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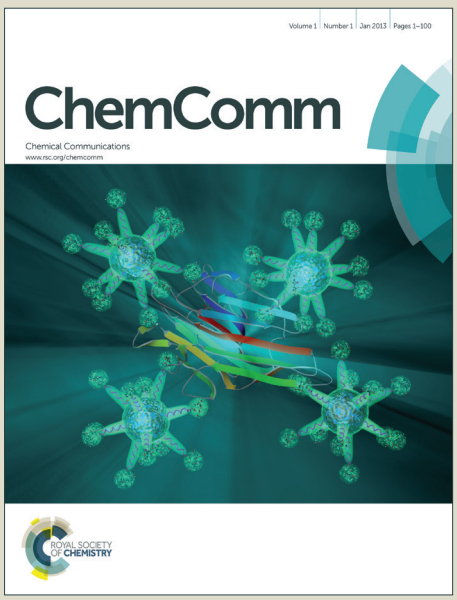

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In Situ Generation of Redox Active Peptides Driven by Selenoester Mediated Native Chemical Ligation

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Redox active peptides are generated through selenoester mediated native chemical ligation (NCL). Distinct nanostructures such as nanotubes to nanofibrillar architectures were observed for self-assembling soft materials.

In nature, complex soft materials are created through hierarchical self-assembly of nanoscale biomolecules.[1] Low molecular weight organic molecules are used in directed self-assembly, which further forms three dimensional network structures. Various functional groups can be incorporated into the molecular structure[2] to mimic and develop the biological processes in the laboratory. However, rational and universal molecular design remained challenge to fit them for biomaterial applications. Self-assembly of short peptides has sought great attention due to their vast applications in drug delivery,[3] tissue engineering[4] and supramolecular electronics.[5] Several physical stimuli[6] such as pH, temperature, light and enzymes are used to explore peptide self-assembly.[7]

Redox active peptide self-assembly is still remained area of interest due to their potential applications in drug delivery. Redox reactions are prevalent in nature to regulate various biological functions. Redox active[8] nature mimicking dynamic self-assembly could be achieved using chemoselective native chemical ligation (NCL) reactions. Peptide self-assembly could be explored through native chemical ligation reaction because (i) this method is orthogonal, (ii) self-assembly through NCL reaction is particularly interesting for the development of controlled dynamic self-assembly and (iii) the presence of free sulfhydryl group in peptides which can offer multiple applications after NCL reactions.

NCL reactions are the most revolutionary method for the total or semi synthesis of proteins.[9] Beside its useful applications in protein synthesis, NCL reactions can be used as effective and efficient methods for the development of soft biomaterials. Collier *et al*. reported an excellent method for the stiffing of a hydrogel via thioester mediated NCL reaction.[10] Messersmith and coworkers described a strategy, which forms covalently cross-linked polymer hydrogels.[11] In general, the conceptual approach for NCL is based on the reaction between two unprotected peptides one bearing C-terminal thioester and another N-terminal cysteine residue based peptide. The sulfhydryl group of N-terminal cysteine residue undergoes trans-thioesterification with C-terminal thioester[12] and forms thioester-linked intermediate. Thioester-linked intermediate simultaneously and rapidly undergoes intramolecular S→N acyl transfer to form native amide bond.[13]

Inspired by this chemoselective ligation reaction,[14] our objective was to develop a simple and efficient approach which can direct dynamic peptide self-assembly. In order to reach our goal, we have synthesized compounds **1-4** with N-terminal capped with aromatic naphthalene-2-methoxycarbonyl (Nmoc) group. The C-terminals of **1-4** is protected with phenyl selenoester, which can readily undergo NCL reaction at room temperature with N-terminal cysteine and N- terminal cysteine based peptide Cys-Gly. Selenoesters are efficient acyl donor and easy to synthesis than the corresponding thioesters due to the better leaving group characteristics of a selenolate versus a thiolate.[15]

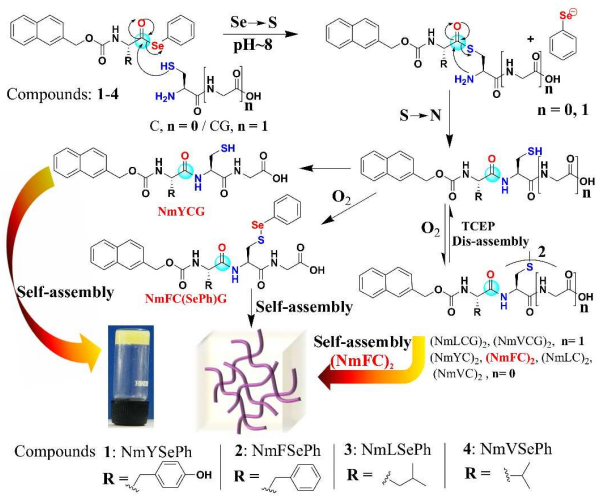

Fig. 1 Selenoester mediated native chemical ligation. Ligated products were formed upon NCL of selenoesters **1-4** with Cys-Gly and cysteine at pH~8. NmYCG self-assembled in its reduced form while oxidized NmFC(SePh)G (sulfur linked with selenophenol) and $(NmFC)_2$ self-assembled to form self-supporting soft materials.

L-cysteinylglycine, a prooxidant is generated from the extracelluer glutathione through the catalytic activity of an enzyme γ-glutamyltransferase.[16] Cys-Gly is known as a highly reactive metabolite which is directly related to induce the

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oxidative stress[17] in the cells. Lin *et al.* reported that higher cysteineglycine level were marginally associated with an increased risk of breast cancer in women.[18] Here, we have used Cys-Gly dipeptide in the development of dynamic peptide self-assembly through NCL reaction. The selenoester easily undergoes NCL reaction with Cys-Gly dipeptide, which could be an assay in further clinical research.

Table 1. Native chemical ligation under inert as well as air conditions

Entry	Substrate	CG[a] and C[b]	Conversion in inert atm.[c] [%]	Conversion in air[c] [%]	Gel[d]
1	**1**	CG	>99 NmYCG	>99 NmYCG	G
2	**2**	CG	22.64/76[e] NmFCG/ NmFC(SePh)G	77.36 NmFC(SePh)G	G
3	**3**	CG	>99 NmLCG	>99 $(NmLCG)_2$	S
4	**4**	CG	65.79 NmVCG	63.35 $(NmVCG)_2$	S
5	**1**	C	>99 NmYC	95.09 $(NmYC)_2$	S
6	**2**	C	94.31NmFC	84.90 $(NmFC)_2$	G
7	**3**	C	>99 NmLC	>99 $(NmLC)_2$	S
8	**4**	C	>99 NmVC	79.28 $(NmVC)_2$	S

[a]CG = Cys-Gly dipeptide, [b]C = Cysteine, [c]The percentage conversions were calculated by integrating the peak areas from HPLC. [d]G = gel, S = solution, Nm = Nmoc. [e]Entry **2** yield in inert atm.: The combined yield for NmFCG and NmFC(SePh)G is 98.64% (22.64+76%).

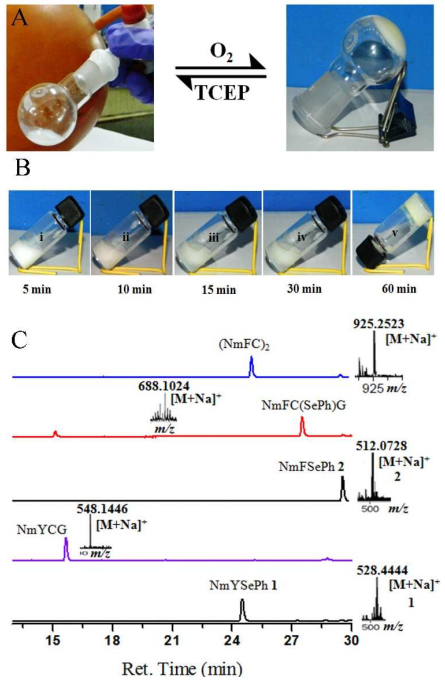

Fig. 2 A) Dynamic self-assembly of $(NmFC)_2$. NCL reaction of compound NmFSePh **2** with cysteine gives NmFC under inert atmosphere and subsequently formed dynamic $(NmFC)_2$ gel upon exposure to air. B) Visual changes of reaction progress initially mixing of compound NmYSePh **1** (20 mmol L^{-1}) with Cys-Gly (20 mmol L^{-1}) in phosphate buffer at pH 8. i) Milky white solution at 5 min, (ii) turbid at 10 min, (iii) colorless solution at 15 min, (iv) colorless viscous solution at 30 min and v) self-supporting gel formed after 60 min. C) HPLC trace analysis of a representative ligation of compound **1** with Cys-Gly with corresponding ESI-MS of ligated product after 1h and **2** with Cys-Gly and Cysteine after 1 h with corresponding ESI-MS of ligated products.

The NCL reaction proceeds through thioester-linked intermediate where acyl transfers from Se→S[15] followed by intramolecular S→N acyl transfer to give a peptide bond (Fig. 1). Compounds **1-4** (20 mmol L^{-1}) were dissolved in 100 µL of ethanol and Cys or Cys-Gly (20 mmol L^{-1}) was dissolved in 900 µL of phosphate buffer (pH = 8, 100 mmol L^{-1}). 900 µL of Cys (C) or Cys-Gly (CG) was added to the reaction vial containing compounds **1** to **4** (entries 1-8). The reaction vial was allowed to leave undisturbed and self-supporting gels were observed after 1 h of reaction for entries **1**, **2** and **6**. The ligation reactions of Nmoc (Nm) capped amino acid based selenoesters with Cys or Cys-Gly was monitored with reverse phase high performance liquid chromatography (HPLC) and the corresponding products were analyzed by ESI-MS (Fig. 2C and S4-S30). We also monitored the visual changes of solution **1** upon mixing with dipeptide Cys-Gly in phosphate buffer (Fig. 2B). The gels were stable towards the pH range of 4-10 and the minimum gelation concentration for NmYCG, NmFC(SePh)G and $(NmFC)_2$ gels was 5 mmol L^{-1}. Gel melting temperature (T_{gel}) was also measured by test tube inversion method at different concentrations of gelators. T_{gel} was observed as 70 °C, 71 °C and 65 °C for NmYCG, NmFC(SePh)G and $(NmFC)_2$ gels at 20 mmol L^{-1} (Table S1) concentration.

The self-assembly of small peptides bearing N-terminal naphthalene moieties has been reported by several groups.[19] The compound **1** gives >99% ligated peptide with Cys-Gly and similarly gives >99% with cysteine (Table 1). Thus, NCL reaction with selenoesters is the easiest and simplest way to synthesis peptides. However, the thioesters with similar amino acid sequences (compounds **5-8**) yielded poor conversion (Fig. S2-S3) of peptides (Table S2) and were unable to form gel under similar conditions. The control experiment with Nmoc capped valine selenoester **3** with alanine confirms the requirement of C-terminal cysteine group in ligation reaction with selenoester (ESI).

Typically, NCL reactions were carried out in presence of reductant such as *tris*(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT). TCEP or DTT helps to avoid the formation of disulfides. Surprisingly, we have not used any reductant for the reaction of compound **1** with Cys-Gly (entry 1). The reduced form of NmYCG was observed after 16 h in aerobic condition (Fig. S5).[20] However, entries **3-8** showed exactly reverse behavior to entry **1**. Oxidized forms of NCL products were observed for entries **3-8** in similar conditions to NCL reaction of entry **1** (Table 1). A self-supporting gel for entry **1** was observed with 99% synthesized peptide NmYCG. Another self-supporting gel for entry **6** was also observed for the newly synthesized 84.9 % oxidized peptide $(NmFC)_2$. However, in case of entry **2**, a dipeptide Cys-Gly reacted with compound **2** and formed the corresponding tripeptide NmFCG. NmFCG further reacted with cleaved selenophenol and formed sulfur-selenium bond (Fig. 1). The resulting product NmFC(SePh)G turned to a self-supporting gel. Our data indicates that reduced and oxidized form of ligated peptides can self-assemble and lead to self-supporting gels.[21] The self-assembly of ligated products drives self-selection and self-organization into reduced and oxidized form of peptides.

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The dis-assembly phenomenon of $(NmFC)_2$ was observed using a reductant TCEP (40 mmol L^{-1}) after 2 h which further explores gelators as versatile candidates for drug delivery (Fig. 2A). The dynamic gel-sol reversal behaviour of $(NmFC)_2$ was monitored by HPLC and rheology (Fig. 3A and S37). The HPLC analysis of $(NmFC)_2$ after addition of TCEP showed 87% conversion of NmFC after 6 h of reaction. The rheology experiment showed that the gel starts to break after 1 h of TCEP addition. Gel formation for $(NmFC)_2$ upon NCL reaction was indicated by time sweep experiment (Fig. S36). The disulfide reduction leads to gel-sol transition of $(NmFC)_2$, which was also monitored with oscillatory time sweep experiment after addition of TCEP (Fig. S37).

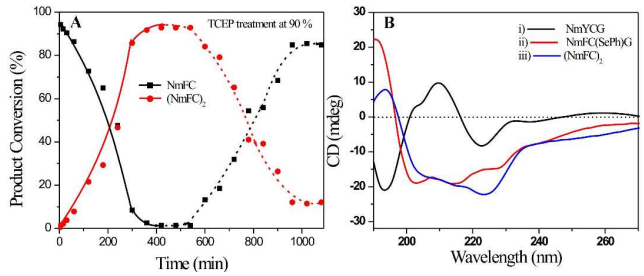

Fig. 3 A) Real time HPLC analysis for $(NmFC)_2$ formation upon exposure to air followed by addition of TCEP at 90% showed converting back into reduced form of NmFC. B) CD spectra of self-assembled peptides (i) NmYCG, (ii) NmFC(SePh)G and (iii) $(NmFC)_2$ (concentration = 2 mmol L^{-1}) formed upon NCL reaction at pH~8.

Circular dichroism (CD) was used to elucidate peptide conformation in gel phase medium (Fig. 3B). The CD spectrum of NmYCG (entry 1) shows a positive peak at 208 nm and a negative peak at 221 nm corresponding to the co-existence of random-coil and β-sheet conformation.[22] However, CD spectra of NmFC(SePh)G and $(NmFC)_2$ in gel state show characteristics twisted conformation. The two negative peaks at 222 nm and 205 nm indicate twisted conformation of self-assembled peptides which may be attributed from the supramolecular ordering of peptide molecules.[23] The CD spectrum of gel $(NmFC)_2$ upon treatment with TCEP indicates the change in conformation of the reduced dis-assembled peptide (Fig. S38). Fourier transform infrared spectroscopy (FTIR) was also used to support the secondary structures of self-assembled peptides (Table S3). The ligated peptide NmYCG shows peaks at 1640 cm^{-1} and 1688 cm^{-1}, which correspond to turn type structures.

Fluorescence spectra were recorded to understand molecular arrangement of NmYCG, NmFC(SePh)G and $(NmFC)_2$ in gel phase. The characteristic emission peak for naphthalene double rings and aromatic side chains appears in the range of 310-330 nm in solution (Fig. S40). NmYCG gel showed emission peak at 337 nm and a broad emission peak at 461 nm indicating efficient overlapping of naphthalene double rings and higher order π-π stacking aggregation in gel phase medium (Fig. S41). Similarly, NmFC(SePh)G and $(NmFC)_2$ gels showed emission peak at 337 nm and 334 nm respectively. Broad emission peak at 465 nm suggests higher order aggregate state for NmFC(SePh)G gel (Fig. S41). Here, aromatic naphthalene ring plays pivotal role in self-assembly process via π-π stacking interactions.

Wide angle powder X-ray diffraction studies were performed to examine the self-assembly of NmYCG, NmFC(SePh)G and $(NmFC)_2$ in gel phase (Fig. S42-S44). The diffraction peak appeared at 2θ = 18.13°, 19.53°, 17.99° with corresponding *d* spacing of 4.88 Å, 4.5 Å and 4.9 Å for NmYCG, NmFC(SePh)G, $(NmFC)_2$ dried gels respectively. These values indicate the spacing between two peptides within the β-sheet structures. The reflection peak appeared at 2θ = 23.14°, 24.46°, 24.16 with corresponding *d* spacing values of 3.84 Å, 3.64 Å, 3.68 Å for dried gels of NmYCG, NmFC(SePh)G and $(NmFC)_2$ respectively.[24] These values clearly show the π-π stacking interaction among the self-assembled peptides. The self-assembly mechanism is shown in Fig. S31.

Time resolved fluorescence study was acquired to investigate the higher order aggregation of the fluorophore groups (Nmoc) of NmYCG, NmFC(SePh)G and $(NmFC)_2$ gels. We measured fluorescence decay traces of the gels at an excitation of 376 nm and the emission was monitored at 470 nm. The average lifetime 1.14 ns of NmYCG, 3.39 ns of NmFC(SePh)G and 1.06 ns of $(NmFC)_2$ were observed (Table S4, Fig. S45). The average fluorescence lifetime of the gel samples indicates a more dense aggregated nanofibrous network in the gel state.

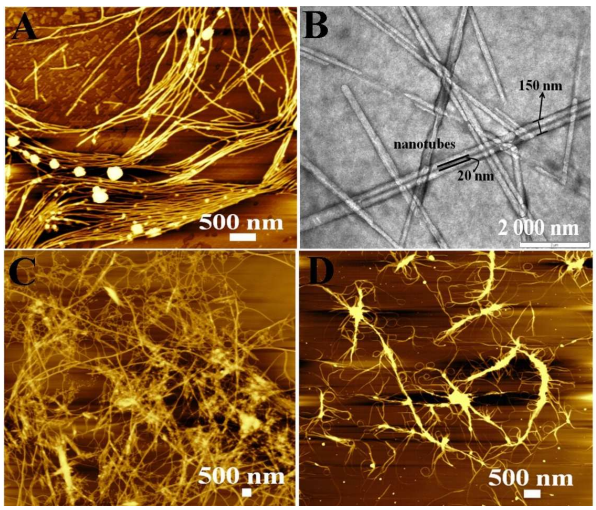

Fig. 4 AFM images indicate nanofibrillar structures of self-assembled gels of A) NmYCG, C) NmFC(SePh)G and D) $(NmFC)_2$. B) TEM image indicates nanotubular structure of self-assembled gels NmYCG with inner diameter of 20 nm and outer diameter of 150 nm.

Transmission electron microscopy (TEM) was used to characterize the morphology of the self-assembled architectures in gel phase. The nanotubular[25] structures were observed for a gel formed by NmYCG (entry **1**) with average diameter of 150 nm. The average inner diameter of nanotubes was observed as 20 nm in TEM image (Fig. 4B). However, gels formed by NmFC(SePh)G (entry **2**) and $(NmFC)_2$ (entry **6**) showed entangled nanofibrillar networks with the diameter ranging from 10 to 60 nm (Fig. S47). The atomic force microscopy (AFM) studies of gels also showed nanofibrillar morphology (Fig. 4). The NmYCG gel exhibited highly aligned nanostructures in gel phase medium with the height of 3 to 6 nm (Fig. 4A). However, the gel formed by NmFc(SePh)G showed elongated thin nanofibrous morphology with average width of 2 nm. The AFM

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analysis of $(NmFC)_2$ indicated that nanofibers originated from thick fibers. The AFM result for gel-sol transition indicates the breaking of nanofibers which leads to dis-assembly of self-assembled gel $(NmFC)_2$ (Fig. S46).

In summary, we have demonstrated selenoester mediated native chemical ligation to explore dynamic redox active peptide self-assembly. Selenoester mediated native chemical ligation is a rapid ligation process over thioester mediated native chemical ligation. Cysteine and Cys-Gly peptide have provided mechanistic insight into the NCL driven self-assembly process. We have also shown the redox active dynamic peptide gels, which are formed via oxidation and reduction of the Nmoc-protected peptide synthesized via NCL reactions. Here, gel-sol transition is highly dependent on the oxidation and reduction of cysteine and cystine based peptides. Peptides are self-assembled via hydrogen bonding and π-π stacking interactions and peptides are redox active in nature. Here, selenoester mediated NCL reaction are responsible for nanofibrillar structure.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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