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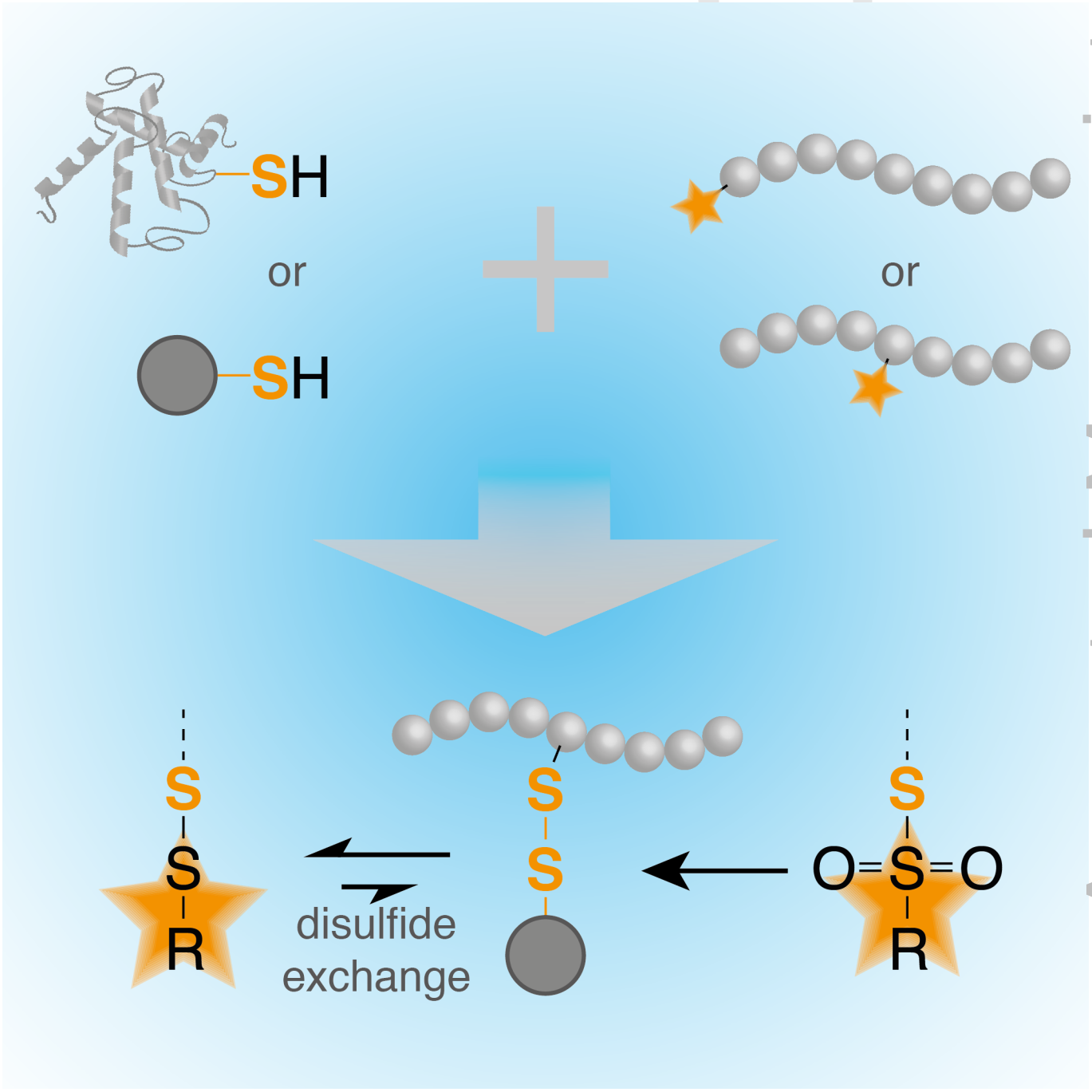
Of Thiols and Disulfides: Methods for Chemoselective Formation of Asymmetric Disulfides in Synthetic Peptides and Polymers

Olga Schäfer[a] and Matthias Barz*[a]

Dedicated to Prof. Horst Walter Kunz



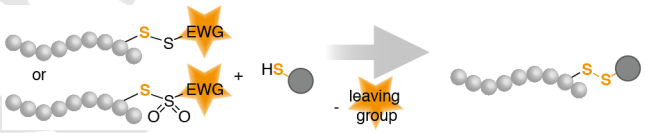
Abstract: With regard to protein- or peptide chemistry, thiols are frequently chosen as a chemical entity for chemoselective modification reactions. And while it is a well established methodology to address cysteines and homocysteines in aqueous media to form S-C bonds, possibilities for the chemoselective formation of asymmetric disulfide bonds are much less approached. Focusing on bioreversibility in conjugation chemistry, the formation of disulfide bonds is highly desirable for the attachment of thiol-bearing bioactive agents to proteins or in cross-linking reactions, since disulfide bonds can combine stability in blood with degradability inside cells. In this concept article recent approaches in the field of activating groups for thiol moieties incorporated in polymeric and polypeptide materials are highlighted. Advantageous combinations of stability during synthesis of the material with high reactivity towards thiols are explored focusing on simplification and prevention of side reactions as well as additional deprotection and activation steps prior to disulfide formation. Moreover, applications of this chemistry are highlighted and future perspectives are envisioned.

Introduction

A thiol moiety in natural peptides as well as in chemically or recombinant engineered peptide materials offers a wide range of options for site specific chemical modification.[1] The reactivity profile of thiols is mainly directed by their relatively low pK_a, which distinguishes them as excellent nucleophiles under physiological conditions,[2,3] as well as their ability to participate in redox processes to form disulfide bonds upon oxidation.[4] This high chemical reactivity of thiols, their abundance in biological systems and the ease of modification gives reason for the significance of reactions addressing thiols in organic chemistry as well as in the life sciences.[5–7]

In this context, the only thiol bearing proteinogenic amino acid cysteine is of great importance. The cysteine moiety offers a gateway to a broad range of bioconjugation techniques[8] as well as the possibility to introduce disulfide bonds, both in natural peptides[9] and in peptidie-based materials.[10,11] Bioconjucation, defined as the covalent attachment of biological and synthetic macromolecules, is a growing area of interest in biological as well as material sciences.[12–14] Central to this field is the site selective conjunction of macromolecules based on two moieties with distinct directed reactivity. This growing toolbox includes chemoselective reactions at cysteine residues such as disulfide exchanges, alkylation, native chemical ligation (NCL) with thioester reagents, Michael addition with maleimides or thiol-ene components following a free radical or Michael addition mechanism.[8,15] In contrast to NCL,[16,17] thiol-ene[18] and maleimide coupling strategies,[19,20] which operate efficiently but yield irreversible bonds, disulfides can be considered as dynamic-covalent bonds.[21–23] Upon a redox environment present in an extracellular setting, disulfide bonds are stable, unless subject to disulfide exchange, and are cleaved intracellularly due to the more potent redox environment.[24,25] Especially in the development of vaccines, drug or gene delivery systems this bioreversibility of disulfides provides enormous benefits for the design of drug delivery systems.[26–29]

While there are numerous pathways which are encountered upon introduction of a disulfide-containing component in a biological environment,[30] creating materials for biomedical applications with the option of chemoselective disulfide formation is a demanding task on its own. One challenge is the mandatory use of protective groups in the synthesis of reactive polythiol containing polymers, since free thiols interfere with most polymerization techniques according to their nucleophilic and redox-active nature (radical transfer).[31] Further, disulfide formation can be directly achieved by a nucleophilic attack of thiol or thiolate-bearing target molecules at nucleophilic sulfur atoms in the synthesized polymer. To ensure a chemoselective formation of disulfides, this process requires soft, often sulfur based leaving groups structurally based on thiosulfonates or disulfides (Scheme 1 and Table 1).



Scheme 1. Synthesis strategy for the chemoselective formation of asymmetric disulfides (EWG = electron withdrawing group).

Paired with these activating group techniques, this article will focus on the synthetic strategies for the incorporation of reactive thiols for chemoselective disulfide formation by post polymerization modification reactions. In the next chapters we describe the synthesis of such polymers obtained by either controlled radical polymerization (CRP), solid phase peptide synthesis (SPPS) and ring-opening polymerization of α-amino acid *N*-carboxy anhydrides (NCA). Since other polymerization techniques are seldom exploited in the context of chemoselective disulfide formation, these approaches are briefly outlined before introducing the main areas of chemoselective disulfide formation in polymer synthesis.

Aside from the illustrated approaches in Scheme 1 for chemoselective disulfide formation, incorporation of disulfides in the main chain is also possible and it is an attractive motif for polymer networks with self-healing properties such as polysulfides obtained by disulfide metathesis under alkaline catalysis,[32] as well as polyurethane[33] or poly(urea-urethane)[34] networks obtained by polyaddition reaction. Further,

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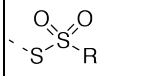
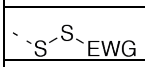
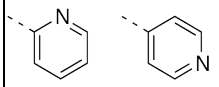
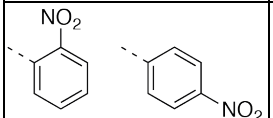
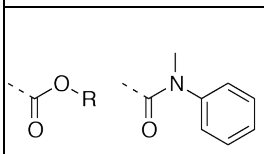
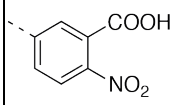
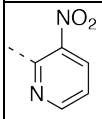
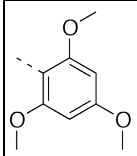
polycondensation reactions yield polyesters as well as cysteine based poly(disulfide-amides)[35] for applications in the field of self-healing materials[36] or drug delivery systems.[37] Thiol reactivity in the side chain of polyesters can be introduced for example by ring-opening co-polymerization of lactides, followed a post polymerization conversion of a trityl-thiol protective group into a thiol reactive pyridyl disulfide groups (PDS).[38]

Table 1. Activating groups for chemoselective disulfide formation.

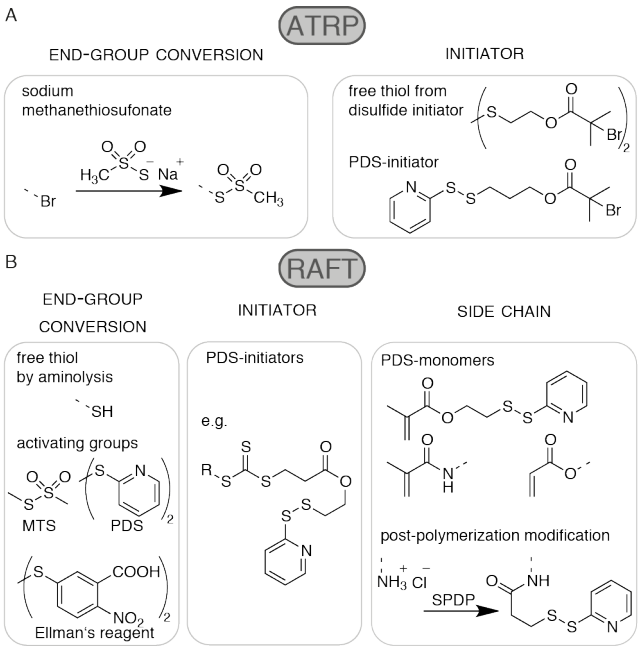
structure	group	synthesis strategy
	alkane thiosulfonate- R = Me (**MTS**) = Et, ^{i}Pr = ONa Thiosulfate- (**Bunte salts**)[39]	ATRP[40] RAFT[41] SPPS[42] NCA[42,43]
		
	2- or 4-pyridyl- (**S-Pyr or PDS**)	ATRP[44] RAFT [45] SPPS[46–48] NCA[49]
	2- or 4-nitrophenyl- (**S-Nps**)	SPPS[50–52]
	Alkoxycarbonyl sulfenyl- (R= Me **Scm**) N'-methyl-N'-phenylcarbamoyl] sulfenyl) (**Snm**)	SPPS[53–55]
	5-thio-2-nitrobenzoic acid- (**Ellmans reagent**)	RAFT[56] SPPS[57]
	3-nitro-2-pyridyl- (**S-Npys**)	SPPS[58]
	*tert*-butylthio (**S-^{t}Bu**)	SPPS[59]
	Trimethoxyphenylthio (**S-Tmp**)	SPPS[60]

Controlled radical polymerization (CRP)

Controlled radical polymerization techniques such as atom transfer radical polymerization (ATRP),[61] nitroxide-mediated polymerization (NMP),[62] and reversible addition-fragmentation chain transfer (RAFT) polymerization[63] provide versatile tools for the preparation of well-defined polymers and offer the great possibility to introduce a multitude of different functionalities into polymers.[64] Directed disulfide formation is known for ATRP and RAFT polymerizations and is achieved by thiol transformation processes following two main strategies: the thiol-alkanethiosulfonate reaction and the thiol-disufide reaction mediated by activated disulfides with electron withdrawing groups (compare Scheme 1).[65]

An initial protection step, however, is mandatory for the use of redox sensitive functional groups such as thiols, since they interfere severely with the controlled radical polymerization process. Thus, the chemical strategies surrounding the thiol moiety are numerous and in the following section disulfide reactivity in ATRP and RAFT techniques will be examined with focus on chemoselective disulfide formation. Thiol end-functionalized polymers synthesized by ATRP are realized either by a post polymerization conversion of the bromo end-group or by protected initiator pathways (Scheme 2A). An end-group conversion can yield the free thiol upon reaction with thiourea and NaOH,[66] or a thiol reactive methane thiosulfonate (MTS) group upon reaction with sodium methanethiosulfonate for directed disulfide formation.[40] Polymerization routes by functional initiators employs either disulfide initiators which yield bioreducible disulfides in the main chain,[67,68] or a 2,4-dinitrophenyl group in the initiator, which gives the free thiol upon base mediated thiolysis.[69] Directed disulfide formation is achieved by incorporating a pyridyl disulfide group (PDS) in the initiator, which gives asymmetric disulfides upon reaction with thiols.[44,70]

RAFT polymerization is another versatile technique yielding well-defined polymers and offers several routes for introduction of disulfide bonds and thiol reactive moieties.[65,71,72] Disulfide bonds can be incorporated by self-condensing vinyl polymerization of RAFT disulfide monomers, yielding hyperbrasnched architectures with bioreducible moieties.[73–75] Further, cross-linking monomers containing disulfide bonds can be implemented in numerous RAFT crosslinking polymerization strategies[76] e.g. in arm-first nanogel fromation[77–79] or core-first approaches.[80] Synthetic strategies for a bioreducible, detachable corona of nanoparticles for biomedical applications[81,82] also rely on the incorporation of disulfide bonds between two blocks during RAFT polymerization. In terms of end-group functionality, materials synthesized by RAFT polymerization have the advantage of the thiocarbonylthio end-group, which can already be considered a protected thiol group. Thus, many end-group transformation methods involve the free thiol, which is obtained by reduction or through reaction with nucleophiles e.g. aminolysis.[65,83] Functionality is then introduced by thiol-ene, thiol-yne, Michael and maleimide reactions either as a separate reaction step or in a one-pot process.[84–87]



Scheme 2. Entries for directed disulfide formation in ATRP and RAFT polymerization techniques.

In analogy to ATRP, strategies for directed thiol transformations in RAFT polymerization are mediated by the MTS[41] and PDS group (Scheme 2B).[45] Both moieties can be introduced by aminolysis of the thiocarbonylthio end-group as a post-polymerization modification and, after directed disulfide formation, were utilized in encapsulation of gold nanoparticles,[88] protein-polymer conjugates[89] and drug attachment by disulfides.[90] Reaction with Elmann's reagent (5,5'-dithiobis-2-nitrobenzoic acid)[56,91] also yields activated thiols after removal of the thiocarbonylthio moiety.[92] Further, thiol reactivity by a PDS group can be introduced during the RAFT polymerization as an end-group by mono- or bifunctional chain transfer agents[93–95] or in the side chain. In the latter strategy, polymerization of PDS modified monomers allowed for particle formation and stabilization by disulfide cross-linking.[96–98] In addition, side chains can also be functionalized in a post-polymerization reaction with N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP).[99]

Concluding, ATRP and RAFT as controlled radical polymerization techniques allow for directed disulfide formation by conversion of thiol moieties into thiol reactive methane thiosulfonates or into activated disulfides. However, since the thiol-reactive compounds are often amine incompatible, a separate step may be required during end-group conversion. Further, upon generating free thiol end-groups, undesired disulfide formation and thiol exchange can pose a synthetic challenge.[100] Alternatively, the PDS moiety, which proves to be stable during the controlled radical polymerization process[101], can be introduced upon polymerization via the initiator in ATRP and RAFT or as a functionalized RAFT-monomer.

Synthesis of polypeptides

While the incorporation of activated thiols into polymers was established over the last decade for controlled radical polymerization techniques, their use in polypeptide synthesis was mainly limited to postpolymerization modification techniques in synthetic or natural polypeptides. Researchers encountered complex requirements for protective groups and needed to choose between options in multistep procedures of protection, deprotection, activation and conjugation reactions. A suitable protective group avoiding this complexity, should allow direct conversion into a disulfide and needs to provide the delicate balance of stability towards amines and other hard nucleophiles with reactivity towards thiols as soft nucleophiles.

Solid phase peptide synthesis (SPPS)

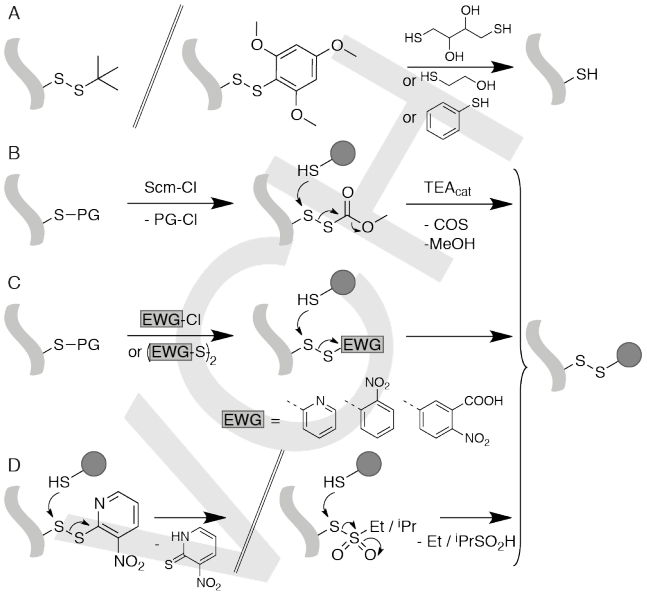
Chemical design of peptide materials is a substantial goal in solid phase peptide synthesis (SPPS) and enables the formation of sequence defined peptides containing one or several disulfide bridges, as well as peptide materials with disulfides for stabilization of drug and [102,103] gene delivery systems[104,105] or peptide based molecular transporters.[106] Disulfides are commonly introduced via the amino acid cysteine. However, side-chain protection is essential, since the nucleophilic thiol interferes with the peptide coupling reaction and free thiols are generally incompatible with hydrolytic protecting groups, as it poisons the catalyst.[107] And while a broad spectrum of protective groups for cysteine in SSPS is available, strategies for disulfide formation rely either on (i) orthogonal reactive protecting groups, symmetrical removal and formation of disulfide by oxidation, (ii) direct oxidative symmetrical removal of protective groups mostly by I_2 or (iii) asymmetric activation and disulfide formation in a displacement reaction.[10,108]

Oxidation of free thiols after peptide synthesis and removal of protecting group will yield symmetrical disulfides when (homo)cysteines react with each other. Various oxidants are known from aerial oxygen,[109] DMSO[110] and supported methionine sulfoxide in analogy to DMSO oxidation with the advantage of simple removal of the oxidizing agent,[111] chlorotrimethylsilane-nitrate salts[112] and oxidation of free SH groups by immobilized metal complexes.[113] Further selective oxidants are trans-3,4-dihydroxyselenolaneoxide (DHS)[114,115] and N-Chlorosuccinimide (NCS), enabling on resin oxidation with good compatibility to other protecting groups.[116] Direct oxidation from the protected cysteine is also a popular pathway, employed with the S-acetamidomethyl group (S-Acm) which is removed by I_2 forming disulfide bonds,[117,118] or (microwave-assisted) disulfide formation from S-triphenylmethyl protected cysteine (S-Trt) by I_2 oxidation.[119] Other strategies include reduction the of p-nitrobenzyl protective group (S-pNB) to a p-aminobenzyl group (S-pAB) prior to oxidation by I_2,[120] oxidation of the S-^{t}Bu group by O_2 in a large excess of cysteine and a chaotropic salt[121] and enzymatic cleavage of phenylacetamidomethyl groups (S-Phacm) followed oxidation in the presence of DMSO in aqueous conditions.[122] However, disulfide formation by oxidation allows only the formation of symmetrical disulfides and suffers from drawbacks such as

removal of the oxidizing agent, low yield, long reaction time, and the formation of side products.[123,124]

Directed asymmetrical disulfide formation, as required for bioconjugation, remains chemically challenging terrain since a displacement reaction of a free, nucleophilic thiol with a second, activated thiol to form a disulfide needs to be coordinated. Especially in intermolecular disulfide bridging, as in bioconjugation techniques employing native cysteine residues,[125,126] formation of homodimers has to be avoided in favor of the desired heterodimer. An approach employing N-terminal cysteine enables coupling forming a cleavable thiazolidine heterocycle upon reaction with an aldehyde moiety.[127] Additionally, free thiols can be activated by an electron withdrawing group e.g. by chlorination[128] or as nitroso-thiols,[129,130] yielding, highly electrophilic species prone to side reactions. A strategy involving mixed aliphatic disulfides relies on the S-^{t}Bu protective group,[59] which is cleaved by thiol containing compounds such as benzenethiol, ß-mercaptoethanol or dithiothreitol (DTT) yielding the free thiol.[131] The trimethoxyphenylthio group (S-Tmp)[60] is another thiol labile alternative, since S-^{t}Bu removal proves to be occasionally difficult[132] (Scheme 3A). A similar approach relies on thiol free reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) which liberates the free cysteine thiol for further coupling reactions from an S-^{i}Pr protective group.[133] The advantage of the pathways outlined above is the orthogonal reactivity to most cysteine protective groups, the compatibility with the Fmoc strategy and relatively mild deprotection procedures. However, the thiol reactivity of these groups is limited to the thiol moieties employed in the deprotection and they do not mediate a directed disulfide formation.

Mixed acyl and aryl disulfides are suitable for regiospecific displacement by free thiol groups and yield ultimately unsymmetrical disulfides. Activation by electron-withdrawing S-alkoxycarbonyl sulfenyl groups e.g. the S-methoxycarbonylsulfenyl group (Scm),[53,134,135] ethyl (Sce), benzyl (Sz), tert-butyl (Scb) derivatives[54] or S-([N'-methyl-N'-phenylcarbamoyl]sulfenyl) (Snm),[55] is accomplished by reaction of the free or protected thiol with alkoxycarbonyl sulfenyl chloride (Scheme 3B). Upon reaction with DTT the free thiol is obtained and mixed disulfides with a thiol component upon a mild base catalysis.[136] The driving force for disulfide formation is the irreversible decomposition of the Scm group in carbonyl sulfide and the respective alcohol. Limiting factors are, however, a possible reaction with the N-terminus and the base sensitivity of this class of activating groups.[55] Further electron-withdrawing activating group such as 2- and 4-nitrophenyl (S-Nps)[50–52] and 2- and 4-pyridyl groups (S-Pyr)[46–48] have been used, since the disulfide formation is promoted by the low pK_a of the aromatic thiol leaving group (Scheme 3C). The introduction of thiol reactive moieties by 2-pyridinesulfenyl chloride is however susceptible to hydrolysis.[108,137] An alternative strategy includes N-terminal thiol deprotection followed by activation with excess 2,2'-dipyridyldisulfide and coupling with a free thiol in the presence of DTT at pH 7.[138] In analogy, reaction with 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) gives also a suitable activated disulfide.



Scheme 3. Approaches for directed disulfide formation in SPPS. A) The free thiol is obtained upon cleavage with thiol containing compounds. B-C) Protective group interconversion and disulfide formation. D) Direct disulfide formation from a protective and activating group.

Although the performance of the above specified activating reagents is efficient in terms of disulfide formation, the additional deprotection and activation step complicates the overall synthetic strategy. An alternative approach operates with the 3-nitro-2-pydidyl group (S-Npys) combining characteristics of both, an activation and protective group (Scheme 3D left).[58] Introduction of the Npys group is performed by reaction of either the protected cysteine[139] or conversion of the free thiol with Npys halides.[140] An alternative pathway includes conversion with 2,2'-dithiobis(5-nitropyridine) (DTNP) into Npys and quantitative disulfide formation upon addition of DTT (with and without addition of thioanisole).[141] Limiting factors are the high stability which complicates removal and requires enhanced temperatures and an access of DTNP. Further studies include Npys disulfide coupling assisted by microwave irradiation[142] and immobilized Npys on resin enabling disulfide formation and facilitated work-up.[143] The chemical stability of the Npys group allows for Boc/benzyl synthesis conditions, but cannot be applied to "low-high" cleavage protocols or to bases employed in the Fmoc-strategy.[144] Thus, in the Fmoc-strategy, the Npys group can either be incorporated at N-terminal position, introduced after solid phase peptide synthesis or by conversion of other protective groups.[145]

The S-alkylsulfonyl group is an alternative protective and activating group for directed disulfide formation and is based on the thiosulfonate motif (Scheme 3D right).[42] Ethyl and isopropyl derivatives have shown outstanding chemoselective thiol reactivity, while remaining thermodynamic inert towards various amines, allowing standard Fmoc SPPS protocols. Thus the site of the cysteine with a thiol-reactive protective group is not limited to a terminal position. The thiosulfonyl groups remained intact

during conversion into the corresponding Fmoc derivatives, coupling by *N,N*-diisopropylcarbodiimide (DIC) with OtBu-alanine to give the dipeptide and cleavage protocolls of the C- and N-terminal protective group. However, coupling protocols employing 1-hydroxybenzotriazole (HOBt) or related O-nucleophiles pose a challenge, since partial cleavage of the activating group resulted in free thiols.

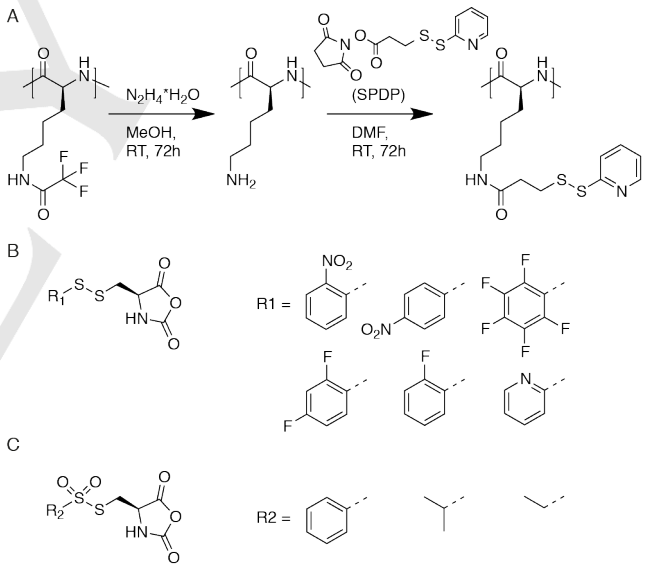
In summary, SPPS offers a well-supplied toolbox for disulfide formation. However, deprotection steps or interconversion to suitable activating groups can increase the synthetic effort. The Npys and S-alkylsulfonyl group fall in the advantageous category of protective and activating groups, which enables asymmetric disulfide formation without further modifications, thus simplifying the overall peptide synthesis procedure. In this context, O- and N-nucleophiles can pose a challenge when present besides thiol activating groups, since the reactivity towards the activated electrophilic sulfur is often high enough to cause side reactions. Strategies in SPPS rely on amine mediated Fmoc-deprotection, which is compatible with the S-alkylsulfonyl group but not the Npys group. However, most activating routes also employ O-nucleophiles derived from triazoles, rather than sole carbodiimides due to the risk of racemization,[146] which results in partial cleavage of the S-alkylsulfonyl group due to the oxophilic character of sulfur.

NCA Polymerization

The ring-opening polymerization of α-amino acid *N*-carboxy anhydrides (NCA) is a well-established methodology for rapid and large scale preparation of synthetic polypeptides.[147–162] In contrast to SPPS, NCA polymerization techniques lack the possibility for sequence-defined polypeptides, provides, however, the possibility of (multi) block copolymer synthesis, graft copolymers and high molecular weight polypeptides. In addition, O-nucleophiles are absent during polypeptide synthesis. Polypeptides synthesized by this method have already entered clinical trails or, as in the case of copaxone, became multi-billion dollar drugs.[155,156]

The high demand for reactive thiols enabling bioreversible conjugation to polypeptides by disulfide bonds is eminent, as illustrated by the well established incorporation of disulfide bonds in initiators, in the main or side chain of polypeptides[164–167] In addition, reactive polypeptides enable a variety of post polymerization modification techniques.[157,168] A multitude of chemoselective modifications, e.g. alkylation,[158] azide-alkyne,[159] thiol-ene and -yne reactions[160–162] is available. However, in analogy to CRP and SPPS, most post polymerization modifications in NCA polymerization result in irreversible bonds.[169] It is evident how implementations of reactive thiols applicable to nucleophilic ring-opening polymerization are scarce, since most activated disulfide moieties are prone to nucleophilic initiators as a consequence of their pronounced reactivity required for disulfide formation. A sequential NCA polymerization followed by sequential deprotection procedures allows for a post-polymerization modification approach and enables chemoselective cross-linking by dithiols (Scheme 4A).[49] Here, activated pyridyl disulfide moieties were introduced into a poly-L-lysine segment in a triblock polypeptide-*block*-polypeptoid copolymer (polypept(o)id)[170,171] employing SPDP.

However, to bypass the required deprotection and activation steps after polymerization, a protective group for thiols, which combines stability during NCA peptide synthesis with the ability to form disulfide bonds in a chemoselective reaction, appears highly desirable. Thus, activated cysteines were employed in NCA synthesis and polymerization, first equipped with protective groups of varying potency as electron withdrawing groups (Scheme 4B).[163] The findings were, however, humbling, since the protective groups in question are highly reactive like most activated thiols and disulfides and were prone to hydrolysis and aminolysis during NCA synthesis as well as during polymerization. At this point, a related class of sulfur-sulfur containing bonds known for its potency in thiol activation was explored: the thiosulfonyl group.[39,172,173] Starting with *S*-pheylsulfonyl-L-cysteine[163] and followed by *S*-alkylsulfonyl-L-cysteines[42] (Scheme 4C), thiosulfonyl protective groups were first explored in NCA polymerization to successfully bridge the gap between stability and reactivity.



Scheme 4. A) Post-polymerization strategy for incorporation of pyridyl disulfide moieties in polypeptide side chains. B) L-cysteine NCAs with disulfide-based side chains of decreasing electron deficiency. C) S-phenylsulfonyl-, *S*-ethylsulfonyl- and *S*-isopropylsulfonyl-L-cysteine NCA. Reproduced with permission from refs. [49,163]. Copyright 2016 and 2017 Elsevier.

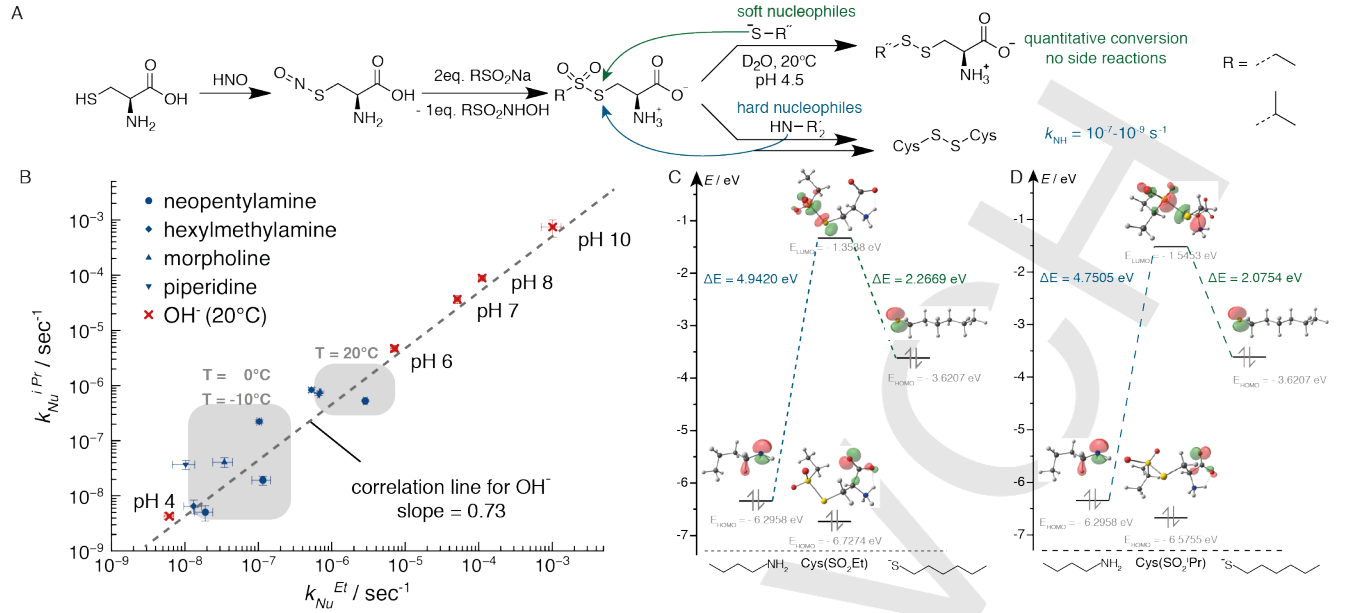


Figure 1. A) Synthesis of *S*-alkylsulfonyl-L-cysteines (Cys(SO_2R)) and reactivity profile towards hard and soft nucleophiles with R' = neopentylamine, hexylmethylamine, morpholine, piperidine, 1-butanamine; R'' = d-penicillamine, cysteamine, glutathione, 1-hexanethiol. B) Plot of log (k_{Nu}) for Cys(SO_2R) under alkaline hydrolysis (red) and aminolysis conditions (blue) at different temperatures. C) DFT calculations of HOMO and LUMO of Cys(SO_2Et) and D) Cys(SO_2 ^{i}Pr) each in relation to HOMOs of butylamine and hexanethiol in a nucleophilic attack. Reproduced with permission from ref. [42]. Copyright 2016 Wiley-VCH.

The synthesis of *S*-alkylsulfonyl-L-cysteines is straightforward and involves the *in situ* formation of the S-nitrosocysteine followed by conversion with either ethanesulfinic or isopropanesulfinic acid sodium salt forming the corresponding thiosulfonate with retained stereochemistry.[42] The desired asymmetric disulfides are obtained upon reaction with soft nucleophiles, like thiols, exhibiting exceedingly fast reaction rates, while hard nucleophiles like amines result in low reaction rates so that a conversion is virtually inhibited[174] (Figure 1A). Thus, both protective groups are stable against aminolysis by primary and secondary amines in a low temperature regime, which enables their use in amine-initiated NCA polymerization (Figure 1B). In aqueous conditions, base-mediated hydrolysis needs to be taken into account due to the the oxophilic character of sulfur, as reflected in increasing rate constants upon higher pH levels. As a result, mildly acidic conditions are preferred when in aqueous media.

In the case of a nucleophilic attack, the reactivity profile of the *S*-alkylsulfonyl protective group is directed by the difference between the energy level of the lowest unoccupied molecular orbital (LUMO) of the electrophile and the highest occupied molecular orbital (HOMO) of the nucleophile. Figures 1C and D show density functional theory (DFT) calculations of the frontier orbitals of *S*-ethylsulfonyl- as well as *S*-isopropylsulfonyl-L-cysteine in relation to hexanethiolate as a soft nucleophile and butylamine as a hard nucleophile.[175,176] For both protective groups the energy gap between the LUMO and the HOMO of the amine (left) significantly exceeds the gap to the HOMO of the thiol (right). A smaller energy gap, however, facilitates an orbital controlled nucleophilic reaction and the DFT calculations are well in line with the observed reactivity of the *S*-alkylsulfonyl protective group towards thiols and the hindered aminolysis.

After ensuring the stability of the *S*-alkylsulfonyl protective group under amine-initiated controlled ring-opening polymerization conditions, both protected L-cysteine derivatives were converted into the corresponding NCAs and polymerized with neopentylamine as initiator (Figure 2A). The resulting poly(*S*-alkylsulfonyl-L-cysteines) display a narrow, symmetrical molecular weight distribution with low polymer dispersity (Đ<1.2) (Figure 2B). Further, MALDI-TOF mass spectrometry studies of homopolymers verified the integrity of the protective group during the NCA polymerization and absence of side reaction.[43] Full agreement between simulated and measured molecular weights clearly confirms the absence of chemical chain termination or other side reactions due to protective group cleavage and emphasize the highly controlled polymerization of Cys(SO_2R) NCAs.

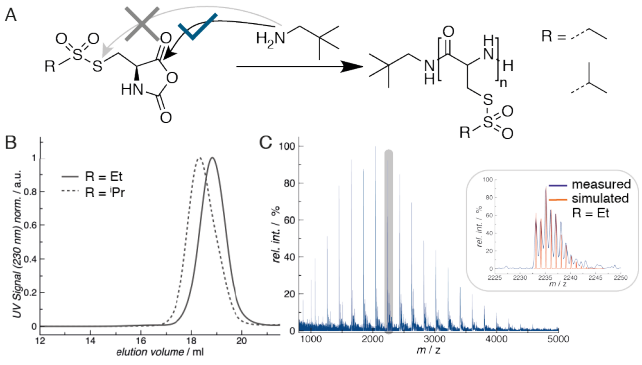


Figure 2. A) Polymerization of Cys(SO_2R) NCA with neopentylamine as initiator, B) HFIP GPC traces of PCys(SO_2R) and C) MALDI-TOF spectrum of PCys(SO_2Et). Reproduced with permission from refs. [42] and [43]. Copyright 2016 Wiley-VCH and American Chemical Society.

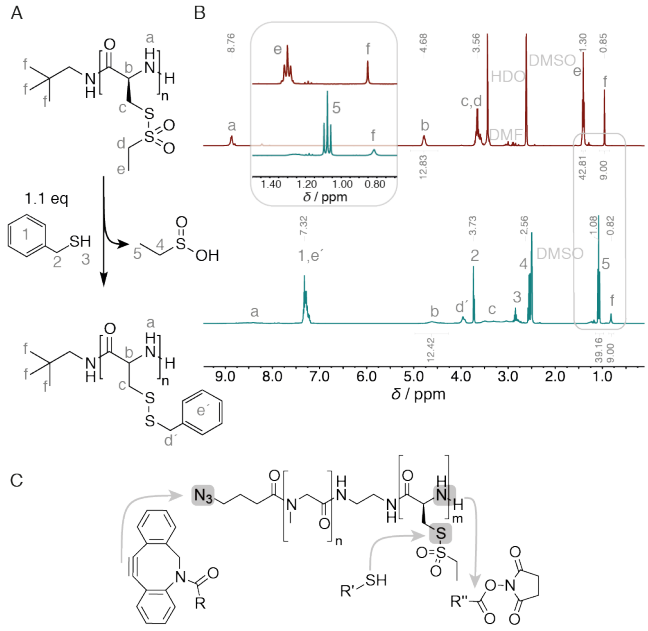


Figure 3. A) Reaction of PCys(SO_2Et) with benzylmercaptan. B) ^{1}H NMR spectrum of PCys(SO_2Et) prior to conversion (red) and after conversion with benzylmercaptan (blue). Enlarged comparison of both ^{1}H NMR spectra emphasizing the quantitative conversion (disappearance of polymer associated protecting group signal at 1.30 ppm and emerging signal at 1.08 of the fully converted protecting group). C) Scheme of PSar$_n$-*b*-PCys(SO_2Et)$_m$ end group conversion with NHS-ester and DBCO moieties, respectively, and thiol reactivity of the *S*-ethylsulfonyl protective group in the side chain. Reproduced with permission from ref. [43,177]. Copyright 2016 and 2017 American Chemical Society.

The chemoselective formation of asymmetric disulfides as mediated by the *S*-alkylsulfonyl protective group in the polymer was carefully verified by conversion with a thiol moiety (Figure 3A). As evaluated by ^{1}H NMR, full chemoselective conversion into the disulfide is achieved with high reaction rates and without detectable side products (Figure 3B).[43] Subsequently, block-copolypept(o)ides incorporating PCys(SO_2R) as a cross-linkable segment were realized by sequential polymerization. The orthogonality of the thiol-reactive moiety of the *S*-alkylsulfonyl protective group towards strain-promoted azide−alkyne cycloaddition (SPAAC) and activated ester-mediated amidation was investigated in amphiphilic polysarcosine-*block*-poly(S-alkylsulfonyl)-L-cysteine block copolymers (Figure 3C). All three functionalities can be addressed independently, thus allowing for functional group interconversion of chain ends, as well as directed disulfide formation in the side chain of the PCys(SO_2R) block in one step.[177]

A combination of this orthogonal direct disulfide formation with further functional polypeptidic segments, such as a cationic poly-L-lysine block as mentioned above in the strategy for post-polymerization modification for disulfide stabilized-polyplexes,[49] adumbrates a gateway to numerous multifunctional polypept(o)idic systems and envisions their use as material platform for biomedical applications.

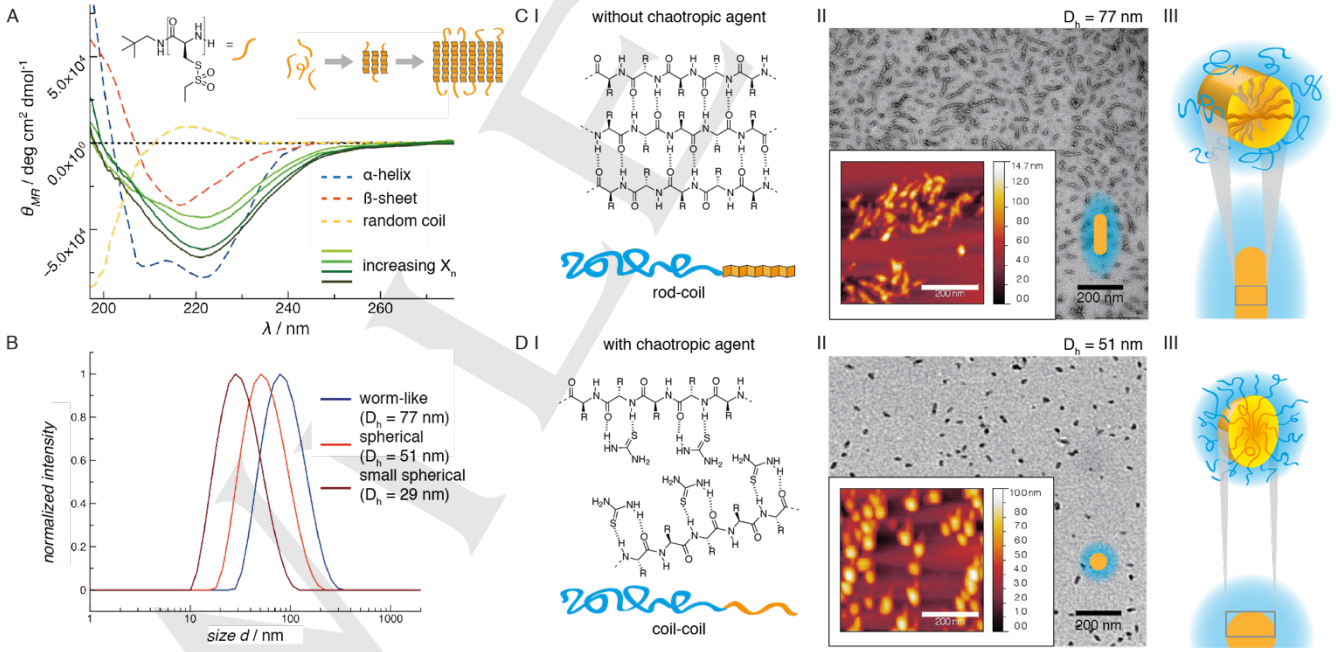


Figure 4. A) CD spectrum of PCys(SO_2Et) of varying degrees of polymerization in HFIP confirming β-sheets in solution. B) Size range of core-cross-linked nanohydrogels as shown in dynamic light scattering experiments. C) Properties of core-cross-linked nanohydrogels in the absence of a chaotropic agent: I) intermolecular hydrogen bond formation resulting in β-sheets and thus rod-coil polymers, II) elongated shapes due to secondary structure stabilization (visualized by AFM/TEM), III) illustration of the core framework of worm-like particles with twisted β-sheets. D) Properties of core-cross-linked nanohydrogels in the presence of a chaotropic agent such as thiourea: I) intermolecular hydrogen bond formation is repressed resulting in coil-coil polymers, II) spherical structures as modulated by the absence of secondary structure stabilization (visualized by AFM/TEM), III) illustration of the core framework of spherical particles with intertwined polymer chains in random coil conformation. Reproduced with permission from refs.[43,184]. Copyright 2016 American Chemical Society and 2017 Wiley-VCH.

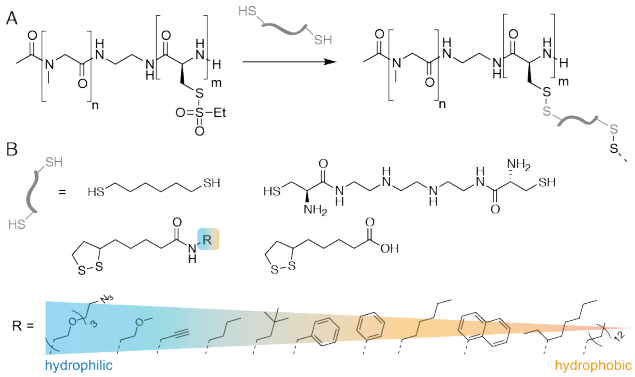


Figure 5. A) Illustration of the cross-linking reaction utilizing the thiol reactivity of the S-ethylsulfonyl group with various dithiols B) such as hexanedithiol, triethylenetetramine α,ω-di(cysteine)diamide (TETA), lipoic acid and lipoic acid derived cross-linkers prior to reduction with side chains of varying polarity. Reproduced with permission from ref. [184]. Copyright 2017 Wiley-VCH

Applications of Thiol-reactive Polypeptides

The incorporation of activated thiols into polypeptides provides access to reactive copolymers, which can be applied to postpolymerization modification reactions[168,169,178,179] leading to the formation of disulfide bonds. Since these bonds are intrinsically reversible and possess a certain dynamic nature, the formation of disulfide bonds is used for bioreversible cross-linking of micelles, polyplexes or protein polymer conjugates or for the attachment of bioactive agents, e.g. antibody or protein drug conjugates[29,180–183] In another recent example multiple functionalities were introduced into polypept(o)ides bearing poly(*S*-alkylsulfonyl-L-cysteine) segments for the formation of asymmetric disulfides.

In addition, self-assembly of polypeptides in solution and of poly(S-alkylsulfonyl)-L-cysteine containing block copolymers in particular, provides access to compelling micellar morphologies (see Figure 4). The S-alkylsulfonyl)-L-cysteine block adapts a β-sheet conformation (Figure 4A), adding cooperative effects to the self-assembly process. Thus, control over self-assembly in solution is not longer solely directed by block length ratios of the hydrophilic to the hydrophobic block, but can be modulated by hydrogen bond formation between polypeptides of a certain secondary structure. Keeping in mind that the secondary structure of proteins can be denatured by different external stimuli,[187] the self-assembly of such polypeptides can coherently be controlled by modulation of hydrogen bond formation. In the most prominent case, a rod–coil block copolymer (intact β-sheet conformation, Figure 4C I) can be converted to a coil–coil polymer with help of a chaotropic agent (disturbed β-sheet conformation, Figure 4D). While rod–coil block copolymers ($PSar_{407}$-*block*-$PCys(SO2Et)_{28}$) form worm-like micelles in aqueous solution (without thiourea, Figure 4C II) the same polymer can lead to the formation of spherical micelles in the presence of a chaotropic agent (with thiourea, Figures 4D II). Disturbing the formation of inter-chain hydrogen bonds, the chaotropic agent converts the rod–coil conformation of the block copolymer to an rod–coil block copolymer and subsequently alters the resulting nanoparticle morphology.

Moreover, the reactive poly(*S*-alkylsulfonyl-L-cysteine) block ensures stabilization of the formed morphologies and the introduction of functionality in the micellar core. The formation of disulfide linkages proceeds at the core-forming block in a chemoselective fashion, whenever the cross-linking is realized by the reaction of di- or oligothiols in aqueous solution (Figure 5A). Interestingly, the size and morphology of the previously assembled micellar structures are not affected by this cross-linking reaction even if the core polarity is inverted by hydrophilic dithiols. Therefore, a complex goal, the complete decoupling of aggregate formation and core-functionalization as well as –stabilization, is realized. As a consequence, core functionality as well as polarity can be easily adjusted according to the implemented dithiol in one single step (Figure 5B). Here, the lipoic acid derivatives are implemented after equimolar reduction with tris(2-carboxyethyl)phosphine (TCEP)

In summary, the implementation of the *S*-alkylsulfonyl protective group in the core-forming block block leads to adjustable size- and morphology regimes in nanoparticle formation by self-assembly. Further, independent core-functionalization in respect to the desired biomedical application is achieved as well as bioreversible core stabilization and thus creating a highly versatile nanoparticle platform.

Concluding Comments

Activated thiols moieties have already drawn the attention of researchers in the fields of organic chemistry and polymer science as a possibility to introduce functionalities into macromolecules by bioreversible disulfide formation. However, a lack of chemoselectivity in complex proteins for side specific disulfide formation in the presence of amines or other nucleophiles is limiting the use of activated disulfides. In contrast, *S*-alkylsulfonyl protected thiols can overcome these limitations since they enable the desired chemoselective formation of disulfide linkages between macromolecules and peptides or other low molecular weight compounds. We believe that in poly(S-alkylsulfonyl)-L-cysteines in particular, an exciting combination of properties is merged to enable asymmetric disulfide formation in a chemoselective manner, while promoting directed self-assembly in solution by β-sheet formation. These properties allow the straightforward synthesis of core-shell nanoparticles with decoupled control over morphology and functionality. Therefore, we anticipate that the *S*-alkylsulfonyl protective group for thiol-activation will develop into a useful tool in the synthesis of functional nanoparticles and in bioconjugation chemistry.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: protecting groups • S-alkylsulfonyl cysteine • nucleophilic substitution • chemoselective disulfide formation • self-assembly

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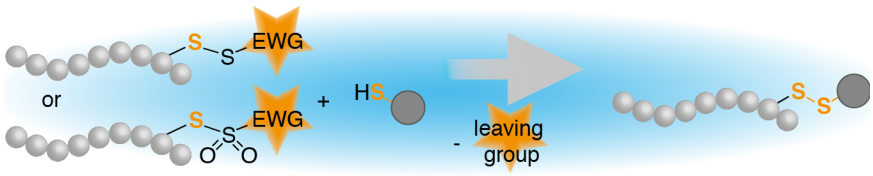

*Olga Schäfer, Matthias Barz**

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Of Thiols and Disulfides: Methods for Chemoselective Formation of Asymmetric Disulfides in Synthetic Peptides and Polymers

How to: directed disulfides in macromolecules. With regard to protein- or peptide chemistry, thiols are the chemical moiety of choice for chemoselective introduction of asymmetric disulfide bonds. An overview on techniques in directed disulfide formation in synthetic peptides and polymers is given focusing on simplification and prevention of side reactions as well as additional deprotection and activation steps prior to disulfide formation.