

Chemiluminescence

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Introduction

Inflammatory cells such as neutrophils, eosinophils, and macrophages produce a multitude of reactive oxygen species (ROS) when they are activated. Of the reactive oxygen species, superoxide anion radical (SOD , O_2^-), a precursor of several oxidants such as hydrogen peroxide (H_2O_2), hydroxy radical (OH), singlet oxygen ($^1\text{O}_2$), and hypochlorite (HOCl), can be formed in, and released from, each of three cell lines. On the other hand, $^1\text{O}_2$ (singlet oxygen, metastable oxygen) and hypohalides can be generated by myeloperoxidase (MPO)-mediated reactions in the neutrophil phagosome at pH 4.5, and by the eosinophil peroxidase-mediated reaction in eosinophils at physiological pH. It is, therefore, important to detect and measure the generation of O_2^- and $^1\text{O}_2$ in small amounts in such biological systems. Because production of highly bactericidal agents such as $^1\text{O}_2$ and HOCl is a result of myeloperoxidase activity, simple and sensitive methods to measure this enzyme activity are required. Herein, we describe the measurement of O_2^- , $^1\text{O}_2$ generation, and myeloperoxidase activity, in leukocytes by chemiluminescence using cypridina luciferin analogues as chemiluminescence probes.

O_2^- Production by Stimulated Leukocytes

When granulocytes or macrophages are stimulated, they produce and release O_2^- . The O_2^- released from these cells reacts efficiently with cypridina luciferin analogue (CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2- α]pyrazin-3-one. MCLA, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one) (Figure 1) to produce an excited carbonyl which emits light in the visible region; i.e., maximum emission at 380 nm for CLA and at 457 nm for MCLA [1-3].

Protocol

1. Dissolve powdered Hanks' balanced salt solution (HBSS) for tissue culture (Nissui Pharmaceutical Co. Ltd, Tokyo) in

distilled water (9.8 g/L) and adjust to pH 7.4 by use of aqueous Na_2HPO_4 solution (280 mOsm).

2. Prepare granulocytes or macrophages by standard methods.
3. Dissolve MCLA ($E_{430\text{ nm}}=9600\text{ M/cm}$) or CLA ($E_{410\text{ nm}}=8900\text{ M/cm}$) in distilled water at a concentration of $40\text{ }\mu\text{M}$.
4. Dilute granulocytes or macrophages with HBSS at 5×10^4 cells for MCLA or 4×10^5 cells for CLA.
5. Add MCLA or CLA ($50\text{ }\mu\text{L}$, final concentration: $1\text{ }\mu\text{M}$).
6. Preincubate at 37°C for 3 min.
7. Add opsonized zymosan (OZ, in water, 20 mg/mL , $80\text{ }\mu\text{L}$), formyl-methionyl-leucyl-phenylalanine (fMLP in Ca^{2+} - and Mg^{2+} -free HBSS/ DMSO = 1:1, v/v, $200\text{ }\mu\text{M}$, $10\text{ }\mu\text{L}$), or 4β -phorbol myristate acetate (PMA in Ca^{2+} - and Mg^{2+} -free HBSS/DMSO = 1:1, v/v, $2\text{ }\mu\text{g/mL}$, $20\text{ }\mu\text{L}$) (Total volume 2.0 mL).
8. Measure chemiluminescence.

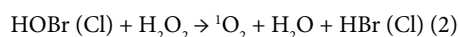
MPO in granulocytes $^1\text{O}_2$ measurements

When peripheral leukocytes derived from bone marrow are separated the neutrophils contain abundant granule myeloperoxidase as a host enzyme. Under acidic conditions, myeloperoxidase (MPO) catalyses the production of HOBr and HOCl in the presence of Br^- and Cl^- , respectively (Reaction 1). The HOBr or HOCl can efficiently react with H_2O_2 to generate $^1\text{O}_2$ (Reaction 2) [4].

MPO



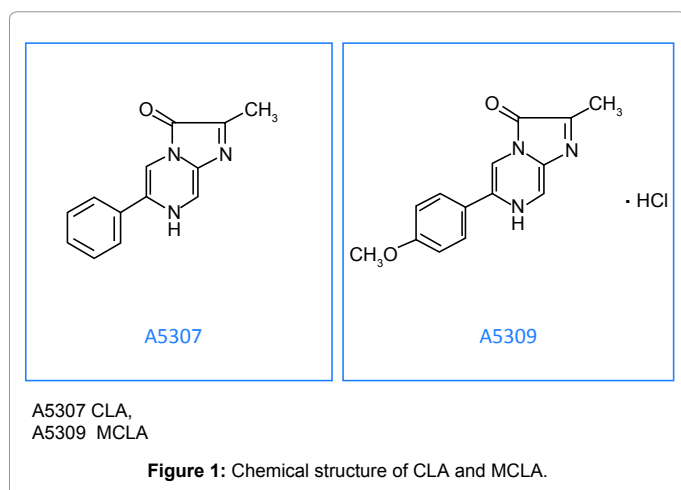
pH 4.5-5.0



MCLA reacts not only with O_2^- but also with $^1\text{O}_2$ to emit light with maximum intensity at 457 nm . The addition of $0.5\text{ }\mu\text{M}$ SOD to the system, in which both O_2^- and $^1\text{O}_2$ are generated, completely quenches the O_2^- dependent luminescence leaving only the $^1\text{O}_2$ -dependent luminescence.

Protocol

1. Mixing human neutrophils and hexadecyltrimethylammonium bromide (0.02% w/v) in potassium phosphate buffer (pH 6.0, 50 mM) for 10 min.
2. Sonicate the mixture on ice for 30 s (0.5-s bursts at 0.5-s



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intervals) at an output level of 30 W (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT, USA).

3. Centrifuge at 40000 g for 20 min at 4°C (The supernatant is used as the sample).
4. Preincubate a standard solution containing MCLA (in water, 200 µM, 100 µL) SOD (in water, 50 µM, 20 µL), KBr (in water, 50 µM, 20 µL), desferrioxamine (in water, 2 mM, 20 µL), H₂O₂ (40 mM, 25 µL), and acetate buffer (0.2 M, 1 mL) (Total volume 1.95 mL).
5. Measure chemiluminescence until baseline is constant.
6. Inject samples rapidly (50 µL) by means of a microsyringe.
7. Measure chemiluminescence.

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