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RESEARCH NOTES

General Spectroscopic Protocol to Obtain the Concentration of the Superoxide Anion Radical

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Nitro Blue Tetrazolium (NBT) is a widely used chemical for the determination of superoxide anion radical (O_2^-) and superoxide dismutase activity in many fundamental biological processes. Its reduction reaction with superoxide and the associated spectral absorption data of its reduction products, i.e., monoformazan (MF) and diformazan (DF), were studied in the current research. The molar extinction coefficient, ε, of each of these two reduction products in dimethyl sulfoxide (DMSO) were calculated to be 120 000 and 235 000 M^{-1} cm^{-1}, respectively. We propose quantifying these species as a means of obtaining superoxide anion radical concentration or as a general screening protocol for the efficacy of an antioxidant.

Introduction

Superoxide anion ($O_2^{-\bullet}$) is the major reactive oxygen species that has been implicated in many biological processes.[1] Typically, any accumulation of superoxide in living systems is catabolized to avoid complications arising from cascading oxidation processes. If the superoxide species are not quenched, they can engender a host of other byproducts, including peroxides and hydroxyl radicals that are generally quite reactive. To obtain a fuller understanding of the role of the superoxide anion in living systems, it is necessary to track its concentration. Yet, it is a challenge to detect its local concentration because of its intrinsically low concentration, short lifetime, and high reactivity. Nitro Blue Tetrazolium (NBT) is one of the most widely used reagents for the activity determination (colorimetric) of superoxide anion radicals ($O_2^{-\bullet}$) and superoxide dismutase (SOD).[2] NBT (3, 3′-(3, 3′-dimethoxy [1, 1′-biphenyl]-4, 4′-diyl)bis [2-(4-nitrophenyl)-2H-tetrazolium dichloride) possesses a nitro group on its benzene ring that enhances its solvation in aqueous solutions because it can form a dication (NBT^{2+}). In a protic solvent, NBT^{2+}, as Scheme 1 illustrates, can be reduced by a stepwise two-electron reduction to monoformazan cation (MF^+) via the production of intermediate, transient free radical cations ($NBT^{+\bullet}$). MF^+ can then be further reduced to DF if there is sufficient reducing power available in the system.[3] Yet, both MF and DF are stable organic compounds and, therefore, precipitate under aqueous conditions.

It has been conjectured that NBT can specifically detect superoxide anion radicals ($O_2^{-\bullet}$) because it has been demonstrated that it does not react with other active oxygen species except •OH.[1] Nevertheless, •OH does not exist to any appreciable extent due to its exceedingly short life. In general, $O_2^{-\bullet}$ loses one electron and becomes ground state oxygen so that there is no •OH formation. The molar reaction between NBT and $O_2^{-\bullet}$ can be quantified as follows:

$$NBT + 2O_2^{-\bullet} \rightarrow MF \quad (1)$$

$$NBT + 4O_2^{-\bullet} \rightarrow DF \quad (2)$$

Although NBT does indeed have merit as a qualitative probe for superoxide, its use as a quantitative reagent in aqueous systems has been less successful because its reduction products (MF and DF) are virtually insoluble.

Nevertheless, because NBT can be reduced to MF, Beauchamp[4] suggested it as part of a potential detection method for the determination of $O_2^{-\bullet}$ in aqueous solution. NBT reacts with O_2^- producing MF which has a maximum absorbance in water at 530 nm. The concentration of O_2^- can therefore be obtained by comparing the absolute absorbance of NBT and MF. This type of methodology has already seen wide use for the screening of antioxidants in biochemical applications, food chemistry, and medicinal technology.[5−7] Also because the concentration of O_2^- can be controlled by SOD, NBT can also be used to determine SOD activity.

However, there still remain serious obstacles to its application which must be addressed. The reaction products (MF and DF) as shown in eqs 1 and 2 are poorly soluble in water, yielding precipitates that hinder the accuracy of any measurements. Also, the molar extinction coefficient of MF (ε_{MF} at 530 nm) at neutral pH water is only 12 800 M^{-1} cm^{-1} and 25 400 M^{-1} cm^{-1} at alkaline pH ($pK = 7.85$).[3] Thus, any measurement at 530 nm is not nearly sensitive enough for detection of transient $O_2^{-\bullet}$ species.

Therefore, the development of any satisfactory detection system for $O_2^{-\bullet}$ according to Beauchamp will first require a solvent to dissolve MF or DF. We have obtained the following evidence to support the development of a suitable system: it has already been reported that dimethyl sulfoxide (DMSO) dissolves KO_2 via the use of a crown ether, while $O_2^{-\bullet}$ in DMSO is known to be stable.[10] Additionally, and most importantly, it has been found that DMSO is a suitable organic solvent to dissolve MF and DF. *However, we currently have no values for the molar extinction coefficients in DMSO for these two species.*

The current research provides one of the first thorough studies of the reduction reaction of NBT with superoxide and the

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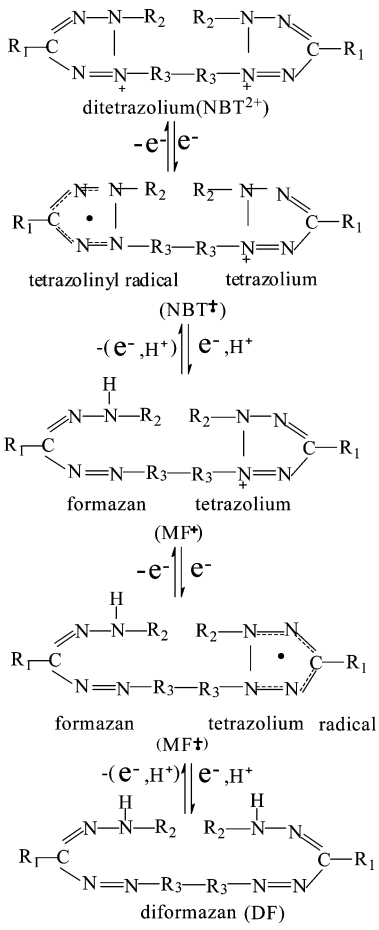
Scheme 1. Sequence of Redox Steps Leading to the Reduction of NBT



R_1=C_6H_5; R_2=p-$NO_2C_6H_4$; R_3=m-$CH_3OC_6H_4$

ensuing spectral characteristics of its reduction products, monoformazan (MF) and diformazan (DF), to provide a universal detection and quantitation method for O_2^- that has high sensitivity.

Experimental Section

NBT ($C_{40}H_{30}N_{10}O_6Cl_2$, purity 99%) was obtained from Sigma-Aldrich chemical company and used as received. 18-Crown-6 ether was obtained from Alfa Products (99%) and was also used as received. KO_2 (purity 96%) was obtained from Fluka and was ground into a powder in a nitrogen atmosphere (glovebox) before dissolving. DMSO was dried over molecular sieves and bubbled with N_2 to completely remove oxygen.

NBT was dissolved in DMSO directly with an N_2 blanket before detection. A 0.04 g portion of KO_2 powder was added into DMSO (100 mL) together with 0.3 g 18-crown-6-ether (the molar ratio of KO_2 to 18-crown-6-ether was approximately 1:2) under stirring at room temperature and ambient conditions. The stock KO_2 solution was kept under nitrogen in a refrigerator. The stock KO_2 solution was diluted 50 times with DMSO before use.

The absorbance of all reaction products were measured by an Agilent 2854 UV/vis spectrophotometer. All of the experiments were run at ambient temperature (19−20 °C).

Results and Discussion

We began by examining the absorbance data for the reaction of NBT with O_2^- and calculating the molar extinction coefficient

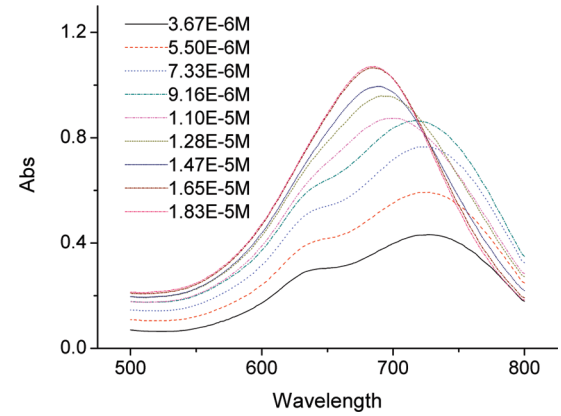


Figure 1. UV/vis spectrum of monoformazan (MF) and diformazan (DF).

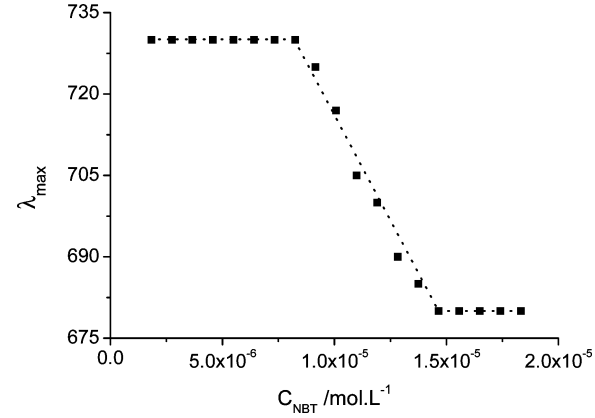


Figure 2. Change in maximum wavelength for the reaction product of NBT with $O_2^{-\bullet}$.

of MF and DF in DMSO which can then be used to quantitate O_2^-. As described by eqs 1 and 2, the product distribution is different when the ratios of the reactants ($O_2^{-\bullet}$/NBT) differ. When the ratio of $O_2^{-\bullet}$/NBT is less than 2, the product is mainly MF whose molar weight is determined by the dosage of O_2^-. When the ratio of $O_2^{-\bullet}$/NBT is more than 4, the reaction is controlled by NBT and the only reaction product is DF. When the ratio of $O_2^{-\bullet}$/NBT is between 2 and 4, different ratios of MF and DF are obtained. Figure 1 presents a typical UV/vis absorbance spectrum of the reaction system containing NBT and O_2^-.

It can be seen in Figure 1 that when the amount of $O_2^{-\bullet}$ is maintained constant while the concentration of NBT is increased, the absorbance of the reaction product DF increases gradually.

When the molar ratio of O_2^-/NBT is more than 4, the spectrum clearly illustrates two peaks at 680 and 730 nm. The higher amplitude peak appeared at 730 nm, but as the dosage of NBT increased, the profile of the absorbance changed as evidenced by the bimodal absorbance distribution at 680 and 730 nm. This is due to the formation of MF and DF, respectively, in accordance with the explanation by Bielski.[3,10] When the molar ratio of $O_2^{-\bullet}$/NBT changes from 4 to 2, the molar ratio of MF (680 nm) to DF (730 nm) (MF/DF) ensuingly became larger as evidenced by the absorbance signatures. However, at this stage, the change in the maximum absorbance could not be estimated. When the dosage of NBT was high enough, the only product was MF; after that point, the maximum absorbance of the product became constant because all of the $O_2^{-\bullet}$ was consumed. Figure 2 displays the maximum wavelength

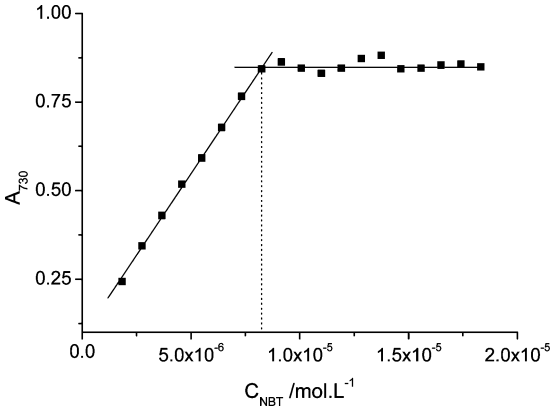


Figure 3. Absorbance of reaction product at 730 nm as a function of NBT concentration.

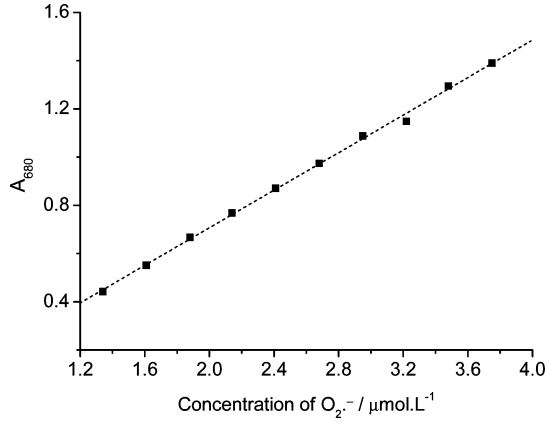


Figure 4. Relationship of absorbance of monoformazan (MF) at 680 nm as a function of the concentration of $O_2^{-\bullet}$.

variation in the reaction system independent of the NBT concentration.

The molar extinction coefficient of MF and DF ($\varepsilon_{MF\ and}\ \varepsilon_{DF}$) can be calculated according to the Beer−Lambert Law, $A = \varepsilon bC$, where A is the absorbance; ε is the molar absorptivity or extinction coefficient; b is the path length for the UV/vis light (1 cm); and C is the concentration of the sample.

Once the absorbance (A) is obtained, ε can be calculated if the concentration of the sample is known. In general, the concentration of $O_2^{-\bullet}$ cannot be obtained by simply weighing KO_2 gravimetrically because of its hydrolytic instability. However, the concentration of NBT can be obtained from its mass.

In Figure 2, there are two inflection points in the entire spectrum due to the change in concentration of NBT when the amount of $O_2^{-\bullet}$ is fixed in the reaction system. The first inflection point is DF where, as shown previously, the molar ratio of $O_2^{-\bullet}$/NBT should be 4. At the second point, the chromophore is MF and, therefore, the molar ratio of $O_2^{-\bullet}$/NBT should be 2. Thus, the molar concentration of the two species should be the same as that of NBT at these points. On the basis of the inflection points, it is possible to calculate the concentration of superoxide anion radical if the concentration of NBT is known.

Therefore, if the extinction coefficient of DF is known under specific conditions, the concentration of NBT can be calculated from the absorbance at 730 nm. In the same way, the concentration of superoxide anion radical can be calculated from the absorbance at 680 nm again using the Beer−Lambert Law (knowing the extinction coefficient of MF) when the ratio of $O_2^{-\bullet}$/NBT is less than 2.

The reaction product absorbance at 730 nm is shown in Figure 3.

The absorbance of the product showed a good linear relationship with the dosage of NBT when the ratio of $O_2^{-\bullet}$/NBT > 4 (where only DF was produced). The equation is given as follows:

$$A_{DF}^{730} = (0.085 \pm 0.0066) + (92600 \pm 1200)C_{NBT} \quad (R^2 = 0.999) \quad (3)$$

When the ratio of $O_2^{-\bullet}$/NBT < 4, MF begins to accumulate, but as already known, the absorbance of product in the reaction system at 730 nm does not change much. An inflection point in the absorbance curve is obtained by changing the dosage of NBT. This point is the critical point of DF, at which the molar ratio of $O_2^{-\bullet}$/NBT = 4 is described by the following equation:

$$A_{730} = \varepsilon_{DF}^{730} b C_{DF} \quad (4)$$

C_{DF} is the concentration of NBT at the critical point and ε_{DF}^{730} is the extinction coefficient of DF, which can be calculated by this equation. From a series of absorbances at 730 nm for the critical points, ε_{DF}^{730} was obtained and was equal to 120 000 M^{-1} cm^{-1} in DMSO.

When the ratio of $O_2^{-\bullet}$/NBT ≤ 2, the only product is monoformazan. At the point where the molar ratio of O_2^{-}/MF = 2, the following equation was obtained:

$$A_{680} = \varepsilon_{MF}^{680} b C_{MF} \quad (5)$$

The concentration of MF was known from the concentration of NBT, and thus, the value of ε_{MF}^{680} in DMSO was found to be 235 000 M^{-1} cm^{-1}.

Kim[11] calculated the extinction coefficient of $O_2^{-\bullet}$ in DMSO at 250 nm (ε_{250}) to be 2686 ± 29 M^{-1} cm^{-1}, which can be used for the determination of $O_2^{-\bullet}$. Obviously, the colorimetric method that uses NBT is far more sensitive for the determination of $O_2^{-\bullet}$ concentration.

As known, when the molar ratio of $O_2^{-\bullet}$/NBT < 2, 2 mol of $O_2^{-\bullet}$ react with 1 mol of NBT to produce 1 mol of MF. An equation describing the absorbance of MF and the concentration of $O_2^{-\bullet}$ was developed using the data from Figure 4 in which the concentration of super oxide radical was between 1.34 × 10^{-6} and 1.23 × 10^{-5}:

$$A_{680}^{MF} = (-0.0712 \pm 0.0147) + (127\,700 \pm 1800)C_{SOR} \quad (R^2 = 0.999) \quad (6)$$

Here, C_{SOR} is the concentration of superoxide anion radical ($O_2^{-\bullet}$). For the method, there are still factors influencing its final determination, such as the reaction time and temperature. During the reaction of NBT and $O_2^{-\bullet}$, the absorbance of the product was stable after 4 min; therefore, the determination of absorbance was carried out at the 5 min mark after starting the reaction because obtaining the absorbance after too short of a time does not ensure complete reaction. Also, the reaction temperature had a significant effect on the stability of $O_2^{-\bullet}$. When the temperature was greater than 24 °C, $O_2^{-\bullet}$ became unstable; thus, the reaction temperature was maintained at 19−20 °C (this temperature is close to the freezing point of DMSO) during the entire experiment.

Conclusions

The reduction process of NBT is complex, and this finding has been presented in this communication. Two reduction

substances (monoformazan and diformazan) are produced relative to the dosage of reductant. The molar extinction coefficient of MF and DF have been calculated to be 120 000 and 235 000 M^{-1} cm^{-1} in DMSO, respectively. When the concentration of NBT is sufficiently high, the absorbance of monoformazan correlates with the concentration of superoxide anion radical with good linearity giving an equation of $A_{680}^{MF} = (-0.0712 \pm 0.0147) + (127\,700 \pm 1800)C_{SOR}$ having an $R^2 = 0.999$. Note that because the absorbance maxima for MF and DF are relatively close, a mixture of the two can complicate the final superoxide anion radical concentration measurement and will therefore require increasing or decreasing the NBT concentration accordingly. This method nevertheless represents a potential new avenue for O_2^- determination, antioxidant screening, and probing oxidation reactions.

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