



Cite this: *Org. Biomol. Chem.*, 2017, **15**, 7339

## Selective lysine modification of native peptides via aza-Michael addition†

Hongli Chen, \* Rong Huang, Zhihong Li, Wei Zhu, Jiakang Chen, Yuexiong Zhan and Biao Jiang\*

A series of vinylsulfonamides were synthesized and screened for site-selective modification of the  $\epsilon$ -amino group of lysine-bearing free  $\alpha$ -amine residues. *N*-Methyl-*N*-phenylethanesulfonamide has emerged as an applicable reagent and has been developed for efficient and highly selective modification of the lysine residue of native peptides in the presence of a free N-terminus via aza-Michael addition. We demonstrated that functional *N*-phenylvinylsulfonamide derivatives with a fluorescent moiety or drug could also be conjugated to the lysine residue of octreotide and insulin with high specificity, without modifying the N-terminus. Our method provides a promising strategy for site-selective lysine functionalization in native peptides with a free N-terminus.

Received 27th July 2017,  
Accepted 21st August 2017

DOI: 10.1039/c7ob01866e

rsc.li/obc

### Introduction

The modification of native peptides at a single amino acid or specific site with synthetic moieties is a significant challenge.<sup>1–3</sup> Among the approximately 20 primary amino acids that compose proteins, only a subset can serve as appropriate targets for bioconjugation. Many strategies have been developed to target nucleophilic natural amino acid residues, of which cysteine and lysine residues are the most popular labeling sites. Cysteine is capable of being involved in a chemoselective reaction due to its superior nucleophilic properties.<sup>4–7</sup> However, the low abundance of free cysteine residues due to its tendency to form disulfide bonds limits its application. Lysine residues are abundant in native proteins, and methods to modify primary amines can provide versatile techniques to label lysine residues.<sup>8,9</sup> However, lysine conjugation methods often modify the N-terminus.<sup>10,11</sup> Site-specific modification of the N-terminus is easier to achieve because the  $\epsilon$ -amino group of lysine is more difficult to deprotonate due to its higher  $pK_a$  ( $\sim 10$ ) compared to the N-terminal  $\alpha$ -amino group ( $pK_a \sim 8$ ). A number of strategies for selective N-terminal modification of native peptides or proteins have been developed.<sup>12–18</sup> In contrast, site-specific functionalization of the  $\epsilon$ -amino group of lysine has rarely been studied.<sup>19,20</sup> Typically, the most common reagents for amine modification, *N*-hydroxysuccinimides (NHS) esters, also react with serine,

tyrosine and histidine, affording heterogeneous bioconjugates.<sup>21</sup> Although the stepwise addition of the substoichiometric amounts of an NHS ester can achieve a single lysine-selective modification, tediousness and low efficiency are the problems.<sup>22</sup> Indeed, low reactivity and the generation of byproducts are the major problems encountered when attempting to selectively label an amine. The Michael-type addition is also an attractive strategy for protein conjugation. Michael acceptors, such as maleimides, vinyl sulfones and acrylamides, have been frequently utilized in the modification of proteins. Vinyl sulfonamides, with a similar structure to vinyl sulfones, are easier to prepare and have also received some attention in applications for bioconjugation.<sup>23–26</sup>

Although the lysine  $\epsilon$ -amino group requires a higher pH to deprotonate, it has higher intrinsic nucleophilicity. Therefore, selective lysine modification can be achieved by carefully adjusting the nucleophilicity and basicity. Herein, we report a class of vinylsulfonamides which site-specifically modify the lysine residue in native peptides rather than the N-terminus via aza-Michael addition.

### Results and discussion

We first synthesized a series of vinylsulfonamides (Fig. 1, compounds 1–7). We found that activated phenylamines were the easiest to use to produce divinylsulfonamides (Fig. 1, compounds 8–10), which would be developed as efficient reagents for peptide stapling in our coming research. Then, we began to screen the abilities of different vinylsulfonamides to promote selective  $\epsilon$ -amino group modification. Our initial study found that triethylenediamine (DABCO) buffer was a good medium

The institute for Advanced Immunochemical Studies, ShanghaiTech University, 99 Haike Road, Pudong, Shanghai, 201210, P.R. China.

E-mail: chenhl@shanghaitech.edu.cn, jiangbiao@shanghaitech.edu.cn

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ob01866e



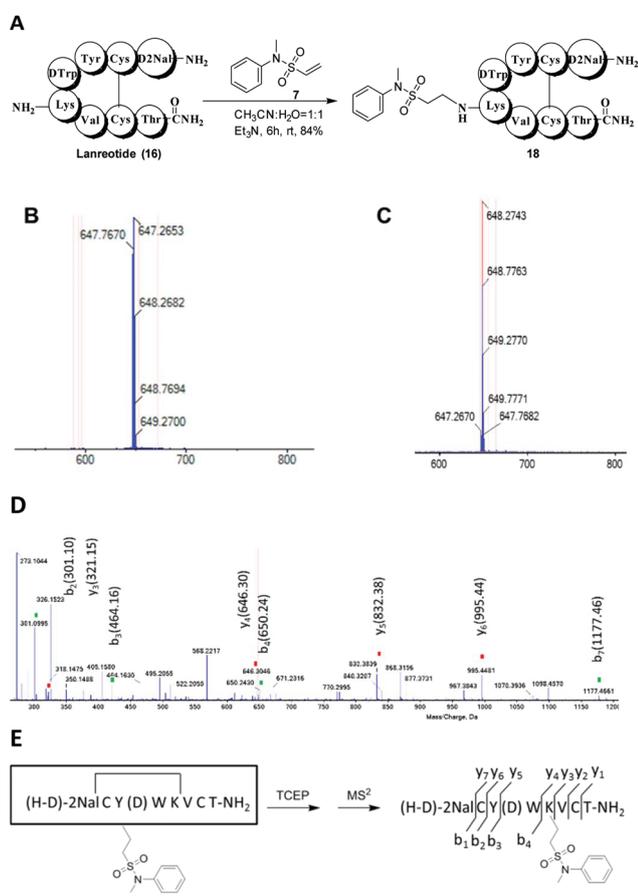


analyzed by RP-HPLC (Fig. 2B). The HRESIMS spectrum showed that product **14** was mono-labeled, with signals at 1216.4995  $[M + H]^+$  and 608.7549  $[M + 2H]^{2+}$  (Fig. 2C). After the reduction of product **14** with TCEP, peptide fragments were obtained and identified by MS<sup>2</sup> (Fig. 2D). The peptide fragment Lys-Thr-Cys-Thr-ol with  $m/z$  635.28  $[M + H]^+$  carried the Michael acceptor moiety, because it corresponds to the calculated M.W. of 634.28 g mol<sup>-1</sup>. The other peptide fragment H<sub>2</sub>N-D-Phe-Cys-Phe-D-Trp with  $m/z$  584.23  $[M + H]^+$  was not modified (Fig. 2E). Therefore, the lysine rather than the N-terminus was selectively functionalized. Compound **14** could be further labeled with fluorescein isothiocyanate (FITC) at the N-terminus to produce compound **15** with high yield (87%). The MS and MS<sup>2</sup> experiment for compound **15** confirmed its structure and attachment site based on an  $m/z$  of 1606.5349  $[M + H]^+$  and 803.7729  $[M + 2H]^{2+}$  and the presence of the fragment FITC-NH-D-Phe with  $m/z$  537.11 (Fig. 2F-H).

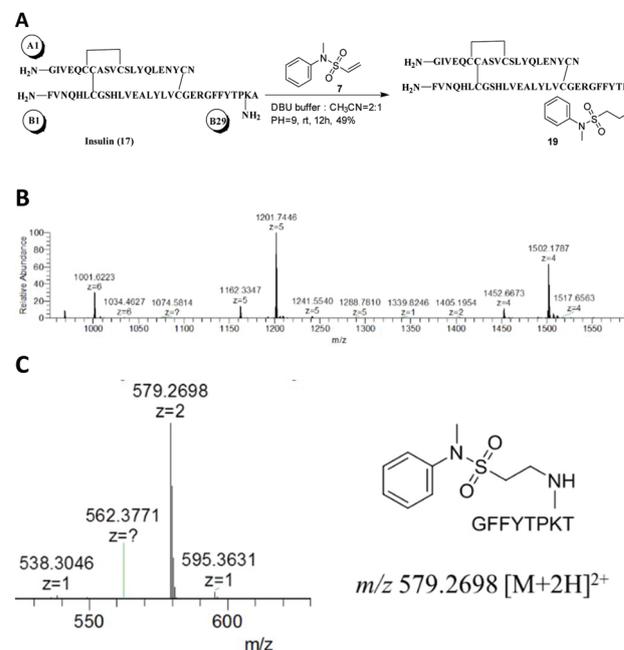
To demonstrate the general applicability of our method to selectively modify native peptides at lysine residues, we also investigated the reaction of compound **7** with lanreotide (**16**) and insulin (**17**). Lanreotide, similar to octreotide, is a somatostatin analog with the sequence H-D-2Nal-Cys(2)-Tyr-D-Trp-Lys-

Val-Cys(7)-Thr-NH<sub>2</sub>. It was modified with compound **7** in a similar fashion to obtain the lysine-modified product **18** with high yield (84%) (Fig. 3A). The HRESIMS spectrum showed that product **18** was mono-labeled, with signals at 647.2653  $[M + 2H]^{2+}$  (Fig. 3B). After the reduction of product **18** with TCEP (Fig. 3C), peptide fragments were obtained and identified by MS<sup>2</sup> (Fig. 3D and E). The result showed that the lysine residue was modified.

Insulin (**17**), a peptide hormone consisting of two polypeptide chains – the A chain (glycine-A1) and B chain (phenylalanine-B1) – has a lysine in the B chain (lysine-B29). The B29 position is generally tolerant with respect to the insulin receptor affinity, and B29-modified analogues such as detemir and degludec (FDA approved drugs) have demonstrated excellent safety profiles.<sup>31</sup> B29 was found to react at a much slower rate than the N-termini of A1 and B1 when insulin was treated with FITC at pH 9.1, producing a mono-A1, mono-B1, di-A1B1, and tri-substituted A1B1B29-labeled mixture.<sup>32</sup> Studies have revealed that NHS ester-based reagents are preferable to selectively modify B29,<sup>33,34</sup> however, there is also a report on the difficulty to selectively attach a single group to the available amine sites.<sup>35</sup> Here, the ability of vinylsulfonamide-based aza-Michael addition to site-specifically label insulin B29 was investigated. To strengthen the applicability of this reaction, more suitable reaction conditions were explored. First, insulin was treated with compound **7** in a buffer series containing various bases at approximately pH 7 and room temperature. The buffer adjusted with DBU was optimal at promoting the site-specific B29 modification of insulin. Although the majority of the conversion yielded a single product, the conver-



**Fig. 3** Selective lysine modification of lanreotide. (A) The reaction scheme. (B) The HRESIMS spectrum of product **18**. (C) The HRESIMS spectrum of the reduced product of compound **18**. (D) The MS<sup>2</sup> spectrum of the reduced product of compound **18**. (E) Peptide fragments.

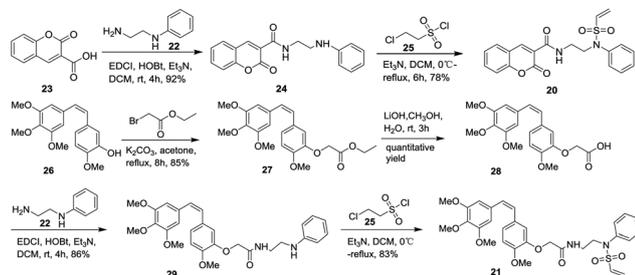


**Fig. 4** Selective modification of insulin B29 lysine. (A) The reaction scheme. (B) The HRESIMS spectrum of product **19**. (C) The MS<sup>2</sup> spectrum of product **19**.



sion of insulin was poor (<10%). The effect of pH was examined based on the DBU buffer. By screening buffers from pH 7 to pH 12, we found that the conversion of insulin was low

when the pH was below 8, and the reaction became complicated when the pH was more than 10. Moderate conversion (52%) and excellent selectivity (almost a single product **19** in 49% yield and 90% yield based on the recovered insulin) were observed in pH 9 buffer adjusted with DBU (Fig. 4A). The iso-



Scheme 2 Synthesis of functional vinylsulfonamides **20** and **21**.

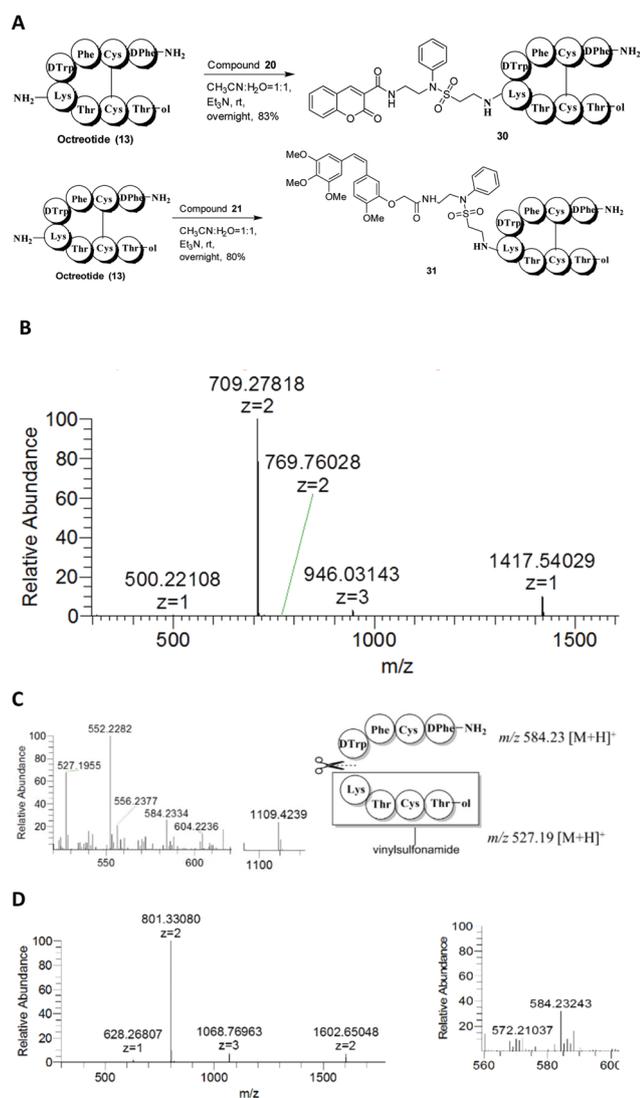


Fig. 5 Selective lysine modification of octreotide with functional vinylsulfonamides **20** and **21**. (A) The reaction scheme. (B) The HRESIMS and MS<sup>2</sup> spectrum of product **30**. (C) The MS<sup>2</sup> spectrum of product **30**. (D) The HRESIMS and MS<sup>2</sup> spectrum of product **31**.

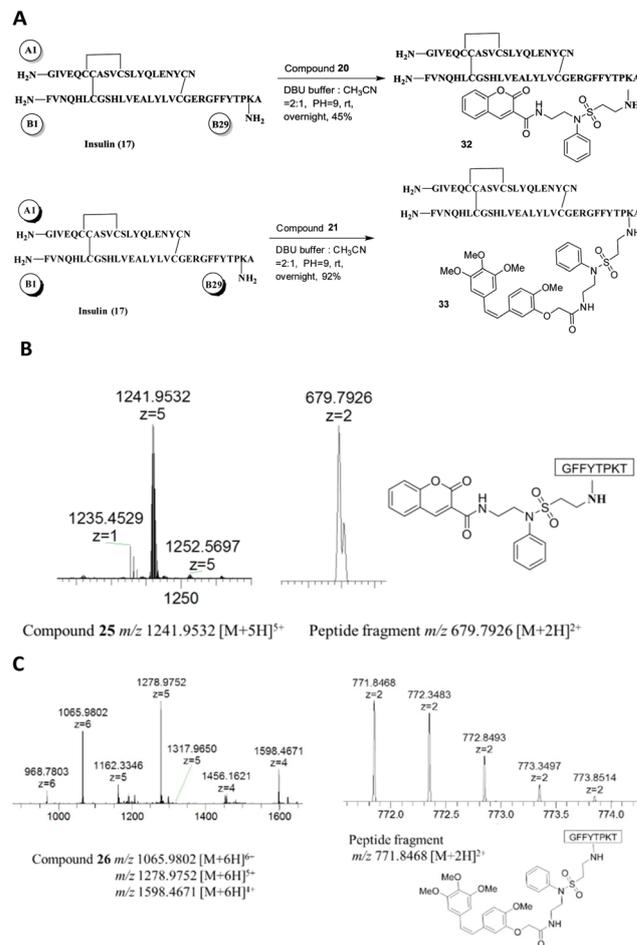


Fig. 6 Selective lysine modification of insulin with functional vinylsulfonamides **20** and **21**. (A) The reaction scheme. (B) The HRESIMS and MS<sup>2</sup> spectrum of product **32**. (C) The HRESIMS and MS<sup>2</sup> spectrum of product **33**. (D) RP-HPLC of the reaction showing that compound **21** was conjugated to insulin.





peptides, octreotide and insulin was investigated. As previously described, octreotide was allowed to react with compounds **20** and **21** in the presence of  $\text{Et}_3\text{N}$ , leading to the mono-labeled derivatives **30** and **31** with high yield and successful selectivity (Fig. 5A). For compound **30**, the mass peak of  $m/z$  709.2782  $[\text{M} + 2\text{H}]^{2+}$  and 1417.5403  $[\text{M} + \text{H}]^+$  was found in the HRESIMS spectrum, matching its calculated M.W. of 1416.5341  $\text{g mol}^{-1}$  (Fig. 5B). After in-gel digestion by chymotrypsin, the  $\text{MS}^2$  spectrum of compound **30** was obtained. The degradation product ( $m/z$  1109.4239) was detected under  $\text{MS}^2$  conditions due to the instability of the attached probe **20**. Compound **20** was degraded to a residue with  $m/z$  308.1147 when it was directly used for the  $\text{MS}^2$  experiment under the same conditions. The modified peptide fragment Lys-Thr-Cys-Thr-ol with  $m/z$  527.1955 included a fragment from probe **20** ( $m/z$  399.04 – 308.11 = 90.93) and the unmodified peptide fragment  $\text{H}_2\text{N-D-Phe-Cys-Phe-D-Trp}$  with  $m/z$  584.2334, indicating labeling at lysine (Fig. 5C). For compound **31**, the mono-lysine-modified feature was also determined by HRESIMS with signals at  $m/z$  801.3308  $[\text{M} + 2\text{H}]^{2+}$  and 1602.6505  $[\text{M} + \text{H}]^+$  (calculated value: 1600.6441) and  $\text{MS}^2$  with an unmodified peptide fragment  $\text{H}_2\text{N-D-Phe-Cys-Phe-D-Trp}$  ( $m/z$  584.2324) (Fig. 5D).

In the case of insulin, to conjugate with compound **20** or **21**, specific mono-modification of the B29 lysine was performed using the above optimized method with DBU buffer at  $\text{pH} = 9$  (Fig. 6A). Products **32** and **33** were also confirmed by MS and  $\text{MS}^2$  (Fig. 6B and C). Compound **21** was an excellent coupling reagent for insulin that resulted in successful conversion (>95%), yield (92%) and selectivity (almost a single product) (Fig. 6D).

Somatostatin (**34**) which has a free N-terminus and two lysine residues with the sequence H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH (disulfide bridge Cys3–Cys14) was employed to investigate whether there was a preference for a single lysine residue. Somatostatin was reacted with compound **7** in DBU buffer ( $\text{pH} = 9$ ) (Fig. 7A). The resulting HRESIMS spectrum revealed that a mono-labeled product **35** (9%) was obtained, with signals at 612.2921  $[\text{M} + 3\text{H}]^{3+}$  and 917.9351  $[\text{M} + 2\text{H}]^{2+}$  (Fig. 7B). Similarly, after the reduction of product **35** with TCEP (Fig. 3C), peptide fragments were obtained and identified by  $\text{MS}^2$  (Fig. 7D and E). The single K9 residue of somatostatin was modified. However, the conversion was low and the reaction system became complicated with the prolonged reaction time. When somatostatin (**34**) was reacted with compound **7** (3 eq.) in the presence of  $\text{Et}_3\text{N}$  (6 eq.) in a  $\text{CH}_3\text{CN}$  and  $\text{H}_2\text{O}$  (1 : 1), a main di-labelled product (**36**) was yielded (63%) with signals at 678.3126  $[\text{M} + 3\text{H}]^{3+}$  and 1016.9655  $[\text{M} + 2\text{H}]^{2+}$  (Fig. 7F). After the reduction of product **36** with TCEP (Fig. 7G), the resulting HRESIMS spectrum showed that A1 and K9 were modified (Fig. 7H–I).

## Conclusions

Selective and effective modification of a lysine residue in the presence of an N-terminus is anticipated to be difficult due to

the lower  $\text{pK}_a$  of the N-terminal position. Here, a useful and efficient method for utilizing vinylsulfonamide-based aza-Michael addition to selectively modify lysine instead of the N-terminus on peptides was developed. Furthermore, we demonstrated that functional vinylsulfonamide derivatives could also be produced *via* reaction with a fluorescent moiety or drug and achieve the selective bioconjugation. The CA4-conjugated probe showed excellent reactivity and selectivity to modify the B29 lysine of insulin. This is a promising method for native peptide modification where the N-terminus is crucial for peptide bioactivity. However, peptides that contain multiple lysine residues will result in a complicated situation. And our method is more beneficial for peptides that lack an accessible free Cys residue which has superior reactivity. Efforts to develop more useful vinylsulfonamide reagents and application of this method to modify peptides or proteins are currently underway.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

This work was supported by ShanghaiTech university.

## Notes and references

- 1 N. Krall, F. P. da Cruz, O. Boutureira and G. J. Bernardes, *Nat. Chem.*, 2016, **8**, 103–113.
- 2 O. Koniev and A. Wagner, *Chem. Soc. Rev.*, 2015, **44**, 5495–5551.
- 3 O. Boutureira and G. J. Bernardes, *Chem. Rev.*, 2015, **115**, 2174–2195.
- 4 A. Abbas, B. Xing and T. P. Loh, *Angew. Chem., Int. Ed.*, 2014, **53**, 7491–7494.
- 5 I. M. Serafimova, M. A. Pufall, S. Krishnan, K. Duda, M. S. Cohen, R. L. Maglathlin, J. M. McFarland, R. M. Miller, M. Frodin and J. Taunton, *Nat. Chem. Biol.*, 2012, **8**, 471–476.
- 6 D. Kalia, P. V. Malekar and M. Parthasarathy, *Angew. Chem., Int. Ed.*, 2016, **55**, 1432–1435.
- 7 S. B. Gunnoo and A. Madder, *ChemBioChem*, 2016, **17**, 529–553.
- 8 H. G. Lee, G. Lautrette, B. L. Pentelute and S. L. Buchwald, *Angew. Chem., Int. Ed.*, 2017, **56**, 3177–3181.
- 9 C. L. Tung, C. T. Wong, E. Y. Fung and X. Li, *Org. Lett.*, 2016, **18**, 2600–2603.
- 10 S. T. Larda, D. Pichugin and R. S. Prosser, *Bioconjugate Chem.*, 2015, **26**, 2376–2383.
- 11 S. Diethelm, M. A. Schafroth and E. M. Carreira, *Org. Lett.*, 2014, **16**, 3908–3911.
- 12 D. Chen, M. M. Disotuar, X. Xiong, Y. Wang and D. H.-C. Chou, *Chem. Sci.*, 2017, **8**, 2717–2722.



- 13 J. I. MacDonald, H. K. Munch, T. Moore and M. B. Francis, *Nat. Chem. Biol.*, 2015, **11**, 326–331.
- 14 Z. W. Lai, A. Petrera and O. Schilling, *Curr. Opin. Chem. Biol.*, 2015, **24**, 71–79.
- 15 A. C. Obermeyer, J. B. Jarman and M. B. Francis, *J. Am. Chem. Soc.*, 2014, **136**, 9572–9579.
- 16 A. O. Chan, C. M. Ho, H. C. Chong, Y. C. Leung, J. S. Huang, M. K. Wong and C. M. Che, *J. Am. Chem. Soc.*, 2012, **134**, 2589–2598.
- 17 G. Xu, S. B. Shin and S. R. Jaffrey, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 19310–19315.
- 18 J. M. Gilmore, R. A. Scheck, A. P. Esser-Kahn, N. S. Joshi and M. B. Francis, *Angew. Chem., Int. Ed.*, 2006, **45**, 5307–5311.
- 19 M. Chilamari, L. Purushottam and V. Rai, *Chem. – Eur. J.*, 2017, **23**, 3819–3823.
- 20 L. Purushottam, S. R. Adusumalli, M. Chilamari and V. Rai, *Chem. Commun.*, 2017, **53**, 959–962.
- 21 N. Abello, H. A. Kerstjens, D. S. Postma and R. Bischoff, *J. Proteome Res.*, 2007, **6**, 4770–4776.
- 22 X. Chen, K. Muthoosamy, A. Pfisterer, B. Neumann and T. Weil, *Bioconjugate Chem.*, 2012, **23**, 500–508.
- 23 J. Dadova, M. Vrabel, M. Adamik, M. Brazdova, R. Pohl, M. Fojta and M. Hocek, *Chem. – Eur. J.*, 2015, **21**, 16091–16102.
- 24 J. L. Furman, M. Kang, S. Choi, Y. Cao, E. D. Wold, S. B. Sun, V. V. Smider, P. G. Schultz and C. H. Kim, *J. Am. Chem. Soc.*, 2014, **136**, 8411–8417.
- 25 E. H. Suh, Y. Liu, S. Connelly, J. C. Genereux, I. A. Wilson and J. W. Kelly, *J. Am. Chem. Soc.*, 2013, **135**, 17869–17880.
- 26 J. Dadova, P. Orsag, R. Pohl, M. Brazdova, M. Fojta and M. Hocek, *Angew. Chem., Int. Ed.*, 2013, **52**, 10515–10518.
- 27 H. Y. Zhang, W. Q. Xu, Y. W. Wang, E. Omari-Siaw, Y. Wang, Y. Y. Zheng, X. Cao, S. S. Tong, J. N. Yu and X. M. Xu, *Int. J. Pharm.*, 2016, **502**, 98–106.
- 28 M. Huo, Q. Zhu, Q. Wu, T. Yin, L. Wang, L. Yin and J. Zhou, *J. Pharm. Sci.*, 2015, **104**, 2018–2028.
- 29 D. H. Na, K. C. Lee and P. P. DeLuca, *Pharm. Res.*, 2005, **22**, 743–749.
- 30 T. Yin, Q. Wu, L. Wang, L. Yin, J. Zhou and M. Huo, *Mol. Pharm.*, 2015, **12**, 3020–3031.
- 31 A. N. Zaykov, J. P. Mayer and R. D. DiMarchi, *Nat. Rev. Drug Discovery*, 2016, **15**, 425–439.
- 32 D. Jacob, M. Joan Taylor, P. Tomlins and T. S. Sahota, *J. Fluoresc.*, 2016, **26**, 617–629.
- 33 D. H. Chou, M. J. Webber, B. C. Tang, A. B. Lin, L. S. Thapa, D. Deng, J. V. Truong, A. B. Cortinas, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 2401–2406.
- 34 T. H. Jensen, S. Havelund, P. K. Nielsen and J. Markussen, *J. Am. Chem. Soc.*, 2005, **127**, 6158–6159.
- 35 K. Thibaudeau, R. Leger, X. Huang, M. Robitaille, O. Quraishi, C. L. Soucy, N. B. Gagnon, P. V. Wyk, V. Paradis, J. P. Castaigne and D. Bridon, *Bioconjugate Chem.*, 2005, **16**, 1000–1008.
- 36 A. Kamal, A. Mallareddy, M. Janaki Ramaiah, S. N. Pushpavalli, P. Suresh, C. Kishor, J. N. Murty, N. S. Rao, S. Ghosh, A. Addlagatta and M. Pal-Bhadra, *Eur. J. Med. Chem.*, 2012, **56**, 166–178.

