Pyridyl Disulfide Reaction Chemistry: An Efficient Strategy towards Redox-Responsive Cyclic Peptide–Polymer Conjugates

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ABSTRACT: Cyclic peptide–polymer conjugates are capable of self-assembling into supramolecular polymeric nanotubes driven by the strong multiple hydrogen bonding interactions between the cyclic peptides. In this study, we have engineered responsive nanotubes by introducing a cleavable bond that responds to a reductant utilizing pyridyl disulfide reaction chemistry. Reactions between a cysteine containing cyclic peptide (CP-SH) and pyridyl disulfide containing polymers were initially studied, leading to the quantitative formation of cyclic peptide–polymer conjugates. An asymmetric cyclic peptide–polymer conjugate (PEG-CP-S-S-*p*PEGA) was then synthesized via orthogonal pyridyl disulfide reaction chemistry and NHS coupling chemistry. The disulfide linker formed by the pyridyl disulfide reaction chemistry was then selectively reduced to thiols in presence of a reductant, enabling the transition of the conjugates from non-assembling unimers to self-assembled supramolecular polymeric nanotubes. It is anticipated that the pyridyl disulfide reaction chemistry will not only enrich the methodology towards the synthesis of cyclic peptide–polymer conjugates, but also lead to the construction of a new family of redox-responsive cyclic peptide–polymer conjugates and supramolecular polymeric nanotubes with tailored structures and functionalities.

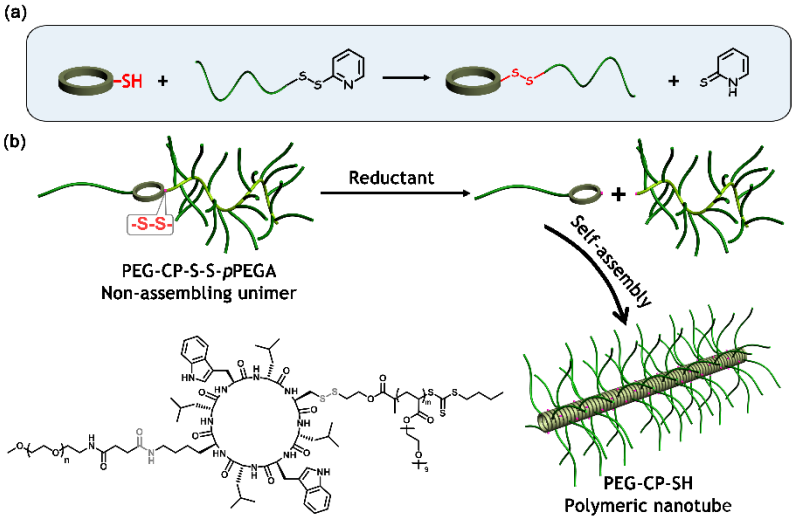
Conjugating synthetic polymers to biomolecules has become a highly prolific field of research since the concept was introduced by Ringsdorf in 1975.[1] Non-naturally modulating these biomolecules with synthetic polymers leads to changes of their structural and functional properties, enabling chemists to tune their inherent activity and stability.[2-3] Peptide/protein–polymer conjugates are of particular interest since they provide efficient strategies towards mediating peptide/protein conformation, improving solubility and stability, facilitating specific interactions, and increasing functionality, and therefore have applications in a wide range of fields, including drug delivery and materials science.[4-14]

Cyclic peptide–polymer conjugates present an example of functional supramolecular polymeric nanotubes (SPNTs).[15] Driven by the strong multiple hydrogen bonding interactions between the flat cyclic peptide rings duo to the alternating D- and L-amino acid configuration, the cyclic peptides self-assemble into β-sheet nanotubes.[16-20] Conjugating macromolecules onto these cyclic peptides prevents their aggregation and improves their stability and solubility in solution, forming SPNTs with well-defined structures.[21-25] In the past few years, several efficient conjugation methodologies have been developed, such as alkyne-azide cycloaddition, activated ester-mediated chemistry (NHS coupling, for example), and thiol-ene click chemistry. Using these conjugation strategies, various cyclic peptide–polymer conjugates with different compositions and architectures have been synthesized. [26-29] In this context, the development of new conjugation strategies is key to the development of these materials, with emphasis on efficiency, orthogonality, and mild reaction conditions.[30-32] In addition, the introduction of a responsive linker between the cyclic peptide and polymer would endow these materials with stimuli-responsive properties, offering great potential regarding the application of on demand drug delivery systems and antimicrobial drugs, but has been rarely reported.[33]

The pyridyl disulfide reaction chemistry, one of the most widely used conjugation chemistry, has been reported to be highly efficient, orthogonal to other conjugation chemistry, and applicable under mild conditions. In addition, this chemistry offers unique properties.[34-39] The reaction produces pyridine-2-thione as side product, which can be measured spectrophotometrically, the reaction can therefore be monitored conveniently *in situ* by UV/vis spectroscopy; the synthesis can be undertaken in most of organic solvents as well as in aqueous solutions over a broad pH range; the resulting disulfide bond formed as the linker can be reversibly cleaved by redox reactions.

Herein, we explored the use of pyridyl disulfide reaction chemistry to fabricate cyclic peptide–polymer conjugates. As depicted in Scheme 1, the reaction between a cysteine containing cyclic peptide (CP-SH) and pyridyl disulfide containing polymers was firstly studied, leading to the synthesis of several different conjugates. An asymmetric cyclic peptide–polymer conjugate (PEG-CP-S-S-*p*PEGA) was then designed and synthesized. Specifically, a brush polymer poly (poly ethylene glycol acrylate) (*p*PEGA) was conjugated to the cyclic peptide via a disulfide linker by pyridyl disulfide reaction chemistry, while a linear polymer poly ethylene glycol (PEG) was conjugated orthogonally via an amide linker by NHS coupling chemistry. Considering the steric hindrance of the brush polymer *p*PEGA, its self-assembling behavior into polymeric nanotubes is anticipated to be greatly restricted. However, benefiting from the disulfide linker, the conjugate PEG-CP-S-S-*p*PEGA can respond to redox stimuli. Once the disulfide linker between cyclic peptide and *p*PEGA is cleaved with the addition of a reductant, the

resultant one arm conjugate, PEG-CP-SH, is expected to form longer polymeric nanotubes as a result of reduced steric hindrance.[38] In this way, we can trigger a transition from non-assembling unimers to self-assembling SPNTs.



Scheme 1 (a) Reaction between a thiol-containing cyclic peptide and a pyridyl disulfide-containing polymer to fabricate cyclic peptide–polymer conjugate; (b) Chemical structure and redox-responsiveness of the asymmetric conjugate PEG-CP-S-S-*p*PEGA.

We first evaluated the efficiency of the pyridyl disulfide reaction for polymer–polymer conjugation. To this regard, pyridyl disulfide containing *p*PEGA-PDS (M_n = 9,000 g mol^{-1}) was synthesized by RAFT polymerization using PABTC-PDS as RAFT agent. Methoxy polyethylene glycol thiol (mPEG-SH, M_n = 10,000 g mol^{-1}) was mixed with *p*PEGA-PDS in a molar ratio of 1:1 in DMF (DMSO-*d* for ^{1}H NMR spectroscopy). After 24 h, the reaction mixture was analyzed by HPLC, GPC and ^{1}H NMR spectroscopy. As indicated by HPLC in Figure 1(b), after reaction, a new retention peak at 18.3 min appeared (*p*PEGA-S-S-PEG) while no trace of unreacted *p*PEGA-PDS was observed at 20.0 min, indicating the high efficiency of the pyridyl disulfide reaction. GPC analysis (Figure S16) gave an increased $M_{n,\,GPC}$ of 35,600 g mol^{-1}, while the values for *p*PEGA-PDS and mPEG-SH were 11,500 and 23,600 g mol^{-1}, respectively, further confirming the reaction between *p*PEGA-PDS and mPEG-SH. Finally, the reaction was monitored by ^{1}H NMR spectroscopy. As shown in Figure S17, after reaction, clear shifts were observed at 6 ~ 9 ppm, corresponding to the change from pyridyl disulfide group to pyridine-2-thione during the reaction process. Moreover, the unique absorption spectrum of pyridine-2-thione allows us to monitor the reaction by UV/vis spectroscopy. An absorption band peaked at 375 nm appeared after reaction (Figure 1(c)). The reaction kinetics could be obtained by measuring the UV/vis spectra of the reaction mixture at different time intervals and plotting the absorbance at 375 nm against reaction time. As shown in Figure S18, the reaction underwent completion within 60 min.

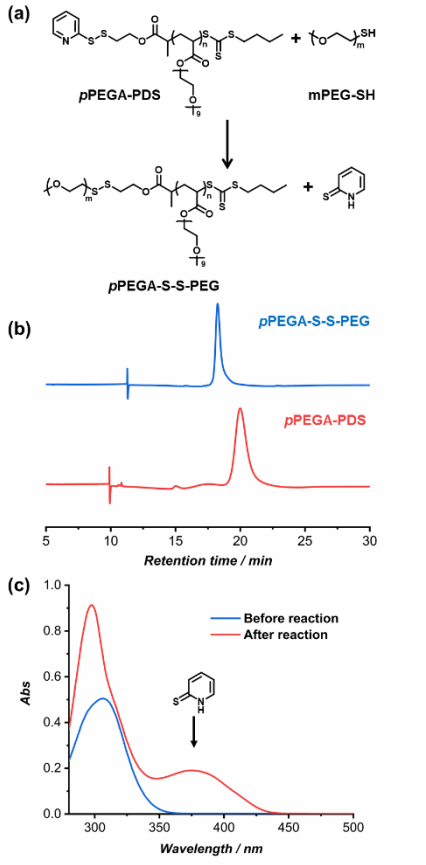


Figure 1 (a) Reaction scheme between *p*PEGA-PDS and mPEG-SH; (b) HPLC spectra for *p*PEGA-PDS and *p*PEGA-S-S-PEG monitored by UV detector at 309 nm; (c) UV/vis spectra of *p*PEGA-PDS and mPEG-SH before and after reaction ([*p*PEGA-PDS]=0.2 mM, [mPEG-SH]=0.4 mM]).

The reaction between thiol-containing cyclic peptide and pyridyl disulfide containing polymers was then investigated. To this end, a linear peptide with the sequence of H_2N-L-Cys(Trt)-D-Leu-L-Trp(Boc)-D-Leu-L-Trp(Boc)-D-Leu-L-Trp(Boc)-D-Leu-COOH was synthesized by solid-phase peptide synthesis using Fmoc-deprotection chemistry. The cyclic peptide CP-SH was then obtained by cyclization reaction of the linear peptide under dilute conditions, followed by deprotection of the protecting groups. Pyridyl disulfide containing polymers, poly (*N*, *N*-dimethyl acrylamide) (*p*DMA-PDS) (M_n = 2,400 g mol^{-1}) and *p*PEGA-PDS (M_n = 9,000 g mol^{-1}), were synthesized by RAFT polymerization using PABTC-PDS as RAFT agent. The reactions between CP-SH and PABTC-PDS, *p*DMA-PDS and *p*PEGA-PDS were monitored by UV/vis spectroscopy, respectively. The reactions underwent completion within 60 min for all cases, showing very good efficiency (Figure 2(b)). Conjugates CP-PABTC, CP-*p*DMA, and CP-*p*PEGA were synthesized by reacting CP-SH and the corresponding PABTC-PDS, *p*DMA-PDS and *p*PEGA-PDS in a molar ratio of 1:1.5 in DMF. After 24 h, the reaction mixture was analyzed by ESI-MS (for CP-PABTC and CP-*p*DMA, Figure S19-20) or HPLC (for CP-*p*PEGA, Figure S21), and no unreacted CP-SH was detected, suggesting the high efficiency of the pyridyl disulfide reaction chemistry. The conjugates were then purified by precipitation to remove unreacted PDS-containing reactants and characterized thoroughly by ESI-MS or GPC (Figure 2(c), 2(d)). This facile and highly efficient pyridyl disulfide reaction chemistry offers a new conjugation strategy towards the synthesis of cyclic peptide–polymer conjugates.

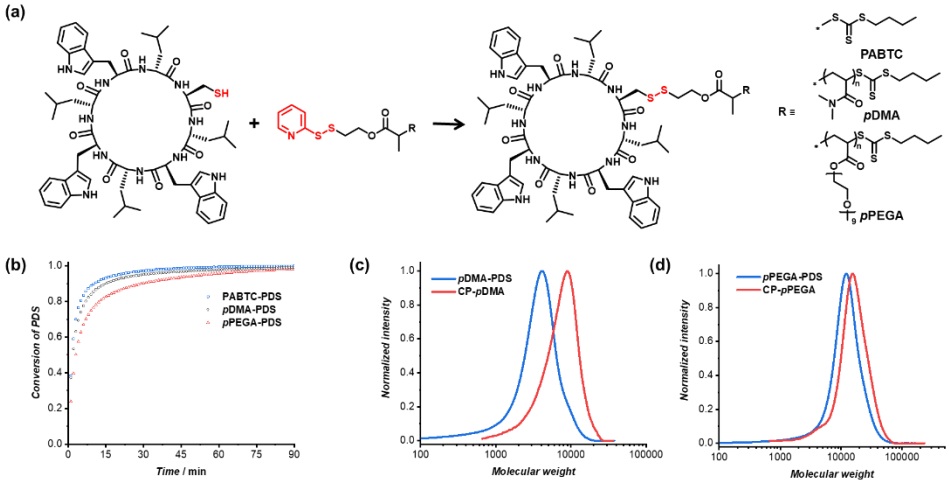


Figure 2 (a) Reaction between CP-SH and PDS containing compounds; (b) Evolution of PDS conversion as a function of time measured by UV/vis spectroscopy ([*p*PEGA-PDS]=0.2 mM, [*p*DMA-PDS]=0.2 mM, [PABTC-PDS]=0.2 mM, [CP-SH]=0.4 mM]); (c) GPC traces (DMF+0.1% LiBr) of *p*DMA-PDS and CP-*p*DMA; (d) GPC traces (DMF+0.1% LiBr) of *p*PEGA-PDS and CP-*p*PEGA.

Considering the few examples of asymmetric cyclic peptide–polymer conjugates,[30-32] we then explored if the presented conjugation chemistry is orthogonal to other conjugation reactions. To this end, an asymmetric cyclic peptide H_2N-CP-SH was designed, to fabricate an asymmetric cyclic peptide–polymer conjugate (PEG-CP-S-S-*p*PEGA). Conjugate H_2N-CP-S-S-*p*PEGA was first synthesized by reacting *p*PEGA-PDS with H_2N-CP-SH in a molar ratio of 1.5:1 in DMF for 24 h, and purified by precipitation in methyl *tert*-butyl ether. From GPC analysis, as shown in Figure 3(a), the conjugate H_2N-CP-S-S-*p*PEGA showed an increased $M_{n,\,GPC}$ of 14,800 g mol^{-1}, while $M_{n,\,GPC}$ of pPEGA-PDS was 10,200 g mol^{-1}.

We tested its orthogonality with NHS coupling chemistry by reacting the conjugate H_2N-CP-S-S-*p*PEGA with mPEG-NHS (M_n = 2,000 g mol^{-1}) to obtain an asymmetric conjugate PEG-CP-S-S-*p*PEGA. The conjugate PEG-CP-S-S-*p*PEGA was synthesized by reacting H_2N-CP-S-S-*p*PEGA with mPEG-NHS in a ratio of 1:1.5 in DMF with the addition of *N*-methylmorpholine as base and purified by dialysis. As shown in Figure 3, GPC analysis of the conjugate PEG-CP-S-S-*p*PEGA gave an increased $M_{n,\,GPC}$ of 19,600 g mol^{-1}, while HPLC analysis showed a decreased retention time of 19.8 min, suggesting that the asymmetric conjugate PEG-CP-S-S-*p*PEGA was successfully synthesized. Moreover, we also showed that PEG-CP-S-S-*p*PEGA could be made by one-pot synthesis (Figure S22), further proving the orthogonality between pyridyl disulfide chemistry and NHS coupling chemistry.

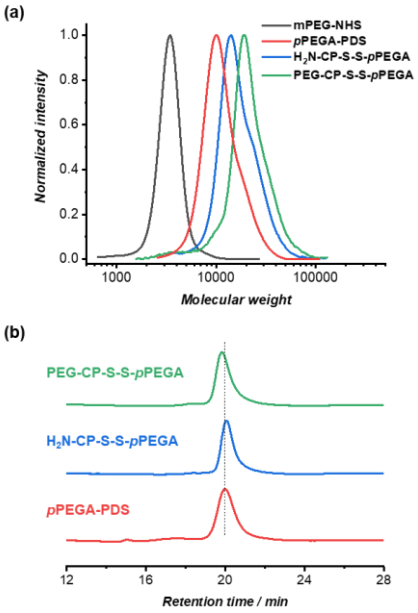


Figure 3 (a) GPC traces (DMF+0.1% LiBr) of mPEG-NHS, *p*PEGA-PDS, H_2N-CP-S-S-*p*PEGA and PEG-CP-S-S-*p*PEGA; (b) HPLC spectra of *p*PEGA-PDS, H_2N-CP-S-S-*p*PEGA and PEG-CP-S-S-*p*PEGA monitored by UV detector at 309 nm.

Another important feature for the pyridyl disulfide conjugation chemistry is that it forms redox-responsive disulfide bond which links the cyclic peptide and polymer. This feature can be exploited on the asymmetric conjugate PEG-CP-S-S-*p*PEGA, which does not assemble into long nanotubes due to the steric hindrance of the brush polymer *p*PEGA.[40] However, once the disulfide linker between cyclic peptide and *p*PEGA is cleaved, the one arm conjugate PEG-CP-SH obtained is expected to form long polymeric nanotubes.

This strategy was illustrated by HPLC and GPC using PEG-CP-S-S-*p*PEGA. Tris(2-carboxyethyl)phosphine (TCEP) was used as reducing agent to break the disulfide bond between cyclic peptide and *p*PEGA, resulting in the formation of PEG-CP-SH and *p*PEGA-SH. As indicated by HPLC in Figure 4, after the treatment with TCEP, two new species with retention time of 16.7 min and 18.6 min were detected, which could be assigned to PEG-CP-SH and *p*PEGA-SH on the basis of their UV/vis spectra. Moreover, GPC analysis showed a decreased molecular weight compared to that of PEG-CP-S-S-*p*PEGA (Figure S23).

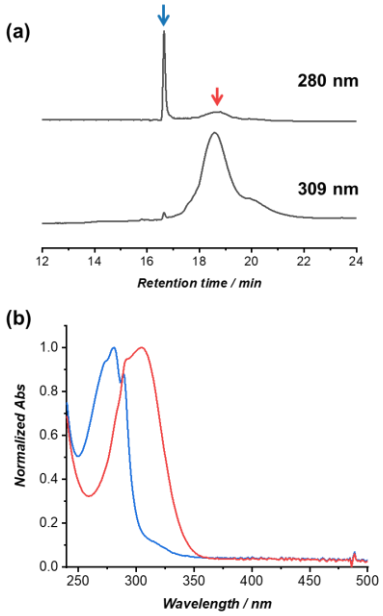


Figure 4 (a) HPLC spectra of PEG-CP-S-S-*p*PEGA after the addition of TCEP monitored by UV detector at 280 nm and 309 nm; (b) UV/vis spectra from HPLC.

The self-assembling properties of the two conjugates PEG-CP-S-S-*p*PEGA and PEG-CP-SH were studied by static light scattering (SLS) and small angle neutron scattering (SANS). SLS experiments were first performed to study the molecular weight (M_a) and number of aggregation (N_{agg}) of both conjugates at different concentrations. The data showed that conjugate PEG-CP-S-S-*p*PEGA formed assemblies with M_a ~ 6.5×10^4 g mol^{-1} and N_{agg} ~ 5, while conjugate PEG-CP-SH formed larger assemblies with M_a ~ 5.8×10^5 g mol^{-1} and N_{agg} ~ 250 (Figure S24). The significant difference in N_{agg} clearly indicated the change of self-assembling structures under the stimuli of reducing agents.

Detailed information about structural parameters of the assemblies in solution was obtained by SANS. Figure 5(a) shows the reduced and corrected scattering data for PEG-CP-S-S-*p*PEGA and PEG-CP-SH conjugates in D_2O. Using the SasView software,[41] the data for both conjugates could be fitted with a core–shell cylinder model,[42-43] thus suggesting they both formed polymeric nanotubes. From the parameters obtained *via* model-dependent structural analysis (Table S1), the average length of the SPNTs formed by PEG-CP-S-S-*p*PEGA is only 8.2 nm, while the average length for PEG-CP-SH is >200 nm (a finite value for SPNT length could not be obtained, as it exceeds observable window for SANS). As a supplementary technique, TEM was used to confirm the formation of long SPNTs (Figure 5(b), Figure S25). Such an increase in length supports our hypothesis that PEG-CP-S-S-*p*PEGA conjugate only forms short nanotubes, but can form long SPNTs when the disulfide linker is cleaved by a reducing agent, resulting in decreased steric hindrance.

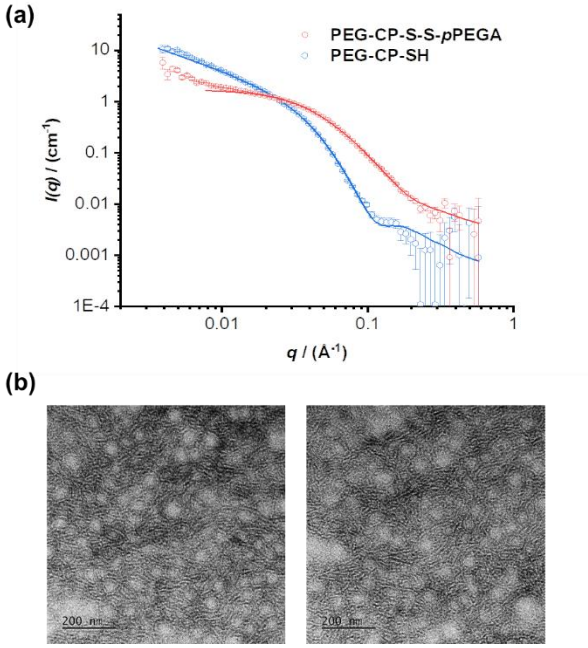


Figure 5 (a) Reduced SANS scattering data for PEG-CP-S-S-*p*PEGA and PEG-CP-SH. The lines correspond to fits to the core–shell cylinder model; (b) TEM images of PEG-CP-SH stained with UOAc.

In conclusion, we have demonstrated the utilization of pyridyl disulfide reaction chemistry to construct cyclic peptide–polymer conjugates. Benefitting from its high efficiency and mild reaction condition, several conjugates were successfully synthesized, including CP-PABTC, CP-*p*DMA and CP-*p*PEGA. The orthogonality was further revealed by synthesizing an asymmetric conjugate PEG-CP-S-S-*p*PEGA, using both pyridyl disulfide reaction chemistry and NHS coupling chemistry. More importantly, the formed disulfide linker between cyclic peptide and polymer endows PEG-CP-S-S-*p*PEGA with redox-responsiveness, leading to the change of self-assembling structures under the stimuli of reductant. It is anticipated that the pyridyl disulfide reaction chemistry will lead to the fabrication of a new family of redox-responsive cyclic peptide-polymer conjugates and SPNTs with tailored structures and functionalities.

ASSOCIATED CONTENT

Supporting Information.
Experimental details and characterization data
This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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