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"Clickable" Polymer-Caged Nanobins as a Modular Drug Delivery Platform

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Abstract: Modularly clickable polymer-caged nanobins (PCNs) were prepared from liposome templates using a *drop-in* cholesterol-modified poly(acrylic acid) reagent followed by cross-linking with alkyne-functionalized diamine linker that allows for the conjugation of azide-modified targeting ligands via click ligation. These PCNs possess pH-responsive characteristics that can be used to trigger the release of encapsulated doxorubicin (DXR) payload inside the liposomal core under mild acidic conditions. After click-conjugation with azide-modified folate as an active targeting ligand, the resulting folate-conjugated, DXR-loaded PCNs (f-PCN_{DXR}) demonstrated enhanced potency to folate receptor (FR)-positive tumor cells such as KB and OvCa432 over FR-negative MCF7 cells. f-PCN_{DXR} can readily discriminate FR-positive tumor cells as a function of the level of cellular FR-expression, showing different degrees of potentiation in each cell. With both targeting functionalities and pH-sensitive drug-releasing triggers, f-PCN_{DXR} was fifty-times more potent than the untargeted agent toward cancer cells that overexpress the folate target receptors.

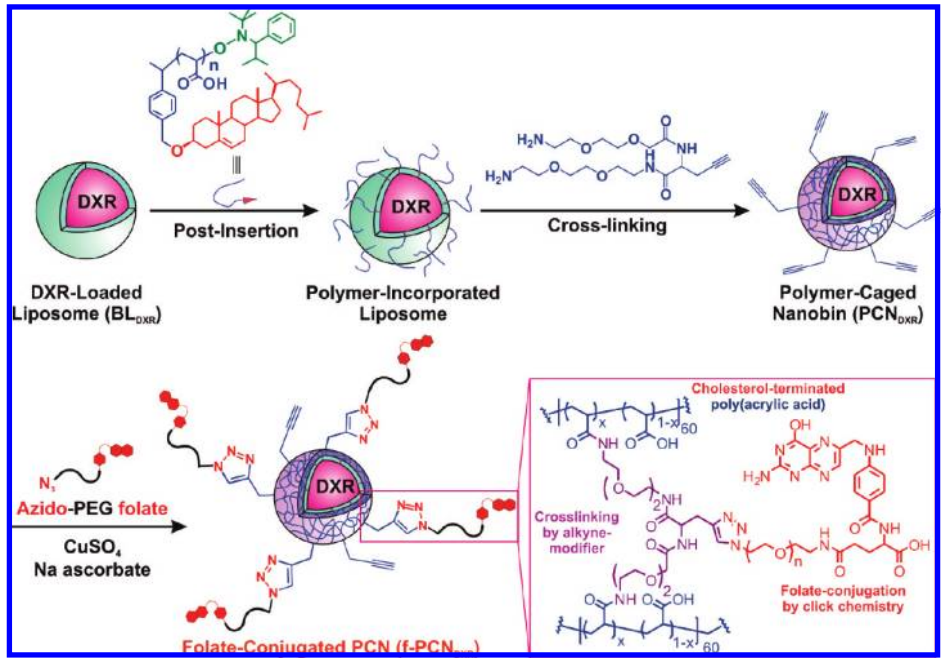
Introduction

Liposomes composed of pH-sensitive, amine-modified lipids have recently attracted much attention due to their enhanced drug-releasing profile in acidic environments[1−4] such as those generally observed in tumor tissues. However, the narrow range of conditions for the formation of such liposomal constructs[5] often limits their compatibility with ion gradient-mediated (IGM) drug loading processes.[6,7] In addition, it can be cumbersome to modify the surfaces of these delivery vehicles with targeting ligands and/or imaging moieties. Previously, we described a *drop-in* strategy for constructing highly stable polymer-caged nanobins (PCNs) around liposome templates whose payloads can be triggered to release under acidic conditions.[8] In PCN fabrication, the cross-linked polymer shell can be applied to virtually any preformed, drug-encapsulating liposomal systems and additionally provides a multitude of functional groups on the liposome surface that can be further modified with imaging and targeting agents.

In targeted delivery, the attachment of targeting ligands to the surface of drug carriers is best accomplished under mild reaction conditions[9] that preserve the integrity of the sensitive targeting molecules. This requirement is easily fulfilled by click chemistry,[10,11] which has been used in many biological applications such as the modification of mosaic virus,[12,13] bacterial cells,[14] and proteins[15] without compromising the structures and functions of these entities. In addition, click chemistry can provide orthogonal reactivity[16,17] that complements other biocompatible ligation methods[18] such as nucleophilic displacement,[9] imine formation,[19] and amide coupling.[20] For PCN fabrication, click chemistry provides a functionalization method that can be integrated exquisitely into our overall *drop-in* strategy using cholesterol-terminated poly(acrylic acid) (Chol-PAA) modifier (Scheme 1). After the Chol-PAA has been inserted into the liposomal membrane, PCNs possessing terminal alkyne groups on its surface can be prepared simply by cross-

(1) Slepushkin, V. A.; Simoes, S.; Dazin, P.; Newman, M. S.; Guo, L. S.; de Lima, M. C. P. *J. Biol. Chem.* **1997**, *272*, 2382–2388.
(2) Gerasimov, O. V.; Boomer, J. A.; Qualls, M. M.; Thompson, D. H. *Adv. Drug Delivery Rev.* **1999**, *38*, 317–338.
(3) Ishida, T.; Kirchmeier, M. J.; Moase, E. H.; Zalipsky, S.; Allen, T. M. *Biochim. Biophys. Acta* **2001**, *1515*, 144–158.
(4) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145–160.
(5) Allen, T. M.; Hong, K.; Papahadjopoulos, D. *Biochemistry* **1990**, *29*, 2976–2985.
(6) Allen, T. M. *Drugs* **1998**, *56*, 747–756.
(7) Chen, H.; MacDonald, R. C.; Li, S.; Krett, N. L.; Rosen, S. T.; O'Halloran, T. V. *J. Am. Chem. Soc.* **2006**, *128*, 13348–13349.
(8) Lee, S.-M.; Chen, H.; Dettmer, C. M.; O'Halloran, T. V.; Nguyen, S. T. *J. Am. Chem. Soc.* **2007**, *129*, 15096–15097.
(9) Bertin, P. A.; Gibbs, J. M.; Shen, C. K. F.; Thaxton, C. S.; Russin, W. A.; Mirkin, C. A.; Nguyen, S. T. *J. Am. Chem. Soc.* **2006**, *128*, 4168–4169.
(10) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.
(11) Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128–1137.
(12) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192–3193.
(13) Destito, G.; Yeh, R.; Rae, C. S.; Finn, M. G.; Manchester, M. *Chem. Biol.* **2007**, *14*, 1152–1162.
(14) Link, A. J.; Tirrell, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 11164–11165.
(15) Deiters, A.; Cropp, T. A.; Mukherji, M.; Chin, J. W.; Anderson, J. C.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 11782–11783.
(16) Joralemon, M. J.; O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2005**, *127*, 16892–16899.
(17) Hawker, C. J.; Wooley, K. L. *Science* **2005**, *309*, 1200–1205.
(18) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996.
(19) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760–1763.
(20) Huang, H.; Kowalewski, T.; Remsen, E. E.; Gertzmann, R.; Wooley, K. L. *J. Am. Chem. Soc.* **1997**, *119*, 11653–11659.

Scheme 1. Click-Based Modular *Drop-in* Strategy for the Preparation of DXR-Loaded, Folate-Conjugated Polymer-Caged Nanobin (f-PCN_{DXR})



linking the PAA chains with alkyne-functionalized diamine cross-linkers. The alkyne groups on the cross-linked polymer shells of the PAA are then ready for "clicking" with azide-containing targeting ligands.[16]

The folate receptor (FR) is a promising target for the development of anticancer drug delivery systems.[21−24] FR is localized on the cell surface, where it binds folic acid in the media, undergoes internalization into acidic endosomes, releases folate and eventually recycles to the cell surface. High levels of FR expression are observed in many carcinomas such as ovarian, lung, and uterine tumors,[25−27] relative to normal tissues. For example, FR expressions of less than 0.1−1 pmol per 10^6 cells ($\leq 10^4-10^5$ receptors/cell) were observed in normal epithelial cells and fibroblasts in contrast to malignant cells which have more than 20 pmol FR per 10^6 cells.[28] Given these attributes of FR, folic acid has been conjugated to many delivery systems such as core−shell type polymeric micelles,[29,30] liposomes modified with acid-triggered releasing devices,[31,32] and Pt prodrug-conjugated carbon nanotubes[33] to facilitate the targeted delivery of anticancer drugs.

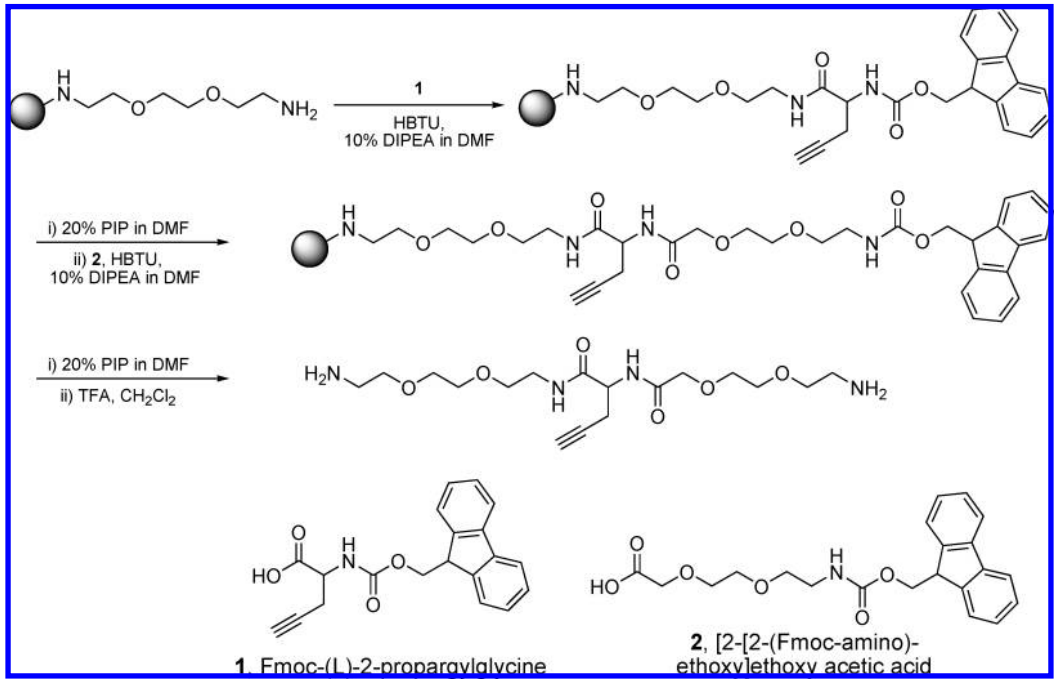
Herein, we report a facile click-based method for modularly functionalizing the surfaces of PCNs, demonstrate its compatibility with the IGM drug-loading process, and provide a targeted delivery system for the potent but toxic drug doxorubicin (DXR). As one of the most potent chemotherapeutic agents, DXR has a significant dose-limiting cardiotoxicity that prevents its widespread clinical application in free form.[34,35] While liposomal formulation of DXR, known commercially as Doxil, is available to clinicians,[36] this encapsulated agent still exhibits significant off-target toxicity. We endeavor to improve the therapeutic index of DXR and other agents by developing a more robust delivery platform that is readily modified with targeting agents and that undergoes a pH-triggered release at the target environment. The proof-of-concept of such a platform is folate-targeted doxorubicin-loaded PCNs (f-PCN_{DXR}), which exhibits significantly enhanced drug efficacy and discriminates cell lines with high selectivity as a function of increased expression of FR by multivalent interactions.

Results and Discussion

DXR was first loaded into bare liposomes using an ammonium sulfate gradient.[37] The resulting DXR-loaded bare liposomes (BL_{DXR}, DXR/lipid = 0.3 mol/mol) was then modified with 10 mol % (compared to the total amount of phospholipids in liposome) of Chol-PAA ($\bar{M}_n$: 3700 Da, PDI: 1.2), previously synthesized via nitroxide-mediated controlled radical polymer-

(21) Sudimack, J.; Lee, R. J. *Adv. Drug Delivery Rev.* **2000**, *41*, 147–162.
(22) Leamon, C. P.; Reddy, J. A. *Adv. Drug Delivery Rev.* **2004**, *56*, 1127–1141.
(23) Gabizon, A.; Shmeeda, H.; Horowitz, A. T.; Zalipsky, S. *Adv. Drug Delivery Rev.* **2004**, *56*, 1177–1192.
(24) Low, P. S.; Henne, W. A.; Doorneweerd, D. D. *Acc. Chem. Res.* **2008**, *41*, 120–129.
(25) Parker, N.; Turk, M. J.; Westrick, E.; Lewis, J. D.; Low, P. S.; Leamon, C. P. *Anal. Biochem.* **2005**, *338*, 284–293.
(26) Ross, J. F.; Chaudhuri, P. K.; Ratnam, M. *Cancer* **1994**, *73*, 2432–2443.
(27) Lu, Y.; Low, P. S. *Adv. Drug Delivery Rev.* **2002**, *54*, 675–693.
(28) Weitman, S. D.; Lark, R. H.; Coney, L. R.; Fort, D. W.; Frasca, V.; Zurawski, V. R. J.; Kamen, B. A. *Cancer Res.* **1992**, *52*, 3396–3401.
(29) Pan, D.; Turner, J. L.; Wooley, K. L. *Chem. Commun.* **2003**, 2400–2401.
(30) Nayak, S.; Lee, H.; Chmielewski, J.; Lyon, L. A. *J. Am. Chem. Soc.* **2004**, *126*, 10258–10259.
(31) Vogel, K.; Wang, S.; Lee, R. J.; Chmielewski, J.; Low, P. *J. Am. Chem. Soc.* **1996**, *118*, 1581–1586.
(32) Rui, Y.; Wang, S.; Low, P. S.; Thompson, D. H. *J. Am. Chem. Soc.* **1998**, *120*, 11213–11218.
(33) Dhar, S.; Liu, Z.; Thomale, J.; Dai, H.; Lippard, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 11467–11476.
(34) Waterhouse, D. N.; Tardi, P. G.; Mayer, L. D.; Bally, M. B. *Drug Safety* **2001**, *24*, 903–920.
(35) Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L. *Pharmacol. Rev.* **2004**, *56*, 185–229.
(36) Lyass, O.; Uziely, B.; Ben-Yosef, R.; Tzemach, D.; Heshing, N. I.; Lotem, M.; Brufman, G.; Gabizon, A. *Cancer* **2000**, *89*, 1037–1047.
(37) Haran, G.; Cohen, R.; Bar, L. K.; Barenholz, Y. *Biochim. Biophys. Acta* **1993**, *1151*, 201–215.

Scheme 2. Synthesis of Alkyne-Modified Diamine Crosslinker[a]



[a] HBTU = *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine; PIP = piperidine; TFA = trifluoroacetic acid.

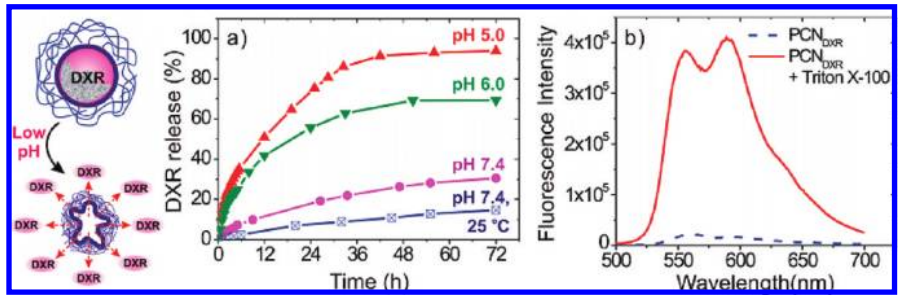


Figure 1. (a) Time-dependent DXR-releasing profiles of polymer-caged nanobin (PCN_{DXR}) under pH 5.0, 6.0, and 7.4 at 37 °C (closed symbols) and 25 °C (open symbol). (b) Fluorescence emission spectra of PCN_{DXR} before and after treatment with Triton X-100 showing significant quenching of DXR fluorescent intensity inside the liposome.

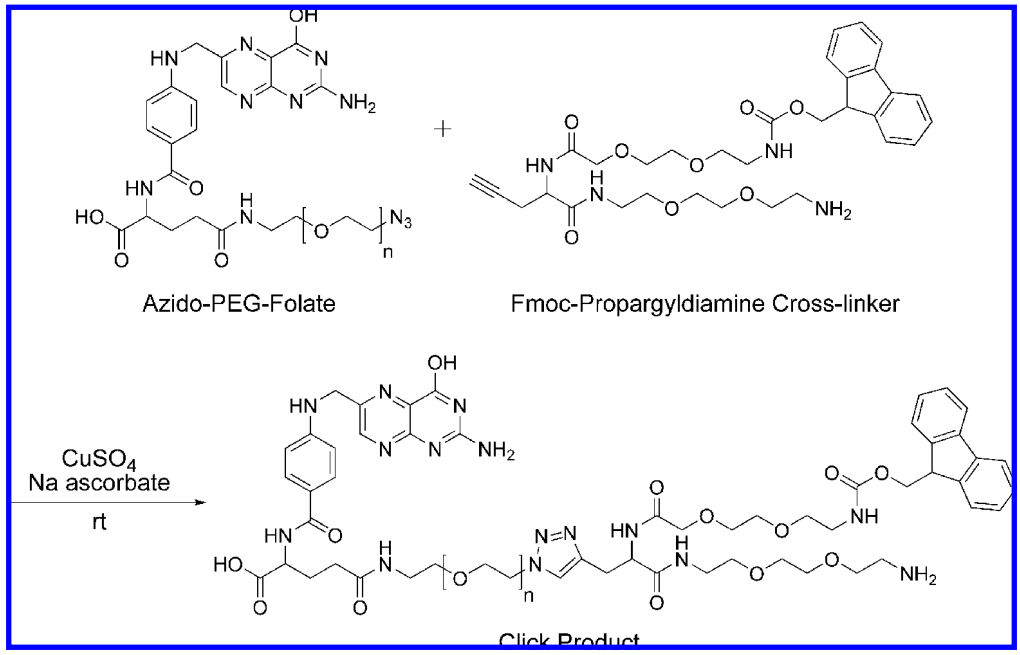
ization.[38] The grafted polymers on the liposomal surface were subsequently cross-linked with a newly designed alkyne-modified diamine linker (Scheme 2) to yield DXR-loaded polymer-caged nanobin (PCN_{DXR}; zeta potential $\zeta = -15.04 \pm 0.96$ mV at pH 7.4, 25 °C; hydrodynamic diameter $D_H = 124 \pm 21$ nm as measured by dynamic light scattering, Figure S1 in Supporting Information (ESI)). After PCN_{DXR} formation, the majority of encapsulated DXR was still present inside the intact liposome (DXR/lipid = 0.28−0.30 mol/mol).

The drug-releasing kinetics for PCN_{DXR} was monitored under acidic conditions (Figure 1a). Because the emission intensity from PCN_{DXR} was substantially self-quenched when DXR is highly loaded inside the liposome,[39] the amount of drug released is conveniently monitored using fluorescent spectroscopy against the total release obtained by treating the liposomes with Triton X-100 (Figure 1b).[3,8] As expected, PCN_{DXR} was quite stable at pH 7.4, with 30% release of DXR after 3 days at 37 °C. At pH 5.0 and 37 °C, however, ~75% of DXR was released from PCN_{DXR} during the initial 24 h and complete release was achieved at 36 h (>90% release). In contrast, 55% release was achieved from BL_{DXR} at pH 5.0 and 37 °C after 3 days (Figure S2, see also Table S1 in Supporting Information for a list of apparent DXR-release rates from PCN_{DXR} at different pH conditions). The enhanced releasing property of the PCN platform under acidic condition, comparing to that of BL, has been ascribed to the structural change of encapsulating polymer cage, resulting in the formation of transient pores on liposomal membrane.[8,40]

Although we have previously observed acid-triggered release of the anionic probe calcein from PCN,[8] the release rate of DXR from PCNs, as reported herein, is significantly faster and may be additionally attributed to both the intrinsic lipophilicity of DXR and the cationic character of protonated DXR inside liposome.[37] While similar preference for cation permeation has been observed for a lipid membrane modified with acrylate-

(38) Benoit, D.; Chaplinski, V.; Braslau, R.; Hawker, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 3904–3920.
(39) Barenholz, Y.; Amselem, S.; Goren, D.; Cohen, R.; Gelvan, D.; Samuni, A.; Golden, E. B.; Gabizon, A. *Med. Res. Rev.* **1993**, *13*, 449–491.
(40) Binder, W. H. *Angew. Chem., Int. Ed.* **2008**, *47*, 3092–3095.

Scheme 3. Model Click Reaction between the Azido-PEG-folate Reagent and Alkyne-Modified Diamine Crosslinker



based polymers,[41] the noncross-linked precursor of PCN does not have significant acid-triggered releasing property.[8] In this sense, the cross-linked PAA cages in the PCN system can play a very important role in triggering the release of DXR under acidic condition. We note that acid-triggered release of physically entrapped doxorubicin has been observed in shell-cross-linked knedel-like naoparticles[42] and PEGylated nanogels,[43] albeit with different physical mechanisms.

Folic acid was next conjugated to alkyne-functionalized PCN_{DXR} via an azide-terminated poly(ethylene glycol) (PEG) spacer (Scheme 1). The PEG spacer was included to enhance aqueous solubility; its inclusion has also been shown to enhance the targeting activity of surface-attached folate.[44−46] The required azido-PEG-folate reagent was synthesized in one step from folic acid and a commercially available azido-PEG amine via DCC-mediated amide coupling. To establish the conditions for the click reaction, equimolar amounts of azido-PEG-folate reagent and alkyne-functionalized diamine cross-linker were reacted together in the presence of $CuSO_4$ and sodium ascorbate (Scheme 3), with the triazole product verified by reverse-phase HPLC analyses (Figure 2a), MALDI-TOF mass spectrometry (Figure 2b), and FT-IR spectroscopy (Figure S3 in Supporting Information). The parent alkyne-functionalized PCN_{DXR} were then treated with 1 mol % (compared to total lipids in PCNs) of the azido-PEG-folate reagent under similar conditions. (The level of folate-conjugation on PCNs was kept below 1 mol % compared to the amount of lipids, because ~0.5 mol % of folate per liposome was reported as an optimized value for targeting.[47])

After click reaction and purification, the resulting folate-conjugated PCNs (f-PCNs) contains about 0.73 mol % of folate ligand as determined by UV−vis spectroscopy (Figure S4 in Supporting Information). The modular versatility of the alkyne groups on the surface of PCNs was also demonstrated with click-conjugation of an azido-ethidium dye to empty PCNs (Figure S5 in Supporting Information).

To verify that f-PCN_{DXR} can be uptaken into cells via FR-mediated endocytosis, FR-overexpressing KB human epithelial nasopharyngeal carcinoma cells (~2.8 × 10^5 FRs per cell)[47] were exposed to f-PCN_{DXR}, either alone or in the presence of 2-mM free folate ligand for 2 and 4 h. Confocal laser-scanning fluorescent microscopy (CLSM) images were obtained for all incubated cells based on the fluorescence of DXR (λ_{em} > 570 nm, no observable background fluorescence was detected from the cell lines at this wavelength, Figure S6 in Supporting

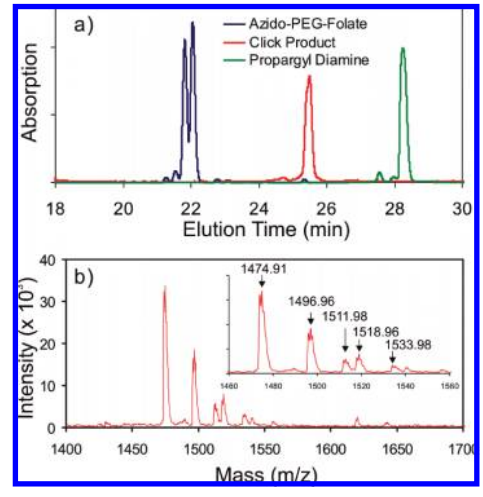


Figure 2. (a) Reverse-phase HPLC chromatogram of the crude reaction mixture. (b) MALDI-TOF mass spectrum of the click product. m/z = 1474.91 [$M_{n=8}$ + $3H^+$], 1496.96 [$M_{n=8}$ + $2H^+$ + Na^+], 1511.98 [$M_{n=8}$ + H^+ + K^+], 1518.96 [$M_{n=9}$ + $3H^+$], 1533.98 [$M_{n=8}$ + Na^+ + K^+] observed; 1471.70 calculated for $[M_{n=8}]^+$.

(41) Thomas, J. L.; Tirrell, D. A. *J. Controlled Release* **2000**, *67*, 203–209.
(42) Nyström, A. M.; Xu, Z.; Xu, J.; Taylor, S.; Nittis, T.; Stewart, S. A.; Leonard, J.; Wooley, K. L. *Chem. Commun.* **2008**, 3579–3581.
(43) Oishi, M.; Hayashi, H.; Iijima, M.; Nagasaki, Y. *J. Mater. Chem.* **2007**, *17*, 3720–3725.
(44) Lee, R. J.; Low, P. S. *J. Biol. Chem.* **1994**, *269*, 3198–3204.
(45) Gabizon, A.; Horowitz, A. T.; Goren, D.; Tzemach, D.; Mandelbaum-Shavit, F.; Qazen, M. M.; Zalipsky, S. *Bioconjugate Chem.* **1999**, *10*, 289–298.
(46) Leamon, C. P.; Cooper, S. R.; Hardee, G. E. *Bioconjugate Chem.* **2003**, *14*, 738–747.

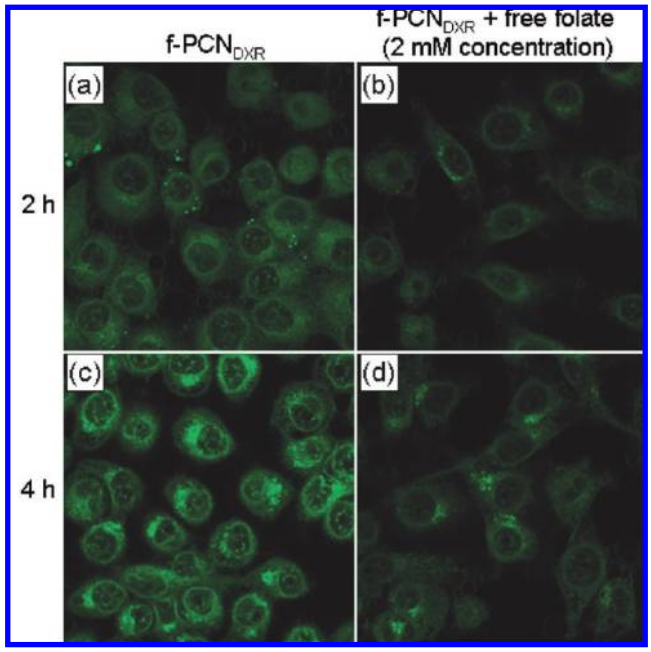


Figure 3. Confocal laser-scanning fluorescent microscopy images of KB cells after exposure to f-PCN$_{DXR}$ in folate-free medium (a and c) or in the presence of free folic acid (2 mM concentration, b and d). (a and b) after 2 h exposure; (c and d) after 4 h exposure.

Information). More DXR fluorescence was detected from the KB cells exposed to f-PCN$_{DXR}$ alone (Figure 3a) than from those incubated with free folate (Figure 3b), suggesting the internalization of f-PCN$_{DXR}$ was drastically hindered by free folate ligand. After 4 h of incubation, increased accumulation of DXR in the cells that were incubated with f-PCN$_{DXR}$ alone was very apparent: they have more intense cytosolic fluorescence (Figure 3c),[48] suggesting that more f-PCN$_{DXR}$ have been internalized via FR-mediated endocytosis, In contrast, cytosolic fluorescence from the cells that were incubated with both f-PCN$_{DXR}$ and free ligand remains low, indicating that FR-mediated endocytosis of f-PCN$_{DXR}$ was mostly blocked by free folates (Figure 3d).

The cellular internalization efficiencies of f-PCN$_{DXR}$, PCN$_{DXR}$, and BL$_{DXR}$ were evaluated by incubating them with KB cells and OvCa432 epithelial ovarian carcinoma cells that are known to have expressed FR but at a level that is lower than that of KB cells.[49,50] Incubations with free DXR were also carried out as controls. After 4 h, CLSM images were obtained for all incubated cells based on DXR fluorescence (Figure 4). Among the images shown in Figure 4, the cultured cells that have been exposed to free DXR are the brightest (Figures 4d and h), consistent with free DXR being able to enter the cells rapidly by free diffusion.[51] For both KB and OvCa432 cells, the intracellular fluorescence from PCN$_{DXR}$ (Figures 4b and f) was significantly higher than those from BL$_{DXR}$ (Figures 4a and e). While both types of liposome might be internalized by nonspecific adsorptive endocytosis[52] into the endosomes which have acidic environment (pH = 5−6),[53] the pH-sensitive polymer cages in PCN$_{DXR}$ can more efficiently transfer DXR into the cytoplasm by destabilizing both liposomal and endosomal membrane.[30,54,55]

For KB cells incubated with f-PCN$_{DXR}$ (Figure 4c), brighter fluorescence intensities than those incubated with PCN$_{DXR}$ (Figure 4b) were observed, due to the enhanced cellular uptake via FR-mediated endocytosis. While similar cellular fluorescence images with dark nuclear regions were also observed in OvCa432 cells that has been incubated with f-PCN$_{DXR}$ (Figure 4g), the fluorescence intensity was lower than that observed for the KB cells, consistent with the lower level of FR expression in OvCa432. Analogous low associations of folate-targeted carriers have been reported for HeLa cells that have lower levels of FR expression compared to KB cells.[56,57]

The cytotoxicities of the PCN formulations were evaluated in FR-positive KB and OvCa432 cells, and in FR-deficient MCF7 human mammary carcinoma cells.[49,56] Each cell line was exposed to the drug preparations (free DXR, BL$_{DXR}$, PCN$_{DXR}$, or f-PCN$_{DXR}$) in folate-free growth media under two different exposure regimes: (1) a 2-h pulsed exposure to drugs followed by a 46-h incubation in drug-free media, or (2) a continuous 48-h incubation in drug-containing media. The absolute numbers of both viable and dead cells were counted using Guava ViaCount assays[58,59] and the relative dose-responsive cell survival percentages compared to the drug-free control were plotted against the total DXR concentration (Figures 5a−f). Half-maximum inhibitory concentrations (IC_{50} values as determined from the dose−response curves, Table 1) and degrees of potentiation[60] (DOP = [IC_{50}(free drug)/IC_{50}(drug in carrier)] × 100, Figure 5g) clearly show the relative potency of BL$_{DXR}$, PCN$_{DXR}$, and f-PCN$_{DXR}$ compared to free DXR. For KB cells, f-PCN$_{DXR}$ (DOP = 144%) is about 50-fold more potent (potency

(47) Saul, J. M.; Annapragada, A.; Natarajan, J. V.; Bellamkonda, R. V. *J. Controlled Release* **2003**, *92*, 49–67.

(48) In the folate-mediated uptake of folate-functionalized gold nanoparticles into KB cells, many of the internalized particles were observed to aggregate into multivesicular bodies (MVBs), also known as late endosomes (Turek, J. J.; Leamon, C. P.; Low, P. S. *J. Cell Sci.* **1993**, *106*, 423−430). Thus, if the internalized f-PCN$_{DXR}$s also aggregate into MVBs, they would appear as 'bright blobs' in the fluorescence microscopy images. In addition, folate-functionalized particles can also be collected into transgolgi network (TGN), which are known to undergo extensive vesicular traffic with MVBs (Maxfield, F. R.; McGraw, T. E. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 121−132), and can also appear as bright perinuclear spots when observed by fluorescence microscopy. Uneven "blob-like" cellular distribution of fluorescent virus particles internalized by folate-mediated endocytosis has been observed in KB cells, in contrast to HeLa cells that show evenly dispersed fluorescence in the cytoplasm.[13]

(49) The amount of DXR (encapsulated inside f-PCN$_{DXR}$) uptaken via FR-mediated endocytosis in the three cell lines used in this study (KB, OvCa432, and MCF7) was quantified using fluorimetry (Figure 6). Assuming that the DXR-uptake levels for each cell type is proportional to the number of folate receptors (FR) on the cell surface, the relative expression levels of FR on each cell line correspond to the order of (KB > OvCa432 ≫ MCF7). This result is consistent with the previously reported level of FR-expression.[50]

(50) Campbell, I. G.; Jones, T. A.; Foulkes, W. D.; Trowsdale, J. *Cancer Res.* **1991**, *51*, 5329–5338.

(51) Goren, D.; Horowitz, A. T.; Tzemach, D.; Tarshish, M.; Zalipsky, S.; Gabizon, A. *Clin. Cancer Res.* **2000**, *6*, 1949–1957.

(52) Luo, L.; Tam, J.; Maysinger, D.; Eisenberg, A. *Bioconjugate Chem.* **2002**, *13*, 1259–1265.

(53) Mukherjee, S.; Ghosh, R. N.; Maxfield, F. R. *Physiol. Rev.* **1997**, *77*, 759–803.

(54) Yessine, M.-A.; Leroux, J.-C. *Adv. Drug Delivery Rev.* **2004**, *56*, 999–1021.

(55) Oh, K. T.; Yin, H.; Lee, E. S.; Bae, Y. H. *J. Mater. Chem.* **2007**, *17*, 3987–4001.

(56) Chen, H.; Ahn, R.; Bossche, J. V. d.; Thompson, D. H.; O'Halloran, T. V. *Mol. Cancer Ther.* 2009, in press, DOI: 10.1158/1535-7163.MCT-09-0045.

(57) Lee, R. J.; Low, P. S. *Biochim. Biophys. Acta* **1995**, *1233*, 134–144.

(58) Donaldson, M.; Antignani, A.; Milner, J.; Zhu, N.; Wood, A.; Cardwell-Miller, L.; Changpriroa, C. M.; Jackson, S. H. *Cell Death Differ.* **2009**, *16*, 125–138.

(59) http://www.guavatechnologies.com/cm/Life%20Science%20Research/Assays/Viability%20&%20Cell%20Counting.html (Access Date: Feb. 23, 2009).

(60) Kramer, R. A.; Zakher, J.; Kim, G. *Science* **1988**, *241*, 694–697.

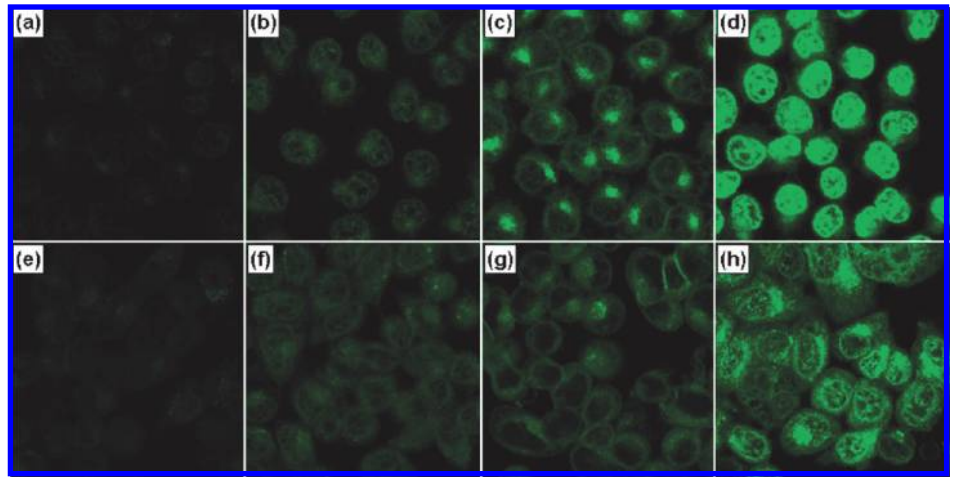


Figure 4. Confocal laser-scanning fluorescence microscopy images of KB cells (a to d) and OvCa432 cells (e to h) after 4-h incubation with BL_{DXR} (a and e), PCN_{DXR} (b and f), f-PCN_{DXR} (c and g), and free DXR (d and h). Because free DXR is a small hydrophobic molecule, it can directly pass through the cell membrane, accumulate in compartments such as nuclei,[51] and give rise to intense fluorescent staining patterns. As such, it should not be compared directly against the different liposome formulations, which can only be taken into the cells via macromolecular transport pathways such as endocytosis. (Exposure dose = 20 μM of DXR.)

= DOP of drug formulation 1/DOP of drug formulation 2)[61] than non-targeted PCN_{DXR} (DOP = 3.1%) in exposure regime 1.

In the presence of free folate ligands (1 mM), the cytotoxicity of f-PCN_{DXR}, as indicated by the IC_{50} value, was reduced about four-folds but not completely blocked, suggesting an enhanced binding affinity[62] resulting from the multivalent binding nature of f-PCN_{DXR}. In exposure regime 2 (continuous 48-h incubation with the drug formulations), the difference in cytotoxicities toward KB cells between f-PCN_{DXR} and PCN_{DXR} was reduced, due to the sufficient internalization of non-targeted PCN_{DXR} (through non-specific adsorptive endocytosis)[52,63] over this long period (Figure 5b). Consistent with a mechanism where targeting can enhance drug efficacy, FR-negative MCF7 cells show no measurable difference in cell viability responses toward f-PCN_{DXR} and PCN_{DXR} for both incubation regimes (Figure 5e and f). However, both formulations still showed better efficacy than BL_{DXR}, possibly due to the acid-triggered drug-releasing property of PCNs.

For OvCa432 cells, where the level of FR-expression is low, a slight enhancement in targeting efficiency was observed for f-PCN_{DXR} over non-targeted PCN_{DXR} in exposure regime 1 (Figure 5c). Presumably, the low level of expressed FRs on OvCa432 was not sufficient to allow for large enhancements in cellular uptake of DXR from f-PCN_{DXR} within a short exposure time. While the exact mechanism is still under debate,[64] such uptake has been shown to depend on the density of receptor on cell surface.[45,47] In addition, the net cellar accumulation of drugs via FR-mediated endocytosis depends on the time required for the internalized FRs recycling back to cell surface after unloading the ligands inside endosomal compartments, which ranges from 8 to 12 h.[24,65] Given these criteria, an appropriately longer incubation should allow for enhanced uptake and lead to improved therapeutic efficacy of f-PCN_{DXR} over nontargeted PCN_{DXR} (Figure 5d) for OvCa432. We note that similar low targeting efficiency has been reported for a low FR-expressing cell line during short exposure to a folate-toxin conjugate.[66] In addition, a 10^5 receptors/cell level has been suggested as a threshold below which targeted therapy become ineffective.[67]

The bar graphs of Figure 5h provide several comparisons of the DOP values for each of the three cell lines in this study. Because the intrinsic toxicity of DXR is different for every cell line, DOP is a more appropriate indicator of the targeting effectiveness of f-PCN_{DXR} than IC_{50} value. However, this parameter depends greatly on the exposure time. In the short-exposure regime 1, the DOP clearly reflects the level of FR-expression in each cell line, suggesting that f-PCN_{DXR} can discriminate the cell lines depending on the level of FR-expression by multivalent interactions.[68] In contrast, little selectivity was observed in the long-exposure regime 2 where non-specific absorption became the main contributor to the cellular uptake of PCN_{DXR}.

The above data suggests that the optimized therapeutic window for any drug formulations must be derived separately for each cell type depending on the level of target receptor expression. The exposure time of a specific cancer cell to multivalent receptor-targeted therapeutics such as f-PCN_{DXR} should be long enough to maximize effective receptor-mediated endocytosis but not so long as to have the uptake advantages being nullified by nonspecific processes. In this sense, our click-based *drop-in* strategy for the preparation of f-PCN_{DXR} provides a facile and modular strategy for tuning the density and morphology of targeting ligands to match the spectrum of biological receptor expression on the cell surface and arrive at the optimum therapeutic window.

Conclusions

In conclusion, we have demonstrated a facile, modular strategy for the production of a targeted drug delivery system based on polymer-caged nanobins. Our data shows that these

(61) This is equivalent to the ratio of the two corresponding IC_{50} values, a commonly used parameter to compare relative drug potencies.[57]

(62) Hong, S.; Leroueil, P. R.; Majoros, I. J.; Orr, B. G.; Baker, J. R.; Banaszak Holl, M. M. *Chem. Biol.* **2007**, *14*, 107–115.
(63) Bae, Y.; Jang, W.-D.; Nishiyama, N.; Fukushima, S.; Kataoka, K. *Mol. BioSyst.* **2005**, *1*, 242–250.
(64) Parton, R. G. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 162–167.
(65) Paulos, C. M.; Reddy, J. A.; Leamon, C. P.; Turk, M. J.; Low, P. S. *Mol. Pharmacol.* **2004**, *66*, 1406–1414.
(66) Leamon, C. P.; Pastan, I.; Low, P. S. *J. Biol. Chem.* **1993**, *268*, 24847–24854.
(67) Chari, R. V. J. *Acc. Chem. Res.* **2008**, *41*, 98–107.
(68) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.

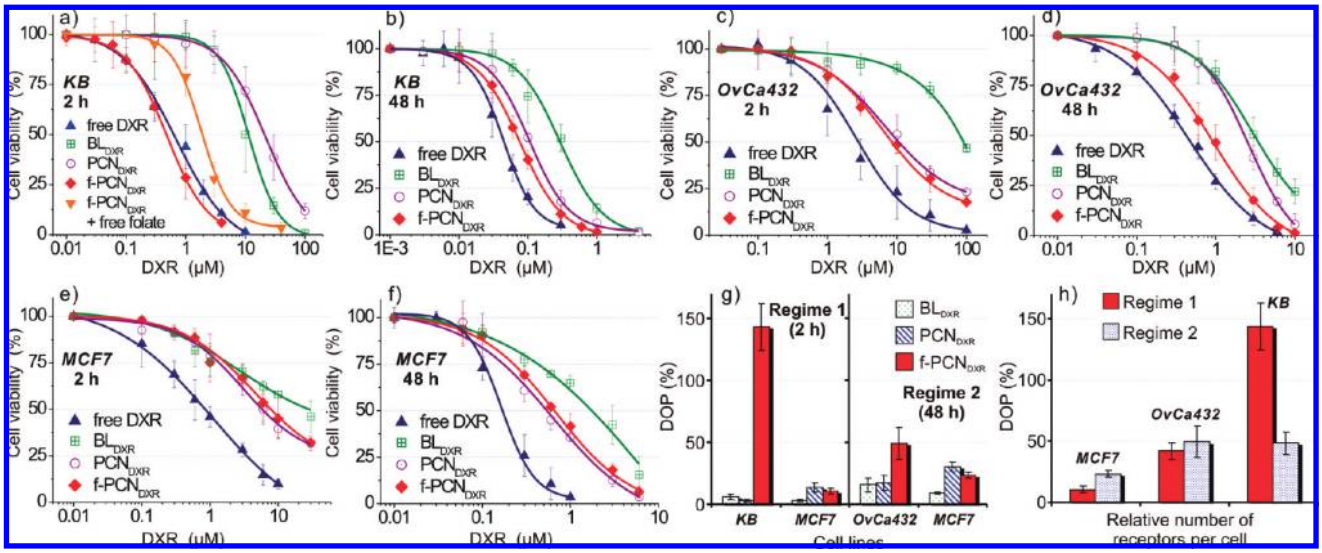


Figure 5. (a−f) Cytotoxicity profiles for various DXR formulations against FR-overexpressing KB human epithelial nasopharyngeal carcinoma cells, OvCa432 epithelial ovarian cancer cells (FR-expressing but at an amount that is less than that for KB), and FR-negative MCF7 breast cancer cells. Regime 1: (a) KB, (c) OvCa432, (e) MCF7 cells that were exposed to drug formulations for 2 h followed by a 46-h post-incubation in drug-free media. Regime 2: (b) KB, (d) OvCa432, (f) MCF7 cells that were incubated with drug formulations for 48-h. All cells were incubated with either free doxorubicin (DXR), DXR-encapsulated bare liposome (BL_{DXR}), DXR-encapsulated polymer-caged nanobin (PCN_{DXR}), or DXR-encapsulated, folate-conjugated PCN (f-PCN_{DXR}). (g) Relative degree of potentiation (DOP) of various DXR-loaded liposomal formulations compared to the free drug (calculated as [IC_{50}(free drug)/IC_{50}(drug in carrier) × 100] for each cell line. (h) Bar graph showing the DOP of f-PCN_{DXR} for each cell type in short and long incubation regime.

nanobins can effectively deliver a significant bolus of therapeutic agent to cancer cells. Once inside the cell, these nanobins undergo triggered-release of the drug in cellular acidic microenvironments such as endosome. The incorporation of a terminal alkyne handle into the polymer cage enables a modular, *drop-in* strategy for PCN functionalization and allows for the conjugation of virtually any azide-modifiable targeting group onto PCNs without the loss of structural integrity or multivalent targeting capability. Coupled to the pronounced pH-sensitive release trigger of the polymer cage, the "clickable" PCN platform can facilitate the synthesis of a broad range of targeted therapeutics. As a proof of concept shown herein, folate-conjugated PCNs can be engineered to deliver drug payload to specific receptor-positive tumor cells with high selectivity. The ability to engender stability, multivalent targeting capability, release trigger, and other functionalities into nanoscale drug delivery vehicles in a facile and modular fashion should make PCN a highly versatile platform that can significantly enhance the utility of liposomal delivery technology in tumors.

Experimental Section

Materials. Unless otherwise noted, all reagents and materials were purchased from commercial sources and used as received. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Doxorubicin is purchased from Polymed Therapeutics, Inc. (Houston, TX). *O*-bis-(aminoethyl)ethylene glycol trityl resin and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were purchased from EMD Biosciences (San Diego, CA). ICP calibration standard solutions of phosphorus (1000 μg/mL P), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC·MeI), piperidine, folic acid, *O*-(2-aminoethyl)-*O*′-(2-azidoethyl)octaethylene glycol, and all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). *Tert*-butyl acrylate was stirred over CaH_2 under nitrogen and fractionated by vacuum transfer right before use. Cholesterol-terminated poly(acrylic acid) was prepared using a literature procedure.[8] Ultrapure deionized water was obtained from a Millipore system (18.2 MΩ cm resistivity).

Measurements. Fourier-transformed nuclear magnetic resonance (NMR) spectroscopy was performed on a Varian INOVA-500 MHz spectrometer in the Northwestern Integrated Molecular Structure Education and Research Center (IMSERC) facilities. Chemical shifts of ^{1}H NMR spectra are reported in ppm against residual solvent resonance as the internal standard ($CHCl_3$ = 7.27 ppm, CHD_2COCD_3 = 2.05 ppm, CHD_2OD = 3.31 ppm).

Fourier-transformed infrared (FTIR) spectroscopy was performed on a Bio-Rad FTS-60 FTIR spectrometer. FTIR spectra of small-molecule compounds were measured by dropping a CH_2Cl_2 solution of the compound on a NaCl plate and allowing the solvent to evaporate prior to measurements. KBr-pellets were prepared for FTIR measurements of azido-PEG-folate, alkyne-modified diamine cross-linker, and click products. Fluorescence emission spectra were obtained on a Jobin Yvon Fluorolog fluorometer (λ_{ex} = 475 nm, λ_{em} = 596 nm, slith width = 3 nm for doxorubicin; λ_{ex} = 475 nm, λ_{em} = 610 nm, slith width = 5 nm for ethidium bromide monoazide). UV−vis absorption spectra were obtained on a CARY

Table 1. Half-Maximal Inhibitory Concentration (IC_{50}/μM) Values of Free DXR, BL_{DXR}, PCN_{DXR}, and f-PCN_{DXR} against KB, OvCa432, and MCF7 Cell Lines

	regime 1 (2-h pulsed exposure)				regime 2 (48-h continuous exposure)			
cell lines	free DXR	BL_{DXR}	PCN_{DXR}	f-PCN_{DXR}	free DXR	BL_{DXR}	PCN_{DXR}	f-PCN_{DXR}
KB	0.69 ± 0.08	11.5 ± 2.2	22.0 ± 5.2	0.48 ± 0.15[a]	0.035 ± 0.005	0.29 ± 0.04	0.10 ± 0.01	0.077 ± 0.008
OvCa432	2.65 ± 0.86	90.9 ± 1.3	10.7 ± 2.0	8.42 ± 1.33	0.46 ± 0.07	2.94 ± 0.44	2.62 ± 0.72	0.93 ± 0.13
MCF7	0.83 ± 0.17	28.0 ± 1.41	6.10 ± 1.95	7.80 ± 2.31	0.16 ± 0.03	1.80 ± 0.20	0.53 ± 0.09	0.68 ± 0.12

[a] IC_{50} value with the presence of 1-mM free folic acid is 1.83 ± 0.36 μM.

300 Bio UV−vis spectrophotometer. Confocal Laser Scanning Microscopy (CLSM) studies were peformed on a Carl Zeiss LSM 510 META microscope.

Electrospray-ionization mass spectrometric (ESIMS) data were obtained on a Micromass Quattro II triple quadrupole mass spectrometer. Phosphorus concentration was determined using a Varian Vista MPX simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES). Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a PE Voyager DE-Pro MALDI-TOF mass spectrometer in positive ionization mode, using 3-indoleacrylic acid as a matrix.

Polymer molecular weights were measured relative to polystyrene standards on a Waters gel-permeation chromatograph (GPC) equipped with Breeze software, a 717 autosampler, Shodex KF-G guard column, KF-803 L and KF-806 L columns in series, a Waters 2440 UV detector, and a 410 RI detector. HPLC-grade THF was used as an eluent at a flow rate of 1.0 mL/min and the instrument was calibrated using polystyrene standards (Aldrich, 15 standards, 760−1 800 000 Da).

High performance liquid chromatography (HPLC) was performed on an Agilent 1100 instrument equipped with a Jupiter 4u Proteo 90 Å semiprep reverse-phase column (250 mm × 10.0 mm) at a flow rate of 2 mL/min, using gradient eluent derived from two different solvent mixtures: A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). Method 1 (for purification of azido-PEG-folate and analyses of click reaction products): at 0 min, solvent mixture A/B = 95/5 v/v; at 25 min, solvent mixture A/B = 50/50 v/v; at 35 min, solvent mixture A/B = 10/90 v/v; at 40 min, solvent mixture A/B = 0/100 v/v. Method 2 (for purification of alkyne-modified diamine cross-linker): at 0 min, solvent mixture A/B = 95/5 v/v; at 30 min, solvent mixture A/B = 5/90 v/v; at 40 min, solvent mixture A/B = 0/100 v/v.

Zeta potential and dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Marvern Instruments, Malvern, UK) with a He−Ne laser (633 nm). Non-invasive backscatter method (detection at 173° scattering angle) was used. Correlation data were fitted, using the method of cumulants, to the logarithm of the correlation function, yielding the diffusion coefficient, D. The hydrodynamic diameters (D_H) of the BLs and PCNs were calculated using D and the Stokes−Einstein equation ($D_H = k_BT/3\pi\eta D$, where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity (η = 0.8872 cP for water)). The polydispersity index (PDI) of liposomes—represented as $2c/b^2$, where b and c are first- and second-order coefficients, respectively, in a polynomial of a semilog correlation function—was calculated by the cumulants analysis. Size distribution of vesicles was obtained by the non-negative least-squares (NNLS) analysis.[69] Unless noted otherwise, all samples were dispersed in 10 mM HEPES solution (pH 7.4, 150 mM NaCl) for DLS measurements. The data reported represent an average of ten measurements with five scans each.

Synthesis of Alkyne-Modified Diamine Cross-Linker (2-(2-(2-(2-Aminoethoxy)ethoxy)-acetamido)-*N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)pent-4-ynamide) (Scheme 2). The alkyne-modified cross-linker was synthesized using a solid-phase methodology on *O*-bis(aminoethyl)ethylene glycol trityl resin and a fluorenylmethoxycarbonyl (Fmoc)-based double-coupling strategy (Scheme 2) on a CS-Bio CS136 peptide synthesizer (Menlo Park, CA). N_α-Fmoc-L-2-propargylglycine was first coupled to the resin mediated by HBTU in DMF. After deprotection of the Fmoc carbamate group (by the addition of 20% v/v piperidine (PIP) in DMF), subsequent coupling of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid with HBTU was carried out. The synthesized cross-linker was detached from the resin using trifluoroacetic acid (TFA) and purified by preparative reverse-phase HPLC using method 2 (see measurements). The final Fmoc group was not removed so that it can serve as a UV−vis tag in further analyses (see below). IR(CH_2Cl_2): 2934, 1682, 1539, 1203, 1136, 837, 800, 721 cm^{-1}. ESIMS: m/z = 389.92 observed for M^{2+}, 388.23 calculated.

Preparation of Alkyne-Modified, Doxorubicin-Loaded Polymer-Caged Nanobins. Doxorubicin-loaded bare liposome was prepared using a modified literature procedure.[37] To a cylindrical glass vial (15 mm × 45 mm) was added DPPC (18.048 μmol), DOPG (1.152 μmol), and cholesterol (12.8 μmol, 40 mol % of the total membrane components; this number is chosen to eliminate the thermal instability of the liposomes that is attributable to the intrinsic phase-transition temperature of the lipid[70,71]), followed by chloroform (0.5 mL) to make a colorless solution. After vortexing (30 s), the solvent was removed by passing a stream of nitrogen over the solution while the vial was warmed in a 50 °C water bath. The resulting dry film was further dried under vacuum on a Schlenk line (<30 mTorr) for one hour. Next, the dry lipid films were hydrated in 300 mM aqueous ammonium sulfate solution (500 μL) followed by vigorous vortexing (3−5 min on a Vortex Mixer, American Scientific Products) to form a dispersion of multilamellar vesicles. After this dispersion was subjected to 10 freeze−thaw cycles, it was extruded ten times through two stacked polycarbonate extrusion membranes (100-nm pore-size) that are maintained at 50 °C in a mini-extruder (Avanti Polar Lipids). The excess ammonium sulfate outside liposome was removed by Sephadex G-50 (10 mL) gel-filtration chromatography pre-equilibrated with 150 mM NaCl solution. To the collected liposome solution (~600−800 μL of a solution with 4 mM lipid concentration) was added doxorubicin (DXR, 0.35 equiv of the total lipid content) followed by incubation at 50 °C for 24 h. The excess DXR outside of the liposome was then removed by Dowex 50WX4 cation-exchange resin.

The loading of the DXR was determined by breaking up the DXR-loaded liposome in a 75-mM HCl solution in 90% 2-propanol and measuring the dissolved doxorubicin concentration using UV−vis spectroscopy based on the extinction coefficient (ϵ) of DXR (11207 $M^{-1}cm^{-1}$ at λ_{max} = 480 nm). Mean hydrodynamic diameter (D_H) of 99 ± 17 nm was determined by DLS measurements (Figure S1 in the Supporting Information). The DXR-loaded bare liposomes (BL_{DXR}) is next subjected to the PCN fabrication process as reported previously.[8] For this process, 10 mol % of the Chol-PAA modifier was chosen to maximize the amount of the modifier while preventing local phase-segregation of all the cholesterol in the membrane. Additionally, 50% of acrylate repeating units in Chol-PAA chains were cross-linked with alkyne-modified diamine cross-linker (25 mol %; if >35 mol % of the cross-linker is used, precipitation will occur, presumably due to interparticle cross-linkings). Mean D_H of 124 ± 21 nm was determined by DLS measurements (Figure S1 in the Supporting Information). The resulting alkyne-modified, DXR-loaded PCN (PCN_{DXR}) can then be used directly in the conjugation with azido-PEG-folate (see below).

DXR-Release Assay under Various pH Conditions (Figure 1). Solutions of BL_{DXR}, PCN_{DXR}, and f-PCN_{DXR} (1 mM of lipids in 20 mM acetate buffer (pH 5.0, 150 mM NaCl), 20 mM MES buffer (pH 6.0, 150 mM NaCl), and 20 mM HEPES buffer (pH 7.4, 150 mM NaCl)) were incubated in a 2-mL Quarz SUPRASIL fluorescence cell (Hellma Cells Inc., Plainview, NY) at either 37 or 25 °C with magnetic stirring. The fluorescence from the liposome-encapsulated DXR was self-quenched due to its high concentration inside the liposome.[39] Hence, only the fluorescence from the DXR that has released out of the liposome was measured as a function of incubation time. Afterward, 5% aqueous Triton X-100 (reduced form, 5.0 μL) was added to totally break up the liposomes and the final DXR fluorescence was measured to give the 100% release value. The extent of release was observed by comparing to the

(69) Stock, R. S.; Ray, W. H. *J. Polym. Sci. Pt. B: Polym. Phys.* **1985**, *23*, 1393–1447.
(70) Hinz, H.-J.; Sturtevant, J. M. *J. Biol. Chem.* **1972**, *247*, 3697–3700.
(71) McMullen, T. P.; Lewis, R. N.; McElhaney, R. N. *Biophys. J.* **1994**, *66*, 741–752.

maximum release value determined by addition of 5% aqueous Triton X-100 (reduced form, 5.0 μL).[8]

Conjugation of Azido-Ethidium to Alkyne-Modified PCN by Click Chemistry (Figure S5 in Supporting Information). Due to the duplication of fluorescence spectra between ethidium and DXR, empty PCNs were used in this experiment. To a solution containing the alkyne-modified PCNs (1.0 mL of a 10.642 mM solution), ethidium bromide monoazide (2 mg, 4.758 μmol), $CuSO_4 \cdot 5H_2O$ (2 mM), and a freshly prepared sodium ascorbate solution (2.0 mg, 10 μmol) was added. The reaction mixture was wrapped with aluminum foil and stirred at room temperature for 5 h in the dark. The resulting ethidium-conjugated PCN_{DXR} solution was purified by Sephadex G-50 gel-filtration chromatography that has been pre-equilibrated with HEPES buffer (20 mM; [NaCl] = 150 mM, pH 7.4) (10 mL total volume for the equilibrated stationary phase). The fluorescent spectrum of the isolated product was then obtained to determine the extent of conjugation. As a control experiment, the same conjugation described above was carried out without Cu catalyst.

Synthesis of the Azido-PEG-folate Targeting Ligand. Azido-PEG-folate ligand was synthesized by reacting *O*-(2-aminoethyl)-*O*′-(2-azidoethyl)octaethylene glycol (456.1 μmol, Aldrich) with folic acid (547.3 μmol, Aldrich) in a dimethylsulfoxide (DMSO) solution (5 mL) containing dicyclohexylcarbodiimide (DCC, 547.3 μmol) and 4-(dimethylamino)pyridine (DMAP, 45.6 μmol). The reaction mixture was stirred overnight in the dark at room temperature during which time dicyclohexylurea formed as a precipitate. After the urea byproduct was removed by filtration, the product was precipitated from the reaction mixture by addition of an excess amount of cold diethyl ether. The precipitated crude product was purified by silica gel flash chromatography, eluting with a stepwise gradient of methanol (20−80% v/v) in chloroform that has been modified with triethylamine (0.1% v/v). The solid product was further purified by preparative reverse-phase HPLC using method 1. In HPLC analysis, two different α- and γ-modified folate isomers were observed (in the ratio of 46 and 54%) and both were used as targeting ligands without further separation due to the well-known targeting ability of both isomers to FR-positive cells.[27,46] IR(KBr): 3295, 2114, 1697, 1609, 1514, 1304, 1109 cm^{-1}. ESI-MS: m/z = 862.46 observed; 862.40 calculated. The concentration of azido-PEG folate ligand was determined by quantitative UV−vis spectroscopy in water based on the extinction coefficient (ϵ) of folic acid (27022 $M^{-1}cm^{-1}$ at λ_{max} = 278 nm).

Conditions for the Click Reaction between Azido-PEG-folate Ligand and Alkyne-Modified Diamine Cross-Linker (Scheme 3). In the search for conditions to carry out the 'click' reaction between the azido-PEG-folate reagent and the alkyne-modified diamine cross-linker, the Fmoc-protected diamine cross-linker was used without removal of protecting group so that it can serve as a UV−vis tag in HPLC analysis (ϵ = 14246 $M^{-1}cm^{-1}$ at λ_{max} = 263 nm). To a solution containing the alkyne-modified diamine cross-linker (1.146 μmol), azido-PEG-folate (1.146 μmol), and $CuSO_4 \cdot 5H_2O$ (2 mM) in 20 mM HEPES buffer (500 μL, pH 7.4, [NaCl] = 150 mM) was added a freshly prepared sodium ascorbate solution (0.5 mL of a 300 mM solution; final concentration of ascorbate is 50 mM in water). The resulting mixture was stirred at room temperature for 12 h before being filtered through a syringe filter. The collected filtrate was analyzed by reverse-phase HPLC (method 1, Figure 2a), MALDI-TOF MS (Figure 2b), and FTIR (Figure S3 in Supporting Information), which show complete formation of the desired product.

Conjugation of Azido-PEG-folate Ligand to Alkyne-Modified PCN_{DXR} by Click Chemistry (Preparation of f-PCN_{DXR}). To a solution containing the alkyne-modified PCN_{DXR} (570 μL of a 2.169-mM solution), azido-PEG folate ligand (24.72 nmol, ∼1 mol % of the total lipid content), $CuSO_4 \cdot 5H_2O$ (2 mM), and a freshly prepared sodium ascorbate solution (1.2 mg in 300 μL of water, 6.18 μmol) was added and the reaction mixture was stirred at room temperature for 12 h. The resulting folate-conjugated PCN_{DXR} solution was purified by Sephadex G-50 (10 mL) gel-filtration chromatography that has been pre-equilibrated with HEPES buffer (20 mM; [NaCl] = 150 mM, pH 7.4). To determine the final concentration of DXR in the as-prepared f-PCN_{DXR}, a small amount of this solution was first broken up in 20-mM HEPES buffer solution (pH 7.4, 150 mM NaCl) including reduced Triton X-100 (∼5 μL of a 5 vol% aqueous solution). The dissolved doxorubicin concentration was then measured using UV−vis spectroscopy based on the extinction coefficient (ϵ) of free DXR at known isosbestic point[72] (3530.6 $M^{-1}cm^{-1}$ at λ = 543 nm).

To determine the yield of the click reaction, the same conjugation described above was carried out with alkyne-modified empty PCNs (vide supra). This was necessary to avoid the overlapping UV−vis absorbances between DXR and folate in f-PCN_{DXR}. The yield of the conjugation with folate was determined by quantitative UV−vis spectroscopy based on the extinction coefficient (ϵ) of folic acid (27022 $M^{-1}cm^{-1}$ at λ_{max} = 278 nm, Figure S4 in Supporting Information).

Cell Culture. a. Medium. Folate-free Roswell Park Memorial Institute (RPMI)-1640 cell culture medium (containing L-glutamine and phenol red) and trypsin solution (0.25%, containing EDTA) was purchased from Invitrogen (San Diego, CA). Penicillin-Streptomycin, L-glutamine, Fungizone, and phosphate-buffered saline (PBS, 1X without calcium and magnesium, Catalog # 21−040) solutions were purchased from Mediatech (Manassas, VA). Eagle's Minimum Essential Medium (EMEM) was purchased from ATCC (Manassas, VA).

b. Cell Lines. Human epithelial nasopharyngeal carcinoma (KB) cells and epithelial ovarian carcinoma (OvCa432) cells were continuously cultured in folate-free RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 0.5% (v/v) Penicillin-Streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2. Human mammary carcinoma (MCF7) cells were continuously cultured in EMEM supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin solution, 1% (v/v) L-glutamine solution, and 1% (v/v) Fungizone solution at 37 °C in a humidified atmosphere containing 5% CO_2.

Confocal Laser Scanning Microscopy (CLSM) Study (Figures 3 and 4). KB and OvCa432 cells were planted in 12-well plate containing a sterilized glass coverslip (2 mL/well, 25 kcell/mL) for 24 h at 37 °C and 5% CO_2 before each experiment. Each cell was incubated with the appropriate drug formulation in folate-free medium (500 μL, [DXR] = 20 μM) for 2 or 4 h at 37 °C and 5% CO_2. After the removal of drug-containing medium, the cell layers were washed with cold PBS (4 × 2 mL) and fixed with PBS-buffered 4% formaldehyde for 5 min, then washed with PBS (3 × 2 mL). Next, the coverslips were placed on slides (Superfrost plus, 25 × 75 × 1 mm, VWR) coated with PBS (50 μL). CLSM images were observed with inverted confocal laser scanning microscope (Carl Zeiss LSM 510 META) with excitation at 488 nm. At this wavelength, no observable background fluorescence was detected from the cell lines (Figure S6 in Supporting Information). A water immersion objective, C-Apochromat 40×/1.20 W korr. UV−vis-IR M27 was used. Obtained images were converted to TIFF format by using ZEN 2007 Light Edition SP1 software (Carl Zeiss).

Cytotoxicity Assays (Figure 5). The cells were seeded in 48-well plates (200 μL/well) with a concentration of 100 000 cells/mL in folate-free RPMI-1640 medium and were incubated to grow for 24 h. The media in the wells were replaced with the preprepared growth media containing the appropriate drug formulation (200 μL of solution at the appropriate doxorubicin concentrations). For exposure regime 1, the drug-treated cells were then further incubated for 2 h in a humidified atmosphere containing 5% CO_2 at 37 °C, after which the drug-containing media were removed by aspiration. The remaining cell layers were washed with PBS buffer (2 × 250

(72) Chaires, J. B.; Dattagupta, N.; Crothers, D. M. *Biochemistry* **1982**, *21*, 3927–3932.

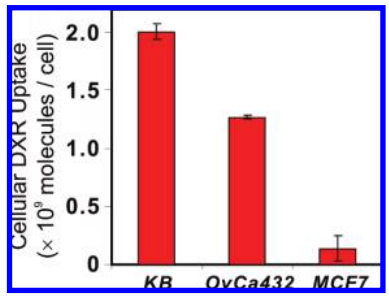


Figure 6. Relative comparison of folate receptor levels in KB, OvCa432, and MCF7 cells expressed in terms of folate-mediated DXR (dosed in the form of f-PCN_{DXR}) uptake.

μL) followed by replacement with fresh growth media (200 μL). The plates were then maintained in the incubator at 37 °C for a further 46 h. For exposure regime 2, the drug-treated cells were incubated for 48 h in a humidified atmosphere containing 5% CO_2 at 37 °C, after which the cells were washed with PBS buffer (2 × 250 μL).

The viable and nonviable cells from cultures were differentially stained with Guava ViaCount reagent (Guava Technologies, Hayward, CA) which contains two fluorescence dyes (a cell membrane-permeable dye and an impermeable dye except for the cells that have lost membrane integrity), allowing assessment of the viable, dead, and midapoptotic cells.[58] Then the absolute number of viable cells was counted by a Guava EasyCyte Mini flow cytometer[59] and the relative cell survival percentages compared to the drug-free control were plotted against the total DXR concentration in logarithmic scale. The data reported represent an average of five measurements from different batches. The dose−response curves were obtained by sigmoidal logistic fitting using *Origin 6.1*[73] and the half-maximal inhibitory concentration (IC_{50}) values were determined on the basis of the fitted data.

Relative Determination of Folate Receptor Levels in Cell Lines via Quantification of f-PCN_{DXR} Uptake (Figure 6). KB, OvCa432, and MCF7 cells were plated in two 12-well plates for 24 h before experiment (2 mL/well, 250 kcells/mL, plate 1 for quantification of DXR and plate 2 for cell counting). Cells were exposed to f-PCN_{DXR} ([DXR] = 10 μM) for 2 h and washed with cold PBS buffer (3 × 2 mL). After trypsinized, each cell line was washed with cold PBS (2 mL/well) and pelleted by centrifugation (1500 rpm (500 *g*, Beckman-Coulter Allegra 6R Centrifuge), 4 °C, 5 min). After removing the supernatant, cells were resuspended in cold (4 °C) PBS solution (2 mL) followed by centrifugation. After washing by suspension/centrifugation process three times, cells from plate 1 were solubilized by adding an aqueous solution of Triton-X 100 (~10 μL or more of a 5 vol% solution until the cells are completely dissolved) and diluted to 200 μL total volume with EDTA-modified HEPES buffer (20 mM; [NaCl] = 150 mM, [EDTA] = 1 mM, pH 7.4). The cell-associated DXR fluorescence was measured (λ_{ex} = 478 nm, λ_{em} = 580 nm, slit = 14 nm) and the amount of DXR was calculated using a predetermined calibration curve. The numbers of DXR molecules per cell were determined against the total number of cells obtained with Guava ViaCount Assay[59] using the samples from plate 2.

(73) http://www.originlab.com/index.aspx?s=9&lm=115&pid=106 (Access Date: Feb. 23, 2009).

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Supporting Information Available: DLS data of PCN_{DXR} and BL_{DXR}, time-dependent DXR-release profile of BL_{DXR}, pH-dependent apparent DXR-release rates, FTIR spectra, UV−vis spectra, click-conjugation experiments of azido-ethidium dye to empty PCNs (scheme and fluorescence spectra) and background fluorescence and bright-field transmission confocal laser-scanning fluorescence microscopy images of KB and OvCa432 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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