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

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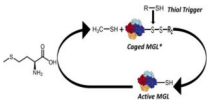
## Abstract

We present an autocatalytic system for the detection and amplification of thiols termed the methionase chain reaction (MCR). MCR is based on the reversible modification of the thiol producing enzyme methionine gamma-lyase (MGL). MCR was able to amplify the concentration of thiols by a factor of 560 and was able to visually detect thiols at concentrations as low as 50 nM.

## Graphical Abstract

The methionase chain reaction is developed; thiols are detected at nanomolar concentrations through the autocatalytic reactivation of methionine gamma-lyase.




Autocatalytic amplification systems have the potential to revolutionize the development of diagnostics by amplifying small amounts of analyte into concentrations that can be detected.[1] For instance, the polymerase chain reaction (PCR) amplifies DNA and is the gold standard for autocatalytic amplification systems.[2] Though useful for a wide variety of applications, PCR is restricted to the detection of nucleic acids.[3] Developing auto-catalytic amplification systems that can amplify non-nucleic acids has been challenging, due to the lack of chemical systems that have autocatalytic properties.

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Conflicts of interest

There are no conflicts to declare.

Motivated by the utility of such systems, researchers have developed several elegant chemical systems that exhibit self-amplifying behavior, which have been based upon the reversible activation of caged compounds.[1,4] For example, Shabat, et. al., developed a boronate-based amplification system that amplifies the concentration of hydrogen peroxide.[5] This amplification system, termed the Dendritic Chain Reaction, was able to convert 5 μM hydrogen peroxide into 500 μM of a signal molecule (4-nitroaniline) in around 40 minutes. Similarly, Anslyn, et. al. developed an autocatalytic amplification system that amplifies the concentration of thiol-containing compounds.[6] This amplification system was able to amplify 3.2 μM of thiol to 160 μM in around 40 minutes. Both of these systems had a limit of detection in the range of 1–10 μM of their analyte molecule.

A drawback of these chemical-based amplification systems is their high limit of detection. Chemical systems are limited by the stoichiometry of their reaction cascade. Enzyme-based systems, however, can produce many signal molecules per enzyme, due to their ability to rapidly turn over their substrate.[7] Despite their potential, autocatalytic amplification strategies that are based on enzymatic catalysis have not been developed to our knowledge.

In this report, we present an enzyme-based autocatalytic system for the amplification of thiols, termed the Methionase Chain Reaction (MCR). The mechanism by which MCR amplifies thiols is shown in Figure 1 and is based upon the reversible inhibition of the enzyme methionine gamma-lyase (MGL). MGL, an enzyme from *Pseudomonas putida*, is an ideal enzyme for developing autocatalytic amplification systems because it produces methanethiol by catalyzing the hydrolysis of methionine.[8] In addition, the active site of MGL contains a cysteine (Cys-116), which is essential for activity.[9,10]

The MCR amplification system is composed of MGL that has been inactivated with a disulfide at Cys-116, termed MGL*, and methionine. In the presence of a thiol target analyte, a small fraction of the MGL* becomes uncaged through a thiol-disulfide exchange reaction. This small fraction of reactivated MGL turns over its substrate methionine and produces methanethiol. Methanethiol then activates additional MGL* via thiol-disulfide exchange. This generates an auto-catalytic loop where the activated MGL generates methanethiol and the methanethiol generates more activated MGL. At the completion of the amplification cascade the generated methanethiol is assayed colorimetrically by the addition of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), which allows for instrument-free detection.[11] To our knowledge, this is the first example of a signal amplification system based upon the autocatalytic reactivation of an enzyme.

The inactive MGL* is the key reagent in the Methionase Chain Reaction (Fig. 1). We began by testing whether the formation of a disulfide on MGL would diminish its activity. MGL was expressed in *E. coli* and purified via a nickel column as has been done previously.[10,12,13] Caged MGL* was synthesized by reacting MGL with a 20x molar excess of DTNB (Fig. 2a).[14] Successful reaction of MGL Cys-116 with DTNB was confirmed using whole protein ESI-MS (ESI† Fig. S4), as well as by enzymatic activity. We measured the catalytic rate of MGL*, using a 100 nM solution of MGL* with 10 mM methionine. As seen in

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Figure 2b, introducing a bulky disulfide at Cys-116 inhibits methionase activity. We therefore used MGL* as a platform for developing the MCR. For further experiments, MGL* was purified from excess DTNB by dialysis (for methods, see ESI† 1.1−1.6).

In order for the MGL* to be used in an autocatalytic system, it has to regain its activity upon deprotection of Cys-116, and be capable of activating other caged MGL*. We tested whether MGL* could be reactivated when mixed with a small amount of active MGL in the presence of methionine. Therefore, to a 500 nM solution of MGL* was added a low concentration of active MGL (5–50 nM) and methionine (10 mM) and the combined activity of the sample was assayed. These starting conditions are representative of the autocatalytic scheme after a small amount of enzyme is reactivated by analyte thiol. The final signal for samples containing MGL* spiked with MGL were significantly higher than those with either MGL or MGL* alone (Fig. 3). This suggests that the methanethiol formed from the low amount of free MGL present does in fact reactivate the rest of the MGL*. This demonstrates the MCR exhibits autocatalytic behavior as predicted by the mechanism of the system.

To further analyze the autocatalytic nature of the MCR, we define an amplification efficiency $\eta_{amp}$. This efficiency refers to the number of measurable signal molecules (in this case, methanethiol) achieved for every enzyme directly activated by the target analyte. This does not include enzymes activated later in the autocatalytic cascade.

$$\eta_{\text{amp}} = \frac{[\text{ Signal Molecules }]}{[\text{ Enzyme Activated }]}$$

For a traditional amplification system utilizing an enzymatic reaction, the amplification efficiency is limited by the substrate turnover of the activated enzyme.[15] Therefore, in traditional (i.e. not autocatalytic) enzyme amplification systems, the amplification efficiency can be described as:

$$\eta_{\text{amp}} \leq k_{\text{cat}}t$$

For an autocatalytic system, however, the amplification efficiency can exceed this value. In an autocatalytic system, active enzymes will activate additional caged enzymes, in addition to creating signal by their enzymatic reaction. This is consistent with what we see in our system where:

$$\eta_{\text{amp}} \gg k_{\text{cat}}t$$

Due to the autocatalytic nature of the MCR, the amplification efficiency, $\eta_{\text{amp}}$, is a nonlinear function of both initial MGL concentration ($[\text{MGL}]_0$), and time (t). We calculated the amplification efficiency from the data in Fig. 3 as the difference between the amount of methanethiol generated in the sample and in the negative control, divided by the concentration of free MGL at the start of the assay. The data described in Figure 3b show that free MGL at a concentration of 5 nM produces a final thiol concentration of 42 μM over background, which gives an amplification efficiency of 8400. In the same time span, 5 nM

of MGL alone would only produce 1.5 μM of methanethiol (Fig 3b). Our system displays signal amplification with clear advantages over traditional enzyme-based assays, namely that it is not limited by substrate turnover.

To show the thiol limit of detection, the MCR was used to amplify the concentration of dithiothreitol (DTT). A 500 nM solution of MGL* was added to 100 μL of DTT solutions at variable concentrations, followed by incubation at 37 $^{O}$C for 30 minutes. Methionine was then added at a 10 mM final concentration, and the resulting amount of methanethiol generated was determined by DTNB after 20 minutes. DTT concentrations between 1–1000 nM were investigated. These concentrations of DTT are undetectable by DTNB, which has a thiol detection limit on the order of 10 μM.

Figure 4b demonstrates that MGL* can amplify the concentration of thiols. For example, MGL* was able to amplify a 100 nM concentration of thiol into a 56 μM concentration above background, within 20 minutes. We quantified the overall efficiency of the system, which was defined as the ratio between the concentration of methanethiol assayed (subtracting the concentration in negative controls) and the concentration of thiol initially in the system (DTT concentration). We define this overall amplification efficiency, $\eta_{\mathrm{tot}}$, to be the concentration of signal molecules produced by the system, divided by the concentration of analyte molecules being measured:

$$\eta_{\mathrm{tot}} = \frac{[\text{Signal Molecules}]}{[\text{Analyte Molecules}]}$$

This efficiency peaked at a DTT concentration of 100 nM, with an overall efficiency ($\eta_{tot}$) of 560.

In addition, Figure 4c demonstrates that the DTNB signal is proportional to the logarithm of the DTT concentration, providing a method to interpolate and quantify the concentration of unknown samples. Therefore, it is possible the MCR can be used for the measurement of low thiol concentrations in addition to their detection. This behavior is predicted by the model of the system described in the Electronic Supplemental Information (Fig. S5−6)†. This suggests that the system follows the mechanism described in Fig. 1. We can see that this efficiency when activating the system with thiols ($\eta_{tot}$=560) is much lower than when activating with active MGL ($\eta_{amp}$=8600). This result suggests that a limiting factor for this system is the reactivation of caged MGL*. To examine this, we define the activation efficiency ($\eta_{act}$) as the ratio between the number of enzymes activated by the target analyte and the initial number of target analytes present.

$$\eta_{\mathrm{act}} = \frac{[\text{Enzyme Activated}]}{[\text{Analyte Molecules}]}$$

Comparing this definition to those of $\eta_{\mathrm{amp}}$ and $\eta_{\mathrm{tot}}$,

$$\eta_{\mathrm{tot}} = \eta_{\mathrm{act}}\,\eta_{\mathrm{amp}}$$

We examined the activation efficiency by comparing the overall efficiency obtained in Fig. 4 and the amplification efficiency obtained in Fig. 3. It can be seen that MGL*, in the presence of 5nM of active MGL, produces a large amount of methanethiol (42 μM over background). To achieve the same signal by activating MGL* with DTT, a DTT concentration of 80 nM is required. This suggests that 80 nM of DTT can only activate 5 nM (~6% of maximum) of MGL* in the 30-minute incubation period (i.e $\eta_{act}$=0.06). This low efficiency is reasonable as the activation of MGL* follows second-order disulfide exchange kinetics, which will be slow at low concentrations of MGL* and DTT.[16]

In this report we describe the Methionase Chain Reaction and show its function as an autocatalytic amplification system. MGL* was able to amplify the thiol analyte signal by a factor of 500–600. This amplification efficiency is considerably higher than previously reported small molecule-based autocatalytic systems. The limit of detection of our system for DTT was 50 nM, which is around two orders of magnitude below the limits of detection of small-molecule systems. Both of these results confirm our hypothesis that enzyme-based autocatalytic systems have significant advantages over chemical-based systems.

We anticipate that the MCR system is limited by the incomplete inactivation of the caged MGL*, as even a 0.1–0.01% level of residual activity in MGL* will cause reactivation of the MGL due to the auto-catalytic nature of the amplification system. The methanethiol produced by this residual catalytic activity will then be amplified exponentially by the system. As the concentration of $[MGL]_0$ becomes very low, the amplified background signal begins to dominate the system, making the absorbance between sample and control wells difficult to distinguish, greatly diminishing the signal-to-noise ratio. Spontaneous reactivation is commonly encountered in other autocatalytic systems, and generally limits the capability of these systems.[5,6]

One possible factor in reducing the background signal of this system is the caging group used to block activity. Of the four different caging disulfides tested, MGL caged with a TNB disulfide (as shown in Fig. 2a) demonstrated the highest signal to background ratio (ESI† Fig. S14). It appears that larger caging groups produce lower background. It may be the case that larger molecules are better at blocking the substrate methionine from entering the active site of MGL. Regardless, it is demonstrated that the caging agent is an important factor when designing a system such as this. As of now, TNB is the most effective blocking group for MGL* with respect to the signal-to-background ratio.

In this study, DTT is used as a model thiol. However we predict the system can work with other thiol-containing molecules. The detection of thiols at low levels has a wide variety of potential applications. Thiol-sensing systems have been employed in the detection of chemical warfare agents such as VX.[6,17] Additionally, substrate probes that release thiols upon hydrolysis by enzymes show promise in diagnostics for antibiotic resistance.[18] The detection of hydrogen sulfide would allow early detection of sulfate-reducing bacteria, a common environmental contaminant in well water.[19] The MCR is able to detect hydrogen sulfide (ESI† Fig. S15), albeit with a higher limit of detection (100 nM) and lower amplification efficiency compared to DTT, suggesting the MCR may detects a variety of target thiols.

In conclusion, we demonstrate that autocatalytic amplification systems can be developed based upon the reversible chemical modification of enzymes. Additionally, we have defined two factors that lead to the overall efficiency of the system: the activation efficiency and the amplification efficiency. Finally, we have identified the amplification of background signal as the key barrier that limits the detection sensitivity of enzyme autocatalytic amplification systems. The results shown in this report demonstrate that autocatalytic reactivation of enzymes can be used for signal amplification of analytes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## References

1. Goggins S and Frost CG, Analyst, 2016, 141, 3157–3218. [PubMed: 27116941]
2. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N and Patel HRH, Expert Rev. Mol. Diagn, 2005, 5, 209–219. [PubMed: 15833050]
3. Arnheim N and Erlich H, Ann. Rev. Biochem, 1992, 61, 131–156. [PubMed: 1379788]
4. Mohapatra H, Schmid KM and Phillips ST, Chem. Commun, 2012, 48, 3018–3020.
5. Sella E and Shabat D, J. Am. Chem. Soc, 2009, 131 9934–9936. [PubMed: 19569684]
6. Sun X and Anslyn EV, Angew. Chemie - Int. Ed, 2017, 56, 9522–9526.
7. Aydin S, Peptides, 2015, 72, 4–15. [PubMed: 25908411]
8. Sato D and Nozaki T, IUBMB Life, 2009, 61, 1019–1028. [PubMed: 19859976]
9. Fukumoto M, Kudou D, Murano S, Shiba T, Sato D, Tamura T, Harada S And Inagaki K, Biosci. Biotechnol. Biochem, 2012, 76, 1275–1284. [PubMed: 22785484]
10. Yamashita M, Inagaki K, Esaki N, Tamura T, Misaki S And Kudou D, Biosci. Biotechnol. Biochem, 2008, 72, 1722–1730. [PubMed: 18603802]
11. Ellman GL, Arch. Biochem. Biophys. 1959, 82, 70–77. [PubMed: 13650640]
12. Inoue H, Inagaki K, Adachi N, Tamura T, Esaki N, Soda K, Tanaka H, Biosci. Biotechnol. Biochem, 2000, 64, 2336–2343. [PubMed: 11193400]
13. Goyer A, Collakova E, Shachar-Hill Y, Hanson AD, Plant Cell Physiol, 2007, 48, 232–242. [PubMed: 17169919]
14. Tawfik DS, Protein Protocols Handbook, 2002, 2, 483–484.
15. Singh R, Blättler WA and Collinson AR, Methods Enzymol, 1995, 251, 229–237. [PubMed: 7651201]
16. Shaked Z, Szajewski RP and Whitesides GM, Biochemistry, 1980, 19, 4156–4166. [PubMed: 6251863]
17. Zheng X, Okolotowicz K, Wang B, MacDonald M, Cashman JR and Zhang J, Chem. Biol. Interact, 2010, 187, 330–334. [PubMed: 20097185]
18. DeBoer TR, Tarlton NJ, Yamaji R, Adams-Sapper S, Wu TZ, Maity S, Vesegna GK, Sadlowski CM, DePaola P, Riley LW, Murthy N, ChemBioChem, 2018, 19, 2173–2177. [PubMed: 30079487]
19. Qi P, Zhang D and Wan Y, Talanta, 2014, 129, 270–275. [PubMed: 25127594]

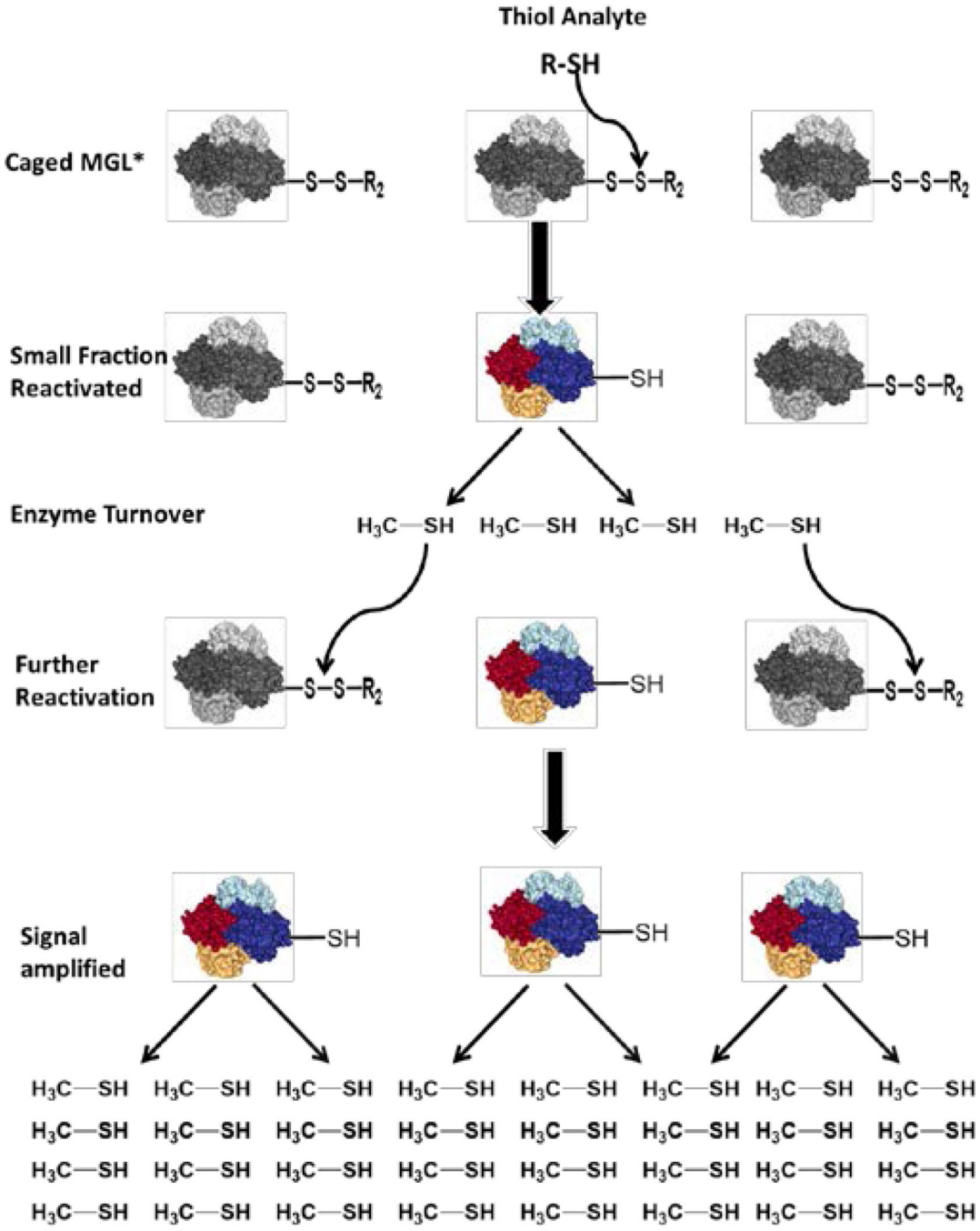


**Fig. 1.**

Description of the Methionase Chain Reaction (MCR). The system consists of disulfide-caged methionine gamma-lyase (MGL*) and methionine. MGL* is initially inactive, and will not catalyze the hydrolysis of methionine. A thiol-containing analyte present at low concentrations acts as a trigger to the system. A small fraction of the caged MGL* will become reactivated by the thiol via thiol-disulfide exchange. Active MGL then produces methanethiol from methionine through its enzymatic activity. Some of the produced methanethiol will further reactivate MGL*. This produces a positive feedback loop in which

all caged enzymes are eventually activated. The reactivated enzymes then produce a large amount of methanethiol, which is assayed colorimetrically.

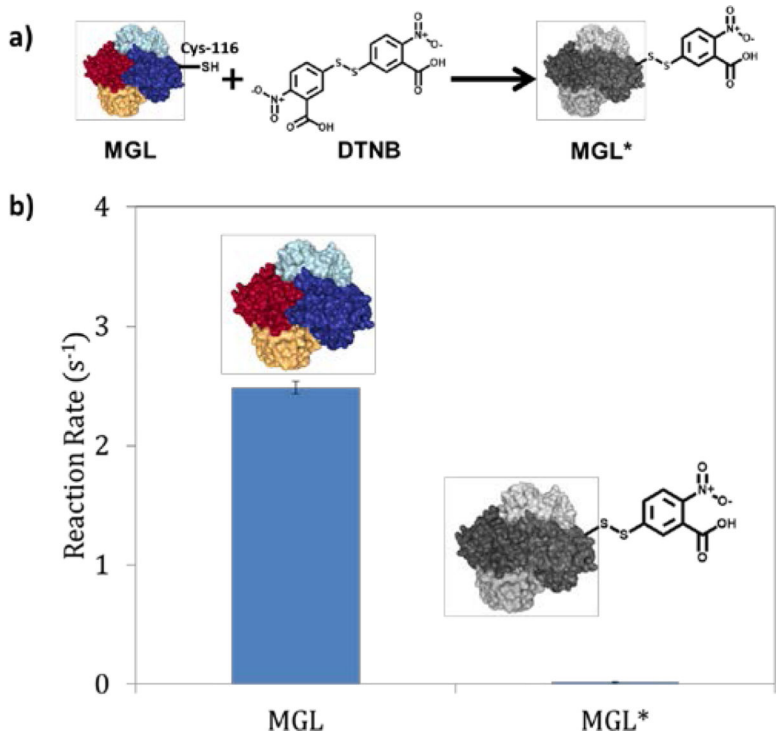


**Fig. 2.**

MGL activity is significantly reduced by the formation of a disulfide. (a) Reaction scheme in which expressed MGL is incubated with 20x DTNB to create caged MGL*. (b) MGL in the presence of excess DTNB shows very little catalytic activity compared to its native activity.

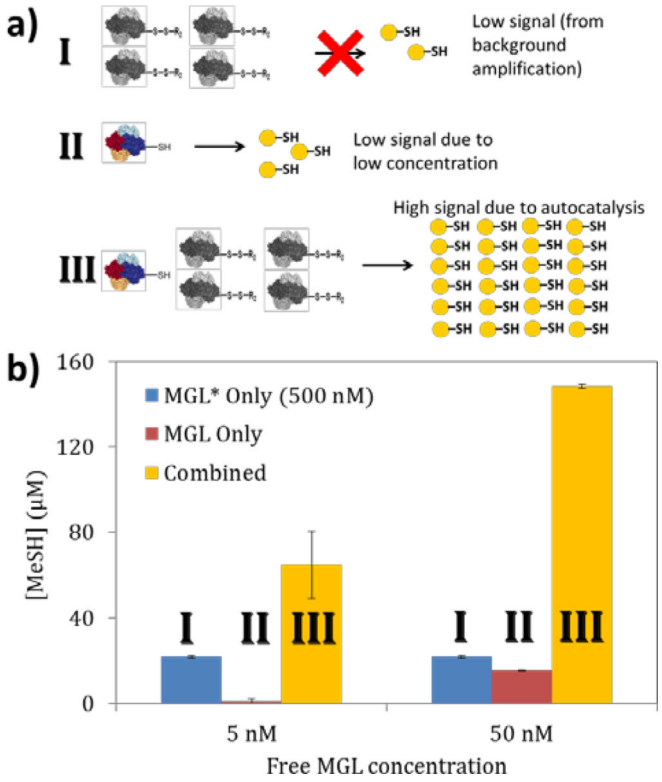


**Fig. 3.**

A solution of caged 500nM MGL* spiked with low concentrations of active MGL in the presence of methionine leads to autocatalytic activation of MGL*. a) (I) MGL* gives a low signal due to its activity being inhibited by DTNB. (II) Active MGL gives a low thiol signal due to its low concentration. (III) When MGL* is spiked with active MGL, the thiol produced by active MGL further activates MGL*, triggering an autocatalytic cascade. b) Both MGL* (500 nM) and low concentrations of MGL produce low thiol signals (blue and red bars respectively). However, when combined, the signal is significantly amplified (yellow bar), demonstrating the autocatalytic cascade predicted in Fig. 1 occurs.

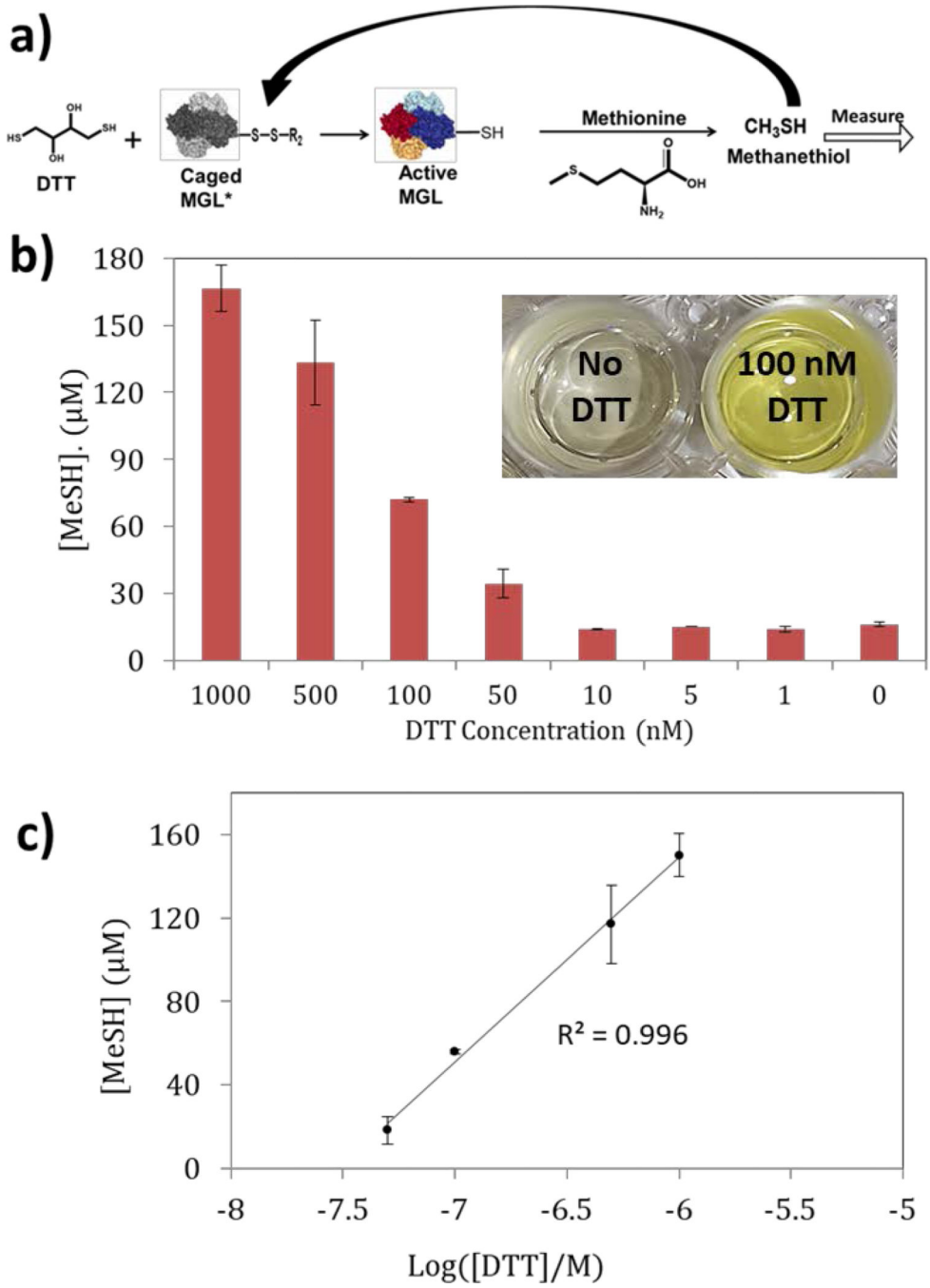


**Fig. 4.**

MGL* can amplify low concentrations of thiol. (a) DTT can reduce the disulfide bond formed in MGL*, reactivating the enzyme. Active enzyme produces methanethiol, which reduces additional MGL*, causing autocatalytic amplification of thiols. (b) Signal generated by the MCR is dependent on initial analyte concentration. Concentrations as low as 50 nM DTT can be detected by this method. The MCR provides a visually detectable readout. Visual detection of 100 nM DTT is shown. (c) A logarithmic relationship between the

concentration of DTT and the signal generated is seen. This is consistent with behavior described by the model of this system (see ESI† Fig. S5−6).