Covalent and Noncovalent Loading of Doxorubicin by Folic Acid-Carbon Dot Nanoparticles for Cancer Theranostics

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ABSTRACT: With special properties such as excellent fluorescence features, low toxicity, good biocompatibility, permeability, and easy clearance from the body, carbon dot (CD)-based nanoparticles (NPs) have the potential to deliver drugs and use in vivo diagnostics through molecular imaging. In this work, folic acid-CD (FA-CD) NPs were prepared to deliver doxorubicin (Dox) covalently and noncovalently as cancer theranostics. FA was conjugated to the surface of CDs for targeting cancer cells with overexpressing folate receptors. CDs prepared with various amounts of precursors lead to their associated NPs with different photoluminescence properties and drug release profiles. The loading of Dox and its releasing data depends on the linkage of drug Dox to FA-CD and CD composition. All NPs were characterized by UV–vis, Fourier transform infrared spectroscopy, and dynamic light scattering. The noncovalent FA-CD-Dox NPs were preferred with a simple preparation process, excellent photoluminescence, and in vitro drug release properties. The noncovalent FA-CD-Dox showed the best efficacy against MDA-MB-231 compared to the CD-Dox and covalent FA-CD-Dox.

INTRODUCTION

Nanoparticles (NPs) have shown a significant impact on the delivery of drugs and bioimaging areas. Recently, the targeted delivery drugs with fluorescence (FL) nanomaterials have attracted lots of interest.1 Some of them are chosen as cancer theranostics. As reported, one of the main challenges in cancer therapy is to effectively detect cancer at its early stage.5 The reasons could be lower concentrations of biomarkers at the cancer site and in bodily fluids at an early stage of the natural progression path of cancer. Moreover, as most cancers are detected relatively late, scientists cannot ideally characterize the true properties of early cancers, which are quite different from late cancers. Other limitations of conventional cancer treatments include poor specificity and low accumulation of drugs at therapeutic sites.7 Those drugs can cause tremendous damage not only to the cancer cells for which their administration is intended but also to unrelated healthy cells. Since 2000, cancer nanotechnology has made steady progress because NPs have remarkable potential in dual functions of diagnosis and therapy of cancer tumor sites1 because they can increase the chances of detecting cancer at the earliest stage and enhance the anticancer drug specificity.5 By specifically targeting cancer cells with the aid of nanomaterials, it is expected that overall drug dosages can be lowered due to higher drug efficacies, causing decreased side effects and increased patients’ quality of life.6

Among different NPs, carbon dots (CDs) have been developed to target the delivery of anticancer drugs due to their physicochemical properties, facile synthetic route, and high quantum yield.1 CDs are generally classified as quasispherical NPs with sizes less than 10 nm and have been extensively used in drug delivery,4,6 biological imaging,7 light-emitting devices,10 and photocatalysts.11 Compared to inorganic quantum dots (QDs), such as CdSe and CdTe, CDs are much safer and are more biocompatible.12 CDs can be prepared by various methods, including laser passivation,13 pyrolysis/hydrothermal,14 and ultrasonic/microwave.15 Depending on the synthetic methods and precursors, CD surfaces can be functionalized with different chemical groups, permitting post-synthesis modifications.16 In particular, CDs can form covalent or noncovalent interactions with therapeutic agents and targeting agents. Leblanc and co-workers developed transferrin-CD-Dox conjugates to target pediatric brain tumor cells.17 Sharon et al. used folic acid (FA) to attach bovine serum albumin-protected CDs to deliver Dox for cancer
treatment. Zhao et al. described the preparation of FA-CDs from different starting materials for cancerous cell recognition and diagnosis. For this purpose, we would like to report the highly advantageous FA-CD NPs to deliver the anticancer drug Dox directly. It is the first time to compare FA-CDs covalently and noncovalently carrying Dox, such as their FL properties, the in vitro drug release efficiency, and their potential against breast cancer cells. The new NP series are prepared with the easiest and most economical methods. The fluorescent CDs were synthesized with a one-step hydrothermal method from citric acid (CA) to ethylenediamine (EDA). To improve the targeted specificity of the tumor cells, FA was covalently linked to the amine group on the surface of CDs via an amide bond. As a targeting agent, FA is a water-soluble vitamin, which is essential for cell survival and has a high affinity for folate receptors (FRs). FR is overexpressed in various tumor cells at sites, such as breast, kidney, ovarian, cervical, lung, colorectal, and brain. Therefore, the FA-CD NPs can be used to target the delivery of anticancer drugs. For example, Dox is widely used to treat certain types of leukemia, lymphomas, and sarcomas. Because of the poor water solubility and low affinity to certain tumor cell nuclei, free Dox anticancer therapy is often limited. Hence, we developed the FA-CD NPs to carry Dox via electrostatic attraction or amide bonding and obtained its drug loading capacity (DLC) and drug loading efficiency (DLE). We also tested all FA-CD-Dox in terms of spectral property, in vitro drug release, and cytotoxicity in breast cancer cell lines and found the best module for future investigation.

**EXPERIMENTAL SECTION**

**Materials.** EDA, CA, doxorubicin hydrochloride (Dox-HCl), dialysis bag (MWCO = 1000–3500 Da), N-hydroxysuccinimide (NHS), 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide (EDC), FA, and Gibco 1× phosphate-buffered saline (PBS) pH 7.4 were obtained from Fisher Scientific, USA.

**Synthesis of CDs.** Two series of CDs were prepared as described by Zhu et al. with minor modifications. The typical procedure is first mixing of 2.00 g (10.41 mmol) of CA with 63.74 μL (0.9540 mmol) of ethylenediamine (EDA) (ratio 9:1) in 10.00 mL of deionized water. The sample was transferred into an autoclave to react at 250 °C for 5 h. After cooling the solution, it was dialyzed in a 3500 Da dialysis bag in 500 mL of water for 6 h. The final sample was collected and lyophilized to obtain the brown-black product with around 30–35 wt % yields.

**Synthesis of FA-CDs.** FA-CDs were prepared following the procedure described by Zhao et al. For the typical procedure, first, 20 mg of FA was dissolved in 8 mL of 1× PBS buffer (pH 7.4) to obtain a clear yellow solution. Then, 4 mL of aqueous solution containing 0.0260 g (0.1356 mmol) of EDC and 0.0156 g (0.1355 mmol) of NHS was added to the FA solution. The mixture was sonicated at room temperature overnight. Then, it was mixed with 2 mL of CDs (22 mg/mL). The reaction solution was sonicated at room temperature for another 24 h. Next, the solution was dialyzed (MWCO 1000 Da) against deionized (DI) water for 1 day to remove the excess FA or CDs. Lyophilization of the resulting product was then carried out to obtain a yellow powder product with around 40 wt % yields.

**Synthesis of Noncovalent CD-DOX.** CD-Dox were prepared as per the procedure described by Yuan et al. For the typical procedure, 1 mL of CD solution (8 mg mL⁻¹), 1 mL of DOX solution (0.4 mg mL⁻¹), and around 2 mL of PBS at pH 7.4 were mixed to form a final 4 mL solution. This solution was then stirred for 24 h at 25 °C and 200 rpm in the dark. To remove the unreacted Dox or CDs, the produced solution was dialyzed with a dialysis membrane (MWCO = 3500 Da) against 500 mL of DI water for 2 h. The reaction was carried out in the dark to prevent photodegradation of Dox. The final solid product was obtained with around 40% yields after the solution was lyophilized for 48 h.

**Synthesis of Noncovalent FA-CD-DOX.** FA-CD-Dox were prepared as per the procedure described by Yuan et al. For the typical procedure, 1 mL of FA-CDs solution (8 mg mL⁻¹), 1 mL of DOX solution (0.4 mg mL⁻¹), and around 2 mL of PBS at pH 7.4 were mixed to form a final 4 mL solution. This solution was then stirred for 24 h at 25 °C and 200 rpm in the dark. To remove the unreacted Dox or FA-CDs, the produced solution was dialyzed with a dialysis membrane (MWCO = 3,500 Da) against 500 mL of DI water for 2 h. The reaction was carried out in the dark to prevent photodegradation of Dox. The final solid product was obtained with around 25% yield after the solution was lyophilized for 48 h.

**Synthesis Covalent of FA-CD-DOX.** The covalent FA-CD-DOX complex was completed using an EDC/NHS coupling reaction. In this procedure, 1 mL of FA-CD solution (8 mg mL⁻¹) was dissolved in 3 mL of phosphate-buffered solution (PBS, pH 7.4) and sonicated for 15 min. To this solution, 0.5 mL of 17 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloric acid (44.3 μMol EDC·HCl) was added and stirred for 30 min. Then, 0.5 mL of 10.2 mg/mL N-hydroxy succinimide (44.3 μMol NHS) was also added and stirred for another 30 min. The ratio of EDC to NHS was kept at 1:1 in the synthesis. Next, 1 mL of DOX solution (0.4 mg mL⁻¹) was added and stirred overnight in the dark to prevent the breakdown of DOX-HCl by light (DOX-HCl is photosensitive). The final solution was dialyzed against 500 mL of DI water for 2 h. The final solid product was obtained with around 29 wt % yields after the solution was lyophilized for 48 h.

**Characterization.** The spectral properties of all the NPs, such as CDs, FA-CDs, and FA-CD-Dox, were studied by UV–vis spectroscopy (PharmaSpec UV-1700, PerkinElmer, USA) and FL spectroscopy (FluoroMax-3, Jobin Yvon Inc, USA) with a standard glass quartz cuvette. The FL emission spectra were taken in the aqueous solution with a slit width of 10 nm for both excitation and emission from 300 to 390 nm.

The samples were also analyzed by Fourier transform infrared (FT-IR) spectroscopy by an IR Prestige-21 spectrometer (Shimadzu, USA) with a Pike Miracle ATR sampling accessory. The spectra were obtained from 600 to 4000 cm⁻¹ with 16 scans. The hydrodynamic diameter of the NPs was measured in an aqueous dispersion with dynamic scattered light (DLS, BI-200SM) at room temperature.

**Drug Loading Capacity (DLC) and DLE.** DLC and DLE were determined using the method as described by Kong et al. with few modifications. The concentration of unbounded Dox was calculated based on the standard calibration curve of DOX (linear line equation = 2.27x + 0.2229) after measuring the free Dox absorption at 485 nm. The amount of Dox loaded in FA-CDs was calculated by subtracting the free Dox from the
initial amount of Dox. The DLC and DLE were calculated using the following equations:

\[
\text{DLC} (\%) = \frac{\text{Amount of DOX loaded in FA - CDs}}{\text{Initial amount of FA - CDs}} \times 100\%
\]

\[
\text{DLE} (\%) = \frac{\text{Amount of DOX loaded in FA - CDs}}{\text{Initial amount of the DOX}} \times 100\%
\]

In Vitro Drug Release. The pH-dependent release of Dox from the FA-CDs-Dox and CD-Dox complexes was also investigated. For a typical procedure, 3 mL of FA-CD-DOX was sealed in a dialysis bag (MWCO = 1000 Da) and in 120 mL of PBS solution at pH 5.0 and heated to 37 °C under mild intermittent stirring. We collected the samples every 30 min for 3 h and then at intervals of 24 h for 3 days. At regular intervals, 2 mL of the release medium in PBS had been replaced with the same volume of fresh PBS solution. The amount of released Dox was determined by using the standard calibration curve of Dox at 485 nm spectrophotometrically. We determined the concentration of the drug released by using the formula \(y = mx + b\), where \(y\) is the absorbance of the unknown, \(m\) is the slope, \(b\) is intercepted, and \(x\) is the concentration (mg/mL). We obtained slope = 22,000 and intercept = –0.0467 with \(R^2 = 0.9988\). The same procedure was repeated at pH 7.4.

Cytotoxicity Assays. The FL of cytotoxicity assays was measured in the following procedure. MDA-MB-231 and MDA-MB-468 cells were plated in 48-well plates at a concentration of 200,000 cells/mL and grown in Leibovitz’s medium supplemented with 15% PBS and 1% penicillin/streptomycin. They were dosed when approximately 75% of confluence cells were treated with either CD-Dox or FA-CD-Dox at a range of Dox concentrations (0.0333 – 0.0001 mg/mL) diluted in serum-free media. Forty-eight hours after treatment, the medium was changed, and 5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT (Sigma-Aldrich), was added. Four hours later, the formazan crystals were dissolved in isopropanol 0.1 N HCl, and absorbance was measured using a SpectraMax Plus (Molecular Devices) absorbance plate reader at 570 nm.

RESULTS AND DISCUSSION

NP Preparation. Three different types of FA-CD-Dox NPs were prepared by the reported protocol with slight modifications. Specifically, FA-CDs were prepared from CD (A) and CD (B) using noncovalent interactions with Dox and covalent conjugation-Dox (Scheme 1).

The complete preparation route is listed in the experimental part and the Supporting Information. Two kinds of fluorescent CDs were synthesized from CA to EDA with different ratios.\(^{20}\) CD (A) and CD (B) were prepared with a ratio of 9:1 and 1:1 of CA/EDA, respectively. The CD (B) solution was light yellow under the ambient light and was bright blue FL under
the UV light (λ = 365 nm). According to the earlier reports, the source of CD fluororesces came from the defects in their structure organization, such as the sp² and sp³ carbon clusters.26 Modification of the CD surface by –OH, –COOH, and –NH₂ groups produced the electron–hole pairs, which led to the appropriate wavelength fluoresces under UV light.27 During the lyophilization process, removing water from CD sample B was less time-consuming compared to sample A. It is attributed to the hydrophilic carboxylic acid groups on the surface of CD (A), which increase the difficulty of water to escape from the NPs. The yield of CD (B) was around 40 wt %, which is also much higher than 12 wt % of CD (A). To improve the targeted specificity for the tumor cells, both CD samples were covalently linked to FA via an amide bond from the classic Carbodiimide Crosslinker Chemistry.28 This method allowed for the creation of a noncleavable covalent bond between CDs and FA. The FA-CD (from CD (B)) solution was less bright blue FL under the UV light (λ = 365 nm) than pure CD (B) with the same weight concentration. Afterward, the anticancer drug Dox was loaded on the FA-CD complex via noncovalent or covalent bonding. The procedure was carried out in the dark. Dox acted as a cation in the aqueous solution of pH 7.4 when the amino group was protonated. Therefore, Dox can be noncovalently loaded on the negatively charged NPs via electrostatic interaction and π–π stacking.29 On the other hand, Dox was also loaded onto FA-CDS covalently from EDC/NHS chemistry. The noncovalent and covalent FA-CD-Dox (from CD (B)) solution was purple-blue, and milky purple color, respectively, under the UV light (λ = 365 nm) compared to pure CD (B) (Figures 1 and S1).

Figure 1. Photograph from left to right of synthesized CDs, FA-CDS, FA, Dox, water, noncovalent (all from CD (B)). All of them were 0.05 mg/mL solution (except water) and observed under a 365 nm UV light. Photograph courtesy of “Michael T. Tetteh”. Copyright 2022. Reusing the image needs permission.

Size. A DLS analyzer was used to determine the sizes of all the NPs. The approximate size ranged from 3 to 4 nm in diameters (Table 1). The EDA and CA composition ratio had little effect on the size and size distribution of all NP particles. There was a noticeable increase in size following FA conjugation but no change following Dox conjugation. It may contribute to the possibility of Dox conjugate with CDs in the gap of FA-CD linkage. Since most of the NPs were dialyzed with a dialysis membrane (MWCO = 3500 Da), the standard deviation was consistent. The exception was the FA-CDS prepared from CD (B) with the membrane (MWCO = 1000 Da), which removed more variable-sized NPs. The covalent FA-CD-Dox from CD (B) also had a small StDev, which may contribute to the covalent bonds of CDs with FA and Dox. At the same time, DLS method limitations,30 porous morphology, and surface charges were from the terminal groups on the NP surface.31

The optical properties of all the NPs were characterized by UV–vis absorption, photoluminescence spectroscopy, and IR. The collected spectra and data were used to confirm the NPs and their functional groups and to calculate their concentration.

UV–Vis Spectra. Figure 2 shows the UV–vis of the NPs prepared. The UV–vis spectrum of CD (A) revealed characteristic absorption peaks at 201 and 240 nm. For CD (B), the UV–vis spectrum showed the characteristic absorption peaks at 206, 240, and 337 nm. The peak for both CDs at around 200 nm represented π → π* electron transition in C=O bonds while that of 240 nm peak was n → π* electron transition. Also, the peak for CD (B) at around 350 nm was attributed to the C=O and C=N bonds with electron transition at n → π* transition in sp² hybridization of carbonyl groups on CD surface.26 The absorption at 337 nm of CD (B) had a much higher absorbance than CD (A) in the same region due to the possible hypochromic effect of the N– H functional group close to the carbonyl group.

As shown in Figure 2, both noncovalent FA-CD-Dox (blue) show strong peaks at around 210, 300, 350, and 480 nm. The covalent FA-CD-Dox (green) shows strong peaks at around 264, 345, and 485 nm. All the FA-CD-Dox NPs had an obvious absorption peak at around 480 nm, which is at the same characteristic peak range of Dox alone (Figure S2). They also had characteristic peaks of FA and CDs at around 350, 210, and 240 nm. These observations elucidated that the product structure of all the FA-CD-Dox NPs contained all the major UV–vis properties of reactants CDs, FA, and Dox (Figure S2). Compared with the spectra of FA-CDS (yellow), noncovalent FA-CD-Dox (from CDs B) (blue) indicated a higher absorption in the 475–500 nm range. Moreover, the peak absorption intensity for the covalent FA-CD-Dox (from CDs B) was much lower than that of the noncovalent series. It may be caused by the quenching effect.

FL Spectra. Figure 3a,c displays the FL spectra of the CDs, in which CD (A) and CD (B) had distinct emission peaks at 460 nm (λem = 360 nm) and 440 nm (λem = 360 nm), respectively. The FL came from the carboxylate groups on the particle surface and the precursors’ oxygen-containing groups. As two kinds of CDs were prepared with different ratios of precursors, the functional group proportion on each CD surface was different. The FL spectra confirm why the aqueous solution of CDs showed a blue luminescence (460 nm) under a 365 nm UV lamp. The noticeable difference was the emission intensity, which yielded 10 times higher from CD (B) than CD (A). The emission intensity of FA-CDS (from CD (A)) was enhanced compared to that of CD (A). Both FA-CDS samples exhibited an excitation-dependent wavelength property by having a maximum emission peak at 450 nm at the excitation wavelengths of 360 and 340 nm correspondingly. The emission peak intensity of FA-CDS from CD (B) was also much higher than that of FA-CDS from CD (A) at all excitation wavelengths.

| NPs             | A size (nm) | StDev (nm) | B size (nm) | StDev (nm) |
|-----------------|------------|------------|------------|------------|
| CDs             | 3.4        | 0.6        | 3.42       | 0.4        |
| FA-CDS          | 4.4        | 0.4        | 4.3        | 0.1        |
| noncovalent     | 4.4        | 0.6        | 4.3        | 0.5        |
| FA-CD-Dox       | 3.74       | 0.15       |             |            |
All FA-CD-Dox composites showed similar excitation peaks (Figure 3b–e) as the components CDs, FA, and Dox were examined separately. For example, all of them had one FL range similar to Dox, which had typical emissions of around 560 and 590 nm. The FL of the final products FA-CD-Dox and their intermediates FA-CDs similarly showed the typical emission and excitation wavelength-dependent shifting property of the corresponding starting CDs due to their special nature, such as the quantum confinement effect and the surface/edge effect. The intensity increased for NPs from CD (A) while the intensity decreased for NPs from CD (B) after conjugation. In the presence of a 365 nm UV lamp, it could also be observed that the luminescence intensity trend for NPs prepared from sample B is FA-CD-Dox (both Figures 3d,e and S3) < FA-CDs < CDs. The possible reason is that FA-CD-Dox prepared from CD (B) with the amide bond quenching effect is in the wavelength of 320–360 nm.

(Figure S3).
FT-IR Spectra. FT-IR spectra revealed that the CD (A) were prepared with an excess carboxylic acid than the primary amine (Figure 4a). Figure 4d shows the strong and intense peak at 1600 cm$^{-1}$ (−NH$_2$ bend), which indicates more primary amines on the surface of CD (B). There was a much broader peak at around 3000−3600 cm$^{-1}$ carboxylic acid (−OH) and another strong and more intense peak at around 1690 cm$^{-1}$ (carboxylic acid −C=O) in Figure 4a compared to Figure 4d. The reason is that CD (A) were prepared with a bigger ratio of carboxylic acid to the amine. The spectra of FA-CDs (Figure 4b,e) were very similar even though they came from different CDs. They both had bands at 3100−3600 (carboxylic acid −OH and amide), 1700 cm$^{-1}$ (carboxylic acid −C==O), and 1600 cm$^{-1}$ (−NH$_2$ bend and C==C), which is similar to FA alone.34 The only small difference is the peak intensity in the figure print region. Both noncovalent FA-CD-Dox (Figure 4c,f) had similar peaks to the corresponding FA-CDs as well as Dox.35 The prominent difference is the peak intensity at around 1650 cm$^{-1}$ (−C==O) in Figure 4c, much lower than that shown in Figure 4f, because more Dox was attached to FA-CDs-Dox from CD (B). It can also be confirmed by the calculated higher DLC and DLE of B1 compared to A with the same amount of CDs and FA in Table 2.

In the covalent FA-CD-Dox FT-IR spectrum (Figure 5 top), the predominant peaks were at around 3348 and 1643 cm$^{-1}$ and medium peaks at 1200 and 1080 cm$^{-1}$. The broad and strong peaks occurred at 1643 cm$^{-1}$ that overlapped 1700 cm$^{-1}$ corresponding to C==O bond stretching in the amide bond and the amide primary −NH$_2$ bend. The peak at 1643 cm$^{-1}$ in the covalent complex was stronger than the NH$_2$ bending peak found in pure CDs, FA, and DOX. This could be a result of various amide bonds formed on different sites of the CD surface, confirming the covalent attachment of FA and DOX to the CDs.31,32 The characteristic peaks at around 1223 cm$^{-1}$ showed the presence of C−N stretching vibration in typical amide bonds.36 The existence of a peak at 1643 cm$^{-1}$ corresponded to the amide bound (RCONHR′) in the spectrum of covalent FA-CD-Dox, while it was absent in the noncovalent FA-CD-Dox, another proof for the formation of covalent bonds between FA-CDs and Dox.

Drug Loading Capacity and DLE. We accessed the loading capacity of Dox using UV−vis absorption spectroscopy at a wavelength of 485 nm, the characteristic peak of free Dox. The DLC and DLE of Dox for all the FA-CD-Dox samples were around 3–6 and 60–80%, respectively (Table 2). These results demonstrated that the prepared FA-CDs showed a comparable DLC for anticancer drug DOX noncovalently and covalently.7,37 Because of the amide bonding, the covalent FA-CD-Dox exhibited slightly higher DLE and DLC with the same amounts of precursors as compared to B1 and B5.

Drug Release at Extracellular Condition. Understanding the drug release profile in extracellular physiological environments is a critical factor in understanding the “switch on” mode of anticancer molecules to activate drug release from the NPs.38 According to the literature, the tumor cell lysosomes and the tumor tissues show lower pH around 4.5−5.5 and pH 6.5−7.2, respectively.39 Therefore, low pH was used as the ideal trigger for a drug-controlled release study. To examine the stability of drugs under extracellular conditions, drug release in neutral PBS (pH 7.4) buffer was analyzed, and results showed that all types of NPs exhibited high drug stability (e.g., noncovalent FA-CD-Dox showed around 20% drug release after 75 h) (Figure 6a,b). In particular, covalent conjugation exhibited the most stable drug stability in PBS (Figure 5c) (i.e., less than 15% release in PBS at 75 h). In contrast, DOX was abruptly released

Table 2. DLC and DLE of NP Samples

| NPs samples | FA-CDs mg | Dox mg | DLC % | DLE % |
|-------------|-----------|--------|-------|-------|
| A1          | 8         | 0.4    | 3.21  | 64.2  |
| B1          | 8         | 0.4    | 3.73  | 74.5  |
| B2          | 12        | 0.6    | 4.06  | 81.2  |
| B3          | 8         | 0.6    | 6.06  | 80.8  |
| B4          | 8         | 0.8    | 6.67  | 66.7  |
| B5          | 8         | 0.4    | 3.95  | 84.4  |

"A1 is noncovalent FA-CD-Dox (from CDs A); B1, B2, B3, and B4 are noncovalent FA-CD-Dox (from CDs B); and B5 is covalent FA-CD-Dox (from CDs B)."
in acidic buffer (i.e., pH of 5.0 in PBS buffer) and almost 80% of the DOX detached from the noncovalent FA-CD-Dox (Figure 6b). Drug stability in a hematologic environment is important because the release of drugs from the nanocarrier before cell uptake will minimize the anticancer efficacy of nanocarriers in cancer cells. Most of Dox in noncovalent FA-CD-Dox was released within around 20 h by the acidic environments (i.e., at both pH of 5.0 and 7.2) (Figure 6). The results confirmed that noncovalent FA-CD-Dox (from CD (B)) exhibited selective drug release in an acidic environment, while both noncovalent NPs exhibited identical drug release patterns under neutral conditions in PBS buffer (Figure 6a,b).
Thus, the noncovalent series might provide a pH-dependent switch mechanism, which is especially useful for acidic tumor surfaces.40

Differential Cytotoxicity on Cancer Cells. In this study, three types of NPs (i.e., noncovalent, covalent FA-CD-Dox, and noncovalent CD-Dox) yielded different cytotoxicities for cancer cells (Figure 7a,b), for noncovalent FA-CD-Dox and CD-Dox, and (Figure S4) for covalent FA-CD-Dox. Breast cancer cell lines MDA-MB 231 and MDA-MB 468 corresponded to triple-negative breast cancer, which often caused low survival rates.41 As those breast cancer cell lines were well studied involving FA targeting, they were chosen to test our FA-CD-Dox NPs.42 The MTT dose–response data were obtained spectrophotometrically after dosing the cells with NPs and incubating the cells for 48 h (Figure 7). Since CDs and FA are not toxic, our results suggested that both noncovalent and covalent FA-CD-Dox had efficacy to kill breast cancer cells at low concentrations of Dox. Specifically, the noncovalent series showed higher efficacy toward the MDA-MB-231 cell line than the CD-Dox (Figure 7a). The reason is that the FR is overexpressed for MDA-MB-231 cancer cells more than MDA-MB-468.43

Two of the curves (MDA-MB-231 + CD-Dox and MDA-MB-468 + CD-Dox) did not converge when fitted to a four-parameter sigmoidal equation (most likely due to the lack of high-dose/low-viability tails), so an IC50 could not be calculated. The IC50 for MDA-MB-231 + FA-CD-Dox was estimated to be 0.001549 mg/mL. The IC50 for MDA-MB-468 + FA-CD-Dox was estimated to be 0.04619 mg/mL. Because an IC50 could not be calculated for all curves, no statistical analysis could be performed. Further study will be carried out.

CONCLUSIONS

As biosafe materials, CDs are one of the most desirable NP candidates for theranostic applications. In our research, the simplest FA-CD NP module was designed and established to carry Dox noncovalently and covalently. The best choice noncovalent FA-CD-Dox (from CDs B) showed the highest efficacy toward the breast cancer cell line MDA-MB 231. By comparing this series with CD-Dox noncovalently, it proves our hypothesis that FA maximizes the intracellular targeting efficacy, especially toward the FR-overexpressed cancer cells. The noncovalent FA-CD-Dox (from CD (B)) also released the drug Dox almost 4-folds in acidic conditions than the neutral condition, which can dramatically reduce unwanted cytotoxicity in normal cells. By adjusting the ratio of FA-CDs to Dox, the comparable high DLE (60–80%) and DLC (3–6%) of anticancer drug Dox were achieved. Our work demonstrated the plausibility of using our FA-CDs for delivering other active chemotherapeutic agents covalently or noncovalently through controlled intracellular trafficking.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01482.

Photograph of water, CD (B), covalent FA-CD-Dox (from CD (B)) observed under a 365 nm UV light; UV–vis spectra of NPs (0.01 mg mL⁻¹): CD (A), CD (B), FA, FA-CDs (from CD (A)), FA-CDs (from CD (B)), FA-CD-Dox (from CD (A)), and FA-CD-Dox (from CD (B)); FL spectra of the NPs (0.01 mg mL⁻¹): FA-CDs (from CD (A)) and FA-CDs (from CD (B)); and MDA-MB 231 and MDA-MB 468 breast cancer cell response to covalent FA-CD-Dox (from CD (B)) (PDF)

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S.D., G.B., and M.T. performed the synthesis, spectra, and calculation. Z.W., V.P., K.K., T.W., and E.G. conceived and performed the FA-CD-Dox cytotoxicity test. Z.C., A.K., and G.L. performed the size analysis. All authors discussed the results and commented on the manuscript drafted by H.M. and V.P.

Notes
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