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Multifunctional Cholesterol-modified Dendrimers for Targeted Drug Delivery to Cancer Cells Expressing Folate Receptors

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Electronic Supplementary Information

Abstract We present here the development of cholesterol (Chol)-modified dendrimer system for targeted chemotherapy of folate (FA) receptor-expressing cancer cells. In our study, poly(amidoamine) (PAMAM) dendrimers of generation 5 (G5) were functionalized step-by-step with Chol, fluorescein isothiocyanate (FI), and FA via a poly(ethylene glycol) (PEG) spacer (PEG-FA), and then acetamide to shield their remaining surface amines. The synthesized G5.NHAc-Chol-FI-PEG-FA (for short, G5-CFPF) dendrimers were utilized to encapsulate 10-hydroxycamptothecin (HCP), a hydrophobic anticancer drug. We find that each G5-CFPF dendrimer can encapsulate 13.8 HCP molecules. The complexes show a slower release profiles of HCP in a pH-dependent manner than the control complexes formed using the same dendrimers without Chol under the same conditions. Thanks to the targeting role played by FA, the complexes display a specific inhibition efficacy to FA receptor-expressing cervical cancer cells. The designed Chol-modified dendrimers may be adopted as a promising carrier for application in targeted cancer therapy.

Keywords PAMAM dendrimers; Folate; Cholesterol; 10-Hydroxycamptothecin; Targeted cancer therapy

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INTRODUCTION

Among various cancer treatments, chemotherapy has been considered as a common strategy in clinical settings. However, traditional chemotherapy faces a number of limitations, such as nonspecific distribution, systemic side effects, rapid clearance, and drug resistance, *etc.*^[1] With the development of nanomedicine, various drug nanocarriers have been introduced to enhance the anticancer efficacy and reduce side effects.^[2–5] The commonly developed nanocarriers, including liposomes,^[6,7] dendrimers,^[8,9] micro- or nanoparticles,^[10–13] nanocapsules,^[14,15] carbon nanotubes,^[16,17] inorganic nanoparticles,^[18–20] and polymers^[21,22] have been subjected to different functionalization in order to target tumors through either passive enhanced permeability and retention effect or active targeting after ligand modification to realize the minimized side effects and maximized thera-

peutic efficacy. Although some multifunctional nanocarriers for drug delivery system have been used, the development of a new platform with distinct advantages is still necessary.

Dendrimers have been widely used to develop targeted drug delivery systems because of their monodispersity, highly branched interior, and well-defined architecture/composition. In addition, owing to the abundant functional groups on their surface, dendrimers can be easily rendered with other functionalities (*e.g.*, targeting, antifouling, and fluorescence imaging, *etc.*),^[23–28] which is beneficial for construction of specific drug delivery systems. Furthermore, dendrimers with hydrophobic interior can be utilized for encapsulation of anticancer drugs with hydrophobicity, such as doxorubicin, which can significantly improve the water solubility and bioavailability of the drugs.^[29–32]

Potent drug delivery system usually requires an excellent antifouling property^[33–36] to make sure the system escapes from the uptake by reticuloendothelial system (RES) after intravenous injection. Modification of nanoparticles (NPs) with poly(ethylene glycol) (PEG) is a well-established strategy to render the NPs with prolonged half-decay life in blood and limited RES clearance.^[37,38] For instance, Zhang and co-workers discovered that generation 5 (G5) poly(ami-

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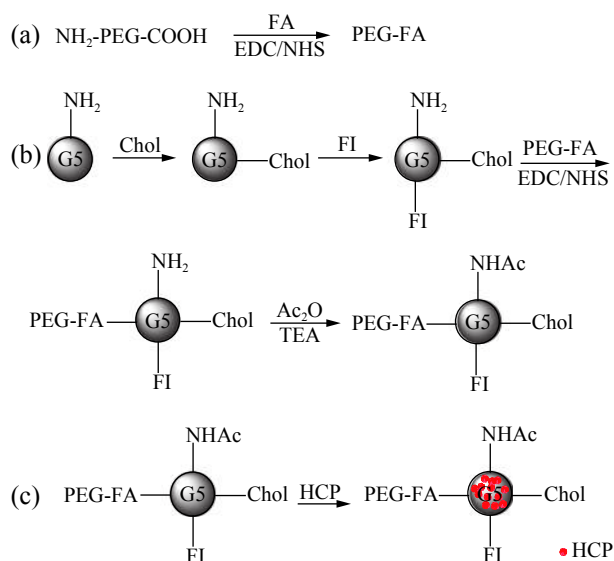
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doamine) (PAMAM) dendrimers modified with folate (FA) *via* a PEG spacer displayed more significantly enhanced specific tumor uptake than G5 dendrimers directly modified with FA.^[39] Recently, we demonstrated that G5 dendrimers modified with PEGylated lactic acid (LA) had a better targeting specificity to cancer cells expressing asialoglycoprotein receptors than the same dendrimers directly modified with LA.^[8] Hence, it is important to link the NP-based carriers *via* a PEG spacer for improved targeting specificity.

As is known, Chol is the key part of the cell membrane, which can regulate the level of lipid organization.^[40] Therefore, Chol has been widely used to improve the cell-penetrating ability for drug delivery systems.^[41,42] For instance, Chol-linked PEG-TAT peptide can self-assemble into biological core/shell nanoparticles, which can load ciprofloxacin (as a model antibiotic) efficiently for drug delivery across the blood-brain barrier.^[43] Chol modification is beneficial for Chol-modified up-conversion nanoparticles to across the blood-brain barrier and applied in photothermal/photodynamic therapies of brain glioblastoma.^[44] Therefore, the advantages of Chol may be utilized in a dendrimer-based drug delivery system.

In this work, G5 PAMAM dendrimers were modified with Chol, fluorescein isothiocyanate (FI) and PEG-FA, and the remaining dendrimer terminal amines were finally acetylated (Scheme 1). The multifunctional G5.NHAc-Chol-FI-PEG-FA (for short, G5-CFPF) dendrimers were loaded with anticancer drug 10-hydroxycamptothecin (HCP). We characterized the dendrimers and complexes systematically, and assessed the release kinetics, therapeutic efficacy, targeting specificity, and effect of targeted drug delivery of the G5-CFPF/HCP complexes. To the best of our knowledge, this could be the first example associated to Chol-modified dendrimer system for targeted drug delivery applications.



Scheme 1 Preparation of (a) FA-PEG-COOH segments, (b) G5-CFPF dendrimers, and (c) the G5-CFPF/HCP complexes. Ac₂O and TEA are acetic anhydride and triethylamine, respectively.

EXPERIMENTAL

G5.NH₂ dendrimers were first conjugated with cholesteryl chloroformate to form G5.NH₂-Chol according to the literature.^[28] Briefly, G5 dendrimers (50 mg, 0.002 mmol) were dissolved in methanol (10 mL), and mixed with *N,N*-diisopropylethylamine (10.34 mg, as a catalyst) and cholesteryl chloroformate (0.02 mmol, 8.98 mg) co-dissolved in dichloromethane (5 mL) under strong stirring for 3 h at 50 °C. The mixture was dialyzed and lyophilized to obtain the G5.NH₂-Chol product.

The formed G5.NH₂-Chol was stepwise reacted with FI, PEG-FA, and acetytic anhydride.^[22,37] In brief, FI (3.5 mg, 0.009 mmol, in 2 mL of DMSO) was dropwise added into an aqueous solution of G5.NH₂-Chol (43.7 mg, 0.0015 mmol, in 20 mL of DMSO) under stirring, and this reaction was continued for 24 h. Afterwards, the mixture was dialyzed and lyophilized to get the G5.NH₂-Chol-FI product.

Next, G5.NH₂-Chol-FI was used to synthesize the conjugates of G5.NH₂-Chol-FI-PEG-FA. The carboxylic acid of the PEG-FA was first activated by EDC/NHS according to the literature.^[22,45] Then the activated PEG-FA was added into the G5.NH₂-Chol-FI aqueous solution (31.1 mg, 0.001 mmol, 10 mL) under stirring for 3 days. The obtained raw product of G5.NH₂-Chol-FI-PEG-FA was finally acetylated and purified according to the literature^[9] to get the product of G5-CFPF.

Owing to the hydrophobicity of the dendrimer internal cavity and free HCP, the formed G5-CFPF can be used to load HCP *via* hydrophobic interaction. The G5-CFPF solution (30 mg, 5 mL of water) was mixed with HCP (3 mg) dissolved in NaOH solution (2 mL, 40 mg/L, pH = 8), and stirred for 24 h to form the G5-CFPF/HCP complexes. G5.NHAc-FI-PEG-FA (for short, G5-FPF)/HCP complexes without Chol were also prepared under similar experimental conditions for comparison. The full experimental detail is available in electronic supplementary information (ESI).

RESULTS AND DISCUSSION

Preparation and Characterization of G5-CFPF/HCP Complexes

Similar to our prior studies,^[46,47] G5 PAMAM dendrimers were used as a scaffold to be modified with different functionalities for anticancer drug delivery applications. Herein, we developed a Chol-modified dendrimer platform to incorporate the advantages of cholesterol. The as-formed G5-CFPF dendrimers and dendrimer/HCP complexes were characterized systematically.

Firstly, the PEG-FA segment was synthesized and characterized *via* NMR spectroscopy (Fig. S1a in ESI). For the conjugated COOH-PEG-FA, a strong peak around 3.5 ppm was attributed to the $-\text{CH}_2-$ proton signal of PEG, and peaks at 6.5–8.5 ppm were assigned to the proton signals of FA. The number of FA attached to each NH₂-PEG-COOH was calculated to be about 0.8 after NMR integration, similar to our previous report.^[22] For G5.NH₂-Chol, the peak at 0.87 ppm was attributed to the proton signals of $-\text{CH}_3$ of Chol, and peaks at 2–3.5 ppm were assigned to the $-\text{CH}_2-$

proton signals of G5 dendrimers (Fig. 1a). The number of Chol attached to each dendrimer reached about 7.0. For G5.NH₂-Chol-FI-PEG-FA, peaks at 6–8 ppm were related to the benzene ring proton signals of FI, and a strong peak (3.5 ppm) was attributed to the $-\text{CH}_2-$ proton signal of PEG (Fig. 1b). By NMR peak integration, we found that 5.2 FI and 8.3 FA-PEG moieties were linked to each G5 dendrimer. After acetylation, a new peak at 1.87 ppm appeared for the G5-CFPF dendrimers, which was associated with the formed acetyl protons,^[48,49] indicating the success of acetylation reaction (Fig. 1c).

The conjugation of FI and PEG-FA to the G5 dendrimer surface was confirmed by UV-Vis spectrometry (Fig. 2). The synthesized G5-CFPF dendrimers display two absorption peaks, of which one at 500 nm can be attributed to the conjugated FI, and the other at 280 nm is related to the attached FA. It should be noted that G5-FPF without Chol conjugation was also prepared and validated by ¹H-NMR (Fig. S2 in ESI) and UV-Vis spectrometry (Fig. S3 in ESI). The linked numbers of FI (5.0) or PEG-FA (8.5) moieties onto each dendrimer were almost identical to those of G5.NH₂-Chol-FI-PEG-FA dendrimers.

The hydrophobic interior of G5-CFPF dendrimers enabled encapsulation of hydrophobic HCP drug.^[31,50] The formed complexes showed a new absorption peak at 375 nm (Fig. 2), which is the characteristic peak of HCP. However,

free HCP had a characteristic absorption peak at 420 nm (Fig. 2). This may be due to an interaction existing between HCP and Chol after Chol modification onto the surface of the dendrimers or between HCP and the hydrophobic dendrimer internal cavity, which caused blue shift of HCP peak (from 420 nm to 375 nm). According to HCP absorption-concentration calibration curve, each G5-CFPF or G5-FPF dendrimer encapsulated 13.8 HCP molecules. In addition, the hydrodynamic size of G5-CFPF reduced from (264.9 ± 74.8) nm to (144.1 ± 34.1) nm after HCP drug loading, showing a strong interaction between HCP and multifunctional G5-CFPF (Table S1 in ESI). Finally, the dendrimer/HCP complexes were quite stable under different pH conditions (pH = 5.0, 7.0, and 10.0) and no sedimentation took place for at least two weeks at 4 °C, which is similar to other dendrimer/drug complexes reported in the literature.^[46,47]

In Vitro Release Kinetic Study

As shown in Fig. 3, HCP was released sustainably from the two complexes. Specifically, the G5-CFPF/HCP complexes displayed a relatively low release of $(38.37 \pm 1.78)\%$ (pH = 5.0) and $(44.36 \pm 1.48)\%$ (pH = 7.4) at 48 h, respectively. In contrast, $(43.10 \pm 1.85)\%$ (pH = 5.0) and $(49.1 \pm 1.65)\%$ (pH = 7.0) of HCP were released from the G5-FPF/HCP within 48 h. The slower release of HCP from the G5-CFPF/HCP complexes than that from G5-FPF/HCP com-

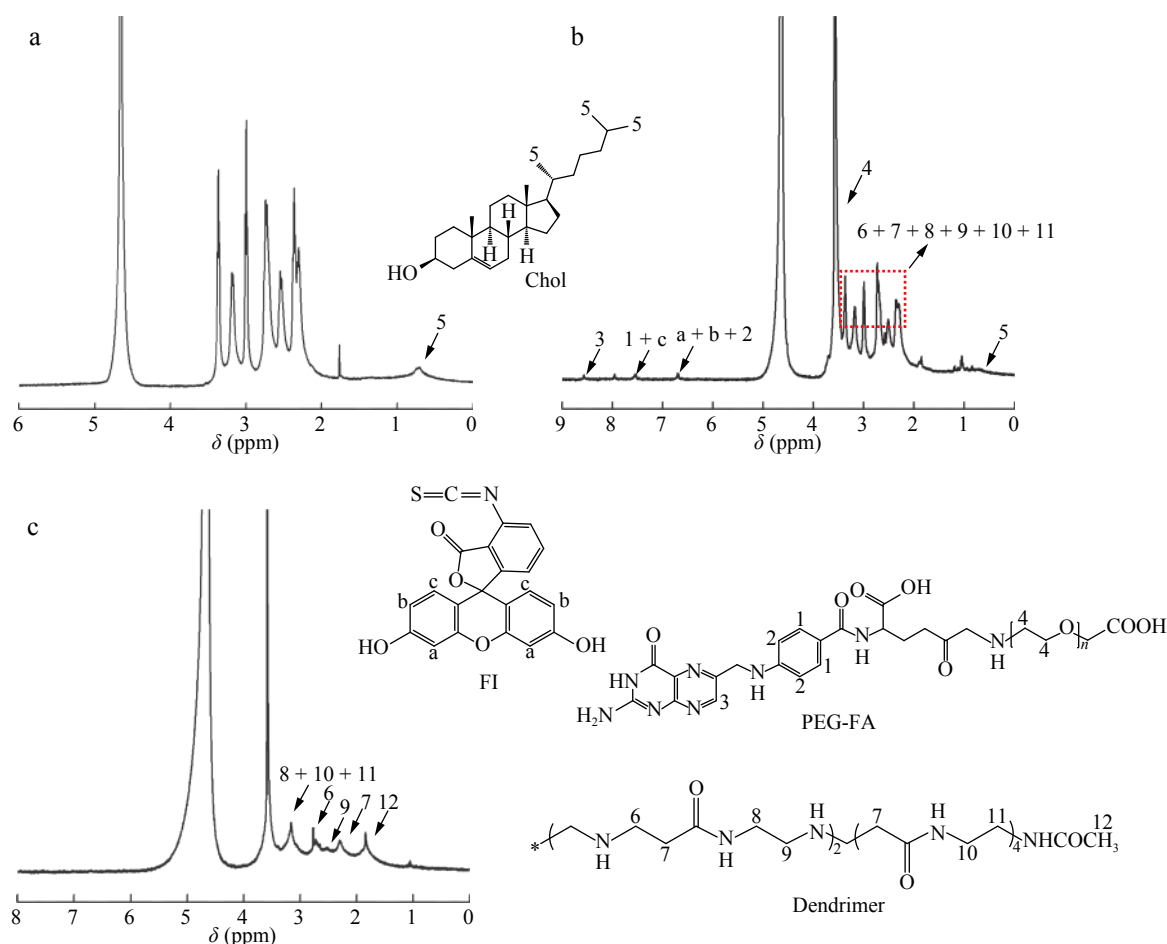


Fig. 1 ¹H-NMR spectra of (a) G5.NH₂-Chol, (b) G5.NH₂-Chol-FI-PEG-FA, and (c) G5-CFPF, respectively

plexes under the same conditions may be attributed to the attached Chol moieties which have a strong hydrophobic interaction with the drug, thereby slowing down the drug disassociation process. Under acidic condition (pH 5.0), the release of HCP was much slower than that under pH 7.4 at the same time points, due to the low water solubility of HCP.

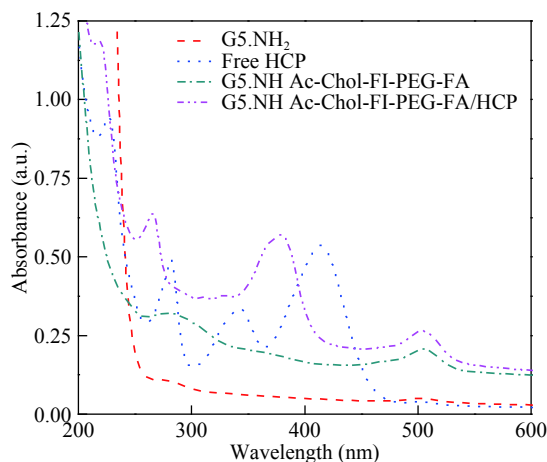


Fig. 2 UV-Vis spectra of free HCP dissolved in PBS, G5.NH₂, G5-CFPF, and G5-CFPF/HCP dispersed in PBS

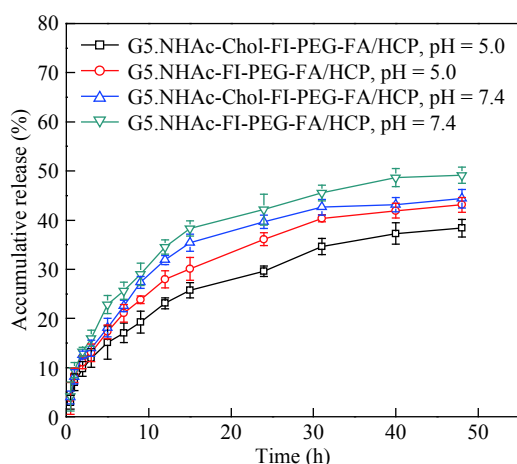


Fig. 3 Accumulative release of HCP from the G5-FPF/HCP or G5-CFPF/HCP complexes in PBS (pH = 7.4) and acetate buffer (pH = 5.0) at 37 °C (Mean $\pm$ S.D., $n = 3$)

Cytotoxicity Assay and Cell Morphology Observation

The therapeutic activity of the G5-CFPF/HCP and G5-FPF/HCP complexes was assessed by *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Fig. 4). Just the same as free HCP, both complexes exhibited a significant cytotoxicity to cancer cells in a dose-dependent manner, compared with PBS control. For example, the cell viability was $(15.05 \pm 1.3)\%$ for free HCP, $(8.5 \pm 4.9)\%$ for G5-CFPF/HCP, and $(13.23 \pm 5.3)\%$ for G5-FPF/HCP, respectively, at the HCP concentration of 10 mg/L. The greater cytotoxicity of G5-CFPF/HCP than G5-FPF/HCP may be attributed to the Chol modification onto the surface of G5-CFPF, which improves the cell-

penetrating ability for drug delivery systems. In addition, the half maximal inhibitory concentration (IC₅₀) values of free HCP, G5-CFPF/HCP, and G5-FPF/HCP were measured to be 0.85, 1.4, and 1.55 mg/L, respectively. It is worth noting that the higher IC₅₀ values of the dendrimer/HCP complexes than those of free HCP could be due to the sustained release of drug from the complexes. Moreover, the G5-CFPF and G5-FPF dendrimers without HCP loading were noncytotoxic under the same conditions. This implies that the therapeutic activity of dendrimer/HCP complexes was just associated with the loaded HCP drug. The therapeutic activity of the dendrimer/HCP complexes was further confirmed by cell morphology observation (Fig. S4 in ESI). Therefore, it seems that modification of Chol onto dendrimers did not alter the therapeutic activity of complexes when compared to the dendrimers without Chol. However, with the Chol modification, the drug can be slowly released, thereby having an improved drug release profile for delivery applications.

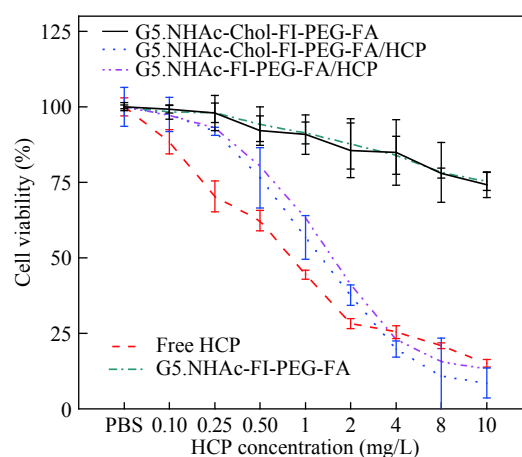


Fig. 4 MTT assay of HeLa cells treated with PBS, G5-FPF, G5-CFPF, free HCP, G5-FPF/HCP, and G5-CFPF/HCP at different HCP concentrations for 24 h (Mean $\pm$ S.D., $n = 3$). The concentrations of G5-FPF and G5-CFPF dendrimers were similar to those of the dendrimers in the corresponding dendrimer/HCP complexes.

Targeted Cellular Uptake of the Complexes

To validate the targeting role played by the linked FA, HeLa cells expressing high-level folate receptors (HeLa-HFAR) and HeLa cells expressing low-level folate receptors (HeLa-LFAR) were treated with the G5-CFPF/HCP complexes (Fig. 5), respectively. The green fluorescence signal of FI was used for flow cytometric assay of the cellular uptake of complexes (Fig. S5 in ESI). Under similar complex concentrations, HeLa-HFAR cells incubated with the G5-CFPF/HCP complexes displayed a much stronger fluorescence intensity than HeLa-LFAR cells did ($p < 0.001$). This should be due to the FA-mediated targeting of the dendrimer/HCP complexes to FAR-overexpressing HeLa cells, similar to our previous work.^[25,26] The targeting specificity of the G5-CFPF/HCP complexes was further verified *via* confocal microscopic imaging of the cells (Fig. S6 in ESI).

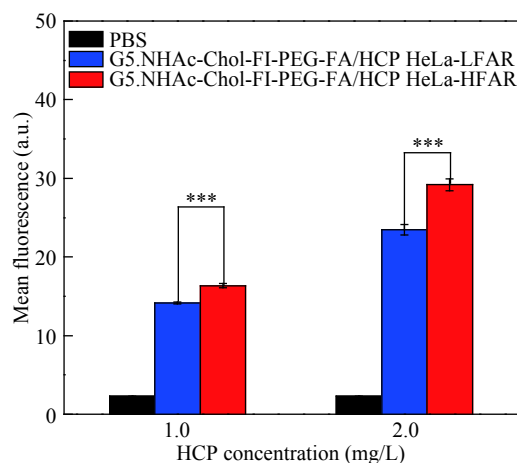


Fig. 5 Flow cytometric analysis of the mean fluorescence of HeLa-HFAR and HeLa-LFAR treated with dendrimer/HCP complexes at different HCP concentrations for 4 h (Mean $\pm$ S.D., $n = 3$). HeLa cells treated with PBS were used as control (** $p < 0.001$).

Targeted Anticancer Efficacy of G5-CFPF/HCP Complexes *in Vitro*

To further compare the targeted anticancer efficacy of G5-CFPF/HCP complexes, the viability of HeLa-HFAR and HeLa-LFAR cells was evaluated by MTT assay (Fig. 6). After 4 h incubation with the G5-CFPF/HCP, the HeLa-HFAR and HeLa-LFAR cells were continually cultured for another 48 h with fresh medium. It can be observed that at the HCP concentration of 1 mg/L, HeLa-HFAR cells treated with the complexes showed a significant decrease of viability ($(80.42 \pm 1.54)\%$), which was much lower than those of HeLa-LFAR cells treated with the same complexes ($(85.53 \pm 1.67)\%$, $p < 0.01$). Clearly, with the FA-mediated targeting specificity, the complexes were able to exert a preferential killing effect to FAR-expressing cancer cells *in vitro*.

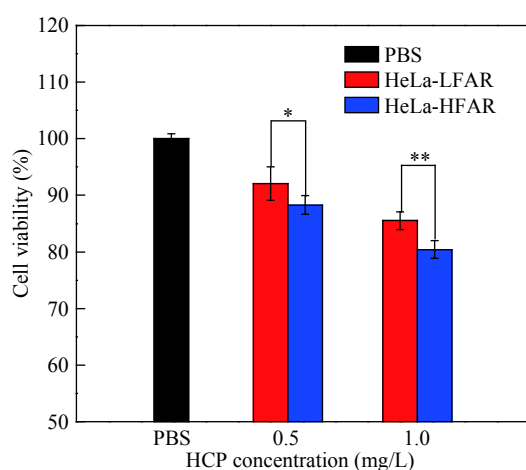


Fig. 6 MTT assay of HeLa-LFAR cells and HeLa-HFAR cells treated with G5-CFPF/HCP complexes at different HCP concentrations for 4 h, followed by incubation with HCP-free fresh medium for another 48 h (Mean $\pm$ S.D., $n = 3$). HeLa cells treated with PBS were used as control (* $p < 0.05$, ** $p < 0.01$).

CONCLUSIONS

In summary, multifunctional G5-CFPF/HCP complexes were successfully synthesized with a reasonable HCP loading efficiency for targeted anticancer drug delivery. It seemed that the modification of Chol did not alter the drug loading behaviour of the dendrimers when compared to the counterpart materials without Chol modification. Importantly, modification of Chol rendered the dendrimers with slower release profile of the HCP drug. The developed G5-CFPF/HCP complexes reserved the anticancer therapeutic efficacy, and can target FAR-expressing cancer cells with a preferential inhibition effect to the cancer cells. The designed Chol-modified dendrimers may be used as a promising carrier system for targeted chemotherapy of other FAR-expressing cancer cells.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-019-2172-9>.

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