

EDGE ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#)



Cite this: *Chem. Sci.*, 2020, **11**, 11266

All publication charges for this article have been paid for by the Royal Society of Chemistry

Solid-phase fluorescent BODIPY-peptide synthesis via *in situ* dipyrin construction†

Yue Wu,^a Wing-Sze Tam,^a Ho-Fai Chau,^a Simranjeet Kaur,^a Waygen Thor,^a Wei Shen Aik,^a Wai-Lun Chan,^a Markus Zweckstetter^{*bc} and Ka-Leung Wong^a  

Traditional fluorescent peptide chemical syntheses hinge on the use of limited fluorescent/dye-tagable unnatural amino acids and entail multiple costly purifications. Here we describe a facile and efficient protocol for *in situ* construction of dipyrins on the N-terminus with 20 natural and five unnatural amino acids and the lysine's side chain of selected peptides/peptide drugs through Fmoc-based solid-phase peptide synthesis. The new strategy enables the direct formation of boron-dipyrromethene (BODIPY)-peptide conjugates from simple aldehyde and pyrrole derivatives without pre-functionalization, and only requires a single-time chromatographic purification at the final stage. As a model study, synthesized EBNA1-targeting BODIPY₁-Pep₄ demonstrates intact selectivity *in vitro*, responsive fluorescence enhancement, and higher light cytotoxicity due to the photo-generation of cytotoxic singlet oxygen. This work offers a novel practical synthetic platform for fluorescent peptides for multifaceted biomedical applications.

Received 3rd September 2020
Accepted 23rd September 2020

DOI: 10.1039/d0sc04849f
rsc.li/chemical-science

Introduction

Fluorescent peptide/peptidomimetic-based targeting and visualization of the subcellular localization,^{1,2} and probing and modulation of intra/inter-biomolecular interactions,^{3,4} of the protein target of interest provides a versatile platform, either *in vitro/vivo* or in assays,^{5–7} for fundamental biochemistry/chemical biology research,^{8–10} as well as drug discovery and development.^{11–14} With the growing significance and recent successes in peptide (and peptide-containing) therapeutics,^{15–20} by virtue of their (i) scalable modular synthetic accessibility and tunability, (ii) target-specific selectivity, potency and efficacy, (iii) good biocompatibility, low systemic toxicity and easy clearance, there is a great demand for more general and flexible synthetic methodologies and ligation protocols to make peptides fluorescent, upon functional *de novo* design, for in-depth biomedical investigations.

To date, despite the development of automated flow-based approaches,^{21,22} solid-phase peptide synthesis (SPPS) remains

the most common method for peptide preparation at both academic and industrial research settings.^{23–27} In SPPS, amino acids with the protected N-terminus (as well as the protected side chain if necessary) are used as building blocks: upon the covalent attachment onto insoluble resin beads and the iterative amidation and N-Fmoc deprotection, tailor-designed peptides of known sequence can be prepared in very good to excellent yields. Meanwhile, all the excess reactants and unbound side-products can be removed by simple washing after each single step, with the only purification step being required until the final cleavage of the peptide from the resin. However, when it comes to synthetic fluorescent peptides, *i.e.* fused with fluorescent proteins (FPs) by molecular biology and site-specific protein labelling protocols,^{28–30} or labelled/modified with small-molecule fluorophores (*e.g.* FITC, TRITC, AMCA, 5/6-FAM, Cy3/5, Lucifer Yellow, *etc.*) at either the N-terminus or at the internal lysines/cysteines/unnatural amino acids' linkable/Clickable side chains with straightforward functional group coupling reactions and bioorthogonal ligation,^{31–38} multiple transformation steps followed by tedious and costly purifications are required. In addition, relatively large fusion FPs and steric fluorophores can alter the physio-chemical properties of short peptides and adversely interfere with their interactions with targets *in vitro/vivo*.³⁹ Therefore, employing an optimal spacer/linker is technically a key routine solution for this “fluorescent post-labeling” approach (Fig. 1A).

To circumvent the above-mentioned limitations, the “fluorescent building block” approach has emerged as a highly promising alternative for fluorescent peptide production

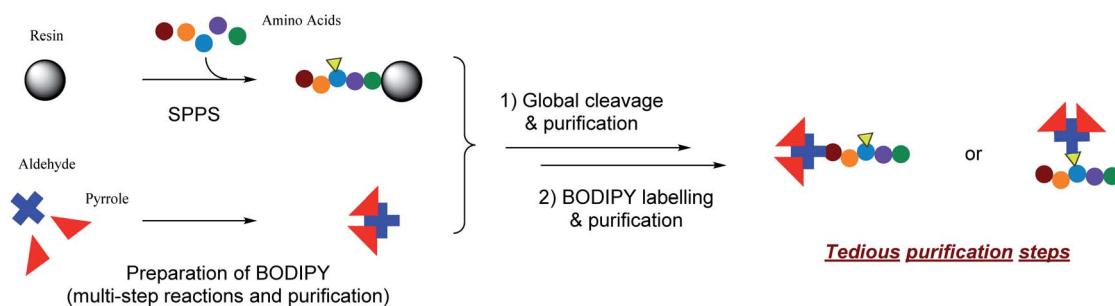
^aDepartment of Chemistry, Hong Kong Baptist University, Kowloon, Hong Kong SAR, China. E-mail: kulicechan@hkbu.edu.hk; klwong@hkbu.edu.hk; wai-lun.chan@mpibpc.mpg.de

^bDepartment for NMR-based Structural Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany. E-mail: mazw@nmr.mpg.de

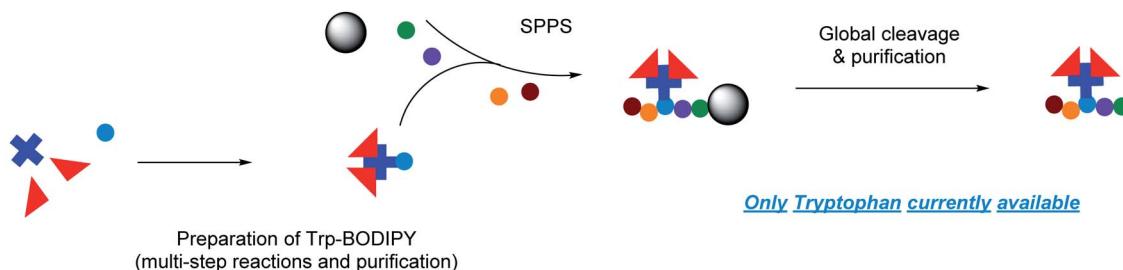
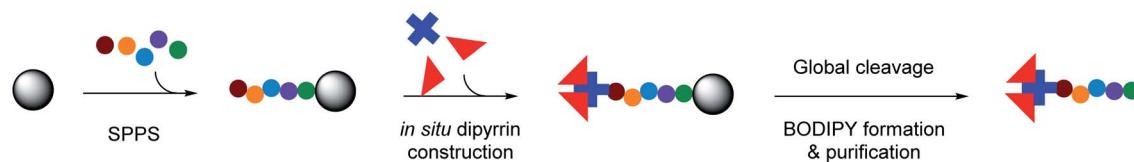
^cGerman Center for Neurodegenerative Diseases (DZNE), Von-Siebold-Str. 3a, 37075 Göttingen, Germany. E-mail: markus.zweckstetter@dzne.de

† Electronic supplementary information (ESI) available. See DOI: [10.1039/d0sc04849f](https://doi.org/10.1039/d0sc04849f)

A "Fluorescent Post-Labeling" Approach



B "Fluorescent building block" Approach

C This work: *In situ* dipyrromethane construction on resin-bound peptides

- ✓ No dipyrromethane building block preparation required
- ✓ Single chromatographic purification at the last step

- ✓ Diverse dipyrromethane available for BODIPY colour tuning
- ✓ 20 natural + 5 unnatural N-terminal AAs & Lys side chain

Fig. 1 Schematic representation of strategies for BODIPY-based fluorescent peptide synthesis. (A) Post-SPPS ligation of fluorescent BODIPY dyes on peptides requiring multistep transformations and purifications. (B) Use of BODIPY-derived fluorescent unnatural amino acid/pre-functionalized building blocks during SPPS. (C) This work: *in situ* dipyrromethane construction during SPPS and then boron complexation to form BODIPY dyes with a single chromatographic purification step.

(Fig. 1B).⁴⁰ For instance, Schultz *et al.* pioneered the use of genetic encoding to introduce fluorescent unnatural amino acids into proteins at specific sites.^{41,42} In addition, Imperiali *et al.* developed an SPPS-based approach for incorporation of fluorescent unnatural amino acids into peptides by design.^{43–45} Since then, a kaleidoscope of novel organic fluorophore-based fluorescent unnatural amino acids with different photophysical properties and structures have been developed.⁴⁰ However, interdisciplinary expertise in synthetic biology and chemistry, experience in careful and judicious peptide design, and sufficient research budget are prerequisites for these approaches.

Over the past few decades, fluorinated boron-dipyrromethane (BODIPY) and its derivatives⁴⁶ have lent themselves to multifarious functional fluorophore scaffolds for diverse

biomedical applications,⁴⁷ *e.g.* fluorescence sensing and imaging (*via* the Förster resonance energy transfer (FRET) or photoinduced electron transfer (PeT) mechanisms), positron emission tomography imaging, photodynamic therapy, as well as *in vitro* and *in vivo* assays, due to their high synthetic availability and cost-effectiveness, tunable photophysical properties and neutral total charge for better cell permeability. BODIPY-peptide conjugates are therefore widely used as dual targeting-imaging tools in life sciences,^{7,48–54} although a "fluorescent post-labeling" strategy has to be used since BODIPY derivatives are generally vulnerable to strong acid and usually not so adaptable to SPPS conditions.^{55–57} To this end, Vendrell *et al.* documented the first "fluorescent building block" approach by developing a fluorogenic Trp-BODIPY amino acid in a spacer-free manner (*i.e.* direct carbon–carbon bond linkage between a tryptophan's

indole moiety at the C2-position and a BODIPY dye by palladium-catalyzed $C_{sp^2}-C_{sp^2}$ Heck reaction).^{58,59} Both linear and cyclic peptides can be prepared.^{60–62} In addition, Ackermann *et al.* disclosed a palladium-catalyzed bioorthogonal late-stage $C_{sp^3}-H$ activation strategy to append BODIPY dyes to the alanine and phenylalanine residues within a peptide.⁶³

Because tryptophan might not be present in the peptide sequence of interest, while phenylalanine and alanine are often involved in functional interactions and conformational controls of the peptide's secondary/tertiary structures, *i.e.* should best not be perturbed, complementary and more universal methods are required to create BODIPY-peptide conjugates. For the BODIPY precursor, there are two common ways to synthesize dipyrin derivatives:⁶⁴ first, Method A, condensation between one aldehyde and two pyrroles, followed by oxidation, which yields symmetrical dipyrins; second, Method B, reaction of an α -ketopyrrole/ α -formylpyrrole with another pyrrole, giving non-symmetrical dipyrins. For both methods, a lot of impurities, including excess pyrroles, intra-pyrrole crossing products, and the oxidant and its derivatives, are mixed with desired product, which renders the purification step a laborious and tedious task. Inspired by the advantage of SPPS, we hypothesize that SPPS and dipyrin synthesis, in this regard, can be perfectly integrated (Fig. 1C).

Herein, we describe a new facile and efficient protocol for *in situ* dipyrin construction on the N-terminus and the side chains of peptides through Fmoc-based solid-phase peptide synthesis (SPPS), where simple aldehyde and pyrrole derivatives can be directly utilized as building blocks without pre-functionalization in SPPS, and only one final-stage chromatographic purification step is required. In connection to our ongoing research program to develop peptide-based EBNA1 inhibitors against Epstein–Barr virus (EBV)-associated malignancies,^{65–67} we constructed dipyrin moieties on our own library of established EBNA1-targeting peptide **Pep₄** and synthesized **BODIPY-Pep₄**, which manifests no erosion of biotargeting performance *in vitro* but enhanced light cytotoxicity over dark cytotoxicity. This can be correlated to the photodynamic therapy (PDT) effect of BODIPY by photoinduced cytotoxic singlet oxygen (1O_2) generation. Taken together, this study offers a new pragmatic SPPS methodology for fluorescent peptide production and facilitates a cornucopia of biomedical applications.

Results and discussion

To construct dipyrin on the N-terminus of resin-bound **Pep₁** ($H_2N-YFMVF-COOH$), which was previously used as an EBNA1-targeting peptide,^{65–67} the benzaldehyde moiety was installed on the N-terminus through routine SPPS, followed by condensation with 2,4-dimethylpyrrole under the catalysis of $BF_3 \cdot OEt_2$ on the resin. Subsequently, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was added to oxidize the newly formed dipyrrolomethane into dipyrin *in situ*: the resin turned red after this step indicating the formation of dipyrin. After global cleavage, the dipyrin-peptide conjugate **DP₁-Pep₁** was obtained with 51% yield, which is comparable to the yield of the

unconjugated peptide (**Pep₁**, 60%). The reaction was monitored by HPLC, ESI-MS and 1H -NMR (Fig. 2).

We next explored the substrate diversity of various aldehydes and pyrroles under the above-mentioned procedure to synthesize a series of dipyrin conjugates of **Pep₁** (Fig. 3). Various simple pyrrole derivatives and indole derivatives were tried, and most of them yielded corresponding symmetrical dipyrin conjugates in good both absolute and relative yields. The failures of **DP₇** and **DP₁₁** suggest that the reaction can be hindered by the steric effect, while the trace amount of **DP_{8/17}** hints towards the sensitivity of the conjugated vinyl group towards the conditions. Since functionalized α -ketopyrroles/ α -formylpyrroles (*e.g.* $-COOH$ group containing) have very few commercial sources and are hard to synthesize as building blocks, 5-formyl-2,4-dimethyl-3-pyrrolecarboxylic acid (FDMPA) attracted our attention for its commercial availability and low cost (below 10 USD per g). FDMPA was able to act as an aldehyde analogue to prepare **DP₁₄**. For the unsymmetrical cases, we adopted the Method B. Treated by $POCl_3$ with other pyrrole derivatives, **DP₁₅** and **DP₁₆** were obtained in good yields.

We then verified the scope of this methodology for peptides with different amino acid compositions (Fig. 4). 4-Formylbenzoic acid and 2,4-dimethylpyrrole were used to construct 1,3,7,9-tetramethyldipyrin (**DP₁**) for a series of peptides that cover all 20 natural amino acids as well as five commonly used unnatural amino acids. All were obtained with comparable yields as the corresponding unconjugated peptides. The synthesized peptides can be used in a variety of studies, such as EBNA1-targeting peptides (**Pep₁–Pep₆**), STAT3-targeting peptides (**Pep₇** and **Pep₈**), as well as some FDA-approved peptide drugs including cetrorelix (**Pep₉**, as GnRH antagonists for treating prostate cancer, endometriosis, uterine fibroids), angiotensin II (**Pep₁₀**, used in treatment of sepsis, septic shock, diabetes mellitus, and acute renal failure) and prenatide (**Pep₁₁**, a copper chelator with potential applications in wound healing and other different functions). In addition to N-terminal conjugation, the dipyrin moiety can also be installed on the side chain of the lysine residue of **Pep₁₁** (the lysine with a removable protecting group on the amine group on the side chain), which substantiates that our procedure can be applied "generally and flexibly" on side chain modifications as well.

Next, we investigated SPPS boron complexation, which is a long-standing challenge for dipyrin-peptide conjugates to deliver BODIPY-peptide conjugates (Fig. 5A). Traditionally, this complexation step is conducted in less-polar solvents. However, peptides often suffer from poor solubility in these solvents, while polar solvents resulted in no complexation. Upon systematic solvent screening, we found acetonitrile (ACN), which displays moderate solubility for peptides, to be optimal: both the purified dipyrin-peptide conjugates and the crude product from SPPS were converted in ACN into the corresponding BODIPY conjugates within 10 minutes with acceptable yields. Notably, bulky base DIPEA should be used and the products should be separated from the reacting mixture rapidly, in order to avoid racemization. 1H -NMR spectra of **DP₁-Pep₁** and **BODIPY₁-Pep₁** are shown in Fig. 5C and S8–S11.[†] In addition, BODIPY-conjugates with different colours of fluorescence



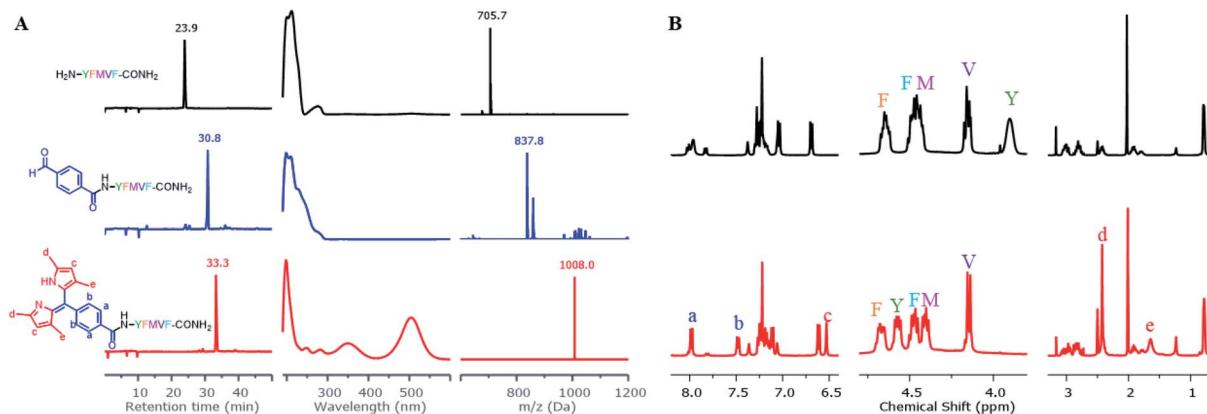


Fig. 2 Direct construction of dipyrin on resin-bound Pep₁. (A) The crude samples (resin) were taken and cleaved, with the resulting solutions being monitored by HPLC and ESI-MS. From top to bottom: unconjugated peptide (black), aldehyde-peptide conjugate (blue), dipyrin-peptide conjugate DP₁-Pep₁ (red); from left to right: their HPLC chromatographs, UV-Vis spectra corresponding to the peak on HPLC, and ESI-MS spectra. (B) Comparisons of ¹H-NMR spectra of Pep₁ and DP₁-Pep₁: four protons from the aldehyde building block (a and b) and two protons from the pyrrole (c) were found in the aromatic region; the α -H of tyrosine (Y, near the N-terminal) shifts largely after conjugation; 12 protons from the four methyl groups of dipyrin (d and e) were found at 2.4 and 1.7 ppm.

were prepared (Fig. 5B), further illustrating the possibility to achieve fast fluorescence labelling of *de novo* peptides with diverse BODIPY dyes for bioimaging and biosensing. It is

axiomatic that the photophysical properties of BODIPY dyes are largely influenced by the extent of electron delocalization around the boron centre (e.g. most BODIPY dyes emitting

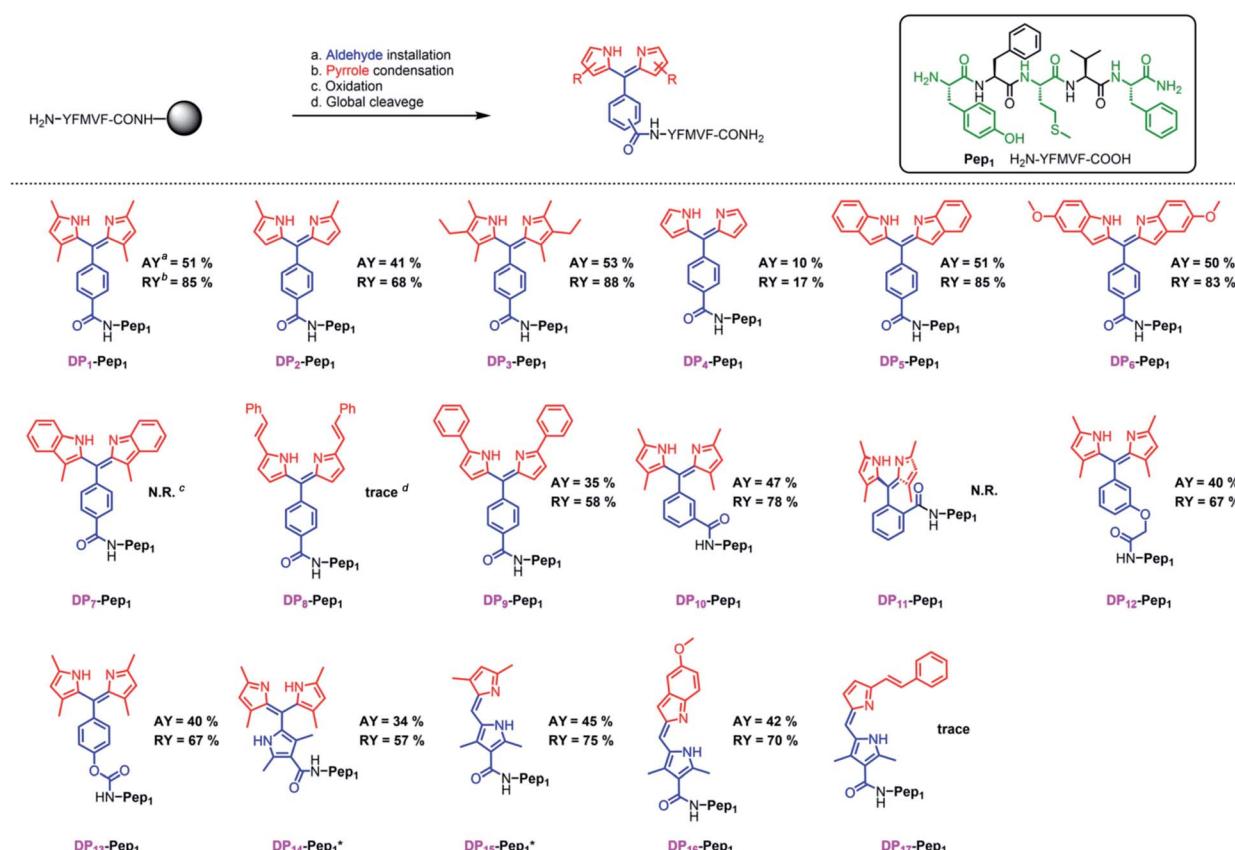


Fig. 3 *In situ* construction of dipyrin derivatives on the peptide YFMVF. Condition: (a) aldehyde-containing carboxylic acid, PyBOP, DIPEA, DMF, 3 h. (b) Pyrrole derivatives, BF₃·OEt₂, r.t., DMF, overnight; (c) DDQ, 1 h, DCM; (d) TFA/TIPS/H₂O, v/v/v, 95/2.5/2.5, r.t., 2 h. ^aAbsolute isolated yield compared with the substitution value of the resin-bound peptide. ^bRelative yield compared with the isolated yield of the unconjugated peptide. ^{c,d}No/Very few desired products were detected by LC-MS.



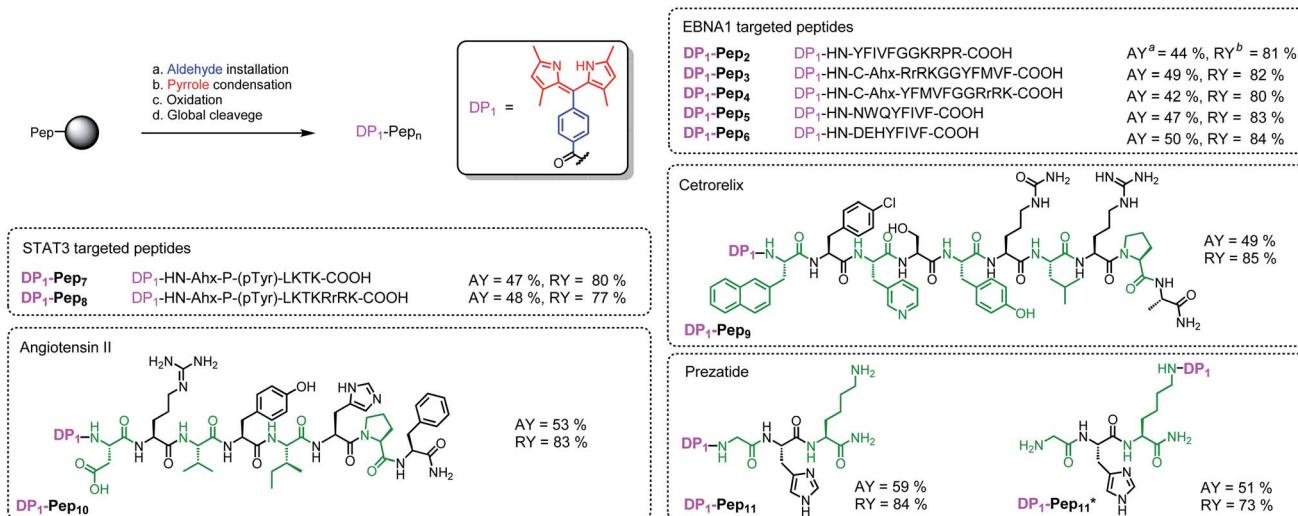


Fig. 4 *In situ* construction of DP_1 on different peptides. ^aAbsolute isolated yield compared with substitution value of the resin-bound peptide. ^bRelative yield compared with the isolated yield of the unconjugated peptide.

around 510 nm in green colour, like **BODIPY₁-Pep₁** by peripheral substitutions on the dipyrrom to (i) extend the π -conjugation system with vinyl/ynyl/aromatic groups (e.g. highly red-shifted **BODIPY₉-Pep₁** to red colour) and (ii) subtly fine-tune it with either electron-donating (e.g. aliphatic groups in **BODIPY₃-Pep₁** for slightly shifting to yellow colour) or electron-withdrawing groups (e.g. halogens).^{46,68}

To support the practical use of the BODIPY-peptide conjugates synthesized by our protocol, we performed confocal imaging for **BODIPY₁-Pep₄** (where **Pep₄** had been designed as a nucleus-penetrating EBNA1-specific peptide in our previous research)^{65–67} with both a HeLa cell line (EBNA1[−]) and the C666 cell line (EBNA1⁺) (Fig. 6A and S6†). As expected, the signal of BODIPY accumulated relatively fast in the nucleus of C666 cells

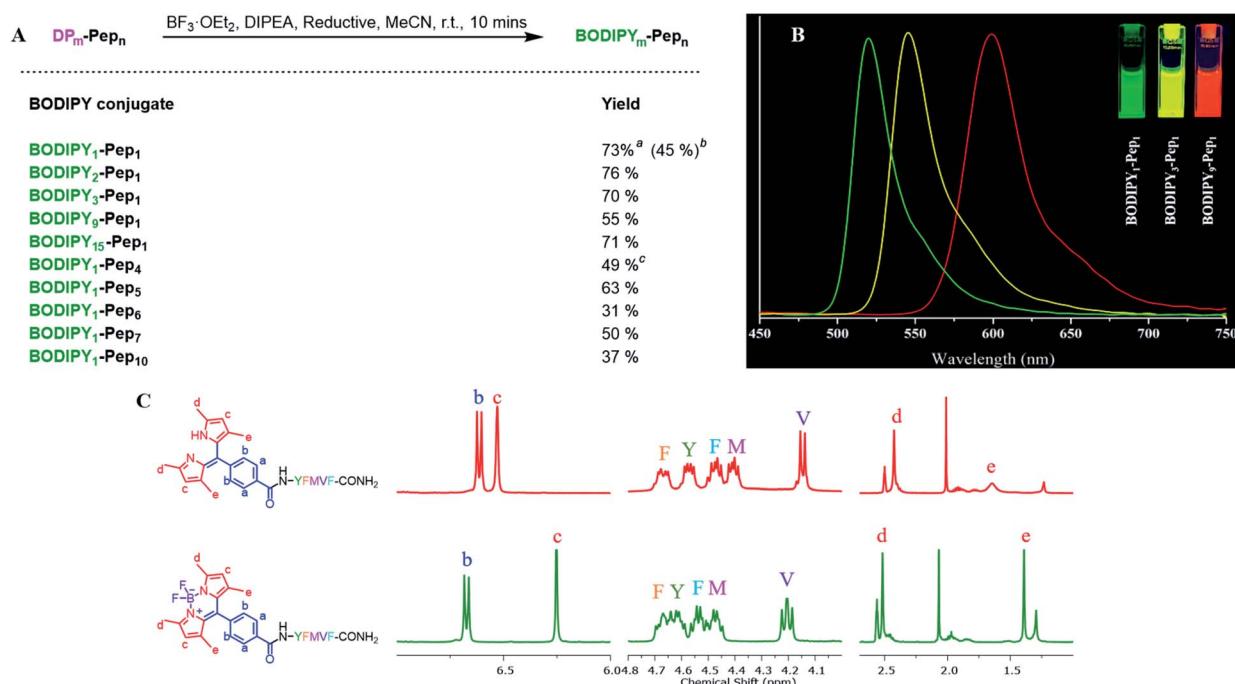


Fig. 5 (A) Boron complexation for dipyrrom-peptide conjugates. ^aPurified dipyrrom conjugate was used; isolated yield compared with the dipyrrom conjugate. ^bCrude dipyrrom conjugate was used; isolated yield compared with the substitution value of the resin-bound peptide. ^cEDT was used as the reductant. (B) The fluorescence spectra of 10 μM BODIPY-peptide conjugates (derived from DP_1 , DP_3 and DP_9 respectively) in DMSO under 365 nm UV light excitation. (C) Comparison of ^1H -NMR spectra of $\text{DP}_1\text{-Pep}_1$ and **BODIPY₁-Pep₁**. The chemical shifts of both pyrrole-H (c) and methyl-H (e) are shifted towards the high field.

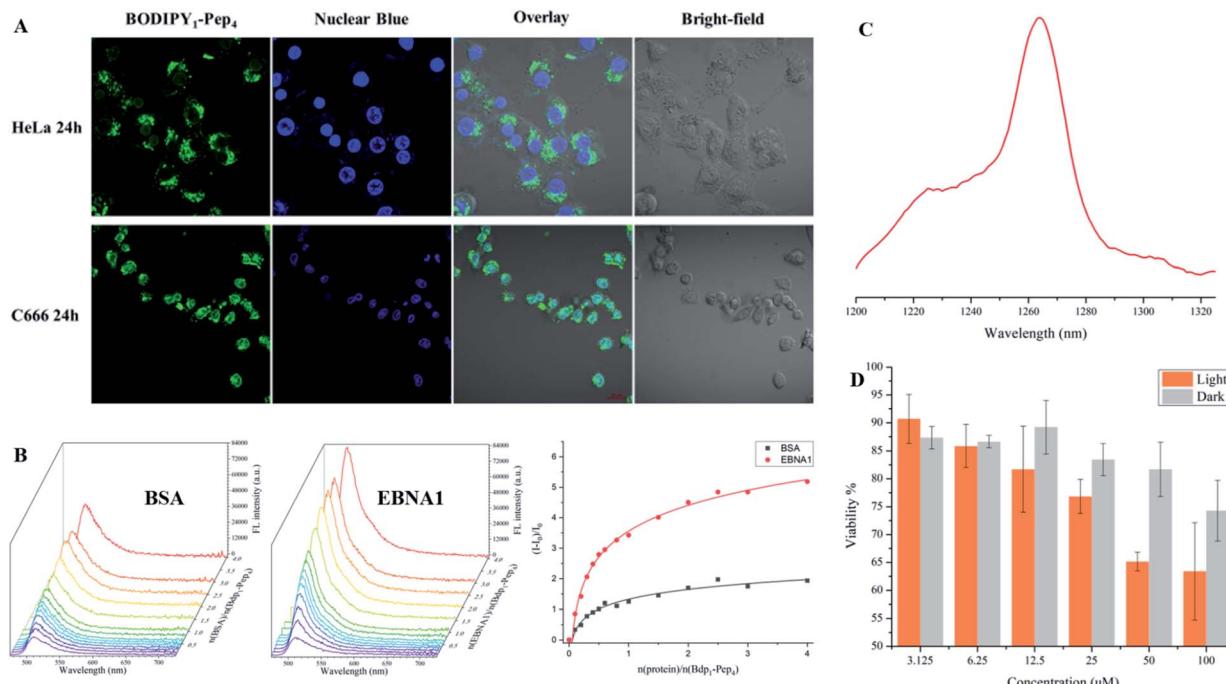


Fig. 6 Imaging of fluorescent peptides. (A) Confocal imaging of BODIPY₁-Pep₄ in HeLa and C666 cell lines. The green fluorescence signal was detected in the nucleus of C666 (EBNA+) but not in the nucleus of HeLa (EBNA-) cells. (B) Fluorescence titration of BSA (left) and EBNA1 (middle) proteins, both at 1.2 μ M, with BODIPY₁-Pep₄. A nearly 5-times enhancement over the course of the titration with EBNA1 was observed (right). (C) Singlet oxygen signal illustrating the potential of BODIPY₁-Pep₄ as a PDT agent. (D) Comparison of dark/light cytotoxicity of BODIPY₁-Pep₄ towards C666 cell line. Light toxicity was significantly higher than the dark toxicity. Error bars represent the standard derivation of the mean.

where the EBNA1 proteins are located, while the uptake of BODIPY into the nucleus of HeLa cells was slow. The confocal imaging experiments confirmed selective EBNA1-targeting performance of BODIPY₁-Pep₄ *in vitro* despite the use of another chromophore. In addition, BODIPY₁-Pep₄ demonstrated an almost 5-fold binding-responsive fluorescence enhancement (Fig. 6B, S1 and S2†), as well as a dark/light cytotoxicity difference due to the photo-generation of cytotoxic singlet oxygen ($^1\text{O}_2$) from the BODIPY moiety (Fig. 6C and D).

Conclusion

We have developed an efficient and convenient methodology to conjugate peptides with dipyrrin moieties during SPPS that can be further derived into highly emissive bioactive BODIPY-peptide conjugates for multicolour imaging. Various dipyrrin derivatives can be constructed on either the N-terminus or the side chain of peptides in both symmetrical and unsymmetrical ways and the substrate scope comprises all 20 natural and five unnatural amino acids with good yield. The workload and cost for synthesizing dipyrrin/BODIPY-peptide conjugates is greatly reduced by this protocol, which therefore holds tremendous promise for expediting the screening of peptide-based fluorescent probes, as well as for the development of high-throughput (HTS) fluorescence screening platforms and the creation of novel metal-peptide nano-frameworks. The BODIPY-labelled EBNA1-specific peptide (BODIPY₁-Pep₄) obtained from this methodology exhibited excellent *in vitro* performance and can

serve as a potential photosensitizer for PDT. This work furnishes a new pragmatic alternative SPPS methodology for fluorescent peptide production that can leverage and impact multifaceted biomedical applications.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Hong Kong Research Grants Council (HKBU12300319) and Hong Kong Health and Medical Research Fund (18170022). M.Z. was supported by the advanced grant '787679 - LLPS-NMR' of the European Research Council.

Notes and references

- 1 M. Staderini, A. Megia-Fernandez, K. Dhaliwal and M. Bradley, *Bioorg. Med. Chem.*, 2018, **26**, 2816–2826.
- 2 S. Lee, J. Xie and X. Chen, *Biochemistry*, 2010, **49**, 1364–1376.
- 3 A. D. Cunningham, N. Qvit and D. Mochly-Rosen, *Curr. Opin. Struct. Biol.*, 2017, **44**, 59–66.
- 4 L. Nevola and E. Giralt, *Chem. Commun.*, 2015, **51**, 3302–3315.
- 5 N. N. Patwardhan, Z. Cai, C. N. Newson and A. E. Hargrove, *Org. Biomol. Chem.*, 2019, **17**, 1778–1786.



- 6 J. Zhang, S. J. Yang, F. Gonzalez, J. Yang, Y. Zhang, M. He, N. Shastri and N. Murthy, *Chem. Commun.*, 2018, **54**, 7215–7218.
- 7 E. Pazos, O. Vázquez, J. L. Mascareñas and M. E. Vázquez, *Chem. Soc. Rev.*, 2009, **38**, 3348–3359.
- 8 O. T. Johnson, T. Kaur and A. L. Garner, *ChemBioChem*, 2019, **20**, 40–45.
- 9 J. A. González-Vera, D. Bouzada, C. Bouclier, M. Eugenio Vázquez and M. C. Morris, *Chem. Commun.*, 2017, **53**, 6109–6112.
- 10 D. Baechle, A. Cansier, R. Fischer, J. Brandenburg, T. Burster, C. Driessens and H. Kalbacher, *J. Pept. Sci.*, 2005, **11**, 166–174.
- 11 M. D. Hall, A. Yasgar, T. Peryea, J. C. Braisted, A. Jadhav, A. Simeonov and N. P. Coussens, *Methods Appl. Fluores.*, 2016, **4**, 022001.
- 12 T. Kim, H. M. Jeon, H. T. Le, T. W. Kim, C. Kang and J. S. Kim, *Chem. Commun.*, 2014, **50**, 7690–7693.
- 13 M. Pelay-Gimeno, A. Glas, O. Koch and T. N. Grossmann, *Angew. Chem., Int. Ed.*, 2015, **54**, 8896–8927.
- 14 W. A. Lea and A. Simeonov, *Expert Opin. Drug Discov.*, 2011, **6**, 17–32.
- 15 D. J. Drucker, *Nat. Rev. Drug Discovery*, 2020, **19**, 277–289.
- 16 A. P. Davenport, C. C. G. Scully, C. de Graaf, A. J. H. Brown and J. J. Maguire, *Nat. Rev. Drug Discovery*, 2020, **19**, 389–413.
- 17 D. Al Shaer, O. Al Musaimi, F. Albericio and B. G. de la Torre, *Pharmaceuticals*, 2020, **13**, 40.
- 18 A. C. L. Lee, J. L. Harris, K. K. Khanna and J. H. Hong, *Int. J. Mol. Sci.*, 2019, **20**, 2383.
- 19 J. L. Lau and M. K. Dunn, *Bioorg. Med. Chem.*, 2018, **26**, 2700–2707.
- 20 K. Fosgerau and T. Hoffmann, *Drug Discovery Today*, 2015, **20**, 122–128.
- 21 N. Hartrampf, A. Saebi, M. Poskus, Z. P. Gates, A. J. Callahan, A. E. Cowfer, S. Hanna, S. Antilla, C. K. Schissel, A. J. Quartararo, X. Ye, A. J. Mijalis, M. D. Simon, A. Loas, S. Liu, C. Jessen, T. E. Nielsen and B. L. Pentelute, *Science*, 2020, **368**, 980–987.
- 22 A. J. Mijalis, D. A. Thomas, M. D. Simon, A. Adamo, R. Beaumont, K. F. Jensen and B. L. Pentelute, *Nat. Chem. Biol.*, 2017, **13**, 464–466.
- 23 S. H. M. Gorissen, J. J. R. Crombag, J. M. G. Senden, W. A. H. Waterval, J. Bierau, L. B. Verdijk and L. J. C. van Loon, *Amino Acids*, 2018, **50**, 1685–1695.
- 24 R. Behrendt, P. White and J. Offer, *J. Pept. Sci.*, 2016, **22**, 4–27.
- 25 J. M. Palomo, *RSC Adv.*, 2014, **4**, 32658–32672.
- 26 V. Mäde, S. Els-Heindl and A. G. Beck-Sickinger, *Beilstein J. Org. Chem.*, 2014, **10**, 1197–1212.
- 27 M. Amblard, J. A. Fehrentz, J. Martinez and G. Subra, *Mol. Biotechnol.*, 2006, **33**, 239–254.
- 28 X. Chen, J. L. Zaro and W. C. Shen, *Adv. Drug Delivery Rev.*, 2013, **65**, 1357–1369.
- 29 X. Charest-Morin, J. P. Fortin, M. T. Bawolak, R. Lodge and F. Marceau, *Pharmacol. Res. Perspect.*, 2013, **1**, e00004.
- 30 N. A. Bindman and W. A. Van der Donk, *J. Am. Chem. Soc.*, 2013, **135**, 10362–10371.
- 31 C. Bednarek, I. Wehl, N. Jung, U. Schepers and S. Bräse, *Chem. Rev.*, 2020, **120**, 4301–4354.
- 32 N. K. Devaraj, *ACS Cent. Sci.*, 2018, **4**, 952–959.
- 33 O. Koniev and A. Wagner, *Chem. Soc. Rev.*, 2015, **44**, 5495–5551.
- 34 C. D. Spicer and B. G. Davis, *Nat. Commun.*, 2014, **5**, 4740.
- 35 K. Lang and J. W. Chin, *Chem. Rev.*, 2014, **114**, 4764–4806.
- 36 R. F. Wissner, S. Batjargal, C. M. Fadzen and E. J. Petersson, *J. Am. Chem. Soc.*, 2013, **135**, 6529–6540.
- 37 J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279.
- 38 P. Vidal, L. Chaloin, J. Méry, N. Lamb, N. Lautredou, R. Bennes and F. Heitz, *J. Pept. Sci.*, 1996, **2**, 125–133.
- 39 S. F. Hedegaard, M. S. Derbas, T. K. Lind, M. R. Kasimova, M. V. Christensen, M. H. Michaelsen, R. A. Campbell, L. Jorgensen, H. Franzky, M. Cárdenas and H. M. Nielsen, *Sci. Rep.*, 2018, **8**, 6327.
- 40 Z. Cheng, E. Kuru, A. Sachdeva and M. Vendrell, *Nat. Rev. Chem.*, 2020, **4**, 275–290.
- 41 J. Wang, J. Xie and P. G. Schultz, *J. Am. Chem. Soc.*, 2006, **128**, 8738–8739.
- 42 D. Summerer, S. Chen, N. Wu, A. Deiters, J. W. Chin and P. G. Schultz, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 9785–9789.
- 43 P. Venkatraman, T. T. Nguyen, M. Sainlos, O. Bilsel, S. Chitta, B. Imperiali and L. J. Stern, *Nat. Chem. Biol.*, 2007, **3**, 222–228.
- 44 B. Imperiali and M. Sainlos, *Nat. Protoc.*, 2007, **2**, 3210–3218.
- 45 M. E. Vázquez, D. M. Rothman and B. Imperiali, *Org. Biomol. Chem.*, 2004, **2**, 1965–1966.
- 46 A. Loudet and K. Burgess, *Chem. Rev.*, 2007, **107**, 4891–4932.
- 47 T. Kowada, H. Maeda and K. Kikuchi, *Chem. Soc. Rev.*, 2015, **44**, 4953–4972.
- 48 A. Sajid, N. Raju, S. Lusvarghi, S. Vahedi, R. E. Swenson and S. V. Ambudkar, *Drug Metab. Dispos.*, 2019, **47**, 1013–1023.
- 49 N. E. M. Kaufman, Q. Meng, K. E. Griffin, S. S. Singh, A. Dahal, Z. Zhou, F. R. Fronczek, J. M. Mathis, S. D. Jois and M. G. H. Vicente, *J. Med. Chem.*, 2019, **62**, 3323–3335.
- 50 A. C. Schulz-Fincke, M. Blaut, A. Braune and M. Gütschow, *ACS Med. Chem. Lett.*, 2018, **9**, 345–350.
- 51 N. Zhao, T. M. Williams, Z. Zhou, F. R. Fronczek, M. Sibrian-Vazquez, S. D. Jois and M. G. H. Vicente, *Bioconjugate Chem.*, 2017, **28**, 1566–1579.
- 52 A. Paulus, P. Desai, B. Carney, G. Carlucci, T. Reiner, C. Brand and W. A. Weber, *EJNMMI Res.*, 2015, **5**, 43.
- 53 S. Banappagari, A. McCall, K. Fontenot, M. G. H. Vicente, A. Gujar and S. Satyanarayananjois, *Eur. J. Med. Chem.*, 2013, **65**, 60–69.
- 54 J. Yang, H. Chen, I. R. Vlahov, J. X. Cheng and P. S. Low, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 13872–13877.
- 55 S. Krajcovicova, J. Stankova, P. Dzubak, M. Hajduch, M. Soural and M. Urban, *Chem. -Eur. J.*, 2018, **24**, 4957–4966.
- 56 A. M. Hansen, A. L. Sewell, R. H. Pedersen, D. L. Long, N. Gadegaard and R. Marquez, *Tetrahedron*, 2013, **69**, 8527–8533.



- 57 M. Vendrell, G. G. Krishna, K. K. Ghosh, D. Zhai, J. S. Lee, Q. Zhu, Y. H. Yau, S. G. Shochat, H. Kim, J. Chung and Y. T. Chang, *Chem. Commun.*, 2011, **47**, 8424–8426.
- 58 L. Mendive-Tapia, R. Subiros-Funosas, C. Zhao, F. Albericio, N. D. Read, R. Lavilla and M. Vendrell, *Nat. Protoc.*, 2017, **12**, 1588–1619.
- 59 L. Mendive-Tapia, C. Zhao, A. R. Akram, S. Preciado, F. Albericio, M. Lee, A. Serrels, N. Kielland, N. D. Read, R. Lavilla and M. Vendrell, *Nat. Commun.*, 2016, **7**, 10940.
- 60 R. Subiros-Funosas, V. C. L. Ho, N. D. Barth, L. Mendive-Tapia, M. Pappalardo, X. Barril, R. Ma, C. B. Zhang, B. Z. Qian, M. Sintes, O. Ghashghaei, R. Lavilla and M. Vendrell, *Chem. Sci.*, 2020, **11**, 1368–1374.
- 61 R. Subiros-Funosas, L. Mendive-Tapia, J. Sot, J. D. Pound, N. Barth, Y. Varela, F. M. Goñi, M. Paterson, C. D. Gregory, F. Albericio, I. Dransfield, R. Lavilla and M. Vendrell, *Chem. Commun.*, 2017, **53**, 945–948.
- 62 R. Subiros-Funosas, V. C. L. Ho, N. D. Barth, L. Mendive-Tapia, M. Pappalardo, X. Barril, R. Ma, C.-B. Zhang, M. Sintes, O. Ghashghaei, R. Lavilla and M. Vendrell, *Chem. Sci.*, 2020, **11**, 1368–1374.
- 63 W. Wang, M. M. Lorion, O. Martinazzoli and L. Ackermann, *Angew. Chem., Int. Ed.*, 2018, **57**, 10554–10558.
- 64 N. A. M. Pereira and T. M. V. D. Pinho E Melo, *Org. Prep. Proced. Int.*, 2014, **46**, 183–213.
- 65 L. Jiang, R. Lan, T. Huang, C.-F. Chan, H. Li, S. Lear, J. Zong, W.-Y. Wong, M. M.-L. Lee, B. D. Chan, W.-L. Chan, W.-S. Lo, N.-K. Mak, M. L. Lung, H. L. Lung, S. W. Tsao, G. S. Taylor, Z.-X. Bian, W. C.-S. Tai, G.-L. Law, W.-T. Wong, S. L. Cobb and K.-L. Wong, *Nat. Biomed. Eng.*, 2017, **1**, 1–10.
- 66 L. Jiang, Y.-L. Lui, H. Li, C.-F. Chan, R. Lan, W.-L. Chan, T. C.-K. Lau, G. S.-W. Tsao, N.-K. Mak and K.-L. Wong, *Chem. Commun.*, 2014, **50**, 6517–6519.
- 67 L. Jiang, H. L. Lung, T. Huang, R. Lan, S. Zha, L. S. Chan, W. Thor, T.-H. Tsoi, H.-F. Chau, C. Boreström, S. L. Cobb, S. W. Tsao, Z.-X. Bian, G.-L. Law, W.-T. Wong, W. C.-S. Tai, W. Y. Chau, Y. Du, L. H. X. Tang, A. K. S. Chiang, J. M. Middeldorp, K.-W. Lo, N. K. Mak, N. J. Long and K.-L. Wong, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 26614–26624.
- 68 G. Ulrich, R. Ziessel and A. Harriman, *Angew. Chem., Int. Ed.*, 2008, **47**, 1184–1201.