A Colorimetric and Fluorometric Chemosensor Based on Quinizarin-Metal Complexes for Alkaline Phosphatase Activity

Yong-Goo Son, Cheol Hee Lee, Ji-Soo Seo, Bum Jun Park, and Jong-Man Kim*

*Department of Chemical Engineering, Hanyang University, Seoul 133-791, Korea. *E-mail: jmk@hanyang.ac.kr*

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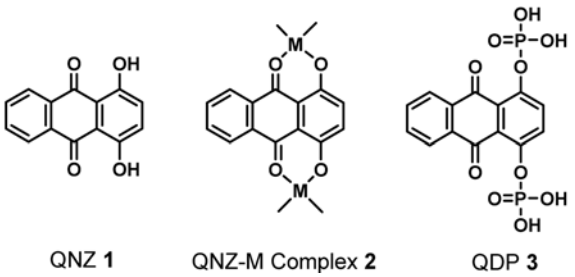
Hydroxyanthraquinones are well known colorimetric indicators for pH and metal ions.[1-6] In high pH media, these substances exist in phenolate anionic forms, which have absorption maxima in the visible region. In addition, hydroxyanthraquinone phenolates readily bind metal ions, such as magnesium and aluminum, to produce highly fluorescent metal-anthraquinone complexes. For example, a greater than 30 fold increase of fluorescence intensity is observed when 1,4-dihydroxyanthraquinone (quinizarin, QNZ, **1**) complexes with aluminum ion.[1] Tight binding by these quinizarins is due to metal ion interactions with the ionized hydroxyl groups and neighboring carbonyl moieties, as depicted in **2**. If the hydroxyl groups of these substances are protected, metal-QNZ complex formation does not take place. Moreover, significant differences in absorption maxima and fluorescence intensities are expected for protected and metal complexed QNZ. Thus, appropriately protected QNZs should serve as dual signal (colorimetric and fluorometric) chemosensors for processes (*e.g.*, enzymatic) that promote cleavage of the hydroxyl protecting groups. In this communication, we report, the results of initial studies leading to the development of a hydroxyquinone-based, enzyme-cleavable colorimetric and fluorometric chemosensor, quinizarin diphosphate (QDP, 3), and its application to a method for assaying alkaline phosphatase activity.



The diphosphorylated quinizarin QDP was readily prepared in two steps from quinizarin.[7] The sequence (Figure 1) begins with reaction of quinizarin with diethyl chlorophosphate in the presence of sodium hydride to yield the phosphate ester **4**. The phosphate ester **4**, generated in this manner, is converted to QDP by treatment with bromotrimethylsilane in chloroform followed by aqueous work-up. Recrystallization of the anilinium salt of QDP **3** from MeOH-acetone gives pure material.

Employment of QDP as an alkaline phosphatase-cleavable colorimetric and fluorometric chemosensor[8-10] was evaluated. Owing to their high turnover numbers, low cost, and broad substrate specificity, enzymes in the alkaline phosphatase family play key roles in a number of important enzyme immunoassay procedures and related affinity sensing methods.[11-12] Solutions of QDP (0.1 mM) in diethanolamine (DEA) buffer (0.1 M, pH 10.0, 20 mM $MgCl_2$) containing alkaline phosphatase (40 μg/mL, from bovine intestinal mucosa) were incubated at 37 °C. The reaction was monitored by UV-visible and fluorescence spectroscopy.

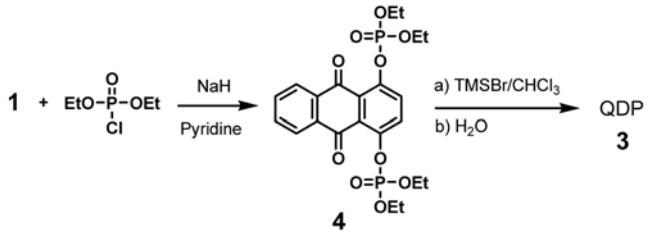


Figure 1. Preparation of QDP **3**.

As displayed in Figure 2(A), incubation of QDP with alkaline phosphatase results in the formation of two new absorptions in the visible region at 535 and 575 nm, which are characteristic of metal complexed forms of quinizarin. This suggestion was confirmed by comparing absorption spectra of quinizarin in the presence and absence of magnesium ion in aqueous basic media. Importantly, incubation of QDP in DEA buffer solutions did not produce the 535 and 575 nm absorption bands when alkaline phosphatase was not present. Michaelis-Menten treatment of rate versus QDP concentration data gave K_m and V_{max} values of 110 μM and 4.2 μM/s, respectively.

Hydrolysis of QDP by alkaline phosphatatase is also accompanied by the formation of a fluorescence emission at 595 nm (excitation at 535 nm, Figure 2(B)) which reaches a maximum intensity after approximately 20 min reaction time. The observed fluorescence corresponds to the metal-quinone complexes since the dianion form of quinizarin itself is nonfluorescent.

In order to demonstrate that the spectroscopic changes are specific for alkaline phosphatase catalyzed reaction, QDP was incubated with other hydrolytic enzymes such as phosphodiesterase and chymotrypsin, as well as the non-specific protein, bovine serum albumin. As presented in viewing Figure 3, only the buffered solution of QDP containing alkaline phosphatase develops the purple color associated with the metal-quinone complexe (Figure 3, B).

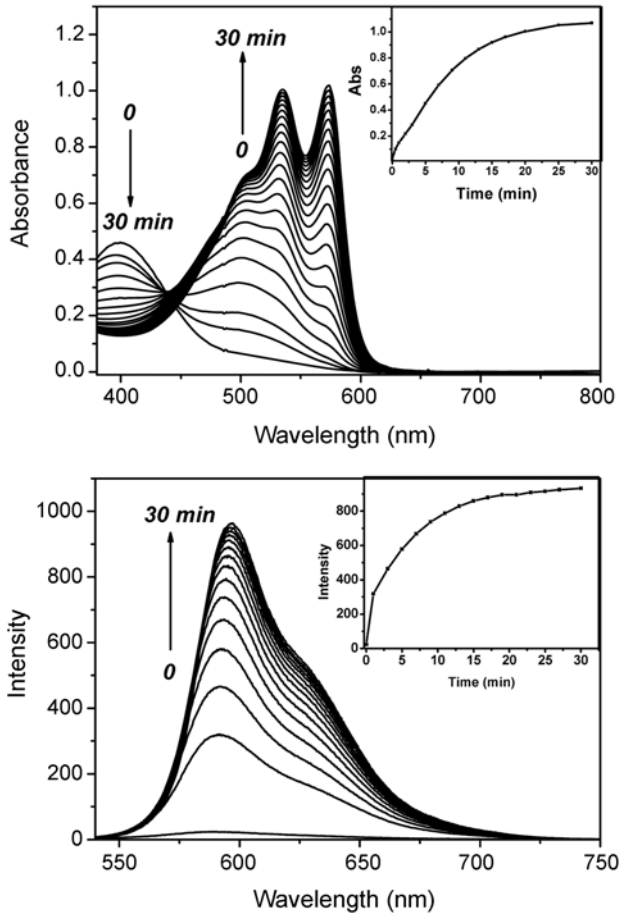


Figure 2. UV-visible (A) and fluorescence (B, excitation at 535 nm) spectroscopic monitoring of DEA buffer solutions (0.1 M, pH 10.0, 20 mM $MgCl_2$) of QDP **3** in the presence of alkaline phosphatase (40 μg/mL) at 37 °C. Insets are changes of absorption (A, measured at 575 nm) and emission (B, measured at 595 nm, excitation at 535 nm) intensities as a function of time.

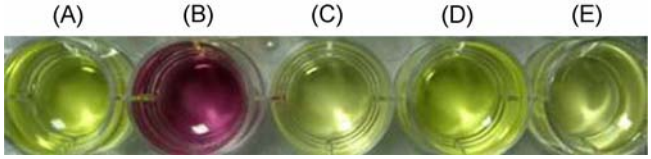


Figure 3. Photographs of solutions of QDP in DEA buffers (0.1 M, pH 10.0, 0.5 mM $MgCl_2$) at 37 °C after 5 min incubation times in the absence of enzyme (A), and in the presence of alkalin phosphatase (0.1 mg/mL) (B), phosphodiesterase (C), chymotrypsin (D), and bovine serum albumin (E). Reaction with chymotrypsin was conducted in HEPES buffer (5 mM, pH 8.0, 0.5 mM $MgCl_2$).

None of other enzymes and BSA (Figures 3, C-E) promote hydrolytic cleavage of QDP.

Comparison of QDP with commercial probe molecules such as 4-nitrophenylphosphate (4-NP) and 4-methylumbelliferyl phosphate (4-MUP) revealed that QDP was comparable to 4-NP and 4-MUP in terms of colorimetric response. However, QDP was found to be less effective than 4-MUP as a fluorometric chemosensor.

In summary, the current study has led to the development of a new strategy for the design of enzyme-cleavable chemosensor. The diphosphorylated anthraquinone QDP, readily prepared from quinizarin, undergoes alkaline phosphatase promoted cleavage to generate fluorescent and intensely purple-colored quinone-metal complexes in aqueous solution. QDP is well suited to alkaline phosphatase assays since magnesium ions, necessary for quinone-metal complex formation after hydrolysis, is a required cofactor of the enzyme. Although this study focused on alkaline phosphatase activity, by proper modification of the anthraquinone backbone, it should be possible to construct dual signal chemosensors for a wide variety of enzyme catalyzed hydrolytic reactions.

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7. To a solution of quinizarin (**1**) (1.00 g, 4.16 mmol) in dry pyridine (100 mL) at room temperature was added sodium hydride (0.40 g, 16.65 mmol) and the resulting solution was stirred for 30 min. To the solution was added diethyl chlorophosphate (2.40 mL, 16.65 mmol) and the mixture was stirred for 3 h at room temperature, poured into *n*-hexane. After the precipitate was filtered off, the filtrate was concentrated in vacuo to give a residue which was redissolved in ether. The ether layer was washed with water and dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, the residue was subjected to silica gel column chromatography (60% ethyl acetate/hexane) to give the bisphosphate ester **4** (1.53 g, 72%) as a yellow powder. m.p. 79-80 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ= 1.41 (t, 12H), 4.38 (m, 8H), 7.77 (m, 4H), 8.19 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ= 16.05, 65.03, 125.61, 126.59, 128.54, 133.65, 133.79, 146.80, 181.44. To a solution containing the bisphosphate ester **4** (1.00 g, 1.95 mmol) in chloroform at room temperature was added bromotrimethylsilane (1.01 mL, 7.80 mmol). The resultant mixture was allowed to stir for 1 h at room temperature, concentrated *in vacuo*. The residue was dissolved in a mixture of ether and water. After collecting the aqueous layer, aniline (0.71 mL, 7.80 mmol) was added and concentrated *in vacuo* to give a solid residue which was recrystallized from methanol-acetone (1 : 4 v/v) to afford the desired product QDP (0.37 g, 33%) as a yellow powder. m.p. 124-125 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ= 6.56 (t, 2H), 6.63 (d, 4H), 7.04 (t, 4H), 7.72 (s, 2H), 7.86 (m, 2H), 8.03 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ= 115.67, 118.02, 125.14, 126.02, 129.00, 131.91, 133.79, 145.43, 147.06, 147.14, 181.34.
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