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Article

Mechanistic deconvolution of autoreduction in tetrazolium-based cell viability assays

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SUMMARY

Optical reporters for biochemical activities within cells are a powerful way to quantify the physiological responses of cellular systems to experimental conditions. For example, tetrazolium dyes, such as 5-diphenyl tetrazolium bromide (MTT), can be used to quantify the number of living cells present in a sample. This is useful for the determination of the toxic or antiproliferative activity of certain conditions that a cell might be exposed to, such as the administration of anticancer compounds. Inside of an active, living cell, tetrazolium salts, such as MTT, are reduced to brightly-colored formazan dye counterparts, which can be measured spectrophotometrically as a proxy for cellular viability. However, it has been reported that small molecules may interfere with the results of an MTT assay, at times impacting the accuracy or reproducibility of the results of the experiment. In this study, to examine if reducing agents were a source of inaccuracy in the MTT assay, we tested common reducing agents in cellular media, which are commonly used in biological assays, for unforeseen autoreduction and determined that ascorbic acid, cysteine, and glutathione (GSH) were reactive with MTT under basic conditions. We also found pH accelerated autoreduction, with a pH of 10 correlating with the most autoreduction. We advise to use media specially depleted of ascorbic acid, cysteine, and GSH, or a higher cellular density for such assays, in order to prevent interference through autoreduction.

INTRODUCTION

Drug discovery campaigns that seek to quantify cellular responses to the administration of a compound are generally dependent on various biochemical assays that give optical readouts for cellular viability (1-3). Cell viability assays involve the exposure of cells to a compound, after which they are incubated and the number of viable cells is measured, typically through the use of dyes that are either produced in a cellular chemical reaction or are selectively localized in dead or living cells (4). For example, the Neutral Red uptake assay uses how many cells have absorbed a supravital red dye into their lysosomes as a measure of membrane damage and cell viability, and the FRAME cytotoxicity test uses a Kenacid blue dye to measure cell viability through the cells' ability to form a protein-dye complex (5-7). These results can be subsequently measured through flow cytometry.

Reduction of proliferation is an important indicator of suppression of cellular growth and can be measured through a multitude of biological assays. Among the many options available for biological testing, the MTT (5-diphenyl tetrazolium bromide) assay has become one of the most widely used means of measuring anti-proliferative cellular activity because it is rapidly deployable, easily scalable for measuring a large quantity of samples, convenient, and easily quantifiable by a plate reader or spectrophotometer (8-10). This assay relies on the reduction of tetrazolium salts, such as MTT by intracellular oxido-reductase enzymes to their formazan counterparts which, being brightly colored, provides an optical readout for the redox activity in living cells (11,12). MTT is a yellow tetrazolium salt that is water soluble and is reduced in metabolically active cells' mitochondria (13). Through succinate dehydrogenase, MTT is converted into a purple water-insoluble formazan crystal, a colorimetric change that can be measured on solubilization with a spectrometer (**Figure 1**). The number of viable cells in the culture is directly proportional to the amount of formazan produced (14). However, the assay assumes that participation of MTT in non-cellular redox reactions is negligible; interference in the assay from media components that react with MTT has created challenges in data reproducibility and assay standardization (15,16).

Cell culture medium, in which cells are grown, typically contains a variety of components that support optimal cell growth and are specific to a particular cell type (17). For example, DMEM (Dulbecco's Modified Eagle Medium) is a common media which contains up to four times the amount of amino acids and vitamins compared to BME (Basal Medium Eagle), as well as increased glucose levels. However, it lacks proteins and growth factors and therefore requires serum supplementation.

Previously, our group and others have performed numerous MTT experiments, in which we noticed that our negative control wells of the media components alone, without the addition of compound, sometimes turned purple without the presence of cells, suggesting the formation of formazan in the absence of biological oxido-reductase activity (18-20). Others have found that compounds such as flavonoids, botanical extracts, and antioxidants sometimes interfere with the MTT assay, leading to false results (18-20). Moreover, it has been reported that reductants present in cellular media, such as

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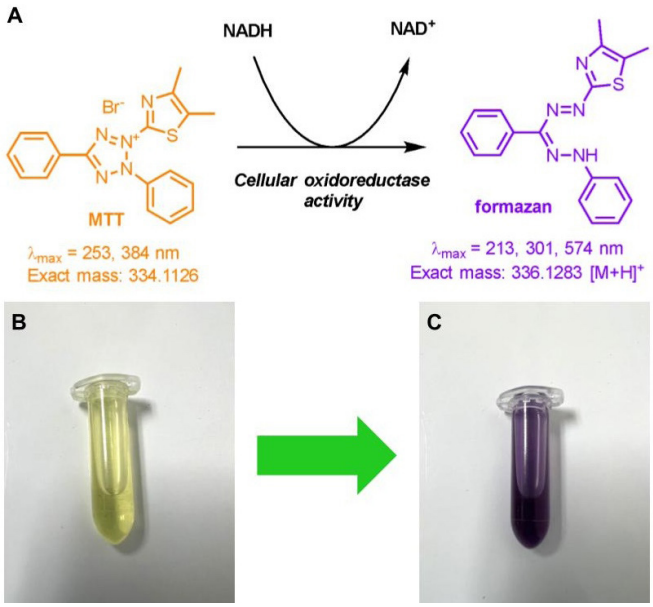

Figure 1: Intracellular reduction of MTT to formazan leads to an optical readout that is proportional to cellular metabolic activity. A) MTT tetrazolium (orange, left) is reduced to MTT formazan (purple, right) in the presence of oxidoreductase enzymes; B) MTT tetrazolium salts (authentic sample shown) are a yellow color with maximal absorbances at 253 and 384 nm; C) MTT formazan (authentic sample shown) is a deeply colored purple dye which can be measured at 574 nm.

free thiols, can reduce MTT under basic conditions (18-20). Furthermore, the reduction of MTT by these compounds is influenced by the type and pH of the medium used (18-20).

Subsequently, we hypothesized that cell culture media components that possess a free thiol might be most reactive as reducing agents and that glycine, which lacks an oxidizable side chain, would be inactive in autoreductions with MTT (20). Here, in an effort to identify the nature and mechanism through which this phenomenon occurs, we screened common reducing agents, such as reducing sugars, ascorbic acid, and oxidation-prone sulfurous amino acids, that are present in commonly used cell culture media (**Figure 2**) (21). These reducing agents, being present in cell culture media, are therefore also common in assays, which may cause unforeseen interference. We aimed to identify the extent to which and the mechanism by which these common reducing agents cause interference in cell-free MTT autoreduction. We chose cysteine and methionine as model amino acids that can be easily oxidized, which contain a thiol and a sulfide functional group respectively, as well as glutathione (GSH), a tripeptide containing a free thiol (**Figure 2**) (22). Finally, we screened ascorbic acid, a commonly used anti-oxidant, as well as glucose, a model reducing sugar (**Figure 2**) (23). Additionally, we screened glycine as a control to verify that the amine or carboxylic acid of amino acids does not react with MTT, and we identified the mechanism of thiol-mediated redox reactions of MTT through liquid chromatography tandem mass spectrometry (LC-MS) (24,25). Through these studies we ultimately determined that ascorbic acid, cysteine, and glutathione (GSH) were reactive with MTT under basic conditions, and we found that pH expedited autoreduction. Therefore, it should be considered when performing MTT assays that reducing agents common to cellular media may alter MTT results. To combat this issue, we advise to use

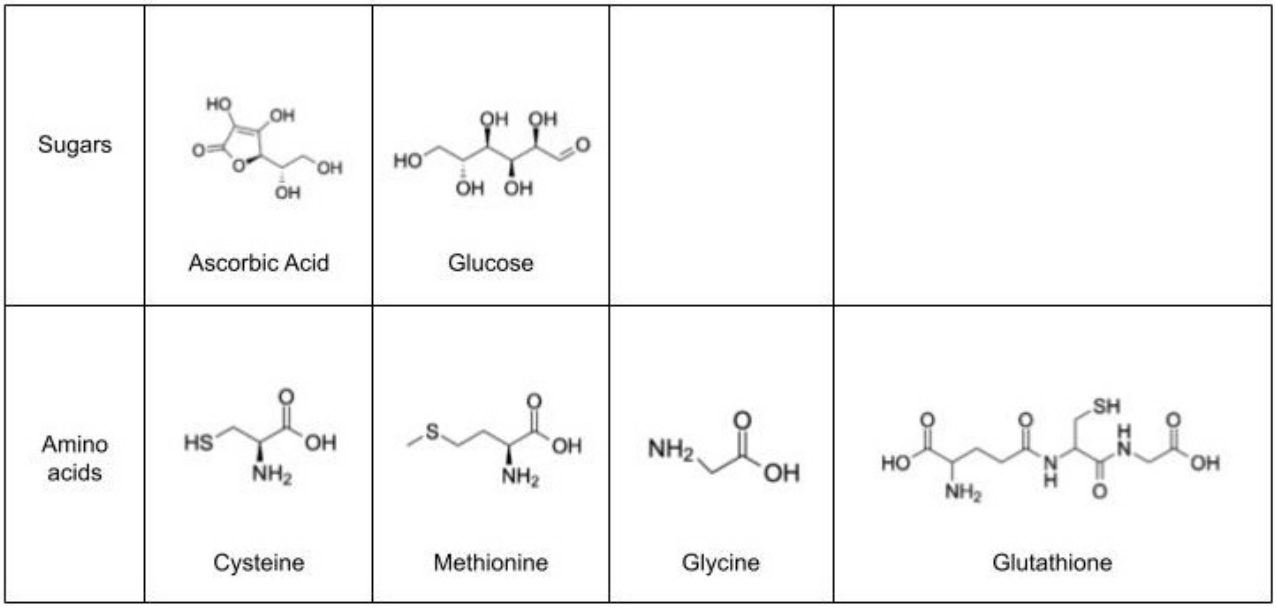

Figure 2: Chemical structures of the different compounds we screened. We screened ascorbic acid and glucose (top row; left to right), as well as three amino acids: cysteine, methionine, and glycine (bottom row; left to right). Finally, we screened a tripeptide, glutathione (bottom right)

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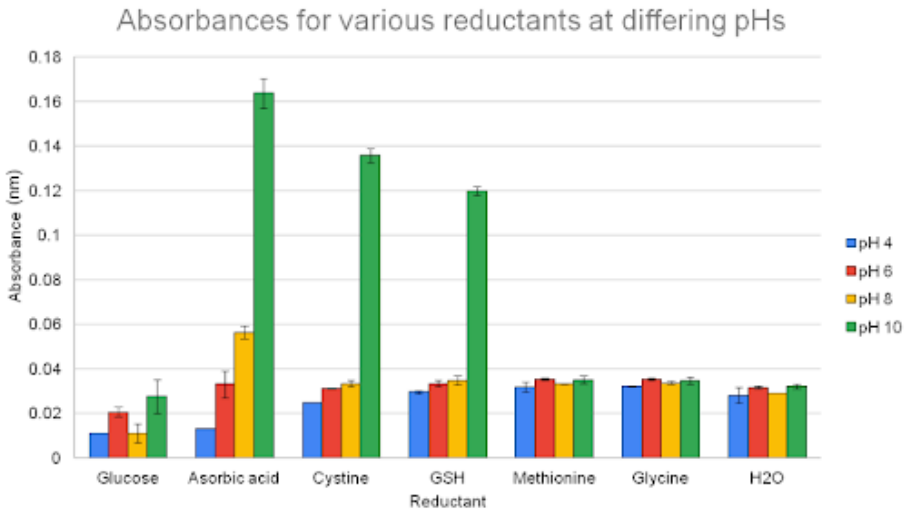

Figure 3: The absorbances for various reductants at differing pHs (pH 4, 6, 8, and 10). Elevated absorbance for ascorbic acid, cysteine, and glutathione at pH 10 measured at 570 nm with standard deviation error bars, suggesting their interference with the assay, and a correlation between pH and autoreduction.

media depleted of ascorbic acid, cysteine, and GSH, or a higher cellular density for such assays, in order to prevent interference through autoreduction.

RESULTS

We screened six reducing agents in cellular media to test whether the agents were reactive with MTT in the absence of cells. To do this, we used a plate reader to measure the absorbance at 570 nm after incubating the crude reaction mixture of each reductant (glucose, ascorbic acid, cysteine, methionine, glycine, and glutathione), MTT, DMSO and H_{2O} as negative controls, and four different pH buffers per reductant (**Figure 2**). We then used UV-visible spectrophotometry to validate the formation of MTT formazan, and LC-MS analysis to determine a mechanism of action.

The elevated absorbances of ascorbic acid, cysteine, and GSH when compared to absorbances of H2O at a pH of 10 in an MTT assay performed on the cellular media autoreductants alone, without the presence of cells, indicate these selected components common in cellular media have influence in the reproducibility and reliability of the assay (**Figure 3**). This also indicates a correlation between pH and autoreduction, specifically pH 10 to a significant increase in autoreduction. We observed negligible autoreduction at a pH of 8 or below, compared to observed absorbances at pH 10 (**Figure 3**). Most mammalian cell lines are cultured at a pH of 7.4 (27).

In order to verify the identity of the purple dye generated in these autoreduction experiments, we analyzed the crude reaction mixture by UV-Vis spectrophotometry and LC-MS. Both UV-Vis and LC-MS analysis of the crude reaction mixture showed that MTT formazan was the major product of the autoreduction reaction as we found that the purple dye that was produced was spectroscopically identical to an authentic standard of MTT formazan (**Figure 4, 5**).

Unreduced MTT dye absorbs strongly at 253 and 384 nm (yellow curve), while an authentic standard of MTT formazan absorbs strongly at 213, 301, and 574 nm (blue curve, **Figure 4**). Consistent with our expectation, the crude reaction mixture also had wavelengths of maximum absorbance at 213, 301, and 574 nm, suggesting that MTT formazan was the major chromophore produced in this reaction.

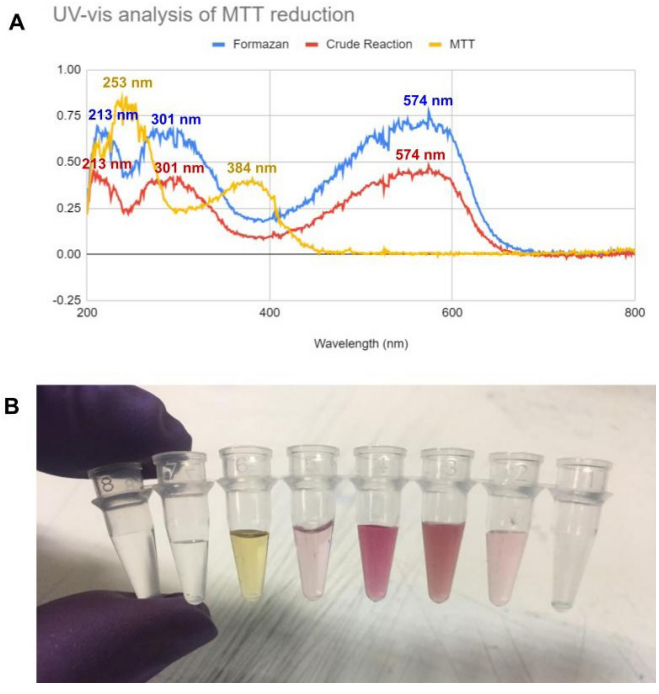

Figure 4: UV-Vis analysis of crude reaction mixture against an authentic standard of MTT and formazan. A) Both formazan and the crude reaction mixture had high absorbances at 213, 301, and 574 nm, suggesting that MTT is produced in this reaction. The wavelength was plotted against absorbance. B) Upon addition of base, the autoreduction reaction (exemplified: cysteine) instantaneously turns from yellow (third tube from left) to purple (right tubes).

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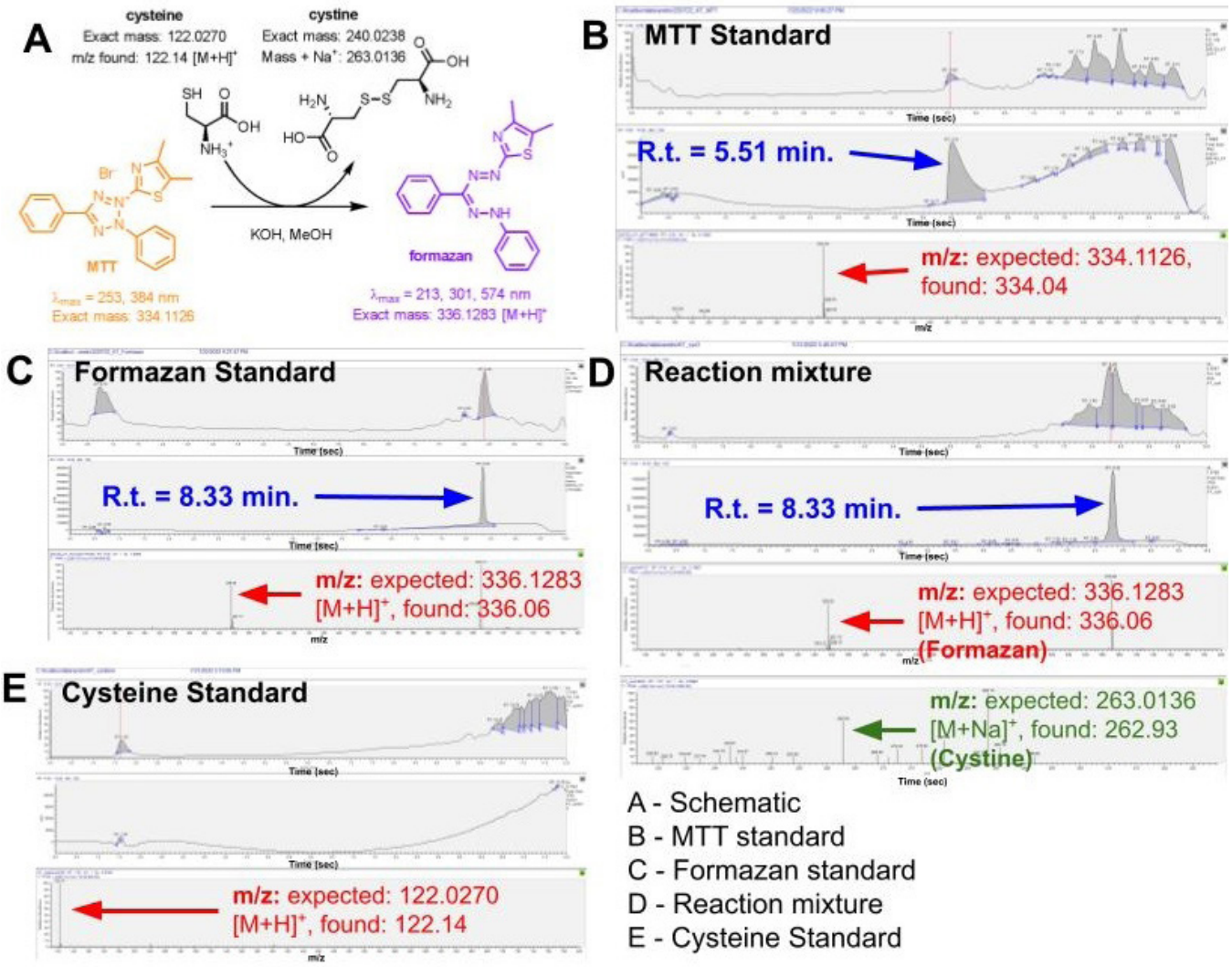

Figure 5: LC-MS analysis verifies the identities of the products of the autoreduction reaction. A) Reaction conditions of experimental set up using cysteine as a model autoreductant. B) An authentic standard of unreduced tetrazolium was found to have a retention time of 5.51 minutes. A mass of 334.04 verifies the exact mass of the tetrazolium cation. C) An authentic standard of MTT formazan was found to have a retention time of 8.33 minutes. The expected mass of 336.06 was identified as the protonated formazan [M+H]+ as well as the protonated dimer at m/z = 670.77 [2M+H]+. D) MTT formazan was positively identified as the major product of the autoreduction reaction (retention time = 8.33 min; m/z found = 336.06 [M+H]+). No MTT was observed at r.t. = 5.51 minutes, indicating full consumption of the tetrazolium. Additionally, cystine was identified in the crude reaction mixture as a sodium salt (expected m/z = 263.0136; found: 262.93). E) Authentic standard of cysteine with a retention time of 1.63.

Using cysteine as a model autoreductant, we analyzed the products of autoreduction through LC-MS. An authentic standard for tetrazolium salt (MTT) had a retention time of 5.51 minutes and a mass of 334.04 m/z, which aligns with the exact mass of the tetrazolium cation (**Figure 5B**). We found that an authentic standard of MTT formazan had a retention time of 8.33 minutes and an expected mass of 336.06 m/z, which we identified as the protonated formazan [M+H]+, as well as the protonated dimer at m/z = 670.77 [2M+H]+ MTT formazan (**Figure 5C**). When we subjected the crude reaction mixture to LC-MS analysis, we positively identified MTT formazan to be the major product of the autoreduction reaction (retention time = 8.33 min; m/z found = 336.06 [M+H]+). We did not observe MTT tetrazolium at retention time (r.t.) = 5.51 minutes, indicating full consumption (**Figure 5B**). Additionally, we identified cystine, the oxidized dimer form of cysteine, in the mass spectrum of the crude reaction mixture, to have a sodium counterion (expected m/z = 263.0136; found: 262.93), suggesting that cysteine oxidizes into cystine in the presence of MTT. It is well-reported that ascorbic acid oxidizes into dehydroascorbic acid, which is unstable to mass spectrometry analysis, and we could not identify any discrete product from the crude mass spectrometry (28) (**Figure 6**). Therefore, we performed a duplicate set of experiments in deuterium oxide (D2O) to probe the mechanism of oxidation in ascorbic acid by nuclear magnetic resonance (NMR) spectroscopy (assigned 1H and 13C NMR spectra of reduced ascorbic acid respectively) **(Figure 7B and 7C)**. The absence of a very downfield C3 proton resonance (**Figure 7D**) and the presence of only one detectable new ketone carbonyl peak

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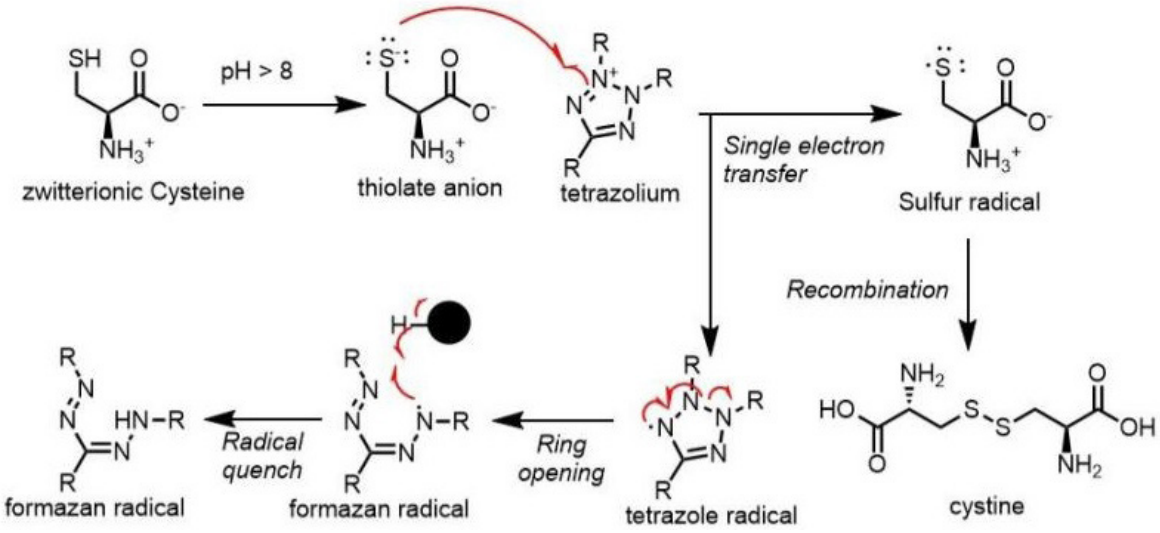

Figure 6: Proposed mechanism for single electron mediated reduction of tetrazoliums by free thiols, whereby a thiol radical source undergoes single electron transfer with a tetrazolium oxidant. The resulting tetrazole radical undergoes ring fragmentation followed by postulated solvent quenching of the nitrogen-centered radical, while two thiol radicals undergo recombination to form disulfides, such as that in cystine.

in 13C NMR of the reaction mixture (**Figure 7E**) suggests that the bicyclic dehydroascorbic acid is the predominant species present. Consistent with expectations and with our hypothesis, free thiols cysteine, glutathione, and ascorbic acid underwent efficient reduction of MTT. Basic pHs (pH = 10) yielded elevated absorbances, indicating a correlation between pH and autoreduction. Autoreduction therefore occurs under selective basic conditions in these three media components.

DISCUSSION

We studied the autoreduction of multiple media components that participate in spontaneous redox reactions with MTT and identified conditions under which acellular formation of formazan dye occurs. Consistent with expectations, cysteine, GSH, and ascorbic acid were reactive with MTT under basic conditions. Methionine, interestingly, did not react despite containing an oxidizable sulfide group, whereas cysteine and GSH have free thiol functionalities. This suggests that the oxidation pathway of thiols does not proceed through sulfoxide or sulfone oxidation, but rather through oxidation to disulfides. Various mechanisms have been proposed for the reduction of ascorbic acid, but we were not able to identify discrete redox products. As we expected, glucose and glycine also did not react. Not surprisingly, glycine, which does not contain any oxidizable functionalities, does not participate in oxidizable redox reactions. We hypothesize that glucose does not participate because it has a higher oxidation potential than the other reductants tested.

Through UV-visible spectrophotometry and LC-MS, it was determined that the product of the reduction of MTT was MTT formazan, and that free thiols were converted into their corresponding disulfides upon oxidation by MTT.

Through our studies, we observed that this reaction is greatly accelerated under basic conditions (pH = 10). We hypothesize that, as the pH approaches the pKa of a typical thiol, there is a greater proportion of thiolate anions present in solution, which, being more electron rich, are more likely to undergo single electron reduction of MTT. Likewise, the second pKa of ascorbic acid is 11.6. Knowing this, we hypothesize that the reaction occurs through the dianionic state of ascorbic acid which is in equilibrium between closed butenolide alkoxide forms and opened α-keto carboxylate forms. Thus, thiols and ascorbic acid are most likely to participate in spontaneous redox reactions with MTT (**Figure 6**). These processes are accelerated under basic conditions. We hypothesize that in all applications this occurs through single electron transfer processes. Taken together, this study provides insight into the mechanisms of autoreduction related interference of MTT assays. Moreover, these results provide a rational basis for the design of future MTT experiments to improve reproducibility by minimizing interference of results.

An experimental error that may have been a confound in our study is the lack of standardization in the time reagents spent incubating. This was due to only being able to pipette the MTT solution into a certain number of wells at a time. Though we strived to maintain proper pipetting technique, having bubbles in the solutions may have altered the absorbance readings as well, causing potential error in absorbance analysis.

Additionally, something to consider is the pH of the MTT assay being performed. If an MTT assay is conducted where the pH is not at a notably basic level, such as a pH 10, autoreduction of these reducing agents in the cellular media may not be as much of an issue. Because the basis of MTT assays involve testing different compounds for their cytotoxicity levels, pH may vary from assay to assay depending on what compounds are involved.

In future studies, further experimentation with the use of reducing agent-depleted cellular media should be conducted to ensure that MTT assays are carried out with minimal interference. Another factor to investigate is the effect of cellular density in the MTT assay, and whether a certain high density may negate the effects of autoreduction by reducing agents in cellular media.

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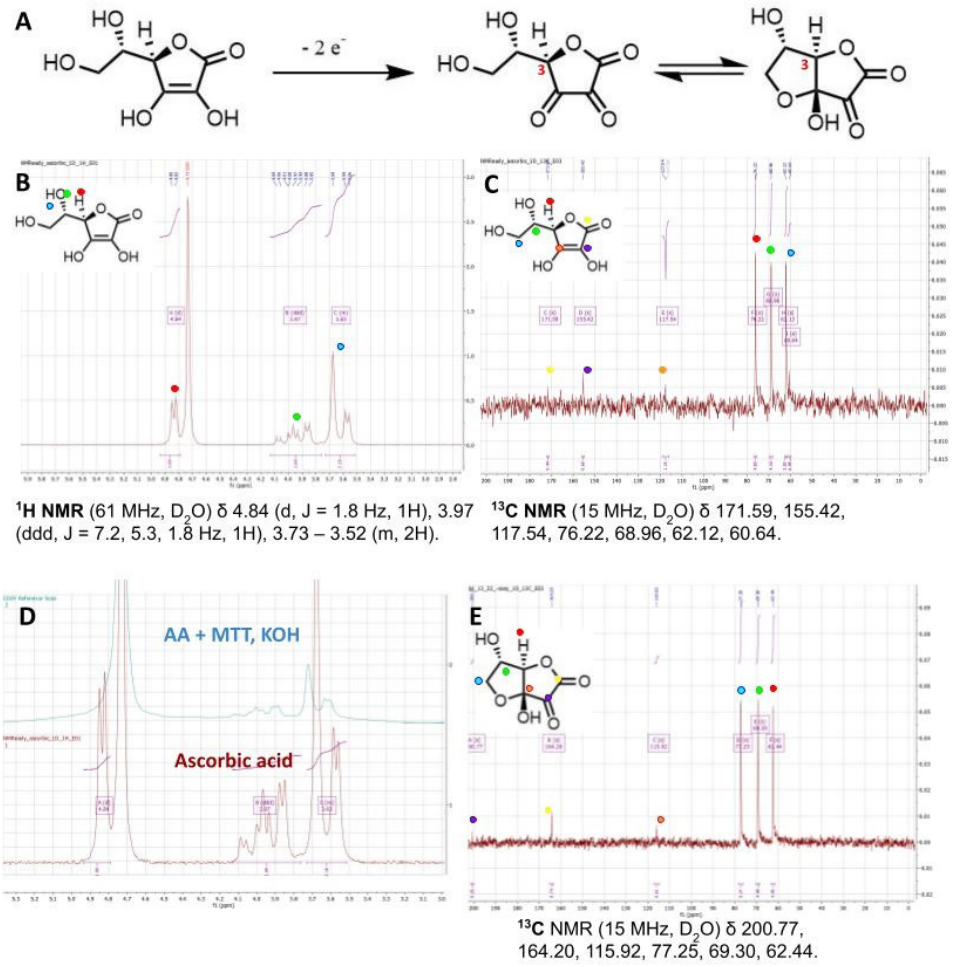

Figure 7:NMR spectra of ascorbic acid oxidation to dehydroascorbic acid. A) Reaction schematic for the oxidation of ascorbic acid into dehydroascorbic acid B) 1H NMR of ascorbic acid C) 13C NMR of reduced form ascorbic acid D) stacked 1H NMR spectra of ascorbic acid (red, bottom) and reaction mixture in D2O after addition of MTT and base E) 13C NMR of crude reaction mixture.

MATERIALS AND METHODS

MTT Dilutions & Solutions

MTT media (AK Scientific) components glucose (99.5%, Sigma Aldrich), ascorbic acid (98%, Sigma Aldrich), cysteine (>98.5%, Fisher Bioreagents), GSH (Sigma Aldrich), methionine (98%, Sigma Aldrich), and glycine (98%, Acros Organics) were diluted in 10 mL of distilled water. 0.1 mM dilutions were made of each component and run in triplicate in a 96 well plate.

MTT Benchmark Assay

We added 10 µL of a reductant, 10 µL of MTT, and 20 µL of DMSO to each well plate. We then added 160 µL of buffer to each well. Each well was identified by buffer pH, with a triplicate of each reducing agent in each buffer pH: pH 4 buffer, pH 6 buffer, pH 8 buffer, or pH 10 buffer. Each buffer contained sodium hydroxide and hydrochloric acid. The plates were incubated for 7.5 minutes, then scanned in a plate reader at 570 nm. All reported values are the average of at least three runs, measured three times each, and error bars indicate the standard deviation of measurements.

UV-visible spectrophotometry

UV-vis were acquired on a BioRad SmartSpec 3000 UV-visible spectrophotometer in quartz cuvettes from a range of 200 nm to 800 nm and at a resolution of 1 nm. All UV-vis experiments were carried out in methanol (HPLC grade, JT Baker).

Liquid Chromatography Mass Spectroscopy (LC-MS)

To determine the mechanism of the thiol-mediated redox reactions of cysteine and GSH, we monitored changes of the reaction through LC-MS. LC-MS solvents were spiked with 0.1% v/v formic acid (LC-MS grade, >95%, AK Scientific). We then performed LC-MS on a Thermo Surveyor HPLC unit coupled to a Thermo LTQ-XL linear ion trap mass spectrometer with an electrospray ion source (ESI). We performed liquid chromatography with a 10 minute linear gradient of 9:1 water:MeOH to 1:9 water:MeOH in a Thermo scientific C18 reverse phase column.

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