ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2024, **60**, 4810

Received 5th February 2024,
Accepted 11th March 2024

DOI: 10.1039/d4cc00602j

rsc.li/chemcomm

Adamantylglycine as a high-affinity peptide label for membrane transport monitoring and regulation†

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The non-canonical amino acid adamantylglycine (Ada) is introduced into peptides to allow high-affinity binding to cucurbit[7]uril (CB7). Introduction of Ada into a cell-penetrating peptide (CPP) sequence had minimal influence on the membrane transport, yet enabled up- and down-regulation of the membrane transport activity.

The molecular recognition of biomacromolecules such as peptides, proteins, nucleic acids, and other biologically active molecules by synthetic receptors is of significant interest for life science applications, for example, in proteomics, imaging, diagnostics, and drug delivery.[1,2] Various classes of synthetic host molecules have been identified in this regard, among which cucurbit[*n*]urils (CBs) stand out due to their very high binding affinities with well-designed guest molecules.[3,4] Ultra-high host–guest binding affinities in water are key for biological applications, for example in pull-down assays, stabilization of biopharmaceuticals, and bioimaging.[3,5] Within recent years, attempts have been made to identify peptide binding epitopes with a strong affinity to CBs, in particular to the highly water-soluble homologue cucurbit[7]uril (CB7), which included sequences of naturally occurring, proteinogenic amino acids,[6] as well as artificial binding epitopes[7] and non-canonical amino acids.[2,8–10]

Stable CB7 complexes with noncanonical amino acids were first explored by Urbach and co-workers and included the *p-tert*-butyl and *p*-aminomethyl derivatives of phenylalanine (*t*BuPhe and AMPhe, Fig. 1).[8] Compared to phenylalanine (Phe), the dissociation constants, K_d, were remarkably enhanced and peptides with an N-terminal AMPhe showed an exceedingly high affinity of K_d = 0.95 nM. Genetic encoding of these unnatural amino acids was subsequently demonstrated by Liu and co-workers and proved to be more efficient for *t*BuPhe than for AMPhe.[2] This enabled the regulation of protein functions by host–guest complexation of the protein-incorporated *t*BuPhe. Recently, AMPhe was engineered into the N-terminus of the B-chain of the diabetes hormone insulin providing a semisynthetic insulin variant that can be bound by CB7 with a K_d value of 99 nM.[10]

As a useful complement, we report herein (*S*)-adamantylglycine (Ada) as a high-affinity CB7 binding motif in peptides. The unmodified amino acid Ada has a picomolar affinity to CB7,[11] which is retained when it is appended to heptaarginine (R7). R7 is a well-known cell-penetrating peptide (CPP) and was previously investigated by us as a model CPP for counterion-activated membrane transport in large unilamellar phospholipid vesicles (LUVs).[12,13] We compare herein R7, Phe-R7, and Ada-R7 in fluorescence-based membrane transport assays and show that Ada can serve as a high-affinity peptide label with minimal effects on the peptide translocation efficacy and kinetics. The picomolar affinity, in conjunction with the convenient introduction into peptides by solid-phase peptide synthesis, establishes Ada as a highly useful peptide label, for example, in peptide-based pull-down assays with CB7-functionalized beads[14] or for intracellular peptide localization experiments with CB7-based fluorescent probes.[15]

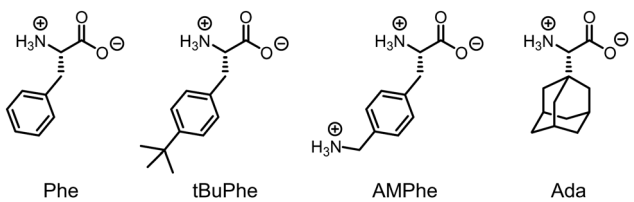

Fig. 1 Structures of the proteinogenic amino acid phenylalanine (Phe) and the noncanonical amino acids *t*BuPhe, AMPhe, and Ada.

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† Electronic supplementary information (ESI) available: Synthesis, supporting results, and details on assay procedures. See DOI: https://doi.org/10.1039/d4cc00602j

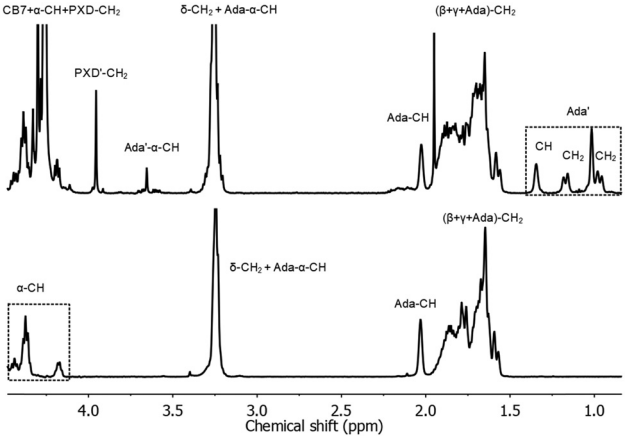

Fig. 2 ^{1}H NMR spectra of Ada-R7 (bottom) and of 0.56 mM Ada-R7, 0.5 mM CB7, and 2.5 mM of the competitor *p*-xylylenediamine (PXD) after 10 days of equilibration (top). Both spectra were measured at 25 °C in D_2O, pD 7.4.

Complexation of Ada-R7 (see ESI† for synthesis) by CB7 was first confirmed by ^{1}H NMR spectroscopy (Fig. 2). The spectrum of Ada-R7 shows multiplets from the α-CH protons in the range from *ca.* 4.1–4.5 ppm and the δ-CH_2 protons of the Arg side chain at 3.25 ppm, which overlap with the α-CH proton of the Ada amino acid.[11] The methylene proton signals of Ada overlap with the β- and γ-CH_2 protons of the Arg residues in the range from 1.55–1.95 ppm, whereas the methine (–CH) group of the unbound adamantyl residue shows up at 2.03 ppm as a clearly separated broad singlet.

In the presence of CB7, the ^{1}H NMR spectra showed clear indications of inclusion of the adamantyl residue in the CB7 cavity. New signals appeared in the range from 0.9–1.4 ppm, which are assigned to the upfield-shifted CB7-bound adamantyl residue.[11] Cavity binding of the Ada residue was also apparent from the appearance of a new singlet at 3.65 ppm, which is assigned to the α-CH proton of the bound Ada residue in accordance with previous observation of the adamantane amino acid.[11] The position and shape of the peaks assigned to the arginine residues remained largely unaffected by the presence of CB7, whereas a new shoulder appeared at the position of the δ-CH_2 protons of the Arg side chain suggesting a loose association of the guanidinium side chain with the remaining free portal of the CB7 cavity.[16]

The binding constant of Ada-R7 was determined next, by NMR competition using *p*-xylylenediamine (PXD) as a reference (Fig. S9, see Chart S1 for a structure overview, ESI†).[11,17] By comparing the integrated peak areas corresponding to the protons of free PXD and CB7-bound PXD as well as free and bound α-CH and alkyl-protons of the Ada residue, the binding constant of Ada-R7 to CB7 was determined as $(4.8 \pm 0.8) \times 10^{11}$ M^{-1}. Complete equilibration of the mixture was ensured by comparing a spectrum, in which Ada-R7 was first mixed with CB7 before addition of PXD, with a second spectrum, in which Ada-R7 was added to the preformed CB7/PXD complex. Identical spectra of both NMR samples indicated that equilibrium had been reached. The binding constant was further confirmed by competitive ITC titrations with PXD (Fig. S10, ESI†), which gave an affinity of 7.0×10^{11} M^{-1}, in good agreement with the NMR value. Competitive fluorescence titrations with the host-dye reporter pair composed of CB7 and berberine (BE) afforded a linear fluorescence decrease indicative of quantitative displacement of BE with increasing peptide concentrations, as expected for the very high binding affinity of the Ada residue to CB7.[11]

The next goal was to clarify to which extent peptide translocation across the lipid bilayer membrane of LUVs is affected by introducing the adamantylglycine amino acid into a known peptide sequence. We have therefore selected heptaarginine (R7) as a well-established CPP, which crosses liposome membranes after counterion activation.[12,13] The membrane transport activity was investigated on one hand with the well-established efflux assay based on carboxyfluorescein (CF) and, on the other hand, with more recently reported supramolecular tandem assays that use liposome-encapsulated host-dye reporter pairs, either CX4/LCG or CB7/BE (see Fig. 3, Chart S1 and Fig. S17–S26, ESI†).[18] The first two assays show a ''switch-on'' fluorescence response upon CPP entry, while the CB7/BE assay is of the ''switch-off'' type.

First, benchmark values were established with R7 and Phe-R7 (Table 1). These hydrophilic, polycationic peptides require an amphiphilic counterion activator, *e.g.* amphiphilic calixarenes,[12,13,19] to translocate across the hydrophobic membrane of liposomes. We have selected here the lower-rim substituted pentyl ether derivative of *p*-sulfonatocalix[4]arene (CX4-C5) with a reported activator efficiency in the nanomolar range,[12] and measured the effective concentrations (EC_{50}) to afford 50% peptide-mediated dye efflux in the CF assay or 50% dye displacement in the CX4/LCG and CB7/BE assay. In excellent agreement with previous results (Table 1),[12,13] this revealed EC_{50} values in the range of 0.1 to 1 μM for the membrane activity of R7 and Phe-R7 with CX4-C5 counterion activation in all three assays. The only exception was R7 with the CB7/BE assay, which showed a very high EC_{50} (>10 μM) due to the lack of a distinctive CB7 binding motif in the R7 peptide. The latter result is also relevant with respect to the mechanism of membrane-active peptides such as CPPs and antimicrobial peptides (AMPs),[13,20,21] since it confirms that R7/CX4-C5 peptide-counterion complexes shuttle across the lipid membrane without causing any membrane perturbations through which hydrophilic molecules can escape from the liposomes.[13] Dye efflux, as in the case of the CF assay, most likely proceeds *via* ternary activator/peptide/CF complexes that can shuttle back from the vesicle lumen to the liposome exterior as previously suggested (Fig. 3a).[21] We therefore conclude that counterion activation with CX4-C5 affords peptide translocation without membrane rupture.[12,13]

Subsequently, the membrane activity was investigated with the three assays for Ada-R7. Notably, the attachment of the adamantylglycine residue to the N-terminus of the R7 peptide had only a negligible influence on the measured membrane activity as obtained by the three assays (Table 1). When comparing the results for the same heptaarginine peptide (either

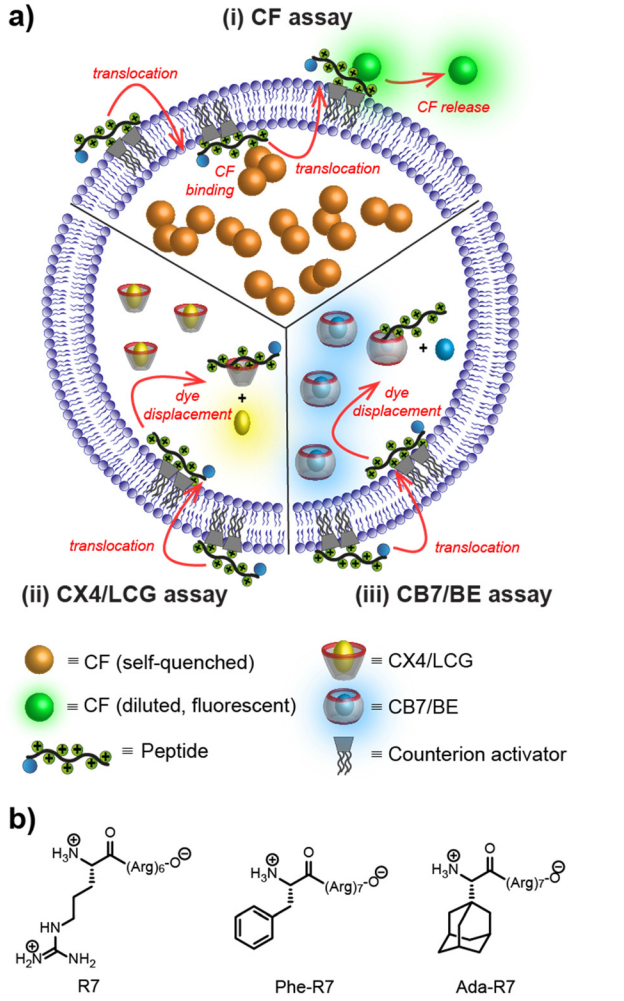

Fig. 3 (a) Principles of membrane transport assays to assess the membrane activity of cell-penetrating peptides (CPPs) with (i) self-quenched carboxyfluorescein (CF assay) and with the supramolecular host-dye reporter pairs (ii) CX4/LCG and (iii) CB7/BE. (b) Structures of the investigated peptides.

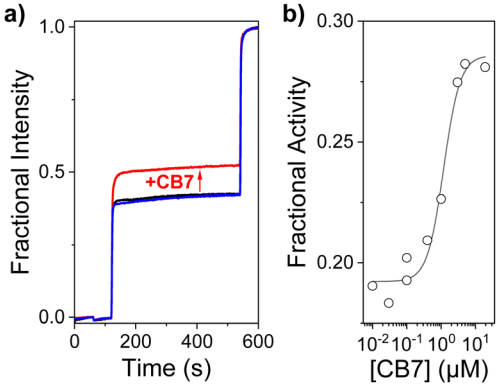

Fig. 4 Regulation of counterion-activated membrane transport of Ada-R7 with externally added CB7. (a) Transport activity was determined with the CX4/LCG assay and 1 μM Ada-R7 in absence (black) and presence (red) of 1 eq. CB7. In the blue trace, 1 μM CB7 was pre-mixed with 1 eq. 1-aminomethyladamantane to block the cavity of CB7. (b) Concentration dependence of the fractional activity on CB7 concentration in the CX4/LCG assay with 0.4 μM Ada-R7. All experiments were performed with POPC/POPS(9 : 1)–CX4/LCG vesicles in presence of 0.8 μM CX4-C5.

with an N-terminal phenylalanyl or adamantylglycinyl residue or without N-terminal amino acid residue), the EC_{50} values showed very good consistency. This clearly demonstrates that adamantylglycine, unlike other amino acids such as tryptophan,[22] has a negligible influence on membrane activity, classifying adamantylglycine as a minimally invasive peptide label for transport studies.

Lastly, we were interested whether binding of externally added CB7 had an influence on the membrane transport activity with the adamantylglycine peptide. Addition of CB7 had indeed a small, but sizable influence, whereas the transport activity systematically increased with increasing CB7 concentration (Fig. 4). This was surprising since an increased size should commonly lead to reduced membrane permeation on account of hindered diffusion. A control experiment in presence of aminomethyladamantane (AMADA) confirmed that CB7 binding to Ada-R7 is indeed responsible for the increased activity. AMADA has a very high affinity to CB7 and very slow dissociation kinetics,[11] which irreversibly blocks the CB7 cavity during the transport experiment. This led to the restoration of the much lower transport activity (red trace in Fig. 4), suggesting that the transport activity of the Ada-R7 peptide can be up- and down-regulated by CB7 binding.

In conclusion, we have introduced herein adamantylglycine (Ada) as a high-affinity amino acid to facilitate peptide-CB7 binding. The small size of Ada renders it minimally invasive in peptide transport experiments. This will be useful for peptide

Table 1 Membrane transport activities and binding affinities of peptides[a]

	R7		Phe-R7		Ada-R7	
Assay	EC_{50} (μM)	K_a (M^{-1})	EC_{50} (μM)	K_a (M^{-1})	EC_{50} (μM)	K_a (M^{-1})
CF	0.33 ± 0.03	n.a.	0.15 ± 0.01	n.a.	0.16 ± 0.02	n.a.
CX4/LCG	1.08 ± 0.06 [0.93 ± 0.06][b]	(7.2 ± 0.5) × 10^7	0.84 ± 0.03 [0.59 ± 0.05][b]	(3.3 ± 0.9) × 10^8	1.01 ± 0.06	(1.2 ± 0.7) × 10^9
CB7/BE	>10	(1.7 ± 0.1) × 10^5	0.15 ± 0.02 [0.05 ± 0.03][c]	(2.9 ± 0.5) × 10^7	0.15 ± 0.02	7.0 × 10^{11}[d] [4.8 × 10^{11}][d]

[a] All transport experiments were conducted with POPC/POPS (9 : 1) LUVs (20 μM total phospholipid concentration) in 10 mM NaH_2PO_4, pH 7.2. Binding constants were determined by competitive fluorescence titration in the same buffer, unless otherwise noted. [b] With 12 μM POPC LUVs in 10 mM Hepes, pH 7.0; see ref. 13. [c] With 12 μM POPC/POPS LUVs in 10 mM $(NH_4)_2HPO_4$, pH 7.0; see ref. 13. [d] K_a of Ada-R7 to CB7 was determined by competitive ITC in H_2O, pH 7.0 and by ^1H NMR in D_2O, pD 7.4 (in square brackets); error *ca.* 10%.

binding to CB7 in competitive environments, *e.g.*, in biological fluids or inside cells. Furthermore, the very high affinity will enable a biorthogonal up- and down-regulation of membrane transport activity of cell-penetrating peptides.

The work was funded by the Deutsche Forschungsgemeinschaft (DFG HE 5967/6-1 and NA 686/15-1).

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 H. Yin, Q. Cheng, D. Bardelang and R. Wang, *JACS Au*, 2023, **3**, 2356–2377; Q. Wu, Q. Lei, H.-C. Zhong, T.-B. Ren, Y. Sun, X.-B. Zhang and L. Yuan, *Chem. Commun.*, 2023, **59**, 3024–3039; D. V. D. W. Kankanamalage, J. H. T. Tran, N. Beltrami, K. Meng, X. Zhou, P. Pathak, L. Isaacs, A. L. Burin, M. F. Ali and J. Jayawickramarajah, *J. Am. Chem. Soc.*, 2022, **144**, 16502–16511; J. Chen, R. J. Hooley and W. Zhong, *Bioconjugate Chem.*, 2022, **33**, 2245–2253; N. Barooah, J. Mohanty and A. C. Bhasikuttan, *Langmuir*, 2022, **38**, 6249–6264; A. Som, M. Pahwa, S. Bawari, N. D. Saha, R. Sasmal, M. S. Bosco, J. Mondal and S. S. Agasti, *Chem. Sci.*, 2021, **12**, 5484–5494; C. J. Addonizio, B. D. Gates and M. J. Webber, *Bioconjugate Chem.*, 2021, **32**, 1935–1946; Y.-H. Liu, Y.-M. Zhang, H.-J. Yu and Y. Liu, *Angew. Chem., Int. Ed.*, 2021, **60**, 3870–3880; A. Kataki-Anastasakou, S. Hernandez and E. M. Sletten, *ACS Chem. Biol.*, 2021, **16**, 2124–2129; W. Xue, P. Y. Zavalij and L. Isaacs, *Angew. Chem., Int. Ed.*, 2020, **59**, 13313–13319; L. Liu, Y. You, K. Zhou, B. Guo, Z. Cao, Y. Zhao and H.-C. Wu, *Angew. Chem., Int. Ed.*, 2019, **58**, 14929–14934; C. L. Schreiber and B. D. Smith, *Nat. Rev. Chem.*, 2019, **3**, 393–400; R. Sasmal, N. Das Saha, M. Pahwa, S. Rao, D. Joshi, M. S. Inamdar, V. Sheeba and S. S. Agasti, *Anal. Chem.*, 2018, **90**, 11305–11314; S. van Dun, C. Ottmann, L.-G. Milroy and L. Brunsveld, *J. Am. Chem. Soc.*, 2017, **139**, 13960–13968; J. Murray, K. Kim, T. Ogoshi, W. Yao and B. C. Gibb, *Chem. Soc. Rev.*, 2017, **46**, 2479–2496.

2 W. Cao, X. Qin, Y. Wang, Z. Dai, X. Dai, H. Wang, W. Xuan, Y. Zhang, Y. Liu and T. Liu, *Angew. Chem., Int. Ed.*, 2021, **60**, 11196–11200.

3 J. An, S. Kim, A. Shrinidhi, J. Kim, H. Banna, G. Sung, K. M. Park and K. Kim, *Nat. Biomed. Eng.*, 2020, **4**, 1044–1052; D. Shetty, J. K. Khedkar, K. M. Park and K. Kim, *Chem. Soc. Rev.*, 2015, **44**, 8747–8761.

4 W. Liu, S. K. Samanta, B. D. Smith and L. Isaacs, *Chem. Soc. Rev.*, 2017, **46**, 2391–2403; L. Cao, M. Šekutor, P. Y. Zavalij, K. Mlinarić-Majerski, R. Glaser and L. Isaacs, *Angew. Chem., Int. Ed.*, 2014, **53**, 988–993; S. Moghaddam, C. Yang, M. Rekharsky, Y. H. Ko, K. Kim, Y. Inoue and M. K. Gilson, *J. Am. Chem. Soc.*, 2011, **133**, 3570–3581.

5 M. J. Webber, E. A. Appel, B. Vinciguerra, A. B. Cortinas, L. S. Thapa, S. Jhunjhunwala, L. Isaacs, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 14189–14194; D.-W. Lee, K. M. Park, M. Banerjee, S. H. Ha, T. Lee, K. Suh, S. Paul, H. Jung, J. Kim, N. Selvapalam, S. H. Ryu and K. Kim, *Nat. Chem.*, 2011, **3**, 154–159; K. L. Kim, G. Sung, J. Sim, J. Murray, M. Li, A. Lee, A. Shrinidhi, K. M. Park and K. Kim, *Nat. Commun.*, 2018, **9**, 1712.

6 L. C. Smith, D. G. Leach, B. E. Blaylock, O. A. Ali and A. R. Urbach, *J. Am. Chem. Soc.*, 2015, **137**, 3663–3669; J. W. Lee, H. H. L. Lee, Y. H. Ko, K. Kim and H. I. Kim, *J. Phys. Chem. B*, 2015, **119**, 4628–4636; S. Sonzini, A. Marcozzi, R. J. Gubeli, C. F. van der Walle, P. Ravn, A. Herrmann and O. A. Scherman, *Angew. Chem., Int. Ed.*, 2016, **55**, 14000–14004; F. Ma, X. Zheng and Z. Li, *Phys. Chem. Chem. Phys.*, 2021, **23**, 13724–13733; F. Ma, X. Zheng, L. Xie and Z. Li, *J. Mol. Liq.*, 2021, **328**, 115479; Y. Zhao, F. Li, F. Ma, J. Zhi, G. Wu and X. Zheng, *Phys. Chem. Chem. Phys.*, 2023, **25**, 7893–7900.

7 R. Meudom, N. Zheng, S. Zhu, M. T. Jacobsen, L. Cao and D. H.-C. Chou, *Curr. Res. Chem. Biol.*, 2022, **2**, 100013.

8 L. A. Logsdon, C. L. Schardon, V. Ramalingam, S. K. Kwee and A. R. Urbach, *J. Am. Chem. Soc.*, 2011, **133**, 17087–17092.

9 X. Chen, Z. Huang, R. L. Sala, A. M. McLean, G. Wu, K. Sokołowski, K. King, J. A. McCune and O. A. Scherman, *J. Am. Chem. Soc.*, 2022, **144**, 8474–8479.

10 H. R. Anderson, W. L. Reeves, A. T. Bockus, P. Suating, A. G. Grice, M. Gallagher and A. R. Urbach, *Bioconjugate Chem.*, 2023, **34**, 212–217.

11 M. A. Alnajjar, W. M. Nau and A. Hennig, *Org. Biomol. Chem.*, 2021, **19**, 8521–8529.

12 S. Peng, A. Barba-Bon, Y.-C. Pan, W. M. Nau, D.-S. Guo and A. Hennig, *Angew. Chem., Int. Ed.*, 2017, **56**, 15742–15745.

13 A. Barba-Bon, Y.-C. Pan, F. Biedermann, D.-S. Guo, W. M. Nau and A. Hennig, *J. Am. Chem. Soc.*, 2019, **141**, 20137–20145.

14 J. Murray, J. Sim, K. Oh, G. Sung, A. Lee, A. Shrinidhi, A. Thirunarayanan, D. Shetty and K. Kim, *Angew. Chem., Int. Ed.*, 2017, **56**, 2395–2398.

15 A. T. Bockus, L. C. Smith, A. G. Grice, O. A. Ali, C. C. Young, W. Mobley, A. Leek, J. L. Roberts, B. Vinciguerra, L. Isaacs and A. R. Urbach, *J. Am. Chem. Soc.*, 2016, **138**, 16549–16552; A. Norouzy, Z. Azizi and W. M. Nau, *Angew. Chem., Int. Ed.*, 2015, **54**, 792–795; Y. Liu, C. Hu, J. A. Serna, F. Biedermann and P. A. Levkin, *RSC Chem. Biol.*, 2023, **4**, 760–764.

16 A. Hennig and W. M. Nau, *Front. Chem.*, 2020, 8; J. Hostaš, D. Sigwalt, M. Šekutor, H. Ajani, M. Dubecký, J. Řezáč, P. Y. Zavalij, L. Cao, C. Wohlschlager, K. Mlinarić-Majerski, L. Isaacs, R. Glaser and P. Hobza, *Chem. – Eur. J.*, 2016, **22**, 17226–17238.

17 L. Cao and L. Isaacs, *Supramol. Chem.*, 2014, **26**, 251–258.

18 A. Hennig, *Org. Mater.*, 2022, **4**, 53–60.

19 Y.-C. Pan, A. Barba-Bon, H.-W. Tian, F. Ding, A. Hennig, W. M. Nau and D.-S. Guo, *Angew. Chem., Int. Ed.*, 2021, **60**, 1875–1882; J. N. Martins, B. Raimundo, A. Rioboo, Y. Folgar-Cameán, J. Montenegro and N. Basílio, *J. Am. Chem. Soc.*, 2023, **145**, 13126–13133; F. Perret, M. Nishihara, T. Takeuchi, S. Futaki, A. N. Lazar, A. W. Coleman, N. Sakai and S. Matile, *J. Am. Chem. Soc.*, 2005, **127**, 1114–1115.

20 J. C. Espeche, R. Varas, P. Maturana, A. C. Cutro, P. C. Maffía and A. Hollmann, *Peptide Sci.*, 2023, e24305; H. T. Kratochvil, Robert W. Newberry, B. Mensa, M. Mravic and W. F. DeGrado, *Faraday Discuss.*, 2021, **232**, 9–48; S. Guha, J. Ghimire, E. Wu and W. C. Wimley, *Chem. Rev.*, 2019, **119**, 6040–6085; M. Vazdar, J. Heyda, P. E. Mason, G. Tesei, C. Allolio, M. Lund and P. Jungwirth, *Acc. Chem. Res.*, 2018, **51**, 1455–1464; M. Gestin, M. Dowaidar and Ü. Langel, in *Peptides and Peptide-based Biomaterials and their Biomedical Applications*, ed. A. Sunna, A. Care and P. L. Bergquist, Springer International Publishing, Cham, 2017, pp. 255–264; S. Futaki and I. Nakase, *Acc. Chem. Res.*, 2017, **50**, 2449–2456; N. Chuard, K. Fujisawa, P. Morelli, J. Saarbach, N. Winssinger, P. Metrangolo, G. Resnati, N. Sakai and S. Matile, *J. Am. Chem. Soc.*, 2016, **138**, 11264–11271; W. B. Kauffman, T. Fuselier, J. He and W. C. Wimley, *Trends Biochem. Sci.*, 2015, **40**, 749–764; R. Brock, *Bioconjugate Chem.*, 2014, **25**, 863–868; E. G. Stanzl, B. M. Trantow, J. R. Vargas and P. A. Wender, *Acc. Chem. Res.*, 2013, **46**, 2944–2954; C. Ciobanasu, J. P. Siebrasse and U. Kubitscheck, *Biophys. J.*, 2010, **99**, 153–162.

21 T. Miyatake, M. Nishihara and S. Matile, *J. Am. Chem. Soc.*, 2006, **128**, 12420–12421.

22 S. Khemaissa, A. Walrant and S. Sagan, *Q. Rev. Biophys.*, 2022, **55**, e10.