Experimental Details.

Sequence Design

The sequences used were adapted from a walker system,[1] and modified using program SEQUIN[2] to minimize sequence symmetry when necessary.

Synthesis and purification of DNA

Unmodified oligonucleotides were purchased from Integrated DNA Technology (Coralville, IA). Purification of single strand DNA was performed via 10-20% denaturing polyacrylamide gel electrophoresis (PAGE). Targeted bands were cut and subjected to an elution buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA at 4 ℃ overnight. The eluates were then extracted with n-butanol to remove the ethidium bromide (EB), and the DNA was recovered by ethanol precipitation.

Modified oligonucleotides were synthesized on an Applied Biosystems 394 synthesizer, removed from the support, and deprotected using routine phosphoramidite chemistry.[3] The OPeC™ reagent package, including pentafluorophenyl S-benzyl-thiosuccinate (PMR) and O-trans-4-(N-Fmoc-S-tert-butylsulfenyl-l-cysteinyl) amino-cyclohexyl O-2-cyanoethyl-N,N-diisopropyl-phosphoramidite (OMR), was purchased from Glen Research (Sterling, VA) and used to modify strands. The OMR was used in the final coupling step in standard phosphoramidite solid-phase oligonucleotide assembly to make 5'-S-tert-butylsulfenyl-L-cysteinyl functionalized oligonucleotide, S2-Cys-S-tBu.[4] The crude S2-Cys-S-tBu was purified by HPLC and the target

fraction was lyophilized overnight.

The thioester modified strand, S1-thioester, was synthesized as shown in Figure S1. To 120 μL of 200 mM PMR in anhydrous acetonitrile (MeCN), 100 μL of 200 mM N-hydroxysuccinimide (NHS) in MeCN was added, and the mixture was incubated at room temperature (RT) for 30 min. To 100 μL of PMR-NHS solution, 100 nmole of HPLC-purified amino-S1 in 480 μL of 1X PBS (pH 7.4) and 20 μL of MeCN were added. The mixture was incubated overnight and the resulting S1-thioester strand was purified by HPLC. The target fraction was lyophilized.[5]

The 4-mercaptophenylacetic acid (MPAA) modified strand, S1-MPAA, was synthesized as shown in Figure S2. The quantity 86.0 mg of MPAA was dissolved in 410 μL of H_2O and 102 μL of 5 M NaOH was added to adjust the pH to about 7. The MPAA solution was left in the air for 2 weeks and doubly deionized water (DD water) was added every day to maintain the volume of the solution. Dimerization of MPAA was confirmed by thin layer chromatography (TLC), eluting with 95:5 v/v dichloromethane (DCM)/methanol (MeOH). The MPAA dimer ($MPAA_2$) was redissolved in DD water to 1 M. To 160.5 μL of 373.86 μM amino-S1 in DD water, 75 μL of 1 M $MPAA_2$, 375 μL of 200 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 22 mM hydroxybenzotriazole (HOBt) in DD water, 75 μL 1 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7) and 64.5 μL H_2O were added. The reaction mixture was incubated at RT for 2.5 h, and then washed with DD water in Amicon Ultra centrifugal filters (NMWL 3 kDa, AUC-3) to get rid of excess reagents. The crude S1-$MPAA_2$ was purified with 20%

denaturing PAGE, following the same protocol as regular strands. Prior to annealing with S0 and S2-Cys-tBu, 100 μL of 800 μM S1-$MPAA_2$ in annealing buffer (40 mM Tris-HCl, pH 8.0, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate, TAE) was mixed with 25 μL of 0.5 M dithiothreitol (DTT), incubated at RT for 3 h and washed 5 times with DD water in AUC-3 to get rid of excess DTT and cleaved MPAA to yield ready-to-use S1-MPAA.

In all cases, the concentration of DNA was determined by OD_{260}.

Sample preparation of oligonucleotides prior to MALDI-TOF using ZipTipC_{18} pipette tips

The quantity 100-200 ng of oligonucleotide was dissolved in 10 μL of the equilibration solution, 0.1 M triethylammonium acetate (TEAA), pH 7.0. ZiptipC_{18} from Millipore (Billerica, MA) was used to desalt the sample and remove impurities.[6] The purified oligonucleotides were eluted with 10 μL matrix from ZiptipC_{18} and directed deposit on the MALDI target. Matrix composition was 9 parts of 50 mg/mL 3-hydroxypicolinic acid in 50% MeCN 50% H_2O, and 1 part of 50 mg/mL ammonium citrate in DD water.

Formation of the 3 strand system for templated ligation

The complex was formed by annealing a mixture of stoichiometric quantity of each component strand to the concentration of 1 μM in TAE buffer. Annealing was initiated at 45 ℃ for 20 min, followed by 37 ℃ for 20 min and room temperature for

20 min.

For native chemical ligation with tris (2-carboxyethyl) phosphine (TCEP) cleavage, annealing was followed by addition of 20 mM TCEP and reaction for 20 min to fully reduce the disulfide bond on S2-Cys-S-tBu. The reaction was carried out with or without addition of exogenous thiols, at 37 ℃ or RT, and for the desired time duration. The reaction was filtered by G25 column (GE healthcare, Little Chalfont, UK) to remove the thiols before it was subjected to gel electrophoresis or MALDI analysis. The best yield (~90%) obtained was with S0-42mer (with a 2-T spacer) or S0-43mer (with a 3-T spacer), 1% v/v thiophenol at 37 ℃, and reacting for 72 h (Figure 2). For TCEP-free reaction, annealing was followed by addition of exogenous thiol, and the reaction was incubated at RT for the desired time duration. The obtained yield was about 87%, from the 3-h reaction with S0-42mer (with a 2-T spacer), 1% v/v thiophenol at RT (Figure 5).

For the disulfide formation system, annealing was followed by incubating the complex solution at RT for 24 h, followed by characterization. The yield was about 94% from the reaction with S0-40mer (no spacer) and 8mer S2'-Cys-S-tBu (Figure 6).

Calculation of templated native chemical ligation yield

EB stained denaturing gel was scanned using a Kodak Image Station. The intensity of each band was measured with GelQuant.NET software (provided by biochemlabsolutions.com). When the two starting strands were distinguished on the gel, the yield was estimated by the formula:

$$\text{yield} = \frac{\text{intensity of product} \times 0.5}{\text{intensity of product} \times 0.5 + \text{intensity of lighter starting strand}} * 100\%$$

When the two starting strands were not distinguished on the gel, the yield was estimated by the formula:

$$\text{yield} = \frac{\text{intensity of conjugate product}}{\text{intensity of conjugate product} + \text{intensity of starting strand}} * 100\%$$

Dithiothreitol (DTT) treatment of the conjugation product with thiols

Dithiothreitol was dissolved in 1X PBS buffer to make 200 mM stock solution. The quantity 100~200 ng oligonucleotide was dissolved in 10 μL of 200 mM DTT solution and incubated at 37 ℃ for 2 hours. ZiptipC_{18} was used to prepare the sample for MALDI-TOF MS.

Denaturing Polyacrylamide Gel Electrophoresis

The sample was dried, followed by addition of 10 μL solution containing 10 mM NaOH, 1 mM EDTA, and 0.1% each of bromophenol blue and xylene cyanol FF tracking dye. The sample tube was vortexed to fully dissolve the DNA; it was run on a denaturing polyacrylamide gel at 55 ℃. The gel contained 8.3 M urea and 10%-20% acrylamide (19:1 acrylamide: bisacrylamide). The running buffer consisted of 89 mM Tris (pH 8.0), 89 mM boric acid, and 2 mM EDTA (TBE). Gels were run on a Hoefer SE 600 electrophoresis unit at 50 V/cm constant powers. Following electrophoresis, the gel was stained with stains-all dye (0.01% strains-all from Sigma, 45% formamide) for 5 minutes. Excess dye was washed away with tap water. The dyed DNA bands on the gel are scanned with an Epson perfection 1660 scanner.

Otherwise, the gel was stained with ethidium bromide for 5 min and scanned by Kodak Image Station.

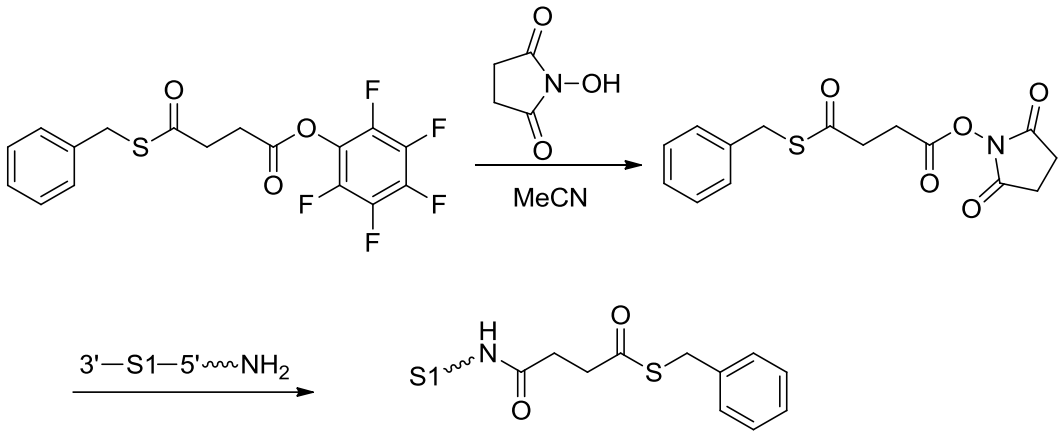

Figure S1. Synthetic route for preparation of S1-thioester (S1_1).

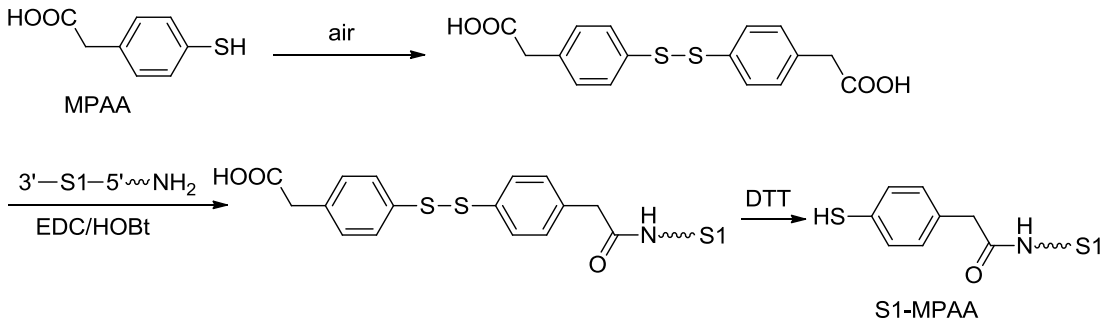

Figure S2. Synthetic route for preparation of S1-MPAA (S1_2).

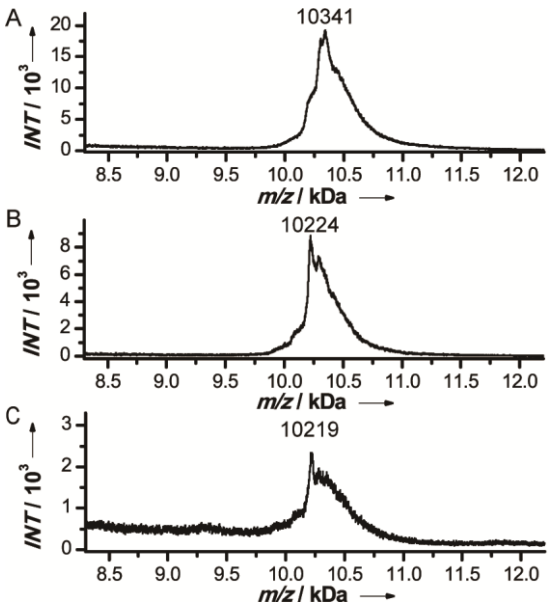

Figure S3. MALDI-TOF spectra of NCL conjugates. (A) Conjugation product after 24h reaction at 37 ℃ with 1% v/v thiophenol: *m/e* calculated (S1_1 - S2 - PhSH, M-H^+): 10321, found: 10341; (B) conjugation product after 24h reaction at 37 ℃ with 1% v/v thiophenol and treated with DTT for 2 h: *m/e* calculated (S1_1 - S2, M-H^+): 10213, found: 10224; (C) conjugation product after 3h reaction at 37 ℃ with no thiol added: *m/e* calculated (S1_1 - S2, M-H^+): 10213, found: 10219. The resolution at 10 kDa is about 100 Da.

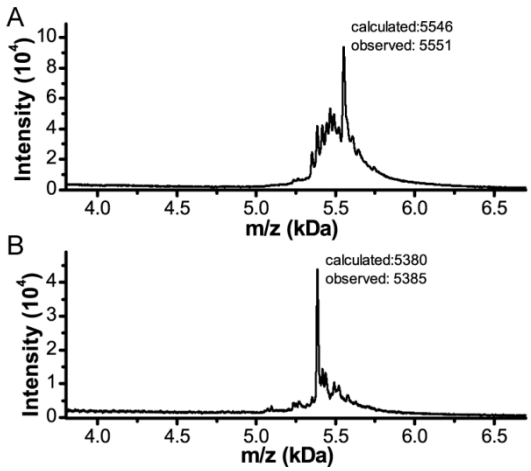

Figure S4. MALDI-TOF MS of conjugation product from reactions with S1'-thioester (S1'_1) and S2'-Cys (S2') after 21 h reaction at RT with 200 mM MPAA. (A) before DTT treatment; (B) after DTT treatment.

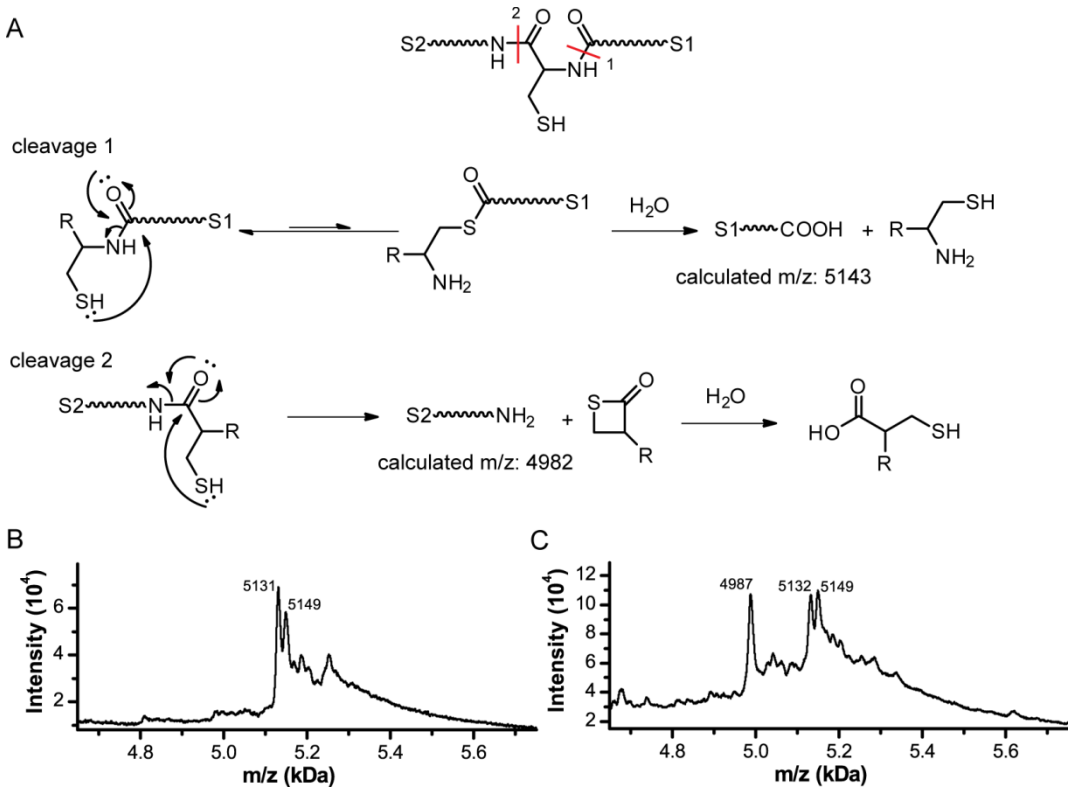

Figure S5. Decomposition of uncapped NCL product. (A) Proposed cleavage of uncapped NCL product. The cleavage 1 is the reverse NCL and yields S1-COOH, which is identical to the hydrolyzed product of S1-thioester. The cleavage 2 involves a 4-member-ring intermediate, which may be rare but similar intermediates were reported.[7] (B) MALDI-TOF spectrum for the reaction mixture of S0, 16mer S1-thioester (S1_1), and 16mer S2-Cys (S2) in the presence of 1% v/v thiophenol after 24-h incubation. (C) MALDI-TOF spectrum for the reaction mixture of S0, S1_1, and S2 in the absence of any exogenous thiol after 24-h incubation. Peak at 5149 may be a result of hydrolysis of S1-thioester, or from cleavage 1. Peak at 4987 corresponds to the product from cleavage 2.

Supporting references

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[5] S. Takeda, S. Tsukiji, H. Ueda, T. Nagamune, *Org. Biomol. Chem.* **2008**, *6*, 2187-2194.

[6] Protocol: Sample Preparation of Oligonucleotides Prior to MALDI-TOF MS Using $ZipTip_{C18}$ and $ZipTip_{\mu\text{-}C18}$ Pipette Tips. http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/3992c159e1f6b98b852575de0063313c/$FILE/TN225.pdf. (accessed 10/01/2010).

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