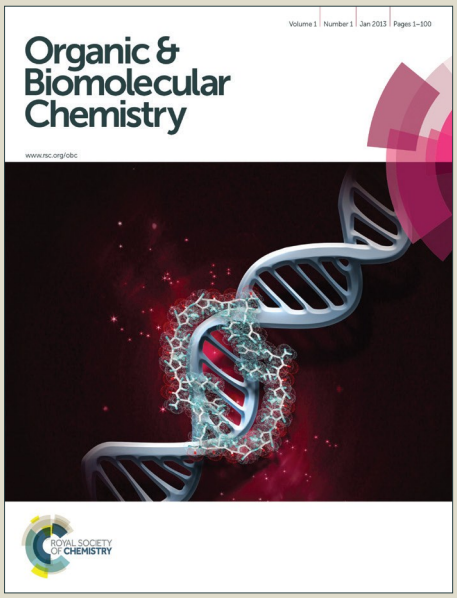
Organic & Biomolecular Chemistry

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: A. Wagner, S. A. Jacques, G. Leriche, M. Mosser, M. Nothisen, C. D. Muller and J. Remy, *Org. Biomol. Chem.*, 2016, DOI: 10.1039/C6OB00846A.



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

View Article Online
DOI: 10.1039/C6OB00846A

From solution to in-cell study of the chemical reactivity of acid sensitive functional groups: a rational approach towards improved cleavable linker for biospecific endosomal release

Sylvain A. Jacques,[a]† Geoffray Leriche,[a]† Michel Mosser,[a] Marc Nothisen,[b] Christian D. Muller,[c] Jean-Serge Remy,[b] and Alain Wagner[a*]

[a.] *LFCS laboratory, CAMB UMR 7199 CNRS University of Strasbourg, LabEx Medalis, icFRC, Faculty of Pharmacy, 74 route du Rhin, 67400 Illkirch, France. E-mail: alwag@unistra.fr*

[b.] *V-SAT laboratory, CAMB UMR 7199 CNRS University of Strasbourg, LabEx Medalis, icFRC, Faculty of Pharmacy, 74 route du Rhin, 67400 Illkirch, France.*

[c.] *Laboraroire d'Innovation Thérapeutique, UMR 7200, CNRS University of Strasbourg, Faculty of Pharmacy, 74 route du Rhin, 67400 Illkirch, France.*

† Both authors contributed equally to the work.

View Article Online
DOI: 10.1039/C6OB00846A

Abstract

pH-sensitive linkers designed to undergo selective hydrolysis at acidic pH compared to physiological pH can be used for selective release of therapeutics at their site of action. In this paper, the hydrolytic cleavage of a wide variety of molecular structures that have been reported for their use in pH-sensitive delivery systems was examined . A wide variety of hydrolytic stability profiles was found among the panel of tested chemical functionalities. Even within a structural family, a slight modification of the substitution pattern has an unsuspected outcome on the hydrolysis stability. This work lead us to establish a first classification of these groups based on their reactivities at pH 5.5 and their relative hydrolysis at pH 5.5 vs. pH 7.4. From this classification, four representative chemical functions belonging eand compare lysosomal cleavage in living cells..and revealed that only the most reactive functions underwent significant lysosomal cleavage, according to flow cytometry measurements. These last results question the acid-based mechanism of action of known drug release systems and advocate for the importance of in-depth structure-reactivity study, using tailored methodology, for the rational design and development of bio-responsive linkers.

Introduction

Specific release of bioactive compounds has become a major issue in modern drug development. The efficacy and the selectivity of the bond breaking process is a paramount component for the success of a prodrug[1] and targeted therapeutics such as Antibody Drug Conjugates (ADC).[2] For a delivery system to be effective, it must remain stable and active until reaching its site of action. As a strategy for selective bond cleavage mechanisms, one can exploit singularities linked to pathologies or sub-cellular compartments including enzymatic activity[3] or high glutathione level.[4]

In the field of pH-sensitive drug delivery systems, several acid-sensitive linkers have been developed to respond to a pH-shift. Typical examples are shown in **Error! Reference source not found.**. For delivery systems, hydrazone,[5] *cis*-aconityl[6] and trityl bonds[7] have been the most widely investigated, but other linkers such as dialkyl and diaryldialkoxysilane,[8] orthoester,[9] acetal,[10] β-propionate,[11] phosphoramidate,[12]

View Article Online
DOI: 10.1039/C6OB00846A

imine,[13] vinyl ether,[14] imidazole[15] and polyketal[16] have also been described and reviewed.[17]

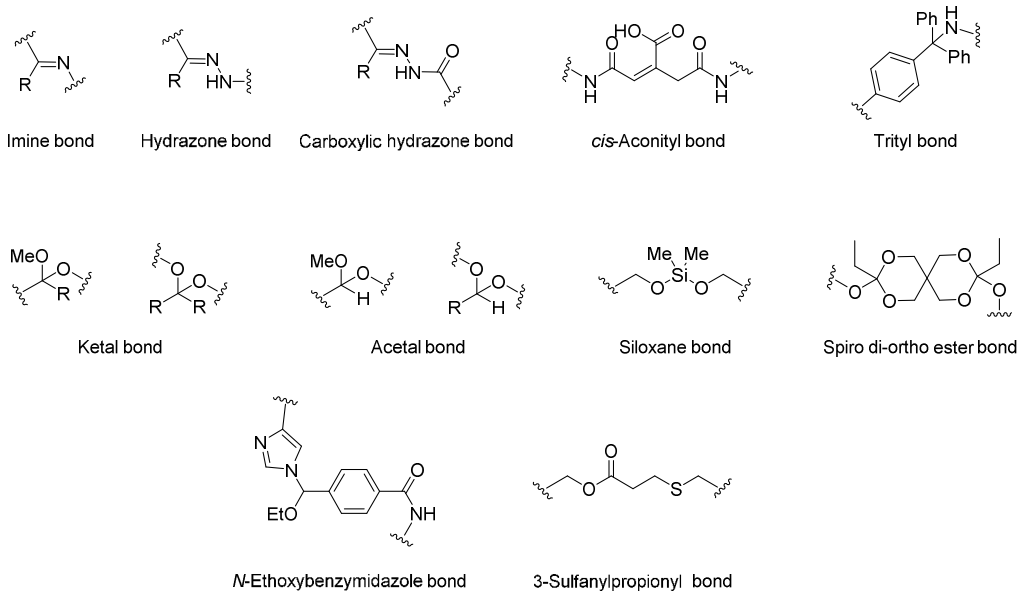


Fig. 1 Examples of acid sensitive bonds.

We have recently published a study on a homo-bifunctional spiro di-orthoester (SpiDo) linker derivative which has shown a fast lysosomal hydrolysis and a high stability in Human plasma.[18] In the course of this work we have noticed that despite many reports on acid-labile delivery systems, the choice of a suitable starting point for the design of biospecific release system remains mostly intuitive.

Most of the acid-sensitive linkers have been described for different applications and in different model systems. Each study mostly focused on the use of one type of acid-sensitive linker, for one particular application, in one specific biological system. The majority of the time, the pH-sensitive linkers are first characterized in solution with their hydrolysis profiles at pH 5.5 and 7.4, and then directly used in specific biological applications. The scarce and disparate data do not provide a precise and comprehensive overview on the chemical reactivity, allowing the direct comparison of chemical structures and guiding the rational design of acid-sensitive biospecific linkers. For this reason, we report herein the pH sensitivity profiles of the main acid-

View Article Online
DOI: 10.1039/C6OB00846A

labile structures under standardized conditions in solution, and further in cellular environments using turn-on fluorescent probes.

Results and discussion

Determination of hydrolysis profiles

Following a literature review, we designed analogs representative of known acid-sensitive chemical linkers. For the most widely reported linkers, several derivatives have been also synthesized to further expand the Structure-Acidlability-Relationship. Each of these derivatives includes a UV traceable moiety to allow HPLC-UV detection. Hydrolytic profiles of all compounds have been monitored in aqueous buffer solution at pH 5.5 (to simulate lysosomal pH) and pH 7.4 (to simulate serum conditions). A 10 mM solution of each molecule was prepared in DMSO and diluted in buffer to obtain a 1 mM solution. For pH 7.4 NaH_2PO_4/Na_2HPO_4 phosphate buffers and for pH 5.5 KH_2PO_4/Na_2HPO_4 were prepared at 100 mM. Each solution was immediately analyzed by analytical HPLC (Eluent A/B, A: NH_3HCOOH solution (10 mM, pH 8.5)/B: ACN. Gradient: 5% B to 95% B in 20 minutes and 10 minutes of re-equilibration). Reaction was performed at 25 °C and crude was injected every 30 minutes for up to 8 hours. The first measurement t_1 was recorded after 30 seconds which is a result of the delay of sample preparation. For each recorded time t_x the amount of non-hydrolyzed product was measured by the calculation of the area under the peak. Comparing with the calculated area at t_1 the percentage of hydrolyzed product was calculated following the formula:

$$\%\ \text{hydrolysis} = 100 \times \frac{(\text{At1} - \text{Atx})}{\text{At1}}$$

For each pH the variation of % hydrolysis within the time of the experiment was plotted. The half-live times $t_{1/2}$ were estimated and the hydrolysis selectivity was measured by the ratio:

$$\frac{t\left(\frac{1}{2}\right)\text{pH7.4}}{t\left(\frac{1}{2}\right)\text{pH5.5}}.$$

When compounds were completely hydrolyzed at the first measurement t_1, a half-life time of less than 0.01 hour (0.5 minute) was attributed. In these particular cases we

View Article Online
DOI: 10.1039/C6OB00846A

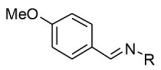
were not able to determine if the compounds were hydrolyzed within the 10 seconds before the analysis (pH 5.5 or 7.4) or during the analysis itself (pH 8.5). However considering the required plasma stability (pH = 7.4) such functional groups were not considered as potent leads to efficient delivery systems.

Synthesis of the model compounds is straightforward (See SI for a full description of their syntheses) but requires careful attention, requiring the use of mild acidic conditions when handling pH-sensitive groups especially upon purification.

Carbazate, oxime, imine, hydrazone and acylhydrazone derived compounds

Compounds containing a carbon-nitrogen double bond (e.g. hydrazone) are the most widely used structures for acid-labile pro-drugs and controlled delivery of doxorubicin, cisplatin and other agents.[5n-5v] In a similar manner, the imine (Schiff base) itself has already been used to achieve acid-triggered drug release.[19] With compounds **1-8** in hand, we studied the lability of the carbon-nitrogen double bond towards hydrolysis at pH 5.5 and 7.4 (Fig. 2). This particular case gave binary results. On one hand, oxime **1**, carbazate **4** and hydrazone pyridine **7** were completely stable at both pH; on the other hand imines **2**, **3** and acylhydrazones **5** and **6** were completely hydrolyzed at the first measurement. It is known that pyridine can be protonated in acidic media and thus can lead to a specific acidic cleavage.[20] However, in our conditions we found that replacing a benzene ring (**2**) to a pyridine ring (**7**) did not improve the stability.

View Article Online
DOI: 10.1039/C6OB00846A



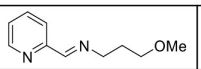
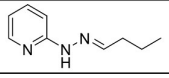
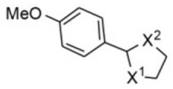
Mol.	R	$t_{1/2}$ (h)	
		pH 5.5	pH 7.4
1	OEt	S	S
2	$(CH_2)_3$-OMe	< 0.01	< 0.01
3	$CH(CH_2\text{-}OH)_2$	< 0.01	< 0.01
4	NH-CO_2Et	S	S
5	NH-CO-Me	< 0.01	< 0.01
6		< 0.01	< 0.01
7		S	S

Fig. 2 Hydrolysis kinetics of compounds **1** – **7** at pH 5.5 and 7.4. (S = Compound stable during the time of the experiment (0 % hydrolysis)).

Substituted heterocycles derived from benzaldehyde

Kinetics stability studies of heterocycles **9** to **11** were done to further examine the effect of the heteroatoms on the hydrolytic profiles of five membered rings in aqueous media (Fig. 3 and Fig. S1). At t_1, imidazoline **9** and oxazolidine **10** showed complete hydrolysis at both pH, while dioxolane **11** displayed distinct hydrolysis profiles. At pH 5.5, 50% of hydrolysis was detected after two hours while less than 5% of hydrolysis was observed at pH 7.4. Extrapolation of the data recorded at pH 7.4 gave a half-life of about 33 hours.

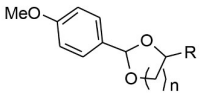
View Article Online
DOI: 10.1039/C6OB00846A



Mol.	X^1	X^2	$t_{1/2}$ (h)		Ratio
			pH 5.5	pH 7.4	
9	N-Me	N-Me	< 0.01	< 0.01	-
10	N-Me	O	< 0.01	< 0.01	-
11	O	O	2.1	33	15.7

Fig. 3 Influence of heteroatoms on the hydrolysis of five membered ring systems. Hydrolysis kinetics of compounds **9** – **11** at pH 5.5 and 7.4.

The influence of ring size and steric effects was studied using dioxolane compounds **11** to **13** (Fig. 4 and Fig. S2). Compound **12** bearing one extra methyl compared to compound **11** showed a better stability at pH 5.5 with a half-life of 4.2 hours. However, it led to a 2-fold decrease in half-life at pH 7.4. 1,3-dioxane type compound **13** revealed to be the most stable of this series at both pH, with no trace of *p*-anisaldehyde being observed after 6 hours. This result is consistent with previous work by Harper[21] and others.[22]



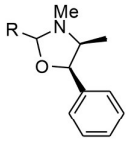
Mol.	n	R	$t_{1/2}$ (h)		Ratio
			pH 5.5	pH 7.4	
11	1	H	2.1	33	15.7
12	1	Me	4.2	16	3.8
13	2	Me	6.1	S	-

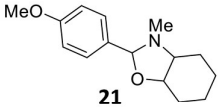
Fig. 4 Influence of steric effects and size of the ring on dioxane and dioxolane type ring systems. Hydrolysis kinetics of compounds **11** – **13** at pH 5.5 and 7.4. (S = Compound stable during the time of the experiment (0 % hydrolysis)).

Following the work by Bundgaard and co-workers who reported the hydrolysis kinetics of several oxazolidines, and assessed their suitability as prodrug forms for beta-amino alcohols

View Article Online
DOI: 10.1039/C6OB00846A

and/or carbonyl-containing compounds,[22] oxazolidines **15** - **21** were synthesized and their pH sensitivity recorded. Derivatives **15** and **16** showed complete hydrolysis at t_1 at both pH (Fig. 5 and Fig. S3 and S4). Oxazolidine **17** was very reactive toward hydrolysis with a half-life time of less than 20 min at pH 5.5. The addition of a second electron donating group did not increase the rate of hydrolysis, and half-lives of **18** were very similar at both pH. As expected, the substitution of benzaldehyde with an electron withdrawing group in **19** decreased the rate of hydrolysis at both pH. A similar effect was also observed with the pyridine derivative **20**. It is known in the literature that the stability of the oxazolidine can be tuned by the steric hindrance on the heterocycles.[23] However in this case, derivative **21** revealed to be very unstable at both pH.





21

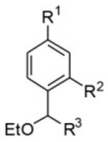
Mol.	R	$t_{1/2}$ (h)		Ratio
		pH 5.5	pH 7.4	
15	Ph	< 0.01	< 0.01	-
16	Ph-*p*Me	< 0.01	< 0.01	-
17	Ph-*p*OMe	0.1	0.4	4.0
18	Ph-*p,o*OMe	0.3	0.2	1.5
19	Ph-*p*NO_2	0.5	2.0	4.0
20	*o*-pyridine	0.3	0.8	2.8
21	-	< 0.01	< 0.01	-

Fig. 5 Effects of the substitution on the benzaldehyde moiety on the hydrolysis rate of oxazolidine derivatives. Hydrolysis kinetics of compounds **15** – **21** at pH 5.5 and 7.4.

We next turned our attention to the hydrolysis of the ketal moiety (Fig. 6 and Fig. S5 and S6). They are well known acid-sensitive moieties that were originally found to be useful as protecting groups for aldehydes.[24] Derivative **22** had a half-life of 0.8 hours and 12.2 hours at pH 5.5 and 7.4 respectively. The rate of hydrolysis was increased by adding an electron donating methoxy group **23**. Moreover, both compounds showed a good stability at pH 7.4. Derivative **24** presented the highest ratio of hydrolysis between both pH. The *N*-

View Article Online
DOI: 10.1039/C6OB00846A

ethoxybenzymidazole derivative **25** showed a slower hydrolysis kinetics at pH 5.5 and a better stability at pH 7.4 compared to its parent diacetal compound **24**. These last results are in agreement with previous study by Yang and coworkers.[15a]



Mol.	R^1	R^2	R^3	$t_{1/2}$ (h)		Ratio
				pH 5.5	pH 7.4	
22	H	H	OEt	0.8	12.2	15.3
23	OMe	H	OEt	0.1	5.3	53
24	H	OMe	OEt	0.1	19.9	199
25	H	OMe		1.1	28.8	31.7

Fig. 6 Effects of the substitution on the hydrolysis rate of ketal derivatives. Hydrolysis kinetics of compounds **22** – **25** at pH 5.5 and 7.4.

Siloxane, acetal, β-thiopropionate THP and carbamate derived compounds

Widely used in organic chemistry as protecting groups,[24] silyl ethers have also been used as hydrolysable monomers in biodegradable polymers,[8] and it is known that the increase in the size of the substituents on the silicon atom significantly reduces the linker hydrolysis rate under acidic conditions. Derivative **26** hydrolyzed 4 times slower at physiological pH as compared to pH 5.5. (Fig. 7 and Fig. S7). Two additional siloxane acetals derived from **26** (where the two phenyl rings in **26** were substituted by two methyl or two *n*-butyl groups) were synthesized but could not be purified due to their high instability. Based on a similar scaffold, acetonide **27** was found to be very unstable and was completely hydrolyzed at t_1. In 2004, Hubbell and co-workers were able to show that 3-sulfanylpropionyl or 4-sulfanylbutyryl esters could be used for oligodesoxynucleotide vectorisation.[25] In our study, β-thiopropionate derivative **28** showed moderate stability at both pH but suffered from poor selectivity with a ratio of 1.5. Finally, THP-based and carbamate compounds **29** and **30** were completely stable at both pH as expected.

View Article Online
DOI: 10.1039/C6OB00846A

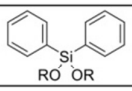
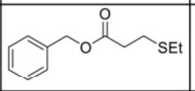
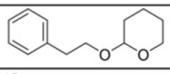
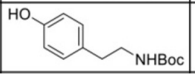
Mol.	Structure	$t_{1/2}$ (h)		Ratio
		pH 5.5	pH 7.4	
26		3.3	12.2	4.0
27		< 0.01	< 0.01	-
28		9.4	14.3	1.5
29		S	S	-
30		S	S	-

Fig. 7 Hydrolysis rates of siloxane, acetal, β-thiopropionate, tetrahydropyranyl ether and carbamate derivatives. Hydrolysis kinetics of compounds **26** – **30** at pH 5.5 and 7.4. (S = Compound stable during the time of the experiment (0% hydrolysis)) R = $(CH_2)_2$-O-$(CH_2)_2$-NHCO-C_5H_6.

Our results reveal that albeit these molecular structures were described to possess good stability at pH 7.4 and increased lability at pH 5.5, important discrepancies in their relative profiles could be observed. The hydrolysis rate can change dramatically, not only from one family to the other, but also within a specific family depending on the substituent pattern of the tested derivatives. However based on that study it seems possible to categorize the different structural molecular motif according to their average hydrolysis pattern.

All tested structures have thus been ranked according to the following criteria (Fig. 8):

- Non-hydrolysable when no hydrolysis was observed under our conditions at pH 5.5
- Fast hydrolysis when the half-life time at pH 5.5 was below 1 hour.
- Highly selective hydrolysis when the ratio $\frac{t_{1/2(\text{pH 7.4})}}{t_{1/2(\text{pH 5.5})}}$ was more than 15.
- Selective hydrolysis when the ratio $\frac{t_{1/2(\text{pH 7.4})}}{t_{1/2(\text{pH 5.5})}}$ was between 2 and 15.
- Non-selective hydrolysis when the ratio $\frac{t_{1/2(\text{pH 7.4})}}{t_{1/2(\text{pH 5.5})}}$ was below 2.

We then decided to go further in this study and validate if the in-solution reactivity could be translated to usable data for the design of endosomal release systems. For that purpose, we decided to incorporate selected acid-labile structures into turn-on FRET-based probes that

View Article Online
DOI: 10.1039/C6OB00846A

will enable us to measure in-cell endosomal release under standardized and comparable conditions. More specifically, we chose four derivatives belonging to four different categories:

- An acylhydrazone derivative which hydrolyzes rapidly but not selectively according to pH.
- A dialkyl acetal derived from *o*-anisaldehyde with kinetic profiles similar to the spiro di-orthoester already studied but showing an improved selectivity for low pH.
- A tetrahydropyranyl ether derivative which showed no hydrolysis in our conditions.

hydrolysable pH 5.5	**fast hydrolysis** $t_{1/2(pH5.5)}$ < 1 h	**highly selective hydrolysis** *Ratio > 15*	*Spiro di-orthoester* *Dialkyl acetal derived from o-anisaldehyde*
		selective hydrolysis *2 < Ratio < 15*	Oxazolidine
		non-selective hydrolysis *Ratio < 2*	Imidazolidine Acetal derived from acetone Hydrazone derived from benzaldehyde *Acylhydrazone* Imine
	slow hydrolysis $t_{1/2(pH5.5)}$ > 1 h	**highly selective hydrolysis** *Ratio > 15*	1,3-dioxane 1,3-dioxolane *N*-ethoxybenzylimidazole
		selective hydrolysis *2 < Ratio < 15*	Siloxane
		non-selective hydrolysis *Ratio < 2*	β-thiopropionate
non-hydrolysable pH 5.5			Oxime Pyridine hydrazone Carbazate derivative *Tetrahydropyranyl ether* Carbamate

Fig. 8 Classification of pH-sensitive structures depending on rate of hydrolysis at pH 5.5 and selectivity of hydrolysis measured by the ratio of hydrolysis at pH 7.4 *vs*. pH 5.5.

View Article Online
DOI: 10.1039/C6OB00846A

Design and synthesis of FRET-type probes

On the basis of the previously reported TAMRA-Orthoester-(BHQ)-2 probe,[18] we designed four FRET-based probes including the acid-sensitive linker between a fluorophore TAMRA, and a quencher (BHQ)-2 (TAMRA-Acid sensitive linker-(BHQ)-2). It is worth noting that particular attention has to be taken when purifying the acid sensitive intermediates and final probes. Synthesis of TAMRA-Acylhydrazone-(BHQ)-2, TAMRA-Orthoester-(BHQ)-2 and TAMRA-Amide-(BHQ)-2 have been previously reported (Fig. 9).[18]

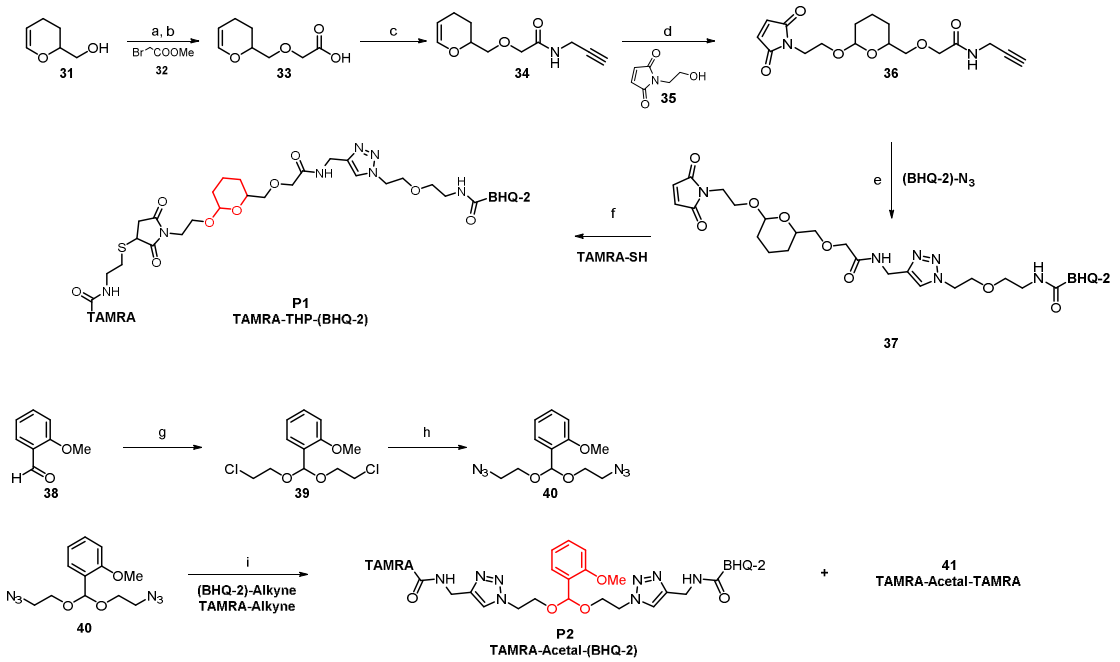
Synthesis of TAMRA-THP-(BHQ-2) probe P1

The synthesis started from (3,4-dihydro-2H-pyran-2-yl)methanol **31** which was converted to carboxylic acid **33** in two steps (Scheme 1). Coupling of **33** with propargylamine gave the corresponding amide **34** in good yield. Reaction of **34** with alcohol **35** in the presence of catalytic amount of p-TsOH gave the THP derivative **36**. A microwave assisted Click reaction between alkyne **36** and an azide derivative of BHQ-2 gave **37** which was further functionalized by Michael addition with TAMRA-SH to give probe TAMRA-THP-(BHQ-2) **P1** after careful purification by preparative HPLC (NH_3HCO_2H/ACN, pH 8.5).

Synthesis of TAMRA-Acetal-(BHQ-2) probe P2

Following the procedure described by Zhang and Zhao,[26] *o*-Anisaldehyde **38** was reacted with chloroethanolamine at 90 °C to give the corresponding acetal **39** which was further functionalized to the corresponding bis-azide **40** after treatment with sodium azide. Finally, a double copper catalyzed cycloaddition of bis-azide **40** with (BHQ-2)-alkyne and TAMRA-alkyne derivatives[26] gave TAMRA-Acetal-(BHQ-2) probe **P2** in 10% yield. The yield of the last reaction was unfavorably low because of solubility issues which favored the formation of TAMRA-acetal-TAMRA side product in 72% yield.

View Article Online
DOI: 10.1039/C6OB00846A



Scheme 1 Synthesis of TAMRA-THP-(BHQ)-2 and TAMRA-Acetal-(BHQ-2) probes **P1** and **P2**. Reagents and conditions: a) **32**, NaH, THF, 0 °C, 3 h, 77%; b) LiOH, MeOH, H_2O, rt, 5 h, quant.; c) propargylamine, HBTU, DIEA, DMF, rt, 16 h, 71%; d) **35**, p-TsOH, CH_2Cl_2, 0 °C to rt, 2 h, 77%; e) Sodium ascorbate, $CuSO_4$, ^tBuOH, H_2O, THF, 4Å molecular sieves, microwave, 50 °C, 1 h, 74%; f) CH_2Cl_2, MeOH, Et_3N, rt, 2 h, 62%; g) chloroethanolamine, p-TsOH, benzene, Dean-Stark, 90 °C, 16 h, 40%; h) NaN_3, DMF, 80 °C, 16 h, 26%; i) $CuSO_4$, sodium ascorbate, ^tBuOH, THF, H_2O, rt, 3 h, 10%.

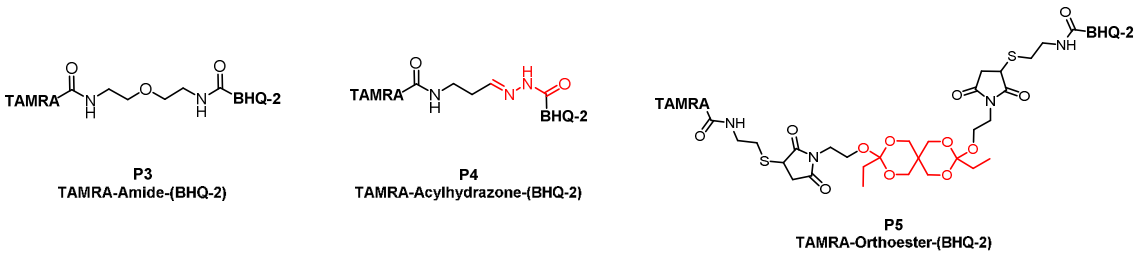


Fig. 9 Previously reported acid sensitive FRET based probes[18]

Effect of pH on hydrolysis of FRET-based probes

Before engaging in-cell studies, we validated that the FRET probes had similar in-solution hydrolytic profiles to their parent molecules. Kinetic fluorescence measurements were conducted at pH 3 to 7 to evaluate the stability of the different probes for 15 hours in

View Article Online
DOI: 10.1039/C6OB00846A

aqueous buffer solution (Fig. 10). For the THP-based probe, no significant hydrolysis was observed within the range of tested pH. The acetal-based probe **P2** was respectively hydrolyzed at 34 % and 16 % at pH 3 and 4 after 15 hours and showed little hydrolysis at pH 5 and 6. In agreement with the results of the small analogs, the hydrolysis of the acylhydrazone derivative did not display a strong pH-selectivity; only two different profiles were observed from pH 3-7. Alternatively, the hydrolysis of the Spiro diorthoester based probe **P5** showed a strong pH-dependency. It was totally and instantly hydrolyzed below pH 3, whereas 1 and 4.5 hours were necessary to get complete hydrolysis at pH 4 and 5, respectively. At pH 6 and 7, 65 % and 24 % of hydrolysis, respectively, were observed after 15 hours. As expected the general profile of hydrolysis rate is in agreement with data obtained by HPLC for the corresponding analogs of THP (non hydrolysable), Acylhydrazone (hydrolysable, non selective), Orthoester and Acetal (hydrolysable, selective).

However, the lability of the pH-sensitive linkers under acidic conditions appears to be much less important after incorporation into the FRET-based probe. Interestingly, these four probes showed slightly different profiles (from completely stable to quickly hydrolysable and from selective to non-selective). The next goal was to reveal which one will be the most suitable to achieve specific endosomal release.

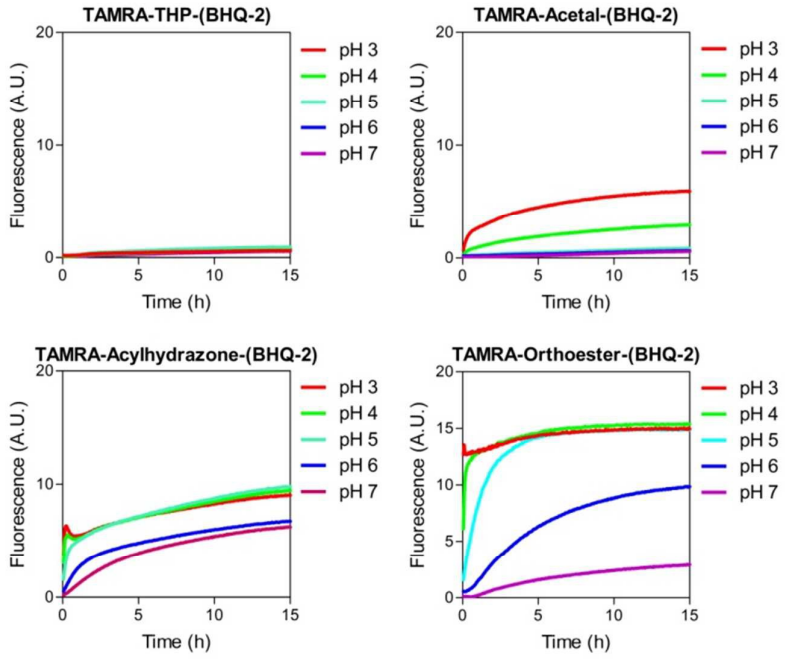


Fig. 10 Hydrolysis kinetics of FRET-based probes **P1**, **P2**, **P4** and **P5** at pH 3 (red lines), 4 (green lines), 5 (light blue lines), 6 (dark blue lines) and 7 (purple lines). All data

View Article Online
DOI: 10.1039/C6OB00846A

were recorded in triplicate at room temperature on a microplate reader and with a probe concentration of 0.5 µM in buffers (100 mM citric acid/phosphate buffer; pH 3 - 7).

Confocal microscopy and flow cytometry analysis

The biosensitivities of the different probes (**P1** – **P5**) were further studied in tissue cultures and cells were analyzed by both confocal microscopy to confirm lysosomal cleavage, and flow cytometry to provide quantitative information about intracellular probe hydrolysis. First BNL-CL2, a mouse liver cell line, was loaded with the different probes (1 µM, 90 min) and the nuclei were stained with Hoechst 33258. The cells were imaged by confocal microscopy and representative images are shown in Fig. 11 **TAMRA-Amide-(BHQ-2)** probe **P3** was used as non-hydrolysable control. Preliminary control experiments were performed using a small TAMRA derivative, such as the released reporter generated after hydrolysis of the acid-sensitive probes. This derivative was incubated in the presence of BNL CL.2 hepatocytes at 1 µM for 2 hours and imaging of the cells revealed an absence of fluorescence compare with the acid-sensitive probe **P5** (Fig. S8). Therefore, the intracellular fluorescence signal can be directly correlated to the intracellular hydrolysis of the probes, even if extracellular hydrolysis of the probe happens.

TAMRA-Orthoester-(BHQ-2) and **TAMRA-Acylhydrazone-(BHQ-2)** probes **P5** and **P4** displayed strong staining distributed predominantly in the peri-nuclear region of cytoplasm that are consistent with lysosomal cleavage (Fig. 11).[18] To get quantitative evaluation of the probe hydrolysis, NIH/3T3 cells were incubated with probes **P1** – **P5** for 4 hours, suspended in media and analyzed by flow cytometry (Fig. 12). This experiment showed that **TAMRA-Orthoester-(BHQ-2)** probe **P5** was the most efficient probe followed by **TAMRA-Acylhydrazone-(BHQ-2)** probe **P4**. **TAMRA-Acetal-(BHQ-2)** probe **P2** showed only very little hydrolysis compared to the non-hydrolysable control probe. These results are in good agreement with hydrolysis rates measured for probes **P1** – **P5** in solution (Fig. 10). Indeed acylhydrazone and orthoester-based probes showed significant hydrolysis at pH 5 and 6 after 1.5 hours while the acetal-based probe showed little to no hydrolysis at pH 5 and 6 after the same period of time. **TAMRA-Acetal-(BHQ-2)** probe **P2** did not shown any lability with little to no fluorescence observed (Fig. 11). Interestingly, **TAMRA-THP-(BHQ-2)** probe **P1** show a fluorescence signal similar to the **TAMRA-Acylhydrazone-(BHQ-2)** probe **P4**.

View Article Online
DOI: 10.1039/C6OB00846A

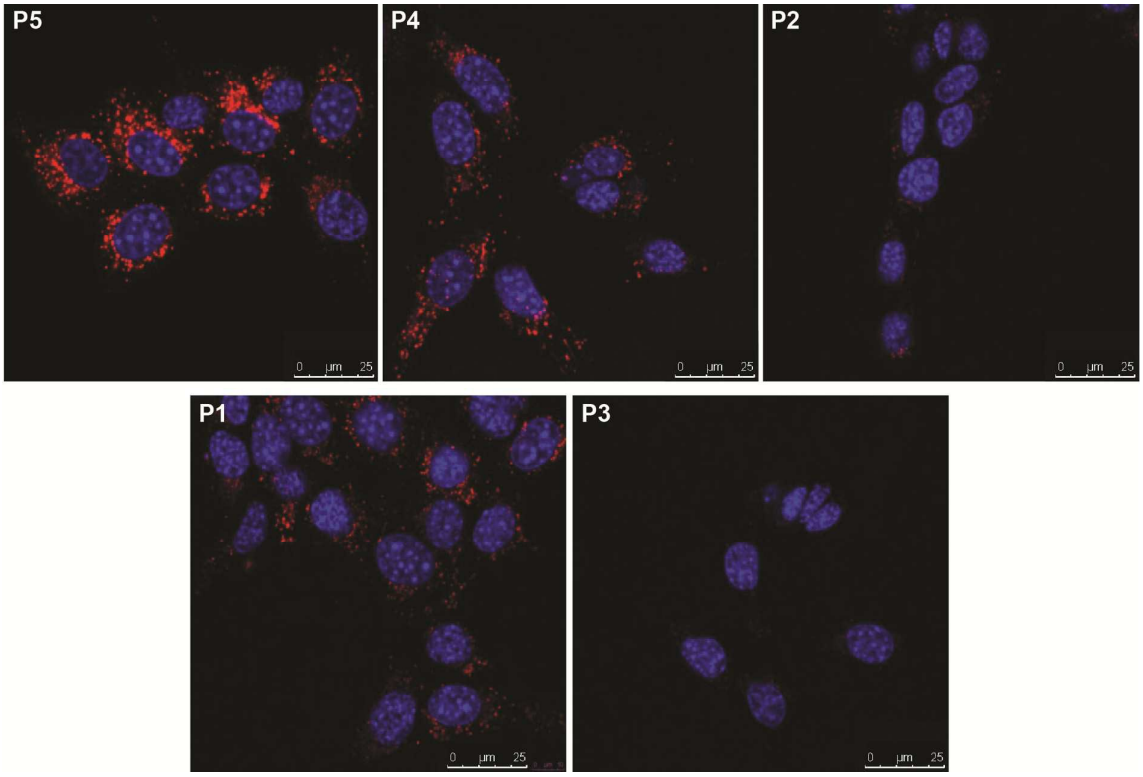


Fig. 11 *In*-vitro imaging of BNL CL.2 cells loaded with probes **P1** – **P5**; Red channel: activable probe (1 µM, 90 min), blue channel: Hoechst nuclei staining (5 µg/mL, 30 min); Scale bar: 25 µM.

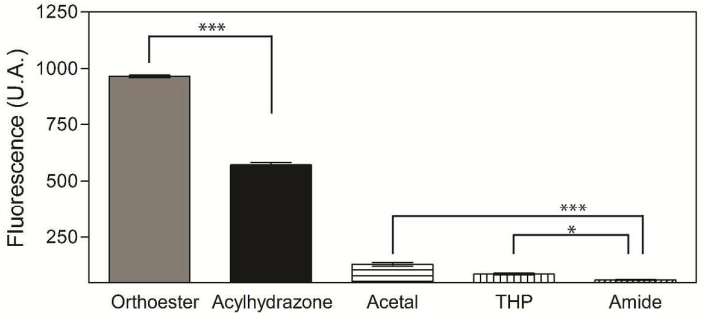


Fig. 12 Flow cytometry analysis (2000 cells/assay) of NIH/3T3 cells loaded with probes **P1** – **P5** (1 µM, 4h). One-way ANOVA using Dunnett's post-test were performed to compare all probes to the control amide and the orthoester to the acylhydrazone; *$p < 0.05$, ***$p < 0.001$.

View Article Online
DOI: 10.1039/C6OB00846A

Conclusion

In this study, the hydrolytic cleavage of a wide variety of molecular structures that were reported for their use in pH-sensitive delivery systems was carefully examined. Standardized data measured by HPLC enabled the establishment of a first classification of these groups based on their hydrolysis profiles at pH 5.5 and their relative hydrolysis at pH 5.5 *vs.* pH 7.4. From this classification four chemical functions belonging to three different categories were selected and used as core motifs to design FRET-based probes. These probes were used to quantify and compare the lysosomal cleavage of the different acid-sensitive structures (orthoester, acylhydrazone, acetal and tetrahydropyranyl ether).

Surprisingly, a wide variety of hydrolytic stability profiles could be found among the chemical functionality supposedly suitable for pH release systems. Even within a structural family, a slight modification of the substitution pattern could have an unsuspected outcome on the hydrolysis stability. Furthermore, we also showed that incorporating these small motifs in larger molecular constructs slowed down their hydrolysis without modifying their relative reactivity. These observations underline the need to carefully design a reporting probe to validate the actual stability of the cleavable group in the environment of the delivery system. Our tests also showed that only the most reactive functional groups underwent significant lysosomal cleavage according to flow cytometry measurements. Interestingly, these are the motifs that proved to be very difficult to synthesize and to store. They showed almost complete hydrolysis in our HPLC assay after 30 seconds. These last results question the validity of the claimed acid-based mechanism of action of some described drug release systems. We believe that structure-reactivity study and the methodology assessing in-cell hydrolysis of the main functional groups will provide a stimulating insight for the rational design and development of improved acid-sensitive linkers.

Acknowledgments

This work was supported through a research contract funded by the French Ministry of Research. The International Center for Frontier Research in Chemistry (icFRC) is acknowledged for financial support to LFCS.

View Article Online
DOI: 10.1039/C6OB00846A

View Article Online
DOI: 10.1039/C6OB00846A

Notes and references

1. J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Järvinen, J. Savolainen, *Nat. Rev. Drug. Discov.*, 2008, **7**, 255; (b) K. M. Huttunen, H. Raunio, J. Rautio, *Pharmacol. Rev.*, 2011, 63, 750; (c) S. Jana, S. Mandlekar, P. Marathe, *Curr Med Chem.*, 2010, **17**, 3874; (d) R. Mahato, W. Tai and K. Cheng, *Adv. Drug Delivery Rev.*, 2011, **63**, 659.
2. P. Sapra, A. T. Hooper, C. J. O'Donnell, H. – P. Gerber, *Expert Opin. Invest. Drugs*, 2011*, **20**, 1131.*
3. (a) S. O. Doronina, T. D. Bovee, D. W. Meyer, J. B. Miyamoto, M. E. Anderson, C. A. Morris-Tilden, P. D. Senter, *Bioconjugate Chem.*, 2008, **19**, 1960; (b) T. Legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. Le Pape, S. Lerondel, S. Papot, *Angew. Chem. Int. Ed.,* 2012*,* **51***,* 11606.
4. K. Takanuki, T. Igarashi, K. Hata, H. Hori, T. Shibata, H. Kitagawa, T. Satoh, S. Inayama. *Biochem Int.,* 1988, **17,** 155.
5. (a) G. Di Stefano, M. Lanza, F. Kratz, L. Merina, L. Fiume, *Eur. J. Pharm. Sci.*, 2004, **23**, 393; (b) P. Sapra, A. T. Hooper, C. J. O'Donnell, H. – P. Gerber, *Expert Opin. Invest. Drugs*, 2011, **20**, 1131; (c) Y. Shama, L. Adar, G. Ashkenasy, A. David, *Biomaterials*, 2011, **32**, 1377; (d) P. C. A. Rodrigues, K. Scheuermann, C. Stockmar, G. Maier, H. H. Fiebig, C. Unger, R. Mülhaupt, F. Kratz, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 355; (e) A. A. Kale, V. P. Torchilin, *Bioconj. Chem.*, 2007, **18**, 363; (f) J. M. Lehn, *Chem. – Eur. J.*, 1999, **5**, 2455; (g) J. M. Lehn, A. V. Eliseev, *Science*, 2001, **291**, 2331; (h) J. Shao, J. P. Tam, *J. Am. Chem. Soc.*, 1995, **117**, 3893; (i) M. J. Vicent, R. Duncan, *Trends Biotechnol.*, 2006, **24**, 39; (j) M. J. Vicent, F. Greco, R. I. Nicholson, A. Paul, P. C. Griffiths, R. Duncan, *Angew. Chem., Int. Ed.*, 2005, **44**, 4061; (k) R. Narang, B. Narasimhan, S. Sharma, *Curr. Med. Chem.*, 2012, **19**, 569; (l) K. Ulbrich, T. Etrych, P. Chytil, M. Jelinkova, B. Rihova, *J. Controlled Release*, 2003, **87**, 33; (m) Y. Bae, S. Fukushima, A. Harada, K. Kataoka, *Angew. Chem., Int. Ed.*, 2003, **42**, 4640; (n) S. Aryal, C. M. J. Hu, L. Zhang, *ACS Nano*, 2009, **4**, 251; (o) S. Liu, Y. Guo, R. Huang, J. Li, S. Huang, Y. Kuang, L. Han, C. Jiang, *Biomaterials*, 2012, **33**, 4907; (p) D. G. van der Poll, H. M. Kieler-Ferguson, W. C. Floyd, S. J. Guillaudeu, K. Jerger, F. C. Szoka, J. M. J. Fréchet, *Bioconjugate Chem.*, 2010, **21**, 764; (q) L. Zhu, C. Tu, B. Zhu, Y. Su, Y. Pang, D. Yan, J. Wu, X. Zhu, *Polym. Chem.*, 2011, **2**, 1761; (r) X. Yang, J. J. Grailer, S. Pilla, D. A.

View Article Online
DOI: 10.1039/C6OB00846A

Steeber, S. Gong, *Bioconjugate Chem.*, 2010, **21**, 496; (s) M. Talelli, M. Iman, A. K. Varkouhi, C. J. F. Rijcken, R. M. Schiffelers, T. Etrych, K. Ulbrich, C. F. van Nostrum, T. Lammers, G. Storm, W. E. Hennink, *Biomaterials*, 2010, **31**, 7797; (t) Y. Lee, S. Y. Park, H. Mok, T. G. Park, *Bioconjugate Chem.*, 2007, **19**, 525; (u) M. Prabaharan, J. J. Grailer, S. Pilla, D. A. Steeber, S. Gong, *Biomaterials*, 2009, **30**, 5757; (v) B. Chen, D. G. van der Poll, K. Jerger, W. C. Floyd, J. M. J. Fréchet, F. C. Szoka, *Bioconjugate Chem.*, 2011, **22**, 617.

6. (a) W. C. Shen, H. J. P. Ryser, *Biochem. Biophys. Res. Commun.*, 1981, **102**, 1048; (b) C. Du, D. Deng, L. Shan, S. Wan, J. Cao, J. Tian, S. Achilefu, Y. Gu., *Biomaterials*, 2013, **12**, 3087; (c) H. Wu, L. Zhu, V. P. Torchilin, *Biomaterials*, 2013, **34**, 1213; (d) Y. Kakinoki, Y. Kaneo, T. Ikeda, K. Tanaka, K. Fujita, *Biol. Pharm. Bull.*, 2008, **31**, 103; (e) S. Zhu, M. Hong, L. Zhang, G. Tang, Y. Jiang, Y. Pei, *Pharm. Res.*, 2010, **27**, 161; (f) N. Lavignac, J. L. Nicholls, P. Ferruti, R. Duncan, *Macromol. Biosci.*, 2009, **9**, 480; (g) M. Meyer, A. Philipp, R. Oskuee, C. Schmidt, E. Wagner, *J. Am. Chem. Soc.*, 2008, **130**, 3272; (h) K. Maier, E. Wagner, *J. Am. Chem. Soc.*, 2012, **134**, 10169.
7. V. F. Patel, J. N. Hardin, J. M. Mastro, K. L. Law, J. L. Zimmermann, W. J. Ehlhardt, J. M. Woodland, J. J. Starling, *Bioconjugate Chem.*, 1996, **7**, 497.
8. (a) L. Bildstein, C. Dubernet, P. Couvreur, *Adv. Drug Delivery Rev.*, 2011, **63**, 3; (b) M. C. Parrott, J. C. Luft, J. D. Byrne, J. H. Fain, M. E. Napier, J. M. Desimone, *J. Am. Chem. Soc.*, 2010, **132**, 17928; (c) A. Pichon, *Nat. Chem.*, 2011, **3**, 97; (d) M. C. Parrot, M. Finniss, J. C. Luft, A. Pandya, A. Gullapalli, M. E. Napier, J. M. DeSimone, *J. Am. Chem. Soc.*, 2012, **30**, 7978.
9. (a) C. Masson, M. Garinot, N. Mignet, B. Wetzer, P. Mailhe, D. Scherman, M. Bessodes, *J. Controlled Release*, 2004, **99**, 423; (b) J. Heller, *Biomaterials*, 1990, **11**, 659 and references herein; (c) J. Heller, J. Barr, S. Y. Ng, K. Schwach Abdellauoi, R, Gurny, *Advanced Drug Delivery Reviews*, 2002, **54**, 1015; (d) X. Guo, F. C. Szoka Jr., *Bioconjugate Chem.*, 2001, **12**, 291; (e) X. Guo, F. C. Szoka Jr. US 6,897,196 B1.
10. (a) R. Liu, Y. Zhang, X. Zhao, A. Agarwal, L. J. Mueller, P. Feng, *J. Am. Chem. Soc.*, 2010, **132**, 1500; (b) T. Fife, L. Jao *J. Org. Chem.*, 1965, **30**, 1492; (c) E. R. Gillies, A. P. Goodwin, J. M. J. Fréchet, *Bioconjugate Chem.* 2004, **15**, 1254; (d) E. R. Gillies, J. M. J. Fréchet, *Bioconjugate Chem.* 2005, **16**, 361; (e) E. M. Bachelder, T. T. Beaudette, K. E. Broaders, J. Dashe, J. M. J. Frechet, *J. Am. Chem. Soc.*, 2008, **130**, 10494; (f) J. Heller, J.

View Article Online
DOI: 10.1039/C6OB00846A

Barr, S. Y. Ng, K. S. Abdellauoi, R. Gurny, *Adv. Drug Delivery Rev.*, 2002, **54**, 1015; (g) E. R. Gillies, J. M. J. Fréchet, *Chem. Commun.*, 2003, 1640; (h) N. Murthy, Y. X. Thng, S. Schuck, M. C. Xu, J. M. J. Fréchet, *J. Am. Chem. Soc.*, 2002, **124**, 12398; (i) J. L. Cohen, A. Almutairi, J. A. Cohen, M. Bernstein, S. L. Brody, D. P. Schuster, J. M. J. Fréchet, *Bioconjugate Chem.*, 2008, **19**, 876; (j) V. Knorr, V. Russ, L. Allmendinger, M. Ogris, E. Wagner, *Bioconjugate Chem.*, 2008, **19**, 1625; (k) Z. Liu, M. Zheng, F. Meng, Z. Zhong, *Biomaterials*, 2011, **32**, 9109; (l) Y. J. Kwon, E. James, N. Shastri, J. M. J. Fréchet, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 18264; (m) W. Chen, F. Meng, R. Cheng, Z. Zhong, *J. Controlled Release*, 2010, **142**, 40–46; (n) J. Zou, G. Jafr, E. Themistou, Y. Yap, Z. A. P. Wintrob, P. Alexandridis, A. C. Ceacareanu, C. Cheng, *Chem. Commun.*, 2011, **47**, 4493.

11. (a) M. Oishi, S. Sasaki, Y. Nagasaki; K. Kataoka, *Biomacromolecules*, 2003, **4**, 1426; (b) M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* 2005, **127**, 1624.
12. J. H. Jeong, S. W. Kim, T. G. Park, *Bioconjugate Chem.*, 2003, **14**, 473.
13. (a) Y. – L. Zhao, Z. Li, S. Kabehie, Y. Y. Botros, J. F. Stoddart, J. I. Zink, *J. Am. Chem. Soc.*, 2010, **132**, 13016; (b) Y. Jia, J. Fei, Y. Cui, Y. Yang, L. Gao, J. Li, *Chem. Commun.* 2011, **47**, 1175.
14. (a) J. Shin, P. Shum, D. H. Thompson, *J. Controlled Release*, 2003, **91**, 187; (b) H. – K. Kim, J. Van den Bossche, S. – H. Hyun, D. H. Thompson, *Bioconjugate Chem.*, 2013, **23**, 2071.
15. (a) S. D. Kong, A. Luong, G. Manorek, S. B. Howell, J. Yang, *Bioconjugate Chem.*, 2007, **18**, 293; (b) A. Luong, T. Issarapanichkit, S. D. Kong, R. Fong, J. Yang, *Org. Biomol. Chem*., 2010, **8**, 5105; (c) E. S. Lee, K. Na, Y. H. Bae, *Nano Lett.*, 2005, **5**, 325; (d) E. S. Lee, D. Kim, Y. S. Youn, K. T. Oh, Y. H. Bae, *Angew. Chem., Int. Ed.*, 2008, **47**, 2418.
16. (a) M. J. Heffernan, N. Murthy, *Bioconjugate Chem.,* 2005, **16,** 1340; (b) J. Sankaranarayanan, E. A. Mahmoud, G. Kim, J. M. Morachis, A. Almutairi, *A. C. S. Nano,* 2010, **4,** 5930.
17. V. Balamuralidhara, T. M. Pramodkumar, N. Srujana, M. P. Venkatesh, N. Vishal Gupta, K. L. Krishna, H. V. Gangadharappa, *American Journal of Drug Discovery and Development*, 2011, **1**, 24; (b) G. Leriche, L. Chisholm, A. Wagner, *Bioorg. Med.*

View Article Online
DOI: 10.1039/C6OB00846A

Chem., 2012, **20**, 571; (c) K. Ulbrich, V. Subr, *Adv. Drug Delivery Rev.*, 2004, **56**, 1023; (d) Z. Ge, S. Liu, *Chem. Soc. Rev.*, 2013, **42**, 7289.

18. G. Leriche, M. Nothisen, N. Baumlin, C. D. Muller, D. Bagnard, J. – S. Remy, S. A. Jacques, A. Wagner, *Bioconjugate Chem.*, 2015, **26**, 1461.
19. Y. Yu, C. - K. Chen, W. - C. Law, E. Weinheimer, S. Sengupta, P. N. Prasad, C. Cheng, *Biomacromolecules*, 2014, **15**, 524.
20. G. F. Walker, C. Fella, J. Pelisek, J.Fahrmeir, S. Boeckle, M. Ogris, E. Wagner, *Mol. Ther,.* 2005, **11**, 418.
21. M. S. Newman, R. J. Harper, *J. Am. Chem. Soc.,* 1958, **80**, 6350.
22. (a) O. Ceder, *Arkiv. Kemi.*, 1954, **6**, 354; (b) R. Leutner, *Monatsh.*, 1932, **60**, 317; (c) R. Leutner, *Monatsh.*, 1935, **66**, 222.
23. (a) M. Johansen, H. Bundgaard, *J. Parm. Sci.*, 1983, **72**, 1294; (b) A. Buur, H. Bundgaard, *Int. J. Pharm.,* 1984, **18**, 325.
24. P. G. M. Wuts, T. W. Greene, Greene's Protective Groups in Organic Synthesis, 4th ed., Wiley: New York, 2007.
25. R. G. Schoenmakers, P. van de Wetering, D. L. Elbert, J. A. Hubbell, *J. Controlled Release*, 2004, **95**, 291.
26. S. Zhang, Y. Zhao, *J. Am. Chem. Soc,* 2010, **132**, 10642.
27. See the Supporting Information for described procedures.
28. Y. H. Kim, W. E. Stites, *Biochemistry,* 2008, **47**, 8804.