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Gadolinium Metallofullerene-Based Activatable Contrast Agent for Tumor Signal Amplification and Monitoring of Drug Release

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Abstract

Activatable imaging probes are promising to achieve increased signal-to-noise ratio for accurate tumor diagnosis and treatment monitoring. Magnetic resonance imaging (MRI) is a non-invasive imaging technique with excellent anatomic spatial resolution and unlimited tissue penetration depth. However, most of the activatable MRI contrast agents suffer from the metal ion-associated potential long-term toxicity, which may limit their bioapplications and clinical translation. Herein, an activatable MRI agent with efficient MRI performance and high safety is developed for drug (doxorubicin) loading and tumor signal amplification. The agent is based on pH-responsive polymer and gadolinium metallofullerene (GMF). This GMF-based contrast agent shows high relaxivity and low risk of gadolinium ion release. At physiological pH, both GMF and drug molecules are encapsulated into the hydrophobic core of nanoparticles formed by the pH-responsive polymer and shielded from the aqueous environment, resulting in relatively low longitudinal relaxivity and slow drug release. However, in acidic tumor microenvironment, the hydrophobic-to-hydrophilic conversion of the pH-responsive polymer leads to amplified MR signal and rapid drug release simultaneously. These results suggest that the prepared activatable MRI contrast agent holds great promise for tumor detection and monitoring of drug release.

Abstract

An activatable magnetic resonance imaging (MRI) contrast agent, which based on gadolinium metallofullerene and pH-responsive polymer, is developed for tumor detection and drug delivery. Once the agent reaches tumor area, the pH-responsive polymer allows activation of MR contrast and drug release (ON state). Therefore, the agent can be used for tumor signal-amplification and monitoring of drug release.

Keywords

gadolinium metallofullerene; magnetic resonance imaging; activatable agent; signal-amplification; drug release

1. Introduction

Activatable imaging probes, which can respond to certain stimuli and achieve different signal intensities, have attracted more and more attention.^[1] Compared to conventional “always ON” probes, one of the main advantages of activatable probes is the increased signal-to-noise ratio, making the feature of target easier to distinguish.^[2] In recent years, several types of activatable probes have been reported for biomedical applications. For instance, tumor microenvironment-responsive nanoprobe could amplify their signals in tumor tissues and thus differentiate tumors from healthy tissues, achieving accurate tumor detection with improved sensitivity and specificity.^[3] Therefore, the development of activatable tumor imaging probes is of great benefit to early diagnosis of cancer, appropriate disease management and achievement of personalized medicine. Beyond tumor diagnosis, activatable probes can also potentially be used to monitor certain process (e.g. drug delivery process) by establishing a connection between their signal and behavior.^[4]

In the past few decades, various biomedical imaging techniques have been used for tumor diagnosis.^[5] Among these techniques, magnetic resonance imaging (MRI) is a non-invasive

imaging technique with excellent anatomic spatial resolution and unlimited tissue penetration depth.^[6] Through the use of contrast agents, the specificity and sensitivity of MRI could be dramatically improved.^[7] Recently, activatable MRI contrast agents, which could rapidly detect solid tumors and even small metastatic tumors, have been developed and used for tumor-specific MRI.^[8] However, these activatable contrast agents are mainly based on gadolinium (Gd)-chelator or manganese ion (Mn^{2+}); thus the metal ion-associated potential long-term toxicity of these agents may limit their bioapplications and clinical translation.^[9] Therefore, it's highly desirable to develop activatable MRI contrast agents with high safety.

Gadolinium metallofullerene (GMF) is a type of promising MRI contrast agent with ultra high relaxivity.^[10] Furthermore, GMF has much lower risk of toxicity than Gd-chelator based agents because toxic Gd^{3+} ions are confined inside the fullerene cages.^[11] Herein, we developed a pH-responsive GMF-based activatable MRI contrast agent (denoted as RNP) for tumor signal-amplification and monitoring of drug release (Figure 1). In this system, GMF, as well as a hydrophobic antitumor drug doxorubicin (DOX), were encapsulated into the pH-responsive polymer nanoparticles, obtaining DOX-RNPs. In physiological environment (pH 7.4), both the GMF and DOX are trapped inside the hydrophobic core and shielded from the aqueous environment; therefore, the longitudinal relaxivity and drug release rate of DOX-RNPs are relatively low (OFF state). However, once the DOX-RNPs reach tumor area, the tumor microenvironment-induced hydrophobic-to-hydrophilic conversion of the pH-responsive polymer allows activation of MR contrast and drug release (ON state). The DOX-RNPs exhibit the following advantages: i) the GMF-based contrast agent allows high safety; ii) the pH-activated tumor MR signal-amplification permits accurate tumor detection; iii) the pH-triggered on-demand drug release realizes effective cancer chemotherapy; (iv) the connection between MR signal and drug release behavior achieves monitoring of drug release. Therefore, the as-prepared DOX-RNP is a promising agent for cancer theranostics.

2. Results and Discussion

2.1. Preparation of DOX-RNPs

To prepare the pH-responsive polymer, a PEG-based macro-RAFT agent was first synthesized according to a previous report.^[12] Then the poly(ethylene glycol)-poly (2-(diisopropylamino)ethyl methacrylate)- (2-(diethylamino)ethyl methacrylate)- methacrylic acid N-hydroxysuccinimide ester (PEG-PDPA-DEA-NHS) was synthesized *via* reversible addition fragmentation chain transfer (RAFT) polymerization (Scheme S1, Supporting Information). The non-responsive polymer poly(ethylene glycol)-poly (2-ethylhexyl methacrylate) methacrylic acid N-hydroxysuccinimide ester (PEG-PEHA-NHS) was also synthesized as a control group (Scheme S2, Supporting Information). Nuclear magnetic resonance results confirmed the chemical structures of synthesized polymers (Figures S1–S3, Supporting Information). Then amino-functionalized GMF, $\text{Gd}_3\text{N}@C_{80}\text{-NH}_2$, was prepared according to a previous report (Figure S4, Supporting Information).^[13] Then the pH-responsive polymer was conjugated to $\text{Gd}_3\text{N}@C_{80}\text{-NH}_2$ by the reaction between NHS groups and amino groups. After self-assembly and drug loading, the obtained DOX-RNPs were purified by ultrafiltration. As shown in Figure S5 (Supporting Information), the

characteristic peak of DOX at about 480 nm was observed in the absorption spectrum of DOX-RNPs, indicating successful DOX loading. The DOX loading efficiency was determined to be ~6% (Figure S6, Supporting Information). Subsequently, transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used to investigate the size and morphology of the DOX-RNPs. As shown in Figures 2a and 2b, the DOX-RNPs had a core-shell structure with the hydrodynamic diameter of 146.2 ± 14.9 nm. However, at pH 6.6, the pH-induced DOX-RNPs dissociation was observed, which was due to the protonation of pH-responsive PDPA-DEA segments (Figure S7, Supporting Information). Furthermore, as shown in Figure S8 (Supporting Information), the DOX-RNPs had good colloidal stability. The non-responsive control group (denoted as NRNPs) was also prepared under the same experimental conditions except that the pH-responsive polymer PEG-PDPA-DEA-NHS was replaced with non-responsive PEG-PEHA-NHS (Figure S9, Supporting Information).

2.2. *In Vitro* Relaxivity

To demonstrate the acidic environment-induced MR signal amplification of DOX-RNPs, the longitudinal relaxivity (r_1) changes of both GMF and DOX-RNPs in response to pH were monitored by using a 7 T MR scanner. As shown in Figure 2c, the GMF showed high r_1 values (more than $20 \text{ mM}^{-1} \text{ s}^{-1}$) at both pH 7.4 and pH 6.6. Compared to the commercial Gd-chelator agents (such as Gd-DTPA), the GMF exhibits much higher (~ 5 times) relaxivity, indicating its efficient MRI performance. However, for DOX-RNPs, the GMF was encapsulated inside a hydrophobic core at pH 7.4 and thus shielded from the aqueous environment, resulting in minimal water interaction. In acidic condition (pH 6.6) simulating intratumoral microenvironment, the hydrophobic PDPA-DEA chains would be protonated and converted to hydrophilic, permitting high water interaction. Thus the DOX-RNPs showed a remarkable increase of longitudinal r_1 relaxivity, augmented from $5.7 \text{ mM}^{-1} \text{ s}^{-1}$ at pH 7.4 to $17.8 \text{ mM}^{-1} \text{ s}^{-1}$ at pH 6.6. The high relaxivity and pH-responsive signal amplification property suggested that RNPs could be used as an activatable contrast agent. In contrast, the relaxivities of GMF and DOX-NRNPs showed no significant change at different pH (Figure 2c and Figure S10, Supporting Information).

2.3. *In Vitro* Drug Release

To investigate the pH-responsive drug release behaviors of DOX-RNPs and DOX-NRNPs, the samples were dialyzed against HEPES buffer (pH 7.4) and MES buffer (pH 6.6). At different time points, the amount of released DOX was calculated by UV-vis calibration curve. As shown in Figure 2d, for DOX-RNPs, only ~27% DOX release was observed at pH 7.4 after 24 h incubation. However, at pH 6.6, up to ~80% DOX release was achieved upon 24 h incubation. This pH-responsive drug release behavior of DOX-RNPs may be attributed to hydrophilic conversion of the pH-responsive polymer and increased solubility of protonated DOX in acidic environment. In contrast, for the non-responsive DOX-NRNPs, the drug release rates at different pH showed no significant difference (Figure S11, Supporting Information). These results demonstrated that both the relaxivity and drug release rate of the DOX-RNPs were related to the environment pH. At physiological pH, the DOX-RNPs was in stable “OFF” state with low relaxivity and slow drug release rate; while in acidic environment, the “ON” state of MRI indicates rapid drug release state. The good

correlation between relaxivity and drug release rate suggests that the DOX-RNP is a promising agent for monitoring drug release.

2.4. Cytotoxicity, Cellular Uptake and Antitumor Effect

The cell cytotoxicities of RNPs and NRNPs were then evaluated on HeLa cells by the methyl thiazolyl tetrazolium (MTT) assay. Both RNPs and NRNPs exhibited negligible toxicity to cells up to a tested concentration of 200 mg L^{-1} after 24 h of incubation (Figure S12, Supporting Information). The pH-responsive cellular uptake behaviors of free DOX hydrochloride and DOX-RNPs were also evaluated on HeLa cells by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM) analysis. As shown in Figure 3, after incubation with free DOX hydrochloride, cells showed high DOX fluorescence intensity inside nucleus at both pH 7.4 and pH 6.6, indicating rapid cellular uptake of small molecule free DOX hydrochloride. However, for the cells incubated with DOX-RNPs, different drug distributions were observed in different conditions. At pH 6.6, higher DOX fluorescence intensity was observed inside cell nucleus when compared to that at pH 7.4, which may be caused by the pH-triggered drug release behavior of DOX-RNPs. At pH 6.6, the released small molecule DOX can enter cells and nucleus rapidly. In contrast, low fluorescence intensities were observed in cells incubated with non-responsive DOX-NRNPs at both conditions of pH 7.4 and pH 6.6.

To further confirm the pH responsiveness of DOX-RNPs, the *in vitro* antitumor activities of different nanomedicines against HeLa cells were investigated. The cells were incubated with samples at different pH for 1 h and then fresh medium for additional 23 h. Afterwards, MTT assay was used to measure the relative cell viabilities of different groups. As shown in Figure 4a, free DOX hydrochloride showed high antitumor activities at both pH values due to rapid cellular internalization. For DOX-RNPs, higher antitumor activity was observed at pH 6.6 when compared to that at pH 7.4. The increased antitumor activity in acidic condition may be due to the pH-induced drug release and subsequent enhanced cellular internalization of drug. Compared to DOX-RNPs, the DOX-NRNPs showed low antitumor activity even at pH 6.6 due to the inefficient drug release (Figure 4b).

2.5. *In Vivo* tumor uptake and MR Imaging

The tumor accumulation and biodistribution of RNPs were evaluated by positron emission tomography (PET) imaging. The PEG-DFO-PDPA was synthesized (Scheme S3, Supporting Information) and then encapsulated into RNPs for radionuclide ^{89}Zr labeling. Then the ^{89}Zr -RNPs were intravenously injected into HeLa tumor-bearing nude mice. After injection, an obvious tumor-to-background contrast was observed, indicating tumor uptake of the RNPs (Figure 5a). As shown in Figure 5b, at 24 h post-injection, the tumor uptake efficiency of ^{89}Zr -RNPs reached 4.79 percent of injected dose per gram of tissue (% ID g^{-1}). At 48 h post-injection, biodistribution study was performed by quantification of radioactivity (Figure 5c). The results demonstrated that the RNPs could accumulate into tumor tissue through EPR effect.

Then T_1 -weighted MRI was performed to evaluate the activatable MR imaging property of DOX-RNPs. The non-responsive DOX-NRNPs served as a negative control. The acquired

T_1 -weighted MR images of HeLa tumor-bearing mice are shown in Figure 6a and 6c. As shown in the MR images, the signal intensities of tumor areas before injection of contrast agents were relatively low. However, after the injection of contrast agents, the signal intensities at tumor sites gradually increased over time due to tumor accumulation of the contrast agents. As shown in Figure 6b, an obvious MR contrast enhancement in tumor was achieved at 24 h post-injection of DOX-RNPs. The calculated signal-to-noise ratio ($\text{SNR}_{\text{post}}/\text{SNR}_{\text{pre}}$) was ~ 1.8 , which was much higher than that of non-responsive control group (Figure 6d). Due to the similar particle size and surface modification, the DOX-NRNPs are expected to have similar *in vivo* behaviors to DOX-RNPs. Therefore, the higher $\text{SNR}_{\text{post}}/\text{SNR}_{\text{pre}}$ value of DOX-RNPs treated group indicated the pH-activated signal-amplification capacity of the DOX-RNPs. All of these results suggested that the DOX-RNPs can be used as an activatable MR contrast agent for signal amplification of tumor area.

2.6. In Vivo Chemotherapy

The *in vivo* antitumor effect was further investigated on the HeLa tumor-bearing mice. Mice were treated with free DOX or DOX-loaded nanoparticles every 3 days for 2 times (dose of DOX equivalents: 5 mg kg^{-1}). As shown in Figure 7a and 7b, effective inhibition of tumor growth was observed in the DOX-RNPs treated group, which could be attributed to the effective DOX-RNPs accumulation at the tumor site and subsequent pH-triggered drug release. Mice treated with DOX-RNPs did not show obvious weight loss, indicating no systemic toxicity was caused by DOX-RNPs (Figure 7c). Furthermore, no noticeable histological changes were observed in major organs of mice treated with RNPs and NRNPs, demonstrating good biocompatibility of the agents (Figure 8).

3. Conclusion

In summary, an activatable MRI agent based on pH-responsive polymer modified GMF nanoparticles (RNPs) was developed for tumor signal amplification and monitoring of drug release. At physiological pH (7.4), both GMF and DOX were encapsulated into the hydrophobic core of RNPs and shielded from the aqueous environment; therefore, the DOX-RNPs showed relative low longitudinal relativity and slow drug release. However, in acidic tumor microenvironment, the hydrophobic-to-hydrophilic conversion of the pH-responsive polymer allows efficient water interaction, resulting in amplified MR signal and rapid drug release. The *in vitro* and *in vivo* results demonstrated effective tumor accumulation, activatable MR imaging capacity and effective antitumor effect of the DOX-RNPs.

4. Experimental Section

Preparation and Characterization of DOX-RNPs and DOX-NRNPs:

The DOX-RNPs was prepared as follows: PEG-PDPA-DEA-NHS (20 mg) THF solution (300 μL) was added into $\text{Gd}_3\text{N@C}_{80}\text{-NH}_2$ DMF solution (700 μL). After stirring for 3 h at room temperature, DOX (3 mg) was added into the above mixture solution. Then the solution was added into distilled water (4 mL) dropwise under stirring. Unencapsulated DOX and organic solvents were removed through ultrafiltration. The DOX-NRNPs was prepared under the same experimental conditions except that the PEG-PDPA-DEA-NHS was

replaced with PEG-PEHA-NHS. The longitudinal relaxivities were measured by an MRI system (7 T, Bruker, Germany).

In Vitro Drug Release:

The amount of DOX was determined by measurement of absorbance at 480 nm by using an UV-Vis spectrophotometer. The loading efficiency (LE) of DOX was calculated by the following equation: $LE = (\text{initial amount of feeding drugs} - \text{free drugs}) / \text{amount of DOX-loaded NPs}$. The *in vitro* drug release behaviors of the samples in different conditions were evaluated at 37 °C with shaking. DOX-loaded samples (2 mL) were added to dialysis bags (MWCO: 12000 Da) and then the bags were placed in 10 mL of different environmental media (pH 7.4 or 6.6). At specific time points, 5 mL of the medium was taken out for measurement of release drug amount. Afterwards, same amount of fresh medium was added to the environmental medium.

In Vitro Cell Experiments:

The *in vitro* cell cytotoxicity, cellular uptake study and antitumor activities of samples were assessed on HeLa cells. To evaluate the cellular uptake of samples at different pH, HeLa cells were incubated with free DOX, DOX-RNPs and DOX-NRNPs (DOX concentration: 5 $\mu\text{g mL}^{-1}$) at pH 7.4 or 6.6 for 1 h. Then the cells were investigated by CLSM. To assess the cytotoxicity and *in vitro* antitumor activities of samples, cells were seeded into 96-well plates at a density of 3×10^3 cells per well. For cytotoxicity evaluation, cells were incubated with samples for 24 h. For antitumor activity evaluation, cells were incubated with free DOX, DOX-RNPs and DOX-NRNPs at different pH for 1 h; after that, the samples were removed and the cells were incubated with fresh culture medium for an additional 23 h. After the incubation, MTT assay was used to evaluate the relative cell viabilities.

In Vivo PET Imaging and MR Imaging:

All animal experiments were performed under a protocol approved by National Institutes of Health Animal Care and Use Committee (NIHACUC). PEG-DFO-PDPA was synthesized (Scheme S3) for ^{89}Zr labeling. Then HeLa tumor-bearing mice were treated with ^{89}Zr -RNPs solution (100 μL , 140 μCi) by intravenous injection. At specific time points, the mice were scanned by using an Inveon small-animal PET scanner (Siemens, Erlangen, Germany). All mice were sacrificed at 48 h post-injection for biodistribution study. The radioactivities of major organs were assayed by using a gamma counter. To assess the activatable MR capacity, samples (150 μL , Gd concentration: $0.5 \times 10^{-3} \text{ M}$) were intravenously injected into HeLa tumor-bearing mice. MR images were acquired on a high magnetic field micro-MR scanner (7.0 T, Bruker, Pharmascan) before and after injections. Signal intensities were measured in defined regions of interest with Image J software.

In vivo Therapy:

When the tumor volume reached 60 mm^3 , the mice were randomly divided into 4 groups ($n = 5$) and treated with saline, free DOX, DOX-RNPs and DOX-NRNPs (dose of DOX equivalents: 5 mg kg^{-1}) *via* intravenous injection every 3 days for 2 times. Tumor sizes and

mice body weights were measured every three days. Tumor volume was calculated by $V = (\text{major axis}) \times (\text{minor axis})^2/2$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic illustration of DOX-RNPs for tumor signal-amplification and monitoring of drug release.

Figure 2.

(a) TEM image of DOX-RNPs. (b) Particle diameter of DOX-RNPs. (c) The r_1 relaxivities and T_1 -weighted MR images of GMF and DOX-RNPs at different pH. (d) *In vitro* drug release behaviors of DOX-RNPs at different pH values.

Figure 3. CLSM images and FCM analyses of HeLa cells incubated with different samples at different pH. The DOX concentration was $5 \mu\text{g mL}^{-1}$.

Figure 4.
The cytotoxicities of free DOX, DOX-RNPs (a) and DOX-NRNPs (b) against HeLa cells at different pH.

Figure 5.

(a) Representative PET images of mice intravenously injected with ^{89}Zr -RNPs. (b) Quantitative volume-of-interest analysis of tumor area at different time points. (c) Biodistribution study performed at 48 h post-injection.

Figure 6. Representative MR images and tumor SNR changes of mice after intravenous injection of DOX-RNPs (a, b) or DOX-NRNPs (c, d).

Figure 7.

(a) Relative tumor volume of the mice after different treatments. (b) The relative tumor weights at the end of treatments. (c) Body weight changes of the mice.

Figure 8.
Hematoxylin and eosin (H&E) staining of major organs. Scale bar is 200 μ m.