Cell-Penetrating Streptavidin: A General Tool for Bifunctional Delivery with Spatiotemporal Control, Mediated by Transport Systems Such as Adaptive Benzopolysulfane Networks

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ABSTRACT: In this report, cell-penetrating streptavidin (CPS) is introduced to exploit the full power of streptavidin–biotin biotechnology in cellular uptake. For this purpose, transporters, here cyclic oligochalcogenides (COCs), are covalently attached to lysines of wild-type streptavidin. This leaves all four biotin binding sites free for at least bifunctional delivery. To maximize the standards of the quantitative evaluation of cytosolic delivery, the recent chloroalkane penetration assay (CAPA) is coupled with automated high content (HC) imaging, a technique that combines the advantages of fluorescence microscopy and flow cytometry. According to the resulting HC-CAPA, cytosolic delivery of CPS equipped with four benzopolysulfanes was the best among all tested CPSs, also better than the much smaller TAT peptide, the original cell-penetrating peptide from HIV. HaloTag-GFP fusion proteins expressed on mitochondria were successfully targeted using CPS carrying two different biotinylated ligands, HaloTag substrates or anti-GFP nanobodies, interfaced with peptide nucleic acids, flipper force probes, or fluorescent substrates. The delivered substrates could be released from CPS into the cytosol through desthiobiotin−biotin exchange. These results validate CPS as a general tool which enables unrestricted use of streptavidin−biotin biotechnology in cellular uptake.

INTRODUCTION

Streptavidin1−4 is a 52 kDa β-barrel tetramer that binds one biotin per monomer with exceptionally high affinity because the barrels close upon binding (Figure 1A). The advantages of such encapsulation combined with tetravalency, stability, and ease of use empower the high versatility of biotin−streptavidin technology. For cellular uptake, streptavidin has been used to noncovalently couple biotinylated substrates to biotinylated transporters such as cell-penetrating peptides (CPPs)5 and other dynamic covalent systems,6 including cell-penetrating poly(disulfide)s,7,8 related disulfide-containing systems,9,10 and cyclic oligochalcogenides (COCs, Figure 1B).11−15 However, this approach is limited to the reliable delivery of only one functionality because it is difficult to control the interfacing of more than two different ligands with the streptavidin tetramer. This limitation is occasionally overcome by covalent linking of fluorophores to proteins.14 A more powerful solution would be cell-penetrating streptavidin (CPS) with covalently attached transporters and all four binding sites free to harness the full power of streptavidin−biotin technology for bifunctional delivery (Figure 1C) with, for example, retention-using-selective-hooks (RUSH)-like16 spatiotemporal control (Figure 1D).

Covalent protein modification has been used previously for cellular uptake. Examples include supercharging of proteins by either addition of positive or removal of negative charges17 or simple covalent conjugation to possible new transporters such as boronic acids or halogen-bond donors.5−10,18 To elaborate on the idea of CPS, we selected COCs as transporters. COCs such as asparagusic acid (AA),11,12 epidiketodithiopiperazines,13 diselenolipoic acid (DSL),14 or the most recent benzopolysulfanes (BPS)15 are currently being explored to access increasingly unorthodox dynamic covalent oligochalcogenide exchange chemistry on the way into the cytosol. Such thiol-mediated uptake of COCs11−15 and related transporters7−10 has allowed delivery of not only small molecules but also larger substrates such as DNA,9c antibodies,9c quantum dots,9 other nanoparticles,9c liposomes, and polymersomes11d to the cytosol without significant capture.
within endosomes. Mechanistic hypotheses envision COCs as molecular walkers, walking along disulfide tracks in membrane proteins through the transient micellar pores known from CPPs but also from disulfide-rich scramblases or voltage-gated ion channels (Figures 2B and 2C). Driving the growing impact of dynamic covalent chemistry to cellular uptake is introduced as an adaptive network including macrocycles as large as 1 containing up to 19 sulfur atoms for cells to select from (Figure 2D). BPS are known to occur in marine natural products have attracted early attention in total synthesis, and appeared top in recent library screens to reverse cognitive defects in mouse models.

The objective of this study was to create a general tool which warrants unrestricted availability of streptavidin−biotin technology in cellular uptake. With COCs as a timely example of freely variable transporters, bifunctional delivery with spatiotemporal controllability is tackled as a functional challenge of biological relevance (Figure 1D). Specifically, HC-CAPA, a new combination of the chloroalkane penetration assay (CAPA) with automated high content fluorescence microscopy, is introduced first to secure direct quantitative and mechanosensitive fluorescent flipper probes with HaloTag-GFP fusion proteins and their controlled release through intracellular desthiobiotin−biotin exchange are demonstrated.

**RESULTS AND DISCUSSION**

Design and Synthesis of CPS. Wild-type streptavidin tetramer 2 contains 16 lysine residues (Figures 1A and 2A), which were partially derivatized to give CPS with different numbers of COCs. We chose to chemically modify amines through intracellular desthiobiotin−HaloTag-GFP fusion proteins, and their controlled release select from (Figure 2D). BPS are known to occur in marine macrocycles by forming adaptive networks of rare sulfur species such as reverse cognitive defects in mouse models. Driving the growing impact of dynamic covalent chemistry to cellular uptake is introduced as an adaptive network including macrocycles as large as 1 containing up to 19 sulfur atoms for cells to select from (Figure 2D). BPS are known to occur in marine natural products have attracted early attention in total synthesis, and appeared top in recent library screens to reverse cognitive defects in mouse models.

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Thus, the delivery of chloroalkanes can be quantified reliably based on function. This is important because the commonly used fluorescence intensity-based assays are often significantly affected by the environment and, in general, demonstrate neither cytosolic location nor intact functionality. CAPA operates with the power of automated high-content quantitative data of the highest possible quality, we adapted the method to an image-based assay using automated high-content fluorescence microscopy (referred to also as high-content screening (HCS) or high-content analysis (HCA)), preserving the power of flow cytometry to record and analyze thousands of cells in a short period of time without losing the subcellular spatial resolution of fluorescence imaging. For HC-CAPA, the mitochondria with HaloTags were top-hatted applying a top-hat preparation (Figures 3, Scheme S9). The absence of unbound ligand per CPS and by meticulous purification using centrifugal filters. Results from HC-CAPA were quantified as CP50

Figure 3. Structure of monomers, CPS with n transporters T, CPS complexes with n substrates S1 and S2, and controls made and used in this study with representative assembly of 39 and indication of pertinent figures (Fig) reporting on activity. n is the stoichiometry used for complex preparation (35: n = 2−4, see text); 47 and 49 are a mixture of m/p-carboxyfluorescein isomers.
mechanistic studies on BPS-mediated uptake was less relevant and off topic. Nevertheless, retention on and thiol-mediated release from thiol-affinity columns, arguably the most compelling test for thiol-mediated uptake,14,15 was confirmed for intact BPS4 23 (Figure S7). As previously noted with AA,12 the presence of 10% serum in Leibovitz’s media did not affect the activity much, resulting in a less than doubled CP50 of BPS4 23.

Targetless Delivery of CPS into Various Cells. The delivery of four biotinylated fluoresceins 32,34 in BPS4 33 into unmodified HeLa cells afforded confocal laser scanning microscopy (CLSM) images with diffusely emitting cells, including their nuclei (Figures S8 and S9).18 Similar images were obtained with biotinylated rhodamine 34 in BPS4 35 inside C2C12, HeLa, MCF7, or MDCK cells (Figures 5A and S9; n44 = 2). Weaker fluorescence was observed in CLSM images of AA4 36 in HeLa cells (Figure 5C). The added cations in RAA4 37 and RDSL4 38 did not help to improve the activity significantly, confirming that CPP-like mechanisms7–11 are negligible for COC-mediated uptake (Figures SD and S8). Contrary to BPS4 33, images of AA4 36 and RAA4 37 contain bright punctate patterns on a diffuse background, which usually indicate contributions from endolysosomes and perhaps also

and number of transporters is rather subtle. Goldilocks behavior further supported that simple passive diffusion does not account for the top activity of BPS4 23. Corroborative experimental support for this important conclusion was secured with myristyl control MA4 31, which is as hydrophobic as BPS but completely inactive (Figure S6, Table S3, and Scheme S9).

We previously reported the mechanistic insights in support of thiol-mediated uptake of BPS, including partial inhibition with Ellman’s reagent which inactivates cell surface thiols, insensitivity toward endocytosis inhibitors (cytochalasin B, Wortmannin, chlorpromazine, or methyl-β-cyclodextrin), the formation of complex adaptive networks of extreme sulfur species in the presence of thiols and disulfides (up to heptamers 1, Figure 1D), and stability in PBS buffer for >2 weeks.19 In the current context focusing on cell-penetrating streptavidin, which is a general and unrestricted availability of biotin–streptavidin biotechnology for cellular uptake independent of the transporter used, repetition of these
precipitating aggregates besides emission from the cytosol. With flow cytometry, such contributions from endosomal capture are recorded as false positives. The here introduced HC-CAPA is powerful because all eventual false positives are eliminated without losses in fast acquisition of statistically relevant high-content data.

**Targeted Delivery of Fluorophores, Nanobodies, and PNA.** Using the best CPS, that is, BPS₄, we explored the simultaneous delivery of two functions, here a reporter and a targeting unit. BPS₄ was loaded with biotinylated chloroalkane 19 and rhodamine 34 (Figures 3 and S5F, Scheme S12). Incubation of HeLa cells expressing HaloTags on mitochondria with the resulting complex 39 afforded CLSM images with fluorescently labeled mitochondria (Figure SF, top). The diffuse emission from cytosol and nuclei observed without targeting (Figures 5A and 5B) disappeared, thus confirming that delivery of complex 39 to the HaloTag on the surface of mitochondria is as specific as expected. A good colocalization with GFP emission demonstrated the targeted delivery of the complex to HaloTags (Figures SF, bottom, S10A, and S10B). HaloTag targeting was substrate independent; the substitution of rhodamine 34 with flipper 40 gave similar images for delivery of the respective complex 41 to HaloTags on mitochondria (Figures 3, 5E, and S11). This result was of interest for the fluorescence imaging of precisely localized membrane tension within living cells. Targeted delivery of biotinylated and Cy5-labeled 18-mer PNA 42 in complex 43 was also accomplished in HGM cells (vide infra, Figure S13).

Biotinylated anti-GFP nanobodies 44 were probed as alternative targeting units in complex 45, recognizing GFP instead of HaloTag of the fusion protein on mitochondria. The resulting images showed good colocalization with GFP together with less important endolysosomal capture and/or precipitates (red dots, Figures S5G and S12). These off-targeted, endolysosomal capture and/or precipitates probably originate from the larger oligomers of complex 45 cross-linked through the multivalent anti-GFP nanobodies 44 with more than one biotin (Figures 3 and S5G'). Shifts in uptake mechanism from cytosolic delivery to endosomal capture have been observed frequently with increasing substrate size, although much more for CPPs 5 than for thiol-mediated uptake. To minimize these side effects, complex 45 was prepared by incubation of BPS₄ with 3 equiv of fluorophores 34 before incubation with the multivalent nanobodies 44, a procedure that should in turn increase formation of the targetless CPS side product 35 loaded with four fluorophores 34 only (Figure 3, n₄₄ = 4). However, eventual contributions from off-targeted delivery with CPS 35 were very minor (diffuse red as in Figures 5A, S5G, and S12). Considering this situation, the targeted delivery mediated by anti-GFP nanobodies 44 to GFP expressed on the surface of mitochondria was remarkably efficient and supported, with red fluorescence originating from ligand 34, that the majority of complex 45 reaches the cytosol in intact functional form (yellow, Figures S5G, S5G', and S12). The control complex 46 without COCs was unable to penetrate cells and showed no colocalization with GFP (Figures S5H, S10C, and S10D).

**Controlled Intracellular Release of Substrates from CPS.** Once delivered in the cells, the substrate would ideally be detached from transporter and target at a well-defined time to ensure and follow its proper function on demand (Figure 1D). To elaborate on the timed release of substrates from CPS, fluorescein as a responsive model substrate was equipped with desthiobiotin 47, N-ethylbiotin 48, and biotin 49 (Figures 3 and 6, Schemes S4 and S13). Desthiobiotin (Kₐ = 10⁻¹¹ M)³,⁴ and N-ethylbiotin (Kₐ = 10⁻⁹ M)⁴ bind to streptavidin but with reduced affinity compared to biotin (Kₐ = 10⁻¹⁴ M).³,⁴ The corresponding complexes 50–52 were readily accessible by incubation with BPS₄ 5 (Figures 3 and 6).

Desthiobiotin 47 was first delivered in complex 50; then biotin 53, a nontoxic vitamin, or its methyl ester 54 was added at a 40 μM concentration as recommended by the RUSH technology⁶ to diffuse into the cell, exchange, and release substrate 47 in the cytosol (Figure 6A). As streptavidin strongly quenches the fluorescence of bound carboxyfluorescein, intracellular desthiobiotin–biotin exchange was detectable by fluorescence recovery of the released substrate 47, whereas the biotin–streptavidin side product 55 or 56 passes undetected.
Quantitative release kinetics were obtained by automated high-content fluorescence microscopy of wells containing thousands of cells with an increasing incubation time with biotin 53 and its methyl ester 54 (Figures 6B–D and S14). Identical t10 = 17 min obtained for controlled release of desthiobiotin 47 from CPS 50 with 53 and 54 demonstrated that passive diffusion of the protonatable carboxylate 53 into the cell is not rate limiting (Figures 6C and S14). In clear contrast, the release of N-ethylbiotin 48 from complex 51 was spontaneous and not affected by the presence of either 53 or 54 (Figure 6B). The lower fluorescence intensity observed with 48 (Figure 6B) compared with 47 (Figure 6C) suggested that 48 dissociated from CPS already during incubation time, diffused out of cells, and was washed away before the start of the acquisition (Figure S14C). Similar spontaneous release of desthiobiotin 47 was slow on the time scale of biotin-controlled release (Figures 6C and S14B). This difference in spontaneous release was in agreement with the poorer binding affinity of N-ethylbiotin 48 (Kd = 10−9 M) compared to desthiobiotin 47 (Kd = 10−11 M). On the other hand, the controlled release of 49 from 52 by biotin–biotin exchange was negligible even after an extended incubation period (Figure 6D), consistent with much stronger binding of biotin 49 compared to desthiobiotin 47. Insufficient release of biotin 49 and excessive release of N-ethylbiotin 48 added up again in Goldilocks behavior, identifying desthiobiotin 47 with neither too strongly nor too weak binding as the ligand of choice for controlled release within cells.

To finally combine targeted delivery and controlled release, PNA was selected as a prototypical example of a useful reagent that has been limited in scope by its cellular permeability. In complex 57, CPS 5 was loaded with desthiobiotinylated 18-mer PNA 58 and chloroalkane 59 (Figures 3 and 6E, Schemes S5, S6, and S13). The good colocalization (yellow) of Cy5 attached to PNA 58 (red) and GFP (green) demonstrated targeted delivery of complex 57 to HaloTags on the mitochondria of HGM cells in intact form and with excellent selectivity (Figures 6F and S15). Subsequent addition of biotin 53 induced PNA release, detectable as weakened emission of Cy5 on the mitochondria due to dilution of the liberated PNA 58 into the cytosol (Figures 6G, 6E, and S15). These results thus supported the compatibility of CPS delivery with spatiotemporal control using biotin analogs.

**CONCLUSION**

This study introduces CPS as a privileged scaffold for cellular uptake, simple and robust, with biotin–streptavidin technology made fully available for general bifunctional delivery with spatiotemporal control. HC-CAPA, the recent CAPA combined with automated high-content fluorescence microscopy27 is devised as the method of choice to quantify cytosolic delivery; the currently emerging benzopolysulfanes are validated as COCs of choice for cytosolic delivery. Their performance supports the integration of increasingly unorthodox oligochalcogenide exchange chemistry to find new ways to enter into cells and encourages studies on mechanistic hypotheses, i.e., molecular walkers and dynamic-covalent adaptive networks.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b13621.

Materials and methods; small-molecule synthesis; cell lines and plasmids; CPS synthesis; combining CAPA with automated HC microscopy; cellular uptake of CPS; targeted delivery; supporting references; NMR spectra; characterization of PNA derivatives; summary of structures (PDF)

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**Notes**

The authors declare no competing financial interest.

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