Synthesis of Self-Targeted Carbon Dot with Ultrahigh Quantum Yield for Detection and Therapy of Cancer

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ABSTRACT: This study aims to engineer a new type of ultrahigh quantum yield carbon dots (CDs) from methotrexate (MTX-CDs) with self-targeting, imaging, and therapeutic effects on MDA-MB 231 breast cancer cells. CDs were synthesized via a straightforward thermal method using a methotrexate (MTX) drug source. The physicochemical characteristics of the prepared MTX-CDs were studied using Fourier transform infrared (FT-IR) spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), X-ray powder diffraction (XRD), and X-ray photoelectron spectroscopy (XPS). TEM and DLS revealed which MTX-CDs have homogeneous spherical morphology with a smaller average size of 5.4 ± 2.2 nm, polydispersity index (PDI) of 0.533, and positive surface charge of around +3.93 mV. Results of FT-IR spectroscopy and high-resolution XPS indicated the presence of residues of MTX on CDs. Therefore, the synthesized MTX-CDs could be targeted and be taken up by FR-positive cell lines without the aid of additional targeting molecules. In vitro epifluorescence images demonstrated high-contrast cytoplasm biodistribution of MTX-CDs after 2 h of treatment. A much stronger fluorescent signal was detected in MDA-MB 231 compared to MCF 7, indicating their ability to precisely target FR. The highest cytotoxic and apoptotic effects were observed in MTX-CDs compared to free MTX obtained by the MTT assay, cell cycle arrest, and annexin V-FITC apoptosis techniques. Results revealed that the novel engineered MTX-CDs were capable of inducing apoptosis (70.2% apoptosis) at a lower concentration (3.2 μM) compared to free MTX, which was proved by annexin V and cell cycle. This work highlights the potential application of CDs for constructing an intelligent nanomedicine with integration of diagnostic, targeting, and therapeutic functions.

INTRODUCTION

In the past decade, considerable effort has been expended to develop self-targeted nanotheranostic platforms to overcome shortcomings of conventional cancer therapy systems. These shortcomings include severe side effects, poor bioavailability, and drug resistance due to nonspecific drug biodistribution.1,2 To date, tremendous effort has been expended in the designing of multtarget nanoparticles that are capable of targeting, imaging, and ablating cancer cells through noninvasive imaging-guided therapeutics. Typically, materials proposed for this application consist of noble metals, organic dyes, and metal oxide nanoparticles.3,4 The major challenge of targeted theranostic systems is their complexity. For example, in such systems, targeting molecules need to attach to the tumor site to concentrate the therapeutic agent and enhance the therapy result and imaging contrast.5 This complexity affects the toxicity, biodistribution, blood circulation time, and overall therapeutic outcome.6 Furthermore, it is a burdensome process to obtain an excellent targeted theranostic nanoplatform to achieve an early diagnosis and therapy of cancer. Thus, it is

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highly desired to synthesize a nontoxic and highly biocompatible nanoplatform whose combined targeting function in a simple route to achieve effective diagnosis and therapy of tumors in the early stages.

Among all types of nanomaterial, interest in the use of CDs as a theranostic nanoplatform has been steadily increased in recent years due to their low toxicity, physicochemical properties, tunable fluorescence spectrum, water solubility, surface functionalization, biocompatibility, cell membrane permeability, and high photostability.10–12 CDs can be synthesized from organic and inorganic sources by top-down and bottom-up synthetic approaches and can be produced in large scales.11,12 CDs rarely have inherent targeting moiety and require at least one biomarker, such as molecular target (e.g., folic acid), antibody, or aptamer as a recognition moiety to identify cancer cells.13–15 Conjugating of targeting, imaging, and therapeutic agents and integrating them onto the CDs is challenging and can affect their fluorescence quantum yields, complexation capacity, color of fluorescence, and quenching capability.16,17

To overcome targeting challenges, Haifang et al. used folic acid (FA), the molecular agent which has the potential to target folate receptors (FR) on cancer cells, as a source for the synthesis of highly luminescent CDs.18 The result of this study has shown that the residues of FA on CDs specifically recognized and targeted FR on cancer cells and promoted folate receptor-mediated cellular uptake. In another study, Zheng et al. synthesized novel fluorescent Asp-CDs with self-targeting ability via a simple thermal route using β-glucose and l-aspartic acid. These Asp-CDs, besides the biocompatibility and tunable full-color emission, can target C6 glioma cells without the aid of any extra targeting molecules. Therefore, Asp-CDs could act as a self-targeted fluorescence imaging agent for noninvasive glioma diagnosis. The results of this study demonstrated that Asp-CDs have potential to become an intelligent nanoplatform with integration of targeting, diagnostic, and therapeutic functions.19 Zheng et al. synthesized theranostic CDs for efficient cancer imaging and therapy. They used a hydrophobic cyanine and poly(ethylene glycol) (PEG800) as a source. Their as-prepared CDs have become an intelligent nanoplatform with integration of targeting, diagnostic, and therapeutic functions.19

RESULTS AND DISCUSSION

Structural, Morphological, and Photoluminescence Properties of the Developed MTX-CDs. The hydrodynamic size and zeta potential of the synthesized MTX-CDs are shown in Figure 1. Based on DLS data, MTX-CDs are mostly monodispersed (PDI = 0.533) with hydrodynamic particle size and zeta potential of around 43 nm and +3.93 mV, respectively. Previously reported studies have shown that CDs can be a conjugate or load in nanomaterials for personalized nanomedicine.22 The present work paves the way for designing multifunctional CD-based theranostic platforms for simultaneously targeting, imaging, and treating cancers.

Figures 2a and 2b show the UV−vis absorption spectrum of the synthesized MTX-CDs and the photoluminescence spectrum, respectively. The photoluminescence excitation wavelength of the synthesized MTX-CDs is 220 nm. The photoluminescence emission of the synthesized MTX-CDs is centered at 650 nm with a lifetime of 1.6 μs (Figure 2b). The photoluminescence spectrum shows that the synthesized MTX-CDs have high photostability and good photothermal stability, especially, after coating with thiol-polyethylene glycol and fluorescent molecules. Their result indicated that CuCD NSs could selectively target tumor tissues and realize multimodal imaging-guided cancer therapy.23

Inspired by the previous studies, we hypothesize that “MTX is a chemotherapeutic FA analog that acts on over-expressed folate receptors in various cancer cells.”24 Hence, MTX can be used to synthesize carbon dots that retain their therapeutic effect as an anticancer agent. Therefore, novel fluorescent MTX-CDs with self-targeting theranostic ability were synthesized via the thermal method using MTX as starting materials. We evaluated the internalization and cytotoxicity of MTX-CDs on the MDA-MB-231 cells. MTX, the analogue of folic acid and an anticancer drug, is used as a precursor for the synthesis of CDs. MTX not only exhibits cytotoxic effects but can also act on cancer cells that overexpress folate receptor. Nevertheless, drug resistance of cancer cells to MTX has generally limited its applications.17 To overcome this problem, MTX can be a conjugate or load in nanomaterials for personalized nanomedicine.25–27 The present work paves the way for designing multifunctional CD-based theranostic platforms for simultaneously targeting, imaging, and treating cancers.
One of the important factors that could affect the QY and also can enhance the conjugation degree of conjugated systems. This increases the electron transition probability from the ground state to the lowest excited singlet state and finally contributes to higher QY of the MTX-CDs indirectly.38 In addition, MTX-CDs possess robust surface a
definitions realizing the cancer cell target ability of the synthesized MTX-CDs.35,36 O 1s and N 1s peaks suggest that MTX-CDs are wrapped outside containing −NH2, −OH, and −COOH. −COOH and −NH2 of MTX molecules have high reaction efficiency under hydrothermal conditions. Previous proof-of-concept studies have demonstrated that MTX is a chemotherapeutic agent that acts as an analogue of FA to targeting of overexpressed folate receptors (FRs) in various cancer cells.24 Chen and co-workers have demonstrated that chemical groups on the surface of the MTX (analogue of FA) have a critical role in binding to FR.37 In this study, the FT-IR and high-resolution XPS results demonstrate the chemical groups of MTX that exist on the synthesized MTX-CDs. Therefore, we expect the prepared CDs to have potential for targeting of FR.

The synthesized MTX-CDs exhibit strong photoluminescence emission. The rich amino groups on the MTX-CDs surface affect the QY and also can enhance the conjugation degree of conjugated systems. This increases the electron transition probability from the ground state to the lowest excited singlet state and finally contributes to higher QY of the MTX-CDs indirectly.38 In addition, MTX-CDs possess robust stability and demonstrate stable fluorescence at pH values between 4 and 11. Moreover, compared to the reported fluorescent materials, the as-prepared MTX-CDs showed superior photostability.

Furthermore, the MTX-CD solution exhibits long-term homogeneity (one phase) without any allusion at room temperature, and strong and stable fluorescence. These results indicate that the manufactured MTX-CDs have very good physicochemical stability.

Possible Origins of the Photoluminescence Properties of MTX-CDs. One of the important factors that could influence the optical properties of MTX-CDs with high QY (87.3%) is pyrolysis temperature. The previously reported synthesis of CD with a high QY indicated that the used source must be heated to a temperature above the melting point.39 Therefore, for this study, MTX-CDs with a high QY pyrolysis temperature of MTX solution reached 240 °C (above the melting point of MTX (185–204 °C)). As shown in Figure 6, the QY of the synthesized MTX-CDs under acidic and basic conditions is relatively low. This phenomenon could be due to a number of possible reasons. One hypothesis is that the MTX...
might break down and the partial activity of MTX residues was shielded or the hydrothermal reaction was incomplete under strong acidic or basic conditions. The weakly acidic condition, which was obtained directly by dissolving MTX in normal water, is an important factor for the synthesis of ultrabright MTX-CDs. Indeed, when the reaction was carried out in normal pH, the activity of hydroxide (OH) was shielded and the dehydration condition could have existed between the amino and carboxyl functional groups of MTX. However, under strong acidic or basic conditions, COOH and NH2 were partially shielded, so the concentration of MTX in normal pH is greatly more effective, and subsequently, the QY of MTX-

Figure 3. Crystalline phase of MTX-CDs investigated by XRD. Four sharp diffraction peaks centered at 17.53, 23.08, 27.17, and 40.42° are indicative of disordered carbon atoms and crystalline nature.

Figure 4. UV–vis and PL spectroscopy results. (a) UV–vis absorption (black line) and PL excitation (red line). (b) Emission spectra of the obtained MTX-CDs. (c) Emission spectra of MTX-CDs at different excitation wavelengths (from 270 to 310 nm with a 10 nm increment). (d) PL decay curve of MTX-CDs.
CDs is much higher. Therefore, the system pH is important for MTX-CD synthesis and directly affects the QY of the obtained MTX-CDs.41

Cytotoxicity Study of the Synthesized MTX-CDs. The cytotoxicity of free MTX and MTX-CDs was studied by the MTT assay using MDA-MB 231 cells. As demonstrated in Figure 7, both free MTX and MTX-CDs inhibited MDA-MB 231 proliferation in a time- and dose-dependent manner. As the concentration of MTX-CDs and free MTX increases from 0.0 to 6 μM, the relative cell viability of MDA-MB 231 decreased from 100 to 34.6% at 24 h. Meanwhile, the half-maximal inhibitory concentration (IC50) of MTX-CDs (MDA-MB, 231:3.2 μM) was lower than that of free MTX (MDA-MB, 231:3.7 μM) at 24 h (Figure 7b). The results revealed that MTX has a cytotoxicity effect on MDA-MB 231 and the MTX-CDs improved its cytotoxic activity. These results could be due to: (a) a higher cellular uptake due to smaller size of the nanoparticles, which leads to stability of the suspension and deeper penetration into the cancerous tissues42,43 and (b) a change in the surface morphology of MTX-CDs in comparison to MTX that could overcome drug resistance.44

Targeted Cancer Cell Imaging Using MTX-CDs. Synthesized MTX-CDs have high QY, stability, and folic acid-induced targeting ability. These properties of the as-prepared MTX-CDs allow it to be utilized for biomedical applications. As a proof concept, the MTX-CD powder was

Figure 5. FT-IR and XPS spectra of the synthesized MTX-CDs. (a) FTIR spectra of the precursor (MTX, red line) and the synthesized MTX-CDs (black line). (b) X-ray photoelectron spectra (XPS) of MTX-CDs. XPS spectra of raw data showing the carbon, nitrogen, and oxygen elemental signatures. (c) Deconvoluted C 1s spectrum. (d) Deconvoluted N 1s spectrum. (e) Deconvoluted O 1s spectrum.
dissolved in the RPMI 1640 medium and used for self-targeted cancer cell imaging and therapy. Under this condition, passivation will cause red-shifting and MTX-CDs will interact with the mixed with cell culture media. As previously described, MTX is a known analogue of FA. We used MDA-MB 231 cell lines (with overexpressed folate receptor) as positive control and MCF-7 cells as negative control cell lines, with deficit of FR receptors.

Epifluorescence and fluorescence cell imaging results demonstrated that MTX-CDs have potential for targeting and bioimaging of cancer cells. FR on the surface of MDA-MB 231 can bind and uptake MTX-CDs in a short time. To evaluate these properties, cellular uptake of MTX-CDs was performed with an epifluorescence microscope (Figure 8). In vitro epifluorescence imaging revealed the high-contrast cytoplasm biodistribution of MTX-CDs after 2 h of treatment.

More evaluation of cell target and uptake potential of MTX-CDs was investigated by the fluorescence cell imaging of MDA-MB 231 and MCF-7 cell lines (positive and negative controls, respectively) after 1 h (Figure 9a,d) and 4 h (Figure 9b,e) of incubation with MTX-CDs. The result showed that MCF7 cells showed a lower fluorescence intensity than MDA-MB 231 cells. As additional control, the MDA-MB 231 cells were pretreated with excess free FA and subsequently incubated with MTX-CDs (100 ppm) for 4 h, and the result showed a relatively weak fluorescence image (Figure 9c). MCF7 cells blocked with FA exhibit no essential change in fluorescence image intensity (Figure 9f). FT-IR and high-resolution XPS data show the existence of chemical targeting active groups on the surface of synthesized MTX-CDs. Besides, in vitro epifluorescence and fluorescence imaging data show that the synthesized MTX-CDs have FR targeting potential. In fact, comparing the images of MCF7 and MDA-MB 231 cells (blocked (with FA) and unblocked groups) proves that self-targeting potential was saved on the synthesized MTX-CD surfaces. In addition, our results are in complete agreement with the literature. Besides, other groups have previously demonstrated the self-targeting of Asp-CDs in vivo. In this work, we did not study the self-targeting in vivo, but we successfully demonstrated the self-targeting of MTX-CDs in vitro. We anticipate that our synthesized MTX-CDs will have self-targeting characteristics in vivo as well. Furthermore, these results show that FA residues in MTX-CDs are essential for cell targeting and uptake. This is demonstrated by the fact that MTX-derived CDs can effectively target FR-positive cancer cells via an FR-mediated procedure. Almost all types of receptors are common between the normal and cancerous cells, but some of these receptors may be overexpressed in cancerous cells. Recent studies have shown that any type of common cell surface receptor, when increased three times or greater, has potential for use as a cancer targeting receptor. Other studies showed that the amount of FR on the cancer cell surface is significantly higher compared to normal cells. In this work, in vitro fluorescence imaging of MCF7, negative control, and blacked MDA-MB 231 cells with MDA-MB 231 cells, positive control, shows excellent cancer cell targeting properties of the synthesized MTX-CDs. Therefore, this self-targeted theranostic system can be expected to perform well in vivo, which requires further investigation.

Cell Cycle Analysis via Flow Cytometry. Cell cycle tests were carried out using flow cytometry. In this method, DNA duplication at G1, S, and G2/M was determined. The effects of MTX-CDs were assessed on MDA-MB 231 cell proliferation in different cell cycle phases. As depicted in Figure 10, free MTX did not show a considerable difference in the cell cycle pattern compared to the control group. A comparison between free...
MTX and MTX-CDs shows a significant difference in the sub-G1 phase. The percentage of cells arrested in the sub-G1 phase when treated with free MTX is higher than in the control group (control group: 0.47%, free MTX: 21.55%). On the other hand, the synthesized MTX-CDs demonstrated an increase in the percentage of cells arrested in the sub-G1 phase (54.18%). The high percentage of sub-G1 population, which verifies apoptotic occurrence in the cell cycle, showed that the cell death was through apoptosis in MTX-CD (54.18%)-treated group. In previous studies, apoptosis was evaluated by the sub-G1 peak in the cell cycle analysis of propidium-iodide-stained nuclei.

Evaluation of the Induction of Cell Apoptosis Studied Using Annexin V-FITC Assay. An annexin V/FITC test was carried out using the flow cytometry technique as a quantitative test for the study of early apoptotic, late apoptotic, and necrotic cells. Test results demonstrated the cell apoptosis of MDA-MB 231 cells induced by free MTX and MTX-CDs. Figure 11 shows the quantitative results of the Annexin V-FITC assay. Viable cell population of free MTX-treated group was near to the control group. The populations of all early and late apoptotic cells in control, free MTX, and MTX-CDs groups were 7.57, 4.74, and 70.2%, respectively. Therefore, the highest population of apoptotic cells among all treatments over 72 h was observed in the MTX-CDs (3.2 μm) group. MTX-CDs could induce more MDA-MB 231 cell apoptosis than other groups.

CONCLUSIONS

In this work, the theranostic capability of ultrahigh-fluorescence MTX-derived CDs with unique surface features is reported for the first time. These MTX-derived CDs are capable of FR self-targeting, are highly fluorescent, and can destroy cancer cells based on targeting features. The MTX-CDs were synthesized through a facile one-step approach by importing MTX as a carbon source, nitrogen source, and surface passivation agent. The as-prepared CDs show excellent PL activity and excitation-independent emission. These nanoparticles offer several distinct advantages: (1) high fluorescence QY of 87.3%; (2) robust photostability and chemostability; (3) the residues of MTX on CDs can specifically target, image, and kill malignant cells. Overall, this work demonstrates that CDs could be used as a platform for constructing an intelligent nanomedicine with the integration of diagnostic, targeting, and therapeutic functions.

EXPERIMENTAL SECTION

Material. Methotrexate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Company (St. Louis, MO). Water (18.2 MΩ cm at 25 °C) was purified using a Millipore system. Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin-EDTA, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies. The dialysis bags (MWCO = 1000) were purchased from Sigma-Aldrich Company. All other chemicals used in these experiments were of analytical reagent grade and were purchased from Sigma-Aldrich Company (St. Louis, MO).

Methods. Apparatus and Characterization. MTX-CD particle size was determined by transmission electron microscopy (TEM, ZEISS EM900) and dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). X-ray diffraction (XRD) was used to investigate the crystallinity of carbon dots. XRD patterns were obtained with a Siemens D500 diffractometer with Cu Kα irradiation (λ = 1.5418 Å) at room temperature, operating at a voltage of 35 kV. These experiments were performed at a scan rate of 1° min⁻¹ in the scan range of 2θ = 2–70°. XPS was performed on an ESCALAB MK II X-ray photoelectron spectrometer using Mg as the excitation source. Fluorescence emission spectra were collected on a NOVA fiber-coupled spectrometer. Optical absorption spectra and FT-IR spectra were recorded on a Shimadzu UV-1800 Series and Bruker (Tensor 27) IR spectrophotometer instrument.

Synthesis of MTX-CDs. The MTX-CDs were synthesized using a hydrothermal method (Figure 12). For this reaction, 13 mg of MTX (as a precursor) was dissolved in 20 mL of deionized water. The mixture, as a clear light yellow solution, was transferred into a 50 mL reactor and heated in an oven for 6 h at 240 °C. After carbonization, a clear yellow-brown
monodisperse solution was obtained. The solution was dialyzed against deionized water through a dialysis tubing (MWCO = 1 kDa) for 24 h to remove the precipitate. Then, an MTX-CD powder was obtained by lyophilizing the solution and stored at 4 °C for further use.

Characterization of the Quantum Yield of Methotrexate Carbon Dots. The quantum yield of the MTX-CDs was measured using the following equation

\[
Q = \frac{Q_r I_r}{I_r OD n_r^2}
\]

where \(Q\) is the quantum yield, \(I\) is the measured integrated emission intensity, \(n\) is the refractive index, and OD is the optical density, which is measured on a UV–vis spectrophotometer. The subscript R refers to the reference fluorophore of known QY, for example, quinine sulfate (QS) in the present work. QS (literature \(U = 0.54\)) was dissolved in 0.1 M H2SO4 (\(n = 1.33\)), and the MTX-CDs were dissolved in distilled water (\(n = 1.33\)).

Cytotoxicity Assays. The cytotoxicity of MTX-CDs was evaluated on MDA-MB-231 cells using a standard MTT assay. For these measurements, the cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin. The cells were maintained at 37 °C, 5% CO2. Confluent cells were removed with 0.05% (w/v) trypsin-EDTA (Gibco), seeded at 2 × 10^3 cells/100 μL/well in 96-well plates, and grown overnight at 37 °C, 5% CO2. The next day, the cells were washed two times with PBS (Gibco) and incubated with free MTX and MTX-CDs corresponding to 0 (0.0 μM), 3.06 (0.68 μM), 6.125 (1.37 μM), 12.5 (2.75 μM), 25 (5.5 μM), and 50 (11 μM) mg 10 mL\(^{-1}\) for 24 h. The culture medium was then discarded. The MTT solution (20 μL, 0.5 mg/mL\(^{-1}\) in RPMI) was added to each well. The cells were incubated for another 4 h, and then the supernatant was abandoned. DMSO (Sigma-Aldrich) (150 μL) was used to liberate the formed formazan. After shaking the plate for 10 min, the absorbance at 570 nm was measured to calculate the cell viability rate (VR) by Origin software.

Epifluorescence and Fluorescence Microscopy Imaging of Cancer Cells with Self-Targeted MTX-CDs. MDA-MB-231 cells were cultured in cell culture dishes at 37 °C using RPMI 1640 growth medium with 10% FBS, 100 units per mL penicillin, and 100 μg/mL streptomycin. The cells were then incubated in a humidified incubator at 37 °C with 5% CO2. All of the cells were incubated at approximately 70% confluence until normal morphology was achieved. Then, the mixture of MTX-CDs (100 μg/mL) in RPMI 1640 medium was added to each dish. The cell dish was placed in an incubator for the desired time. The cells were washed three times with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) to remove unattached compounds. For epifluorescence and fluorescence imaging, a Nikon E1000M (Nikon, Tokyo, Japan) research fluorescence microscope equipped with the Plan Apo apochromatic objectives (Nikon, Tokyo, Japan) was used. The best fluorescence excitation was observed when mirror cube units for 480–510 and 510–550 nm and a fluorescence microscope (Olympus microscope Bh2-FCA, Japan) were used.

Cell Cycle Analysis via Flow Cytometry. One of the important aspects of cell biology is the cell cycle. This method was utilized to determine the DNA duplication at G1, S, and G2/M stages. One can change the normal cycle of cancer cells in cancer therapy. Cell cycle distribution after treatment, in the presence of either MTX-CDs or MTX, was studied with MDA-MB 231 cells using flow cytometry and propidium iodide (PI) staining of DNA. In cell cycle studies, DNA content is used as an index of cell generation. For this purpose, the cells were seeded in six-well plates with a density of 5 × 10^5 cells per well and subsequently incubated under culture conditions for 24 h. The attached cells were treated

![Figure 10. Cell cycle analysis performed by staining the DNA content of the cell followed by flow cytometry. The percentage of cells in the G0/G1, S, or G2/M phase is indicated by (a) untreated cells as a control, (b) MTX (3.2 μm), and (c) MTX-CDs (3.2 μm). (d) Quantitative results of cell cycle arrest and distribution.](https://dx.doi.org/10.1021/acsomega.0c03215)
with MTX-CDs (3.2 μM, IC50 value) and MTX and incubated for 24 h (nontreated cells were considered as a control group). Twenty four hours after treatment, the cells were trypsinized and centrifuged to remove the supernatant and washed with cold PBS to remove all trypsin. Afterward, the cells were suspended in 300 μL PBS and 700 μL ethanol and stored in a dark place (5–7 days, 4 °C). The samples were then centrifuged and resuspended in 700 μL of PBS, and this cycle was repeated and the cells were suspended in 300 μL of PBS. This was followed by adding 7 μL of ribonuclease A to the samples and incubating for 45 min at 37 °C. Finally, 10 μL of PI was added to the samples, which were stored for 10 min at room temperature. Changes in the cell cycle were investigated by a FACSCalibur flow cytometer (Becton Dickinson).

**Apoptosis Analysis by Flow Cytometry.** To evaluate the therapeutic efficacy of MTX-CDs and free MTX, the induction of apoptosis in MDA-MB 231 cells were studied. Specifically, the cells were seeded in six-well plates with a density of 5 × 10^5 cells per well and subsequently incubated under the culture condition for 24 h. The attached cells were treated with MTX-CDs (3.2 μM, IC50 values) and free MTX and incubated for 24 h (nontreated cells were considered as a control group). Twenty four hours after treatment, the cells were trypsinized and centrifuged to remove the supernatant and washed one more time with cold PBS to remove all trypsin. Afterward, the cells were suspended in 100 μL of annexin binding buffer. To stain the apoptotic cells, 5 μL of annexin V and 5 μL of PI were added to all groups and incubated for 15 min at room temperature. Then, the percentage of early and late apoptosis was investigated by a FACSCalibur flow cytometer (Becton Dickinson).
This approach was carried out as specified by the manufacturer.

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**Notes**

The authors declare no competing financial interest.

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