Electronic Supplementary Information

The Methionase Chain Reaction: An enzyme-based autocatalytic amplification system for the detection of thiols

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Methods

Materials

DNA encoding Methionine gamma-lyase with a C-terminal 6x His tag was synthesized by Thermo Fisher Scientific (Waltham, MA, USA). Additionally, B-PER bacterial lysis reagent, Slide-A-Lyzer dialysis cassettes, and a BCA Protein Assay Kit were purchased from Thermo Fisher Scientific. A Gibson Cloning Kit and Phusion DNA Polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Plasmid spin miniprep and PCR purification kits were purchased from Qiagen (Venlo, Netherlands). HisTrap Nickel Affinity columns were purchased from GE Healthcare (Chicago, IL, USA). Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) was purchased from Chem-Impex Int'l. Inc (Wood Dale, IL, USA). DTT (dithiothreitol) was purchased from Fisher Bioreagents (Waltham, MA, USA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Flash chromatography was performed using SiliaFlash G60 silica (70-230 mesh) purchased from SiliCycle (Quebec, Canada). NMR spectra were recorded in deuterated methanol or chloroform on a Bruker Avance 400 console with an Oxford Instruments 9.4 T magnet. Absorbance was measured on a Tecan Infinite M200 microplate reader. Modelling work was done using MATLAB, a product of MathWorks (Natick, MA, USA). Mass spec data was gathered at the QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley (Berkeley, CA).

Cloning

PCR was used to linearize the vector pET29-b and add 5’ and 3’ overhang regions matching the 3’ and 5’ end, respectively, of the MGL DNA. The vector and gene were assembled via Gibson Assembly. Proper plasmid construction was confirmed via Sanger sequencing, and the plasmid was transformed into BL21 *E. coli* cells for expression.

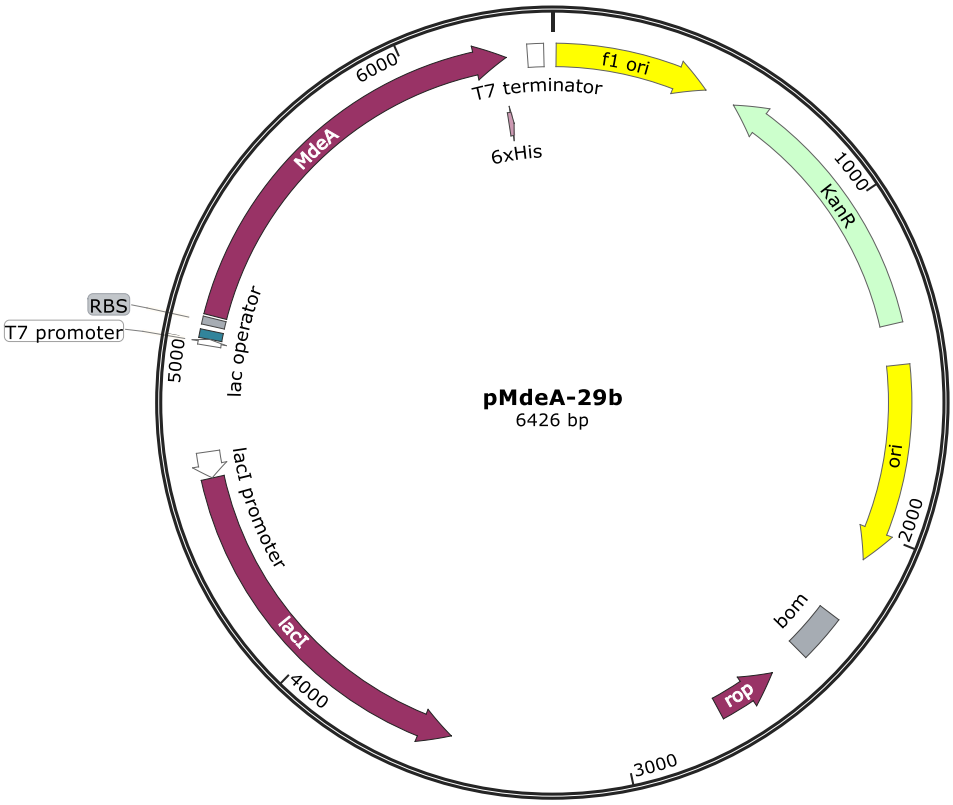


Fig. S1. MGL Expression Vector Map

Protein Expression and Purification

MGL was expressed in 2xYT media. MGL expression was induced in mid-exponential phase of cell growth (A600 ~0.5) by the addition of 0.3 mM IPTG. The cell pellet was harvested after 4 hours of growth post-induction at 37 ºC. Cells were lysed using B-PER bacterial lysis reagent following the manufacturer's instruction. MGL was purified from the soluble fraction of the cell lysate via a nickel-NTA column. The buffer was exchanged to MGL Buffer (PBS, 1 mM EDTA, 10 µM PLP, 5% glycerol) by dialysis. MGL purification was confirmed via SDS-PAGE. The concentration was measured using the BCA assay against known BSA standards. Protein activity was confirmed by the assay described below.

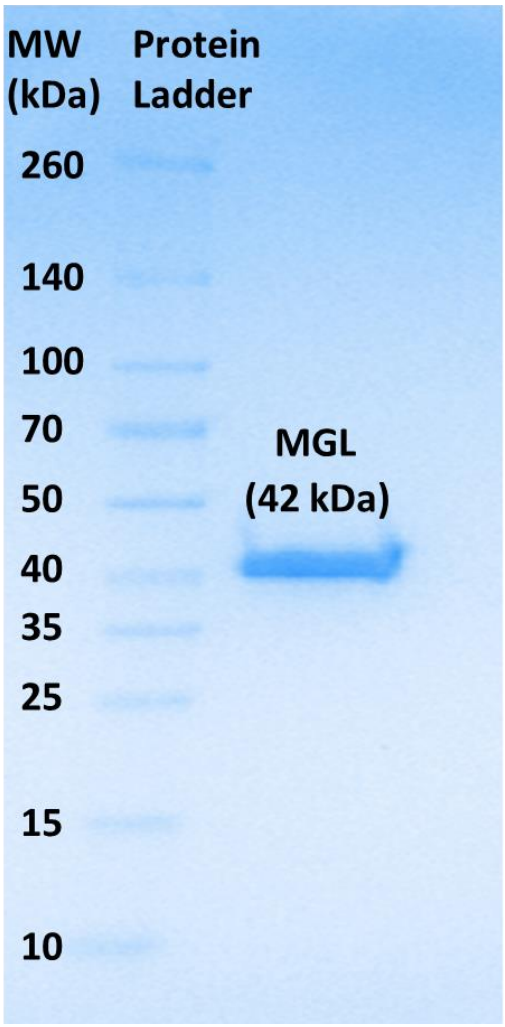


Fig. S2. MGL Expression SDS-PAGE

Methionase Activity Assay

Variable amounts of enzyme and methionine were added to PCR tubes, and the total volume was adjusted to 100 uL total volume with PBS at pH 8.0. The reaction was carried out at 37 ºC. Total thiol content was then measured using DTNB (Ellman's Reagent). The addition of DTNB quenches the reaction. The absorbance was measured at 412 nm and was compared to a cysteine standard curve to determine methanethiol content. All assays were carried out in triplicate for a given set of conditions.

The amount of methanethiol produced by 100 nM MGL in five minutes was used to estimate the initial rate of the enzymatic reaction. The assay was carried out over a range of initial methionine concentrations (0.2 mM to 10 mM). Lineweaver-Burke analysis was used to determine the kcat and Km to be 5.1 s^{-1} and 570 uM respectively.

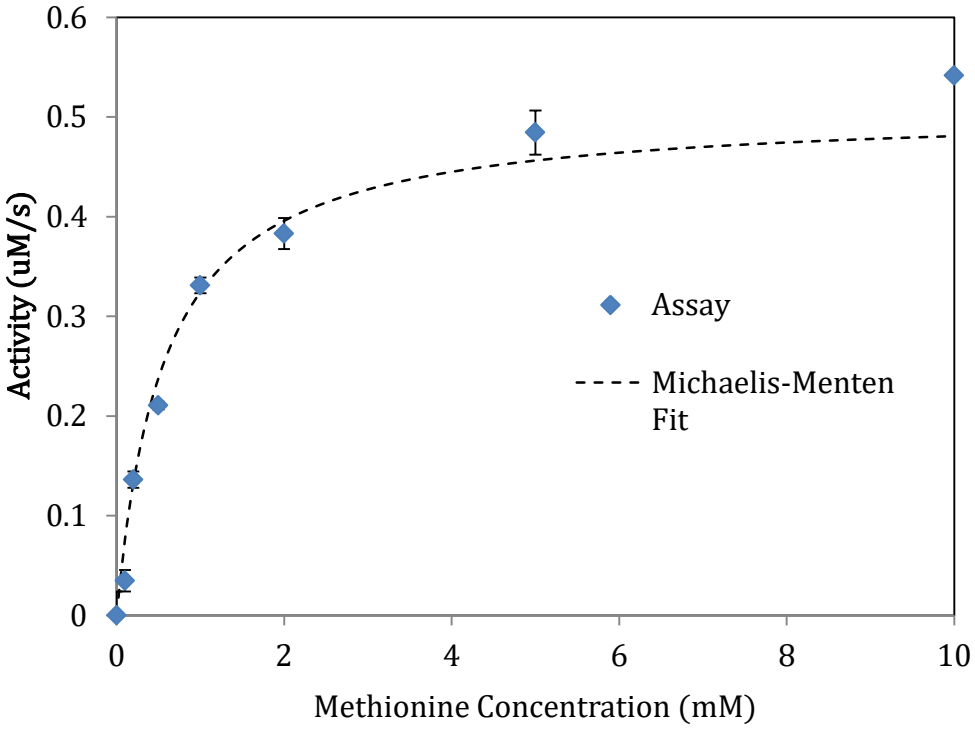


Fig. S3. MGL Activity Assay

Synthesis of MGL*

1 mL of a 24 µM MGL solution in MGL Buffer was mixed with 250 µL of 2 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) and left to react for 1 hour on ice. The mixture was then dialyzed (10k MWCO) against MGL buffer for 16 hours at 4 ºC with buffer exchanges every 4 hours. A 500:1 buffer volume to reaction mixture was used. MGL* was stored at 4 ºC for near term use (1-3 days) or was mixed into a 50% glycerol solution and stored at -20 ºC for up to a month.

Mass Spectrometry Analysis of MGL*

In order to determine the degree of modification on MGL, MGL* was analyzed using intact-mass LC-MS and compared to unmodified MGL. This revealed that MGL* is a mixture of mono- and di-modified MGL. Furthermore, the crystal structure of MGL (UniProtKB#: P13254) shows that of the four cysteines present on MGL, only two are surface exposed (Cys49 and Cys116). The other two (Cys190 and Cys270) are buried in the hydrophobic core, and therefore inaccessible to modification. The two possible modification sites are therefore Cys49 and Cys116, with complete modification of Cys116, as confirmed by the lack of enzymatic activity of MGL*. This is also confirmed by previous studies[1,2] on the modification of MGL using Ellman's reagent, which shows that Cys116 is preferably modified over Cys49.

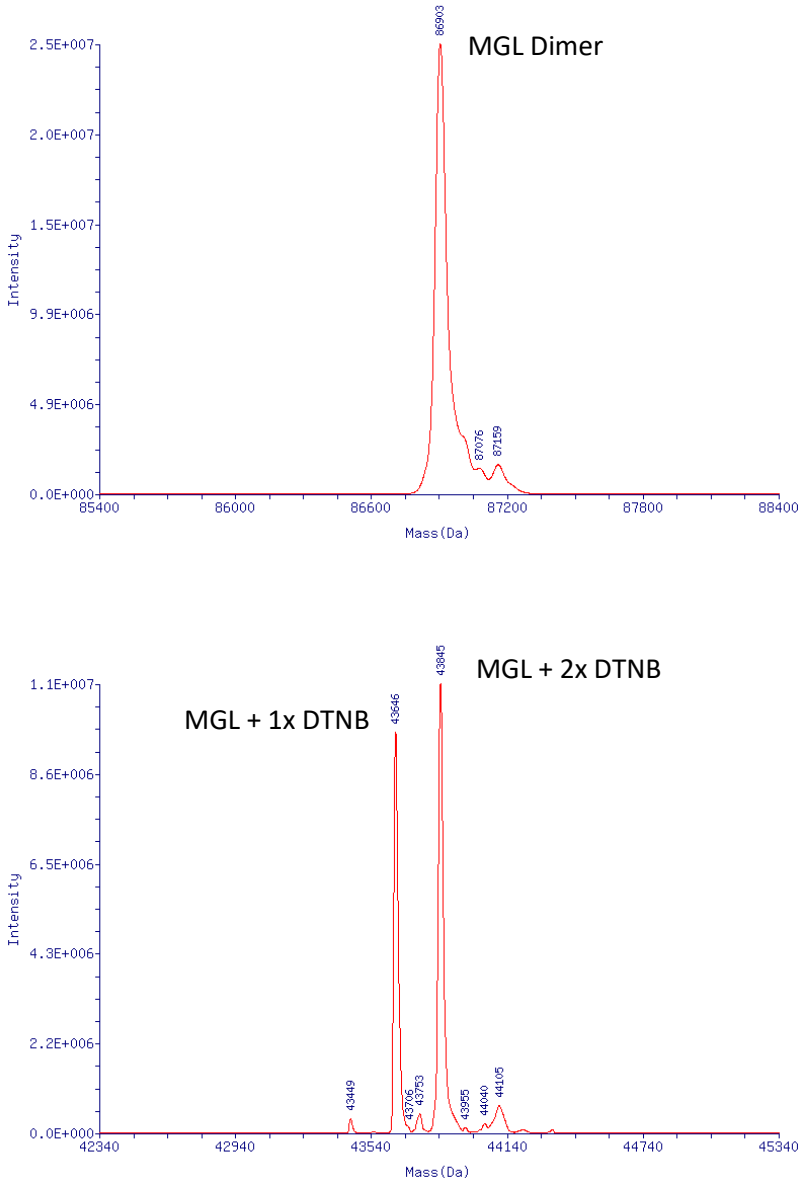


Figure S4: Mass Spectrum of unmodified MGL (above) and MGL covalently modified with DTNB (below)

Autocatalytic System Model

Kinetic Model

Our kinetic model of the autocatalytic MGL system consists of four coupled differential equations relating four quantities: the concentration of methionine, concentration of free thiol, concentration of caged MGL, and the concentration of free MGL.

$$\frac{d[P]}{dt} = k_{cat}[E]\frac{[S]}{K_m + [S]} - k_2[E*][P]$$

$$\frac{d[S]}{dt} = -k_{cat}[E]\frac{[S]}{K_m + [S]}$$

$$\frac{d[E]}{dt} = -\frac{d[E*]}{dt} = k_2[E*][P]$$

[P]= concentration of free thiol
[E]= concentration of free MGL
[E*]= concentration of caged MGL
[S]= concentration of methionine

k_{cat} is the turnover number of MGL for methionine
K_m is the Michaelis-Menten constrant
k_2 is the second-order rate constant for the thiol-disulfide exchange between MGL* and methanethiol

Our kinetic model makes the following assumptions:

1) The enzymatic reaction converting methionine to methanethiol follows classic Michaelis-Menten kinetics.
2) The thiol-disulfide exchange reaction between methanethiol and caged MGL follows second-order kinetics.
3) The rate of thiol-disulfide exchange between methanethiol and caged MGL is comparable to the rate of exchange between DTT and caged MGL.
4) Only uncaged MGL is capable of turning over methionine.

Parameter Estimation

Our kinetic model depends on knowledge of three key parameters described above: K_m, k_{cat}, and k_2. We estimated the 2nd-order thiol-disulfide reaction rate from the empirical value of the activation efficiency η_{act}. We solved for a rate constant k_2 such that the extent of reaction after 30 minutes is equal to 6% of the initial concentration of thiol. We determined the rate constant to be

70 $M^{-1}s^{-1}$. This is a reasonable order of magnitude for a thiol disulfide exchange, based on the pKa of methanethiol and TNB^{2-} (10.4 and 4.38).[3]

Although we could measure the value of k_{cat} from out in vitro kinetic assay, we observed a difference in reaction rates between freshly expressed MGL and that which has undergone treatment to create MGL* and is later reactivated. This is reasonable, as the caging reaction and subsequent dialysis requires the protein to be kept in solution for around 24 hours. Therefore, the k_{cat} observed in the Methionase Chain Reaction is less than that measured in the in vitro assay. However, for the purposes of the model, we will assume the value of K_m is 570 μM even after MGL modification. The system is less sensitive to the value of this parameter as the substrate methionine is held in excess. To estimate the value of k_{cat} for our model, we iterated around different values, until the signal created by 1 μM DTT matches the experimentally determined value. By this method, we estimated the k_{cat} of MGL following reactivation to be 0.5 s^{-1}, which, as expected, is less than the measured turnover rate of unmodified MGL.

Behavior Predicted by the Model

We found numerical solutions to the differential equations described in the previous section. We did this to simulate our model under the same conditions that the experiment described in Figure 4 of the main text was done. The initial concentration of methionine [S] was set to zero and the initial concentration of caged enzyme [E*] was set to 500 nM. Initial concentration of thiol [P] was set to the DTT concentration used in each trial, and free enzyme concentration [E] was set initially to zero. The differential equation was solved for 30 minutes to reflect the pre-incubation period before the substrate was added. Final values of [P], [E], and [E*] were then used as initial conditions for the next numerical solution, while the value of [S] was increased to 10 mM, modelling the point in which the system was spiked with methionine. The system of differential equations was then solved for a time period of 20 minutes. This was done for every initial DTT concentration tested in the experiment. The solutions are shown in figure S4.

The plot below shows how we envision the system behaves. There is an initial period in which no signal is detected, as the MGL is still mostly inactivated. After a period of time, the system becomes activated fairly quickly, leading to asymptotically linear behavior that corresponds to the period in which all MGL has been reactivated. Also seen, a certain concentration of DTT is required to distinguish the sample from the control in this period. Concentrations ranging from 50-1000 nM are easily detected by the system as described in this model. Concentrations lower than this range cannot be easily distinguished from the background. This matches the limit of detection that was determined experimentally.

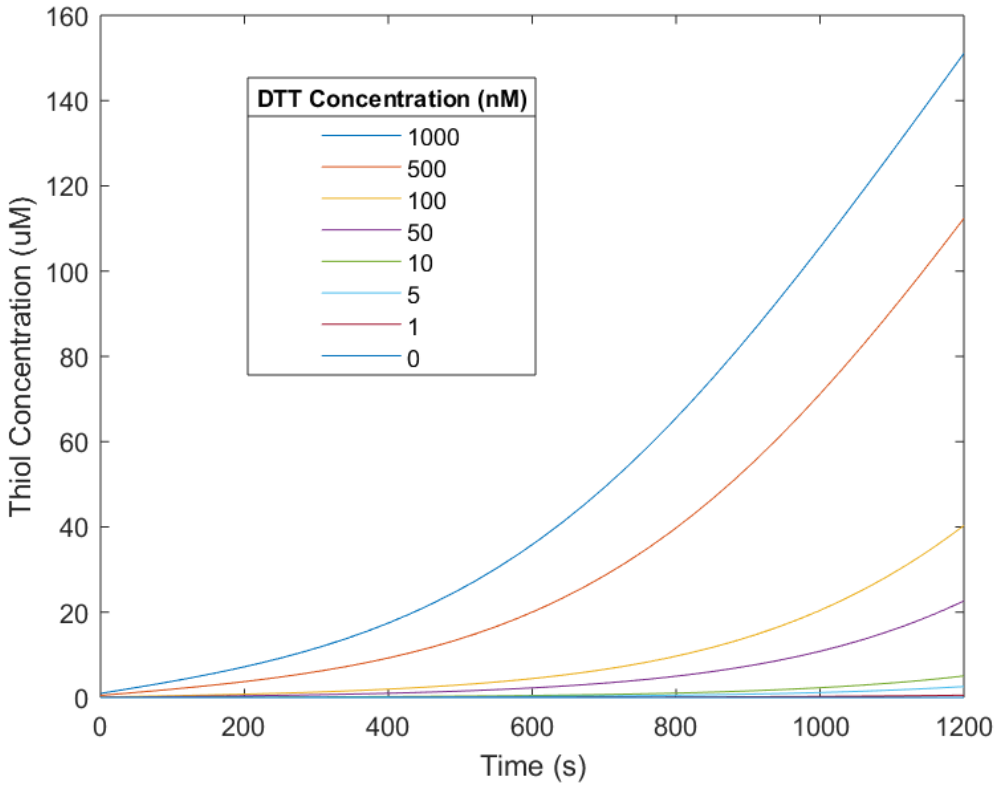


Fig. S5. DTT Detection Assay Simulation

We then compared our experimental data to the results of the model more directly. One thing our model does not account for is the background amplification described in the text. Therefore, to make a better comparison, we processed our experimental data by subtracting the output signal by the signal generated with no DTT added. The model matches the experimental data fairly well, as shown in Fig. S5. This suggests our model does describe the behavior of the MCR system fairly well, reinforcing evidence that the system behaves as we propose in Fig. 1 of the main text.

Additionally, we found a linear relationship exists between the signal generated and the logarithm of the initial DTT concentration, for all values that generated significant signal (Fig. S6). The linear relationship did not hold for concentrations so low the system was not activated in the timespan of the experiment/simulation. This is logical, as it takes a certain time for the system to become activated, after which the completely uncaged MGL will begin generating signal. The time it takes for the system to become completely reactivated is proportional to the logarithm of analyte trigger, as seen in other exponential systems such as qPCR.[4] However, after this point, signal will be generated linearly with respect to time, causing the behavior seen. This explains why this relationship only holds for samples which have become significantly reactivated by the time the concentration of methanethiol is assayed. This important result allows for interpolation to measure unknown quantities using the MCR. However, it also imposes a

limit on when linear interpolation is possible. Importantly, this behavior is demonstrated by both our model and experimental results, suggesting our model does accurately describe the kinetics of the Methionase Chain Reaction.

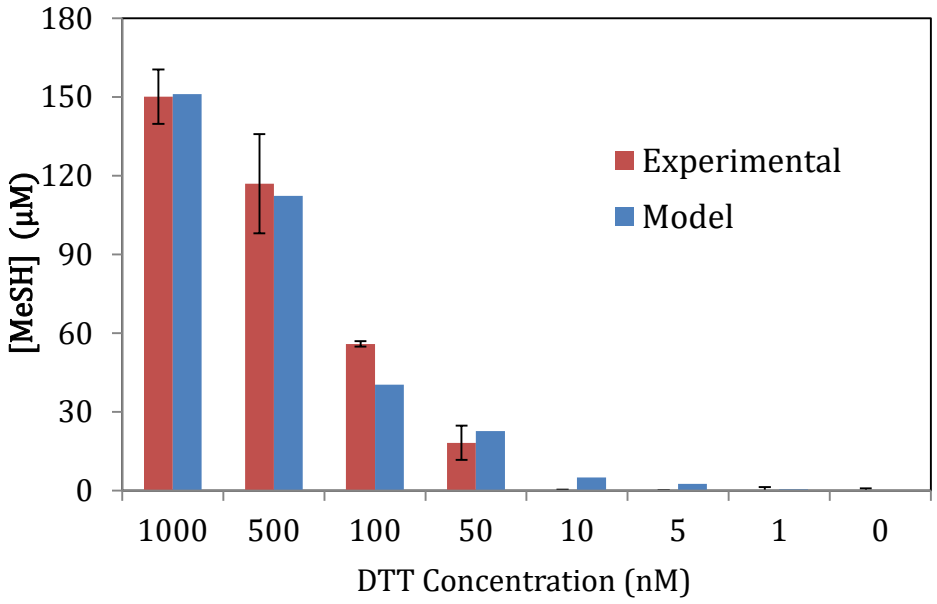


Fig. S6. Comparison of Experimental and Simulated Results of DTT Detection Assay

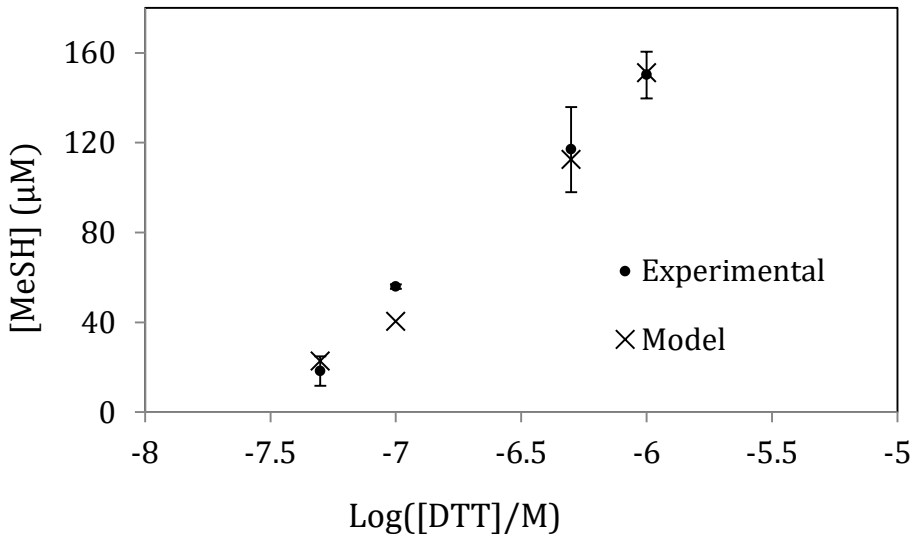


Fig. S7. Comparison of Experimental and Simulated Results of DTT Detection Assay: Semi-log Plot

Testing Disulfide Protecting Groups

Given the problem of background amplification, we sought to test whether different blocking groups could reduce the signal generated by caged MGL*. In addition to TNB (5-thio-2-nitrobenzoic acid) as a disulfide blocking group, we also caged MGL with methanethiol, thiophenol, and t-butylthiophenol disulfides as shown below. DTNB and methyl methanethiosulfanate can be used to cage MGL with TNB and methanethiol respectively. However, for thiophenol and t-butylthiophenol to be used as activity-blocking disulfides, an activated caging disulfide was first synthesized.

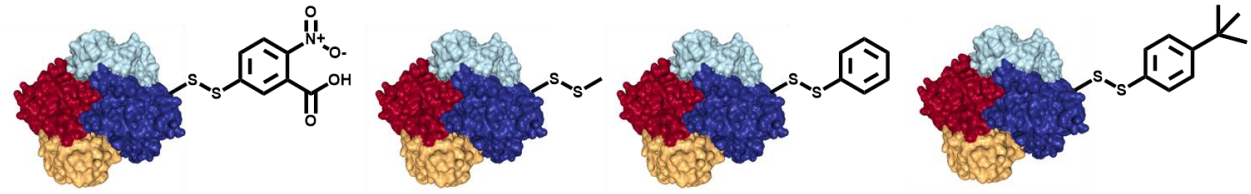


Fig. S8. MGL Caged with 4 Distinct Disulfides

Blocking Group Synthesis

Synthetic Scheme

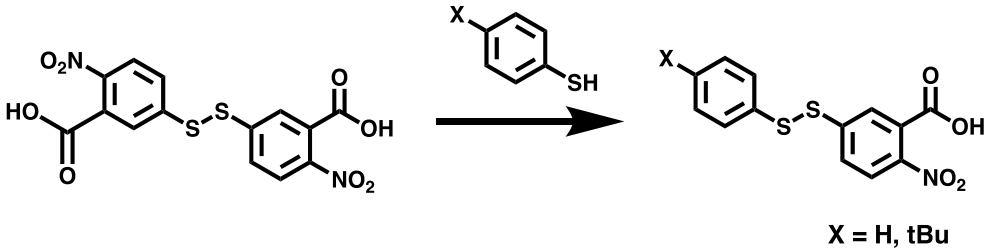
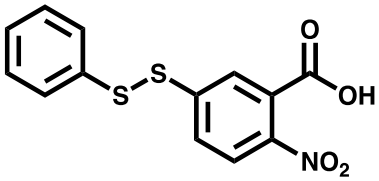


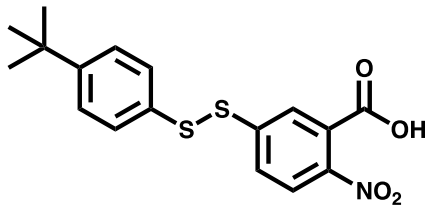
Fig. S9. Caging Reagent Synthesis Scheme

Protocols



2-nitro-5-(phenyldisulfaneyl)benzoic acid: To a mixture of Ellman's reagent (100.9mg, 0.25mmol, 1eq) in MeCN (0.75mL) and EtOH (0.75mL), was added thiophenol (19.3uL, 20.7mg, 0.19mmol, 0.75eq). The mixture was left to stir overnight at room temperature, before adding ethyl acetate (40mL) and DI H_2O (20mL). The organic phase was collected, dried with Na_2SO_4, and concentrated. The resulting crude was purified by column chromatography

(95:5:0.05 / DCM:MeOH:AcOH) to yield a yellow-tinted solid (43.9mg, 0.14mmol, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 – 8.09 (m, 2H), 8.02 (d, J = 8.5 Hz, 1H), 7.79 (m, 2H), 7.63 (m, 3H), 1.58 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.52, 145.73, 144.39, 134.94, 130.39, 129.21, 127.88, 127.85, 127.39, 126.22, 124.40. HRMS: (ESI-) Found: 305.9900 m/z, Calculated: 305.9900 m/z.



5-((4-(tert-butyl)phenyl)disulfaneyl)-2-nitrobenzoic acid: To a solution of Ellman's reagent (103.6mg, 0.25mmol, 1eq) in MeCN (0.5mL), EtOH (0.5mL) and DI H_2O (0.5mL), was added 4-tert-butylthiophenol (32uL, 30.9mg, 0.19mmol, 0.75eq). The mixture was left to stir overnight at room temperature, before adding ethyl acetate (40mL) and DI H_2O (20mL). The organic phase was collected, dried with Na_2SO_4, and concentrated. The resulting crude was purified by column chromatography (95:5:0.05 / DCM:MeOH:AcOH) to yield a yellow-tinted solid (27.9mg, 0.077mmol, 41%). ^{1}H NMR (400 MHz, MeOD) δ 7.95 – 7.87 (m, 2H), 7.80 (dd, J = 8.6, 2.2 Hz, 1H), 7.48 – 7.38 (m, 4H), 1.29 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ145.68, 133.11, 129.88, 129.34, 127.69, 127.60, 125.86, 35.52, 31.56. HRMS: (ESI-) Found: 362.0526 m/z, Calculated: 362.0526 m/z.

NMR of Synthesized Compounds

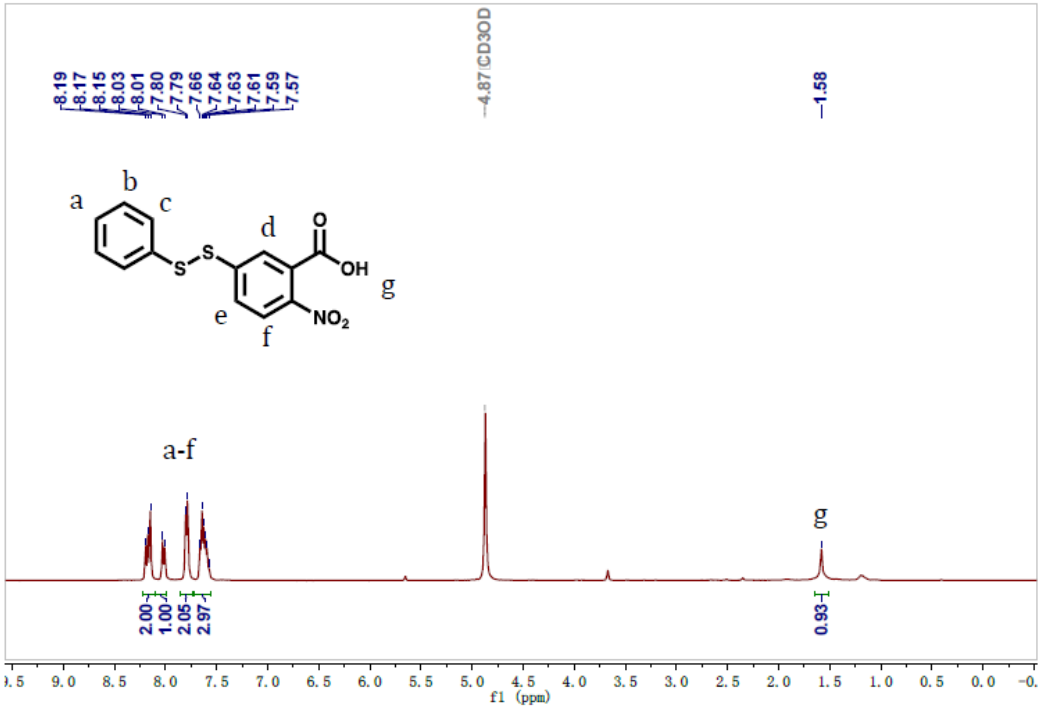


Fig. S10. ^{1}H NMR of *2-nitro-5-(phenyldisulfaneyl)benzoic acid*

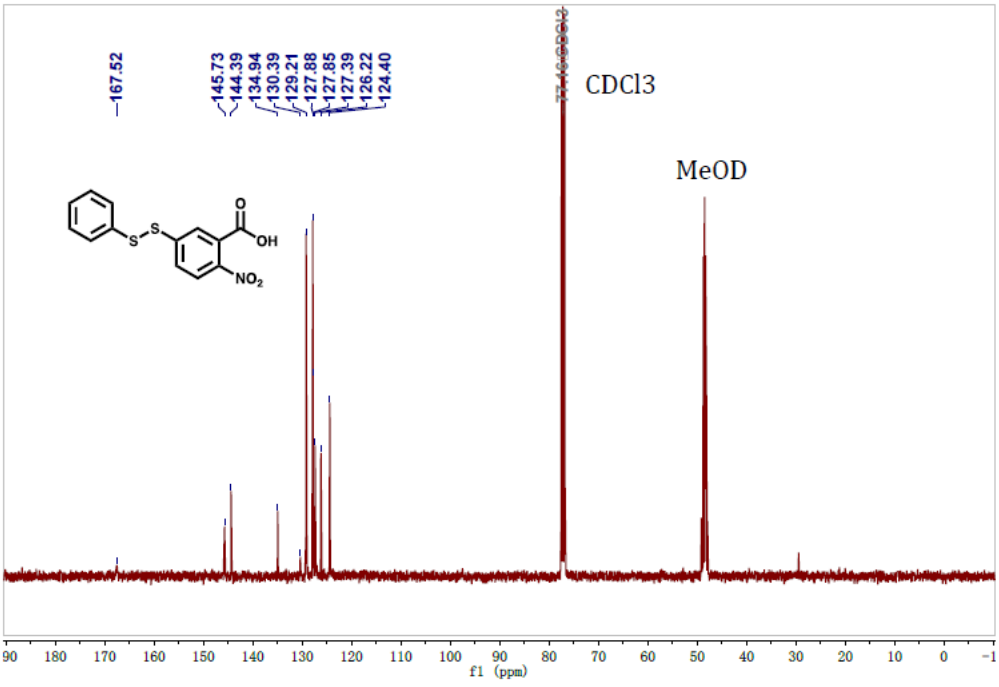


Fig. S11. ^{13}C NMR of *2-nitro-5-(phenyldisulfaneyl)benzoic acid*

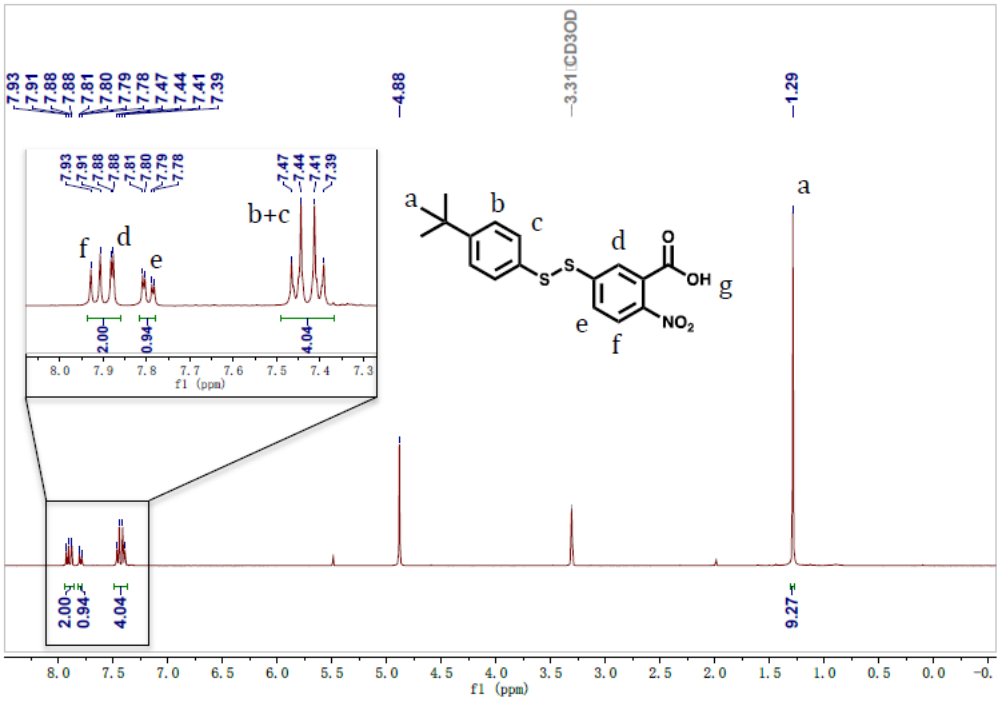


Fig. S12. ^{1}H NMR of *5-((4-(tert-butyl)phenyl)disulfaneyl)-2-nitrobenzoic acid*

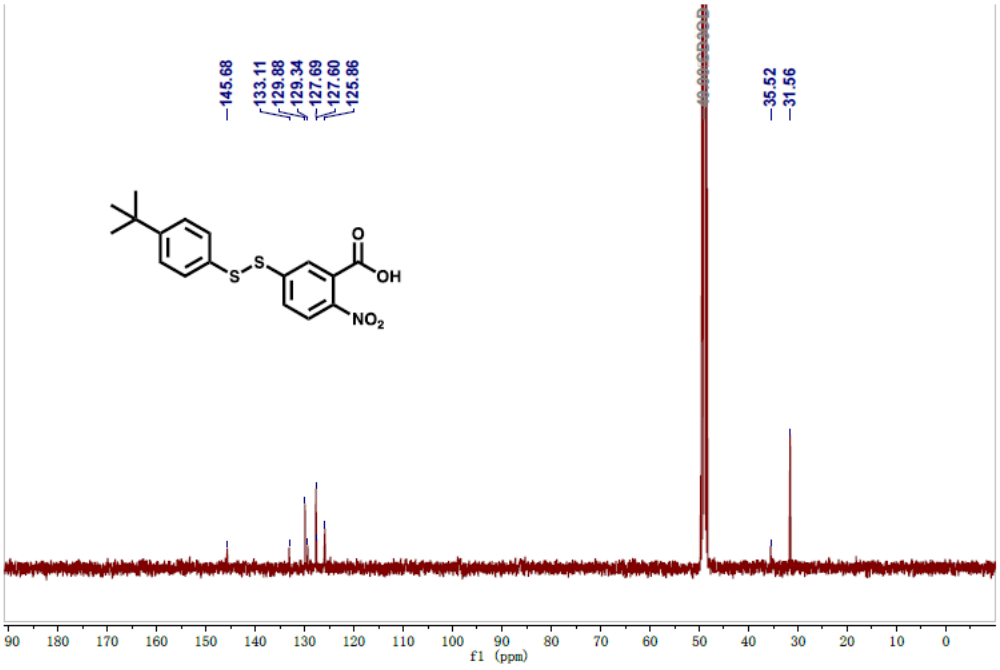


Fig. S13. ^{13}C NMR of *5-((4-(tert-butyl)phenyl)disulfaneyl)-2-nitrobenzoic acid*

Signal-Background Ratio Experiments

MGL-TNB was created by the protocol described in the "Synthesis of MGL*" section. MGL-methanethiol was created using an identical protocol, except with methyl methanethiosulfonate as caging agent.[5] MGL caged with thiophenol and t-butylthiophenol were both made by reacting MGL with the TNB-activated version of that respective thiol and purified as the others were.

For these four different versions of MGL*, the signal given by 100 nM DTT was compared to the background signal when no DTT was added. It was found that MGL-TNB had the highest signal-to-background ratio, with results similar to that found in Fig. 4 of the main text. Methanethiol and thiophenol both had poor signal-to-background ratios, with no statistically significant difference between signal and background. This suggests the limit of detection of the autocatalytic system with thiophenol and methanethiol blocking groups is greater than 100 nM. The signal-to-background ratio for t-butylthiophenol was intermediate. It appears that the larger the caging group, the higher the signal-to-background ratio is. This does enforce the claim that the source of background is residual MGL activity even when caged. It is possible larger blocking groups prevent substrate binding due to steric hindrance. Although this is likely not the only factor to consider when reducing background signal, the size and shape of the protecting group seem to be an important factor.

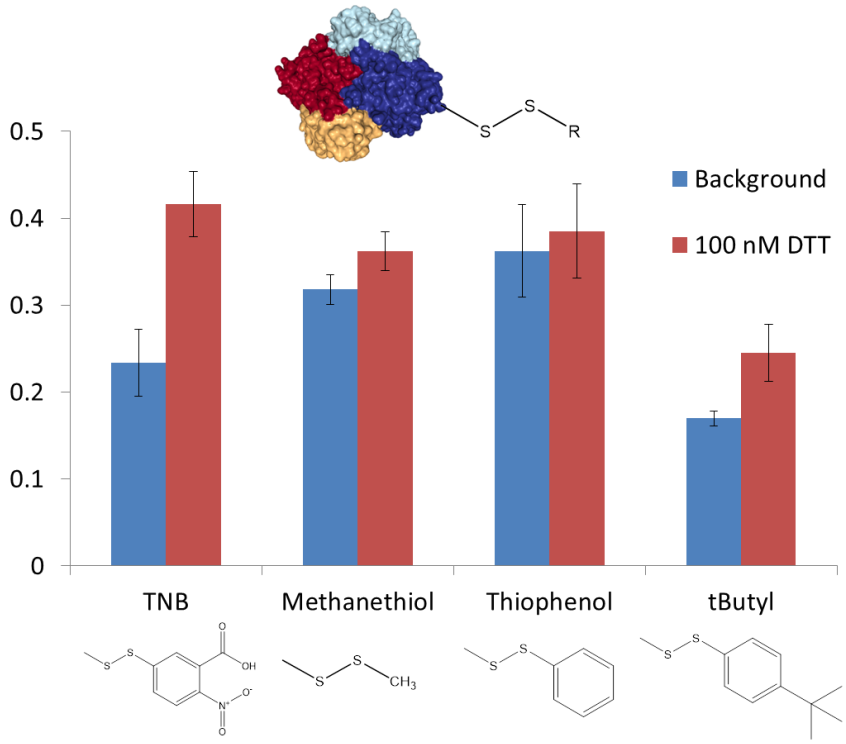


Fig. S14. Signal and Background of MGL* with Various Disulfides

MCR Sensitivity Depends on Thiol Measured

Hydrogen Sulfide Detection

We sought to examine whether the MCR could be applied to different thiols. We repeated the MCR assay described in Figure 4 of the main text, except with sodium hydrosulfide (NaHS) rather than DTT. NaHS is used as a hydrogen sulfide donor in solution. The results of the assay are shown in Figure S15 below.

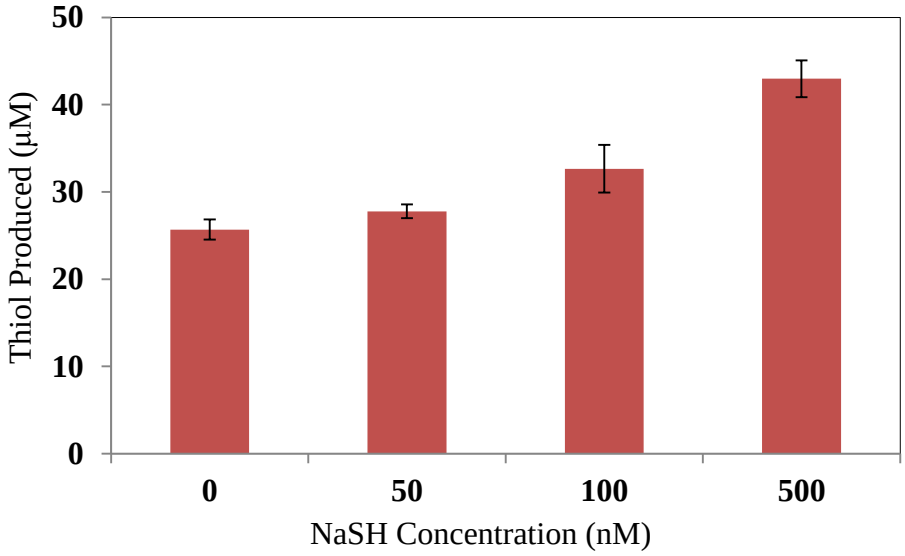


Fig. S15. Detection of Sodium Hydrosulfide via MCR

Only sodium hydrosulfide concentrations 100 nM and above produced a statistically significant ($p<0.01$) signal above the background signal. This limit of detection is higher than when DTT is the thiol examined (50 nM). Additionally, the system produces less overall signal, and therefore has a lower overall amplification efficiency in comparison to DTT. The η_{tot} of the MCR when amplifying DTT is 560, whereas with NaSH, the η_{tot} is 70.

This result provides two interesting conclusions. First, the MCR is not limited to the detection of DTT, and may therefore be used as a detection system for various thiol-containing compounds. The use of the MCR in the detection of sulfate-reducing bacteria, which produce hydrogen sulfide,[6] is one promising potential application of this system.

Second, this result demonstrates that the effectiveness of the MCR depends on the thiol being detected. Therefore, it is critical to evaluate the ability of the MCR on each individual thiol being measured. However, its ability to discriminate between different thiols is a potentially useful feature uncommon in most thiol-sensing probes. In the example of detecting sulfate-reducing bacteria, it would be useful to discriminate hydrogen sulfide from glutathione and other cellular thiols.

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