Article

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Abstract

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Reference

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Sidechain Engineering in Cell-Penetrating Poly(disulfide)s

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Cell-penetrating poly(disulfide)s (CPDs) have been introduced recently to explore new ways to enter into cells. In this report, we disclose a general method to covalently modify the sidechains of CPDs. Compatibility of copper-catalyzed alkyne-azide cycloaddition (CuAAC) with the addition of either strained cyclic disulfides of varied ring tension or increasing numbers of guanidinium and phosphonium cations is demonstrated. Reloading CPDs with disulfide ring tension results in an at least 20-fold increase in activity with preserved sensitivity toward inhibition with the Ellman's reagent. The cumulation of permanent positive charges by sidechain engineering affords Ellman-insensitive CPDs with similarly increased activity. Co-localization experiments indicate the CPDs reach endosomes, cytosol and nucleus, depending on their nature and their concentration. Supported by pertinent controls, these trends confirm that CPDs operate with combination of counterion- and thiol-mediated uptake, and that the balance between the two can be rationally controlled. For the most active CPDs, uptake can be observed at substrate (fluorophore) concentrations as low as 5 nM.

Keywords: Cell-Penetrating Poly(disulfide)s • Thiol-Mediated Uptake • Disulfide Ring Tension • Cell-Penetrating Peptides • Membranes •

Introduction

Most cationic cell-penetrating peptides (CPPs) are guanidinium-rich oligomers and polymers (e.g. **1**, *Fig. 1*).[1–6] The ability of CPPs to cross lipid bilayer membranes originates from the poor acidity of the guanidinium cation.[2] They bind to cell membranes by repulsion-driven ion-pairing interactions with anionic lipids or activators,[2] cross the membrane through dynamic micellar defects,[3] and detach from the membrane by ion exchange with intracellular polyanions (*Fig. 1a*). This productive, counterion-mediated delivery into the cytosol (*Fig. 1aA*) is in kinetic competition with endocytic uptake and endosomal capture (*Fig. 1aB*).

In cell-penetrating poly(disulfide)s (CPDs) such as **2**,[7] the peptide backbone of CPPs is replaced by a disulfide polymer.[8–13] This is of interest to a) prepare the transporters in situ by ring-opening disulfide-exchange polymerization,[7] b) destroy the transporters upon arrival in the cytosol by reductive depolymerization to minimize toxicity and liberate the native substrate,[8] and c) integrate new uptake mechanisms (*Fig. 1a*).[8] Namely, disulfide exchange with exofacial thiols attaches CPDs covalently to the cell surface, disulfide exchange with glutathione releases them into the cytosol. Existence and significance of contributions from this thiol-mediated uptake mechanism[9] has been demonstrated by partial CPD inhibition upon removal of exofacial thiols with Ellman's reagent.[8] Moreover, cellular uptake of monomeric disulfides has been shown to increase with increasing ring tension from lipoic acid with a CSSC dihedral angle of 35° in **3** to asparagusic acid with 27° in **4** (*Fig. 1b*).[14] Uptake of activated acyclic disulfides **5** without ring tension is clearly less efficient.[14]

Sidechain modification of polymers is of general interest to avoid tedious optimization of polymerization conditions with every structural modification and to produce comparable functional systems with identical scaffold.[4-6,12,15-21] Synthetic strategies that in part have been

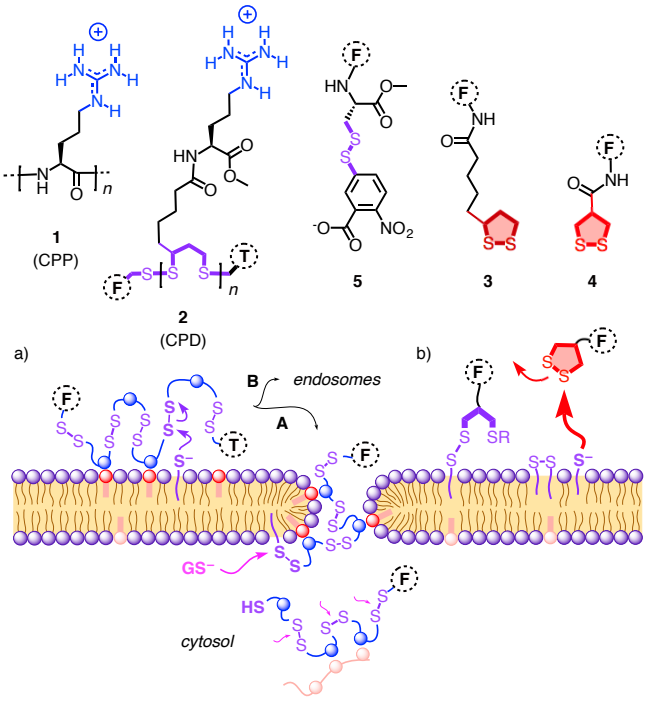
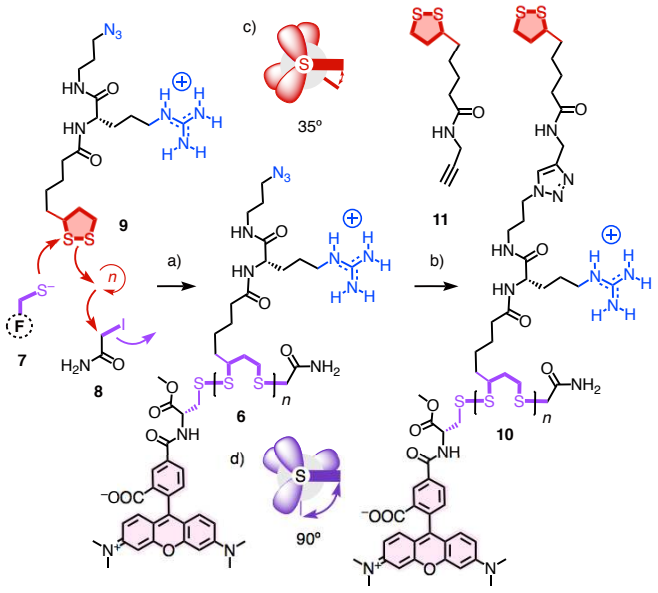


Figure 1. General structure of CPPs (**1**), CPDs (**2**) and monomeric transporters **3**–**5** with activated disulfides (T = terminator; F = fluorophore / initiator. a) CPDs combine counterion- and thiol-mediated uptake: Binding to cell membranes repulsion-driven ion-pairing and disulfide exchange with exofacial thiols is followed by translocation through micellar defects and release and destruction in the cytosol by counterion and disulfide exchange. This direct cytosolic delivery (A) is in kinetic competition with endocytic delivery to endosomes (B). b) Strain-promoted thiol-mediated delivery of monomeric disulfides.

applied previously to synthetic transport systems include the formation of hydrazones,[4,15,16] sulfonium cations,[6] boronate esters,[17] amides,[12] thioesters,[18] disulfides,[19] diselenides,[20] triazoles,[5,21] and so on. In the following, we introduce a general method for sidechain modification of



Scheme 1. a) Synthesis and b) sidechain modification of representative CPDs (*n* = 18), with indication of CSSC dihedral angles for c) high (red) and d) low tension (purple). a) Release of ring tension during substrate-initiated ring-opening disulfide-exchange polymerization (DMF, TEOA buffer, pH 7.0, 70 °C, 30 min). b) Reloading of CPDs with ring tension by sidechain modification with CuAAC ($CuSO_4$, sodium ascorbate, TBTA, 25 °C, 4 h).

CPDs, apply the secured synthetic access to comparable systems to tackle the intriguing challenge to reload poly(disulfide)s from ring-opening disulfide-exchange polymerization with disulfide ring tension (*Scheme 1, TOC graphic*) and dissect contributions from counterion-mediated (*Fig. 1a*)[2] and thiol-mediated (*Fig. 1b*)[8,9,14] uptake to end up with the most active CPDs reported so far.

Results and Discussion

The challenge to reload CPDs with tension is of conceptual interest because during the preparation of polymers **6** by ring-opening disulfide-exchange polymerization from initiators **7** to terminators **8**, all tension present in monomers **9** is naturally released (*Scheme 1*). The idea to synthesize CPDs with ring tension was thus chemically intriguing. However, the availability of reloaded CPDs such as **10** was also of practical interest because increasing uptake activity of monomers **3**–**5** with increasing tension suggested that similar increases in activity could be achieved on the level of the intrinsically much more active polymers. Because of much experience with orthogonal dynamic covalent chemistry in different contexts,[15] hydrazone exchange was initially envisioned for sidechain modification of CPDs. However, the results were not convincing (not shown). CuAAC (copper(I)-catalyzed alkyne-azide cycloaddition),[5,21,22] compatible with CPDs,[23] was chosen next. In monomers **9**, azides were added without changing the arginine motif in original CPD **2** (*Scheme 1*; monomers with alkynes instead of azides gave poorly soluble CPDs, not shown). The synthesis of monomers **9** was straightforward, details can be found in the Supporting Information (*Scheme S1*). Ring-opening disulfide-exchange polymerization[7] of azide monomers **9** under modified conditions in DMF at 70 °C was initiated with the previously reported, thiolated and red-emitting tetramethylrhodamine (TAMRA) fluorophore **7** (*Fig. 2*), and terminated with iodoacetamide **8** (*Scheme 1*). Characterization of the obtained azide CPDs **6** by GPC gave reproducibly an average M_w = 8.6 ± 0.8 kDa, an M_n = 9.5 ± 1.0 kDa, and an excellent PDI = 1.03 ± 0.3 (*Fig. 2b*). An average of M_w = 8.6 ± 0.8 kDa obtained for CPDs **6** corresponded to 18 monomers per polymers (*n* = 18).

For sidechain modification, alkyne **11** with a less strained lipoic acid was prepared first (*Schemes 1, S2*). CuAAC conditions and product analysis by RP-HPLC after disulfide reduction were established with monomer **9** (*Schemes 1, S5*; *Table S1*). Application of the lessons learned called for sidechain modification of azide CPDs **6** in H_2O/THF 9:1 in the presence of sodium ascorbate, $CuSO_4$, tris(benzyltriazoylmethyl)amine (TBTA), and an excess of alkyne **11** (*Scheme 1*). RP-HPLC analysis after reductive depolymerisation of the reloaded CPD **10** revealed triazole **12** as main product together with traces of unreacted azide **13** (*Fig. 2a*). The yield of sidechain modification determined by this method calculated to 80-95% for this and most CPDs described in the following.

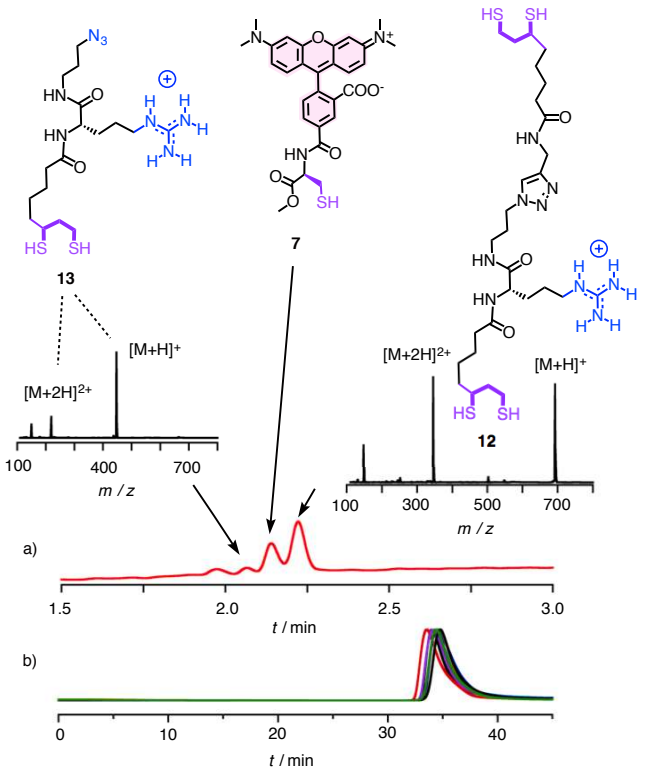


Figure 2. (a) RP-HPLC of CPD **10** after reductive depolymerization with DTT, with ESI MS of pertinent peaks. (b) GPCs of several, independently prepared CPDs **6**.

Following the developed procedure, the synthesis of CPD **14** with increased tension in the sidechain was unproblematic (*Fig. 3, S2, S7*; *Schemes S2, S5*; *Table S1*). As expected from strained disulfide monomers,[14] the introduction of ring tension by sidechain modification resulted in significant quenching of the TAMRA fluorescence in CPDs **10** and **14** by a factor of 40 and 52, respectively (*Fig. 4*). Fluorescence recovery in response to disulfide reduction confirmed that the strained cyclic disulfides in CPDs **10** and **14** are intact (*Fig. 4*).

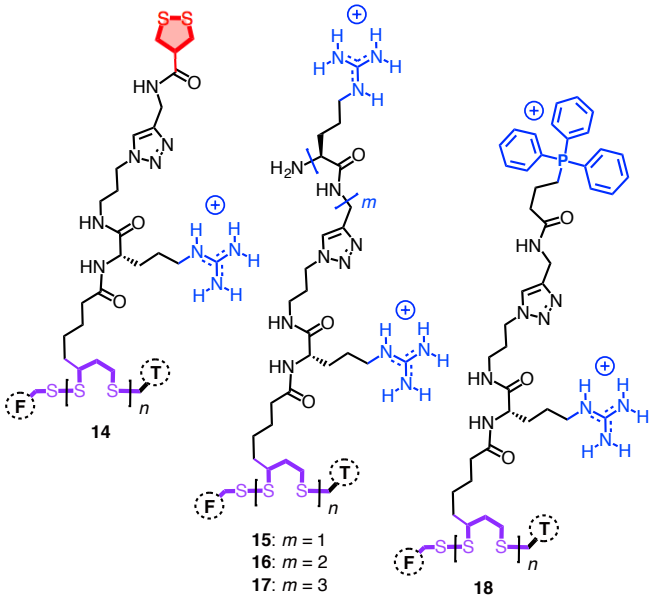


Figure 3. Structure of sidechain modified CPDs (n = 18). T = $CONH_2$, F from TAMRA **7**.

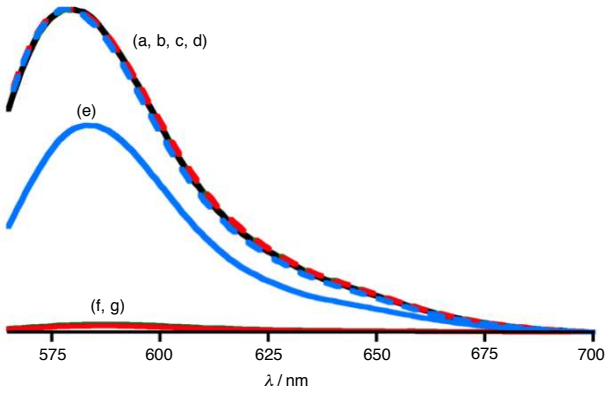
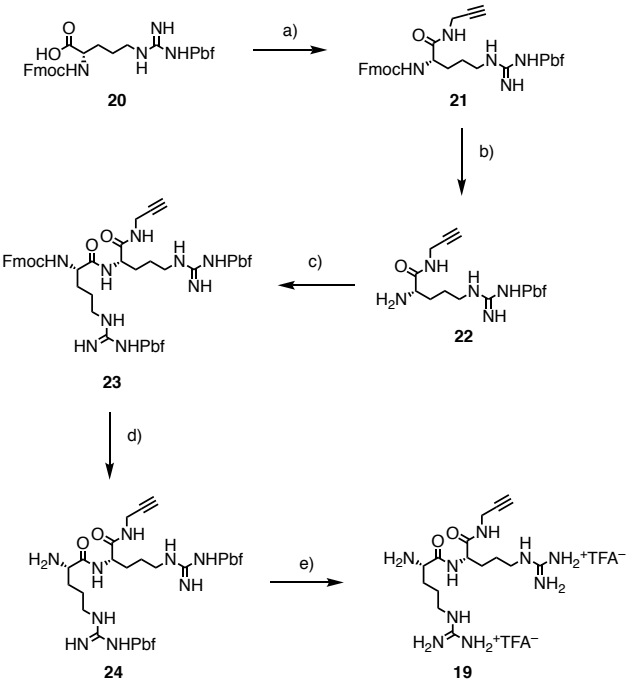


Figure 4. Normalized emission intensity of TAMRA **7** (a, black), CPDs **15** (b and e, blue), **10** (c and f, green) and **14** (d and g, red) before (e, f and g, solid) and after depolymerization (b, c and d, dashed).

Complementary to the reloading of ring tension in CPDs **10** and **14**, sidechain engineering in CPDs **15**–**18** was used to explore the cumulation of positive charges on a constant, comparable polymer backbone. CPDs **15**–**17** carry increasing numbers of arginine residues in their sidechains, and CPD **18** a more hydrophobic triphenyl phosphonium cation (*Fig. 3*). The synthesis of the respective sidechain modifiers was straightforward (*Schemes S3, S4*). The preparation of alkyne **19** is shown as a representative example (*Scheme 2*). The synthesis of CPDs **15** and **18** by CuAAC sidechain engineering as outlined above (*Scheme 1*) occurred quantitatively (*Figs. S8*, *S11*; *Scheme S5*; *Table S1*). Consistent with the literature,[24] CuAAC yields for sidechain engineering in CPD **6** decreased gradually with increasing bulk of the sidechain modifiers. Namely, CPD **16** was obtained in 75% yield by reacting the alkyne **19** containing two more arginines with the azides in CPD **6**, CPD **17** with three arginines in only 37% yield (*Figs. 3, S9*, *S10*; *Scheme S5*; *Table S1*). Contrary to the situation with strained disulfides in **10** and **14**, sidechain engineering with cumulative charges did not cause significant quenching of the fluorophores in **15**–**18** (*Figs. 4*, *S12*).



Scheme 2. Representative synthesis of sidechain modifiers: synthesis of alkyne **19**. a) Propargylamine, HATU, DIPEA, DMF, rt, 3 h, 71%; b) piperidine, DMF, rt, 0.2 h, quant.; c) **20**, HATU, DIPEA, DMF, 0.5 h, 93%; d) piperidine, DMF, rt, 0.2 h, 74%; e) TFA, $CHCl_3$, rt, 0.4 h, quant.

Fluorescence quenching up to a factor of 52 in CPDs with reloaded ring tension but not with cumulated charges complicated quantitative studies on cellular uptake. Studies on uptake into HeLa Kyoto cells by flow cytometry[10] were less meaningful under these circumstances, particularly because the extent of intracellular fluorescence recovery by disulfide reduction, although expected to be complete, was impossible to assess with full confidence. Studies on cellular uptake therefore had to rely on the more qualitative but also more informative confocal laser scanning microscopy (CLSM). CPD concentrations were estimated from the absorbance of TAMRA in solution. At concentrations as low as 100 nM, incubation with HeLa Kyoto cells in Leibovitz medium for four hours at 37 °C revealed uptake for all polymers (*Figs. 5*, *S13*). With relatively short CPDs at very low concentrations, the resulting images were naturally dominated by puctate emission, usually associated with inefficient delivery to endosomes (see below). This was consistent with previous results, delivery into cytosol and nucleoli is observed at higher concentration and/or with longer polymers.[25] However, most images also contained diffuse emission from the cytosol. At high dilution near detection limit, this diffuse cytosolic emission can be difficult to see against background from outside the cells. It is best appreciated from the contrast provided by the nuclei, appearing as large dark circles. This clean contrast provided by the "black" nuclei also supported that neither diffuse nor puctate emission originate from CPD absorbed outside of the cells,[26] possibly also resisting the routinely applied heparin extraction before imaging.[27] The presence of the "black" nuclei naturally confirmed not only the presence of CPDs in cytosol but also their absence in the nucleus when delivered at these very low concentrations.

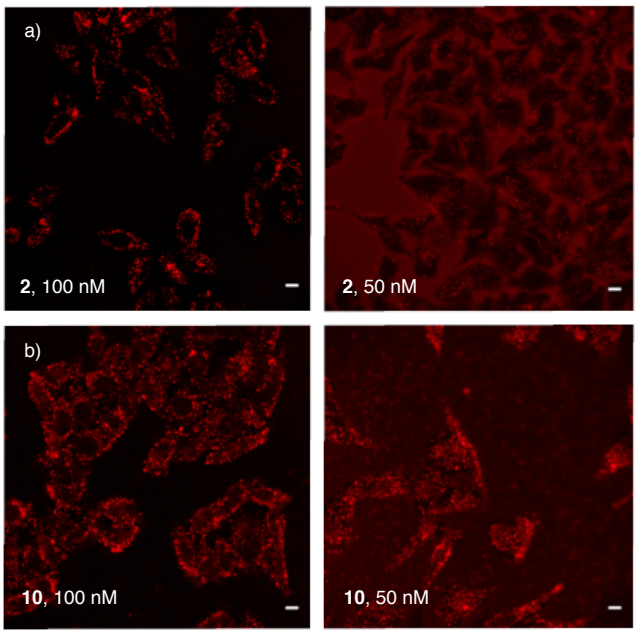


Figure 5. CLSM images of HeLa Kyoto cells after 4 h incubation with 100 nM (left, laser power: LP = 10%) and 50 nM (right) **2** (a, LP = 8%) and **10** (b, LP = 15%) at 37 °C in Leibovitz's medium. Scale bar 10 µm.

At 100 nM, similar results were obtained for all CPDs tested. At 50 nM, however, the original CPD **2** and the new azide precursor **6** could not enter the cells anymore (*Figs. 5a, S14a-b*). In clear, consistent and reproducible contrast, all sidechain-engineered CPDs tested remained active at 50 nM (*Figs. 5b, S14c-e*). Continuing dilution revealed activity down to 5 nM of CPD **15** with additional guanidinium cations from sidechain engineering (*Fig. 6b*). Similarly intriguing activity was obtained for CPDs **16** and **17** with additional guanidiniums as well as reloaded ring tension in their sidechain for CPDs **10** (*Fig. 6a*) and **14**. Even at 1 nM concentration, uptake of sidechain-engineered CPDs **14**–**17** was detectable, although image quality at detection limit became quite poor (not shown). Uptake activity of CPD **10** with reloaded tension at high dilution appeared inferior to that of CPD **15** with cumulated charges (*Fig. 6a vs 6b*). However, these measurements were performed near detection limit, ultrahigh dilution, and emission intensities depend much on the release of ring tension (*Figs. 4*, *S12*). Apparent differences between CPD **15** with additional guanidiniums and CPD **10** with reloaded ring tension should thus not be overestimated. What can be said with certainty is that sidechain-engineered CPDs, either with reloaded tension or cumulated charges, are at least 20 times more active than the original CPDs with regard to minimal deliverable substrate concentration, with activity being detectable down toward the detection limit of the fluorophore used.

Inhibition by the Ellman's reagent DTNB is a hallmark of all thiol-mediated uptake.[8,9,14] Oxidation of exofacial thiols will destroy their ability to react with cell-penetrating poly(disulfide)s. Preincubation of the HeLa cells with DTNB resulted in complete inhibition of the original CPD **2** (*Fig. 7a*). This result confirmed the validity of previous results from flow cytometry by fluorescence imaging at higher dilution.[8] DTNB inhibition of the more active CPD **10** with reloaded tension was as powerful as inhibition of the original CPD **2** (*Fig. 7b*). The same was true for the azide precursor **6** and CPD **14** with increased tension. However, DTNB failed to inhibit the uptake of CPDs **15** (*Fig. 7c*), **16** (*Fig. 7d*) and **17** with one to three additional guanidiniums in their sidechain. These complementary trends suggested that the Ellman-sensitive CPDs with reloaded tension enter cells preferably by thiol-mediated uptake,[8,9,14] whereas the Ellman-insensitive CPDs with cumulated charges enter cells preferably by counterion-mediated uptake (*Fig. 1*).[2] This interpretation supported that a dual mechanism accounts for the entry of CPDs into cells, and that the balance between entering cells by thiol-mediated and counterion-mediated uptake depends on their structure and, presumably, also other

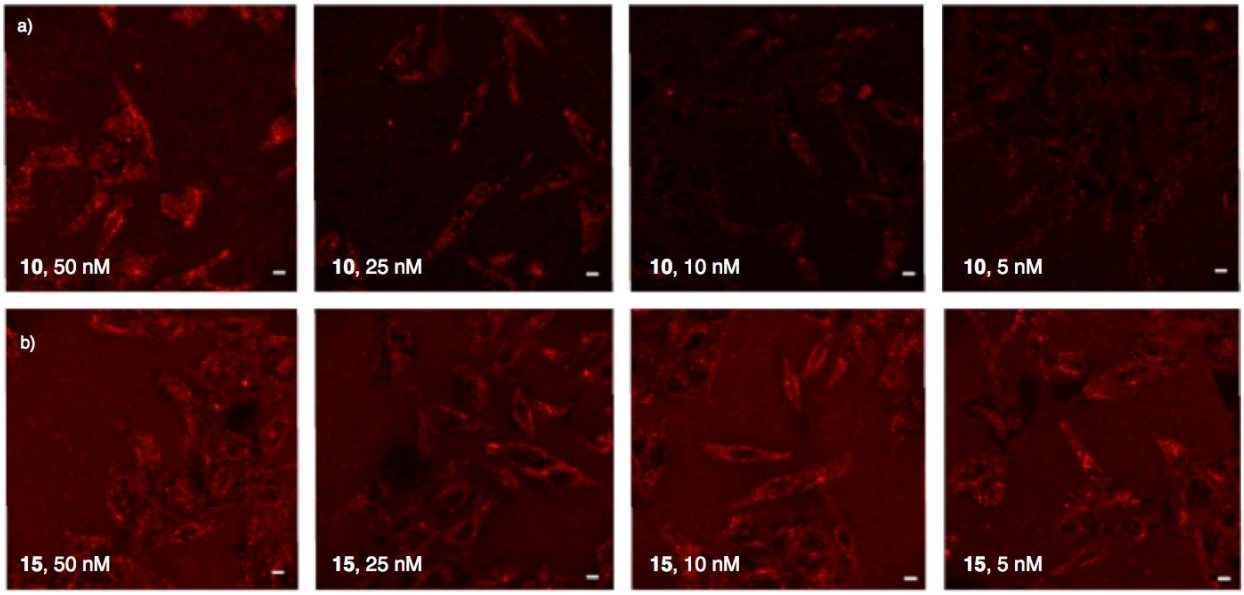


Figure 6. CLSM images of HeLa Kyoto cells after 4 h incubation with **10** (a) and **15** (b) at, from left to right, 50 nM, 25 nM, 10 nM, and 5 nM concentration, 37 °C, Leibovitz's medium. LP = 15%. Scale bar 10 µm.

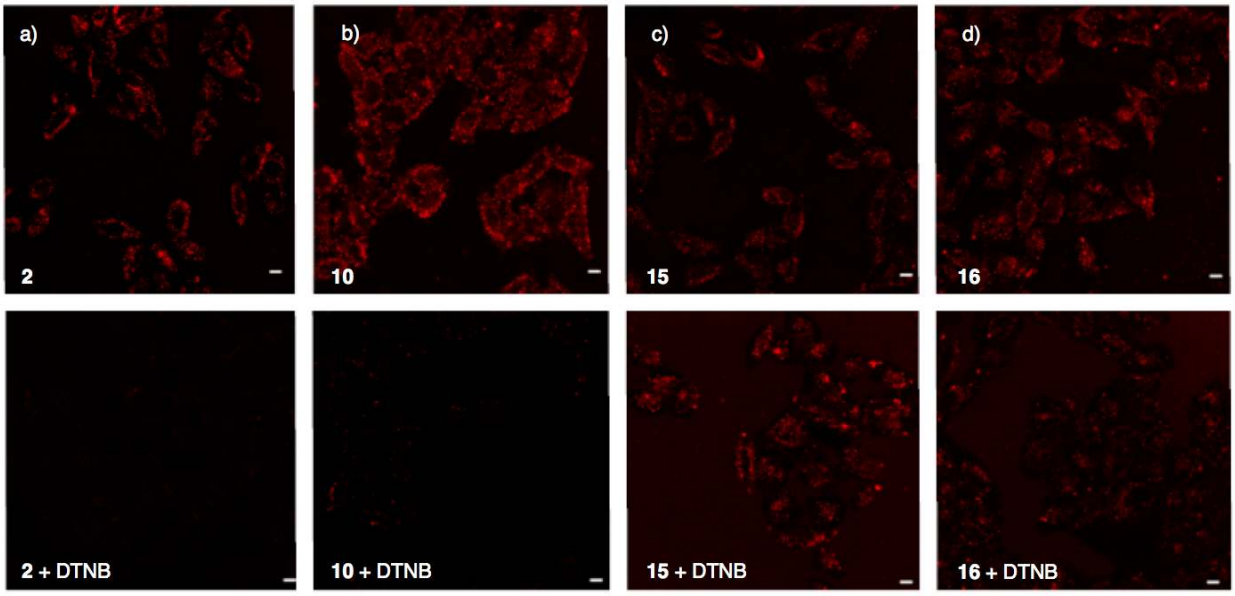


Figure 7. CLSM images of HeLa Kyoto cells after 4 h incubation with 100 nM **2** (a, LP = 10%), **10** (b, LP = 10%), **15** (c, LP = 12%) and **16** (d, LP = 12%) without (top) and with (bottom) pre-incubation with 1.2 mM DTNB for 30 min before addition of CPDs; all at 37 °C, Leibovitz's medium. Scale bar 10 µm.

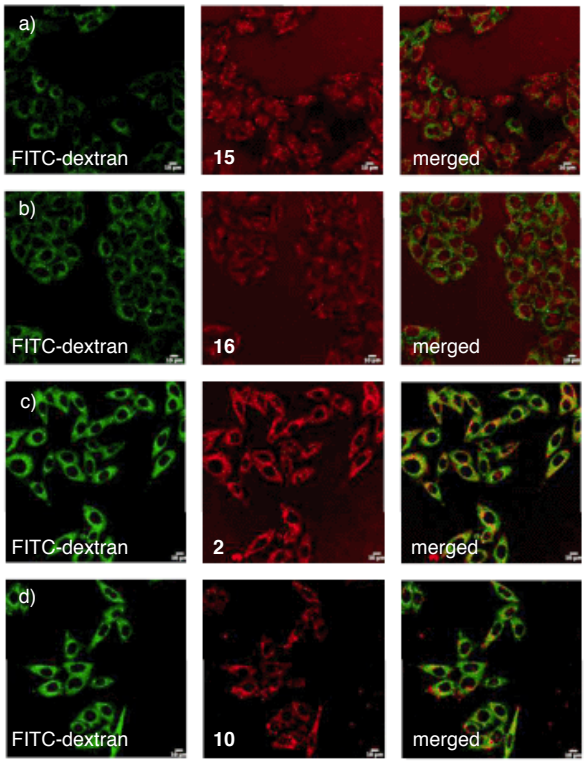


Figure 8. CLSM images of HeLa Kyoto cells after 4 h incubation with 100 nM **15** (a, PCC 0.04), **16** (b, PCC 0.12), **2** (c, PCC 0.28) and **10** (d, PCC 0.28) at 37 °C in Leibovitz's medium, followed by 15 min post-incubation with 100 µM FITC-dextran 40 kDa. Left: FITC-dextran 40 kDa, LP = 10%; center: CPDs, LP = 4%; right: merged. Scale bar 10 µm.

parameters, including their environment.

Co-localization experiments were in qualitative agreement with this conclusion. FITC-dextran 40 kDa was selected to probe for endosomal delivery. CPDs **15**-**17** with additional guanidiniums showed poor co-localization with the endosomal probe (*Fig. 8a, b, S15*). Quantitative co-localization analysis afforded Pearson's correlation coefficients[28] PCC = 0.04 – 0.12. In clear contrast, the original CPD **2** and CPD **10** with reloaded tension localized better with endosomes (*Fig. 8c, d*). Considering that PCC > 0.5 is generally accepted as convincing evidence for co-localization, the found PCC = 0.28 was still relatively low. However, yellow areas were clearly visible in the merged images (*Fig. 8c, d*). This clear increase in co-localization with endosomes compared to cation-rich CPDs supported a shift toward thiol-mediated uptake with disulfide-rich CPDs **2** and **10**. Although to be interpreted with much caution, particularly with high dilution experiments, these results were in good agreement with the clear differences seen for Ellman inhibition. Namely, CPDs enter cells with a dual mechanism, counterion-mediated uptake dominates with cation-rich, thiol-mediated uptake with disulfide-rich CPDs. The latter, most likely, also involves receptor-mediated endocytosis,[9,29] the former mostly direct translocation (*Fig. 1*).[2] Important to remember that these results apply to high dilution only. At higher concentrations, original CPDs **2** accumulate mostly in nucleoli.[8]

Co-localization with mitochondria was particularly interesting with CPDs **18** with phosphonium cations in their sidechains (*Fig. 3*). These hydrophobic cations have been observed frequently to be attracted by these highly polarized organelles with a strongly negatively charged surface.[30] The PCC = 0.36 obtained in co-localization experiments with MitoTracker Green for CPD **18** was indeed much higher than the PCC = 0.09 of CPD **6** and all other CPDs tested (*Figs. 9, S16*). However, the PCC = 0.36 of CPD **18** remained below the PCC > 0.5 threshold, and distinctly yellow areas were difficult to identify in the merged images obtained (*Fig. 9b*). These results overall suggested that CPD **18** with sidechain-engineered phosphonium cations has indeed some preference for

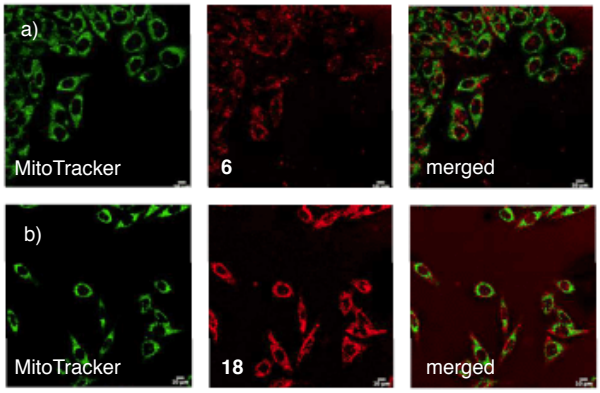


Figure 9. CLSM images of HeLa Kyoto cells after 4 h incubation with 100 nM **6** (a, PCC 0.07) and **18** (b, PCC 0.36) at 37 °C in Leibovitz's medium, followed by 15 min post-incubation with 100 nM MitoTracker Green. Left: MitoTracker, LP = 6%, center: CPDs, LP = 6%, right: merged. Scale bar 10 µm.

mitochondria. More significant selectivity could possibly be prevented by CPD depolymerization in the cytosol before reaching the mitochondria or endosomal capture.[25] Considering the low PCCs with endosomal trackers, the latter appeared less likely.

Conclusions

The availability of synthetic methods to modify the sidechains of cell-penetrating poly(disulfide)s (CPDs) is of central importance to identify the best performers. It would be difficult to develop conditions for ring-opening disulfide-exchange polymerization for each new sidechain. This is particularly true for sidechains that are incompatible with polymerization conditions, such as strained disulfides. Moreover, it would be difficult to compare results for different sidechains that are attached to different polymer backbones. In this report, we disclose a general synthetic method for CPD sidechain engineering. CuAAC is shown to work reliably and in high yield, despite the presence of reactive disulfides and positive charges at very high effective concentrations. As far as the minimal deliverable substrate concentration is concerned, the reloading of disulfide ring tension and the cumulation of cations along a constant CPD scaffold provides access to the most active CPDs reported so far. Significant uptake still observed at detection limit around 5 nM suggests that sidechain engineering increases CPD activity more than 20 times. This increase in activity obtained from reloaded disulfide tension in CPDs occurs with preserved sensitivity to Ellman inhibitors and partial localization in endosomes, whereas similar increases from cumulated charges on the same CPD scaffold is accompanied by Ellman insensitivity and little localization in endosomes. These consistent differences confirm that CPDs enter cells by a dual mechanism, and demonstrate that contributions from thiol-mediated and counterion-mediated uptake can be balanced by design, that is sidechain engineering. These results provide clear perspectives to further improve the efficiency of this new way to enter cells. Current efforts focus on increased solubility at high concentrations to, perhaps, end up with neutral CPDs.

Experimental Section

See Supplementary Material.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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