A high throughput spectrophotometric method for singlet oxygen quenching

Suaib Luqman,, Kaneez Fatima & Nusrat Masood

Suaib Luqman

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Abstract

An easy spectrophotometric method for evaluating quenchers of singlet oxygen is described which can be used for high throughput assessment of large number of samples in a 96-well plate format. Singlet oxygen is a higher energy state molecular oxygen species produced in various intracellular reactions, extracellular reactions by the aid of photo sensitizer, light source and/or by chemical means. In this protocol, we have developed a simple and accurate procedure for generating singlet oxygen with no side reactions at physiological pH. The second aspect of this assay is detection by p-nitroso dimethylaniline (RNO), which is an already established suitable trap with imidazole and/or its derivatives for singlet oxygen. We have synchronized the assay system by performing various combinations of reagent used and also by plotting their spectra, pH and time-dependence curve monitored spectrophotometrically at 440nm.

Figures at a glance
Subject terms: Biochemistry

Keywords: Singlet oxygen, Hypochlorous acid, Hydrogen peroxide, RNO, L-Histidine

Introduction

Singlet oxygen (1O2) is a high energy appearance of oxygen exists in the singlet state with a total quantum spin of zero1. In 1931, it was predicted as meta-stable intermediate state in dye-sensitized photo-oxygenations2. However, in 1964, it was fully established that 1O2 was same whether generated in sensitized photo-oxygenations3, through radio frequency4 or chemical reactions (e.g. H2O2/NaOCl)5. It forms a crucial part in almost every field of science including biological (food oxidation, cell signalling, oxidative stress)6-8, medical (photodynamic therapy for microbes, malaria or cancer)9-11, environmental (as an air pollutant due to ozone decomposition)12, chemical (chemical production of 1O2 and reactions involving 1O2)13 and physical (the best part to explain the properties of 1O2, especially its reactivity and stability in various solvents and how it influences numerous aspects like light-related properties with the help of quantum theory)14 sciences (Figure 1 & Figure 2).

In this protocol, we are reporting a simple high throughput procedure for singlet oxygen quenching which can be easily determined spectrophotometrically in a 96 well plate format at 440 nm. This protocol is an improved and customized version wherein the system used to generate singlet oxygen can be done chemically (equimolar H2O2/NaOCl) or biologically (photosensitizer and light/UV source) with RNO (p-nitroso dimethylaniline), a strong yellow color dye and imidazole or its derivatives (L-Histidine)15. The bleaching is a consequence of 1O2 capture by the imidazole ring which results in the formation of a trans-annular peroxide intermediate complex16. In the absence of RNO, 1O2 decomposes or rearranges into the final oxygenation product, therefore, the imidazole system along with RNO can be used as a sensitive and selective test for the presence of 1O2 in aqueous solutions (Figure 3). Singlet oxygen can also partially bleach RNO even in the absence of imidazole derivatives. In such a case, the bleaching of RNO is strongly increased by the presence of imidazole molecules with a characteristic dependence on their concentration. The separation of the product of RNO bleaching by thin layer chromatography also serves as an additional proof of the presence of 1O2 in the system. The imidazole with RNO technique has been applied to a number of sensitizing and non-sensitizing dyes15-20. Various other methods have also been reported (Table 1)19-45 but they all require highly specialized instruments, costly fluorescent/luminescent probes, and/or highly dangerous radioactive probes still being more sensitive and efficient. If the sample size is huge and has to be assessed on a large scale, our method is more useful, easy, simple, accurate and cost-effective.

Reagents

• 25mM Disodium hydrogen phosphate (Na2HPO4, 61755005001046, Merck Ltd.)
• 25mM Sodium dihydrogen phosphate (NaH2PO4, 61784505001046, Merck Ltd.)
• 100mM Sodium hypochlorite (239305-500ml, Sigma-Aldrich)
• 100mM Hydrogen peroxide (107209, Merck Ltd.)
• 12.5 mM L-Histidine (73767-100mg, Sigma-Aldrich)
• 100µM N,N-Dimethyl-4-Nitrosoaniline [RNO] (D172405-25g, Sigma-Aldrich)
• Positive control: Rose Bengal (330000-1g, Sigma-Aldrich)
• Negative control: DABCO (D27802-25g, Sigma-Aldrich)

Equipment

• 25mM Disodium hydrogen phosphate (Na2HPO4, 61755005001046, Merck Ltd.)
• 25mM Sodium dihydrogen phosphate (NaH2PO4, 61784505001046, Merck Ltd.)
• 100mM Sodium hypochlorite (239305-500ml, Sigma-Aldrich)
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• Positive control: Rose Bengal (330000-1g, Sigma-Aldrich)
• Negative control: DABCO (D27802-25g, Sigma-Aldrich)
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**Troubleshooting • Anticipated Results • References • Acknowledgements • Figures • Associated Publications • Author Information**

- Pipettes from Thermo-Fisher Scientific India Pvt Ltd., Noida, India.
- 96 well plates from M/s Tarsons Products Pvt Ltd., New Delhi, India.
- Microlips (10µl) from M/s Tarsons Products Pvt Ltd., New Delhi, India
- Microlips (100µl) from M/s Tarsons Products Pvt Ltd., New Delhi, India
- Microlips (1000µl) from M/s Tarsons Products Pvt Ltd., New Delhi, India
- Centrifuge 15 ml tube from M/s Tarsons Products Pvt Ltd., New Delhi, India
- Eppendorf tubes from Genaxy Scientific Solan, Himachal Pradesh, India.
- Microplate Reader from Molecular Devices Corporation, California, USA.
- pH Meter from Eutech Instruments, Navi Mumbai, India.

**Procedure**

**Reagent Preparation**

Sodium Phosphate Buffer (25mM, pH 7.0)
Separate solutions of disodium hydrogen phosphate in 100ml deionized water (25mM, by dissolving 0.445g) and sodium hydrogen phosphate monobasic in 100ml deionized water (25mM, by dissolving 0.39gm) are made and then pH 7.0 is maintained by adding two solutions slowly and measuring it by pH meter.

RNO Preparation (100 µM)
It is prepared by dissolving 0.15 mg in 10ml of 25mM sodium phosphate buffer. Wrap the bottle containing RNO solution with aluminium foil.

L-Histidine (12.5mM)
Dissolve 48.5mg in 25 ml of 25mM sodium phosphate buffer.

Sodium hypochlorite (100mM)
Commercially available sodium hypochlorite (Sigma) has molarity of 0.7M. We use 100mM, so add 28.5µl in the 200µl reaction system.

Hydrogen Peroxide (100mM)
Commercially available H2O2 has 9.7Molarity. We prepare 1M stock solution from which we add 20µl for 200µl reaction mixture.

Sample Preparation
Positive control (Rose Bengal) and negative control (DABCO) is prepared in deionized water likewise other samples either a crude extract from plant, cell, tissue, pure samples can be prepared in their respective solvent i.e. DMSO, ethanol or water.

Methodology
1. Mark 96 well plate in such a way that one row in triplicate is used for L-histidine followed by another row in triplicate without L-histidine. Rows with L-Histidine contain 25µl L-histidine solution while rows without L-histidine contains same amount of buffer.

2. Add the test sample/standard in triplicate in both marked row of 96-well plate and keep control wells without any sample or standard.

3. For a standard sample, we used 100µM, 50µM, 25µM concentrations in triplicate in both L-histidine
and without L-histidine rows.

4. For control wells (without any sample) we have added 106.5µl of 25mM sodium phosphate buffer for 200µl reaction mixture.

5. Add 20µl RNO in each well and keep the plate in a dark place.

CRITICAL STEP: wrap the RNO tube with aluminum foil because it is light sensitive.

6. Then, add 20µl of 100mM H₂O₂ in each well followed by immediate addition of 28.5µl of 100mM NaOCl.

CRITICAL STEP: Add NaOCl immediately after H₂O₂ (there should be no time gap). Also first add NaOCl, then H₂O₂ otherwise RNO dye will be bleached as it is sensitive to hypochlorous acid and this may give false positive results.

7. Cover the 96-well plate with lid and wrap with aluminum foil then incubate the plate in the dark for 10 min at room temperature.

8. Finally, take the absorbance or kinetic reading (for 10 min) at 440nm in a microplate reader. Kinetic reading taken for 10 min with 5 sec orbital shaking, medium and read at 30-sec interval.

9. The graph is drawn with respect to control. Bleaching of RNO decreases the intensity of yellow color with decrease in optical density which indicates that singlet oxygen has not been quenched by the sample otherwise if there is no bleaching, it means that singlet oxygen has been quenched by the sample.

Percentage of singlet oxygen inhibition by sample is calculated by

\[ \text{Percentage} = \left( \frac{\text{control O.D} - \text{sample O.D}}{\text{control O.D}} \right) \times 100 \]

10. IC50 values can be derived using curve-fitting methods with statistical analysis.

Timing

Time line estimated for nine samples and two standards along with control.
Step 1-3 : 30 min
Step 4-8 : 30 min
Step 9-13: 90 min to 120 min

Troubleshooting

 ISSUE EXPLANATION SOLUTION
RNO bleaching on addition of NaOCl RNO is sensitive to hypochlorous acid but when used with H₂O₂,
reactions take place resulting in HCl and singlet oxygen production Use H2O2 first then NaOCl. Stock solution of RNO decolourizes in light. RNO is light sensitive Wrap RNO with aluminium foil. Variation in reading Two possible reasons: Both H2O2 and NaOCl are not added immediately after one another. Plate does not incubate in dark/ experiment not performed in dark. Use H2O2 first and then immediately add NaOCl.

Incubate plate in dark/experiment should be performed in a dark place. RNO bleaching gives false positive result. NaOCl and H2O2 bleach RNO on prolonged exposure. Record reading within 10 min. Kinetic reading shows saturated line. Kinetic reading start after 10-15 min, it means reaction is already completed. Kinetic reading should start within given time i.e. 10 min. Variation in kinetic graph Sample is not properly dissolved or may be precipitated in well. Sample should be dissolved properly. Use different solvent to check their solubility and also run a vehicle control.

**Anticipated Results**

In this protocol RNO with L-Histidine functions as a 1O2 trap (Table 2). We have chosen different concentrations of buffer, RNO, L-Histidine and equimolar solution of NaOCl/H2O2 in order to find the appropriate combination of concentrations which can give least absorbance value (Table 3; Figure 4-5). In short, if this system is working properly then more absorbance means more quenching of singlet oxygen. In Figure 4, spectra of various molarities of RNO and buffer were taken and λ max was observed. By this experiment, we have established that λ max for 100, 50, 25µm of RNO was 440nm; while at other concentration it was found to be at 350nm. We have chosen 100µm RNO concentration for our further experiments as it shows maximum absorbance while there was no significant changes obtain at different molarities of buffer. We also wish to judge the best concentration of L-Histidine and NaOCl/H2O2 which can be used for the assay procedure. We have taken five different concentrations of L-Histidine and three different concentrations of NaOCl/H2O2 and tried all possible permutation and/or combinations as reflected in Figure 5. It was observed that a combination of 12.5mM of L-Histidine and 100mM of NaOCl/H2O2 gives a minimum optical density (absorbance), hence it was used for further experimentation. The equimolar concentration of NaOCl/H2O2 generates singlet oxygen which is captured by L-Histidine forming trans-annular complex with RNO resulting in the bleaching of RNO as a consequence a decrease in the optical density (absorbance) was observed. The experiment was also performed at different pH in order to get the optimum pH and the least optical density (absorbance). At pH 7 which also represent the physiological pH (Figure 6) significant and required values were revealed. Spectral analysis was also performed with standard antioxidant in a range of 340-600nm and it was observed that 440nm was the best wavelength for all the studied antioxidant compounds (Figure 7). Time-dependent experiments revealed (Figure 8) that initial change occurs within 2 min after the reaction starts and saturation occurs after 10 min which is the endpoint.

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Figures

Figure 1: Conversion of triplet state of oxygen into singlet state on excitation
Figure 2: Factors for singlet oxygen generation and their effect on biological system

Figure 3: Diagrammatic representation of singlet oxygen generation in presence and/or absence of L-histidine
Figure 4: Estimation of $\lambda_{\text{max}}$ at different concentration of RNO and buffer system

![Figure 4: Estimation of $\lambda_{\text{max}}$ at different concentration of RNO and buffer system](image)

Figure 5: Estimation of least optical density for L-Histidine and singlet oxygen generating system (NaOCl/H$_2$O$_2$)

![Figure 5: Estimation of least optical density for L-Histidine and singlet oxygen generating system (NaOCl/H$_2$O$_2$)](image)
Figure 6: Effect of pH on L-Histidine and singlet oxygen generating system (NaOCl/H2O2)

Figure 7: Spectra of standard antioxidants on singlet oxygen quenching
Figure 8: Time-dependent effect of standard antioxidants on singlet oxygen quenching
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**Associated Publications**

This protocol is related to the following articles:

**Quenching of singlet oxygen by natural and synthetic antioxidants and assessment of electronic UV/Visible absorption spectra for alleviating or enhancing the efficacy of photodynamic therapy**
Kaneez Fatima, Nusrat Masood, and Suaib Luqman
*Biomedical Research And Therapy* 3 (2) 29/02/2016 | doi:10.7603/s40730-016-0008-6

**Eclipta yellow vein virus enhances chlorophyll destruction, singlet oxygen production and alters endogenous redox status in Andrographis paniculata**
Asifa Khan, Suaib Luqman, Nusrat Masood, Dhananjay Kumar Singh, Sana Tabanda Saeed, and Abdul Samad
*Plant Physiology And Biochemistry* 104 () 165 - 173 | doi:10.1016/j.plaphy.2016.03.027

**Author information**

**Affiliations**
Suaib Luqman
Suaib Luqman

Unaffiliated
Kaneez Fatima & Nusrat Masood

**Competing financial interests**

None

**Corresponding author**
Correspondence to: Suaib Luqman (s.luqman@cimap.res.in)

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