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Cellular Uptake: Lessons from Supramolecular Organic Chemistry

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The objective of this Feature Article is to reflect on the importance of established and emerging principles from supramolecular organic chemistry to contribute to one of the most persistent problems in the life sciences. The main topic is dynamic covalent chemistry on cell surfaces, particularly disulfide exchange for thiol-mediated uptake. Examples for boronate and hydrazone exchange are added for contrast, comparison and completion. Of equal importance are the discussions of proximity effects in polyions and counterion hopping, and more recent highlights on ring tension and ionpair-π interactions. These lessons from supramolecular organic chemistry apply to cell-penetrating peptides, particularly the origin of "arginine magic" and the "pyrenebutyrate trick," and the currently emerging complementary "disulfide magic" with cell-penetrating poly(disulfide)s. They further extend to the voltage gating of neuronal potassium channels, gene transfection, and the delivery of siRNA. The collected examples illustrate that input from conceptually innovative chemistry is essential to address the true challenges in biology beyond incremental progress and random screening.

1. Introduction

The discovery of new ways to enter into cells is a central challenge in the life sciences. The tantalizing problem is to bring hydrophilic compounds across the hydrophobic biomembrane barriers without causing significant damage. Over the years, most intense efforts worldwide have resulted in impressive progress with oligonucleotides, including siRNA and gene transfection,[1] with liposomes,[2] polymersomes[3] and related larger assemblies,[4] and with cell-penetrating peptides

(CPPs) and their mimics.[5] The discovery of the latter in the late 80s has caused much excitement.[6] It was found that the so-called protein transduction domain (PTD) of the TAT protein of the HIV virus can easily enter into cells. Interestingly, the virus seems to use the PTD to interact with oligonucleotides, cellular uptake is achieved by membrane fusion instead. Attachment of the short CPPs to β-galactosidase made the enzyme distribute evenly in liver, kidney, lung, heart, spleen and even in the brain.[7]

These intriguing observations stimulated intense research on CPPs. CPP mimics of varied length, sequence, backbone, functionalities and labels have been prepared and explored with regard to the covalent or non-covalent delivery of small drugs and probes, proteins, DNA, RNA, and so on. Results are reviewed regularly from different points of view.[5] Such comprehensive coverage is not intended in the first section of this Feature Article. Only a few highlights on innovative chemistry will be introduced first to illustrate principles, potential and problems before focusing on the main topic, that is the role and use of counterions. The second section of the account moves from "arginine magic" to "disulfide magic," that is from counterion hopping to dynamic covalent chemistry on cell surfaces. This conceptually innovative, currently emerging approach to enter cells promises to overcome central problems with CPPs, particularly their toxicity.

2. Counterion-mediated uptake

2.1. Cell-penetrating peptides and their mimics

The HIV-1 TAT peptide **1** is composed of nine amino acids, six of them are arginines, two are lysines, the only residue without formal positive charge is a glutamine in position 6 (Figure 1). The simplest CPP mimics thus are oligoarginines **2**, best with a length around octapeptides. The peptide backbone has been replaced with a great variety of scaffolds.[5,8-11] Examples reach from synthetic polymers such as the oxanorbornene **3**[9] or polyacrylates **4**[10] to G-PNAs **5**.[11] Co-polymers **3** with

variable cationic, aliphatic and aromatic sidechains are easily accessible by Grubbs ring-opening metathesis polymerization. Possible contributions from aromatic groups, including pyrene, can thus be evaluated in much detail.[9] Guanidinium-rich peptide-nucleic acids (G-PNAs) **5** are interesting because of the general usefulness of PNAs in intracellular DNA/RNA chemistry.[11] Although (or because) they are uncharged, original PNAs were however found to poorly enter into cells. The replacement of the original glycines with arginines in the backbone of G-PNAs **5** has solved this problem with surprisingly little disturbance of their function, i.e., duplex or triplex formation with RNA or DNA.

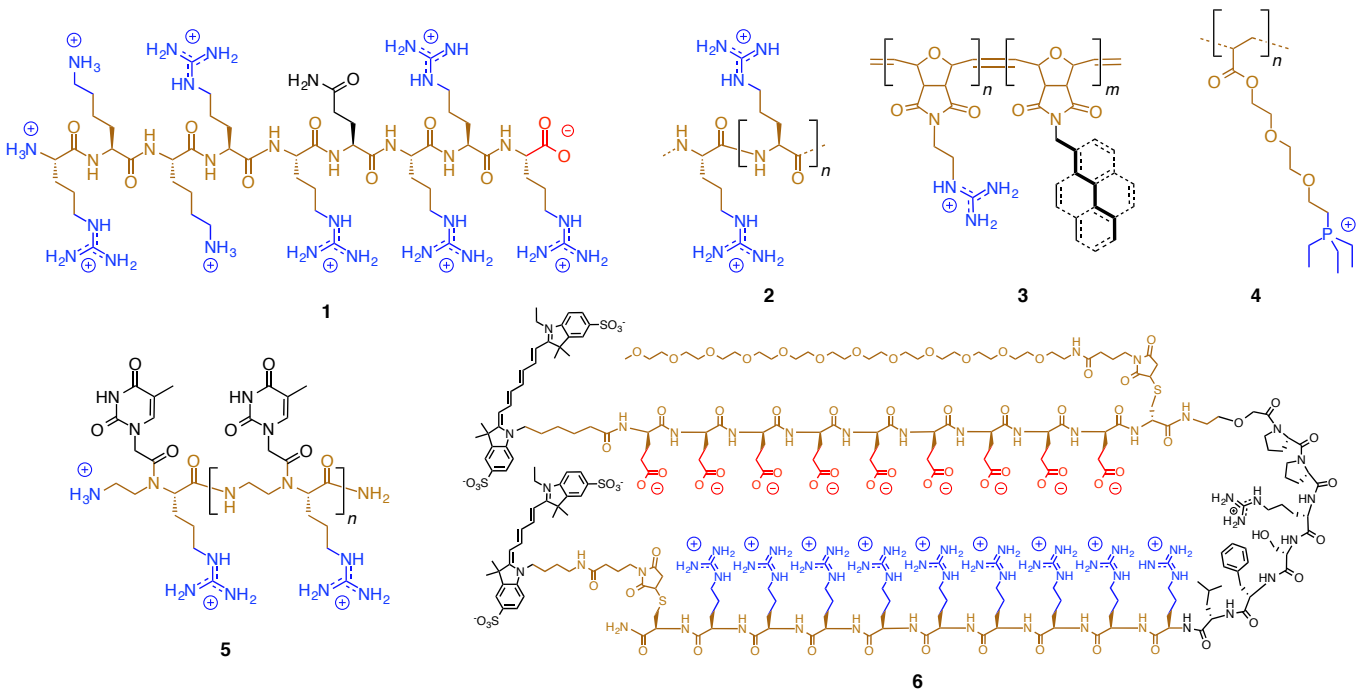

Fig. 1. The original CPP **1** from the TAT protein together with oligo-/polyarginine **2** and selected CPP mimics designed to probe for contributions from aromatics (**3**) and phosphonium cations (**4**), to recognize cytosolic oligonucleotides (**5**) or to sense thrombin in vivo (**6**).

Replacement of the guanidinium cations is more difficult because "arginine magic" is important for the function of CPPs (see below). Ammonium cations are often tested instead.[5] Intriguing

acylguanidinium cations with increased acidity have been introduced by the group of Schmuck.[12] The more demanding phosphonium cations in the sidechains of polyacrylates **4** from the Fréchet group have been proposed recently to offer a non-toxic alternative to ammonium cations in the context of siRNA delivery.[10a] Very recently, this nice series was enriched with polypeptides that have hydrophilic sulfonium cations in their sidechains.[10b]

The thrombin sensor **6** from the Tsien group is shown as recent illustration for the potential of CPPs to address important biological questions.[13] In **6**, a nonaarginine functions as CPP. The positive charges of the CPP are neutralized by a nonaglutamate. *R* Stereoisomers are used in these two domains to prevent degradation by proteases, and the neutralizing domain is PEGylated to assure solubility. The two domains are linked together with a short peptide with a PPR-SFL substrate for thrombin. *S* Stereoisomers are used in this domain to allow enzymatic cleavage. In the presence of thrombin, the nonaarginine CPP is separated from its nonaglutamate inhibitor and thus enters and stains the nearby cells. For detection, a Cy5 donor is attached to the CPP and a Cy7 acceptor to the inhibitor, the presence of thrombin causes to disappearance of FRET from Cy5 to Cy7. This CPP sensor reported correctly on blood coagulation in wounded mice tails as well as inhibition by hirudin, a protein from the salivary gland of leeches. Most importantly, atherosclerotic plaques in carotid arteries could be imaged in vivo.

2.2. Arginine magic

The question how the most hydrophilic, polycationic CPPs can move across the hydrophobic membrane barriers has intrigued the community since their appearance in the literature.[6] Because of the elusive, dynamic nature of the relevant processes, the debate on their mode of action is continuing until today. Highlighting the central role of arginines, the "mysterious" activity of CPPs has been informally referred to in the community as "arginine magic."

Early on, we have proposed that this arginine magic is a counterion effect.[14] This suggestion was based on puzzling observations with synthetic ion channels.[15] Whereas pores formed by lysine-

rich artificial β-barrels showed the expected anion selectivity, arginine-rich pores were cation selective. This observation of polycationic pores that attract cations was counterintuitive. The apparent paradox was explained by the binding of phosphate counterions that invert the internal charge of the pore from positive to negative and thus result in the selectivity for cation.[15a] This explanation was intriguing because, apparently, the binding of phosphate counterions to arginine-rich pores is that strong that they behave like part of the molecular structure of the pore.

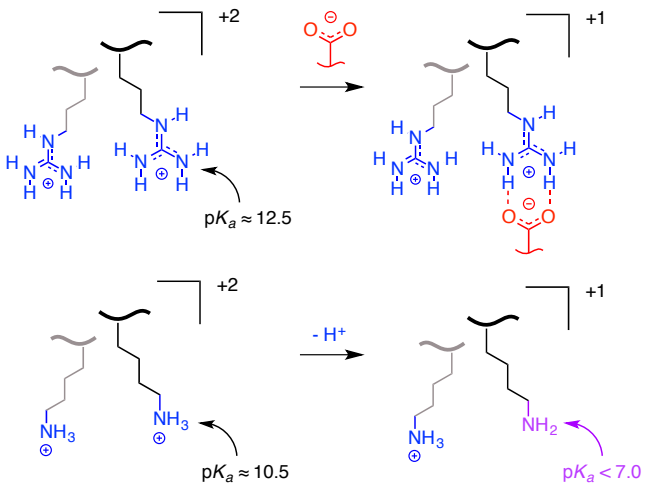

Fig. 2. The origin of "arginine magic": The more acidic ammonium cations can overcome charge repulsion between proximal lysine residues by deprotonation. The only solution for the less acidic guanidinium cations to overcome charge repulsion between proximal arginine residues is the thermodynamically stable yet kinetically labile binding of counterions, preferably carboxylates for additional support from perfectly preorganized hydrogen bonding.

The origin of this exceptionally strong binding of counterions to arginine-rich but not to lysine-rich functional systems is the weak acidity of the guanidinium cation. Two or more cations in close proximity suffer from charge repulsion (Figure 2). With the more acidic ammonium cations from lysine, characterized by an intrinsic pK_a = 10.5, this charge repulsion can be eliminated by deprotonation. As a result, the acidity of an ammonium cation in the proximity of another one

increases significantly. The pK_a drops to a value around 7. This proximity effect is very well known in chemistry and biology, accounting, for example, for the mode of action of enzymes that work with imine and enamine intermediates, e.g., class I aldolases.[16] The reverse proximity effect is operational for acid catalysis with carboxylic acids in neutral water, present, for example, in glycosidases or selected proteases.[16]

With proximal arginines, charge repulsion cannot be eliminated by deprotonation because the acidity of the guanidinium cation, with an intrinsic pK_a = 12.5, is too weak (Figure 2). The only solution to overcome charge repulsion in guanidinium-rich functional systems is the permanent presence of counterions. With arginine-rich CPPs, counterions will always be there and matter, but they can be exchanged rapidly. In other words, polyion-counterion complexes are (thermodynamically) very stable but (kinetically) very labile.[17] This dual nature of polyion-counterion complexes determines their characteristics. Their highly dynamic nature complicates systematic studies and presumably accounts for much of the apparently conflicting behavior often reported for CPPs in the literature. They cannot be isolated and characterized like covalent molecules by standard methods such as NMR, and they also change their composition during function, depending on conditions and local environment. Most importantly, the same fundamental principles of supramolecular organic chemistry are likely to account for their mode of action. A reasonable counterion hopping mechanism looks as follows (Figure 3): CPPs approach biomembranes loaded with hydrophilic counterions (e.g., phosphates, chlorides). Counterion exchange with anionic lipids in the membrane binds CPPs to the cell surface and increases their overall hydrophobicity. The most convincing mechanism for their translocation to the other side of the membrane involves micellar pores. At low concentration, they are short-lived enough to avoid high toxicity and flexible enough to let covalently attached large objects enter into the cell as well. Pore formation with CPPs has been demonstrated in planar bilayer conductance experiments,[18] and cellular uptake of larger objects, proteins and beyond, has been documented

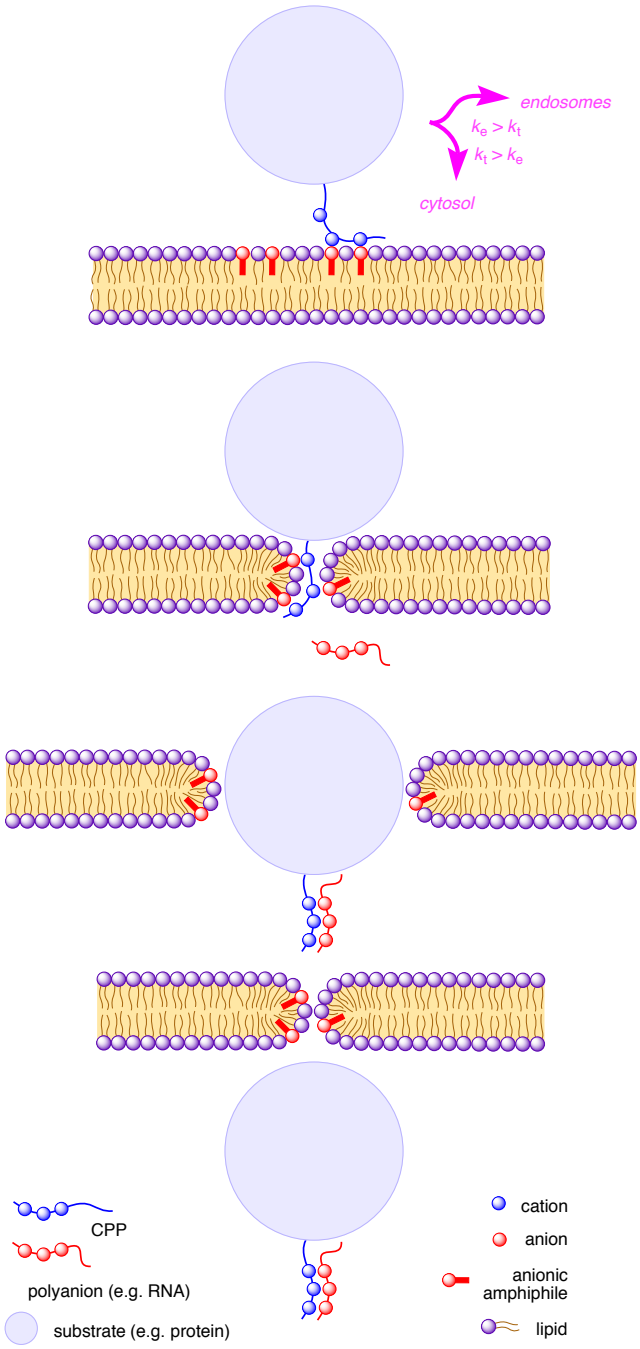

Fig. 3. Counterion-mediated uptake: CPPs bind to the outer membrane surface by counterion exchange to anionic lipids, move across through transient micellar pores and detach from the inner surface by counterion exchange to hydrophilic polyions. The competing endocytosis occurs if direct, counterion-mediated translocation is too slow ($k_e > k_t$) and results in endosomal capture (k_e = rate of endocytosis, k_t = rate of translocation).

extensively.[5] In the cytosol, CPPs detach from the membrane by another counterion exchange. In the spirit of Le Chatelier for coupled equilbria, binding to intracellular polyanions, either RNAs, NTPs, etc in the cytosol or DNA in the nucleoli, is conceivable as ultimate driving force for the cellular uptake of CPPs.

2.3. The pyrenebutyrate trick

Understanding counterion hopping on and of CPPs, we immediately thought: If counterions matter so much, why not use them? One important problem with CPPs and their mimics is that, depending on conditions and their nature, they can fail to really penetrate cells and end up trapped in endosomes

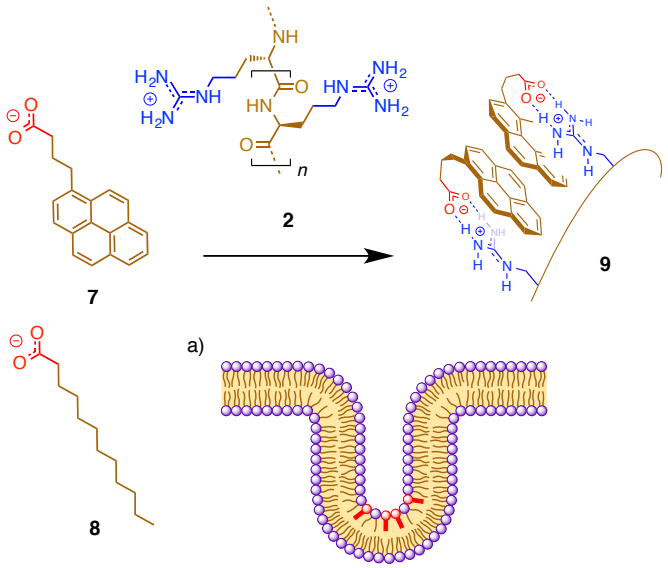

Fig. 4. The "pyrenebutyrate trick": Pyrenebutyrate **7** mediates the direct cytosolic delivery of CPPs **2**, whereas other anionic amphiphiles such as laurate **8** fail to do the same. a) Pyrenebutyrate **7** also induces negative membrane curvature in model membranes (red symbols, compare Figure 3).

instead.[19] According to the counterion-mediated uptake model, such non-productive endocytosis should dominate if the direct transmembrane translocation to either cross the plasma membrane or

to escape from the endosome is too slow ($k_e > k_t$, Figure 3). The addition of hydrophobic counterions should thus accelerate transmembrane translocation and deliver CPPs directly into the cytosol. Extensive counterion screening afforded pyrenebutyrate **7** as the best "catalyst" of direct translocation,[19] many other anions failed,[20] including fullerenes or the laurate **8** explored for some time in the Wender group (Figure 4).[5b,20,21a] In a broader context, the concept of counterion-mediated translocation can be considered as related to phase-transfer catalysis in organic synthesis or the counterion-assisted asymmetric catalysis that emerged soon after the introduction of the "pyrenebutyrate trick".[22]

Today, the cytosolic delivery of underperforming CPPs with pyrenebutyrate is referred to as the "pyrenebutyrate trick" in the community. The proposed counterion-mediated penetration mechanism of guanidinium-rich molecules has been frequently corroborated by results from our lab[19,20] and other research groups.[21b,24-29] Pyrenebutyrate was shown to redirect octaarginine from endosomes to cytosol and nucleus.[19] With pyrenebutyrate, cytosolic delivery of functional peptides and proteins (including EGFP, apoptosis-inducing peptide) attached to octaarginine was achieved in cell lines such as COS-7, PC12, CHO-K1 or RAW264.7, HeLa cells and neurites including cell bodies of hippocampal primary cultured neuronal cells.[19] Activity at 4 ºC, where endocytosis does not work, indicates that the pyrenebutyrate trick is energy independent and thus supports direct penetration of the membrane.[19] The presence of lipidic counterions, and the importance of the pH gradient in their net anionic charge, have been recently confirmed as an universal driving force for peptide penetration *in vitro* and *in silico*.[21b] When protein tagged short oligo(Arg)s were tested, $(Arg)_3$-GFP and $(Arg)_3$-RFP could translocate into cells in the presence of pyrenebutyrate.[24] Other examples from the same group include transdermal delivery of $(Arg)_{11}$ conjugated small molecular tyrosine inhibitor (hydroxyquinone)[25] and $(Arg)_{11}$ fused tyrosine inhibitory peptides[26] using pyrenebutyrate. However, pyrenebutyrate failed to enhance the uptake of non-covalent complexes of CPPs and quantum dots, presumably because the anionic QDs act as competitors.[27] Concepts

related to the pyrenebutyrate trick include the delivery of impermeable phosphorylated peptides with Zn-Dpa transporters with a pyrene moiety.[28]

Recent observations in giant vesicles suggest that pyrenebutyrate can induce negative membrane curvature (Figure 4a).[29] The observed invaginations resemble the ones found with Shiga and cholera toxin, lectins and noroviruses.[30] These observations indicate that the molecular basis of "pyrenebutyrate trick" might be more complex than expected, and that a more complete understanding would be desirable, also to explain and overcome occasional failures.

2.4. Ionpair-π interactions

The active structure **9** of CPPs bound to pyrenebutyrate activators was early proposed to feature pyrene dimers with ion pairs binding on their free aromatic surface (Figure 4).[14] Whereas the occurrence of pyrene dimers could be supported by excimer emission, the proposal of ion pairs sitting together on aromatic surfaces was purely speculative at that time. Cation-π interactions were of course well understood,[31] as was the necessary presence of nearby counterions.[32] However, the possibility to place anions on π-acidic surfaces had just been brought up by theoreticians, essentially without any experimental support.[33]

Meanwhile, anion-π interactions are better understood[34] and confirmed to contribute to binding,[35] transport[36] and even catalysis.[37] Moreover, the possibility of anion-π and cation-π interactions to occur on the same surface has received experimental support last year.[38] Push-pull chromophores were considered as ideal to elaborate on the possibility of such ionpair-π interactions. Amino-naphthalimides were selected as most accessible and most compact example (Figure 5). Guanidinium-carboxylate pairs were covalently positioned in antiparallel and parallel orientation with respect to the push-pull dipole. The antiparallel ionpair-π interactions in **10** generated a red

shift of the absorption maximum (+44 nm). This shift was not present in the parallel system **11**, because ionpair-π

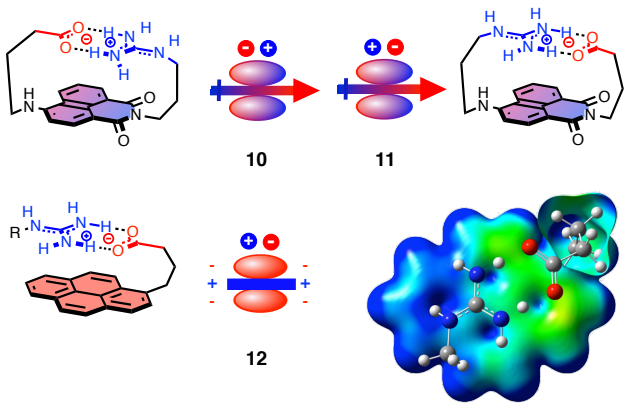

Fig. 5. Ionpair-π interactions: Covalent positioning of anions and cations on push-pull chromophores in antiparallel (**10**) and parallel (**11**) orientation with respect to the push-pull dipole (top), and model of complex **12** with pyrenebutyrate pairing with a guanidinium cation on the polarized pyrene surface (bottom). Adapted form ref 38 with permission, © Wiley 2014.

repulsion on the polarized excited chromophore is removed by proton transfer from the poorly acidic guanidinium cation to the carboxylate. The observation of this proton transfer from guanidinium cations on the polarized surface of excited push-pull chromophores is intriguing with regard to protein chemistry in general and the mode of action of arginine-rich CPPs in particular (compare Figure 2).[39]

With evidence that ionpair-π interactions exist and matter, we were of course curious to see if the early, fully hypothetical structure of pyrenebutyrate-CPP complex **9** would be scientifically reasonable (Figure 4). To our delight, computational simulations of complex **12** gave a stable minimum for the ionpair-π complex (Figure 5). However, convincing complexes from computational simulations should not be confused with experimental evidence that ionpair-π interactions really contribute to the pyrenebutyrate trick. Alternative or additional possible

contributions reach from aromatic interactions at the membrane interface, most frequent with membrane proteins,[40] to the membrane invaginations[29,30] mentioned in the previous section.

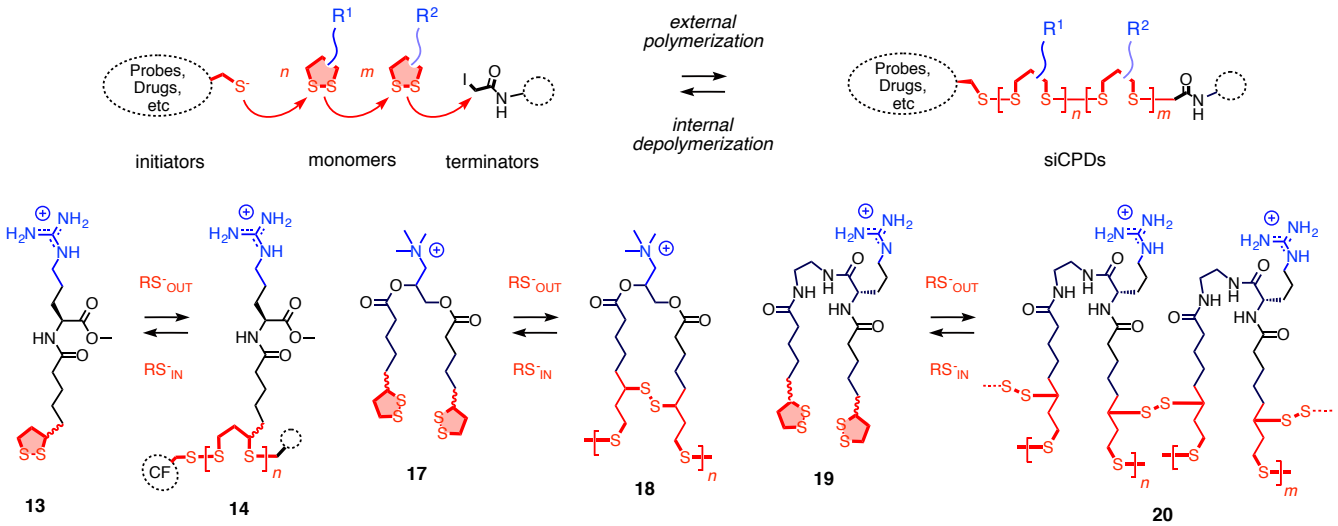

Fig. 6. Substrate-initiated cell-penetrating poly(disulfide)s (siCPDs) are grown directly on substrates of free choice right before and destroyed right after uptake to minimize toxicity and release the substrate. Representative propagators (**13**, **19**) and more (**14**) and less active siCPDs (**20**) are shown together with related cationic poly(disulfide)s **18** that have been of interest in the context of gene transfection.

2.5. Voltage-gated ion channels

Counterion-mediated translocation is obviously not limited to cellular uptake of CPPs. The finding that the voltage gating of neuronal potassium channels originates from arginine-rich paddles caused a discussion similar to the older and more intense debate about the mode of action of CPPs. Some time after the discovery of the pyrenebutyrate trick,[14] it was confirmed that, not surprisingly, anionic lipids account for voltage gating with arginine-rich paddles.[41]

2.6. Gene transfection and siRNA

Counterion-mediated uptake is best appreciated with oligonucleotides. From gene transfection to cellular uptake of siRNA, cationic amphiphiles are used routinely to accelerate transmembrane translocation.[1] A delightfully rich collection of cationic amphiphiles is available, and several different modes of action are known. Why then is it that counterion-mediated uptake is routine with polyanions and subject of debate with the charge-inverted systems?[42] The difference is that biomembranes contain anionic lipids. These intrinsic counterion activators can make CPPs appear as if they would move across membrane barriers without any help, and their variability presumably accounts for puzzling, sometimes contradictory results with CPPs. With charge inversion to polyanions, the intrinsic activators are missing and cannot confuse the situation: No question here that cationic counterion activators are needed.

3. Thiol-mediated uptake

Reports on improved cellular uptake in the presence of thiols or disulfides can be found scattered throughout the literature.[43-45] Perhaps because these observations often came as a surprise in the context of studies on other topics, it took remarkably long time to formulate the concept of thiol-mediated uptake, and much remains to be confirmed, explored and possibly exploited. The concept of thiol-mediated uptake as defined by Mike Gait in 2012 focuses on dynamic covalent disulfide exchange chemistry to covalently attach the transporters to the cell surface for uptake.[43] The possibility of thiol-mediated uptake has arguably received most attention in the context of gene transfection with cationic poly(disulfide)s, particularly by the Oupicky group.[44] Scope and limitations of thiol-mediated uptake are currently mapped out with cell-penetrating polydisulfides (CPDs)[46-48] and the application of ring tension to this "disulfide magic".[49] These more recent developments are briefly summarized in the following.

3.1. Cell-penetrating poly(disulfide)s

The inspiration for substrate-initiated cell-penetrating poly(disulfide)s (siCPDs) came from the finding that ring-opening disulfide-exchange polymerization is best to grow multicomponent architectures directly on solid oxide surfaces.[50] Somewhat related to protein folding, this underappreciated process turned out to be delightfully robust. We thus wondered whether or not this wonderful, nearly forgotten ring-opening disulfide-exchange polymerization[51,52] could also be used to grow CPDs directly on any substrate that has to be delivered into cells (Figure 6).[48] Arrived in the cytosol via counterion-mediated uptake (Figures 2-4), the siCPDs would be destroyed right after entry by reductive depolymerization to liberate the substrate and eliminate toxicity, one of the main disadvantages of CPPs.

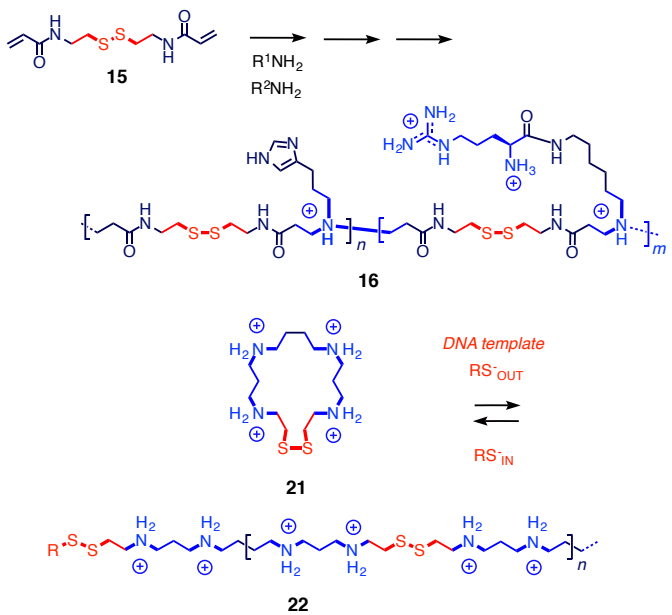

Fig. 7. Selected cationic poly(disulfide)s that have been explored in the context of gene transfection.

The concept of siCPDs has been elaborated with thiolated carboxyfluorescein (CF) and related probes as initiators and iodoacetates as terminators (Figure 6).[48] As propagators, several derivatives

of lipoic acid were tested. The simplest propagator **13** is composed of lipoic acid and arginine only. Ring-opening disulfide-exchange polymerization on thiolated CF gave siCPD **14** within five minutes at room temperature in neutral water. The generality of the approach is well documented with a series of propagators, and co-polymerization with different propagators is unproblematic (R^1, R^2, Figure 6).[48] According to the established MTT assay, also the most performant siCPDs were not toxic at concentrations as high as 10 μM.[46] At this concentration, the control CPPs killed all HeLa cells. This clear difference was in support of the concept that destruction right after work will minimize the toxicity of the transporter.

Before the introduction of siCPDs, cationic poly(disulfide)s have been studied quite extensively in the context of gene transfection.[44,51,53-57] These cationic poly(disulfide)s are usually not prepared by disulfide-exchange polymerization. In contrast, the disulfide is already present as in the representative propagator **15** (Figure 7). Polymerization and co-polymerization are achieved by conjugate addition of amines to Michael acceptors as in propagator **15**. Several examples for post-modification of poly(disulfide)s prepared this way exist. In cationic poly(disulfide)s **16**, for example, the arginine has been attached to the final polymer.[54] Cationic poly(disulfide)s **16** is shown as example because it is quite similar to siCPD **14**. However, it has only been studied in the context of gene delivery by endocytosis. The imidazoles and the guanidinium residues have both been attached to mediate the escape from the endosome, either by the proton sponge effect or by direct translocation similar to CPPs. Direct translocation across the endosomal membrane was found to be more effective.

Many variations have been reported for this approach based on conjugate addition.[44,45,53] In clear contrast, examples for disulfide-exchange polymerization prior to the introduction of siCPDs are rare. In a pioneering study, propagators **17** have been polymerized with a thiolate as initiator (Figure 6).[55] The resulting cationic poly(disulfide)s **18** have been successfully applied to gene delivery, with emphasis on gene release upon reductive depolymerization. Applied to cellular

uptake of siCPDs, divalent propagators **19** (with decreased uptake and increased toxicity) were much less impressive than propagators such as **13** with one strained disulfide only.[46] This poor performance was rationalized with the formation of branched, more complex polymers **20** that are less useful for thiol-mediated uptake and more difficult to depolymerize. A very recent example for disulfide-exchange polymerization from the Miller group uses cationic macrocycles such as **21** as propagators (Figure 7).[56] This example is important because disulfide-exchange polymerization was explored after the binding of propagators **21** to DNA templates. The resulting cationic poly(disulfide)s **22** were again tested exclusively for gene transfection, applications as CPDs were not reported.

3.2. Disulfide magic

The uptake of the new siCPDs **14** into HeLa cells depended strongly on their length (Figure 6).[47] Short siCPDs **14** (and too hydrophobic variations) localized in endosomes. Longer polymers were found mostly in the cytosol, whereas very long siCPDs **14** accumulated with high selectivity in the nucleoli. This important length dependence of siCPDs revealed much about their mode of action. Namely, siCPDs are expected to bind to the cell surface by counterion exchange to anionic lipids, exactly like other CPPs (Figure 8, compare Figures 2 and 3). The ability of too short and too hydrophobic siCPDs to form micellar pores and move across the membrane barrier is insufficient to compete with endocytosis ($k_e > k_t$, Figure 8).[14,19,46,47] As a result, they end up trapped in endosomes. Longer siCPDs can move faster across membranes, also confirmed in model studies in fluorogenic vesicles. As a result, direct translocation kinetically outcompetes endocytosis, and the siCPDs can reach the cytosol ($k_t > k_e$). The velocity of reductive depolymerisation by glutathione in the cytosol decreases with increasing polymer length.[47] Polymers of intermediate length are thus destroyed fast enough to release the substrates in the cytosol ($k_d > k_n$, Figure 8). Longer polymers can proceed into the nucleus before complete depolymerization and, being polycations, bind very strongly to the DNA in the nucleoli ($k_n > k_d$, Figure 8). Long enough siCPDs

were not fully inactivated at 4 ºC.[46] This excluded that only energy dependent uptake processes are involved. Moreover, inhibitors of macropinocytosis (chlorpromazine), clathrin-mediated endocytosis (worthmannin) and caveolar endocytosis (methyl-β-cyclodextrin) did not inactivate siCPDs.[46] These forms of endocytosis do thus not account for the uptake of siCPDs.

However, the most intriguing aspect of the mode of action of siCPDs is that they do not only bind to the cell surface by the proximity-enhanced counterion exchange known from CPPs as "arginine magic" (Figures 2, 3): During uptake, siCPDs bind covalently to the cell surface.[46] Cells express thiols on their surface to protect against an oxidative environment.[43,44] Disulfide exchange with these exofacial thiols attaches siCPDs covalently to the cell surface (Figure 8, right side). After translocation, perhaps by counterion-mediated micellar pores, perhaps through other pathways (see below), siCPDs are cleaved from the inner surface by disulfide exchange with glutathione.

The existence of these contributions from thiol-mediated uptake to the activity of siCPDs can be easily demonstrated. Disulfide exchange with Ellman's reagent DTNB (5,5′-dithiobis-2-nitrobenzoate), converts all thiols on the cell surface into activated disulfides. After incubation with Ellman's reagent, the uptake activity of siCPDs was strongly inhibited because only the counterion-mediated mechanism was left available.[46] This sensitivity to surface oxidation suggested that siCPDs enter cells essentially via thiol-mediated uptake, i.e., by dynamic covalent chemistry on the surface of the cell.

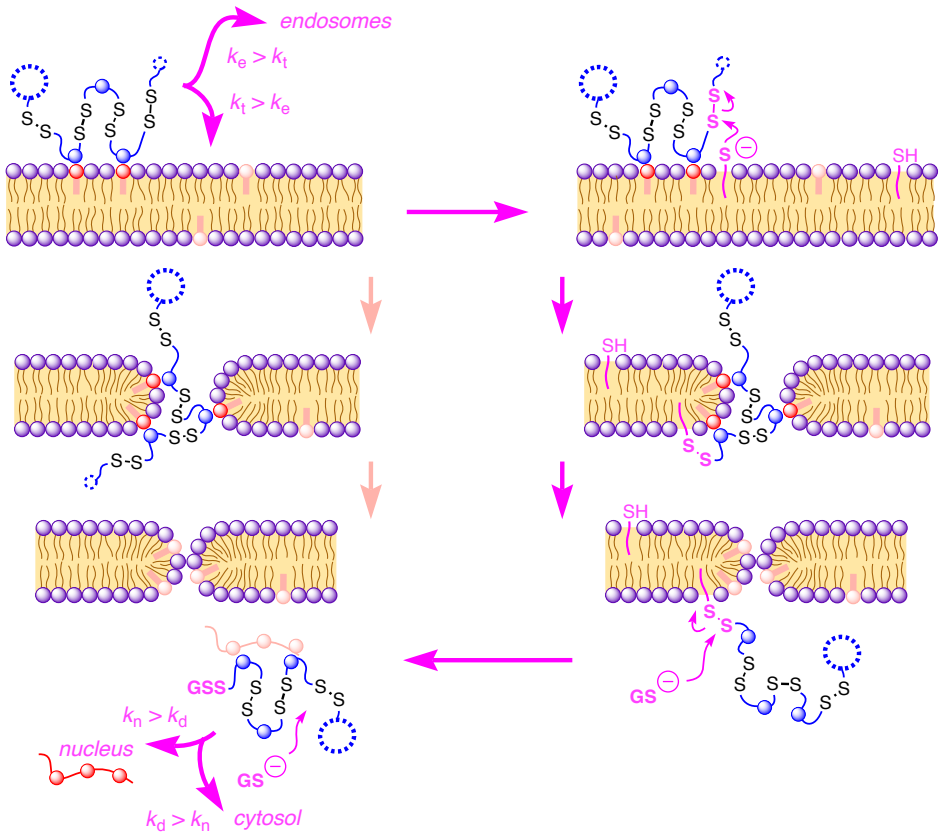

Fig. 8. Comparison of counterion-mediated (left) and thiol-mediated (right) uptake of cell-penetrating poly(disulfide)s. In counterion-mediated uptake, non-covalent binding of siCPDs to the surface by proximity-enhanced counterion exchange is followed by translocation through micellar pores and internal release by counterion exchange to multivalent acceptors in the interior. In thiol-mediated uptake, binding of siCPDs to the surface by dynamic covalent disulfide exchange is covalent. This can be followed by translocation through micellar pores and internal release by disulfide exchange with cytosolic glutathione. Slow translocation with, e.g., too short siCPDs results in dominant endocytosis and endosomal trapping, fast internal depolymerization in cytosolic delivery and slow internal depolymerization with, e.g., too long siCPDs in delivery to the nucleoli (k_e = rate of endocytosis, k_t = rate of translocation, k_d = rate of depolymerization, k_n = rate of nuclear uptake).

3.3. Ring tension

Seeing this decisive importance of thiol-mediated uptake for the activity of siCPDs, we immediately thought: Lets apply tension! Ring tension is more than explosives[58] or intramolecular activation of leaving groups. Ring tension has been helpful for chemical interference with biological systems in the broadest sense, with examples reaching from bioorthogonal labeling to β-lactam or β-peptide antibiotics or antitumor drugs such as calicheamicins, dynemicin A, mitomycin C, and so on.[59] To explore ring tension in the context of thiol-mediated uptake, disulfides with increasing tension were attached to CF, a fluorescent probe that cannot enter cells without help.[49] Disulfides without tension have a CSSC dihedral angle of 90º (Figure 9).[60] The acyclic disulfide **23** with an Ellman leaving group was used as an example for activated disulfides without tension. The dihedral angle of 62º in DTT derivative **24** was selected to probe for cyclic disulfides with weak ring tension. Intermediate tension was accessible with derivative of lipoic acid **25**, featuring a dihedral angle of 35º. The 27º angle in asparagusic acid provided access to maximal ring tension in conjugate **26**.

According to flow cytometry analysis, uptake into HeLa Kyoto cells increased gradually with increasing ring tension.[49] Even the difference of 8º in tension between lipoic acid in **25** and asparagusic acid in **26** clearly mattered. This high sensitivity to small changes in ring tension was consistent with previous results with multicomponent architectures on oxide surfaces.[61] Activated leaving groups in conjugate **23** were clearly less powerful than ring tension in **25** and **26**.[49]

Several experiments were conceived to explore existence, scope and limitations of dynamic covalent chemistry on cell surfaces during uptake with “disulfide magic.” Incubation of cells with iodoacetamides converts all surface thiols into inactive sulfides (Figure 9a). As a result, the activity of all transporters **23**-**26** operating with disulfides was strongly reduced.[49] Interestingly, the activity of controls with thiols in place of disulfides decreased as well. This suggested that disulfides on the cell surface are coupled to thiols, possibly by exofacial protein disulfide isomerases (PDI).[43,44]

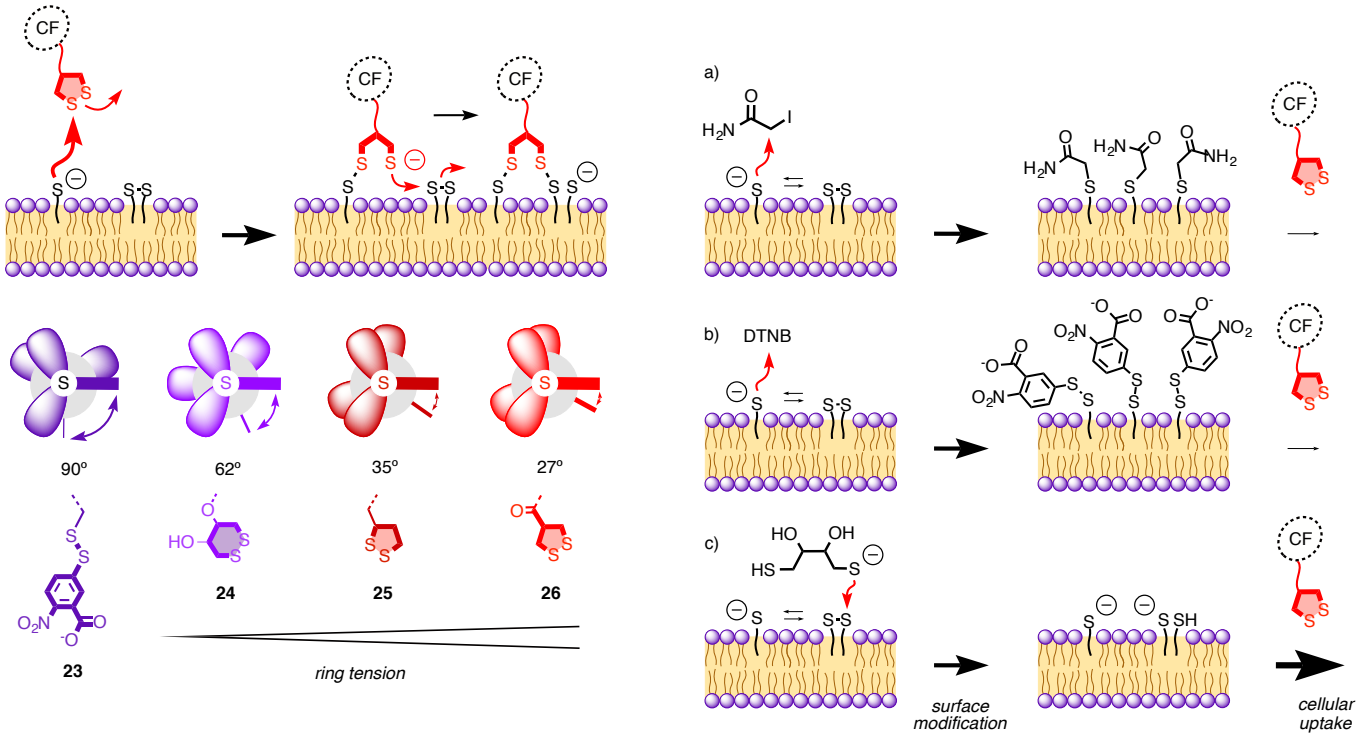

Fig. 9. Cellular uptake under tension (*left*): Increasing uptake of CF-conjugates **23**-**26** with decreasing CSSC dehedral angle indicates that strained disulfides react best with exofacial thiolates for covalent binding to the cell surface. "Disulfide magic" (*right*): a) Conversion of exofacial thiols and disulfides into sulfides inhibits uptake of transporters with disulfides and thiols. b) Conversion of exofacial thiols into activated disulfides inhibits uptake of transporters with disulfides and increases uptake transporters with thiols. c) Conversion of exofacial disulfides into thiols increases the uptake of transporters with disulfides and inhibits uptake transporters with thiols.

Incubation of cells with Ellman's reagent converted all thiols into activated disulfides.[49] As a result, activity of all transporters operating with disulfides, from monomers **23**-**26** to siCPD **14**, was strongly reduced (Figures 9b, 6). As expected from the presence of activated disulfides on the cell surface, the activity of controls with thiols in place of disulfides increased strongly.

Most interesting was the reduction of the cell surface with DTT (Figure 9c). The activity of transporters operating with disulfides increased, whereas thiol-containing controls became less

active. Strongest activation was found for **23** with activated leaving groups, followed by **26** with highest ring tension. This difference suggested that uptake under ring tension can also involve disulfides on the cell surface. This conclusion implied that the first exchange to release the tension can be followed by a second disulfide exchange that binds the transporters with two disulfide bonds to the surface (Figure 9, left side). The removal of disulfides from the cell surface inhibits the uptake of thiol-containing controls as expected.

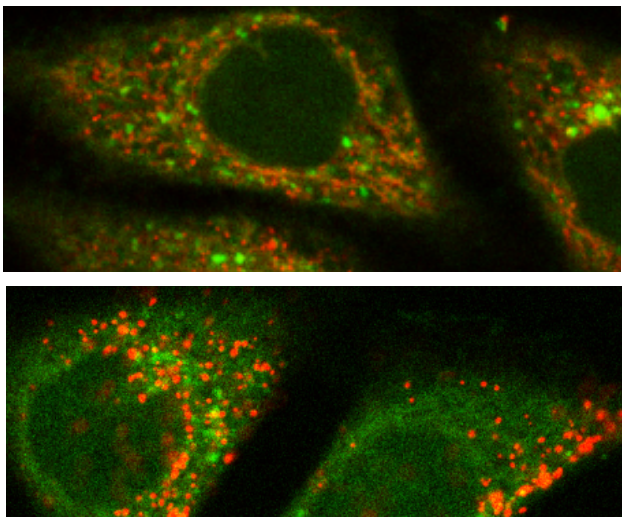

Fig. 10. Co-localization studies for thiol-mediated uptake under tension: Uptake into HeLa cells of **26** (green) together with Dextran Red to track endosomes (red, top) and LysoTracker Red DND-99 (red, bottom). Adapted form ref 49 with permission, © Wiley 2015.

Thiol-mediated uptake under highest tension in **26** was reduced at low temperature but insensitive to standard inhibitors of endocytosis such as chlorpromazine (macropinocytosis), worthmannin (clathrin-mediated endocytosis) or methyl-β-cyclodextrin (caveolar endocytosis). Co-localization experiments revealed partial delivery to the endosomes but no specific accumulation in lysosomes and mitochondria (Figure 10). Incomplete co-localization with endosomes and significant presence in cytosol support the most intriguing perspective that thiol-

mediated uptake could possibly occur through so far unknown pathways. Contributions from counterion-mediated uptake can of course be excluded with anionic monomers. The implication that "disulfide magic" provides indeed a new, conceptually innovative entry into cells is also supported by the less exciting results from orthogonal[62] dynamic covalent chemistry[51,63,64] on cell surfaces with boronic ester exchange.[64]

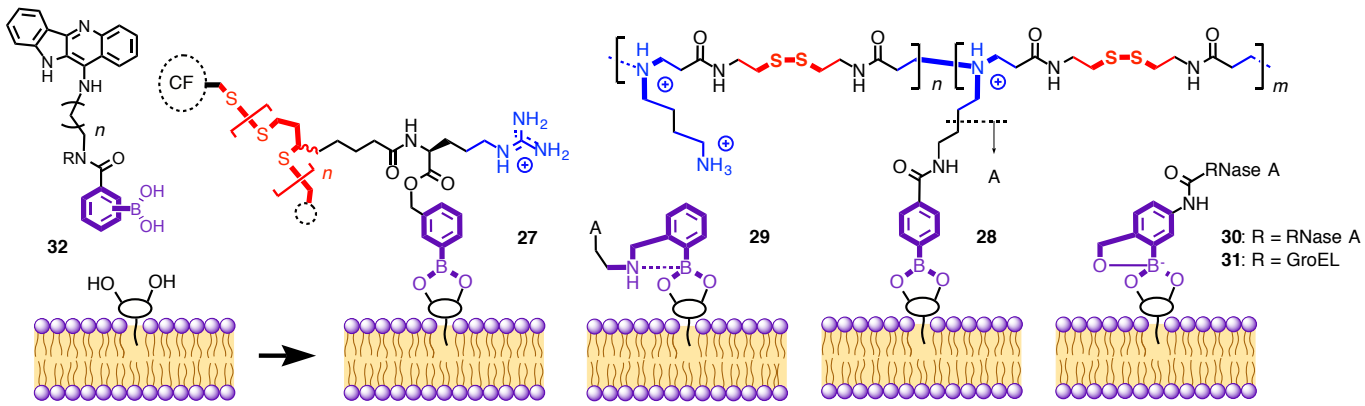

Fig. 11. Boronic ester exchange with glycosaminoglycans on cell surfaces hinders uptake of siCPDs **27** and gene transfection with cationic poly(disulfide)s **28** and **29** but enables uptake of proteins and small drugs attached to benzoboroxoles in **30**-**31** and boronic acids in conjugate **32** (*n* = 1, 2, 5; R = H, Me).

4. Boronate-mediated uptake

Dynamic covalent chemistry[62-64] between boronic acids and the carbohydrates on cell surfaces has been considered in several groups. Unexpected high complexing ability is present with sialic acid, one of the most abundant saccharide derivatives on cell surfaces.[65] The perspective to use boronic ester exchange[64] for covalent uptake is certainly as appealing as "disulfide magic." In the context of siCPDs, the potential of boronic ester exchange has been explored with **27** (Figure 11).[46] Added to HeLa cells, siCPDs **27** localized mainly on the surface and appeared also in endosomes. This finding suggested that dynamic covalent binding to cell surfaces with boronic esters occurs but it

does not lead to uptake beyond endosomes. This clearly different behavior indicated that "disulfide magic" is more than just dynamic covalent chemistry on cell surfaces and could indeed open a new pathway to enter into cells.

Similar trends were reported for gene transfection with cationic poly(disulfide)s **28**.[57] The presence of boronic acids was not beneficial for uptake and increased toxicity, probably because the transporters could disturb the membrane for longer time before their destruction in the cytosol. Attempts to stabilize the boronic esters with proximal amines in **29** did not improve the situation.

Replacement of the ammonium cations in poly(disulfide) **29** with primary alcohols was interesting to address gene transfection with less charged transporters.[66] Boronic acids, presumably binding to phosphodiesters in the DNA backbone, generated more stable polyplexes. However, binding of boronic acids to the surface of the cells was so strong that it had an unfavorable effect on the DNA transfection efficiency. This problem could be addressed with carbohydrates (e.g. D-sorbitol and dextran) that compete with oligosaccharides at the cell surface for the formation of boronic esters.

Removal of the disulfides rather than the positive charges in transporter **29** was explored with phenylboronic acid-modified polyethylenimine (PEI).[67] The presence of boronic acids enhanced both DNA condensation and *in vivo* transfection (2 to 3 orders of magnitude) in three different cell lines (HepG2, COS-7 and HeLa). However, this came at cost of higher cytotoxicity compared to unmodified PEI. To explore the role of boronic acids, cellular uptake of fluorescently labeled polymers was monitored by confocal microscopy. The obtained images showed increased PEI uptake after the attachment of boronic acids, and their localization inside the nuclei. The presence of interactions between boronic acid and the cell membrane was tested with transfection experiments in the presence of glucose. The efficiency decreased with the increase of glucose concentration, indicating the competitive inhibition of boronic ester exchange on the surface of the cell.

To probe for the importance of both disulfides and positive charges in **28**, a commercially available triblock polyether polymer was decorated with phenylboronic acid groups.[68] The neutral, disulfide-free polymers were studied as non-viral gene delivery vectors. The presence of boronic acid moieties increased DNA complexation, ascribed to coordination between phosphate and boronic acid groups. The functionalization had also a positive impact in cellular uptake, since an increased transfection efficiency, up to 1000 times, was observed in different cell lines, also in presence of serum. However, all the positive effects due to the installation of boronic acid derivatives in the polymer were partially overwhelmed by the increase of cytotoxicity by almost one order of magnitude.

Boronic acids attached to RNase **30** were found to mediate the uptake of the enzyme.[69] This breakthrough could originate from the use of benzoboroxoles, i.e. boronic acids with proximal alcohols that afford tetrahedral borate anions upon reaction with diols or catechols.[70] Controls with boronic acids lacking proximal alcohols were inactive. In a nice follow up on this study, the same benzoboroxoles were attached to barrel-shaped chaperonin GroEL mutants.[71] In the presence of Mg^{2+}, the resulting conjugate **31** assembles into nanotubes that can be loaded with guests. Intracellular ATP hydrolysis disassembles the aggregates and liberates the cargo. No cellular uptake was observed in the absence of benzoboroxoles.

In a systematic study of the effect of boronic acid substitution on the cellular uptake and cytotoxicity in two different cancer cell lines, different quindoline derivatives such as **32** were prepared and studied.[72] In the presence of boronic acids, decreased uptake and enhanced localization closer to the cell membrane were observed.

5. Hydrazone-mediated uptake

The hydrazone bond, i.e., the third main dynamic covalent bond besides disulfides and boronic esters,[62,63] is less attractive for dynamic covalent chemistry on cell surfaces because both involved

functional groups are rare. Hydrazone exchange can be considered in the context of endosomal escape because the pH within endosome is lowered and thus promotes the hydrolysis of hydrazones.[73]

In an alternative approach, hydrazone sensitivity towards acid catalyzed hydrolysis has been elegantly applied in the stimuli-responsive selective delivery of cytotoxic molecules (Figure 12a).[5e,74] In this strategy, drug-loaded liposomes were premixed with artificial lipids that were linked, through dynamic hydrazones, to polyethylene glycol (PEG) chains. The PEG moieties functioned as physiological stabilizers of the liposomes vehicles and, at the same time, masked TAT pendants located at the vesicle surface (Figure 12a). The liposomes were further decorated with antibodies for selective cellular recognition. Upon exposure to the lowered pH conditions, typical of cancer cells, the pH-triggered release of the PEG chains allowed the selective, CPP mediated penetration of the liposome and the intracellular delivery of the cargo. Dynamic hydrazone bonds have been also applied for the reversible, pH sensitive attachment of hydrophobic and cationic side chains to block anionic copolymers (Figure 12b).[75] These grafted polymers **33** performed well in the intracellular delivery of anti-GAPDH siRNA.

Recently, hydrazones became interesting for cellular uptake for a different reason.[76] The topic emerged during the creation of an artificial nose that operates based on hydrazone exchange.[77] This differential sensor was constructed based on the observation that many odorants are hydrophobic aldehydes and ketones (Figure 13). Hydrazone formation with a collection of small peptide dendrons containing one to six hydrazides and one or two positive charges afforded cationic amphiphiles. These amphiphiles can activate DNA as cation transporter in fluorogenic vesicles.[42] Different activation with different peptide hydrazides attached to a given odorant was then used to generate patterns. Pattern recognition with routine methods afforded overlap-free identification in the virtual principal component space for all tested odorants and perfumes, including mixtures enantiomers (muscone, etc), *cis*-*trans* isomers (cucumber aldehyde), and so on.[77]

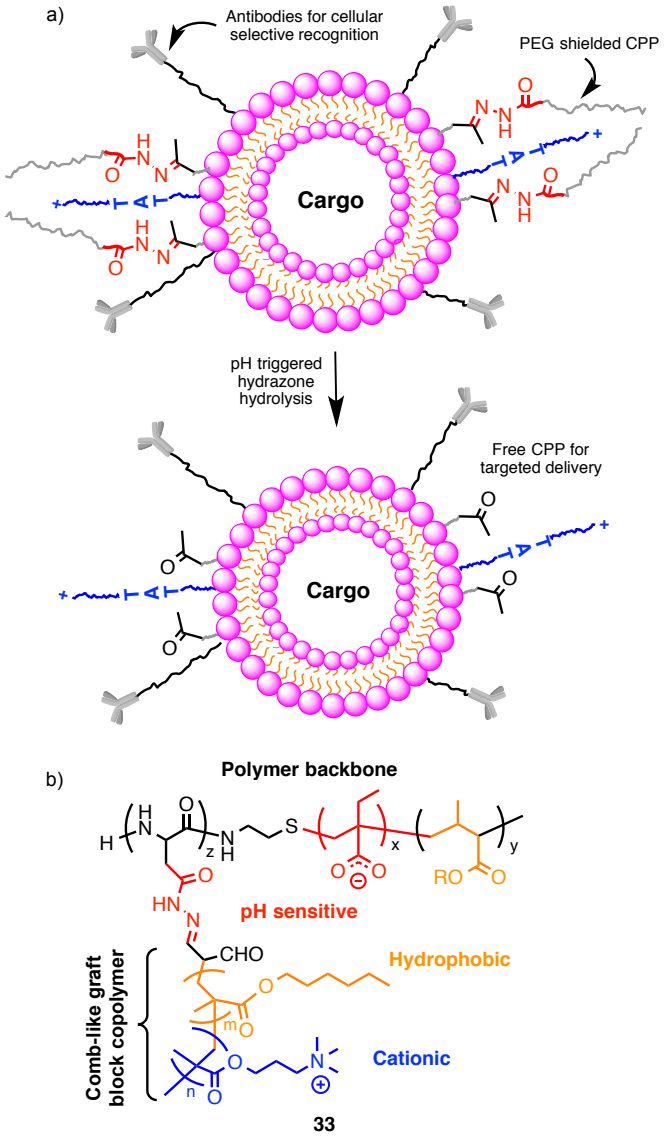

Fig. 12. pH sensitivity of hydrazone dynamic bonds in cellular uptake. a) Polyethylene glycol (PEG) chains enhance the physiological stability of drug-loaded liposomes while masking the CPP (TAT) moiety. Upon lowered pH exposure (typical of cancer cells) the hydrazone linked PEG is hydrolyzed and the TAT moiety is unmasked. b) Hydrazone linkages for grafting block copolymers **33** with suitable hydrophobic/hydrophilic pendants for nucleic acid delivery.

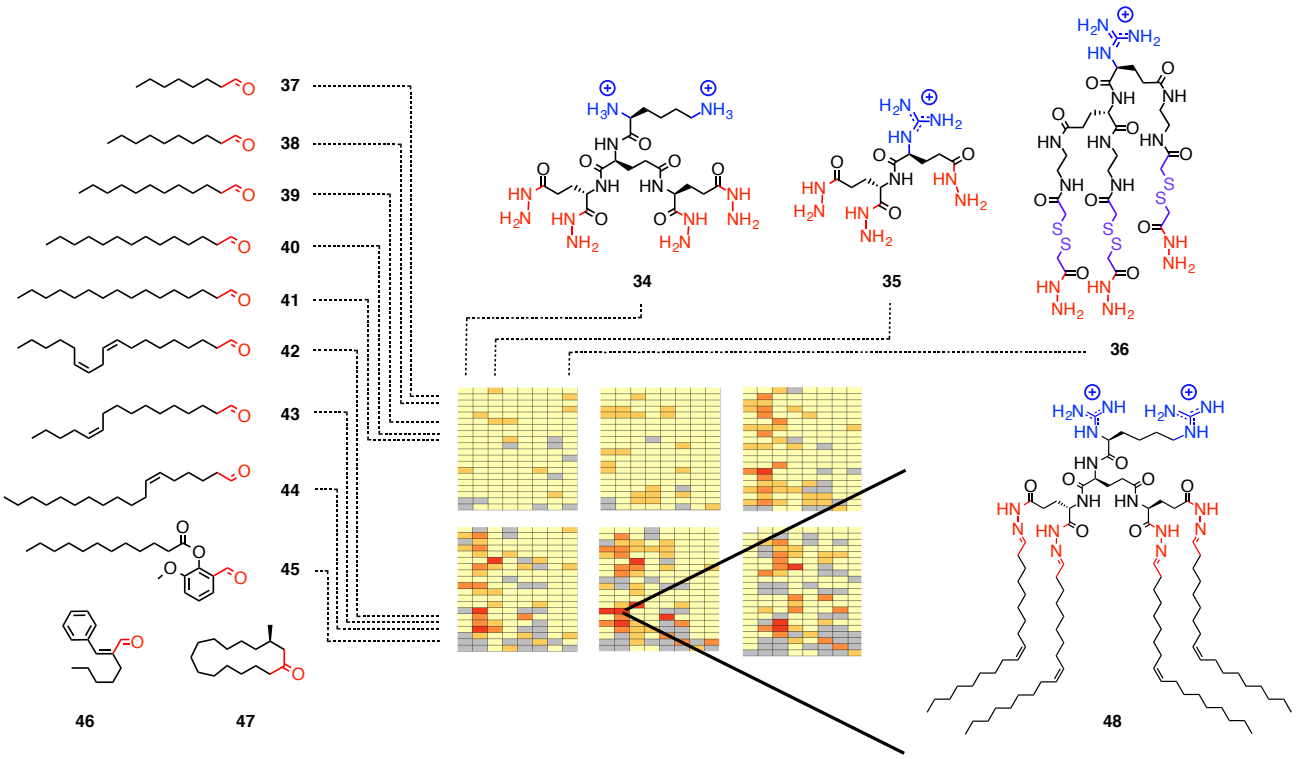

Fig. 13. Hydrazone formation explored for rapid access to large libraries of cationic amphiphiles composed of cationic heads such as **34-36** and hydrophobic tails such as **37-47**. In automated screens for siRNA delivery, amphiphile **48** emerged as best. Adapted from reference 76 with permission, © 2013 American Chemical Society.

According to the concept of counterion-mediated uptake, the large libraries of cationic amphiphiles generated for the construction of this artificial nose should contain potential candidates for the delivery of oligonucleotides into cells.[42] With predictions being impossible, an automated screening assay for the delivery of siRNA into HeLa cells was developed.[76] Some cationic peptide hydrazides **34-36** and hydrophobic aldehydes **37-47** used for the construction of the library are shown to illustrate the approach (Figure 13). We were hoping that pleasant odorants like jasmine aldehyde **46** or muscone **47** would emerge as useful for siRNA delivery, and that disulfide magic in **36** would help here as well. To our disappointment, robotic library screening identified

amphiphile **48** with four ordinary oleyl tails attached to a disulfide-free peptide mini-dendron with two guanidinium cations as best. However, uptake efficiencies found for **48** were impressive. Used as a single compound without further optimization, siRNA delivery with tetrahydrazone **48** was better than the commercial standard lipofectamine, also when applied to cell lines that are hard to transfect (human primary skin fibroblasts).[76] Uptake mechanism including possible contributions for hydrazone exchange are so far unknown, endocytosis is likely and hydrazone-mediated endosomal escape possible.

6. Summary and Outlook

This Feature Article reflects on lessons from established and emerging principles of supramolecular organic chemistry for cellular uptake. A counterion hopping mechanism is first introduced to explain not only the mode of action of cell-penetrating peptides but also the selectivity of synthetic ion channels and the voltage gating of neuronal potassium channels. Counterion hopping originates from proximity effects between intramolecular permanent charges. The understanding of counterion hopping of and on cell-penetrating peptides, i.e. the fundamental principles of supramolecular organic chemistry, is essential to improve their performance. An example is the addition of pyrenebutyrate to accelerate direct translocation across the membrane into the cytosol and thus overcome endosomal capture by endocytosis.[19]

Thiol-mediated uptake, currently emerging as most promising alternative to counterion-mediated uptake of cell-penetrating peptides, is another wonderful example of lessons from supramolecular organic chemistry applied to cellular uptake. Dynamic covalent chemistry, *en vogue* in several disciplines as winning strategy to combine the best of non-covalent and covalent chemistry,[62-64] is made to take place on cell surfaces and drive the concept of covalent uptake to the extreme. Namely, disulfide exchange with thiols on the cell surface covalently binds the transporter to the cell before uptake. After uptake in the cytosol, disulfide exchange with glutathione in the cytosol detaches and destroys the transporter to liberate the substrate and eliminate toxicity.

Compared to counterion hopping with cell-penetrating peptides, disulfide hopping during thiol-mediated uptake appears much more promising because it operates with covalent chemistry and does not need multiple charges, i.e., does not suffer from the notorious unreliability of dynamic polyion-counterion complexes. The understanding of the fundamental principles of supramolecular organic chemistry is once again essential to harness thiol-mediated uptake: Activities increase with ring tension - what an "explosive," "dynamite" entry into cells![49] Concerning the broader perspectives, many intriguing questions invite for answers: Is thiol-mediated uptake under tension generally applicable to different cells? Is it compatible with the delivery of larger substrates such as proteins, DNA, liposomes, polymersomes, quantum dots, and so on? Most importantly, can ring tension be amplified by multivalency? And finally the key question: How does it really work? Does thiol-mediated uptake under tension occur through a new pathway? Dependence on temperature, independence on endocytosis inhibitors and poor co-localization indicate that the answer could be yes,[49] pull-down proteomics will be needed to clarify if and which proteins are involved.

Compared to the excitement with thiol-mediated uptake under tension, complementary approaches with dynamic covalent boronic esters, hydrazone exchange and even counterion exchange appear less promising today. However, they all together illustrate that input from chemistry is essential to address the really major challenges in biology and beyond, cellular uptake being one of them.

As stated throughout the text, this Feature Article focuses on lessons from supramolecular organic chemistry for cellular uptake. Many other important aspects related to this most complex topic were thus naturally beyond the scope of the article. For instance, with direct translocation as energy-independent pathway of preference in most studies, other uptake mechanisms are not further elaborated. The different types of endocytosis, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, phagocytosis or receptor-mediated endocytosis are

mentioned but not discussed in detail.[78] Moreover, routine experiments to identify these known pathways, covering energy dependence, specific inhibitors and colocalization probes, are not described in detail.[19,46,49,53,54,78] Differences between different cell types are not explicitly covered either.[19,53,76,78] These omissions have not been made because the concerned topics would be less important. In the contrary, they have already been described in many excellent reviews.[1-5,44,53,78] Important contributions from supramolecular organic chemistry to cellular uptake have not been reviewed, at least not with similarly high frequency. One objective of this Feature Article is to attract attention to these arguably less recognized approaches from chemistry and their enabling power for conceptual innovation at the most fundamental level.

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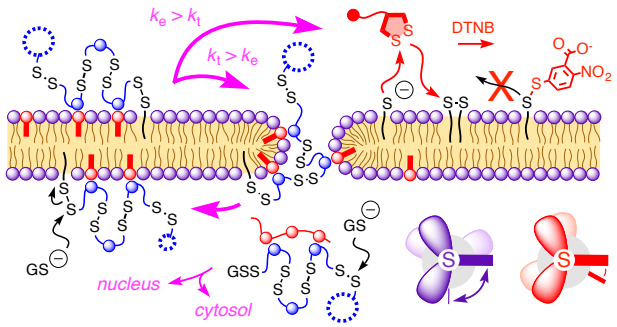
TOC graphic



This Feature Article summarizes contemporary supramolecular chemistry approaches to find conceptually innovative ways to enter into cells.