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Guidelines for Using 3-Nitro-L-Tyrosine as an Antidegradation Reagent of H_2O_2 in the Cold Atmospheric Plasma-Stimulated Solutions

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ABSTRACT: Over the past decade, cold atmospheric plasma (CAP)-stimulated solutions (PSS) have shown promise in medicine and agriculture. The degradation of important CAP-originated reactive species, particularly H2O2 in PSS during storage, weakens the application potential of PSS. In this study, the guidelines for the use of 3-nitro-L-tyrosine as an antidegradation reagent of H2O2 in PSS have been proposed through preliminary investigations.

KEY WORDS: cold plasma, 3-nitro-L-tyrosine, antidegradation

I. INTRODUCTION

Cold atmospheric plasma (CAP) is a near–room-temperature ionized gas composed of electrons, positive charged ions, and neutral particles.[1] Dozens of reactive species are generated in the gas phase of CAP, including hydroxyl radicals, singlet oxygen, superoxide, ozone, and nitric oxide.[2] CAP has shown a promising application in medicine through its selective anticancer capacity on dozens of cancer cell lines[3,4] and also in agriculture through protecting the food and seeds from the threat of pathogen as well as improving the germination of seeds.[5,6] Recently, the CAP-simulated biologically adaptable solutions (PSS) such as medium and simple buffered solutions have also been demonstrated to be an effective selective anticancer tool.[7–9]

Particularly for the *in vivo* application, PSS can be directly injected into the tumorous tissues in the mouse, which cannot be assessed directly by CAP.[10,11] In agriculture, PSS can also be used to inactivate the yeasts on grapes[5] and to improve the seeds' germination rate.[6] The stable storage of PSS over a relatively long time (e.g., 1 week) is nec-

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essary for its application in both medicine and agriculture. Because of the complicated reactions between the reactive species in PSS, the degradation of reactive species is a natural drawback of PSS during storage.[7,8,12,13] The freeze at an adequately low temperature such as −80°C was a strategy to resist the degradation of PSS.[12,13] A stable storage of PSS at about 2°C to 8°C is the ideal condition for many biologically adaptable solutions with a low cost. However, this has been a challenge for both plasma medicine and plasma agriculture.

Recent breakthroughs in understanding the degradation mechanism of PSS during storage revealed that the consumption of reactive species such as H_2O_2 in PSS was mainly due to the specific components in solutions such as cysteine and methionine.[14] The H_2O_2 concentration in the PSS without cysteine and methionine was much more stable than that of the PSS containing cysteine and methionine during storage at 8°C, 22°C, and −25°C.[14] More importantly, a specific tyrosine derivative such as 3-nitro-L-tyrosine (3NT) could significantly resist the H_2O_2 degradation in the CAP-stimulated medium at about 8°C.[14]

In PSS, the CAP-stimulated buffered solution is made by treating a simple buffered saline solution such as phosphate-buffered saline (PBS). PBS does not contain highly reactive chemicals such as cysteine, methionine, and pyruvate. However, the degradation in the CAP-stimulated PBS still persists during storage over a relatively long time if stored at about 8°C.[14] Although 3NT is the only confirmed antidegradation reagent for H_2O_2 in PSS at about 8°C,[14] the guidelines for the effective use of this antidegradation reagent are still largely unknown. This article presents the optimized guidelines for the application of 3NT in the storage of CAP-stimulated PBS over 1 week at 4°C.

II. METHODS

A. Making CAP-Stimulated PBS, H_2O_2-Containing PBS (H_2O_2-PBS), and 3NT-Containing PBS (3NT-PBS)

The plasma device was a CAP jet generator using helium as the carrying gas. Detailed information about the device has been provided in previous studies.[8,9,14] One milliliter of PBS in a well on a 12-well plate (Falcon) was treated by CAP for 1 or 2 minutes. The gap between the end of the dielectric plasma tube and the bottom of the 12-well plate was 3 cm. The H_2O_2–PBS was made by adding the purchased 30 wt % H_2O_2 solution (Sigma-Aldrich) in PBS. The 3NT-PBS was prepared by dissolving 3-nitro-L-tyrosine powder (Sigma-Aldrich, N7389) in PBS. All solutions were transferred into 1.5-mL centrifuge tubes by a pipette immediately after preparation. For the storage experiments, these centrifuge tubes were stored in a refrigerator at 4°C for 7 days without ambient light.

B. Extracellular NO_2^- and H_2O_2 Assay

The NO_2^- and H_2O_2 concentrations in PBS were measured using the Griess Reagent System (Promega, G2930) and Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich,

MAK165-1KT) according to the protocols provided by the respective manufacturers. For the reactive nitrogen species (RNS) and H_2O_2 measurements, the sample solutions and controls were measured by an H1 microplate reader (Hybrid Technology) at 540 nm (absorbance) and 540 nm (excitation)/590 nm (emission), respectively. The final absorbance or fluorescence of the experimental group was obtained by deducting the measured absorbance or fluorescence of the control group from the measured absorbance or fluorescence of the experimental group. The NO_2^- or H_2O_2 concentration was obtained based on the standard NO_2^-/H_2O_2 concentration–absorbance/fluorescence curves.

III. RESULTS AND DISCUSSION

The concentration of 3NT in PBS is a key factor for its antidegradation capacity during storage at 4°C. The solubility of 3NT in PBS was approximately 9 mM. Thus, the antidegradation effect of 3NT in PBS was investigated through comparing the H_2O_2 concentration in the CAP-stimulated PBS and the CAP-stimulated 3NT-PBS (0–9 mM) before and after storage.

First, the H_2O_2 in the CAP-stimulated PBS was not stable and decreased about 32% after the 7 days of storage at 4°C (Fig. 1a). This degradation trend was ceased and even was completely reversed by increasing the concentration of 3NT in PBS from 0.2 to 0.8 mM. When the concentration of 3NT in PBS was higher than 1 mM, more H_2O_2 was generated in the CAP-stimulated PBS during the 7 days of storage. In other words, 3NT regenerated H_2O_2 in solution during storage at 4°C. However, 3NT affected the generation of H_2O_2 in the CAP-stimulated PBS, particularly when the concentration of 3NT was higher than 1 mM. Due to these two basic features of 3NT, a maximum antidegradation effect should be achieved when the concentration is relatively small. In this study, such maximum effect appeared when 1 mM 3NT was dissolved in PBS. Moreover, the biologically adaptable solution with a high concentration of 3NT may be toxic to cells.[14] Thus, a low concentration of 3NT is also important for the safety application of PSS in both medicine and agriculture.

We further investigated the impact of NO_2^- on the H_2O_2 concentration in the PBS solution during storage. The concentration of NO_2^- in the 1 minute of CAP-stimulated PBS was about 10 μM. A noticeable degradation of H_2O_2 was not observed, even as the concentration of NO_2^- increased to 10 times larger than that generated in the CAP-stimulated PBS (Fig. 1b). The natural degradation of H_2O_2 in PBS causes a 13% decrease of H_2O_2 concentration. However, the decrease of H_2O_2 concentration in the CAP-stimulated PBS decreases 32% (Fig. 1a). Thus, the degradation of H_2O_2 in the CAP-stimulated PBS at 4°C is mainly due to other unknown mechanisms, such as the reaction between H_2O_2 and other reactive species, rather than the reaction between H_2O_2 and NO_2^- in the CAP-stimulated PBS.

It should be noted that the direct CAP treatment is necessary for the antidegradation capacity of 3NT. We confirmed that merely mixing the CAP-stimulated PBS with the untreated 3NT-PBS would not resist the degradation of H_2O_2. In addition, the mixture of

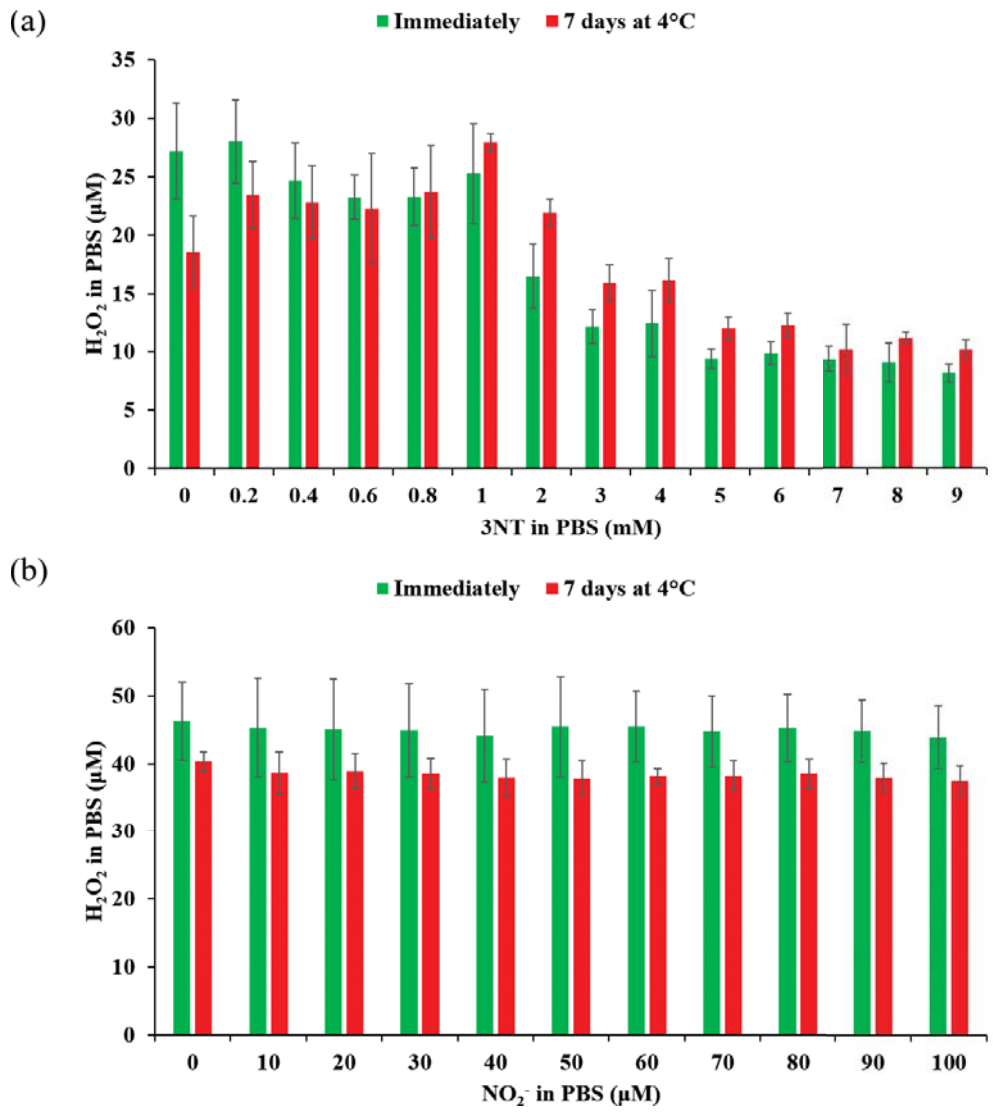

FIG. 1: 3NT is an antidegradation agent for H_2O_2 in the CAP-stimulated PBS. (a) The change of the H_2O_2 concentration in the CAP-stimulated 3NT-PBS after 7 days of storage at 4°C. The CAP-stimulated 3NT-PBS solution was made by the protocols described in Methods. (b) The change of the H_2O_2 concentration in the H_2O_2-NO_2^- PBS solution after the 7 days of storage at 4°C. The H_2O_2-NO_2^- PBS solution was made by adding specific volume of 0.1 M nitrite ion standard solution (72586, Sigma-Aldrich) in the H_2O_2-PBS solution. For all cases, the H_2O_2 concentration in the sample solution was measured immediately after the CAP treatment and 7 days after storage at 4°C without lighting. Results are presented as the mean ± standard deviation of three independently repeated experiments performed in triplicate.

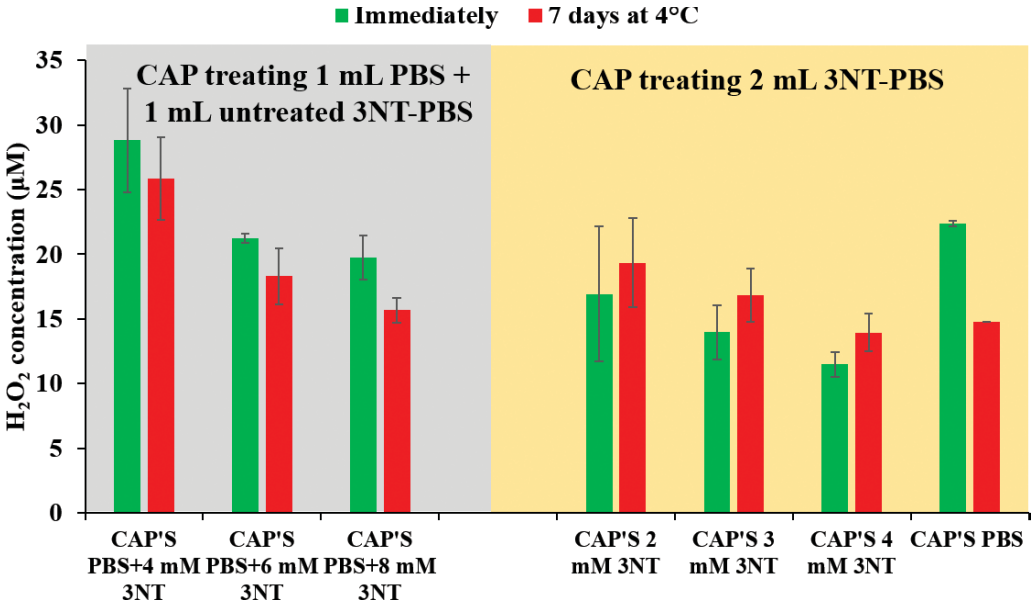

FIG. 2: Direct treatment is necessary for antidegradation of H_2O_2 in the CAP-stimulated PBS (CAP's PBS). The H_2O_2 concentration in the mixture of the untreated 3NT with the CAP-stimulated PBS was compared with the H_2O_2 concentration in the CAP-stimulated 3NT-PBS solution (CAP's 3NT) after 7 days of storage at 4°C. For the first case, 1 mL of the CAP's PBS was mixed with 1 mL of the untreated 3NT-PBS solution. Considering the dilution effect of the mixture process, the concentration of 3NT was 4 mM, 6 mM, and 8 mM, respectively. The CAP treatment time was 2 minutes. For the second case, 2 mL of 3NT-PBS solution was treated by CAP for 2 minutes. The concentration of 3NT was 2 mM, 3 mM, and 4 mM, respectively. The H_2O_2 concentration in the sample solutions were measured immediately after the CAP treatment and 7 days after the storage at 4°C without lighting. Results are presented as the mean ± standard deviation of three independently repeated experiments performed in triplicate.

the CAP-stimulated PBS with the untreated 3NT-PBS could not significantly affect the generation of the CAP-originated H_2O_2 (Fig. 2). Based on these results, two basic features of 3NT, the antidegradation of H_2O_2 during the storage and the interference on the H_2O_2 formation, both rely on direct CAP treatment. This result indicates that the CAP-originated short-lived reactive species may be related to the above unique features of 3NT.

We investigated the role of the short-lived reactive species in the H_2O_2 formation, which might provide clues to understand the weakened H_2O_2 generation during CAP treatment due to 3NT. Methylene blue (MB) is highly reactive with short-lived reactive species such as hydroxyl radicals ($OH^{\cdot}$) in aqueous solutions.[15–18] MB is not highly reactive with H_2O_2. The effect of MB on the H_2O_2 generation in the CAP-stimulated PBS was investigated by increasing the concentration of MB in PBS during CAP treatment. We found that a high concentration of MB largely inhibits H_2O_2 generation (Fig. 3a)

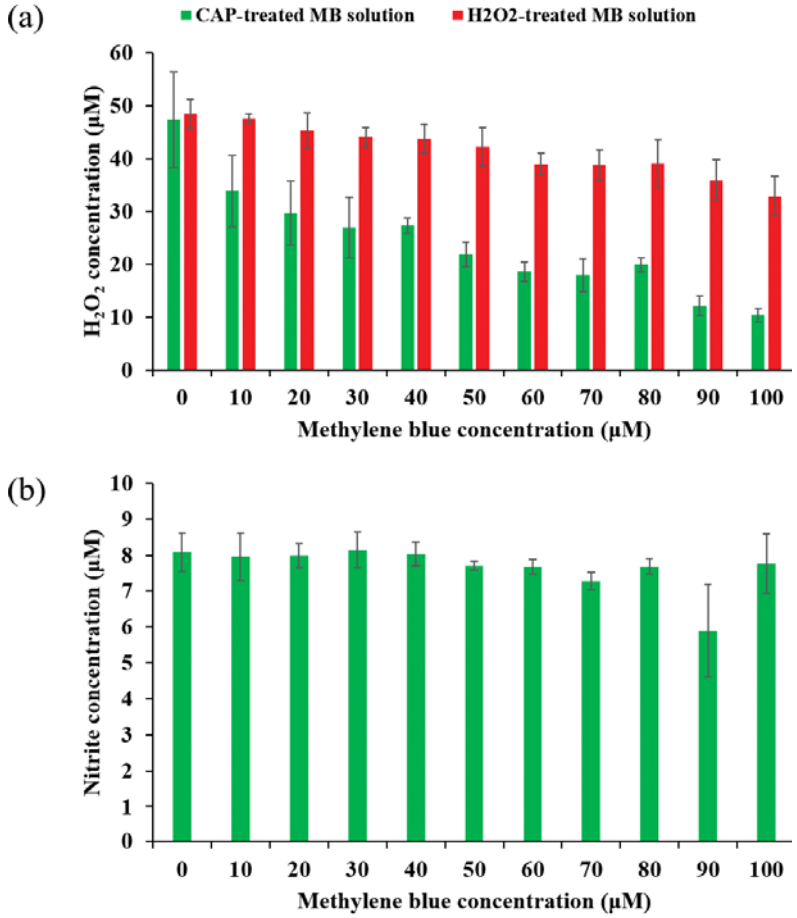

FIG. 3: The short-lived reactive species in CAP mainly contribute to the generation of H_2O_2 in the CAP-treated buffered solution. (a) H_2O_2 concentration in the CAP-treated MB-PBS solution and the H_2O_2-treated MB-PBS solution. The H_2O_2 concentrations in the CAP-treated 100 μM MB-PBS solution and the H_2O_2-treated 100 μM MB-PBS solution were 10.4 μM and 32.9 μM, respectively. Thus, about at least 22.5 μM of short-lived reactive species-based H_2O_2 generation has been inhibited by MB. Considering the H_2O_2 concentration of the CAP-treated 0 μM MB-PBS solution was about 47.4 μM, the short-lived reactive species contribute to at least 47.5% of total H_2O_2 generation. (b) NO_2^- concentration in the CAP-treated MB-PBS solution. The CAP treatment was performed on 1 mL of sample solution in a well of 12-well plate. Results are presented as the mean ± standard deviation of three independently repeated experiments performed in triplicate.

but not NO_2^- generation (Fig. 3b) in the CAP-stimulated PBS. The H_2O_2 in the CAP-stimulated PBS is mainly due to reactions based on short-lived reactive species. Similarly, 3NT may consume the CAP-originated short-lived reactive species, which could form H_2O_2 rather than consume the formed H_2O_2 after CAP treatment. The mechanism

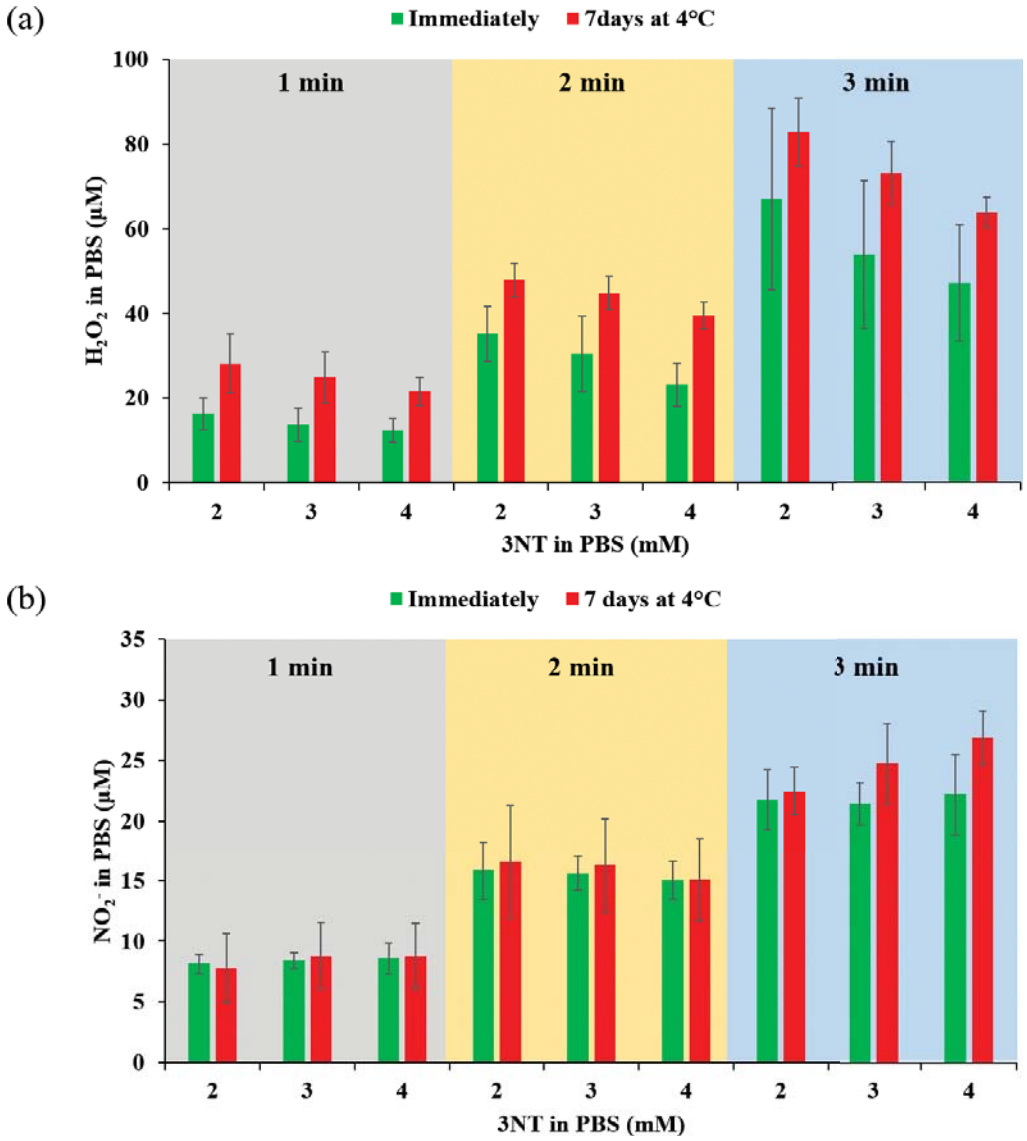

FIG. 4: 3NT can be an effective antidegradation reagent of H_2O_2 in CAP-stimulated PBS with different treatment times. (a) H_2O_2 in the CAP-stimulated PBS. (b) NO_2^- in the CAP-stimulated PBS. The H_2O_2 and $NO2^-$ concentration in sample solutions were measured immediately after CAP treatment and 7 days after storage at 4°C without lighting. Results are presented as the mean ± standard deviation of three independently repeated experiments performed in triplicate.

of regeneration of H_2O_2 in the CAP-stimulated 3NT-containing PBS during storage at 4°C is still unknown. It may be related to the specific modification on 3NT during CAP treatment. It remains a challenge in the research of stable PSS.

Finally, our results show that the antidegradation capacity of 3NT is independent of CAP treatment time. The CAP treatment was assessed between 1 and 3 minutes; the two primary effects of 3NT, the interference on the CAP-originated H_2O_2 formation and the regeneration of H_2O_2 during storage, were not affected (Fig. 4a). Thus, 3NT can be widely used in the potential application of PSS. In addition, the corresponding NO_2^- generation in the CAP-stimulated 3NT-PBS has also been investigated (Fig. 4b). The results indicate that 3NT will neither change the NO_2^- generation during CAP treatment nor trigger the regeneration of NO_2^- during storage. The only exception occurs when CAP treatment is 3 minutes. In that case, the NO_2^- concentration slightly increases during storage. The antidegradation capacity of 3NT may not correlate with the NO_2^- generated by CAP. This finding is consistent with the observation shown in Fig. 1b that NO_2^- does not cause the degradation of H_2O_2 in PBS during storage.

IV. CONCLUSIONS

To date, 3NT is the only confirmed antidegradation reagent for the H_2O_2 generated in PSS. Through an unknown mechanism, 3NT causes the regeneration of H_2O_2 during storage at 4°C, although it will also decrease the generation of H_2O_2 during CAP treatment. Based on these two features of 3NT, two guidelines for using 3NT have been demonstrated. First, the direct CAP treatment on a 3NT-containing solution is necessary for resisting the degradation of H_2O_2 during storage at 4°C. Alternatively, simple mixing 3NT with PSS does not eliminate the H_2O_2 degradation. Second, to achieve the maximum antidegradation effect, only a relatively small concentration of 3NT (as low as 1–2 mM in solution) is necessary. In this study, H_2O_2 in the CAP-stimulated 1 mM 3NT-PBS solution could be stably stored for 7 days at 4°C in the absence of ambient light.

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