at 2000 rpm for 2 min to remove larger aggregates, and a

drop of the supernatant was carefully deposited onto a copper TEM grid. The grid was then dried at room temperature under ambient conditions. No staining agent was applied, as the inherent electron density of halloysite nanotubes provided sufficient contrast.

Additionally, elemental analysis of HNTs-FA and MHNTs-FA can be done using the energy dispersive X-ray (EDX) method. FESEM devices typically come equipped with EDX capabilities for elemental analysis, aiding in determining the accuracy of synthesis by identifying the percentage of atoms that make up the compounds.

Cell viability assay by MTT protocol

The common MTT method was utilized to assess the cell viability rate of FR-positive HT29 colon cancer cells and FR-negative LoVo colon cancer cells exposed to HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, and the well-known drug cisplatin for comparison. The cells were cultured in appropriate conditions, including a specialized culture medium at 37 °C with adequate humidity, then transferred to 96-well plates at a density of 15,000 cells *per* well. They were then treated with five different concentrations of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (ranging from 0.44 to 7 mg mL^{-1}), five different concentrations of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (ranging from 0.38 to 6 mg mL^{-1}), five different concentrations of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex (ranging from 0.008 to 0.13 mg mL^{-1}), and cisplatin (ranging from 0.008 mg mL^{-1} to 0.13 mg mL^{-1}). Cell viability was assessed after 48 and 72 h using the common MTT protocol.³¹ Three repetitions ($n = 3$, $\pm$ standard deviation (SD)) were conducted for each concentration to minimize error. All concentrations were calculated in terms of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, ensuring that the modified HNTs contained an equal amount of loaded $[\text{Pd}(\text{dipic})_2]^{2-}$ complex.

Cell viability assay by sulforhodamine B

For this study, HT29 cells were transferred to a 96-well plate and incubated with five different concentrations of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, and free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, similar to the concentrations used in the MTT method, for 72 h in a standard incubator. To begin, the culture medium was removed from the wells, and then 100 μL of cold 10% TCA were added to each well. The plate was placed in a refrigerator at 4 °C, away from light, for 60 min. Subsequently, excess TCA was washed away with sterile distilled water, and the wells were dried thoroughly by gently blowing air over them. Next, 100 μL of 0.5% SRB solution were added to each well, and the plate was incubated

for another 30 min in the dark at room temperature. Excess SRB was dissolved using a 0.5% acetic acid solution, and the wells were dried once more by gently blowing air over them. Finally, 200 μL of Tris base (10 mM) were added to each well and shaken for 20 min. The absorbance of the pink-stained cells was then measured at 510 nm.

Investigating the expression of genes involved in apoptosis by real-time PCR method

Five separate flasks containing one million HT29 colon cells were prepared. The first flask was treated with 0.0047 mg mL^{-1} of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, the second with 0.017 mg mL^{-1} of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, the third with 0.43 mg mL^{-1} of free $[\text{Pd}(\text{dipic})_2]^{2-}$, the fourth with 0.49 mg mL^{-1} of cisplatin, and the last one was considered a control. The concentrations of components were selected based on half-maximal inhibitory concentration (IC_{50}), and the cells were incubated for 12 h. After incubation, the treated cells were washed with PBS (2 $\times$), detached from the bottom of the flasks with trypsin, and collected in five separate falcons by centrifugation (6 min at 4500 rpm). The cells were then re-suspended in PBS, and RNA isolation was performed following the protocol of the SinaPureTM RNA kit.

To isolate RNA, 400 μL of Lysis solution were added to each falcon and homogenized by vortexing for one minute. Then, 300 μL of precipitation solution were added, and the falcons were shaken ten times. The solutions were transferred to five tubes with collector columns and incubated with 100 μL of 10% DNase solution for 15 min at 25 °C. After centrifugation (12000 g) for one minute, the bottom solutions were discarded. Washing steps were performed with washing solutions 1 and 2, followed by elution with RNase-free water.

For cDNA synthesis, the SambioTM cDNA Synthesis Kit was used. The reaction mixture included 2 μL of SamScript Enzyme, 10 μL of 2 $\times$ reaction buffer, and 8 μL of RNA isolated from cells. The mixture was incubated at specific temperatures for cDNA synthesis. Five cDNA samples were synthesized from the five RNAs isolated.

Gene expression analysis was conducted using the Evagreen qPCR dye with corresponding primers and synthesized cDNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize cycle threshold (CT) values, and the fold change in gene expression was calculated using the delta delta cycle threshold ($\Delta\Delta\text{CT}$) method. Real-time polymerase chain reaction (PCR) was performed to evaluate the expression of Bclx, Bak1, AKT1, and Caspase-3 genes from cancer cells using the Applied Biosystems StepOneTM system (Thermo Fisher Scientific, Massachusetts, USA).³²

Quantification of apoptosis via flow cytometry

HT29 human colorectal adenocarcinoma cells were seeded at a density of 6.0×10^5 cells *per* T-25 flask and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cell attachment and stabilization.³³ Following incubation, cells were treated with 0.017 mg mL^{-1} of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ for 24 h. Untreated cells cultured under identical conditions served as the negative control. After treatment, both control and treated cells were harvested using trypsinization, washed twice with cold phosphate-buffered saline (PBS), and resuspended in 100 μL of binding buffer provided in the Annexin V Apoptosis Detection Kit. Subsequently, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added to each sample. The mixtures were gently vortexed and incubated for 15 min at room temperature in the dark. After incubation, 400 μL of binding buffer were added to each tube. Samples were immediately analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Lipid peroxidation assay in erythrocytes

About 200 mL of heparinized blood were obtained from Zabol Blood Transfusion Organization. It was then centrifuged at 3000 rpm for half an hour, twice, to separate pure erythrocytes from the blood.³⁴ Next, the pure erythrocytes were washed twice with PBS, and a mixture of 1:9 was prepared from erythrocytes and PBS, respectively. Using this erythrocyte mixture, two different concentrations of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (3.5 and 7 mg mL^{-1}), MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (3 and 6 mg mL^{-1}), and free $[\text{Pd}(\text{dipic})_2]^{2-}$ (0.065 and 0.13 mg mL^{-1}) were prepared with a final volume of 5 mL and incubated at 37 °C for 24 h. At time intervals of 0 and 24 h, 1 mL was removed from each of the samples and added to a mixture of 2:1 trichloroacetic acid (20%) and thiobarbituric acid (0.028 M), then incubated at 100 °C for 15 min. The supernatant was separated by centrifugation, and its absorbance was measured at a wavelength of 532 nm to determine the amount of malondialdehyde (MDA) produced by erythrocytes. All steps were repeated three times to eliminate possible errors ($n = 3$).

Results and Discussion

Characterization of HNTs-FA and MHNTs-FA

The unique hollow tubular structure of HNTs (interlayer and intracavity), along with their modifiable surface, has made them widely used in drug delivery.³⁵ Their compatibility, good physicochemical properties,

naturalness, and excellent solubility²⁶ led us to choose HNTs as carriers of a Pd complex to improve solubility, targeting delivery, performance, and reduce side effects. For the first time, we synthesized two types of HNTs drug delivery systems (HNTs-FA and MHNTs-FA) to deliver anionic $[\text{Pd}(\text{dipic})_2]^{2-}$ complex to HT29 colon cancer cells using FA.

The surface of HNTs was modified using FA as a targeting agent and decorated with MNPs to simplify purification and use. To achieve this, we used ferric chloride hexahydrate and ferrous chloride tetrahydrate, introducing HNTs into the reaction. The crucial step involved adding sodium hydroxide, which transformed the Fe^{2+} and Fe^{3+} ions into superparamagnetic Fe_3O_4 nanoparticles (NPs).³⁶ It was essential for the reaction environment to be devoid of oxygen, so we used argon gas during the reaction to ensure an oxygen-free environment. As a result, the MHNTs-FA took on a dark brown hue, allowing for easy separation from the reaction solution using the Dynamag magnet device. This simplified the purification process, making it efficient and straightforward. Furthermore, the potential of iron in targeting overexpressed TfR1 on colon cancer cells is also under consideration.

The surface modification agent used was APTES to introduce amine groups onto the surface of HNTs,³⁷ allowing us to conjugate the FA through an amide bond. We also used a routine method to magnetize the surface of HNTs for easy purification using a magnetic device (Dynamag). Additionally, the presence of Fe_3O_4 nanoparticles (MNPs) on the surface of HNTs was intended to aid in cancer treatment. It is noted in all sources that the outer surface charge of HNTs is naturally negative, allowing Fe^{2+} and Fe^{3+} ions in iron chloride solutions to be electrostatically attracted to this negative charge.³⁸ One indication of the formation of MNPs on the surface of HNTs is the change in color of the initial solution after adding NH_4OH . The initial color of the solution containing HNTs and Fe ions was colorless, which changed to dark brown after adding the reducing agent.

The accuracy of the synthesis can be verified to some extent by the FTIR spectrum. Figure 1a, related to the initial HNTs, confirms that Si–O vibrations are present in the regions of 470, 1033, and 1090 cm^{-1} . The OH groups of the inner aluminum surface of HNTs are confirmed by visible bands at 910, 3626, and 3695 cm^{-1} . Additionally, the band at 540 cm^{-1} is attributed to Al–O vibration.

Therefore, the addition of any functional group to the initial HNTs will result in the appearance of additional peaks. For instance, when decorating the surface of HNTs with Fe_3O_4 (create MHNTs), the peak in the 3442 cm^{-1} region becomes stronger, indicating an increase in bending

OH groups due to Fe_3O_4 (Figure 1b). Furthermore, the presence of a peak in the 1404 cm^{-1} region suggests the stretching vibration of OH, confirming the presence of Fe_3O_4 on the surface of HNTs. Additionally, Fe–O stretching vibrations can be observed in the region of 555 cm^{-1} , overlapping with Si–O vibrations.

Before examining modified HNTs containing FA, it was first observed the spectrum of pure FA (Figure 1c). The FTIR spectrum of FA reveals characteristic absorption bands corresponding to its functional groups. Peaks around 3400 cm^{-1} indicate O–H and N–H stretching vibrations, typical of hydroxyl and amine groups. Additionally, peaks in the 2839 and 2970 cm^{-1} regions are attributed to the methylene groups. Sharp bands near 1691 cm^{-1} are attributed to C=O stretching of carboxylic acid. Peaks in the region of 1600–1500 cm^{-1} suggest aromatic C=C stretching, while signals around 1300–1000 cm^{-1} correspond to C–N and C–O stretching vibrations, confirming the presence of pteridine and *para*-aminobenzoic acid moieties.

The conjugation of FA to HNTs and the formation of HNTs-FA can be observed by the appearance of strong peaks in the 3448 cm^{-1} region, associated with the stretching vibrations of the hydroxyl groups of FA, as well as peaks in the 2854 and 2931 cm^{-1} regions, attributed to the methylene groups. Absorption bands at 1627 and 1686 cm^{-1} further confirm the presence of FA carbonyl groups (Figure 1d).

The conjugation of FA to MHNTs can be confirmed in Figure 1e, where the absorption of 1404 cm^{-1} , confirming Fe_3O_4 , is observed alongside peaks confirming FA.

The FTIR spectrum of the palladium complex $[\text{Pd}(\text{dipic})_2]^{2-}$, shown in Figure 1f, displays distinct vibrational bands that confirm its coordination environment. A broad absorption around 3300–3400 cm^{-1} corresponds to O–H stretching, likely originating from residual water or hydrogen bonding interactions. The sharp band near 1512 cm^{-1} indicates asymmetric stretching of coordinated carboxylate groups. Peaks near 1111 and 586 cm^{-1} are attributed to C–N and Pd–O/Pd–N vibrations, respectively, supporting the chelation of dipic through both nitrogen and oxygen atoms. These spectral features closely resemble those of the original synthesis source of this complex.³⁹

The spectrum of the final composition, immediately after loading the anionic palladium complex into the modified nanotube pores (MHNTs-FA), is depicted in Figure 1g. It bears resemblance to Figure 1e, with some broadening in certain areas, possibly due to overlap. Overall, the similarity to the original nanocarrier somewhat confirms the presence of the complex within the pores.

Figure 2 shows images of HNTs-FA and MHNTs-FA captured by a TESCAN MIRA3 XMU FESEM device. Upon comparing the two images, we can clearly see the

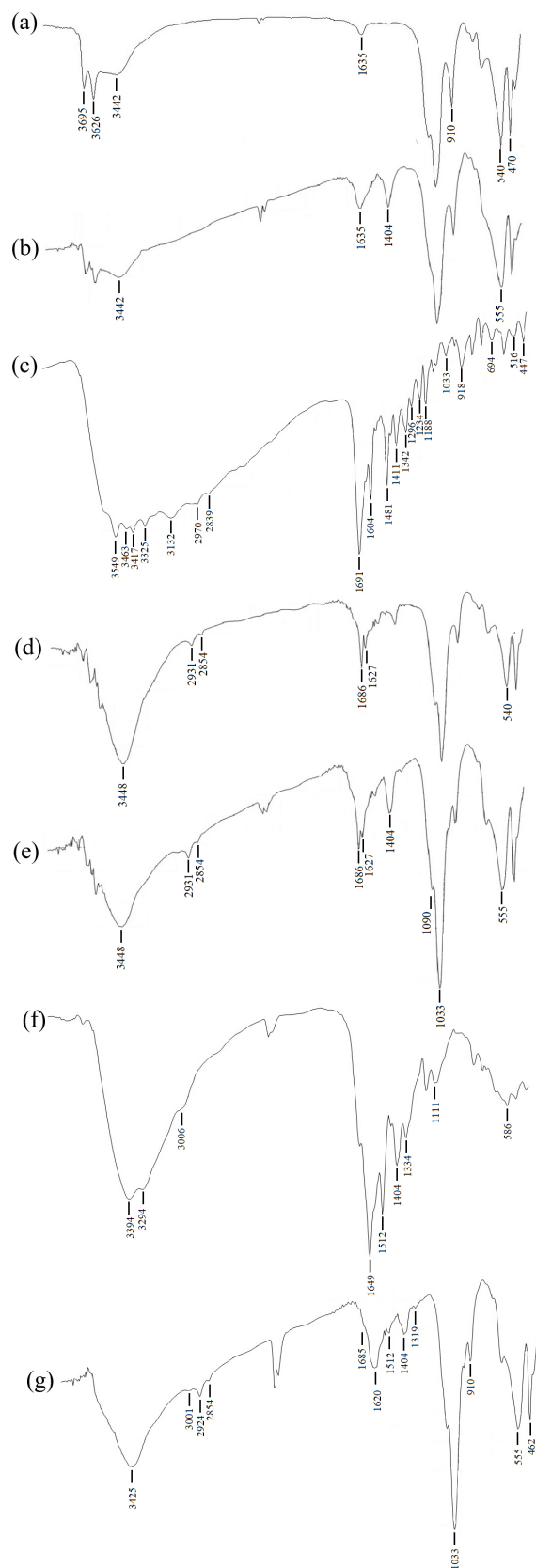


Figure 1. FTIR spectra of (a) free HNTs, (b) MHNTs, (c) FA, (d) HNTs-FA, (e) MHNTs-FA, (f) $[\text{Pd}(\text{dipic})_2]^{2-}$, and (g) MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$. The spectra were taken in KBr with a resolution of 16 cm⁻¹.

presence of Fe_3O_4 on the surface of HNTs. In Figure 2b, which pertains to MHNTs-FA, the Fe_3O_4 MNPs adhered to the surface are distinctly visible. In contrast, in Figure 2a, the surface of HNTs is devoid of any magnetic particles.

The images were prepared with a magnification of 50000 $\times$ and a scale of 500 nm. In general, the diameter of HNTs after surface modification with FA was measured to be 49 nm and their length was calculated to be about 390 nm.

To better understand the structure of the final synthesized compound (MHNTs-FA), TEM was also used to confirm the previous results. According to the scale presented in Figure 2c, the nanotubes had lengths ranging from approximately 300 to 600 nm and diameters between 30 and 50 nm. The spherical nanoparticles observed on the surfaces of the nanotubes had diameters ranging from approximately 10 to 20 nm. Image analysis was conducted using ImageJ software (version 1.54q)⁴⁰ to measure the average length and diameter of the nanotubes. This image confirms the successful deposition of Fe_3O_4 nanoparticles on the surface of HNTs, as the original tubular morphology remains intact. The relatively narrow size distribution and the absence of significant aggregation indicate good colloidal stability, which is crucial for biomedical applications, including drug delivery systems.

Another method that can confirm the correctness of the synthesis of modified HNTs is elemental analysis by EDX. By comparing Figures 3a and 3b, it is possible to understand the presence of MNPs and FA on the surface of HNTs. Basically, the building blocks of HNTs include oxygen, alumina, and silica,⁴¹ but after conjugation to FA, additional peaks of carbon and nitrogen, as well as a change in weight percentage of oxygen, alumina, and silica, confirm the presence of FA on the surface of HNTs-FA (Table 1). The reason for the reduction of the O, Al, and Si percentage was the addition of new elements to the structure of the HNTs, proving that HNTs-FA has been synthesized.

By looking at Table 1, it can be seen that iron has been added to the structure of the composition and the surface of HNTs has been decorated with MNPs. In addition to the previous peaks, the peak related to Fe is also evident in the Figure 3b, proving that MNTs-FA are well-formed.

Cytotoxicity test by MTT

After conducting an investigation using UV-Vis spectroscopy, it was determined that 60.1% of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex was loaded inside the HNTs-FA cavities, while 69.3% of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex was loaded inside the MHNTs-FA cavities. Consequently, the quantity of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex required for

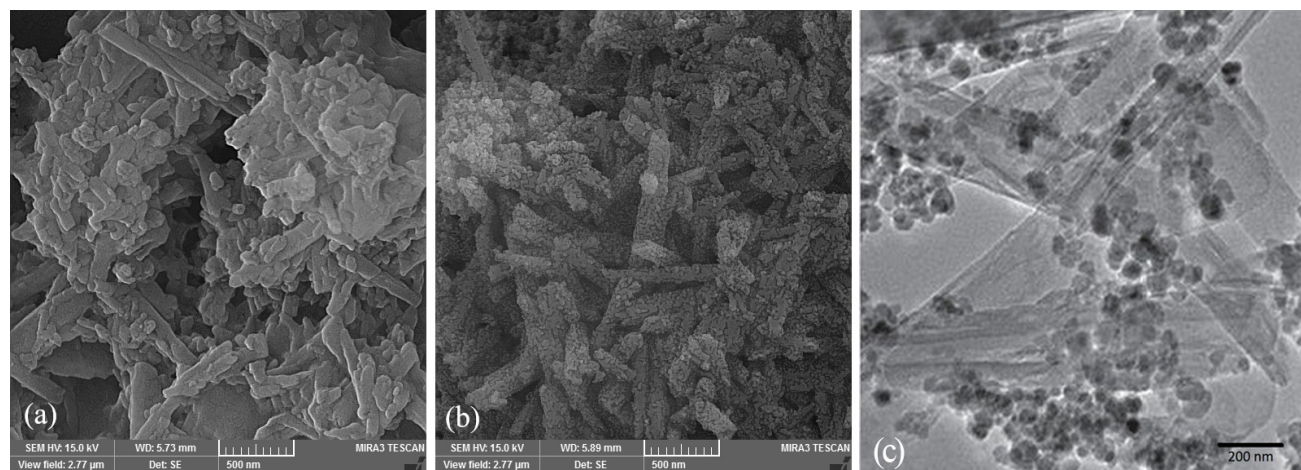


Figure 2. Morphology of nanotubes using FESEM. The tubular structure of (a) HNTs-FA, with a diameter of approximately 49 nm and a length of around 390 nm, is clearly visible; (b) MHNTs-FA, the Fe_3O_4 NPs adhered to the surface are distinctly visible; (c) TEM image of MHNTs-FA to confirm the FESEM findings.

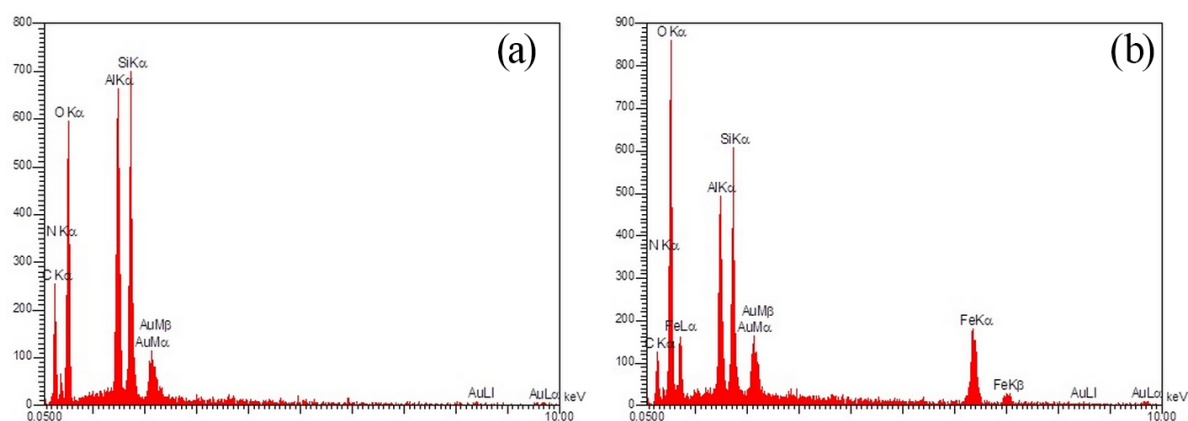


Figure 3. EDX diagram of (a) HNTs-FA and (b) MHNTs-FA.

Table 1. Elemental analysis obtained from EDX spectra

	HNTs / %	HNTs-FA / %	MHNTs-FA / %
Oxygen	61.6	42.82	43.06
Silica	20.2	8.17	7.42
Aluminum	8.2	7.45	5.85
Carbon	—	26.95	16.92
Nitrogen	—	14.61	7.88
Iron	—	—	18.86

HNTs: halloysite nanotubes; HNTs-FA: folic acid-modified halloysite nanotubes; MHNTs-FA: folic acid-modified magnetic halloysite nanotubes.

cytotoxicity was found to be directly related to the amount of complex loaded within the cavities of modified HNTs.

Loading the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex into the cavities of modified HNTs will enhance systemic distribution, solubility, and, most importantly, active penetration into cancer cells via FRs and endocytosis.

The FA in the structure of modified HNTs can effectively target the overexpressed FRs on cancer cell

surfaces and enter the cells through receptor-mediated endocytosis.⁴² FA exerts multifaceted effects on HT29 colorectal cancer cells, influencing proliferation, genomic integrity, epigenetic landscape, and drug response. Several studies have shown that FA supplementation can modulate the growth and differentiation of HT29 cells. For example, Kim *et al.*⁴³ reported that folate deficiency in colorectal cancer cells results in uracil misincorporation in DNA, leading to chromosomal breakage and genomic instability that may enhance carcinogenesis. However, the relationship between FA and cancer cell proliferation is complex and dose-dependent. While physiological concentrations of FA may have protective effects, supraphysiological levels have been shown to enhance the proliferation of established cancer cells.⁴⁴ In HT29 cells, high-dose FA exposure was associated with increased cell viability and increased expression of proliferation markers such as Ki-67 and PCNA (proliferating cell nuclear antigen), suggesting a potential tumor-promoting effect under certain conditions.⁴⁵ FA also influences epigenetic regulation

through its role in one-carbon metabolism and DNA methylation. Abnormal methylation patterns are hallmarks of colorectal cancer, and folate availability can alter the expression of tumor suppressor genes and oncogenes. For example, Crider *et al.*⁴⁶ highlighted that folate-mediated methylation changes may affect the expression of genes involved in cell cycle regulation and apoptosis. In addition to its direct effects on cell growth, FA may modulate the sensitivity of HT29 cells to chemotherapeutic agents. 5-Fluorouracil (5-FU), a common drug in the treatment of colorectal cancer, targets thymidylate synthase, a folate-dependent enzyme. Studies have shown that FA can enhance the cytotoxicity of 5-FU by stabilizing the ternary complex between the drug, the enzyme, and the folate cofactor, thereby improving therapeutic efficacy.⁴⁷ Therefore, we expect that the low amount of FA loaded on the HNTs will not cause tumor growth and will only play the role of targeting and cofactor to assist the palladium complex.

Previous research demonstrated that the CC_{50} value (the 50% cytotoxic concentration) for the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex is 0.11 mM.¹⁵ It appears that the level of cytotoxicity is influenced by the negative charges on the metal complex ions. The greater the negative charge, the weaker the potency. This was supported by the discovery that Pd complexes synthesized with dipic, which possess fewer or neutral negative charges, exhibited a more favorable effect on MCF-7 breast cancer cells. The findings from the previous study¹⁵ clearly indicated that the cytotoxicity of the uncharged complex was superior to that of the complex with two negative charges. The diminished performance may be attributed to negative charge repulsion between DNA and the complex, or reduced penetration into the lipophilic membrane of the cell.

Therefore, we decided to use HNTs because their internal lumen is positively charged due to $\text{Al}-\text{OH}$ groups.³⁴ This allows them to accept negatively charged compounds and act as a carrier. This neutralizes the negative charge of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex and improves its passage through the lipophilic membrane. Furthermore, the presence of FA on surface of HNTs enables active targeting and reduces the side effects of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex on normal cells. To test this hypothesis, FR-positive HT29 cells were chosen to study the impact of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ on colon cancer, and to be compared with the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex and the anticancer drug cisplatin. Also, the FR-negative LoVo cell line was chosen to help determine the role of FA in improving the performance of the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex.

The concentrations of modified HNTs used were chosen based on the amount of $[\text{Pd}(\text{dipic})_2]^{2-}$ complex

loaded into them to compare their cytotoxic effects with free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex. Conversely, low concentrations were selected to observe the positive effects of HNTs on cytotoxicity. In previous research, the CC_{50} value was 0.11 mM,¹⁵ so in this study, we considered concentrations in micromolar and reduced them by about 1000 times.

As shown in Figures 4a and 5a, free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex only reduced cell death below 50% at the highest concentration after 72 h of exposure to HT29 and LoVo colon cancer cells, and was not effective in inhibiting growth at other concentrations. However, when encapsulated within HNTs-FA, they showed improved performance in inhibiting HT29 cancer cell growth due to the presence of FA and active passage through folate receptors. Figure 4b clearly confirms that most concentrations of HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ can inhibit the growth of HT29 cells, especially after 72 h. The presence of Fe_3O_4 MNPs on the surface of MHNTs-FA may have contributed to its better performance, as shown in Figure 4c, where all concentrations reduced cell death below 50% even after 48 h of exposure. The enhanced effect of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ may be attributed to active transport through pathways other than folate receptors. It has been proven that most metals, especially iron, can enter cancer cells through the copper transporter 1 (CTR1) and transferrin receptor-1 (TfR1).⁴⁷ Because cancer cells have a higher expression of TfR1 than normal cells,⁴⁹ it is a potential target for the treatment of cancers. Therefore, it can be assumed that the multiple entry of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ is the reason for the better performance of this composition compared to others.

An interesting result was obtained when MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ were tested on the LoVo cell line. Since this colon cancer cell line lacks the FRs but overexpresses TfR1, it was observed that HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ could not inhibit cancer cells at any concentration (Figure 5b). However, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, due to the presence of MNPs, was able to reduce the cell death rate below 50% at high concentrations after 48 and 72 h of treatment (Figure 5c). Therefore, it is hopeful that MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ can use both FRs and TfR1 receptors to enter cancer cells.

In this research, the cytotoxic effects of the compounds were compared with cisplatin. In the past, it has been proven that the IC_{50} of cisplatin is equal to 6.3 μM after the proximity with HT29.⁵⁰ Considering the low amount of cisplatin utilized in the current study, it was not far from the expectation that cisplatin could also inhibit the growth of cancer cells. Figure 4d shows that no concentration of cisplatin used reduced the rate of cell death of HT29 cells below 50%. This is also true for the LoVo cell line.

Only after 72 h of exposure to cisplatin at the highest concentration did the cell death rate fall below 50% (Figure 5d). The data were analyzed using one-way analysis of variance (ANOVA) and Tukey's *post hoc* test, with a significance level set at $P < 0.05$.

Table 2 illustrates the superior inhibitory effect of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ on the growth of HT29 colon cancer cells compared to cisplatin and free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex. It shows that the IC_{50} values calculated after 48 and 72 h of treatment with HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ were much lower than those obtained for free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex and cisplatin.

Table 2 also shows that the IC_{50} values obtained for the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ on LoVo cancer cells are nearly identical. Therefore, it can be concluded that the absence of the FRs in LoVo colon cancer cells does not affect the efficacy of the HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ compared to free complex. In contrast, it proved that the IC_{50} value of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ is superior to that of cisplatin. This improvement is attributed to the presence of the

TfR1 receptor on LoVo cells, allowing the compound to effectively enter the cancer cells through MNPs.

Therefore, a key finding can be stated as loading $[\text{Pd}(\text{dipic})_2]^{2-}$ into HNTs modified with FA and MNPs can target FRs and TfR1 on colon cancer cells, providing a dual route for the entry of the Pd complex into the target cell. Furthermore, it was demonstrated that FA does not promote tumor proliferation. This conclusion is supported by morphological changes observed in HT29 cancer cells following treatment with modified HNTs. Specifically, the cells transitioned from an elongated, myocyte-like morphology (Figure 6a) to a rounded, apoptotic-like form (Figure 6b), suggesting the induction of cell death.

Cytotoxicity test by SRB

Sometimes, different environmental factors or the presence of specific compounds can lead to inaccurate results in the MTT method. This is due to the fact that the conversion of MTT to formazan by the cell is a metabolic process that can be affected by external factors, leading to fluctuations in the results.⁵¹ To address this issue, we

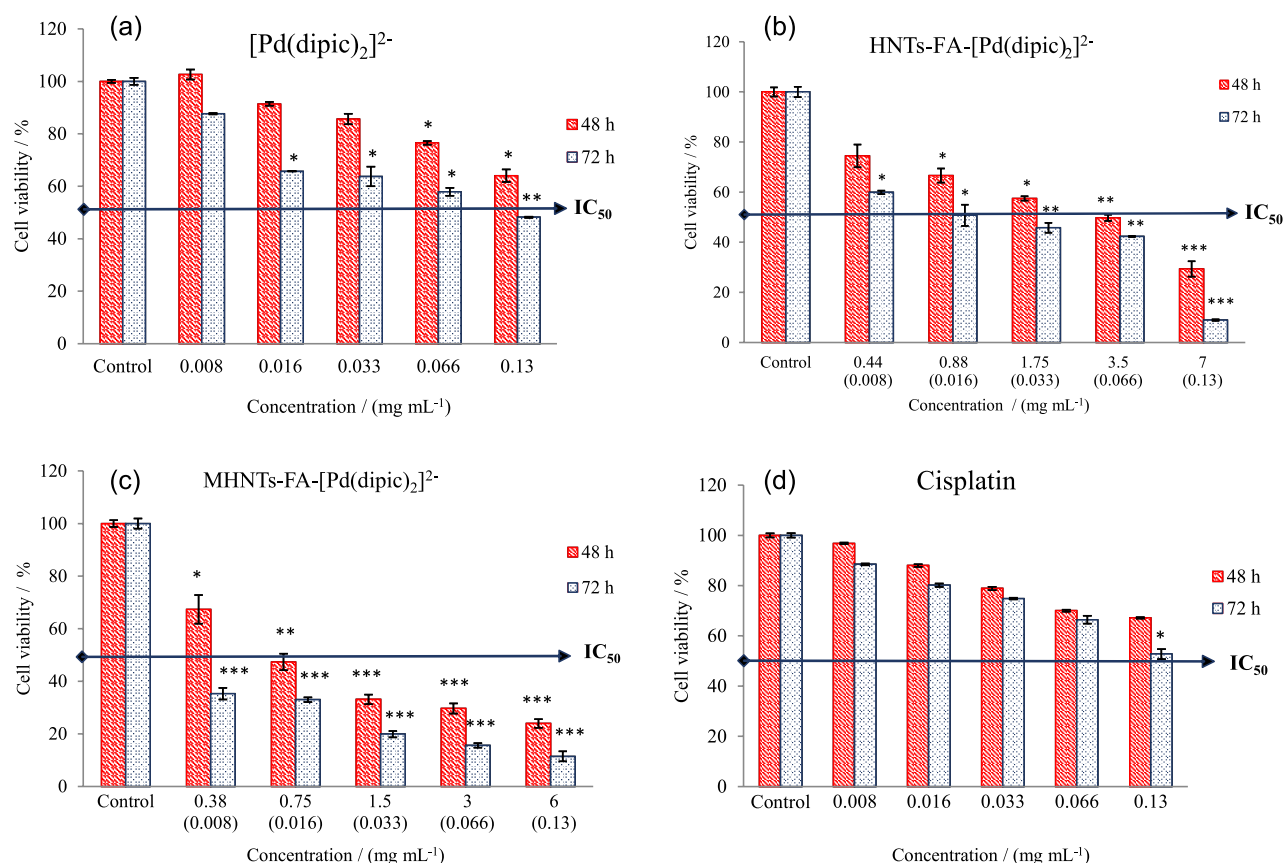


Figure 4. Results of the cell survival assay for (a) the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, (b) HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, (c) MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, and (d) cisplatin, conducted using the MTT method on HT29 colon cancer cells. The number in parentheses on the x-axis for panels b and c corresponds to the concentration of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex loaded into the modified HNTs to ensure equal values in terms of effective composition. MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ exhibited a significant positive response at all concentrations and time points, with the significance level set at $P < 0.05$.

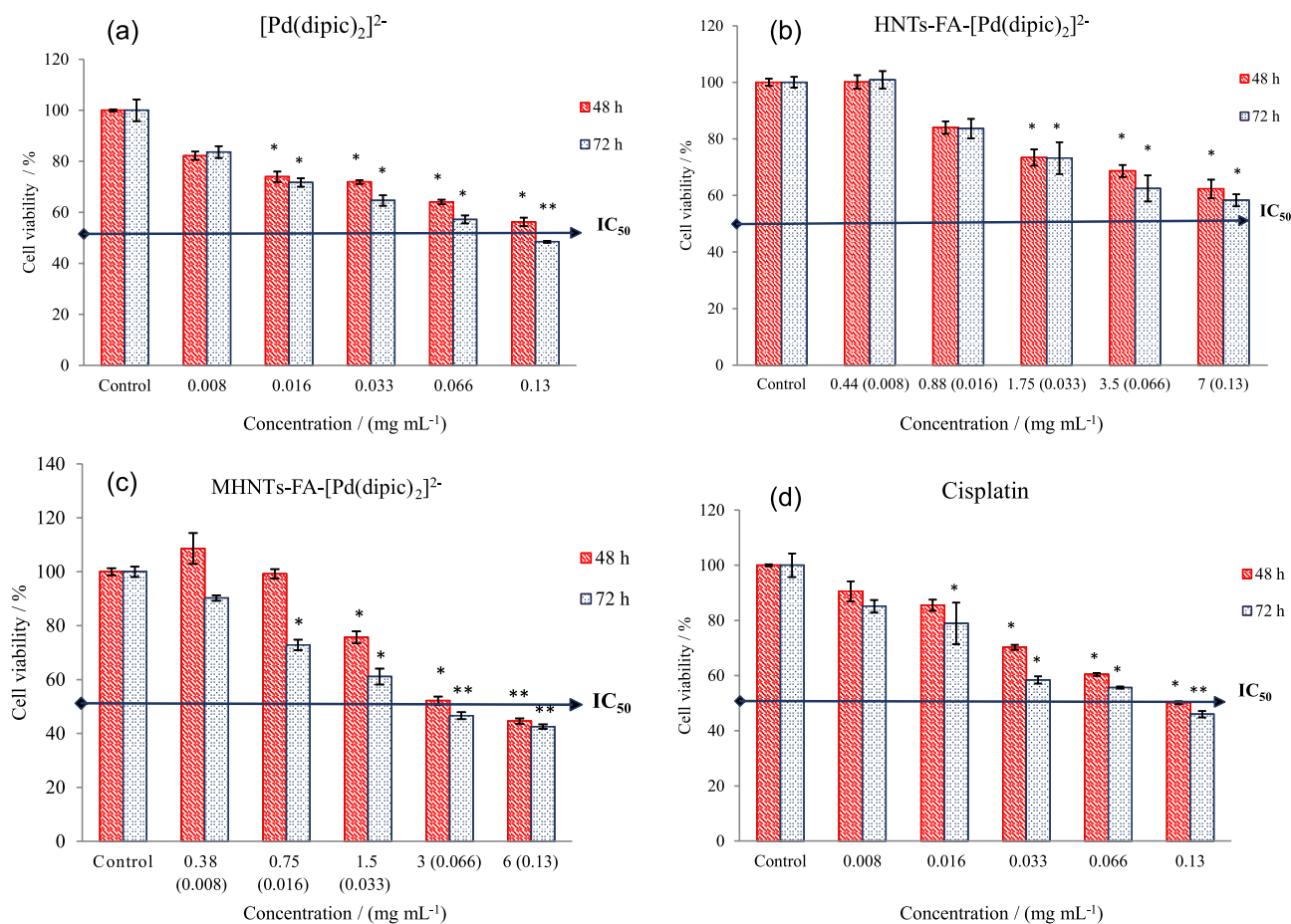


Figure 5. Cell survival assay results for (a) the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, (b) HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, (c) MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, and (d) cisplatin, conducted using the MTT method on LoVo colon cancer cells. The number in parentheses on the x-axis for panels b and c corresponds to the concentration of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex loaded into the modified HNTs to ensure equal values in terms of effective composition. The performance of all compounds is nearly identical, with no significant superiority observed when the significance level is set at $P < 0.05$.

Table 2. Half-maximal inhibitory concentration (IC_{50}) value of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex, and cisplatin. The IC_{50} values were calculated from the graph drawn based on the logarithm of the complex concentration relative to the percentage of cell viability (Figures S1 and S2)

	HT29 colon cancer cells		LoVo colon cancer cells	
	IC_{50} of 48 h / (mg mL ⁻¹)	IC_{50} of 72 h / (mg mL ⁻¹)	IC_{50} of 48 h / (mg mL ⁻¹)	IC_{50} of 72 h / (mg mL ⁻¹)
MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	0.017	0.0018	0.092	0.067
HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	0.047	0.019	0.27	0.18
Free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex	0.43	0.11	0.30	0.11
Cisplatin	0.49	0.21	0.13	0.089

MHNTs-FA: magnetic halloysite nanotubes; HNTs-FA: folic acid-modified halloysite nanotubes; dipic: dipicolinic acid.

decided to assess cell viability by measuring the protein content of cells and using SRB staining to evaluate the ability of modified HNTs to inhibit cancer cells. As shown in Figure 7a, both HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ were effective in inhibiting HT29 colon cancer cells and reducing the protein content of the cancer cells.

However, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ exhibited stronger

inhibition, likely due to the presence of FA and MNPs in the modified HNTs structure. This is further supported by the IC_{50} value obtained, as shown in Figure 7b, which confirms the superior inhibitory capacity of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$. It is worth noting that the IC_{50} values obtained from the SRB staining method were lower than those obtained from the MTT method, which is a common occurrence.

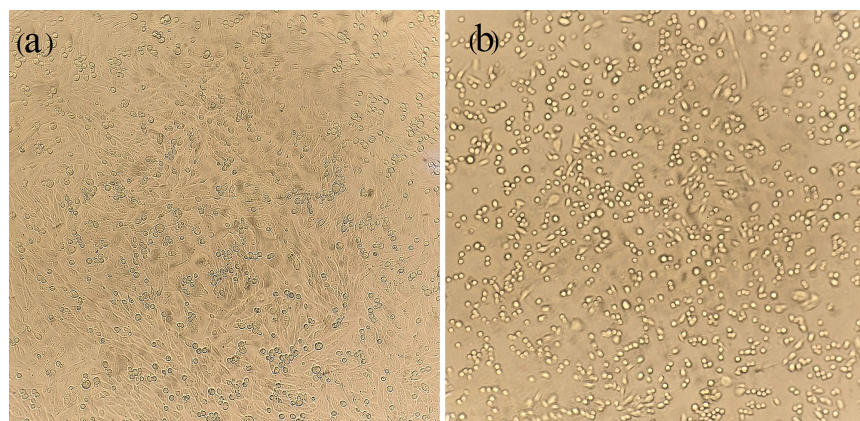


Figure 6. Microscopic image of HT29 cells (a) before and (b) after treatment with modified HNTs.

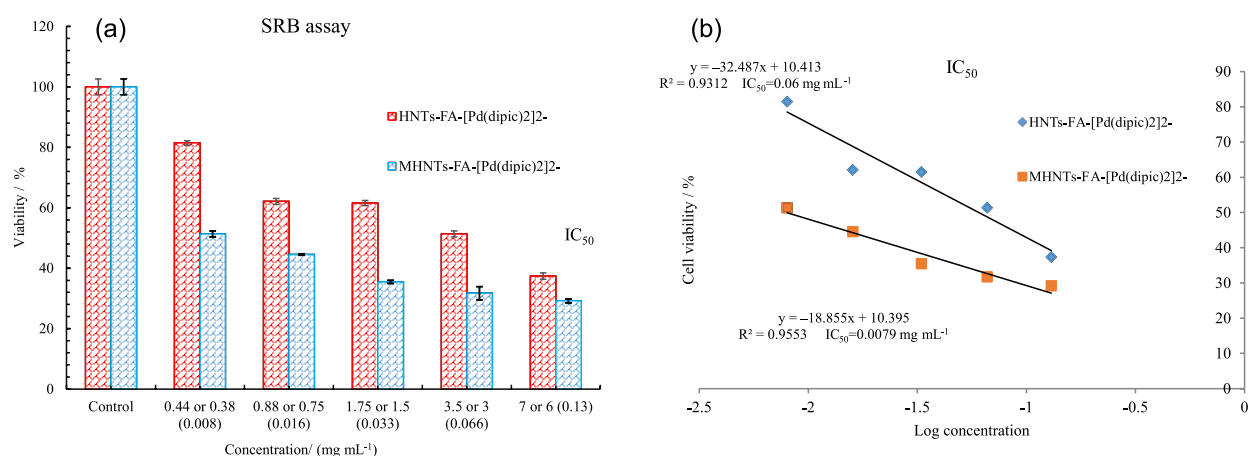


Figure 7. (a) The evaluation of inhibition of HT29 cells treated with HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ using the SRB staining method. (b) The calculation of IC_{50} was done by plotting the logarithm of the concentration of the loaded anionic complex *versus* cell viability percentage. The number in parentheses on the *x*-axis corresponds to the concentration of free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex loaded into the modified HNTs to ensure equal values in terms of effective composition.

Investigating the expression levels of genes involved in apoptosis induction and apoptosis inhibition using real-time PCR

It has been confirmed that colorectal cancer is the second most common cancer in women and the third most common cancer in men.⁵² Given that 5-fluorouracil is currently the only drug approved for the treatment of colon cancer, researchers and oncologists face a significant challenge.⁵³ This is due to the numerous side effects it causes in patients, leading to an increased demand for alternative treatment strategies. In one study,⁵⁴ it was demonstrated that a Pd^{II} complex increases the expression of Bax in lung cancer cells (A549) through a mitochondrial-dependent pathway mediated by reactive oxygen species (ROS). This is followed by the destruction of the mitochondrial outer membrane and the release of cytochrome c, leading to the activation of Caspase-3. In another study,⁵⁵ researchers found that the Pd^{II} saccharinate complex can increase Caspase-3 gene

expression and induce apoptosis in the MCF-7 breast cancer cell line. The ability of the Pd complex to induce apoptosis in Balb-c mice was confirmed.⁵⁶ It was found to increase the expression of Caspase-3 and Bax genes, while simultaneously decreasing the expression of p53, PCNA, and Bcl-2 genes. Therefore, we decided to investigate the ability of modified HNTs to induce apoptosis in HT29 colon cancer cells. The concentrations of all compounds used were selected based on their IC_{50} values, and the cells were exposed to the compounds for 24 h.

Both HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ contain folic acid, which targets folate receptors and facilitates the specific penetration of the complex into cancer cells. They were compared to the free $[\text{Pd}(\text{dipic})_2]^{2-}$ and cisplatin to assess the impact of the carrier and targeted drug delivery. Changes in the expression of two proteins, Bak1 and Bclx, are crucial as they can indicate mitochondrial damage when treated with modified HNTs. An increase in the ratio of Bak1 to Bclx leads to the release of Cyt-c and the formation of apoptosis protease activating

factor 1 (Apaf-1), signaling the initiation of apoptosis through the mitochondrial-dependent intrinsic pathway.⁵⁷

The results from Figure 8 indicate that both the free $[\text{Pd}(\text{dipic})_2]^{2-}$ and modified HNTs were able to enhance the expression of the Bak1 gene and elevate its levels compared to the control. The x -axis in the diagram represents the expression of control genes, which is set to one. It was observed that cisplatin did not have the same effect on increasing the expression of the pro-apoptotic gene Bak1. However, analyzing this gene in isolation may not be sufficient, and the Bak1/Bclx ratio should be taken into account. The data presented in Table 3 demonstrates that HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ caused a 6.5-fold increase, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ resulted in a 1500-fold increase, free $[\text{Pd}(\text{dipic})_2]^{2-}$ showed a 1.7-fold increase, and cisplatin led to a 1.6-fold increase in the Bak1/Bclx ratio. This suggests that free $[\text{Pd}(\text{dipic})_2]^{2-}$ and cisplatin have similar effects and do not possess a unique ability to induce apoptosis through the intrinsic pathway. Therefore, it can be concluded that the induction of apoptosis is enhanced when free $[\text{Pd}(\text{dipic})_2]^{2-}$ is placed inside the cavities of HNTs-FA, especially when placed in the magnetic cavities of MHNTs-FA. The presence of FA in the structure of HNTs may be the reason for inducing intrinsic apoptosis. It has been proven that consuming folic acid is important in preventing stomach cancer. As a result, the expression of the tumor suppressor p53 gene in the stomach mucosa significantly increases after its use.⁵⁸ Attias *et al.*²¹ also confirmed that FA causes a dose-dependent reduction of IGF-IR protein in colon cancer cells in a p53-dependent manner. However, it has no effect on wild-type p53-depleted cells. The increase in p53 gene expression is directly linked to the increase in Bak1 gene expression.⁵⁹ Therefore, it can be inferred that FA plays a role in activating the mitochondrial intrinsic pathway and increasing the Bak1/Bclx ratio.

Apoptosis is initiated by mitochondrial outer membrane permeabilization (MOMP). An increase in extracellular iron leads to a rise in the concentration of intracellular iron-sensitive pools, promoting MOMP and intracellular ROS production. Iron-mediated ROS production can trigger intrinsic apoptosis. Additionally, cardiolipins in the mitochondrial outer membrane can be oxidized by ROS. Cytochrome c released through Bax during MOMP can activate Caspase 3, inducing apoptosis through the extrinsic pathway.⁶⁰ For this reason, MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ had the highest level of Bak1 gene expression and subsequently the highest level of Caspase-3 gene expression. Although free $[\text{Pd}(\text{dipic})_2]^{2-}$ induced the extrinsic pathway of apoptosis, similar to cisplatin, the presence of magnetic iron on the surface of the MHNTs-FA further increased the expression of Caspase-3 gene by 304 times compared to the control (Table 3).

AKT1 is one of the three widely used serine/threonine-protein kinases responsible for various processes such as metabolism, proliferation, cell survival, growth and angiogenesis.⁶¹ Therefore, it can be said that there is a significant relationship between drug resistance and AKT1 gene expression.⁶²

In Figure 8, it is evident that free $[\text{Pd}(\text{dipic})_2]^{2-}$ increased the expression of the AKT1 gene and raised its level to above 6. However, when the complex was loaded into the cavities of MHNTs-FA and HNTs-FA, the expression of the AKT1 gene did not increase, despite its significant impact on cancer cells and its ability to induce apoptosis in them.

The key point is that the expression of the AKT1 gene is correlated with the Bclx gene, and only free $[\text{Pd}(\text{dipic})_2]^{2-}$ increased these two apoptosis-inhibiting genes (Table 3). Synergism between the PI3Kinase/AKT pathway and Bcl-xL in inhibiting apoptosis in adenocarcinoma cells has been confirmed by Qian *et al.*,⁶³ which aligns well with our

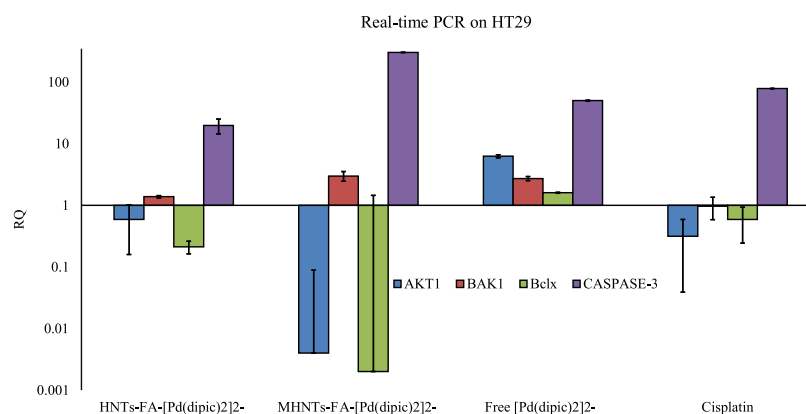


Figure 8. Expression levels of genes related to apoptosis and treatment resistance. Each sample was tested twice, and the average cycle threshold (CT) value was adjusted based on the housekeeping gene GAPDH. The fold change in expression was determined using the $\Delta\Delta\text{CT}$ method, with RQ equaling $2^{-\Delta\Delta\text{CT}}$. The middle line signifies 1 and reflects the expression level of the control.

results. Therefore, the beneficial role of the modified HNTs is easily understood.

Table 3. $\Delta\Delta\text{CT}$ and RQ values of genes investigated during real-time PCR on HT29 cancer cells

Gene	Compound	$\Delta\Delta\text{CT}$	RQ
AKT1	control	0	1
	HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	0.75	0.59
	MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	7.945	0.004
	free $[\text{Pd}(\text{dipic})_2]^{2-}$	-2.65	6.28
	cisplatin	1.665	0.315
BAK1	control	0	1
	HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	-0.47	1.38
	MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	-1.585	3
	free $[\text{Pd}(\text{dipic})_2]^{2-}$	-1.45	2.73
	cisplatin	0.05	0.97
Bclx	control	0	1
	HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	2.235	0.212
	MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	8.985	0.002
	free $[\text{Pd}(\text{dipic})_2]^{2-}$	-0.675	1.6
	cisplatin	0.76	0.59
Caspase-3	control	0	1
	HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	-4.31	19.84
	MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$	-8.25	304.4
	free $[\text{Pd}(\text{dipic})_2]^{2-}$	-5.65	50.21
	cisplatin	-6.3	78.79

MHNTs-FA: folic acid-modified magnetic halloysite nanotubes; HNTs-FA: folic acid-modified halloysite nanotubes; dipic: dipicolinic acid; $\Delta\Delta\text{CT}$: delta delta cycle threshold; RQ: relative quantification.

MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ reduced the expression of antiapoptotic genes (AKT1 and Bclx) and were successful in inducing apoptosis through the intrinsic pathway. The superiority of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ in reducing the expression of the AKT1 gene can be attributed to folic acid. Several studies^{64,65} have confirmed that folic acid can prevent the expression of AKT proteins. For instance, Wang *et al.*⁶⁴ treated breast

cancer cells (MDA-MB-231) with folate and found that folic acid inhibited the growth of MDA-MB-231 cells, suppressed the expression of Bcl-2 and p-AKT proteins, and simultaneously increased the expression of Bax, PTEN (phosphatase and tensin homolog), and P53 proteins. Since AKT1 is a cell response protein that can promote cell survival and resistance to tumor treatment, inhibiting its expression with MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ shows promise in cancer treatment and prevention of cancer metastasis.

It can be concluded that the encapsulation of $[\text{Pd}(\text{dipic})_2]^{2-}$ within HNTs-FA and MHNTs-FA cavities was able to downregulate the AKT1 gene, while the free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex was not capable of reducing AKT1, which is responsible for drug resistance.

Flow cytometric analysis of apoptosis

To evaluate the apoptotic potential of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ on HT29 cells and confirm the results from the gene expression section, a double staining assay using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) was performed, followed by flow cytometric analysis. In Figures 9a and 9b, the resulting dot plots are divided into four quadrants, each representing distinct cell states: Q4 (Annexin V⁻ / PI⁻) for viable cells with intact membranes and no phosphatidylserine leakage, Q3 (Annexin V⁺ / PI⁻) for early apoptotic cells exposed to phosphatidylserine while maintaining membrane integrity, Q2 (Annexin V⁺ / PI⁺) for late apoptotic cells with damaged membranes and phosphatidylserine leakage, and Q1 (Annexin V⁻ / PI⁺) for necrotic cells with disrupted membranes but no apoptosis markers.

Treatment with MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ resulted in a significant increase in the percentage of cells in Q3 (Figure 9b) compared to the untreated control group (Figure 9a). This showed a 44.5% *versus* 1.37% difference, indicating a

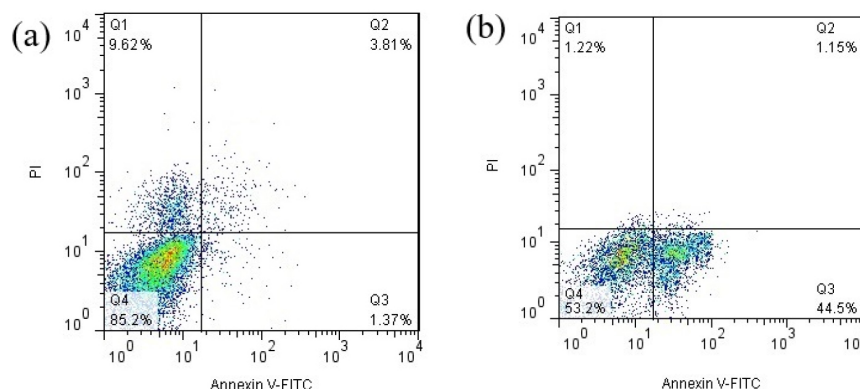


Figure 9. Flow cytometry analysis of (a) the untreated HT29 cells and (b) treated with MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$.

significant induction of early apoptosis. Additionally, the decrease in Q4 indicated a reduction in the viable cell population, further confirming the cytotoxic efficacy of this compound. Conversely, the increase in Q1 in the control group suggested potential necrotic effects in cancer cells. These findings suggest that MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ effectively induces programmed cell death in HT29 cells, supporting its potential as a targeted anticancer agent.

Investigation of oxidative stress on erythrocytes

Exogenous compounds that enter the bloodstream, such as drugs or drug carriers, can be harmful to different types of blood cells, including platelets, erythrocytes, and granulocytes.⁶⁶ Although few studies have been conducted on the adverse effects of Pd^{II} complexes on erythrocytes, it can be assumed that they may cause harm to blood cells due to the inability to differentiate between normal and cancer cells. Conversely, loading the complex onto the surface of NPs or placing it inside the cavities of NPs may also pose problems. NPs, with their unique structure, function, and size, can induce cytotoxicity and oxidative stress in red blood cells.⁶⁷ Since the typical approach for assessing oxidative stress involves monitoring the level of MDA production through the lipid peroxidation of unsaturated fatty acids, we chose to examine the impact of MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$, and free $[\text{Pd}(\text{dipic})_2]^{2-}$ on erythrocytes using this method. The results of this analysis can be seen in Figure 10.

The graphic shows that erythrocytes exposed to both MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$

at high concentrations can reduce MDA production, demonstrating protective effects.

A significant finding is the minimal change in MDA production by erythrocytes when exposed to free $[\text{Pd}(\text{dipic})_2]^{2-}$. The free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex itself does not have a significant effect on erythrocytes because of its mechanism of action, which is more effective on nucleated cells with DNA. Hemolysis studies have shown that cisplatin, a widely used chemotherapeutic agent, causes hemolysis only at high concentrations, especially when exposed to excessive amounts of erythrocytes. A report⁶⁸ has shown that high concentrations of cisplatin may cause oxidative damage and increase hemolysis by disrupting cell membranes. This is because its primary mechanism of action is on nucleated cells, causing cytotoxicity through DNA cross-linking in nucleated cells.¹ Red blood cells lack a nucleus and are not subject to genotoxic effects such as DNA adduct formation or micronucleus production,⁶⁹ making them less susceptible to oxidative and structural damage caused by cisplatin. This indicates that none of the concentrations of free $[\text{Pd}(\text{dipic})_2]^{2-}$ had a notable impact on erythrocytes or induced oxidative stress. In conclusion, prolonged exposure of modified HNTs to red blood cells does not lead to complications or lipid peroxidation. In fact, it may have a protective effect and reduce oxidative stress.

Conclusions

In this research, palladium anionic complex ($[\text{Pd}(\text{dipic})_2]^{2-}$) was loaded into the cavities of halloysite nanotubes (HNTs) for the first time. HNTs were modified

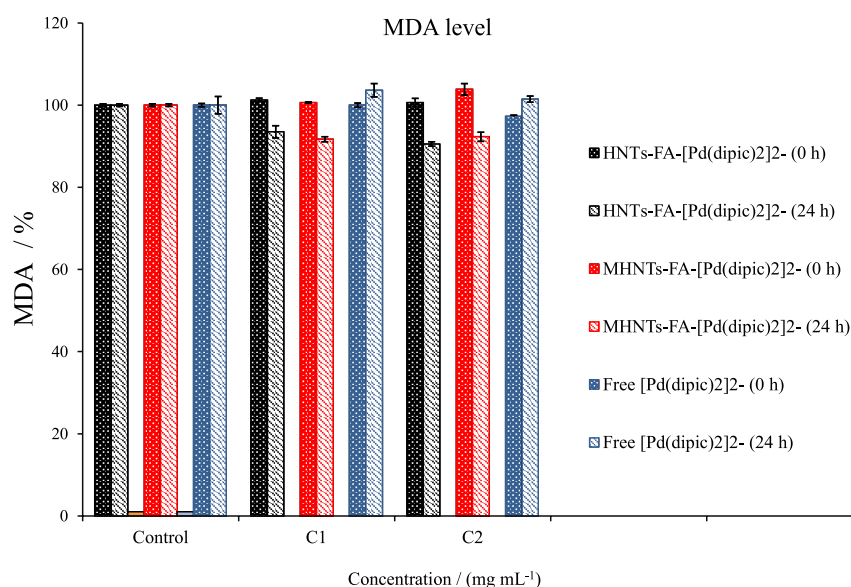


Figure 10. Evaluation of malondialdehyde (MDA) production by erythrocytes after 0 and 24 h exposure to HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (C1 = 3.5 and C2 = 7 mg mL^{-1}) (black columns), MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ (C1 = 3 and C2 = 6 mg mL^{-1}) (red columns), and free $[\text{Pd}(\text{dipic})_2]^{2-}$ (C1 = 0.065 and C2 = 0.13 mg mL^{-1}) (blue columns).

with folic acid (FA) and magnetic nanoparticles (MNPs) to actively enter cancer cells and deliver the complex. Cytotoxicity tests were conducted using the MTT assay and SRB staining method. Results confirmed that MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ have the ability to inhibit the growth of HT29 colon cancer cells. In contrast, free $[\text{Pd}(\text{dipic})_2]^{2-}$ complex and even cisplatin at low concentrations were not successful in inhibiting cancer growth. Real-time PCR tests showed that MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ and HNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ can activate both intrinsic and extrinsic pathways of apoptosis, leading to a significant decrease in the expression of AKT1, a protein responsible for cell survival and treatment resistance. Flow cytometric analysis confirmed that MHNTs-FA- $[\text{Pd}(\text{dipic})_2]^{2-}$ could induce early apoptosis in HT29 cells, leading to programmed cell death in this cell line. Furthermore, it was discovered that modified HNTs did not induce oxidative stress in erythrocytes, making them a promising candidate for targeted drug delivery of the $[\text{Pd}(\text{dipic})_2]^{2-}$ complex to enhance its effectiveness against cancer cells.

Supplementary Information

Supplementary information (IC₅₀ plots) is available free of charge at <http://jbcs.sbgq.org.br> as PDF file.

Data Availability

All data and findings from this study are available in the text.

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Author Contributions

HD contributed to designing, writing, and editing. LS was responsible for laboratory work. SAH collaborated in editing the text and conducting additional tests. MHM designed the study and supervised the experiments. All authors made substantial contributions to the final manuscript and have approved its submission. They are also aware of the order of authorship and have approved the submitted version of the manuscript.

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