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Article

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BANG, Eun Kyoung, *et al*.

Abstract

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Reference

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Poly(disulfide)s

Eun-Kyoung Bang, Marco Lista, Giuseppe Sforazzini, Naomi Sakai and Stefan Matile*

Don't forget poly(disulfide)s. There is a rich literature pointing out the advantages of the dynamic nature of single disulfide bridges to explore self-sorting, biomolecular engineering, biomembrane analysis, and so on. Disulfide bonds between polymer chains are essential for protein folding, materials properties and the stabilization of various supramolecular architectures. However, poly(disulfide)s with disulfide bonds in the main chain are rarely used today to create interesting structures or functions. To attract attention and outline scope and limitations of poly(disulfide)s to build modern supramolecular systems, the rather eclectic recent literature on the topic is summarized. The review is moving from fascinating basic studies including photoinduced metathesis, polycatenanes and polyrotaxanes to applications in biosupramolecular systems such as micelles, membranes, tubes, gels, carriers, pores, sensors, catalysts and photosystems.

Introduction

Poly(disulfide)s are dynamic polymers with disulfide repeats in their main chain.[1] The disulfide bond is a dynamic covalent bond, which can be easily cleaved and reformed on demand, but is stronger than the non-covalent interactions present in supramolecular polymers.[2] The dynamic nature of disulfides accounts for unique properties in poly(disulfide)s such as adaptability, stress resistance, self-repair or degradability in response to physical or chemical stimulation.

Without disulfides in their main chain, neither proteins nor vulcanized rubber are poly(disulfide)s. Proteins are polyamides with disulfide bridges cross-linking polymer backbones (Figure 1). The reversibility of thiol-disulfide exchange is essential to direct proteins to fold into their active conformation **1**. On the macroscopic level, the reversibility of disulfide reduction and thiol oxidation is used by the hair dresser to produce curls in hair by unfolding protein (keratin) into the reduced form **2** followed by refolding into reshaped oxidized form **1**.

Rubber **3** is a poly(isoprene). During vulcanization, rubber is exposed to sulfur **4**, and oligosulfides are inserted to bridge the main poly(isoprene) chains. Because it is less sticky and has improved mechanical properties, vulcanized rubber **5** is used to make tires, shoe soles, bowling balls, and so on.

Although the two arguably best known disulfide-containing polymers, proteins and rubbers, are not poly(disulfide)s but polymers with di- or oligosulfide crosslinks, their properties reveal much of the advantages of poly(disulfide)s. These advantages are centered around the dynamic covalent bonds that can form and break reversibly in response to redox chemistry, nucleophilic substitution, light or mechanical force. Many other examples exist for the crosslinking of polymers with disulfide bridges, including polymer micelles, nanoparticles, and so on.[3,4] For poly(disulfide)s with disulfides as repeat units in the main chain, similarly attractive advantages such as resistance to environmental degradation, good adhesion, flexural strength or thermal curing have been recognized early on in academia and industry.[1] However, today's applications of disulfides in research on functional biosupramolecular systems involve mainly monomeric disulfides, whereas examples for oligo(disulfide)s and particularly poly(disulfide)s are rare, with the noteworthy exception of gene delivery (see below).

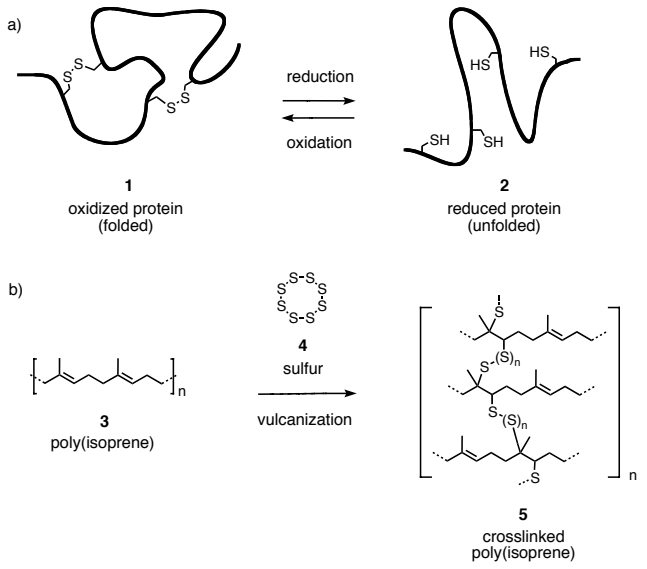

Figure 1. Dynamic disulfide and trisulfide bridges in (a) proteins and (b) vulcanized rubber can be broken and reformed with redox chemistry and introduced by vulcanization of rubber with sulfur.

We have recently experienced the unique advantages of poly(disulfide)s in our search for simple, user-friendly approaches to sophisticated multicomponent photosystems and were surprised to realize how little poly(disulfide)s are used in other groups. Don't forget poly(disulfide)s! With the following review, we try to stimulate interest in this unique family. We first recapitulate briefly the widespread use of monomeric disulfides in the field. Then we move on over

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oligo(disulfide)s to the main part on poly(disulfide)s, summarizing a rather eclectic but fascinating collection of examples from basic studies on synthesis, solubility, metathesis, polycatenanes and polyrotaxanes toward applications in functional micelles, membranes, tubes, pores, photosystems, catalysts and gene carriers.

Disulfide Monomers

The disulfide bond is one of the most intriguing dynamic covalent bonds. On the monomer level, thiol-disulfide exchange is extensively used to covalently capture systems such as host-guest, ligand-receptor or enzyme-substrate complexes.[5-7] Thiol-disulfide exchange is ideal to study self-sorting (Figure 2a).[8] In general, two possible partners are linked together either as heterodimer **6** or as homodimers **7** and **8**. Then, disulfide exchange is initiated by catalytic amounts of thiolates, and the concentration of hetero- and homodimers is determined at equilibrium. This strategy has been most successfully applied to analyze the organization of lipids in biomembranes by nearest-neighbor recognition.[9] Protein-protein interactions have been dissected by equilibrating disulfide terminated helix bundles or β-sheet dimers.[10]

Another example for the usefulness of dynamic disulfide chemistry on the monomer level is cysteine scanning to, e.g., determine amino acid residues that are exposed to the interior of transmembrane ion channels and pores.[11] In this approach, single cysteines are converted in situ into disulfides, and the change in conductance is measured. In the light of the abundant and successful use of disulfide exchange on the monomer level, it is surprising to realize that oligo(disulfide)s and poly(disulfide)s are used only occasionally for similar purposes.

Oligo(disulfide)s

The synthesis of macrocyclic oligo(disulfide)s is attracting scientific attention since long time because of their importance as starting materials in ring-opening disulfide polymerization (see below). More recently, oligo(disulfide)s received some attention in the context of dynamic combinatorial chemistry (DCC).[5,6] In this approach, dithiols are oxidized and equilibrated to produce a dynamic combinatorial mixture or library (DCL). This mixture is then exposed to external stimulation to amplify certain members of the library. Highlights from this approach include the synthesis of oligo(disulfide) catenanes **9**-**11** (Figure 2b).[12,13] Another milestone is the identification of macrocyclic oligo(disulfide) receptors for ephedrine from a DCL of more than 9000 components.[14] The amplified tetra(disulfide) receptors reached dissociation constants up to 67 μM in water. These values are among the best obtained for ephedrine.

Selected macrocyclic oligo(disulfide)s such as **12** or **13** used for polymer synthesis or obtained by depolymerization of poly(disulfide)s such as **14** by thiol-disulfide exchange with dithiols such as **15** will appear in the next chapters on the main topic of this review (Figure 3).

Poly(disulfide)s

After this short introduction on disulfide crosslinking as well as monomeric and oligomeric disulfides, a more comprehensive description on current progress with poly(disulfide)s will be delivered in the following sections. Recent synthetic approaches will be covered first, before moving on to rotaxanes, catenanes, vesicles, micelles and tubes. These chapters on structure will be followed by functional poly(disulfide)s, covering photosystems, pores, catalysis as well as the most developed field of gene carriers.

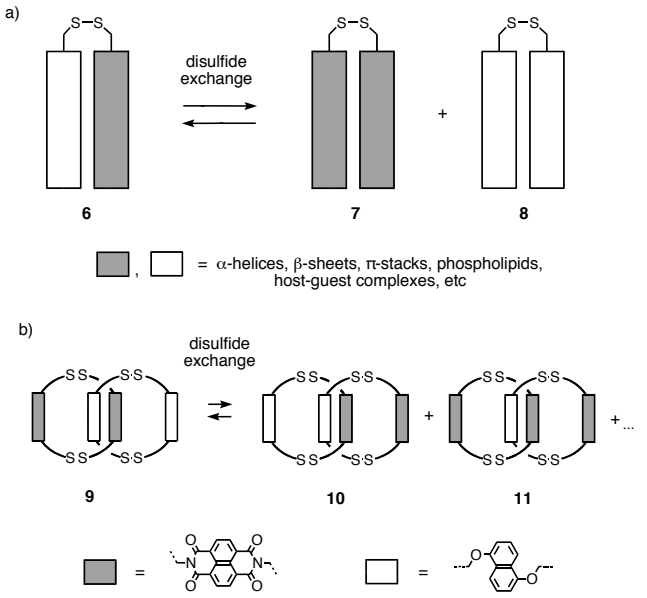

Figure 2. (a) Thiol-disulfide exchange between disulfide monomers is used extensively to study molecular recognition and self-sorting, (b) Disulfide exchange between oligo(disulfide)s has been used in dynamic combinatorial libraries to synthesize catenanes.

Synthesis

The synthesis of poly(disulfide)s has been investigated since the second half of the 1940's.[1] Three general routes exist. The first is ring-opening thiol-disulfide exchange polymerization of disulfide monomers or larger oligo(disulfide)s. The second is oxidative polymerization of dithiols. The third approach uses unrelated polymerization methods such as conjugate addition to polymerize monomeric disulfides. Ring-opening disulfide metathesis can occur with thiolate initiators or with heat. The former proceeds by nucleophilic substitution with sulfur acting as both electrophile and nucleophile, the latter via sulfenyl radicals.

Alkyl and aryl poly(disulfide)s usually are adhesive and thermally curable solids with high flexural strength. To further elaborate on these properties, the synthesis of poly(disulfide)s without side products has attracted some also more recent interest. Aromatic poly(disulfide)s have been particularly problematic because of their high melt viscosity. To synthesize these sturdy solids without side products, ring-opening polymerization of cyclic aromatic oligo(disulfides) such as **12** emerged as particularly attractive approach because it can be initiated by heat only (Figure 3).[15-19]

Homolytic disulfide cleavage into sulfur radicals at elevated temperature initiates the radical polymerization into poly(disulfides) **14**. Several other aromatic oligo(disulfides) such as **13** have been prepared to study ring-opening radical polymerization. In the presence of dihalogenated arenes such as biphenyl **16**, ring opening oligo(disulfide) polymerization irreversibly affords the corresponding poly(sulfides) **17**.

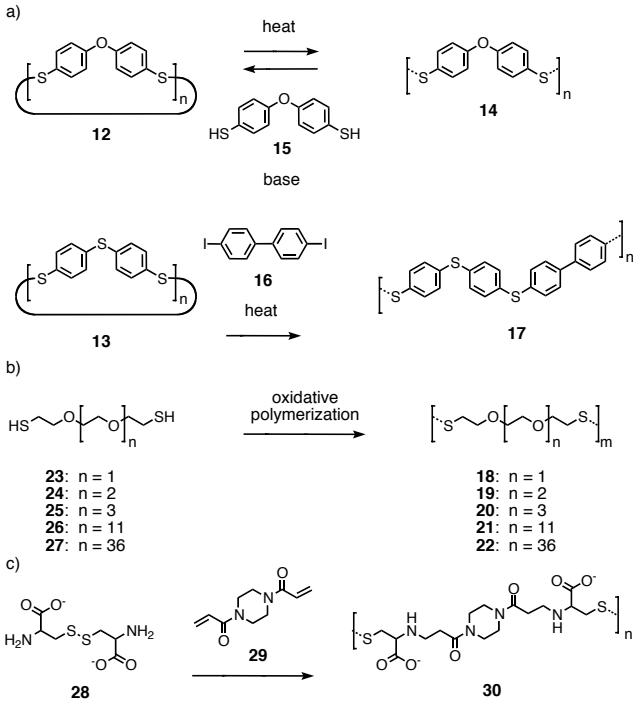

Figure 3. Polymerization of a) cyclic oligo(disulfide)s, b) linear dithiols and c) linear disulfides into poly(disulfide)s.

Several strategies to synthesize poly(disulfides) with improved solubility have been reported. Polyethylene glycols (PEG), appreciated as universal solubilizer, has been incorporated in poly(disulfide)s **18**-**22** (Fig. 3b).[20,21] Synthesized from dithiols **23**-**27** in the presence of first base and then hydrogen peroxide, polymerization proceed via macrocyclic intermediates and living ring-opening polymerization controlled by sulfur radical recombination. Extension with ethanedithiol and depolymerization with DTT demonstrated the living and reversible nature of the polymerization (DTT: (*S*,*S*)-dithiothreitol). Poly(disulfide) **18** obtained under these conditions had high molecular weight up to M_n = 250000, polydispersity as low as M_w/M_n = 1.15, and was soluble in THF (M_w: Weight-average molecular weight; M_n: Number-average molecular weight). DSC revealed rubbery polymers with a glass temperature T_g = -50 ºC.

Poly(disulfide)s **19**-**22** with increasing PEG domains were obtained in DMSO at room temperature. M_n = 19000 was observed with 20% DMSO, increasing DMSO concentrations gave shorter polymers. The short poly(disulfide)s depolymerized within one hour in the presence of 5 mM glutathione.

An example for the synthesis of poly(disulfide)s without thiol oxidation and thiol-disulfide exchange is given with cystine **28** (Fig. 3c).[22] Michael addition to the acceptors in diamide **29** afforded polydisulfide **30** in up to 60% conversion. Size exclusion chromatography revealed polymers with M_n = 11900-51800, depending on the Michael acceptors used. Depolymerization in water followed the kinetics of other poly(amido amine)s but was dramatically accelerated by reducing agents. Water soluble poly(disulfides) **30** and **18**-**22** were non-toxic. The synthesis of more, usually less thoroughly characterized, mostly cationic poly(disulfide)s will be described in the chapter on gene delivery.

The different methods available for poly(disulfide) synthesis offer different advantages. Oxidative and thiol-disulfide exchange polymerization are mild and tolerate the presence of many functional groups, whereas disulfide exchange polymerization initiated by heat requires harsher conditions. All three approaches are reversible and thus compatible with reactivation, self-repair, recycling and responsiveness to templates. Disadvantages include in all cases the occurrence of macrocyclic side products, originating from "backbiting," that is intramolecular disulfide exchange, and thus difficult to avoid.

Ring-opening thiol-disulfide exchange polymerization is arguably the most attractive method because the process is directional and requires neither oxidative conditions nor heat. Initiators and terminators can be used for control and analysis to e.g., suppress macrocyclic side products, polymerize from surfaces (electrodes, pores), introduce labels or create sequences in multicomponent co-polymers (see below). Processes unrelated to disulfide chemistry are interesting to bypass disadvantages during polymerization (e.g., macrocyclization, reversibility) and benefit from the advantages afterwards. This approach has been strongly favored in the context of gene delivery with poly(disulfide)s (see below).

Rotaxanes, catenanes and photoinduced metathesis

Although ring-opening polymerization of cyclic disulfide monomers has been reported in many variations, structural studies of the obtained poly(disulfide)s are relatively rare. In a series of recent studies, the Endo group collected evidence in support of polycatenane structures.[23-28] The polymerization of bulk 1,2-dithiane **31** was achieved in a sealed tube at 80 ºC (Figure 4). The poly(disulfide)s **32** with M_n = 83800 showed no resonances for terminal groups in the NMR spectra. Linear control polymers **33** obtained with dithiol or monothiol terminators showed the corresponding terminator signals in their NMR spectra. This suggested that poly(disulfide)s **32** are cyclic.

The unique materials properties of poly(disulfide)s **32** further suggested that the macrocycles are mechanically interlocked. Evidence in support of polycatenane structure includes independence of the glass temperature on polymer length, a rubber plateau of the storage modulus above melting in dynamic viscoelastic analysis, and reversible stress resistance up to 3000% elongation. None of these properties were seen with linear control **33** and cyclic poly(oxyethylene)

without polycatenane structure. Photoinduced disulfide metathesis of poly(disulfide) **32** caused a rapid loss in molecular weight until $M_W \sim 5000$. This value was identical with the molecular weight entanglement obtained from the modulus strength of the rubbery plateau in dynamic viscoelastic analysis. This suggested that photoinduced disulfide metathesis in dilute solution converts the polycatenane structure of poly(disulfide) **32** into disconnected cyclic polymer **34** of naturally much lower molecular weight. The reproducibly obtained $M_W \sim 5000$ implied the presence of macrocycles containing about 40 monomers in average. Copolymers made from dithiane **31** and lipoic acid **35** showed similar polycatenane structure. Addition of divalent metal cations produced insoluble material with, however, preserved and detectable melting temperature.

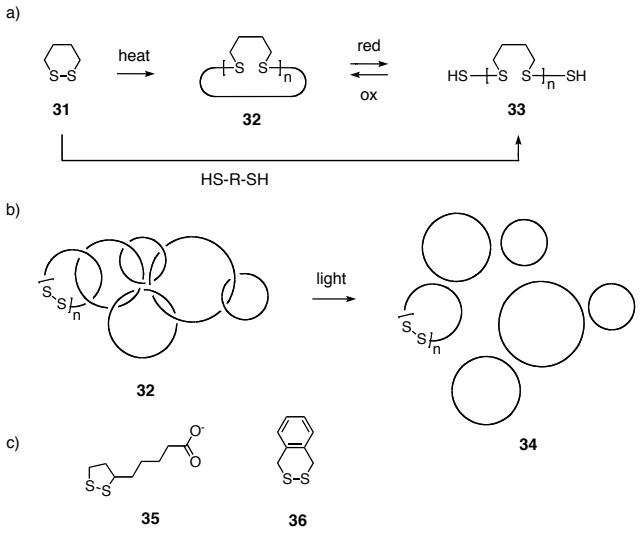

Figure 4. Linear poly(disulfide)s, cyclic poly(disulfide)s as well as polycatenanes. a) Formation of polycatenanes with heat and reductive opening into linear polymers. b) Transformation of polycatenanes into cyclic polymers with light. c) Alternative monomer substrates.

The generality of polycatenane formation from thermal ring-opening disulfide metathesis polymerization was confirmed recently with aromatic disulfide **36**. In these studies, polycatenane formation is favored because of the solvent- and terminator-free reaction conditions. Under these conditions, termination occurs mostly by macrocyclization, and the maximized proximity of the polymer chains maximizes the probability to form mechanically interlocked macrocycles.

Photoinduced disulfide metathesis was recently studied in depth on the polymer level with the presumably linear polyester **37** (Figure 5).[29] Upon UV irradiation of a drop-casted poly(disulfide) film with a 400 W high-pressure mercury lamp, the polydispersity increased from $M_w/M_n = 1.2$ to $M_w/M_n = 2.0$, whereas the average polymer length remained constant around $M_n = 16000$. Separation of longer polymers **38** with $M_n = 63800$ and shorter polymers **39** and $M_n = 8700$ provided access to corroborative evidence for photoinduced poly(disulfide) metathesis. Within one hour of photoirradiation, the two peaks in the size exclusion chromatogram merged into one peak in the middle. The $M_n = 16000$ was that of the original poly(disulfide) **37**.

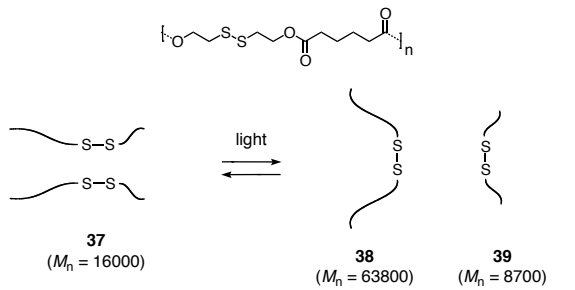

Figure 5. Photoinduced disulfide metathesis in poly(disulfide) films.

Polyrotaxane poly(disulfide)s **40** have been prepared by slipping disulfide **41** and crown ethers **42** into each other (Figure 6).[30] Equilibrium was reached within one week at room temperature. The formation of mechanical bonds is driven by the recognition of ammonium cations within the crown ethers. High molecular weight polymers of $M_w = 28000$ were isolated in 64% yield by gel permeation chromatography. Poly(disulfide) formation was demonstrated by depolymerization with reducing agents.

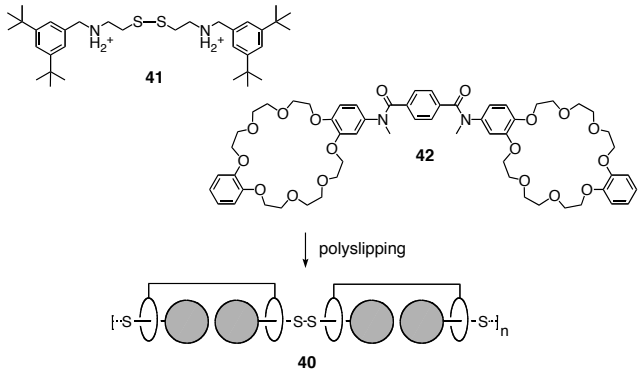

Figure 6. Poly(disulfide) polyrotaxanes.

Vesicles, micelles and tubes

The first poly(disulfide) vesicle **43** was prepared from 1,2-bis(mercaptoundecanoyl)-*sn*-glycero-3-phosphatidylcholine **44** by oxidative polymerization of the linear dithiols (Figure 7).[31] Many variations of the theme followed quickly.[31-35] Phospholipid **45** has elongated hexadecanoyl tails, **46** has the polymerizing thiols moved from the middle toward the surface of the lipid bilayer membrane. Phospholipid **47** has the strained cyclic disulfides from lipoic acid **35** in place of thiols to polymerize vesicles by thiol-disulfide exchange rather than by oxidation. Lipids **48** and **49** are the singly derivatized analogs.

Poly(disulfide) vesicles were obtained by polymerizing preformed vesicles. The obtained polymerized vesicles were generally longer lived than unpolymerized ones. Polymerized vesicles obtained from thiol-disulfide exchange were resistant to lysis by the detergent sodium dodecylsulfate (SDS), whereas vesicles obtained by oxidative polymerization were

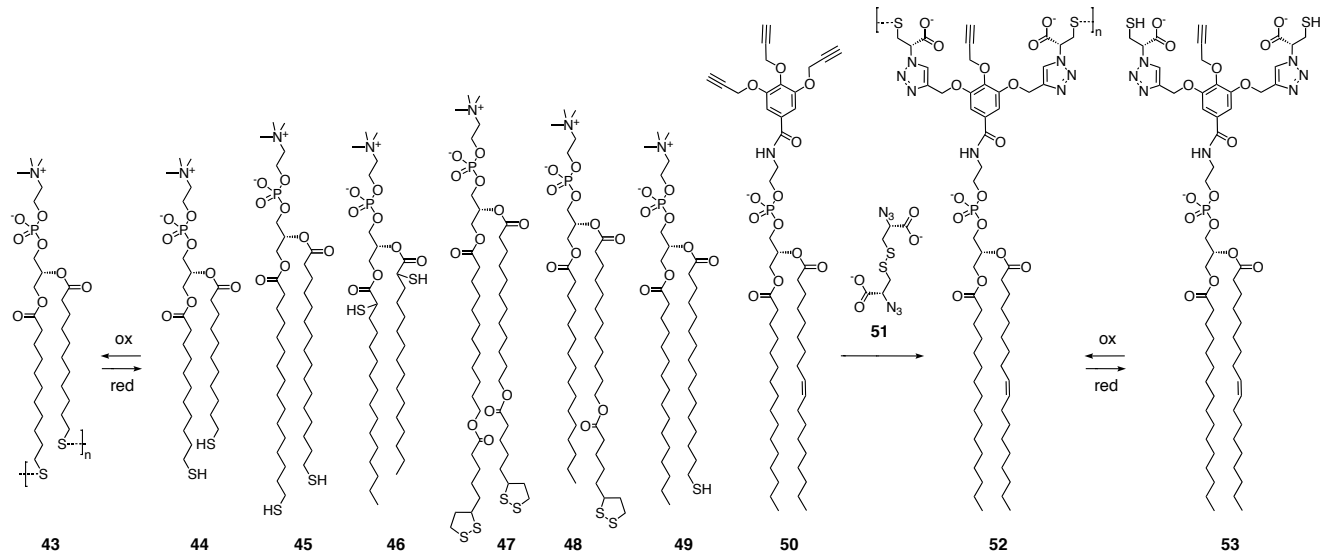

Figure 7. Poly(disulfide) vesicles such as **43** and **52** obtained by vesicle polymerization with the three main methods, that is disulfide metathesis, thiol oxidation, and unrelated processes.

not. According to the dithiolane band in the UV spectra, polymerization of vesicles from **47** initiated by DTT or thiol **49** proceeded to completion. Polymerization and depolymerization with reducing agents could also be followed by monitoring disappearance and reappearance of the monomers by thin layer chromatography. Negative-stain transmission and freeze-fracture electron micrographs revealed intact unilamellar vesicles. Polymerization generally reduced the leakage of [^{14}C]glucose. Permeabilities could be fine-tuned in mixed vesicles and partial, switchable polymerization with precisely administered pH pulses from 6.4 (unreactive) to 8.4 (reactive) and back.

More recent contributions to poly(disulfide) vesicles focused on polymerization at the surface of the bilayer.[36] Polymerization of phospholipids **50** with diazide **51** was achieved not by disulfide chemistry but by Cu-catalyzed cycloaddition. Depolymerization with DTT gave mainly dithiol **53** together with singly but not triply derivatized analogs. Mixed vesicles with DOPC became increasingly leaky for carboxyfluorescein (CF) in the presence of increasing mole fractions of **50** (DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine). In pure vesicles of **50**, all content was lost in 20 h. Polymerization efficiently inhibited CF leakage, reduction with DTT reactivated CF leakage. Leakage decreased with decreasing pH.

The cone-shaped peptide amphiphile **54** was designed to self-assemble into cylindrical micelle fibers below pH 4 but disassemble in neutral water (Figure 8).[37] Micelle cross-linking by oxidation of the central cysteines with 10 mM iodine produced poly(disulfide) fibers that were also stable at higher pH but could be rapidly disassembled with reducing agents. Polymerization occurred without visible change of the global fiber architecture. The phosphorylated serines were placed at the surface of the fiber to initiate the mineralization of hydroxyapatite with the crystallographic c axes aligned with the long axes of the fibers. This directionality was of interest because it is the same as that observed with collagen fibrils in bone. The RGD terminus of the amphiphile **54** was taken from the collagen-associated protein fibronectin to enable integrin-mediated cell adhesion (RGD: Arginine (Arg) - glycine (Gly) – aspartate (Asp)).

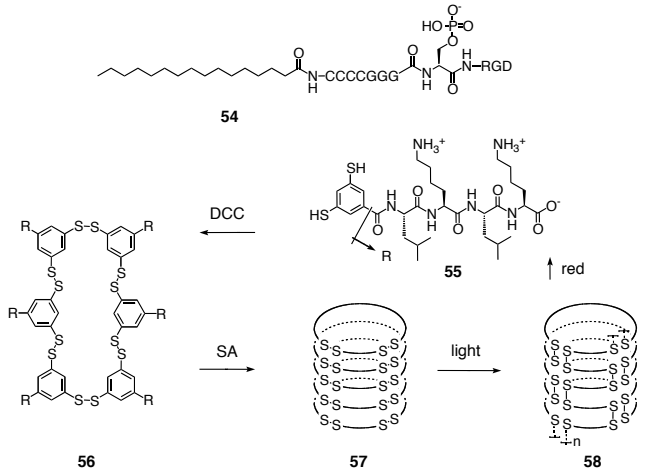

Figure 8. Poly(disulfide) micelles and tubes. DCC: Dynamic combinatorial chemistry; SA: self-assembly.

Oxidative polymerization of dithiol **55** produces a large mixture of products.[38] However, application of the principles of DCC allowed amplification of the cyclic hexamer **56**. This amplification was possible because the macrocycle **56** is the only oligomer that self-assembles into mechanosensitive tubes **57** with parallel, amphiphilic β-sheets on their outer surface. Irradiation of the tubes **57** for three days at 365 nm with a 8 W UV lamp gave a hydrogel.[39] Photoinduced disulfide metathesis as described above was identified to account for gelation, converting the fragile stacks of oligo(disulfide)s **57** into the stable poly(disulfide)s **58**. According to cryo-TEM

and CD spectroscopy, disulfide metathesis occurred without changes in the global architecture of the stacks (TEM: Transmission electron microscopy). Treatment with 10 equivalents DTT afforded the monomeric dithiol starting material **55**. Gel electrophoresis demonstrated that the poly(disulfide)s have $M_w > 30000$.

Photosystems

Ring-opening disulfide metathesis emerged as the ideal reaction to realize self-organizing surface-initiated polymerization (SOSIP, Figure 9).[40-42] To add directionality to functional surface architectures, it will be unavoidable to learn how to construct them directly on the solid substrates. This is challenging because self-assembly procedures require extensive synthetic efforts on the monomer level to reach appreciable sophistication, whereas classical polymer brushes tend to stop growing as soon as more demanding systems are involved. A broad screen of meaningful alternative revealed SOSIP with disulfide exchange polymerization as methodology of choice for facile access to complex surface architectures.

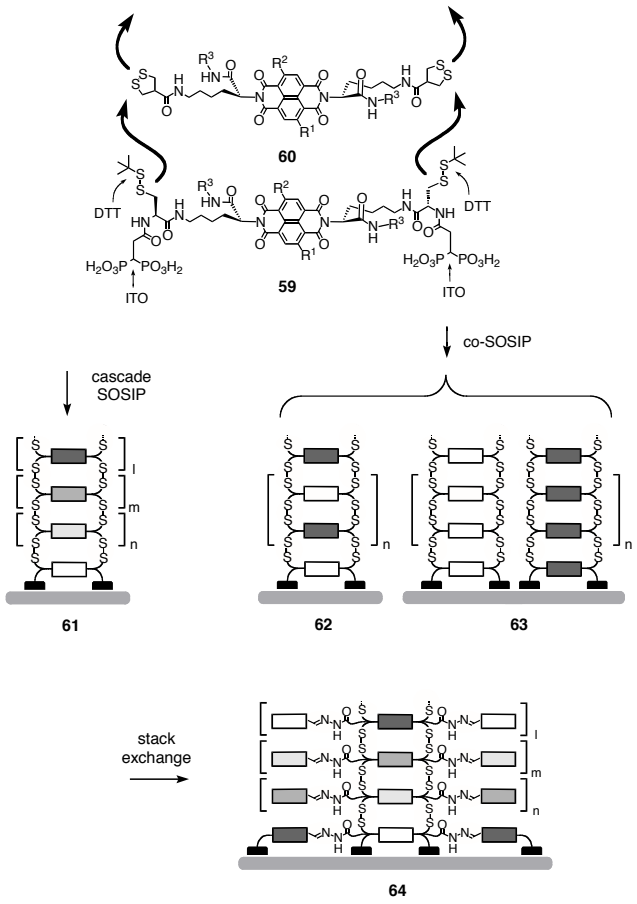

Figure 9. Poly(disulfide) photosystems obtained by SOSIP (self-organizing surface-initiated polymerization) methodology: Gradients by cascade-SOSIP, double-channel architectures by uniform self-sorting during co-SOSIP,

For SOSIP, initiators such as **59** are bound to indium tin oxide (ITO) surfaces and activated with DTT and base (Figure 9).[40] Propagators such as **60** are then expected to recognize the initiators on the surface by π-stacking of the aromatic cores and hydrogen bonding within the surrounding amide network. This molecular recognition places the strained disulfide of asparagusic acid directly on top of the thiolate nucleophiles generated on the surface. Ring-opening disulfide exchange will covalently capture the propagator on top of the initiator and produce at the same time new thiolates on the surface of the bilayer for ongoing polymerization. SOSIP was found to be reversible as well as reactivatable with DTT, and experimental evidence in support of self-repair during SOSIP was secured.[41] SOSIP produces oriented photosystems with smooth surfaces that are much more active than disordered controls.

With SOSIP, it became very straightforward to engineer oriented redox gradients into single-channel photosystems such as **61**.[40] The substrate is simply dipped into different propagator solutions in the desired order to obtain the designed gradient by cascade SOSIP. To transport holes and electrons in different co-axial molecular channels, co-SOSIP of two different monomers was considered.[41] Aromatics with different π-acidity but almost identical structure were found to undergo alternate (or social) self-sorting into inactive photosystems **62**. However, already very minor structural differences between two propagators were sufficient to achieve uniform (or narcissistic) self-sorting into highly active double-channel photosystems **63**. Compared to single-channel analogs, activities increase up to 40-times. With large structural differences between propagators, uniform lateral self-sorting of double-channel photosystems into inactive microdomains was observed. Whereas propagator concentrations are fixed during co-SOSIP, initiator concentrations are freely variable. This flexibility was used to build photosystems of freely variable composition by surface-templated co-SOSIP.[41]

Post-SOSIP stack exchange was introduced to build covalent double-channel photosystems **64** with antiparallel redox gradients.[42] In brief, a central stack with an oriented gradient was prepared first by cascade SOSIP. Chemoorthogonal hydrazone exchange was used next to drill giant pores into SOSIP architectures and fill them with new π-stacks of free choice. These new stacks were then partially removed and replaced by the respective partners, donors or acceptors, to build the oriented, antiparallel gradients. The poly(disulfide) backbone of SOSIP photosystem was stable under these conditions.

Conjugated poly(disulfide)s **65** with high molecular weight ($M_W > 1000000$) have been prepared by oxidation of triazine **66** (Figure 10).[43] Inspired by light-harvesting complexes in algae, the disulfides in this interesting 2D polymer were expected to lead to high charge mobility. The conjugated nature of the amorphous solid was confirmed by a broad band with a maximum around 400 nm and a tail up to 650 nm or 1.91 eV in the diffuse reflectance spectrum. Mott-Schottky plots demonstrated n-semiconducting properties in the dark and revealed a flatband potential at -3.8 eV. This was consistent with the reduction of water (-4.6 eV), whereas the obtained -5.7 eV were considered as too high for photocatalytic water oxidation (-5.9 eV).

Hydrogen evolution occurred upon irradiation in neutral water. Addition of Ce(III)/Ce(IV) was beneficial to accept holes and capture OH^- as CeO_2, thus preventing photocorrosion of the polymer to increase turnover. Occurrence of the latter process was confirmed by a decrease in pH. Doping with Ru or carbon nanotubes as co-catalysts further increased efficiency up to 8.1 μmol/h H_2 for at least 18 h. The wavelength dependence of the overall quantum efficiency, maximal at 0.20% with 280-400 nm light, confirmed that the conjugated poly(disulfide) accounts for activity.

Poor photocurrent generation was attributed to insufficient contact to ITO surfaces. Doping with ruthenium or carbon nanotubes increased photocurrents and quenched photoluminescence at 550 nm (excitation at 400 nm). Both effects indicated contributions to photoinduced charge separation and charge translocation.

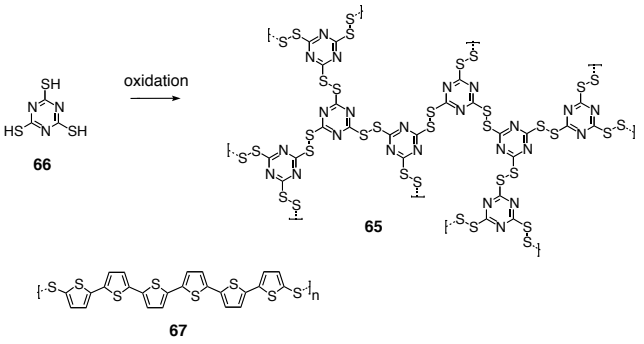

Figure 10. Artificial photosystems with fully or partially conjugated poly(disulfide)s.

Poly(disulfide) **67** was prepared as part of a series of polymers with alternating sexithiophene sequences and bridge moieties of varied conjugation.[44] The disulfide was of interest because of a possible participation of the electron-rich S-S moiety to the conjugated path. However, the conductivities of p-doped **67** suggested that conduction proceeds via a direct hopping between stacked sexithiophene moieties of cofacial polymer chains. A better conductivity was observed for polymer with a simple double bond as bridge, presenting itself as the most efficient connection between coplanar sexithiophene moieties.

Pores

Stochastic sensing with pores is a unique method to detect label-free, "native" chemistry in action on the single-molecule level. In brief, it is fairly straightforward to measure the currents flowing across single ion channels and pores. The binding of analytes or reactions taking place within pores can thus be detected as changes in the conductance of this single pore. To detect disulfide polymerization on the single-molecule level, pore sensors were equipped with a single cysteine in the ion-transporting pathway (**68**, n = 0; Figure 11).[45] Reaction of this thiol with an activated 5,5'dithiobis(2-nitrobenzoic acid) probe **69** gave pore **70**, n = 0. Because of this bulky and charged group, the conductance of this pore was clearly reduced (Figure 11). Thiol/disulfide exchange with (mercaptoethyl)ether **71** gave pore **68**, n = 1, with a regenerated, free thiol. The decrease in bulk and charge within pore **68**, n = 1, was correctly reflected as an increase in conductance (Figure 11). Continuing polymerization resulted in a steady decrease in conductance until complete block was reached with n = 8.

The lifetime of the individual oligomers decreased slightly with increasing poly(disulfide) length. Characteristic values moved from 18 to 3.3 seconds for pore **68** with free thiols at the terminus and 3.3 to 0.21 seconds for pore **70** with activated disulfides at the terminus. This suggested that polymer growth experienced little environmental hindrance, perhaps becoming a bit more accessible with increasing length. The movements of single poly(disulfide) were detectable as extremely short-lived, transient blocks with lifetimes below detection limit. During propagation, the poly(disulfide) was occasionally broken, presumably by intrachain cyclization or internal disulfide exchange with **71**.

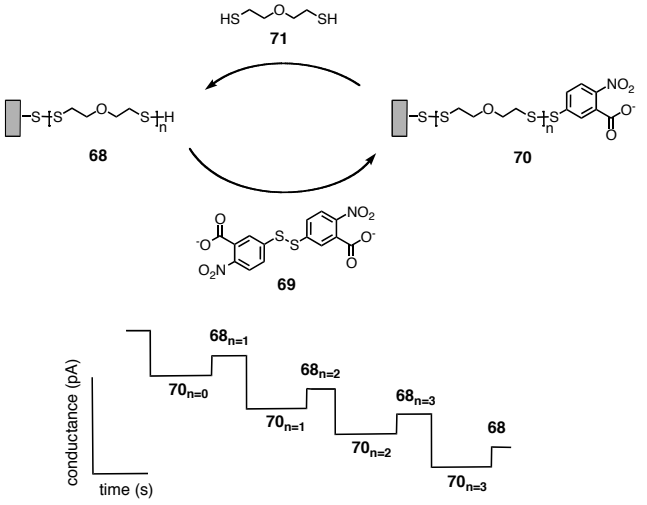

Figure 11. Detection of the polymerization of poly(disulfide)s within pores on the single-molecule level.

The detection of poly(disulfide) formation on the single-molecule level is complementary to research focusing on the detection of protein unfolding on the single-molecule level.[46] In these much more common experiments, folded proteins such as **72** are bound with one terminus to a surface and with the other terminus to the tip of an force-clamp microscope (Figure 12). The opening of single disulfide bonds in response to pulling is then seen as an instantaneous elongation of the partially unfolded protein by a few nanometers.

A recent milestone paper describes the detailed analysis of the unfolding of protein **72** with two disulfide bridges (Figure 12).[46] Breaking of the first disulfide bridge by force or assisted by thiol-disulfide exchange with free cysteines yielded an elongation by 4 nm that could be assigned to the partially unfolded protein **73**. At this point, intramolecular disulfide exchange to **74** or **75** can compete with full unfolding into **76**. Statistical analysis of the single-molecule kinetics revealed that without additional free cysteine in solution, the formation of **74** is preferred (Figure 12a), whereas additional cysteines push for direct full unfolding.

From dwell time analysis, the rate constants of intramolecular thiol-disulfide exchange could be determined for the first time. The obtained values are in the range of intracellular disulfide reduction by glutathione and thus biologically relevant and in need of enzymatic control.

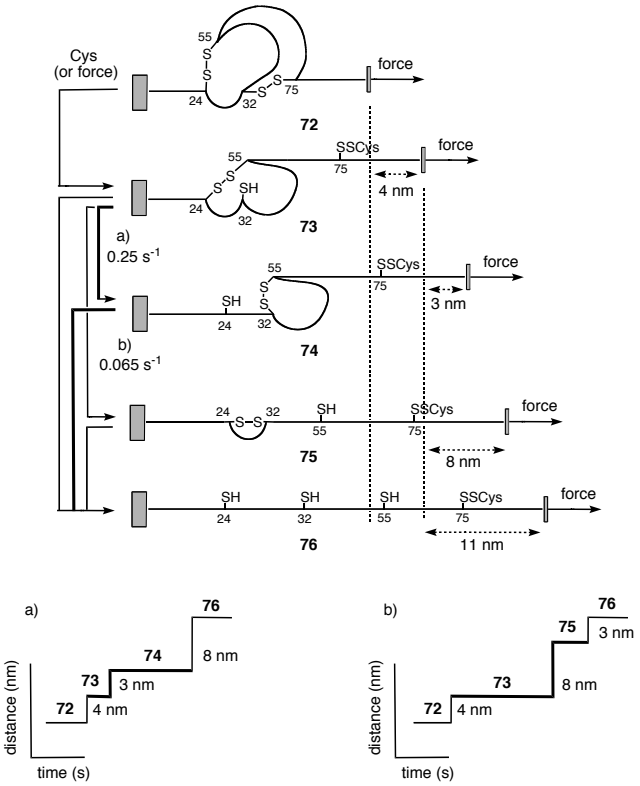

Figure 12. Detection of protein unfolding pathways on the single-molecule level by force clamp spectroscopy. a) Favored thiol-disulfide exchange to **74**. b) Disfavored thiod-disulfide exchange to **75**.

Catalysis

Several poly(disulfide)s have been used in catalytic systems. However, they mostly served to produce polymer effects, disulfide chemistry was presumably not involved in catalysis. Disulfide **77**, obtained by oxidative polymerization of dithiol **78**, is an artificial DNase (deoxyribonulcease) with roughly doubled activity compared to monomeric 1,4,7,10-tetraazacyclododecane or "cyclen" (Figure 13).[47] This polymer effect was explained with improved binding to DNA duplexes. The melting temperature of calf-thymus DNA at 73.5 ºC increased by 3.4 ºC in the presence of monomeric and by 4.0 ºC in the presence of polymeric catalyst.

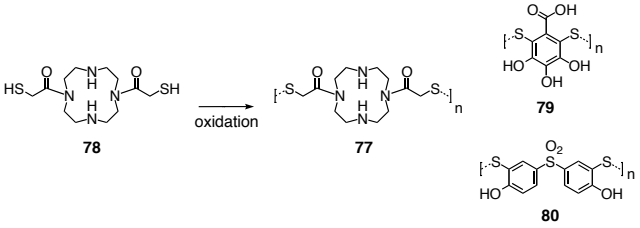

Figure 13. Recent poly(disulfide)s involved in catalysis.

Poly(disulfide)s such as **79** or **80** modulate the activity of peroxidase to oxidize amines.[48] Very short polymers were used in the study (n ~ 7). Poly(gallate) **79** accelerated peroxidase about 35.6-times, monomeric gallate 2.6-times. The K_i dropped from 13.3 μM for monomer to 1.3 μM for polymer. Poly(disulfide) **80** activated peroxidase by a factor of 12 (monomer: 8). The activation or inactivation of peroxidase by polyphenols was rationalized with an enzyme-independent equilibrium between amine and phenol radicals, specific poly(disulfide) chemistry doesn't appear to contribute to activity.

Gene Delivery

Poly(disulfide)s are increasingly popular to mediate the cellular uptake of genes and siRNA (small interfering RNA).[48-52] With ideal properties of apparently exceptional promise, cationic poly(disulfide)s are predicted to be the next generation of non-viral gene delivery systems. Depending on their structure, cationic poly(disulfide)s can improve on gene compression and the extracellular stability of the resulting polyplexes toward ionic strength, acids, bases, enzymes or other proteins. More hydrophobic poly(disulfide)s are expected to interact better with lipid bilayer membranes. However, the most significant contribution of poly(disulfide)s to gene delivery is intracellular release by reductive depolymerization with glutathione. This step further accounts for minimized cytotoxicity. In this field, poly(disulfide) research has reached appreciable maturity and is being reviewed regularly.[48-52] This review thus only summarizes structural aspects and selected concepts and results of more general interest. The extensive field of crosslinked cationic polymers, mainly poly(ethyleneimine)s (PEIs), and crosslinked polyion complex micelles will not be covered because they do not involve pure poly(disulfide)s with disulfides in their backbone.

Among the three main synthetic routes to polydisulfide carriers (see above), two are extensively used to prepare gene carriers, whereas ring-opening thiol-disulfide exchange polymerization is completely missing. Most common is the polymerization of monomeric disulfides such as cystaminebisacrylamide (CBA) **81** by methods unrelated to disulfide chemistry such as conjugate addition (Figure 14). Efforts toward a fully controlled sequence-specific solid-phase synthesis of disulfide-containing poly(amido amine)s (PAAs) have been reported recently.[53,54]

Usually, PAAs **82-105** obtained by conjugate addition to CBA **81** are mainly characterized focusing on aspects and methods relevant for function. This includes the characterization of polyplexes with DNA or RNA as well as functional assays for gene delivery and cytotoxicity, sometimes complemented by fluorescence imaging. As with all complex systems, the correlation of design and result is sometimes not very clear. However, biodegradable PAAs gave overall very high transfection efficiency combined with low toxicity. The former was attributed to DNA condensing properties and endosomal buffering, the latter to intracellular reductive depolymerization. Experimental evidence for the functional relevance of this concept include reduced activity

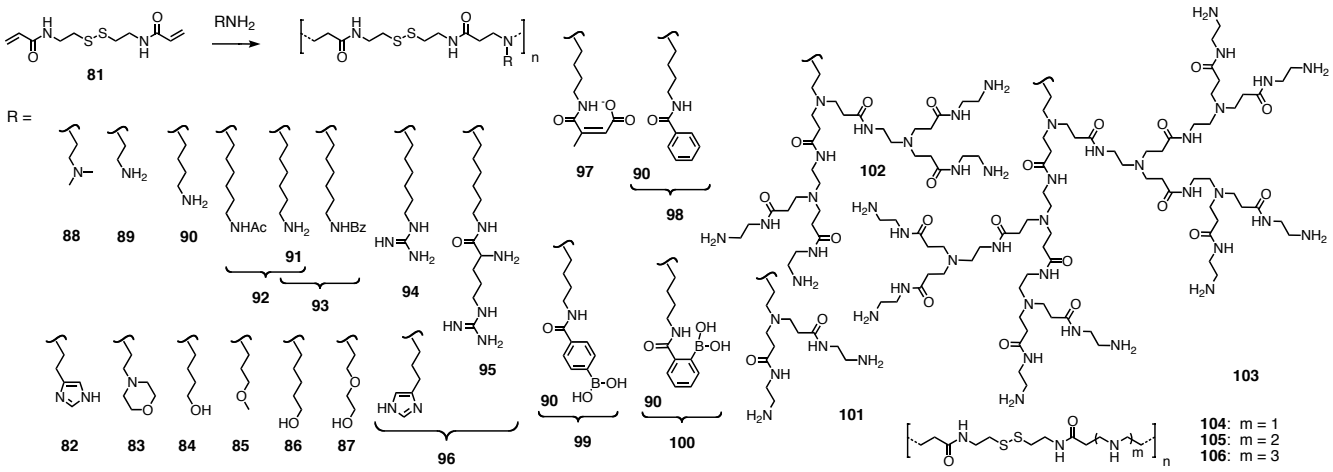

Figure 14. Bioreducible cationic poly(disulfide) PAA carriers for gene delivery. The polymers are shown in neutral form. However, proximity effects suggest that amines are partially protonated at pH = 7, whereas proximal guanidine bases are fully protonated but partially neutralized by tightly bound couterions, and imidazoles remain at mostly neutral.

in the presence of buthionine sulfoximine, depleting intracellular glutathione.[55,56] Moreover, intracellular depolymerization has been followed by dequenching of doubly-labeled carriers.[57]

The cationic charges in the PAA backbone are essential to form polyplexes with DNA. Imidazoles in polymer **82** were added as additional proton sponges for endosomal escape.[58,59] In the "proton sponge" approach, polymers with weak bases with pK_a between 7.4 and 5.1 (e.g., proximal amines, imidazoles) are expected to bind multiple protons in the more acidic endosomes. This might either induce osmotic stress and rupture of the endosomal membrane ("proton sponge effect"), or cause conformational changes in the polyplexes to improve interaction with and translocation across the membrane.

In polymers **83**-**87**, increasing lipophilicity was tested as concept to facilitate the translocation across bilayer membranes; polymer **86** with lateral pentanol chains turned out best.[59-64] The same was done at increasing charge density with alkylamine sidechains in **88**-**91**. The most active hexylamine polymer **91** was further partially capped in copolymers **92** and **93** to balance charge density against lipophilicity. Targeted delivery of Fas siRNA into rat cardiomyocytes was achieved using endocytosis via prostaglandin E_2 receptors.[65] Identical siRNA targeting was achieved by modifying poly(disulfide) **91** with a primary cardiomyocyte specific peptide.[66] Guanidinylation in polymers **94** and **95** resulted in much improved performance, presumably because of improved cell penetration and other factors such as nuclear localization.[67-72] This hexylamine sidechain was also combined with RGD peptide motifs for tumor targeting.[71]

In the most recent copolymer **96**, potential cell-penetration properties and high charge density were unified with the proton sponge effect from the weaker imidazole bases.[72] This study is most interesting. Copolymers with guanidinium/imidazole (G/I) ratios 2:1, 1:1 and 1:2 of all ~6 kD molecular weight were compared. With increasing imidazole content, the buffering capacity increased and toxicity decreased. However, DNA compacting power also decreased, and zeta potentials at saturation dropped from +25 mV to +12 mV. According to the flow cytometry, cellular uptake also decreased from 69% to 44% with decreasing G/I ratios. Most importantly, transfection efficiency dropped from 162-times (!) to 79-times that of the PEI standard. In the presence of the endosomolytic chloroquine, transfection efficiencies decreased for all copolymers **96** but increased for the imidazole-free polymer **95**. This result demonstrated that copolymers **96** operate with their own sponge effect. Nigericin inhibits the proton sponge effect by cation antiport and hinders transport to the lysosome. Different to other bufferless carriers, the transfection efficiencies of polymer **95** increased 10-times, much more than that of copolymers **96**. This important finding demonstrated that guanidinium-rich carriers escape from the endosome by direct membrane penetration, providing invaluable experimental evidence that cell-penetrating peptides[73] mediate endosomal escape and that direct passive diffusion is much more powerful for transfection than the sponge effect.

In polymer **97,** an effective intracellular protein delivery system was developed with negatively charged citraconic side groups that can be intramolecularly hydrolyzed to afford charge reversal upon pH decrease.[74] The release of complexed proteins is triggered by disulfide reduction. Control **98** and copolymers **99** and **100** were made to study the effects of the presence of two different types of phenylboronic acids as side groups for gene delivery.[75] The transfection efficiency of polyplexes of **99** was approximately similar to that of control **98** and commercial PEI, both in the absence and the presence of serum, indicating that **99** and **98** are potent gene carriers. However, the polymers with phenylboronic acids showed increased cytotoxicity, caused by increased membrane disruptive interaction as was indicated by the increased hemolytic activity observed for these polymers.

Dendritic poly(amidoamine)s (PAMAM)s **101-103** were introduced into PAA poly(disulfide) **89** via repetitive Michael addition and amidation.[76] The bioreducible poly(amidoamine)s with dendritic poly(amidoamine)s showed high buffer capacity, low cytotoxicity and strong DNA binding ability at low DNA/polymer ratios.

To prepare PAAs such as **89-91** with primary amines in their side chain, Boc protection was used during polymerization.[77,78] Polyamine polymers **104-106** were prepared without protection, yielding polyamine chains incorporated in the linear main chain as well as branched side products from conjugate addition of the secondary amines. Polymer **106** showed higher RNAi than linear analogs when used to deliver VGEF-directed siRNA to human prostate cancer cells (VEGF, vascular endothelian growth factor).

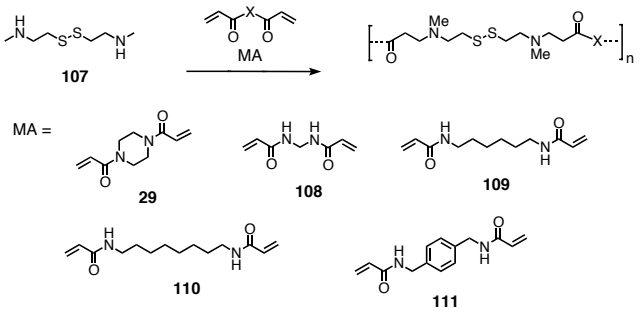

Figure 15. An alternative approach to gene delivery with bioreducible polymers. MA = Michael acceptor.

To vary also the Michael acceptor part, the disulfide motif was moved to the diamine nucleophile **107** (Figure 15).[79] This has the advantage that in polymers obtained with Michael acceptors **29** and **108-111**, the backbone amines are nearer to the disulfide and thus more acidic. The result is an improved proton sponge effect.

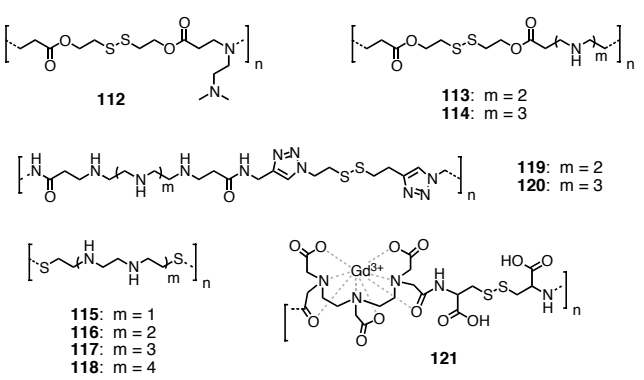

Figure 16. Poly(disulfide) carriers with esters, amines, triazoles and Gd^{3+} complexes.

The bioreducible poly(β-aminoesters) **112-114** have different amino monomers in the main chain (Figure 16).[80] High accumulation in tumors and high silencing efficiency of intratumor EGFP expression was observed when RNA complexes were intravenously injected into mice bearing U-87 MG-GFP tumor (EGFP: Enhanced green fluorescent protein).

Linear poly(ethylenimine)s (PEIs), a classic in the field, were also prepared with disulfides in the main chain.[57] The degradation of **115-118** in HeLa cells was visualized by fluorescence microscopy using the probe-to-probe dequenching effect of BODIPY-FL fluorescence dyes.

An alternative yet trendy cycloaddition approach was used to prepare cationic poly(disulfide) carriers **119** and **120**.[81] The buffering capacity and DNA binding ability of these polymers were evaluated by acid-base titration, gel retardation, and ethidium bromide exclusion assay.

Poly(disulfide)s **121** with Gd^{3+} complexes in the main chain have been tested in vitro and in vivo as a biodegradable MRI contrast agents. The clearance of disulfide-containing polymers and its degradation was tested in vitreous humor.[82] Polymer **121** showed faster clearance than controls, suggesting depolymerization into smaller fragments in the vitreous humor, possibly due to disulfide bond degradation.

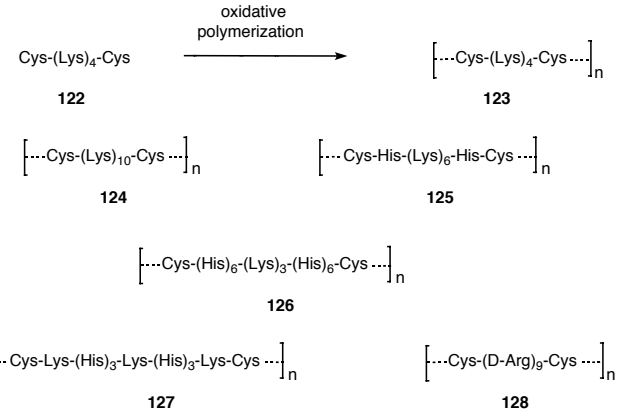

Figure 17. Bioreducible polymers as gene carriers from oxidative polymerization of short peptides.

Oxidative polymerization was used to prepare poly(disulfide)s from short peptides. Although they contain disulfides in their backbone, these systems can be considered as at the borderline to crosslinked polyamide polymers. Oxidative polymerization of the minimal peptide CK_4C **122** gave poly(disulfide) **123** (Figure 17).[83] This biodegradable cationic polymer could condense DNA and mediate transgene expression in HepG2 cells. Polyplexes of the elongated $CK_{10}C$ polymer **124** could be further crosslinked to enhance extracellular stability without losses in gene release upon reductive depolymerization with 2.5 mM DTT.[84] Increased transgene expression was the result. The same was true with histidines in polymer **125**. The increased buffering capacity was demonstrated by enhanced in vitro gene expression in the absence of the endosomolytic chloroquine.

In polymer **126**, the histidine content is increased to 70%.[85] This polymer was used to deliver GFP mRNA into PC-3 cells and siRNA in rat neurons. Oxidative copolymerization of the similarly His-rich polymer **127** was explored to infiltrate nuclear locaization sequences (NLS) with a thiol at each terminus ($CGAGPK_3RKVC$).[86] Increasing NLS content decreased translocation activity, and no targeting was observed, possibly because depolymerization in the cytosol occurred before DNA transport into the nucleus.

Whereas polyarginine is cytotoxic, depolymerizable C(D)R_9C polymer **128** was non-toxic for 1 week after injection of polyplexes into mouse lung, and showed no morphological or inflammatory changes.[87] Gene expression levels achieved with **128** were higher than with PEI standards. Longer motifs of the R-rich cell-penetrating peptide (CPP) family were subjected to oxidative disulfide crosslinking as well. Dynamically polymerized Tat-10H peptide, for example, was 7000-times more active than the monomer without increase in toxicity.[88] The same crosslinking strategy was applied to another classic in the field, the fusogenic peptide KALA.[89]

Concluding Remarks

The working title of this review was: Poly(disulfide)s - more than mere rubber! Indeed, this fairly comprehensive review of recent work on the topic shows that poly(disulfide)s have been used to create a broad variety of functional supramolecular materials. Many different topics and functions have been touched upon with poly(disulfide)s in a rather eclectic manner. The specific topics covered are listed in abstract and introduction. The success of these studies on the one hand and their comparably small number and applied nature on the other hand suggest that poly(disulfide)s are today a niche domain where nice discoveries are waiting to be made.

Poly(disulfide)s are advantageous to build functional supramolecular systems because their chemistry is robust, mild, directional and reversible. *This is chemistry that really works!* Lessons from nature corroborate that poly(disulfide) chemistry is tolerant toward the presence of many functional groups. Known from protein chemistry and confirmed with examples covered in this review, this quite general chemoorthogonality and mild reaction conditions are ideal to construct and use more sophisticated architectures without complications from protecting group chemistry (e.g., grow polymers within protein pores or multicomponent photosystems on solid surfaces). Thiol-disulfide exchange polymerization is particularly attractive in this context because it includes directionality.

Essential is the reversibility of disulfide bond formation in response to diverse stimuli, that is, redox pulses, thiol nucleophiles, heat or light. This property is advantageous to reactivate incomplete architectures to continue their construction or to destroy functional systems after use (e.g., detoxify gene delivery). The reversibility of disulfide bond formation is the key to adaptability and self-repair. Known from the macroscopic level as quite unique elasticity, curability, stress resistance and flexural strength, this ability to correct errors and respond to new environments allows functional poly(disulfide) architectures to shift from kinetic traps to thermodynamic minima and to adapt to templates.

Taken together, these attractive advantages of poly(disulfide)s have been essential to successfully secure access to the functional systems summarized in this review. Increased attention in the community to this precious chemistry promises much excitement in the future.

Acknowledgement

Dedicated to Steve Regen, a pioneer of the field and constant source of inspiration, on the occasion of his 65th birthday. We thank coworkers and collaborators who contributed to our research on the topic, S. Otto (U Groningen) for advise and the University of Geneva, the European Research Council (ERC Advanced Investigator), the National Centre of Competence in Research (NCCR) Chemical Biology and the Swiss NSF for financial support. G.S. is a Curie fellow.

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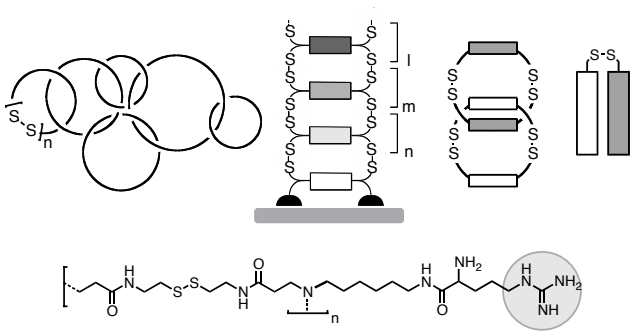

Recent progress with poly(disulfide)s is summarized comprehensively; highlights include dynamic topological plasticity, single-molecule detection methods, artificial photosystems as well as much excitement about gene delivery.