Water-Dispersible Fullerene Aggregates as a Targeted Anticancer Prodrug with both Chemo- and Photodynamic Therapeutic Actions

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Prodrug therapy is one strategy to deliver anticancer drugs in a less reactive manner to reduce nonspecific cytotoxicity. A new multifunctional anticancer prodrug system based on water-dispersible fullerene (C60) aggregates is introduced; this prodrug system demonstrates active targeting, pH-responsive chemotherapy, and photodynamic therapeutic (PDT) properties. Incorporating (via a cleavable bond) an anticancer drug, which is doxorubicin (DOX) in this study, and a targeting ligand (folic acid) onto fullerene while maintaining an overall size of approximately 135 nm produces a more specific anticancer prodrug. This prodrug can enter folate receptor (FR)-positive cancer cells and kill the cells via intracellular release of the active drug form. Moreover, the fullerene aggregate carrier exhibits PDT action; the cytotoxicity of the system towards FR-positive cancer cells is increased in response to light irradiation. As the DOX drug molecules are conjugated onto fullerene, the DOX fluorescence is significantly quenched by the strong electron-accepting capability of fullerene. The fluorescence restores upon release from fullerene, so this fluorescence quenching–restoring feature can be used to track intracellular DOX release. The combined effect of chemotherapy and PDT increases the therapeutic efficacy of the DOX–fullerene aggregate prodrug. This study provides useful insights into designing and improving the applicability of fullerene for other targeted cancer prodrug systems.

1. Introduction

Prodrug therapy provides a means to deliver anticancer drugs in a less reactive manner in order to reduce nonspecific cytotoxicity. Prodrugs are the pharmacologically inert derivatives of drugs that can be converted into active drug molecules in vivo, either enzymatically or nonenzymatically. One major objective of prodrug design is to ensure active targeting and specific activation by conjugating drugs to suitable targeting ligands, that is, those that display high binding affinity for their receptor(s). Many prodrug research efforts have concentrated on conjugating anticancer drugs onto different carriers. These carriers transport the drugs to tumors and are subsequently released either intracellularly or extracellularly. Among these carriers, nanoparticles are promising candidates because of their enhanced permeability and retention (EPR) effect. Several particle-based prodrugs have been developed. For a nanoparticulate delivery system, one challenge lies in successfully combining therapeutic, targeting, and other desirable actions into one system; it is difficult to couple multiple functional groups in sufficient concentrations since the number of attachment sites on the particle surface is limited. Fullerenes and their derivatives have been extensively investigated as candidates for clinical drug applications. To
improve the aqueous solubility of fullerenes, several methods have been developed to synthesize water-soluble (e.g., fullerol) or water-dispersible fullerenes into drug carriers. For example, Sun et al. covalently linked an anticancer drug (doxorubicin, DOX) onto fullerene derivatives and obtained fullerene–spacer–DOX conjugates. These conjugates exhibited improved aqueous compatibility, thus supporting the utilization of fullerenes in prodrg delivery systems.

Another advantage of fullerene \( (C_{60}) \) is related to its photoexcitation properties. In response to light irradiation, fullerene generates singlet oxygen \( \text{^1O}_2 \) or other reactive oxygen species; these by-products are highly cytotoxic. In regard to the use of water-soluble fullerenes for photodynamic therapeutic (PDT) applications, the groups of Yamakoshi, Tabata, Detrembleur, Jerome, and others have conducted pioneering work. Detrembleur and Jerome et al. synthesized a well-defined poly(vinyl alcohol)/fullerene nanohybrid through bulk cobalt-mediated radical polymerization. In addition, they grafted poly(vinyl acetate) and poly(N-vinylpyrrolidone)-co-poly(vinyl acetate) onto fullerene and determined that these types of nanohybrids are deprived of intrinsic cytotoxicity in the dark, whereas they induced cell death following light stimulation. Yamakoshi et al. prepared the first \( C_{60}-N\)-vinylpyrrolidone (NVP) copolymer, which is highly water-soluble; the confirmed biological activities of this copolymer include photoinduced \( \text{^1O}_2 \) generation and DNA cleavage. Tabata et al. synthesized Gd\textsuperscript{3+}-chelated \( C_{60}\)-polymethylene glycol (PEG) as a novel photosensitizer with both tumor targetability and magnetic resonance imaging activity. The singlet-oxygen-generating property of fullerenes renders them potential photosensitizers for use in PDT for cancers and other malignant diseases. However, the limited tumor selectivity of PDT agents has been the main drawback for clinical application, because nonspecific activation of singlet oxygen generation from photosensitizers is also induced in healthy, nonmalignant cells.

In regard to particle-based drugs, size and stability are important properties that govern their in vivo performances. Stable and smaller particle sizes (\(<200\) nm) can reduce uptake by the reticuloendothelial system (RES), thus increasing permeability and retention in the body. However, particles smaller than 100 nm can also cause cytotoxicity as well as nonspecific cellular uptake. Due to fullerene’s small size (around 1 nm), fullerene (or its aggregates, which are generally smaller than 100 nm)-based drugs can undergo nonspecific cellular uptake with or without targeting ligands. Thus, the potential of fullerene for clinical applications has not yet been fully realized.

Herein, we report a new multifunctional anticancer prodrg system based on water-dispersible fullerene aggregates; these agents feature active targeting and pH-responsive chemotherapeutic and PDT properties. There are several advantages of the multifunctional nanoparticle–drug platform. First, use of modified fullerene as the carrier, which has abundant sites for modification, allows the covalent incorporation of multiple functional elements into one system. This results in high drug loading (\(\approx 23\) wt% by absorption spectroscopy, and \(\approx 26\) wt% by high-performance liquid chromatography (HPLC)) as well as the ability to track intracellular drug release. Second, controlling the size of the water-dispersible fullerene aggregate to ensure that it is greater than 100 nm (135 nm), and then incorporating the anticancer drug (through a cleavable bond) and targeting ligand (folic acid) onto fullerene results in an anticancer prodrg with an active targeting effect. Third, this fullerene-based system also exhibits PDT properties; cytotoxicity towards folate receptor (FR)-positive cancer cells is further enhanced upon light exposure.

2. Results and Discussion

2.1. Synthesis of the Fullerene-Aggregate-Based Targeted Prodrug (DOX-Hydrazone-Fullerenol-Folic Acid)

DOX, a potent first-line cytotoxic chemotherapeutic agent, was selected as the anticancer drug agent in this study. For synthesis of the fullerene-aggregate-based targeted prodrg, succinic acid was first linked onto fullerenol, and the hydrophilic linker oligo(ethylene glycol) [EG3, namely triethylene glycol or tri(ethylene oxide)] was covalently incorporated onto the conjugates. DOX molecules were then conjugated onto the fullerene via the acid-sensitive carboxylic hydrazone, which is cleavable under acidic conditions. Moreover, an active targeting ligand (folic acid, FA) was also linked onto the fullerene through an amidation reaction. Large amounts of EG3 were incorporated onto fullerenes as linkers for several reasons. First, EG3 may minimize the nonspecific interaction between nanoparticles and biological molecules, since EG3 exhibited neutral and hydrophilic properties and did not bind nonspecifically to proteins, DNA, and RNA. This results in a prolonged circulation time. Second, the EG3 linkers can easily undergo chemical modification to covalently link ligand molecules; a short-chain ethylene glycol with a well-defined length is preferred for coating nanoparticles. Third, the large amounts of hydrophilic EG3 linkers, hydrazone groups, and amide groups on the surface of fullerene promotes strong intermolecular interactions (such as hydrogen bonding), so prodrg aggregates will readily form in water.

The average size of the aggregates was maintained at approximately 135 nm by optimizing the amounts of functional groups. It is well known that water-soluble fullerene derivatives tend to form aggregates in aqueous solutions. The prodrg (DOX-hydrazone-fullerenol-FA) developed herein formed dispersible aggregates in water with an average diameter of around 135 nm within a concentration range of 4–4000 \(\mu\)g mL\(^{-1}\) (Figure S1, Supporting Information). These data suggest that the prodrg aggregates are quite stable in water.

The fullerene-aggregate-based targeted prodrg (DOX-hydrazone-fullerenol-FA) and the control (DOX-hydrazone-fullerenol; prodrg without targeting ligand) were synthesized as outlined in Schemes S1 and S2 (Supporting Information). These compounds were characterized by \(^1\)H nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), and mass spectrometry (MS) (Figures S2–S7). A schematic illustration of the multifunctional anticancer system is shown in
Fullerene Aggregates as a Targeted Anticancer Prodrug

Figure 1. From the MS data, there are on average 21 hydroxyl groups on each fullerene, 20 succinic acid groups coupled onto one fullerenol, and 16 EG3-BOC-hydrozine (BOC = tert-butoxycarbonyl) chains on each C60-based conjugate. Based on estimation from HPLC data, there is an average of five DOX groups on each conjugate. Illustrative structures of the prodrug (DOX-hydrazone-fullerenol-FA) and the control (DOX-hydrazone-fullerenol) are provided in Scheme 1.

The loading capacity of the fullerene-aggregate-based targeted prodrug was assessed using absorption spectroscopy and HPLC. For the absorption spectroscopy method, DOX loading in the aggregates was determined by using the DOX molar extinction coefficient $11,500 \ \text{cm}^{-1} \ \text{M}^{-1}$ at $\lambda_{\text{max}} = 485 \ \text{nm}$. As shown in Figure S8, the aggregated fullerene and DOX overlap in the absorption spectrum; the net absorption of DOX can be obtained by deducting the absorption value of fullerene, and the loading of the DOX was calculated as 23.3 wt%. From the HPLC data, the drug loading was determined to be around 26 wt% (Figure S9). The high loading capacity was attributed to the high number of hydroxyl groups on fullerenol, which can be further modified for conjugation with DOX. The content of the targeting ligand FA was calculated as 2.33 wt% based on $^1$H NMR measurement.

In order for a drug-delivery device to achieve the desired benefits, it must be present in the bloodstream long enough to reach its therapeutic site of action. However, the opsonization or removal of nanoparticulate drug carriers from the body by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES), is a major therapeutic obstacle. The macrophages, which are typically Kupffer cells, cannot directly identify the nanoparticles themselves, but rather recognize specific opsonin proteins bound to the surface of the particles. The removal of nanoparticles from the body’s circulatory system by the MPS begins with the adsorption of plasma proteins (i.e., opsonins including complement proteins and immunoglobulins) on the particle surface. Many nanoparticle drug systems utilize surface treatments that interfere with the binding of opsonin proteins to the particle surface as a means of imparting stealth properties, or MPS-avoidance characteristics to nanoparticles.

Figure 1. Schematic illustration of the fullerene-based aggregate with dual anticancer actions. A) High-resolution transmission electron microscopy (HRTEM) image of a DOX-hydrazone-fullerenol-FA aggregate. B) Fluorescence microscopy image of HeLa cells in which DOX molecules have been released from their carriers.

Scheme 1. Structures of the prodrug (DOX-hydrazone-fullerenol-FA) and control (DOX-hydrazone-fullerenol). The five attached chains are for illustration purposes only.
for example, surface grafting of PEG or oligo(ethylene glycol).\[14] In this study, we incorporated EG3 linkers onto the particles to ensure that they could evade host defenses. We evaluated the nonspecific binding capacity of the synthesized prodrug nanoparticles using serum albumin (the most abundant blood protein). The total amount of bovine serum albumin (BSA) adsorbed on the prodrug (DOX-hydrazone-fullerenol-FA) is 21 μg mg⁻¹ in pH 7.4 phosphate-buffered saline (PBS) solution, whereas the amount of BSA adsorbed on the control prodrug (DOX-hydrazone-fullerenol) is 19 μg mg⁻¹ in pH 7.4 PBS buffer solution (Figure S10). These BSA adsorption values are comparable to those of some PEG (or OEG)-modified nanoparticles of similar diameters,\[14,15] thus indicating that the fullerene aggregates may be resistant to opsonization. Addition of EG3 linkers could promote the resistance of the prodrug to nonspecific protein binding.\[11]

In addition, we measured the zeta potential (Z-potential) of the prodrug. The Z-potential values were obtained as mean ± standard deviation (SD) based on triplicate independent experiments. The Z-potential of fullerol dispersion in pH 7.4 PBS buffer is −40 ± 1.5 mV, which is consistent with the literature,\[15] while that of fullerene-aggregate-based targeted prodrug (DOX-hydrazone-fullerenol-FA) in pH 7.4 PBS buffer is −29 ± 0.7 mV. The Z-potential of the control prodrug (DOX-hydrazone-fullerenol) is −33 ± 1 mV; this negative charge indicates that the prodrug nanoparticles are not electrophysiologically capable of binding to negatively charged proteins.

2.2. In Vitro Drug Release

The fullerene-based prodrug system is designed for endosomal/lysosomal release of DOX. Since the pH of blood and normal tissues is around 7.4, and the pH values of endosomal and lysosomal compartments are much lower (at pH 5.0–5.5),\[16] the pH-sensitive releasing property of the C60-based prodrug will facilitate drug delivery.

To verify the pH-sensitive release of DOX in vitro, DOX-hydrazone-fullerenol-FA samples were incubated at a simulated physiological condition (PBS, pH 7.4) and in an acidic environment (acetic acid buffer, pH 5.3 and 6.0) at 37°C. The supernatants were collected and examined. The DOX release amounts were determined by optical absorbance measurements of the supernatant solutions at 485 nm (Figure 2A). The rates and amounts of DOX released from the DOX-hydrazone-fullerenol-FA prodrug strongly depended on the pH of the medium. DOX-hydrazone-fullerenol-FA showed a much faster DOX release at pH 5.3 and 6.0 than at pH 7.4. The release of DOX from the DOX-hydrazone-fullerenol-FA in an acidic environment was governed by the acid-cleavable characteristics of the carboxylic hydrazone linkage between the DOX molecules and the nanoparticles. The carboxylic hydrazone linkage can undergo hydrolysis under acidic conditions, thus increasing DOX release from the prodrug at pH 5.3 and 6.0.

In addition, the MS data (Figure S11) confirm that the released chemical is in fact DOX. Moreover, as the DOX molecules were conjugated onto fullerene, the fluorescence of DOX was quenched due to the strong electron-accepting capability of fullerene. DOX fluorescence was restored upon release from fullerene (Figure 2B and Figure S12), so this fluorescence quenching–restoring feature can be used as a tracker for intracellular DOX release.

The pH-dependent releasing behavior is highly desirable for achieving tumor-targeted DOX delivery for effective anticancer treatment.\[17] The very slow DOX release rate observed at pH 7.4, which mimics the physiological conditions of the bloodstream, ensures minimal DOX release during blood circulation. Following internalization, the prodrug will be localized to the acidic endosomal compartments where DOX can be cleaved from the nanoparticles. The DOX will then diffuse into the cytosol and later into the nucleus.\[18] Therefore, toxic side effects are greatly reduced because only tumor cells will be affected by the drug.

2.3. Cytotoxicity and FR-Mediated Cellular Uptake of Nanoparticles

The in vitro cytotoxicity of hydrazine-fullerenol-FA against HeLa, L929, and A549 cells was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. Figure 3 shows the cytotoxicity of hydrazine-fullerenol-FA nanoparticles at different concentrations (0, 6, 12, 18, 24, and 30 μg mL⁻¹). The hydrazine-fullerenol-FA nanoparticles have no cytotoxic effect on cell viability, thus indicating that the carrier itself exhibits no cytotoxicity.

To confirm the selective targeting ability of the fullerene-based prodrug with FA as the targeting ligand against FRs on the cells, we investigated cellular uptake of the prodrug system by FR-positive and FR-negative cell lines: FR-positive HeLa, FR-negative A549, and FR-negative L929 cells (Figure 4 and Figure S13). Figure 5 shows, after 2 h of incubation in FA-free medium, few DOX-hydrazone-fullerenol nanoparticles were internalized by the three cell lines as evidenced by the weak intracellular fluorescence. After 2 h of incubation with the DOX-hydrazone-fullerenol-FA nanoparticles, the FR-positive
HeLa cells exhibited much brighter fluorescence than the two FR-negative cells (L929 and A549). Therefore, more DOX-hydrazone-fullerenol-FA nanoparticles were internalized by FR-positive cells than by FR-negative cells. These results demonstrate that the DOX-hydrazone-fullerenol-FA nanoparticles (diameter of around 130 nm) do not exhibit obvious nonspecific cellular uptake, and the cellular uptake of these nanoparticles by FR-positive cells can be increased by cell surface FA expression. The DOX-hydrazone-fullerenol-FA nanoparticles were transported into the HeLa cells through a FA-receptor-mediated endocytic process. Moreover, the uptake of DOX-hydrazone-fullerenol-FA by HeLa cells after 2 h of incubation in FA-containing and FA-free culture medium provides additional evidence for the selective targeting ability of the prodrug system (Figure S14). HeLa cells exposed to DOX-hydrazone-fullerenol-FA nanoparticles in FA-free RPMI 1640 culture medium showed much brighter fluorescence than that observed for the normal RPMI 1640 medium (FA-containing medium). Therefore, the cellular uptake extent of DOX-hydrazone-fullerenol-FA nanoparticles in FA-free medium was significantly higher than that in FA-containing medium. These data suggest that free FA present in the medium may inhibit the binding between the FA-conjugated nanoparticles and FRs on the HeLa cells through competitive inhibition. These data support the notion that more efficient uptake by FR-positive cells can be achieved with conjugation of FA onto the nanoparticles.

In addition, flow cytometry analysis was performed to compare the endocytosis of DOX-hydrazone-fullerenol-FA nanoparticles with that of FA-free DOX-hydrazone-fullerenol nanoparticles using three cell lines: L929, A549, and HeLa. The results are shown in Figure 6. Cells without any DOX treatment were used as a negative control to control for nonspecific autofluorescence. For HeLa cells exposed to an equivalent DOX concentration and incubation time, the DOX-hydrazone-fullerenol-FA nanoparticles exhibited higher fluorescence intensity than DOX-hydrazone-fullerenol nanoparticles, which indicates that the amount of cellular uptake of DOX-hydrazone-fullerenol-FA nanoparticles was higher than that for DOX-hydrazone-fullerenol nanoparticles. In regard to the FR-negative cell lines (A549 and L929), the results indicate that the amount of cellular uptake of DOX-hydrazone-fullerenol-FA nanoparticles was close to that of DOX-hydrazone-fullerenol nanoparticles. These results provide further evidence that cellular uptake of fullerenol nanoparticles by FR-positive cells can be increased by attaching FA to the particle surface. In addition, the DOX-hydrazone-fullerenol-FA nanoparticles were transported within cells via a FR-mediated endocytic process.

A fluorescence microscope was also used to monitor the intracellular fluorescence of HeLa cells after incubation with DOX-hydrazone-fullerenol-FA nanoparticles for increasing time periods (Figure 7). With increasing incubation time, the intracellular fluorescence intensity increased, and after 6 h of incubation the fluorescence was very intense. The fluorescent DOX molecules entered the nuclei upon 6 h of incubation (Figure 7), which indicates that some of the DOX molecules were cleaved from their carriers, because 130 nm DOX-hydrazone-fullerenol-FA nanoparticles are unlikely to translocate into nuclei.

The cytotoxicities of DOX-hydrazone-fullerenol-FA, DOX-hydrazone-fullerenol nanoparticles (aggregates), and free DOX were evaluated and compared using HeLa, L929, and A549 cell lines. The three cell lines were treated with free DOX, DOX-hydrazone-fullerenol-FA, or DOX-hydrazone-fullerenol nanoparticles, respectively, with an equivalent concentration of DOX for 48 h, and the cell viabilities were determined (Figure S15). For the FR-positive HeLa cells, DOX-hydrazone-fullerenol-FA
nanoparticles exhibit superior cytotoxicity as compared to DOX-hydrazone-fullerenol nanoparticles. There is no significant difference in terms of cell viability when the FR-negative L929 and A549 cell lines are exposed to DOX-hydrazone-fullerenol-FA and DOX-hydrazone-fullerenol nanoparticles (Figure 5B and C). These data support the view that the FA moieties in DOX-hydrazone-fullerenol-FA nanoparticles play an important role in enhancing the cytotoxic effect as they increase the binding to FR-expressing cells. This high-affinity binding subsequently increases their intracellular uptake as a result of receptor-mediated endocytosis. The FA molecules present on the surface of the nanoparticle prodrug do not have a remarkable effect on cellular uptake and/or cytotoxicity for FR-negative L929 and A549 cell lines. Figure 5 also indicates that free DOX demonstrated higher cytotoxicity against all three cell lines than the two fullerene-conjugated DOX materials. The lower cytotoxicity for the fullerene-based drugs has been previously reported by Bogdanovic and Rade; they
attributed the reduced cytotoxicity to the antioxidative activity of fullerenols. Perhaps, the antioxidative activity may help to reduce the cytotoxicity of DOX in the fullerene-based prodrug. The rate of drug uptake by different cell lines could also contribute to the difference in cytotoxicities. Free DOX is a small molecule, so it is easily internalized by the cell via the fast pinocytosis process. However, the nanoparticle-conjugated DOX with a diameter of approximately 130 nm can only be internalized via the slower endocytosis process. Thus, free DOX exhibited higher cytotoxicity towards all three cell lines in a time-dependent manner. In addition, endocytosis can be enhanced by a FR-mediated process; the DOX-hydrazone-fullerenol-FA nanoparticles may be quickly internalized by FA-positive HeLa cells and exhibit higher cytotoxicity than DOX-hydrazone-fullerenol.

Previously, Sun et al. prepared several fullerene–spacer–DOX conjugates. They found that the intrinsic properties of both DOX and fullerene were preserved following preparation. The introduction of hydrophilic ethylene glycol spacers between DOX and fullerene moieties improved the aqueous compatibility and structural flexibility of the conjugates. For this DOX-hydrazone-fullerenol-FA system, the hydrophilic oligo(ethylene glycol) also served as hydrophilic linker, thus resulting in a flexible C60-based prodrug with readily accessible DOX units. Moreover, using the 130 nm fullerene-based nanoparticles as carriers conjugated to FA promotes the internalization of the prodrug system into FR-positive cells rather than FR-negative cells, which results in reduced cytotoxicity to healthy, nonmalignant cells. Our strategy is a further improvement on the system proposed by Sun et al.

2.4. Singlet Oxygen Generation from DOX-Hydrazone-Fullerenol-FA Nanoparticles and Their PDT Effect

Fullerene is a well-known photosensitizer capable of producing reactive oxygen species in response to light illumination. In this study, we also made use of this photodynamic property to further enhance the cytotoxicity towards FR-positive targeted cells (HeLa). By using a chemical method involving the photooxidation of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), we verified the capability of generating singlet oxygen by the fullerene-aggregate-based targeted prodrug (DOX-hydrazone-fullerenol-FA), as shown in Figure S16. The photodynamic cytotoxic effect of DOX-hydrazone-fullerenol-FA and hydrazine-fullerenol-FA aggregates towards HeLa cells was evaluated using MTT assays (Figure 8). To rule out potential cytotoxicity solely induced by light irradiation, we irradiated HeLa cells in the absence of DOX-hydrazone-fullerenol-FA or hydrazine-fullerenol-FA nanoparticles (Figure S17). We determined that irradiation alone did not exhibit any significant cytotoxicity towards the cells. Figure 8A shows that the cells exposed to blue light (460–485 nm) exhibited prominent cytotoxicity in a dosage-dependent manner, thus indicating that the nanoparticles exhibit a photodynamic cytotoxic effect. Furthermore, cytotoxicity from a combination of chemotherapy and PDT was investigated. HeLa cells were treated with DOX-hydrazone-fullerenol-FA nanoparticles and exposed to light. As shown in Figure 8B, HeLa cells exhibited even lower cell viabilities upon 30 min of light irradiation in the presence of DOX-hydrazone-fullerenol-FA nanoparticles, as compared to those without light irradiation. These data indicate that combined chemotherapy and PDT promoted extensive cell death at various concentrations. For the DOX-hydrazone-fullerenol-FA nanoparticles at a DOX concentration of 8 μg mL⁻¹, the nanoparticles’ concentration is close to 30 μg mL⁻¹; hence the therapeutic actions of DOX-hydrazone-fullerenol-FA are additive (Figure 8). Thus, the fullerene-aggregate-based targeted system functions as a dual therapeutic (chemo and photodynamic) anticancer drug.

The PDT effects of water-soluble (dispersible) fullerenes have been previously reported. The current fullerene-aggregate-based targeted system exhibits a water solubility of 10 mg mL⁻¹, which is lower than that of the C60–NVP copolymer system (up to 240 mg mL⁻¹) created by Yamakoshi et al., who greatly improved the solubility of their systems by adjusting the C60/NVP ratio. In regard to the photoinduced cell cytotoxicity, the fullerene–PEG conjugates prepared by

![Figure 7](image-url) Fluorescence microscopy images for HeLa cells incubated with DOX-hydrazone-fullerenol-FA for increasing incubation times: A) 0, B) 1, C) 2, D) 6 h.
In summary, we have covalently incorporated DOX-based prodrugs as well as targeting ligands onto fullerene. The drug- and ligand-containing fullerene formed nanosized (~130 nm) aggregates in water. The aggregates released DOX in an acidic environment due to acid-mediated cleavage of the bond between the carrier and drug. With FA as the targeting ligand, aggregates could be preferably internalized by FA-positive cells; this feature makes the FA-conjugated fullerene-aggregate-based prodrug an ideal specific anticancer drug system. FR-positive cells (HeLa cells) are selectively killed by DOX over FR-negative cells. Moreover, the fullerene-aggregate-based carriers themselves exhibited a photodynamic effect upon light exposure, further enhancing the cytotoxicity towards HeLa cells. Compared with our previously reported prodrug system with silica nanoparticles as the carriers,[8] the current system not only exhibits photodynamic action and a much higher loading capability but also allows tracking of intracellular DOX release. This drug design approach provides useful insights for designing and improving the applicability of fullerene in other prodrug systems for targeted cancer therapy.

3. Conclusion

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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