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pH-Sensitive, *N*-ethoxybenzylimidazole (NEBI) bifunctional crosslinkers enable triggered release of therapeutics from drug delivery carriers†

Alice Luong, Tawny Issarapanichkit, Seong Deok Kong, Rina Fong and Jerry Yang*

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This paper presents a pH-sensitive bifunctional crosslinker that enables facile conjugation of small molecule therapeutics to macromolecular carriers for use in drug delivery systems. This *N*-ethoxybenzylimidazole (NEBI) bifunctional crosslinker was designed to exploit mildly acidic, subcellular environments to trigger the release of therapeutics upon internalization in cells. We demonstrate that an analog of doxorubicin (a representative example of an anticancer therapeutic) conjugated to human serum albumin (HSA, a representative example of a macromolecular carrier) *via* this NEBI crosslinker can internalize and localize into acidic lysosomes of ovarian cancer cells. Fluorescence imaging and cell viability studies demonstrate that the HSA-NEBI-doxorubicin conjugate exhibited improved uptake and cytotoxic activity compared to the unconjugated doxorubicin analog. The pH-sensitive NEBI group was also shown to be relatively stable to biologically-relevant metal Lewis acids and to serum proteins, supporting that these bifunctional crosslinkers may be useful for constructing drug delivery systems that will be stable in biological fluids such as blood.

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Introduction

Bifunctional crosslinkers are widely used for modification of natural and synthetic macromolecules.[1,2] A missing tool in bioconjugation chemistry, however, is a bifunctional crosslinker that exhibits controlled hydrolytic properties in mild acidic environments. Such crosslinkers could be useful for generating novel acid-responsive materials for a range of applications including drug delivery. A common characteristic of many macromolecular- or nanoparticle-based drug delivery systems (DDSs), for instance, is the rapid internalization and intracellular localization of the DDS into acidic endosomes (pH 5.5–6.0) and lysosomes (pH 4.5–5.0) of cells upon arrival to the targeted tissue.[3,4] Due to this common pathway of cellular uptake, there has been significant interest in methods to engineer acid-triggered responses from DDSs for rapid intracellular release of therapeutics.[4–15] Acid-sensitive groups, for instance, have been previously explored for covalent conjugation and controlled release of specific drugs from macromolecular carriers for various drug delivery applications.[12,16–24] Although many previously reported acid-sensitive groups exhibit accelerated hydrolysis in acidic solutions, only hydrazones have thus far made it into clinical applications in an FDA-approved drug as a pH-sensitive linker for conjugating a hydrazine-containing drug to a ketone-containing antibody.[25,26] In order to broaden the use of acid-sensitive linkers in DDSs, several challenges remain to be addressed including: (1) stability of the linker in biological fluids, (2) synthetic accessibility of DDSs comprising acid-cleavable linkers, and (3) capability of using the linkers for conjugating a broad range of therapeutics to drug delivery carriers under mild, biocompatible conditions.

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, MC 0358, La Jolla, CA, 92093-0358, USA. E-mail: jerryyang@ucsd.edu; Fax: (+1) 858-534-4554; Tel: (+1) 858-534-6006

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Here, we evaluate a novel, acid-sensitive, bifunctional crosslinker, based on an *N*-ethoxybenzylimidazole[27] (NEBI) platform, for conjugation of drugs to a protein carrier. We designed this bifunctional NEBI crosslinker (**1**, Fig. 1A) on one side to carry a carboxylic acid functionality for conjugation to amine- or alcohol-containing drugs through standard amidation or esterification conditions,[28,29] and on the other side to carry an azide functionality for, *e.g.*, conjugation to macromolecules through "click" reactions.[30,31] We evaluated the utility of this acid-sensitive linker in DDSs by analyzing the uptake and activity of an analog of doxorubicin (an FDA-approved cancer therapeutic[32]) on human ovarian cancer cells, when the doxorubicin analog was conjugated to human serum albumin (HSA). In this work, we used HSA as a model drug delivery carrier since this protein is known to enter cancer cells through a endocytotic mechanism.[33] HSA is also currently used as an FDA-approved drug carrier for paclitaxel for the treatment of breast cancer, and therefore is an excellent model for these studies.[34]

Results and discussion

Fig. 1 shows the structure of an acid-sensitive bifunctional crosslinker (**1**) derived from a NEBI platform. In previous work, we demonstrated that this class of molecules undergoes accelerated hydrolysis in mild acid (*i.e.*, at pH 5.5) compared to solutions at physiological pH of 7.4.[27] Fig. 1 outlines the procedure used to conjugate doxorubicin to HSA *via* NEBI linker **1**. We incorporated alkyne functionalities on the lysines of HSA by reacting the NHS ester of 4-pentynoic acid with HSA in 0.1 M HEPES buffer (pH 8.3)[35] to afford HSA-alkyne **2**.[36] After coupling of the amine of doxorubicin to the carboxyl functionality on **1** using standard amide coupling conditions, we reacted this NEBI-doxorubicin conjugate with **2** under copper-mediated "click" conditions to afford HSA-NEBI-doxorubicin conjugate **3**. This procedure resulted in a loading of ~1 doxorubicin molecule

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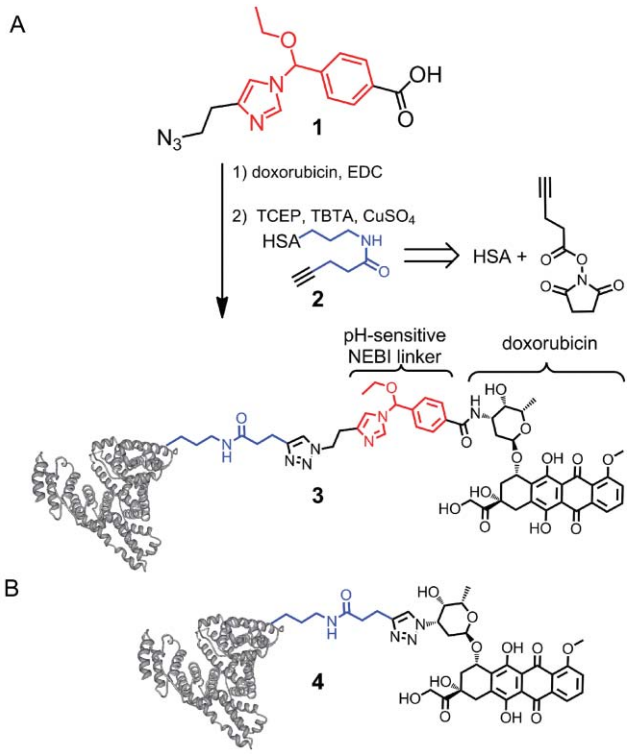

Fig. 1 Structure of a pH-sensitive bifunctional crosslinker and of two doxorubicin-human serum albumin (HSA) conjugates. (A) Synthetic scheme for the synthesis of HSA-doxorubicin conjugate **3** comprising a pH-sensitive NEBI crosslinker **1**. (B) Structure of an HSA–doxorubicin conjugate **4** comprising acid-stable amide and triazole groups. EDC = 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, TCEP = tris(2-carboxyethyl)phosphine, TBTA = tris(benzyltriazolylmethyl)amine.

per HSA protein (see the ESI† for details of the synthesis and characterization of **3**). In order to provide a comparison for the utility of acid-sensitive linker **1** for drug delivery applications, we also synthesized an HSA-doxorubicin conjugate **4** (Fig. 1B), which did not comprise an acid-labile group.

Previous studies showed that a NEBI group carrying doxorubicin **5** hydrolyzes in mildly acidic solutions to produce doxorubicin analog **6** (Fig. 2), with a half-life at pH 5.5 of 55 h at 37 °C.[27] At pH 7.4, however, **5** hydrolyzed with a much slower half-life of 1150 h at 37 °C. These results suggest that doxorubicin molecules conjugated to drug delivery carriers *via* NEBI linkers would hydrolyze with accelerated rates inside of cells if these doxorubicin-NEBI conjugates are internalized and localized within the acidic compartments of the cells.

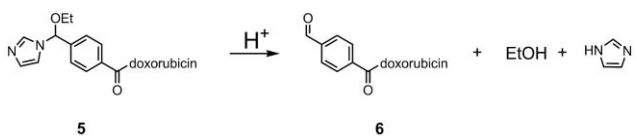

Fig. 2 Schematic illustration for the hydrolysis of NEBI-doxorubicin conjugate **5** in mildly acidic solutions.

To determine whether the HSA-NEBI-conjugate **3** could internalize and localize within acidic compartments of cells, we examined the uptake and subcellular localization of **3** in human ovarian cancer cells. Fig. 3B illustrates that incubation of cells with **3** (red) for 24 h resulted in significant cellular uptake. Subsequent treatment of these cells with Lysotracker Blue revealed the location of the lysosomes (blue regions in Fig. 3C). Fig. 3D shows that a significant fraction of **3** co-localized (green) with Lysotracker Blue in these cells, suggesting that **3** indeed localized primarily within the lysosomes of these cells. This result is in agreement with previous reports for the uptake of HSA into the cells *via* an endocytotic mechanism.[33] Since these imaging studies confirm that HSA-NEBI-doxorubicin conjugate **3** internalizes and localizes in acidic compartments of cancer cells, and since NEBI groups exhibit accelerated hydrolysis in mild acidic solutions,[27] we hypothesized that incubation of these cells with **3** will result in intracellular release of doxorubicin analog **6**, resulting in reduced viability of the cells.

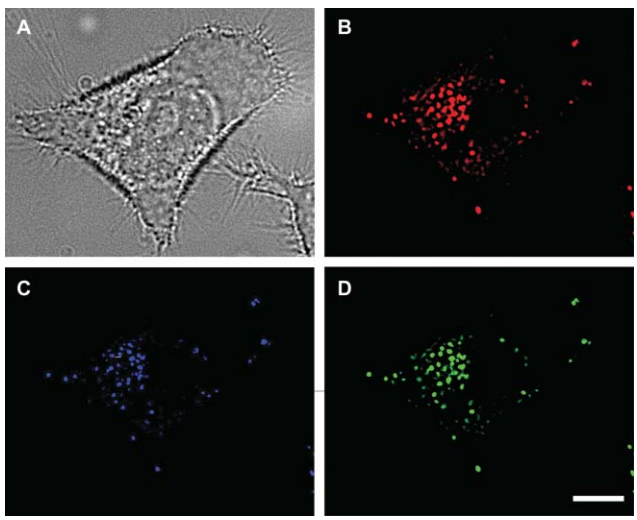

Fig. 3 Fluorescence imaging studies for the internalization and localization of HSA-NEBI-doxorubicin conjugate **3** in human ovarian carcinoma 2008 cells. (A) Differential interference contrast image of a human ovarian cancer 2008 cell. (B) Fluorescence micrograph from a z-slice through the cell showing the location of **3** (red) inside the cell. The subcellular distribution of **3** was determined by monitoring the intrinsic fluorescence of doxorubicin. (C) Fluorescence micrograph from the same z-slice through the cells as shown in (B), except showing only the location of the lysosomes, which were stained with Lyostracker blue. (D) A merged fluorescence micrograph of (B) and (C) indicating areas of co-localization of **3** and the Lysotracker Blue. Scale bar = 5 μm.

In order to assess whether **3** exhibits cytotoxic activity to cancer cells, we determined the viability of human ovarian carcinoma 2008 cells [38] that were exposed to HSA-NEBI-doxorubicin conjugate **3**. Indeed exposure of cells to increasing concentrations of **3** resulted in reduced cell viability with an IC_{50} of 1.6 μM (Fig. 4A).[39] As expected, control experiments revealed that native HSA and HSA-alkyne conjugate **2** were not toxic to cells within the same protein concentration range (Fig. 4B). Additionally, Fig. 4A shows that conjugate **3** was ~4 times more toxic than doxorubicin analog **6** alone [40,41] (*i.e.*, the doxorubicin analog expected to accumulate in cells after hydrolysis of the NEBI group, IC_{50} = 5.8 μM). The HSA-NEBI-doxorubicin conjugate **3** was also 10 times more potent than HSA-doxorubicin conjugate **4** (IC_{50} = 16 μM), which lacked a pH-sensitive linker.[42] These results from cytotoxicity experiments demonstrated that a drug conjugate comprising

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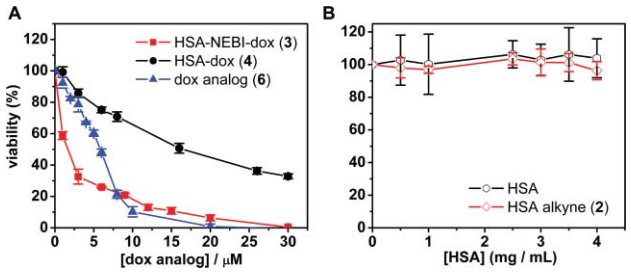


Fig. 4 Cytotoxicity studies of HSA, HSA conjugates, and doxorubicin analog **6** on human ovarian carcinoma 2008 cells. (A) Graph of the percent viability of cells upon exposure to HSA-NEBI-doxorubicin **3** (IC_{50} = 1.6 μM), HSA-doxorubicin conjugate **4** (IC_{50} = 16 μM), and doxorubicin analog **6** (IC_{50} = 5.8 μM). (B) Control experiments showing the viability of cells upon exposure to HSA or HSA-alkyne **2**. In all experiments, cells were exposed to the samples for 3 days prior to analysis for viability using a standard SRB cell viability assay[37] and compared to the viability of untreated cells.

pH-sensitive NEBI linkers was more potent compared to a conjugate that did not comprise an acid-labile group (**4**) or compared to free **6** alone.

We hypothesized that the observed improved toxicity of HSA-NEBI-doxorubicin **3** compared to free **6** was due to the capability of **3** to exhibit improved uptake of doxorubicin in the cells through an HSA-mediated, endocytotic process.[33] To support this hypothesis, we incubated the ovarian cancer cells with conjugate **3** or doxorubicin analog **6** (with an equal concentration of total doxorubicin in both experiments). After 3 h,[43] the cells were washed to remove excess extracellular molecule **3** or **6** and the cells were examined for the uptake of doxorubicin by deconvolution microscopy (Fig. 5A and B). This analysis revealed that cells incubated with conjugate **3** had an average of ~5.5 times the amount of doxorubicin within the cells compared to cells incubated with molecule **6** (Fig. 5C). This increased uptake of

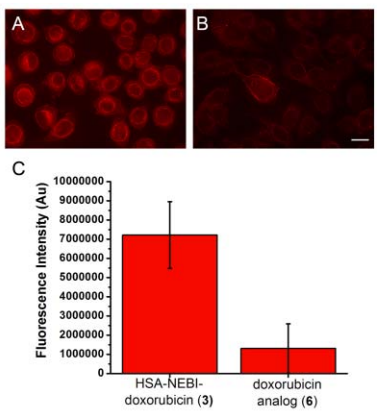
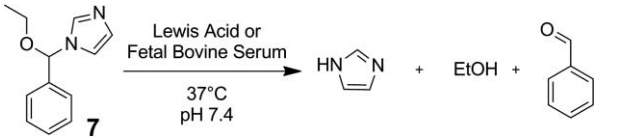


Fig. 5 Analysis of the uptake of HSA-NEBI-doxorubicin (**3**) and doxorubicin analog (**6**) by human ovarian carcinoma 2008 cells. (A) and (B) are micrographs of representative z-slices through cells that were incubated with molecule **3** (A) or molecule **6** (B) for 3 h. Here, the intrinsic fluorescence of doxorubicin is shown in red. Scale bar = 15 μm. (C) The bar graph indicates that there is approximately 5.5 times the amount of doxorubicin in the cells incubated with molecule **3** compared to cells incubated with molecule **6**. Average total fluorescence per cell was determined from analysis of 10 cells in each sample image. Analysis of the data in (C) revealed a p-value of 3.2×10^{-9}.

Table 1 Hydrolysis of NEBI groups in buffered solutions containing biologically-relevant Lewis acids or FBS



Additive	Total concentration in blood/mM	k/h^{-1}	k_{rel}
None	N/A	0.0211	1
Zn(II)	0.107	0.0241	1.14
Cu(II)	0.016	0.0249	1.18
Mg(II)	1.56	0.0222	1.05
Fe(III)[a]	8.00	0.0228	1.08
Ca(II)	1.51	0.0238	1.13
Na(I)	85.6	0.0222	1.13
K(I)	41.5	0.0221	1.05
10% Serum	N/A	0.0221	1.05

[a] [$FeCl_3$] used in these experiments was 0.01 mM due to solubility limitations of $FeCl_3$ in buffer.

doxorubicin conjugated with HSA *via* the NEBI linker correlated well with the observed increased toxicity of conjugate **3** compared to doxorubicin analog **6** (Fig. 4A). Additionally, to attempt to gain some insight for the origin of the observed toxicity from conjugate **3**, we incubated the ovarian carcinoma 2008 cells with **3** for 72 h. The cell lysate was then analyzed by LC-MS. This analysis revealed a complex mixture of doxorubicin-derived species, with at least three known metabolites[44,45] of doxorubicin identified by MS (see Fig. S3 in the ESI† for details).[46] These combined results support that conjugating doxorubicin to an HSA carrier *via* the pH-sensitive NEBI crosslinker can result in improved uptake and toxicity compared to unconjugated molecule, with cytotoxic activity originating from the release of active derivatives of doxorubicin in the cells.

Finally, since it is important for a pH-sensitive linker in DDSs to be stable in biological fluids, we examined the hydrolysis of the parent NEBI (**7**) in solutions containing biologically relevant factors (other than protons) that are present in blood. For instance, since protons are Lewis acids that catalyze the hydrolysis of NEBI groups in solution (Fig. 2),[27] we tested the hydrolytic stability of NEBI groups in the presence of common metal Lewis acids found in the body. Table 1 shows the measured rate constants (k) for the hydrolysis of **7** in buffered solutions (pH 7.4, 37 °C) containing various concentrations of biologically-relevant metal Lewis acids.[47] In these studies, we used metal ion concentrations corresponding to their reported total concentration in blood,[48] although it is expected that the concentrations of free metal ions in the blood are much lower than these values since the metal ions are likely associated with various biomolecules under physiological settings. These hydrolysis studies revealed that the metal Lewis acids at these high concentrations did not significantly affect the rate of hydrolysis of **7** relative to hydrolysis in the absence of these metal ions (k_{rel}). Table 1 also shows that the rate constant for hydrolysis of **7** in a solution containing 10% fetal bovine serum (FBS) and 90% phosphate buffered saline (PBS, pH 7.4, 37 °C) remained relatively unchanged compared to hydrolysis of **7** in solutions of pure buffer. These results collectively demonstrate that pH-sensitive NEBI groups are relatively stable to biological fluids such as blood.

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Conclusion

This work demonstrates that a new pH-sensitive, bifunctional NEBI crosslinker (**1**) can be employed for facile modification of macromolecules with small molecules. Here, we present one application for such an acid-sensitive crosslinker by demonstrating its use for conjugating an anticancer agent (here, doxorubicin analog **6**) to a protein-based drug carrier (here, HSA). The resulting DDS (**3**) exhibited improved cellular uptake and cytotoxic activity in cancer cells compared to free molecule **6** alone. Conjugate **3** also exhibited 10-fold improved cytotoxic activity compared to a conjugate that did not comprise an acid-labile group (**4**), supporting that the cleavable, pH-sensitive NEBI group can have a strong influence on the potency of therapeutics conjugated to HSA. Although several factors may contribute to the observed cytotoxic activity of **3** in ovarian cancer cells (Fig. 4A), the results presented here are consistent with the hypothesis that the activity of **3** may be due to the accelerated hydrolysis of the NEBI crosslinker upon uptake and intracellular localization of **3** in the acidic lysosomes of cancer cells.

Bifunctional crosslinkers based on NEBI groups such as **1** possess several attractive features as a tool for generating acid-responsive materials. For instance, they afford facile methods to attach a broad range of amine- and alcohol-containing molecules to macromolecules through standard amide or ester bond-forming reactions.[12,28,29,49–51] Additionally, the ability to incorporate functional groups on NEBI crosslinkers with orthogonal reactivity to amide or ester bond-forming reactions (*e.g.*, alkyne or azide functional groups for "click" reactions) facilitates conjugation of small molecules to a range of natural and synthetic macromolecules. Moreover, the previously reported capability to tune the rates of hydrolysis of NEBI groups under mild aqueous acidic conditions[27] makes it possible to optimize the rates of release of small molecules for specialized applications. Finally, these bifunctional NEBI crosslinkers are attractive since they are synthetically accessible from readily available and cheap starting materials. These advantageous features make it possible, for instance, to rapidly synthesize a range of DDSs carrying a variety of therapeutic agents that can be released from drug delivery carriers at controlled rates within acidic compartments of targeted cells.

Experimentals

Measurement of cellular uptake and subcellular localization of 3 by fluorescence microscopy

Human ovarian carcinoma 2008 cells were plated with Roswell Park Memorial Institute-1640 (RPMI-1640) phenol red free media supplemented with 10% fetal bovine serum (FBS) on 35 mm glass bottom dishes (MatTek Co., Ashland, MA) and incubated overnight. A solution containing 10 μM of HSA-NEBI-doxorubicin (**3**) was added to the cells and allowed to incubate for 24 h. Lysotracker Blue (Invitrogen, L7525) was added to the cells and incubated for 30 min. The remaining living cells were washed three times with phenol red free RPMI-1640 media without FBS and then immediately imaged with a Delta Vision Deconvolution Microscope System (Applied Precision, Issaquah, WA).

Cytotoxicity studies

Human ovarian carcinoma 2008 cells were plated into each well of a 96 well plate (3000 cells/well) using RPMI-1640 media with 10% FBS. The cells were incubated for 24 h at 37 °C with 5% CO_2. After the incubation period, cells were dosed with various concentrations of HSA, **2–4**, **6**, or 4-carboxybenzaldehyde. The cells were allowed to incubate with the molecules for 72 h. After incubation, cells were washed 3 times with 200 μL of PBS (Mediatech, 46-013CM) pH 7.4. Cells were fixed with 200 μL of PBS and 50 μL of 50% trichloroacetic acid for one hour at 4 °C. After fixation, cells were washed 5 times with water and allowed to dry. A 0.4% sulforhodamine B (SRB, Sigma Aldrich, S1402) solution in 1% acetic acid was added to the cells and incubated for 15 min at room temperature. The cells were washed 3 times with 1% acetic acid and the 96 well plate was allowed to dry. Tris base solution (10 mM, 200 μL) was added to the wells for 15 min prior to measuring the absorbance at 515 nm. All data points represent the UV absorbance of SRB relative to a sample of cells that were fixed at time zero of the incubation period (*i.e.*, 24 h after introduction of cells to the wells of the 96 well plate). Ref. 37 reports a similar protocol for estimating cell viability.

Measurement of hydrolysis of *N*-ethoxybenzylimidazole 7

Solutions containing 0.5 mM *N*-ethoxybenzylimidazole **7** was incubated in 0.1 M HEPES (pH 7.4) buffer and incubated at 37 °C. The hydrolysis of **7** in the presence of various metal Lewis acids was monitored by observing the formation of benzaldehyde product by UV absorbance at 235 nm. For measurement of the hydrolysis of **7** in the presence of FBS, FBS was dialyzed against the solution of 0.5 mM **7** (in 0.1 M PBS buffer, pH 7.4) during the course of the hydrolysis experiment. The dialysis chamber was removed only during measurement of the formation of benzaldehyde product by UV spectroscopy; the dialysis chamber was necessary for these studies since the UV absorption of serum proteins in FBS interfered with the analysis of the hydrolysis of **7**.

Acknowledgements

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Notes and references

1 *Thermo Scientific Pierce Crosslinking Technical Handbook*, Thermo Scientific, 2009.
2 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, 2008.
3 L. A. Bareford and P. W. Swaan, *Adv. Drug Delivery Rev.*, 2007, **59**, 748–758.
4 E. R. Gillies, A. P. Goodwin and J. M. J. Frechet, *Bioconjugate Chem.*, 2004, **15**, 1254–1263.

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5 C. C. Lee, E. R. Gillies, M. E. Fox, S. J. Guillaudeu, J. M. J. Frechet, E. E. Dy and F. C. Szoka, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 16649–16654.
6 K. T. Oh, H. Q. Yin, E. S. Lee and Y. H. Bae, *J. Mater. Chem.*, 2007, **17**, 3987–4001.
7 V. F. Patel, J. N. Hardin, J. M. Mastro, K. L. Law, J. L. Zimmermann, W. J. Ehlhardt, J. M. Woodland and J. J. Starling, *Bioconjugate Chem.*, 1996, **7**, 497–510.
8 W. C. Shen and H. J. P. Ryser, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 1048–1054.
9 M. Guo, Y. Yan, H. Zhang, H. Yan, Y. Cao, K. Liu, S. Wan, J. Huang and W. Yue, *J. Mater. Chem.*, 2008, **18**, 5104–5112.
10 H. Yin and Y. H. Bae, *Eur. J. Pharm. Biopharm.*, 2009, **71**, 223–230.
11 A. Bajaj, B. Samanta, H. Yan, D. J. Jerry and V. M. Rotello, *J. Mater. Chem.*, 2009, **19**, 6328–6331.
12 A. Malugin, P. Kopeckova and J. Kopecek, *J. Controlled Release*, 2007, **124**, 6–10.
13 G. J. Tong, S. C. Hsiao, Z. M. Carrico and M. B. Francis, *J. Am. Chem. Soc.*, 2009, **131**, 11174–11178.
14 E. R. Gillies and J. M. J. Frechet, *Chem. Commun.*, 2003, 1640–1641.
15 R. E. Kohman and S. C. Zimmerman, *Chem. Commun.*, 2009, 794–796.
16 E. M. Bachelder, T. T. Beaudette, K. E. Broaders, J. Dashe and J. M. J. Frechet, *J. Am. Chem. Soc.*, 2008, **130**, 10494–10495.
17 W. M. Choi, P. Kopeckova, T. Minko and J. Kopecek, *J. Bioact. Compat. Pol.*, 1999, **14**, 447–456.
18 T. Etrych, M. Jelinkova, B. Rihova and K. Ulbrich, *J. Controlled Release*, 2001, **73**, 89–102.
19 R. S. Greenfield, T. Kaneko, A. Daues, M. A. Edson, K. A. Fitzgerald, L. J. Olech, J. A. Grattan, G. L. Spitalny and G. R. Braslawsky, *Cancer Res.*, 1990, **50**, 6600–6607.
20 J. Hongrapipat, P. Kopeckova, J. Liu, S. Prakongpan and J. Kopecek, *Mol. Pharmaceutics*, 2008, **5**, 696–709.
21 S. E. Paramonov, E. M. Bachelder, T. T. Beaudette, S. M. Standley, C. C. Lee, J. Dashe and J. M. J. Frechet, *Bioconjugate Chem.*, 2008, **19**, 911–919.
22 X. Shi, S. H. Wang, M. Shen, M. E. Antwerp, X. Chen, C. Li, E. J. Petersen, Q. Huang, W. J. Weber and J. R. Baker, *Biomacromolecules*, 2009, **10**, 1744–1750.
23 M. M. Stevens, S. Allen, M. C. Davies, C. J. Roberts, J. K. Sakata, S. J. B. Tendler, D. A. Tirrell and P. M. Williams, *Biomacromolecules*, 2005, **6**, 1266–1271.
24 P. A. Wender, E. A. Goun, L. R. Jones, T. H. Pillow, J. B. Rothbard, R. Shinde and C. H. Contag, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 10340–10345.
25 P. R. Hamann, L. M. Hinman, I. Hollander, C. F. Beyer, D. Lindh, R. Holcomb, W. Hallett, H.-R. Tsou, J. Upeslacis, D. Shochat, A. Mountain, D. A. Flowers and I. Bernstein, *Bioconjugate Chem.*, 2002, **13**, 47–58.
26 P. D. Senter, *Curr. Opin. Chem. Biol.*, 2009, **13**, 235–244.
27 S. D. Kong, A. Luong, G. Manorek, S. B. Howell and J. Yang, *Bioconjugate Chem.*, 2007, **18**, 293–296.
28 M. K. Dhaon, R. K. Olsen and K. Ramasamy, *J. Org. Chem.*, 1982, **47**, 1962–1965.
29 I. M. Shiina and Ryo, *Heterocycles*, 2008, **76**, 1313–1328.
30 T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, *Org. Lett.*, 2004, **6**, 2853–2855.
31 A. E. Speers and B. F. Cravatt, *Chem. Biol.*, 2004, **11**, 535–546.
32 C. Carvalho, R. X. Santos, S. Cardoso, S. Correia, P. J. Oliveira, M. S. Santos and P. I. Moreira, *Curr. Med. Chem.*, 2009, **16**, 3267–3285.
33 V. Tuan Giam Chuang, U. Kragh-Hansen and M. Otagiri, *Pharm. Res.*, 2002, **19**, 569–577.
34 N. Desai, V. Trieu, Z. W. Yao, L. Louie, S. Ci, A. Yang, C. L. Tao, T. De, B. Beals, D. Dykes, P. Noker, R. Yao, E. Labao, M. Hawkins and P. Soon-Shiong, *Clin. Cancer Res.*, 2006, **12**, 1317–1324.
35 J. Yang, I. Gitlin, V. M. Krishnamurthy, J. A. Vazquez, C. E. Costello and G. M. Whitesides, *J. Am. Chem. Soc.*, 2003, **125**, 12392–12393.
36 Compound **2** represents a mixture of isomers, since there are multiple lysine residues present in HSA.
37 V. Vichai and K. Kirtikara, *Nat. Protoc.*, 2006, **1**, 1112–1116.
38 P. A. Andrews, M. P. Murphy and S. B. Howell, *Cancer Res.*, 1985, **45**, 6250–6253.
39 The IC_{50} values were determined after incubation of cells with HSA or doxorubicin conjugates for 3 days. This incubation time was chosen so that the pH-sensitive NEBI group could hydrolyze to an appreciable extent during these cytotoxicity studies (*i.e.*, more than one half-life according to the data in Fig. 2).
40 The IC_{50} value for compound **6** (5.8 mM) in ovarian carcinoma 2008 cells is different than the IC_{50} value that was previously reported in ref. 27. We attribute the variation in these values to: (1) the experimental differences between the previously used colony formation assay and the SRB viability assay used here, and (2) to difference in the time of exposure of compound **6** to cells.
41 The IC_{50} of native doxorubicin is 8.3 nM using the same cell viability assay.
42 We hypothesize that the observed, weak toxicity of **4** arises from the natural degradation of HSA in the lysosomes, which results in the release of a small amount of some active derivatives of doxorubicin in the cell over the course of the 3 day incubation.
43 We chose a 3 h incubation time for the uptake studies because we required high concentrations (20 μM) of **3** and **6** in these experiments to clearly observe the fluorescence of the doxorubicin conjugates in the cells. Longer incubation times leads to substantial death of cells, which significantly lowers the density of cells within the field of view in the deconvolution microscope.
44 D. J. Taatjes, G. Gaudiano, K. Resing and T. H. Koch, *J. Med. Chem.*, 1996, **39**, 4135–4138.
45 D. J. Taatjes, G. Gaudiano, K. Resing and T. H. Koch, *J. Med. Chem.*, 1997, **40**, 1276–1286.
46 Since benzaldehyde has been reported to have potential adverse effects in cells at millimolar concentrations (A. Ishida, N. Miwa and S. Mizuno, *Cancer Res.*, 1983, **43**, 4216–4220), we investigated whether the benzaldehyde functionality attached to doxorubicin in **6** could account for the observed toxicity of **3**. We found that a 50 mM concentration of 4-carboxybenzaldehyde was not toxic to the ovarian carcinoma 2008 cells (see Fig. S2 in the ESI†), suggesting that the putative formation of a benzaldehyde functionality after intracellular hydrolysis of the NEBI group in **3** was likely not by itself responsible for the observed toxicity of conjugate **3** at the concentrations used for the cell viability experiments.
47 The rate of hydrolysis of compound **7** in the absence of any additive was previously estimated in ref. 27 to be 0.028 h^{-1} by ^1H NMR in buffered D_2O (pD 7.4). To obtain the values for relative rates shown in Table 1, we monitored the change in UV absorption upon hydrolysis of compound **7** in buffered H_2O (pH 7.4) in the presence of various additives. The small difference in the estimated value of the rate constants of compound **7** (here, 0.021 h^{-1} in the absence of any additive) compared to the previous estimate by NMR may reflect differences between these two analytical methods.
48 G. V. Iyengar, W. E. Kollmer and H. J. M. Bowen, *The Elemental Composition of Human Tissue and Bodily Fluids*, Verlag Chemie, New York, 1978.
49 R. C. Larock, *Comprehensive Organic Transformations*, John Wiley & Sons, 1999.
50 D. Yu, P. Peng, S. S. Dharap, Y. Wang, M. Mehlig, P. Chandna, H. Zhao, D. Filpula, K. Yang, V. Borowski, G. Borchard, Z. Zhang and T. Minko, *J. Controlled Release*, 2005, **110**, 90–102.
51 Z. Xie, H. Guan, X. Chen, C. Lu, L. Chen, X. Hu, Q. Shi and X. Jing, *J. Controlled Release*, 2007, **117**, 210–216.