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Cellular Uptake of Substrate-Initiated Cell-Penetrating Poly(disulfide)s

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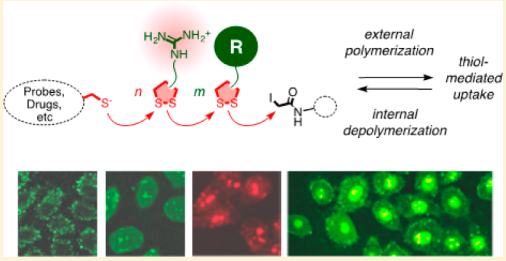
Supporting Information

ABSTRACT: Substrate-initiated, self-inactivating, cell-penetrating poly(disulfide)s (siCPDs) are introduced as general transporters for the covalent delivery of unmodified substrates of free choice. With ring-opening disulfide-exchange polymerization, we show that guanidinium-rich siCPDs grow on fluorescent substrates within minutes under the mildest conditions. The most active siCPD transporters reach the cytosol of HeLa cells within 5 min and depolymerize in less than 1 min to release the native substrate. Depolymerized right after use, the best siCPDs are nontoxic under conditions where cell-penetrating peptides (CPPs) are cytotoxic. Intracellular localization (cytosol, nucleoli, endosomes) is independent of the substrate and can be varied on demand, through choice of polymer composition. Insensitivity to endocytosis inhibitors and classical structural variations (hydrophobicity, aromaticity, branching, boronic acids) suggest that the best siCPDs act differently. Supported by experimental evidence, a unique combination of the counterion-mediated translocation of CPPs with the underexplored, thiol-mediated covalent translocation is considered to account for this decisive difference.



INTRODUCTION

Efficient cellular delivery is one of the key problems that hampers discovery and development of novel drugs and probes.[1] The problem is most pronounced for but not limited to larger substrates, including oligonucleotides, proteins, and nanoparticles. Since the discovery of the TAT peptide more than 20 years ago,[2] a broad variety of arginine-rich cell-penetrating peptides (CPPs) and CPP mimics have been introduced for this purpose.[3] Attached or complexed to CPPs, otherwise undeliverable molecules such as drugs, fluorophores, proteins, siRNA, plasmid DNA, or quantum dots are able to cross the cellular barrier. However, CPPs can be cytotoxic, are often trapped in the endosomes, and do not always work reliably. Counterion-mediated translocation has been introduced to improve direct cytosolic delivery and bypass endosomal capture.[4] To reduce toxicity, the sequence as well as the peptidic backbone of CPPs have been varied extensively.[3] One of the most promising modifications is represented by cell-penetrating poly(disulfide)s (CPDs).[5,6] Despite the growing interest in CPDs, synthetic methods to produce them are mainly focused on the postmodification of existing polymers (such as polyethylenimine, PEI) or the reaction of monomers already containing a disulfide, and their use has been mainly limited to noncovalent gene transfection.

Inspired by the robustness of the recently discovered ring-opening disulfide-exchange polymerization to grow complex functional architectures directly on solid surfaces,[7] we have proposed to grow CPD transporters directly on molecular substrates in solution, in situ, right before delivery (Figure 1).[8] The term "substrate" is used here to designate any object in need of assistance to enter cells (e.g., drugs, probes, peptides, proteins, DNA, RNA, etc.). Reductive depolymerization of the obtained substrate-initiated (si) CPDs by endogenous glutathione (GSH) in the cytosol would then eliminate toxicity and liberate the native substrate. The siCPD concept promises access to a general, fast, and noninvasive method for the covalent delivery of unmodified substrates, nontoxic, traceless, avoiding noncovalent formulations, and applicable to any substrate of free choice.

The growth of the CPD transporter on the molecular substrate in solution is initiated by a thiol (or a complementary functional group converter) that acts as an initiator **I** of the disulfide-exchange polymerization (Figure 1). Nucleophilic disulfide exchange opens the strained disulfide in the otherwise freely variable monomer **M**, forms a covalent disulfide bond between **I** and **M**, and regenerates a reactive thiol to attack the next **M**. The polymerization is terminated with an iodoacetamide **T**. The simplest possible terminator **T** is used in this study, but the introduction of additional drugs or probes with the freely variable **T** is of course inviting for the future. In this study, siCPDs are grown on fluorescent substrates to monitor,

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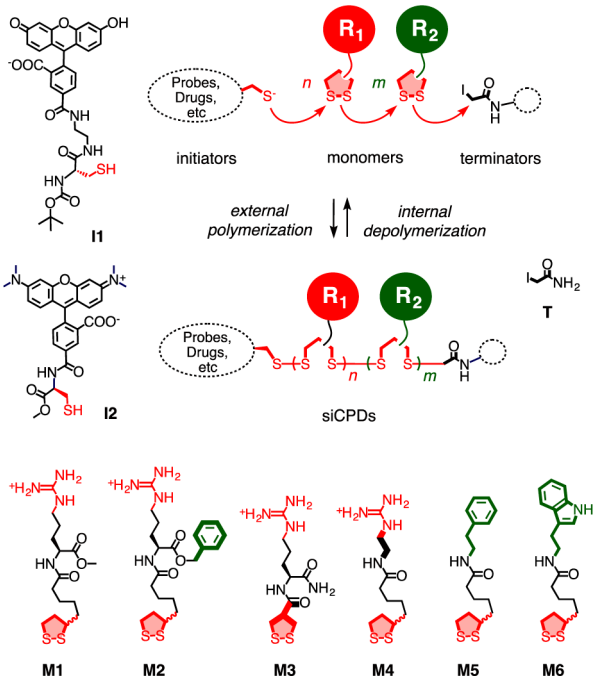

Figure 1. Concept of substrate-initiated, self-inactivating CPD transporters. Fluorescent initiators (**I1** derived from carboxyfluorescein and **I2** derived from rhodamine) were used to generate polymers through ring-opening disulfide-exchange polymerization or copolymerization of monomers **M1**−**M6**. The polymerization process was controlled using iodoacetamide **T** as the terminator.

for the first time, their entry into HeLa cells. Experimental evidence is provided that siCPDs (1) enter cells within 5 min, (2) can depolymerize in less than 1 min after uptake, (3) are much less toxic than CPPs, (4) operate independent of the attached substrate, (5) can target different organelles on demand, and (6) can enter cells by endocytosis or direct translocation across the membrane barrier, depending on their structure. Presumably, their uptake is mediated by a so far underexploited disulfide exchange with endogenous thiols at the membrane surface,[1,6] i.e., a conceptually innovative, dynamic-covalent[9] way to enter cells.

RESULTS AND DISCUSSION

Design and Synthesis. To study the capability of siCPDs to enter cells, fluorescent initiators **I1** derived from carboxyfluorescein (CF) and **I2** from 5-carboxytetramethylrhodamine (TAMRA) and monomers **M1**−**M6** were envisioned first (Figure 1). In **M1**, racemic lipoic acid offers the strained disulfide needed for ring-opening disulfide-exchange polymerization initiated by the fluorescent thiols **I1** and **I2**, whereas L-arginine offers the guanidinium cation needed to obtain polymers that can cross bilayer membranes like CPPs. In **M2**, the methyl ester of **M1** is replaced by a benzyl ester to increase hydrophobicity and add π-basicity to the polymer. In **M3**, the lipoic acid of **M1** is replaced by a more reactive asparagusic acid. In **M4**, the spacer between the guanidinium cation and lipoic acid is as short as possible. The new **M5** and **M6** contain the essence of phenylalanine (Phe) and tryptophan (Trp) with similarly minimalist spacers for copolymerization with the cationic **M4**. The synthesis of all initiators and monomers was very straightforward (Scheme S1, Supporting Information).[10]

Substrate-initiated polymerization[8] and copolymerization[11] with **M1**−**M6** were best in buffer at pH 7.0−7.5 in 5−30 min at room temperature, depending significantly on the nature of the monomer. The formation of siCPDs was routinely followed by the appearance of transport activity in fluorogenic vesicles (see below).[8,10,11] All new siCPDs were characterized by gel permeation chromatography (GPC; Figure S1, Supporting Information) to determine molecular weight and polydispersity and MALDI MS to also confirm the composition of the copolymers (Figures S2−S4, Supporting Information). Under these conditions, 200 mM **M1**−**M4** polymerized with 5 mM **I1**, a thiolated derivative of CF, for example, afforded fluorescent siCPDs **1**−**4** with an average molecular weight (M_w) of 6000−10000 and a polydispersity index (PDI) of ∼1.5 (Table S2, Supporting Information). The length of the polymers was variable on demand; shorter polymers were obtained with higher initiator concentrations or shorter reaction time. All siCPDs tested were purified by GPC before use to remove

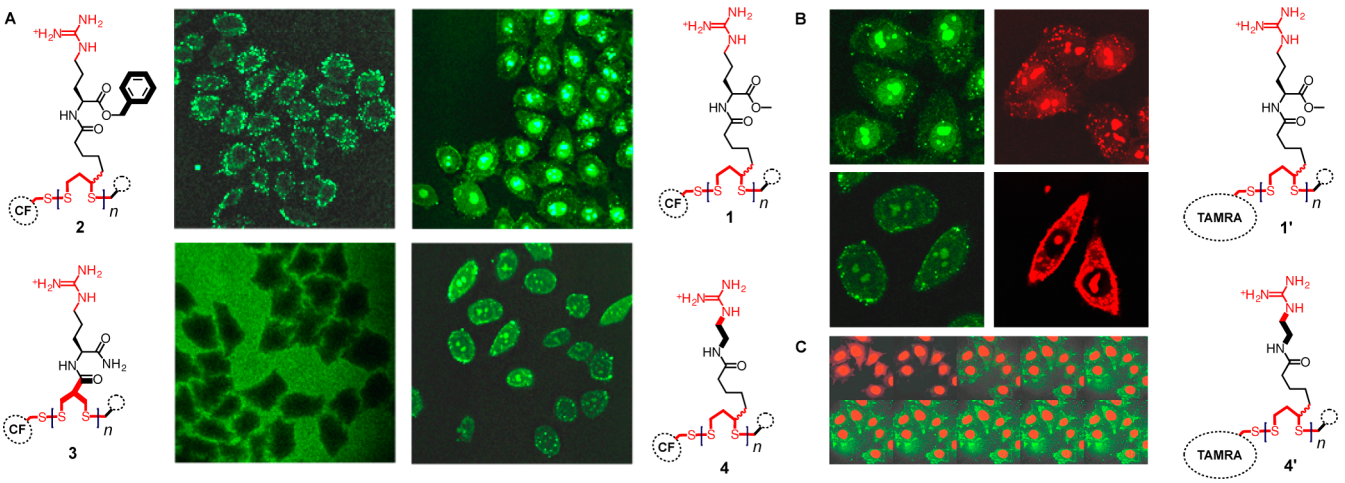

Figure 2. Cellular uptake of fluorescent siCPDs. (A) CLSM images of HeLa cells after 15 min of incubation with CF-labeled polymers **1**−**4**, 500 nM polymer in Leibovitz medium at 37 °C. (B) Same for polymers **1** and **4** prepared from CF (**I1**) or TAMRA (**I2**). (C) Spinning disk microscopy kinetics of the uptake of CF-labeled polymer **4** into HeLa cells transfected in the presence of DRAQ5 (500 nM **4** in Leibovitz medium, 37 °C). Images were taken with a time interval of 1 min. The first image corresponds to t = 0.

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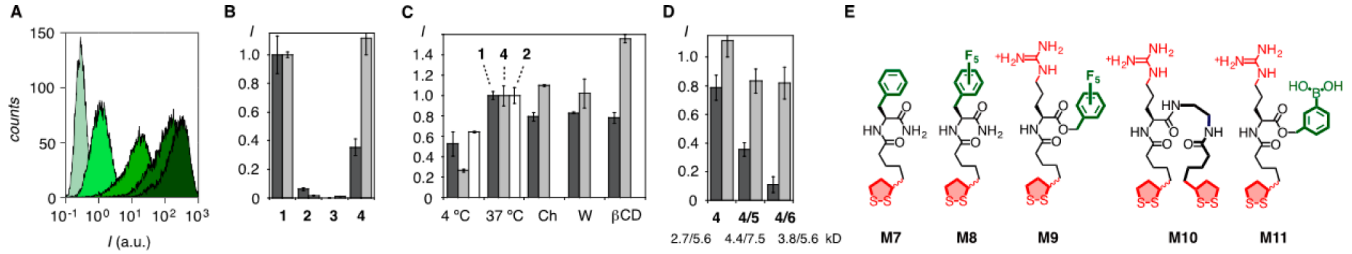

Figure 3. Mechanistic and structural insights into the entry of siCPDs into HeLa cells. (A) Flow cytometry analysis counting fluorescent HeLa cells after incubation with 500 nM initiator **I1** (left, light green, overlap with blank) and CF-labeled (co)polymers **1**/**2** (0:1, 2:1, 8:1, 1:0, with increasing fluorescence instensity *I*). Surface-bound material was removed by washing three times with heparin-containing PBS. (B) Uptake quantification for CF-labeled polymers **1**−**4** with CLSM (dark) and flow cytometry (light), normalized to fluorescence intensity *I* with **1**. (C) Flow cytometry data for the uptake of CF-labeled polymers **1** (dark), **4** (light), and **2** (empty) into HeLa cells at 4 and 37 °C and, for **1** and **4** only, also in the presence of chlorpromazine (Ch), wortmannin (W), and methyl-*β*-cyclodextrin (*β*CD), normalized to *I* at 37 °C. (D) Flow cytometry data for the uptake of low (light) and high (dark) molecular weight, CF-labeled polymer **4** and copolymers **4**/**5** (8:1) and **4**/**6** (8:1) into HeLa cells, normalized to *I* with **1** (A). Error bars indicate the mean ± standard deviation, $n \geq 3$. (E) Structure of additional monomers prepared to grow siCPDs (compare Figure 1).

unused reagents and eventual side products with low molecular weight.

Cellular Uptake. The ability of the siCPDs **1**−**4** to transport a fluorescent CF substrate into HeLa Kyoto cells was examined using confocal laser scanning microscopy (CLSM). After incubation for 15 min at 37 °C with a 500 nM solution of the desired polymer in Leibovitz medium, the cells were washed with PBS containing heparin (20 U/mL) to remove siCPDs that are bound reversibly at the cell surface (or can exit the cells as easily as they entered). Significant intracellular fluorescent signals were recorded from cells incubated with siCPDs **1**, **2**, and **4** (Figure 2A). Only polymer **3** grown with asparagusic instead of lipoic acid did not carry CF substrates into HeLa cells. The inactivity of polymer **3** in HeLa cells perfectly reflected the inactivity of polymer **3** as a transporter in fluorogenic vesicles.[8] This finding thus corroborated the validity of model studies in fluorogenic vesicles to, at least in part, predict the activity of siCPDs as well as CPPs.[8,11] The CF and TAMRA initiators **I1** and **I2** alone did not enter HeLa cells under experimental conditions (Figure S7, Supporting Information).

The intracellular distribution of CF-labeled siCPDs **1**−**4** differed significantly. The original siCPD **1** accumulated mainly inside the nucleus, especially in the nucleoli (Figure 2A, top right, B, top left). The minimalist siCPD **4** localized mainly in the cytoplasm (Figure 2A, bottom right, B, bottom left). The more hydrophobic, *π*-basic siCPD **2** remained mainly trapped inside the endosomes (Figure 2A, top left). The localization of different siCPDs in different organelles was relatively independent of the concentration and incubation time.

To explore the dependence of siCPD uptake on the attached substrate, polymers **1**′ and **4**′ were grown with initiator **I2**, a thiolated derivative of TAMRA. HeLa Kyoto cells were incubated under the conditions used for the CF-labeled siCPDs **1** and **4**. The results demonstrated that siCPDs **1**′ and **4**′ grown with the neutral TAMRA **I2** behave exactly like siCPDs **1** and **4** grown with the anionic CF **I1** (Figure 2B). Namely, siCPDs delivered their substrates reliably to the nucleoli and cytoplasm, respectively, independent of the nature of the substrates.

The kinetics of cellular uptake and localization of CF-labeled siCPD **4** were measured with HeLa Kyoto cells that were treated with DRAQ5 to visualize their nuclei in the far red. Spinning disk microscopy images were recorded immediately after the addition of the polymer solution and with a time interval of 1 min during incubation with 500 nM in Leibovitz medium at 37 °C (Figure 2C). Already after 2 min of incubation, intracellular fluorescence could be observed. Within 10 min, siCPD **4** accumulated in the cytosol but was unable to significantly enter into the nucleus. Intracellular fluorescence intensity was preserved after removal of the polymer solution used to incubate the cells. This irreversible accumulation of siCPD **4** in the cytosol was in excellent agreement with fast reductive depolymerization of siCPD **4** in the cytosol (see below).

Flow cytometry was used for rapid access to quantitative data (Figure 3A). Comparison with CLSM data for polymers **1**−**4** revealed comparable trends but clear underestimates with CLSM for cytosolic emission from siCPD **4**, which performs the best according to flow cytometry (Figure 3B).

Significant uptake of siCPDs **1**, **2**, and **4** was observed at 4 °C (Figure 3C). This demonstrated that uptake does not occur exclusively by endocytosis.[3,4b] Compared to that at 37 °C, reduced activity found at 4 °C could originate simply from less favorable direct translocation across the rigidified membranes,[12] although losses from missing contributions from endocytosis cannot be excluded.

Comparably high activity was obtained with siCPD **2**, although endosomal location at 37 °C demonstrated exclusive uptake by endocytosis, but CLSM images obtained at 4 °C showed the hydrophobic siCPD **2** mainly at the surface, probably too deeply buried in the hydrophobic core of the membrane to be removed by washing with heparin (Figure S6B, Supporting Information).[13] Preserved localization for siCPDs **1** and **4** in the nucleoli and cytosol, respectively, at 4 °C was in agreement with a preserved uptake mechanism, i.e., dominant direct translocation across the membrane (Figure S6A,C). The validity of this interpretation was further supported by insensitivity of siCPDs **1** and **4** to selective endocytosis inhibitors, i.e., wortmannin for macropinocytosis, chlorpromazine for clathrin-mediated endocytosis, and methyl-*β*-cyclodextrin (M*β*CD) for caveolar endocytosis.[14] The distinct increase in activity of siCPDs **4** with M*β*CD could originate from facilitated translocation across cholesterol-poor membranes.[15]

The activity of siCPD **4** increased with increasing length of the polymer (Figure 3D). The same trend, even more pronounced, was found for the more hydrophobic copolymers **4**/**5** and **4**/**6**. Significant length dependence was as expected because both transport activity in membranes[4a,16] and

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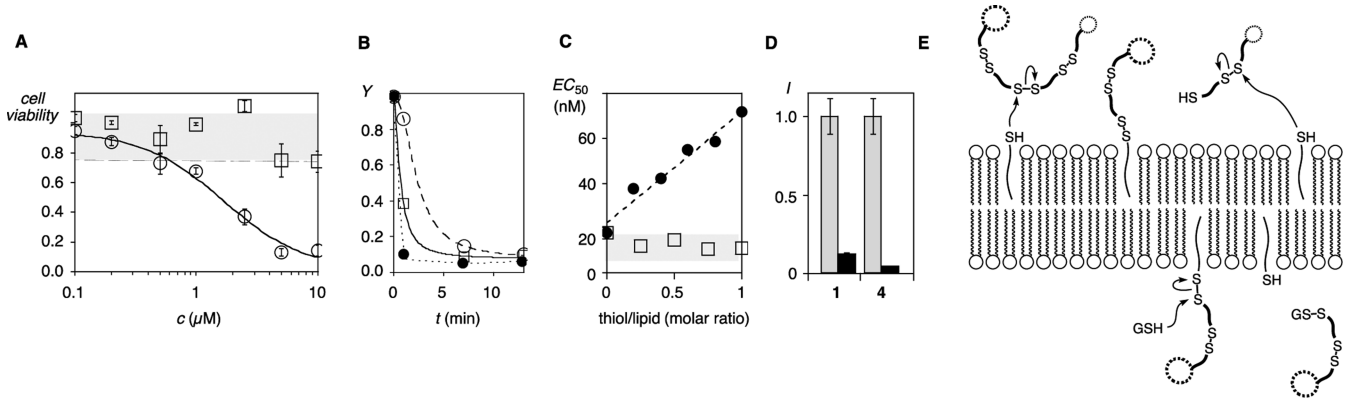

Figure 4. Toxicity, depolymerization, and thiol-mediated translocation of siCPDs. (A) Cell viability measured with the MTT assay for polymer **4** (squares) and poly-L-arginine (circles) at different concentrations. (B) Relative transport activity *Y* of polymer **4** (filled symbols) and **1** (empty symbols) in fluorogenic vesicles after incubation with 2.5 mM (circles) and 5 mM (squares) glutathione (GSH) for time *t*. (C) Effective concentration of polymer **4** in fluorogenic vesicles in the presence of increasing amounts of octadecanethiol (circles) and GSH (squares). (D) Flow cytometry data for the uptake of CF-labeled polymers **1** and **4** into HeLa cells with (dark) or without (light) pretreatment with 1.2 mM DTNB for 30 min before addition of siCPDs. Uptake for each polymer is normalized to 1.0 in the absence of DTNB. (E) Proposed mechanism for thiol-mediated uptake of siCPDs.

contributions from endocytosis (and toxicity) generally increase with the length of related polymers, including CPPs.[3]

However, the introduction of hydrophobic benzyl and indolyl residues in copolymers **4/5** and **4/6** neither increased the activity of siCPD **4** nor changed the preferential accumulation in the cytosol (Figure 3D). Consistently decreasing activity with increasing hydrophobicity, in sharp contrast to many observations with CPPs and the general importance of Phe and Trp for membrane protein function, already provided first indications that siCPDs might be fundamentally different and function by a different mechanism (see below). Similarly decreasing activity with increasing hydrophobicity was found in the amino acid series with π-basic copolymers **1/2** (Figure 3A) and **1/7** and, slightly less pronounced, also with superhydrophobic π-acids in copolymers **1/8** and **1/9** (Figure S8, Supporting Information). The branched polymer **10** or copolymers **1/10** obtained with the divalent monomer **M10** showed particularly poor activity and high toxicity (Figures S8 and S9, Supporting Information). Also boronic acids in copolymers **1/11** did not increase activity despite the possibility to assist uptake with the formation of dynamic covalent boronic ester bonds with glycosaminoglycans at the cell surface, thus enhancing the local concentration and promoting uptake.[5f,17] Quite the contrary, a less desired increase of copolymers **1/11** permanently located at the cell surface and in the endosomes was noted in CLSM images (Figure S6D, Supporting Information). This general failure to improve performance with fairly standard structural modifications suggested that the cellular uptake of siCPDs **1** and **4** is already near maximum levels for this class of transporters and possibly occurs by a different mechanism compared to that of other CPP mimics, i.e., thiol-mediated uptake.[1,6]

Toxicity, Internal Depolymerization, and Exofacial Thiols. Why are siCPDs better? What really makes the difference? Toxicity was quantified first. MTT assays were performed with the cytosolic siCPD **4** in comparison with poly-L-arginine as a comparable CPP. In this assay, the metabolic activity of the cells was assessed by their ability to enzymatically convert the tetrazolium dye MTT into formazan.[18] The polymers were incubated with HeLa cells for 15 min at a concentration ranging from 0.1 to 10 μM. Then the polymer solution was removed, and the cells were washed with heparin sulfate. After addition of culture media, the cells were incubated for 24 h prior to execution of the MTT assay. Under these conditions, siCPD **4** showed negligible cytotoxicity up to 10 μM, whereas polyarginine (16.4 kDa) exhibited an EC_{50} below 2 μM (Figure 4A; hexaarginine was also cytotoxic at 10 μM). The nontoxicity of siCPD **4** was in excellent agreement with rapid depolymerization as soon as the cytosol is reached (see below). All siCPDs tested had good cell viability in the concentration used for the cellular uptake measurements (Figure S9, Supporting Information). However, contrary to the nontoxic siCPD **4** in the cytosol, siCPD **1** was increasingly cytotoxic at higher concentrations. This finding was consistent with incomplete depolymerization before leaving the cytosol (see below) and subsequent interference with cellular function after binding to the oligonucleotides in the nucleoli.

Cytosolic depolymerization was quantified next. It is difficult to quantify in cells, so transport studies in fluorogenic vesicles were selected to secure direct evidence. In this firmly validated assay (see above, Figure S5, Supporting Information),[8,11] large unilamellar vesicles (LUVs) composed of egg yolk phosphatidylcholine (EYPC) are loaded with CF at concentrations high enough to ensure self-quenching. Local dilution upon CF export by siCPDs,[8,11] CPPs,[3,4,15] or other anion transporters is then observed as fluorescence recovery. Consistent with perfect cellular uptake, siCPDs **1** and **4** showed maximal transport activity in the CF assay (without activation by amphiphilic counterions, polyarginine is inactive in EYPC vesicles).[4a] Incubation with GSH at cytosolic concentrations resulted in rapid loss of activity. The cytosolic siCPDs **4** was completely inactivated within less than 1 min by 2.5 mM GSH (Figure 4B, ●). Depolymerization of the nucleolar siCPDs **1** by 2.5 mM GSH was complete within about 5 min (Figure 4B, □). The lifetime of the transporter in 5 mM GSH was clearly shorter (Figure 4B, ○).

Sensitivity toward exofacial thiols was explored last. The presence of octadecanethiol in the EYPC membrane significantly changed the transport activity of siCPD **4** (Figure 4C, ●). At a molar ratio octadecanethiol/lipid of 1:1, the EC_{50} of **4**, i.e., the effective siCPD concentration to reach 50% activity, was weakened by a factor of 4. A similar increase of the EC_{50}

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was not observed in the presence of equivalent concentrations of GSH (Figure 4C, □; these GSH concentrations are far below the ones needed to depolymerize siCPD **4** as in Figure 4B, ●). Activation by thiols at the membrane surface but insensitivity to equimolar thiols in the water indicated that disulfide exchange between siCPD **4** and exofacial thiols occurs. In fluorogenic vesicles, the resulting shortening of siCPD **4** caused a loss in activity. In cells, this covalent binding to the surface is expected to increase the local concentration of the siCPD and thus accelerate direct, counterion-mediated translocation across the membrane (Figure 4E). Experimental evidence for thiol-mediated translocation from fluorogenic vesicles is unprecedented. To evaluate the validity of this implication from fluorogenic vesicles, the inhibition of thiol-mediated translocation with Ellman's reagent (i.e., 5,5′-dithiobis-2-nitrobenzoic acid, or DTNB) was explored directly in HeLa cells. The cells were incubated with the cell-impermeable DTNB for 30 min to convert all free thiols at the surface into disulfides. According to flow cytometry measurements, cellular uptake of siCPDs **1** and **4** was significantly reduced in the absence of exofacial thiols (Figure 4D). Similarly reduced uptake has been observed previously for other cell-penetrating poly(disulfide)s.[1,6] The inactivation by Ellman's reagent was more pronounced for siCPD **4** than for siCPD **1**. Considering that siCPD **4** depolymerizes faster than siCPD **1** (Figure 4C), this difference implied that the efficiency of thiol-mediated translocation is determined by the velocity of disulfide exchange.

CONCLUSIONS

This study introduces substrate-initiated cell-penetrating poly(disulfide)s as general, nontoxic, self-inactivating transporters for the covalent delivery of native substrates of free choice. We provide experimental evidence that the formation of siCPDs can be initiated by fluorescent probes and occurs within minutes under the mildest conditions (water, room temperature, pH 7) and that the most active siCPDs reach the cytosol of HeLa cells in 5 min, where they depolymerize in less than 1 min. The most active siCPDs are nontoxic at all tested concentrations (up to 10 μM), whereas comparable CPPs are toxic. Intracellular localization and the uptake mechanism are independent of the substrate and can be varied on demand by varying the hydrophobicity and disulfide-exchange kinetics. Namely, more hydrophobic siCPDs enter mainly by endocytosis and accumulate in endosomes. More hydrophilic siCPDs with fast disulfide-exchange kinetics accumulate in the cytosol because they depolymerize as soon as they arrive. In clear contrast, the lifetime of siCPDs with slow disulfide-exchange kinetics is sufficient for them to proceed from the cytosol to the nucleus and accumulate on the anionic oligonucleotides in the nucleoli.

The simplest siCPDs are the best. The most compact siCPD **4** is derived from lipoic acid and a guanidinium cation, the original siCPD **1** simply from lipoic acid and L-arginine. Classical structural modifications (hydrophobicity, aromaticity, branching, boronic acids) do not improve performance. This unresponsiveness suggests that the best siCPDs act differently. Insensitivity toward inhibitors demonstrates that endocytosis is almost irrelevant. Significant dependence on the presence of exofacial thiols suggests that the counterion-mediated translocation known from CPPs[4] is coupled with thiol-mediated translocation.[1,6] Namely, siCPDs bind covalently to the membrane surface by disulfide exchange with exofacial thiols, cross the membrane like CPPs along transient micellar defects, and detach into the cytosol by disulfide exchange with intracellular glutathione (Figure 4E). This fascinating, conceptually innovative thiol/counterion-mediated uptake mechanism drives the concept of covalent delivery of unmodified substrates to the extreme: The self-inactivating transporters not only grow covalently on the molecular substrate in solution, they also bind covalently to the membrane they are crossing. The inability of both initiators **I1** and **I2** to enter HeLa cells demonstrates that, contrary to predictions,[1] simple thiolation is insufficient to turn on thiol-mediated uptake. Permanent capture at the surface and in endosomes found with boronic acids in copolymers **1**/**11** confirmed that thiol-mediated translocation requires more than dynamic covalent bonds at the cell surface. For covalent translocation, the simplest siCPDs are then best because they offer disulfide bonds and guanidinium cations at the highest effective concentration for thiol/counterion-mediated translocation.

Once the siCPD has arrived in the cytosol, disulfide-exchange kinetics seem to determine its final destination. Whereas the instantaneous depolymerization of transporter **4** liberates the unmodified substrates in the cytosol, a lifetime of less than 5 min is sufficient for transporter **1** to proceed into the nucleus. Rapid self-inactivation right after uptake also explains nicely why transporter **4** is completely nontoxic. Most importantly, it appears that thiol/counterion-mediated translocation is also controlled by disulfide-exchange kinetics. However, the origin of the different depolymerization kinetics is unknown. Differences in polymer length are insufficient to account for their extent. Possibly, the guanidinium cations in **4** are in the best position to activate the thiolate leaving groups by intramolecular ion pairing, but this is just a hypothesis. Apparently essential to fully understand and exploit the unique advantages of siCPDs, the origin, the variability, and the functional consequences of disulfide-exchange kinetics of siCPDs are currently explored in the greatest detail, with particular emphasis on thiol/counterion-mediated translocation.

ASSOCIATED CONTENT

Supporting Information

Experimental details and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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