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Acid-Labile Traceless Click Linker for Protein Transduction

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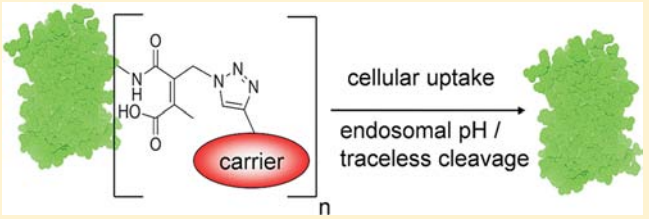
Supporting Information

ABSTRACT: Intracellular delivery of active proteins presents an interesting approach in research and therapy. We created a protein transduction shuttle based on a new traceless click linker that combines the advantages of click reactions with implementation of reversible pH-sensitive bonds. The azidomethyl-methylmaleic anhydride (AzMMMan) linker was found compatible with different click chemistries, demonstrated in bioreversible protein modification with dyes, polyethylene glycol, or a transduction carrier. Linkages were stable at physiological pH but reversible at the mild acidic pH of endosomes or lysosomes. We show that pH-reversible attachment of a defined endosome-destabilizing three-arm oligo(ethane amino)amide carrier generates an effective shuttle for protein delivery. The cargo protein nlsEGFP, when coupled via the traceless AzMMMan linker, experiences efficient cellular uptake and endosomal escape into the cytosol, followed by import into the nucleus. In contrast, irreversible linkage to the same shuttle hampers nuclear delivery of nlsEGFP which after uptake remains trapped in the cytosol. Successful intracellular delivery of bioactive ß-galactosidase as a model enzyme was also demonstrated using the pH-controlled shuttle system.



INTRODUCTION

In recent years click chemistry reactions, especially the copper-catalyzed 1,3-dipolar cycloaddition (CuAAC) and the Staudinger ligation, became useful tools for conjugating biomolecules.[1−4] Both reactions have great advantages compared to other linking strategies, like high efficiency and bioorthogonality. Nevertheless, for some applications, such as drug delivery, a completely bioreversible bond between the two conjugated molecules would be favorable. Dimethylmaleic anhydride is known to form amide bonds with amines that are cleaved under mild acidic conditions.[5−10] Herein we describe the synthesis and applications of a new heterobifunctional linker based on substituted dimethylmaleic anhydride that combines the advantages of click chemistry with implementation of a pH-sensitive bond between conjugated biomolecules. This linker is labile under mild acidic conditions, which are typical for tumor tissue[11] or early endosomes.[12] Moreover, this linker is cleaved off traceless, resulting in an unmodified molecule of interest. The utility in protein modification is demonstrated in examples of reversible dye labeling of proteins, pH-sensitive modification with polyethylene glycol (PEG), and intracellular transduction of proteins in bioactive form.

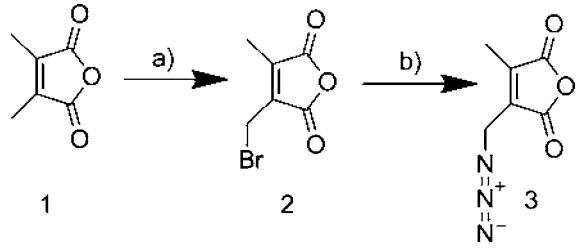
RESULTS AND DISCUSSION

The azidomethyl-methylmaleic anhydride linker **3** (AzMMMan) was synthesized from dimethylmaleic anhydride **1** by two simple reaction steps (radical substitution with *N*-bromosuccinimide resulting in **2** followed by reaction with sodium azide, see Scheme 1).

The heterobifunctional linker was used to introduce acid-labile azido groups into human serum albumin (HSA), EGFP, or ß-galactosidase (ß-Gal) (Scheme 2, step a) by reaction of the maleic anhydride moiety with amino groups of the proteins. The AzMMMan−HSA conjugate **4** was coupled with an alkyne-bearing hemicyanine dye **12** (synthesized as shown in Scheme S1, Supporting Information) in a copper-catalyzed fashion (Scheme 2, step b), resulting in conjugate **7**. Alkyne dye **12** was used, on the one hand, to determine the degree of protein modification with the new linker **3** (Figure S2, Supporting Information) and, on the other hand, to investigate cleavage kinetics under acidic conditions (Figure 1).

As expected, the number of azides introduced in HSA was linear to the initial concentration of **3** (Figure S2, Supporting Information). Figure 1 displays the kinetics of pH-dependent cleavage of dye−conjugate **7**. Dye-labeled HSA **7** was incubated in buffers of different pH. Cleavage of dye **12** from the protein

Scheme 1. Synthesis of Linker 3 (AzMMMan)[a]



[a](a) *N*-Bromosuccinimide, benzoyl peroxide, 56%; (b) sodium azide, 88%.

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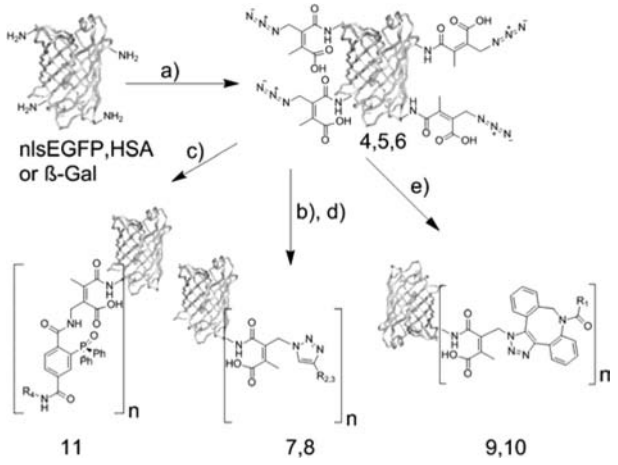
Scheme 2. pH-Reversible Modification of Proteins with Dyes, PEG, or Transduction Oligomer[a]



[a](a) Modification of nlsEGFP, HSA, or ß-Gal with AzMMMan linker **3**. CuAAC conjugation of AzMMMan–HSA **4** with (b) alkyne–hemicyanin dye **12** or (d) alkyne–PEG, resulting in conjugate **7** or **8**, respectively. (c) Staudinger ligation of Dylight 488 (R4) to AzMMMan–HSA resulting in conjugate **11**. (e) Conjugation of AzMMMan–nlsEGFP **5** or AzMMMan–ß-Gal **6** with carrier polymer **386** (R1) via copper-free cycloaddition resulting in conjugate **9** or **10**, respectively.

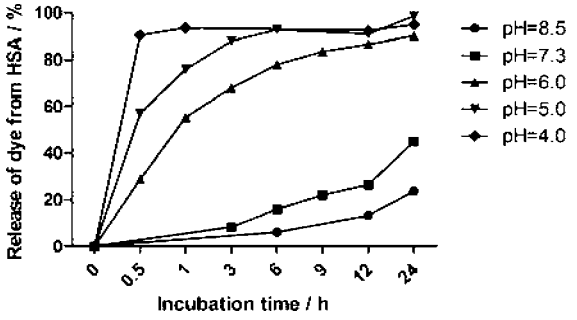


Figure 1. Acid-catalyzed release of dye **12** from conjugate **7** at 37 °C. Samples of **7** were incubated in buffers of different pH. At each time point, the fraction (%) of dye remaining conjugated with HSA was determined after protein purification. Percentage of released dye is calculated as (100 − % HSA conjugated dye).

was determined at various times by measuring residual dye absorbance of purified protein fractions. At basic pH of 8.5 the conjugate was quite stable with only about 25% cleavage after 24 h. In contrast, at pH 6 the same amount was released already after 30 min. The half-life at pH 5 is about 0.5 h, whereas at pH 4 already 90% of the linker was cleaved in that time. Cleavage of the linker appears to follow pseudo-first-order kinetics.

Figure 2 reveals that conjugate **7** is quite stable under physiological serum conditions at 37 °C. After 2 h incubation in buffer containing 30% FCS still 94% of the dye remained coupled to HSA. Even after an incubation time of 12 h 60% of the bonds still are intact. The cleavage rate does not differ much from incubation in pure PBS buffer at physiological pH (Figure 1).

Also, the so-called Staudinger ligation can be performed with azido-proteins.[3] A phosphine-containing dye was coupled to AzMMMan–HSA **4** (Scheme 2, step c), resulting in conjugate **11**. Figure S3, Supporting Information, shows similar pH-dependent cleavage of the two HSA conjugates independent of being coupled by Staudinger ligation or CuAAC.

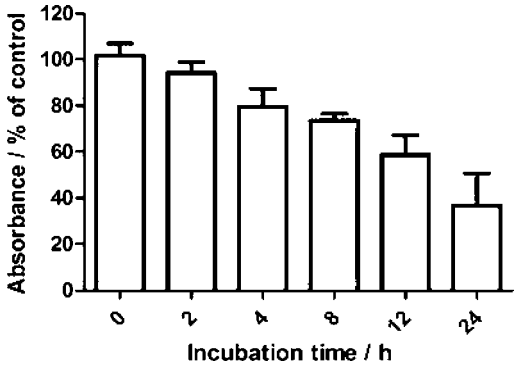


Figure 2. Serum stability of conjugate **7** at 37 °C. Samples of **7** were incubated in PBS buffer pH 7.4 containing 30% FCS. Dye remaining conjugated with the protein was determined by measuring the absorbance of the conjugate after purification from released dye.

Another application of linker **3** is reversible PEGylation of proteins (Scheme 2, step d). Figure 3 gives evidence that HSA can be effectively coupled with alkyne-bearing PEG (lane 3). The PEG conjugate **8** shows extended stability at physiological pH (lane 4) but is cleaved under very mild acidic conditions of pH 6 (lane 5). At pH 5 or 4 almost all PEG is released (lanes 6 and 7).

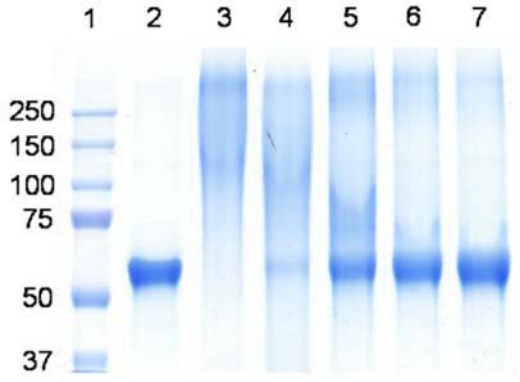


Figure 3. Acid-catalyzed release of PEG from HSA conjugate **8**. SDS–polyacrylamide gel electrophoresis after 16 h incubation: lane 1 marker, lane 2 unmodified HSA, lane 3 pH 8.5, lane 4 pH 7.3, lane 5 pH 6, lane 6 pH 5, lane 7 pH 4.

A more detailed cleavage kinetic of PEG–AzMMMan–HSA at the endosomal pH of 5 is displayed in Figure 4. For this purpose PEG was distally end labeled with tetramethylrhodamine (TMR) dye. Release of TMR–PEG from HSA conjugate was determined at various times by measuring the absorbance of TMR dye remaining incorporated in purified PEGylated protein conjugate. In contrast to the type of coupling reaction which has a minor influence on cleavage kinetics (Figure S3, Supporting Information) the coupled molecule has more impact on acidic bond cleavage. PEG release from the protein was considerably slower compared with dye released from conjugate **7** (Figure 1).

Protein transduction, i.e., the intracellular delivery of proteins, can take advantage of the endosomal acidification process.[13−15] nlsEGFP bearing a nuclear localization sequence (which mediates natural active nuclear import once a protein

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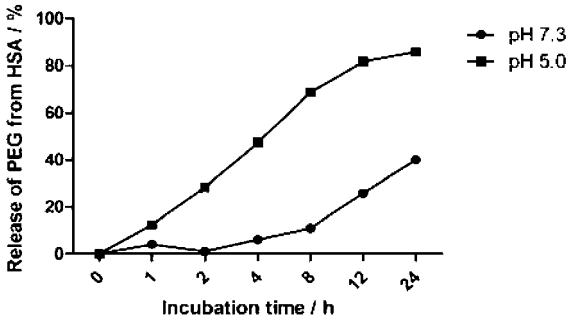


Figure 4. Kinetics of PEG release from HSA conjugate at an endosomal relevant pH of 5, and stability of the conjugate at physiological pH of 7.3. TMR-labeled PEG released from HSA protein was determined by comparing the absorbance of the intact PEG–HSA conjugate before and after incubation.

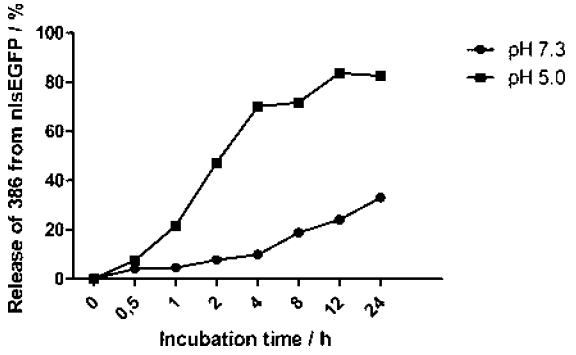
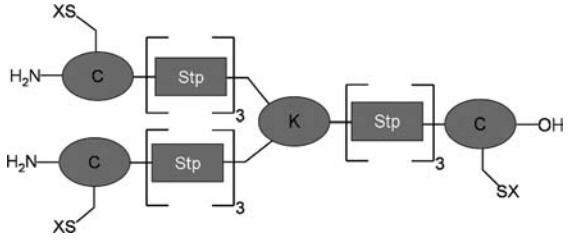


Figure 5. Acid-catalyzed release of TMR–**386** from conjugate **9** at 37 °C. Samples of TMR-labeled **9** were incubated in PBS buffers of pH 7.3 and 5.0. Dye-labeled **386** released from the protein was determined indirectly by measuring the absorbance of the conjugate after purification.

resides in the cytosol) was bound to the three-arm cationic oligo(aminoethane) amide **386** (Scheme 2, step e). Oligomer **386** (Scheme 3) was previously developed as a precise nontoxic

Scheme 3. Structure of Transduction Oligomer 386[a]



[a]Structure of **386** (x = H; Stp, succinoyl tetraethylene pentamine; C, cysteine; K, α,ε-modified branching lysine).[16]

carrier for cytosolic siRNA delivery.[16,17] Cationic carriers like **386** containing diaminoethane motifs bind cells and act as proton sponges which upon endosomal acidification become increasingly positively charged, triggering endosome disruption and release of its content.[18,19]

For intracellular delivery of nlsEGFP protein, two different linking strategies, conventional irreversible conjugation and pH-reversible conjugation, were compared. For the synthesis of an irreversible conjugate, the commercial linker SMCC was used, resulting in thioether linkage. In the new strategy, oligomer **386** was coupled with AzMMMan–nlsEGFP **5** by copper-free cycloaddition via dibencylcyclooctyne (see Scheme 2e). Thus, **9** contains an acid-labile bond between carrier **386** and nlsEGFP, which is expected to be reversible in the endosomal microenvironment before transfer into the cytosol.

Successful modification of the nlsEGFP protein with transduction oligomer **386** and acidic lability of the resulting conjugate **9** is shown in Figure S5, Supporting Information. The cleavage kinetics was determined in more detail (Figure 5) by labeling the **386** carrier with TMR dye and further processing analogously as in Figure 4. As already observed for the PEG–HSA conjugate, coupling of carrier **386** to nlsEGFP is retarding the cleavage kinetics. Nevertheless, after 1 h incubation at endosomal pH 5.0, >20%, and after 2 h almost 50% of the transduction oligomer was released from the nlsEGFP protein.

The native conformation of nlsEGFP after release of **386** from conjugate **9** was investigated by circular dichroism spectroscopy. Figure S6, Supporting Information, reveals that the tertiary structure of nlsEGFP is not significantly affected by coupling and consecutive cleavage of the carrier **386**.

The cell culture experiment shown in Figure 6 demonstrates how important intracellular cleavage can be for protein delivery in the case of covalent binding to a carrier molecule using the

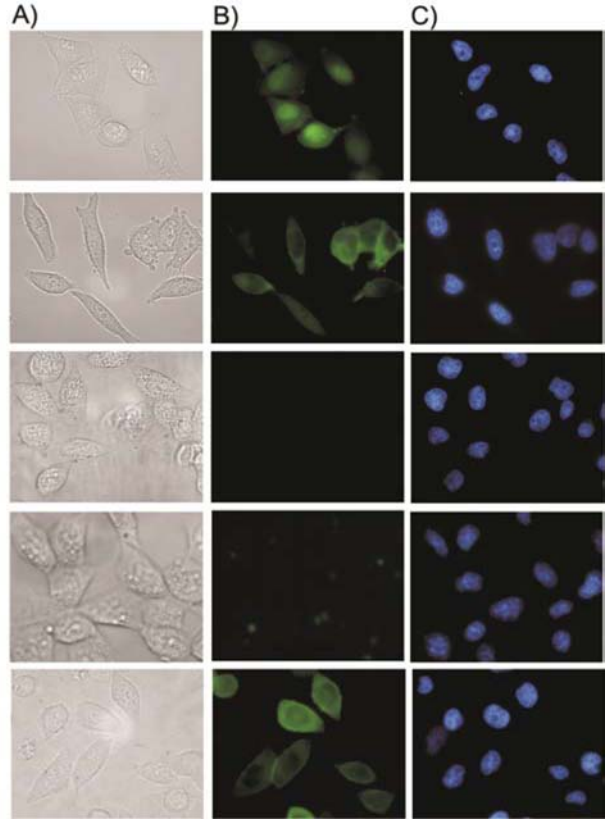


Figure 6. Transduction of HeLa cells with **386**-modified nlsEGFP. Row 1: carrier **386** was coupled with nlsEGFP via the cleavable AzMMMan linker **9**. Row 2: carrier **386** was coupled by irreversible SMCC thioether linkage. Row 3: transfection was done with unmodified nlsEGFP. Row 4: transfection with conjugate **9** at 4 °C in the presence of endocytosis inhibitors. Row 5: transfection with conjugate **9** in the presence of endosome acidification inhibitors. (A) Brightfield picture of the transfected cells, (B) EGFP fluorescence of the transfected cells, and (C) Hoechst 33342 DNA stain of the cell nucleus.

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novel linker **3**. Both constructs, based on the pH-reversible conjugation or on conventional irreversible conjugation by SMCC thioether linkage, were able to deliver nlsEGFP into the cytosol of HeLa cells (Figure 6, rows 1 and 2). Flow cytometry experiments suggest that the stable thioether construct seems to be slightly more efficient (data not shown). Cells in row 3 were transfected with unmodified nlsEGFP as a control. The fluorescence image shows that no protein was internalized.

However, only nlsEGFP which had been coupled to the carrier by acid-labile bonds was further translocated into the nucleus to a large extent (Figure 6, row 1). The lack of nuclear import of the irreversibly modified nlsEGFP is consistent with direct (by chemical modification of the nls lysines) or indirect (by sterical shielding) inactivation of the nuclear localization signal. Alternatively, active nuclear transport may be hampered by unspecific cytosolic retention of the polycationic transduction shuttle. In contrast, the nuclear import of the pH-labile click conjugate **9** can be explained by a release of unmodified nlsEGFP in the acidifying endosome before escape into the cytosol, followed by nls-mediated nuclear import.

Transfection of cells with conjugate **9** at 4 °C in the presence of three different endocytosis inhibitors (amiloride (inhibitor for macropinocytosis), chloroproamzine (inhibitor for clathrin-mediated endocytosis), β-cyclodextrin (inhibitor for caveolae-mediated endocytosis)) almost completely eliminates internalization (Figure 6, row 4). This observation suggests an active, energy-dependent internalization mechanism.

The experiment shown in Figure 6, row 5, highlights acidification in the endosome as a critical requirement for intracellular release of the carrier. Cells were preincubated and transfected in the presence of chloroquine and ammonium chloride. Both substances prevent acidification of the cellular endosomes. Transfection under these conditions results in a similar observation that was made for the noncleavable construct. The conjugate is taken up by the cell, and the balanced distribution suggests at least some endosomal escape, but only minor translocation of the protein into the nucleus was observed.

Transduction oligomer **386** has been shown to exhibit low cytotoxicity when used as a carrier for siRNA or DNA.[16,17] To verify the nontoxic properties also in the case of being covalently bound to a protein, a cell viability test was performed that confirmed this assumption. Figure 7 shows the low toxicity of the transduction shuttle, even at high transduction concentrations. It exhibited only marginally higher toxicity than the unmodified nlsEGFP protein. With the standard concentration used for transfections above (10 μg/mL) a decline in metabolic activity of only 10% in comparison to untreated cells was observed. Even after transfection with high amounts of 80 μg of **386** nlsEGFP conjugate cell viability decreased by only 33%. The toxicity profile of stable and reversible bound carrier does not differ much. However, at the highest tested concentration the irreversible SMCC construct seems to be slightly more toxic.

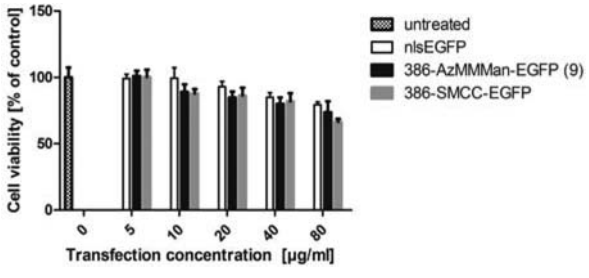

Figure 7. Cell viability assay. HeLa cells were transfected with different amounts of **386**–nlsEGFP conjugates. Unmodified nlsEGFP was used for comparison.

The fluorescence properties and CD spectra of construct **9** indicate that the protein conformation is not significantly affected by modification with the linker and transduction oligomer. Therefore, we examined whether the activity of an enzyme would be maintained and survive modification, delivery, and intracellular release steps. As a model enzyme we chose ß-galactosidase (ß-Gal). It is of rather big size (119 kDa), and the activity of the enzyme in the cytosol can easily be investigated by use of the fluorescent substrates.

Carrier **386** was covalently bound to the ß-Gal protein over the AzMMMan linker (Scheme 2, step e), resulting in conjugate **10**. This construct was able to transport biologically active ß-galactosidase into the cytosol of HeLa cells. ß-Gal activity was detected using the fluorescent substrate C12-FDG (C12-fluorescein-dibeta-D-galactopyranoside), which exhibits FITC fluorescence after cleavage. This fluorescence was determined by flow cytometric analysis. Already with a transfection concentration of 1 μM a significant raise in fluorescence was observed. Transfection with 2.5 μM ß-Gal further raised the fluorescent properties. In contrast, cells treated with 2.5 μM unmodified ß-Gal (without shuttle) did not show a significant shift in fluorescence. This experiment evidences that the transduction shuttle is able to transduce biologically active proteins into the cytosol of cells.

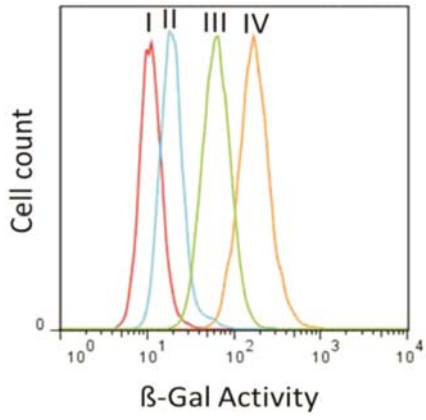

Figure 8. HeLa cells were transfected with conjugate **10** (**386**–AzMMMan–ßGal), washed and incubated with C12-FDG substrate, and evaluated by flow cytometry. I: untreated cells. II: cells transfected with 2.5 μM unmodified ß-Gal. III: cells transfected with 1 μM conjugate **10**. IV: cells treated with 2.5 μM of conjugate **10**.

CONCLUSION

In summary, we present a new pH-sensitive linker **3** which combines the advantages of click chemistry with implementation of a traceless cleavable bond between two conjugated molecules. Three different click chemistries were performed which all are compatible with the acid-labile properties. Traceless cleavage may be a particularly important feature in protein transduction strategies to maintain full bioactivity of enzymes and other proteins. The current example of **386**

carrier-mediated cytosolic delivery and subsequent nuclear import of released nls-EGFP demonstrates the advantage of the traceless linker. To demonstrate that the modification does not irreversibly affect the structure and biological activity of proteins, we transported **386**-modified ß-galactosidase as a model enzyme which exhibited cytosolic activity in transduced cells far higher than without shuttle. Aside from these encouraging options for protein delivery and modification, the linker might have broader use in the design of novel programmed, acid-labile, and biodegradable drug delivery systems.[19,20] Targeted therapeutics could, after delivery into acidic tumor areas or upon cellular uptake into endosomes, be dismantled from their outer shell including targeting ligands. Besides drug delivery, the linker may also be of interest for other applications such as reversible labeling of various biological and also chemical molecules.

■ ASSOCIATED CONTENT

Supporting Information

Figures, analytical data, and experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Saxon, E.; Bertozzi, C. R. *Science (New York, N.Y.)* **2000**, *287*, 2007.

(2) Yu, H.; Nie, Y.; Dohmen, C.; Li, Y.; Wagner, E. *Biomacromolecules* **2011**, *12*, 2039.

(3) Vallee, M. R.; Majkut, P.; Wilkening, I.; Weise, C.; Muller, G.; Hackenberger, C. P. *Org. Lett.* **2011**, *13*, 5440.

(4) Dohmen, C.; Frohlich, T.; Lachelt, U.; Rohl, I.; Vornlocher, H.-P.; Hadwiger, P.; Wagner, E. *Mol. Ther. Nucleic Acids* **2012**, *1*, e7.

(5) Meyer, M.; Philipp, A.; Oskuee, R.; Schmidt, C.; Wagner, E. *J. Am. Chem. Soc.* **2008**, *130*, 3272.

(6) Meyer, M.; Zintchenko, A.; Ogris, M.; Wagner, E. *J. Gene Med.* **2007**, *9*, 797.

(7) Nieto, M. A.; Palacian, E. *Biochim. Biophys. Acta* **1983**, *749*, 204.

(8) Meyer, M.; Dohmen, C.; Philipp, A.; Kiener, D.; Maiwald, G.; Scheu, C.; Ogris, M.; Wagner, E. *Mol. Pharmaceutics* **2009**, *6*, 752.

(9) Rozema, D. B.; Lewis, D. L.; Wakefield, D. H.; Wong, S. C.; Klein, J. J.; Roesch, P. L.; Bertin, S. L.; Reppen, T. W.; Chu, Q.; Blokhin, A. V.; Hagstrom, J. E.; Wolff, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12982.

(10) Rozema, D. B.; Ekena, K.; Lewis, D. L.; Loomis, A. G.; Wolff, J. A. *Bioconjugate Chem.* **2003**, *14*, 51.

(11) Engin, K.; Leeper, D. B.; Cater, J. R.; Thistlethwaite, A. J.; Tupchong, L.; McFarlane, J. D. *Int. J. Hyperthermia* **1995**, *11*, 211.

(12) Murphy, R. F.; Powers, S.; Cantor, C. R. *J. Cell Biol.* **1984**, *98*, 1757.

(13) Lee, Y.; Ishii, T.; Kim, H. J.; Nishiyama, N.; Hayakawa, Y.; Itaka, K.; Kataoka, K. *Angew. Chem., Int. Ed. Engl.* **2010**, *49*, 2552.

(14) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Dashe, J.; Frechet, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 10494.

(15) Murthy, N.; Campbell, J.; Fausto, N.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2003**, *14*, 412.

(16) Schaffert, D.; Troiber, C.; Salcher, E. E.; Frohlich, T.; Martin, I.; Badgujar, N.; Dohmen, C.; Edinger, D.; Klager, R.; Maiwald, G.; Farkasova, K.; Seeber, S.; Jahn-Hofmann, K.; Hadwiger, P.; Wagner, E. *Angew. Chem., Int. Ed. Engl.* **2011**, *50*, 8986.

(17) Frohlich, T.; Edinger, D.; Klager, R.; Troiber, C.; Salcher, E.; Badgujar, N.; Martin, I.; Schaffert, D.; Cengizeroglu, A.; Hadwiger, P.; Vornlocher, H. P.; Wagner, E. *J. Controlled Release* **2012**, DOI: http://dx.doi.org/10.1016/j.jconrel.2012.03.018.

(18) Miyata, K.; Nishiyama, N.; Kataoka, K. *Chem. Soc. Rev.* **2012**, *41*, 2562.

(19) Wagner, E. *Acc. Chem. Res.* **2011**, DOI: http://dx.doi.org/10.1021/ar2002232.

(20) Wagner, E. *Expert Opin. Biol. Ther.* **2007**, *7*, 587.