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Modular Polymer-Caged Nanobins as a Theranostic Platform with Enhanced Magnetic Resonance Relaxivity and pH-Responsive Drug-Release**

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Abstract

'Clickable' polymer-caged nanobins enable the combination of Gd^{III} MRI contrast agents and an anticancer drug (gemcitabine, GMC) into a single theranostic platform. The resulting Gd^{III}-conjugated, GMC-loaded PCNs (Gd^{III}-PCN_{GMC}) exhibit significant enhancements in r_1 relaxivity, drug uptake, and pH-sensitive drug release.

Keywords

theranostic; gadolinium; gemcitabine; magnetic resonance imaging; drug-delivery

Recent advances in nanoscience have spurred new developments in the field of theranostics (the combination of both therapeutic and diagnostic functionalities in a single system).^[1] These nanoscale platforms possess improved pharmacokinetic profiles and targeting ability for specific diseases.^[2, 3] Additionally, these integrated systems have been shown to selectively deliver therapeutic agents to target tissues while simultaneously monitoring biological responses to the therapy, providing important feedbacks in the treatment of highly heterogeneous diseases, particularly cancer.^[4, 5] Such concurrent evaluation of tumor response can be of crucial importance in clinical cancer therapy: treatments could be more successful if the prescribed regimens can be adjusted accordingly from the timely feedback information.^[6]

Since magnetic resonance imaging (MRI) can provide detailed high-resolution, tomographic information of disease tissue in real time and *in vivo*, it has become a powerful diagnostic tool for staging primary and recurrent solid tumors and for assessing suitable treatment regimens. Therefore, MRI is a good technique to be incorporated into theranostic platforms for the post-treatment evaluation of solid tumors.

MRI diagnoses are often supplemented with paramagnetic Gd^{III} complexes, which enhance the signal intensity by reducing the longitudinal relaxation time (T_1) of water protons nearby

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the Gd^{III} center.^[7] Despite the biomedical potential of MRI, clinically available Gd^{III} contrast agents are hampered by their intrinsic low efficiency, resulting in a need to administer high doses of contrast agents.^[7] Conjugating a large number of Gd^{III} ions onto nanoscale structures, such as gold^[8, 9] and titanium oxide^[10] nanoparticles, lipid nanoparticles,^[11] and viral capsids,^[12] has led to enhanced MR relaxivity. However, many of these systems do not have effective drug-loading capabilities, which limit their potential as theranostic platforms.

We have demonstrated polymer-caged nanobins (PCNs) as a nanoscale delivery platform^[13] that can be surface-modified with targeting groups by ‘click’ chemistry^[14] in a facile manner. This platform is constructed based on a liposomal template which allows for the encapsulation of a high dose of small-molecule drugs via an ion gradient-mediated (IGM) drug loading process.^[15] Since the polymer shell of PCN contains many ‘clickable’ terminal alkyne groups on the surface, azide-modified Gd^{III} complexes can be easily conjugated to drug-loaded PCNs, resulting in highly effective theranostic agents. The immobilization of Gd^{III} complexes on the surface of the PCN results in enhanced per-Gd^{III} relaxivity due to an increase in rotational correlation time (τ_R).^[16, 17] Additionally, conjugating a large number of Gd^{III} complexes onto a single PCN leads to a high local concentration of contrast agents and significantly enhanced per-particle relaxivity compared to small-molecule Gd^{III} agents.^[9]

Herein, we demonstrate drug-loaded, Gd^{III}-conjugated PCNs as a versatile theranostic platform with excellent drug uptake, high Gd^{III}-loading, and enhanced MR relaxivity (both per-Gd^{III} and per-particle). The IGM drug-loading capability of the PCN system was expanded in this study to include gemcitabine (GMC, Gemzar[™] Lilly, Greenfield, IN), a nucleoside analogue for an antimetabolite of deoxycytidine.^[18] Clinically, GMC has been used as a first-line chemotherapeutic agent for non-small-cell lung cancer,^[19] pancreatic cancer,^[20] metastatic breast cancer, and recurrent ovarian cancer.^[21] Despite the clinical success of GMC, its short plasma half-life (9–13 min for human)^[22] and adverse toxicity such as myelosuppression greatly limit its chemotherapeutic efficacy.^[19] By encapsulating GMC in PCN, the therapeutic index of GMC can be greatly improved due to the protection of the drug from renal clearance and prolonged circulation half-life.^[23]

In addition, the well-known enhanced permeation and retention (EPR) effect^[24] will allow for preferential accumulation of the PCN-packaged drug in solid tumor tissues, facilitating local drug uptake as well as release of drugs in the acidic environment of the tumor.^[25] Indeed, our Gd^{III}-conjugated, GMC-loaded PCNs (Gd^{III}-PCN_{GMC}) exhibit significant enhancement in r_1 relaxivity and pH-sensitive drug-release properties as a proof-of-concept theranostic platform (Scheme 1).

The mild basicity of GMC and its moderate lipophilicity^[26] enable this drug to be remotely loaded into the core of a liposome using the IGM drug-loading strategy. Therefore, GMC was added to a solution of bare liposomes whose core contains ammonium sulfate (300 mM solution) and incubated at 50 °C for 24 h^[27] before the unloaded GMC molecules were removed by gel filtration. The resulting GMC-loaded bare liposomes {BL_{GMC}, GMC/lipid = 0.25 mol/mol, GMC concentrations were measured by UV-vis spectroscopy (Figure S1 in Supporting Information (SI)) and lipid concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES)} was then modified with cholesterol-terminated poly(acrylic acid) ($M_n \sim 5,100$ Da) followed by *in situ* cross-linking with alkyne-functionalized diamine linkers^[13] to prepare GMC-loaded ‘clickable’ PCNs (PCN_{GMC}) with 50% of the carboxyl groups of Chol-PAA cross-linked.

The diameter of the resulting PCN_{GMC} was 108 ± 6 nm as measured by dynamic light scattering (DLS, Figure S2 in SI) and transmission electron microscopy (TEM, Figure S3 in SI) with a zeta potential (ζ) of -15.02 ± 0.86 mV. As a large number of terminal alkyne groups are present on the surface of PCN particles ($\sim 150,000$ /particle, 25% of the total number of carboxyl groups of the Chol-PAA), Gd^{III}-conjugated PCN_{GMC} with several Gd^{III}-to-lipid ratios were prepared by Cu^I-catalyzed 'click' ligation^[14] with [10-(3-azido-2-hydroxypropyl)-1,4,7-tris(acetic acid) tetraazacyclododecane]gadolinium(III) (HPN₃-DO3A-Gd^{III}). After purification by gel filtration, Gd^{III}-PCN_{GMC} with final Gd^{III}-to-lipid ratios ranging between 0.032 and 0.45 were obtained (determined by ICP-OES, Table 1).

The aforementioned flexible click-based strategy allows for very high loading of Gd^{III} complexes per PCN (up to 45,000 Gd^{III}/particle) without significantly changing the diameter of the PCN (see Figure S2 in SI). As described above, due to the large number of available terminal alkynes, the loading of Gd^{III} complex can be increased further with higher percentages of cross-links or with the use of cross-linkers possessing multiple free alkynes.

To evaluate the relaxivity of each Gd^{III}-PCN_{GMC}, T_1 relaxation times were measured on a Bruker Minispec relaxometer at 37 °C and 60 MHz (1.41 T, which is comparable to the magnetic field strength of clinically used MR scanners).^[30] The relaxivity (r_1) was calculated as the slope of a linear fit of $1/T_1$ (in s⁻¹) versus mM Gd^{III} (Figures S4 and S5, Table S1 in SI). The relaxivity (r_1) was significantly increased with higher Gd^{III}-to-lipid ratios and plateaus at 0.45 with an approximately five-fold enhancement of r_1 (15.9 mM⁻¹s⁻¹) compared to DOTA-Gd^{III} (3.21 mM⁻¹s⁻¹)^[17] (Table 1).

Presumably, the enhanced relaxivity of Gd^{III}-PCNs is a result of a longer rotational correlation time (τ_R) due to anchoring of the Gd^{III} complexes on the polymer-cages, consistent with Solomon-Bloembergen-Morgan theory.^[31] Given that $\sim 10^5$ lipid molecules are present in a 100-nm liposome particle,^[28, 29] a single Gd^{III}-PCN particle with 0.45 Gd^{III}-to-lipid ratio can exhibit a relaxivity of 715,500 mM⁻¹s⁻¹ per particle at 60 MHz (220,500 mM⁻¹s⁻¹/particle at 300 MHz, Table 1). T_1 -weighted MR images of Gd^{III}-PCN_{GMC} in solution were acquired at 7 T and 25 °C, showing significant contrast enhancement with Gd^{III}-PCN platform (Figure S6 in SI).

Since drug delivery vehicles with negligible cargo-release profiles often have low therapeutic potencies in both *in vitro*^[32] and *in vivo*^[33] trials, triggered drug-release properties under specific conditions are an important criteria in the design of efficient therapeutic and theranostic platforms. Given the known acidity in tumor interstitium^[25] and cellular endosomes,^[34] acid-sensitivity has been shown to be a good trigger for drug release from delivery vehicles in cancer therapy. As PCNs possess acid-triggered cargo-release characteristics due to their pH-responsive polymer shells,^[35] the drug-release profiles of all samples of Gd^{III}-PCN_{GMC} were monitored at pH 5.0 and 37 °C, in comparison to those at physiological condition (pH 7.4, 37 °C). To this end, Gd^{III}-PCN_{GMC} was incubated in acetate (pH 5.0) or HEPES (pH 7.4) buffers (both contain 150 mM NaCl) and the drug-release kinetics were measured by comparing the GMC-to-lipid ratios in the remaining PCNs after purification by filtration. As expected, the release rates were clearly pH-dependent for Gd^{III}-PCN_{GMC} (Figure 1 and Table S2 in SI): $\sim 75\%$ of GMC was released from Gd^{III}-PCN_{GMC}4 during the initial 24 h and near-complete release was achieved by 48 h. In comparison, under neutral condition, $\sim 20\%$ and $\sim 60\%$ of GMC was released from Gd^{III}-PCN_{GMC}4 in 24 h and 48 h, respectively. This is similar to the release profile of BL_{GMC}, where no significant differences in release were observed under either pH 7.4 or 5.0 (Figure 1). This acid-sensitive drug-release property of Gd^{III}-PCN_{GMC} demonstrated its excellent potential as a cancer theranostic agent.

The *in vitro* therapeutic potency of Gd^{III}-PCN_{GMC} was evaluated using HeLa human cervical cancer cells (Figure 2 and Figure S7 in SI). Surprisingly, Gd^{III}-PCN_{GMC} exhibited significantly higher potency (IC₅₀ of 14.4 μ M) than free GMC (IC₅₀ of $\sim$ 100 μ M). This is attributed to the enhanced cellular uptake of the encapsulated drug, which can be internalized into the cell via endocytosis of the nanoscale delivery vehicles.^[36] In contrast, because GMC uptake is mostly mediated by human concentrative nucleoside transporters (NTs, transmembrane proteins that normally mediate the cellular uptake of nucleosides^[21]), such as hCNT1 and hCNT3, that are absent in HeLa cells,^[37, 38] its uptake into these cells can be relatively low. Indeed, GMC has been shown to have very limited potency in HeLa cell lines^[39] without the specific expression of target transporters.^[40] In this sense, PCN encapsulation provides a novel venue for small-molecule nucleoside-analogue drugs to confer cytotoxicity to cancer cells that lack the expression of specific target transporters. Once being uptaken into the cell via endocytosis, the pH-sensitive polymer cage of PCN can destabilize the endosomal membrane under acidic conditions, leading to the rupture of endosomal vesicles.^[41] Such polymer-mediated membrane perturbation can substantially facilitate the delivery of drug molecules from the endosomes into the cytosol, resulting in enhanced therapeutic efficacy.

Supporting the aforementioned hypothesis are quantification studies that show the enhanced Gd^{III} cellular uptake of Gd^{III}-PCNs over DOTA-Gd^{III}. To determine the cellular internalization efficacy, HeLa cells were incubated for 24 h in complete media containing either GMC-free Gd^{III}-PCNs or DOTA-Gd^{III} molecules. Cellular Gd^{III}-uptake was determined by ICP-MS after repeated (3 $\times$) washings with Dulbecco's modified phosphate-buffered saline (DPBS) followed by harvesting, centrifugation, supernatant removal and further cell pellet washing to remove any non-specifically bound Gd^{III}-PCN. Results show 30- to 70-fold higher Gd^{III} uptake for cells incubated with Gd^{III}-PCNs (0.45 Gd-to-lipid ratio) compared to cells treated with DOTA-Gd^{III} (Figure 3). This cellular uptake enhancement of Gd^{III}-PCNs was observed in dose- and time-dependent manners in both HeLa cells and non-cancerous NIH/3T3 mouse fibroblast cells (Figures S9 and S10 in SI), suggesting a typical endocytosis-internalization mechanism.^[42] The enhanced cellular uptake of Gd^{III}-PCNs, compared to the corresponding small-molecule agents, helps to explain the aforementioned enhanced potency of Gd^{III}-PCN_{GMC}.

In sharp contrast to Gd^{III}-PCN_{GMC}, drug-free Gd^{III}-PCNs show negligible cytotoxicity (Figures S11 and S12 in SI) even with their enhanced cellular uptake. Therefore, conjugating Gd^{III} complexes to PCNs provides not only a theranostic platform but a highly efficient cellular-labeling agent that can be used for long-term cell tracking and lineage studies *in vivo*. The delivery and cellular contrast enhancement is an improvement over previously tested small-molecule contrast agents such as polyarginine due to the enhanced cellular uptake and minimal non-specific binding of PCNs.^[43]

The enhanced cellular internalization of Gd^{III}-PCNs via endocytosis suggests their potential as non-invasive cell-tracking agents for MRI.^[44] Indeed, the high uptake of Gd^{III}-PCNs was clearly reflected in the significant contrast enhancement observed in the *T*₁-weighted MR images of NIH/3T3 cells exposed to Gd^{III}-PCNs compared to those of cells exposed to DOTA-Gd^{III} (Figure 4, see also Table S3 in SI). Such enhanced contrast was also observed in the *T*₁-weighted MR images of HeLa cells (Figure S13 and Table S4 in SI). Together, with the improved therapeutic efficacy observed for Gd^{III}-PCN_{GMC}, these results demonstrate the promising potential of the Gd^{III}-PCN_{DRUG} platform as a cell-permeable theranostic agent.

In conclusion, we have demonstrated the versatility of new 'clickable' polymer-caged nanobins for the simultaneous incorporation of high doses of Gd^{III}-based MRI contrast

agents and an anticancer drug, gemcitabine, in a single delivery theranostic platform. The surface-conjugation of a high density of Gd^{III} complexes onto the PCN via ‘click’ ligation significantly enhanced the per-Gd^{III} MR-relaxivity, resulting in very high per-particle relaxivity.

Notably, the ‘nano-packaging’ of gemcitabine inside the PCN circumvents the membrane transporter-specific cellular-uptake pathway, which often limits the therapeutic effectiveness of nucleoside analogue drugs, making the combined Gd^{III}-PCN_{GMC} materials a much better theranostic agent than either of its components. Our ‘clickable’ PCN strategy is highly specific for tuning of the Gd^{III}/GMC loading ratio (0.128 to 1.8). This allows for the specific matching of the proportions of active drugs and MR contrast agents that can remedy the detrimental sensitivity and concentration mismatches between therapeutics and diagnostics in combined theranostic platforms.^[1]

Beyond the enhanced r_1 relaxivity reported herein, the most obvious benefit from attaching an imaging agent to a nanoscale drug delivery agent like our PCN platform is the ability to verify if the drug delivery agent has reached its targeted diseased organs or tissues. Several other advantages are also apparent: first, if an MRI contrast agent can be co-delivered with a drug in a single nanoscale package that can target to a specific diseased site, a broader range of organs and tissues can be imaged beyond the current limited group of vasculature network, liver, and kidney.^[45] Second, because nanoparticle delivery is quite specific in cancer chemotherapy, whether through the EPR effect or active targeting, a much smaller amount of the imaging agent is needed, lessening the material cost and any potential side effects due to the imaging agent. A third advantage of a theranostic platform such as our PCN is the macroscopic nature of the Gd^{III}-attached polymer cage, which should result in an increased residence time for the Gd^{III} MRI contrast agent at the diseased site, allowing for long-term repeated imaging to evaluate the benefits of the treatment. In such a manner, the prescribed regimen can be adjusted in a timely fashion from ongoing feedback information, allowing for more successful treatment.

Experimental Section

The preparation of PCNs and other experimental procedures are available in the Electronic Supporting Information (ESI).

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Figure 1. Time-dependent GMC-releasing profiles of bare liposome (BL_{GMC}, open symbols) or Gd^{III}-PCN_{GMC} (0.45 Gd^{III}-to-lipid ratio, closed symbols) under either pH 5.0 or 7.4 and at 37 °C. The linear release profiles observed for BL_{GMC} are attributed to the moderate lipophilicity of GMC.

Figure 2. Plots of dose-responsive cell viability of HeLa cells exposed to free GMC and Gd^{III}-PCN_{GMC} (0.45 Gd^{III}-to-lipid ratio) after a 72-h incubation.

Figure 3. Concentration-dependent, cellular-uptake profile of Gd^{III}-PCNs (0.45 Gd^{III}-to-lipid ratio) compared to DOTA-Gd^{III} by HeLa human cervical cancer cells. Cells were incubated in complete media containing Gd^{III}-PCN or DOTA-Gd^{III} at given Gd^{III} concentrations for 24 h at 37 °C.

Figure 4.

(A) T_1 -weighted MR image of NIH/3T3 mouse fibroblast cells incubated with solutions of Gd^{III}-PCNs (10 μ M and 40 μ M Gd^{III} concentrations, Gd^{III}-to-lipid ratio = 0.39) and DOTA-Gd^{III} (10 μ M and 40 μ M Gd^{III} concentrations) for 24 h at 7 T (300 MHz) and 25 °C (TR/TE = 500/11 ms). (B) The corresponding image-intensity color map for panel A where maximum and minimum image intensities are shown in the calibration bar on the right hand side. The scale bars at the bottom left corners in both images correspond to 1.0 mm.

Scheme 1.

Preparation of gemcitabine-loaded, Gd^{III} -conjugated polymer-caged nanobins ($Gd^{III}-PCN_{GMC}$) by 'click' ligation.

Table 1Relaxivities of Gd^{III}-PCNs of varying Gd^{III}-to-lipid ratios at 60 MHz and 37 °C in water.

Agents	Gd ^{III} /lipid ratio	Per-Gd ^{III} r_1 (mM ⁻¹ s ⁻¹)	No. of Gd ^{III} /particle ^a	Per-particle r_1 (mM ⁻¹ s ⁻¹)
DOTA-Gd ^{III}	-	3.21	-	-
Gd ^{III} -PCN _{GMC1}	0.032	11.3	3.2×10^3	36,160
Gd ^{III} -PCN _{GMC2}	0.11	14.5	1.1×10^4	165,300
Gd ^{III} -PCN _{GMC3}	0.24	15.0	2.4×10^4	352,500
Gd ^{III} -PCN _{GMC4}	0.45	15.9 (4.9) ^b	4.5×10^4	715,500 (220,500) ^b

^aThe number of lipid molecules per 100-nm liposome was assumed to be 10^5 based on Ref. [28, 29].^bRelaxivity measured at 300 MHz (7 T) and 25 °C in water.