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A novel fluorescent method for determination of peroxynitrite using folic acid as a probe

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Abstract

A novel method for the determination of peroxynitrite using folic acid as a fluorescent probe is described. The method is based on the oxidation of the reduced, low-fluorescent folic acid by peroxynitrite to produce a high-fluorescent emission product. The fluorescence increase is linearly related to the concentration of peroxynitrite in the range of 3×10^{-8} to 5.0×10^{-6} mol L^{-1} with a correlation coefficient of 0.998, and the detection limit is 1×10^{-8} mol L^{-1}. Interferences from some metal ions normally seen in biological samples, and also some anions structurally similar to peroxynitrite were studied. The optimal conditions for the detection of peroxynitrite were evaluated.

Keywords: Peroxynitrite; Folic acid; Fluorimetry; Probe

1. Introduction

Peroxynitrite chemistry is of remarkable interest with increasing evidences showing the importance of peroxynitrite in the development of oxidative damage in various pathologies [1,2]. Peroxynitrite, generated from the diffusion-controlled reaction between the nitrogen monoxide and superoxide radicals [3,4], can cause lipid peroxidation [5], chemical cleavage of DNA [6,7], inactivation of key metabolic enzymes (e.g., aconitase [8,9], succinate dehydrogenase, ribonucleotide reductase, and cytochrome oxidase of the mitochondrial electron transport chain [10,11]), and reduction in cellular antioxidant defenses by oxidation of thiol pools [12]. Peroxynitrite can also nitrate protein tyrosine residues, possibly leading to inactivation of tyrosine kinase [13] or the disruption of key cytoskeletal components that may contribute to the pathogenesis of diseases, including inflammatory processes, ischemia-reperfusion, septic shock, and neurodegenerative disorders [14–16].

Due to the great importance of the research mentioned above, the point of interest was concentrated upon the measurement of peroxynitrite in both pathological and normal conditions in biological systems. However, the peroxynitrite assay is extremely difficult because of the low concentration, high activity, and elusive natures of peroxynitrite. Currently, most of techniques for peroxynitrite measurement are indirectly based on chemical detection of the decomposition products removed from biological systems. Peroxynitrite generation is usually measured by ultraviolet–visible (UV–vis) spectroscopy [17], chemiluminescence [18], immunohistochemistry [19], amperometry [20,21], electron spin resonance (ESR) [22,23], and fluorescence [24–26].

Two fluorogenic probes, dihydrodichlorofluorescein (DCFH) and dihydrorhodamine-123 (DHR-123), which are considered to be ideal, have been widely employed to monitor peroxynitrite in various systems [27,28]. The above methods are based on the oxidation of the reduced, non-fluorescent forms of fluorescent dyes such as fluorescein and rhodamine by peroxynitrite to produce the parent dye molecule, resulting in a dramatic increase in fluorescence response. However, the synthesis of these probe molecules is rather difficult and inconvenient [29]. Additionally, the use of organic dyes is very likely to result in environmental pollution, which should be avoided as possible as we can. Thus, a cheap, fast, and simple method to determine the peroxynitrite is needed.

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Folic acid is made up of a pterin moiety (purine and pyrazine fused together) that is linked to the side chain containing *p*-aminobenzoic acid (pteroic acid) and glutamic acid. Folic acid functions as a cofactor in the transfer and utilization of one carbon groups, which plays a key role in the biosynthesis of purines and pyrimidines and regeneration of methionine [30]. Recent studies showed that the pathogenesis of cardiovascular, hematological and neurological diseases and cancer are associated with the antioxidant activity of folic acid. Folic acid can act as a peroxynitrite scanvenger [31,32] due to its high reaction rate with peroxynitrite. Folic acid is a low-fluorescent substance, but the oxidation folic acid by peroxynitrite can give high-fluorescent emission product. Comparing with the above mentioned two dyes, folic acid is relative inexpensive, not toxic to biological system and stable in solution. The present study was designed to build a new fluorometric method for peroxynitrite determination.

2. Experimental

2.1. Chemicals

Folic acid (Shanghai Chem. Agent, Inc., Shanghai, China) was prepared by dissolving appropriate amount of folic acid in 0.001 mol L^{-1} NaOH and kept frozen, and the working barbital buffer solution was prepared by dissolving 4.125 g barbital sodium in 500 mL distilled water and add 0.7 mL 1.0 mol L^{-1} HCl. All the reagents were of analytical reagent grade and were used without further purification, unless stated otherwise. Doubly distilled water was used throughout.

2.2. Synthesis of peroxynitrite

Peroxynitrite was synthesized according to the previous description [33]. An aqueous solution of 0.6 mol L^{-1} sodium nitrite was rapidly mixed with an equal volume of 0.7 mol L^{-1} H_2O_2 containing 0.6 mol L^{-1} HCl and then immediately quenched with the same volume of 1.5 mol L^{-1} NaOH. Then some MnO_2 powder was added to the mixture solution to eliminate the excess H_2O_2, then the mixture was filtered and stored at −18 °C. Peroxynitrite concentration was determined by UV spectrometry at 302 nm ($\varepsilon = 1670$ L mol^{-1} cm^{-1}) [29].

2.3. Apparatus

Absorption spectra were obtained on a cary-100 UV–visible spectrophotometer (Varian, USA). The fluorescence spectra and relative fluorescence intensity were measured with a fluorescence-4500 (Hitachi, Japan) with a 10 mm quartz cuvette, the excitation and the emission wavelength slits were respectively set at 5.0 and 10.0 nm. All pH values were measured with a pH S-301 digital ion meter.

2.4. Procedure

In a set of 10 mL-volumetric tubes containing pH 9.4 barbital buffer solution, 1.0 mL of folic acid (1.0×10^{-4} mol L^{-1}) and different amount of peroxynitrite were added. The tubes were closed and then quickly and carefully shaken. The reaction solution was kept at room temperature for 5 min. The fluorescence intensity of the solution was recorded at 460 nm with the excitation wavelength set at 380 nm.

2.5. Detection of peroxynitrite in biological samples

At first, the Hela cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) which contains 10% heat-inactivated fetal calf serum, penicillin (100 U mL^{-1}) and streptomycin (50 μg mL^{-1}) in a 5% CO_2 environment at 37 °C. Next, these cells were plated on a six-well chamber. When growing up to 80% confluency, they were treated with various concentration of adriamycin for 6 h. Then the adherent cells were detached by trypsin treatment and washed twice with PBS containing 1% BSA. After that, these cells were added to barbital buffer solution containing 1.5×10^{-5} mol L^{-1} folic acid and were broken using an ultrasonic disintegrator. Finally, the suspension was taken for analysis under the optimally experimental conditions.

3. Results and discussion

3.1. Spectra characteristics

Fig. 1 shows the fluorescence excitation and emission spectra of folic acid and the mixture of folic acid with peroxynitrite in barbital buffer solution (pH 9.4). Folic acid has low fluorescence excitation and emission spectra. However, high fluorescence product generated by the introduction of peroxynitrite into the solution of folic acid, resulting in dramatic increase in spectra characteristics with excitation maximum at 380 nm and fluorescence emission maximum at 460 nm.

3.2. Optimization of the general procedure

The effect of pH on the fluorogenic reaction was studied in the range of 7–9.4 in barbital buffer solution, and the results are

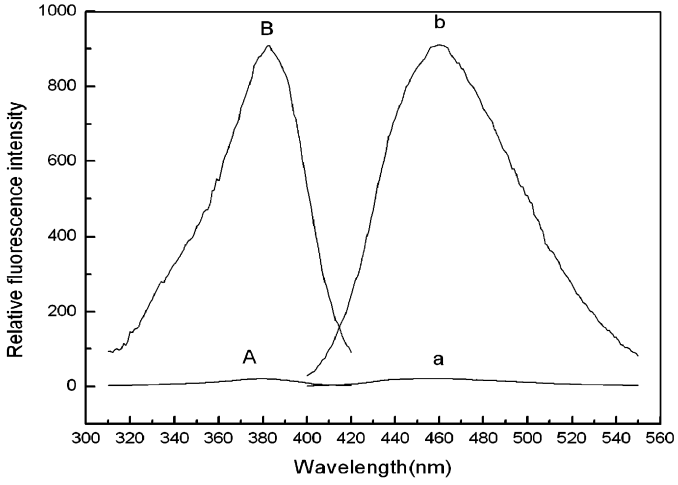

Fig. 1. Fluorescence excitation and emission spectra of the system. (A and a) Folic acid, 1.5×10^{-5} mol L^{-1}; (B and b) folic acid, 1.5×10^{-5} mol L^{-1} + peroxynitrite, 2.0×10^{-6} mol L^{-1}; pH 9.4 barbital buffer solution, 25 °C.