

# Water Soluble Peroxyoxamide Reagent: Evaluation for Fluorescent Silica Nanoparticles Chemiluminescence

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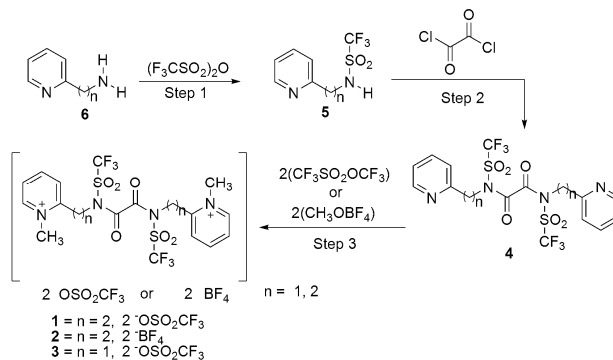
## ABSTRACT

Enzymatic and nonenzymatic chemiluminescence immunoassay methods are widely used as an analytical tool in various bio-molecular detection applications. Here, we demonstrate the synthesis and <sup>1</sup>H NMR analysis of peroxyoxamide reagents and its application for characterization of covalently linked texas red dye with goat anti-mouse IgG antibody and generic goat IgG antibody on mesoporous silica-particles through luminometer and CCD imaging in (90%) aqueous solution. Further, fluorescein dye doped silica-nanoparticles (F-SiNPs) were synthesized and characterized through SEM and TEM images. The function of F-SiNPs was analyzed in nanomolar range through chemiluminescence reaction of peroxyoxamide reagent with hydrogen peroxide in aqueous medium.

**Keywords:** peroxyoxamide, fluorescent dye, goat antibody, silica nanoparticles, chemiluminescence

## 1 INTRODUCTION

The chemiluminescence reactions are widely used as analytical tools in various chemical and biological applications [1], because they offer a practical simplicity and significantly reduce back ground interference, high sensitivity and short response time. Among the chemiluminescence reactions, peroxyoxalate chemiluminescence (POCL) is one of the most efficient synthetic chemiluminescence systems, which was first introduced by Chandross in 1963 [2]. POCL reaction involves hydrogenperoxide oxidation of an aryl oxalate ester, like bis(2,4,6-trichlorophenyl)oxalate (TCPO) or bis(2,4-dinitrophenyl)oxalate (DNPO) in the presence of fluorophore to produce a high energy intermediate (1,2-dioxetanedione), which excites a fluorophore in organic solvent (tetrahydrofuran), then the fluorophore quickly emits light. The key feature of this reaction is the capability of excite a wide range of fluorophores from the ultra-violet



**Scheme 1:** Synthetic steps for peroxyoxamide reagents

to the near infrared region. Therefore, this technology is used for the detection of a large rang of fluorophores [3].

The application of POCL in the detection of biological agents in aqueous medium is very limited due to partial solubility and low stability of TCPO and other oxalate esters in aqueous bio-friendly medium. Although, several water soluble-oxalate esters and amides have been reported, initially by Mohan [4] and Neil W. Barnett co-workers [5]. But no development has been reported in biodetection using water soluble POCL, except Daban's group has been reported the detection of a biotin-avidin interaction on a PVDF membrane using a water soluble peroxyoxalate reagent [6]. Here we demonstrated the synthesis and evaluation of water soluble peroxyoxamide compounds (scheme 1) and their application for fluorescent organic dye labeled biomolecules detection in 90% aqueous solution and florescent organic dye doped silica nanoparticles chemiluminescence.

## 2 EXPERIMENTAL

### 2.1 Reagents and Instrumentation

All the chemical reagents used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA, Fisher Scientific, New Jercey, USA), Invitrogen molecular probes

(Eugene, Oregon, USA) and antibodies was purchased from Immunology Consultant Laboratory, Newberg, OR USA.

A 500 MHz NMR (Bruker Avance 500) was used to for structural characterization of oxamide reagents and their kinetic study. Mass spectra were recorded using Jeol-JMS-AX505HA. Perkin Elmer LS-5, luminescence detector, Beckman Coulter LA-400, and CCD imaging from Apogee Inst. Inc. E-260; s/n-A6148 digital camera were used in the test. The imaging software (MaxIm DL and CCD 4.51) used for generating the images.

## 2.2 Synthesis of peroxyoxamide reagent

**2.2.1.** 2,2'-Oxalyl-bis[(trifluoromethanesulfonyl)imino]ethylene-bis(N-methylpyridinium)trifluoromethane sulfonate (oxamide **1**)

The synthesis of oxamide **1** was carried out as reported by Neil W. Barnett co-workers. [5]

**2.2.2.** 2,2'-Oxalyl-bis[(trifluoromethanesulfonyl)imino]ethylene-bis(N-methylpyridinium)tetrafluoroborate (oxamide **2**)

*Step 3.* Trimethyloxoniumtetrafluoroborate (0.18 g, 0.0012 mol) was added to a stirred solution of *N,N'*-bis [2-(2'-pyridyl)ethyl]-*N,N'*-bis(trifluoromethylsulfonyl) oxamide **3** in dry dichloromethane (5 ml). After the addition was completed, the reaction mixture was stirred at room temperature for 4 h. The resultant white solid was separated by filtration and washed with dichloromethane several times to obtain the white amorphous powder **2** with a yield of 90%. Melting point 164 - 166° C. In  $d_6$  acetone  $^1\text{H}$  NMR  $\delta$  3.70 (2H, b), 4.43 (1H, b), 4.64 (3H, s), 4.75 (1H, b), 8.13 (1H, t), 8.22 (1H, d), 8.71 (1H, t), 9.02 (1H, d),  $^{13}\text{C}$  NMR  $\delta$  33.0, 47.3, 118.6 (q,  $^1J = 324$  Hz), 121.2 (q,  $^1J = 320$  Hz), 126.8, 127.8, 143.7, 146.9, 153.4, 160.3  $^{19}\text{F}$  NMR  $\delta$  -74.0, -79.0, -151.7. FAB positive ion mass spectrum for  $\text{C}_9\text{H}_{12}\text{F}_3\text{N}_2\text{O}_2\text{S}^+$   $m/z = \text{Calc. } [M]^+ 269.0566$ , obs. 269.7575, light green crystals melting point 78 - 80° C

## 2.3. Fluorescent labeling of antibody on mesoporous silica

Mesoporous silica particles were covalently linked with fluorescent organic (alexa fluor 555) dye labeled goat anti-mouse IgG and goat IgG as reported previously by our group [7].

## 2.4 POCL reaction in aqueous solution

The POCL reaction mixture in each test was the same as the following unless specified. The fluorescent texas red dye was dissolved in DMSO (10 mg/mL) and the solutions were made in 2 mM NaOH for final concentration 100  $\mu\text{g}/\text{mL}$ . The oxamide **1** or **2** was dissolved in THF:CH<sub>3</sub>CN (1:1) to a final concentration of 50 mM. 10  $\mu\text{L}$  dye and 10  $\mu\text{L}$  of 50 mM oxamide reagents solutions were added into the well of a 96 well plate. 0.3% and 0.03% hydrogen

peroxide solutions 100  $\mu\text{L}$  ) in sodium phosphate (PBS) buffer (10 mM, pH 7.2) containing 150 mM NaCl, was injected through luminometer into the well to initiate the POCL reaction. The measurements were taken immediately (ten cycles with 10 seconds per cycle) after oxamide injected. The relative luminescence units in the results represent the integral of the curve formed from the 10 cycles.

To check the stability of oxamide reagent in aqueous media, freshly made 50 mM oxamide reagent **1** was used in the test at time zero (start point). The same solution was then used in the POCL reactions at 0, 5, 10, 15, 20, 25, 30, 40, 45, 65, 75, 90, 110, 130, 150, 170, and 190 min. from the start point. Measurements were taken immediately at each time point till 100 sec. To check the effect of polyethylene glycol (PEG) on POCL reaction, hydrogen peroxide was dissolve in 10%, 25% and 50% PEG in PBS buffer solution and 100  $\mu\text{L}$  of this solution was injected into to reaction mixture. To observe surfactant effect on the POCL reaction, hydrogen peroxide was dissolve in 1%, 0.1% and 0.01% triton and tween-20 in PBS buffer solution.

In case of POCL reaction of labeled mesoporous silica particles, 0.5  $\mu\text{L}$  suspension in PBS was dropped on cellulose membrane followed by 0.5  $\mu\text{L}$  oxamide **1** solution in THF:CH<sub>3</sub>CN (1:1) to a final concentration of 50 mM and finally 4.0  $\mu\text{L}$  0.3% hydrogen peroxide solution in (PBS) buffer (10 mM, pH 7.2) to initiate the reaction. The images were taken immediately with 60 seconds exposure time.

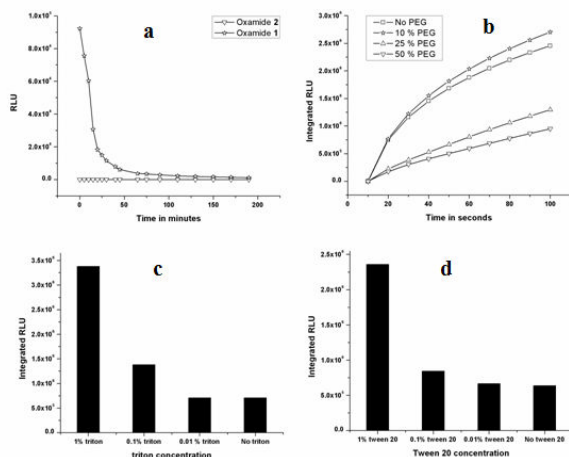
## 2.5. Chemiluminescence of Fluorescent silica nanoparticles

We have synthesized uniform fluorescein dye doped silica nanoparticles (F-SiNPs) using a reverse microemulsion method [8] for bioanalysis applications as labeling reagents. The first monomer precursor was prepared according to method described by Wensheng Yang et al. [9]. The fluorescein isothiocyanate (FITC) was covalently attached to the coupling agent 3-aminopropyltriethoxysilane (APS) by an addition reaction of amine group with the isothiocyanate group followed by F-SiNPs were synthesized from TEOS and monomer precursors in W/O microemulsion [10] and finally encapsulation with TEOS. All the synthesis with F-SiNPs performed in the dark to avoid photobleaching. POCL reaction of F-SiNPs was carried out in aqueous suspension as described in section 2.4 using 100 ng F-SiNPs for each experiment.

## 3 RESULT AND DISCUSSION

The kinetics of chemiluminescence of oxamide **1** and **2** were shown in figure 1a. A considerable loss of chemiluminescence intensity was observed when a stock solution of **1** was used over the time for its POCL reaction. The observed half life of **1** was observed less than 20 minutes which agreed the result reported by Neil et al. [5].

To understand the change in structure of **1**, we performed  $^1\text{H}$  NMR kinetic analysis in  $\text{D}_2\text{O}$  over the different time period using same sample and observed a dynamic change in its conformation from one structural isomer to another as shown in figure 2. To resolve the cause of this phenomenon, we synthesize two more oxamide, by substituting trifluoromethylsulfonate with tetrafluoroborate cation **2** and reducing one carbon from ethyl to methyl group using 2-(aminomethyl)pyridine **3** (data not shown)



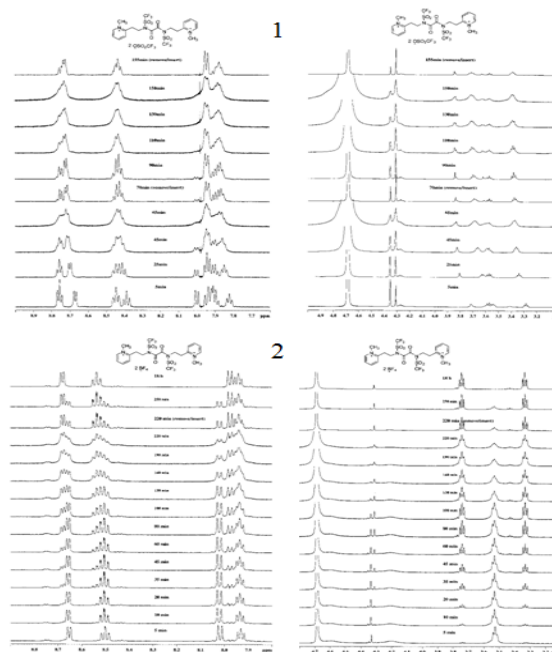
here).

**Figure 1:** Analysis of aqueous POCL reaction of oxamide **1** using 0.03 % hydrogen peroxide (a) time titration graph of stability of oxamide **1** and **2** in 90% aqueous solution, (b) Effect of PEG solution (c) effect of surfactant with triton, and (d) effect of surfactant with tween -20.

The oxamide **2** also exhibit a dynamic change in its conformation as is clear from  $^1\text{H}$  NMR, the two hydrogen peaks between 3.5 to 3.6 ppm were getting separated, the two peaks one at 3.28 and another 3.72 ppm were becoming broad, the two peaks between 4.3 to 4.38 ppm were changing their height ratio, the aromatic hydrogen peaks were become broad and broad. In the same fashion a beautiful dynamic change was observed in  $^1\text{H}$  NMR of oxamide **2**, the peaks at 3.32 and 3.74 ppm were slowly getting clear triplet while 3.48 to 3.6 ppm were disappearing, the peaks at 4.32 to 4.36 were changing their height ration and also aromatic peaks were getting closer and broader. These dynamic change in oxamide **1** and **2** clearly indicated that trifluoromethyl sulfonate and tetrafluoroborate both cations are not participating in dynamic behavior of **1**. Further, oxamide **3** didn't show any change  $^1\text{H}$  NMR. That resulted the two carbon chain of 2-(2-Aminoethyl)-pyridine could be one of the factor responsible for dynamic behavior of **1** and **2**.

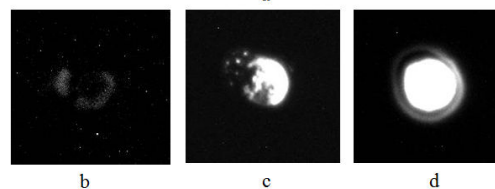
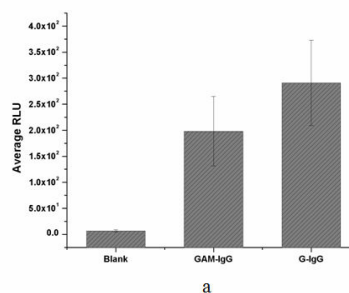
Further, the change in trifluoromethylsulfonate anion to tetrafluoroborate in compound **2** did not improve the reactivity of **1**. To improve the stability and reactivity of oxamide **1**, poly ethylene glycol and two surfactant triton, tween - 20 were added in 90% aqueous solution. Three

concentrations (10%, 25% and 50% PEG) of aqueous solution were used to optimize the best condition for chemi-



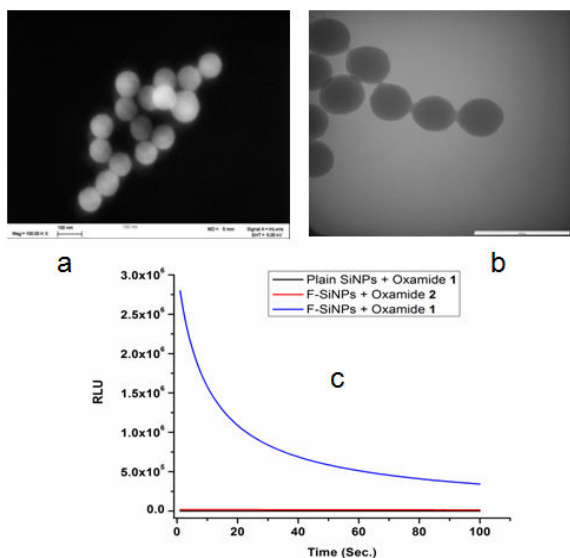
**Figure 2:**  $^1\text{H}$  NMR kinetic study of oxamide **1** and **2** at different time interval in water ( $\text{D}_2\text{O}$ ).

-luminescence intensity over 100 sec. Apparently from figure 1b, 10% PEG aqueous solution was giving more luminescence reading than 25 and 50%. The addition of minute quantities of surfactant (1% triton or 1% tween-20) in to the reaction solvent slow down the reactivity of oxamide **1** and increases longevity of reaction as shown in fig 1c, and d respectively.



**Figure 3a:** Luminometer readings for blank, and CCD imaging characterization of (b) non-fluorescent labeled mesoporous silica particles, (GAM-IgG)/(c) Alexa Fluor 555 dye labeled goat anti-mouse-IgG and (G-IgG)/(d) Alexa Fluor 555 dye labeled goat IgG antibody, covalently linked with mesoporous silica surface.

Aqueous solution of oxamide **1** was used to analyze the covalent linkage of fluorescent dye labeled goat anti-mouse IgG antibody and generic goat IgG antibody with mesoporous silica using chemiluminescence. The bar graph of figure **3a** shows relative luminescence of fluorescent labeling of both antibodies compared to non-labeled mesoporous silica particles in more than 95% aqueous and bio-friendly medium in 250 nM of dye concentrations. The CCD images (Figure **3b, c** and **d**) of non-fluorescent labeled and fluorescent labeled silica nano-particles also support and corresponds the luminometer results.



**Figure 4a:** SEM, and **(b)** TEM images of fluorescein doped silica nanoparticles (F-SiNPs) ~ 100 nm. **(c)** Luminescence graph of POCL reaction (F-SiNPs) with oxamide **1, 2**.

Further, POCL reaction of oxamide **1** was applied for the analysis of F-SiNPs. These nanoparticles were in the range of 100 nm in diameter and spherical in shape (fig 4a, b). The photostability of F-SiNPs is very high and exhibit very high luminescence upon treating with oxamide **1** in POCL reaction at 0.03 % hydrogen peroxide. These F-SiNPs can be efficiently applied for covalent labeling of biomolecules for immunoassay based sensor method. According to the graph (fig 4c), which shows that only oxamide **1** can excite the fluorophore for luminescence while oxamide **2** has all most negligible activation properties.

#### 4 CONCLUSION

Here, we have synthesized three peroxyoxamide reagents **1, 2**, and **3** and characterized as previously reported and first time, we have reported <sup>1</sup>H NMR analysis of these reagents in aqueous solution to check its structural changes. We believe that the reagent **1** works very well for

aqueous chemiluminescence and can illuminate fluorescent organic dye labeled biomolecules or fluorescent organic-dye-doped silica nanoparticles also. Due to its properties, oxamide **1** can be used for developing optical sensor in diagnostic fields.

#### 5 ACKNOWLEDGEMENTS

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