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A self-immolative linker that releases thiols detects penicillin amidase and nitroreductase with high sensitivity via absorption spectroscopy

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Abstract

This article reports the synthesis and characterization of a novel self-immolative linker, based on thiocarbonates, which releases a free thiol upon activation via enzymes. We demonstrate that thiocarbonate self-immolative linkers can be used to detect the enzymes penicillin G amidase (PGA) and nitroreductase (NTR) with high sensitivity using absorption spectroscopy. Paired with modern thiol amplification technology, the detection of PGA and NTR were achieved at concentrations of 160 nM and 52 nM respectively. In addition, the PGA probe was shown to be compatible with both biological thiols and enzymes present in cell lysates.

Graphical Abstract

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Author Contributions

E.M.E., J.J.R., L.R. and N.M. conceived of and presented the idea. E.M.E., A.K.A., P.U., D.L.K. and E.R. did the synthesis. M.H., I.L., H.H., N.J., M.R.S., and D.C. did characterization studies including HPLC and DTNB assays. E.M.E. and J.J.R. did the DETECT assays. J.J.R and H.H. performed the cell lysate DETECT studies.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Conflicts of interest

There are no conflicts to declare.

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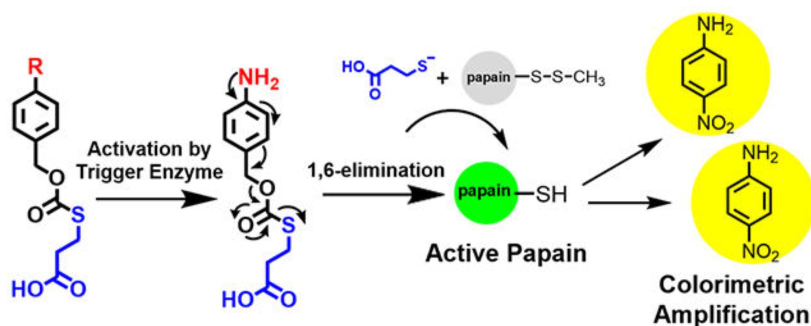

Chemical probes that can detect enzymes play a central role in biotechnology and biochemistry and have enabled the development of a wide variety of diagnostics, imaging, and biochemical assays.[1–3] Current chemical probes for enzymatic detection are primarily based upon fluorescent compounds. The fluorescent probes act as selective enzyme substrates and undergo changes in fluorescence after enzymatic transformation.[1–4] Due to their low limits of detection (LOD), fluorophores have been preferred over chromophores. However, a key challenge with developing fluorescent turn-on probes for enzymatic detection is that the enzyme probe must be a compatible substrate for the enzyme and consequently must be chemically similar to the enzyme's natural substrate. This is challenging because sfluorophores are large planar hydrophobic molecules and bear little similarity to the vast majority of enzyme substrates found in nature.

Chromogenic probes are also being considered as reagents for detecting enzymes, but have not gained widespread use because of their lower sensitivity in comparison to fluorescent probes. Chromogenic probes, however, have several advantages over fluorescent probes such as low background and the potential for visual detection. If strategies for increasing their detection sensitivity were developed, they would impact multiple areas of biotechnology.

Amplification strategies will likely play a central role in the effort to increase the detection efficiencies of chromogenic probes to a level comparable to fluorogenic probes.[5–8] We recently demonstrated that a dual-enzyme amplification system, termed DETECT, enhanced the detection sensitivity of TEM-1 beta-lactamase by 4 orders of magnitude compared to nitrocefin-based detection.[5] DETECT is based upon a beta-lactamase substrate that releases a thiol upon cleavage, which then activates a disulfide-protected caged papain. The generated free papain then reacts with N-Benzoyl-L-arginine 4-nitroanilide (BAPA), leading to release of 4-nitroaniline which allows for absorption-based detection (Fig. 1). Although DETECT is able to increase the detection sensitivity of beta-lactamases,[5,7,8] engineering DETECT for other enzymes is challenging because strategies for making enzyme substrates that release thiols have not been developed.

In this report we present a general strategy for increasing the detection sensitivity of chromogenic probes using DETECT. A tunable self-immolative linker that releases a free thiol was developed to bridge the gap between DETECT and the enzyme substrate. This self-immolative linker releases a thiol upon a 1,6-elimination from a thiocarbonate and can be easily tailored to various enzymes by modifying the functional group para to the

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thiocarbonate (Fig. 1). Using this technology, we were able to detect penicillin G amidase (PGA) *in situ* at concentrations down to 160 nM using a trigger phenyl amide group. The probe was shown to be compatible with biological thiols, and detected PGA in the presence of up to 5 mM glutathione (GSH). The probe is also compatible with complex biological conditions, showing a PGA detection limit of 160 nM in HEK 293 cell lysate. The versatility of the probe was then illustrated using a nitroreductase (NTR) probe, containing a nitro group to trigger the 1,6-elimination, which provided a limit of detection (LOD) of 52 nM. To our knowledge this is the first report of a free thiol releasing self-immolative probe using a thiocarbonate that does not undergo an intramolecular cyclization. [9,10] The development of a simple enzyme probe that can be tuned by minor modifications of the R group constitutes a versatile addition to the field of sensitive enzyme-selective probes.

The thiocarbonate linker is tailored to contain a functional group which is cleaved by the appropriate enzyme and releases an anilinic amine. Para to the functional group is a thiocarbonate containing a carboxylic acid for solubility (Fig. 2A). In the presence of the trigger enzyme, a 1,6-elimination is initiated thus leading to the release of a free thiol. The aromatic and alkyl thiol probes **1** and **3,** along with their control analogues **2** and **4** were investigated for their ability to release free thiol in the presence of PGA, using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), commonly known as Ellman's reagent, to quantify released thiol (Fig. 2B). The alkyl thiol probe **3** was found to form significantly larger thiol concentrations compared to **1**, with 120 μM thiol released from 404 μM of **3**, compared to roughly 30 μM generated from **1**. To understand why DTNB is able to detect the presence of free thiols from the alkyl thiocarbonate **3**, liquid chromatography-mass spectrometry (LC-MS) was employed.

LC-MS chromatograms reveals that when using the aromatic thiocarbonate **1**, the released 4-mercaptobenzoic acid reacts with several intermediates, as well as starting material, thus consuming the generated free thiol. Upon the release of a free thiol via a 1,6-elimination the aromatic thiol can either react with the aza-P-quinone methide, starting material, or dimerize. Interestingly, the (4-aminophenyl)methanol that would be produced from the reaction of aza-P-quinone methide with water is not detected by LC-MS (Fig. S7). Conversely, while the alkyl thiocarbonate **3** did show some minor dimerization of the released 3-mercaptopropionic acid, it is free from most side products formed by **1**, and free thiol is still observed after incubation with enzyme (Fig. S8). Due to the reactive nature of the aromatic thiocarbonate the alkyl thiocarbonate is necessary for the probe design.

Conventional thiol detection (DTNB) was then compared with DETECT (Fig. 3A) for PGA-triggered thiol release. DETECT shows an improvement in thiol detection by a factor of 3 after 60 minutes compared to DTNB (Fig. 3A). After 60 minutes a plateau in signal is observed using DTNB; however, using DETECT we observe an exponential rise of the thiol signal by a factor of 8 after 120 minutes (Fig. 3B). Using DETECT we were able to detect PGA concentrations as low as 160 nM (Fig. 3B), compared to 16 μM using DTNB, while using 1/16 of the probe concentration (25 μM vs 404 μM).

In order for the tunable probes to be utilized for enzyme detection, it is important for them to be compatible under biological conditions. We therefore investigated if PGA probe **3** could

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detect PGA in the presence of biological thiols. The probe was incubated with PGA in the presence of various concentrations of GSH up to 5 mM, which is in the upper range of biological thiol concentrations. Thiol detection was amplified and measured using DETECT. It was found that probe **3** caused a significant signal increase at GSH concentrations up to 5 mM (Figure S11), which allows for the use of the tunable probe when detecting enzymes in the presence of biological systems containing excess thiol.

In addition to thiols, biological systems contain other potentially interfering factors, such as proteases, that can limit the use of the thiocarbonate probes *in vitro*. To assess the ability of probe **3** to detect PGA in biological systems, HEK 293 cell lysate was spiked with a varying amount of PGA and probe **3** was added to the cell lysate in the presence of a protease inhibitor cocktail. Probe **3** successfully detected PGA in cell lysate at PGA concentrations of 160 nM, corresponding to 18 μg PGA/mg cell lysate (Fig. 4). This illustrates that the tunable probes can indeed be used to detect enzymes in biological systems.

The LOD achieved by the thiol-releasing probe **3** compares to reported fluorescent probes. For example, Tian et al.[11] developed a DDAP NIR probe which showed a fluorescence detection of PGA between 0.001-0.004 U/mL PGA. While fluorogenic probes are indeed able to detect low concentrations of enzymes via a 1:1 enzyme to fluorescent probe ratio, by using DETECT we are able to detect 0.00014 U/mL PGA (160 nM) via absorption spectroscopy.

To examine the versatility of the self-immolative probe we synthesized a probe, **5,** for the detection of nitroreductase (NTR). NTR plays a central role in numerous metabolic processes and is being considered as a diagnostic probe for a variety of infectious and metabolic diseases. However, developing sensitive probes for NTR has been challenging, and existing NTR probes have been limited to fluorescent probes,[1,2,12–15] with many of the probes possessing weak fluorescence.[12,16,17] To the best of our knowledge, there is no established chromogenic NTR probe that changes from colorless to colored in the presence of NTR. While there have been reports on some type of color change for potential NTR probes in literature[16,17] a full analysis on the chromogenic limitations has not been reported to determine the limit of detection.

To improve on these limitations, the self-immolative NTR probe **5,** which has a nitro group para to the thiocarbonate (Fig. 5A), was monitored using DETECT. The NTR probe **5** undergoes a similar chemistry to **3** to release a thiol which can be detected chromogenically. Initial experiments with **5** using DNTB for thiol detection did not show signal even after 120 minutes. This might be due to low thiol release or due to the reduction of the nitro group on DTNB by NTR. However, by using DETECT even low levels of thiol release from the NTR probe were sufficient to activate DETECT and give a detectable chromogenic signal (Fig. 5B). Furthermore, the LOD of NTR using **5** was found to be 52 nM (Fig. 5C), which is similar to fluorogenic probes taken *in vivo* at the same detection timescale[1,2,15,16] and is able to outperform commercially available fluorogenic coumarin probes that have been used to detect microbial NTR[18] (Fig. S8). Unlike PGA, NTR requires a flavin nucleotide, nicotinamide adenine dinucleotide phosphate (NADPH), as a reducing agent for the reduction of nitro groups to amine. For *in situ* studies this becomes problematic when

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using fluorescent probes. For example, many of the coumarin fluorescent probes rely on ultraviolet wavelength excitation which overlaps with the absorption of NADPH and thus results in false positive fluorescent signals. Therefore, relying on absorption signals in the visible spectrum is a superior alternative when monitoring for NTR.

Tunable self-immolative linkers based on novel thiocarbonates indeed possess the properties needed to release a free thiol in the presence of an enzyme trigger. This probe design makes it possible to modify the functional group of the self-immolative probe for the detection of a variety of desired enzymes. Using an alkyl thiocarbonate rather than an aromatic thiocarbonate minimizes side reactions and improves detection sensitivity. Using DETECT we were able to detect 52 nM of NTR, an enzyme that is challenging to detect chromogenically, and 160 nM of PGA at probe concentrations similar to those used for fluorogenic detection. Furthermore, we showed that thiol-releasing probes can be used to detect PGA in the presence of biological thiols such as glutathione, as well as in the presence of biological enzymes present in HEK 293 cells. This tunable probe design will allow for the development of a range of chromogenic enzyme probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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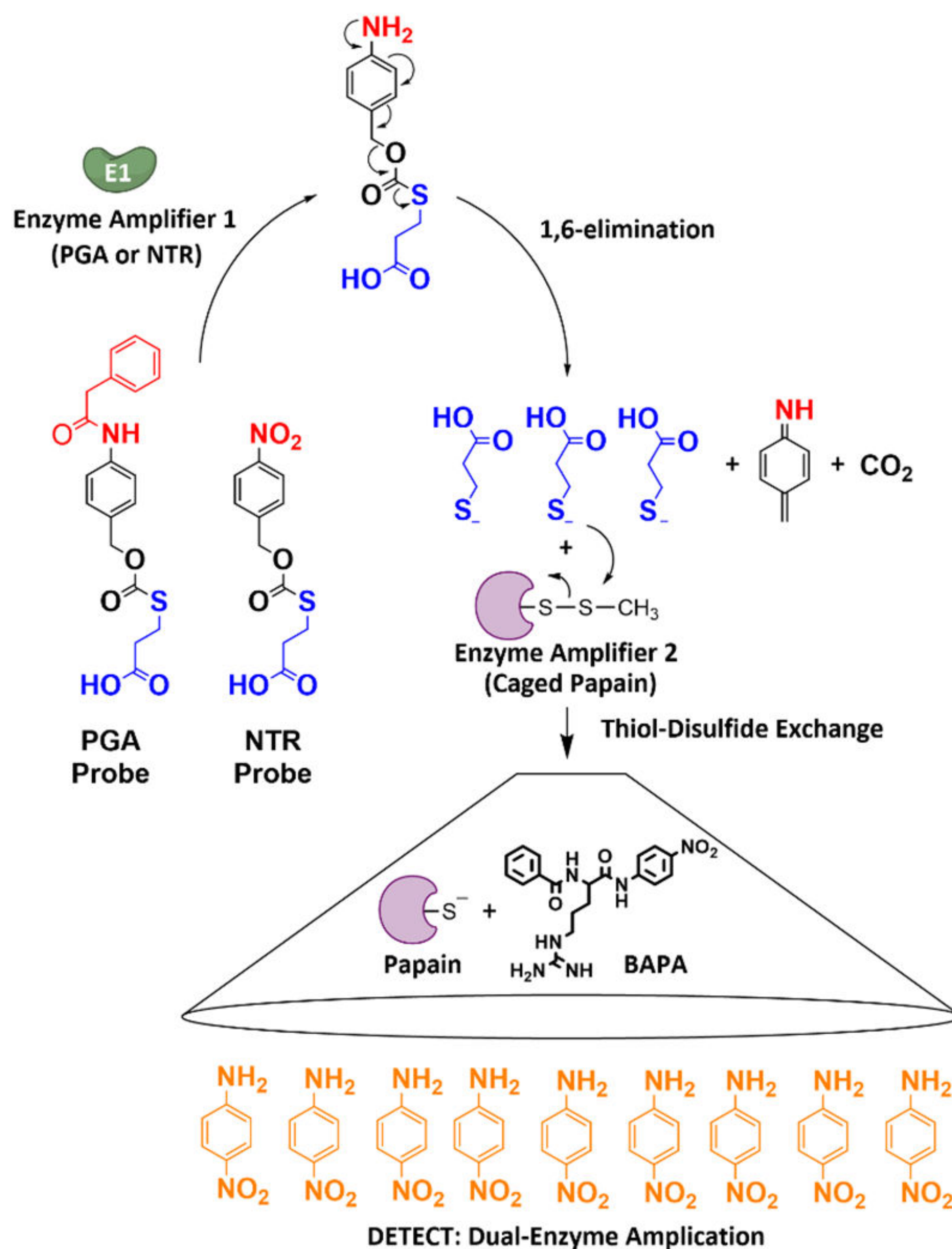

Figure 1.

The thiol-releasing probes contain a trigger functionality for either penicillin G amidase (PGA) or nitroreductase (NTR), which generates an anilinic amine, triggering a 1,6-elimination resulting in thiol release. The thiol then activates papain by undergoing a disulfide exchange with caged papain. Free papain then reacts with BAPA, releasing 4-nitroaniline which can be detected by absorption spectroscopy.

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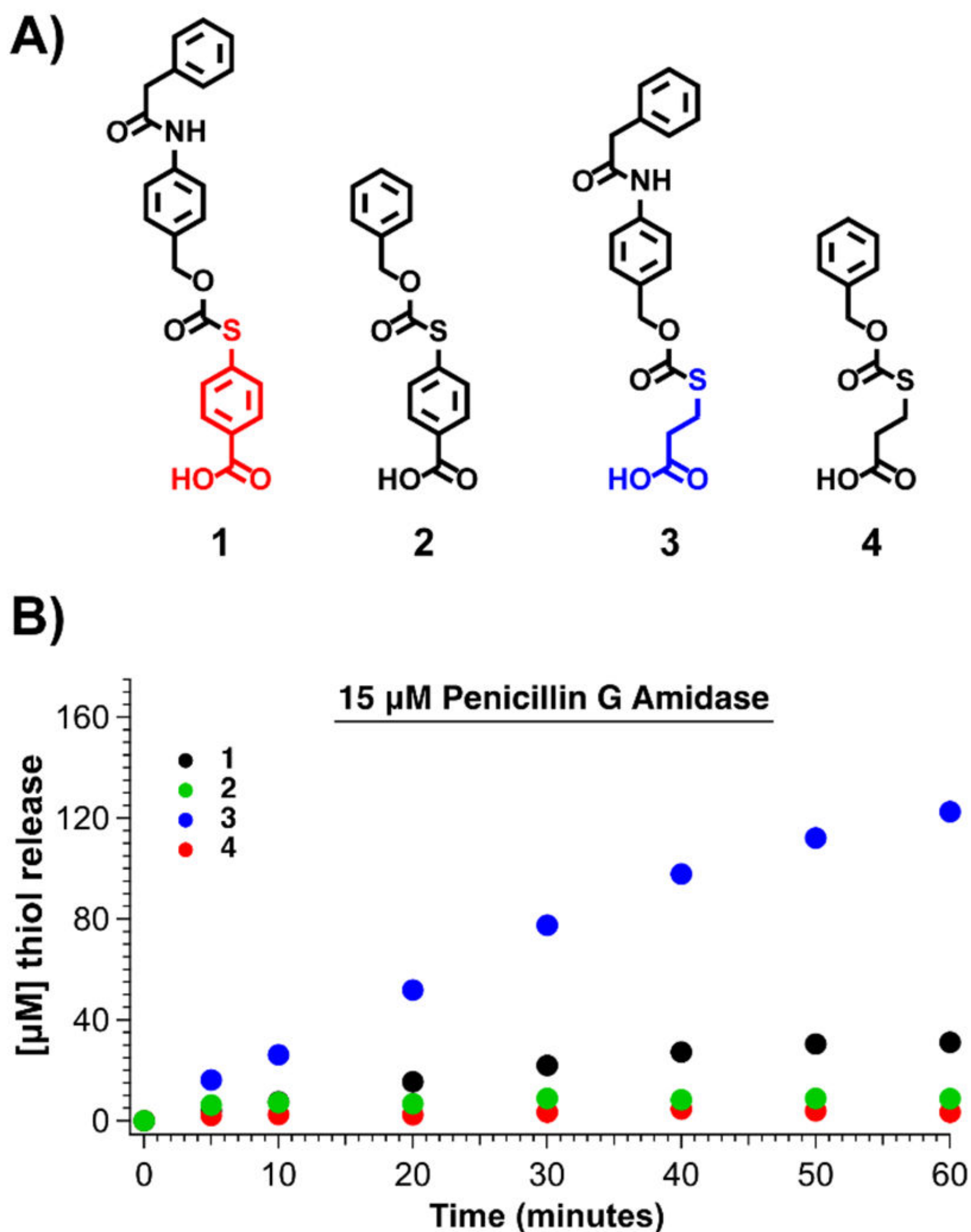

Figure 2: Alkyl thiocarbonate PGA probes efficiently release free thiols after a 1,6-elimination.
A) Structures of alkyl and aromatic thiocarbonate PGA probes as well as control probes used in the study. B) Comparison of thiol release between alkyl vs. aromatic thiocarbonate PGA probe using Ellman's reagent (or DTNB), a common chromogen to detect thiols, at a probe concentration of 404 μM.

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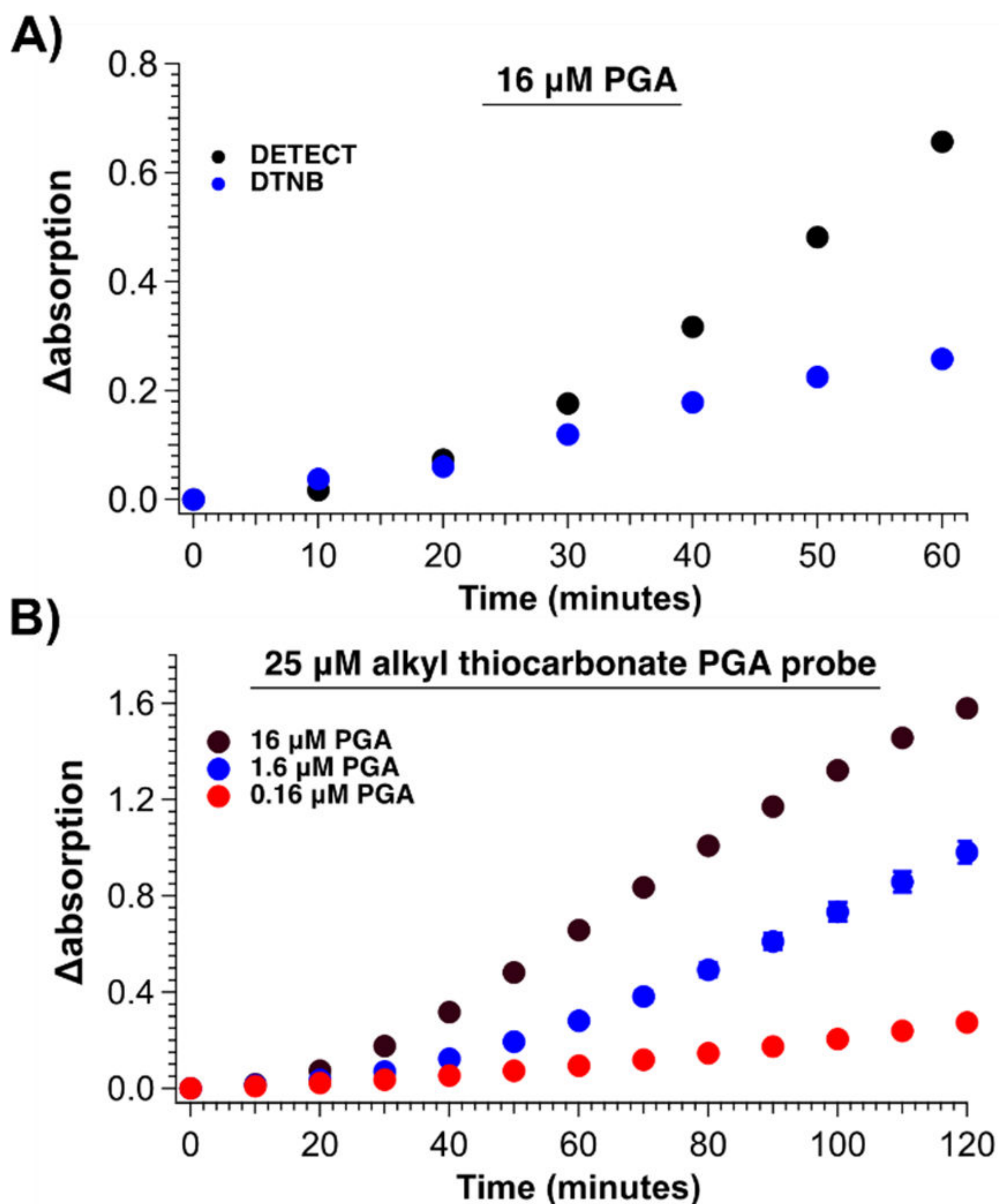

Figure 3: Thiocarbonate PGA probe efficiently detects PGA at 160 nM concentrations.

A) Comparison of DTNB vs DETECT, in which 25 μM of probe was used instead of 404 μM, reveals that after 60 minutes there is a 3 times signal enhancement. B) Using DETECT it is possible to measure the presence of 160 nM of PGA.

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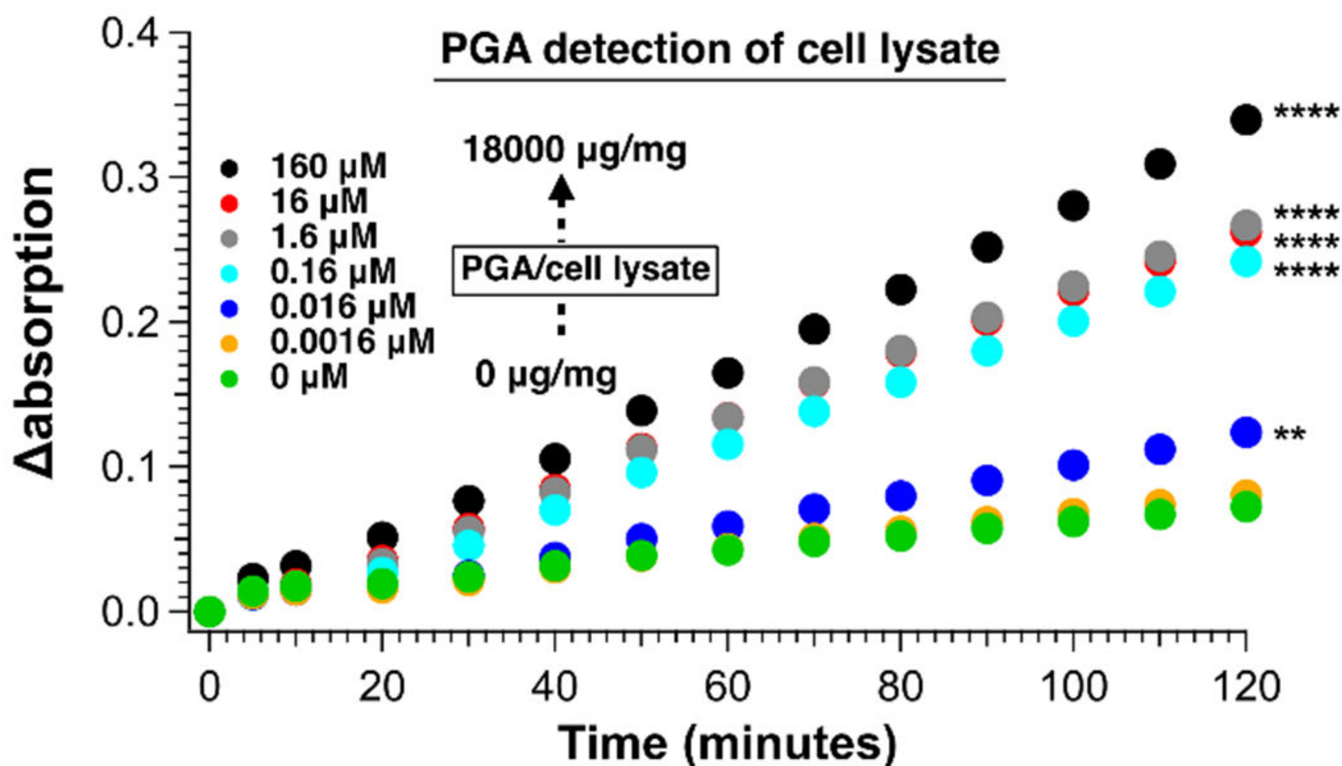

Figure 4: Thiocarbonate PGA probe can detect PGA in cell lysate.

A) PGA at increasing concentrations was added to HEK 293 cell lysate and the PGA probe **3** was added. The PGA probe **3** successfully detected with high accuracy for PGA concentrations down to 160 nM PGA, corresponding to 18 μg PGA per mg of cell lysate. Indicated statistical significance is in relations to 0 μM.** P 0.01, **** P 0.0001.

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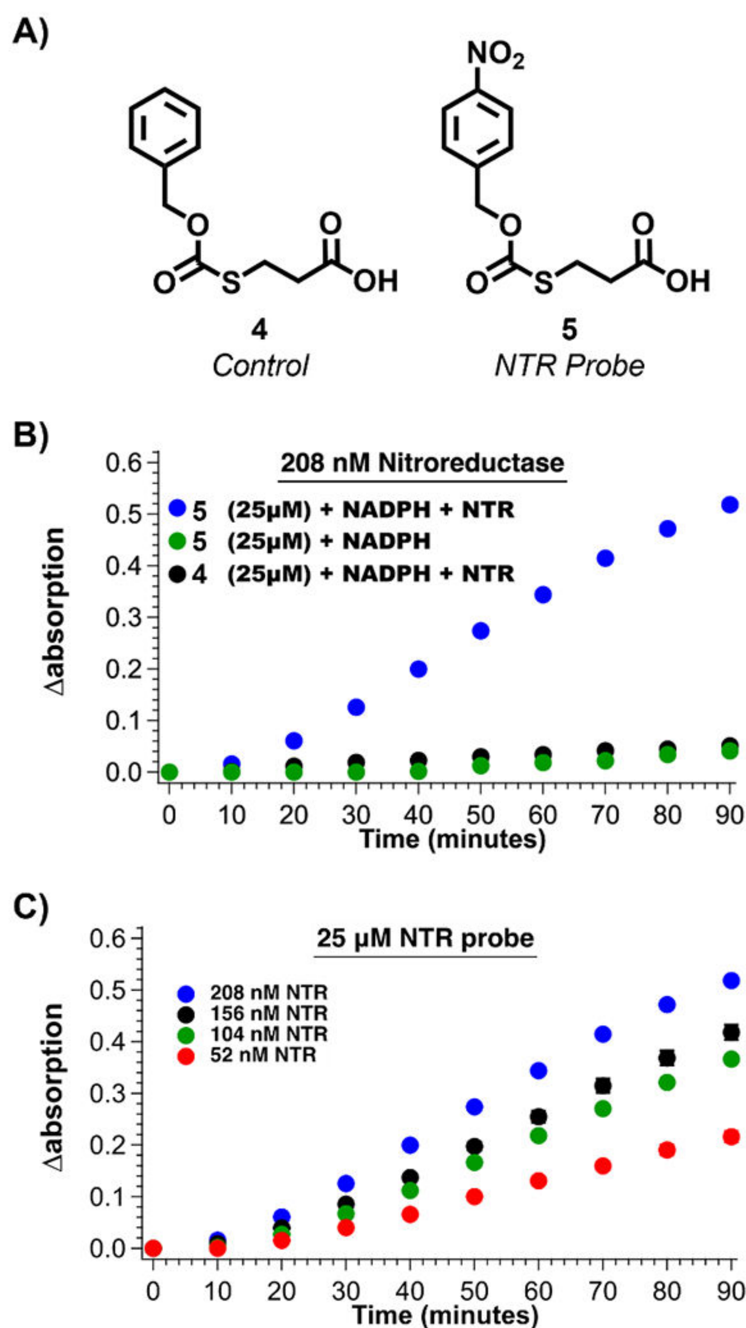

Figure 5: Thiocarbonate NTR probe efficiently detects NTR at 52 nM concentrations.
A) NTR and control probe for NTR used for studies. B) Using DETECT to detect the presence of 208 nM NTR. C) DETECT assay revealed that NTR probe could detect the presence NTR up 52 nM.